UNIVERSITAT DE BARCELONA

Caracterización citogenético-molecular de la leucemia linfocítica crónica con cariotipo complejo.

Identificación de subgrupos con relevancia clínica

Silvia Ramos Campoy

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IMIM
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Institut Hospital del Mar d'Investigacions Mèdiques

UNIVERSITAT DE BARCELONA

Caracterización citogenético-molecular de la leucemia linfocítica crónica con cariotipo complejo.
Identificación de subgrupos con relevancia clínica

Memoria presentada por SILVIA RAMOS CAMPOY para optar al grado de Doctora por la Universitat de Barcelona

Programa de Doctorado en Biomedicina
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Barcelona, diciembre 2022

Tesis realizada bajo la dirección de las Dras. **Blanca Espinet Solà** y **Anna Maria Puiggos Metje**, en el Grup de Recerca Translacional en Neoplàsies Hematològiques (GRETNHE) del Institut Hospital del Mar d'Investigacions Mèdiques (IMIM) y la tutoría del Dr. **Elías Campo Güerri** del Departament de Fonaments Clínics de la Universitat de Barcelona.

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CERTIFICAN

Que la tesis doctoral titulada "Caracterización citogenético-molecular de la leucemia linfocítica crónica con cariotipo complejo. Identificación de subgrupos con relevancia clínica", presentada por Silvia Ramos Campoy para acceder al título de Doctora por la Universitat de Barcelona, se ha realizado bajo su supervisión y cumple los requisitos formales y científicos para ser defendida delante del tribunal correspondiente.

El trabajo se ha llevado a cabo en el Grup de Recerca Translacional en Neoplàsies Hematològiques del Institut Hospital del Mar d'Investigacions Mèdiques, en el Parc de Recerca Biomèdica de Barcelona.

Barcelona, diciembre 2022

Dra. Blanca Espinet Solà   Dra. Anna Maria Puiggros Metje

A Rosa y Reyes, mis padres, mis pilares

A Oscar

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Listado de Abreviaturas

ADN: Ácido desoxirribonucleico

ARN: Ácido ribonucleico

ATM: *Ataxia telangiectasia mutated*

B2M: β2-microglobulina

BCL2: *B-cell lymphoma 2*

BCR: Receptor de célula B (del inglés *B-cell receptor*)

BIRC3: *Baculoviral IAP repeat containing 3*

BR: Bendamustina-rituximab

BTK: *Bruton's tyrosine kinase*

CC: Citogenética convencional

CDR3: Región determinante de complementariedad 3

CK: Cariotipo complejo (del inglés *complex karyotype*)

CLL-IPI: *Chronic Lymphocytic Leukemia International Prognostic Index*

DAPI: 4',6-diamino-2-fenilindol

DLBCL: Linfoma difuso de célula B grande (del inglés *diffuse large B-cell lymphoma*)

DLEU1: *Deleted in lymphocytic leukemia 1*

DLEU2: *Deleted in lymphocytic leukemia 2*

ESMO: *European Society for Medical Oncology*

FCR: Fludarabina-ciclofosfamida-rituximab

FISH: Hibridación *in situ* fluorescente (del inglés *fluorescence in situ hybridization*)

Hb: Hemoglobina

ICC: *International Consensus Classification*

IG: Gen de las inmunoglobulinas

IGHV: Gen de la región variable de la cadena pesada de las inmunoglobulinas
IL-2: Interleucina 2
ISCN: *International System for Human Cytogenomic Nomenclature*
LBM: Linfocitosis B monoclonal
LDH: Lactato deshidrogenasa
LLC: Leucemia linfocítica crónica
LOH: Pérdida de heterocigosidad (del inglés *loss of heterozigosity*)
Mb: Megabases
MG: *Microarrays* genómicos
MYD88: *Myeloid differentiation primary response gene 88*
NGS: Secuenciación de nueva generación (del inglés *Next Generation Sequencing*)
OGM: Mapeo óptico del genoma (del inglés O*ptical Genome Mapping*)
OMS: Organización Mundial de la Salud
Pb: Pares de bases
PI3K: *Phosphatidylinositol 3-kinase*
PLCγ2: *Phospholipase C gamma 2*
RB1: *Retinoblastoma 1*
R/R: Refractario o en recaída
SF3B1: *Splicing factor 3B, subunit 1*
SG: Supervivencia global
SLP: Supervivencia libre de progresión
SNP: Polimorfismo de un solo nucleótido (del inglés *Single Nucleotide Polymorphism*)
SYK: *Spleen tyrosine kinase*
TP53: *Tumor protein p53*
TPA: 12-tetradecanoilforbol-13-acetato

TPT: Tiempo al primer tratamiento

TR: Transformación de Richter

ZAP-70: *Zeta-chain-associated protein kinase 70kDa*

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Resumen

La complejidad genómica, detectada por citogenética convencional (CC) en forma de cariotipos complejos (CK) o por *microarrays* genómicos (MG), incluyendo los patrones de cromotripsis, se ha asociado a un curso clínico más agresivo y a una peor respuesta al tratamiento en pacientes con leucemia linfocítica crónica (LLC) tratados con esquemas de quimioinmunoterapia. En dos grandes estudios retrospectivos se ha demostrado que el punto de corte que mejor predice un peor pronóstico es tener cinco o más alteraciones tanto por CC como por MG. No obstante, la estratificación en distintos grupos de riesgo según la complejidad genómica detectada por ambas técnicas aún no ha sido comparada en una gran cohorte de LLC. Además, existe poca información disponible acerca de los mecanismos subyacentes y el impacto clínico de la cromotripsis en el desarrollo y la evolución de esta entidad, especialmente en un contexto de alta complejidad genómica.

En esta tesis se ha llevado a cabo el mayor estudio hasta la fecha en el que se ha analizado de manera paralela por CC y MG una cohorte de 340 pacientes con LLC enriquecida en casos con CK. Los resultados obtenidos sugieren que tanto la CC como los MG son útiles en la detección de complejidad genómica. Sin embargo, entre ambas técnicas se evidenció un grado de acuerdo moderado, con un tercio de los pacientes clasificados en categorías de riesgo discordantes e incluso un 3% categorizados en grupos opuestos. Estas diferencias se debieron principalmente a limitaciones propias de cada técnica. Además, se ha demostrado que las recomendaciones actuales para el análisis de MG son adecuadas para el estudio de la complejidad en LLC. Por lo tanto, las

dos metodologías son útiles pero no equivalentes en la estratificación pronóstica de los pacientes con LLC basada en la complejidad genómica.

Por otro lado, en un segundo trabajo se estudiaron 33 pacientes con patrones sugestivos de cromotripsis, con siete o más cambios en el estado de número de copia localizados en un solo cromosoma. Estos casos fueron comparados con una cohorte de 129 pacientes con CK sin cromotripsis para explorar el impacto de la cromotripsis en un contexto de alta complejidad genómica. Todos ellos presentaron una elevada complejidad genómica (CK por CC y/o al menos tres alteraciones ≥5Mb por MG) y una mayor frecuencia de alteraciones en *TP53* que los pacientes con CK sin cromotripsis. Además, este fenómeno se asociaba a un tiempo al primer tratamiento inferior excepto cuando la cohorte se dividía según el estado de *TP53*. Estos hallazgos sugieren que el pronóstico adverso observado en pacientes con LLC con CK y cromotripsis podría deberse en realidad a la mayor prevalencia de deleciones y/o mutaciones en *TP53* y no a la presencia de cromotripsis *per se*. Asimismo, el análisis mediante mapeo óptico del genoma demostró ser de utilidad al detectar la mayoría de las alteraciones previamente descritas e identificar nuevos reordenamientos asociados a la cromotripsis, permitiendo distinguir dos patrones de reordenamientos y aportando una mejor visión de la complejidad genómica de estos pacientes.

I. INTRODUCCIÓN

1. Leucemia Linfocítica Crónica

1.1. Aspectos generales

La leucemia linfocítica crónica (LLC) es una neoplasia hematológica caracterizada por la proliferación y acumulación de linfocitos B maduros clonales, típicamente CD5+, en la sangre periférica, médula ósea, ganglios linfáticos y otros órganos linfoides (Rozman and Montserrat, 1995; Zenz *et al.*, 2010). Es la leucemia más frecuente en los adultos de los países occidentales, representando el 30% de las leucemias, con una incidencia estimada en 4-5 casos por 100.000 habitantes y año (Eichhorst *et al.*, 2021). Afecta principalmente a individuos mayores de 50 años, siendo la edad mediana al diagnóstico de aproximadamente 70 años, y es más habitual en hombres que en mujeres. Se ha visto que los familiares de primer grado de individuos afectos por LLC presentan un mayor riesgo de desarrollar LLC u otras neoplasias linfoproliferativas que la población general. Sin embargo, y a pesar de que en estudios de familias se han identificado variantes que podrían aumentar el riesgo de padecer la enfermedad, aún se desconocen los factores exactos que promueven esta susceptibilidad (Speedy *et al.*, 2014; Goldin *et al.*, 2016; Law *et al.*, 2017; Speedy *et al.*, 2019).

Pese a que la mediana de supervivencia global (SG) es de alrededor de 10 años, la LLC se caracteriza por presentar un curso clínico altamente variable. Así, mientras que algunos pacientes presentan un curso clínico indolente y se mantienen estables durante mucho tiempo, otros desarrollan una enfermedad agresiva y progresan rápidamente, requiriendo tratamiento. Por este motivo, ha sido necesario el estudio de factores pronósticos que permitiesen categorizar a los pacientes según el riesgo de progresión (González-Gascón *et al.*, 2021).

1.2. Diagnóstico

La clasificación de 2016 de la Organización Mundial de la Salud (OMS) así como la nueva propuesta de la OMS 2022 y la *International Consensus Classification* (ICC) actual incluyen a la LLC dentro de la categoría de neoplasias de células B maduras (Swerdlow *et al*., 2016; Alaggio *et al*., 2022; Campo *et al*., 2022). El primer requisito para su diagnóstico es la presencia de ≥5x10^9/L de linfocitos B clonales en sangre periférica en un período mantenido de mínimo 3 meses (Hallek *et al.*, 2018). Debido a que la mayoría de los pacientes son asintomáticos en el momento del diagnóstico, es común que la enfermedad se detecte de manera casual en un análisis de sangre rutinario. Tras el hallazgo de linfocitosis, es imprescindible confirmar que el paciente presenta una LLC y no otra neoplasia de célula B madura. Para ello, el diagnóstico definitivo de una LLC se basa en los resultados obtenidos en el estudio morfológico e inmunofenotípico de la sangre.

En cuanto a su morfología, las células de la LLC observadas en un frotis de sangre periférica son típicamente linfocitos maduros de pequeño tamaño, con un citoplasma escaso, un núcleo denso en el que es difícil visualizar un nucléolo y cromatina compactada (**Figura 1**). A veces se pueden encontrar entremezcladas células de aspecto atípico o prolinfocitos. El inmunofenotipo es imprescindible para confirmar la clonalidad de los linfocitos B mediante la demostración de la restricción de las cadenas ligeras kappa (κ) o lambda (λ) en la inmunoglobulina de superficie (Hallek *et al.*, 2018). Aparte de κ y λ, el panel del estudio inmunofenotípico necesario para diagnosticar una LLC, diferenciándola así del resto de síndromes linfoproliferativos de células B, debe incluir el antígeno de célula T CD5 (expresado de manera aberrante en las LLC) y los marcadores de célula B CD19, CD20 y CD23 (**Figura 1**) (Rawstron *et* *al.*, 2018). De manera característica, las células LLC muestran una expresión débil de la inmunoglobulina de superficie, CD20 y CD79b. Algunos casos pueden tener un inmunofenotipo atípico (por ejemplo, con CD5-, CD23- o expresión fuerte de las inmunoglobulinas). Adicionalmente, en algunos casos puede estudiarse qué territorios ganglionares están afectos mediante técnicas de imagen.

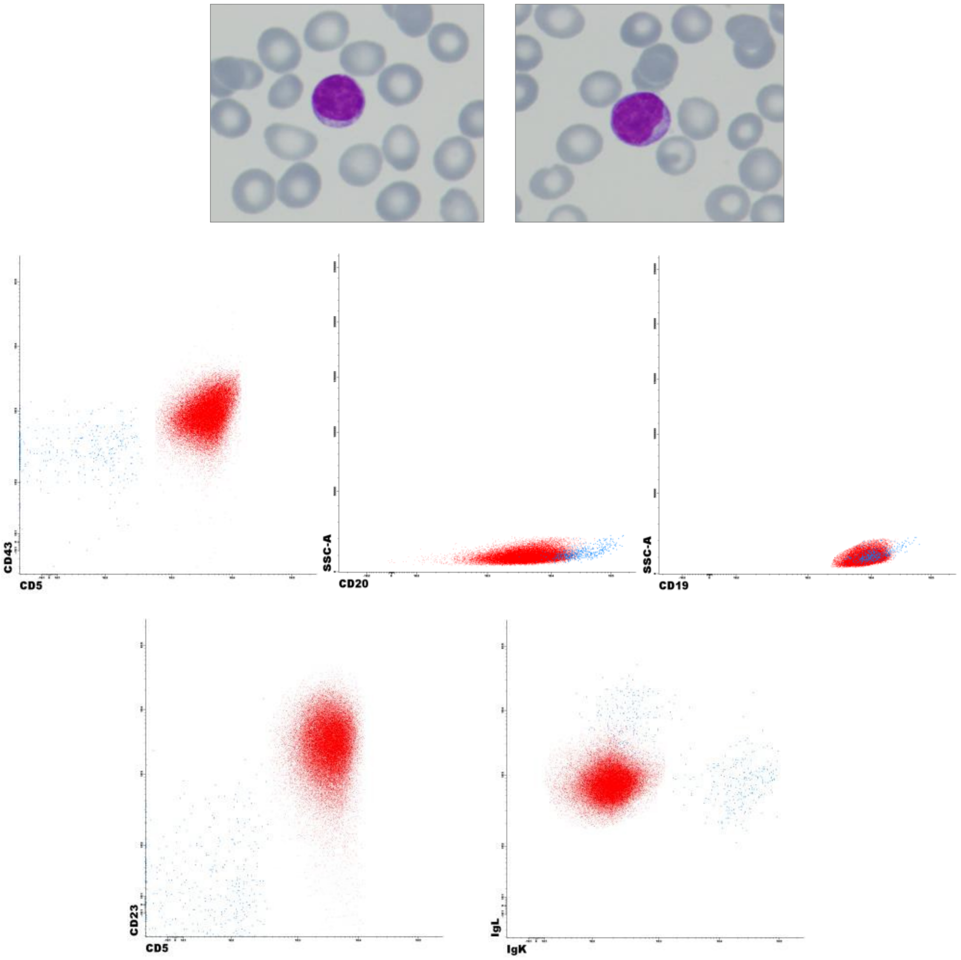


Figura 1. Morfología e inmunofenotipo característicos de la leucemia linfocítica crónica. En los plots de citometría, los linfocitos tumorales de la LLC aparecen destacados en rojo; los linfocitos normales están destacados en azul. *Imágenes cedidas por el Laboratorio de Citología Hematológica del Hospital del Mar.*

A pesar de que la mayoría de los pacientes no presenta síntomas específicos en el momento del diagnóstico, entre un 5-10% sí presenta síntomas B, que incluyen pérdida de peso, fiebre superior a 38ºC, astenia o sudoración nocturna. Asimismo, los individuos afectados también pueden presentar adenopatías, que pueden o no aumentar de tamaño, esplenomegalia o hepatomegalia, fallo de la médula ósea e incluso una mayor tendencia a infecciones de repetición (Sharma and Rai, 2019).

Cabe destacar que entre un 5-15% de la población mayor de 60 años presenta un estado considerado como predecesor de la LLC denominado linfocitosis B monoclonal (LBM). La LBM se define como la presencia de <5x10^9/L linfocitos B clonales con fenotipo de LLC en sangre periférica durante un mínimo de 3 meses junto con la ausencia de otras manifestaciones clínicas de la LLC como son las adenopatías, las visceromegalias, las citopenias o la afectación extramedular (Marti *et al.*, 2005; Alaggio *et al.*, 2022; Campo *et al.*, 2022). En función de si los niveles de células B se encuentran por debajo o por encima de 0,5x10^9/L se denomina LBM de bajo recuento, que raramente progresa a LLC, o LBM de alto recuento, que puede progresar a LLC e incluso requerir tratamiento (Rawstron *et al.*, 2010; Vardi *et al*., 2013; Criado *et al.*, 2018). La tasa anual de progresión de una LBM a una LLC que requiere tratamiento es de alrededor del 1-2% (Rossi *et al.*, 2009; Stratri and Shanafelt, 2015).

Por otro lado, existe otra entidad denominada linfoma linfocítico de células pequeñas (LLP) que se define por la presencia de una población linfocitaria con inmunofenotipo de LLC en ganglios linfáticos de tamaño aumentado, sin estar presentes en sangre periférica (<5x10^9/L linfocitos clonales en sangre periférica). El LLP y la LLC son diferentes presentaciones de una misma enfermedad. En este caso, el diagnóstico debe realizarse mediante biopsia del ganglio o de otros tejidos afectos (Hallek *et al.*, 2018). Asimismo, la LLC puede evolucionar a un tipo de linfoma con fenotipo agresivo denominado Transformación de Richter (TR). Esta transformación sucede con una incidencia del 0,5-1% entre los pacientes con LLC y presenta dos variantes según si el linfoma es difuso de célula B grande (DLBCL) o linfoma de Hodgkin, siendo más común el primero (Parikh *et al.*, 2013; Mouhssine and Gaidano, 2022). En un 85% de los casos el DLBCL asociado a una TR está clonalmente relacionado con la población LLC y confiere un pronóstico muy desfavorable y pobre respuesta al tratamiento. Por el contrario, en un 15% de los pacientes el linfoma no está clonalmente relacionado con la LLC. En estos casos su impacto en la supervivencia es similar al del DLBCL originado *de novo* (Parikh *et al.*, 2014; Rossi *et al.*, 2018).

1.3. Factores pronósticos

1.3.1. Estadios clínicos

Entre los factores pronósticos mejor establecidos en la LLC se encuentran los sistemas de estadiaje definidos por Rai y Binet (Rai *et al.*, 1975; Binet *et al.*, 1981). Ambos son ampliamente utilizados tanto en la práctica clínica como en ensayos clínicos y se basan en datos obtenidos tras la exploración física y en ciertos parámetros del hemograma (**Tabla 1**). El sistema de Rai permite la clasificación de los pacientes en cinco grupos de riesgo (0-IV) teniendo en cuenta la presencia de linfocitosis, adenopatías, hepatomegalia y/o esplenomegalia y los niveles de plaquetas y hemoglobina (Hb) (**Tabla 1**). Del mismo modo, la clasificación de Binet permite la categorización de los pacientes en tres estadios clínicos (A-C) según el número de áreas ganglionares afectadas y según la presencia o ausencia de anemia y/o plaquetopenia (**Tabla 1**). Ambos sistemas permiten identificar aquellos pacientes que presentan una peor evolución, con fallo incluso en la médula ósea, pero no permiten predecir qué pacientes con estadios iniciales van a desarrollar una enfermedad más agresiva.

Tabla 1. Sistemas de estadiaje de Rai y de Binet con los rasgos que definen los estadios de cada uno.

Estadios de RAI

Estadio	Descripción	Riesgo
Rai 0	Linfocitosis en sangre periférica o médula ósea	Bajo
Rai I	Linfocitosis + adenopatías	Intermedio
Rai II	Linfocitosis + esplenomegalia y/o hepatomegalia	
Rai III	Linfocitosis + anemia (Hb <11 g/dL)	Alto
Rai IV	Linfocitosis + trombopenia (plaquetas $<100\text{x}10^9$/L)	

Estadios de BINET

Estadio	Descripción	Riesgo
Binet A	Una o dos áreas ganglionares afectadas* No anemia (Hb ≥10 g/dL) o trombopenia (plaquetas $\geq 100\text{x}10^9$/L)	Bajo
Binet B	Tres o más áreas ganglionares afectadas* No anemia (Hb ≥10 g/dL) o trombopenia (plaquetas $\geq 100\text{x}10^9$/L)	Intermedio
Binet C	Presencia de anemia (Hb <10 g/dL) y/o trombopenia (plaquetas $<100\text{x}10^9$/L)	Alto

*Áreas ganglionares: cabeza y cuello, axilar, inguinal, esplenomegalia y/o hepatomegalia palpable

1.3.2. Marcadores séricos y fenotípicos

Existen determinados marcadores séricos cuyos niveles en sangre ayudan a predecir el riesgo de progresión de los pacientes. Los principales marcadores descritos en la LLC son la lactato deshidrogenasa (LDH), la β2-microglobulina (β2M), la timidina cinasa y el CD23 soluble. Niveles elevados de estos factores se asocian a enfermedad avanzada y a un pronóstico desfavorable (Matthews *et al.*, 2006; Tam *et al.*, 2008; Letestu *et al.*, 2010; Piñeyroa *et al.*, 2022).

Aparte de los marcadores séricos, los niveles de expresión de ciertos marcadores fenotípicos de las células neoplásicas también se han utilizado para la estratificación pronóstica de la LLC. En este sentido, niveles elevados de expresión de la molécula de superficie CD49d, que es la subunidad α4 del heterodímero de la integrina α4β1 (>30% de las células tumorales), de la proteína citoplasmática *zeta-chain-associated protein kinase 70* (ZAP-70) (≥20%) y/o de la proteína transmembrana CD38 (≥30%) se han correlacionado con la ausencia de mutaciones en el gen de la región variable de la cadena pesada de las inmunoglobulinas (IGHV) y tiempo al primer tratamiento (TPT) y SG más cortos (Crespo *et al.*, 2003; Guia *et al.*, 2003; Rassenti *et al.*, 2008; Bulian *et al.*, 2014).

1.3.3. Alteraciones genéticas

En la LLC no existe ninguna alteración patognomónica que permita su diagnóstico. Además, a diferencia de otros síndromes linfoproliferativos B, la presencia de reordenamientos recurrentes es muy poco habitual. No obstante, gracias a los estudios llevados a cabo con el fin de caracterizar genéticamente la enfermedad utilizando diferentes metodologías, como la citogenética convencional, la hibridación *in situ* fluorescente (FISH), los *microarrays* genómicos o las técnicas de secuenciación, se han ido identificando una gran variedad de alteraciones genómicas. De hecho, aplicando la técnica de FISH se ha podido identificar al menos una alteración en aproximadamente un 80% de los pacientes. En este sentido, en el año 2000, Döhner y colaboradores desarrollaron un modelo jerárquico que permitía estratificar a los pacientes en diferentes grupos de riesgo basándose en la detección de tan solo cuatro anomalías cromosómicas por FISH (Döhner *et al.*, 2000). Las cuatro alteraciones cromosómicas que conforman este modelo y cuyo impacto clínico está ampliamente demostrado son la deleción de 13q14 aislada [del(13q)], la trisomía del cromosoma 12 [+12], la deleción en 11q22q23 [del(11q)], afectando al gen *ATM*, y la deleción de 17p13 [del(17p)], que incluye el gen *TP53*. Ser un modelo jerárquico implica que el grupo de pacientes con del(17p) puede tener también cualquiera de las otras alteraciones; el grupo con del(11q) puede tener cualquiera excepto del(17p); el grupo con +12 puede tener también del(13q) pero no del(11q) ni del(17p) y en el grupo con del(13q), ésta debe estar aislada, sin presencia de ninguna de las otras alteraciones. Así pues, según este modelo se establecen las siguientes categorías de riesgo en función de las alteraciones citogenéticas presentes (**Figura 2**):

- **Deleción de 13q14 como única alteración**: pronóstico favorable
- **Cariotipo normal o trisomía 12**: pronóstico intermedio
- **Deleción de 11q22 (*ATM*) o 17p13 (*TP53*):** pronóstico desfavorable

Desde entonces, el estudio de estas cuatro alteraciones por FISH se ha incorporado en el manejo de los pacientes con LLC. De hecho, las guías actuales del *International Workshop on Chronic Lymphocytic Leukemia* (iwCLL) establecen el FISH como test obligado para orientar el pronóstico de los pacientes que van a recibir tratamiento, tanto en la práctica clínica como en ensayos clínicos. Del mismo modo, las guías de la *European Society for Medical Oncology* (ESMO) recomiendan realizar un FISH tan solo para la detección de del(17p) (y el análisis de mutaciones en *TP53*) antes del inicio de tratamiento y establecen como técnica adicional deseable la realización de un FISH más extenso para el estudio de las otras alteraciones citogenéticas (Hallek *et al.*, 2018; Eichhorst *et al.*, 2021).

En el apartado 3 de esta introducción se explicaran con más detalle las características de estas alteraciones y otras anomalías descritas en la LLC así como su impacto en el pronóstico de la enfermedad.

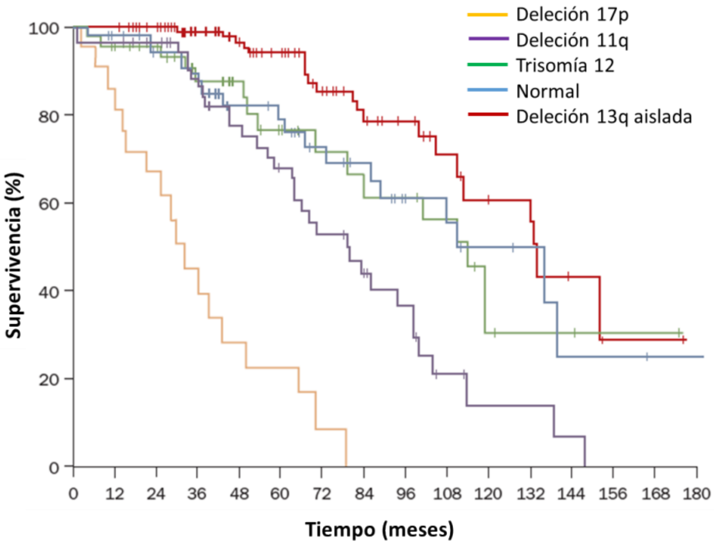


Figura 2. Supervivencia global de los pacientes con LLC según las categorías citogenéticas establecidas por el modelo jerárquico propuesto por Döhner y colaboradores basándose en la detección de cuatro alteraciones citogenéticas por hibridación *in situ* fluorescente (Döhner *et al.*, 2000; adaptada de Cramer *et al.*, 2011).

Con respecto a las mutaciones génicas, la LLC no se caracteriza por presentar unas mutaciones específicas. Sin embargo, gracias a los estudios llevados a cabo con técnicas de *Next-Generation Sequencing* (NGS) en los últimos años se han descrito variantes en hasta 40 genes. No obstante, cabe destacar que la incidencia de estas mutaciones, observadas en alrededor de un 3-15% de pacientes en el momento del diagnóstico, es menor con respecto a otras neoplasias linfoides y otros tumores. Los principales genes que presentan mutaciones con relevancia clínica probada en LLC son *TP53*, *ATM*, *NOTCH1*, *SF3B1*, *BIRC3* y *MYD88* (**Tabla 2**) (Fabbri *et al.*, 2011; Puente *et al.*, 2011; Baliakas *et al.*, 2015b; Landau *et al.*, 2015; Puente *et al.*, 2015; Nadeu *et al.*, 2016; Tausch *et al.*, 2020). De todas ellas, la única con un claro valor pronóstico y predictivo de respuesta al tratamiento es la mutación en *TP53*. Por este motivo, es la única mutación cuyo estudio es obligatorio antes de iniciar tratamiento (Hallek *et al.*, 2018; Eichhorst *et al.*, 2021).

- **Mutaciones en *TP53***: son las más relevantes clínicamente por su impacto negativo en pacientes tratados con quimioinmunoterapia. Son poco frecuentes en el momento del diagnóstico (4-8%) pero su incidencia aumenta en pacientes refractarios o en recaída (R/R) (30-40%). El 60% de los casos con mutaciones en *TP53* tienen el otro alelo delecionado. No obstante, las mutaciones en *TP53* por sí mismas, en ausencia de del(17p), se han asociado a refractariedad a la quimioinmunoterapia y SG más corta (Malcikova *et al.*, 2018; Blakemore *et al.*, 2020). Además, se ha visto que incluso las mutaciones presentes en subclones se asocian a mal pronóstico (Rossi *et al.*, 2014; Nadeu *et al.*, 2018; Malcikova *et al.*, 2021). Por eso, actualmente, es la única mutación que debe analizarse en la rutina diagnóstica en pacientes que deban iniciar tratamiento puesto que los que porten esta alteración deben evitar la quimioterapia y beneficiarse de otros tratamientos, como los inhibidores de BTK o BCL2 (Campo *et al.*, 2018; Hallek *et al.*, 2018).

- **Mutaciones en *NOTCH1***: se detectan en un 10-15% de los casos con LLC al diagnóstico. Son más frecuentes en estadios avanzados y en portadores de +12. Se han asociado a pronóstico desfavorable y a mayor riesgo a desarrollar TR (Larráyoz *et al.*, 2017; Tausch *et al.*, 2020; Edelmann *et al.*, 2022; Pozzo *et al.*, 2022). Además, se ha visto que las mutaciones en *NOTCH1* no solo tienen impacto pronóstico sino también predictivo, promoviendo resistencia a la inmunoterapia con anticuerpos anti-CD20 (D'Agaro *et al.*, 2018; Tausch *et al.*, 2020). De manera característica, en un estudio se observó que hasta un 3% de los pacientes con LLC presentan mutaciones en la región 3'UTR de *NOTCH1* que promueven errores en el proceso de *splicing* (Puente *et al.*, 2015).

- **Mutaciones en *ATM*:** se encuentran en un 10% de los pacientes al diagnóstico y en un 30-40% de los casos con del(11q). Se asocian a pronóstico adverso cuando se encuentran de manera aislada y en presencia de del(11q) (Stankovic *et al.*, 2014; Hu *et al.*, 2019).

- **Mutaciones en *SF3B1*** *(splicing factor 3b subunit 1):* se detectan en una baja proporción de los casos (5-10%) al diagnóstico y provocan una alteración en el mecanismo de *splicing* (Tang *et al.*, 2020). Suelen ir acompañadas de del(11q) o mutaciones en *ATM* y se asocian a pronóstico desfavorable, con un tiempo de progresión inferior tras el tratamiento con fludarabina, ciclofosfamida y rituximab (Te Raa *et al.*, 2015; Pozzo *et al.*, 2021).

- **Mutaciones en *BIRC3*:** son las menos frecuentes, encontrándose en un 2-5% de los pacientes al diagnóstico. La presencia de mutaciones en este gen se ha relacionado con refractariedad a la fludarabina, estadios avanzados, IGHV no mutado y del(11q) (Blakemore *et al.*, 2020; Diop *et al.*, 2020).

- **Mutaciones en *MYD88*** *(myeloid differentiation primary response 88):* están presentes en alrededor de un 3-5% de los casos, especialmente en pacientes jóvenes. A diferencia de las mutaciones anteriores, éstas se asocian a un pronóstico favorable y otros factores de buen pronóstico como la del(13q) o IGHV mutado (Martínez-Trillos *et al.*, 2014; Shuai *et al.,* 2020).

Tabla 2. Resumen de las mutaciones génicas más frecuentes en pacientes con LLC observadas al diagnóstico.

Mutación	Región cromosómica	Función biológica	Pronóstico	Frecuencia
SF3B1	2q33.1	*Splicing* del ARN mensajero	Malo	5-10%
MYD88	3p22.2	Señalización NF-κB	Bueno	3%
NOTCH1	9q34.3	Señalización de NOTCH1	Malo	10-15%
ATM	11q22.3	Reparación del ADN	Malo	10-12%
BIRC3	11q22.2	Señalización NF-κB	Malo	2-5%
TP53	17p13.1	Reparación del ADN	Muy malo	5-10%

1.3.4. Estado mutacional del gen de la región variable de la cadena pesada de las inmunoglobulinas (IGHV)

Durante el proceso de maduración de un linfocito B se producen de manera fisiológica hipermutaciones somáticas en el gen que codifica para la región variable de la cadena pesada de las inmunoglobulinas (IGHV). Cuando los linfocitos B maduros circulantes son expuestos a un antígeno, migran hacia los centros germinales de los órganos linfoides secundarios, donde se producen las hipermutaciones somáticas con el fin de adecuar la respuesta inmune. De esta manera, el receptor de la célula B (BCR) adquiere una elevada afinidad y especificidad para diferentes antígenos (Young and Brink, 2021) (**Figura 3**).

En la LLC, el estado mutacional de IGHV representa uno de los principales factores con impacto pronóstico (Damle *et al.*, 1999; Hamblin *et al.*, 1999). Además, éste se mantiene estable durante el curso de la enfermedad. La LLC se ha clasificado en dos tipos moleculares en función del grado de identidad con la línea germinal, es decir, en función de la presencia o ausencia de mutaciones en IGHV:

- **IGHV no mutado**: porcentaje de homología ≥98% (<2% de mutaciones) con respecto a la línea germinal. En estos casos se ha observado mayor activación de la vía de señalización del BCR, promoviendo una mayor proliferación y supervivencia celular. Además, se ha asociado la presencia de IGHV no mutado con otros factores genéticos de alto riesgo. En consecuencia, estos casos se asocian con pronóstico adverso así como a peor respuesta al tratamiento (Damle *et al.*, 1999; Hamblin *et al.*, 1999; Burger *et al.*, 2013; Rozovski *et al.*, 2018).

- **IGHV mutado**: porcentaje de homología <98% (≥2% de mutaciones). Aproximadamente el 60% de los casos al diagnóstico tienen IGHV mutado. Las células tumorales derivan de un linfocito B que ha estado en contacto con un antígeno. En estos pacientes, los linfocitos se encuentran en un estado de anergia. Por este motivo, se asocian con un pronóstico favorable (Rozovski *et al.*, 2018).

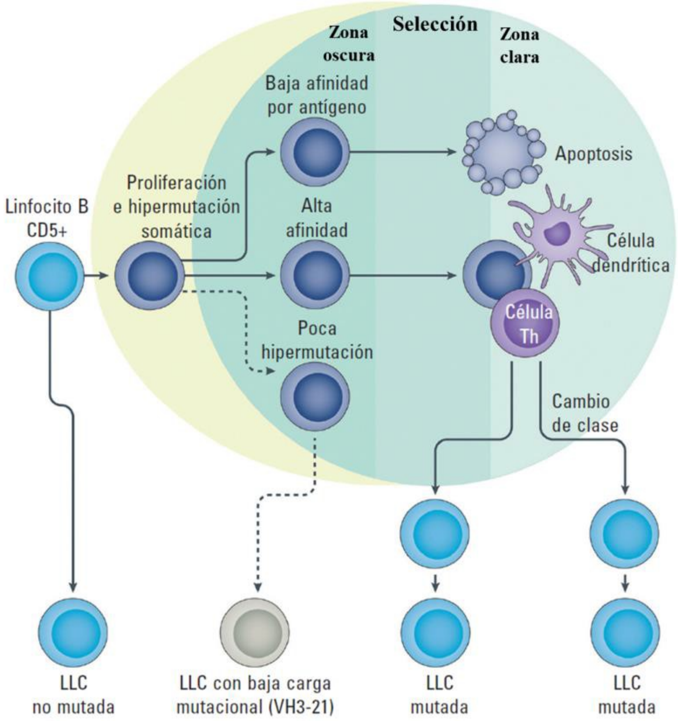

Zona oscura
Selección
Zona clara
Baja afinidad por antígeno
Apoptosis
Proliferación e hipermutación somática
Linfocito B CD5+
Alta afinidad
Célula dendrítica
Célula Th
Poca hipermutación
Cambio de clase
LLC no mutada
LLC con baja carga mutacional (VH3-21)
LLC mutada
LLC mutada

Figura 3. Proceso de maduración del linfocito B a su paso por el centro germinal. Las células LLC con IGHV no mutado se originan a partir de células CD5+ que no han experimentado el proceso de hipermutación somática, mientras que las células LLC con IGHV mutado derivan de células B CD5+ que han pasado por el centro germinal (adaptada de Kipps *et al.*, 2017).

No solo el estado mutacional del gen IGHV se asocia con el pronóstico en LLC. Se ha visto que el repertorio de genes de inmunoglobulinas (IG) producidos por los linfocitos B tumorales es limitado. Entorno a un tercio de los pacientes no emparentados presenta secuencias casi idénticas (estereotipadas) de la región determinante de la complementariedad 3 (CDR3, del inglés *complementary determining region 3*), la principal región del BCR implicada en la unión al antígeno. Actualmente, se han descrito alrededor de 200 subgrupos o *subsets* estereotipados, siendo 19 los principales. Estos *subsets* están asociados con unos rasgos clínicos y biológicos determinados así como con distinto pronóstico (Agathangelidis *et al.*, 2012; Baliakas *et al.*, 2014b; Vardi *et al.*, 2014; Stamatopoulos *et al.*, 2017; Jaramillo *et al.*, 2020; Agathangelidis *et al.*, 2021). Uno de los estereotipos que mejor ejemplifica esto es el *subset* 2, caracterizado por el uso del gen IGHV3-21, asociado a un pronóstico adverso independientemente de si el estado de IGHV es mutado o no mutado (Baliakas *et al.*, 2015).

Tal y como se ha resumido a lo largo de la sección 1.3 de la introducción, existe una gran plétora de biomarcadores útiles para determinar el pronóstico de la LLC, algunos de los cuales también ayudan a predecir la respuesta al tratamiento (**Figura 4**). Es importante destacar que a nivel genético actualmente en la práctica clínica tan solo es obligado estudiar la presencia de del(17p) y de mutaciones en *TP53* y el estado mutacional de las IGHV, puesto que han probado ser los factores con mayor impacto en el curso clínico de los pacientes con LLC.

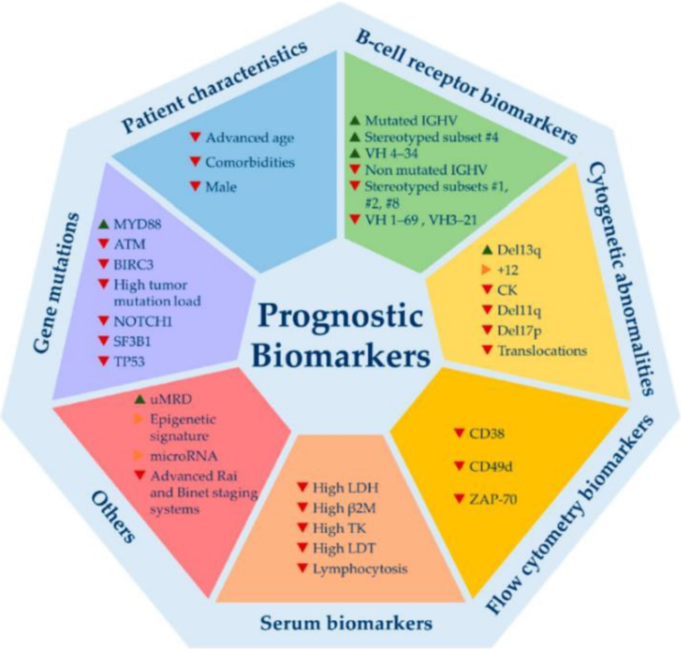

Prognostic Biomarkers
Patient characteristics
▼ Advanced age
▼ Comorbidities
▼ Male
B-cell receptor biomarkers
▲ Mutated IGHV
▲ Stereotyped subset #4
▲ VH 4–34
▼ Non mutated IGHV
▼ Stereotyped subsets #1, #2, #8
▼ VH 1–69 , VH3–21
Cytogenetic abnormalities
▲ Del13q
► +12
▼ CK
▼ Del11q
▼ Del17p
▼ Translocations
Flow cytometry biomarkers
▼ CD38
▼ CD49d
▼ ZAP-70
Serum biomarkers
▼ High LDH
▼ High β2M
▼ High TK
▼ High LDT
▼ Lymphocytosis
Others
▲ uMRD
► Epigenetic signature
► microRNA
▼ Advanced Rai and Binet staging systems
Gene mutations
▲ MYD88
▼ ATM
▼ BIRC3
▼ High tumor mutation load
▼ NOTCH1
▼ SF3B1
▼ TP53

Figura 4. Resumen de los biomarcadores pronósticos utilizados en los pacientes con LLC (González-Gascón *et al.*, 2021).

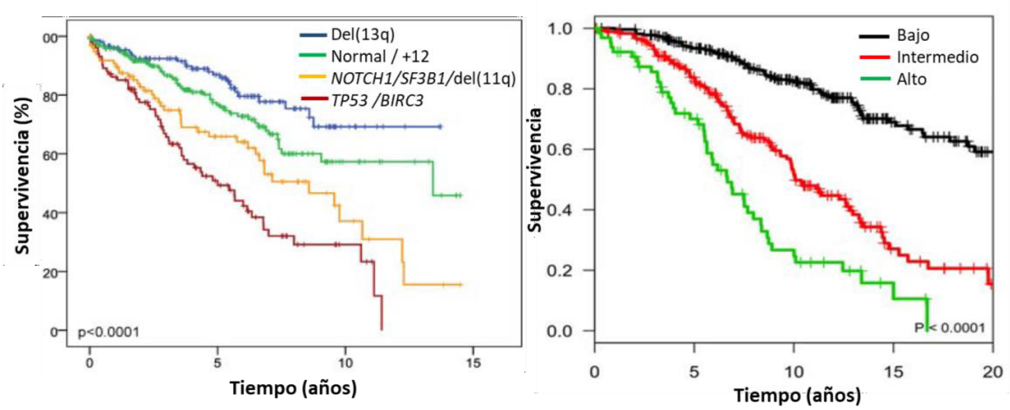
Teniendo en cuenta todos estos parámetros con impacto clínico, se han desarrollado varios algoritmos pronósticos y predictivos con el fin de facilitar la estratificación de los pacientes en distintos subgrupos según su riesgo de progresión y la necesidad de recibir tratamiento. Entre estos índices destaca el *CLL International Prognostic Index* (CLL-IPI) (International CLL-IPI working group, 2016), el cual permite otorgar una puntuación determinada en base al riesgo de evolución del paciente considerando cinco variables clínicas, bioquímicas y moleculares, con una implicación pronóstica distinta. Concretamente, el sistema incluye la presencia de alteraciones en *TP53*, tanto deleciones como mutaciones, la presencia de IGHV no mutada, los niveles elevados de B2M, la edad >65 años y el estadio clínico de Rai >0. A cada una de ellas se le otorga una puntación que difiere según el peso desfavorable que suponga en el pronóstico (**Tabla 3**).

Tabla 3. Índice Pronóstico Internacional LLC (CLL-IPI).

Factor pronóstico	Puntuación
Edad >65 años	1
Estadio Rai I-IV o Binet B-C	1
Niveles de B2M >3,5mg/L	2
IGHV no mutado	2
Del(17p) o mutación en *TP53*	4

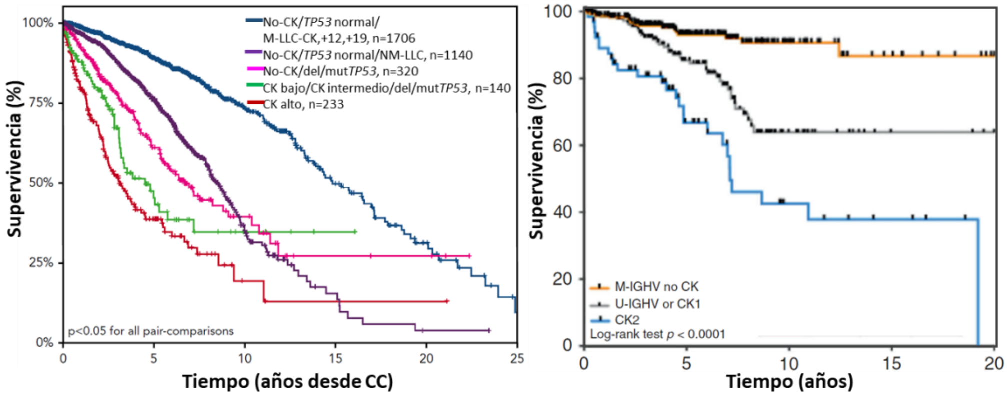
Riesgo	Puntuación	SG a los 5 años
Bajo	0-1	93%
Intermedio	2-3	79%
Alto	4-6	63%
Muy alto	7-10	23%

Adicionalmente, se han propuesto distintos índices en los que se han incluido diferentes parámetros. La **figura 5** recoge algunos ejemplos de estos algoritmos. Por ejemplo, Rossi y colaboradores evidenciaron asociaciones entre determinadas alteraciones citogenéticas y mutaciones génicas de manera que propusieron un nuevo modelo integrado en el que establecieron cuatro grupos pronósticos teniendo en cuenta ambos tipos de anomalías (Rossi *et al.*, 2013). Así, los pacientes podían presentar riesgo alto, en presencia de alteraciones en *TP53* y/o mutaciones en *BIRC3*; riesgo intermedio, con mutaciones en *NOTCH1* y/o *SF3B1* y/o del(11q); riesgo bajo, si tenían +12 o cariotipo normal o bien riesgo muy bajo, si únicamente presentaban del(13q). El *Barcelona-Brno score* estratifica a los pacientes en riesgo alto, intermedio o bajo considerando simplemente el estado mutacional de IGHV y el análisis por FISH de la del(11q) y la del(17p) (Delgado *et al.*, 2017). Baliakas y colaboradores integraron tres categorías de complejidad genómica establecidas según el número de alteraciones detectadas por CC (0-2 alt., 3-4 alt. o ≥5 alt.), la presencia de anomalías en *TP53* y el estado mutacional de IGHV (Baliakas *et al.*, 2019) mientras que el grupo italiano incluyó el estado mutacional de IGHV en combinación con la presencia o ausencia de CK y/o reordenamientos desequilibrados (Visentin *et al.*, 2019).



a) Modelo de Rossi *et al.* (2013)

b) Modelo de Delgado *et al.* (2017)



c) Modelo de Baliakas *et al.* (2019)

d) Modelo de Visentin *et al.* (2019)

Figura 5. Ejemplos de índices pronósticos propuestos para el manejo de los pacientes con LLC (adaptada de Rossi *et al.*, 2013; Delgado *et al.*, 2017; Baliakas *et al.*, 2019; Visentin *et al.*, 2019).

En global, se han desarrollado múltiples índices pronósticos que incluyen otras variables genéticas (aparte de *TP53* e IGHV) para predecir la evolución de los pacientes con LLC. Sin embargo, ninguno de ellos ha sido implementado en la práctica clínica. Es importante destacar que las variables incluidas en estos sistemas han mostrado tener valor pronóstico independiente en estudios llevados a cabo en la era de la quimioinmunoterapia. Por lo tanto, es necesario reevaluar el valor pronóstico y predictivo de los biomarcadores incluidos en estos índices a medida que se vayan aprobando e incorporando nuevos agentes terapéuticos en el manejo de los pacientes con LLC.

1.4. Tratamiento

1.4.1. Criterios de tratamiento

Los pacientes con LLC presentan un curso clínico extremadamente variable. Aproximadamente un tercio de los pacientes no necesitará ser tratado nunca mientras que otros casos requerirán tratamiento a los pocos meses. Por este motivo, a los pacientes con un diagnóstico de LLC que no presentan sintomatología ni signos de progresión debe realizarse un seguimiento que consiste en "observar y esperar" (*watch and wait*), es decir, no deben tratarse ya que no se ha demostrado que un tratamiento precoz, en estadios iniciales, suponga un beneficio para la supervivencia de estos casos (Herling *et al.*, 2020; Muchtar *et al.*, 2021). En cambio, los pacientes que presentan enfermedad activa con síntomas o signos de progresión son candidatos a iniciar tratamiento. Según los criterios establecidos por el iwCLL, para iniciar tratamiento los pacientes deben presentar al menos uno de los siguientes síntomas (Hallek *et al.*, 2018):

- Insuficiencia medular progresiva que se manifiesta con el desarrollo o el empeoramiento de anemia (Hb <10g/dL) y/o trombocitopenia (<100 x 10^9/L)
- Esplenomegalia masiva (>6cm), progresiva o sintomática
- Adenopatías masivas (≥10cm) o de crecimiento progresivo o sintomático
- Tiempo de duplicación linfocitaria <6 meses o incremento progresivo de ≥50% de la linfocitosis en un periodo de dos meses
- Anemia y/o trombocitopenia autoinmunes que no responden a tratamiento con corticoesteroides
- Afectación sintomática o funcional de otros órganos o tejidos como la piel, el riñón o el pulmón
- Síntomas relacionados con la enfermedad (síntomas B): pérdida de peso (>10% en los 6 meses previos), astenia significativa (ECOG ≥2), fiebre >38ºC persistente durante >2 semanas sin evidencia de infección o sudoración nocturna en ausencia de infección (≥1 mes)

1.4.2. Estrategias terapéuticas en LLC

A pesar de que la LLC se considera una enfermedad incurable, actualmente, existen múltiples opciones terapéuticas cuya elección va a depender del estado del paciente, así como de la presencia de factores pronósticos adversos (alteraciones en *TP53*, del(11q), IGHV no mutado) y de la línea de tratamiento.

a) Quimioterapia / quimioinmunoterapia

En el pasado, la combinación de agentes alquilantes (clorambucilo) o análogos de purina (fludarabina, bendamustina, ciclofosfamida) con anticuerpos monoclonales anti-CD20 (rituximab, ofatumumab, obinutuzumab), convirtiendo la quimioterapia en quimioinmunoterapia, supuso una revolución en el tratamiento de la LLC (Hallek *et al.*, 2010; Robak *et al.*, 2010; Abrisqueta *et al.*, 2013; Eichhorst *et al.*, 2016; Fischer *et al.*, 2016; Thompson *et al.*, 2016). En este sentido, el esquema FCR (fludarabina, ciclofosfamida y rituximab) ha sido el tratamiento estándar en LLC durante las últimas décadas. Sin embargo, a pesar de las mejorías observadas con la quimioinmunoterapia, determinados factores como las alteraciones en *TP53, ATM,* IGHV no mutado o los cariotipos complejos, daban lugar a resistencia (Delgado *et al.*, 2020). En los últimos años, con la aparición de los nuevos tratamientos dirigidos, el uso de esquemas de quimioinmunoterapia, como FCR, bendamustina-rituximab o clorambucilo-obinutuzumab, entre otros, es cada vez menos habitual.

b) Nuevos agentes dirigidos

El abordaje terapéutico de la LLC ha evolucionado de manera considerable en la última década con la incorporación de nuevos fármacos cuyo mecanismo de acción difiere de los agentes citotóxicos y anticuerpos tradicionales. Gracias a los múltiples estudios enfocados en la patogénesis de la LLC se ha podido entender mejor la biología de la enfermedad, identificando nuevas dianas terapéuticas implicadas en distintas vías de señalización y con un rol importante en la supervivencia y proliferación de la célula tumoral (**Figura 6**). Las principales vías de señalización inhibidas por los nuevos fármacos son la vía del receptor de célula B (BCR) (ibrutinib, acalabrutinib), esencial para la proliferación y supervivencia así como para la migración de las células LLC, y la vía de *B-cell lymphoma 2* (BCL2), una proteína anti-apoptótica que contribuye a la supervivencia de la célula LLC (venetoclax). Los estudios iniciales han probado que ambos tipos de fármacos son eficaces incluso en los pacientes de alto riesgo (con del(17p)/mutación *TP53)*, tradicionalmente relacionados con refractariedad y resistencia al tratamiento.

