Caracterización histopatológica y pronóstica del carcinoma escamoso de vulva

Núria Carreras Dieguez

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CARACTERIZACIÓN HISTOPATOLÓGICA Y PRONÓSTICA DEL CARCINOMA ESCAMOSO DE VULVA

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Listado de abreviaturas y acrónimos

- **ADN:** ácido desoxirribonucleico
- **ARN:** ácido ribonucleico
- **CEV:** carcinoma escamoso de vulva
- **CI:** intervalo de confianza (por sus siglas en inglés, *confidence interval*)
- **DEVIL:** lesión intraepitelial exofítica vulvar diferenciada (por sus siglas en inglés, *differentiated exophytic vulvar intraepithelial lesion*)
- **dVIN:** neoplasia vulvar intraepitelial de tipo diferenciado (por sus siglas en inglés, *differentiated vulvar intraepithelial neoplasia*)
- **FIGO:** *International Federation of Gynecology and Obstetrics*
- **HR:** cociente de riesgo (por sus siglas en inglés, *hazard ratio)*
- **HSIL:** lesión escamosa intraepitelial de alto grado (por sus siglas en inglés, *high grade squamous intraepithelial lesion*)
- **LAST:** terminología de patología escamosa del tracto genital inferior (por sus siglas en inglés, *lower anogenital squamous terminology*)
- **OMS:** Organización Mundial de la Salud
- **PCR:** reacción en cadena de la polimerasa (por sus siglas en inglés, *polymerase chain reaction*)
- **VAAD:** acantosis vulvar con diferenciación alterada (por sus siglas en inglés, *vulvar acantosis with altered differentiation*)
- **VPH:** virus del papiloma humano

Enumeración de los artículos de la tesis

Esta tesis doctoral se presenta en forma de compendio de artículos y consta de 10 objetivos y 2 artículos de investigación original, que se enumeran a continuación. Además, se adjunta en la introducción de la tesis un artículo preliminar de revisión de la literatura.

1. AUTORES: **Carreras-Dieguez N**, Saco A, Del Pino M, Pumarola C, López del Campo R, Manzotti C, Garcia A, Marimon L, Diaz-Mercedes S, Fusté P, Rodrigo-Calvo MT, Vega N, Torné A, Rakislova N

   TÍTULO: Vulvar squamous cell carcinoma arising on human papillomavirus-independent precursors mimicking high-grade squamous Intraepithelial lesion: a distinct and highly recurrent subtype of vulvar cancer

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1 | Introducción

1.1. Carcinoma escamoso de vulva: aspectos generales

El carcinoma escamoso de vulva (CEV) es una neoplasia genital poco común, con una incidencia global de 1-2 casos por 100.000 mujeres/año (1), que típicamente se presenta en edades avanzadas, aunque en las últimas décadas se ha observado un aumento de incidencia en mujeres jóvenes (2). Además, con el aumento en la esperanza de vida, se prevé una mayor incidencia de esta neoplasia en los próximos años (2). El CEV es el tipo histológico más prevalente y representa el 90% de los tumores malignos de la vulva, siendo el melanoma el segundo en frecuencia (3).

En la Figura 1 se muestra la incidencia mundial del carcinoma de vulva.

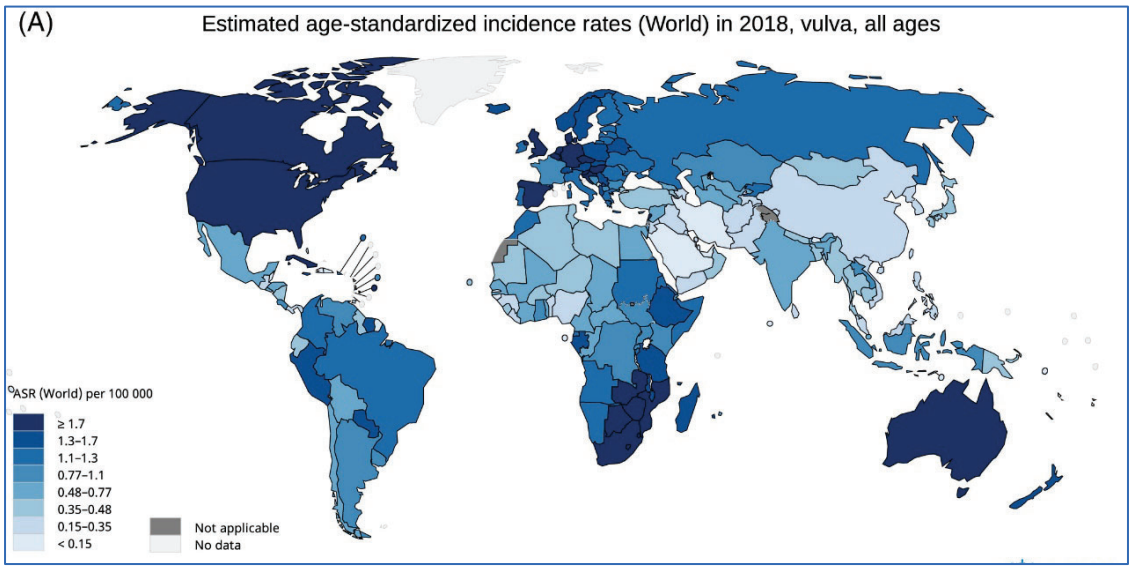

Figura 1. Incidencia mundial del carcinoma de vulva.

Bray F, et al. Int J Cancer. 2020; 147(10):2764-2771 (4)

Clásicamente, el CEV se ha clasificado según sus características histológicas en diferentes variantes entre las que destacan el subtipo basaloide, condilomatoso y el queratinizante. En septiembre de

2020 la Organización Mundial de la Salud (OMS) publicó una nueva clasificación del CEV (5), basada en las dos vías de carcinogénesis claramente identificadas en la vulva: una vía asociada al virus del papiloma humano (VPH) y una vía independiente del VPH. En parte, el fundamento de esta nueva clasificación estriba en que, mientras la clasificación histológica no siempre se correlaciona con las características clínicas y el comportamiento del tumor, los tumores asociados al VPH e independientes de VPH sí han mostrado características clínicas e historia natural claramente diferentes. El CEV suele estar precedido durante un período de tiempo variable por una lesión intraepitelial premaligna, que se considera precursora del carcinoma invasivo. Los tumores asociados a VPH e independientes de VPH están asociados a un tipo particular de lesión precursora(5).

Desafortunadamente, todavía existen muchos aspectos poco conocidos de la historia natural del CEV y sus lesiones precursoras, particularmente de los tumores independientes de VPH. Gran parte de este desconocimiento se debe a la baja frecuencia de estos tumores y a las limitaciones inherentes a la clasificación clásica del CEV. Además, las lesiones precursoras del CEV independiente de VPH son difíciles de caracterizar tanto clínica como histológicamente. A fecha de hoy, la conducta clínica y tratamiento del CEV son homogéneos, independientemente de su asociación o no a VPH o de su tipo histológico específico (6,7). Prácticamente no existe disponibilidad de terapias dirigidas específicas para el CEV (6,7), y hay un número muy limitado ensayos clínicos publicados en este campo (8,9).

1.2. Carcinoma escamoso de vulva asociado e independiente del virus del papiloma humano

La prevalencia de VPH como agente etiopatogénico del CEV varía entre el 9% y el 100% en las diferentes series publicadas (10–13). El mayor estudio internacional acerca de la implicación de VPH en el CEV describe una prevalencia del 25% entre los 1709 casos de CEV evaluados (11). En Europa, América del Norte y Oceanía, la mayoría de los CEV se desarrollan a través de la vía independiente de VPH. Datos de nuestro grupo indican que menos del 20% de los CEV tratados en el Hospital Clínic de Barcelona están asociados al VPH (14). En cambio, en otros países en vías

de desarrollo en los que además existe una elevada prevalencia global de infección VPH, los CEV predominantes son los asociados a este virus (11,12). Además de las variaciones en la prevalencia de la infección VPH en las distintas áreas geográficas, otro factor responsable de esta variabilidad entre la proporción de CEV asociado e independiente de VPH es el uso de diferentes técnicas de detección de VPH, su precisión diagnóstica, o la posibilidad de contaminación de las muestras, que pueden resultar en una subestimación o sobreestimación del papel etioptatogénico del VPH (13).

Desde el punto de vista histológico, los CEV asociados a VPH se han caracterizado tradicionalmente como basaloides o condilomatosos, mientras que los tumores independientes de VPH se han caracterizado como queratinizantes (15,16). Los tumores de tipo basaloide presentan células pequeñas, basaloides, con escaso citoplasma y poca queratinización; mientras que los de tipo condilomatoso suelen presentar una apariencia condilomatosa y cambios coilocíticos (13). Los tumores queratinizantes se conforman por células diferenciadas en ausencia de coilocitosis y frecuentemente muestran perlas de queratina(13). La anterior clasificación de la OMS del CEV (17) (basaloide, condilomatoso, queratinizante, no queratinizante y verrucoso) se veía limitada por una cierta superposición entre algunas de las características histológicas de estos subtipos, por lo que la clasificación de estas lesiones no siempre era clara. Además, también se ha descrito una superposición considerable entre los tipos histológicos y su relación con el VPH: aproximadamente un tercio de los tumores asociados al VPH son queratinizantes, mientras que una quinta parte del CEV independiente de VPH muestra características histológicas basaloides o condilomatosas (10,16). En los últimos años, se ha demostrado que, desde el punto de vista clínico y pronóstico, es más relevante la asociación o no con el VPH que el tipo histológico (18,19). Los CEV asociados a VPH se presentan a edades más tempranas que los independientes de VPH (59 *versus* 72 años) (18), y hay estudios que sugieren que tienen mejor pronóstico que los tumores independientes de VPH(18,20,21). Así pues, la nueva clasificación de la OMS, se fundamenta en la caracterización etiopatogénica del CEV, estableciendo como prioridad el *status* VPH frente a la clasificación puramente morfológica.

En los últimos años, algunos autores han propuesto una sub-clasificación de los tumores VPH independientes en función de p53 (mutado o *wild-type*) (19,21,22). Los tumores VPH independientes p53 *wild-type* tienen frecuentemente lesiones precursoras asociadas específicas,

llamadas acantosis vulvar con diferenciación alterada (23) y lesión intraepitelial exofítica vulvar (24) (VAAD y DEVIL respectivamente, por sus siglas en inglés). En cambio, los tumores con alteraciones en p53 son habitualmente de tipo queratinizante y su lesión precursora es frecuentemente la neoplasia vulvar intraepitelial de tipo diferenciado (dVIN). Sin embargo, existe poca evidencia entre las diferencias clínicas y pronósticas de estos dos subtipos de tumores independientes de VPH (21).

1.3. Lesiones precursoras del carcinoma escamoso de vulva

Las lesiones intraepiteliales precursoras son distintas para cada tipo etiopatogénico de CEV (asociado a VPH e independiente de VPH). La clasificación de las lesiones intraepiteliales asociadas a VPH se realiza mediante la terminología LAST (*Lower Anogenital Squamous Terminology*), propuesta en 2012 y reconocida en la clasificación de la OMS 2020. Esta terminología unifica la nomenclatura para designar a las lesiones asociadas a VPH en todo el tracto anogenital (25). Así pues, las lesiones precursoras asociadas a VPH se denominan "lesiones intraepiteliales escamosas de alto grado" (HSIL por sus siglas en inglés). El término HSIL vulvar reemplaza las antiguas denominaciones de uVIN (neoplasia vulvar intraepitelial de tipo usual) y VIN 2-3. Las lesiones HSIL se caracterizan por una clara atipia citológica, una ausencia de maduración celular que afecta a todo el espesor del epitelio, un elevado ratio núcleo-citoplasma, la hipercromasia y por presencia de mitosis en todo el grosor de la epidermis, que está engrosada y suele mostrar hiper- y/o paraqueratosis (26). Es relativamente frecuente la afectación de los anejos cutáneos en este tipo de lesiones (27). Se han descrito dos variantes morfológicas de HSIL, una denominada basaloide y otra denominada condilomatosa. La variante basaloide presenta una marcada inmadurez de las células en todo el grosor del epitelio, con atipia citológica más prominente y marcados cambios coilocíticos. La variante condilomatosa se caracteriza por la presencia de crestas anchas y profundas que resultan en su apariencia condilomatosa, con marcado pleomorfismo, agrandamiento nuclear y figuras mitóticas atípicas, siendo fácilmente reconocibles los signos de la infección por VPH (coilocitosis, multinucleación, gránulos gruesos de cromatina). Sin embargo, muchas lesiones HSIL tienen características mixtas (13). Es frecuente la asociación de estas

lesiones con lesiones premalignas en otras áreas del tracto anogenital, como el cuello uterino, ano o vagina (28). Las lesiones precursoras asociadas a VPH muestran un riesgo relativamente bajo de progresión a carcinoma invasivo, entre el 9 y el 16% según las series en pacientes no tratadas (29,30), y alrededor del 3% en las pacientes tratadas (30).

Las lesiones precursoras independientes de VPH se denominan "neoplasia intraepitelial vulvar de tipo diferenciado" (dVIN), y se originan a menudo sobre piel vulvar previamente afecta por dermatosis inflamatorias crónicas (liquen escleroso, liquen plano u otras). Las lesiones dVIN se caracterizan por la presencia de queratinocitos atípicos. El epitelio es altamente diferenciado y la atipia se limita a los estratos basales y parabasales, donde las células presentan un citoplasma amplio y forman las llamadas "perlas abortivas". Las crestas papilares suelen estar elongadas y ramificadas. La extensión a anejos cutáneos es poco habitual (31). El reconocimiento histológico de dVIN se ve dificultado por el alto grado de diferenciación celular y su difícil diferenciación de las lesiones inflamatorias (32). Esta entidad es frecuentemente infradiagnosticada por la sutileza de sus características histológicas, que supone un desafío para la mayoría de patológos (32). A diferencia de las lesiones asociadas a VPH, la tasa de progresión de dVIN a carcinoma invasivo es más alta (32-41%) y el proceso de progresión es más rápido (13,31,33–35). Aunque la mayoría de CEV en nuestro ámbito son independientes de VPH, las lesiones dVIN de forma aislada son raramente diagnosticadas en la práctica asistencial, siendo más frecuente su diagnóstico en asociación con las lesiones de CEV. Este hecho se explica, en parte, por la dificultad en su diagnóstico para clínicos y patólogos, así como por su rápida progresión desde lesión premaligna a carcinoma invasivo (32,36).

Igual que en el CEV, se ha descrito un cierto grado de solapamiento entre las características histológicas de las lesiones precursoras asociadas a VPH (HSIL) y las independientes de VPH (dVIN). Varios autores muestran que una proporción pequeña de lesiones dVIN pueden tener características morfológicas de HSIL (37,38), y a la inversa (39). Un trabajo reciente de nuestro grupo, describe que el 6% de tumores independientes de VPH surgen de una lesión precursora morfológicamente indistinguible de una lesión HSIL (38). En semejanza al HSIL asociado a VPH, este precursor independiente de VPH presenta una maduración celular anormal en todo el grosor del epitelio, con células de morfología basaloide y cambios coilocíticos. Estas lesiones han recibido

el nombre de dVIN basaloide o lesiones HSIL-*like*(38). A diferencia del HSIL asociado a VPH, estas lesiones no presentan positividad para p16 y son negativas para VPH de alto riesgo (véase apartado 1.4); en cambio, frecuentemente presentan alteraciones en p53, de forma similar al dVIN convencional (37,38). Dado que estas lesiones se han descrito recientemente, no hay estudios que analicen su relevancia clínica o pronóstica.

Las lesiones premalignas asociadas a VPH con características de dVIN son muy infrecuentes y escasamente descritas en la literatura. En 2018, Rakislova *et al.* identificaron un 1,4% de lesiones con características dVIN o de liquen simple adyacentes a CEV asociado a VPH de forma concluyente (39) ; puesto que un resultado falso positivo en la determinación de VPH podría haber alterado de forma sustancial los resultados de este estudio, los autores definen como tumor asociado a VPH de forma concluyente aquel que presenta positividad para ácido desoxirribonucleico (ADN) y ácido ribonucleico (ARN) de VPH de alto riesgo y p16. Los autores describen morfológicamente las lesiones premalignas asociadas a este infrecuente subgrupo de tumores, caracterizado por una sobreexpresión de p16. Sin embargo, no describen las posibles implicaciones clínicas ni pronósticas de este tipo de lesiones(39).

Así pues, en una proporción de pacientes con lesiones premalignas de vulva, los criterios puramente histológicos no son útiles para el diagnóstico, y, por consiguiente, algunos casos de dVIN con características HSIL-*like* pueden ser incorrectamente diagnosticados y/o infratratados. A la inversa, las pacientes con HSIL vulvar y características dVIN-*like*, probablemente sean tratadas de forma más agresiva de lo que sería necesario (véase apartado 1.7). Desafortunadamente, se desconoce si estas lesiones con características histológicas inusuales y los carcinomas que se originan sobre ellas presentan distinto pronóstico y, por lo tanto, deberían tratarse de forma diferente.

Las lesiones inflamatorias de la vulva (liquen escleroso, el liquen simple crónico y el liquen plano, principalmente), a pesar de no mostrar signos de displasia, se consideran el sustrato para el desarrollo de CEV independiente de VPH. Aunque su prevalencia es elevada en pacientes postmenopáusicas, su etiología y patogénesis es incierta y su potencial oncológico constituye un campo particularmente complejo y poco explorado (40). Mientras que algunos autores reportan

que una proporción significativa de estas lesiones inflamatorias pueden progresar a CEV (41), otros observan lo contrario (42).

1.4. Clasificación del carcinoma escamoso de vulva y sus precursores: determinación del virus del papiloma humano e inmunohistoquímica

Una de las herramientas utilizadas para la distinción entre los carcinomas y las lesiones precursoras asociados o no a VPH son los test moleculares que detectan secuencias de ADN del VPH, generalmente basadas en la reacción en cadena de la polimerasa (PCR), puesto que los criterios puramente histológicos carecen de precisión. Las técnicas basadas en PCR tienen una sensibilidad muy alta, pero la contaminación y la ubicuidad del VPH son causa de resultados falsamente positivos. Además, dado que la detección de VPH en lesiones vulvares habitualmente se realiza en tejidos fijados en formol y parafinados, la fragmentación del ADN secundaria a la fijación del tejido puede causar falsos negativos (10).

Además de la prueba VPH, la detección de p16 se ha usado como biomarcador subrogado de la infección por VPH (43). p16 es una proteína de la célula humana que se encuentra sobreexpresada de forma selectiva en células infectadas por VPH. Se ha propuesto que, para clasificar de manera concluyente un tumor como asociado a VPH, se requiere la detección de ADN de VPH y una tinción inmunohistoquímica positiva para p16, mientras que lo contrario (VPH negativo en pruebas moleculares y tinción inmunohistoquímica negativa para p16) sería el requisito para clasificar una lesión como independiente de VPH. De hecho, en un estudio publicado por nuestro grupo, la mayoría de casos de CEV se incluyeron en una de estas dos categorías (10).

Desafortunadamente, no existe un marcador subrogado para las lesiones independientes de VPH. Sin embargo, el CEV independiente de VPH y las lesiones dVIN muestran a menudo un patrón de expresión anormal para p53 en la inmunohistoquímica, lo que sugiere mutación en el gen *TP53*. Hay estudios que demuestran que la tinción inmunohistoquímica para p53 tiene una buena correlación con el status del gen *TP53* (19,21,22): los patrones de inmunohistoquímica de tipo basal o sobreexpresión difusa (inmunohistoquímica para p53 alterada) corresponden a *TP53*-mutado, y los patrones disperso (*scattered*) y medio-epitelial (inmunohistoquímica para p53

normal) corresponden a *TP53 wild-type* (44,45). Las alteraciones de p53 se presentan en aproximadamente dos tercios de los tumores independientes de VPH (46). Por lo tanto, existe un tercio de CEV independientes de VPH que presentan una expresión normal de p53 (perfil inmunohistoquímico normal o *wild-type)*. Este subgrupo de CEV p16 y p53 negativos presenta un difícil diagnóstico y un comportamiento poco conocido. Así pues, la inmunohistoquímica para p53 presenta limitaciones importantes para diferenciar lesiones malignas y premalignas independientes de VPH de otras lesiones inflamatorias benignas (47). Se necesitan estudios moleculares que identifiquen marcadores subrogados de las lesiones independientes de VPH, permitiendo un diagnóstico más fiable.

1.5. Alteraciones genómicas en el carcinoma escamoso de vulva y sus lesiones precursoras

La carcinogénesis del CEV asociado a VPH se conoce bien, pues tiene muchos puntos en común con la carcinogénesis del cáncer de cuello de útero, que es el tumor por antonomasia asociado a VPH. En cambio, los mecanismos moleculares del CEV independiente de VPH son, en gran parte, desconocidos. Dichos mecanismos parecen ser más complejos que los de la vía de carcinogénesis asociada a VPH (48). Los pocos estudios moleculares publicados incluyen un número pequeño de casos y se centran únicamente en la evaluación de genes implicados en la carcinogénesis de los tumores de cabeza y cuello, mucho más prevalentes (21). Extrapolar esta información al CEV puede se inexacto, ya que se trata de tumores muy diferentes. Las mutaciones más frecuentemente reportadas en CEV incluyen *TP53*, *PTEN* y *CDKN2A*, independientemente del *status* VPH. Los pocos estudios que comparan los perfiles genómicos del CEV asociado e independiente de VPH presentan resultados discordantes. Algunos describen que los CEV independientes de VPH presentan más mutaciones que los asociados a VPH (49), mientras otros encuentran una proporción similar de mutaciones en ambos grupos (21). La mayoría de estudios que sugieren que las mutaciones identificadas con más frecuencia en el CEV independiente de VPH y sus lesiones precursoras son en *TP53*, seguidas por *NOTCH-1*, *PICK3CA*, y *HRAS* (21). Dos

estudios recientes concluyeron que el CEV independiente de VPH con un perfil de *TP53* mutado se asocia a peor pronóstico que el CEV independiente de VPH con perfil *TP53 wild-type* (19,21).

En cuanto a las lesiones precursoras independientes de VPH, la mayoría de estudios se han centrado en evaluar las mutaciones de *TP53*, con resultados variables. Los estudios moleculares más completos demostraron que al menos un tercio de las lesiones premalignas independientes de VPH presentan mutaciones en *TP53* (50). Frecuentemente, se han observado mutaciones en los genes *NOTCH-1, HRAS* y *PICK3CA* (21,50). No obstante, la principal limitación de estos estudios es que se han basado en paneles de genes evaluados en los carcinomas de cabeza y cuello. Son escasos los estudios que exploran los perfiles moleculares de las lesiones inflamatorias de la vulva asociadas a lesiones premalignas y carcinoma invasor (51).

1.6. Presentación y conducta clínica en las lesiones precursoras de carcinoma escamoso de vulva

La presentación clínica de las lesiones premalignas de la vulva (dVIN y HSIL vulvar) es muy heterogénea. El 50% de las pacientes son asintomáticas, y el diagnóstico suele realizarse de forma incidental en un examen ginecológico rutinario, remarcando la importancia de realizar una exploración vulvar sistemática. El síntoma más frecuente del dVIN y el HSIL vulvar es el prurito vulvar seguido por dolor, escozor, dispareunia o disuria (52,53).

Las lesiones HSIL presentan una apariencia macroscópica variable, suelen ser sobreelevadas o papilomatosas y pigmentadas (13). Clínicamente, las lesiones dVIN son difíciles de reconocer, y se presentan en forma de placas blanquecinas mal definidas, típicamente menos voluminosas que las lesiones tipo HSIL (13). Estas lesiones se suelen asociar a procesos dermatológicos inflamatorios, principalmente el liquen escleroso y el liquen simple crónico, y pueden tener manifestaciones clínicas como la irritación local o el dolor (13).