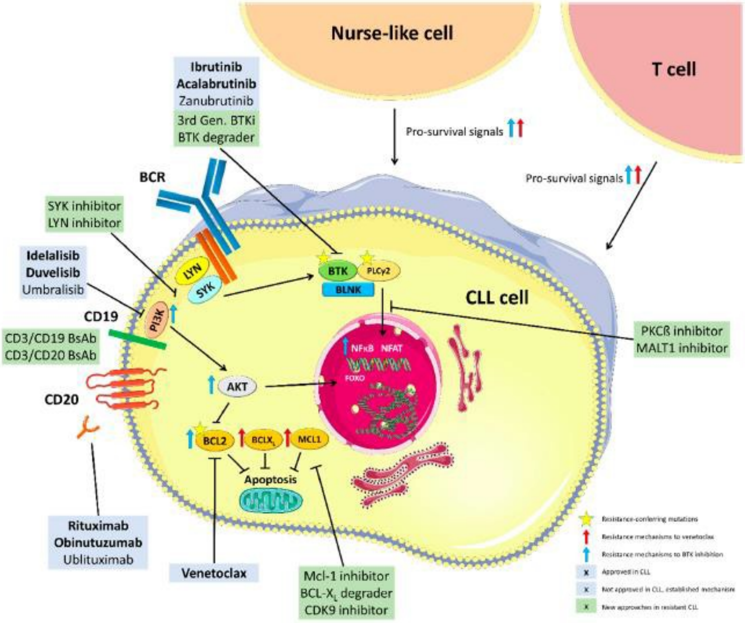

Nurse-like cell
T cell
Ibrutinib
Acalabrutinib
Zanubrutinib
3rd Gen. BTKi
BTK degrader
Pro-survival signals
Pro-survival signals
BCR
SYK inhibitor
LYN inhibitor
Idelalisib
Duvelisib
Umbralisib
CD19
CD3/CD19 BsAb
CD3/CD20 BsAb
CD20
LYN
SYK
PI3K
BTK
PLCγ2
BLNK
CLL cell
PKCß inhibitor
MALT1 inhibitor
NFκB
NFAT
FOXO
AKT
BCL2
BCLX$_L$
MCL1
Apoptosis
Rituximab
Obinutuzumab
Ublituximab
Venetoclax
Mcl-1 inhibitor
BCL-X$_L$ degrader
CDK9 inhibitor
Resistance-conferring mutations
Resistance mechanisms to venetoclax
Resistance mechanisms to BTK inhibition
Approved in CLL
Not approved in CLL, established mechanism
New approaches in resistant CLL

Figura 6. Esquema de las diferentes opciones terapéuticas con fármacos dirigidos aprobados y en estudio para el tratamiento de la LLC (Fürstenau and Eichhorst, 2021).

- **Inhibidores de la vía del BCR – inhibidores de BTK**

Ibrutinib fue la primera molécula diseñada como un inhibidor covalente irreversible de BTK, impidiendo la supervivencia y la migración de las células LLC (Woyach *et al.*, 2014). A pesar de haber demostrado ser una terapia efectiva para los pacientes con LLC, en ocasiones se desarrollan resistencias al fármaco debidas a la aparición de mutaciones en el sitio de unión de ibrutinib a BTK (cisteína 481) o en el gen que codifica para la *phospholipase C gamma 2* (PLCγ2), otra molécula que actúa posteriormente en la vía de señalización del BCR (Walliser *et al.*, 2016;

Woyach *et al.*, 2018; Shanafelt *et al.*, 2019; Abrisqueta *et al.*, 2021). Actualmente, en España está aprobado como opción terapéutica tanto en primera línea como en pacientes R/R durante un tiempo ilimitado aunque debido a la elevada toxicidad cardiovascular asociada a su uso se están priorizando otras opciones (Archibald *et al.*, 2021; Pellegrini *et al.*, 2021).

Acalabrutinib es un inhibidor de BTK de segunda generación que también se une de manera irreversible al mismo sitio de unión que ibrutinib. Esta molécula presenta una selectividad mayor para BTK, lo que conlleva un mejor perfil de toxicidades que ibrutinib (Awan *et al.*, 2019; Woyach *et al.*, 2020; Byrd *et al.*, 2021; Ghia *et al.*, 2022; Sharman *et al.*, 2022). Actualmente, y dado el beneficio económico que supone ofrecer acalabrutinib en vez de ibrutinib, es una opción terapéutica disponible para todos los pacientes en primera línea y en R/R.

- **Inhibidores de la vía de BCL2**

Venetoclax es una pequeña molécula mimética del dominio BH3 presente en BCL2 que, al unirse a dicho dominio, inhibe su función permitiendo la liberación de las proteínas pro-apoptóticas (Souers *et al.*, 2013). Debido a los resultados obtenidos en diversos estudios, en España está aprobado en combinación con obinutuzumab como tratamiento de fija duración en primera línea y en combinación con rituximab (VR) para todos los pacientes en R/R con independencia del estado de *TP53* (Seymour *et al.*, 2018; Fischer *et al.*, 2019; Kater *et al.*, 2019; Al-Sawaf *et al.*, 2020). La resistencia a venetoclax es debida al desarrollo de mutaciones que reducen la afinidad del fármaco por BCL2, siendo la más común la Gly101Val, en el dominio de unión de BCL2 (Blombery *et al.*, 2020).

- **Inhibidores de la vía del BCR – inhibidores de PI3K**

Idelalisib es un inhibidor irreversible de la subunidad δ de la porción catalítica de la *phosphatidylinositol 3-kinase* (PI3K). Debido a la alta toxicidad asociada al fármaco, aumentando el riesgo a padecer infecciones oportunistas, actualmente solo está indicado en primera línea como última alternativa para aquellos pacientes que no puedan seguir otros tratamientos con inhibidor de BTK o BCL2 o en pacientes R/R tras haber recibido ibrutinib o venetoclax (Furman *et al.*, 2014; O'Brien *et al.*, 2015; Jones *et al.*, 2017).

Todos estos hallazgos han cambiado el paradigma de tratamiento de la LLC en España, tal y como queda reflejado en las últimas guías del Grupo Español de LLC (**Figura 7**). No obstante, es un ámbito en continua evolución. En este sentido, se están llevando a cabo múltiples ensayos clínicos para estudiar la eficacia de nuevos agentes, como zanubrutinib, pirtobrutinib, tirabrutinib o nemtabrutinib, tanto en monoterapia como en combinación, e incluso nuevas combinaciones de fármacos ya aprobados, como ibrutinib con venetoclax, y combinaciones de éstos con nuevos anticuerpos monoclonales para intentar conseguir respuestas más duraderas con una terapia de tiempo limitado (Hillmen *et al.*, 2019; Jain *et al.*, 2021a; Jain *et al.*, 2021b; Niemann *et al.*, 2021; Wierda *et al.*, 2021; Hillmen *et al.*, 2022; Muhowski *et al.*, 2022; Naeem *et al.*, 2022; Tam *et al.*, 2022).

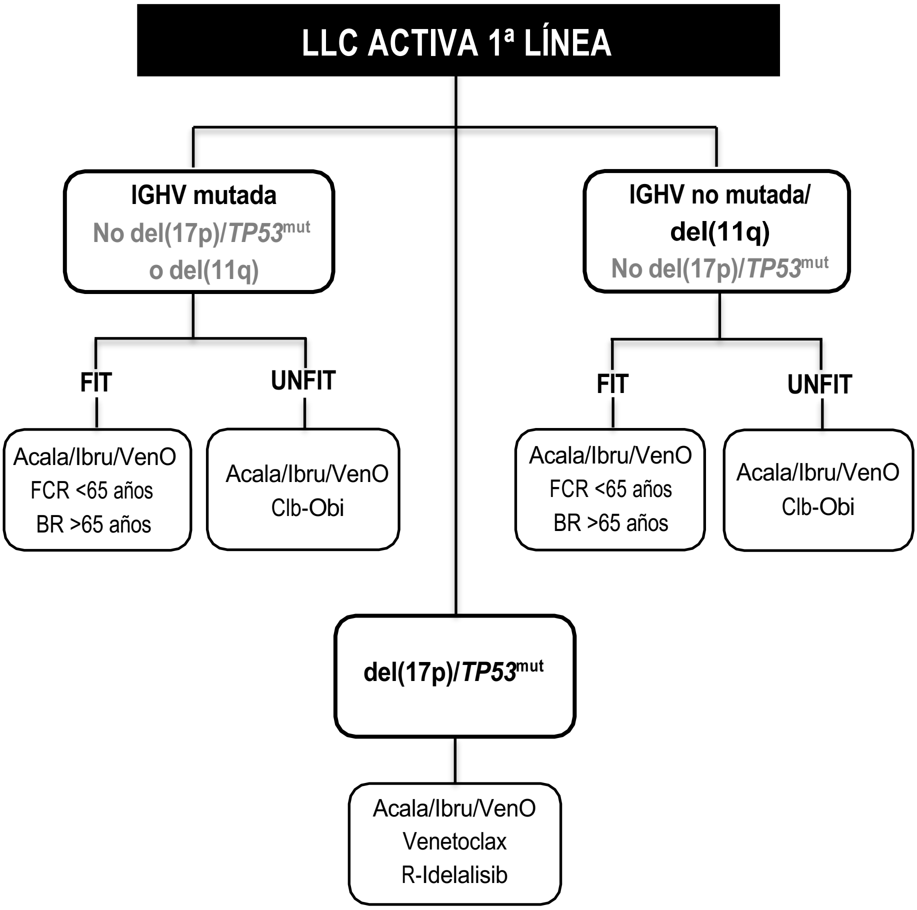

LLC ACTIVA 1ª LÍNEA
IGHV mutada
No del(17p)/TP53mut
o del(11q)
IGHV no mutada/
del(11q)
No del(17p)/TP53mut
FIT
UNFIT
FIT
UNFIT
Acala/Ibru/VenO
FCR <65 años
BR >65 años
Acala/Ibru/VenO
Clb-Obi
Acala/Ibru/VenO
FCR <65 años
BR >65 años
Acala/Ibru/VenO
Clb-Obi
del(17p)/TP53mut
Acala/Ibru/VenO
Venetoclax
R-Idelalisib

Figura 7. Algoritmo de tratamiento de la LLC en primera línea establecido por el Grupo Español de Leucemia Linfocítica Crónica. Acala: acalabrutinib; Ibru: ibrutinib; VenO: venetoclax + obinutuzumab; Clb-Obi: clorambucilo + obinutuzumab; FCR: fludarabina + ciclofosfamida + rituximab; BR: bendamustina + rituximab.

▪ Trasplante alogénico de progenitores hematopoyéticos

Debido a que la mayoría de pacientes con LLC son de edad avanzada y dada la elevada morbimortalidad que supone, el trasplante de progenitores hematopoyéticos se considera una opción terapéutica en tan solo una minoría de pacientes. En concreto, aquellos pacientes jóvenes, sin comorbilidades, con una LLC agresiva por presencia de alteraciones en *TP53* o por no responder tras varias líneas de tratamiento con inhibidores de BTK y BCL2 pueden recibir un trasplante alogénico en caso de que exista un donante histocompatible. En algunos casos incluso se podría plantear la opción de llevar a cabo terapia celular con linfocitos T quiméricos (CAR-T) en el contexto de un ensayo clínico (Kharfan-Dabaja *et al.*, 2016; Krämer *et al.*, 2017; Dreger *et al.*, 2018; Geyer *et al.*, 2019; Roeker *et al.*, 2020; Siddiqi *et al.*, 2022).

2. Técnicas de análisis citogenómico aplicadas en la LLC

2.1. Citogenética convencional

La citogenética convencional (CC) fue desarrollada a finales de los años cincuenta y consiste en la tinción de los cromosomas para el estudio de su estructura. Gracias a la posibilidad que ofrece de visualizar todo el genoma, pudiendo así identificar tanto alteraciones numéricas como estructurales, sean equilibradas o desequilibradas, actualmente sigue siendo la técnica de elección para la mayoría de estudios citogenéticos en neoplasias hematológicas.

Para poder visualizar un cromosoma en un microscopio óptico es necesario que la célula se encuentre en metafase, ya que ésta es la fase del ciclo celular en la cual el material genético adquiere la condensación adecuada. Por este motivo, el principal paso del protocolo de la CC consiste en cultivar la muestra con un mitógeno específico para estimular la división de las células de interés y así obtener metafases valorables. Tradicionalmente, los linfocitos B se cultivaban con el mitógeno 12-O-tetradecanoilforbol-13-acetato (TPA). No obstante, se ha demostrado que en la LLC esta aproximación está limitada por la baja tasa de división de las células tumorales *in vitro*. Los primeros estudios realizados en esta entidad demostraron que los mitógenos utilizados no eran muy efectivos y permitían detectar clones alterados en tan solo un 30-40% de los casos (Juliusson *et al.*, 1990; Oscier *et al.*, 2002; Puiggros *et al.*, 2017). Posteriormente, en los años 2010, se incorporaron nuevos mitógenos, como la combinación de oligonucleótidos CpG (DSP30) con interleucina 2 (IL-2), que mejoraron considerablemente el rendimiento de la técnica, incrementando la tasa de casos alterados hasta en un 60-80% (Dicker *et al.*, 2006; Haferlach *et al.*, 2007; Put *et al.*, 2009; Heerema *et al.*, 2010). De hecho, las guías actuales del ERIC recomiendan realizar dos cultivos en paralelo utilizando dos mitógenos diferentes (TPA y DSP30+IL-2) para cada paciente, con el fin de detectar una mayor proporción de casos con cariotipos alterados (Baliakas *et al.*, 2022). Una vez realizado el cultivo, las células son expuestas a colcemid, que impide la formación del huso mitótico durante la división celular provocando la inmovilización de la metafase, y a una solución hipotónica que aumenta el volumen del núcleo. A continuación, las células se fijan con Carnoy (3 partes de metanol/1 parte de ácido acético) para eliminar la membrana citoplasmática y el citoplasma y facilitar la observación de los cromosomas. Tras la realización de una extensión, la identificación de éstos se realizará teniendo en cuenta su tamaño y el patrón de bandas que presenten. Este patrón característico de cada cromosoma se obtiene cuando son teñidos con un colorante, usualmente Wright o Giemsa, que permite diferenciar unas bandas más oscuras, correspondientes a regiones enriquecidas en Adenina (A) - Timina (T), de otras más claras, ricas en regiones Guanina (G) – Citosina (C). El análisis del cariotipo se lleva a cabo visualmente. Así, la detección de cambios en los patrones de bandas refleja la presencia de alteraciones (Espinet *et al.*, 2021). El cariotipo, en el cual se identifican los 22 pares de cromosomas autosómicos y los cromosomas sexuales, es finalmente descrito siguiendo el Sistema Internacional para la Nomenclatura Citogenómica Humana (**Figura 8**) (McGowan-Jordan *et al.*, 2020).

Aparte de la necesidad de llevar a cabo un cultivo, la principal limitación de la CC es su baja resolución, establecida en 10Mb. Esto dificulta la identificación de alteraciones pequeñas, como las deleciones típicas en LLC en la región 13q14 que a menudo por su tamaño no pueden detectarse, o de translocaciones crípticas que impliquen los extremos de los cromosomas.

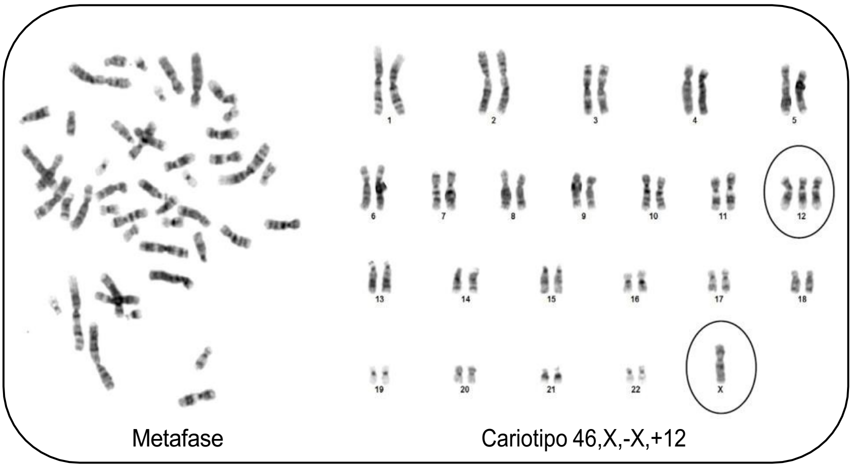


Figura 8. Imagen de una metafase de un paciente con LLC con su correspondiente cariotipo. Corresponde a un individuo de sexo femenino con trisomía del cromosoma 12 y pérdida de un cromosoma X.

En cuanto a sus ventajas, aparte de permitir la identificación de alteraciones en todo el genoma, también permite definir la arquitectura clonal, es decir, describir las alteraciones presentes en clones individuales. Esto es relevante puesto que permite identificar la presencia de evolución clonal en un paciente, que consiste en la adquisición de nuevas alteraciones genómicas durante el curso de la enfermedad (López *et al.*, 2013; Bracher *et al.*, 2022).

2.2. Hibridación *in situ* fluorescente (FISH)

La hibridación *in situ* fluorescente (FISH) es una técnica que consiste en la localización de secuencias de ADN específicas en los cromosomas mediante el marcaje de dichas regiones con sondas fluorescentes (pequeña secuencia de ADN purificado de cadena sencilla unida a un fluoróforo) complementarias a la región de interés (Pardue and Gall, 1969; Bauman *et al.*, 1980). Aunque fue desarrollada en los años sesenta, no fue hasta mediados de los noventa cuando se implementó en los laboratorios diagnósticos para la identificación de alteraciones cromosómicas en LLC.

El procedimiento de la FISH implica la desnaturalización, es decir, la separación de las cadenas de la doble hélice del ADN, tanto del ADN de la muestra que queremos analizar como de la sonda marcada, sometiéndolo a elevadas temperaturas (73-90ºC). A continuación, se produce la hibridación, en la cual la sonda se une a la secuencia de ADN de interés por complementariedad de bases. Tras una serie de lavados para eliminar el exceso de sonda que no se ha unido con el ADN de la muestra y la contratinción de todo el ADN con el fluorocromo 4',6-diamidino-2-fenilindol (DAPI), el resultado de la hibridación se visualiza mediante un microscopio de fluorescencia.

La FISH permite detectar tanto alteraciones cromosómicas numéricas como estructurales y puede realizarse en cromosomas en metafase y en núcleos en interfase. Esto hace que no sea necesario cultivar las células. Además, tiene una mayor sensibilidad que la CC, pudiendo detectar alteraciones presentes en un 5% de la población tumoral, aunque el punto de corte exacto para considerar un caso como portador de la alteración debe establecerse en cada laboratorio. Por el contrario, la principal limitación de esta técnica es que solo permite el estudio de aquellas regiones concretas que son complementarias a las sondas que se están utilizando. Como consecuencia, el análisis por FISH subestima la verdadera complejidad genómica de un caso.

En la LLC, este método permitió aumentar la tasa de detección de alteraciones cromosómicas en >80% de los pacientes. Por este motivo, fue implementado como técnica de elección para la estratificación del riesgo citogenético en pacientes con LLC (Döhner *et al.*, 2000). De hecho, actualmente, es la única técnica citogenética que se considera obligatoria para poder orientar el pronóstico del paciente antes de que inicie tratamiento (Hallek *et al.*, 2018).

2.2.1. Tipos de sondas

Existen diferentes tipos de sondas que permiten la visualización de distintos segmentos del genoma (**Figuras 9-13**). Es importante escoger bien el tipo de sonda a utilizar según la alteración que se quiera identificar.

- **Sondas de *locus* específico o secuencia única**: diseñadas para localizar una región genómica concreta, ya sea un gen o una banda cromosómica. Permiten la detección de alteraciones numéricas y estructurales (deleciones o duplicaciones).

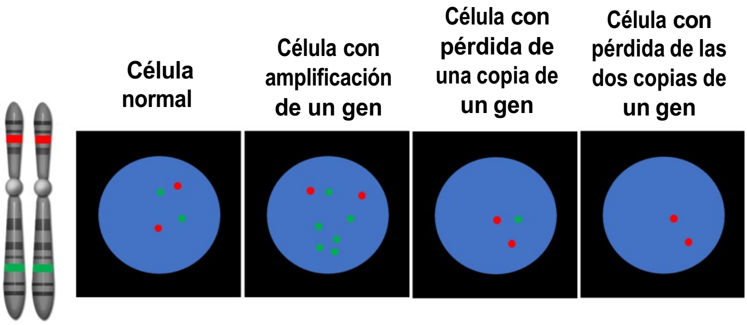

Célula normal
Célula con amplificación de un gen
Célula con pérdida de una copia de un gen
Célula con pérdida de las dos copias de un gen

Figura 9. Patrones de hibridación clásicos obtenidos utilizando sondas de FISH de *locus* específico.

- **Sondas de rotura o *break apart* (BA)**: se utilizan para detectar reordenamientos cromosómicos. Consisten en dos sondas de *locus* específico marcadas con dos fluorocromos distintos, generalmente rojo y verde, que flanquean en sus extremos 5' y 3' un gen de interés implicado en un reordenamiento. En una situación normal, se verá una señal de fusión de tonalidad amarilla equivalente a la localización cercana de las dos sondas, roja y verde, correspondiente a cada cromosoma. Cuando la muestra de estudio presenta el reordenamiento, las sondas de una de estas señales se verán separadas. Pueden emplearse cuando solo se conoce uno de los dos genes involucrados en el reordenamiento.

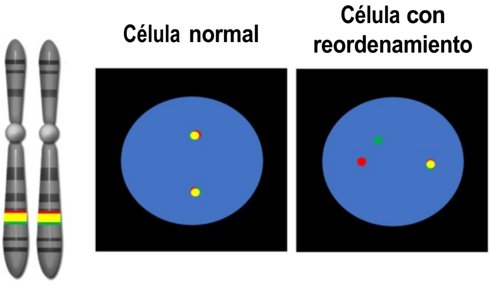

Célula normal
Célula con reordenamiento

Figura 10. Patrones de hibridación clásicos obtenidos utilizando sondas de FISH de *break apart*.

- **Sondas de doble fusión o *dual fusion* (DF)**: también son útiles para detectar reordenamientos cromosómicos. En este caso los dos genes implicados en la translocación son conocidos, de manera que se marcan con dos fluorocromos distintos. Cuando la muestra presenta el reordenamiento, se observa un patrón de dos fusiones por la unión de las dos señales que se han dividido como consecuencia del mismo. Además, se observarán dos señales normales correspondientes a los cromosomas no reordenados.

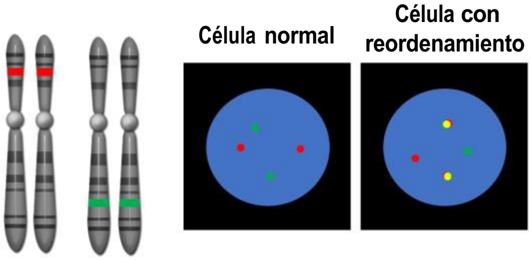

Célula normal
Célula con reordenamiento

Figura 11. Patrones de hibridación clásicos obtenidos utilizando sondas de FISH de *dual fusion*.

- **Sondas centroméricas**: son complementarias a las secuencias repetitivas de ADN características de la región centromérica o pericentromérica de un cromosoma. Estas sondas son útiles para detectar aneuploidías (ganancia o pérdida de un cromosoma).

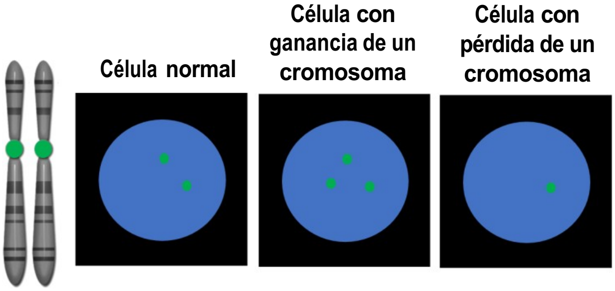

Célula normal
Célula con ganancia de un cromosoma
Célula con pérdida de un cromosoma

Figura 12. Patrones de hibridación clásicos obtenidos utilizando sondas de FISH centroméricas.

- **Sondas de pintado cromosómico**: están constituidas por un conjunto de sondas de *locus* específico que hibridan con un cromosoma entero. De esta manera, se puede marcar todo un cromosoma. Son especialmente útiles para detectar aneuploidías, identificar a los cromosomas implicados en grandes reordenamientos e incluso caracterizar cromosomas marcadores, pero no para detectar alteraciones pequeñas, por debajo de la resolución de la CC, como deleciones que afecten a una pequeña región. Para su correcta interpretación, el análisis de la hibridación debe realizarse sobre metafases.

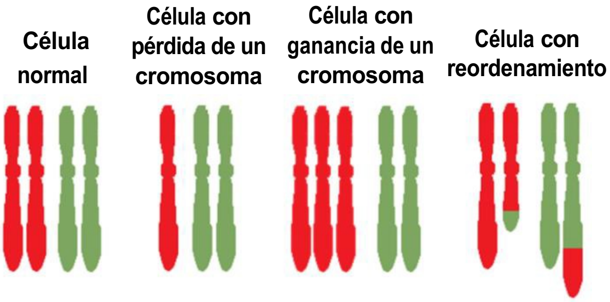

Célula normal
Célula con pérdida de un cromosoma
Célula con ganancia de un cromosoma
Célula con reordenamiento

Figura 13. Patrones de hibridación clásicos obtenidos utilizando sondas de FISH de pintado cromosómico.

2.3. *Microarrays* genómicos

Las plataformas de *microarrays* (*mikro*: del griego, pequeño y *array*: del inglés, distribución ordenada) consisten en un conjunto de biomoléculas ordenadas ortogonalmente en filas y columnas fijadas sobre un soporte sólido miniaturizado. Existen distintos tipos de plataformas de *microarrays* según el tipo de biomolécula presente en el soporte*:* los genómicos (ADN), de expresión (ADN copia), de metilación, de tejido, de células o de proteínas.

Los *microarrays* genómicos (MG) están constituidos por una colección de sondas de ADN. Mediante la hibridación de la muestra a analizar con las sondas fijadas en estas plataformas se pueden detectar alteraciones en el número de copia (CNA), es decir, ganancias y pérdidas de material genético a lo largo de todo el genoma y a una resolución alta. Existen dos tipos de MG:

- ***Microarrays* de CGH o hibridación genómica comparada** (*Comparative Genome Hybridization*): en este tipo de *microarrays* se utiliza un ADN tumoral y un ADN control. Ambos ADN se marcan con distinto fluoróforo y se hibridan conjuntamente (hibridación competitiva) sobre un mismo soporte sólido con millones de sondas de ADN que cubren todo el genoma (Pinkel *et al.*, 1998). La imagen obtenida corresponde a la lectura de los dos fluorocromos. Mediante la comparativa de la intensidad relativa de fluorescencia de ambas muestras se puede extrapolar el número de copias del genoma de la siguiente manera (**Figura 14**):
    - Mayor intensidad de fluorescencia de la muestra de interés indica ganancia de material de esa región.
    - Mayor intensidad de fluorescencia de la muestra control indica pérdida de material en la muestra de estudio.
    - Señal con color neutro (amarillo, por la mezcla de los fluoróforos rojo y verde), indica que no hay diferencias en el número de copia entre el ADN tumoral y el ADN control.
- ***Microarrays* de SNP o polimorfismos de un solo nucleótido** *(Single Nucleotide Polymorphism):* este tipo de plataformas también contienen sondas de oligonucleótidos correspondientes

a variantes alélicas de SNPs (variaciones de la secuencia de ADN que afectan a una sola base) seleccionados. De esta manera, además de poder detectar CNA, estos *microarrays* también permiten obtener el genotipado de la muestra. A diferencia de los *microarrays*-CGH, aquí la muestra problema se hibrida directamente sobre las sondas del *microarray*, sin necesidad de incluir una muestra control (Mei *et al.*, 2000). En caso de que se utilice un control, ambas muestras se hibridarían en *microarrays* independientes (**Figura 14**). Otro rasgo característico de este tipo de *microarrays* es que permiten identificar regiones con pérdida de heterocigosidad (LOH) sin cambio en el número de copia (CN-LOH, del inglés *copy number neutral loss of heterozigosity*). La detección de CN-LOH es relevante, sobre todo si afecta a un gen supresor de tumores, puesto que puede ser indicativa de la presencia de mutaciones puntuales en el gen que pasarían a ser bialélicas por la presencia de una CN-LOH, adquiriendo un estado homocigoto que puede favorecer el desarrollo tumoral.

Una ventaja importante de esta metodología con respecto a la CC es la no necesidad de obtener células en división. Tanto si llevamos a cabo un *microarray* de CGH como de SNP, la muestra de partida es ADN extraído, de manera que no es necesario cultivar los linfocitos. Además, la resolución es mayor, lo cual permite identificar alteraciones de hasta 50Kb. Sin embargo, también tiene ciertas limitaciones, como la incapacidad para detectar reordenamientos equilibrados o definir distintos clones y la sensibilidad limitada, dificultando la identificación de alteraciones presentes en alrededor del 20% de las células tumorales.

La aplicación de los MG en la rutina diagnóstica es restringida en centros españoles, aunque sí han reemplazado a las técnicas clásicas en otros países europeos. En cuanto a su análisis, en 2016 se publicaron unas recomendaciones europeas que recogen los criterios mínimos de interpretación clínica y redacción de informes de MG en el estudio de LLC y otras neoplasias hematológicas (Schoumans *et al.*, 2016). Según estas guías, debemos tener en cuenta aquellas CNA no asociadas a la enfermedad con un tamaño ≥5Mb y aquellas alteraciones clínicamente relevantes en la LLC (del(11q), del(13q), del(17p) y +12) independientemente de su tamaño.

De manera global, el grado de concordancia que se ha observado entre las alteraciones detectadas por MG y FISH en LLC se encuentra entre el 79-98% (Gunnarson *et al.*, 2010; Ouillette *et al.*, 2011a; Tang *et al.*, 2021). Además, el estudio de MG ha permitido caracterizar mejor las alteraciones incluidas en el panel de FISH de LLC, así como describir otras alteraciones recurrentes en LLC no incluidas en el panel estándar de FISH (Edelmann *et al.*, 2012; Urbankova *et al.*, 2014). Los MG también permiten detectar complejidad genómica. Globalmente, los resultados descritos en la literatura han demostrado que las plataformas de *microarrays* son útiles para el estudio genómico de la LLC y para la identificación de nuevas alteraciones que podrían ser crípticas por las técnicas convencionales (CC y FISH).

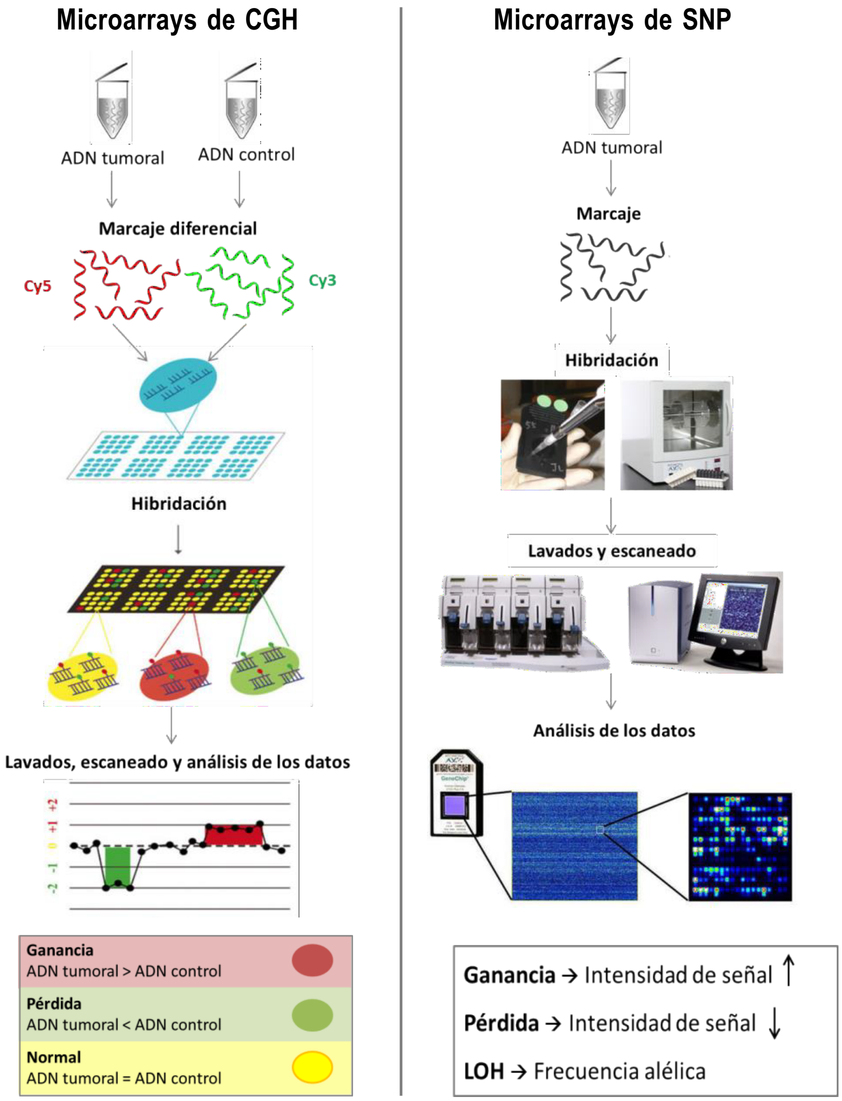

Microarrays de CGH
ADN tumoral
ADN control
Marcaje diferencial
Cy5
Cy3
Hibridación
Lavados, escaneado y análisis de los datos
-2 -1 0 +1 +2
Ganancia
ADN tumoral > ADN control
Pérdida
ADN tumoral < ADN control
Normal
ADN tumoral = ADN control
Microarrays de SNP
ADN tumoral
Marcaje
Hibridación
Lavados y escaneado
Análisis de los datos
Ganancia → Intensidad de señal ↑
Pérdida → Intensidad de señal ↓
LOH → Frecuencia alélica

Figura 14. Esquema comparativo de las técnicas de *microarrays* de hibridación genómica comparada (CGH) y *microarrays* de polimorfismos de un solo nucleótido (SNP).

2.4. Mapeo óptico del genoma

El mapeo óptico del genoma (OGM, del inglés *optical genome mapping*) es una nueva tecnología que ha surgido con el objetivo de poder realizar un estudio citogenómico a nivel de todo el genoma, superando las limitaciones de las técnicas convencionales. El OGM permite identificar con una elevada resolución y en un solo test una gran variedad de alteraciones cromosómicas, tanto numéricas como estructurales (translocaciones, inversiones, deleciones, duplicaciones e inserciones).

Técnicamente, el OGM consiste en la obtención de moléculas de ADN de elevado peso molecular (UHMW, del inglés *ultra-high molecular weight*) extraídas de muestras frescas de sangre periférica, aspirados de médula ósea, líneas celulares o tejido fresco/congelado. La longitud de las moléculas de ADN (>250 Kb) supone la principal diferencia con respecto a las actuales técnicas de secuenciación o de *microarrays*, en las que las moléculas resultantes alcanzan un tamaño máximo de aproximadamente 500 pb. Una vez extraídas, las moléculas de ADN son marcadas enzimáticamente con un fluoróforo en unas secuencias específicas de 6 pb (CTTAAG) repetidas unas 15 veces aproximadamente en 100 Kb. Estas moléculas de ADN largas y marcadas se cargan en un chip que se coloca en el instrumento Saphyr, dentro del cual se hacen pasar por unos nanocanales que las alinean, permitiendo así la obtención de imágenes de cada molécula de manera individual. Gracias al patrón único generado en el marcaje, el *software* puede reconocer inequívocamente la ubicación de cada molécula en el genoma. De manera análoga al estudio del cariotipo, mediante el análisis del patrón de marcas y la comparación con un genoma de referencia se pueden identificar distintos tipos de variantes numéricas y estructurales (**Figura 15**).

En cuanto a las ventajas de esta metodología, uno de sus puntos fuertes es su alta resolución, debida en parte a la elevada densidad de marcas que contienen los fragmentos, lo cual permite la identificación de alteraciones pequeñas de hasta 500 pb. Por otro lado, no es necesario realizar un cultivo celular y, además, es capaz de detectar alteraciones presentes en bajas frecuencias alélicas (hasta 5%). Asimismo, el análisis se lleva a cabo con un *software* fácil de usar e intuitivo, por lo que no es necesario disponer de herramientas bioinformáticas complejas para obtener e interpretar los resultados.

Si bien el OGM ya es considerado como una técnica prometedora, con una gran probabilidad de sustituir a las convencionales, es cierto que aún existen limitaciones técnicas que necesitan mejorar para poder ser implementado en la práctica rutinaria. Una de ellas es la incapacidad de detectar alteraciones en regiones con ADN altamente repetitivo como los centrómeros o telómeros. Además, el OGM no permite detectar la ploidía de la muestra ni definir la arquitectura clonal.

Recientemente, se han publicado diversos estudios utilizando cohortes de pacientes con distintas neoplasias hematológicas, como la leucemia mieloide aguda, los síndromes mielodisplásicos o la LLC, en los cuales se ha demostrado la capacidad de esta metodología para reemplazar las técnicas citogenómicas tradicionales (CC, FISH y MG). En estos primeros trabajos el OGM identificó una alta proporción (90-100%) de las alteraciones que habían sido detectadas previamente con las técnicas de rutina (Neveling *et al.*, 2021; Gerding *et al.*, 2022; Levy *et al.*, 2022; Puiggros *et al.*, 2022; Rack *et al.*, 2022; Yang *et al.*, 2022). Además, también reveló la presencia de anomalías adicionales no detectadas con los métodos rutinarios por ser crípticas o por no haber sido identificadas previamente en esas entidades y cuyo potencial diagnóstico, pronóstico y predictivo debe seguir explorándose. Por otro lado, se ha visto que la aplicación del OGM ayuda a describir con mayor precisión las alteraciones previamente identificadas por las técnicas convencionales.

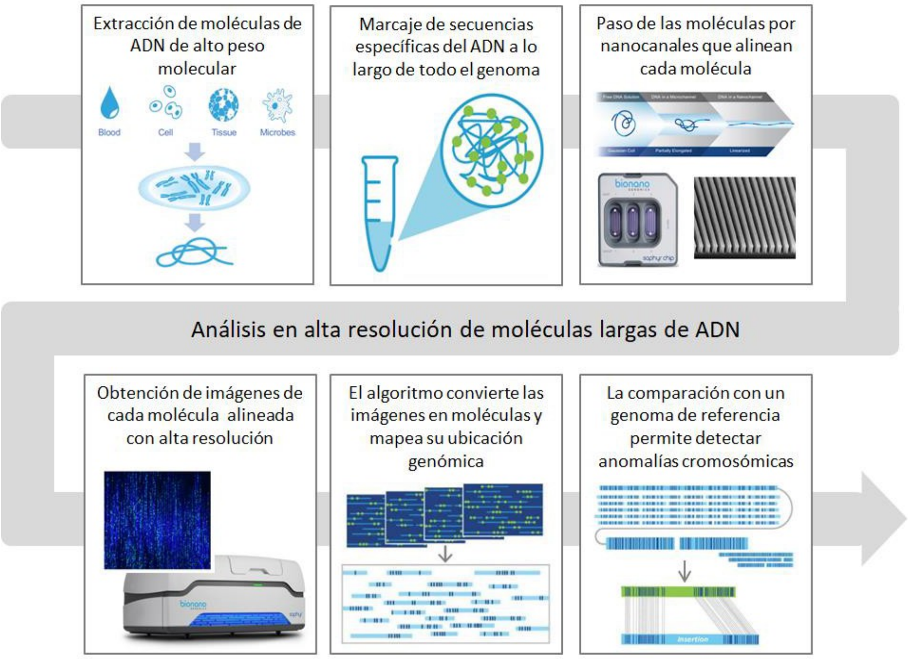
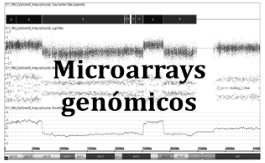

Extracción de moléculas de ADN de alto peso molecular
Blood
Cell
Tissue
Microbes
Marcaje de secuencias específicas del ADN a lo largo de todo el genoma
Paso de las moléculas por nanocanales que alinean cada molécula
Free DNA Solution
DNA in a Microchannel
DNA in a Nanochannel
Gaussian Coil
Partially Elongated
Linearized
bionano
saphyr chip
Análisis en alta resolución de moléculas largas de ADN
Obtención de imágenes de cada molécula alineada con alta resolución
bionano
saphyr
El algoritmo convierte las imágenes en moléculas y mapea su ubicación genómica
La comparación con un genoma de referencia permite detectar anomalías cromosómicas
Insertion

Figura 15. Flujo de trabajo con el instrumento Saphyr para visualizar y analizar moléculas largas de ADN, de alto peso molecular mediante la tecnología de mapeo óptico del genoma.

En la **tabla 4** se resumen las ventajas y las limitaciones de las técnicas citogenómicas disponibles actualmente para el estudio de la LLC.

Tabla 4. Resumen de las ventajas y limitaciones de las técnicas citogenómicas disponibles actualmente para el estudio citogenómico de la LLC.

✓ VENTAJAS	TÉCNICA	✗ LIMITACIONES
✓ Visualización de todo el genoma ✓ Técnicamente sencilla y barata ✓ Detección de alteraciones equilibradas ✓ Identificación de clones individuales	**Citogenética convencional**	× Baja resolución (>10Mb) × Necesidad de muestra fresca para cultivar × Depende de la actividad mitótica *in vitro* de la célula tumoral × Sensibilidad limitada (10%)
✓ Se puede realizar sobre núcleos en interfase ✓ No necesidad de células en división ✓ Sensibilidad de 1-5% ✓ Detecta alteraciones pequeñas (100-200 Kb) ✓ Tasa de detección de alteraciones ~80%	**FISH**	× Información limitada a regiones concretas × Estudio de una o pocas alteraciones a la vez × Subestima la complejidad real
✓ Análisis de todo el genoma ✓ No necesidad de células en división ✓ Alta resolución (50-300 Kb) ✓ Los arrays de SNPs permiten genotipar (LOH)	 **Microarrays genómicos**	× Sensibilidad limitada (~20%) × No detección de alteraciones equilibradas × No distinción de clones individuales × Técnicamente más cara
✓ Análisis de todo el genoma ✓ Detección de alteraciones numéricas y todo tipo de variantes estructurales en un solo test ✓ Alta resolución (>500 pb) ✓ Sensibilidad de 1-5% para variantes estructurales	**Mapeo óptico del genoma**	× Requiere ADN ultra largo × No permite genotipar (LOH) × No distinción de clones individuales × Técnicamente laboriosa y más costosa × Sensibilidad limitada para CNA (15-20%)

3. Alteraciones citogenéticas de la LLC

3.1. Alteraciones recurrentes con valor pronóstico conocido en LLC

Tal y como se ha comentado anteriormente, actualmente existen cuatro alteraciones citogenéticas recurrentes en LLC que conforman el modelo jerárquico de Döhner y cuyo impacto clínico se ha validado en múltiples estudios. En este apartado se detallaran las características de estas alteraciones así como su rol en la evolución clínica de los pacientes.

3.1.1. Deleción 13q14

La del(13q) es la alteración citogenética más frecuente en la LLC, detectándose en un 50-60% de los casos al diagnóstico (Döhner *et al.*, 2000). A menudo se encuentra como única alteración; por este motivo se considera un evento temprano en la patogénesis de la enfermedad, y puede presentarse como deleción monoalélica o bialélica. Aunque su tamaño sea heterogéneo entre los pacientes, todos presentan una región mínima delecionada que contiene los genes *deleted in lymphocytic leukemia-1* y *deleted in lymphocytic leukemia*-2 (*DLEU1* y *DLEU2*, respectivamente) así como los microRNAs *MIR15A* y *MIR16-1*. El rol de estos microRNAs en el desarrollo de la LLC parece estar relacionado con la modulación de la expresión de genes implicados en el ciclo celular y la apoptosis, como el gen anti-apoptótico *BCL2*, lo cual concuerda con la elevada expresión de *BCL2* observada en la LLC (Cimmino *et al.*, 2005; Klein *et al.*, 2010). Esta anomalía confiere muy buen pronóstico si se encuentra de manera aislada; los pacientes portadores de del(13q) pueden tener incluso una supervivencia mayor que los pacientes con cariotipo normal (Döhner *et al.*, 2000; Rossi *et al.*, 2013). Sin embargo, se ha visto que el impacto clínico asociado a la del(13q) es heterogéneo y puede estar modulado en función de las características de la deleción, aunque aún se desconoce la razón exacta de esta heterogeneidad (Durak Aras *et al.*, 2021; Khalid *et al.*, 2021). En este sentido, se han descrito dos tipos de deleciones según el tamaño que ésta presente. Las del(13q) de tipo I son las deleciones cortas y se caracterizan por no incluir el gen del retinoblastoma 1 (*RB1*) mientras que las deleciones de tipo II, o deleciones grandes, son aquellas que sí incluyen *RB1*. La relevancia de identificar estos dos tipos radica en el hecho de que las deleciones de tipo II se han asociado con una peor evolución clínica (Ouillette *et al.*, 2011b; Parker *et al.*, 2011; Mian *et al.*, 2012). Por otro lado, algunos autores han sugerido que tanto el tamaño del clon celular como el tipo de deleción (monoalélica o bialélica) pueden modular el pronóstico asociado a esta anomalía. Se ha demostrado que el porcentaje de núcleos afectados con estas alteraciones tiene implicación en el pronóstico, siendo éste peor en aquellos casos con un porcentaje elevado de células con la deleción (Hernández *et al.*, 2009; Van Dyke *et al.*, 2010; Dal Bo *et al.*, 2011; Rodríguez *et al.*, 2012). Asimismo, existe cierta controversia en relación al efecto del tipo de deleción en el curso clínico. En algunos estudios la presencia de una deleción bialélica se ha relacionado con una evolución clínica más agresiva y TPT más corto, mientras que en otros trabajos no se han encontrado diferencias significativas entre los casos con deleción bialélica y monoalélica (Orlandi *et al.*, 2013; Puiggros *et al.*, 2013b; Durak Aras *et al.*, 2021).

3.1.2. Trisomía 12

La +12 es la segunda alteración más frecuente en LLC, encontrándose en un 15-20% de los casos al diagnóstico. Los pacientes con esta anomalía se asocian con unos rasgos morfológicos e inmunofenotípicos atípicos (Autore *et al.*, 2018). En general, se consideran de pronóstico intermedio y presentan una mayor incidencia de trombocitopenia, IGHV no mutado, TR y de otros cánceres secundarios (Fabbri *et al.*, 2013; Strati *et al.*, 2015a). Aunque habitualmente aparece como única alteración, puede asociarse con otras alteraciones cromosómicas, como trisomías adicionales (especialmente trisomía 19, acompañada en la mayoría de los casos por la trisomía 18) o translocaciones de la región 14q32 (Autore *et al.*, 2018; Roos-Weil *et al.*, 2018). Estos casos con múltiples trisomías constituyen un subgrupo de pacientes con un curso clínico favorable (Baliakas *et al.*, 2014a; Baliakas *et al.*, 2016). No obstante, en un 30-40% de los casos con +12 se pueden detectar mutaciones de *NOTCH1,* confiriendo a estos casos un peor pronóstico (Balatti *et al.*, 2013; Villamor *et al.*, 2013).

3.1.3. Deleción 11q22q23

La del(11q) se encuentra en <10% de los casos con LLC al diagnóstico y en alrededor de un 25-30% de los pacientes en estadios avanzados y en pacientes R/R (Letestu *et al.*, 2010; Gaidano and Rossi, 2017). Suele ser monoalélica y aunque el tamaño puede ser variable, normalmente afecta a un gran fragmento (>20Mb). La región mínima delecionada en este *locus* incluye el gen *ataxia telangiectasia mutated* (*ATM)*, implicado en los mecanismos de reparación de las roturas de doble cadena del ADN. Alrededor de un 20-30% de los casos con esta deleción presenta mutaciones en el otro alelo de *ATM,* resultando en una pérdida de función completa (Skowronska *et al.*, 2012; Lozano-Santos *et al.*, 2017). No obstante, algunos pacientes con del(11q) mantienen el gen *ATM* intacto pero presentan deleción de un gen cercano a *ATM*, *baculoviral*

IAP repeat-containing 3 (*BIRC3*), un regulador negativo de la vía de señalización de NF-κB (Rose-Zerilli *et al.*, 2014; Quijada-Álamo *et al.*, 2021). En cualquier caso, la del(11q) está asociada a IGHV no mutado, peor evolución clínica y TPT y SG más cortos con los tratamientos quimioterápicos estándar. Además, los casos con esta alteración tienden a presentar síntomas B, destacando el desarrollo de adenopatías voluminosas (Stankovic *et al.*, 2014).

3.1.4. Deleción 17p13

La del(17p) es la alteración menos frecuente en el momento del diagnóstico, detectándose en alrededor del 5-8% de los casos. No obstante, su incidencia aumenta hasta el 30-40% en los casos R/R y a un 50-60% en los casos con TR (Gaidano and Rossi, 2017; Bracher *et al.*, 2022). De manera general, se considera un evento tardío en la evolución de la enfermedad. De hecho, se ha demostrado que es la principal alteración adquirida durante el curso de la enfermedad (Bracher *et al.*, 2022). De las cuatro anomalías incluidas en el modelo jerárquico de Döhner, la del(17p) es la que se asocia a peor pronóstico, especialmente en pacientes tratados con quimioinmunoterapia, principalmente por la pérdida del gen *TP53* (Soussi and Baliakas, 2022). *TP53* es un gen supresor de tumores que codifica para la proteína p53, uno de los principales puntos de control del ciclo celular y ampliamente conocida como “guardián del genoma”. Ante la presencia de señales que indican un daño en el ADN, p53 detiene el ciclo celular, permitiendo así que los mecanismos de reparación actúen y reparen el daño.

La del(17p) se asocia a otros factores de mal pronóstico como expresión elevada de CD38 y CD49d, IGHV no mutado o la presencia de cariotipos complejos. Cabe destacar que alrededor de un 70-80% de los pacientes con del(17p) presentan mutación en el otro alelo de *TP53*. En condiciones normales, *TP53* se activaría ante la presencia de un daño en el ADN causado por la quimioterapia. En cambio, cuando *TP53* está alterado y no es funcional, las células LLC con daño en el ADN no pueden desencadenar la apoptosis. Como consecuencia, la pérdida de este gen tanto por del(17p) como por mutación en *TP53* confiere un pronóstico adverso y refractariedad a la quimioterapia (Gaidano and Rossi, 2017; Soussi and Baliakas, 2022) No obstante, en los ensayos clínicos iniciales con los nuevos fármacos, parecía que el impacto negativo de *TP53* podría mitigarse al menos parcialmente. En consecuencia, según las guías actuales del iwCLL y del ESMO, es necesario analizar la presencia de ambas alteraciones ya que en caso de confirmarse una del(17p) y/o mutación en *TP53*, estos pacientes deben ser tratados con las nuevas modalidades terapéuticas (O'Brien *et al.*, 2018; Kater *et al.*, 2019; Al-Sawaf *et al.*, 2020; Burger *et al.*, 2020).

3.1.5. Otras alteraciones con valor pronóstico

Aparte de las cuatro anomalías cromosómicas mencionadas anteriormente, se han detectado otras alteraciones citogenéticas menos frecuentes pero con impacto pronóstico demostrado.

- La **ganancia en 2p** está presente en un 5-15% de los casos, siendo más frecuente en estadios avanzados y en casos R/R. Se ha relacionado con una SG inferior y con mayor riesgo de TR. Entre los genes afectados por esta alteración se encuentran *MYCN*, *REL* y *MSH2* (Chapiro *et al.*, 2010; Cosson *et al.*, 2017; Kostopoulou *et al.*, 2019).
- La **deleción en 6q** se encuentra en un 5-10% de los pacientes con LLC y se ha asociado a un pronóstico intermedio. En la mayoría

de los casos la deleción afecta a la región 6q21-23, provocando una disminución en la expresión del gen *FOXO3*, un factor de transcripción que promueve la apoptosis (Wang *et al.*, 2011; Jarosova *et al.*, 2017).

- La **deleción en 8p** así como la **ganancia en 8q24**, detectadas en un 2-5% de los casos con LLC, se han relacionado con la presencia de CK y alteraciones en *TP53*. De hecho, entre los casos con *TP53* alterado, la frecuencia de deleciones en 8p y ganancias en 8q aumenta hasta un 80% y 44%, respectivamente. Ambas anomalías se asocian a pronóstico desfavorable, incluso en ausencia de alteraciones en *TP53* (Blanco *et al.*, 2016; Chapiro *et al.*, 2018; Ondrousková *et al.*, 2022).