El diagnóstico de confirmación de las lesiones premalignas de la vulva es histológico. Habitualmente la toma de biopsia en estos casos se realiza en la consulta con anestesia local (53,54). En el caso de las lesiones inflamatorias de la vulva, como el liquen plano o escleroso, no

es necesario realizar confirmación diagnóstica histológica sistemática a menos que existan dudas diagnósticas, sospecha de lesión invasiva o preinvasiva, falta de respuesta al tratamiento tópico, o en caso de antecedente de patología maligna o premaligna vulvar o en otras áreas de tracto anogenital (53).

El tratamiento de las lesiones inflamatorias de la vulva (liquen plano y liquen escleroso) consiste en la aplicación de corticoides tópicos de potencia alta. En casos muy evolucionados de liquen escleroso con distorsión anatómica de los genitales externos y secuelas asociadas a la fibrosis y esclerosis, se puede plantear un tratamiento quirúrgico, para restaurar la funcionalidad y anatomía del área genital. El tratamiento de segunda línea del liquen plano, consiste en uso de corticoides sistémicos, antipalúdicos o fármacos inmunosupresores (55). El tratamiento de elección del dVIN es la escisión, principalmente por el riesgo asociado de enfermedad microinvasiva oculta y el mayor riesgo de progresión a CEV. El tratamiento de HSIL puede realizarse con procedimientos escisionales, o en caso que se disponga de biopsias previas y bajo riesgo de invasión oculta, también puede realizarse con terapias destructivas (escisión, vaporización láser CO_2) o tópicas (imiquimod, 5-fluorouracilo). Es importante destacar que en general estas lesiones presentan un elevado porcentaje de recurrencias y riesgo de progresión a lesiones preinvasivas, por lo que es esencial un seguimiento estrecho de las pacientes tratadas (53).

1.7. Presentación clínica, tratamiento y pronóstico del cáncer escamoso de vulva

El principal motivo de consulta de las pacientes con cáncer de vulva es la presencia de una lesión ulcerada en genitales externos, unifocal o mulltifocal, en ocasiones asociada a sangrado o a secreción serosa o purulenta. Es frecuente la historia previa de molestias o prurito vulvar crónico (56). A la exploración física, es importante delimitar correctamente la extensión de la lesión, evaluando la posible afectación de la vagina, uretra o ano, así como la presencia y extensión de lesión premaligna adyacente (56). En estas pacientes, es frecuente diagnosticar la presencia de lesiones inflamatorias asociadas (41,56). También es importante examinar el área inguinal para

descartar la presencia de adenopatías patológicas. El diagnóstico de confirmación del cáncer de vulva es histológico, por lo que debe realizarse una biopsia con anestésico local (56).

En las pacientes con cáncer de vulva, es necesario realizar un estudio de extensión mediante resonancia magnética, ecografía inguinal para evaluar la presencia de metástasis. En caso de objetivar (en la exploración física o por pruebas de imagen) ganglios inguinales sospechosos debe realizarse una biopsia dirigida. Si existe una sospecha clínica o radiológica (o patológica) de enfermedad ganglionar o a distancia, se recomienda realizar una tomografía toracoabdominal o tomografía por emisión de positrones (6). También se recomienda una exploración del cuello del útero, vagina y ano, especialmente en pacientes con patología asociada a VPH (6).

La clasificación TNM y de la FIGO (International Federation of Gynecology and Obstetrics), permiten clasificar a las pacientes con cáncer de vulva en base al tamaño de la lesión y la presencia o no de metástasis ganglionares o a distancia. En 2021, se publicó la última clasificación de la FIGO del cáncer de vulva, basada en el análisis de supervivencia de 12.063 pacientes con cáncer de vulva (figura 2, tabla 1) (57). Las principales características de esta nueva clasificación son:

1. Recomienda describir la afectación ganglionar de forma análoga a la utilizada en cáncer de cuello de útero, distinguiendo entre micrometástasis y macrometástasis (las células tumorales aisladas no contabilizan como metástasis ganglionares). Además del tamaño de la metástasis ganglionar, se contempla la presencia de invasión extracapsular.
2. Indica el uso de técnicas de imagen para la estadificación, de forma similar a la clasificación del cáncer de cuello de útero.
3. Es aplicable a todos los tipos morfológicos de cáncer de vulva, no solo el cáncer escamoso (excluye únicamente al melanoma de vulva).
4. Recomienda documentar la asociación o no a VPH del cáncer de vulva, basada en el resultado de la inmunohistoquímica para p16 y/o de los test moleculares para VPH (57).

El tratamiento principal del CEV es quirúrgico. Se recomienda la escisión completa de la lesión con márgenes libres (márgenes clínicos de 1 cm, aceptándose márgenes más estrechos en las áreas en las que el tumor es próximo a las estructuras de la línea media como clítoris, uretra u ano, con

el objetivo de preservar su función). Es importante incluir las lesiones sugestivas de preinvasión dentro del área de resección (pudiendo realizarse una resección más superficial a este nivel). Si es necesario, se pueden emplear técnicas reconstructivas para facilitar el cierre primario de la herida. En caso de presentar márgenes quirúrgicos en contacto con la lesión, se recomienda realizar una ampliación de márgenes como tratamiento de elección (siendo la radioterapia la segunda opción de tratamiento) (6,7).

En las pacientes con tumores en estadio FIGO mayor a IA, se recomienda realizar una estadificación ganglionar inguinal. Los tumores unifocales menores de 4 cm, sin ganglios sospechosos en el estudio de extensión, se pueden estadificar mediante la técnica de la biopsia selectiva del ganglio centinela, que se deberá realizar de forma uni o bilateral según la distancia del borde medial del tumor a la línea media (mayor o menor de 1 cm). En caso de ganglio centinela positivo o imposibilidad de idenificación del ganglio centinela, se debe realizar una linfadenectomía inguinofemoral homolateral. En el caso de tumores multifocales o mayores o iguales de 4 centímetros, se recomienda realizar una linfadenectomía inguinofemoral de los ganglios superficiales y profundos (unilateral si el margen medial dista más de 1 cm de la línea media o bilateral en caso contrario). Se puede valorar la realización de una linfadenectomía inguinofemoral contralateral en presencia de metástasis ganglionares ipsilaterales (6,7).

El tratamiento adyuvante con radioterapia está indicado en los siguientes casos: 1) presencia de márgenes quirúrgicos afectos, e imposibilidad de ampliación quirúrgica de los mismos; 2) se puede valorar la adyuvancia con radioterapia en casos de margen quirúrgico próximo al tumor, aunque no existe consenso sobre la distancia mínima al margen para su indicación; 3) presencia más de un ganglio inguinofemoral metastásico (radioterapia adyuvante sobre la ingle, se puede valorar añadir tratamiento quimioterápico sensibilizante) (6,7). Las pacientes con enfermedad avanzada no resecable se tratan con quimioradioterapia, que en algunas ocasiones puede ser neoadyuvante para realizar posteriormente tratamiento quirúrgico con el objetivo de evitar una cirugía exenterativa de entrada (6) .

Las pacientes con cáncer de vulva presentan una supervivencia a 5 años superior al 60-70% en estadios iniciales (I-II), que disminuye ostensiblemente ante la presencia de enfermedad metastásica ganglionar (30-60%) o a distancia (<25%). Tanto en pacientes con enfermedad en

estadio inicial o con metástasis ganglionares, la mayoría de recidivas son locales o ganglionares. La tasa de recurrencia local es del 24-36% en pacientes sin metástasis ganglionar y del 36-46% en pacientes con metástasis ganglionar. La recidiva ganglionar aislada ocurre en menos de un 3% de pacientes con ganglios negativos al diagnóstico y en un 8% de pacientes con ganglios positivos al diagnóstico (57,58). Los principales factores pronósticos descritos en el CEV son la presencia de metástasis ganglionares, el número y extensión de las mismas, el estadio FIGO, la presencia de invasión linfovascular, el tamaño tumoral inicial y la edad de la paciente (57–63).

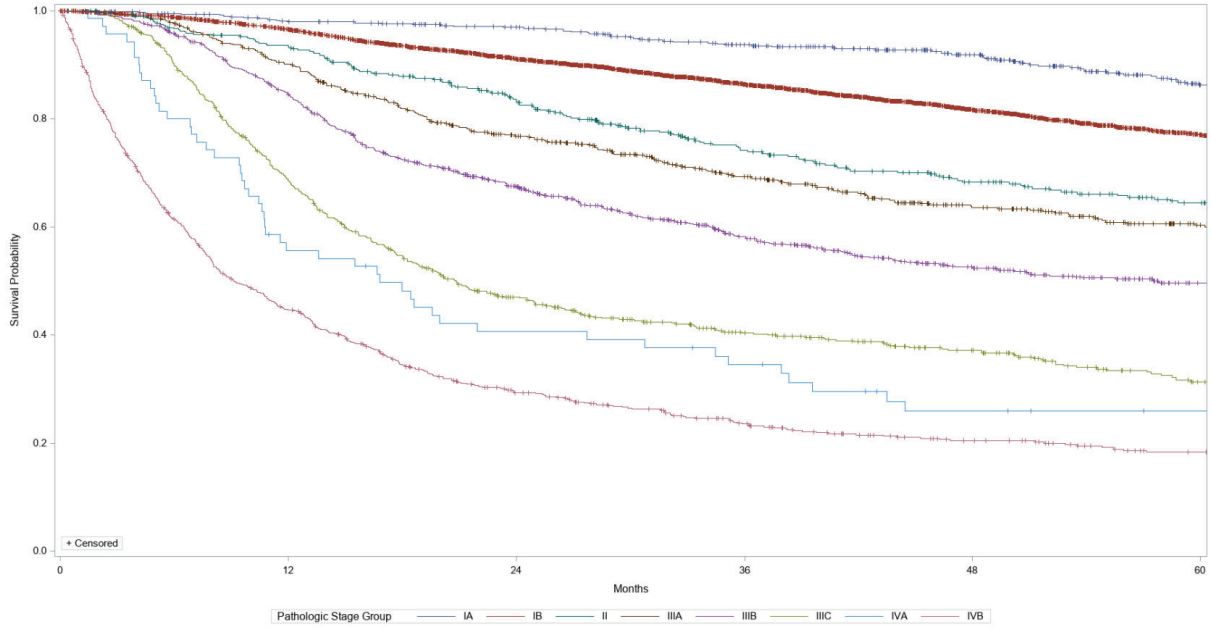

Figura 2. Curva de supervivencia de las pacientes con cáncer de vulva incluidas en el desarrollo de la nueva clasificación de la FIGO 2021

Olawaiye A et al. Int J Gynecol Obstet. 2021;155(1):43–7 (57)

Tabla 1. Clasificación 2021 de la FIGO del cáncer de vulva

I Tumor confinado a la vulva

IA Tamaño tumoral ≤2 cm e invasión estromal ≤1mm[a]

IB Tamaño tumoral >2 cm o invasión estromal >1mm[a]

II Tumor de cualquier medida con extensión al tercio externo de la uretra, tercio externo de la vagina o tercio externo del ano, con ganglios linfáticos negativos

III Tumor de cualquier medida con extensión a la parte superior de las estructuras perineales adyacentes o con cualquier número de ganglios linfáticos afectos, no fijos y no ulcerados

IIIA Tumor de cualquier medida con extensión a los dos tercios superiores de la uretra, a los dos tercios superiores de la vagina, mucosa vesical, mucosa rectal o metástasis ganglionar regional [b] ≤5mm

IIIB Metástasis ganglionar regional [b] > 5mm

IIIC Metástasis ganglionar regional [b] con extensión extracapsular

IV Tumor de cualquier medida fijo al hueso de la pelvis, o con metástasis ganglionares ulceradas o fijas, o metástasis a distancia

IVA Enfermedad fija al hueso de la pelvis, o presencia metástasis ganglionares regionales [b] ulceradas o fijas

IVB Metástasis a distancia

[a] La profundidad de invasión se mide desde la membrana basal de la cresta papilar más profunda libre de tumor al punto de invasión más profundo

[b] Ganglios linfáticos inguinales y/o femorales

Adaptado de Olawaiye A et al. Int J Gynecol Obstet. 2021;155(1):43–7 (57)

1.8. Artículo preliminar: revisión sobre el panorama molecular del carcinoma escamoso de vulva

Con el objetivo de contextualizar a nivel genómico y molecular los dos trabajos de investigación original que conforman la presente tesis doctoral, se realizó una revisión de la literatura sobre el panorama molecular del CEV, que se adjunta a continuación.

AUTORES: **Carreras-Dieguez N**, Guerrero J, Rodrigo-Calvo MT, Ribera-Cortada I, Trias I, Jares P, López del Campo R, Saco A, Munmany M, Marimon L, Ferrando M, Vega N, del Pino M, Torné A, Ordi J, Rakislova N

TÍTULO: **Molecular Landscape of Vulvar Squamous Cell Carcinoma**

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RESUMEN:

Objetivos: El carcinoma escamoso de vulva (CEV) es un tumor infrecuente con dos vías de carcinogénesis descritas (asociada e independiente del virus del papiloma humano [VPH]), cuyo panorama molecular está poco explorado. El objetivo de este estudio es realizar una revisión de la literatura sobre los marcadores moleculares asociados a esta neoplasia.

Metodología: en enero de 2021 realizamos una búsqueda exhaustiva de la literatura usando las plataformas Pubmed Medline y Scopus para identificar publicaciones sobre el perfil molecular del

CEV. Se consideraron elegibles los estudios observacionales, prospectivos y retrospectivos, que evaluaran alteraciones moleculares en el CEV.

Resultados: se incluyeron 14 estudios, con un total de 749 muestras de CEV. Los estudios incluídos son metodológicamente muy heterogéneos en cuanto a estrategias de secuenciación y de determinación del VPH, el tamaño muestral es limitado, así como el número de genes estudiados, y la mayoría no disponen de análisis de supervivencia. Las alteraciones genéticas identificadas son variables entre las series. *TP53* y *CDKN2A* son los genes en los que se han identificado más alteraciones, seguidos por *PIK3CA, HRAS* y *PTEN*. Se han identificado de forma recurrente alteraciones en la vía de *PI3K/AKT/mTOR*. Sin embargo, la heterogeneidad de los estudios no permite extraer conclusiones robustas en cuanto a alteraciones moleculares o vías de carcinogénesis claramente implicadas en el desarrollo del CEV.

Conclusiones: existen pocos estudios sobre las alteraciones moleculares implicadas en la carcniogénesis del CEV, muy heterogéneos y con un tamaño muestral limitado. Ello impide tener una visión completa de las vías de carcinogénesis de este tumor y de su panorama molecular. Se necesitan estudios a gran escala que permitan extraer conclusiones robustas sobre las alteraciones moleculares implicadas en la carcinogénesis del CEV.

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Review

Molecular Landscape of Vulvar Squamous Cell Carcinoma

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Abstract: Vulvar squamous cell carcinoma (VSCC) is a rare malignancy with dual pathogenesis, Human papillomavirus (HPV)-associated and HPV-independent, with a poorly explored molecular landscape. We aimed to summarize the findings of the series analyzing molecular hallmarks of this neoplasm. In January 2021, we conducted a comprehensive literature search using Pubmed Medline and Scopus to identify publications focused on genomic profiling of VSCC. Observational studies, including both prospective and retrospective designs, evaluating molecular alterations in VSCC were deemed eligible. A total of 14 studies analyzing 749 VSCC were identified. The study series were heterogeneous in HPV testing and sequencing strategies, included small sets of tumors and cancer genes, and commonly lacked survival analysis. Only one extensive targeted next-generation sequencing-based study comprised a large cohort of 280 VSCC. The mutated genes, their number, and frequencies were highly variable between the series. Overall, *TP53* and *CDKN2A*, followed by *PIK3CA*, *HRAS*, and *PTEN*, were the most frequently studied and mutated genes. Mutations involved in the PI3K/AKT/mTOR pathway, including *TP53*, *HRAS*, *KRAS*, and *PIK3CA*, have been consistently reported across the studies. However, the role of individual mutations or pathways in the development of VSCC remains unclear. In conclusion, heterogeneity and the small sample size of available molecular series contribute to a limited view of the molecular landscape of VSCC. Large-scale genome- or exome-wide studies with robust HPV testing are necessary to improve the molecular characterization of VSCC.

Keywords: vulvar cancer; vulvar squamous cell carcinoma; molecular analysis; genomic landscape; next generation sequencing; whole-exome sequencing

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1. Introduction

Vulvar squamous cell carcinoma (VSCC) is an uncommon malignancy of the lower genital tract generally regarded as a disease in older women [1]. However, some epidemiological indicators suggest a rising incidence of this tumor in young women, which added to the increasing life expectancy, will likely cause an increase in the rates of this disease in the future [2]. In the last decade of the 20th century, it became clear that there are two different etiopathogenic pathways leading to VSCC: one associated with human papillomavirus

(HPV) and a second carcinogenic pathway independent of HPV infection [3]. A number of studies have provided evidence showing that HPV-associated and HPV-independent VSCC have different clinico-pathological features and natural history [4]. These etiopathogenic and clinical differences between HPV-dependent and HPV-independent tumors have also been seen in other types of tumors that have been studied more than VSCC, such as head and neck carcinomas [5,6]. The geographical distribution of these two types of VSCC is also different [7]: in high-income countries, most VSCC developed through the HPV-independent route [4] and affected mostly post-menopausal women [8], whereas in low- and middle-income countries HPV-associated VSCC were more common [7] and involved younger patients [8].

Classically, VSCCs had been classified according to their morphological features. All previous VSCC classifications included several histological types, namely basaloid, warty, keratinizing and non-keratinizing subtypes, as well as other infrequent variants. The main drawback of these morphology-based classifications was the complete lack of prognostic implications [9]. The publication of the new classification from the World Health Organization (WHO) in September 2020 [10] has resulted in a major conceptual shift in the categorization of VSCC (and also of vaginal and cervical tumors), as for the first time it gives priority to a molecular attribute—i.e., the HPV status—rather than to the histological features. In this new WHO classification VSCC are divided into two major types, HPV-associated and HPV-independent [10]. Increasing evidence indicating that HPV-associated VSCCs have a better prognosis than HPV-independent tumors [5,6] was the rationale leading to this major change in the classification. Nevertheless, despite the clear etiological and clinical differences between these two major types of VSCC, the management of patients with HPV-associated and HPV-independent VSCC remains the same.

There is strong evidence indicating that p16 immunohistochemistry (IHC) can be used as a surrogate marker to establish HPV status in VSCC [11]. Although not perfect, p16 IHC seems to be more reliable than HPV testing, a method that has shown some limitations [9,11–13]. The carcinogenic pathways of HPV-associated VSCC are similar to the carcinogenesis of cervical carcinoma, the model for HPV-associated tumors [14]. Most of these HPV-associated tumors arise in an intraepithelial precursor histologically similar to the cervical precursor and is called a high-grade squamous intraepithelial lesion [15]. However, its mutational landscape is not completely understood. Alternatively, the molecular mechanisms leading to HPV-independent VSCC remain unclear and complex. Inflammatory dermatoses, including lichen sclerosus and lichen simplex chronicus, are considered the main etiologic drivers [16]. An intraepithelial precursor, called a differentiated vulvar intraepithelial neoplasia (dVIN), is frequently identified in the adjacent skin and is thought to precede most HPV-independent VSCC [17]. Mutations in *TP53* have been identified in a significant proportion of these tumors [3]. Recently, a different subset of HPV-independent precursors has been described, namely, differentiated exophytic vulvar intraepithelial lesions (DEVIL) [18], and vulvar acanthosis with altered differentiation (VAAD) [19], which seem to be associated with a particular subset of p53 wild type HPV-independent VSCC; these tumors are frequently classified morphologically as verrucous carcinomas.

Recent advances in next-generation sequencing (NGS) are giving rise to an unprecedented characterization of cancer genomes [20,21]. NGS studies are commonly focused on somatic mutations and copy number variations, major players in cancer development. Molecular research in cancer remains challenging and progress is far more evident in prevalent malignancies, such as breast or lung cancers, than in rare malignancies such as vulvar or penile cancer.

The mutational landscape of VSCC has been poorly investigated over the past three decades. The vast majority of the research on VSCC has focused mainly on the mutations of the tumor suppressor gene *TP53* [22–24] and those genes known to be relevant in head and neck cancer [25]. In contrast, large-scale whole-genome- or whole-exome sequencing studies in VSCC have been absent in the past few decades. Thus, knowledge on the molecular hallmarks of HPV-associated and HPV-independent VSCC is limited to date.

Knowledge on recurrent mutations in VSCC will certainly open doors to better prognostic stratification and the identification of new targets for therapy. Herein, we aimed to review the existing molecular-based study series on VSCC, provide an overview of the available genomic data, and present challenges in the molecular characterization of VSCC.

2. Methodology

In January 2021, we conducted a comprehensive literature search using Pubmed Medline and Scopus to identify publications focused on genomic profiling of VSCC. We used the terms "vulva", "cancer", "carcinoma", "molecular", "genomic", and "mutation". Observational studies, including both prospective and retrospective designs, evaluating molecular alterations in VSCC were deemed eligible. Reviews, meta-analyses, and letters to editors, as well as publications in languages other than English, were excluded. Reference lists from initially selected studies and from reviews were searched to identify additional relevant studies. Selected articles were additionally cross-referenced. Studies in which data on the frequency of the mutated genes were not specifically reported were excluded. Additional exclusion criteria involved articles focusing on non-squamous cell neoplasms, those analyzing only chromosome arm-level alterations, and those not specifically focused on the VSCC molecular landscape.

Study selection was based on the content of the abstract. Two reviewers (NR and NC) independently evaluated the papers. Studies focused on genomic alterations in VSCC were selected. The full text of the articles was, then, reviewed to ensure they met the eligibility criteria. Discrepancies between reviewers were resolved by consultation with a third author (JO) if no agreement could be reached.

The data extracted from the selected articles included the number of VSCC analyzed, the type of HPV testing, HPV prevalence, DNA sequencing technique, the gene panel used (in case of targeted NGS), and the number and frequency of identified mutations.

3. Results

The literature review initially identified 1760 studies following the publication screening workflow described in Figure 1, and from these 886 were excluded (duplicates, book chapters, unavailable full text, and languages other than English). Among the remaining 874 titles and abstracts that were screened, 33 full-text articles were finally assessed for eligibility. Of these, 22 articles that did not meet the selection criteria were excluded, leaving 11 full-text articles, and after reviewing the references, 3 additional articles [26–28] were identified.

A total of 14 studies, which explored the somatic and/or copy number mutational landscape in 749 VSCC samples from 738 patients were finally selected. The publication years ranged from 2005 to 2020. Most of the studies (n = 12; 86%) were published between 2017 to 2020 and more than one-third of them (n = 6; 43%) were released during the first COVID-19 pandemic year (2020). Seven studies (50%) were conducted in North America, six (43%) in Europe, and one (7%) in Asia. Figure 2 shows the geographical distribution of the included studies.

Eight series (57%) were based exclusively on VSCC, whereas six (43%) included both VSCC and premalignant lesions. One study evaluated the molecular profiles in primary and metastatic VSCC in a subset of cases [29]. Eight studies (57%) analyzed only somatic mutations [25,28,30–35], two studies (14%) focused only on copy number alterations [36,37], and four (28%) included both somatic mutation profiling and analysis of copy number alterations [27,29,38,39]. Twelve studies (86%) applied NGS. Nine of the twelve (75%) NGS-based studies used targeted panels, two (17%) performed whole-exome sequencing [31,39], and one (7%) whole-genome (shallow) sequencing [37]. Both whole-exome sequencing studies included analysis of copy number alterations and one [39] additionally included the analysis of mutational signatures. Of the nine studies with targeted NGS panels, five (55%) used commercial panels, two (22%) customized panels, and two studies (22%) have

not specified the panel type. Table 1 shows the main characteristics of the selected study series focused on the genomic alterations in VSCC.