A diferencia de lo que sucede en otras neoplasias hematológicas, las translocaciones cromosómicas son poco frecuentes en la LLC. En estudios previos en los que se ha analizado la presencia de alteraciones estructurales se ha visto que entre los reordenamientos recurrentes en la LLC destacan aquellos que involucran la región 13q14, el gen *BCL2* (18q21) o el gen de las IG (14q32). De hecho, se podría decir que la translocación más común, aunque tan solo se encuentre en un 2% de los casos, es la t(14;18)(q32;q21) (Puente *et al.*, 2015; Costa *et al.*, 2022). En cuanto a su impacto pronóstico, aún sigue siendo incierto. En un primer momento, Mayr y colaboradores sugirieron que la presencia de translocaciones empeoraba el curso clínico de los pacientes (Mayr *et al.*, 2006). Sin embargo, en estudios posteriores este impacto negativo se ha correlacionado con la presencia de translocaciones desequilibradas especialmente en el contexto de un CK (Baliakas *et al.*, 2014a; Herling *et al.*, 2016; Rigolin *et al.*, 2018; Visentin *et al.*, 2019; Heerema *et al.*, 2021). Más recientemente, se ha confirmado que un número creciente de translocaciones se asocia a un pronóstico gradualmente adverso (Costa *et al.*, 2022).

3.2. Complejidad genómica

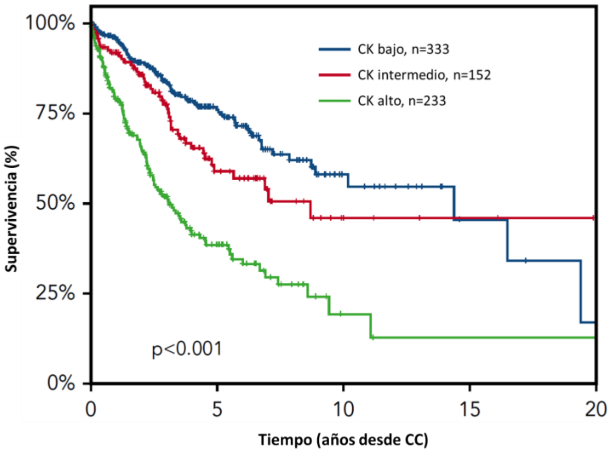
3.2.1. Cariotipo complejo

En los primeros estudios citogenéticos llevados a cabo en pacientes con LLC se observó que un incremento en el número de alteraciones presentes en el cariotipo estaba asociado con un peor pronóstico (Juliusson *et al.*, 1990). Sin embargo, no fue hasta inicios de los años 2000 cuando se utilizó por primera vez el concepto de "cariotipo complejo" en la LLC (Haferlach *et al.*, 2007; Badoux *et al.*, 2011). La definición clásica de CK comprende la presencia de tres o más alteraciones cromosómicas detectadas por CC en un mismo clon celular. Se encuentran en un 10-15% de los pacientes al diagnóstico y en hasta un 30% de los pacientes en recaída tras recibir tratamiento (Baliakas *et al.*, 2014a; Puiggros *et al.*, 2017). Además, se encuentran frecuentemente en pacientes con TR. De manera general, se han asociado a enfermedad avanzada y a otros factores de mal pronóstico, tales como la del(17p) o la mutación en *TP53*, la del(11q) o IGHV no mutado (Baliakas *et al.*, 2014a; Cavallari *et al.*, 2018). Sin embargo, se ha visto que el 50% de los pacientes con CK no presentan alteraciones de alto riesgo (*TP53* y/o *ATM*) y muestran una evolución clínica agresiva equivalente a la de aquellos pacientes con estas alteraciones de alto riesgo que no presentan un CK. Del mismo modo, el CK empeora el pronóstico de los pacientes con *TP53* y/o *ATM* alterados (Blanco *et al.*, 2016; Herling *et al.*, 2016; Puiggros *et al.*, 2017).

A pesar de que tradicionalmente se han relacionado con un curso clínico desfavorable, los pacientes con CK constituyen un grupo heterogéneo, es decir, no todos los CK son iguales en cuanto al tipo de alteraciones numéricas y estructurales que presentan ni confieren el mismo pronóstico. Por este motivo, se han llevado a cabo diversos estudios con el fin de refinar la definición de CK y de determinar su impacto clínico. Gracias a estos trabajos se ha demostrado que el número de alteraciones detectadas por CC como variable continua puede correlacionarse con el curso clínico de los pacientes con LLC (Jaglowski *et al.*, 2012; Travella *et al.,* 2013; Baliakas *et al.*, 2014a). En este sentido, el grupo ERIC (*European Research Initiative on CLL*) llevó a cabo un estudio multicéntrico retrospectivo en el que se incluyeron datos citogenéticos de más de 5000 pacientes con LLC con el objetivo de caracterizar mejor este subgrupo y evaluar su impacto en la práctica clínica. En este trabajo, los pacientes pudieron categorizarse en tres grupos de riesgo según el número de alteraciones que presentaban en el cariotipo. Los autores sugirieron que la presencia de un cariotipo complejo alto, con cinco o más alteraciones cromosómicas, es lo que mejor predice un pronóstico adverso, independientemente de otros biomarcadores como el estadio clínico o el estado mutacional de IGHV o *TP53*. Los casos con tres (cariotipo complejo bajo) o cuatro (cariotipo complejo intermedio) anomalías cromosómicas únicamente tenían impacto clínico en presencia de alteraciones en *TP53* (**Figura 16**) (Baliakas *et al.*, 2019).

Asimismo, no solo el número de alteraciones influye en el impacto clínico del CK; el tipo de alteraciones que lo componen también tiene un papel importante en su valor pronóstico. Respecto a esto último, se ha visto que aquellos pacientes con CK que presentan múltiples trisomías (+12, +19, + otra alteración numérica o estructural) tienen un pronóstico favorable a pesar de tener un CK por definición (**Figura 16**) (Baliakas *et al.*, 2016). En cuanto a la presencia de alteraciones estructurales, Mayr y colaboradores reportaron que los pacientes con CK que presentaban translocaciones tenían una supervivencia libre de tratamiento y una SG inferior a aquellos sin translocaciones (Mayr *et al.*, 2006). Posteriormente, se sugirió que la coexistencia de CK con reordenamientos desequilibrados era lo que empeoraba el curso clínico (Rigolin *et al.*, 2018).

a) Impacto de la complejidad genómica



b) Impacto de las múltiples trisomías

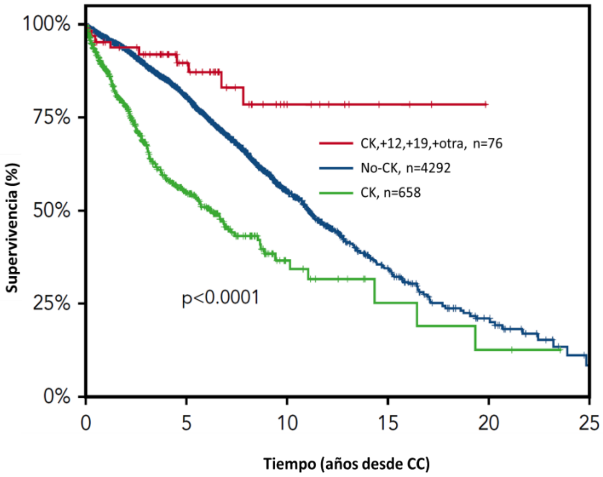


Figura 16. Curvas de supervivencia Kaplan-Meier para la supervivencia global en (A) las tres categorias de riesgo establecidas según el número de alteraciones detectadas por CC y en (B) los pacientes con múltiples trisomías (adaptada de Baliakas *et al.*, 2019).

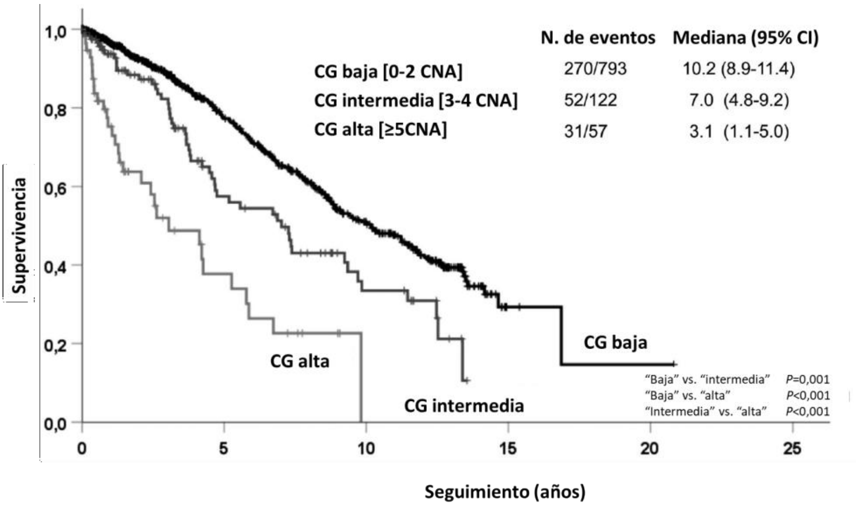
Globalmente, los CK se han asociado también a refractariedad al tratamiento en pacientes tratados con los regímenes estándar de quimioinmunoterapia, tanto en los basados en clorambucilo como en los regímenes FCR (Badoux *et al.*, 2011; Baliakas *et al.*, 2014a; Herling *et al.*, 2016; Le Bris *et al.*, 2017), así como en un estudio llevado a cabo en pacientes con LLC tras un trasplante alogénico (Jaglowski *et al.*, 2012). No obstante, con la incorporación de los nuevos agentes dirigidos, el impacto que tiene la presencia de CK en pacientes que reciben las nuevas modalidades terapéuticas no está del todo establecido. Según los resultados de los primeros ensayos clínicos con los nuevos fármacos (ibrutinib, acalabrutinib, venetoclax), llevados a cabo en pacientes mayores, R/R, los CK parecían tener un valor pronóstico negativo para la SLP y la SG (Thompson *et al.*, 2015, Chanan-Khan *et al.*, 2016; Anderson *et al.*, 2017; O'Brien *et al.*, 2018). Sin embargo, en trabajos más recientes en los que el seguimiento de los estudios iniciales es más largo, los pacientes son tratados en primera línea o en los que se han testado nuevas combinaciones terapéuticas, este impacto negativo en la SLP y SG no es tan evidente (Brown *et al.*, 2018; Mato *et al.*, 2018; Woyach *et al.*, 2018, Kipps *et al.*, 2019; Al-Sawaf *et al.*, 2020; Byrd *et al.*, 2020; Kreuzer *et al.*, 2020).

3.2.2. Complejidad genómica definida por *microarrays* genómicos

Como se ha mencionado en el punto anterior, la definición de CK se basa actualmente en los resultados obtenidos por CC. No obstante, debido a la limitación de necesitar células en división, esta técnica ha sido reemplazada en algunos países europeos por los MG. Los estudios iniciales llevados a cabo utilizando estas plataformas demostraron que, aparte de las alteraciones recurrentes en LLC, los MG eran capaces de detectar complejidad genómica (Sargent *et al.*, 2009; Simons *et al.*, 2012). De hecho, la identificación de un mayor número de CNA se correlacionó con un pronóstico adverso, con peor respuesta al tratamiento y TPT y SG inferiores (Kujawski *et al.*, 2008; Kay *et al.*, 2010; Gunnarsson *et al.*, 2011; Ouillette *et al.*, 2011a). Sin embargo, hasta hace poco tiempo no se había propuesto una definición de complejidad genómica detectada por MG.

Recientemente, con el fin de establecer la definición de complejidad genómica mediante MG y determinar la utilidad clínica de estudiar las CNA en el ámbito de la LLC, el grupo ERIC llevó a cabo un segundo estudio multicéntrico retrospectivo en el que se analizaron un total de 2300 pacientes con datos de MG. En este trabajo, los pacientes pudieron clasificarse en tres categorías de riesgo según el número de CNA detectadas por MG. En concreto, se estratificaron en los grupos de complejidad genómica baja (0-2 CNA), complejidad genómica intermedia (3-4 CNA) y complejidad genómica alta (≥5 CNA). De manera similar a los resultados obtenidos en el anterior estudio del ERIC en el que usaron datos de CC, únicamente la presencia de complejidad genómica alta por MG se asociaba a un curso clínico desfavorable y constituía un factor pronóstico independiente en el análisis multivariado para el TPT y la SG (Leeksma *et al.*, 2021) (**Figura 17**).

a) Supervivencia global



b) Tiempo al primer tratamiento

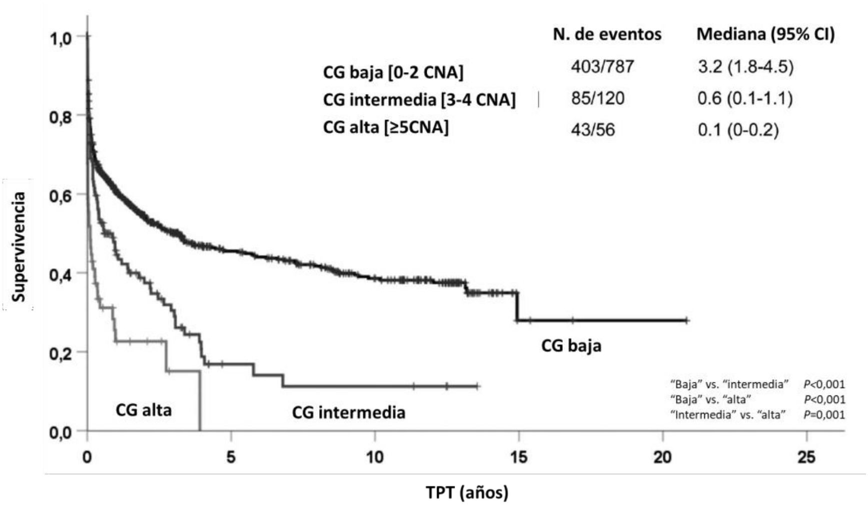


Figura 17. Gráfico de supervivencia Kaplan-Meier donde se muestra el impacto de los tres grupos de riesgo definidos según las CNA detectadas por *microarrays* genómicos en la (A) SG y en el (B) TPT (adaptada de Leeksma *et al.*, 2021).

A pesar de haber obtenido resultados similares por CC y MG en los dos grandes trabajos del ERIC, es importante destacar que hasta la realización de esta tesis doctoral ningún estudio había comparado ambas técnicas en la estratificación pronóstica de los pacientes con LLC. Por otro lado, en el pasado los laboratorios tenían dificultad para analizar e interpretar los resultados obtenidos por MG por una falta de estandarización, lo cual ha impedido la incorporación de esta técnica en los ensayos clínicos de LLC. Por este motivo, la gran mayoría incluyen información de CC pero no de MG. De hecho, el estudio MURANO es el primero en incorporar la complejidad genómica por MG en un ensayo clínico analizando los nuevos fármacos. Según los resultados obtenidos, la complejidad genómica alta (≥5 CNA) se asocia con un curso clínico adverso en pacientes tratados tanto con quimioinmunoterapia (BR) como con la combinación venetoclax + rituximab (Kater *et al.*, 2020).

3.2.3. Cromotripsis

La aparición de nuevas técnicas de mayor resolución, como los MG o la tecnología de NGS, así como el continuo desarrollo de las herramientas bioinformáticas han permitido la identificación de nuevas formas de complejidad genómica. En concreto, gracias a estas nuevas metodologías se han detectado alteraciones genómicas masivas caracterizadas por la presencia de múltiples reordenamientos genómicos que a menudo se originan en un único evento catastrófico. En este sentido, se han descrito hasta tres procesos generados por distintos mecanismos moleculares que darían lugar a múltiples reordenamientos denominados cromotripsis, cromoanasíntesis y cromoplexia (Pellestor *et al.*, 2019; Pellestor *et al.*, 2022). Todos ellos se agrupan bajo el concepto de cromoanagénesis y se distinguen mediante una serie de características específicas de cada proceso (Holland and Cleveland, 2012). Brevemente, la cromotripsis deriva de múltiples roturas de la doble cadena de ADN con la posterior reagrupación aleatoria de los fragmentos (Stephens *et al.*, 2011; Korbel *et al.*, 2013). La cromoanasíntesis se produce tras la replicación errónea del ADN, con fallos de la horquilla de replicación y cambios en la hebra patrón utilizada durante el proceso, dando lugar a múltiples ganancias en el número de copias (duplicaciones y triplicaciones) en combinación con deleciones (Liu *et al.,* 2011; Arlt *et al.*, 2012). Por último, se denomina cromoplexia a un fenómeno descrito por primera vez en cáncer de próstata caracterizado por la presencia de múltiples translocaciones encadenadas entre hasta ocho cromosomas distintos, dando lugar a pocas o ninguna alteración en el número de copia (Baca *et al.*, 2013; Shen *et al.,* 2013). Los mecanismos que originan cada uno de estos fenómenos se ilustran en la **figura 18**.

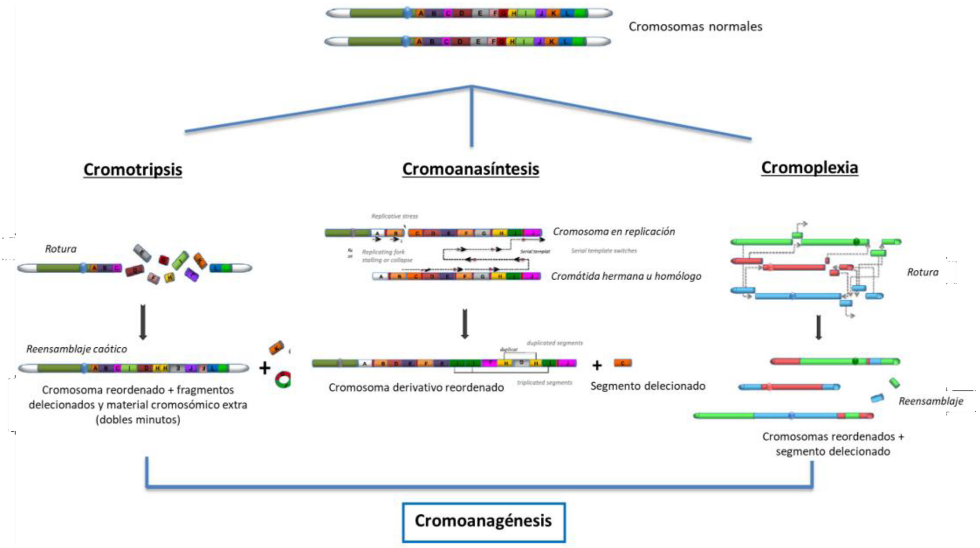

Cromosomas normales
Cromotripsis
Cromoanasíntesis
Cromoplexia
Rotura
Replicative stress
Cromosoma en replicación
Replicating fork stalling or collapse
Serial template switches
Cromátida hermana u homólogo
Rotura
Reensamblaje caótico
Cromosoma reordenado + fragmentos delecionados y material cromosómico extra (dobles minutos)
duplicated segments
triplicated segments
Cromosoma derivativo reordenado
Segmento delecionado
Reensamblaje
Cromosomas reordenados + segmento delecionado
Cromoanagénesis

Figura 18. Esquema del mecanismo de formación de los eventos catastróficos cromotripsis, cromoanasíntesis y cromoplexia, todos ellos englobados bajo el término de cromoanagénesis (adaptada de Pellestor and Gatinois, 2020).

En esta tesis doctoral se ha hecho especial énfasis en el estudio del fenómeno de cromotripsis. La cromotripsis (procedente del griego, "*chromos"* de cromosoma; "*thripsis"*, de fragmentarse en pedazos) se definió por primera vez en 2011 en un caso de LLC estudiado mediante secuenciación del genoma completo (WGS, del inglés *whole genome sequencing*) en el que la paciente presentaba múltiples reordenamientos estructurales en el brazo largo del cromosoma 4 (Stephens *et al*., 2011). Los autores definieron ese fenómeno como un evento catastrófico único por el cual uno o unos pocos cromosomas se rompían en diferentes fragmentos que, tras la acción errónea de los mecanismos de reparación, se juntaban aleatoriamente dando lugar a cromosomas derivativos (Stephens *et al.*, 2011). Este proceso se visualiza en forma de un patrón de al menos diez cambios oscilantes entre dos y tres estados de número de copia cuando se analizan por MG o NGS, aunque algunos autores también consideran como cromotripsis los patrones con al menos siete cambios oscilantes. Estas múltiples CNA pueden dar lugar a la pérdida de genes supresores de tumores, a la activación de oncogenes o incluso pueden derivar en la formación de nuevos genes de fusión. Cualquiera de estas tres situaciones puede promover el inicio o la evolución de un proceso cancerígeno. Aunque se describió por primera vez en un caso con LLC, posteriormente, se observó en muchos otros tipos de tumores, tanto sólidos como hematológicos, e incluso en pacientes con anomalías congénitas (Kloosterman *et al.*, 2011; Magrangreas *et al.*, 2011; Hirsch *et al.*, 2013; Gamba *et al.*, 2015; Notta *et al.*, 2016; Collins *et al.*, 2017; Ratnaparkhe *et al.*, 2017). Globalmente, todos los reordenamientos asociados a la cromotripsis descritos hasta la fecha comparten una serie de características: i) los numerosos reordenamientos generados están focalizados en uno o en unos pocos cromosomas; ii) la reunión de los fragmentos se produce sin ningún orden ni orientación preferencial; iii) no existe homología en las secuencias de los puntos de ruptura o únicamente hay microhomología de unos pocos nucleótidos; iv) hay poco cambio en el número de copias de ADN, generalmente entre una y dos copias; v) se conserva la heterocigosidad en los fragmentos reordenados (Korbel *et al.*, 2013).

En cuanto a las causas subyacentes y los mecanismos implicados, se han propuesto diferentes modelos para explicar el origen de la cromotripsis, desde el efecto de agentes exógenos, como la radiación ionizante o drogas quimioterapéuticas, hasta la acción de mecanismos endógenos como la pulverización de un cromosoma en un micronúcleo, entre otros. No obstante, el desencadenante exacto sigue siendo una incógnita (Crasta *et al.*, 2012; Maciejowski *et al.*, 2015; Zhang *et al.*, 2015; Morishita *et al.*, 2016). Entre las hipótesis sugeridas, los dos procesos celulares más aceptados que podrían explicar el origen de la cromotripsis derivan de una segregación cromosómica defectuosa durante la mitosis (**Figura 19**):

a) Condensación prematura de un cromosoma localizado dentro de un micronúcleo:

Durante la división celular es posible que uno o varios cromosomas no se separen correctamente y queden encapsulados en micronúcleos, que son unas estructuras similares al núcleo principal, con una doble membrana nuclear, pero con algunos defectos como una menor densidad de complejos de poros nucleares y una lámina B nuclear defectuosa que afecta a la integridad estructural de la membrana del micronúcleo. Esto hace que no puedan acceder a éste todos los elementos necesarios para la reparación y replicación del ADN. Así pues, cuando se produce una replicación asincrónica del cromosoma localizado en el micronúcleo en comparación con el núcleo principal de la célula, el primero se condensa de forma prematura, rompiéndose en varios fragmentos que se juntarán erróneamente formando un cromosoma derivativo. El micronúcleo puede permanecer en la célula durante varios ciclos celulares sin ser degradado hasta eliminarse o reincorporarse al núcleo principal, dando lugar a la presencia de cromotripsis en el genoma (Terzoudi *et al.*, 2015; Zhang *et al.*, 2015; Ly and Cleveland, 2017; Pantelias *et al.*, 2019).

b) Fragmentación de cromosomas dicéntricos durante los ciclos de ruptura-fusión-puente:

Los telómeros son regiones de ADN repetitivo, concretamente hexámeros, que protegen los extremos de los cromosomas. Cada vez que una célula se divide, la longitud de los telómeros se va acortando hasta que se vuelven inestables. Cuando los extremos de los cromosomas quedan desprotegidos, se pueden fusionar con otros cromosomas formando cromosomas dicéntricos, con dos centrómeros. Durante la mitosis, los dos centrómeros de un cromosoma dicéntrico son empujados hacia los polos opuestos de la célula formando un puente entre las dos células hijas. La ruptura de este puente al final de la anafase genera dos cromosomas nuevos con los extremos desprotegidos, que volverán a iniciar un nuevo ciclo de ruptura-fusión-puente (Maciejowski *et al.*, 2015; Ernst *et al.*, 2016; Maciejowski *et al.*, 2017).

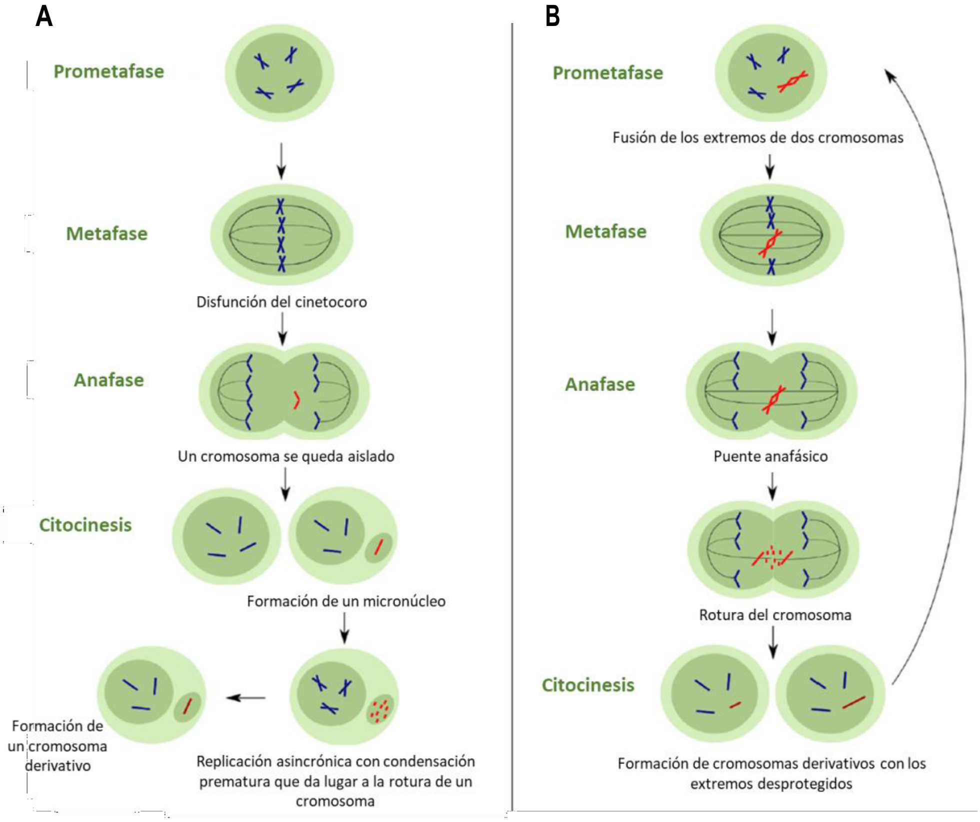

A
Prometafase
Metafase
Disfunción del cinetocoro
Anafase
Un cromosoma se queda aislado
Citocinesis
Formación de un micronúcleo
Formación de un cromosoma derivativo
Replicación asincrónica con condensación prematura que da lugar a la rotura de un cromosoma
B
Prometafase
Fusión de los extremos de dos cromosomas
Metafase
Anafase
Puente anafásico
Rotura del cromosoma
Citocinesis
Formación de cromosomas derivativos con los extremos desprotegidos

Figura 19. Esquema de dos de los modelos propuestos sobre el origen de la cromotripsis. A) Condensación prematura de un cromosoma dentro de un micronúcleo. B) Ciclo de fusión-ruptura-puente iniciado a partir de un cromosoma dicéntrico (adaptada de Zavacka and Plevova, 2021).

Con respecto a su prevalencia, se ha visto que es altamente variable entre los distintos tipos de tumores. En un primer momento se creía que la cromotripsis se podía encontrar con una frecuencia de entre un 2-3% de los cánceres con la excepción de los tumores óseos, donde su prevalencia ascendía a un 25% (Kim *et al.*, 2013; Cai *et al.*, 2014). Sin embargo, con el creciente número de estudios en los que se ha secuenciado el genoma de diferentes tipos de cáncer se ha podido observar que las estimaciones previas en cuanto a la prevalencia de la cromotripsis podrían haber subestimado la frecuencia real de este fenómeno. De hecho, en un estudio llevado a cabo por el *Pan-Cancer Analysis of Whole Genomes* (PCAWG) *Consortium* en el que se analizaron más de 2000 tumores de 38 tipos diferentes mediante secuenciación de todo el genoma, los autores encontraron patrones de cromotripsis en más del 50% de los casos en la mayoría de los cánceres e incluso en el 100% de los sarcomas, sugiriendo que podría jugar un rol importante en la tumorogénesis (Cortés-Ciriano *et al.*, 2020; Voronina *et al.*, 2020). Cabe destacar que este fenómeno se ha asociado a la presencia de alteraciones en *TP53* (Rausch *et al.*, 2012; Fontana *et al.*, 2018), aunque también se ha visto en casos con *ATM* alterado (Ratnaparkhe *et al.*, 2017). En cuanto a su impacto pronóstico, la cromotripsis se correlaciona con enfermedad agresiva y evolución clínica desfavorable en distintas entidades. Así pues, el paradigma tradicional del desarrollo del cáncer, según el cual un tumor surgía por la acumulación progresiva de mutaciones, ha sido cuestionado tras el descubrimiento de estos fenómenos catastróficos mediante los cuales un solo evento puede dar lugar a múltiples reordenamientos.

En el ámbito de la LLC, su prevalencia es de alrededor del 1-3% en cohortes no seleccionadas y también se ha asociado a factores de mal pronóstico, especialmente alteraciones de *TP53,* encontradas en un 70-80% de los casos, IGHV no mutado y complejidad genómica, así como a TPT, SLP y SG más cortas (Stephens *et al.*, 2011; Edelmann *et al.*, 2012; Puente *et al.*, 2015; Salaverria *et al.,* 2015; Leeksma *et al.*, 2021). No obstante, existe poca información disponible en relación a las causas y a las consecuencias de la cromotripsis y su impacto pronóstico en la LLC. Debido a la baja incidencia general de este fenómeno en una población de vida real con LLC, la mayoría de los estudios publicados estaban limitados por un número bajo de casos con cromotripsis. Además, aunque se ha visto especial implicación de los cromosomas 2, 3, 5, 6, 8, 9, 11, 13 y 17 y se han propuesto algunos genes localizados en las regiones mencionadas, en estos estudios no se ha demostrado la implicación de ningún gen específico que pudiera impulsar su formación ni de ningún cromosoma o región genómica particular que estuviesen más comúnmente afectados (Edelmann *et al.*, 2012; Pei *et al.*, 2012; Bassaganyas *et al.*, 2013; Salaverria *et al.,* 2015; Tan *et al.*, 2015; Parker *et al.*, 2016; Leeksma *et al.*, 2021). No obstante, a pesar de que tradicionalmente se ha asociado a mayor complejidad genómica, ningún trabajo ha profundizado en el rol de la cromotripsis en un contexto de genomas complejos.

II. HIPÓTESIS Y OBJETIVOS

1. Hipótesis

Los pacientes con LLC y alta complejidad genómica, detectada tanto por CC como por MG, se asocian generalmente a una enfermedad más agresiva y a otros factores de mal pronóstico. Pese a que tradicionalmente la complejidad genómica se ha definido por la detección de cariotipos complejos mediante CC, los MG también son útiles para su evaluación. Esta última técnica ha permitido la identificación de nuevos patrones de complejidad, como la denominada cromotripsis, cuyas causas e impacto pronóstico en la LLC no están del todo establecidos. Aunque los pacientes con elevada complejidad genómica generalmente se han asociado a un curso clínico desfavorable, a presencia de alteraciones en *TP53* y a IGHV no mutado, se ha visto que constituyen un grupo heterogéneo en el que tanto el número como el tipo de alteraciones presentes podrían modular su impacto clínico.

La hipótesis de esta tesis se basa en que una mejor caracterización de los pacientes con una elevada complejidad genómica no solo contribuirá a determinar mejor su pronóstico sino que también ayudará a encontrar mejores estrategias terapéuticas adaptadas a este subgrupo de pacientes.

2. Objetivos

El objetivo principal de la tesis es caracterizar a nivel genético los pacientes con LLC y complejidad genómica comparando la utilidad de distintas metodologías. Para ello, se definen los siguientes objetivos específicos:

1. Analizar la complejidad genómica de los casos con LLC utilizando las técnicas de citogenética convencional (CC) y *microarrays* genómicos (MG) en una cohorte enriquecida en CK.
2. Comparar la estratificación del riesgo de los pacientes según la complejidad genómica detectada por CC y MG.
3. Caracterizar la complejidad genómica asociada a cromotripsis así como los patrones de reordenamientos relacionados con ésta mediante mapeo óptico del genoma.
4. Evaluar el impacto pronóstico de la cromotripsis en una cohorte de pacientes con LLC y alta complejidad genómica por CC o MG.

III. RESULTADOS

La memoria de la tesis doctoral titulada "Caracterización citogenética-molecular de la leucemia linfocítica crónica. Identificación de subgrupos con relevancia clínica" se presenta en formato de compendio de dos artículos en los que la doctoranda Silvia Ramos Campoy consta como primera autora y son fruto directamente del trabajo de la tesis.

ARTÍCULO 1

Ramos-Campoy, *et al*. Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients. *Haematologica*. 2022;107(3):593-603.
doi: 10.3324/haematol.2020.274456. PMID: 33691382

Índices de calidad: IF (2021) = 11,04; 1r decil de la categoría *Hematology*

ARTÍCULO 2

Ramos-Campoy, *et al*. *TP53* abnormalities are underlying the poor outcome associated with chromothripsis in chronic lymphocytic leukemia patients with complex karyotype. *Cancers* (Basel). 2022;14(15):3715.
doi: 10.3390/cancers14153715. PMID: 35954380

Índices de calidad: IF (2021) = 6,575; 1r cuartil en la categoría *Oncology*

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ARTÍCULO 1

Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients

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La complejidad genómica se ha asociado a un peor pronóstico en pacientes con leucemia linfocítica crónica (LLC). En estudios cooperativos previos se ha establecido el punto de corte que mejor predice un curso clínico adverso en cinco o más alteraciones detectadas tanto por citogenética convencional (CC) como por *microarrays* genómicos (MG). Sin embargo, no se ha comparado la estratificación del riesgo por los dos métodos. En este trabajo, se ha analizado una cohorte de 340 pacientes con LLC previamente no tratados altamente enriquecida en casos con cariotipo complejo (46,5%). Todos ellos se han estudiado de manera paralela mediante CC y MG. Las alteraciones detectadas por ambas técnicas han sido comparadas y se ha analizado la estratificación pronóstica de los pacientes en tres grupos de riesgo según la complejidad genómica (0-2, 3-4 y ≥5 alteraciones).

En general, no se encontraron diferencias significativas en el porcentaje de pacientes en cada grupo de riesgo, pero cuando se analizaron los casos de manera individual, únicamente se observó un grado de acuerdo moderado entre los dos métodos (κ=0,507; p<0,001). La clasificación fue discordante en 100 pacientes (29,4%), incluyendo un 3% que se clasificaron en grupos de riesgo opuestos. La mayoría de las discrepancias se debieron a limitaciones de la propia técnica y no se obtuvo una mejor correlación en el número de alteraciones detectadas cuando se aplicaron diferentes estrategias de filtrado en los resultados de MG. No obstante, ambas técnicas mostraron un índice de concordancia similar para la predicción del TPT (CBA: 0,67 vs. MG: 0,65) y de la SG (CBA: 0,55 vs. MG: 0,57). La detección de una alta complejidad (≥5 alteraciones) mantuvo su significancia en el análisis multivariado para el TPT, en el que se incluyeron el estado de *TP53* y de IGHV, tanto definida por CC (HR: 3,23; p<0,001) como por MG (HR: 2,74; p<0,001). Los resultados sugieren que ambos métodos son útiles pero no equivalentes para la estratificación del riesgo de los pacientes con LLC.

ARTICLE

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Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients

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ABSTRACT

Genome complexity has been associated with poor outcome in patients with chronic lymphocytic leukemia (CLL). Previous cooperative studies established five abnormalities as the cut-off that best predicts an adverse evolution by chromosome banding analysis (CBA) and genomic microarrays (GM). However, data comparing risk stratification by both methods are scarce. Herein, we assessed a cohort of 340 untreated CLL patients highly enriched in cases with complex karyotype (CK) (46.5%) with parallel CBA and GM studies. Abnormalities found by both techniques were compared. Prognostic stratification in three risk groups based on genomic complexity (0-2, 3-4 and ≥5 abnormalities) was also analyzed. No significant differences in the percentage of patients in each group were detected, but only a moderate agreement was observed between methods when focusing on individual cases ($\kappa=0.507$; $P<0.001$). Discordant classification was obtained in 100 patients (29.4%), including 3% classified in opposite risk groups. Most discrepancies were technique-dependent and no greater correlation in the number of abnormalities was achieved when different filtering strategies were applied for GM. Nonetheless, both methods showed a similar concordance index for prediction of time to first treatment (TTFT) (CBA: 0.67 *vs.* GM: 0.65) and overall survival (CBA: 0.55 *vs*. GM: 0.57). High complexity maintained its significance in the multivariate analysis for TTFT including *TP53* and IGHV status when defined by CBA (hazard ratio [HR] 3.23; $P<0.001$) and GM (HR

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2.74; $P<0.001$). Our findings suggest that both methods are useful but not equivalent for risk stratification of CLL patients. Validation studies are needed to establish the prognostic value of genome complexity based on GM data in future prospective studies.

Introduction

Deletions of 17p13 region and/or mutations in *TP53* as well as the mutational status of the variable region of the immunoglobulin heavy chain (IGHV) gene constitute the most important prognostic and predictive factors in chronic lymphocytic leukemia (CLL) in the era of chemoimmunotherapy.[1] However, several studies have highlighted the independent clinical significance of genomic complexity, mainly defined by the detection of complex karyotypes (CK) by chromosome banding analysis (CBA), due to its association with an unfavorable clinical outcome. This has been demonstrated in patients treated not only with standard chemoimmunotherapy regimes[2-5] but also in the initial clinical trials with the novel mechanism-based therapeutic agents such as ibrutinib or venetoclax.[6-8]

Early studies in CLL defined CK as the presence of at least three numerical and/or structural chromosomal abnormalities in the same cell clone detected by CBA.[9,10] Of note, the increasing number of chromosomal abnormalities in the karyotype has been correlated with the worsening of clinical evolution of CLL patients.[11,12] In this context, a large retrospective study from the European Research Initiative on CLL (ERIC) has reported that patients with five or more abnormalities (the so-called high-CK) display an adverse outcome independently of other known biomarkers such as *TP53* abnormalities or the IGHV mutational status.[5] On the other hand, it has been demonstrated that CK might have a different clinical impact in CLL patients according to not only the number, but also the type of aberrations detected in the karyotype. In this regard, it has been described that patients with CK carrying +12, +19 display a particularly favorable outcome while the presence of unbalanced rearrangements define a subset with very aggressive disease.[13-15]

Even though CBA has been the gold standard method to identify CK, in the last decade genomic microarrays (GM) have emerged as a valuable tool for genome-wide screening in CLL.[16-20] Indeed, some studies have correlated the genomic complexity detected by GM to progressive disease and poorer response rates to treatment.[21-23] Nonetheless, although some European countries have replaced conventional techniques by GM, standard criteria to analyze and define genomic complexity by GM are still needed. According to the published guidelines for GM analysis in acquired hematologic neoplasms, recurrent aberrations with known clinical relevance in the disease irrespective of their size as well as other copy number abnormalities (CNA) ≥5 Mb should be considered in order to reduce the detection of benign constitutional variants and avoid the reporting of anomalies with uncertain clinical significance.[24] However, it remains unclear whether this threshold is the optimal to analyze CLL patients or whether potentially relevant chromosomal imbalances are disregarded by applying this highly conservative size cut-off. Besides, another multicenter study conducted by ERIC suggested that CLL patients could be divided into three distinct prognostic subgroups based on the number of CNA.[25] According to Leeksma *et al.*, the so-called high genomic complexity (high-GC) subgroup, which is defined by carrying ≥5 CNA, emerged as prognostically adverse, independently of other biomarkers. Nevertheless, to the best of our knowledge, the comparison of genomic complexity for risk stratification using CBA and GM in parallel has not been performed in a large CLL cohort.

In the present multicenter retrospective study we aimed to compare the usefulness of CBA and GM techniques in a series of 340 CLL patients with and without CK to determine both their concordance and their equivalence in the prognostic stratification of CLL patients with CK. Moreover, we have analyzed the detected aberrations using different counting strategies to ascertain whether other parameters, such as the type of the aberrations or their size, might have an influence on the risk stratification of CLL patients.

Methods

Patient cohort

A total of 340 previously untreated CLL (n=327; 96.2%) and monoclonal B-cell lymphocytosis (n=13; 3.8%) patients from 18 European institutions were included. All had CBA results at diagnosis or before treatment. GM data were already available or obtained from DNA extracted within 1 year. Analyses were performed on peripheral blood (PB) (n=286) or bone marrow (BM) (n=54). Due to the purpose of the study, this cohort was enriched in patients with CK (n=158; 46.5%). Demographic, clinical and biological characteristics are summarized in Table 1. The study was carried out in accordance with national and international guidelines (Professional Code of Conduct, Declaration of Helsinki) and approved by the Hospital del Mar Ethics Committee (2017/7565/I).

Chromosome banding analyses

CBA was performed on G- or R-banded chromosomes. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2016).[26] A complex karyotype was defined as the presence of three or more numerical and/or structural chromosomal abnormalities (abn.) detected in the same cell clone. Patients were stratified in three categories: non-CK (0-2 abn.), low/intermediate-CK (3-4 abn.) and high-CK (≥5 abn.)[5]

Genomic microarray analyses

Microarray platforms used are summarized in the *Online Supplementary Table S1*. All aberrations found irrespective of size were collected, although non-classical CLL abnormalities (other than gain of chromosome 12 and losses of 11q, 13q and 17p) were filtered in the CNA count for prognostic stratification following the 5 Mb cut-off size recommended.[24] Three subgroups were defined according to genomic complexity (GC): low-GC (0-2 CNA), intermediate-GC (3-4 CNA) and high-GC (≥5 CNA).[25] This strategy was compared with other CNA counting methodologies, such as the inclusion of smaller abnormalities (no size filter or 1 Mb as cut-off) or counting as a unique CNA small contiguous abnormalities (with a distance ≤1 Mb between them) or those included in a chromothripsis event.

Statistical analyses

Descriptive statistics were used to provide frequency distributions of discrete variables while statistical measures were used to provide median values and ranges for quantitative variables. Groups were compared using Chi-square or Fisher exact tests for discrete variables and Mann-Whitney U test for continuous variables. The Kappa coefficient was used for assessing the agreement in patients categorization among techniques. Survival analysis was restricted to 259 patients. A total of 81 non-CK cases from three institutions were excluded as CBA was performed at recruitment for clinical trials, introducing a bias in the results. Time to first treatment (TTFT), the end point of the study, was calculated from the date of cytogenetic study to the date of first treatment or last follow-up while overall survival (OS) was defined from date of cytogenetic study until last follow-up or death. Kaplan–Meier method was used to estimate the distribution of TTFT and OS. Comparisons among patient subgroups were performed with the log-rank test. The concordance statistic (C-index) was calculated to assess the accuracy of CBA and GM for predicting TTFT and OS. Multivariate analysis using Cox proportional hazards regression model was used to assess the maintenance of the independent prognostic impact on TTFT and OS. Statistical analyses were performed using SPSS v.23 software (SPSS Inc, Chicago, IL, USA) and R v3.5.2. *P*-values <0.05 were considered statistically significant.

Additional information regarding the methodology of the study is detailed in the *Online Supplementary Appendix*.

Results

Number and type of abnormalities detected by chromosome banding analysis and genomic microarrays

Regarding CBA, 270 of 340 (79.4%) patients showed an abnormal karyotype. Overall, 182 were considered non-CK (0-2 abn.) while 158 displayed a CK (≥3 abn.). The vast majority of non-CK aberrant cases carried only one aberration (75 of 112; 66.9%), while the median number of abnormalities among CK patients was four (range, 3-19). Abnormal karyotypes from the non-CK group mainly included known recurrent CLL aberrations, the most frequent being trisomy of chromosome 12 (15.4% patients). In contrast, the CK group showed a wide variety of abnormalities affecting all chromosomes and included unbalanced structural aberrations (552 of 823; 67.1%), complex abnormalities affecting material of unknown origin (179 of 823; 21.7%) and monosomies (155 of 823; 18.8%). In seven of these, a co-existence of +12 and +19, associated with more indolent course, was found (4.4%). Balanced translocations, potentially missed when studied by GM, were present in only 57 patients (11.5% non-CK; 22.8% CK). Even though they were detected in a minority of cases, 13q14 and 14q32 loci were recurrently involved (in 13 and seven patients, respectively).

GM had the highest detection rate of abnormalities, with 309 of 340 (90.9%) cases carrying at least one CNA when all the abnormalities, irrespective of their size, were considered. No significant differences were observed among the GM platforms used. Expectedly, the non-CK group showed a significantly lower median number of CNA compared with CK patients (2 CNA; range, 0-10 *vs.* 6 CNA; range, 0-51; *P*<0.001). Nearly half of the patients carried at least one small (<5 Mb) non-classical CLL CNA (median size 5.38 Mb; range, 0.019-198 Mb) that would not be taken into consideration following the current microarray recommendations (35.7% non-CK *vs.* 64.6% CK patients; *P*<0.001). Although they were less frequent, similar results were observed regarding the presence of non-classical CLL CNA below 1 Mb (26.9% *vs.* 44.9%; *P*<0.001). Patterns suggestive of chromothripsis or chromothripsis-like were identified in 30 patients (20 and ten cases, respectively).

Fluorescence *in situ* hybridization (FISH), the gold standard method for the detection of the four genetic abnormalities included in the Döhner *et al.* prognostic hierarchical model,[27] confirmed the higher incidence of high-risk aberrations in the CK group. Specifically, del(11q) was found in 12.4% (22 of 177) of non-CK patients and 32.2% (49 of 152) of CK patients (*P*<0.001) while del(17p) was present in 4.5% (eight of 177) and 40.1% (61 of 152), respectively (*P*<0.001). Detection of del(13q), del(11q) and del(17p) was lower by CBA compared to FISH although these loci were involved in different type of abnormalities (*Online Supplementary Table S2*). GM showed a high concordance with FISH results (*Online Supplementary Table S3*).

With regard to commonly detected non-classical CLL abnormalities, similar results were observed by both CBA and GM among non-CK and CK patients. The only recurrent aberrations detected by CBA within the non-CK group were deletions in the long arm of chromosome 14 (6.6%) and unbalanced translocations affecting 2p arm (5.5%), which were detected as losses at 14q and gains of 2p region by GM, respectively. Likewise, despite being distributed

Table 1. **Baseline characteristics of patients at diagnosis and last follow-up.**

	NON-CK GROUP n = 182; n (%)	CK GROUP n = 158; n (%)	*P*-value
Sex			
Men	118 (64.8%)	113 (71.5%)	0.115
Median age at diagnosis	66 years (29-89)	68 years (33-96)	0.056
Stage at diagnosis			
MBL	11 (6.0%)	2 (1.3%)	0.024
CLL	171 (94.0%)	156 (98.7%)	
Binet A	117/159 (73.6%)	80/136 (58.8%)	0.009
Binet B/C	42/159 (26.4%)	56/136 (41.2%)	
Common CLL genomic aberrations*			
del(13)(q14)	103/182 (56.6%)	96/158 (60.8%)	0.437
isolated del(13)(q14)	70/103 (67.9%)	25/96 (26.0%)	<0.001
Trisomy 12	29/182 (15.9%)	27/158 (17.1%)	0.775
del(11)(q22q23)	25/182 (13.7%)	49/158 (31.0%)	<0.001
Aberrations in *TP53***	21/164 (12.8%)	70/156 (44.9%)	<0.001
del(17)(p13)	8/182 (4.4%)	65/158 (41.1%)	<0.001
TP53 mutation	15/161 (9.3%)	45/147 (30.6%)	<0.001
Unmutated IGHV	80/169 (47.3%)	92/138 (66.7%)	<0.001
Median follow-up (range)	68 months (0-261)	29 months (0-160)	<0.001
Time from diagnosis to cytogenetic study	3.5 months (0-242)	0 months (0-298)	<0.001
Treatment			
Treated patients	32/101 (31.7%)	103/151 (68.2%)	<0.001
Median time to first treatment (95% CI)	NR	13 months (8-18)	<0.001
Survival			
Median overall survival (95% CI)	102 months (82-121)	81 months (58-103)	0.367

*Deletions and trisomy detected by fluorescence *in situ* hybridization (FISH) and/or genomic microarrays. **Cases in which *TP53* mutation screening was not performed and FISH and/or genomic microarrays were negative for deletion were not considered. CLL: chronic lymphocytic leukemia; CK: complex karyotype; MBL: monoclonal B-cell lymphocytosis; CI: confidence interval, NR: not reached.

Table 2. **Recurrent copy number abnormalities found by genomic microarrays within the non-complex karyotype and complex karyotype subgroups and minimal common altered regions.**

		Non-CK	
CNA	n (%)	Cytogenetic bands	Minimal deleted/amplified region (GRCh37/hg19)
Gain 2p	16 (8.8)	p25.3-p21	chr2: 29,477 - 45,859,076
Loss 14q	13 (7.1)	q24.1-q32.11	chr14: 69,272,718 - 91,882,259

		CK	
CNA	n (%)	Cytogenetic bands	Minimal deleted/amplified region (GRCh37/hg19)
Gain 2p	39 (24.7)	p24.3-p23.1	chr2: 15,664,402 - 30,125,169
Gain 2p		p22.3-p15	chr2: 32,877,675 - 62,206,329
Loss 3p	13 (8.2)	p21.31-p21.31	chr3: 47,084,224 - 48,321,854
Gain 3q	11 (6.9)	q26.1-q29	chr3: 165,375,394 - 196,284,424
Loss 4p	15 (9.5)	p16.2-p15.2	chr4: 5,481,786 - 25,640,042
Loss 6q	15 (9.5)	q16.3-q21	chr6: 103,468,966 - 112,256,460
Loss 8p	16 (10.1)	p23.1-p22	chr8: 12,617,155 - 15,933,687
Gain 8q	17 (10.8)	q24.21-q24-21	chr8: 128,286,744 - 130,380,043
Loss 14q	13 (8.2)	q24.2-q24.3	chr14: 70,711,555 - 77,202,084
Loss 15q	16 (10.1)	q15.1-q15.1	chr15: 41,755,587 - 42,090,500
Gain 15q	11 (6.9)	q22.31-q26.3	chr15: 66,265,674 - 99,711,975
Gain 17q	12 (7.6)	q22-q25.1	chr17: 56,560,919 - 71,135,799
Loss 18p	24 (15.2)	p11.31-p11.23	chr18: 4,853,926 - 7,717,988
Gain 19q	12 (7.6)	q13.41-q13.42	chr19: 51,943,080 - 54,499,334

In non-complex karyotype (non-CK) group, aberrations were considered recurrent if present in at least ten patients while in CK group, recurrence was set at 10 and 15 patients for gains and losses, respectively. CNA: copy number abnormality.

Table 3. **Classification of patients in the previously suggested risk categories according to the number of aberrations detected by chromosome banding analysis and genomic microarrays.**

		CHROMOSOME BANDING ANALYSIS			
		Non-CK (0-2 abn.)	Low / Intermediate-CK (3-4 abn.)	High-CK (≥5 abn.)	Total
GENOMIC MICROARRAYS	Low-GC (0-2 CNA)	157	27	8	192 (56.5%)
	Intermediate-GC (3-4 CNA)	23	37	23	83 (24.4%)
	High-GC (≥5 CNA)	2	17	46	65 (19.1%)
	Total	182 (53.5%)	81 (23.8%)	77 (22.6%)	340

A moderate agreement was observed between methods (κ=0.507;*P*<0.001). CK: complex karyotype; GC: genomic complexity; CNA: copy number abnormality; abn.: abnormalities.

along the genome, gains of chromosome arms 2p, 3q, 8q, 15q, 17q and 19q and losses at 3p, 4p, 6q, 8p, 14q, 15q and 18p, usually involved in unbalanced translocations or simple deletions in the karyotype, were the most recurrent CNA detected in CK patients (*Online Supplementary Figure S1*). Detailed information regarding recurrent CNA found by GM is shown in Table 2.

Risk stratification of the genomic complexity observed by chromosome banding analysis and genomic microarrays

In order to compare the concordance among risk stratification based on CBA and GM data, patients were classified into those categories suggested by previous large-scale studies.[5,25] Notably, both techniques did not significantly differ in the percentage of patients classified into intermediate-risk categories (3-4 abnormalities; 23.8% by CBA *vs.* 24.4% by GM; *P*=0.923) or those showing the highest risk (≥5 abnormalities; 22.6% and 19.1%, respectively; *P*=0.299). However, when focusing in individual cases, only a moderate agreement was observed between methods (κ=0.507; *P*<0.001). Discordant classification was obtained in 100 patients (29.4%), including eight cases with ≥5 abnormalities in the karyotype which would not be considered complex by GM and two patients with high-GC who did not have CK (2.9%) (Table 3).

Next, we evaluated if the CNA filtering strategy used for GM results could underlie the differences observed in the assessment of the complexity. Regardless of the CNA filtering strategy, the proportion of patients with CNA <5 Mb was similarly represented among those patients with increased complexity scored by CBA (n=58) or by GM (n=42) (55.2% *vs.* 64.3%, respectively; *P*=0.413). When less strict filtering strategies were applied for GM, no greater

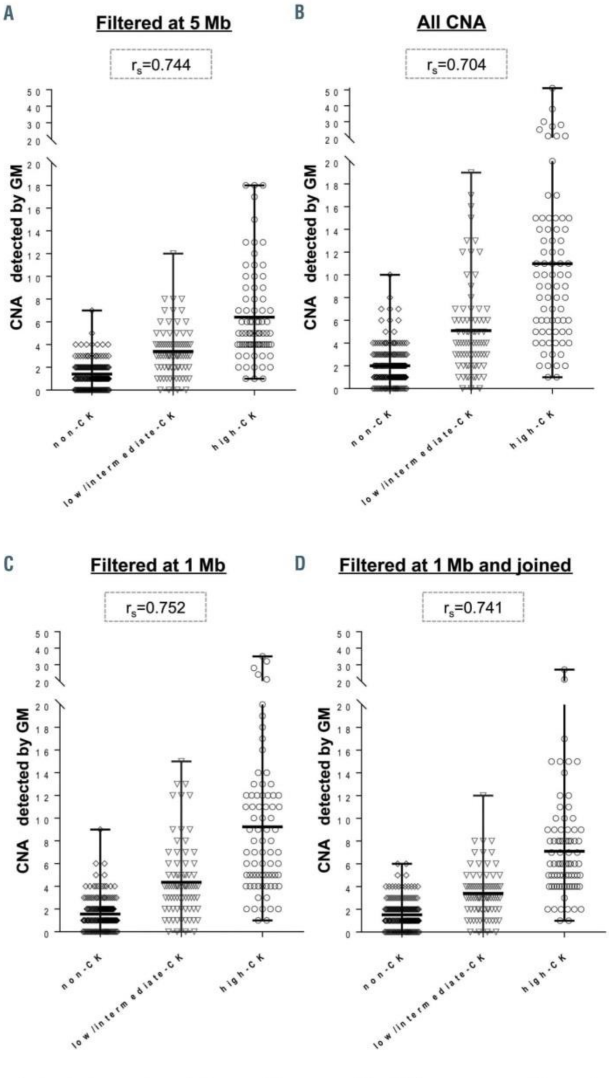

Figure 1. Distribution of the number of copy number abnormalities detected by genomic microarrays among the groups identified by chromosome banding analysis. Patients were divided according to the risk groups defined by chromosome banding analysis (CBA) in non-complex karyotype (non-CK) (0-2 abnormalities [abn.]), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.). Each plot represents copy number abnormality (CNA) counts found when non-classical chronic lymphocytic leukemia (CLL) abnormalities were filtered by different strategies: (A) Current recommended criteria for genomic microarrays (GM) analysis (cut-off size: ≥5 Mb); (B) considering all the CNA irrespectively of their size; (C) considering only those CNA ≥1 Mb; (D) filtering by 1 Mb cut-off and grouping small contiguous abnormalities or considering those CNA included in a chromothripsis event as a unique CNA. Spearman correlation coefficient between CBA and GM counts is shown for each GM analysis.

correlation in the number of abnormalities counted by CBA and GM was achieved. Similar results were observed when including all the abnormalities irrespective of their size, using 1 Mb as a cut-off for non-classical CLL CNA, or if CNA separated by <1 Mb or chromothripsis patterns were counted as one event to evaluate the effect of joining consecutive aberrations (Figure 1).

Parallel analyses of the abnormalities detected by CBA and GM were also performed to identify other potential causes of discrepancy. Among those abnormalities recorded only by CBA, differences were mainly due to the presence of balanced translocations (n=28 patients) or abnormalities represented in a minor proportion of tumor cells probably expanded during the cytogenetics culture which were missed by GM (n=40 patients). Moreover, FISH with chromosome painting probes performed in two high-CK cases by CBA, who showed low-GC, revealed that some apparently unbalanced abnormalities were complex balanced rearrangements that ultimately did not lead to loss of material (*Online Supplementary Figure S2*). On the other hand, when assessed by GM, most of the more complex cases showed aberrations <10 Mb, which is the resolution threshold of CBA, or multiple CNA that corresponded to complex rearrangements recorded as single abnormalities in

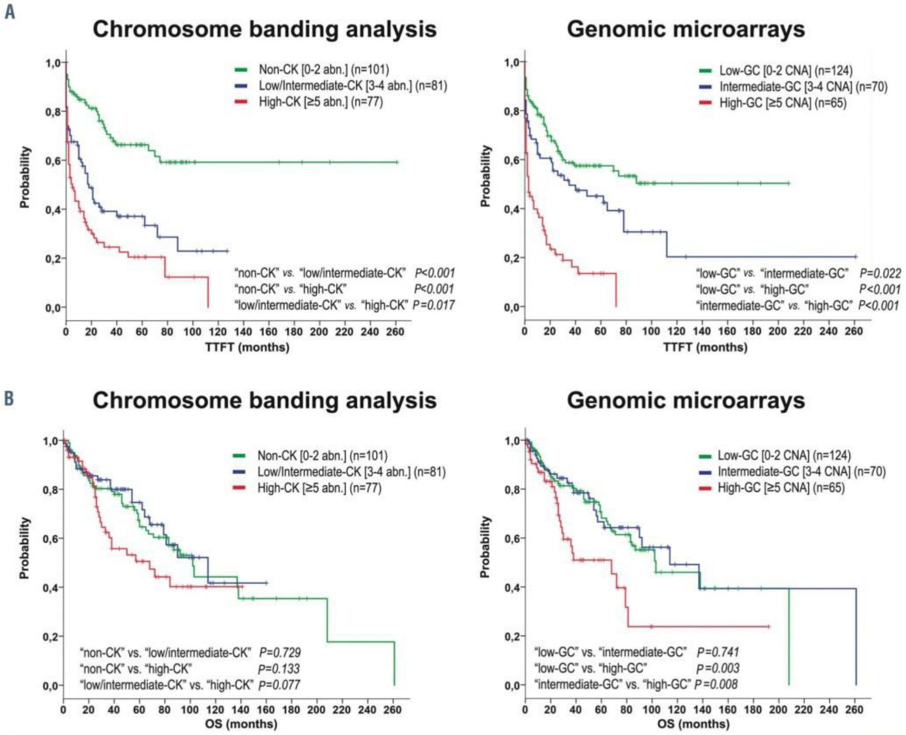

Figure 2. Kaplan-Meier plots for time to first treatment and overall survival based on genomic complexity stratification assessed by chromosome banding analysis and genomic microarrays. Kaplan-Meier estimation for time to first treatment (TTFT) (A) and overall survival (OS) (B) in patients classified in each category based on total number of aberrations found by chromosome banding analysis (CBA): non-complex karyotype (non-CK) (0-2 abnormalities [abn.]), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.) (plots on the left) and based on total number of copy number aberrations (CNA) detected by genomic microarrays (GM): low-genomic complexity (GC) (0-2 CNA), intermediate-GC (3-4 CNA) or high-GC (≥5 CNA)] (plots on the right).

the karyotype (73 and 19 cases, respectively). No division of the tumor clone during the cytogenetics culture is the most feasible explanation for 50 patients who carried CNA ≥10 Mb that should have been identified by CBA, of which 17 presented a normal karyotype. Detailed comparison for the ten patients who only displayed high complexity by one method is shown in the *Online Supplementary Table S4*.

The genetic analysis using both methods allowed the correction of the karyotype in six patients after GM interpretation (*Online Supplementary Table S5*). Although it resulted in a change of the number of abnormalities recorded by CBA for three of them, initial counts were considered for the present analysis.

Prognostic impact of complex karyotype stratification by chromosome banding analysis and genomic microarrays

As previously stated in the ERIC studies, significant differences in terms of TTFT were observed within the three risk groups according to the number of aberrations found by CBA and GM.[5,25] Whereas the highest risk group defined by both techniques displayed a similar short median TTFT (5 and 3 months by CBA and GM, respectively), TTFT was shorter for the intermediate risk group when defined by CBA (18 months *vs.* 35 months) (Figure 2A). Indeed, both methods showed a similar accuracy for predicting TTFT (C-index: 0.67 by CBA *vs.* 0.65 by GM). With regard to OS, only the highest risk groups defined by each technique displayed a poorer outcome (68 months in both cases) (Figure 2B) although differences were only statistically significant in GM defined groups. Equivalent C-indexes were obtained for OS (0.55 by CBA *vs.* 0.57 by GM).

In order to compare the discriminatory power for outcome prediction of both techniques, patients were first classified according to CBA to assess TTFT of GM-defined groups within each category. Of note, those non-CK and low/intermediate-CK patients by CBA who carried ≥5 CNA (high-GC) showed a poor outcome equivalent to that observed in the high-CK by CBA (median TTFT: 2 and 1 months, respectively). However, within the high-CK group, low-GC patients did not show a better evolution (TTFT: 2 months) while cases with intermediate-GC displayed an unexpected median TTFT of 22 months (Figure 3A). When these analyses were performed in the reverse order, CBA

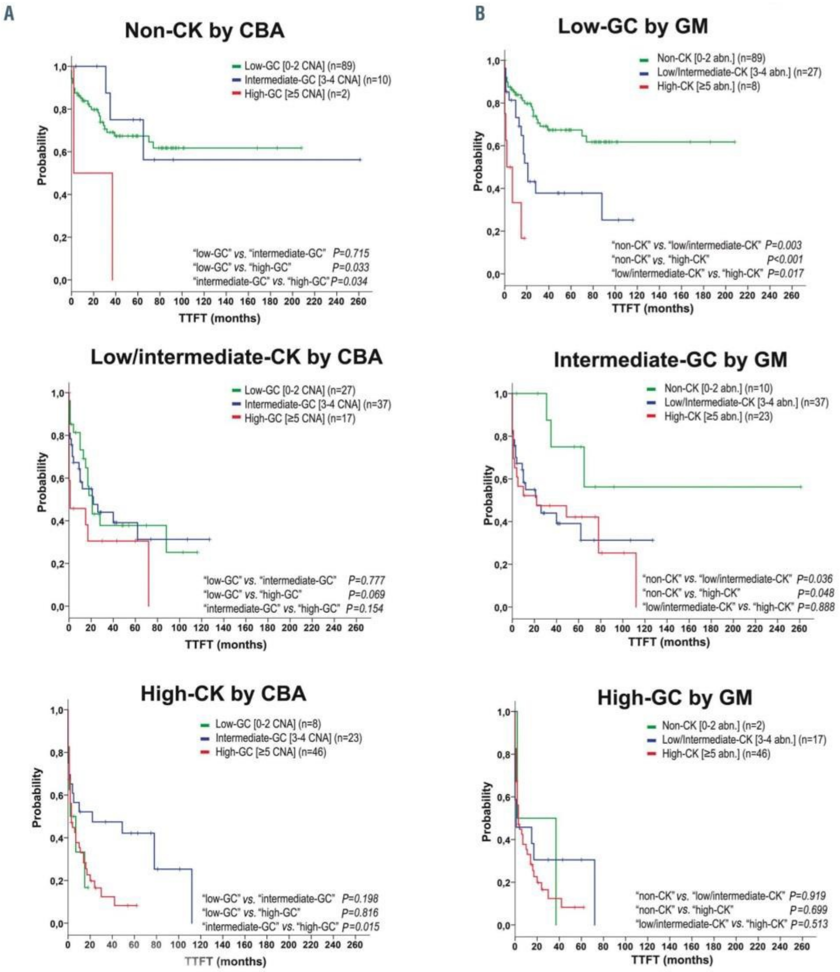

Figure 3. Kaplan-Meier plots for time to first treatment of the genomic risk stratification within each category defined by the alternative technique. (A) Patients classified in each category based on total number of aberrations found by chromosome banding analysis (CBA): non-complex Karyotype (CK) (0-2 abnormalities [abn.]), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.) are represented in different plots. Time to first treatment (TTFT) of genomic microarrays (GM) defined groups was assessed. Within non-CK and low/intermediate-CK, cases classified as high-GC (≥5 copy number abnormalities [CNA] by GM) showed a poor outcome. In the high-CK group, those low-GC patients did not display a better evolution while intermediate-GC cases showed an unexpected median TTFT of 22 months. (B) Each plot represents patients classified in each category based on total number of CNA detected by GM: low-GC (0-2 CNA), intermediate-GC (3-4 CNA) or high-GC (≥5 CNA). Within each subgroup, TTFT of CBA defined groups was assessed. Low-GC patients could be stratified in three risk categories when reclassified by CBA, while no significant differences were observed when intermediate-GC and high-GC subsets were reclassified.

reclassification within the low-GC patients allowed the distinction of three risk categories showing similar outcomes to those observed when applied to the global cohort (*P*<0.001). No significant differences were observed when the intermediate-GC and high-GC categories were reclassified (Figure 3B). It is noteworthy that the ten cases categorized in opposite risk groups displayed the poor prognosis predicted by the technique that classified them in the higher risk category.

Expectedly, the frequency of *TP53* abnormalities (deletions and/or mutations) increased together with the complexity by both methods. In contrast, intermediate and high risk categories showed a similar increased proportion of unmutated IGHV (U-IGHV) and del(11q) compared

Table 4. **Univariate and multivariate analysis for time to first treatment.**

Variable	Univariate analysis		Multivariate analysis for CBA		Multivariate analysis for GM	
	Median TTFT in months (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
CBA						
low/intermediate-CK *vs*. non-CK	18 (11-25) *vs*. NR	<0.001	2.54 (1.47-4.41)	<0.001	-	-
high-CK *vs*. non-CK	5 (1-9) *vs*. NR	<0.001	3.23 (1.81-5.76)	<0.001	-	-
GM						
intermediate-GC *vs*. low-GC	35 (0-74) *vs*. NR	0.022	-	-	1.24 (0.76-2.04)	0.395
high-GC *vs*. low-GC	3 (0-6) *vs*. NR	<0.001	-	-	2.74 (1.61-4.67)	<0.001
Del/mut *TP53*	4 (0-9)	<0.001	1.72 (1.14-2.60)	0.010	1.44 (0.92-2.26)	0.109
U-IGHV	12 (4-20)	<0.001	1.71 (1.12-2.61)	0.012	2.12 (1.39-3.22)	<0.001
del(11)(q22q23)	17 (9-25)	0.111	NA	NA	NA	NA

CBA: chromosome banding analysis; CK: complex karyotype; non-CK: 0-2 abnormalities detected by CBA; low/intermediate-CK: 3-4 abnormalities; high-CK: ≥5 abnormalities; GM: genomic microarrays; GC: genomic complexity; low-GC: 0-2 copy number abnormalities (CNA) detected by genomic microarrays; intermediate-GC: 3-4 CNA; high-GC: ≥5 CNA; del/mut *TP53*: abnormalities in *TP53* include deletion in 17p13 and/or mutation in *TP53* gene; U-IGHV: CLL with unmutated IGHV; NR: not reached; NA: not assessed.

Table 5. **Univariate and multivariate analysis for overall survival.**

Variable	Univariate analysis		Multivariate analysis for GM*	
	Median OS in months (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
CBA				
low/intermediate-CK *vs*. non-CK	114 (65-163) *vs*. 102 (83-121)	0.729	-	-
high-CK *vs*. non-CK	68 (25-111) *vs*. 102 (83-121)	0.133	-	-
GM				
intermediate-GC *vs*. low-GC	114 (64-164) *vs*. 103 (55-151)	0.741	0.69 (0.36-1.34)	0.275
high-GC *vs*. low-GC	68 (32-104) *vs*. 103 (55-151)	0.003	1.51 (0.76-3.01)	0.244
Del/mut *TP53*	50 (29-71)	<0.001	1.89 (1.05-3.42)	0.034
U-IGHV	79 (58-100)	0.008	1.97 (1.15-3.36)	0.013
del(11)(q22q23)	79 (53-105)	0.255	NA	NA

*Multivariate analysis for CBA-defined risk categories was not performed due to the lack of statistical significance in the univariate analysis. OS: overall survival; CBA: chromosome banding analysis; CK: complex karyotype; non-CK: 0-2 abnormalities detected by CBA; low/intermediate-CK: 3-4 abnormalities; high-CK: ≥5 abnormalities; GM: genomic microarrays; GC: genomic complexity; low-GC: 0-2 copy number abnormalities (CNA) detected by genomic microarrays; intermediate-GC: 3-4 CNA; high-GC: ≥5 CNA; del/mut *TP53*: abnormalities in *TP53* include deletion in 17p13 and/or mutation in *TP53* gene; U-IGHV: CLL with unmutated IGHV; NR: not reached; NA: not assessed.

with non-CK/low-GC patients (*Online Supplementary Table S6*). Despite being highly associated with these known prognostic factors, three groups with significant differences on TTFT could be established by CBA and GM when patients were categorized depending on their *TP53* status (*Online Supplementary Figure S3*). Regarding IGHV status, similar results were obtained within the mutated IGHV (M-IGHV) group while no clear discrimination was observed in the U-IGHV subset (*Online Supplementary Figure S4)*. No prognostic impact was observed for del(11q) in the entire cohort (Table 4). High complexity defined by both CBA and GM maintained its significance when a multivariate analysis for TTFT including *TP53* and IGHV status was performed. Conversely, genomic complexity by GM lost its significance in the multivariate analysis for OS (Table 5).

Finally, the impact of other genetic findings was also analyzed. In this regard, the presence of unbalanced rearrangements was associated with shorter TTFT in the entire cohort (11 months *vs*. NR; $P<0.001$) and within the non-CK subgroup (10 months *vs*. NR; $P=0.001$) (*Online Supplementary Figure S5*). A negative impact was also observed for chromothripsis (2 months *vs*. 37 months; $P<0.001$), which was mainly found among CK patients (29 of 30). Indeed, tendency to this worse evolution was maintained within this subset (5 months *vs*. 15 months; $P=0.062$) (*Online Supplementary Figure S6*). As expected, these cases showed a high frequency of abnormalities in *TP53* (22 of 30; 73.3%) and U-IGHV (21 of 29; 72.4%). In the multivariate analysis including these features and genomic complexity, only the latter defined by both CBA and GM retained its negative impact (*Online Supplementary Table S7*).

Discussion

In recent years, there has been a rising interest in identifying CLL patients with CK since they may pursue a more aggressive clinical course and respond less well to treatment.[2,4,28] Large co-operative studies within ERIC have demonstrated that five abnormalities is the optimal cut-off which better predicts an impaired outcome by both CBA and GM.[5,25] However, data comparing the risk stratification based on genomic complexity by both methods in the same patients are scarce. Indeed, a small cohort of 122 patients from the Leeksma *et al.* study was analyzed by GM and CBA, but the proportion of CK cases was very low, as expected in an unselected CLL population. To the best of our knowledge, the present study is the largest report conducted to date in which a cohort of CLL patients enriched in CK cases has been simultaneously analyzed by CBA and GM, comparing the usefulness of both methods in their prognostic stratification.

By clustering patients according to criteria previously defined by ERIC, we confirmed that both techniques did not differ in the proportion of patients classified into each risk category. Notwithstanding, it should be pointed out that only moderate agreement was observed between them. Discordances in the risk assigned to nearly one third of the patients were found, including around 3% of patients classified in either high or low risk groups depending on the methodology employed for their study. We have demonstrated that most of these discordances are the consequence of known limitations intrinsic to each technique. In this regard, the clonal architecture in the sample could mask some alterations by GM, if present in low percentages below their limited sensitivity (~20%), while the CBA result would rely on the *in vitro* proliferative capacity of the altered clones.[29,30] In addition, balanced abnormalities are only detectable by CBA and, on the contrary to expectations, our FISH painting studies confirmed that not all the complex rearrangements described in the karyotype ultimately imply gain or loss of genomic material. On the contrary, some abnormalities recorded as a single monosomy or unbalanced translocation in the karyotype turned out to be multiple CNA or even chromothripsis events when assessed by GM. Thus, our results suggested that discrepancies were not predictable by the type of abnormalities detected by any of the methods. Conversely, we discarded a global underestimation of the genomic complexity associated with the application of the recommended filtering criteria for non-classical CLL CNA by GM.[24] Small abnormalities (<5 Mb) were equally found by GM among concordant and discordant patients, and greater agreement in the number of abnormalities could not be achieved when smaller CNA were also considered. Thus, we have confirmed that the present recommendations for GM analysis are robust for complexity assessment.[24] The observed differences did not represent a poorer performance for CBA or GM in patients risk stratification. In both cases, the established risk groups showed significant differences in terms of TTFT, which were independent of *TP53* and IGHV mutational status. Concerning OS, only high complex groups displayed a dismal evolution. In addition, the heterogeneity regarding the GM platforms employed could be a limitation of this study. However, all GM results were reviewed and uniformly interpreted using the same criteria to filter CNA and similar findings were obtained among different GM companies.

Regarding CBA data, previous publications have investigated whether specific cytogenetic patterns not identifiable by GM (presence of balanced or unbalanced rearrangements) may correlate with dismal outcome. Initial studies suggested that carrying chromosomal translocations was associated with poorer clinical course.[31] More recently, this negative impact has been attributed to the presence of unbalanced rearrangements and its association with CK.[2,32] Indeed, Rigolin *et al.* proved that CK carrying unbalanced rearrangements constituted a very poor risk subset with particular features such as an increased proportion of *TP53* aberrations and a lower frequency of 11q deletions. The presence of these aberrations has also been associated with a deregulated expression of genes involved in cell cycle control and DNA damage response.[14] Visentin *et al.* showed that the combination of the presence of CK and/or unbalanced rearrangements by CBA and IGHV mutational status improved their risk stratification.[15] In our cohort, we observed a shorter TTFT for those patients with unbalanced rearrangements but the poor outcome was not confirmed within CK group. Unexpectedly, GM were unable to detect CNA related to all the apparently unbalanced rearrangements. Indeed, the eight patients with high-CK classified as low-GC by GM carried this type of abnormality and showed a dismal evolution. On the other hand, our GM analyses identified patients with patterns of chromothripsis who showed a short TTFT. As previously reported, there was a high association between chromothripsis and CK or *TP53* aberrations.[19,33,34] Our study is based in a retrospective cohort highly enriched in patients carrying CK, which was necessary to extensively compare both genetic methodologies in the detection of these prognostically relevant highly complex cases. Therefore, as it is not representative of a real-life CLL cohort, it hinders the development of more accurate genetic prognostic scores. Additionally, potential confounding effects of different therapeutic agents could be attributed to the retrospective and multicenter nature of the cohort enriched in treated patients. These could underlie the lack of statistical significance of genome complexity in the analyses for OS.

To date, most of the survival analyses of genomic complexity included in clinical trials have been reported using CBA data. Even though the prognostic/predictive value of CK for TTFT and progression-free survival in patients treated with chemoimmunotherapy has been extensively demonstrated,[2-5,10] its clinical relevance in patients receiving the new treatment modalities has not been fully established. Initial data from clinical trials with novel agents, mainly developed in older relapsed/refractory and/or in high risk patients (*TP53* del/mut, U-IGHV) suggested an adverse significance of CK.[6-8,35] In contrast, a number of recent studies including extended follow-ups of older trials, pooled analyses or new drug combinations have not confirmed its adverse significance.[36-43] However, most of these studies have analyzed CK impact taking into account patients with ≥3 aberrations but not those with high complexity (≥5 aberrations), compared a low number of patients and showed relatively short follow-ups.[44] Thus, additional analyses are needed to clarify the prognostic/predictive impact of genomic complexity.

One particular finding of this study is that, even though overall concordance between FISH and GM is high (90%), GM do not detect around 20% of cases with *TP53* deletion due to their low sensitivity.[23,29] The presence of 17p13 deletions and/or mutations in *TP53* predicts the poorest outcome and its assessment is currently mandatory in CLL study. Our results confirm that FISH should be maintained for the study of CLL patients complemented with one genome-wide technique to assess genome complexity for risk stratification. The choice between CBA and GM will depend on each laboratory, which should take into account the methods and equipment availabilities, personnel expertise and the economic costs, among others.

In conclusion, we have confirmed that both CBA and GM are valuable tools to assess the prognosis of CLL patients based on genomic complexity. Nevertheless, a considerable proportion of cases are discordantly classified depending on the technique employed. Consequently, previous findings generated from CBA, currently the gold standard for cytogenetic assessment, are not directly applicable to GM or other promising cytogenomic methodologies such as optical genome mapping. Additional validation studies are needed to establish the prognostic value of genomic complexity by GM in future prospective studies and clinical trials.

Disclosures

No conflicts of interest to disclose.

Contribution

AP and BE designed the research study; SR, AP and BE provided patients data, analyzed the data and wrote the manuscript; SBo and JS performed a great proportion of genomic microarrays from cases which lacked this information; SBo, JS and SBe were involved in analysis, interpretation and critical discussion of the results; SBe, MJL, DC, HP, GMR, MO, MLB, RC, RS, TB, EG, CM, FB, XC, MJC, AC, JCS, FNK, DO and CH provided patient data and samples. All authors read the last version of the manuscript.

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Data sharing statement

Detailed chromosome banding analyses and genomic microarrays profiles for selected cases are provided in the Online Supplementary Tables. Please contact either bespinet@parcdesalutmar.cat or apuiggros@imim.es for additional data.

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Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients

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SUPPLEMENTARY DATA

Supplementary Methods

Patient cohort

Patients were diagnosed between 1983 and 2018 according to current guidelines.[1-4] Clinical information collected at diagnosis included demographics (age and gender), Binet stage, genetic and molecular data. Regarding information on evolution, dates of treatment administration and last follow-up were collected. Of note, data from CBA or GM of some patients have been included in previous publications although they were not used with the same purpose as the present study.[5-13]

Cytogenetic analyses

Peripheral blood (PB) or bone marrow (BM) samples cultures using either phorbol-12-myristate-13-acetate (TPA) (n=228; 67%), immunostimulatory cytosine guanine dinucleotide (CpG)-oligonucleotide DSP30 plus interleukin 2 (IL-2) (n=19; 5.6%) or both (n=93; 27.4%) as mitogens were established following standard procedures.[14] At least 20 metaphases were analyzed in cases with normal karyotype while for abnormal karyotypes, the minimum were 10. Number and type of abnormalities were recorded. Balanced rearrangements included translocations and inversions, while chromosome additions, duplications, insertions, isochromosomes, as well as derivative, dicentric, ring and marker chromosomes were considered unbalanced rearrangements and were counted as one aberration.

Interphase fluorescence *in situ* hybridization (FISH) results were available in 320/340 (94.1%) cases using probes for the chromosomal regions 13q14, 11q22 (*ATM*) and 17p13 (*TP53*) and the centromere of chromosome 12 (CEP 12). In five cases, whole chromosome painting was performed in order to study the discrepancies observed between CBA and GM.

Genomic microarray analyses

Genomic microarrays data were already available or obtained from DNA extracted in a period of time less than one year from the date of CBA in order to avoid the emergence of additional abnormalities (median time from CBA to GM=0 months; range: 0-12). GM were assessed on DNA from whole PB (n=113; 33%), PB mononuclear cells (n=63; 19%), PB CD19+ purified cells (n=110; 32%) or from BM samples (n=54; 16%). Only DNA that fulfilled quality controls required was amplified, labelled and hybridized to different genomic microarray platforms according to the manufacturer's protocols. Obtained data were visually revised and copy number variants found as benign polymorphisms in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) were excluded. For defining genome coordinates, annotations of genome version GRCh37/hg19 were used. Chromothripsis-like and chromothripsis patterns were defined by the presence of ≥7 and ≥10 oscillating switches, respectively, between two or three copy number states on an individual chromosome.[7,8,15]

Although the objectives of the study did not consider the analysis of copy-number neutral loss of heterozygosity (CN-LOH), in those cases in which the microarray platform included single nucleotide polymorphisms (SNP) probes, a global screening for CN-LOH was performed. CN-LOH were recorded when detected in a region larger than 10Mb and extending to chromosome telomeres. They were not included in the counting of CNAs.

TP53 mutation analysis

A total of 308 (90.6%) cases were screened for *TP53* mutations. For the assessment of *TP53* mutations exons 4-8 were sequenced (exons 9-10 were also included in some centers) following ERIC recommendations.[16] Sixty (19.5%) cases were screened by Sanger sequencing whereas the remaining (n=248; 80.5%) were analyzed by next-generation sequencing. Only mutations with a variant allele frequency >10% were considered.

IGHV mutational analysis

IGHV mutational status was analyzed in 307 (90.3%) patients following established international guidelines.[17] Sequences were examined and interpreted using the IMGT database and the IMGT/V-QUEST tool. Clonotypic IGHV gene sequences with <98% germline identity were defined as mutated (M-IGHV) whereas those with ≥98% identity were classified as unmutated (U-IGHV).

Statistical analyses

As different European centers were involved in the present study, before performing the survival analyses we evaluated the homogeneity of the results in terms of time to first treatment (TTFT). We found out that in three institutions, TTFT in the non-CK group was notably shorter than previously reported in other studies[11] because CBA in these centers were mainly performed at recruitment for clinical trials. Therefore, in order to avoid biases in the results reported herein, 81 cases were not included in the survival analyses. As for the CK group, no differences were observed between the collaborating centers. Consequently, survival analyses were performed in 259 patients.

Supplementary Results

Risk stratification of the genomic complexity observed by CBA and GM

Regarding CBA, when results obtained with each mitogen were considered separately, those cases stimulated with IL-2+DSP30 exhibited a higher proportion of complex cases. Significant differences were observed in the percentage of patients classified into intermediate-risk categories (3-4 abnormalities; 20.6% with TPA vs. 32.1% with IL-2+DSP30) or those showing the highest risk (≥5 abnormalities; 14.9% and 27.7%, respectively) (p<0.001). However, when comparing with GM classification, both methods presented a similar moderate agreement (TPA: κ=0.464; IL-2+DSP30: κ=0.530).

Number and type of abnormalities detected by CBA and GM

Regions with CN-LOH were detected in 23 (7.5%) patients as the microarray platform used in 306 cases also contained SNP probes. Median size of CN-LOH was 50.1Mb (range: 11.9-159Mb) and they were found in several chromosomes. Notably, two of the three cases with CN-LOH affecting 17p arm and the only case with CN-LOH involving *ATM* gene had *TP53* and *ATM* genes mutated, respectively. Nevertheless, CN-LOH data were not included in the analyses.

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Supplementary Tables

Table S1. Genomic microarray platforms used in this study.

Genomic microarray platform	**n (%)**
Whole-Genome 2.7M (ThermoFisher)	62 (18.2)
CytoScan HD array (ThermoFisher)	87 (25.6)
Affymetrix SNP6.0 (ThermoFisher)	82 (24.1)
SurePrint G3 Human CGH 8x60K (Agilent)*	11 (3.2)
SurePrint G3 ISCA CGH+SNP Bundle, 4x180K (Agilent)	75 (22.1)
Illumina Human Omni1-Quad array (Illumina)	12 (3.5)
Illumina Human Omni2.5-Quad array (Illumina)	11 (3.2)

*Custom design described in Salaverria I, Martín-Garcia D, López C, et al. Detection of chromothripsis-like patterns with a custom array platform for chronic lymphocytic leukemia. Genes Chromosomes Cancer. 2015;54(11):668-80.

Table S2. Detection of the four classical CLL abnormalities by chromosome banding analysis in those patients with FISH results available.

	FISH		CHROMOSOME BANDING ANALYSIS				
Locus affected	**Patients tested**	**Altered cases** *n* (%)	**Abnormalities in CLL *loci*** *n* (%)	**Detail of the abnormalities**	n (%)	**Confirmed by FISH**	**Overall concordance**
13q14	327	188 (57.5)	97 (29.7)	Monosomy 13	13 (13.4)	12/13 (92.3%)	50.0%
				Deletion 13q14	66 (68.0)	64/66 (97.0%)	
				Balanced translocation involving 13q14	9 (9.3)	9/9 (100%)	
				Unbalanced translocation in 13q14	9 (9.3)	9/9 (100%)	
Chromosome 12	327	56 (17.1)	54 (16.5)	Trisomy 12	54 (16.5)	54/54 (100%)	96.4%
11q22q23 (*ATM*)	329	71 (21.6)	67 (20.4)	Monosomy 11	1 (1.5)	1/1 (100%)	87.3%
				Deletion 11q22q23	58 (86.6)	55/58 (94.8%)	
				Additional material in 11q22q23	1 (1.5)	1/1 (100%)	
				Balanced translocation involving 11q22q23	2 (2.9)	1/2 (50.0%)	
				Unbalanced translocation in 11q22q23	5 (7.5)	4/5 (80.0%)	
17p13 (*TP53*)	329	69 (21.0)	67 (20.4)	Monosomy 17	14 (20.9)	13/14 (92.9%)	91.3%
				Deletion 17p13	10 (14.9)	10/10 (100%)	
				Additional material in 17p	16 (23.9)	14/16 (87.5%)	
				Isochromosome (17)(q10)	5 (7.5)	5/5 (100%)	
				Dicentric chromosomes affecting 17p	4 (5.9)	4/4 (100%)	
				Unbalanced translocation in 17p	18 (26.9)	17/18 (94.4%)	

Table S3. Detection of the four classical CLL abnormalities by genomic microarrays in those patients with FISH results available.

	FISH		GENOMIC MICROARRAYS				
Locus affected	**Patients tested**	**Altered cases** n (%)	**Altered cases** n (%)	**Median size** Mb (range)	**Minimal abnormal region** Cytobands (coordinates: GRCh37/hg19)	**Overall concordance**	**Detail of discordant cases**
13q14	327	188 (57.5)	171 (52.3)	1.94 (0.035-95.7)	q14.2-q14.2 (50,632,951 - 50,659,544)	88.8% (167/188)	- 21 only positive by FISH (20/21 <30% nuclei) - 4 only positive by GM
Chromosome 12	327	56 (17.1)	55 (16.8)	133.60 (132.3-133.8)	p13.33-q24.33 (192,539 - 132,349,534)	98.2% (55/56)	- 1 only positive by FISH (7% nuclei)
11q22q23 (*ATM*)	329	71 (21.6)	68 (20.7)	25.81 (0.151-54.5)	q22.3-q22.3 (108,125,328-108,276,581)	91.5% (65/71)	- 6 only positive by FISH (5/6 <30% nuclei) - 3 only positive by GM
17p13 (*TP53*)	329	69 (21.0)	58 (17.6)	21.47 (0.470-25.9)	p13.1-p13.1 (7,481,305-7,678,604)	82.6% (57/69)	- 12 only positive by FISH (11/12 <20% nuclei) - 1 only positive by GM

Table S4. Abnormalities detected by chromosome banding analysis and genomic microarrays in the ten patients classified in opposite risk categories depending on the technique employed for their study.

Case	CHROMOSOME BANDING ANALYSIS		GENOMIC MICROARRAYS					
	Karyotype	Number of aberrations	Type	Chr.	Start-end	Size (Mb)	Number of CNA	Number of CNA ≥5Mb
#37	47,XY,add(8)(p23),-9,add(12)(q24),-13,-18,+4mar[41]/46,XY[8]	9	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	2	1
			GAIN	18	p11.31-p11.23 (6929190-8087455)	1,158		
#38	47,XY,-4,del(7)(p?),+12,add(15)(q26),+mar[20]/47,XY,+12[20]/ 46,XY[10]	5	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	1	1
#100	46,XY,add(19)(q13.4)[25]/45,XY,der(2)t(2;6)(p23;p12),del(6)(q?), -20,del(21)(q22)[5]/46,XY[21]	5	GAIN	2	p25.3-p13.3 (0-70151030)	70,151	1	1
#119	46,XX,i(17)(q10)[7]/45,XX,-13,add(14)(q11),i(17)(q10)[4]/ 44,XX,del(6)(q14q24),add(7)(q36),-12,-15,i(17)(q10)[1]/ 43,XX,-5,del(6)(q14q24),add(7)(q36),add(10)(q22),-12, -15,i(17)(q10)[7]/46,XX[4]	9	GAIN	17	q11.1-q25.3 (25270397-81041938)	55,772	2	2
			LOSS	17	p13.3-p11.1 (525-22261792)	22,261		
#121	47,XY,+12[9]/47,XY,del(X)(q25),add(5)(q31),add(8)(q24),+12, del(14)(q22q32)[4]/46,XY[7]	5	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	2	2
			LOSS	14	q23.2-q32.12 (63953105-93505497)	29,552		
#152	46,XX,del(14)(q24q32)[1]/44,XX,der(4;21)(q10;q10),del(14)(q24q32), der(15;22)(q10;q10)[5]/43,XX,der(1)t(1;17)(p11;q11), der(4;21)(q10;q10),der(11)t(1;11)(p11;q25),del(14)(q24q32), der(15;22)(q10;q10),-17[5]/ XY 46,XX[6]	6	LOSS	4	p16.3-p14 (0-39309957)	39,310	4	2
			LOSS	4	p12-p11 (45427534-49174296)	3,747		
			LOSS	4	q11-q12 (52697788-55087565)	2,390		
			LOSS	14	q24.1-q32.33 (69262059-106067093)	36,805		
#180	46,Y,der(X)t(X;2)(q26;p15)[5]/46,XY,der(2)t(2;2)(p24;p15)[5]/ 46,XY,der(5)t(2;5)(p15;q35),r(8)(p11q24),der(11)t(5;11)(?;q24)t(2;5) (p15;?)[4]/46,XY,der(6)t(2;6)(p15;q27)[2]/46,XY[14]	6	GAIN	2	p25.3-p15 (0-62206329)	62,206	2	2
			LOSS	13	q14.2-q14.3 (49874813-51747327)	1,873		
#228	45,X,-Y,del(11)(q?14),del(13)(q14q22)[7]/ 44,idem,der(12)t(12;13)(p?13;q?12)[5]/45,idem,t(1;11)(p?36;q13)[3]/ 46,XY[2]	5	LOSS	11	q14.1-q23.3 (79261152-116172518)	36,911	3	2
			LOSS	13	q14.11-q21.33 (43292880-72523559)	29,231		
			LOSS	X	q27.3-q27.3 (145094655-145177733)	0,083		

#c362	44,XY,der(5;17)t(5;17)ins(5;17)(q1?3;p11q25)[2]/46,XY[12]	1	LOSS	5	q12.3-q13.1 (64558088-66809076)	2,251	10	7
			LOSS	5	q13.2-q23.1 (70908308-119198005)	48,290		
			LOSS	5	q31.3-q32 (144329951-149326255)	4,996		
			LOSS	5	q33.1-q33.2 (149834719-155509902)	5,675		
			LOSS	11	q22.3-q23.3 (105315158-115742438)	10,427		
			LOSS	13	q14.2-q14.3 (48675135-51631607)	2,956		
			LOSS	17	p13.3-p11.2 (1-19149275)	19,149		
			LOSS	17	q11.1-q11.2 (25270425-26179601)	0,909		
			LOSS	18	p11.32-p11.21 (454728-14318059)	13,863		
			LOSS	20	p13-p11.1 (60001-26107860)	26,048		
#c377	46,XY[29]	0	LOSS	1	q42.12-q42.12 (225692953-225845385)	0,152	7	5
			LOSS	1	q42.12-q44 (225961441-249224401)	23,263		
			LOSS	4	p16.3-p15.2 (10001-27127332)	27,117		
			LOSS	4	p14-q21.21 (39184089-81142337)	41,958		
			LOSS	4	q31.3-q31.3 (153332112-154672325)	1,340		
			LOSS	13	q14.13-q14.3 (46725019-52636989)	5,912		
			LOSS	13	q14.2-q14.3 (50339821-51605362)	1,266		

Those CNA highlighted in grey were non-classical CLL abnormalities smaller than 5Mb.

Table S5. Initial and rewritten karyotypes from six patients in which the formula was modified after genomic microarrays analysis.

Case	Initial karyotype	N abn.	Abnormalities by genomic microarrays				Rewritten karyotype	N abn.
			Type	Chr.	Start-end	Size (Mb)		
#16	45,XY,-13,add(15)(p11),der(17)t(13;17)(q11;p11)[10]/ 46,XY[10]	3	GAIN	3	q11.1-q29 (93626178-197851986)	104,226	45,XY,-13,der(15)t(3;15)(q11;p11), der(17)t(13;17)(q11;p11)[10]/46,XY[10]	3
			LOSS	3	p26.3-p26.1 (311066-6061949)	5,751		
			LOSS	13	q14.2-q14.3 (50595391-51485770)	0,890		
			GAIN	15	q22.2-q26.3 (60417426-102345371)	41,928		
			LOSS	17	p13.3-p11.1 (9474-22227062)	22,218		
#43	47,XX,del(11)(q23),-14,+2mar[10]/46,XX[20]	4	GAIN	2	p25.3-p11.2 (12770-89129064)	89,116	47,XX,+i(2)(p10),del(11)(q23),del(13)(q14q22)[10]/ 46,XX[20]	3
			LOSS	11	q14.1-q23.3 (77108160-117201998)	40,094		
			LOSS	13	q14.2-q22.3 (49894796-77764277)	27,869		
			LOSS	14	q23.2-q24.1 (64199833-69665479)	5,466		
#48	47,XY,der(12)(q?),+der(12)(q?),del(13)(q?)[30]	3	GAIN	12	p13.33-q23.2 (173786-102013163)	101,839	47,XY,der(12)(q?),+der(12)t(12;13)(q23;q21), del(13)(q14q21)[30]	3
			GAIN	13	q21.32-q34 (67265752-115107733)	47,842		
			LOSS	13	q14.13-q21.31 (46950690-63774667)	16,824		
			LOSS	17	p12-p12 (14000097-14622477)	0,622		
#58	46,XY,-5,-6,-14,+3mar[9]/46,XY[11]	6	LOSS	5	q14.2-q23.1 (82410385-115495863)	33,085	46,XY,del(5)(q14q23),der(6)inv(6)(?),del(13)(q12q14)[9]/ 46,XY[11]	3
			LOSS	13	q14.2-q14.3 (50140480-51426156)	1,286		
			LOSS	13	q12.3-q14.3 (32145265-52311881)	20,167		
#61	43-45,X,-X,del(2)(p15),+4,-7,add(11)(q21),-12,-13, add(14)(q32),add(17)(p11)[cp6]/46,XX[9]	9	GAIN	2	p25.3-p13.1 (12770-73803026)	73,790	45,X,-X,del(2)(p15),+4,der(7)t(2;7)(p13;q36), del(11)(q21q23),-12,del(13)(q14),der(14)t(2;14)(p13;q32), dic(17;18)(p10;p10),+3mar[6]/46,XX[9]	12
			LOSS	11	q21-q23.2 (95086750-112810693)	17,724		
			LOSS	13	q14.2-q14.3 (50691182-51659251)	0,968		
			LOSS	17	p13.3-p11.2 (525-21565553)	21,565		
			LOSS	18	p11.32-p11.21 (136226-13719291)	13,583		
#62	46,XX,t(2;5)(p16;p15),del(4)(q31),add(15)(q26)[6]/ 46,XX[18]	3	No aberrations				46,XX,t(2;15)(p16;q26),t(4;5)(q31;p15)[3]/46,XX[18]	2

Abbreviations: Abn.= Abnormalities, Chr.= Chromosome

Table S6. Frequency of different genetic features in the three subgroups defined by chromosome banding analysis and genomic microarrays (n=259)

	CHROMOSOME BANDING ANALYSIS					GENOMIC MICROARRAYS				
	Non-CK	**Low/ intermediate-CK**	**High-CK**	***p*-value**	***p*-value** low/int vs high-CK	**Low-GC**	**Intermediate-GC**	**High-GC**	***p*-value**	***p*-value** intermediate vs high-GC
Known CLL genetic prognostic factors										
Del(13)(q14)	63 (62.4%)	47 (58.0%)	49 (63.6%)	0.744	0.470	68 (54.8%)	50 (71.4%)	41 (63.1%)	0.071	0.301
Trisomy 12	18 (17.8%)	19 (23.5%)	8 (10.4%)	0.095	0.029	28 (22.6%)	15 (21.4%)	2 (3.1%)	0.002	0.001
Del(11)(q22)	12 (11.9%)	26 (32.1%)	23 (29.9%)	0.002	0.762	13 (10.5%)	28 (40.0%)	20 (30.8%)	<0.001	0.263
Del(17)(p13)/mutation *TP53* (n=239)	7 (8.4%)	23 (28.7%)	47 (61.8%)	<0.001	<0.001	11 (10.3%)	21 (30.9%)	45 (70.3%)	<0.001	<0.001
U-IGHV (n=227)	25 (28.1%)	42 (61.8%)	50 (71.4%)	<0.001	0.229	40 (36.7%)	35 (60.3%)	42 (70.0%)	<0.001	0.271
Type of abnormality by CBA										
Unbalanced rearrangements	3 (3.0%)	58 (71.6%)	76 (98.7%)	<0.001	<0.001	26 (21.0%)	49 (70.0%)	62 (95.4%)	<0.001	<0.001
Presence of material from unknown origin	1 (1.0%)	32 (39.5%)	45 (58.4%)	<0.001	0.017	15 (12.1%)	26 (37.1%)	37 (56.9%)	<0.001	0.021
Clonal evolution	1 (1.0%)	40 (49.4%)	41 (53.2%)	<0.001	0.627	20 (16.1%)	29 (41.4%)	33 (50.8%)	<0.001	0.277
Type of abnormality by GM										
Common CNA										
Gain 2p	2 (2.0%)	12 (14.8%)	27 (35.1%)	<0.001	0.003	3 (2.4%)	19 (27.1%)	19 (29.2%)	<0.001	0.788
Loss 3p	1 (1.0%)	5 (6.2%)	8 (10.4%)	0.021	0.335	3 (2.4%)	3 (4.3%)	8 (12.3%)	0.015	0.089
Gain 3q	0	6 (7.4%)	5 (6.5%)	0.024	0.822	0	4 (5.7%)	7 (10.8%)	0.002	0.283
Loss 4p	1 (1.0%)	7 (8.6%)	8 (10.4%)	0.019	0.708	1 (0.8%)	2 (2.9%)	13 (20.0%)	<0.001	0.002
Loss 6q	1 (1.0%)	5 (6.2%)	10 (13.0%)	0.004	0.144	1 (0.8%)	5 (7.1%)	10 (15.4%)	<0.001	0.128
Loss 8p	0	6 (7.4%)	10 (13.0%)	0.001	0.245	0	5 (7.1%)	11 (16.9%)	<0.001	0.079
Gain 8q	1 (1.0%)	8 (9.9%)	9 (11.7%)	0.010	0.713	0	4 (5.7%)	14 (21.5%)	<0.001	0.007
Loss 14q	2 (2.0%)	4 (4.9%)	9 (11.7%)	0.021	0.123	5 (4.0%)	4 (5.7%)	6 (9.2%)	0.348	0.521
Loss 15q	0	5 (6.2%)	11 (14.3%)	<0.001	0.091	1 (0.8%)	3 (4.3%)	12 (18.5%)	<0.001	0.009
Gain 17q	0	3 (3.7%)	9 (11.7%)	0.001	0.058	1 (0.8%)	3 (4.3%)	8 (12.3%)	0.002	0.089
Loss 18p	3 (3.0%)	6 (7.4%)	18 (23.4%)	<0.001	0.005	2 (1.6%)	8 (11.4%)	17 (26.2%)	<0.001	0.028
Gain 19q	1 (1.0%)	6 (7.4%)	6 (7.8%)	0.059	0.927	1 (0.8%)	8 (11.4%)	4 (6.2%)	0.004	0.282
Chromothripsis	1 (1.0%)	7 (8.6%)	22 (28.6%)	<0.001	0.001	1 (0.8%)	6 (8.6%)	23 (35.4%)	<0.001	<0.001

Abbreviations: CK = complex karyotype, non-CK = 0-2 abnormalities detected by chromosome banding analysis, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, U-IGHV = CLL with unmutated IGHV

Table S7. Univariate and multivariate analysis for time to first treatment (TTFT)

Variable	**Univariate analysis**		**Multivariate analysis for CBA**		**Multivariate analysis for GM**	
	Median TTFT in months (95% CI)	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**
CBA						
low/intermediate-CK vs. non-CK	18 (11-25) vs. NR	<0.001	2.85 (1.53-5.31)	<0.001	-	-
high-CK vs. non-CK	5 (1-9) vs. NR	<0.001	4.54 (2.18-9.44)	<0.001	-	-
GM						
intermediate-GC vs. low-GC	35 (0-74) vs. NR	0.022	-	-	1.60 (1.05-2.43)	0.029
high-GC vs. low-GC	3 (0-6) vs. NR	<0.001	-	-	3.52 (2.27-5.46)	<0.001
Unbalanced rearrangements	11 (5-17)	<0.001	0.97 (0.53-1.77)	0.916	-	-
Chromothripsis	2 (0-6)	<0.001	-	-	1.35 (0.83-2.20)	0.228

Abbreviations: CBA = chromosome banding analysis, CK = complex karyotype, non-CK = 0-2 abnormalities detected by CBA, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GM = genomic microarrays, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, CI = confidence interval, NR = not reached.

Supplementary Figures

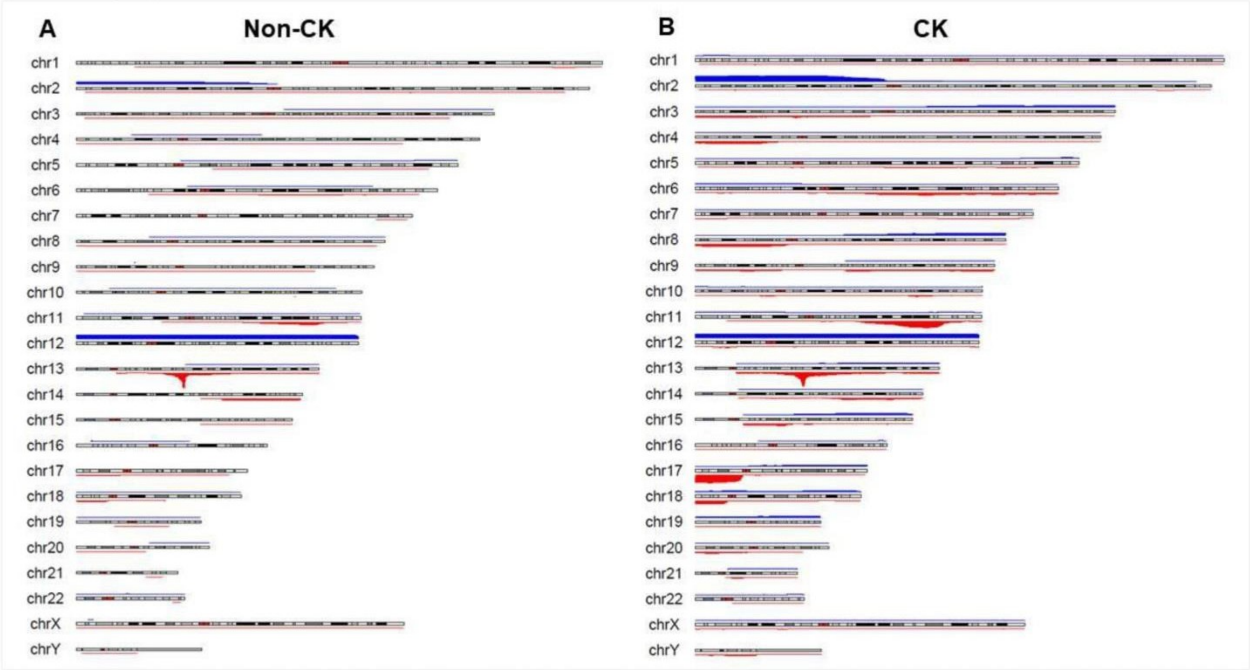
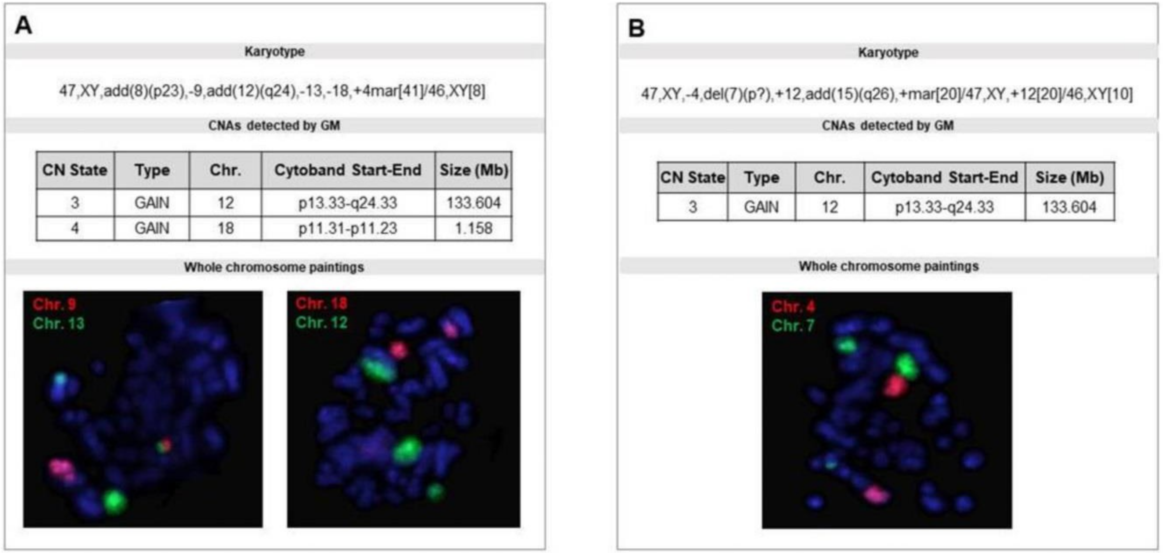


Figure S1. **Distribution of the copy number aberrations detected by genomic microarrays in non-CK and CK groups.** (A) Non-CK subgroup (0-2 abnormalities), (B) CK subgroup (≥3 abnormalities). Gains are represented in blue above and losses in red below the affected chromosomal regions. The thickness of the bars represents the number of cases showing the respective gain or loss. Figures were created by using KaryoploteR package of R.