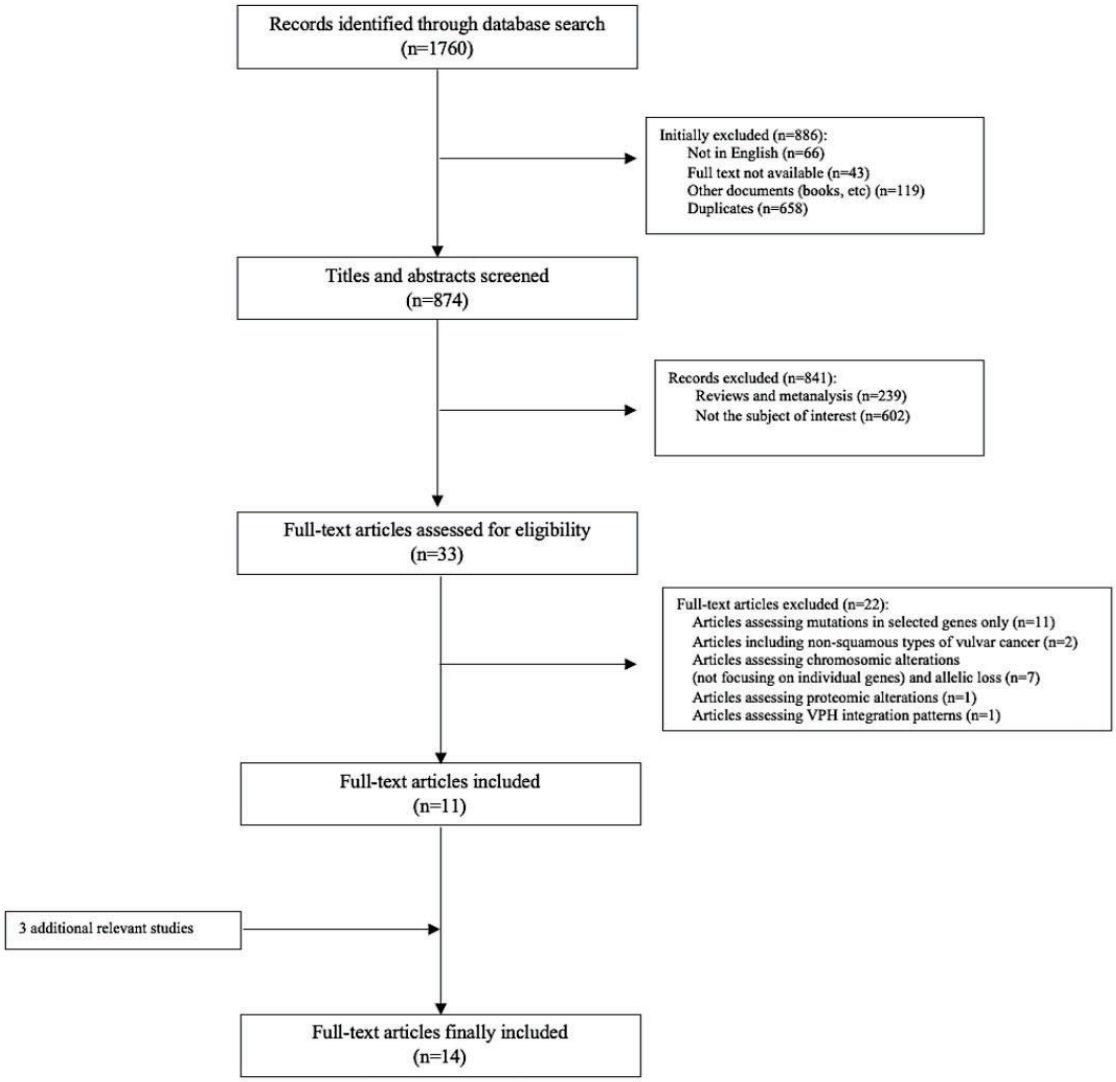

Figure 1. Flow diagram of publication screening and identification.

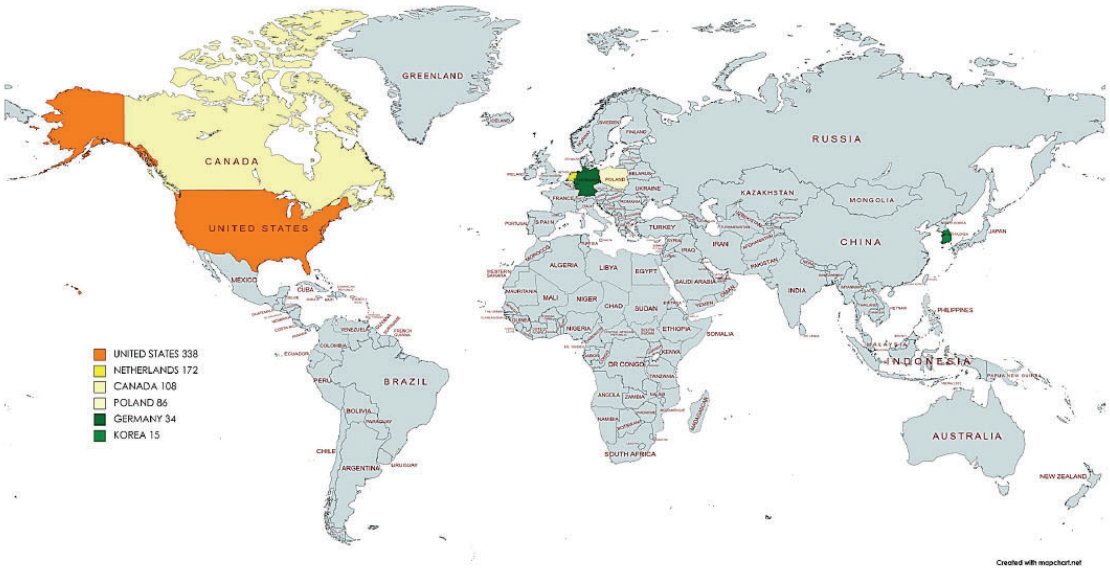

Figure 2. Geographical distribution and numbers of vulvar squamous cell carcinomas (VSCC) analyzed in the included studies by country.

Table 1. Main characteristics of the studies analyzing the genomic alterations in vulvar squamous cell carcinomas (VSCC).

	Source	Research Paper	Year	Country	Type of Sample	Type of Genomic Analysis	Gene Panels	Number of Targeted Genes	N (VSCC Samples)	HPV Prevalence	Most Frequent Individual Gene Alteration	Differences in Overall Mutational Frequency by HPV Status	Genes More Altered in HPV+ VSCC	Genes More Altered in HPV-VSCC
1	[36]	Kunjoonju et al.	2005	USA	Cell lines	MLPA	Customized	122	13	ND	*TMSB10* (92%), *CCND1* (83%), *IL12A* (67%), *CTNNB1* (67%), *BCL2* (58%)	-	N/A	N/A
2	[31]	Trietsh et al.	2014	The Nether-lands	FFPE blocks	Sanger Mass spectometry	GynCarta 2.0 panel	13	107	16.2%	*TP53* (54%), *CDKN2A* (13%), *HRAS* (9%), *PIK3CA* (7%), *PPPR1A* (3%), *KRAS* (1%), *PTEN* (1%)	- (not analyzed)	-	*TP53, CDKN2A, HRAS, PIK3CA, KRAS*
3	[25]	Nooij et al.	2017	The Nether-lands	FFPE blocks	Targeted NGS	Customized	17	36	22.2%	*TP53* (58%), *NOTCH1* (33%), *HRAS* (29%), *KMT2D* (11%)	Yes (higher in HPV-VSCC)	-	*TP53, NOTCH-1, HRAS*
4	[28]	Weberpals et al.	2017	Canada	FFPE blocks	Targeted NGS	Ion AmpliSeq Cancer Hotspot v2 Panel	50	43	51.2%	*TP53* (35%), *PIK3CA* (23%), *HRAS* (14%), *KIT, CDKN2A* (12%), *FGFR3* (9%)	No	*FGFR3*	*TP53, PIK3CA, HRAS, CDKN2A*
5	[27]	Watkins et al.	2017	USA	FFPE blocks	Targeted NGS	N/S	300	14	0.0%	*TP53* (79%), *CDKN2A* (36%), *PIK3CA* (14%), *KMT2D* (14%), *CCND1* (14%), *EGFR* (7%)	N/A	N/A	N/A
6	[39]	Han et al.	2018	Korea	FFPE blocks (n = 14 cases) Frozen tissue (n = 1)	NGS	N/A	Whole exome	15	40.0%	*TP53* (33%), *FAT1* (27%), *APC* (20%), *CASP8* (20%), *PIK3CA* (13%), *FBXW7* (13%), *NOTCH-1* (13%), *BRCA2* (13%), *EP300* (13%)	Yes (higher in HPV- VSCC)	*PIK3CA, FBXW7,*	*TP53, CDKN2A, HRAS, FAT1, APC*
7	[37]	Swarts et al.	2018	The Nether-lands	FFPE blocks	NGS	N/A	Whole genome shallow sequencing	24	45.8%	*TP63* (46%), *JAG1* (33%), *CD44* (54%), *MET* (77%), *DSC/DSG* (38%), *RBFOX1* (38%)	No	*TP63* gains	*CCND1* amplifications
8	[30]	Zieba et al.	2018	Poland	Frozen samples Celll lines	Targeted NGS	Ion AmpliSeq Cancer Hotspot v2 Panel	50	81	64.0%	*TP53* (44%), *CDKN2A* (23%), *PIK3CA* (9%), *FBXW7* (6%), *HRAS* (6%),	No	*AKT1, FGFR3, SMAD4, JAK3*	*FLT3, GNAQ*
9	[34]	Zieba et al.	2020	Poland / The Nether-lands	FFPE blocks	Targeted NGS	Ion AmpliSeq Cancer Hotspot v2 Panel	50	10	40.0%	*TP53* (70%), *CDKN2A* (30%)	No	-	-
10	[35]	Tessier-Cloutier et al.	2020	Canada	FFPE blocks	Targeted NGS	N/S	33	33	6.1%	*TP53* (72%), *PIK3CA* (34%), *HRAS* (28%), *PTEN* (6%), *MET* (9%), *BRAF* (3%)	No	-	-

Table 1. *Cont.*

	Source	Research Paper	Year	Country	Type of Sample	Type of Genomic Analysis	Gene Panels	Number of Targeted Genes	N (VSCC Samples)	HPV Prevalence	Most Frequent Individual Gene Alteration	Differences in Overall Mutational Frequency by HPV Status	Genes More Altered in HPV+ VSCC	Genes More Altered in HPV-VSCC
11	[38]	Prieske et al.	2020	Germany	Frozen tissue	NGS	N/A	Whole exome	34	35.3%	*TP53* (56%), *MUC4* (71%), *TTN* (29%), *ZNF717* (29%), *PIK3CA* (11%), *KMT2D* (11%), *SYNE2* (15%), *SYNE1* (13%), *FBXW7* (9%), *NSD1*, *NBPF1* (21%), *CDKN2A* (6%)	Only for CNA	20q gains	*TP53* 11q gains
12	[33]	Williams et al.	2020	USA	Blood FFPE blocks	Targeted NGS (CGP)	Foundation One platform	406	280	36.3%	*TP53* (55%), *CDKN2A* (36%), *TERTp* (49%), *EGFR* (9%), *PIK3CA* (22%), *CCND1* (15%), *NOTCH-1* (14%), *CDKN2A* (36%), *PTEN* (6%), *FBXW7* (6%)	No	*PIK3CA*, *PTEN*, *EP300*, *STK11*, *AR*, *FBXW7*, *KMT2D*, *BAP1*	*TP53*, *TERTp*, *CDKN2A*, *CCND1*, *FAT1*, *NOTCH1*, *EGFR* *PDL-1/PDL-2*
13	[32]	Pors et al.	2021	Canada	FFPE blocks	Targeted NGS	Customized	33	33	0.0%	*TP53* (645), *HRAS* (6%), *PIK3CA* (6%), *PTEN* (3%), *GNAS* (3%) *EGFR* (3%)	N/A	N/A	N/A
14	[29]	Xing et al.	2020	USA	FFPE blocks	Targeted NGS (CGP)	Ion ampliseq Oncomine Comprehen-sive v.2	143	42 *	37.5%	*TP53* (62%), *CDKN2A* (27%), *PIK3CA* (15%), *HRAS* (8%), *NOTCH-1* (8%)	(not analyzed)	*PIK3CA*	*TP53*, *CDKN2A*, *HRAS*, *NOTCH-1*, *BIRC3* amplifications

CNA; copy number alterations; FFPE: formalin-fixed paraffin-embedded; HPV: human papillomavirus; NGS: Next-generation sequencing. WES: whole-exome sequencing; CGP: comprehensive genomic profiling; N/A: not applicable; ND: not determined; N/S: not specified; MLPA: multiplex ligation-dependent probe amplification assay; USA: United States of America; * only data from 26 VSCC was available to be included this review.

The two whole-exome sequencing cohorts included VSCC matched with normal tissue, with the largest series comprised of 34 VSCC [38]. The study with the largest sample size [33] explored a total of 406 cancer-related genes in 280 VSCC samples using NGS-based hybrid capture genome profiling and analysis of mutational signatures.

HPV analysis was conducted in 13 out of the 14 studies (93%). Of the 14 studies, 9 (69%) used PCR HPV testing: unspecified PCR (3), SPF-10 (2), Amplisense HPV PCR (2), short PCR fragment L1 (1), and HPV risk assay (1). One study (8%) used an NGS-based approach, one study (8%) used HPV in situ hybridization (RNA scope), one study (8%) used only p16, and in one study (8%) HPV testing was not detailed. Of the 9 studies with HPV PCR testing, 6 studies (67%) additionally performed p16 IHC. Neither the whole-genome sequencing nor the largest NGS cohort included p16 IHC. The proportion of HPV-associated VSCC ranged between 0% [27] and 64% [30], and 9 out of the 14 studies (64%) with available HPV data compared molecular abnormalities between HPV-associated and HPV-independent VSCC.

The prognostic implications of the molecular alterations identified in VSCC were evaluated in 7 out of 14 articles (50%). Neither the two whole-exome sequencing studies nor the largest targeted NGS-based study included follow-up data. The total number of cases analyzed, the frequencies of the alterations in each individual gene, and the number of papers in which each particular gene have been evaluated are shown in Table 2.

3.1. Most Frequently Analyzed and Detected Somatic Mutations in VSCC

Mutations in *TP53*, *CDKN2A*, *PIK3CA*, and *HRAS* were the most commonly analyzed and detected abnormalities. *TP53* has been assessed in 12 studies and alterations have been identified in 54% (387/712; range 33–79%) of analyzed samples. *PIK3CA* mutations have been assessed in 12 studies and the overall frequency of the mutation of this gene is 16% (112/712; range 0–34%). *HRAS* and *CDKN2A* mutations have been screened in 11 and 9 studies, respectively, and abnormalities have been identified in 9% (60/678; range 0–28%) and 26% (156/610; range 6–36%) of cases, respectively.

The most frequent (but not the most studied) somatic mutations were identified in *MUC4* (24/34; 71%), followed by *CD44* (13/24; 54%). Each of them was analyzed in a single study, in a different whole-exome-based series [36,38].

3.2. Genomic Differences Based on HPV Status

Among the ten studies that have compared molecular abnormalities based on HPV status, two (20%) [25,39] showed that the mutational load was significantly higher in HPV-associated VSCC. However, one of the whole-exome sequencing cohorts has not identified mutational load differences [38], and two studies, including the largest NGS cohort [33], have not identified differences in terms of mutational load between the two major types of VSCC, but have shown qualitative differences in the mutational profile between HPV-associated and HPV-independent VSCC.

The largest targeted NGS study showed that HPV-associated VSCC harbor alterations in the PI3K/mTOR pathway (*PIK3CA*, *PTEN*, *STK11*, *FBXW7*, and *SOX2*), whereas HPV-independent VSCC showed more frequent mutations in *TP53*, *TERT*, *CDKN2A*, and *CCND1*, as well as amplifications in *EGFR* and *PD-L1*. The same study estimated that at least half of the HPV-associated VSCC have a potentially targetable alteration in the PI3K/mTOR pathway [33].

Three series [28,33,38] have identified statistical differences in *TP53* alterations depending on the HPV status and four [25,29,31,39] have shown a tendency in *TP53* enrichment in HPV-independent VSCC, often combined with *CDKN2A* alterations. Two studies, both conducted by the same group [30,34], have not shown any differences for *TP53* or *CDKN2A* mutations based on HPV status.

Table 2. Frequencies of identified alterations in individual genes stratified by the number of articles that performed molecular analyses in vulvar squamous cell carcinomas (VSCC).

Gene	Number of VSCC with the Gene Alteration	Number of VSCC Assessed	Overall Frequency	Frequency Range	Number of Articles
TP53	387	712	54.4%	33–79%	12
PIK3CA	112	712	15.7%	0–34%	12
HRAS	60	678	8.8%	0–28%	11
CDKN2A	156	610	25.6%	6–36%	9
PTEN	26	647	4.0%	0–6%	9
CTNNB1	12	326	3.7%	0–67%	8
EGFR	33	456	7.2%	0–29%	7
KRAS	10	589	1.7%	0–23%	7
NOTCH1	57	482	11.8%	0–33%	7
FBXW7	27	434	6.2%	0–13%	6
FGFR3	11	474	2.3%	0–9%	6
RB1	13	446	2.9%	0–7%	6
STK11	19	452	4.2%	0–7%	6
ATM	10	164	6.1%	0-67%	5
BRAF	1	267	0.4%	0–3.3%	5
CCND1	63	365	17.3%	0–83%	5
ERBB2	7	437	1.6%	0–3%	5
ERBB4	8	173	4.6%	0–50%	5
MET	14	191	7.3%	0–9%	5
RET	2	171	1.2%	0-7%	5
B2M	6	26	23.1%	0–50%	2
BCL2	14	36	38.9%	29–58%	2
PRKDC	7	27	25.9%	0–58%	2
BIRC2	6	13	46.1%	NA	1
CASP1	5	13	38.5%	NA	1
CASP6	6	13	46.1%	NA	1
CD44	13	24	54.2%	NA	1
CREBBP	3	15	20.0%	NA	1
DSC	9	24	37.5%	NA	1
EMS1	4	13	30.8%	NA	1
HIF1A	7	24	29.2%	NA	1
IL6	6	13	46.1%	NA	1
ILI2A	7	13	61.5%	NA	1
JAG1	8	24	33.3%	NA	1
MUC4	24	34	70.6%	NA	1
NBPF1	7	34	20.6%	NA	1
NCOA3	5	13	38.5%	NA	1
NKFB1	5	13	38.5%	NA	1
NRG1	3	15	20.0%	NA	1
PRFKDC	6	13	46.1%	NA	1
PTPRD	5	24	20.8%	NA	1
RBFOX1	8	24	33.3%	NA	1
RBFOX3	7	24	29.2%	NA	1
TERTp	136	280	48.6%	NA	1
THBS1	5	13	38.5%	NA	1
TMSB10	11	13	84.6%	NA	1
TTN	10	34	29.4%	NA	1
ZFHX3	3	15	20.0%	NA	1
ZNF717	10	34	29.4%	NA	1

N/A: not applicable.

In 2017, Watkins et al. [27] showed a significant increase of *PIK3CA* mutations in DEVIL lesions, described as an HPV-negative precursor. *PIK3CA* mutations were further confirmed not only in DEVIL but also in *TP53* wild-type dVIN [35], which also harbored *HRAS* mutations. Three years later, Tessier-Cloutier et al. focused on HPV-independent tumors, including verrucous VSCC, DEVIL, and VAAD. Strikingly, these cases were always the *TP53*-wild type but consistently harbored *PIK3CA* and *HRAS* mutations. The authors suggested a specific carcinogenic pathway different from the pathway of the typical keratinizing VSCC and dVIN [35].

3.3. Copy Number Variations in VSCC

One of the earliest studies conducted on VSCC focused exclusively on copy number variations in individual genes using a 122-gene panel in VSCC cell lines [36] reported a high prevalence in *TMSB10* losses (9/12, 92%) and gains in *CCND1* (8/12; 66%). The *TMSB10* copy number variations or mutations have not been confirmed in further studies, in contrast with *CCND1* alterations, which have been reported in 17% of VSCC from five studies, although with a broad range from 0% to 83%. Several studies have also shown frequent *CCND1* amplifications in HPV-independent VSCC [33,37]. Alternatively, HPV-associated VSCC harbored *TP63* and *BCL2* gains [37].

Whereas no differences in copy number variations loads were observed between HPV-associated and HPV-independent VSCC in the two whole-exome sequencing studies, Prieske et al. [38] identified gains in 20q and 11q as more abundant in HPV-associated and HPV-independent VSCC, respectively. Han et al. [39] showed 3q gains in HPV-associated VSCC, while the HPV-independent VSCC harbored gains in 7p and 8q and losses in 2q, and additionally identified high rates of copy number variations in *PIK3CA*. Swarts et al. [37] showed that the two types of VSCC display overlapping copy number alterations. Interestingly, this study showed that premalignant lesions and not VSCC differ significantly in terms of copy number variations. In this latter study, gains in chromosome 1 were identified as a risk factor for progression from vulvar high-grade squamous intraepithelial lesions to VSCC.

3.4. Prognostic Role of Molecular Alterations in VSCC

One of the earliest studies [31] showed that mutations in both *TP53* and *HRAS*, or *CDKN2A*, related to HPV-independent VSCC, were associated with a significantly worse prognosis. Zieba et al. [30] reported that neither HPV status nor mutations were associated with VSCC patient progression. Nooij et al. [25] showed a higher local recurrence rate of patients with HPV-independent *TP53*-mutated VSCC, compared with HPV-independent *TP53*-wild type VSCC and HPV-associated VSCC. Tessier-Cloutier et al. [35] reported worse overall survival in cases with *TP53* and *PIK3CA* co-mutations. The remaining three study series have not shown solid evidence of the prognostic impact of the explored gene mutations.

3.5. Potential Molecular Therapeutic Targets to Treat VSCC

In most of the included studies, the authors suggested therapeutic molecular targets based on the molecular alterations identified. Targeting the PI3K/AKT/mTOR pathway was the most frequently proposed strategy among the reviewed series [27–31,33–35]. Williams et al. [33] suggested that patients with *KMT2D* mutations might benefit from aurora kinase inhibitors. A few authors [28,31] proposed that the use of combined regimens (i.e., MEK inhibitors and PI3K inhibitors, mTOR and MEK inhibitors) might be useful to treat VSCC, instead of only targeting the PI3K pathways.

Other identified potential therapeutic targets involved *NOTCH-1* [25], *FGFR* [28], *MET*, and *BRAF* [35]. Kunjoonju et al. suggested that *TMSB10*, *CTNNB1*, *BCL2*, *CCND1*, and *IL12A* might be key molecular targets in VSSC [36], while Watkins et al. [27] highlighted that *EGFR*- mutated patients might benefit from targeted therapy.

4. Discussion

A growing number of research studies have focused on the genomic landscape in VSCC, particularly in the last four years. The analysis of these studies shows a marked variation in the number of mutations, the specific mutated genes, and the frequencies of these mutations. Unfortunately, there were notorious methodological differences between the studies, and consequently, their results might not be comparable, which represents the main limitation of the present study. Notably, the number of cases and the set of genes analyzed was limited in almost all series. Indeed, the two whole-exome sequencing series included no more than 50 samples in total, whereas the largest targeted NGS study explored 406 genes, which constitutes less than 2% of the genome coverage of any of the whole-exome sequencing studies, which might prevent obtaining solid molecular profiles.

While some series have suggested that HPV-independent tumors have a larger mutational load [25,39], other series [33,38] have indicated that the mutational load does not significantly differ by HPV status. However, the variation in the molecular techniques and strategies to detect HPV and, therefore, the comparisons between the different studies, might be biased. More importantly, p16 staining, a well-characterized surrogate marker of HPV status in VSCC [11], has been used only in half of the studies, while the combination of p16 and HPV PCR, probably the best strategy to conclusively assign a case as HPV-associated or HPV–independent [9], has been used in less than half of the series. It is particularly notorious there was a lack of the use of p16 staining in the whole-exome sequencing studies as well as in the largest NGS cohort. Thus, the analysis based on HPV status might be limited. The few studies that exclusively used PCR-based HPV testing [30,34] reported no clear genomic differences between HPV-associated and HPV-independent VSCC, but the authors acknowledge that the HPV tests used in the studies were not designed to be used in formalin-fixed, paraffin-embedded tissue [30]. Therefore, the hypothesis of similar oncogenic mechanisms for HPV-associated and HPV-independent VSCC lacks a solid basis.