A
Karyotype
CNAs detected by GM
Whole chromosome paintings
Chr. 9
Chr. 13
Chr. 18
Chr. 12
B
Karyotype
CNAs detected by GM
Whole chromosome paintings
Chr. 4
Chr. 7

47,XY,add(8)(p23),-9,add(12)(q24),-13,-18,+4mar[41]/46,XY[8]

CN State	Type	Chr.	Cytoband Start-End	Size (Mb)
3	GAIN	12	p13.33-q24.33	133.604
4	GAIN	18	p11.31-p11.23	1.158

47,XY,-4,del(7)(p?),+12,add(15)(q26),+mar[20]/47,XY,+12[20]/46,XY[10]

CN State	Type	Chr.	Cytoband Start-End	Size (Mb)
3	GAIN	12	p13.33-q24.33	133.604

Figure S2. Whole chromosome painting FISH images of two high-CK cases classified as low-GC by genomic microarrays. (A) Nine aberrations were detected by CBA while only two were observed by GM. FISH was performed using chromosome painting probes for chromosomes 9 (red) and 13 (green), on the left image, and for chromosomes 12 (green) and 18 (red), on the right image. FISH revealed that chromosomes apparently lost in the karyotype appeared to be fragmented, either constituting the additional material of other chromosomes or being part of marker chromosomes. (B) Five aberrations were detected by CBA while only gain of chromosome 12 was detected by GM. FISH was performed using chromosome painting probes for chromosomes 4 (red) and 7 (green). According to FISH images, both chromosomes were present in the analyzed metaphases but were fragmented (chr.7) or considered as marker chromosomes (chr.4). Chromosomes were stained with DAPI.

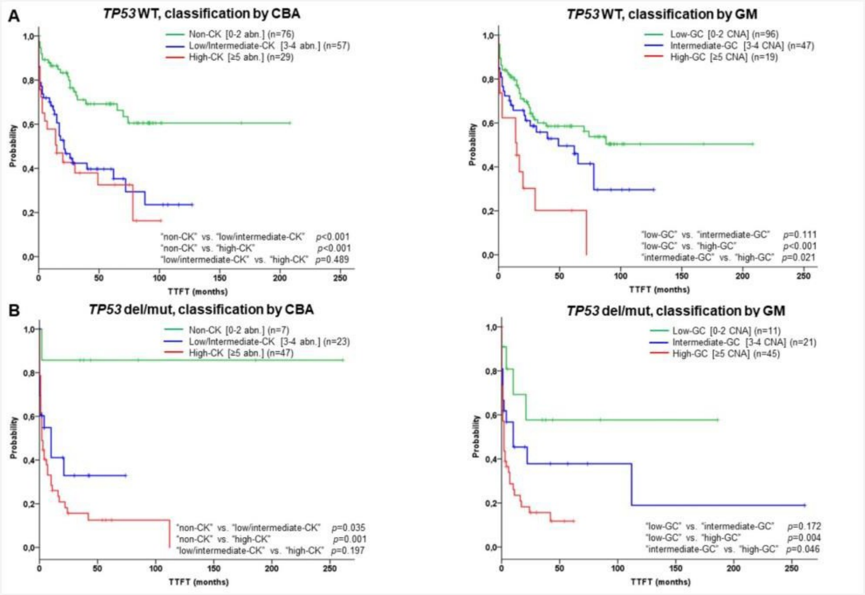


Figure S3. Effect on TTFT of risk categories defined by chromosome banding analysis and genomic microarrays in patients with abnormal *TP53* (deleted and/or mutated). Kaplan-Meier estimation for TTFT in risk categories defined by CBA (plots on the left) or GM (plots on the right) in patients with normal *TP53* (A) and in patients with deleted and/or mutated *TP53* (B).

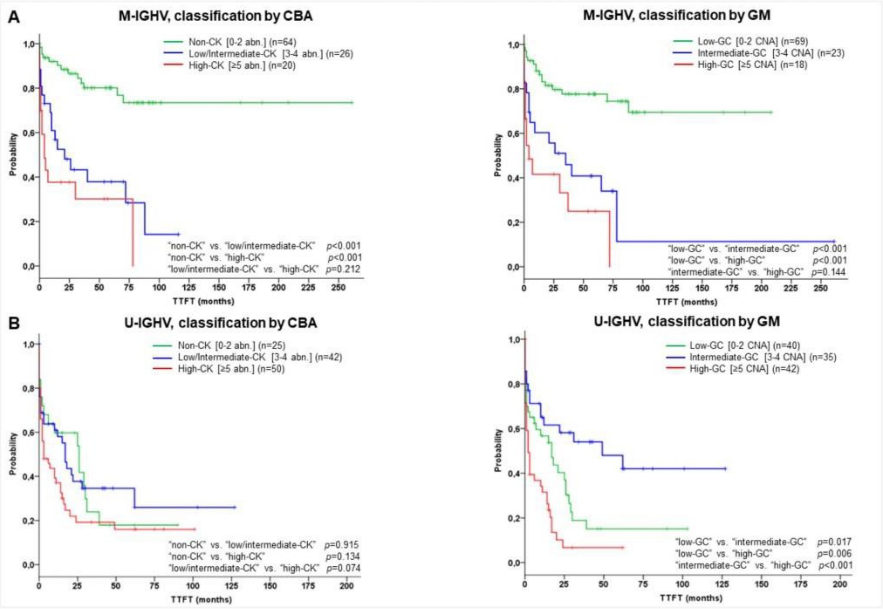


Figure S4. Effect on TTFT of risk categories defined by chromosome banding analysis and genomic microarrays in patients with M-IGHV or U-IGHV. Kaplan-Meier estimation for TTFT in risk categories defined by CBA (plots on the left) or GM (plots on the right) in patients with M-IGHV (A) and in patients with U-IGHV (B).

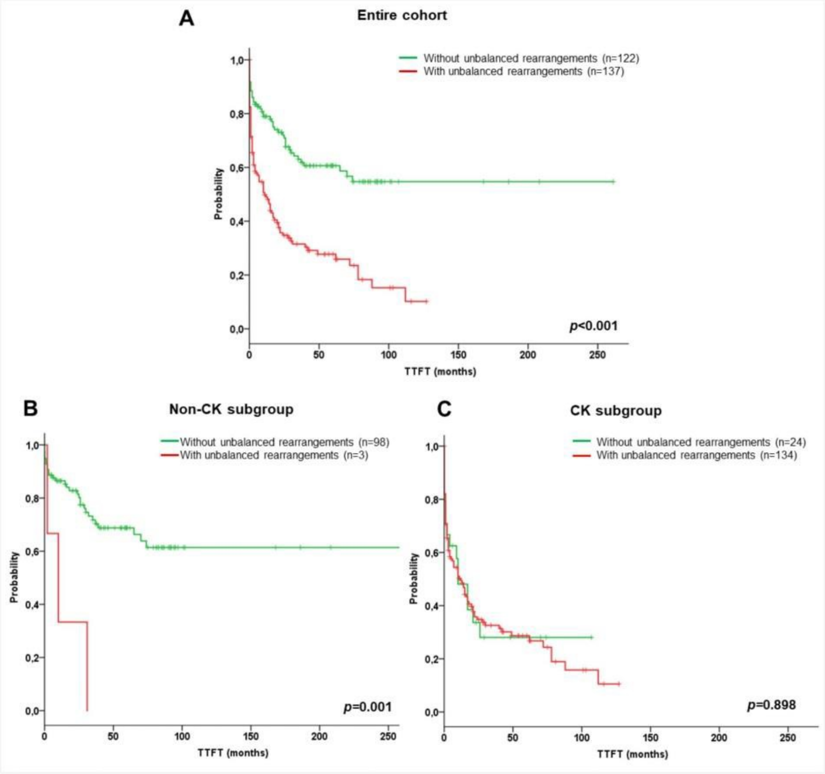


Figure S5. Effect on TTFT of unbalanced rearrangements detected by chromosome banding analysis in the entire cohort and within the non-CK and CK subgroups. Kaplan-Meier estimation for TTFT in patients with and without unbalanced rearrangements in the entire cohort (A) and in non-CK (B) and CK subgroups (C).

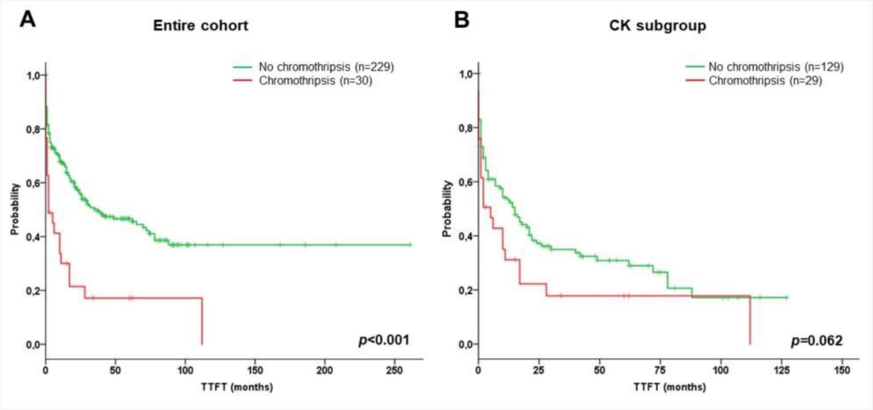


Figure S6. Effect on TTFT of chromothripsis in the entire cohort and within the CK subgroup. Kaplan-Meier estimation for TTFT in patients with and without chromothripsis in the entire cohort (A) and in CK subgroup (B). Survival plot for non-CK subgroup is not shown as only one case displayed chromothripsis.

ARTÍCULO 2

TP53 abnormalities are underlying the poor outcome associated with chromothripsis in chronic lymphocytic leukemia patients with complex karyotype

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La cromotripsis se ha asociado a un curso clínico desfavorable y a factores de mal pronóstico en pacientes con leucemia linfocítica crónica. A pesar de estar relacionada con una elevada inestabilidad genómica, los estudios previos no han explorado el rol de la cromotripsis en un contexto de cariotipo complejo. En este trabajo, se ha analizado una cohorte de 33 pacientes con LLC y cromotripsis y se ha comparado con una cohorte de 129 casos sin cromotripsis con cariotipo complejo. Nueve de los pacientes con cromotripsis también se han analizado utilizando la técnica del mapeo óptico del genoma. Se han comparado los patrones detectados por *microarrays* genómicos en estos pacientes y se ha analizado el valor pronóstico de la cromotripsis.

La cromotripsis estaba distribuida por todo el genoma, siendo los cromosomas 3, 6 y 13 los más frecuentemente afectados. El OGM detectó un 88,1% de las alteraciones de número de copias previamente conocidas y varios reordenamientos adicionales relacionados con la cromotripsis (mediana: 9, rango: 3-26). Además, se pudieron identificar dos tipos de patrones: uno en el que los reordenamientos estaban focalizados en la región cromotríptica (3/9 casos) y otro en el que estaban implicados cromosomas cromotrípticos y no cromotrípticos (6/9). Los casos con cromotripsis mostraron un TPT más corto que los pacientes sin cromotripsis (mediana TPT: 2m vs. 15m; *p*=0,013). Sin embargo, cuando los pacientes se estratificaron según el estado de *TP53*, la cromotripsis no afectó al TPT. Sólo *TP53* mantuvo su significancia en el análisis multivariado para el TPT, en el que se incluyeron la cromotripsis y la complejidad genómica definida por MG (HR: 1,60; *p*=0,029). En conclusión, los resultados sugieren que las alteraciones en *TP53,* en lugar de la propia cromotripsis, subyacen el mal pronóstico observado en este subgrupo de pacientes.

cancers

MDPI

Article

TP53 Abnormalities Are Underlying the Poor Outcome Associated with Chromothripsis in Chronic Lymphocytic Leukemia Patients with Complex Karyotype

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Simple Summary: Chromothripsis, a genomic event that generates massive chromosomal rearrangements, has been described in 1–3% of CLL patients and is associated with poor prognostic factors (e.g., *TP53* abnormalities and genomic complexity). However, previous studies have not assessed its role in CLL patients with complex karyotypes. Herein, we aimed to describe the genetic characteristics of 33 CLL patients with high genomic complexity and chromothripsis. Moreover, we analyzed the clinical impact of chromothripsis, comparing these patients against a cohort of 129 patients with complex karyotypes not presenting this catastrophic event. Nine cases were also assessed via the novel

cytogenomic methodology known as optical genome mapping. We confirmed that this phenomenon is heterogeneous and associated with a shorter time to first treatment. Nonetheless, our findings suggested that *TP53* abnormalities, rather than chromothripsis itself, underlie the dismal outcome.

Abstract: Chromothripsis (cth) has been associated with a dismal outcome and poor prognosis factors in patients with chronic lymphocytic leukemia (CLL). Despite being correlated with high genome instability, previous studies have not assessed the role of cth in the context of genomic complexity. Herein, we analyzed a cohort of 33 CLL patients with cth and compared them against a cohort of 129 non-cth cases with complex karyotypes. Nine cth cases were analyzed using optical genome mapping (OGM). Patterns detected by genomic microarrays were compared and the prognostic value of cth was analyzed. Cth was distributed throughout the genome, with chromosomes 3, 6 and 13 being those most frequently affected. OGM detected 88.1% of the previously known copy number alterations and several additional cth-related rearrangements (median: 9, range: 3–26). Two patterns were identified: one with rearrangements clustered in the region with cth (3/9) and the other involving both chromothriptic and non-chromothriptic chromosomes (6/9). Cases with cth showed a shorter time to first treatment (TTFT) than non-cth patients (median TTFT: 2 m vs. 15 m; $p = 0.013$). However, when stratifying patients based on *TP53* status, cth did not affect TTFT. Only *TP53* maintained its significance in the multivariate analysis for TTFT, including cth and genome complexity defined by genomic microarrays (HR: 1.60; $p = 0.029$). Our findings suggest that *TP53* abnormalities, rather than cth itself, underlie the poor prognosis observed in this subset.

Keywords: chronic lymphocytic leukemia; genomic complexity; chromothripsis; *TP53*; genomic microarrays; optical genome mapping

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1. Introduction

The therapeutic landscape for patients with chronic lymphocytic leukemia (CLL) has expanded with the emergence of new targeted agents. In this context, genomic complexity has become increasingly important due to its controversial role as a predictor of response to therapy. It has been associated with shorter survival and worse response rates in patients treated with standard chemoimmunotherapy [1–4], yet its role in patients receiving new treatment modalities is still not fully established. In the initial trials performed with BTK and BCL2 inhibitors (i.e., Ibrutinib, Acalabrutinib, Venetoclax), it appeared to be an independent prognostic factor [5–9]. However, this negative impact has been controversial in recent trials using different therapeutic combinations and in those previous studies after a longer follow-up [10–13]. Even though genomic complexity has been mainly defined by the detection of complex karyotypes (CK) by chromosome banding analysis (CBA), genomic microarrays (GM) are also a valuable tool to assess genomic complexity in CLL [14,15]. In addition, optical genome mapping (OGM) has arisen as a promising cytogenomic methodology for whole genome screening, able to detect all types of structural and copy number alterations (CNA) at a higher resolution than traditional cytogenetic methods. Recently, several groups have proven that OGM is a useful technique to detect a wide range of clinically significant cytogenomic abnormalities in different hematological neoplasms [16–19].

The emergence of GM and other high-resolution molecular techniques, such as next-generation sequencing, has allowed the identification of massive genomic alterations characterized by the occurrence of multiple genomic rearrangements, often generated in a single catastrophic event. These processes are globally referred to as chromoanagenesis and include chromothripsis, chromoanasynthesis and chromoplexy [20,21]. Chromothripsis (cth) (Greek, "chromo" for chromosome; "thripsis" for shattering into pieces) is a unique catastrophic event in which tens to hundreds of genomic fragments are shattered and randomly stitched together due to the subsequent erroneous repair mechanisms, producing highly derivative chromosomes. This process was initially described in a CLL patient as the presence of $\geq$10 oscillating switches between two or three copy number states in one or a few chromosomes [22]. Nonetheless, some authors also considered those with at least seven copy number switches to be cth events [23–25]. Several models have been proposed in order to explain its origin, including chromosome pulverization within a micronucleus, premature chromosome condensation or fragmentation of dicentric chromosomes during breakage–fusion–bridge cycles, among others [26–28]. However, the mechanisms underlying the formation of these complex patterns are still unknown. Its prevalence is highly variable and ranges between 2 to almost 100% among different tumors [29–31]. In CLL patients, cth prevalence is low (1–3% in unselected cohorts), and most studies are limited to a small number of cases [24,32]. Globally, reported cases present great heterogeneity in terms of the type and number of structural variants but also in the genomic regions and chromosomes affected. Nonetheless, this phenomenon preferentially occurs in certain chromosomes (2, 3, 6, 8, 9, 11, 13 and 17), and some authors have suggested a potential role of genes located in the recurrently abnormal regions in cth development [22–25,31–37]. In addition, no detailed comparison between patterns observed in chromothriptic chromosomes detected by GM or NGS and their corresponding karyotype has been performed to date. As for its clinical impact, it has been related to *TP53* abnormalities (found in approximately 70–80%) and a shorter time to first treatment and overall survival [23–25,32]. Nevertheless, the assessment of cth is not included in the International Workshop on CLL guidelines [38]. It is noteworthy that although it is known that cth is frequently found in the context of complex genomes, none of the aforementioned studies explored the impact of the overall genomic complexity on the evolution of these cases. In this regard, our group recently reported a strong association between cth and CK and the poor prognosis associated with cth, even within the CK subset [15]. However, the impact of *TP53* status and other clinico-biological characteristics in these patients with cth merits further exploration.

The aim of the present study was to describe the clinical and genomic characteristics of a cohort of 33 CLL patients with patterns of cth detected by GM, especially focusing on the relationship of cth with the overall genomic complexity. Furthermore, we compared cth cases with a cohort of non-chromothriptic CLL cases with CK to elucidate whether the presence of these highly complex patterns could have a negative effect on survival in this particular subgroup. Finally, we analyzed nine cases using OGM to determine the utility of this novel technique in the identification of cth.

2. Materials and Methods

2.1. Patient Cohort

A total of 162 CLL patients with genomic complexity detected by GM were selected. Among them, 33 showed patterns of cth by GM. The remaining 129 cases, which also showed CK by CBA ($\geq$3 abnormalities in the same cell clone) but did not display cth, were considered as the control group for the comparison of clinical and biological characteristics [15]. All patients had CBA and GM results available at diagnosis or prior to treatment. Demographic, clinical and biological characteristics are summarized in Table 1. Thirty patients with cth and the control cohort were selected from a previous work from our group [15]. The three additional cases with chromothripsis were identified in another study from our group [39].

Table 1. Baseline characteristics of patients at diagnosis and last follow-up.

	Chromothripsis n = 33; n (%)	**Control Group n = 129; n (%)**	***p*-Value**
Gender			
Men	23 (69.7%)	93 (72.1%)	0.785
Median age at diagnosis	66 years [33–91]	69 years [37–96]	0.177
Complex karyotype by CBA	30 (90.9%)	129 (100%)	0.008
3–4 abnormalities	7 (23.3%)	74 (57.4%)	0.001
≥5 abnormalities	23 (76.7%)	55 (42.6%)	
Stage at diagnosis			
MBL	1 (3.0%)	1 (0.8%)	0.367
CLL	32 (97.0%)	128 (99.2%)	
Binet A	16/30 (53.3%)	66/109 (60.6%)	0.532
Binet B/C	14/30 (46.7%)	43/109 (39.4%)	
Common CLL genomic aberrations *			
del(13)(q14)	19 (57.6%)	80 (62.0%)	0.641
Trisomy 12	1 (3.0%)	26 (20.2%)	0.018
del(11)(q22q23)	9 (27.3%)	42 (32.6%)	0.560
Aberrations in *TP53*	23 (69.7%)	49/127 (38.6%) **	0.001
del(17)(p13)	22 (66.7%)	45 (34.9%)	0.001
TP53 mutation	13/31 (41.9%)	32/119 (26.9%)	0.104
Unmutated IGHV	23/31 (74.2%)	71/110 (64.5%)	0.314
Median follow-up [range] ***	28 months [0–160]	33 months [1–160]	0.490
Time from diagnosis to cytogenetic study	1 month [0–298]	0 months [0–129]	0.163
Treatment ***			
Treated patients ^	29 (87.9%)	86 (66.7%)	0.017
Median time to first treatment [95% CI]	2 months [0–6]	15 months [9–21]	0.013
Survival ***			
Median overall survival [95% CI]	64 months [16–112]	90 months [59–121]	0.132

* Deletions and trisomy detected by FISH and/or genomic microarrays. ** Cases in which *TP53* mutation screening was not performed and FISH and/or genomic microarrays were negative for deletion were not considered. *** Data regarding treatment and follow-up from the control group were updated with respect to the previous publication. ^ Patients treated during the follow-up of the study. All samples used for the analysis, except one, were collected prior to treatment. Abbreviations: MBL = monoclonal B-cell lymphocytosis, CI = confidence interval.

2.2. Genomic Microarray Analyses

DNA was extracted from whole peripheral blood (PB) (n = 7; 21.2%), PB mononuclear cells (PBMCs) (n = 8; 24.2%), PB CD19+ purified cells (n = 13; 39.4%) or from bone marrow samples (n = 5; 15.2%) obtained no more than one year after CBA (median: 0 months; range: 0–12). Only DNA that fulfilled the required quality controls was amplified, labelled and hybridized using different genomic microarray platforms according to the manufacturers' protocols [ThermoFisher Scientific (n = 25; 75.8%), Agilent (n = 5; 15.2%) and Illumina (n = 3; 9.0%)] (Table S1). The number of abnormalities was recorded as previously described [15]. Chromothripsis was defined by the presence of ≥7 oscillating switches between two or three copy number states on an individual chromosome [23–25]. Coordinates were given according to the annotations of genome version GRCh37/hg19.

2.3. Optical Genome Mapping

For each sample, a minimum of 1.5 million PBMCs were used to extract ultra-high molecular weight (UHMW) DNA, following the manufacturer's instructions (Bionano Prep Frozen Cells DNA Isolation Protocol, Bionano Genomics, San Diego, CA, USA). Then, UHMW DNA was enzymatically labeled in a sequence-specific manner using the Bionano Prep Direct Label and Stain (DLS) Protocol (Bionano Genomics). The molecules obtained, labeled around 15 times per 100 Kbp, were cleaned up and loaded onto a Saphyr chip and imaged via the Saphyr instrument (Bionano Genomics). In the chip, molecules were linearized in nanochannels by electrophoresis, and multiple cycles were run to reach an average genome coverage of 300× (approximately 1300 Gb of data per sample). Imaged molecules $\geq$150 Kbp were analyzed using the rare variant pipeline (RVP) included in Bionano Solve software (v.3.5, Bionano Genomics, San Diego, CA, USA) and visualized in Bionano Access software (v1.6, Bionano Genomics, San Diego, CA, USA). The RVP included two algorithms: a structural variant (SV) analysis, based on the comparison of the labeling pattern against a reference assembly (hg19), and a tool to call large CNA inferred from the coverage of labels detected in each genomic interval. Default recommended confidence scores and an OGM control sample dataset provided by Bionano were used to pre-filter the abnormalities initially called by the software. The cut-off was set at 100 Kbp for SVs and 500 Kbp for CNA. Moreover, OGM results were manually reviewed to merge segmented CNA and discard variants found as benign polymorphisms in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home, accessed on 7 March 2022), SVs found to be duplicated in the results and low-quality translocation calls. Finally, the abnormalities detected by OGM in each patient were recorded and those involving chromothriptic regions were compared to the results previously obtained by GM and CBA techniques.

2.4. Whole Chromosome FISH Painting

Whole chromosome FISH painting (WCP) was performed in 6/9 cases analyzed by OGM to validate some selected cytogenomic abnormalities. Whole chromosome painting probes (MetaSystems, Altlussheim, Germany) for chromosomes 1, 2, 3, 6, 8, 9, 11, 13, 15, 17, 19 and Y were used. Chromosomes were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). FISH signals were observed under a fluorescence microscope in order to confirm or discard novel rearrangements revealed by OGM.

2.5. Statistical Analyses

Descriptive statistics were used to provide frequency distributions of discrete variables, while statistical measures were used to provide median values and ranges for quantitative variables. Groups were compared using Chi-square or Fisher exact tests for discrete variables and the Mann–Whitney U test for continuous variables. Time to first treatment (TTFT), the primary end-point of the study, was calculated from the date of cytogenetic study to the date of first treatment or last follow-up, whereas overall survival (OS) was defined from date of cytogenetic study to last follow-up or death. The Kaplan–Meier method was used to estimate the distribution of TTFT and OS. Comparisons among patient subgroups were performed via the Log-rank test. One patient with cth was excluded from survival analyses for having previously received treatment. A multivariate analysis using the Cox proportional hazards regression model was used to assess the independent prognostic impact on TTFT. Statistical analyses were performed using SPSS v.23 software (SPSS Inc., Chicago, IL, USA) and R v3.5.2. p-values < 0.05 were considered statistically significant.

3. Results

3.1. Identification of Chromothripsis Patterns by Genomic Microarrays

A total of 33 patients with cth detected by GM were included. Even though the majority of patients displayed cth in only one chromosome (25/33; 75.6%), eight patients showed complex patterns in several chromosomes (range: 2–4). Among the 46 chromothriptic events detected, 25 (54.3%) included changes that alternated between two copy number states, mostly between one and two copies, resulting in discontinuous deletions of several fragments. In 19/46 (41.3%) events, the oscillations involved both gains and losses, while in 2/46 (4.4%), the rearrangements implied only gains of chromosomal material. Furthermore, these oscillations in the copy number state were located either focally, involving only one chromosome arm, or throughout the whole chromosome (n = 17 and 29, respectively). Interestingly, no differences were observed between the patterns found in those chromothriptic events displaying 7–9 oscillating switches (n = 16) and those with ≥10 switches (n = 30) (Table S2). Cth was found in almost all chromosomes, with the most frequently involved chromosomes being 3, 6 and 13 (five cases each) (Figure S1). Of note, three of the five cases with cth in chromosome 3 carried a deletion of the 3p21.31 locus, which includes the *SETD2*, *CDC25A*, *MAP4*, *FBXW12* and *ATRIP* genes. As for cases with cth in chromosome 6, three of them displayed deletion of 6q21, which includes the *FOXO3a* gene. Regarding cth in chromosome 13, all had the 13q14 CLL common deleted region, which involved the *DLEU1* and *DLEU2* genes as well as the microRNAs miR-16-1 and miR-15a, with several additional deletions throughout the whole chromosome arm (Table S3).

In addition, cth patterns detected by GM were compared to the chromosomal aberrations found in the karyotype to assess whether CBA could suggest the presence of this phenomenon. In most of the chromothriptic events (15/46; 32.6%), the presence of multiple losses detected by GM was reported as monosomies by CBA. Notably, these were accompanied by chromosome markers or additional material of unknown origin, which could explain the apparent loss of the entire chromosome. Moreover, 24/46 (52.2%) of the chromosomes involved had different unbalanced structural aberrations, including unbalanced translocations (12/46; 26.1%), which might suggest the involvement of other chromosomes in the formation of cth, additional material of unknown origin (5/46; 10.9%) and single deletions (7/46; 15.2%). Unexpectedly, seven chromosomes with cth did not show any aberration by CBA. Nonetheless, in one of these cases, the karyotype was normal, suggesting non-division of the tumor clone, while in 6/7 cases, other abnormalities in different chromosomes were detected (Table S4 and Figure S2).

3.2. *Detection of Different Chromothripsis Patterns by Optical Genome Mapping*

Nine patients were analyzed using OGM. This methodology detected almost all the CNA related to cth previously visualized by GM (74/84; 88.1%), showing a high concordance in size and coordinates (Table S5). Remarkably, it also allowed the identification of several rearrangements involving chromothriptic regions (median: 9, range: 3–26), including intra-chromosomal (median: 6, range: 3–12) and inter-chromosomal translocations (median: 5, range: 0–14). Overall, two patterns of rearrangements could be observed. First, in 3/9 cases, the translocations identified by OGM only clustered in the cth region. These findings were in accordance with CBA and FISH results in two of the patients with an abnormal karyotype (cases #8 and #9 in Table S5). In particular, in one patient showing focal cth-related rearrangements on chromosome 6 (case #9), whole chromosome FISH painting (WCP) confirmed the presence of material from this chromosome only in the whole abnormal der(6) and in its normal counterpart (Figure 1A). In the second patient (case #8), OGM rearrangements were also limited to chromosome 6 but CBA identified an unbalanced t(6;19)(q12;p13) that was not detected by OGM, probably due to the limitations of this technique in the detection of abnormalities involving telomeric regions (Figure 1B). Further WCP analyses could not be performed on the third case (case #32), as cells from this patient did not yield abnormal metaphases for CBA. Notably, the three cases with clustered cth-related rearrangements displayed cth in chromosome 6. Notwithstanding, the abnormalities involved were very heterogeneous among these three cases and a commonly deleted region could not be identified. Only small deleted fragments (range: 0.64–2.83 Mb) were common between case #8 and the two remaining cases (cases #9 and #32), with no known gene included (Figure S3).

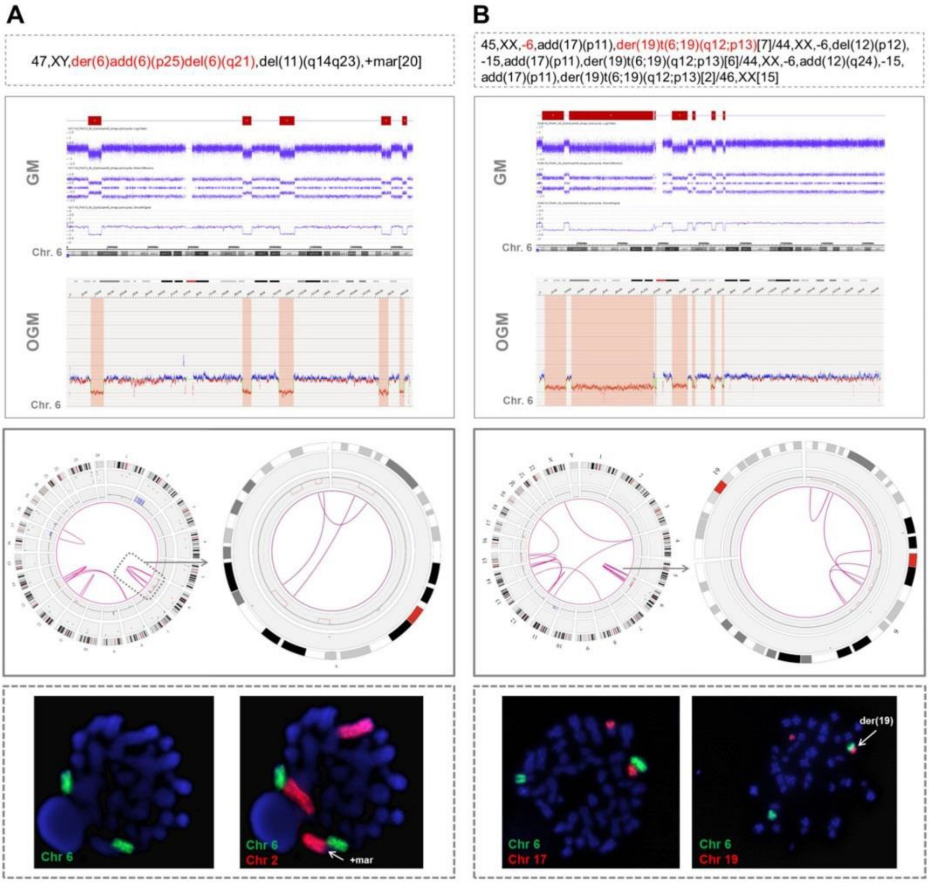
A
47,XY,der(6)add(6)(p25)del(6)(q21),del(11)(q14q23),+mar[20]
GM
Chr. 6
OGM
Chr. 6
Chr 6
Chr 6
Chr 2
+mar
B
45,XX,-6,add(17)(p11),der(19)t(6;19)(q12;p13)[7]/44,XX,-6,del(12)(p12),-15,add(17)(p11),der(19)t(6;19)(q12;p13)[6]/44,XX,-6,add(12)(q24),-15,add(17)(p11),der(19)t(6;19)(q12;p13)[2]/46,XX[15]
GM
Chr. 6
OGM
Chr. 6
Chr 6
Chr 17
der(19)
Chr 6
Chr 19

Figure 1. Two examples of cases analyzed by optical genome mapping showing only intra-chromosomal chromothripsis-related rearrangements. (**A**) A patient with chromothripsis in chromosome 6 in which OGM showed the presence of three intra-chromosomal translocations (case #9). When whole chromosome FISH painting (WCP) was performed, two green signals corresponding to chromosome 6 were observed, confirming the presence of material from this chromosome in the one initially reported as "der(6)add(6)(p25)del(6)(q21)" and in its normal counterpart. To ensure that this metaphase was abnormal, chromosome 2 was also stained, since both GM and OGM detected a duplication of 2p. WCP confirmed that the duplicated 2p was the marker chromosome found by CBA. (**B**) Patient with chromothripsis in chromosome 6 in which CBA identified a monosomy 6 and an unbalanced t(6;19)(q12;p13) (case #8). OGM revealed some rearrangements clustered in chromosome 6, but it did not detect this translocation. It was probably not called by OGM due to the involvement of the telomeric region of chromosome 19, a highly repetitive region in which the OGM detection of structural variants is known to be limited. The hybridization pattern obtained by WCP confirmed the presence of this translocation, since two different signals (orange and green), corresponding to both chromosomes, could be observed together but without showing a mixing of these signals, which

suggests that they rearranged after the process underlying chromothripsis. The abnormalities detected by CBA in chromosomes with chromothripsis are highlighted in red in the karyotype. Chromosome views show the comparison of the CNA profiles identified by GM and OGM in the chromothriptic chromosomes. The Circos plot represents the abnormalities identified by OGM for the whole genome (on the left) and the chromothriptic chromosome involved in this process (on the right). Different layers show, from outer to inner, cytobands of different chromosomes, structural variants (including deletions, duplications, inversions and insertions), copy number alterations and rearrangements, which are represented by lines joining the chromosomes involved.

Second, in 6/9 cases, OGM revealed the presence of rearrangements between the chromothriptic chromosome and other non-chromothriptic chromosomes (Figure 2).

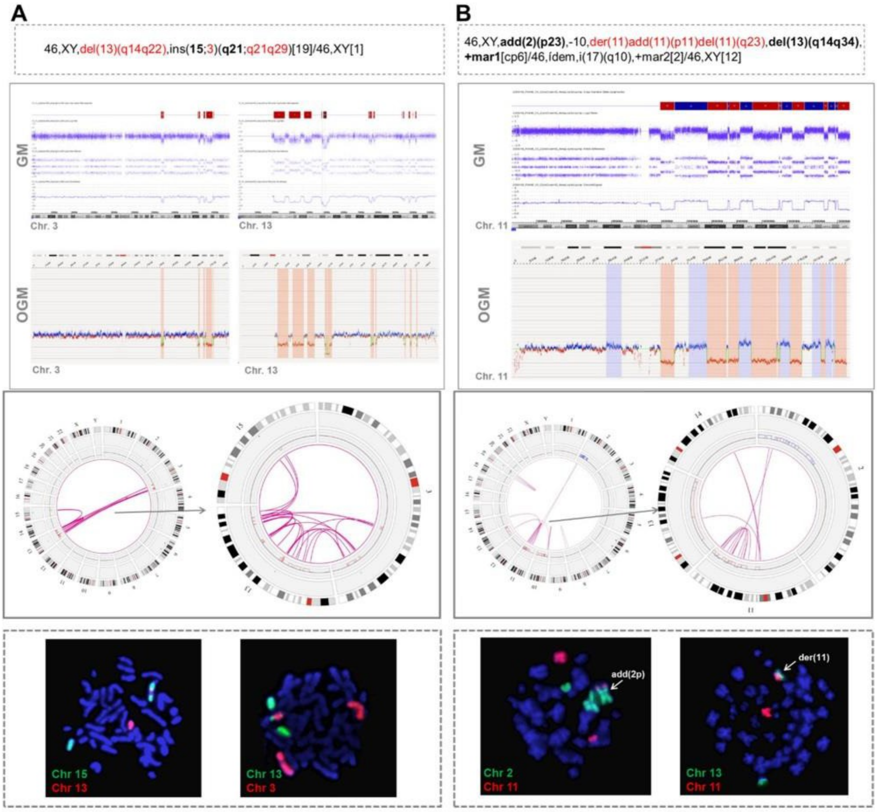

Figure 2. Two examples of cases with chromothripsis analyzed by optical genome mapping showing rearrangements between chromothriptic and non-chromothriptic chromosomes. (**A**) Patient with chromothripsis in chromosomes 3 and 13 (case #31). OGM detected 10 rearrangements between chromosomes 3 and 13 and four rearrangements between chromosomes 13 and 15. Whole chromosome FISH painting (WCP) revealed the presence of material from both chromosomes 3 and 13 inserted in chromosome 15. (**B**) Patient with chromothripsis in chromosome 11 (case #17). OGM revealed the presence of revealed the presence of

intra-chromosomal translocations and several additional rearrangements involving chromosomes 2, 13 and 14. Notably, t(2;11) and t(11;13) were validated by WCP. Conversely, despite showing only one line in the Circos plot, three parallel t(11;14) were identified by OGM and could not be validated by WCP. However, they could not be ruled out with certainty as true translocations since the rearranged fragment located between the breakpoints was very small and could be missed due to the low resolution of the technique. The abnormalities found by CBA in chromosomes with chromothripsis are highlighted in red in the karyotype. Additional chromosomes associated with chromothriptic events are highlighted in bold. Chromosome views show the comparison of the CNA profiles identified by GM and OGM in the chromothriptic chromosomes. The Circos plot represents the abnormalities identified by OGM for the whole genome (on the left) and for chromothriptic and non-chromothriptic chromosomes involved in this process (on the right). Different layers show, from outer to inner, cytobands of different chromosomes, structural variants (including deletions, duplications, inversions and insertions), copy number alterations and rearrangements, which are represented by lines joining the chromosomes involved.

These rearrangements involved 1 to 6 partners. In one case (case #16), 28 novel translocations were observed along the genome, leading to a very highly complex profile, which could suggest the presence of another catastrophic phenomenon known as chromoplexy (Figure 3). OGM results were compared with CBA data to see whether some of these rearrangements could also be detected in the karyotype. Interestingly, CBA could identify the translocations revealed by OGM in only two cases (cases #2 and #31 in Table S5). Nonetheless, most of the non-chromothriptic chromosomes with novel translocations revealed by OGM were altered, displaying either monosomies together with marker chromosomes or carrying deletions or additional material of unknown origin. Likewise, both GM and OGM also detected CNA in the breakpoints of these non-chromothriptic chromosomes related to chromothriptic events. Several genes were found in the breakpoints. However, none of the novel translocations or genes involved in the breakpoints were common among them (Table S5). Of note, WCP was carried out in six cases in order to validate the rearrangements found by OGM. Most of the novel translocations were confirmed, suggesting the involvement of other chromosomes in the development of cth. Only three new rearrangements could not be validated by WCP (t(11;14) in case #17 in Table S5). However, caution should be taken since the rearranged fragment could be missed due to the limited resolution of the WCP technique (Figure 2B).

46-50,XY,**add(1)(p36)**,add(2)(q34),+add(2)(q34),del(3)(p11p22),**del(8)(p21)**,add(9)(q34),**add(10)(p11)**,-12, **-13**,**del(13)(q13)**,+21,+22,+3mar[cp10]/46-50,XY,add(1)(p36),add(2)(q34),+add(2)(q34),del(3)(p11p22), del(8)(p21),-9,add(10)(p11),-12,-13,del(13)(q13),+21,+22,+3mar[cp10]

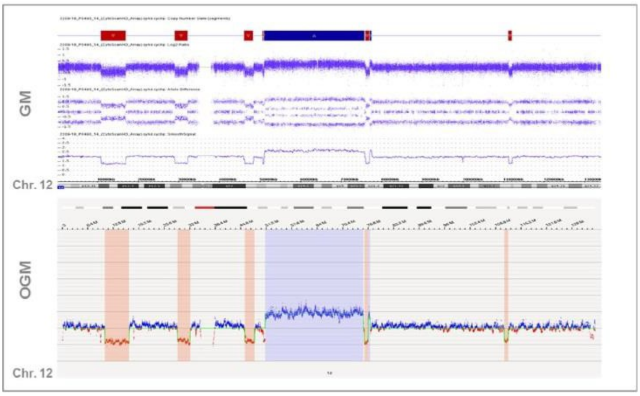

Figure 3. *Cont.*

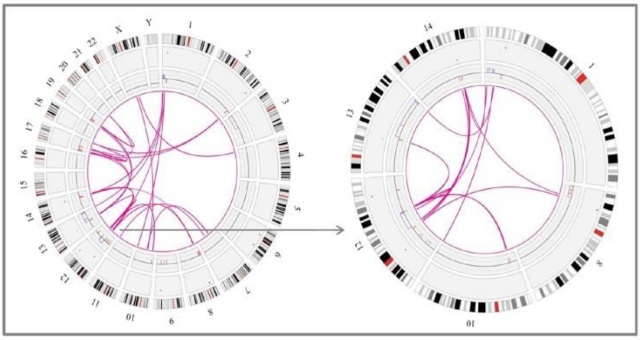

Figure 3. Example of a case with chromothripsis that shows multiple rearrangements with other chromosomes when analyzed by optical genome mapping. Overall, the karyotype of this patient presented a high complexity, with several bits of additional material found in different chromosomes and a few chromosome markers (case #16). Chromosome 12 displayed monosomy by CBA (highlighted in red in the karyotype) but when analyzed by GM and OGM, this chromosome showed an identical pattern of chromothripsis, with >10 switches between 2–3 copy number states. In addition, OGM identified several rearrangements among different chromosomes, as shown in the Circos plot depicted at the bottom of the figure. This highly complex profile could be associated with another catastrophic phenomenon known as chromoplexy, characterized by the presence of multiple chained translocations. Those additional chromosomes associated with chromothriptic events are highlighted in bold in the karyotype.

3.3. Association of Chromothripsis with Other Clinical Characteristics and Prognostic Impact

Clinical and biological features of patients with cth were compared between the different chromothriptic patterns observed. In the total cohort, 8/33 (24.2%) cases showed cth patterns in more than one chromosome. Among them, the majority (7/8; 87.5%) presented a high complexity, with ≥10 switches in at least one of the chromosomes involved, while only 1/8 (12.5%) displayed patterns with 7–9 switches. Conversely, patients with only one chromothriptic chromosome showed a similar frequency of patterns constituted by 7–9 (10/25; 40%) and ≥10 (15/25; 60%) switches. However, when comparing the abnormalities detected by CBA in both subgroups, no differences could be observed (median: 6 abn. [range: 0–16] in patients with one affected chromosome vs. 7.5 [range: 2–11] in those with >1 chromosome; $p = 0.481$). Likewise, no significant differences could be found among both subgroups in terms of gender, age, IGHV status or frequency of deletions and/or mutations in *TP53* (del/mut*TP53)* or del11q22q23 (*ATM)* (data not shown). Then, in the nine patients studied by OGM, clinical characteristics were compared between patients with only intra-chromosomal cth-related rearrangements and those with involvement of both chromothriptic and non-chromothriptic chromosomes. Overall, both subgroups were similar in gender (66.7% men in both groups), age (median: 67 vs. 66 years; $p = 0.362$), IGHV status (2/2, 100% vs. 5/6, 83.3%; $p = 1.000$) or frequency of del/mut*TP53* (66.7% vs. 50%; $p = 1.000$) or del(11q) (33.3% in both groups). However, the median number of abnormalities was higher in those patients with rearrangements involving non-chromothriptic chromosomes compared with those with clustered rearrangements. These differences were found when the number of abnormalities was recorded both by CBA (median: 8.5 [range: 2–16] vs. 0, 3 and 6 abnormalities, respectively) and GM (median: 19.5 [range: 11–30] vs. 15 [range: 10–15], respectively). Notwithstanding, it is important to note that statistical conclusions cannot be drawn from such a small subset of patients studied by OGM. Next, we aimed to determine whether the number of switches in the copy number state, 7–9 or ≥10, could have different impacts on the outcome of patients with cth. In this regard, patients having only 7–9 switches were compared to those with at least one cth event comprising ≥10. No significant differences were observed for TTFT between these two subgroups (median TTFT: 2 m vs. 2 m; $p = 0.924$) (Figure 4). Therefore, they were considered a unique group for subsequent analyses.

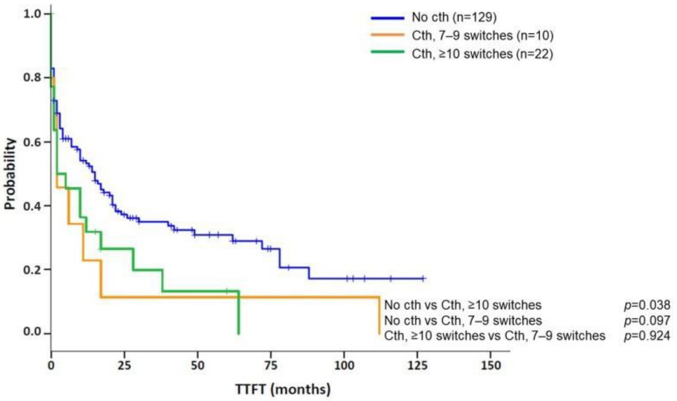

Figure 4. Kaplan–Meier plots for time to first treatment (TTFT) based on the presence of chromothripsis and the number of oscillating switches found in chromothripsis patterns. Kaplan–Meier estimation for TTFT in patients with 7–9 switches between 2–3 copy number states and ≥10 switches between 2–3 copy number states compared to a cohort of CLL cases carrying a complex karyotype (CK) without chromothripsis (cth). Of note, patients were classified into the "Cth ≥ 10 switches" group if they showed at least one chromothripsis event with these characteristics.

Concerning the prognostic impact of cth, patients included in the present series were compared to an aggressive cohort of CLL patients carrying CK without cth (Table 1) [15]. In this context, cth had a negative impact on TTFT compared with the control group (median TTFT: 2 m vs. 15 m; $p = 0.013$) (Figure 5A). Likewise, cases with cth showed a tendency towards a shorter OS (median OS: 64 m vs. 90 m; $p = 0.205$) (Figure 5B).

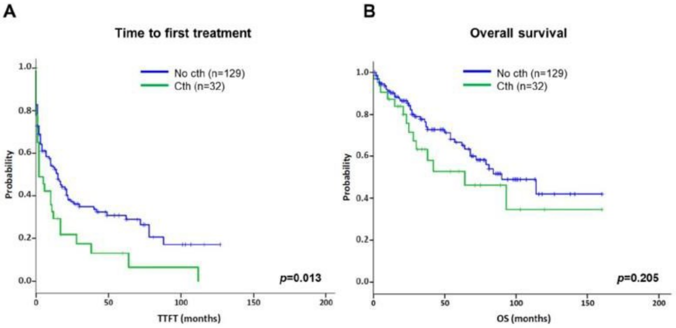

Figure 5. Kaplan–Meier plots for time to first treatment (TTFT) and overall survival (OS) based on the presence of chromothripsis. Kaplan–Meier estimation for TTFT (**A**) and OS (**B**) in patients with chromothripsis (Cth; including cases with 7–9 and ≥10 switches) compared to a cohort of CLL cases carrying complex karyotype (CK) without chromothripsis (No cth).

In addition, cth was associated with high genomic complexity and other genomic poor prognostic factors. Particularly, 30/33 patients carried a CK by CBA, with 23 (76.7%) cases having more than five abnormalities. The three non-CK cases by CBA showed genomic complexity as GM identified several abnormalities (median: 10; range: 10–24), with at least three of them $\geq$5 Mb (range: 3–6). In addition, unmutated IGHV (U-IGHV) and del(11q) were found at frequencies similar to the control group, which is known to be enriched in these poor prognostic markers (74.2% vs. 64.5%, $p = 0.314$, for U-IGHV; 27.3% vs. 32.6%, $p = 0.560$, for del(11q)). In contrast, patients with cth had a higher frequency of del/mut*TP53* than the control group (69.7% vs. 38.6%; $p = 0.001$). Of note, when patients were categorized according to their *TP53* status, cth did not significantly affect TTFT (del/mut*TP53* group, $n = 72$, median TTFT: 4 m vs. 2 m, $p = 0.242$, for non-cth and cth, respectively; WT *TP53* group, $n = 87$, median TTFT: 20 m vs. 17 m, $p = 0.486$, for non-cth and cth, respectively) (Figure 6).

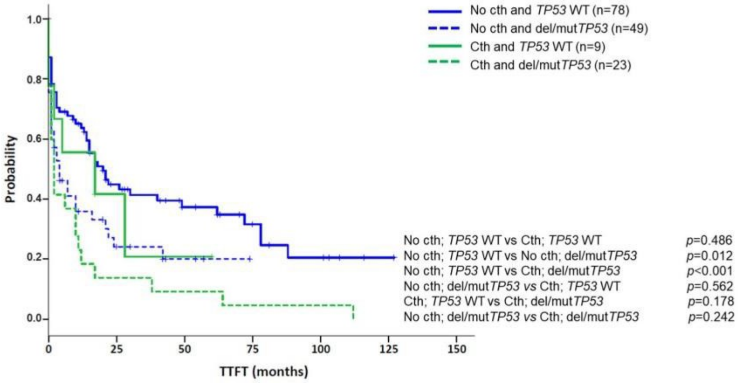

Figure 6. Kaplan–Meier plots for time to first treatment (TTFT) based on the presence of chromothripsis and abnormalities in *TP53* (deletions and/or mutations). Patients were classified according to the presence of chromothripsis (cth) and within each group (No cth vs. Cth), TTFT was assessed based on the presence of aberrations in *TP53* (deletions and/or mutations).

On the other hand, no significant differences were observed based on IGHV status or del(11q) for TTFT in the whole cohort (Table 2) and among patients with cth (Cth group, $n = 30$, median TTFT: 1 m vs. 6 m, $p = 0.412$, for mutated-IGHV and U-IGHV, respectively; Cth group, $n = 32$, median TTFT: 6 m vs. 2 m, $p = 0.079$, for non-del(11q) vs. del(11q), respectively). Notably, results from the mutated IGHV subset should be taken with caution since 6/8 cases also carried del/mut*TP53*. Therefore, the tendency towards a shorter TTFT could be attributed to the confounding effects of *TP53*. In the multivariate analysis for TTFT, including genome complexity defined by GM, *TP53* status and cth, only the presence of del/mut*TP53* retained significance (Table 2).

Table 2. Univariate and multivariate analysis for time to first treatment (TTFT).

Variable	**Univariate Analysis ***		**Multivariate Analysis**	
	Median TTFT in Months (95% CI)	***p*-Value**	**Hazard Ratio (95% CI)**	***p*-Value**
GM				
Intermediate-GC vs. low-GC	21 (4–38) vs. 17 (12–22)	0.941	0.91 (0.53–1.56)	0.719
High-GC vs. low-GC	3 (0–6) vs. 17 (12–22)	0.006	1.45 (0.82–2.57)	0.205
del/mut*TP53*	3 (1–5)	<0.001	1.60 (1.05–2.43)	0.029
U-IGHV	10 (3–17)	0.317	NA	NA
del(11)(q22q23)	14 (7–21)	0.614	NA	NA
Chromothripsis	2 (0–6)	0.013	1.21 (0.76-1.93)	0.422

* In comparison with the CK cohort extracted from Ramos-Campoy et al., 2022. Abbreviations: GM = genomic microarrays, GC = genomic complexity, low-GC = 0–2 copy number alterations (CNA) detected by genomic microarrays, intermediate-GC = 3–4 CNA, high-GC = ≥5 CNA, U-IGHV = CLL with unmutated IGHV, CI = confidence interval, NA = not assessed.

4. Discussion

Several works have focused on the study of cth in different types of hematological neoplasms and solid tumors due to its potential role in cancer onset and progression. In CLL, limited numbers of cases have been reported, and they were mainly associated with poor prognostic factors, such as abnormal *TP53* and genomic complexity and a dismal outcome (Table S6). Strikingly, none of the studies published explored the relationship between cth and overall genomic complexity. To the best of our knowledge, this is the largest cohort of CLL with cth assessed in the context of genomic complexity. Herein, an extensive study of the cytogenomic aberrations observed by genomic microarrays was performed, including a comparison with CBA results and an in-depth analysis with novel optical genome mapping technology.

Cth in CLL is highly heterogeneous in terms of the type and number of structural variants, but also in the genomic regions and chromosomes involved [22–25,31–37]. In this sense, chromothriptic events described in this cohort mostly involved losses of fragments or alternated losses and gains and were located indistinctly throughout whole chromosomes or focalized in one chromosome arm. The most recurrently involved regions in our series were located at 3p21, 6q21 and 13q14, which had been previously reported in CLL cases with cth [24,25,36]. Regarding the 3p21.31 locus, this region contains the *SETD2* gene, which encodes a histone methyltransferase involved not only in the regulation of gene transcription but also in maintenance of genomic stability, and whose inactivation was also found to be associated with genomic complexity, del/mut*TP53*, cth and aggressive disease [24,36]. Besides, the recurrent deletion affecting the 6q21 locus (containing the *FOXO3a* gene) has been described in 6% of CLL patients and associated with shorter progression-free survival [23,40]. In the context of cth, this region was also deleted in the patient studied by Bassaganyas et al. [34]. The deletion in 13q14, involving the *DLEU1* and *DLEU2* genes as well as the microRNAs miR-16-1 and miR-15a, is the most common cytogenetic lesion in patients with CLL, being present in more than 50% of cases at diagnosis and strongly associated with a favorable prognosis when found as the sole abnormality [41]. However, this good outcome vanishes when accompanied by many other anomalies constituting a pattern of cth [24]. On the other hand, we also found involvement of the previously reported 6p21.1 and 10q24 regions (including *NFKBIE* and *NFKB2*, respectively) in two patients, independently [34]. Likewise, our cohort included one previously reported case with a gain in 5p13.33, which had been suggested as a possible mechanism underlying the formation of these complex rearrangements through the increase in telomerase activity by the deregulation of the *TERT* gene [25]. However, no other case carrying this abnormality was found. Overall, our results confirm that no significant correlation could be established between any of these affected regions and cth onset, suggesting that it might not arise from deregulation of one potential driver gene; rather, it would be initiated by other mechanisms, as yet unknown, that could lead to the alteration of distinct genomic regions.

In the present study, we were able to compare the chromothriptic patterns detected by GM with the chromosomal abnormalities reported in the karyotype. As expected, due to its limited resolution, it was not possible to detect cth by CBA. However, our results confirmed that most of the chromothriptic chromosomes were already altered in the karyotype, carrying a monosomy, deletion or being involved in unbalanced rearrangements. Furthermore, although the chromothriptic chromosomes might display a simple aberration or even be normal, the presence of chromosome markers and additional material of unknown origin was a common feature in these cases, leading overall to more complex karyotypes than those without cth. This was reflected by the higher frequency of cases with $\geq$5 abnormalities compared with the control group. Thus, CBA can only suggest the presence of complex rearrangements through the detection of abnormalities involving material of unknown origin that might constitute patterns of cth. On the other hand, taking advantage of the structural information provided by the analysis of nine patients using OGM, we were able to reveal rearrangements associated with chromothriptic events, including intra-chromosomal and inter-chromosomal translocations. Even though some of the breakpoints directly overlapped or were close to coding genes, we did not find any common driver gene that could trigger the formation of cth. The novel rearrangements clustered in the chromothriptic chromosomes or involved different non-chromothriptic chromosomes. OGM also underscored a case with a higher complexity profile, comprising several chained translocations involving several chromosomes, which is characteristic of another catastrophic event called chromoplexy. This phenomenon was first identified in prostate cancer, with a prevalence of ~90%, and is also present in other solid tumors [42,43]. However, despite being described in some hematological neoplasms, such as multiple myeloma, mantle cell lymphoma or in previous reports on CLL [24,44–46], clinical and biological differences between both chromoplexy and cth and their impact on CLL have not been further explored. In this study, no differences in clinical characteristics could be identified among patients showing the aforementioned rearrangement patterns, although it should be noted that the cohort was too small to draw any conclusion. Even though it is still unknown whether the distinct patterns of rearrangements could affect the evolution of the disease, OGM is a novel technology that provides a more detailed description of these catastrophic events than GM and could shed light on the mechanisms involved in the development of cth. In addition, OGM data analysis is based on a user-friendly interface and could be potentially included in clinical practice. In contrast, even though whole genome sequencing methods have been extensively used for the characterization of cth [22,24,31,34,37], several issues, including high costs and the need for more harmonized analysis pipelines, preclude their incorporation in a routine setting.

Regarding clinical impact, we have shown that CLL patients with cth had a shorter TTFT compared with patients with genomic complexity lacking cth. These results are in accordance with prior studies performed in large CLL cohorts, which reported poor outcomes associated with cth in terms of progression-free survival, TTFT and OS [23–25,32]. However, it is noteworthy that the present study only comprised cases with CK and/or genomic complexity detected by GM. Thus, our results should not be extrapolated to a real-life CLL population, where complex cases account for around 10% of patients. Even though cth has been previously associated with genomic complexity, this is the first study focused on this high-risk group. Unfortunately, our series was based on a retrospective and multicenter cohort, including patients receiving different therapeutic agents, which hindered the assessment of overall survival or response to new targeted modalities. As expected, the cth group showed an enrichment of patients with high complexity ($\geq$5 aberrations by CBA) and del/mut*TP53* (both being found in 70% of patients). These frequencies were similar to those defined in previous publications, which, despite not describing results from CBA data, reported high complexity in these cases and a significant association of cth with *TP53* abnormalities (up to 81%) [23–25,32]. Notwithstanding, these known independent poor prognostic markers were significantly increased, even when cth patients were compared with the control group with CK, also known to be related to these features. It is therefore conceivable that the poorer evolution observed in cases with cth might be related not only to the chromothriptic event itself but also to the presence of high complexity by CBA or *TP53* abnormalities. In this regard, cth did not affect TTFT when cases were categorized according to *TP53* status. Indeed, in the multivariate analysis, *TP53* was the only parameter maintaining its prognostic significance, reinforcing this hypothesis. Therefore, cth does not represent an independent prognostic factor for CLL patients with genomic complexity, and its identification is not essential in the clinical setting. In summary, a strong connection between *TP53* and cth has been demonstrated. Nonetheless, it would be very interesting to better explore the characteristics and the clinical evolution of cth in cases with wild-type *TP53*, in which other mechanisms would promote the survival of the clones with cth and the confounding effects of *TP53* dysfunction on survival would be avoided.

5. Conclusions

In conclusion, chromothripsis is a recurrent event in CLL patients with genomic complexity and is strongly associated with an increased frequency of *TP53* abnormalities. In this sense, the short survival observed in CK cases with cth might actually be due to the increased prevalence of del/mut*TP53*. In addition, OGM has proven to be a valuable cytogenomic tool, capable of detecting previously known abnormalities and identifying new rearrangements, affording an improved view of the highly complex genomic landscape of these patients. However, important questions remain regarding the mechanisms underlying cth and the impact of these patterns on the onset and evolution of CLL. Therefore, further studies with larger cohorts, including more cases with cth and preserved *TP53*, are needed to better understand the role of this phenomenon in CLL pathogenesis and prognosis, particularly in patients treated with new therapeutic agents, to better elucidate the potential of cth as a predictive marker.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14153715/s1, Figure S1. Number of cases showing chromothripsis for each chromosome. Chromosomes are represented on the *X*-axis and the total number of cases showing chromothripsis for each chromosome is represented on the *Y*-axis. The chromosomes involved most in the cohort (3, 6 and 13) are highlighted in blue. Figure S2. Copy number profiles of some of the chromothriptic chromosomes. Copy number profiles derived from genomic microarray analyses were available for 18 cases. Figure S3. Chromothripsis detected in chromosome 6 in those cases in which optical genome mapping only revealed intra-chromosomal chromothripsis-related rearrangements. Whole chromosome 6 view of genomic microarray results from cases #8, #9 and #32, which carried intra-chromosomal rearrangements when analyzed by OGM, are represented. Only small deleted fragments were common between case #8 and the other two remaining cases. Specifically, three fragments of 2.83 Mb (10,743,398–13,571,692), 1.20 Mb (16,137,146–17,257,084) and 1.17 Mb (86,948,132–88,115,441) were shared between cases #8 and #9 (highlighted in green), and three fragments of 1.79 Mb (7,651,724–9,438,895), 0.64 Mb (19,953,714–20,591,009) and 1.28 Mb (36,084,473–37,366,801) were common between cases #8 and #32 (highlighted in red). Table S1. Genomic microarray platforms used in this study. Table S2. Patterns of chromothripsis found in cases with 7–9 switches and those with $\geq$10 switches between 2–3 copy number states. Table S3. Detailed information of the chromothriptic events detected by GM, including the genes involved in the rearrangements (*shown in the excel file attached*). Table S4. Detailed information of the chromothriptic events detected by GM and karyotypes obtained by CBA (*shown in the excel file attached*). Table S5. Results obtained by optical genome mapping in those chromosomes with chromothripsis (*shown in the excel file attached*). Table S6. Review of the literature published about chromothripsis in CLL (*shown in the excel file attached).*

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Informed Consent Statement: Informed consent was obtained from all patients involved in the study.

Data Availability Statement: Detailed chromosome banding analyses, genomic microarrays and optical genome mapping profiles for selected cases are provided in Supplementary Tables. Please contact either bespinet@psmar.cat or apuiggros@psmar.cat for additional data.

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TP53 abnormalities are underlying the poor outcome associated with chromothripsis in chronic lymphocytic leukemia patients with complex karyotype

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SUPPLEMENTARY MATERIAL

Supplementary Tables

Table S1. Genomic microarray platforms used in this study.

Genomic microarray platform	n (%)
CytoScan 750K array (ThermoFisher)	2 (6.1%)
CytoScan HD array (ThermoFisher)	16 (48.5%)
Affymetrix SNP6.0 (ThermoFisher)	7 (21.2%)
SurePrint G3 Human CGH 8x60K (Agilent)*	1 (3.0%)
SurePrint G3 ISCA CGH+SNP Bundle, 4x180K (Agilent)	4 (12.1%)
Illumina Human Omni1-Quad array (Illumina)	3 (9.1%)

*Custom design described in Salaverria I, Martín-Garcia D, López C, et al. Detection of chromothripsis-like patterns with a custom array platform for chronic lymphocytic leukemia. Genes Chromosomes Cancer. 2015;54(11):668-80.

Table S2. Patterns of chromothripsis found in cases with 7-9 switches and those with ≥10 switches between 2-3 copy number states.

	Events with 7-9 switches n = 16	**Events with ≥10 switches** n = 30	***P-value***
Type of CNVs			0.850
Only losses of material	8 (50.0%)	17 (56.7%)	
Only gains of material	1 (6.2%)	1 (3.3%)	
Alternating gains and losses	7 (43.8%)	12 (40.0%)	
Location of the CNVs			0.181
Chromosome arm	8 (50.0%)	9 (30.0%)	
Whole chromosome	8 (50.0%)	21 (70.0%)	

Table S3. Detailed information of the chromothriptic events detected by GM including the genes involved in the rearrangements (*shown in the excel file attached*).

Table S4. Detailed information of the chromothriptic events detected by GM and karyotypes obtained by CBA (*shown in the excel file attached*).

Table S5. Results obtained by optical genome mapping in those chromosomes with chromothripsis (*shown in the excel file attached*).

Table S6. Review of the literature published about chromothripsis in CLL (*shown in the excel file attached*).

En las próximas páginas se muestra una adaptación de la tabla S6.

Table S6. Review of the literature published about chromothripsis in CLL**.**

Publication	Disease	Methodology	Cohort of the study		Clinico-biological characteristics	
			Cohort of the study	**Patients with chromothripsis**	**Genomic complexity**	***TP53* deletion and/or mutation**
Stephens et al, 2011	CLL	WGS	10	1 (untreated 62-year-old woman)	Not specified	Not specified
Edelmann et al, 2012	CLL	SNP Array Version 6.0	353 untreated CLL (CLL8 trial) 144 with paired reference DNA	19 cases^ (7/19 with ≥10 switches; 12/19 with 7-9 switches)	Not specified	36.8% (7/19 cases)
Pei et al, 2012	CLL	CBA FISH Affymetrix CytoScan HD array	1	1	Case with CK by CBA and >5 CNA by GM	Deletion in 63% of nuclei Mutation not assessed
Bassaganyas et al, 2013	CLL	CBA FISH SNP Array Version 6.0 WGS, WES RNA-sequencing Targeted re-sequencing	Longitudinal analysis during 11 years of a 59-year-old CLL case with chromothripsis (4 samples)	1	Non-CK by CBA; but >5 CNA by GM	No
Li Tan et al, 2015	SLL / CLL	CBA FISH SNP array	A 62-year-old woman previously untreated	1	Case with CK by CBA and >5 abnormalities by GM	Deletion in 43% of nuclei
Puente et al, 2015	CLL	SNP Array Version 6.0 WGS of 150 tumor/normal pairs WES of 440 cases (84 with both WGS and WES)	452 CLL + 54 MBL Pretreatment tumor and matched non-tumor samples	15/452 CLL cases^	All of them had ≥5CNA	26.7% (4/15 del/mut*TP53*)

^Chromothripsis included patients with at least 7 copy number switches between 2-3 copy number states.

*79% with high-risk genomic aberrations, defined by the presence of del(11)(q22.3), del(17)(p13) and/or TP53 mutation.

**2658 tumors from 38 cancer types studied.

#Data regarding survival is expressed based on the available information in the respective publications.

Clinico-biological characteristics			Main results	
***ATM* deletion**	**Unmutated IGHV**	**Survival [#]**	**Candidate genes in chromothriptic regions**	**Other findings**
Not specified	Not specified	Rapid deterioration	No gene proposed	42 somatically acquired genomic rearrangements involved the long arm of chromosome 4. Nine rearrangements joined chromosome 4 to other chromosomes. She had a rapid deterioration in her clinical course, with quick relapse after treatment with alemtuzumab. The rearrangements persisted in a post-treatment sample collected 31 months after the initial sample, with no emergence of new genomic rearrangements.
42.1% (8/19 cases)	74%	Poor prognosis Median OS: 47m; *p* <0.01 Median PFS: 20m; *p* <0.01	No gene proposed	In 2/7 cases with chromothripsis, a second chromosome carried frequent CNA with 6 and 8 copy numbr changes. More commonly affected chromosomes are not specified.
No	Not specified	Not specified	No gene proposed	CBA detected a del(14)(q22), a t(2;5;7)(q31;q21;q11.2) and a loss of all or part of chromosome 6. SNP arrays profile revealed discontinous deletions found in chromosomes 2, 5, 6 and 7.
Mutated *ATM*	Yes	Not specified	No gene proposed	CBA and FISH only detected a der(18)t(4;18)(q12;p11.2). Chromothripsis patterns were found in chromosomes 1, 6, 10 and 12. Within chromothripsis rearrangements, they found a deletion in 6q21 and a deletion in 10q24 (*NFKB2*). Chromothripsis-related rearrangements led to a fusion transcript of *UBR2-SPATS1* Besides, they also detected an additional somatic *de novo* SNV in *ATM* as well as a mutation in *NFKBIE*. CBA and FISH showed the same profile in the four samples. Chromothripsis was not detected in the additional samples (one pre-treatment; two post-treatment). Chromothripsis subclones did not survive chemotherapy, having no apparent implications for patient prognosis.
Not specified	Not specified	Rapid deterioration	No gene proposed	Chromothripsis patterns detected in chromosomes 8, 17 and 19p.
6.7% deleted (1/15) 13.3% mutated (2/15)	66.7% (10/15 cases)	Inferior TTT (p<0.05)	*SETD2*	The authors did not find a recurrent rearrangement. However, they observed involvement of chromosome 13 in 4/15 cases with chromothripsis, resulting in loss of microRNAs mir-15a and mir-16. *SETD2* inactivation was more frequent among CLL cases with chromothripsis (26% vs 1.4%)

Table S6. Review of the literature published about chromothripsis in CLL (*Cont.*).

Publication	Disease	Methodology	Cohort of the study		Clinico-biological characteristics	
			Cohort of the study	**Patients with chromothripsis**	**Genomic complexity**	***TP53* deletion and/or mutation**
Salaverria et al, 2015	CLL	Custom oligonucleotide-based CGH array	180	8/180 (4.4%)^	Not specified	75% (6/8 cases with deletion 3/6 with concomitant mutation)
Parker et al, 2016	CLL	SNP array	1006 patients from the ADMIRE, ARCTIC, UK CLL4, GCLLSG CLL8, SCSG CLL2O trials	27 (2.7%)	Not specified	Not specified
Burns et al, 2018	CLL	WGS	46	1	>5 CNA by WGS	Yes
Cortés-Ciriano et al, 2020	CLL**	WGS	86 cases with CLL PCAWG Consortium	1^	Not specified	WT
Leeksma, et al 2021	CLL	CBA (122 cases) FISH (260 cases) SNP array CGH array	2293 from 13 European CLL laboratories	32	Associated with high total number of CNA (median 6)	81% (26/32 cases)
Present series	CLL	CBA FISH WCP SNP array OGM	162 cases with CK	33	33/33 with >5CNA by GM (30/33 with CK by CBA)	69.7% (23/33 cases)

Clinico-biological characteristics			**Main results**	
***ATM* deletion**	**Unmutated IGHV**	**Survival [#]**	**Candidate genes in chromothriptic regions**	**Other findings**
6.7% deleted (1/15) 13.3% mutated (2/15)	66.7% (10/15 cases)	Inferior TTT (p<0.05)	*SETD2*	The authors did not find a recurrent rearrangement. However, they observed involvement of chromosome 13 in 4/15 cases with chromothripsis, resulting in loss of microRNAs mir-15a and mir-16. *SETD2* inactivation was more frequent among CLL cases with chromothripsis (26% vs 1.4%)
Not specified	Not specified	Poor prognosis (5-year OS 0% vs 56%, p=0.02)	*TERT*	In 3/8 cases, the authors observed a shattered 5p arm, alternating gains and losses, including gain of *TERT* (confirmed by FISH). In MVA for OS including 17p13 deletions, chromothripsis and >6 CNA, only del(17)(p13) (HR 3.5; 95% CI: 1.7-7.2; p=0.001) showed independent prognostic impact.
Not specified	Not specified	Not specified	*SETD2*	The authors study the presence of deletions and mutations of the *SETD2* gene in large CLL cohorts. They concluded that the inactivation of this gene, involved in the maintenance of genome stability, was associated with genomic complexity, *TP53* abnormalities and chromothripsis.
Yes	Yes	Not specified	No gene proposed	This work focuses on the study by WGS of the landscape of non-coding mutations of both mutated and unmutated IGHV CLL. There is no specific comment on the only case with chromothripsis. It is just mentioned as an additional finding.
Not specified	Not specified	Not specified	No gene proposed	After analyzing several samples from different tumors, the authors concluded that their estimated frequencies of cth are higher than previously reported, maybe due to the increased sensitivity of the algorithm employed for its detection. However, they found that prevalence of cth is highly variable among cancer types. Affected chromosomes in the CLL case are not reported.
19% (6/32 cases)	Not specified	Poor prognosis Median OS: 3.7 years, [95% CI]: 0.6-6.8 years; p<0.001	No gene proposed	Chromosomes most frequently affected with cth were 2 (n=3), 3 (n=6), 6 (n=3), 8 (n=4) and 17 (n=3). Survival of these patients was worse than that of cases with *TP53* abn or del*ATM* without chromothripsis.
27.3% (9/33 cases)	74.2% (23/31 cases)	Poor prognosis Median TTFT: 2 months, [95% CI]: 0-6 months; *p*=0.013	No gene proposed	Chromothripsis was more frequently found in chromosomes 3, 6 and 13. No differences were observed when analyzing patients with 7-9 switches or ≥10. Only *TP53* was significant in the MVA, including *TP53,* chromothripsis and genomic complexity defined by GM.

Las tablas suplementarias S3, S4, S5 y S6 originales se pueden visualizar accediendo en el siguiente enlace:

www.mdpi.com/article/10.3390/cancers14153715/s1

Supplementary Figures

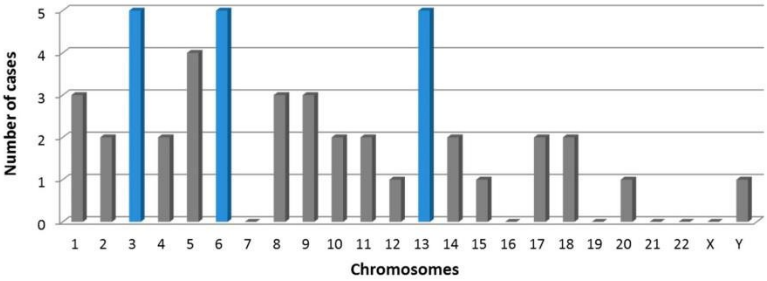
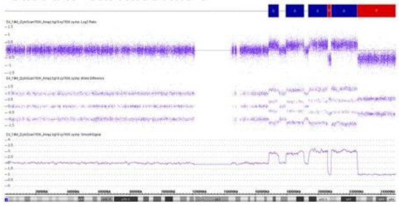
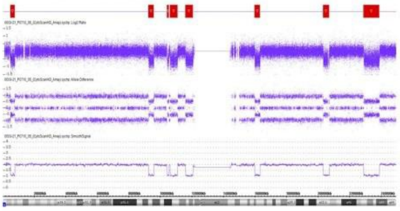


Figure S1. **Number of cases showing chromothripsis for each chromosome**. Chromosomes are represented on the X-axis and the total number of cases showing chromothripsis for each chromosome is represented on the Y-axis. The chromosomes involved most in the cohort (3, 6 and 13) are highlighted in blue.

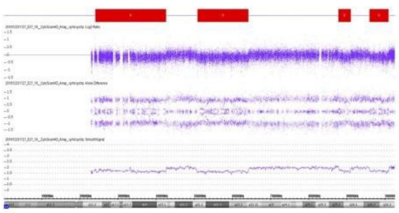
Case #1: Chromosome 1



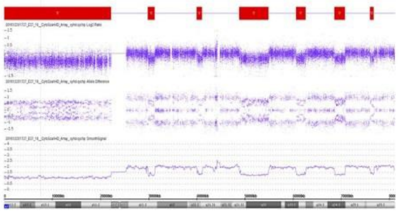
Case #2: Chromosome 1



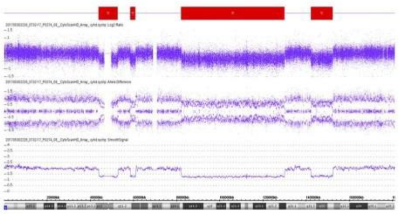
Case #3: Chromosome 15



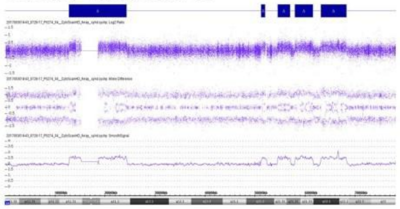
Case #3: Chromosome 17



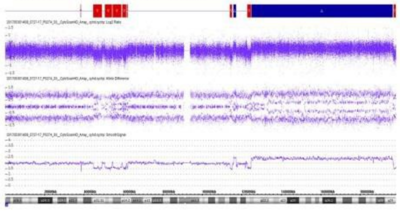
Case #4: Chromosome 3



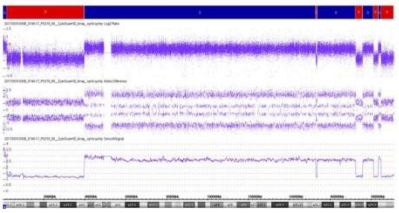
Case #5: Chromosome 18



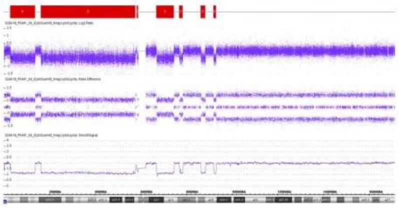
Case #6: Chromosome 5



Case #7: Chromosome 4



Case #8: Chromosome 6



Case #9: Chromosome 6

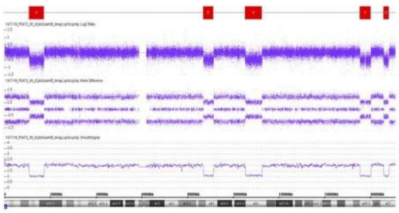
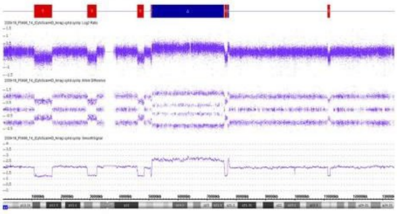
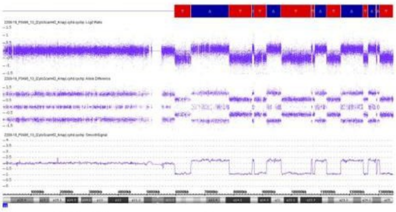


Figure S2. **Copy number profiles of some of the chromothriptic chromosomes**. Copy number profiles derived from genomic microarray analyses were available for 18 cases.

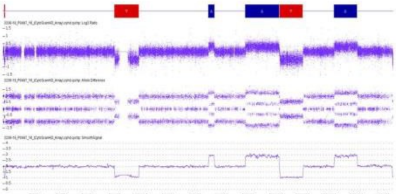
Case #16: Chromosome 12



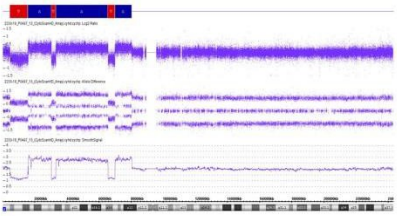
Case #17: Chromosome 11



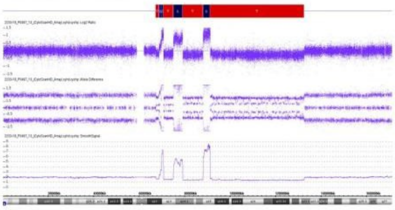
Case #18: Chromosome 8



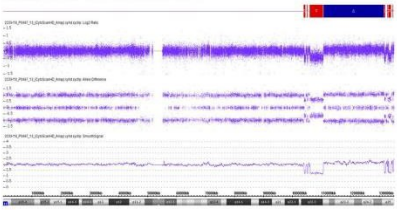
Case #19: Chromosome 2



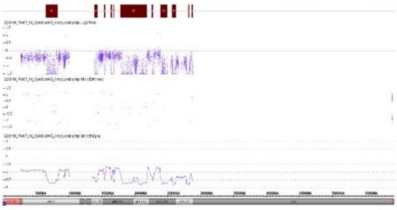
Case #19: Chromosome 6



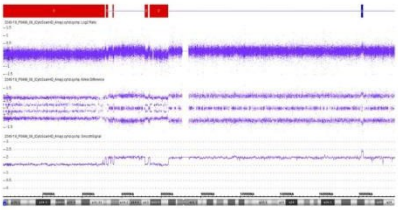
Case #19: Chromosome 11



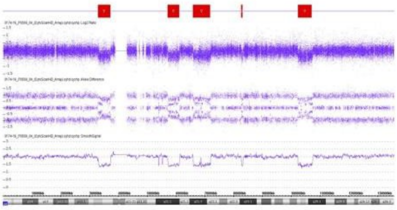
Case #19: Chromosome Y



Case #20: Chromosome 3



Case #26: Chromosome 10



Case #26: Chromosome 13

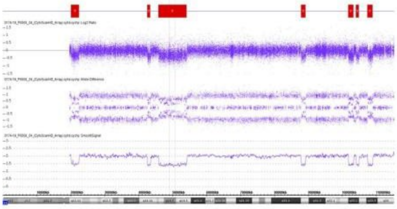
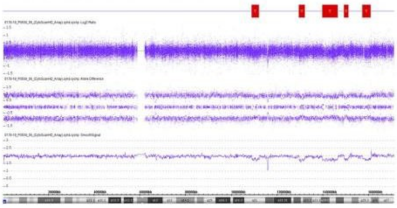
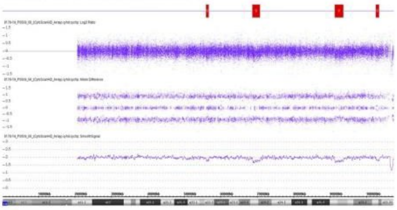


Figure S2 (cont.). Copy number profiles of some of the chromothriptic chromosomes. Copy number profiles derived from genomic microarray analyses were available for 18 cases.

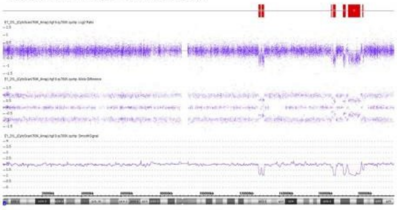
Case #27: Chromosome 6



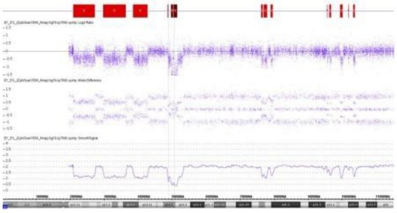
Case #27: Chromosome 14



Case #31: Chromosome 3



Case #31: Chromosome 13



Case #32: Chromosome 6

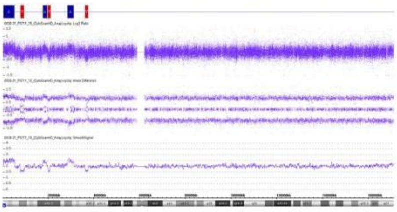


Figure S2 (cont.). **Copy number profiles of some of the chromothriptic chromosomes**. Copy number profiles derived from genomic microarray analyses were available for 18 cases.

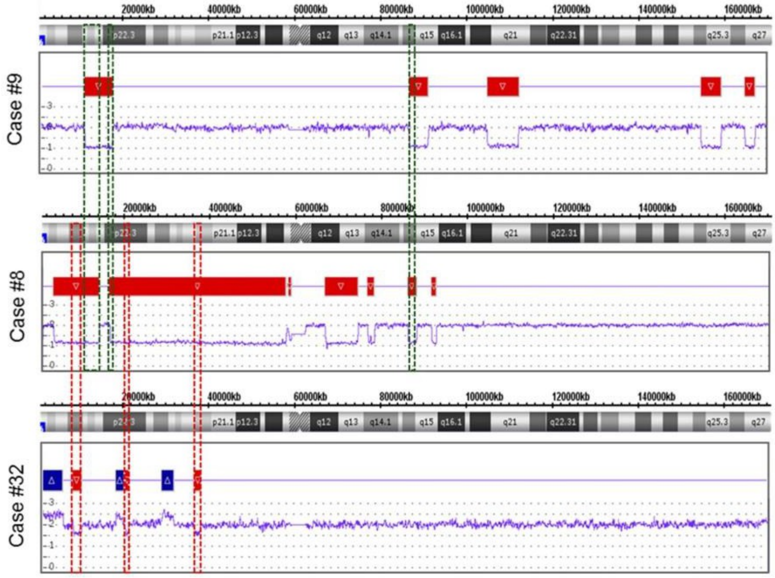


Figure S3. Chromothripsis detected in chromosome 6 in those cases in which optical genome mapping only revealed intra-chromosomal chromothripsis-related rearrangements. Whole chromosome 6 view of genomic microarray results from cases #8, #9 and #32, which carried intra-chromosomal rearrangements when analysed by OGM, are represented. Only small deleted fragments were common between case #8 and the other two remaining cases. Specifically, three fragments of 2.83 Mb (10,743,398-13,571,692), 1.20 Mb (16,137,146-17,257,084) and 1.17 Mb (86,948,132-88,115,441) were shared between cases #8 and #9 (highlighted in green) and three fragments of 1.79 Mb (7,651,724-9,438,895), 0.64 Mb (19,953,714-20,591,009) and 1.28 Mb (36,084,473-37,366,801) were common between cases #8 and #32 (highlighted in red).

IV. DISCUSIÓN

En los últimos años, la complejidad genómica en pacientes con LLC ha ido adquiriendo cada vez una mayor importancia por su asociación con un curso clínico más agresivo y especialmente por su papel controvertido como factor predictivo de la respuesta a los nuevos tratamientos. Actualmente, la complejidad genómica se puede analizar mediante CC y MG, aunque se están desarrollando nuevas metodologías citogenómicas con un gran potencial como el OGM. Debido al gran interés que suscitan los casos con CK y/o complejidad genómica, la presente tesis se ha centrado en este subgrupo de pacientes. Concretamente, se ha explorado la utilidad de las técnicas citogenómicas disponibles en los laboratorios de citogenética en la detección de la complejidad genómica, la comparación de estas técnicas en cuanto a la estratificación pronóstica de los pacientes con LLC, la complejidad asociada a patrones de cromotripsis y el impacto pronóstico de este fenómeno en un contexto de complejidad genómica.

1. Detección de complejidad genómica mediante las técnicas citogenómicas disponibles actualmente

El primer trabajo de esta tesis doctoral se ha centrado en la comparación de la utilidad de la CC y los MG en la detección de complejidad genómica y en la categorización de los pacientes en distintos grupos de riesgo en base a la complejidad genómica detectada por ambas técnicas (Ramos-Campoy *et al., Haematologica* 2022). Anteriormente, tan solo Leeksma y colaboradores habían estudiado un total de 122 pacientes en paralelo por CC y MG, pero la proporción de casos con CK en esta cohorte era muy baja, tal y como se espera en una población de LLC no seleccionada (Leeksma *et al.*, 2021). En este sentido, los pacientes incluidos en el primer artículo constituyen la mayor cohorte de LLC enriquecida en casos con CK analizada de manera simultánea por CC y MG.

Teniendo en cuenta los criterios propuestos en los dos grandes estudios cooperativos retrospectivos del ERIC para categorizar a los pacientes en tres grupos de riesgo en función del número de alteraciones detectadas por CC o MG (0-2, 3-4, ≥5), no se observaron diferencias en el porcentaje de pacientes clasificados en cada categoría. Sin embargo, al analizar los casos de manera individual únicamente se observó un grado de acuerdo moderado entre ambas técnicas. Un 29,4% de los pacientes se clasificó en grupos discordantes, incluyendo alrededor de un 3% que estaban categorizados en grupos de riesgo opuestos en función del método empleado para su estudio. Cabe destacar que las discordancias fueron debidas fundamentalmente a limitaciones intrínsecas propias de cada técnica. Como todas las metodologías, tanto la CC como los MG tienen sus ventajas y sus limitaciones. Esto es esperable puesto que de entrada ambas parten de tipos de muestra distintos. En la CC es necesario cultivar las muestras de sangre periférica, introduciendo así un factor que puede potencialmente modular los resultados obtenidos ya que éstos van a depender de la eficacia del cultivo y de la capacidad proliferativa que tenga el clon anómalo *in vitro*. Esta limitación metodológica ha mejorado con la incorporación de los nuevos mitógenos. De hecho, en las guías actuales del ERIC se recomienda llevar a cabo dos cultivos en paralelo con distinto mitógeno, TPA y DSP30 + IL-2, para cada paciente ya que se ha visto que es la mejor estrategia para identificar un mayor número de casos con cariotipos anómalos (Baliakas *et al.*, 2022). No obstante, en muchos centros todavía no se han incorporado los nuevos mitógenos y se sigue realizando un único cultivo con TPA. En cambio, en los MG la muestra de partida es ADN total de la muestra de sangre periférica, de manera que no es necesario obtener muestras frescas para estimular su proliferación *in vitro*. Además, tienen una mayor resolución que la CC, permitiendo detectar alteraciones crípticas por CC de hasta 50 Kb y definir mejor las regiones con ganancias y pérdidas de material genético. Esto hace que los MG sean capaces de ofrecer una visión más compleja de alteraciones que parecen sencillas por CC como ciertas monosomías o deleciones simples que en realidad están constituidas por múltiples pérdidas o incluso forman parte de eventos de cromotripsis. Por contra, una de las principales desventajas de las plataformas de MG en comparación con la CC es su sensibilidad limitada, lo cual dificulta la detección de alteraciones presentes en menos del 20% de la población tumoral. Aunque en este trabajo se ha utilizado ADN extraído de distintos tipos de muestra (sangre periférica, células mononucleadas, células CD19+ seleccionadas con métodos inmunomagnéticos de sangre periférica y médula ósea), pudiendo influir en la sensibilidad de la técnica, los MG detectaron alteraciones incluso en las muestras de sangre periférica total. Esto es debido a que la LLC se caracteriza por presentar de manera general una gran expansión de la población de células B tumorales en sangre periférica. Por otra parte, los MG no permiten detectar translocaciones equilibradas que sí pueden ser identificadas por CC. A pesar de que este tipo de alteración es poco frecuente en la LLC, la identificación de translocaciones equilibradas puede ayudar en algunas ocasiones en el diagnóstico diferencial entre una LLC y otras neoplasias de célula B madura, como los linfomas foliculares o los linfomas de células del manto. Por otro lado, se ha confirmado que algunos casos con reordenamientos aparentemente desequilibrados en realidad no van acompañados de ganancias o pérdidas de material, por lo que no serían detectados por MG. Otro factor que diferencia los MG de la CC es que los MG no permiten definir la presencia de subclones de modo que no ofrecen información sobre la heterogeneidad citogenética intraclonal. Esto supone que mediante el análisis de MG no se puede detectar la presencia de evolución clonal, correlacionada con la adquisición de alteraciones en *TP53* y con una peor evolución clínica en pacientes con LLC (Condoluci and Rossi, 2020; Bracher *et al.*, 2022). Por el contrario, aunque no es un aspecto que se haya tenido en cuenta en el trabajo presentado, las plataformas de MG que incluyen sondas de SNP permiten la detección de regiones de CN-LOH, una alteración que no se puede identificar ni por CC ni por FISH. La detección de una CN-LOH puede ser indicativa de una mutación en algún gen supresor de tumores relevante para la patología de interés que por la presencia de la CN-LOH pasa a ser bialélica. En consecuencia, la identificación de una CN-LOH orienta a realizar estudios moleculares para analizar la presencia de mutaciones. Asimismo, aunque en la LLC esto no es muy relevante, los *microarrays* de SNP también permiten detectar el tipo de ploidía.

Globalmente, y teniendo en cuenta las ventajas y limitaciones de cada una, en la actualidad la CC es la técnica de elección para el estudio citogenómico de la LLC aunque en algunos centros europeos ha sido reemplazada por los MG. En cambio, en otras hemopatías malignas, como en la leucemia linfoblástica aguda (LAL), los MG se emplean de manera habitual para su estudio citogenético. Sin embargo, en estas patologías los MG no se utilizan para realizar un estudio global de todo el genoma, y así identificar complejidad genómica, sino que se utilizan para la detección de alteraciones clínicamente relevantes, crípticas por CC o FISH, como las pequeñas deleciones en *IKZF1* (Baughn *et al.*, 2015).

Por otro lado, se han publicado unos criterios para analizar e interpretar los resultados derivados de los MG. Así, las guías actuales recomiendan establecer el punto de corte para filtrar las alteraciones no clásicas de la LLC en cuanto a su tamaño en ≥5Mb (Schoumans *et al.,* 2016). De esta manera, se intenta excluir aquellas alteraciones pequeñas sin relevancia clínica y reducir la probabilidad de incluir alteraciones potencialmente benignas. Las CNA <5Mb únicamente se tienen en cuenta si incluyen algún gen conocido cuya relación con la enfermedad esté demostrada. Aunque parece un filtro muy conservador, en el primer artículo no se obtuvo una mejor correlación en el número de alteraciones detectadas por las dos técnicas cuando se aplicaron diferentes estrategias de filtrado por tamaño, incluso considerando las CNA ≥1Mb o todas las CNA detectadas. De esta manera, se ha demostrado que las recomendaciones actuales para el análisis de los MG en neoplasias hematológicas son adecuadas para el estudio de la complejidad en LLC.

En resumen, las técnicas citogenómicas disponibles actualmente en la práctica clínica (CC, FISH y MG) aportan información relevante para el estudio de la LLC pero no equivalente. Por lo tanto, la mejor caracterización de la enfermedad se obtiene cuando se realizan de manera complementaria (Puiggros *et al.*, 2013a). Sin embargo, el coste económico asociado y el tiempo invertido dificultan la combinación de múltiples técnicas en los laboratorios diagnósticos. Un hallazgo interesante del primer artículo es que, a pesar de la elevada concordancia entre FISH y MG (90%), los MG no identificaron alrededor de un 20% de los casos con deleción de *TP53* que sí había sido identificada por FISH. La razón de estas diferencias estaba relacionada con la sensibilidad limitada de los MG (Puiggros *et al.*, 2013a). La presencia de alteraciones en *TP53*, tanto deleciones como mutaciones, debe estudiarse de manera obligatoria en todos los casos con LLC antes de iniciar tratamiento ya que es el biomarcador pronóstico más importante en la patología (Hallek *et al.*, 2018). De hecho, según las guías actuales del iwCLL, la estratificación del riesgo genético en la LLC requiere un análisis por FISH de las cuatro regiones clásicas complementado con el análisis mutacional de *TP53* e IGHV, y exponen que la realización de la CC es “deseable” antes de iniciar tratamiento en el contexto de los ensayos clínicos aunque también es útil en la práctica general (Hallek *et al.*, 2018). Nuestros resultados sugieren que la complejidad genómica debería analizarse siempre, al menos en ensayos clínicos prospectivos, ya sea por CC o por MG, para definir mejor el pronóstico de los pacientes ya que el estudio del cariotipo y/o de los perfiles de MG permite identificar alteraciones no cubiertas por el panel estándar de sondas de FISH, incluyendo la presencia de CK/complejidad genómica. Además, de esta manera, se podría esclarecer el valor pronóstico y predictivo de la respuesta al tratamiento de la complejidad genómica, especialmente en la era de los nuevos fármacos. En este sentido, los hallazgos obtenidos confirman que el FISH debe mantenerse como técnica para el estudio de las alteraciones en *TP53* pero, como este método únicamente ofrece una visión parcial del genoma, debería complementarse con otra técnica que permita el análisis de todo el genoma para evaluar la complejidad genómica y así poder estratificar a los pacientes en distintos grupos según su riesgo de progresión. La técnica empleada para dicho análisis debería elegirse entre la CC y los MG, que son las técnicas actualmente disponibles e implementadas en la práctica clínica, y la decisión final dependerá de cada laboratorio, teniendo en cuenta, entre otros factores, los métodos y equipamientos disponibles, la experiencia del personal y los costes económicos de cada una (Baliakas *et al.*, 2022).

Por otro lado, es interesante destacar el desarrollo de nuevos métodos citogenómicos que podrían potencialmente sustituir la CC y los MG en un futuro. En los últimos años ha surgido una nueva metodología muy prometedora, el mapeo óptico del genoma, capaz de superar las limitaciones de las técnicas convencionales en un solo test. Recientemente, varios estudios han demostrado que el OGM es una técnica útil para el estudio citogenético ya que ha podido identificar entre un 90-100% de las alteraciones citogenómicas clínicamente relevantes en diferentes neoplasias hematológicas, como leucemia mieloide aguda (LMA), síndromes mielodisplásicos (SMD), LAL o mieloma múltiple (Kriegova *et al.*, 2021; Lühmann *et al.*, 2021; Neveling *et al.*, 2021; Gerding *et al.*, 2022; Levy *et al.,* 2022; Rack *et al.,* 2022). Además, es capaz de desvelar alteraciones adicionales cuyo impacto clínico aún se desconoce. En la LLC, el OGM ha sido capaz de detectar hasta un 90% de las alteraciones previamente identificadas por CC, FISH o MG, con una alta concordancia en cuanto a tamaño y coordenadas en comparación con las CNA detectadas por MG. Asimismo, la identificación de nuevas alteraciones ha contribuido a mejorar la descripción de algunas anomalías cromosómicas estructurales identificadas en el cariotipo (Puiggros *et al.*, 2022). En este caso, las diferencias con respecto a las técnicas convencionales son debidas a limitaciones del OGM, como por ejemplo la dificultad para detectar alteraciones en regiones altamente repetitivas, como los centrómeros o los telómeros, o la sensibilidad limitada para identificar alteraciones numéricas. En cuanto a la capacidad para detectar complejidad genómica, en el mayor estudio llevado a cabo hasta la fecha en una cohorte con LLC se ha establecido de manera aleatoria el punto de corte en diez o más alteraciones para discernir el grupo de alta complejidad por OGM asociado a una peor evolución clínica (Puiggros *et al.*, 2022). Sin embargo, la cohorte analizada estaba enriquecida en casos con CK de manera que no se puede extrapolar este valor a una cohorte de LLC no seleccionada. Globalmente, esta técnica permite obtener en un solo test resultados que actualmente requieren la realización de CC junto con múltiples análisis de FISH. Sin embargo, aunque parece que el OGM tiene un gran potencial para el estudio de la complejidad genómica, es necesario estudiar cohortes más amplias para establecer unos criterios estándar que permitan definir la complejidad genómica por OGM antes de incorporar esta técnica en la rutina diagnóstica. Un aspecto interesante del OGM es que su análisis se lleva a cabo con un *software* relativamente sencillo de utilizar, lo cual facilita su incorporación en los laboratorios clínicos. A pesar de estas ventajas, los gastos totales asociados al OGM, incluyendo los costes de los reactivos, el equipo y la infraestructura técnica necesaria así como el tiempo que se debe dedicar a nivel de laboratorio, siguen siendo superiores a los de la CC. En consecuencia, la aplicación del OGM no permite abaratar los costes asociados al análisis citogenómico pero sí ofrecer una mejor caracterización genómica, permitiendo además la identificación de alteraciones adicionales en genomas complejos. Del mismo modo, a pesar de que las técnicas de secuenciación del genoma completo se han utilizado en diversos estudios para la caracterización genética de la LLC, e incluso de la cromotripsis (Stephens *et al.*, 2011; Puente *et al.*, 2015; Burns *et al.*, 2018; Cortés-Ciriano *et al.*, 2020), éstas siguen teniendo ciertas limitaciones que impiden su implementación en la práctica rutinaria, como su elevado coste económico y la falta de estandarización metodológica y armonización en los criterios de análisis e interpretación de resultados.

2. Estratificación pronóstica de los pacientes con LLC según la complejidad genómica detectada por CC y MG

En consonancia con lo que se había reportado anteriormente en las publicaciones del ERIC, las tres categorías citogenéticas definidas por CC y MG en el primer artículo de esta tesis presentaban diferencias significativas en el TPT, independientemente del estado de *TP53* o IGHV. En el caso de la SG, sólo los grupos de mayor riesgo (≥5 alteraciones por CC o ≥5 CNA por MG) presentaron diferencias significativas. Así pues, tanto la CC como los MG han probado ser eficaces en la predicción del riesgo de evolución de los pacientes, sin que ninguna de las técnicas muestre un peor rendimiento en comparación con la otra. Es importante mencionar que aunque la heterogeneidad en cuanto a las plataformas de MG utilizadas en este estudio podría suponer una limitación, todos los casos se revisaron e interpretaron usando los mismos criterios para el filtrado de las CNA y se obtuvieron resultados similares entre las distintas plataformas.

A pesar de que durante décadas los pacientes con CK se han considerado un único subgrupo homogéneo, se ha demostrado que en realidad no todos los CK son equivalentes y su impacto clínico va a depender no solo del número sino también del tipo de alteraciones que presenten. Con respecto al número, aunque tradicionalmente los CK se han definido por la presencia de tres o más alteraciones en un mismo clon celular, su definición sigue generando controversia. Baliakas y colaboradores demostraron que los pacientes con CK se podían clasificar en tres grupos de riesgo según tenían tres (CK bajo), cuatro (CK intermedio), o cinco o más (CK alto) alteraciones, siendo los últimos los que se asociaban a peor pronóstico independientemente del estado de *TP53* o IGHV (Baliakas *et al.*, 2019). Con respecto a los MG, éstos han demostrado ser útiles para el estudio de la LLC ya que no solo permiten la identificación de las anomalías clínicamente relevantes para la enfermedad sino que además pueden detectar alteraciones adicionales, que podrían tener impacto en el pronóstico (por ejemplo, la ganancia de 2p o la ganancia de 8q), e incluso complejidad genómica (Chun *et al.*, 2018). De hecho, en los estudios iniciales ya se demostró que la detección de un mayor número de alteraciones por MG se correlacionaba con un peor pronóstico (Gunnarsson *et al.*, 2011; Ouillette *et al.*, 2011a). Sin embargo, hasta hace poco tiempo ningún grupo había propuesto una definición de complejidad genómica por esta técnica. En este sentido, el ERIC llevó a cabo el estudio con la cohorte más amplia analizada por MG. En este trabajo se reportaron diferencias significativas en el TPT según el número de CNA detectadas por MG. De nuevo, los pacientes se podían segregar en distintos grupos de riesgo según tenían tres (complejidad genómica baja), cuatro (complejidad genómica intermedia) o cinco o más (complejidad genómica alta) CNA. De acuerdo a lo observado con CC, los pacientes con cinco o más CNA detectadas por MG eran los que presentaban una enfermedad más agresiva (Leeksma *et al.*, 2021). Por lo tanto, parece que la idea más aceptada actualmente es que los pacientes con LLC que presenten cinco o más alteraciones detectadas por CC o MG van a desarrollar una enfermedad más agresiva. El curso clínico de los pacientes con tres o cuatro va a depender de la presencia de alteraciones en *TP53*.