Despite these limitations, the genomic landscape of VSCC is expanding beyond the well-known mutations in tumor suppressors *TP53* and *CDKN2A*, biomarkers that are difficult to target [40]. Mutations in the PI3K/AKT/mTOR pathway, apart from *TP53*, including *HRAS*, *KRAS*, *PIK3CA*, *KMT2D*, *PTEN*, and *FBXW7*, have been consistently reported across different study series. One of the whole-exome sequencing studies [39] showed that somatic mutations of *PIK3CA*, combined with the copy number variations in the same gene, comprised more than half (60%) of all molecular alterations, irrespective of the HPV status. Indeed, one of the systematic reviews [41] highlighted the PI3K pathway as the most important genomic abnormality in VSCC. Notably, most of the mutations involved the PI3K/AKT/mTOR pathway were more frequently found in HPV-associated VSCC in the largest study [33]. However, this study was based on the PCR-only strategy for HPV identification, with no p16 IHC. Thus, this correlation with HPV status has to be interpreted cautiously.

Several of the genes of the PI3K/AKT/mTOR pathway, including *PIK3CA*, *PTEN*, and *FBXW7*, have been described in the Drug Gene Interaction database as targetable by known drugs. Accordingly, several authors proposed targeting the PI3K/AKT/mTOR pathway. For instance, patients with *KMT2D* mutations might benefit from aurora kinase inhibitors, as suggested by Williams et al. [33], and recently shown in head, neck, and cervical cancer [42]. Nevertheless, the prognostic or therapeutic roles of the abnormalities in this pathway in VSCC are yet to be elucidated [43].

Besides the genes directly involved in the PI3K/AKT/mTOR pathway, other genes, such as *NBPF1* and *TSC2*, can have activating or inhibiting interactions with this cascade. Although identified in a single study series in this review, *NBPF1* has tumor growth inhibitory effects through the inhibition of the PI3K signaling pathway [44]. *TSC2* losses also lead to the enhancement of mTOR activity [45]. Therefore, the role of the PI3K/AKT/mTOR cascade likely plays a bigger role than originally thought.

Interestingly, the largest NGS cohort [33] identified significant rates of *NOTCH-1* mutations in HPV-independent tumors (19%). The Notch signaling pathway is known as one of the key players in maintaining normal tissue homeostasis [46], but similarly to the PIK3CA/AKT/mTOR pathway, its prognostic and therapeutic implications are far from clear in solid cancers. Interestingly, high rates (71%) of *NOTCH-1* mutations have been shown in a whole-exome sequencing study of squamous cell carcinomas of the penis [47], a male tumor with many similarities with VSCC (dual HPV-associated/HPV-independent pathway, similar precursor lesions). Moreover, the authors identified mutations in the PI3K pathway in one-third of the tumors. These shared findings between VSCC and penile cancer might open possibilities for the enrollment in trials exploring the role of *NOTCH-1* mutations as predictors of response to PI3K/mTOR inhibitors [48].

The evidence of *EGFR* amplifications in 11% of HPV-independent VSCC, as shown by Williams et al. [33], might open doors to prognostic stratification or treatment with Cetuximab [49,50]. A phase II clinical trial assessing the role of erlotinib (anti-*EGFR* tyrosine kinase inhibitor) in VSSC has shown an acceptable toxicity with a significant clinical response (27.5% of patients showed partial response and 40% stable disease), but with limited sustained response rates [51]. Alternatively, as *CCND1* amplifications are also most frequently seen in HPV-independent VSCC [33,36], it might be worth exploring the potential additive oncogenic effects of *EGFR* and *CCND1* alterations, as recently shown in oral squamous cell carcinomas [52].

Another intriguing observation, recognized in both the whole-exome sequencing as well as the largest NGS cohorts, are abnormalities in *FBXW7*, a p53-dependent tumor suppressor gene frequently mutated in other female genital tumors, such as endometrial and cervical cancers [53]. *FBXW7* is a modulator of NOTCH signaling cascade and recent studies have implicated *FBXW7* status in chemoresistance [54]. However, while the largest NGS study [33], and one of the whole-exome sequencing series, identified *FBXW7* mutations predominantly in HPV-associated tumors, in the second whole-exome sequencing study [38] these mutations were restricted to HPV-independent VSCC.

Mutations in mucins, including *MUC16* (formerly known as *CA125*), have been frequently detected in a whole-exome sequencing-based study [38]. Nevertheless, these mutations should be confirmed in new studies using whole-exome sequencing or modern NGS-based tools. Stimulatingly, *MUC16* has been shown to be altered only in gynecological malignancies and other benign conditions [55] and has been recognized as a tumor biomarker and a novel target for cancer therapy [56].

Curiously, none of the series confirmed copy number variations in *TMSB10* and *IL12A*, identified with high frequencies by Kunjoonju et al. [36]. However, this study used a small subset of 12 VSCC cell lines and not formalin-fixed, paraffin-embedded, or frozen tissue as most of the other series did. Similarly, a subset of somatic mutations, including *MUC4* or *CD44*, has been reported only by one of the whole-exome studies. Thus, the methodology employed by these studies might not be robust.

The prognostic role of genomic alterations is limited to the few most recurrent genes. It is of note that the prognostic differences were identified for combinations of mutations rather than for individual alterations. The co-mutations with worse prognoses mostly consisted of *TP53* combined with *HRAS*, *CDKN2A*, or *PIK3CA* mutations [31,35]. As *TP53* is more commonly mutated in HPV-independent VSCC [38], these findings are in line with the increasing evidence on the worse prognoses of HPV-independent VSCC [57].

In 2017, Nooij et al. [25] suggested that a subset of HPV-independent VSCC (HPV-negative, *TP53*-wild type) should be considered as a distinct etiopathogenic, morphologic, and molecular subtype, characterized by *NOTCH-1* and *HRAS* mutations. More recently, Tessier-Cloutier et al. have provided evidence indicating that a particular variant of VSCC, verrucous VSCC, and two precursor lesions, DEVIL and VAAD, might be part of the spectrum of this distinct HPV-negative *TP53*-wild type pathway and that all of these lesions harbor *HRAS* and *PIK3CA* mutations [35]. However, neither the whole-exome sequencing cohorts nor the largest NGS series specifically analyzed these lesions, and the

evidence indicating that they truly represent a specific entity is still limited. Moreover, the clinical behavior of these lesions is still poorly understood [1,16].

In conclusion, although with the recent effort in characterizing the genomic landscape of VSCC, much still remains unknown on the molecular mechanisms involved in the pathogenesis of this tumor. Comparisons between existing series on VSCC are limited by different sample sizes, heterogeneous HPV detection, and tumor DNA sequencing methods. Despite it being known that a number of mutations are druggable, the clinical utility of them is still unknown in patients with VSCC. Large-scale, ideally multicentric studies, with a solid HPV testing strategy p16 and p53 IHC, a strong follow-up component to further analyze possible prognostic implications related to genomic mutations, as well as clinical trials analyzing the possibility of gene-targeted therapies, are needed to elucidate the specific roles of known and newly described mutations, or combinations of them.

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2 | Hipótesis

La reciente clasificación de la Organización Mundial de la Salud (OMS 2020) del carcinoma escamoso de la vulva (CEV), en cuya elaboración nuestro grupo ha jugado un papel de liderazgo, contempla dos tipos etiopatogénicos distintos según su asociación o no con el virus del papiloma humano (VPH): el CEV asociado a VPH y el CEV independiente de VPH. En nuestro medio, la mayoría de casos de CEV de desarrollan de forma independiente del VPH. Habitualmente el CEV se desarrolla sobre lesiones precursoras intraepiteliales que preceden al desarrollo del carcinoma en meses o años. Cada uno de los dos tipos de CEV se desarrolla sobre un tipo específico de lesión precursora, la lesión intraepitelial escamosa de alto grado (HSIL vulvar) para los carcinomas asociados a VPH y el VIN de tipo diferenciado (dVIN) para los carcinomas independientes de VPH. Cada uno de los dos tipos de lesión intraepitelial presenta unas características morfológicas diferenciales.

Nuestro grupo definió en 2009 la existencia de CEV independientes de VPH que se desarrollaban sobre lesiones intraepiteliales morfológicamente indistinguibles de la HSIL vulvar, la lesión precursora de los carcinomas asociados a VPH, y por lo tanto, con características morfológicas claramente diferentes de las lesiones de dVIN. Estas lesiones han sido denominadas como *HSIL-like*. La existencia de estas lesiones se ha confirmado más recientemente, tanto en estudios realizados por nuestro grupo, como en estudios realizados por otros investigadores. Sin embargo, se desconoce si la presencia de esta lesión intraepitelial confiere o no alguna característica clínica o de pronóstico diferencial a los carcinomas independientes de VPH que se desarrollan sobre ella.

Por otro lado, a pesar de la evidente diferencia etiopatiogénica entre los dos tipos de CEV propuestos por la OMS, asociado e independiente de VPH, las evidencias en cuanto a sus diferencias clínicas y de pronóstico han sido poco estudiadas. Además, en los últimos años se ha planteado que los CEV independientes de VPH se deberían sub-dividir, a su vez, según la presencia o ausencia de mutación de *TP53* y que esta subdivisión debería ser considerada en la próxima revisión de la clasificación de la OMS. Sin embargo, la información existente sobre la implicación clínica de subclasificar estos tumores VPH independientes es muy escasa.

La hipótesis general de la presente tesis doctoral es que existen subgrupos de pacientes con CEV identificables en base a las características moleculares y morfológicas que tienen relevancia clínica y pronóstica, más allá de los grupos definidos etiopatogénicamente por la clasificación de la OMS.

La tesis está compuesta por dos trabajos, cuyas hipótesis se detallan a continuación:

1. **Primer trabajo:** las lesiones premalignas adyacentes al CEV condicionan el comportamiento clínico y el pronóstico del tumor. Concretamente, las pacientes con CEV independiente de VPH con lesión precursora de morfología HSIL-*like* tienen un comportamiento clínico diferente del resto de los CEV independientes de VPH.
2. **Segundo trabajo:** la asociación o no a VPH y las alteraciones en p53 tienen relevancia clínica y pronóstica en las pacientes con CEV. La aplicación de técnicas para detectar VPH y evaluar el estado mutacional de p53 definiría tres subgrupos de pacientes: 1) CEV asociado a VPH, 2) CEV independiente de VPH con p53 normal y 3) CEV independiente de VPH con expresión anormal de p53. La identificación de diferencias clínicas y pronósticas entre subgrupos permitiría en el futuro establecer una estrategia terapéutica adaptada a cada subgrupo.

3 | Objetivos

El objetivo general de la tesis doctoral es identificar subgrupos específicos de pacientes con carcinoma escamoso de vulva en base a su caracterización histopatológica (morfológica y molecular), cuya relevancia clínica y pronóstica posibilite en el futuro una mayor individualización en la conducta clínica y terapéutica de estas pacientes.

OBJETIVOS ESPECÍFICOS:

1. Determinar la frecuencia con la que los carcinomas escamosos de vulva independientes del virus del papiloma humano se desarrollan sobre lesiones con características morfológicas de lesión escamosa intraepitelial de alto grado (estudio número 1).
2. Evaluar las características clínicas de las mujeres con carcinoma escamoso de vulva independiente de virus del papiloma humano con lesión precursora escamosa intraepitelial de alto grado-*like,* comparándolas con las de las mujeres con carcinoma escamoso de vulva independiente del virus del papiloma humano con lesión precursora tipo neoplasia vulvar intraepitelial diferenciada y las de las mujeres con carcinoma escamoso de vulva asociado al virus del papiloma humano (estudio número 1).
3. Evaluar las características anatomopatológicas e inmunohistoquímicas del carcinoma escamoso de vulva independiente de VPH con lesión precursora escamosa intraepitelial de alto grado-*like*, comparándolas con las de las mujeres con carcinoma escamoso de vulva independiente del virus del papiloma humano con lesión precursora tipo neoplasia vulvar intraepitelial diferenciada y las de las mujeres con carcinoma escamoso de vulva asociado al virus del papiloma humano (estudio número 1).
4. Analizar el posible impacto pronóstico del carcinoma escamoso de vulva independiente del virus del papiloma humano con lesión precursora escamosa intraepitelial de alto grado-*like* (estudio número 1).

5. Analizar el papel de la detección del virus del papiloma humano y la determinación inmunohistoquímica de p16 y p53 en la clasificación del carcinoma escamoso de vulva (estudios números 1 y 2).
6. Evaluar las diferencias clínicas entre el carcinoma escamoso de vulva asociado al virus del papiloma humano y el carcinoma escamoso de vulva independiente del virus del papiloma humano (estudio número 2).
7. Analizar las diferencias histológicas e inmunohistoquímicas entre el carcinoma escamoso de vulva asociado al virus del papiloma humano y el carcinoma escamoso de vulva independiente del virus del papiloma humano (estudio número 2).
8. Determinar si existen diferencias en cuanto a pronóstico entre el carcinoma escamoso de vulva asociado al virus del papiloma humano y el carcinoma escamoso de vulva independiente del virus del papiloma humano (estudio número 2).
9. Analizar si existen diferencias clínicas y de pronóstico entre las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano en función de la expresión inmunohistoquímica de p53 (normal o alterada) (estudio número 2).
10. Evaluar la utilidad de la nueva clasificación de la OMS de 2020 de los tumores de la vulva, planteando posibles cambios sobre la misma que puedan ayudar a su mejora (estudio número 2).

4 | Material, métodos y resultados

La metodología y los resultados de los dos estudios se encuentran detalladamente descritas en las secciones correspondientes de cada trabajo, que se encuentran a continuación tal y como han sido publicadas en la literatura científica.

4.1. Primer estudio

AUTORES: **Carreras-Dieguez N**, Saco A, Del Pino M, Pumarola C, López del Campo R, Manzotti C, Garcia A, Marimon L, Diaz-Mercedes S, Fuste P, Rodrigo-Calvo MT, Vega N, Torné A, Rakislova N

TÍTULO: **Vulvar squamous cell carcinoma arising on human papillomavirus-independent precursors mimicking high-grade squamous Intraepithelial lesion: a distinct and highly recurrent subtype of vulvar cancer**

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CATEGORÍA: *Pathology*

TIPO DE PUBLICACIÓN: investigación original

RESUMEN:

Objetivos: cada categoría de carcinoma escamoso de vulva (CEV), el CEV asociado a virus del papiloma humano (VPH) y el CEV independiente de VPH, se origina de una lesión intraepitelial precursora específica: la lesión escamosa intraepitelial de alto grado (HSIL) y la neoplasia intraepitelial vulvar diferenciada (dVIN), respectivamente. Sin embargo, existe un subgrupo de CEV independiente de VPH que se origina de un precursor intraepitelial morfológicamente muy similar a HSIL (HSIL-*like*). El objetivo de este estudio es explorar las características clinicopatológicas y pronósticas del CEV independiente de VPH con lesión adyacente HSIL-*like* y compararlas con el CEV independiente de VPH con lesión adyacente dVIN y el CEV asociado a VPH con lesión adyacente HSIL.

Metodología: se identificaron de forma retrospectiva 105 casos de pacientes con CEV con lesiones intraepiteliales adyacentes, tratadas quirúrgicamente en el Hospital Clínic de Barcelona. Los casos fueron clasificados en tres grupos en base a la lesión precursora adyacente: 1) CEV asociado a VPH con lesión precursora tipo HSIL (n=26), 2). CEV independiente de VPH con lesión precursora tipo

dVIN (n=54) y 3) CEV independiente de VPH con lesión precursora tipo HSIL-*like* (n=25). Se analizaron las características histológicas y clínicas, incluyendo la supervivencia libre de enfermedad y la supervivencia específica por enfermedad en los tres grupos, realizando una regresión de Cox con análisis univariado y multivariado.

Resultados: las pacientes con tumores independientes de VPH con lesión adyacente tipo HSIL-*like*, a pesar de presentar características morfológicas y clínicas similares a las pacientes con CEV asociado a VPH, tenían una edad de presentación similar a las pacientes con CEV independiente de VPH y lesión precursora dVIN, mayor que las pacientes con tumores asociados a VPH (76 y 77 años *versus* 66 años, respectivamente, p<0,001). El CEV independiente de VPH con lesión precursora HSIL-*like* se asoció a un mayor riesgo de recidiva (*hazard ratio* [HR] 3,87; p<0,001) que los tumores independientes de VPH con lesión precursora dVIN (HR 2,27; p=0,1) y que los tumores asociados a VPH (HR 1). Esta tendencia a la recidiva se mantuvo significativa en el análisis multivariado. No se observaron diferencias en la supervivencia específica por enfermedad entre grupos.

Conclusiones: el CEV independiente de VPH con lesión premaligna adyacente tipo HSIL-*like* presenta características clínicas diferenciadas y tiene una importante tendencia a la recidiva, por lo que podría beneficiarse de un seguimiento más estrecho después del tratamiento.

Histopathology

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Vulvar squamous cell carcinoma arising on human papillomavirus-independent precursors mimicking high-grade squamous intra-epithelial lesion: a distinct and highly recurrent subtype of vulvar cancer

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Vulvar squamous cell carcinoma arising on human papillomavirus-independent precursors mimicking high-grade squamous intra-epithelial lesion: a distinct and highly recurrent subtype of vulvar cancer

Aims: Each category of vulvar squamous cell carcinoma (VSCC), human papillomavirus (HPV)-associated and HPV-independent, arises on a specific intra-epithelial precursor: high-grade squamous intra-epithelial lesions (HSIL) and differentiated vulvar intra-epithelial neoplasia (dVIN), respectively. However, a subset of HPV-independent VSCC arises on an intra-epithelial precursor closely mimicking HSIL. We aimed to explore the clinicopathological features of the HPV-independent tumours with HSIL-like lesions and compare them with HPV-independent VSCC with dVIN and HPV-associated tumours with HSIL.

Methods and results: We retrospectively identified 105 cases of surgically treated VSCC with adjacent intra-epithelial precursors. The cases were classified into three groups based on the HPV status and the adjacent precursor identified: (i) HPV-associated VSCC with HSIL ($n = 26$), (ii) HPV-independent VSCC with dVIN lesions ($n = 54$) and (iii) HPV-independent VSCC with HSIL-like lesions ($n = 25$). We analysed the histological and clinical features including the recurrence-free survival and disease-specific survival in the three groups. Patients with HPV-independent VSCC with HSIL-like lesions and with dVIN were older than patients with HPV-associated VSCC (76 and 77 versus 66 years, respectively, $P < 0.001$). HPV-independent VSCC with HSIL-like lesions recurred more frequently [hazard ratio (HR) = 3.87; $P < 0.001$] than HPV-independent VSCC with dVIN (HR = 2.27; $P = 0.1$) and HPV-associated VSCC (HR = 1). In the multivariate analysis, HPV-independent VSCC with HSIL-like lesions remained significant for recurrence. No differences in disease-specific survival were observed between the three groups.

Conclusions: Even though VSCC with HSIL-like lesions are not associated with higher mortality, they are more likely to recur and might benefit from more intensive treatment strategies and closer surveillance after treatment.

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Keywords: HPV-independent vulvar cancer, dVIN, HPV, HSIL, HSIL-like lesions, vulvar cancer, vulvar squamous cell carcinoma

Introduction

Vulvar squamous cell carcinoma (VSCC) is a rare neoplasm with high morbidity and non-negligible mortality.[1] The 2020 World Health Organisation (WHO) classification has divided VSCC on the basis of its aetiological relationship with the infection by human papillomavirus (HPV) into two distinct types, HPV-associated and HPV-independent.[2–4] There is growing evidence indicating that patients with HPV-associated VSCC have a better prognosis than those with HPV-independent tumours.[5–7]

HPV-associated VSCC typically affects younger women, commonly shows immature, poorly differentiated, basaloid and/or warty histological features and strong, block type staining for p16 in immunohistochemistry (IHC). HPV-associated VSCC usually develops from an HPV-induced intra-epithelial precursor named high-grade squamous cell lesion (HSIL), characterised by immature basal-appearing cells involving the whole thickness of the epithelium, also known as vulvar intra-epithelial neoplasia (VIN) of usual type.[8] As its invasive counterpart, vulvar HSIL typically overexpresses p16.[9,10] In contrast, HPV-independent VSCC affects older women,[3] commonly shows mature, well-differentiated, keratinising features, does not overexpress p16,[3] frequently shows an abnormal p53 IHC pattern[2,11] and commonly arises in the context of chronic inflammatory lesions of the vulvar skin, such as lichen simplex chronicus and lichen sclerosus.[12,13] HPV-independent VSCC develop from an intra-epithelial precursor named differentiated VIN (dVIN), a lesion characterised by atypical basal cells with normal maturation in the superficial layers. dVIN is a subtle lesion with deceptively bland appearance, frequently overlapping with inflammatory dermatoses.[14,15] In spite of its subtle histological features, dVIN has been shown to be a highly oncogenic lesion that rapidly progresses to invasive VSCC.[16] As the invasive tumour, dVIN does not overexpress p16[3] and frequently shows an abnormal p53 IHC pattern.[2,11]

Recently, it has been described by our group that approximately 6% of the HPV-independent VSCC arise from an intra-epithelial precursor morphologically indistinguishable from HSIL.[17] Similarly to the true HPV-associated HSIL, this HPV-independent intra-epithelial precursor has an immature appearance with whole-thickness abnormal maturation and is composed of cells with basaloid morphology or koilocytotic-like (warty) features. These lesions have been referred to as basaloid dVIN[18] or HSIL-like lesions.[17] In contrast with conventional HSIL, these lesions do not overexpress p16, are negative for high-risk HPV[3,17,18] and frequently show p53 abnormalities,[17] in similarity to conventional dVIN.[15] Due to their recent characterisation, studies analysing the follow-up of VSCC arising on this particular precursor are lacking. Thus, it remains unclear whether HSIL-like lesions should be considered as part of the morphological spectrum of dVIN or whether they have any specific clinicopathological and prognostic features.

In this study we evaluated a large series of VSCC with associated intra-epithelial precursors, including HPV-associated HSIL, HPV-independent dVIN and HPV-independent HSIL-like lesion, aiming at analysing the clinicopathological features and the behaviour of these three groups of VSCC.

Methods

CASE SELECTION

We retrospectively retrieved all VSCC from patients who underwent primary surgery at the Department of Gynaecological Oncology of the Hospital Clinic of Barcelona, Spain, during a 46-year period (February 1975–December 2021). The clinical charts and all the available pathological material were carefully reviewed. The following clinicopathological variables were retrieved from the electronic archives: patient age, type and date/s of treatment/s, tumour size, location, depth of invasion, tumour multifocality, tumour margin status, presence of premalignant lesion in the margin, lymph node involvement, date and site of first cancer recurrence and last follow-up or death.

All available haematoxylin and eosin-stained slides of all cases, including the invasive tumour as well as the adjacent skin, were carefully reviewed by two gynaecological pathologists with expertise in vulvar pathology (N.R. and A.S.), specifically looking for

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confirmation of the diagnosis of invasive carcinoma and presence of any intra-epithelial precursor in the adjacent skin.

The following exclusion criteria were established: (i) neoadjuvant radiotherapy or chemotherapy, (ii) absence of significant atypia in the adjacent skin (thus, lesions showing only inflammatory or reactive changes or intra-epithelial lesions lacking atypia such as vulvar acanthosis with altered differentiation, or differentiated exophytic vulvar intra-epithelial lesion, were excluded), (iii) insufficient tumour tissue for IHC and HPV DNA testing; and (iv) follow-up time shorter than 6 months.

The institutional ethical approval for this study was obtained (registry reference HCB/2020/1198). Written study consent was obtained from all the patients enrolled into the study.

Histological revision of the invasive tumour and adjacent skin

The histological variant of the invasive VSCC (keratinising, non-keratinising, basaloid, warty and verrucous)[19] and the type of atypical intra-epithelial lesion, including HSIL, dVIN and HSIL-like lesions, were recorded. The diagnosis of dVIN was made in the presence of significant basal atypia with preserved maturation in the upper layers.[20] HSIL and HSIL-like lesions were diagnosed when full-thickness epithelial atypia, high nuclear-to-cytoplasmic ratio and marked nuclear pleomorphism was identified[17,18,20,21]; these two lesions could not be differentiated on the basis of pure morphological criteria.

In addition to the atypical precursor, other coexisting lesions in the adjacent skin, including non-atypical precursors (vulvar acanthosis with altered differentiation and differentiated exophytic vulvar intra-epithelial lesion) and inflammatory dermatoses (lichen simplex chronicus, lichen sclerosus and lichen planus), was also recorded. These lesions were diagnosed based on classical pathological criteria.[12,13]

p16 immunohistochemistry

IHC for p16 was performed using a monoclonal antibody (clone E6H4; Roche-mtm-Laboratories, CINtec Histology Kit, Heidelberg, Germany). The staining was classified as either positive (diffuse and block-like staining) or negative (completely negative p16 or patchy staining).[9] p16 IHC was evaluated independently in the invasive tumour and in the premalignant lesion.

HPV DNA detection and genotyping

DNA was extracted from whole sections of a representative formalin-fixed paraffin-embedded block from surgical specimens. The analysed tissue included the invasive tumour and the precursor, but no separated analysis of HPV DNA in the invasive tumour and the precursor was conducted.

SPF10 PCR and the LiPA25 system were used for HPV DNA detection and typing (version 1; Labo Biomedical Products, Rijswijk, the Netherlands). A volume of 10 µl of isolated DNA was PCR-amplified using the INNO-LiPA HPV Genotyping Extra II kit (Fujirebio, Ghent, Belgium). This system allows the genotyping of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 26, 53, 66, 70, 73, 82, 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83 and 89.

Assignment of HPV status

Both p16 IHC staining and HPV testing were considered for HPV status categorisation in invasive tumours and in the intra-epithelial lesions. Specimens with positive staining for p16 and/or high-risk HPV detected in the molecular analysis were classified as HPV-associated. The inclusion of a tumour as HPV-independent required both negative p16 IHC staining and absence of high-risk HPV DNA. Tumours showing low-risk or unclassifiable HPV types or having an invalid result in the HPV testing with negative p16 staining were considered as HPV-independent.

Classification of the tumours into three groups

All the cases complying with study criteria were further classified into three categories in accordance to the HPV status and type of adjacent premalignant lesions: (i) HPV-associated VSCC with adjacent HSIL; (ii) HPV-independent VSCC with HSIL-like lesions; (iii) HPV-independent VSCC with dVIN. The discrimination between HSIL and HSIL-like lesions was based on positive or negative HPV status of the invasive tumour and p16 IHC positive or negative staining in the premalignant lesion, respectively.

p53 immunohistochemistry

p53 IHC was performed with the monoclonal antibody CONFIRM (DO-7; Roche, Heildelberg, Germany). The results were evaluated using the pattern-based interpretation framework recently described.[11,22] p53 staining patterns were classified into two major categories: normal (wild-type) and abnormal (mutant). Normal category included scattered and mid-epithelial staining patterns, whereas abnormal expression included basal or diffuse overexpression, as well as null and cytoplasmic staining.

Results of p53 IHC were not used to categorise either invasive VSCC or vulvar premalignant lesions.

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Treatment and follow-up

All patients underwent surgical treatment with vulvectomy or local wide excision of VSCC. The lymph node approach has been evolving from 1975 to the latest version. Before 1998, all women underwent inguinofemoral lymphadenectomy. In 1998, in our centre, we began to perform sentinel lymph node biopsy followed by inguinofemoral lymphadenectomy in order to validate the technique.[23] From 2003, sentinel lymph node biopsy was considered validated and the procedure was performed as the only staging method in all patients with unifocal VSCC measuring less than 4 cm. Patients with a positive sentinel lymph node underwent an ipsilateral inguinofemoral lymphadenectomy. Adjuvant radiotherapy and chemotherapy were indicated in accordance with the clinical guidelines at the time of diagnosis.

Patient follow-up, which included physical examination, was performed every 4–6 months for the first 2 years and annually afterwards. Imaging techniques (magnetic resonance imaging, inguinal ultrasound or computed tomography scan) were periodically conducted in patients with advanced VSCC or when recurrence was suspected. The patient was considered to have local recurrence when the tumour appeared in the same location after a minimum disease-free period of 6 months.

Given that the International Federation of Gynaecology and Obstetrics (FIGO) classification of VSCC has changed during the inclusion period,[24,25] all the patients were retrospectively restaged using the FIGO 2021 criteria[26] in order to use homogeneous staging criteria throughout the study sample. Information on relapse and cause of death was retrieved from the clinical charts.

Statistical analysis

For all data analyses, StataIC version 15.0.591 was used. χ^2 tests (categorical data) and analysis of variance (ANOVA; numerical data) were run to compare the clinical and histopathological data between the three study groups.

The endpoints of the study were recurrence-free and disease-specific survival, and were calculated from the date of treatment (primary surgery) to the date of first recurrence or death due to disease, respectively. Survival analyses were conducted using the Kaplan–Meier method and differences between survival curves were calculated using the log-rank test. Univariate Cox regression was performed to evaluate the prognostic role of the three study groups. Multivariate Cox regression was run to confirm the associations after accounting for possible confounding factors, and included all significant variables in univariate analysis. Two-sided P-values < 0.05 were considered statistically significant.

Written study consent was obtained from all the patients enrolled into the study. The study data set is available upon request.

Results

CHARACTERISTICS OF THE STUDY COHORT

Two hundred and four VSCC were identified during the study period. Five patients (2.4%) had advanced tumours treated with initial radiation and/or chemotherapy and were consequently excluded from the study. Eighty-eight patients (43.1%) were excluded due to the absence of atypical premalignant lesion in the skin. No case was excluded due to insufficient tumour tissue for IHC and HPV DNA testing. Finally, six patients (2.9%) were discarded because of insufficient follow-up time (less than 6 months). Thus, the final study cohort included 105 patients with VSCC.

The mean age at diagnosis was 72.6 years (range = 30.7–95.4) and the mean follow-up time was 57.8 ± 43.2 months. Forty-eight patients (45.7%) were treated with vulvectomy and 57 (54.3%) with wide local excision. Eighty-eight patients (83.8%) underwent surgical lymph node evaluation either by sentinel lymph node biopsy (n = 39), inguinofemoral lymphadenectomy (n = 28) or both sentinel and inguinofemoral dissection (n = 21). Five patients had IA FIGO stage VSCC, thus negativity of lymph nodes was assumed. Twelve patients (11.4%) did not undergo surgical lymph node evaluation because of poor performance status. Twenty-eight patients (26.6%) received adjuvant radiation therapy after the initial surgical treatment and four (3.8%) received adjuvant chemotherapy.

CLASSIFICATION OF THE TUMOURS INTO THREE GROUPS

Twenty-six VSCC (24.8%) were classified as HPV-associated. All these tumours were p16-positive, and 23 (88.5%) tested positive for high-risk HPV. All the 26 VSCC had HSIL in the adjacent skin, which stained positive for p16.

Seventy-nine VSCC (75.2%) were classified as HPV-independent. All of them stained negative for p16. Seventy-five of 79 HPV-independent VSCC (94.9%) had a negative HPV testing result; three

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cases (3.8%) showed an invalid result and in one case (1.3%) a low-risk HPV (HPV 6) was identified. Among the 79 HPV-independent VSCC, 54 (68.4%) showed dVIN in the adjacent skin. All dVIN lesions were negative for p16 IHC. Twenty-five of 79 HPV-independent VSCC (31.6%) showed HSIL (morphologically) in the adjacent skin. In all 25 cases the adjacent precursor was also negative for p16 (HSIL-like lesions).

No differences in the surgical treatment (vulvectomy versus wide excision or type of lymph node evaluation), in the proportion of patients without nodal evaluation or in the proportion of patients receiving adjuvant treatment were seen between study groups (Supporting information, Table S1).

CLINICAL FEATURES

The mean age at diagnosis was 61.4 ± 16.0 years for the patients with HPV-associated VSCC with HSIL, 76.8 ± 11.5 years for HPV-independent VSCC arising on dVIN and 75.2 ± 9.9 years for the HPV-independent VSCC arising on HSIL-like lesions ($P < 0.001$). Figure 1 shows the clinical appearance, histological and immunohistochemical features of VSCC with adjacent HSIL, HSIL-like and dVIN.

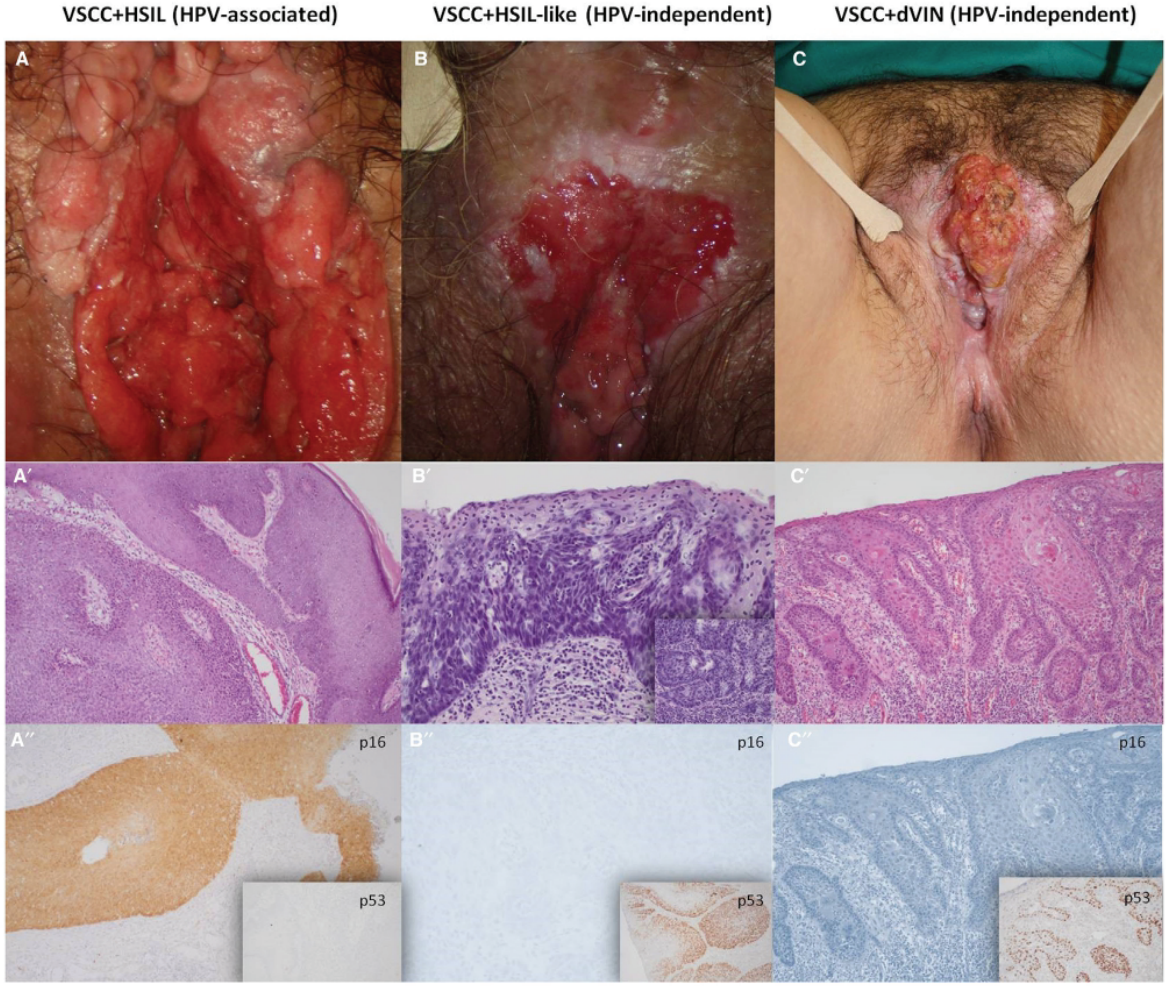

Figure 1. Clinical, histological (haematoxylin and eosin) and immunohistochemical expression of a typical examples of HPV-associated VSCC with HSIL (A, A′, A″), HPV-independent VSCC with HSIL-like lesions (B, B′, B″) and HPV-independent VSCC with dVIN (C, C′, C‴). (A, B, C, clinical pictures of the vulvar squamous cell carcinomas and adjacent skin lesions; A′, B′, C′ haematoxylin and eosin of the tumour and pre-malignant lesions; A″, B″, C″ p16 and p53 immunohistochemistry).

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MACROSCOPIC, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL FEATURES

The macroscopic features of VSCC with HSIL and HPV-independent VSCC with HSIL-like lesion were similar: all the tumours (26; 100%) with HSIL and the majority of those (20; 80%) with HSIL-like lesions were exophytic, showing raised lesions in adjacent skin in all cases. The tumours with dVIN were heterogeneous in appearance [22 (40.7%) exophytic; 32 (60%) flat, deeply ulcerated], with adjacent skin of inflammatory appearance in 10 cases (18.5%).

Table 1 shows the histological subtypes, p53 IHC staining and other associated inflammatory lesions of the three groups included into the study. Most of the invasive tumours in the groups of HPV-associated VSCC with HSIL and HPV-independent VSCC with HSIL-like lesions displayed basaloid, warty or non-keratinising features, while HPV-independent VSCC were mostly keratinising ($P < 0.001$). The histological features were identical in HPV-associated VSCC tumours with HSIL and HPV-independent VSCC with HSIL-like lesion, both in invasive tumour and adjacent lesion: architectural disarray was prominent, mitotic rate was high, atypical mitoses were readily identifiable and koilocytotic changes were common. No cases with coexistent HSIL, dVIN or HSIL-like lesions and other non-atypical precursors (vulvar acanthosis with altered differentiation and differentiated exophytic vulvar intra-epithelial lesion) were identified. Figure 2 shows the morphological spectrum of HSIL-like lesions and of the invasive VSCC developing in the background of these precursors.

The majority of the HPV-associated VSCC showed a wild-type pattern of expression of p53, whereas most of the HPV-independent VSCC (with dVIN or HSIL-like) showed an abnormal pattern of p53 IHC staining ($P < 0.001$). Among patients with abnormal p53 staining, 53 (71.6%) showed a diffuse overexpression pattern, 13 (17.8%) showed null staining, five (6.8%) showed a basal overexpression pattern and three (4.1%) showed cytoplasmatic staining. Twenty-three

Table 1. The histological subtypes, the p53 immunohistochemical staining and other associated inflammatory lesions for the three groups of vulvar squamous cell carcinoma included in the study

	HPV-associated VSCC with adjacent HSIL ($n = 26$)	HPV-independent VSCC with adjacent dVIN ($n = 54$)	HPV-independent VSCC with adjacent HSIL-like lesion ($n = 25$)	P
Histological type [n (%)]				**<0.001**
Keratinising	1 (3.9%)	49 (90.7%)	6 (24.0%)	
Basaloid	21 (80.8%)	0 (0%)	15 (60.0%)	
Warty	2 (7.7%)	3 (5.6%)	3 (12.0%)	
Non-keratinising	2 (7.7%)	0 (0%)	1 (4%)	
Verrucous	0 (0.0%)	2 (3.7%)	0 (0.0%)	
p53 IHC abnormal pattern [n (%)]	2 (7.6%)	51 (94.4%)	21 (84.0%)	**<0.001**
Associated inflammatory lesions [n (%)]				**<0.001**
Lichen simplex chronicus	0 (0.0%)	21 (38.9%)	5 (20.0%)	
Lichen sclerosus	0 (0.0%)	18 (33.3%)	3 (12.0%)	
Lichen simplex and lichen sclerosus	0 (0.0%)	11 (20.4%)	3 (12.0%)	
No inflammatory lesion	26 (100%)	4 (7.4%)	14 (56.0%)	

Bold indicates statistically significant p values.
P-values correspond to multigroup comparison (HPV-associated VSCC with adjacent HSIL versus HPV-independent VSCC with adjacent dVIN versus HPV-independent VSCC with adjacent HSIL-like lesion).
dVIN, Differentiated vulvar intra-epithelial neoplasia; IHC, Immunohistochemical; HPV, Human papillomavirus; HSIL, high-grade squamous intra-epithelial lesion; VSCC, Vulvar squamous cell carcinoma.

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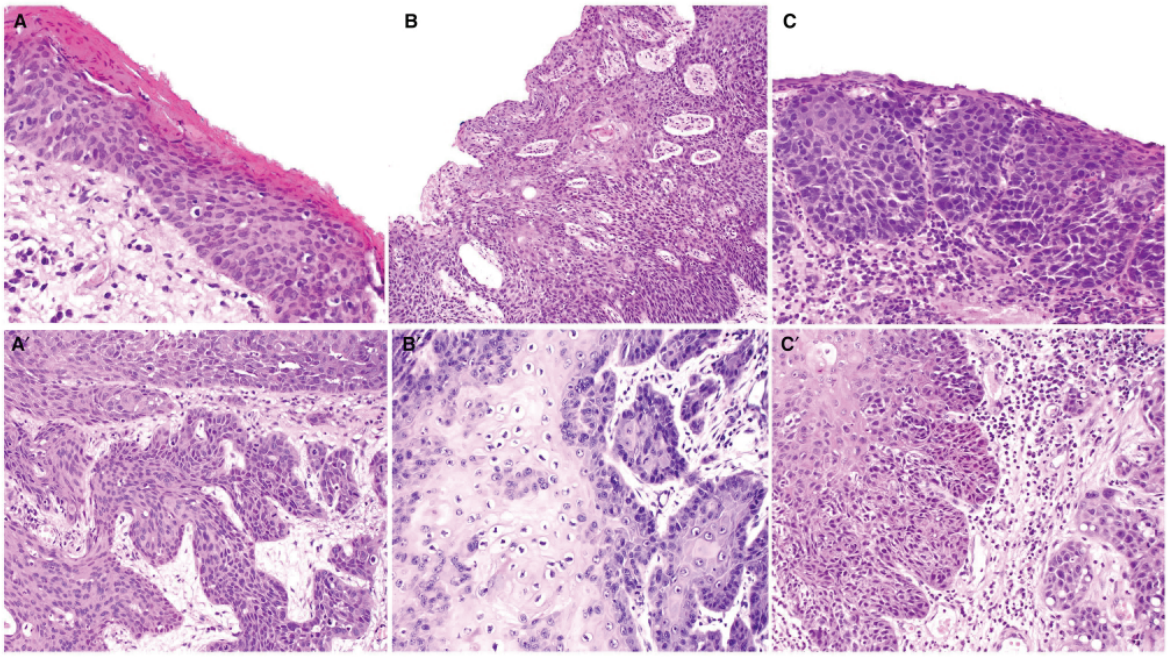

Figure 2. Morphological spectrum of HSIL-like lesions and invasive VSCC. A, B, C, HSIL-like lesions and A′, B′, C′ the invasive carcinoma arising in association with each of these lesions. A, HSIL-like lesion showing diffuse replacement of the epidermis by undifferentiated keratinocytes with scant cytoplasm (basaloid appearance) extending throughout the entire thickness of the epidermis, with a parakeratotic surface reaction (A′) Non-keratinising poorly differentiated invasive squamous carcinoma arising in association with HSIL-like lesion showed in (A). Mitotic figures are abundant. B, HSIL-like lesion showing exophytic, papillomatous architecture (warty appearance) diffuse replacement of entire epidermis by atypical keratinocytes and koilocytotic-like (warty) features. B′, Invasive VSCC with marked koilocytotic-like (warty) features arising in association with lesion depicted in (B). C, HSIL-like lesion showing total replacement of epidermis by large atypical cells with hypercromatic nuclei. Acantholytic features are present in the basal layer. Slight parakeratosis is noted in the outer layer (C′) Undifferentiated invasive VSCC arising in association with the HSIL-like lesion depicted in (C). Large atypical cells with variable amount of cytoplasm, acantholytic features and foci of slight keratinisation are present.

(74.2%) patients with p53 normal staining showed a scattered pattern and eight (25.8%) a mid-epithelial pattern of staining. Inflammatory lesions coexisted in more than one-third of patients with HPV-independent VSCC arising on dVIN or HSIL-like lesions, while no inflammatory lesions were identified in HPV-associated VSCC with adjacent HSIL ($P < 0.001$).

PATHOLOGICAL PROGNOSTIC FACTORS AND FIGO STAGING

Table 2 shows the pathological prognostic features and the FIGO staging of the three groups of patients with VSCC.

HPV-independent tumours with dVIN were larger and more deeply invasive than HPV-associated tumours with HSIL and HPV-independent VSCC with HSIL-like lesions ($P = 0.004$ and $P = 0.003$, respectively). No differences were observed between the groups in terms of location, multifocality, presence of premalignant lesion in surgical margin, lymphovascular invasion or lymph node metastases. A higher proportion of patients with surgical margin affected by invasive carcinoma was seen in HPV-associated VSCC (23.1%, $P = 0.029$). More than 75% of patients within each of the three groups showed early FIGO stage, with no differences between them ($P = 0.456$). No changes in FIGO 2021 staging category (early versus advanced stage VSCC) were identified as a result of implementing FIGO 2021 criteria for VSCC staging.

RECURRENCE-FREE AND DISEASE-SPECIFIC SURVIVAL

More than half (64.0%) of the patients with HPV-independent VSCC with HSIL-like lesions recurred,

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Table 2. The pathological variables and the FIGO staging for the three groups of patients with vulvar squamous cell carcinoma according to the human papillomavirus status and type of the lesion adjacent to the invasive tumour

	HPV-associated VSCC with adjacent HSIL (n = 26)	HPV-independent VSCC with adjacent dVIN (n = 54)	HPV-independent VSCC with adjacent HSIL-like lesion (n = 25)	P
Tumour size (mm)	19.9 ± 14.6	27.8 ± 15.4	16.0 ± 14.9	**0.004**
Location [n (%)]				0.143
Central	21 (80.8%)	32 (59.3%)	15 (60.0%)	
Lateral	5 (19.2%)	22 (40.7%)	10 (40.0%)	
Multifocal invasive carcinoma [n (%)]	6 (23.0%)	14 (25.9%)	7 (28.0%)	0.921
Depth of invasion (mm)	5.0 ± 4.1	7.4 ± 5.0	3.8 ± 3.4	**0.003**
Lympho-vascular invasion [n (%)]	5 (19.2%)	9 (16.7%)	1 (4.0%)	0.287
Surgical margin affected by invasive carcinoma [n (%)]	6 (23.1%)	2(3.7%)	3 (12.0%)	**0.029**
Surgical margin affected by premalignant lesion [n (%)]	10 (38.5%)	16 (29.6%)	11 (44.0%)	0.427
Lymph node metastasis [n (%)]*				0.328
Yes (includes ITC)	6 (30.0%)†	15 (29.4%)‡	3 (13.6%)	
No	14 (70.0%)	36 (70.6%)	19 (86.4%)	
FIGO staging [n (%)]				0.456
Early (I/II)	21 (80.8%)	41 (75.9%)	22 (88.0%)	
Advanced (III/IV)	5 (19.2%)	13 (24.7%)	3 (12.0%)	

Bold indicates statistically significant p values.

P-values correspond to multigroup comparison (HPV-associated VSCC with adjacent HSIL versus HPV-independent VSCC with adjacent dVIN versus HPV-independent VSCC with adjacent HSIL-like lesion).

dVIN, Differentiated vulvar intraepithelial neoplasia; FIGO, The International Federation of Gynaecology and Obstetrics; HPV, Human papillomavirus; HSIL, High-grade squamous intraepithelial lesion; ITC, Isolated tumour cells VSCC, Vulvar squamous cell carcinoma.

*12 patients did not undergo surgical staging;

†One patient with isolated tumour cells;

‡Two patients with isolated tumour cells.

whereas recurrence was documented in only 35.0% of HPV-independent VSCC with dVIN and 19.2% of HPV-associated VSCC with HSIL. A large amount of the recurrences in all the three groups occurred locally, in the same area as the previous tumour: 40% (two of five) of recurrences in HPV-associated VSCC with HSIL, 68.4% (13 of 19) of the recurrences in HPV-independent VSCC with dVIN and 68.8% (11 of 16) in the HPV-independent VSCC with HSIL-like lesions. Table 3 shows the prognostic features of the three main groups of patients.