Las guías actuales de nomenclatura citogenómica del ISCN incluyen un método para el contaje de las alteraciones pero no exponen una definición clara de CK (McGowan-Jordan *et al.*, 2020). Esto es debido a que no existe un consenso, es decir, la definición es específica de cada enfermedad de manera individual en relación a su impacto en el pronóstico y éste varía entre las distintas neoplasias hematológicas e incluso entre diferentes ensayos clínicos de una misma hemopatía, como en el caso de la LMA. En este sentido, el grupo francés de citogenética hematológica ha llevado a cabo una revisión en la que plasman las diferencias que existen en cuanto a la definición y en cuanto al impacto clínico del cariotipo complejo en distintas neoplasias hematológicas (Nguyen-Khac *et al.*, 2022). Mientras que en algunas entidades, como en los SMD o LMA, el CK tiene un claro valor pronóstico e incluso su identificación se incluye en índices pronósticos, en otras como en el linfoma folicular o el linfoma esplénico de la zona marginal, sigue siendo incierto. En los SMD, por ejemplo, el CK se define por la presencia de tres o más alteraciones y es uno de los biomarcadores más importantes de pronóstico adverso (Bersanelli *et al.*, 2021). De hecho, la detección de CK mediante CC está incluida en el índice IPSS-R (*Revised International Prognostic Scoring System*) (Greenberg *et al.*, 2012). En la LMA, el CK es una de las alteraciones más frecuentes. En esta entidad, la presencia de CK también se ha correlacionado con un peor pronóstico y está incluida en el grupo de riesgo desfavorable. Sin embargo, no se ha llegado a un consenso claro para su definición (tres o más alteraciones según los ensayos del *European Leukemia Net* vs cuatro o más alteraciones cromosómicas según el *Medical Research Council*) aunque la mayoría de las clasificaciones pronósticas consideran como CK los casos con tres o más alteraciones (Bager *et al.*, 2018). En la LLC, tanto su definición como su impacto clínico han suscitado un gran debate. No obstante, como se ha mencionado anteriormente, los resultados obtenidos en el estudio de Baliakas y colaboradores, en el que se ha incluido la cohorte más amplia hasta el momento analizada por CC, sugieren establecer el punto de corte en cinco o más para identificar aquellos pacientes con mayor riesgo de progresión (Baliakas *et al.*, 2019). En este sentido, Jondreville y colaboradores sugirieron utilizar el concepto de "CK" para los casos con tres o cuatro alteraciones y el de "alto-CK" para aquellos con cinco o más en todas las neoplasias hematológicas (Jondreville *et al.*, 2020). Por lo tanto, es necesario que se establezcan unos criterios estándar que permitan definir la complejidad genómica en cada entidad, tanto por CC como por MG si se aplica esta técnica, con unos puntos de corte consensuados en cuanto a las alteraciones cromosómicas que la componen, teniendo en cuenta su impacto en el curso clínico de la patología de manera que sea consistente en los diferentes estudios.

En cuanto al impacto del tipo de las alteraciones que componen un CK en LLC, se ha confirmado que los pacientes con múltiples trisomías, aquellos con +12, +19, y otra alteración numérica [normalmente +18] y/o estructural [frecuentemente del(13q)], presentan unos rasgos muy característicos (mayor frecuencia de IGHV mutado, mayor expresión de cadenas ligeras λ, baja prevalencia de alteraciones en *TP53)* y muestran un curso clínico muy favorable a pesar de tener un CK por definición (Baliakas *et al.*, 2014; Baliakas *et al.*, 2016; Baliakas *et al.*, 2019). Por otro lado, varios estudios han explorado si la presencia de reordenamientos equilibrados y desequilibrados, alteraciones que únicamente pueden detectarse por CC, pueden correlacionarse con un pronóstico adverso. Si bien en un inicio Mayr y colaboradores reportaron que aquellos pacientes con LLC y CK tenían una peor evolución clínica especialmente en presencia de translocaciones (Mayr *et al.*, 2006), en estudios más recientes este impacto negativo se ha asociado principalmente a la presencia de reordenamientos desequilibrados y a su relación con los CK (Baliakas *et al.*, 2014; Heerema *et al.*, 2021; Costa *et al.*, 2022). De hecho, en trabajos previos se ha visto que los casos con CK que presentan reordenamientos desequilibrados constituyen un subgrupo de muy mal pronóstico. En este estudio los pacientes con CK y translocaciones desequilibradas presentaban una mayor frecuencia de alteraciones en *TP53*. Sin embargo, el impacto pronóstico negativo de las translocaciones era independiente del estado de *TP53*. Además, los pacientes presentaban una expresión desregulada de genes implicados en el control del ciclo celular y en la respuesta al daño en el ADN (Rigolin *et al.*, 2018). Incluso se ha visto que combinando la presencia de CK y/o reordenamientos desequilibrados detectados por CC y el estado mutacional de las IGHV se podría mejorar la estratificación pronóstica de los casos y la predicción del riesgo a desarrollar una TR (Visentin *et al.*, 2019; Visentin *et al.*, 2022). Además, en un estudio reciente en el que se ha realizado el análisis citogenético de más de 2800 pacientes con LLC se ha demostrado que la presencia de un número creciente de translocaciones se asocia a un pronóstico progresivamente más desfavorable (Costa *et al.*, 2022). En la serie analizada en esta tesis, la presencia de reordenamientos desequilibrados se asociaba a un TPT más corto. Sin embargo, este impacto negativo no se pudo confirmar en el subgrupo de pacientes con CK.

Como se ha mencionado al inicio, el valor pronóstico y predictivo de los CK para el TPT y la SLP ha sido ampliamente demostrado en estudios retrospectivos llevados a cabo en pacientes tratados con los regímenes estándar de quimioinmunoterapia (Badoux *et al.*, 2011; Baliakas *et al.*, 2014a; Herling *et al.*, 2016; Le Bris *et al.*, 2017). Sin embargo, su relevancia clínica en pacientes tratados con los nuevos fármacos dirigidos, como ibrutinib, acalabrutinib y venetoclax, sigue siendo controvertida (Brown *et al.*, 2018; Mato *et al.*, 2018; Woyach *et al.*, 2018; Kipps *et al.*, 2019; Al-Sawaf *et al.*, 2020; Byrd *et al.*, 2020; Kreuzer *et al.*, 2020; Kittai *et al.*, 2021). Cabe resaltar que muchos de estos estudios han analizado el impacto del CK considerando estos pacientes como un grupo homogéneo y teniendo en cuenta los casos con tres o más alteraciones pero no aquellos con alta complejidad (cinco o más alteraciones) (Chatzikonstantinou *et al.*, 2021). Además, han comparado un número bajo de pacientes y tienen unos seguimientos relativamente cortos, motivo por el que se observan discrepancias entre los distintos trabajos. En consecuencia, es necesario que se lleven a cabo estudios adicionales con cohortes más amplias para explorar y esclarecer el impacto pronóstico y predictivo de la complejidad genómica, definida por la presencia de ≥3 y ≥5 alteraciones, en pacientes tratados con las nuevas modalidades terapéuticas. Además, los hallazgos obtenidos han confirmado que los resultados de la CC no se pueden extrapolar a los MG. La gran mayoría de los análisis de supervivencia en los que se ha evaluado el impacto de la complejidad genómica en ensayos clínicos se han llevado a cabo utilizando los resultados derivados de la CC. Recientemente, en el ensayo MURANO se ha incluido por primera vez la complejidad genómica detectada por MG y se ha demostrado que la presencia de alta complejidad genómica, definida por la presencia de cinco o más CNA detectadas por MG, se asocia a un curso clínico adverso tanto en pacientes tratados con quimioinmunoterapia como con venetoclax + rituximab (Kater *et al.*, 2020). No obstante, es necesario incluir también análisis de complejidad genómica detectada mediante

MG en ensayos clínicos prospectivos con el fin de establecer unos criterios estándar que permitan definir complejidad genómica por MG y determinar cuál es realmente su impacto en los pacientes, especialmente en aquellos tratados con los nuevos fármacos.

3. Criterios de definición de cromotripsis y complejidad genómica asociada a patrones de cromotripsis detectados por MG

En el segundo artículo se han analizado 33 casos con patrones sugestivos de cromotripsis detectada por MG (Ramos-Campoy *et al.*, *Cancers* 2022). La prevalencia de este fenómeno en la LLC es baja, encontrándose en alrededor de un 1-3% de los casos en cohortes no seleccionadas, aunque la mayoría de estos estudios están limitados a unos pocos casos con cromotripsis y no la han analizado en un contexto de alta complejidad genómica (Stephens *et al.*, 2011; Edelmann *et al.*, 2012; Puente *et al.*, 2015; Salaverria *et al.*, 2015; Parker *et al.*, 2016; Burns *et al.*, 2018; Leeksma *et al.*, 2021). En la serie descrita en esta tesis se ha encontrado cromotripsis en una mayor proporción de casos (33 de los 162 incluidos). Sin embargo, es importante tener en cuenta que, por los objetivos del estudio, la cohorte estaba constituida en su totalidad por casos con complejidad genómica, ya sea en forma de CK por CC o con más de tres alteraciones ≥5Mb por MG. Como consecuencia, esta proporción no representa la incidencia real de este fenómeno en una población de vida real de LLC no seleccionada.

En cuanto a la definición de cromotripsis, años atrás se definieron unos criterios para que la detección de este fenómeno fuera reproducible entre los distintos estudios (Korbel *et al.*, 2013). No obstante, algunos de estos criterios tienen en cuenta características que únicamente pueden observarse usando WGS, como por ejemplo la orientación de los fragmentos reordenados. Así pues, no hay un consenso claro sobre qué cantidad de cambios en el número de copia detectados por MG deben considerarse para definir un evento como cromotripsis. En este sentido, en el presente estudio se han comparado las características de los patrones y el impacto pronóstico de los fenómenos de cromotripsis formados por 7-9 con respecto a aquellos con diez o más cambios en el número de copia. Los resultados sugieren que el punto de corte puede establecerse en siete o más cambios en el número de copia puesto que no se observaron diferencias en los patrones de CNA que los constituían. Además, tampoco se observaron diferencias en el TPT entre los dos subgrupos, motivo por el cual se han considerado como un único grupo.

Con respecto a las regiones afectadas, los resultados obtenidos concuerdan con las publicaciones previas, en las que se ha visto que la cromotripsis es heterogénea y no presenta un patrón común, ni en cuanto al tipo ni en cuanto al número de alteraciones que constituyen estos patrones complejos. Asimismo, tampoco se ha encontrado una región genómica o cromosoma específico que esté alterado de manera recurrente. En la cohorte descrita, la cromotripsis estaba distribuida por todo el genoma y las regiones que presentaban estos eventos en mayor proporción fueron las localizadas en 3p21 (región que incluye el gen *SETD2*), 6q21 (contiene el gen *FOXO3a* y está presente en un 6% de los casos con LLC) y 13q14 (alteración citogenética más común entre los pacientes con LLC al diagnóstico y asociada con un impacto pronóstico muy favorable si se encuentra de manera aislada). Los tres *loci* han sido previamente relacionados con la patogénesis de la LLC e incluso se han descrito en otros casos con cromotripsis aunque su rol exacto en el desarrollo de estos patrones se desconoce (Edelmann *et al.*, 2012; Bassaganyas *et al.*, 2013; Puente *et al.*, 2015; Parker *et al.*, 2016; Jarosova *et al.*, 2017). Además, también se encontraron eventos de cromotripsis en las regiones previamente reportadas 5p13.33, 6p21 y 10q24, que engloban los genes *TERT*, *NFKBIE y NFKB2,* respectivamente (Bassaganyas *et al.*, 2013; Salaverria *et al.*, 2015). Así pues, estos hallazgos confirman la heterogeneidad de la cromotripsis de manera que no se puede establecer una correlación entre ninguna de las regiones afectadas y el desarrollo de este fenómeno. Esto sugiere que la cromotripsis no se originaría a partir de la desregulación de un gen en concreto sino que debe haber otros mecanismos, aún desconocidos, que desencadenen la disrupción de distintas regiones genómicas.

Es importante mencionar que éste es el primer estudio en el que se han comparado los patrones observados mediante MG con las alteraciones descritas en el cariotipo. Como era de esperar, no se pudo detectar ningún caso con cromotripsis mediante CC. Esto es debido fundamentalmente a la resolución de la CC, limitada a aproximadamente unas 10Mb. Por este motivo, la cromotripsis únicamente puede detectarse con técnicas de mayor resolución que la CC, como los MG, las plataformas de secuenciación o el OGM. No obstante, la mayoría de los cromosomas cromotrípticos aparecían alterados en el cariotipo. Además, uno de los rasgos característicos de los casos con cromotripsis era la presencia de cromosomas marcadores o de material adicional de origen desconocido, dando lugar a cariotipos más complejos que en los casos sin cromotripsis. De hecho, en el subgrupo de pacientes con cromotripsis era más frecuente observar casos con cinco o más alteraciones que en el grupo control. Por lo tanto, la CC no permite detectar un patrón de cromotripsis como tal pero sí puede sugerir la presencia de reordenamientos complejos mediante la identificación de alteraciones que implican material cromosómico de origen desconocido.

Por otro lado, nueve de los casos que presentaban patrones de cromotripsis se analizaron utilizando el OGM para determinar la utilidad de esta técnica en la identificación de patrones complejos. En general, el OGM detectó hasta el 88% de las CNA previamente identificadas por las técnicas convencionales y además, desveló nuevos reordenamientos asociados a la cromotripsis, incluyendo translocaciones intracromosómicas e intercromosómicas. Globalmente, el OGM ofrece una visión más detallada de las alteraciones estructurales, permitiendo así una descripción más minuciosa que los MG de los patrones de cromotripsis. Aun así, tampoco permitió identificar ningún gen común (implicado en los puntos de corte de los reordenamientos o en las regiones con CNA) relacionado con la aparición de la cromotripsis. Sin embargo, sí permitió identificar dos patrones de reordenamientos asociados. En concreto, los reordenamientos adicionales encontrados relacionados con la cromotripsis estaban focalizados en la región con cromotripsis o bien implicaban cromosomas cromotrípticos y no cromotrípticos. Como se ha mencionado anteriormente, la cohorte estudiada por OGM tan solo incluía nueve casos de manera que no se pueden extraer conclusiones, pero sería interesante explorar si estos patrones de reordenamientos diferentes tienen un impacto distinto en la evolución de este subgrupo de pacientes.

Gracias al estudio mediante OGM se pudo identificar un caso con un patrón típico de otro evento catastrófico, englobado dentro del concepto de cromoanagénesis, conocido como cromoplexia. A pesar de que se han reportado casos con cromoplexia con anterioridad, tanto en tumores sólidos como en algunas neoplasias hematológicas, incluyendo MM, linfoma de células del manto o LLC, no se ha descrito el impacto pronóstico de estos eventos catastróficos (Baca *et al.*, 2013; Shen *et al.*, 2013; Nadeu *et al.*, 2020; Neuse *et al.*, 2020; Costa *et al.*, 2022). En el estudio presentado no se observaron diferencias en las características clínicas de los pacientes con cromotripsis con respecto al único caso con cromoplexia pero de nuevo es importante destacar que la cohorte era demasiado pequeña para extraer conclusiones sólidas.

4. Impacto pronóstico de la cromotripsis en una cohorte enriquecida en casos con complejidad genómica

En nuestro estudio, los pacientes con cromotripsis presentaron un TPT más corto que el grupo control. Estos resultados coinciden con lo que se ha publicado previamente en grandes cohortes de LLC, en las que se ha descrito una asociación entre la cromotripsis y una SLP, TPT y SG más cortos (Edelmann *et al.*, 2012; Puente *et al.*, 2015; Salaverria *et al.*, 2015; Leeksma *et al.*, 2021). Cabe destacar que estos trabajos no son equiparables ya que han utilizado definiciones distintas de cromotripsis (siete o más vs diez o más cambios en el número de copia) y han empleado técnicas diferentes para su análisis (MG vs NGS).

A pesar de que la cromotripsis se había asociado previamente con complejidad genómica, el estudio presentado es el primero en analizar la cromotripsis centrándose en su rol en una cohorte de alto riesgo. Tal y como se esperaba, el grupo con cromotripsis estaba enriquecido en casos con alta complejidad (≥5 alteraciones por CC). Además, estos casos también presentaban una mayor frecuencia de alteraciones en *TP53.*

Tanto la alta complejidad como las alteraciones en *TP53* se encontraron en frecuencias similares a las reportadas en publicaciones previas (alrededor de un 70%) en las que ya se había correlacionado la cromotripsis con una mayor complejidad y con mayor incidencia de alteraciones en *TP53* (Puente *et al.*, 2015; Salaverria *et al.*, 2015; Leeksma *et al.*, 2021). Es importante recalcar que la cromotripsis no tuvo impacto en el TPT cuando los pacientes se categorizaron según el estado de *TP53*. Incluso en el análisis multivariado, *TP53* fue la única variable que mantuvo la significancia estadística. Estos hallazgos sugieren que el peor pronóstico observado en los casos con CK y cromotripsis es debido a la mayor frecuencia de alteraciones en *TP53* y no a la presencia de cromotripsis *per se*. Además, como el contexto genómico en el que se desarrolla la cromotripsis es tan complejo y heterogéneo, el impacto negativo asociado a este fenómeno puede estar también modulado por los múltiples genes y regiones genómicas afectadas. Por lo tanto, la cromotripsis no representa un factor pronóstico independiente para los pacientes con LLC y complejidad genómica. En consecuencia, la no identificación de este fenómeno por la utilización de técnicas que no permiten su visualización, como la CC, no tiene especial relevancia en la práctica clínica. Sin embargo, sería interesante estudiar el rol de la cromotripsis en la evolución de los pacientes con el gen *TP53* normal, sin alteraciones, para entender mejor los posibles mecanismos que promueven la supervivencia de los clones con cromotripsis.

La mayoría de los estudios llevados a cabo en pacientes con LLC y cromotripsis se realizan en un momento concreto de la enfermedad. De hecho, en el estudio presentado no se han llevado a cabo estudios longitudinales para explorar la dinámica de la cromotripsis durante la patogénesis de la LLC, entre otras razones por la no disponibilidad de muestras secuenciales de un mismo paciente. Sin embargo, es un aspecto relevante ya que existe cierta controversia con respecto al momento en el que emerge la cromotripsis. Inicialmente, se creía que ocurría de manera temprana en la progresión del tumor (Stephens *et al.*, 2011). Sin embargo, en el trabajo de Bassaganyas y colaboradores, en el que realizaron un análisis longitudinal de un paciente con LLC y cromotripsis a lo largo de once años, los autores concluyeron que la cromotripsis no era un evento temprano durante la patogénesis de la LLC. En el paciente estudiado, la cromotripsis apareció años después del diagnóstico, justo antes de iniciar tratamiento. Este hallazgo concuerda con el hecho de que en la mayoría de los casos reportados, la cromotripsis se ha asociado con una alta complejidad genómica y con factores de mal pronóstico, como las alteraciones en *ATM* o *TP53*, lo cual sugiere que las células que contienen estos defectos tienen mayor predisposición a sufrir este fenómeno. Las alteraciones en ambos genes, *ATM* y *TP53*, son poco comunes en el momento del diagnóstico y su frecuencia aumenta hasta un 25% y 35-40%, respectivamente, en pacientes R/R. No obstante, a pesar de no haber realizado análisis longitudinales en el estudio descrito en la tesis, se han visto patrones de cromotripsis en pacientes que no presentan estas alteraciones de alto riesgo. Por lo tanto, si la cromotripsis es un evento temprano que puede desencadenar el desarrollo del tumor o si es un evento tardío que surge tras las alteraciones de las vías de *ATM* o *TP53*, sigue siendo una incógnita. Así, son necesarios más estudios llevados a cabo en cohortes más amplias con pacientes con cromotripsis analizados de manera longitudinal para esclarecer la dinámica de este fenómeno.

En cualquier caso, es importante destacar que los resultados obtenidos en los dos artículos incluidos en esta tesis doctoral se basan en una cohorte altamente enriquecida en casos con CK puesto que el foco principal del trabajo era caracterizar este subgrupo de pacientes, de especial interés por su pronóstico adverso, y comparar la utilidad de distintas metodologías genéticas en la detección de la complejidad genómica y en la estratificación pronóstica. Por lo tanto, al no ser representativa de una cohorte real de LLC, dificulta el desarrollo de índices pronósticos genéticos más precisos. Además, la naturaleza multicéntrica y retrospectiva de la misma hace que los pacientes hayan recibido diferentes tipos de tratamiento, lo cual dificulta el estudio de la SG o la respuesta a las nuevas modalidades terapéuticas. Esta podría ser una de las razones por las que en el primer estudio la complejidad genómica no tuvo un impacto estadísticamente significativo en la SG.

En resumen, se ha demostrado que el CK y/o la complejidad genómica constituyen biomarcadores pronósticos relevantes en la LLC y también podrían potencialmente ser buenos factores predictivos, incluso en la era de los nuevos fármacos. En este sentido, los resultados obtenidos durante el desarrollo de la presente tesis doctoral demuestran que tanto la CC como los MG son técnicas útiles para evaluar el pronóstico de los pacientes con LLC según su complejidad genómica. No obstante, hay que tener en cuenta que una proporción considerable de los casos se clasifica de manera discordante según la técnica empleada. En consecuencia, aunque la CC es la técnica de elección para el estudio de complejidad genómica, los hallazgos encontrados previamente por CC no deben extrapolarse a otras técnicas como los MG o el OGM. El OGM ha surgido como una herramienta valiosa para el estudio citogenómico de la LLC, capaz de superar muchas de las limitaciones que existen actualmente con las técnicas convencionales empleadas en la práctica clínica. Además, es una herramienta útil para el estudio de la complejidad genómica, y concretamente de la cromotripsis, puesto que permite detectar la mayoría de las alteraciones previamente identificadas por CC, FISH o MG y nuevos reordenamientos asociados a este fenómeno. Se ha demostrado que el pronóstico adverso observado en los pacientes con LLC con complejidad genómica y cromotripsis se asocia a una mayor frecuencia de alteraciones en *TP53*. No obstante, todavía existen muchas preguntas sin resolver con respecto a los mecanismos que promueven la cromotripsis y su rol en el desarrollo y evolución de la LLC. Como consecuencia, son necesarios estudios adicionales en cohortes más amplias para establecer el valor pronóstico de la complejidad genómica definida por otras metodologías, como los MG o el OGM, en estudios prospectivos y en ensayos clínicos. Además, también es necesario estudiar más casos con cromotripsis con *TP53* normal, para entender mejor su impacto en la patogénesis de la LLC y en su pronóstico, especialmente en pacientes tratados con las nuevas modalidades terapéuticas.

V. CONCLUSIONES

1. La citogenética convencional y los *microarrays* genómicos son técnicas útiles para el estudio de la complejidad genómica en pacientes con LLC pero no son equivalentes. Las limitaciones intrínsecas de cada técnica son responsables de estas discordancias.
2. La complejidad genómica tiene un impacto significativo en el tiempo al primer tratamiento teniendo en cuenta las distintas categorías de riesgo establecidas por los dos métodos según el número de alteraciones. Ambas técnicas son igualmente útiles para la estratificación pronóstica aunque el grado de acuerdo entre ellas en la clasificación de los pacientes con LLC es moderado.
3. La cromotripsis es un fenómeno recurrente entre los pacientes con LLC y complejidad genómica y se caracteriza por ser altamente heterogénea, ya que involucra múltiples regiones sin tener un patrón común ni ninguna región o gen afectado con mayor frecuencia.
4. El mapeo óptico del genoma es una técnica útil para el estudio de la cromotripsis en LLC puesto que es capaz de detectar la mayoría de las alteraciones en el número de copia que componen estos eventos. Además, permite la identificación de reordenamientos adicionales relacionados con la cromotripsis que pueden localizarse únicamente en la región cromotríptica o pueden implicar también cromosomas no cromotrípticos. El impacto clínico de estos patrones de reordenamientos se desconoce.
5. El pronóstico adverso observado en los pacientes con LLC y complejidad genómica que muestran patrones de cromotripsis está relacionado con la mayor frecuencia de alteraciones en *TP53* y no con la presencia de cromotripsis *per se*. En consecuencia, el hecho de no identificarlos en la práctica clínica no supone un efecto negativo para el

manejo de los pacientes ya que no representa un factor pronóstico independiente para los pacientes con LLC y complejidad genómica.

6. Los resultados presentados en esta tesis se han obtenido a partir de cohortes retrospectivas, con una gran heterogeneidad en cuanto a los tratamientos recibidos. Por lo tanto, es necesario incluir el estudio de la complejidad genómica mediante citogenética convencional o *microarrays* genómicos en ensayos clínicos prospectivos para refinar su definición y esclarecer su impacto pronóstico y predictivo de la respuesta al tratamiento, especialmente en pacientes tratados con los nuevos agentes dirigidos.

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ANEXOS

Durante la realización de esta tesis doctoral la doctoranda también ha participado en el desarrollo de un estudio del grupo de investigación centrado en demostrar la utilidad de la técnica del mapeo óptico del genoma para el análisis de la complejidad genómica en LLC. Los resultados obtenidos se han recogido en la siguiente publicación:

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Optical Genome Mapping: A Promising New Tool to Assess Genomic Complexity in Chronic Lymphocytic Leukemia (CLL)

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Simple Summary: Genome complexity, detected by chromosome banding analysis or chromosomal microarray analysis, is a poor prognostic factor for chronic lymphocytic leukemia (CLL). Herein, we aimed to assess the performance of optical genome mapping (OGM) for the cytogenomic characterization of CLL patients, with a special focus on risk stratification based on genomic complexity. A cohort of 42 patients enriched in complex karyotypes was assessed by OGM, and the results were compared with those obtained from current methods. Moreover, clinical–biological characteristics and time to first treatment were analyzed according to the OGM-defined complexity. Globally, OGM identified 90% of the known alterations and provided novel structural information about these aberrations in 55% of patients. Regarding genomic complexity, OGM allowed us to identify a complex group ($\geq$10 alterations) displaying enrichment of *TP53* abnormalities and poorer evolution. Altogether, we confirmed that OGM is a valuable tool for the cytogenomic assessment and prognostic stratification of CLL patients.

Abstract: Novel treatments in chronic lymphocytic leukemia (CLL) have generated interest regarding the clinical impact of genomic complexity, currently assessed by chromosome banding analysis (CBA) and chromosomal microarray analysis (CMA). Optical genome mapping (OGM), a novel technique based on imaging of long DNA molecules labeled at specific sites, allows the identification of multiple cytogenetic abnormalities in a single test. We aimed to determine whether OGM is a suitable alternative to cytogenomic assessment in CLL, especially focused on genomic complexity. Cytogenomic OGM aberrations from 42 patients were compared with CBA, FISH, and CMA information. Clinical–biological characteristics and time to first treatment (TTFT) were analyzed according to the complexity detected by OGM. Globally, OGM identified 90.3% of the known alterations (279/309). Discordances were mainly found in (peri-)centromeric or telomeric regions or subclonal aberrations (<15–20%). OGM underscored additional abnormalities, providing novel structural information on known aberrations in 55% of patients. Regarding genomic complexity, the number of OGM abnormalities had better accuracy in predicting TTFT than current methods (C-index: 0.696, 0.602, 0.661 by OGM, CBA, and CMA, respectively). A cut-off of $\geq$10 alterations defined a complex OGM group (C-OGM, $n = 12$), which included 11/14 patients with $\geq$5 abnormalities by CBA/CMA and one patient with chromothripsis (Kappa index = 0.778; $p < 0.001$). Moreover, C-OGM displayed enrichment of *TP53* abnormalities (58.3% vs. 3.3%, $p < 0.001$) and a significantly shorter TTFT (median: 2 vs. 43 months, $p = 0.014$). OGM is a robust technology for implementation in the routine management of CLL patients, although further studies are required to define standard genomic complexity criteria.

Keywords: chronic lymphocytic leukemia; optical genome mapping; chromosome banding analysis; chromosomal microarrays; genomic complexity; prognosis

1. Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the clonal proliferation and accumulation of abnormal mature B cells, typically CD5 positive, in the blood and lymphoid tissues. This disease is the most common leukemia in Western countries and displays a highly variable clinical course, ranging from very indolent cases to patients with aggressive disease and rapid progression [1]. This clinical heterogeneity has important consequences for clinical follow-up, therapeutic strategies, and patient survival. Because of this, several prognostic and predictive biomarkers have been identified over the years [2]; the most relevant being deletions and/or mutations in *TP53*, located at 17p13, and the mutational status of the variable region of the immunoglobulin heavy chain (IGHV) gene [1]. FISH analysis of the four-probe set included in Dohner's hierarchical model [targeting deletions on 13q14, 11q22 (*ATM*), 17p13 (*TP53*), and trisomy of chromosome 12] is currently considered the gold standard for cytogenetic assessment in CLL [3]. However, this strategy underestimates the true genetic complexity of the tumor cells, which also predicts a more aggressive clinical course [4,5]. Although the negative impact of complex karyotypes (CK) has been extensively demonstrated in patients treated with standard chemoimmunotherapy regimes [6–9], its clinical relevance in patients receiving new treatment modalities has not been fully established. While initial data from clinical trials with novel agents suggested an adverse significance of CK [10–13], recent studies, including extended follow-ups of older trials, pooled analyses, or new drug combinations, reported controversial results regarding its adverse significance [14–21]. Thus, additional analyses are required to clarify the prognostic/predictive impact of genomic complexity in the new treatment era.

To date, most of the outcome analyses of genomic complexity published in CLL have been reported using chromosome banding analysis (CBA) data and defined CK as the presence of three or more chromosomal alterations detected in the same cell clone [22,23]. An increasing number of chromosomal abnormalities in the karyotype has been correlated with a worse clinical evolution of CLL patients, and, notably, the types of aberrations detected also modulates its prognostic impact [8,24–26]. Moreover, chromosomal microarray analysis (CMA) has replaced CBA and FISH in some laboratories for the detection of copy number variants (CNVs). Two large retrospective studies from the European Research Initiative on CLL (ERIC) demonstrated that genomic complexity, detected by either CBA or CMA, is an independent adverse prognosticator in CLL, five or more being the number of aberrations that best predicted a worse evolution [9,27]. In addition, our group compared the performance of CBA and CMA to detect genomic complexity in a cohort of 340 patients enriched in CK. Although the prognostic utility of both methods was validated in this study, we demonstrated that risk stratification by CBA and CMA was not fully equivalent due to the intrinsic limitations of each technique [28]. The ERIC group recently published their recommendations, in which CBA still remains the gold standard methodology, capable of providing information on structural and numerical aberrations at a low resolution of the whole genome while also allowing the overview of the clonal landscape and intraclonal hierarchy [29]. It is noteworthy that, while CBA results rely on the in vitro proliferative capacity of the altered clones, CNV profiles can be defined at high resolution by CMA without having to culture samples. In some cases, CMA could reveal multiple CNVs or even chromothripsis events associated with monosomies or unknown cytogenetic elements reported in the karyotype. On the contrary, balanced abnormalities are only detectable by CBA, and subclonal alterations present at low percentages (<20%) could be missed by CMA due to its lower sensitivity [28–31].

Optical genome mapping (OGM) has emerged as a promising novel technique that could potentially overcome the aforementioned limitations in a single test. OGM relies on high-throughput imaging of long DNA molecules (>250 Kb) fluorescently labeled at a specific 6 bp sequence motif, occurring approximately 15 times per 100 Kb in the human genome. The unique labeling pattern throughout the genome confers the ability to unambiguously identify the genomic location of every imaged molecule, generating a local consensus map that can be compared to a reference genome to detect structural variants (SVs). This analysis is performed via the so-called rare variant pipeline, which specifically addresses mosaic samples and automatically detects both balanced and unbalanced SVs from single molecules, genome-wide, starting at 5 Kb and down to a 1% allele fraction. In addition, genome coverage depth information is also used to address copy number variant (CNV) and whole-chromosome aneuploidy identification [32,33]. Several recent publications have demonstrated OGM's good performance in the cytogenomic assessment of different hematological malignancies, especially focused on myeloid neoplasms (acute myeloid leukemia and myelodysplastic syndromes) and acute lymphoblastic leukemia cases. In these studies, OGM effectively detected most of the clinically relevant abnormalities reported by standard methods and, in some cases, also provided new cytogenomic information [34–37]. Conversely, the assessment of genomic complexity by OGM or the global characterization of the cytogenetic abnormalities identified in each patient was beyond the scope of the published studies, which focused on the identification of SVs currently interrogated in diagnostic procedures or that overlapped with genes defined as potentially relevant in leukemogenesis.

In the present study, we aimed to assess the performance of OGM for the cytogenomic characterization of CLL patients in comparison with previous CBA and CMA information, with a special focus on genomic complexity. Moreover, we analyzed the potential use of OGM for the prognostic stratification of CLL patients based on genomic complexity.

2. Materials and Methods

2.1. Patients and Samples

A total of 42 patients diagnosed with CLL ($n = 39$; 92.9%) and monoclonal B-cell lymphocytosis ($n = 3$; 7.1%) with available CBA, FISH and CMA results were included. Due to the purpose of the study, 18 of them presented CK by CBA (42.9%). All analyses were performed on cryopreserved peripheral blood mononuclear cells (PBMCs) obtained simultaneously to CBA or within one year. The median time from diagnosis to sampling was 10 months (range: 0–174 months), and only two patients (4.8%) received treatment prior to the genetic analyses. The main characteristics of the entire cohort are summarized in Table 1. The study was carried out in accordance with national and international guidelines (Professional Code of Conduct, Declaration of Helsinki) and approved by the Hospital del

Mar Ethics Committee (2017/7565/I). Of note, CBA, CMA, and FISH data from 17 patients were included in a previous publication [28].

Table 1. Baseline characteristics of patients at diagnosis and last follow-up.

	Non-CK Group	**CK Group**	**p-Value**
	$n = 24$; n (%)	**$n = 18$; n (%)**	
Gender			
Male	17 (70.8%)	11 (61.1%)	0.530
Median age at diagnosis (range)	66 (42–85)	69 (37–88)	0.297
Stage at diagnosis			
MBL	0 (0.0%)	3 (16.7%)	0.071
CLL	24 (100%)	15 (83.3%)	
Binet A	22 (91.7%)	10 (66.7%)	0.085
Binet B/C	2 (8.4%)	5 (33.3%)	
Common CLL genomic aberrations *			
del(13)(q14)	19 (79.2%)	11 (61.1%)	0.302
Trisomy 12	3 (12.5%)	5 (27.8%)	0.256
del(11)(q22q23)	5 (20.8%)	7 (38.9%)	0.302
Aberrations in *TP53*	0 (0.0%)	8 (44.4%)	<0.001
del(17)(p13)	0 (0.0%)	7 (38.9%)	0.001
TP53 mutation	0 (0.0%)	7 (38.9%)	0.001
Unmutated IGHV	12/23 (52.2%)	12/18 (66.7%)	0.524
Time from diagnosis to cytogenetic study (range)	22.5 months (0–123)	6 months (0–174)	0.867
Median follow-up (range)	44.5 months (0–95)	31.5 months (0–78)	0.065
Treatment			
Treated patients	13 (54.2%)	13 (72.2%)	0.338
Median time to first treatment (95% CI)	43 months (26–54)	9 months (8–30)	0.165

* Deletions and trisomy detected by FISH and/or chromosomal microarray analysis. Abbreviations: MBL = monoclonal B-cell lymphocytosis, CI = confidence interval.

2.2. DNA Extraction, Labelling and Data Collection for Optical Mapping

For each sample, 1.5 million cells were used to extract ultra-high molecular weight (UHMW) DNA following manufacturer's instructions (Bionano Prep Frozen Cells DNA Isolation Protocol, Bionano Genomics, San Diego, CA, USA). The Direct Label Enzyme (DLE-1) was used to enzymatically transfer DL-green fluorescent labeling to UHMW DNA following the Bionano Prep Direct Label and Stain (DLS) Protocol (Bionano Genomics). Labeled molecules were directly loaded onto a Saphyr chip and imaged via the Saphyr instrument (Bionano Genomics). The Saphyr Chip's nano-channels allow only a single linearized DNA molecule to travel through via electrophoretic migration. Multiple cycles were run to generate approximately 1300 Gb of data per sample and reach an average genome coverage of 300×.

2.3. Data Assembly, Structural Variant Calling and Filtering

Genomic analysis was performed with the rare variant pipeline included in Bionano Solve software (v.3.5) and visualized with Bionano Access software (v1.6) (Bionano Genomics). Briefly, the imaged molecules were aligned against a reference assembly (hg19), and two algorithms were applied to identify SVs and large CNVs. The structural variant calling algorithm assessed any deviation in the labeling pattern to detect insertions, inversions, deletions, duplications, and intrachromosomal or interchromosomal translocations. Of note, interstitial deletions or inversions >5 Mb were called as intrachromosomal translocations by this pipeline. Copy number variant calls were inferred from the label coverage detected in each genomic interval, indicating the presence of gains or losses in those regions with a significant increase or decrease from baseline. All abnormalities called by the software were prefiltered according to default recommended confidence scores for Bionano

Access version 1.6 (confidence scores: insertion: 0, deletion: 0, inversion: 0.7, duplication: −1, intra-translocation: 0.3, inter-translocation: 0.65, and CNV: 0.99) or whether they were described in the OGM control sample dataset provided by Bionano Genomics. In addition, as certain regions of the genome had an unusually high variance in their coverage that could be due to an unreliable molecule alignment (often concentrated around centromere and telomere regions), both pipelines masked SV and CNV calls occurring in these regions. Finally, we also applied size filters to further reduce the number of variants and obtain results comparable to those observed by CMA. The cut-off size for SVs (inversions, insertions, deletions, and duplications) was set at 100 Kb, while only those CNVs larger than 500 Kb were called by the CNV tool. Prefiltered SV and CNV data were downloaded and visually reviewed with the Bionano Access software in order to merge segmented CNVs, discard those SVs duplicated in the results, and variants found as benign polymorphisms in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home; accessed on 4 April 2022). In addition, the curated outputs from both algorithms were also compared in order to identify duplicities. In this regard, CNV gains and losses which overlapped with duplications and deletions or intrachromosomal translocation breakpoints, respectively, were considered as the same abnormality, and coordinates from the SV pipeline were taken. The final aberrations found in each patient were recorded for comparison with the results obtained by standard techniques. Additionally, less restrictive filters for confidence scores were applied in four patients with undetected known abnormalities to ascertain whether these were missed due to filtering issues or due to real OGM technical limitations.

2.4. Chromosome Banding Analyses and Fluorescence In Situ Hybridization

Peripheral blood (PB) samples were used to set two parallel cultures using phorbol-12-myristate-13-acetate (TPA) and immunostimulatory cytosine guanine dinucleotide (CpG)-oligonucleotide DSP30 plus interleukin 2 (IL-2) as mitogens. At least 20 metaphases from each culture were analyzed when possible, and a minimum of 10 were evaluated in three patients with abnormal karyotypes. Karyotypes from the most altered culture (more abnormal clones or altered metaphases detected) were collected and described according to the International System for Human Cytogenetic Nomenclature (ISCN 2020) [38]. The number and type of abnormalities were recorded as previously described [28].

Data from the routine FISH panel for *TP53* (17p13), *ATM* (11q22), D13S319 (13q14), and chromosome 12 centromeric region (Metasystems, Altlussheim, Germany) assessed in uncultured PB were also collected. In order to validate some of the OGM findings, additional FISH analyses were performed using commercial locus-specific, whole chromosome painting probes (Metasystems; Abbot Molecular, Abbott Park, IL, USA) or custom bacterial artificial chromosome (BAC) probes from the Children's Hospital Oakland Research Institute library (Oakland, CA, USA). Details regarding the additional FISH probes tested are shown in Table S1.

2.5. Chromosomal Microarray Analyses

Chromosomal microarrays were assessed in DNA from PBMCs ($n = 12$) or PB CD19+ purified cells ($n = 28$). DNA was amplified, labeled, and hybridized to CytoScan HD ($n = 30$) or CytoScan 750K ($n = 10$) platforms (ThermoFisher Scientific, Eugene, OR, USA) according to manufacturer's protocols. Copy number variants found as benign polymorphisms in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home; accessed on 4 April 2022) were excluded. The remaining aberrations, irrespective of size, were collected and reported using annotations of genome version GRCh37/hg19. CNV counting for risk classification of patients was performed using previously described criteria [27,28]. As the current analysis pipelines used for OGM results do not enable copy-number neutral loss of heterozygosity (CN-LOH) detection, these abnormalities were not considered in the present study.

2.6. Comparison among Techniques

First, we assessed the capability of OGM to detect the abnormalities previously defined by CBA, FISH, and CMA. In this regard, results obtained from standard techniques were compiled to set the list of abnormalities to be identified by OGM. The following criteria were applied: (i) for CNVs, coordinates defined by CMA were prioritized while FISH abnormalities or well-defined gains or losses identified in the karyotype but missed by CMA for technical reasons were also included; (ii) for balanced rearrangements (inversions or translocations), breakpoints defined in the karyotype were collected; (iii) among complex abnormalities in the karyotype affecting material of unknown origin, only the chromosomal breakpoints where additional material was identified were recorded to assess the presence of translocations by OGM in these regions. OGM results were considered concordant if the same abnormality was identified, even if it showed some variability in size or breakpoints. The percentage of detection by OGM was recorded, and potential causes of discrepancy were evaluated. Regarding additional abnormalities called by OGM, images from the available karyotypes and CMA data were reviewed to ascertain the potential relevance of OGM findings. Those abnormalities that could be compatible with any element observed in the karyotype were considered 'real' if validated by additional FISH analyses that allowed karyotype reinterpretation. In addition, rearrangements associated with chromothripsis events or CNVs detected by CMA were considered 'real' if validated by FISH or 'potentially real' if they were not specifically studied by FISH. Results from the additional FISH validations performed were used to finally classify some uncertain variants as 'real' or 'false positive' calls and to define custom criteria to identify novel OGM translocations with poor quality. The latter were classified as 'potential false positive' and were not considered in the final OGM abnormalities count.

In addition, we assessed the potential use of OGM for the identification of higher-risk patients based on their genomic complexity. To this end, the number of abnormalities reported by OGM was compared with that identified by standard methods in the whole cohort. Furthermore, patients were categorized based on the complexity detected by OGM and compared with the previously defined risk groups in the two ERIC studies [9,27].

2.7. Statistical Analyses

Descriptive statistics were used to provide frequency distributions of discrete variables, while statistical measures were used to provide median values and ranges for quantitative variables. Chi-square or Fisher exact tests were used to compare discrete variables and the Mann–Whitney U test to assess continuous variables. The number of abnormalities recorded by OGM and CBA or CMA were correlated by Spearman's Rho, and the concordance among techniques was established via Cohen's Kappa index. Time to first treatment (TTFT) was defined as the time interval between the sampling date and the date of first treatment. The Kaplan–Meier method was used to estimate the distribution of TTFT in the 40 treatment-naïve patients. Comparisons among patient subgroups were performed via the Log-rank test. The concordance statistic (C-index) for right-censored data was calculated to assess the accuracy of each technique in predicting TTFT. As only four patients died during follow-up, overall survival analyses were not assessed. Likewise, multivariate analysis could not be performed due to the limited size of the present cohort. Statistical analyses were performed using SPSS v.23 software (SPSS Inc, Chicago, IL, USA) and R v3.5.2 (R Foundation for Statistical Computing, Vienna, Arustria). p-values < 0.05 were considered statistically significant.

3. Results

3.1. Global SV and CNV Detection by OGM

OGM results from all 42 samples run met the recommended quality metrics, showing an average molecule N50 (for molecules > 150 Kb) value of 305 Kb and an average 363-fold effective coverage (Table S2). Globally, a total of 2202 abnormalities were called with the recommended prefiltering criteria for the Bionano Access version 1.6. Nonetheless, when the output was further filtered based on size ($\geq$100 Kb) and visually reviewed, the number of detected abnormalities reduced to 579. In all, 36 deletions, 3 inversions, 4 duplications, 142 intrachromosomal translocations, 185 interchromosomal translocations, 59 CNV gains, and 150 CNV losses were considered for comparison with standard techniques. Of note, 42 of these CNVs were also called as duplications (n = 6), deletions (n = 24), or intrachromosomal translocations (n = 12) by the SV calling pipeline and subsequently not counted in the genomic complexity estimation (Figure S1).

3.2. Concordance Rate of OGM Results with Standard Techniques

In the whole cohort, a total of 309 abnormalities previously detected by CBA, FISH, and/or CMA were considered to explore the concordance with OGM results. While CNVs represented the vast majority of these (193 losses and 67 gains), information from only 49 translocations was previously available. Of note, in 29 of the rearrangements, only one of the translocation breakpoints was identified by CBA, as they involved additional material of unknown origin in the karyotype ("add"). Globally, 90.3% of the previously known alterations (279/309) could be identified by OGM. Of note, no significant differences in the detection rate were observed between patients with non-CK and those carrying a CK by CBA (detection of 83/89 (93.3%) and 196/229 (89.1%), respectively; p = 0.396). In order to reach this concordance, a lower confidence score threshold was applied to properly detect five known translocations, and more permissive filtering criteria were used to pick up 15 alterations initially missed by the CNV pipeline. The latter included nine small CNVs (range: 292–488 Kb) filtered by the 500 Kb cut-off size used for CNVs, and six larger CNVs (range: 1282–22,049 Kb), which had been detected in less than 20% of cells by FISH and/or CMA and could also be visually suspected in the whole chromosome CNV view of the Bionano Access software. It is noteworthy that, when comparing only those abnormalities included in the routine CLL FISH panel, the concordance rate increased to 95.6% (detection of 55/57 known deletions on 13q14, 11q22 (*ATM*), 17p13 (*TP53*) or trisomy 12). In this regard, OGM failed to detect a 13q14 deletion and a *TP53* deletion found in 15% and 17% of nuclei by FISH, respectively, and did not pick up any additional one of these recurrent CLL alterations.

As well as their detection, we could evaluate OGM's accuracy regarding the definition of genomic location and size for 238/247 CNVs previously defined by CMA. To this end, CNVs detected by both techniques showed a high overlap with a median difference of only 22 Kb in the defined coordinates (range: 0–8.1 Mb). Major discordances were due to the known OGM masking of the terminal 2–5 Mb from large CNVs or whole chromosome aneuploidies, which were located at repetitive regions (12/238, 5.0%). Once these discrepancies found to be masked were excluded, differences larger than 2 Mb in size (range: 2.1–5.7 Mb) were only observed in five CNVs (2.1%). It is noteworthy that OGM allowed the definition of the genomic coordinates from eight known CNVs that had been previously detected by FISH and/or CBA but missed by CMA. In addition, similar results were observed when the alterations called by the SV and CNV pipelines were compared.

Among the 30 missed abnormalities, 14 were not expected to be detected by OGM as they were translocations with breaks in the (peri-)centromeric or telomeric regions (n = 11), which are not covered by OGM labels, two relatively small telomeric deletions (1.5 and 2.5 Mb) and one gain involving chromosome Y (4.1 Mb) that were masked by the CNV analysis pipeline to avoid the calling of false positive in these highly variable regions. On the other hand, sensitivity explained the discrepancy in 13 of the remaining 16 aberrations that were susceptible to detection by OGM (Table 2). In this regard, nine CNVs undetected by OGM were identified in a median of 17% of cells by FISH and/or CMA (range: 3–25%). Among these, three were not detected by CMA either. Similarly, sensitivity could explain four missed translocations that had been detected in minor clones by CBA related to the culture selection of abnormal clones intrinsic to this technique. Although the percentage of abnormal cells could not be set for these translocations, it is important to note that the SV pipeline correctly detected some other abnormalities found in only 5% of cells by FISH.

Finally, OGM was not able to identify a small 138 Kb deletion encompassed in a complex chromothripsis event and two additional abnormalities that could not be associated with any feasible cause of discrepancy (Table 2).

Table 2. Known abnormalities not detected by OGM and their potential cause of discrepancy.

Patient ID	Undetected Alteration	Size (Kb)	Potential Cause of Discrepancy	Comments
12	Translocation t(13;?)(p11;?)	-	Involvement of (peri-)centromeric regions	
CK4	Translocation t(17;?)(p11;?)	-	Involvement of (peri-)centromeric regions	
CK8	Translocation t(17;18)(q10;q10)	-	Involvement of (peri-)centromeric regions	
CK9	Translocation t(13;21)(q11;p11)	-	Involvement of (peri-)centromeric regions	
CK10	Translocation t(14;?)(p11;?)	-	Involvement of (peri-)centromeric regions	
CK12	Translocation t(11;?)(p11;?)	-	Involvement of (peri-)centromeric regions	
CK13	Translocation t(14;17)(q11;p11)	-	Involvement of (peri-)centromeric regions	
CK16	Translocation t(14;18)(p11;q11)	-	Involvement of (peri-)centromeric regions	
CK16	Translocation t(15;22)(p11;q15)	-	Involvement of (peri-)centromeric regions	
CK17	Translocation t(15;?)(p11;?)	-	Involvement of (peri-)centromeric regions	
CK4	Translocation t(6;19)(q12;p13)	-	Involvement of telomeric region	
7	Gain Yq11.223q11.23(24637115_28799654)	4163	Masked region (partial CNV on chr. Y)	Detected at 50% by CMA, and visually suggested in the whole genome CNV view
CK2	Deletion 17p13.3p13.3(525_2489182)	2489	Masked region (telomere)	
CK6	Deletion 1p36.33p36.32(1997349_3740109)	1743	Masked region (telomere)	
20	Deletion 6q21	NA	Sensitivity	Detected in 12% of nuclei by FISH (CMA not available)
21	Deletion 13q14 (D13S319)	NA	Sensitivity	Detected in 17% of nuclei by FISH (not detected by CMA)
CK3	Gain 6pterq16	NA	Sensitivity	Detected in 3% of nuclei by FISH, confirmed in metaphases (not detected by CMA)
CK8	Deletion 17p13.3p11.2(526_21347924)	21347	Sensitivity	Detected in 15% of nuclei by FISH (also visually found by CMA)
CK12	Gain 11q14.2q14.2(86177075_86856206)	679	Sensitivity	Detected at 25% by CMA, and visually suggested in the whole genome CNV view
CK12	Gain 11q22.3q22.3(106600681_106991146)	390	Sensitivity	Detected at 25% by CMA, and visually suggested in the whole genome CNV view
CK12	Gain 11q24.3q24.3(129345165_130249509)	904	Sensitivity	Detected at 25% by CMA, and visually suggested in the whole genome CNV view

Table 2. *Cont.*

Patient ID	Undetected Alteration	Size (Kb)	Potential Cause of Discrepancy	Comments
CK12	Gain 19q13.2q13.42(41644540_54499334)	12855	Sensitivity	Detected at 20% by CMA
CK13	Gain 1p22pter	NA	Sensitivity	Detected in 5% of nuclei by FISH (not detected by CMA)
7	Translocation t(X;?)(p22;?)		Sensitivity	Percentage not available, could be a minor clone expanded during CBA culture
CK4	Translocation t(12;?)(q24;?)		Sensitivity	Percentage not available, could be a minor clone ("add(12)(q24)" detected as clonal evolution in only two out of eight abnormal metaphases)
CK6	Translocation t(9;?)(q34;?)		Sensitivity	Percentage not available, could be a minor clone expanded during CBA culture (CK defined as a composite karyotype)
CK8	Translocation t(6;?)(p25;?)		Sensitivity	Percentage not available, could be a minor clone ("add(6)(p25)" detected as clonal evolution from abnormal cells with monosomy of chr. 17, as the *TP53* deletion was detected at 15% the abnormality was probably below that percentage)
8	Deletion 13q32.1(95520821_95658848)	138	Small deletion within chromothripsis	Small deletion, part of a complex CMA profile on chr. 13 properly detected
CK16	Translocation t(11;?)(q23;?)		Unknown cause of discrepancy	The "add(11)(q23)" was present in the main clone. Although not called as SV, some imaged molecules showed fusions between different regions of chr 11; WCP for chr. 11 confirmed hybridization in the whole abnormal chromosome
CK17	Translocation t(9;?)(q34;?)		Unknown cause of discrepancy	Percentage not available; although the "add(9)(q34)" could be a minor clone (detected as clonal evolution in only two out of 13 abnormal metaphases), other abnormalities from the same clone were properly detected. WCP revealed that the additional material was from chr. 17 and, as seen by CBA, the chr. 17 telomeric region could be involved in the fusion (telomeric regions are masked by the SV pipeline)

Abbreviations: NA = Not available; WCP: whole chromosome FISH painting.

Detailed results obtained by each of the standard techniques, the 309 known abnormalities considered, and their detection by each of the OGM analysis pipelines are shown in Table S3.