Figure 3 shows the Kaplan–Meier curves for recurrence-free and disease-specific survival rates for the three groups. With a median recurrence-free survival time of 28.6 months, patients with HPV-independent VSCC and HSIL-like adjacent lesions showed a significantly higher tendency to recurrence (P = 0.01). No differences in disease-specific survival were identified between the three groups.

Table 4 shows the results of univariate and multivariate Cox regression analysis for recurrence-free and disease-specific survival. HPV-independent VSCC with HSIL-like lesion and the presence of premalignant lesion in contact with the surgical margins were independent factors for recurrence. Regarding disease-specific survival, no variables reached the preset 0.05 level of significance in univariate nor multivariate analysis. FIGO stage and the presence of

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Table 3. Mean follow-up, local recurrences and death due to disease and mean time to recurrence or death of the three for the three groups of patients with vulvar squamous cell carcinoma according to the human papillomavirus (HPV) status and type of the lesion adjacent to the invasive tumour

	HPV-associated VSCC with adjacent HSIL (n = 26)	HPV-independent VSCC with adjacent dVIN (n = 54)	HPV-independent VSCC with adjacent HSIL-like lesion (n = 25)	P
Mean follow-up (months)	52.5 ± 38.5	56.7 ± 43.8	65.6 ± 44.2	0.49
Recurrence of VSCC [n (%)]	5 (19.2%)	19 (35.0%)	16 (64.0%)	**<0.001**
Mean time to recurrence (months)	17.2 ± 13.6	32.9 ± 42.0	40.78 ± 46.1	0.31
Deaths due to VSCC [n (%)]	2 (7.6%)	8 (14.8%)	4 (16.0%)	0.61
Median time to death (months)	15.01 ± 2.4	11.2 ± 4.5	10.4 ± 3.5	0.61

Bold indicates statistically significant p values.
P-values correspond to multigroup comparison (HPV-associated VSCC with adjacent HSIL versus HPV-independent VSCC with adjacent dVIN versus HPV-independent VSCC with adjacent HSIL-like lesion).
dVIN, Differentiated vulvar intraepithelial neoplasia; HSIL, high-grade squamous intra-epithelial lesion; VSCC, Vulvar squamous cell carcinoma.

lymph node metastases showed the highest hazard ratio for mortality (3.31 and 3.68 respectively, $P = 0.06$), with wide confidence intervals. In spite of its association with recurrence, HPV-independent VSCC with HSIL-like lesion did not have a significant effect on disease-specific survival.

Discussion

In this study, we have explored the prognostic features of patients with HPV-independent VSCC arising on HSIL-like lesions in a relatively large cohort of VSCC surgically treated in a single institution during a 46-year-long period. To our knowledge, this is the first study to assess the prognostic implications of this unusual subset of patients with HPV-independent VSCC arising on a precursor that closely mimics the HPV-associated precursor, HSIL. Although our group first described this lesion in 2009,[18] and further characterised its histological features in a larger series of cases in 2020,[17] the possible prognostic implications of these lesions remained unknown due to the absence of follow-up in both studies.[17,18] The key finding of this study is that HPV-independent VSCC with adjacent HSIL-like lesions have a strong tendency towards recurrence. Importantly, this observation was maintained after adjustment for confounding factors. These data could have major clinical implications in terms of recurrence risk stratification.

The HPV-independent HSIL-like lesions were initially described as an unusual, basaloid variant of dVIN[18] and briefly mentioned in the last 2020 WHO classification in the dVIN chapter.[19] However, herein we show that VSCC arising on these lesions have a number of distinctive clinicopathological features. First, HPV-independent VSCC with HSIL-like lesions are histologically indistinguishable from HPV-associated VSCC both in the invasive tumour and in the adjacent skin precursor, showing mainly basaloid, warty or non-keratinising histology. Secondly, in similarity to HPV-associated VSCC, these tumours tended to be smaller and more superficially invasive than those of dVIN group. Finally, and most remarkably, in spite of being small and superficially invasive tumours, more than half of the patients with HPV-independent VSCC with HSIL-like lesions showed recurrences.

A possible explanation for the smaller size and more superficial invasion of HPV-associated VSCC and HPV-independent VSCC with HSIL-like lesions is their clinical presentation. HSIL and HSIL-like lesions tended to be raised, coalescent lesions, probably causing significant discomfort to the patient and prompting early medical consultation. In contrast with lesions with HSIL features, dVIN lesions were smaller and poorly defined, which might justify a delay in consultation and diagnosis.

In addition to the features similar to HPV-associated tumours with conventional HSIL described

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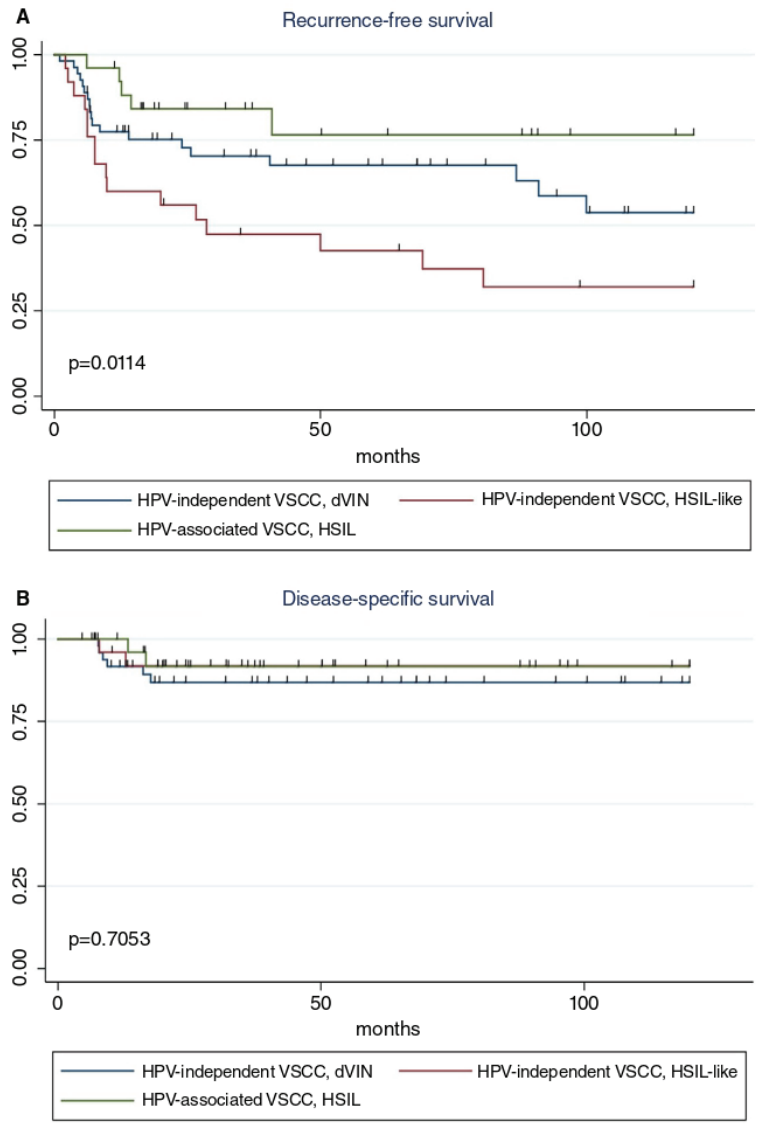

Figure 3. Kaplan–Meier curves for recurrence-free survival (A) and disease-specific survival (B) for the three groups: HPV-associated VSCC with HSIL, HPV-independent VSCC and HSIL-like lesions and HPV-independent VSCC with dVIN.

above, HPV-independent VSCC with HSIL-like lesions show a number of similarities with HPV-independent VSCC arising on dVIN. First, as expected from any HPV-independent VSCC,[2] both groups share high rates of p53 alterations (84.0 and 94.4%) and frequently coexist with adjacent inflammatory lesions (44.0 and 92.6%, respectively), which contrasts with the almost constant wild-type pattern of p53 IHC staining and the absence of inflammatory lesions in HPV-associated tumours. In addition, the patients from the two HPV-independent groups show similar ages (mean age = 75 and 77 years), while patients with HPV-associated VSCC were more than 10 years younger (mean age = 61 years).

Even though we identified no differences in terms of disease-specific survival, HPV-independent tumours with HSIL-like lesions showed a higher recurrence rate in both the univariate and the multivariate analyses. The factors involved in the high recurrence rate of these tumours should be explored in subsequent

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Table 4. Multivariate analysis for recurrence-free survival and disease specific survival

	Recurrence-free survival				Disease-specific survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Study groups								
VSCC with HSIL	1				1			
VSCC with dVIN	1.98 (0.74–5.30)	0.175	2.26 (0.84–6.07)	0.107	1.75 (0.35–8.66)	0.494	1.71 (0.34–8.46)	0.513
VSCC with HSIL-like lesion	3.87 (1.42–10.57)	**<0.001**	3.64 (1.32–9.89)	**0.012**	1.05 (0.15–7.49)	0.958	1.29 (0.18–9.36)	0.802
HPV association								
HPV-associated VSCC	1				1			
HPV-independent VSCC	2.55 (1.00–6.51)	**0.051**	*		1.50 (0.32-7.07)	0.607		
p53 immunohistochemistry								
p53 wild-type	1				1			
p53 mutant	1.83 (0.84–3.98)	0.127			2.01 (0.43–9.47)	0.377		
FIGO stage								
Early FIGO stages (I–II)	1				1			
Advanced FIGO stages (III–IV)	1.16 (0.53–2.52)	0.717			3.31 (0.93–11.77)	0.065	3.22 (0.88–11.75)	0.077
Lymph node metastases								
No	1				1			
Yes	1.29 (0.60–2.79)	0.511			3.68 (0.92–14.81)	0.066	*	
Surgical margin affected by tumour								
No	1				1			
Yes	1.65 (0.69–3.95)	0.257			No events			
Tumour size								
Size <40 mm	1				1			
Size ≥40 mm	0.94 (0.39–2.25)	0.887			1.28 (0.27–6.02)	0.757		
Age at diagnosis (years)								
Age <70	1				1			
Age ≥70	1.22 (0.64–2.35)	0.548			0.91 (0.26–3.22)	0.884		

Bold indicates statistically significant *p* values.
dVIN, Differentiated vulvar intra-epithelial neoplasia; FIGO, The International Federation of Gynaecology and Obstetrics; HR, Hazard ratio; HSIL, High-grade squamous intraepithelial lesion; VSCC, Vulvar squamous cell carcinoma.
*Not included in multivariate analysis because of collinearity.

studies. Strikingly, most of these tumours recurred despite being at initial FIGO stage at diagnosis and with low rates of lymph node involvement, factors usually associated with improved prognosis.[27] Curiously, in a series of patients with superficially invasive VSCC, Preti *et al.*[28] describe a higher recurrence rate in smaller tumours; the authors hypothesise that the potential presence of microscopic satellite

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lesions not clinically visible could be the cause of this observation. Of note, basaloid histology, which was frequent in tumours with HSIL-like adjacent lesion, has been previously associated with aggressive clinical behaviour in other cancer types,[29–31] especially in HPV-negative head and neck squamous cancers.[32] Indeed, basaloid laryngeal squamous cell carcinoma, a predominantly HPV-negative tumour,[33] shows higher rates of recurrence than conventional keratinising tumour.[34]

Although morphological overlap between HPV-associated and -independent VSCC precursors has been described by several authors,[17,18,35,36] a standard terminology for HSIL-like lesions related to HPV-independent VSCC has not been defined due to their poor characterisation and the lack of evidence on their clinical and prognostic implications. The 2021 WHO classification states that HPV-independent precursor lesions can show a basaloid or warty/condylomatous-like morphology simulating HPV-associated HSIL. Due to their morphology closely mimicking HPV-associated HSIL, we prefer the term HSIL-like for the definition of these lesions.

In spite of their HSIL-like morphology, our results suggest HPV-independent VSCC arising on HSIL-like lesions have distinctive clinical behaviour. These tumours can be identifiable using routine haematoxylin and eosin staining and p16/p53 IHC, which are widely available tools in pathology laboratories in settings where HPV-independent VSCC are predominant. Our findings highlight the importance of using basic molecular biomarkers (p53 and p16) for characterisation of VSCC and its precursors. The main strength of this study is the high number of patients with VSCC included, all of them tested for the presence of HPV and stained with p16 and p53 IHC, and especially the accurate clinical information and the long-term follow-up available. The limitations include the retrospective design of the study, the possible selection bias due its single institutional nature and the heterogeneity of treatment strategy related to the long period of inclusion, with different surgical strategies applied in different periods (more radical resection in the earlier cases). Finally, due to the low mortality rate of this cancer, the cohort is probably too small to ascertain statistical differences for disease-specific survival between the three groups.

Conclusion

In conclusion, we show for the first time that HPV-independent VSCC arising on HSIL-like lesions are infrequent but have distinctive clinical, pathological and behavioural features. They are highly recurrent tumours which warrant closer surveillance after surgery. Further prospective studies are needed to determine if this subgroup of patients might benefit from more intensive treatment strategies (i.e. more radical excisions or adjuvant treatment). Importantly, these unusual tumours can be easily identifiable at the pre-malignant stage using routine haematoxylin and eosin staining and p16/p53 IHC. As these tools are widely available in pathology laboratories in settings where HPV-independent VSCC are predominant,[37] these tumours should be routinely reported by pathologists. Advanced age of the patients and the presence of concomitant inflammatory lesions in the adjacent skin should raise the suspicion of this entity to the pathologist. Evaluation of clinicopathological features of these tumours in a larger number of VSCC patients may yield more definitive prognostic information, especially for disease-specific survival.

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Conflict of interest

The authors declare no conflicts of interest.

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Authors contributions

N.C-D.: study design, investigation, methodology, statistical analysis, writing of the first draft, writing and editing; A.S.: investigation, writing and editing; M.d.P.: investigation, writing and editing, statistical analysis; C.P: investigation, writing and editing; R. L. d. C.: investigation, writing and editing; C.M.:

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investigation, writing and editing; A.G.: investigation, writing and editing, L.M.: investigation, methodology, writing and editing; S. D-M.: investigation, writing and editing; P. F.: investigation, writing and editing; M. T. R.-C.: investigation, writing and editing, N.V.: investigation, methodology, writing and editing, A.T.: study design, resources, investigation, methodology, writing and editing, project management; N.R.: study design, resources, investigation, methodology, statistical analysis, writing of the first draft, writing and editing, project management.

Patient consent statement

Written study consent was obtained from all the patients enrolled in the study.

Data availability statement

The study dataset is available upon request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Type of surgical and adjuvant treatment in each of the three study groups.

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4.2. Segundo estudio

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TÍTULO: **Human papillomavirus and p53 status define three types of vulvar squamous cell carcinomas with distinct clinical, pathological and prognostic features**

REVISTA: Histopathology, 2023. 83 (1): 17-30. DOI: 10.1111/his.14925

FACTOR DE IMPACTO: 7,778 (primer cuartil, primer decil)

CATEGORÍA: *Pathology*

TIPO DE PUBLICACIÓN: investigación original

RESUMEN:

Objetivos: La clasificación de la OMS 2020 del carcinoma escamoso de vulva (CEV) divide estas neoplasias en dos tipos: CEV asociado al virus del papiloma humano (VPH) y CEV independiente de VPH. Los tumores independientes de VPH, a su vez, se han dividido recientemente en función de si presentan p53 *wild-type* o mutado. Sin embargo, las implicaciones clínicas y pronósticas de está clasificación no han sido identificadas de forma clara.

Metodología: se identificaron de forma retrospectiva 190 casos de pacientes con CEV tratadas quirúrgicamente en el Hospital Clínic de Barcelona. Se evaluó la detección de VPH y las tinciones inmunohistoquímicas para p16 y p53, definiendo tres grupos de estudio: 1) CEV asociado a VPH (n=33), 2) CEV independiente de VPH con p53 normal (n=20) y 3) CEV independiente de VPH con p53 anormal (n=137). Se analizaron las características histológicas y clínicas, incluyendo la supervivencia libre de enfermedad y la supervivencia por enfermedad en los tres grupos, realizando una regresión de Cox con análisis univariado y multivariado.

Resultados: los dos tipos de tumores independientes de VPH (p53 normal y alterada), además de ser clínicamente e histológicamente distintos, presentaron un mayor riesgo de recidiva que los

tumores asociados a VPH en el análisis multivariado (*hazard ratio* [HR] 3,63; p=0,023 para los tumores p53 normal y HR 2,78; p=0,028 para los tumores con p53 alterada). Aunque las diferencias no alcanzaron la significación estadística, se objetivó una tendencia a una peor supervivencia específica por enfermedad en los tumores independientes de VPH, en comparación con los tumores asociados a VPH. A pesar de presentar mayor riesgo de recurrencia, el CEV independiente de VPH p53 normal no se asoció a un mayor riesgo de muerte por enfermedad que el CEV independiente de VPH con p53 alterado. Únicamente el estadio FIGO avanzado se asoció con un mayor riesgo muerte por enfermedad en el análisis multivariado (HR=2,83; p=0,010).

Conclusiones: el *status* de VPH y de p53 tienen implicaciones clínicas y pronósticas, lo que justifica la clasificación molecular en 3 grupos propuesta para el CEV (CEV asociado a VPH, CEV independiente de VPH con p53 *wild-type* y CEV independiente de VPH con p53 mutado).

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Human papillomavirus and p53 status define three types of vulvar squamous cell carcinomas with distinct clinical, pathological, and prognostic features

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Human papillomavirus and p53 status define three types of vulvar squamous cell carcinomas with distinct clinical, pathological, and prognostic features

Introduction: Based on their etiological relationship with human papillomavirus (HPV), the 2020 WHO classification has divided vulvar squamous cell carcinomas (VSCC) into two distinct types, HPV-associated and HPV-independent, and HPV-independent tumours have recently been divided according to p53 status. Nevertheless, the clinical and prognostic significance of this classification has not been clearly established. We analysed the differential clinical, pathological, and behavioural characteristics of these three types of VSCC in a large series of patients.

Methods and results: VSCC samples from patients who underwent primary surgery at the Hospital Clinic of Barcelona, Spain, during a 47-year period (January 1975 to January 2022) were analysed (n = 190). HPV detection, p16, and p53 immunohistochemical staining were evaluated. We also analysed recurrence-free survival (RFS) and disease-specific survival (DSS). Thirty-three tumours (17.4%) were HPV-associated and 157 (82.6%) HPV-independent. Of these, 20 showed normal and 137 abnormal p53 expression. The two types of HPV-independent tumours showed worse RFS in the multivariate analysis (hazard ratio [HR] = 3.63; P = 0.023 for the HPV-independent p53 normal VSCC and HR = 2.78; P = 0.028 for the HPV-independent p53 abnormal VSCC). Although the differences were not significant, HPV-independent VSCC had worse DSS than HPV-associated VSCC. Although patients with HPV-independent p53 normal tumours had worse RFS than patients with HPV-independent p53 abnormal tumours, the DSS was better for the former group. Only advanced FIGO stage was associated with worse DSS in multivariate analysis (HR = 2.83; P = 0.010).

Conclusion: The association of HPV and p53 status have prognostic implications, reinforcing a three-tier molecular classification of VSCC (HPV-associated VSCC, HPV-independent VSCC with normal p53, HPV-independent VSCC with abnormal p53).

Keywords: HPV, vulvar cancer, vulvar squamous cell carcinoma

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Introduction

Vulvar squamous cell carcinoma (VSCC) represents over 95% of all vulvar malignancies. The 2020 World Health Organization (WHO) classification divides this neoplasia into two distinct pathological types: human papillomavirus (HPV)-associated and HPV-independent, based on their etiological relationship with infection by this oncogenic virus.[1,2] This new classification based on etiological criteria represents a major change compared with previous classifications primarily established according to morphological characteristics.[2]

Several differences support this classification. HPV-associated VSCC typically affects younger women and develops from an intraepithelial precursor named high-grade squamous intraepithelial lesion (HSIL).[3–5] Morphologically, both HPV-associated VSCC and HSIL characteristically show an immature basaloid appearance or warty architecture with koilocytic features.[3,4] Immunohistochemically (IHC), intraepithelial and invasive HPV-associated lesions typically show strong, diffuse, "block type" positivity for p16, and a normal pattern of p53 IHC expression.[6,7] Contrarily, HPV-independent VSCC usually arise in older women, develop from an intraepithelial precursor named differentiated vulvar intraepithelial neoplasia (dVIN), and are commonly associated with chronic inflammatory lesions such as lichen sclerosus and lichen simplex chronicus.[1,3,4] Morphologically, both HPV-independent VSCC and dVIN characteristically have a well-differentiated appearance, with prominent keratinisation. HPV-independent lesions are typically negative for p16, and frequently show an abnormal pattern of p53 IHC expression that correlates with mutated *TP53*.[6,7] Several studies have shown that there is a significant morphological overlap involving not only invasive VSCC, but also intraepithelial proliferations, with occasional HPV-associated lesions showing well-differentiated, keratinizing features and occasional HPV-independent lesions showing an immature, basaloid, or warty architecture.[8–11] Due to this morphological overlap between the two types of VSCC, ancillary tests, such as molecular HPV detection and/or IHC, are required for an adequate classification of these tumours.[2]

Recent reports have suggested that HPV-independent VSCC can be separated into two different categories based on the status of p53: HPV-independent VSCC with wildtype p53 and HPV-independent VSCC with mutated p53, and that these two variants have differential clinical and pathological features.[12–14] The p53 wildtype HPV-independent VSCC are commonly of verrucous type and are preceded by specific precursors named vulvar acanthosis with altered differentiation (VAAD)[15] and differentiated exophytic vulvar intraepithelial lesion (DEVIL),[16] both of which have been recently classified under the term of verruciform acanthotic vulvar intraepithelial neoplasia (vaVIN).[17] In contrast, p53 mutated HPV-independent VSCC are commonly of the keratinizing type and are preceded by dVIN. It has been shown that IHC staining for p53 strongly correlates with the status of the *TP53* gene, with normal patterns of expression indicating a wildtype *TP53* and abnormal patterns correlating with a mutated *TP53*.[12–14]

However, there is limited knowledge of the clinical, pathological, and prognostic features of the two main types of VSCC defined by the WHO classification, i.e. HPV-associated and HPV-independent, and whereas some studies report that HPV-associated VSCC are less aggressive than HPV-independent VSCC, other studies find no significant differences.[18–20] There is controversy over whether HPV-independent carcinomas should be subclassified into two distinct categories: p53 normal (wildtype) and p53 abnormal (mutated), as the number of studies describing HPV-independent tumours as separate categories are very scant. Currently, regardless of HPV and p53 status, all cases of VSCC are equally treated, with surgery (with or without adjuvant radiotherapy) being the main pillar of treatment. Primary chemoradiation is reserved for unresectable lesions or as neoadjuvant treatment in selected patients with advanced-stage disease.[21] Thus, the main objective of this study was to analyse a large series of patients with VSCC and evaluate the clinical, pathological, and behavioural differential characteristics of these three types of VSCC.

Materials and Methods

STUDY DESIGN

We performed a retrospective study in which all VSCC from patients who underwent surgery as primary treatment at the Department of Gynaecological Oncology of the Hospital Clinic of Barcelona, Spain, during a 47-year period (January 1975 to January 2022) were retrieved from the files of the Department of Pathology. In all cases, a thorough revision of the clinical charts and the surgical pathology report was conducted, focusing on age, tumour size, location, uni- or multifocality, lymph node metastases, and International Federation of Gynaecology and Obstetrics (FIGO) staging. All available pathological material was carefully reviewed. The following inclusion criteria were required for this study: (i) histologically confirmed invasive VSCC; (ii) surgery with or without adjuvant radiotherapy as primary treatment; (iii) available paraffin block/s

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representative of the invasive tumour and the adjacent skin with sufficient material for IHC and HPV-DNA testing; and (iv) follow-up time of at least 6 months (or to death). Exclusion criteria were: patients who underwent primary nonsurgical treatment.

Overall, 190 patients with VSCC fulfilling the inclusion criteria were identified. Institutional ethical approval for this study was obtained (registry ref HCB/2020/1198). Informed written consent was obtained from all the patients included in the study.

SELECTION OF THE PARAFFIN BLOCKS FOR IHC AND DNA TESTING

In all cases, HPV-DNA detection by polymerase chain reaction (PCR) and IHC staining for p16 and p53 were performed in formalin-fixed, paraffin-embedded samples (FFPE). A paraffin block showing both invasive tumour and adjacent skin, including any intraepithelial precursors if present, was selected. When no paraffin blocks representative of the two lesions were identified, two different blocks were analysed.

HPV ANALYSIS

The DNA was extracted from whole sections of FFPE blocks. HPV-DNA detection and genotyping were performed using SPF10 PCR and the LiPA25 system (v. 1, Labo Biomedical Products, Rijswijk, The Netherlands). A volume of 10 µl of isolated DNA was PCR-amplified by the INNO-LiPA HPV Genotyping Extra II Amplification (Fujirebio, Gent, Belgium) kit, which was also used for HPV genotyping. This method allows the genotyping of both high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 26, 53, 66, 70, 73, 82) and low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83, and 89).

P16 IMMUNOHISTOCHEMISTRY

p16 IHC staining was performed using a CINtec Histology anti-p16INK4a (clone E6H4) monoclonal mouse antibody (Roche Cat# 705-4793, RRID:AB_2833232; Basel, Switzerland). Diffuse, block-type staining was considered as a positive result, whereas either completely negative or patchy staining was considered as negative for p16.[6,7]

P53 IMMUNOHISTOCHEMISTRY

IHC for p53 was performed using the CONFIRM anti-p53 (clone DO-7) primary monoclonal antibody (Roche Cat# 05278775001, RRID:AB_2892528). The results were evaluated using the recently described IHC pattern-based interpretation framework.[22,23] Staining was evaluated independently in the invasive tumour and in the adjacent skin. p53 staining patterns were classified into two major categories: normal and abnormal. Normal (wildtype) patterns, included scattered and mid-epithelial staining patterns, whereas abnormal (mutant) expression, included basal or diffuse overexpression, as well as null and cytoplasmic staining patterns.

CRITERIA OF VSCC CLASSIFICATION

All the study cases were classified into three main groups based on their association with HPV and the pattern of p53 IHC expression. The groups included: (i) HPV-associated VSCC, (ii) HPV-independent p53 normal VSCC, and (iii) HPV-independent p53 abnormal VSCC.

To categorize a tumour as HPV-associated or -independent, both p16 IHC staining and HPV testing were considered. Tumours with positive staining for p16 and/or molecular testing showing high-risk HPV-DNA, were classified as HPV-associated. The inclusion of a tumour as HPV-independent required negative p16 IHC staining and the absence of high-risk HPV DNA. These HPV-independent tumours were subclassified as p53 normal and p53 abnormal based on the IHC patterns previously described.

HISTOLOGICAL REVISION OF THE INVASIVE TUMOUR

All haematoxylin and eosin (H&E)-stained slides from each tumour were reviewed by two gynaecological pathologists with expertise in vulvar pathology (N.R., A.S.). The histological variant of the invasive VSCC (keratinizing, nonkeratinizing, basaloid, warty, and verrucous) was recorded.[2] The evaluation was conducted blinded to HPV testing and p16 and p53 IHC results.

EVALUATION OF THE ADJACENT SKIN

The skin adjacent to the invasive tumour was reviewed in all cases. The presence and type of intraepithelial lesion (if present), as well as its presence in the resection margin were carefully evaluated and recorded. We specifically looked for intraepithelial precursors, including HSIL,[2] dVIN,[24,25] and vaVIN (VAAD/DEVIL).[15–17] The presence of inflammatory dermatoses (lichen simplex chronicus, lichen sclerosus, and lichen planus) was also recorded.[26] HSIL was

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diagnosed when full-thickness epithelial atypia, high nuclear-to-cytoplasmic ratio, and marked nuclear pleomorphism was identified.[3] dVIN was diagnosed based on significant basal atypia with preserved maturation in the upper layers.[27,28] Acanthotic and/or verruciform architecture, altered squamous differentiation with absent cytological atypia, in the context of negative or patchy p16 and normal p53 staining were used as criteria for the diagnosis of vaVIN (VAAD/DEVIL).[1,17]

TREATMENT AND FOLLOW-UP

All patients underwent surgical treatment with vulvectomy or wide local excision of VSCC lesions. In 1998, our centre started to perform sentinel lymph node biopsy followed by inguinofemoral lymph node dissection in order to validate the technique.[29] In 2002, we began to perform sentinel lymph node biopsy exclusively in patients with unifocal VSCC measuring <4 cm. When a positive sentinel lymph node was identified, ipsilateral inguinofemoral lymph node dissection was performed. Adjuvant radiotherapy and chemotherapy were administrated in accordance with the latest clinical guidelines for the treatment of VSCC prevailing at the time of diagnosis.

The patients were followed in accordance with the protocols of our centre with physical examination every 4–6 months for the first 2 years and annually afterwards. During follow-up, imaging techniques (magnetic resonance imaging, inguinal ultrasound, or computed tomography scan) were periodically performed in patients with advanced stage VSCC or when recurrence was suspected.

Information on surgical and adjuvant treatment, relapse of disease, and causes of death was retrieved from the clinical charts. All cases were retrospectively restaged according to FIGO 2021 criteria.

STATISTICAL ANALYSIS

StataIC/v15.0.591 was used for all the data analyses. The clinical and histopathological data were compared among the different groups of VSCC using χ^2 tests (categorical data) and analysis of variance, ANOVA (numerical data).

Endpoints of the study were recurrence-free survival (RFS) and disease-specific survival (DSS). RFS was defined as the time from treatment (primary surgery) until the patient survived without any sign of disease recurrence (local or distant) or the date of the last follow-up. DSS was calculated as the time from the date of primary surgery to the date of death by VSCC or the date of the last follow-up. Survival analyses were conducted using the Kaplan–Meier method and survival curves were compared using the log-rank test. Survival data are expressed as median RFS and DSS. Given that these data were not computable in all study groups because the survival probability did not reach 50%, we also computed the 10-year recurrence and mortality rates (and compared them with a χ^2 test). Univariate Cox regression was performed to evaluate the role of HPV and p53 status in RFS and DSS. Multivariate Cox regression was used to assess possible confounding factors involved in RFS and DSS; besides the three study groups, all variables with significance in the univariate analysis were included in the multivariate analysis. The results were statistically significant when $P < 0.05$.

Results

CLASSIFICATION OF THE VSCC INTO THE THREE PATHOLOGICAL TYPES

Of the 190 patients with VSCC included in the study, 33 (17.4%) were classified as HPV-associated and 157 (82.6%) were HPV-independent. The 157 HPV-independent VSCC were subclassified as HPV-independent VSCC with normal p53 IHC expression (20 tumours, 10.5% of the total cohort of patients), and HPV-independent VSCC with abnormal p53 IHC expression (137 tumours, 72.1% of the total cohort of patients).

HPV TESTING AND P16 AND P53 IHC EXPRESSION

All HPV-associated VSCC showed diffuse overexpression of p16. HPV testing was positive in 30/33 (90.9%) and negative in 3 (9.1%) HPV-associated tumours. Single HPV types were identified in 25 tumours (66.7%), whereas multiple types were detected in five cases (15.6%, four cases with two HPV types and one with four HPV types). HPV16 was the most frequently identified type (23 [69.7%] tumours, including 18 single and all five multiple infections). HPV33 was identified in four tumours (12.1%, all single infections) and HPV56 in three carcinomas (9.1%, two single infections, one multiple infection). HPV6, HPV42, HPV53, and HPV66 were identified only in multiple infections, in addition to HPV16. p53 showed a normal pattern of expression in 32/33 (96.9%, 22 [66.7%] cases showing a scattered pattern and 10 [30.3%] a mid-epithelial pattern) HPV-associated VSCC and an abnormal pattern in one (3.0%, diffuse overexpression). The latter case

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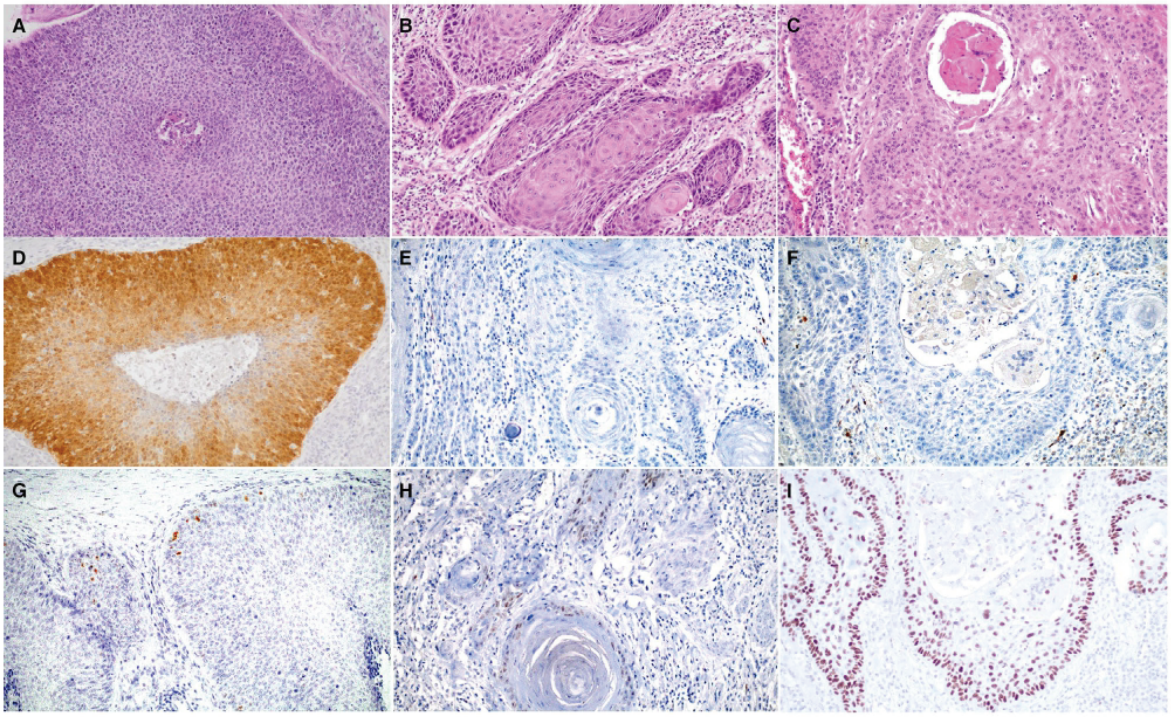

Figure 1. Histological (haematoxylin and eosin) and p16 and p53 immunohistochemical (IHC) expression of a typical example of HPV-associated VSCC (first column), HPV-independent VSCC with normal p53 IHC (second column) expression and HPV-independent VSCC with abnormal p53 IHC expression (third column; **A–C**, haematoxylin and eosin; D–F p16 IHC; **G–I**, p53 IHC).

was positive for high-risk HPV (HPV16), showed basaloid histology, was FIGO IA stage and had favourable behaviour (no recurrence or death because of disease), in similarity with the remaining HPV-associated VSCC.

All HPV-independent VSCC showed negative staining for p16. HPV detection was negative in 155/157 (98.7%) HPV-independent tumours and positive in 2/157 (1.3%). These two cases were single type infections caused by low-risk HPV types (HPV6 and HPV61; one case each). p53 showed a normal pattern of expression in 20/157 HPV-independent VSCC (12.7%, all cases showing a scattered pattern) and an abnormal pattern in 137 (87.3%). In the subset of HPV-independent tumours with abnormal p53 IHC, diffuse overexpression was the most frequent pattern (92/137; 67.6%), followed by a null pattern (20/137; 14.6%), basal overexpression (15/137; 10.9%), and cytoplasmic expression (10/137; 7.3%). The two HPV-independent VSCC with a positive low-risk HPV-DNA test showed an abnormal p53 IHC; all HPV-independent tumours with normal p53 IHC staining had a negative result in the HPV testing.

Figure 1 shows the H&E stain, as well as the p16 and p53 IHC staining of a representative example of each category group defined in the study.

TREATMENT

All patients were treated with primary surgery, either vulvectomy (85 women, 44.7%) or wide local excision (105 women, 55.3%). One hundred sixty patients (84.2%) underwent surgical lymph node evaluation either by sentinel lymph node biopsy ($n = 62$), inguinofemoral lymph node dissection ($n = 54$), or both sentinel and inguinofemoral dissection ($n = 44$). There were no differences in the type of surgery (vulvectomy versus wide local excision, $P = 0.09$) or in the type of lymph node evaluation (sentinel lymph node biopsy versus inguinofemoral lymph node dissection versus both procedures, $P = 0.26$) between study groups. Among the 30 patients who did not undergo surgical lymph node evaluation, seven patients presented FIGO stage IA VSCC (negativity of lymph nodes was assumed), and 23 patients did not undergo surgical nodal evaluation because of poor performance status.

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Table 1. Clinical features and prognostic factors of the three pathological types of vulvar squamous cell carcinomas (VSCC): Human papillomavirus (HPV)-associated, HPV-independent with normal p53 expression and HPV-independent with abnormal p53 expression

		HPV-independent VSCC		
	HPV-associated VSCC (n = 33)	With normal p53 (n = 20)	With abnormal p53 (n = 137)	P
Age (years)	63.6 ± 15.9	76.7 ± 14.8	75.8 ± 11.1	**<0.001**
Tumour size (mm)	20.5 ± 15.3	33.7 ± 19.4	28.3 ± 18.3	**0.024**
Surgical margin affected by invasive tumour	6 (18.2)	0 (0.0)	10 (7.3)	**0.046**
Surgical margin affected by premalignant lesion	12 (36.4)	7 (35.0)	37 (27.0)	0.485
Location				
Central	26 (78.9)	13 (65.0)	81 (59.1)	0.108
Lateral	7 (21.2)	7 (35.0)	56 (40.8)	
Multifocal carcinoma	6 (18.2)	6 (30.0)	28 (20.4)	0.607
Lymph node metastasis n (%)				
Yes	8 (24.2)	5 (25.0)	47 (34.3)	0.219
No	18 (54.6)	11 (55.0)	78 (56.9)	
Not assessed (surgically)	7 (21.2)	4 (20)	12 (8.8)	
FIGO staging n (%)				
Early (I/II)	26 (78.79)	16 (80.0)	92 (67.6)	0.260
Advanced (III/IV)	7 (21.2)	4 (20.0)	45 (32.8)	

FIGO, International Federation of Gynaecology and Obstetrics.
Bold values indicate significant of $P < 0.05$.

There were no differences in the proportion of patients who did not undergo surgical nodal evaluation among the study groups. Sixty-three (34.2%) patients received adjuvant radiotherapy and nine (4.7%) adjuvant chemotherapy. There was no significant difference in the proportion of patients receiving adjuvant treatment (30.3% versus 15.0% versus 37.2%; $P = 0.131$) in HPV-associated and HPV-independent VSCC with normal and abnormal p53. Sixteen patients (8.4%) who should have received radiotherapy according to current guidelines[21,30] did not, but the differences in this proportion were not significant among the study groups.

CLINICAL FEATURES

Table 1 shows the clinical features and prognostic factors of the three types of VSCC. Patients with HPV-associated VSCC were significantly younger than those with HPV-independent (p53 normal and abnormal) tumours. HPV-associated VSCC were smaller and less deeply invasive than the two types of HPV-independent tumours. No differences in terms of percentage of HPV-associated and -independent tumours were observed over the time lapse of the study.

Among patients with surgical evaluation of lymph nodes, 60 (35.9%) presented lymph node metastases (including four cases of isolated tumour cells). One hundred thirty-four patients (70.5%) showed an early FIGO 2021 stage (I or II), whereas 56 (29.5%) were diagnosed at an advanced FIGO 2021 stage. No significant differences were observed among the three groups in terms of percentage of cases with metastatic lymph nodes or advanced FIGO stage, or involvement of the surgical margins by premalignant lesion (Table 1).

HISTOLOGICAL SUBTYPES AND PROGNOSTIC FACTORS

The histological features of VSCC and the associated lesions identified in the adjacent skin are shown in

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Table 2. Histological features of the three pathological types of VSCC (vulvar squamous cell carcinomas): human papillomavirus (HPV)-associated, HPV independent with normal p53 expression and HPV-independent with abnormal p53 expression

	HPV-associated VSCC (n = 33)	HPV-independent VSCC		P
		With normal p53 (n = 20)	With abnormal p53 (n = 137)	
Histological type				
Keratinizing	5 (15.2%)	12 (60.0%)	107 (78.1%)	<0.001
Verrucous	0 (0.0%)	5 (25.0%)	5 (3.7%)	
Basaloid	19 (57.6%)	2 (10.0%)	14 (10.2%)	
Warty	3 (9.1%)	1 (5.0%)	6 (4.3%)	
Mixed basaloid/warty	4 (12.1%)	0 (0.0%)	1 (0.7%)	
Non-keratinizing	2 (6.1%)	0 (0.0%)	4 (2.9%)	
Depth of infiltration (mm)	5.5 ± 4.5	4.9 ± 3.1	7.7 ± 5.4	**0.012**
Lympho-vascular invasion	6 (18.2%)	3 (15.0%)	22 (16.1%)	0.944
Intraepithelial precursors				
dVIN	0 (0.0%)	7† (35.0%)	75‡ (54.7%)	**<0.001**
HSIL	28* (84.8%)	0 (00.0%)	0 (0.0%)	
vaVIN (VAAD/DEVIL)	0 (0.0%)	6 (30.0%)	0 (0.0%)	
No intraepithelial precursor	5 (15.2%)	7 (35.0%)	62 (45.2%)	
Associated inflammatory lesions				
Lichen sclerosus	0 (0.0%)	10§ (52.6%)	59¶ (43.1%)	**<0.001**
Lichen simplex chronicus	0 (0.0%)	1 (5.3%)	14** (10.2%)	
Lichen planus	0 (0.0%)	1 (5.3%)	2 (1.5%)	
No lesion	33 (100%)	7 (36.8%)	62 (45.3%)	

dVIN, differentiated vulvar intraepithelial neoplasia; vaVIN, verruciform acanthotic vulvar intraepithelial neoplasia; VAAD, vulvar acanthosis with altered differentiation; DEVIL, differentiated exophytic vulvar intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.
Bold values indicate significant of $P < 0.05$.
*Two lesions with dVIN-like features were identified in this group.
†Four lesions with HSIL-like features were identified in this group.
‡Twenty-one lesions with HSIL-like features were identified in this group.
§Two cases showed lichen sclerosus and lichen simplex chronicus.
¶Seven cases showed lichen sclerosus and lichen simplex chronicus.
**One case showed lichen simplex chronicus and lichen planus.

Table 2. Twenty-eight out of 33 (84.8%) of the HPV-associated tumours and only 28/157 (17.8%) HPV-independent tumours were of basaloid, warty, mixed warty/basaloid, or nonkeratinizing subtypes ($P < 0.001$). Among the HPV-independent tumours, 75% (119/157) were of the keratinizing subtype. Five out of the 20 (25%) HPV-independent tumours with p53 normal staining were verrucous carcinomas.

HPV-associated VSCC more frequently showed an intraepithelial precursor in the adjacent skin than HPV-independent carcinomas (28/33 [93.9%], versus 88/157 [56.1%]; $P = 0.002$). HSIL was identified in 28/33 (84.8%) HPV-associated VSCC and in all cases showed strong and diffuse positive staining for p16. The most frequent precursor in HPV-independent VSCC was dVIN (82/157 [52.2%]). A high frequency (30.0%) of vaVIN (VAAD/DEVIL) lesions were identified in the group of HPV-independent p53-normal VSCC. All precursors identified in HPV-independent VSCC were negative for p16.

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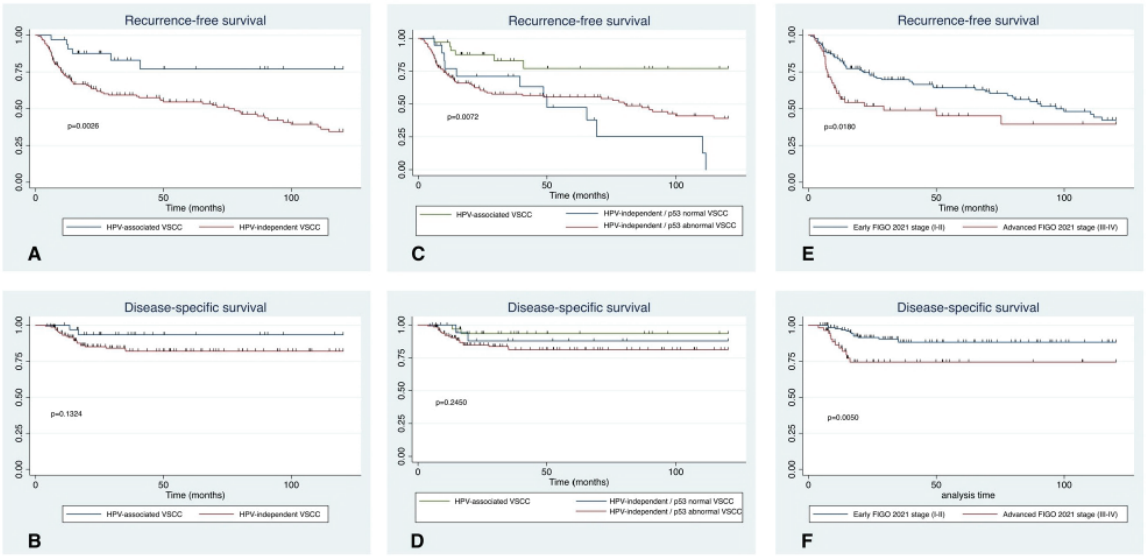

Figure 2. Kaplan–Meier curves for recurrence-free survival (RFS) and disease-specific survival (DSS) for HPV-associated VSCC, HPV-independent VSCC with normal p53 immunohistochemical expression and HPV-independent VSCC with abnormal p53 immunohistochemical expression (**A**–**D**), and FIGO initial stages (I or II) versus FIGO advanced stages (III or IV) (**E**,**F**).

No inflammatory skin lesions were identified in HPV-associated VSCC, whereas a high percentage of these conditions, mainly lichen sclerosus, were identified in HPV-independent carcinomas.

CLINICAL OUTCOMES

The mean follow-up duration was 53.1 ± 43.5 months. There were no differences in terms of time of follow-up among groups ($P = 0.932$). Figure 2 shows the Kaplan–Meier curves for RFS and DSS for HPV-associated versus HPV-independent VSCC with normal and abnormal p53 IHC expression tumours, and early FIGO versus advanced FIGO stages. With a median RFS of 50 months, patients with HPV-independent p53 normal VSCC showed the worst RFS in the log rank test, followed by patients with HPV-independent p53 mutant VSCC and by those with HPV-associated VSCC. Although the differences were not statistically significant, HPV-independent VSCC showed a tendency to a worse DSS than HPV-associated VSCC. Remarkably, patients with HPV-independent normal p53 tumours had a better DSS than patients with HPV-independent abnormal p53 tumours, in spite of having a worse RFS. Patients with an advanced FIGO stage showed a significantly worse DSS.

The 10-year recurrence rate of patients with HPV-associated VSCC was significantly lower than that of patients with HPV-independent abnormal p53 and p53 normal carcinomas (6/33 [18.8%] versus 67/137 [48.9%] versus 12/20 [60.0%], $P = 0.002$). Patients with HPV-independent p53 abnormal VSCC presented the highest mortality rate, followed by those with HPV-independent p53 normal VSCC, and HPV-associated VSCC, although the differences did not reach statistical significance (21/137 [15.3%] versus 2/20 [10%] versus 2/33 [6.1%], $P = 0.334$).