3.3. Novel Abnormalities Detected by OGM

As OGM provided data not only on CNV genomic location but also structural information, we next assessed whether OGM could provide a more comprehensive structural overview of the known aberrations detected compared to a CBA, FISH, and CMA combination. Of note, the number of interchromosomal translocations called by OGM ($n = 194$)

was much higher than expected, taking into account previous CBA data, even in non-CK patients. In this regard, additional analyses were performed to ascertain which calls should be considered real or, on the contrary, filtered as potential false-positive calls. A total of 72 translocations were considered unsuitable for these focused comparisons as they were distributed among only three patients (with 16, 20, and 36 translocations, respectively) that showed highly complex karyotypes including several abnormalities involving material of unknown origin, difficult to compare with OGM or validated by FISH. Thus, these three cases were excluded from the comparison. Among the remaining 122 translocations with CBA and CMA data available, 12 (9.8%) corresponded to previously well-defined translocations (seven known translocations, five of them having two breakpoints identified in each derivative chromosome), and 63 (51.7%) could potentially be reflecting unknown rearrangements associated with previously described abnormalities. Conversely, 47/122 (38.5%) were not supported by CBA and were called in apparently normal regions by CMA (Figure 1). Visual inspection of OGM calls revealed that, contrarily to that observed for those concordant or potentially real calls, most apparently false-positive calls displayed few labels mapped in at least one of the translocation partners ($\leq$10), which also correlated with low 'RawConfidenceLeft' or 'RawConfidenceRight' parameter values (<10) in the SMAP file generated by the rare variant pipeline (Figure 2). Three of these translocations with low labels [t(5;6)(q22.3;q27) called in patients 3 and 5, and t(11;19)(q23.3;p13.3) in patient 13] were assessed by interphase FISH using targeted custom BACs break-apart probes and could not be validated. Moreover, 51 additional calls with high label coverage in both translocation partners were indirectly validated by whole chromosome FISH painting (Table S4). These findings suggested that the final set of translocation calls to be considered could be manually filtered based on these criteria. On the other hand, high variability was observed in the number of self-molecules supporting the translocations called by OGM (median: 45; range: 5–175). A total of 27 of the apparently well-covered translocations were based on less than 10 molecules, which is the cut-off used by some authors in hematological studies [39,40]. Thereby, we next evaluated if these low values could also be associated with a higher rate of false-positive calls, but no conclusive results were obtained. Fourteen translocations were consistent with CBA or whole chromosome FISH painting (WCP) information, whereas two were not validated by interphase FISH with custom BAC probes and were classified as false-positive calls. Notably, five of the latter were subclonal abnormalities by CBA detected in only 5–7% of nuclei by FISH or confirmed in clones initially missed by CBA (Table S4). These results suggested that the number of molecules supporting the translocation calls could be impaired by both potential errors in the mapping of the sample or by intrinsic characteristics of the abnormal clone. Thus, this parameter was not considered in the final filtering of the translocations found in the whole cohort. Likewise, contrarily to that described by other authors [37], as most of the validated translocations failed the assembly chimeric score provided by the software, this parameter was not considered either. Globally, 45/192 (23.4%) interchromosomal and 5/142 (3.5%) intrachromosomal translocation calls were manually discarded from the analysis, and none corresponded to any known abnormality by conventional techniques (Figure 1).

Once data were further manually filtered, novel structural findings on previously known aberrations were found in 23/42 patients (54.8%). On the one hand, when compared with CBA, OGM identified 23 intra- and 37 interchromosomal translocations associated with 33 of the previously known structural rearrangements: 15 called as translocations, inversions, insertions, or deletion, and 18 reported as "add" in the karyotype. Interestingly, OGM showed rearrangements involving at least three chromosomes in 13/18 (72.2%) abnormalities in which only one of the breakpoints could be identified in the karyotype ("add"). Moreover, it also revealed higher complexity in 7/15 (46.7%) apparently well-described abnormalities (Figure 3). On the other hand, 93 intra- and 30 interchromosomal translocations underscored by OGM were associated with CNVs identified by CMA. Apart from unbalanced rearrangements underlying large terminal CNV or rearrangements associated with complex chromothriptic events, unexpected rearrangements between apparently unrelated small interstitial CNV were also recorded. Interestingly, apart from the three translocations with breakpoints at 13q14 defined by CBA, OGM detected novel translocations associated with known 13q14 or 11q22 (including *ATM*) deletions in 5/29 (17.2%) and 2/11 (18.2%), respectively (Figure S2).

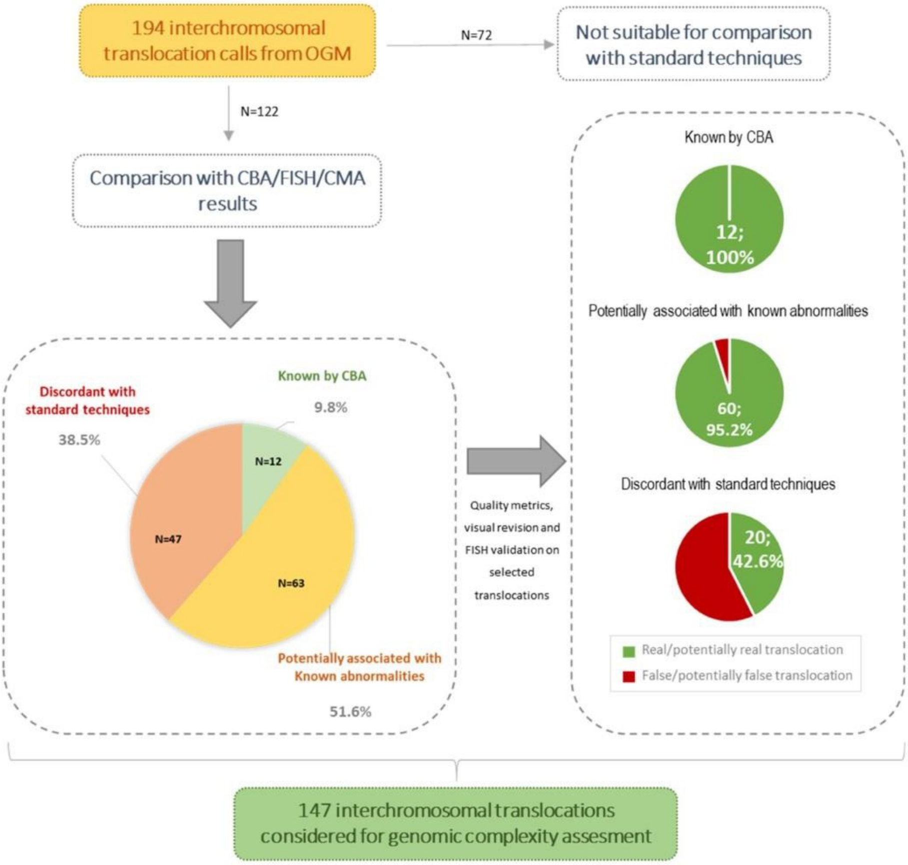
194 interchromosomal translocation calls from OGM
N=72
Not suitable for comparison with standard techniques
N=122
Comparison with CBA/FISH/CMA results
Known by CBA
9.8%
Discordant with standard techniques
38.5%
N=12
N=47
N=63
Potentially associated with Known abnormalities
51.6%
Quality metrics, visual revision and FISH validation on selected translocations
Known by CBA
12; 100%
Potentially associated with known abnormalities
60; 95.2%
Discordant with standard techniques
20; 42.6%
🟩 Real/potentially real translocation
🟥 False/potentially false translocation
147 interchromosomal translocations considered for genomic complexity assesment

Figure 1. Summary of the analyses performed to define the final set of interchromosomal translocations considered as real or potentially real in the present cohort.

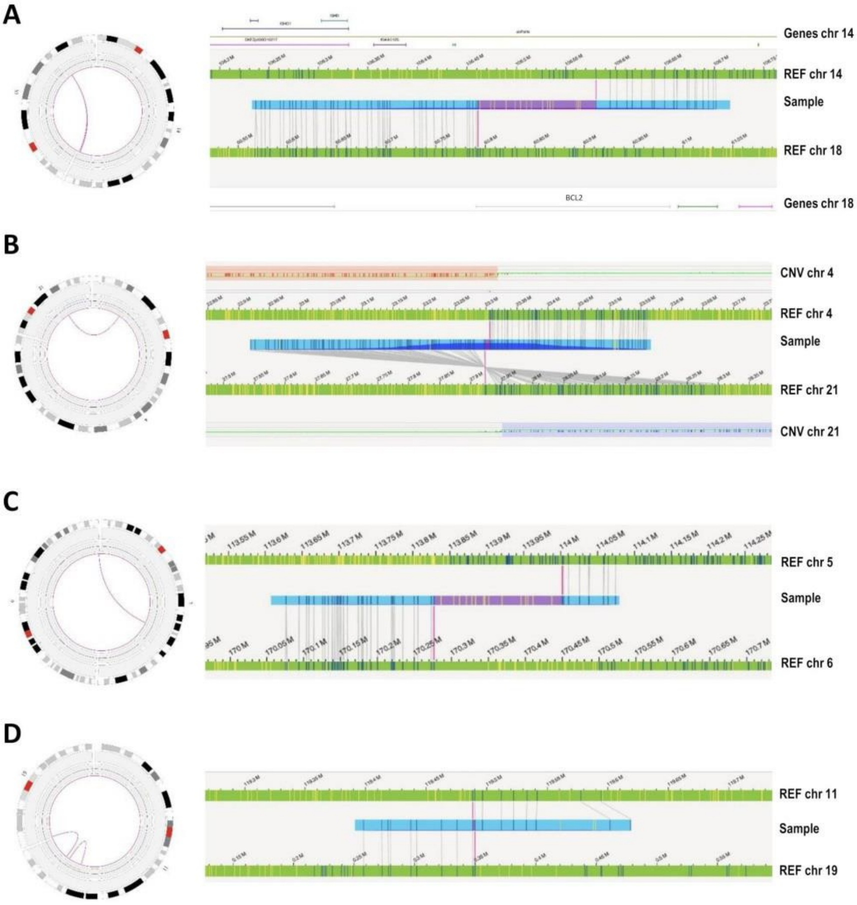
A
Genes chr 14
REF chr 14
Sample
REF chr 18
BCL2
Genes chr 18
B
CNV chr 4
REF chr 4
Sample
REF chr 21
CNV chr 21
C
REF chr 5
Sample
REF chr 6
D
REF chr 11
Sample
REF chr 19

Figure 2. Examples of the Circos plots and genome map views showing two real and two non-validated interchromosomal translocations. Validated translocations displayed a high number of labels mapped on each chromosome involved in the rearrangement: (**A**) Previously known t(14;18)(q32;q21) from patient #16, in which OGM also correctly identified the involvement of the *BCL2* and IGH genes in the rearrangement, and (**B**) t(4;21) with breakpoints at the limit of known CNV by CMA detected in patient #12, which corresponded to an unbalanced translocation that was cryptic in the karyotype and could be validated by WCP. On the contrary, images (**C**,**D**), representing a t(5;6) found in patient #3 and a t(11;19) from patient #13, were not validated by FISH using custom BAC probes for the specific rearrangements. These calls showed a low number of labels (<10) in at least one of the mapped chromosomes, which corresponded to the one showing low confidence score values. Of note, none showed breakpoints affecting regions provided by Bionano Genomics as DLE-1 masked (such as segmental duplications, common false positive regions or gaps).

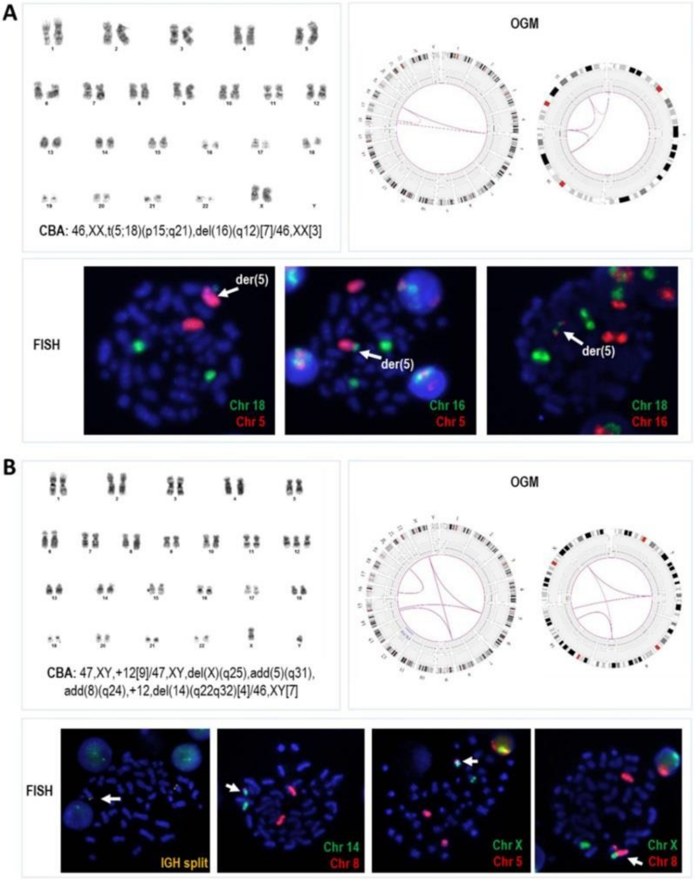
A
OGM
CBA: 46,XX,t(5;18)(p15;q21),del(16)(q12)[7]/46,XX[3]
FISH
der(5)
Chr 18
Chr 5
der(5)
Chr 16
Chr 5
der(5)
Chr 18
Chr 16
B
OGM
CBA: 47,XY,+12[9]/47,XY,del(X)(q25),add(5)(q31),
add(8)(q24),+12,del(14)(q22q32)[4]/46,XY[7]
FISH
IGH split
Chr 14
Chr 8
Chr X
Chr 5
Chr X
Chr 8

Figure 3. Examples of cases in which OGM revealed that abnormalities apparently well-described by CBA were more complex than initially assumed. (**A**) Patient #24 showed an abnormal karyotype described as containing a t(5;18)(p15;q21) and a del(16)(q12). Whole-genome Circos plot and Circos plot from chromosomes 5, 16 and 18 obtained by OGM revealed that the initially reported abnormalities were a three-way translocation between these chromosomes. OGM results were confirmed by WCP, in which material from both chromosomes 16 and 18 was detected in the der(5) chromosome; (**B**) The karyotype defined in patient #CK11 contained two deletions [del(X)(q25) and del(14)(q22q32)] and additional material of unknown origin at chromosomes 5 and 8 [add(5)(q31) and add(8)(q24)]. Circos plots show the four translocations identified by OGM that shared breakpoints on chromosomes X, 5 and 8, and two breakpoints on chromosome 14 were located at the start and end coordinates from a known CNV loss identified by CMA. FISH analysis allowed their validation and karyotype reinterpretation. The corrected ISCN formula for this abnormal clone was finally defined as 47,XY,der(X)t(X;5)(q22.1;q32),der(5)t(5;14)(q32;q32.2),der(8)t(X;8)(q22.1;q23.3),+12,der(14)t(8;14)(q23.3;q24.1).

In addition to the aforementioned translocation calls, OGM also identified three inversions and six deletions ≥100 Kb previously undetected by conventional techniques. Among these deletions, three could be identified in the revision of raw CMA data (sizes: 0.8, 1.8, and 4.1 Mb), and one was validated by FISH (6% of the nuclei presented the deletion using a custom BAC probe within the 4.2 Mb region lost by OGM). The two remaining discordant deletions were of just 230 Kb and 239 Kb and were not further assessed. In addition, in the two patients with no CMA study available, OGM underscored the presence of two large CNVs associated with a cryptic translocation by CBA (validated by FISH) and a 1.2 Mb deletion associated with a known t(13;17)(q14;p13).

3.4. Global Genomic Complexity Found by OGM

Regarding the genomic complexity identified by OGM, after the filtering and manual revision of raw results, the median number of abnormalities among the whole cohort was five (range: 1–70). As expected, those patients showing a CK by CBA displayed a significantly higher median number of alterations compared with non-CK patients (20 [range: 4–70] vs. 3 [range: 1–48]; $p < 0.001$). These differences were related to higher detection of both translocations and CNVs in the CK group (Figure S3). A significant strong correlation with the number of anomalies recorded by CBA and CMA was observed ($r_s = 0.734$ and $r_s = 0.845$, respectively). Nonetheless, it is noteworthy that OGM detected a significantly higher complexity than conventional techniques, which detected a median of two and three abnormalities by CBA and CMA, respectively (range: 0–16 for CBA and 0–30 for CMA) (Figure S3). In terms of prognostic impact, considering the number of abnormalities as a continuous variable, OGM presented a higher accuracy in predicting TTFT than CBA and CMA (C-index: 0.696, 0.602, 0.661; by OGM, CBA, and CMA, respectively).

Next, we evaluated if OGM could be a useful tool for risk stratification based on genomic complexity in CLL. Thus, considering the abnormalities found by OGM and comparing their distribution among the CBA and CMA risk categories defined in previous studies [28], an arbitrary cut-off of ≥10 alterations was used to define two risk groups by OGM. Based on this criterion, genomic complexity by OGM was assigned to 12 patients (28.6%) (Complex by OGM, C-OGM), while the remaining 30 patients were classified into a lower risk category (Non-complex by OGM, NC-OGM). Globally, the C-OGM group included 11/14 patients considered to be in the highest risk category by at least one standard technique (CBA and/or CMA) and one additional patient with a normal karyotype that, despite being considered to be in the CMA intermediate-risk group, displayed chromothripsis (Figure 4). Detailed OGM information from the three higher-risk patients, according to conventional methods, not included in the C-OGM group is shown in Figure 3B (patient CK11) and Figure S4 (patients 12 and CK16). Globally, for the identification of higher-risk patients, a moderate-strong agreement was observed between OGM and the combination of CBA and CMA methods ($\kappa = 0.778$; $p < 0.001$). As expected, the C-OGM group also showed an increased proportion of patients with *TP53* abnormalities (58.3% vs. 3.3%, $p < 0.001$) and a significantly shorter TTFT (median TTFT: 2 vs. 43 months, $p = 0.014$) compared with NC-OGM patients (Figure S5). Conversely, none of the other patient characteristics compared presented significant differences (Table 3). Interestingly, all patients with multiple trisomies by CBA (namely +12, +19, +/− other abnormalities; patients 21, CK1, CK15, CK18), previously described to exhibit very favorable outcomes [24], were categorized into the NC-OGM group with four to eight abnormalities detected by this technique.

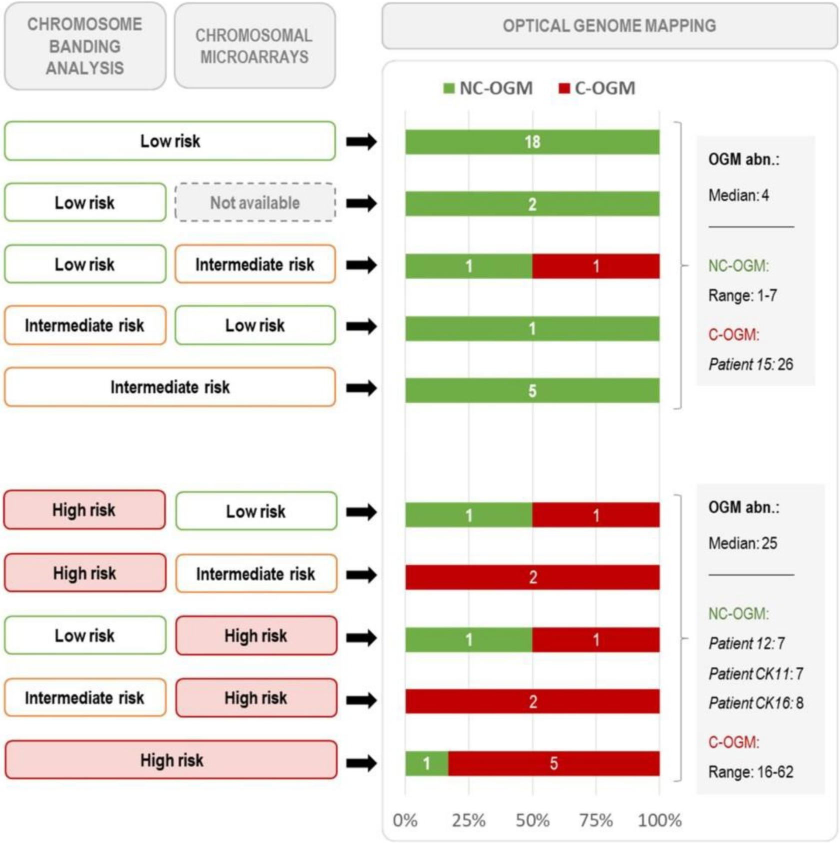

Figure 4. Patient distribution based on genomic complexity risk stratification by CBA, CMA and OGM. CBA and CMA categories were defined as described in previous studies (Ramos-Campoy et al., 2022): Low risk (0–2 abnormalities), Intermediate risk (3–4 abnormalities) and High risk (≥5 abnormalities). Patients were divided into two categories based on OGM results: NC-OGM (Non-complex by OGM, <10 abnormalities) and C-OGM (Complex by OGM, ≥10 abnormalities). Each line represents one possible combination of the risk assigned by CBA and/or CMA (depicted on the left), for the OGM results (right) the number of patients classified into each category is plotted and the number of abnormalities recorded is detailed in the gray boxes. Globally, for the identification of high-risk patients, a moderate-strong agreement was observed between OGM and the combination of CBA and CMA methods ($\kappa = 0.778$; $p < 0.001$).

Table 3. Comparison of the clinical and biological characteristics of CLL patients classified according to the genomic complexity detected by OGM.

Characteristics	NC-OGM (*n* = 30)	C-OGM (*n* = 12)	*p*-Value
Age at diagnosis	68 (37–85)	67 (55–88)	0.944
Males	20 (66.7%)	8 (66.7%)	1.000
Advanced Binet stage (B or C)	3 (10.0%)	4 (33.3%)	0.088
Time from diagnosis to OGM study (months)	8 (0–174)	25 (0–125)	0.854
Number of abnormalities by OGM (filtered data)	4 (1–11)	32 (16–70)	<0.001
Copy number variants (CNV gains and losses)	1 (0–4)	12 (2–25)	<0.001
Translocations (intra and interchromosomal)	2 (0–8)	22 (9–42)	<0.001
Genomic complexity by conventional methods			
Low/intermediate-CK by CBA (3–4 abn.)	6 (21.4%)	2 (7.1%)	1.000
High-CK by CBA (≥5 abn.)	2 (6.7%)	8 (66.7%)	<0.001
Intermediate-GC by CMA (3–4 abn. *) (*n* = 40)	6/28 (21.4%)	3/12 (25.0%)	1.000
High-GC by CMA (≥5 abn. *) (*n* = 40)	2/28 (7.1%)	8/12 (66.7%)	0.001
High complexity by CBA and/or CMA (≥5 abn.)	2 (6.7%)	11 (91.7%)	<0.001
Chromothripsis	0 (0.0%)	9 (75.0%)	<0.001
FISH abnormalities			
del(13q)	22 (73.3%)	8 (66.7%)	0.715
Trisomy 12	7 (23.3%)	1 (8.3%)	0.402
del(11q) [*ATM*]	8 (26.7%)	4 (33.3%)	0.715
del(17p) [*TP53*]	1 (3.3%)	6 (50.0%)	0.001
***TP53* abnormalities (del/mut)**	1 (3.3%)	7 (58.3%)	<0.001
Unmutated IGHV (*n* = 41)	15/29 (51.7%)	9/12 (75.0%)	0.296
Last follow-up (*n* = 40) ¥			
Treated patients	16/30 (53.3%)	8/10 (80.0%)	0.090
Time to first treatment (months, 95% CI)	43 (28.0–52.6)	2 (1.9–23.0)	0.014
Follow-up (months)	42 (0–95)	26 (0–82)	0.070

Values are given as median (range) or number (%). Abbreviations: NC-OGM = non-complex by optical genome mapping, <10 abnormalities), C-OGM = complex by optical genome mapping, ≥10 abnormalities), CBA = Chromosome banding analysis, CMA = Chromosomal microarray analysis, abn. = abnormalities. * Criteria defined by Leeksma et al., 2020 for intermediate genomic complexity (Intermediate-CG): 3–4 CNV; and high genomic complexity (High-CG): ≥5 CNV, including all classic CLL CNV (chromosome 12 and losses of 11q, 13q and 17p) irrespectively of their size and other CNVs > 5 Mb. ¥ Those patients treated prior to genetic testing were excluded from the time to first treatment analyses.

4. Discussion

Over the last decade, several works have highlighted the prognostic and predictive value of genomic complexity in patients with CLL. Recent large ERIC cooperative studies have validated the performance of both CBA and CMA in the risk stratification of CLL and suggested the detection of five or more chromosomal abnormalities as an adverse biomarker to be implemented in routine CLL management [9,27]. As both techniques had some intrinsic limitations that led to discordant risk stratification in up to 30% of patients [28], herein, we aimed to evaluate whether the recently developed OGM technology is a suitable alternative to overcome these discrepancies. To the best of our knowledge, this is the largest cytogenetic characterization via OGM conducted to date in CLL patients.

Overall, we demonstrated the feasibility of OGM in detecting 90% of the alterations previously defined by the combination of standard techniques (CBA, FISH, and/or CMA) in a single assay. In addition, this technique afforded not only high accuracy in the definition of genomic coordinates for CNVs when compared with CMA but also provided additional structural information on previously known aberrations. As expected, the concordance reached was similar to that reported in other hematological malignancies, in which OGM detection of clinically relevant known abnormalities ranged between 90 and 100% [35–37,40–42]. The number of missed abnormalities in the present series was low, but the discrepancies found underscore some OGM limitations that should be taken into account. First, although this is a whole-genome view technology, the analysis is compromised at some locations that could not be properly labeled or mapped (such as centromeres, telomeres, or regions with a high number of repetitions). Thus, nearly half of the 30 abnormalities missed in our study involved these regions and were not expected to be detected by OGM. Considered individually, as none had been previously described as clinically relevant in CLL, their misdetection is not assumed to be highly relevant for patient management. However, as suggested by other authors, manual inspection of OGM imaged molecules is highly recommended for pathologies in which aberrations affecting these regions are well-known prognostic biomarkers (e.g., *CRLF2* rearrangements involving the PAR1 region on chromosome X in acute lymphoblastic leukemia) [37,41,42]. It is noteworthy that these studies were performed against hg19 [GRCh37], as used herein, and some cases would be identified using hg38 [GRCh38] as a reference, which has a more robust assembly for the Xp region [43]. Second, OGM specifications describe that at 300x effective coverage, it allows the detection of SVs and CNVs in up to 5% and 10% of the allele fraction, respectively. In our experience, the SV tool was able to detect some translocations at these low frequencies, but the detection of CNVs was not fully guaranteed if present in less than 20% of cells. In this regard, sensitivity was assumed to be the most feasible cause of the discrepancy in 43% of the missed abnormalities. While these variants had been previously detected in a median of 17% of cells, concordant OGM results also included CNVs found in only 12–15% of nuclei by FISH and/or CMA. Although some authors have set the sensitivity threshold for OGM at 10% [35,41], our results were in accordance with Rack et al. and suggested that detection of abnormalities around or below 15% varies among regions or loci [37]. It is noteworthy that some of the CNVs not called by OGM were visually identified in the whole-genome CNV view with Bionano Access and could potentially be manually added in future software upgrades, as currently performed for CMA analysis.

In contrast to other published OGM studies in hematological malignancies, which focused on the detection of clinically relevant abnormalities, our goal was to evaluate CLL abnormalities genome-wide in order to identify patients with genomic complexity. This strategy allowed us to benefit from the high potential of OGM, underscoring novel aberrations which had not been identified with standard methods and whose clinical relevance deserves further analysis. On the one hand, OGM identified several small SVs (from 3 Kb to 100 Kb) that were considered beyond the scope of this study. Our proof-of-concept analysis aimed to obtain comparable results with the currently used methodologies; therefore, a cut-off size of 100 Kb was set to generate results with a resolution similar to that of CMA. Nonetheless, these small aberrations missed by standard methods could involve clinically relevant genes (novel or already known) and should be analyzed in more depth in subsequent studies and in larger CLL cohorts. On the other hand, a higher number of chromosomal translocations than initially expected was detected by OGM. Indeed, novel translocations allowed us to better characterize known abnormalities in 54.8% of the patients assessed. These included not only abnormalities involving material of unknown origin by CBA (marker chromosomes or "add") but also provided novel structural information related to apparently well-characterized translocations or deletions in the karyotype, as well as CNVs detected by CMA. Notably, not all the novel OGM translocations correlated with the results defined by standard techniques, and nearly 20% of them were classified as false-positive findings. Other authors have also reported similar observations and suggested manual inspection to filter out SV artifacts distinguishable from true translocation calls [35–37]. Following our experience, those translocation calls based on the mapping of $\leq$10 labels in one of the translocation partners should be filtered. In contrast, while we were not able to validate two translocations with less than 10 molecules, 22% of the previously known or validated translocations were supported by only five to nine mapped molecules by OGM. Thus, contrarily to some authors that set a minimum of 10 supporting molecules for the analyses [39,40], we recommend not filtering these variants but performing validations with alternative techniques before reporting them. It is remarkable that, since we processed the samples, a new upgrade of Bionano Access has been developed that has improved the filtering of these types of false-positive calls. In this regard, in two of our samples reprocessed with the most recent update of Bionano Access (1.7 version), nine of the ten inter-translocations assumed as potential false-positive results were no longer called, but novel translocations were also identified in this updated version (data not shown). Globally, we have demonstrated that the custom filtering criteria applied herein are useful for OGM analysis, but the distinction between true translocations and technical artifacts is sometimes challenging. Consequently, guidelines for the filtering and interpretation of OGM variants are urgently needed to implement this technique in a routine setting.

Concerning genomic complexity assessment by OGM, as expected by its high resolution, our results demonstrated that this novel technique was able to identify an overall higher number of abnormalities than conventional methods. Nonetheless, when data were manually curated, and small SVs (<100 Kb) were excluded from the analysis, a strong correlation between techniques was found. Likewise, we demonstrated that occasional discrepancies in the detection of known aberrations did not hinder the accuracy of the number of abnormalities recorded by OGM in predicting TTFT, which was even better than that achieved by CBA and CMA. Indeed, the higher C-index obtained by OGM indirectly proves the clinical importance of the additional aberrations identified by OGM, which allows a better delineation of the genomic complexity of these patients compared with current methodologies. Apart from its predictive value as a continuous variable, the definition of standard criteria to discriminate those higher-risk patients based on complexity is mandatory to incorporate OGM technology in the standard diagnostic algorithm. In this regard, we were able to demonstrate that 91.7% of patients harboring at least 10 abnormalities by OGM (C-OGM group) were also classified into the highest risk group based on the complexity identified by the combination of CBA and CMA. Moreover, this C-OGM group showed a statistically significantly higher proportion of *TP53*$^{del/mut}$ cases and shorter TTFT. It is noteworthy that, as the cohort analyzed herein is limited in size and highly enriched in patients carrying CK, the cut-off used could not be statistically set. Thus, it should only be taken into account as a proof-of-concept of the capability of OGM to stratify patients based on their genomic complexity. Further studies in larger cohorts of CLL patients are required to statistically define robust criteria for genomic complexity stratification by OGM, not only in retrospective series but also to clarify its potential role in the management of patients treated with novel targeted agents.

On the other hand, OGM represents a cost-effective approach to substitute CBA combined with multiple FISH tests that reduce global costs and turn-around times in other hematological malignances (e.g., acute lymphoblastic leukemia and acute myeloid leukemia). In contrast, the total expenses of the OGM technique (technician time dedication, reagents, equipment, and other technical infrastructure required for its implementation) are still superior to those of CBA; currently, the standard-of-care method to detect genomic complexity CLL. Thus, major benefits from OGM application in CLL do not rely on the cost savings but on the better genomic characterization achieved and novel abnormalities that could be uncovered from these highly complex genomes.

5. Conclusions

In summary, we have confirmed that OGM is a valuable tool for the cytogenomic assessment of CLL patients. It not only effectively detects most of the abnormalities defined by the combination of standard methods in a single test but also provides a more comprehensive genomic analysis allowing an enhanced interpretation. Despite a few abnormalities still being missed due to OGM limitations, these are expected to be addressed by the technical and analytical improvements of upcoming Bionano Access upgrades. As for its utility for risk stratification, herein, for the first time, we have demonstrated the association of an increasing number of abnormalities by OGM with a worse clinical evolution of CLL patients. Nonetheless, further studies in larger unselected cohorts are required to define standard genomic complexity criteria by OGM and to define the clinical significance of the novel abnormalities underscored by this technology. In conclusion, OGM is a new methodology that could potentially replace current cytogenomic methodologies for the routine management of CLL patients in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14143376/s1, Figure S1. Flow diagram for filtering SV and CNV calls to define the final set of OGM abnormalities to be compared with standard techniques. Figure S2. Examples of novel translocations called by OGM that were associated with known CNVs previously identified by CMA. Figure S3. Distribution of the number of abnormalities detected in non-CK and CK groups. Figure S4. Example of two of the high-risk patients by CBA and/or CMA with less than 10 abnormalities detected by OGM. Figure S5. Kaplan–Meier plots for time to first treatment (TTFT) based on the presence of genomic complexity detected by OGM. Table S1. FISH probes tested to validate OGM results. Table S2. Technical performance of OGM in the whole cohort. Table S3. Detailed information on the detection of the 309 known abnormalities by OGM. Table S4. Summary of the interchromosomal translocation calls detected by OGM and comparison with CBA and FISH data.

Author Contributions: Conceptualization, A.P. and B.E.; Methodology, A.P., S.R.-C. and B.E.; Formal Analysis, A.P.; Investigation, A.P., S.R.-C., J.K., M.d.l.R., M.S., C.M., M.R.-R., S.B., J.S., T.M. and A.H.; Resources, A.P., S.R.-C., B.E., R.C., E.G., R.G.-S., S.A., M.A.M.-G., M.D.G.-M., X.C., L.A. and A.F.; Data Curation, A.P.; Writing—Original Draft Preparation, A.P., S.R.-C. and B.E.; Writing—Review and Editing, A.P., S.R.-C., J.K., M.d.l.R., M.S., C.M., M.R.-R., S.B., R.C., E.G., R.G.-S., S.A., M.A.M.-G., M.D.G.-M., X.C., L.A., A.F., T.M., A.H., J.S. and B.E.; Visualization, A.P.; Supervision, A.P. and B.E.; Project Administration, B.E.; Funding Acquisition, B.E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Drug Research Ethical Committee (CEIm) of the Hospital del Mar, Barcelona (2017/7565/I).

Informed Consent Statement: Informed consent was obtained from all patients involved in the study.

Data Availability Statement: Detailed chromosome banding analyses, chromosomal microarrays and optical genome mapping profiles are provided in Supplemental Tables. Additional data of the study are available from the corresponding authors on reasonable request.

Conflicts of Interest: No potential conflicts of interest were disclosed.

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Supplementary Materials

Optical Genome Mapping: A Promising New Tool to Assess Genomic Complexity in Chronic Lymphocytic Leukemia (CLL)

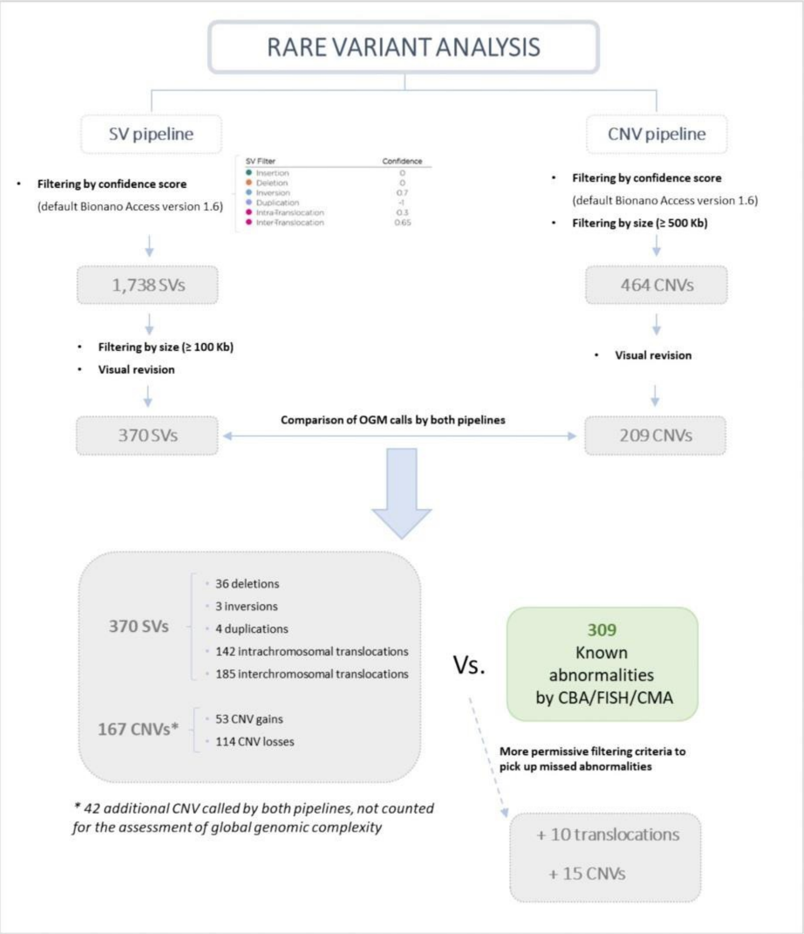

RARE VARIANT ANALYSIS
SV pipeline
CNV pipeline
Filtering by confidence score
(default Bionano Access version 1.6)
SV Filter
Confidence
Insertion 0
Deletion 0
Inversion 0.7
Duplication -1
Intra-Translocation 0.3
Inter-Translocation 0.65
Filtering by confidence score
(default Bionano Access version 1.6)
Filtering by size (≥ 500 Kb)
1,738 SVs
464 CNVs
Filtering by size (≥ 100 Kb)
Visual revision
Visual revision
Comparison of OGM calls by both pipelines
370 SVs
209 CNVs
370 SVs
36 deletions
3 inversions
4 duplications
142 intrachromosomal translocations
185 interchromosomal translocations
167 CNVs*
53 CNV gains
114 CNV losses
Vs.
309
Known
abnormalities
by CBA/FISH/CMA
More permissive filtering criteria to pick up missed abnormalities
* 42 additional CNV called by both pipelines, not counted for the assessment of global genomic complexity
+ 10 translocations
+ 15 CNVs

Figure S1. Flow diagram for filtering SVs and CNVs calls to define the final set of OGM abnormalities to be compared with standard techniques.

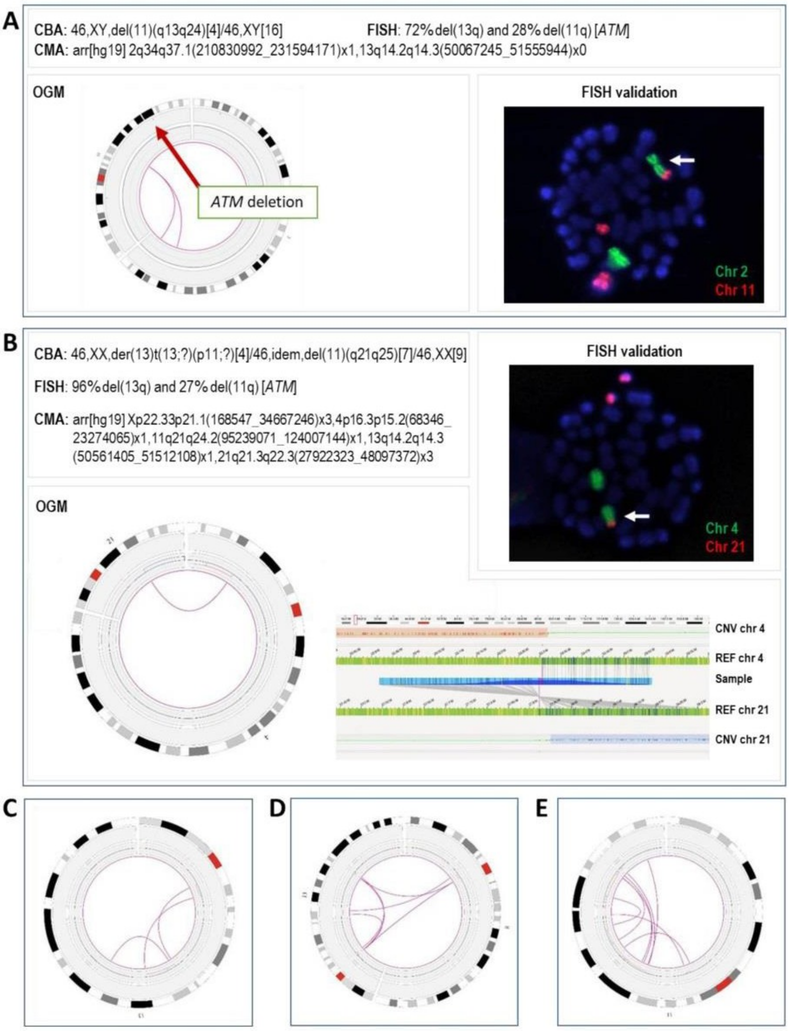

A
CBA: 46,XY,del(11)(q13q24)[4]/46,XY[16]
FISH: 72% del(13q) and 28% del(11q) [ATM]
CMA: arr[hg19] 2q34q37.1(210830992_231594171)x1,13q14.2q14.3(50067245_51555944)x0
OGM
ATM deletion
FISH validation
Chr 2
Chr 11
B
CBA: 46,XX,der(13)t(13;?)(p11;?)[4]/46,idem,del(11)(q21q25)[7]/46,XX[9]
FISH: 96% del(13q) and 27% del(11q) [ATM]
CMA: arr[hg19] Xp22.33p21.1(168547_34667246)x3,4p16.3p15.2(68346_23274065)x1,11q21q24.2(95239071_124007144)x1,13q14.2q14.3(50561405_51512108)x1,21q21.3q22.3(27922323_48097372)x3
OGM
FISH validation
Chr 4
Chr 21
CNV chr 4
REF chr 4
Sample
REF chr 21
CNV chr 21
C
D
E

Figure S2. Examples of novel translocations called by OGM that were associated with known CNV previously identified by CMA. (**A**) Patient #11 showed a deletion 11q affecting bands q13q24 as a single abnormality by CBA, also detected in 28% of nuclei by FISH with the 11q22 probe included in the CLL FISH panel. CMA failed to detect this deletion but revealed the presence of a large 20Mb deletion affecting 2q arm missed in the karyotype. Circos plot shows the abnormalities found by OGM on chromosomes 2 and 11, which suggested that the 11q deletion identified was smaller than assumed by conventional techniques (188Kb, including *ATM*) and that the abnormal chromosome 11 detected in the karyotype was a derivative chromosome generated from a cryptic unbalanced t(2;11). Whole-chromosome FISH painting confirmed OGM translocations; (**B**) OGM analysis from patient #12 showed a novel translocation t(4;21) with breakpoints at the limit of two large terminal CNV previously identified by CMA (23Mb loss at 4p and 20Mb gain at 21q). The

rearrangement was confirmed by FISH, which revealed the presence of an unbalanced translocation that was cryptic by CBA; Circos plots showing novel OGM translocations involving known 13q deletions detected by CMA in patient CK5 (**C**) and patient CK9 (**D**), and multiple translocations revealing the high complexity associated with a known *ATM* deletion previously underestimated in patient CK3 (**E**).

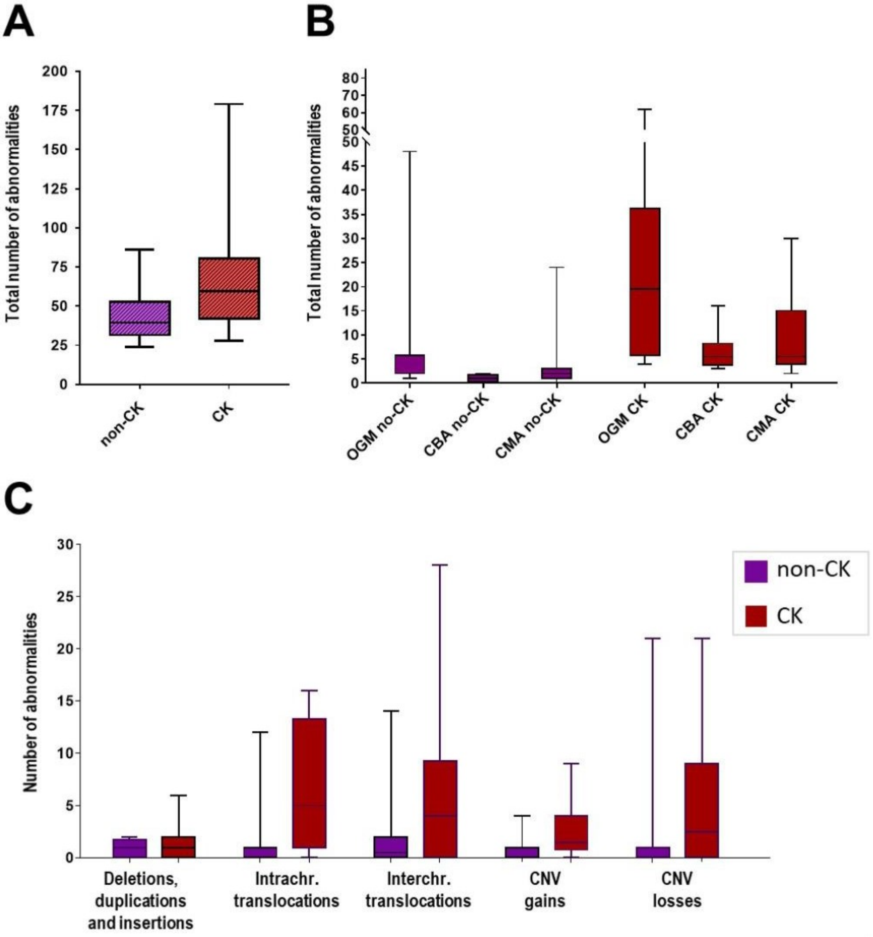
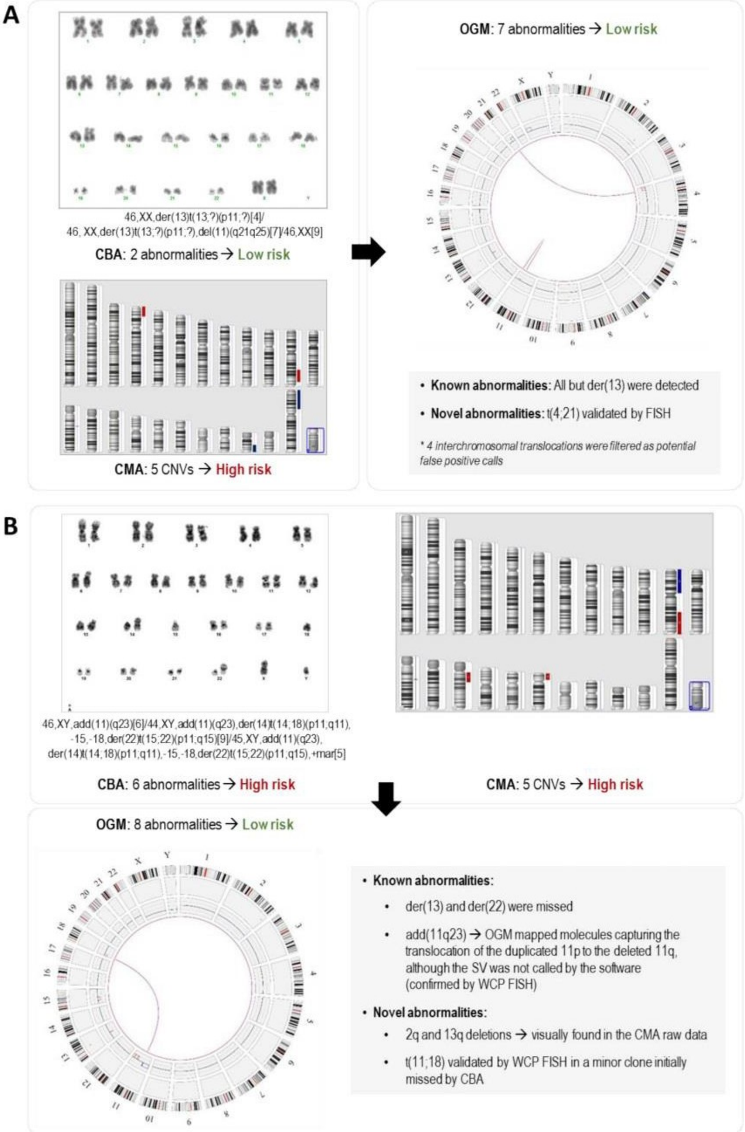


Figure S3. Distribution of the number of abnormalities detected in non-CK and CK groups. (**A**) Total number of SV and CNV called by OGM pipelines; (**B**) Total number of abnormalities by OGM (curated results), chromosome banding analysis (CBA) and chromosomal microarrays (CMA); (**C**) Distribution of the number of the different types of SV and CNV detected by OGM (curated results).


A
CBA: 2 abnormalities → Low risk
CMA: 5 CNVs → High risk
OGM: 7 abnormalities → Low risk
B
CBA: 6 abnormalities → High risk
CMA: 5 CNVs → High risk
OGM: 8 abnormalities → Low risk

46,XX,der(13)t(13;?)(p11;?)[4]/
46, XX,der(13)t(13;?)(p11;?),del(11)(q21q25)[7]/46,XX[9]

- **Known abnormalities:** All but der(13) were detected
- **Novel abnormalities:** t(4;21) validated by FISH

** 4 interchromosomal translocations were filtered as potential false positive calls*

46,XY,add(11)(q23)[6]/44,XY,add(11)(q23),der(14)t(14;18)(p11;q11),
-15,-18,der(22)t(15;22)(p11;q15)[9]/45,XY,add(11)(q23),
der(14)t(14;18)(p11;q11),-15,-18,der(22)t(15;22)(p11;q15),+mar[5]

- **Known abnormalities:**
  - der(13) and der(22) were missed
  - add(11q23) → OGM mapped molecules capturing the translocation of the duplicated 11p to the deleted 11q, although the SV was not called by the software (confirmed by WCP FISH)
- **Novel abnormalities:**
  - 2q and 13q deletions → visually found in the CMA raw data
  - t(11;18) validated by WCP FISH in a minor clone initially missed by CBA

Figure S4. Example of two of the high-risk patients by CBA and/or CMA with less than 10 abnormalities detected by OGM. Detailed results from patient #12 (**A**) and #CK16 (**B**), who were classified in the low complexity group by OGM despite being classified in the highest risk group by at least one of the standard techniques. For each patient karyotype defined by CBA, karyogram generated by CMA and circos plots showing the curated results obtained by OGM are shown. In addition, the number of abnormalities recorded by each technique and a brief explanation of the comparison among techniques are also detailed.

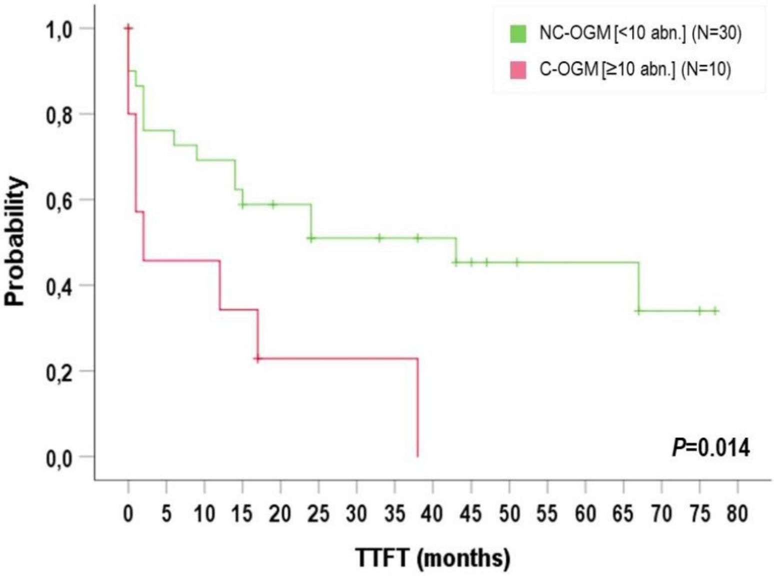


Figure S5. Kaplan-Meier plots for time to first treatment (TTFT) based on the presence of genomic complexity detected by OGM. Patients were divided in two categories based on OGM results: NC-OGM (Non-complex by OGM, <10 abnormalities) and C-OGM (Complex by OGM, ≥10 abnormalities).

Las tablas suplementarias S1, S2, S3 y S4 se pueden visualizar accediendo en el siguiente enlace:

https://www.mdpi.com/article/10.3390/cancers14143376/s1