The univariate and multivariate analyses for RFS and DSS are shown in Table 3. In the univariate analysis, age at diagnosis older than 80, HPV-independent tumours (both p53 normal and abnormal), advanced FIGO 2021 stage, the presence of metastatic lymph nodes and surgical margins affected by premalignant lesion were associated with a worse RFS. HPV-independent p53 normal tumours showed a higher hazard ratio for recurrence (3.63, $P = 0.023$) than HPV-independent abnormal p53 tumours (2.78, $P = 0.028$). In the multivariate analysis, the two types of HPV-independent tumours, metastatic lymph nodes, and surgical margins affected by premalignant lesion were associated with impaired RFS. Regarding DSS, an advanced FIGO 2021 stage and metastatic lymph nodes were associated with worse prognosis in the univariate analysis, and an advanced FIGO stage was associated with worse DSS in the multivariate analysis.

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Figura 2 (detalle)

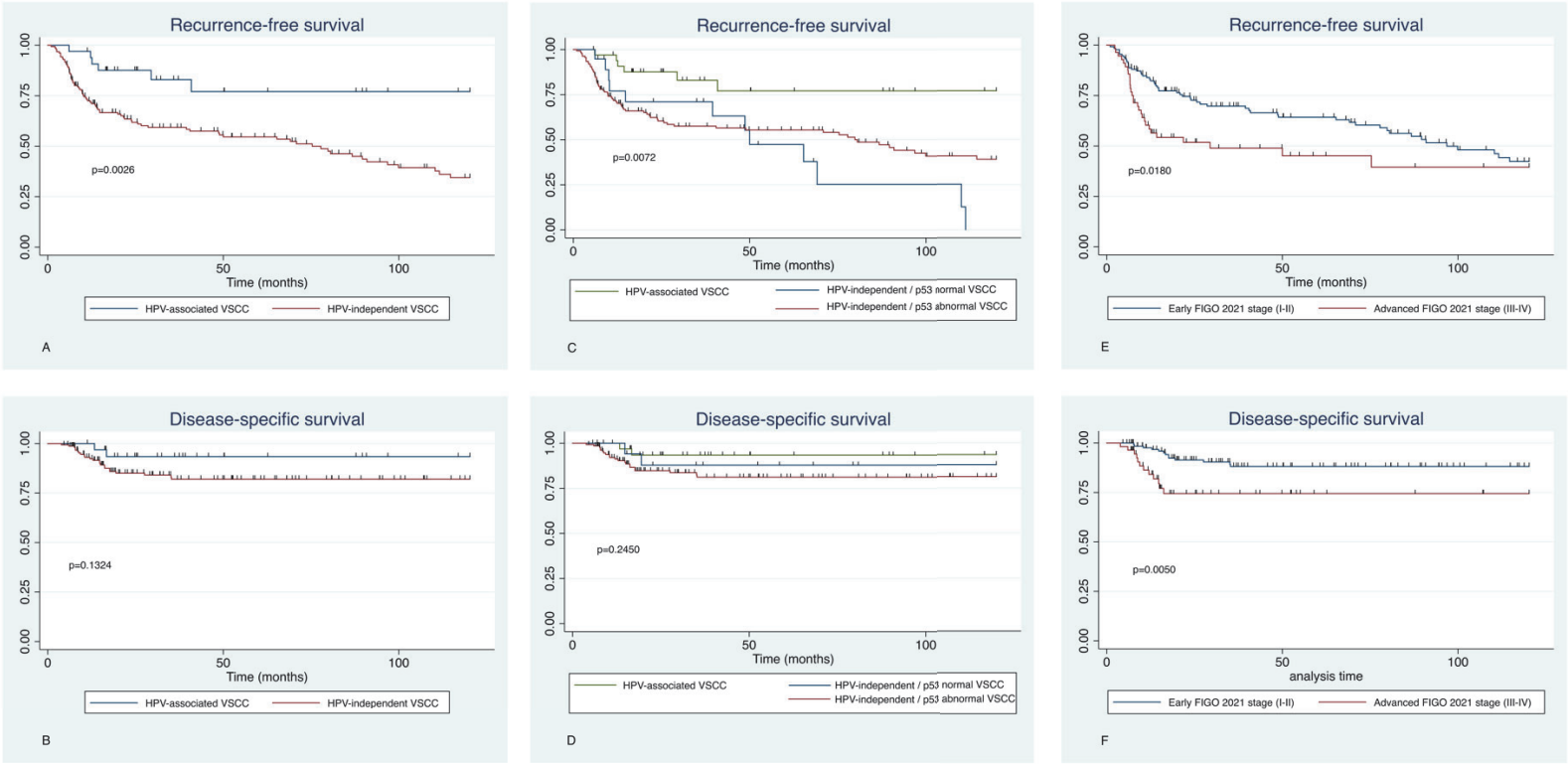

Figure 2. Kaplan–Meier curves for recurrence-free survival (RFS) and disease-specific survival (DSS) for HPV-associated VSCC, HPV-independent VSCC with normal p53 immunohistochemical expression and HPV-independent VSCC with abnormal p53 immunohistochemical expression (**A**–**D**), and FIGO initial stages (I or II) versus FIGO advanced stages (III or IV) (**E**,**F**).

Table 3. Univariate and multivariate analysis hazard ratios for recurrence-free survival and disease-specific survival

	Recurrence-free survival				Disease-specific survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
VSCC type								
HPV-associated	1				1			
HPV-independent with normal p53	4.17 (1.56–11.12)	**0.004**	3.32 (1.10–10.04)	**0.034**	1.84 (0.26–12.05)	0.543	1.82 (0.26–12.92)	0.549
HPV-independent with abnormal p53	3.21 (1.39–7.41)	**0.006**	2.78 (1.11–6.94)	**0.028**	3.05 (0.71–13.01)	0.132	2.85 (0.67–12.19)	0.157
FIGO stage								
Early (stage I-II)	1				1			
Advanced (stage III-IV)	1.72 (1.09–2.69)	**0.019**	*		2.94 (1.3–6.47)	**0.007**	2.83 (1.29–6.23)	**0.010**
Lymph node metastases								
No	1				1			
Yes	1.90 (1.18–3.06)	**0.008**	1.85 (1.14–2.96)	**0.012**	3.36 (1.41–8.02)	**0.006**	*	
Surgical margin affected by tumour								
No	1				1			
Yes	1.24 (0.60–2.57)	0.561			0.47 (0.06–3.44)	0.453		
Surgical margin affected by premalignant lesion								
No	1				1			
Yes	1.56 (1.01–2.42)	**0.046**	1.70 (1.05–2.74)	**0.030**	0.90 (0.38–2.16)	0.816		
Lympho-vascular invasion								
No	1				1			
Yes	1.55 (0.88–2.73)	0.130			2.15 (0.85–5.40)	0.104		
Size								
Less than 25 mm	1				1			
More than 25 mm	1.38 (0.89–2.12)	0.147			1.97 (0.49–4.36)	0.093		

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Table 3. (*Continued*)

	Recurrence-free survival				Disease-specific survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Depth of invasion								
Less than 5 mm	1				1			
More than 5 mm	1.40 (0.91–2.16)	0.124			1.59 (0.71–3.55)	0.255		
Age at diagnosis								
Younger than 80 years	1				1			
Equal or older than 80 years	1.56 (1.00–2.45)	**0.049**	1.51 (0.91–2.50)	0.108	1.41 (0.63–3.14)	0.401		

VSCC, vulvar squamous cell carcinomas; FIGO, International Federation of Gynaecology and Obstetrics; HPV, human papillomavirus; HR, hazard ratio.
Bold values indicate significant of $P < 0.05$.
*Not included in multivariate model because of collinearity.

Discussion

Our study confirms that HPV-associated and HPV-independent VSCC are different entities, not only in terms of clinical and pathological features, but also in terms of prognosis. Thus, the results of our study support the WHO 2020 classification that has divided this neoplasia into two distinct types based on their etiological relationship with HPV.[1,2] Our results are also in keeping with recent reports indicating that HPV-independent VSCC should be separated into two different types based on the status of p53: HPV-independent VSCC with wildtype (normal p53 IHC expression) and with mutated p53 (abnormal p53 IHC expression), because these two pathological types have differential clinical and pathological features and a different behaviour.[12–14]

As shown in previous studies, HPV-associated VSCC are clinically distinct: they arise in younger women and are smaller and less invasive at diagnosis than HPV-independent VSCC.[12,14,31] These parameters have classically been identified as prognostic factors in VSCC.[12,32] Remarkably, in spite of the small size and superficial invasion observed in HPV-associated tumours, no differences were observed in terms of lymph node metastases or FIGO staging at diagnosis when compared to HPV-independent tumours, indicating the potential aggressiveness of the HPV-associated tumours.

HPV-associated and HPV-independent tumours were also histologically different: most HPV-associated tumours showed basaloid or warty features, whereas most HPV-independent tumours were of the keratinizing variant.[3,11] In addition, each tumour type was associated with a specific precursor lesion: HSIL for the HPV-associated, vaVIN (VAAD/DEVIL) for the HPV-independent tumours with normal p53, and dVIN for the HPV-independent tumours with abnormal p53. However, a significant percentage of tumours showed paradoxical morphological features in the invasive tumour (15% of HPV-associated tumours presented keratinizing morphology and 20% of HPV-independent VSCC showed basaloid features) as well as in the precursor lesion, emphasizing the need for ancillary techniques (HPV-DNA detection and/or p16 IHC) to confidently classify VSCC.[11] Interestingly, a high proportion (25%) of patients with HPV-independent p53 normal tumours had a verrucous carcinoma, a subtype of VSCC rarely exhibiting lymph node metastasis or fatal outcomes.[15,33,34]

The most relevant results of this study are related to the prognosis of the three pathological types. Several studies have evaluated the relationship between HPV

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and prognosis in VSCC with controversial results. While some authors have reported that HPV-association is not a prognostic factor,[19,35] recent studies indicate that HPV-associated VSCCs have an improved prognosis.[14,20,31] Curiously, a previous study by our group did not identify differences in prognosis between HPV-associated and -independent VSCC.[18] However, this study included a lower number of patients with a shorter median follow-up time (3.8 years), and classification of the HPV status of VSCC was exclusively based on HPV testing. In the present study we included a large series of patients treated primarily with surgery at a single institution and with a long follow-up period. Women with HPV-associated tumours had a better RFS in the univariate and the multivariate analyses. Some authors suggest that a possible explanation for this phenomenon lies in the better response of the HPV-associated tumours to radiotherapy.[12,31] Indeed, it has been shown that HPV-associated VSCC present better response to radiotherapy,[36,37] a phenomenon which has also been described in HPV-associated tumours of the head and neck.[37,38]

The status of the surgical margins and the radicality of the surgical excision may have an impact on the risk of recurrence, which might lead to bias.[39,40] Interestingly, McAlpine *et al.*[31] noted no differences in outcome between HPV-associated and -independent VSCC for the cohort of patients treated before 1995, when treatment was mainly radical surgery, whereas HPV-independent tumours showed worse prognosis in recent years, when more conservative surgical strategies have been adopted.[31] In our cohort, the proportion of patients who underwent vulvectomy versus wide local excision were not different between the study groups ($P = 0.09$), but patients with HPV-associated VSCC showed a higher rate of margin involvement by invasive carcinoma. In univariate analysis, surgical margin affected by the tumour was not associated with worse RFS or DSS, but surgical margin affected by premalignant lesion was associated with worse RFS both in the univariate and the multivariate analysis. This association has been previously reported by other authors[41,42] and highlights the importance of completely excising the premalignant lesion in addition to the invasive tumour.

The different etiopathogenic pathways involved in HPV-associated and -independent VSCC[3] confer biological plausibility to the differences identified in prognosis, with different molecular alterations involved in each diagnostic category.[43] A large study based on next-generation sequencing has shown that alterations in the PI3K/mTOR pathway are involved in HPV-associated VSCC, while HPV-independent tumours harbour mutations in *TP53*, *TERT*, *CDKN2A*, and *CCND1*.[14,44–47] Although some of these studies have evaluated the prognostic role of the above-mentioned mutations,[14,45,48] the limited number of tumours included in these molecular studies precludes reaching strong conclusions. However, in our study only lymph node metastases and an advanced FIGO stage (III or IV) were associated with a worse DSS.

Several recent studies have suggested that HPV-independent VSCC could be divided into two distinct clinical-pathological and molecular entities, HPV-independent p53-normal tumours and HPV-independent p53-abnormal tumours.[12–14] Morphologically, the former group of VSCC has been associated with vaVIN (VAAD/DEVIL) precursor lesions.[14,16,17,48] Moreover, some authors have molecularly described this subgroup of tumours that have a higher frequency of mutations in *PIK3CA*, *NOTCH1*, and *HRAS*, suggesting that they might have a specific oncogenic pathway.[14,48] Interestingly, in our study patients with HPV-independent, p53 normal tumours, had a high recurrence rate—which is in contrast with the results of the series reported by Nooij *et al.*,[14] who described a lower recurrence rate in patients with HPV-independent, normal p53 tumours than in patients with tumours with abnormal p53. Remarkably, in spite of having a worse RFS, the patients with HPV-independent p53 normal tumours had a better DSS than those with HPV-independent p53 abnormal tumours, indicating that this subgroup might have an intermediate prognosis.

The findings of our study raise the question as to whether all patients with VSCC should undergo the same treatment and follow-up. This is the current approach in most institutions, where all patients with VSCC are treated similarly, irrespective of HPV and p53 status, and with surgery being the main pillar of treatment.[30] Increasing evidence indicating that several types of VSCC have different prognosis and different response to radiation therapy, plus the feasibility of presurgical evaluation of HPV, p16, and p53 status, based on IHC and molecular HPV analysis,[11,22,23,49] suggest that the treatment of VSCC might be stratified based on the different risk in the near future. To the best of our knowledge, no prospective studies have evaluated the possibility of tailoring VSCC treatment in accordance with HPV or p53 status. However, several investigators[12,50] consider HPV or p53 status when assessing the response to treatment of patients with VSCC.

The single HPV-associated VSCC with p53 abnormal diffuse overexpression is intriguing, as p53

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protein is usually degraded in HPV-driven cancers.[51] In this tumour both a high-risk HPV PCR positive result and p16 overexpression were present and, thus, the HPV-associated status seems to be unquestionable. A similar case of p16-positive and abnormal p53 with diffuse overexpression has been identified in a recent series of vulvar cancers.[13] In addition, sequencing studies have also reported occasional HPV-associated VSCC with *TP53* mutations.[14,52] The favourable clinical behaviour of this tumour might indicate that p53 alteration does not confer an adverse outcome in HPV-related VSCC. The main strength of our study is that it is one of largest series of patients with VSCC treated in a single centre in which the prognostic role of HPV and p53 IHC status have been evaluated.

The study also has some limitations. Its retrospective nature and the wide inclusion period of the study might have led to information bias. Indeed, although all patients were treated in accordance with our cancer centre protocols, these were modified during the study period, and this could have affected the outcomes. Nonetheless, the frequency of these patients was balanced among groups.

In conclusion, our results suggest that the association of HPV and p53 status have clinical and prognostic implications, reinforcing the three-tier molecular classification of VSCC, HPV-associated VSCC, HPV-independent VSCC with normal (wildtype) p53, HPV-independent VSCC with abnormal (mutated) p53, described by other authors. Further prospective studies are needed to explore the possibilities of tailoring the treatment of VSCC patients in accordance with these molecular subgroups.

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Authors contributions

Nuria Carreras-Dieguez: study design, investigation, methodology, statistical analysis, writing of the first draft, writing, and editing; Adela Saco: investigation, methodology, writing, and editing; Marta del Pino: investigation, writing, and editing, statistical analysis; Lorena Marimon: investigation, methodology, writing, and editing; Ricardo López del Campo: investigation, writing, and editing; Carolina Manzotti: investigation, writing, and editing; Pere Fusté: investigation, writing, and editing; Clàudia Pumarola: investigation, writing, and editing; Aureli Torné: study design, resources, investigation, methodology, writing, and editing, resources, project management; Adriana Garcia: study design, investigation, writing, and editing, Natalia Rakislova: study design, resources, investigation, methodology, writing, and editing, project management.

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Conflict of interest

The authors declare no conflicts of interest.

Patient consent statement

Study consent was obtained from all the patients enrolled in the study.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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5 | Discusión

Los dos estudios que integran esta tesis doctoral muestran que existen factores histopatológicos en el carcinoma escamoso de vulva (CEV) que tienen relevancia clínica y pronóstica. El primer artículo destaca la importancia pronóstica de la lesión preinvasiva asociada al CEV, centrándose en un subtipo de lesión infrecuente: los tumores independientes del virus del papiloma humano (VPH) con lesiones preinvasivas adyacentes morfológicamente similares a las lesiones intraepiteliales de alto grado (HSIL-*like*), que presentan una importante tendencia a la recurrencia. El artículo hace evidente la superposición de características histológicas que existe entre las lesiones invasivas y preinvasivas asociadas e independientes de VPH y resalta la necesidad de emplear marcadores moleculares (*status* de VPH, p16 y p53) para un correcto diagnóstico de las mismas. El segundo artículo evalúa el rol de VPH y p53 tanto a nivel clínico como pronóstico, confirmando que existen tres subgrupos moleculares de CEV (asociado a VPH, independiente de VPH p53 normal e independiente de VPH p53 alterado), con un comportamiento clínico y pronóstico diferenciado. Adicionalmente, el estudio preliminar sobre las alteraciones moleculares del CEV busca contextualizar molecularmente los dos estudios de investigación original, ofreciendo una revisión de la literatura sobre el panorama molecular del CEV, centrada en sus dos vías de carcinogénesis (asociada e independiente de VPH).

5.1. Lesión premaligna adyacente al carcinoma escamoso de vulva: relevancia clínica y pronóstica

El primer artículo de la tesis doctoral se centra en el estudio de las lesiones premalignas adyacentes al CEV, particularmente de las lesiones HSIL-*like* sobre las que se desarrollan tumores independientes de VPH. Aunque esta entidad ya fue descrita por nuestro grupo en 2009 (37) y caracterizada en una cohorte con mayor número de casos en 2020 (38), las implicaciones pronósticas de este tipo de lesiones no habían sido previamente estudiadas. El principal hallazgo del primer estudio que integra esta tesis es que los tumores independientes de VPH con lesiones premalignas HSIL-*like* tienen una importante tendencia a la recidiva.

Estas lesiones se describieron inicialmente como una variante inusual de dVIN, sin embargo, presentan características clínico-patológicas muy distintas al dVIN convencional. En primer lugar, tanto la lesión premaligna como el tumor son morfológicamente indistinguibles de las lesiones de tipo HSIL y el CEV asociado a VPH, respectivamente; al igual que el CEV asociado a VPH, el CEV independiente de VPH con lesión precursora HSIL-*like* presenta en el momento del diagnóstico un menor tamaño y profundidad de invasión que los casos de CEV independiente de VPH con dVIN adyacente. El menor tamaño y profundidad de invasión del CEV independiente de VPH con lesión precursora HSIL-*like* podría explicarse en parte por su forma de presentación clínica: las lesiones HSIL y HSIL-*like* tienden a ser más sobreelevadas y coalescentes, y más sintomáticas que las lesiones tipo dVIN, que generalmente son menos aparentes clínicamente, con alteraciones más sutiles. Ello podría motivar que la paciente consulte de forma más precoz en los casos de CEV con lesión precursora HSIL o HSIL-*like*.

En cambio, el CEV independiente de VPH con lesión HSIL-*like* presenta también características en común con los tumores independientes de VPH asociados a dVIN. En primer lugar, la edad de presentación de los dos subtipos de tumores es similar (superior a la de las pacientes con tumores asociados a VPH). En segundo lugar, como es esperable en el CEV independiente de VPH, presentan una elevada tasa de alteraciones en p53. En tercer lugar, el CEV independiente de VPH con lesión precursora HSIL-*like* frecuentemente coexiste con lesiones inflamatorias de la vulva, a diferencia de los tumores asociados a VPH.

A nivel pronóstico, aunque no se identificaron diferencias a nivel de supervivencia, el CEV independiente de VPH con lesión precursora tipo HSIL-*like* se asoció a un mayor riesgo de recidiva, tanto en el análisis univariado como en el multivariado. En relación con los resultados del segundo estudio, que se discutirán más adelante, la relativa menor tasa de alteraciones de p53 en el CEV con lesión premaligna HSIL-*like* (84% *versus* 94% en los tumores independientes de VPH con lesión dVIN asociada) es coherente con la mayor tasa de recurrencia que se ha identificado en este subgrupo de tumores. Los CEV con lesión premaligna HSIL-*like* presentaron con frecuencia una histología basaloide, que en tumores de otras localizaciones, como los tumores independientes de VPH de cabeza y cuello, se ha asociado a un comportamiento clínico más agresivo (64–68). Por ejemplo, el carcinoma de células escamosas de laringe de tipo basaloide, un tumor

predominantemente independiente de VPH, presenta tasas de recurrencia superiores al tumor queratinizante convencional (68). Se necesitan estudios prospectivos que evalúen si las pacientes con CEV independiente de VPH con lesión precursora HSIL-*like,* por su tendencia a la recidiva, podrían beneficiarse de pautas de seguimiento más intensivas.

Este estudio pone de manifiesto la importancia del uso de marcadores moleculares (determinación de VPH, inmunohistoquímica de p16 y p53) para la caracterización del CEV y sus lesiones precursoras. El solapamiento morfológico entre los precursores de CEV asociados e independientes de VPH ha sido descrito por varios autores (37,38,46,69). Así pues, el diagnóstico del CEV y sus lesiones precursoras no puede basarse exclusivamente en sus características morfológicas, puesto que es imprecisa y la clasificación de los mismos tiene implicaciones clínicas y pronósticas. A parte de la determinación del VPH por PCR, las tinciones inmunohistoquímicas para p16 y p53 (43,45) son herramientas ampliamente disponibles en los laboratorios de anatomía patológica de nuestro medio, donde el CEV independiente de VPH es más prevalente. Ambos han demostrado ser marcadores subrogados fiables de la infección por VPH y las alteraciones en *TP53*, respectivamente (19,21,22,43).

5.2. Diferencias clínicas y pronósticas entre subgrupos de carcinoma escamoso de vulva: rol de VPH y p53

El segundo estudio de la presente tesis doctoral destaca las diferencias clínicas y pronósticas entre el CEV asociado a VPH e independiente de VPH, lo que apoya la nueva clasificación de la OMS 2020, basada en el *status* VPH como primer elemento de clasificación. Como ya han reportado otros estudios, evidenciamos que los tumores asociados a VPH son clínicamente distintos a los independientes de VPH: la edad de presentación es menor, suelen ser de menor tamaño y presentan una menor infiltración en profundidad que el CEV independiente de VPH (18,19,21,70); estas tres características se han considerado tradicionalmente factores pronósticos del CEV (19,57–63). A pesar de estas diferencias clínicas entre el CEV asociado e independiente de VPH, no observamos diferencias en la tasa de metástasis ganglionares o el estadio FIGO entre los dos subtipos de CEV, en concordancia con otros autores (15,18).

Desde el punto de vista histológico, es conocido que el CEV asociado e independiente de VPH son distintos, siendo los primeros de tipo basaloide o condilomatoso y los segundos predominantemente queratinizantes (15,16). Nuestros resultados fueron concordantes con esta asociación, aunque un porcentaje significativo de tumores mostró características histológicas poco habituales (el 15% de los tumores asociados a VPH presentó una morfología queratinizante y el 20% de los tumores independientes de VPH presentó una morfología basaloide). Este hallazgo, que ya había sido descrito con anterioridad por otros autores (10,16), refuerza una vez más la importancia del uso de técnicas auxiliares (detección del ADN del virus y/o tinción inmunohistoquímica para p16) para poder clasificar con seguridad los casos de CEV en uno de los dos grupos propuestos por la OMS.

Los resultados del segundo estudio confirman que el CEV asociado a VPH presenta mejor pronóstico que el CEV independiente de VPH. Aunque en los primeros estudios relativos al rol del VPH en el pronóstico CEV no se hallaron diferencias entre los tumores asociados e independientes de VPH (15,71,72), estudios más recientes han evidenciado diferencias en la supervivencia libre de enfermedad y la supervivencia global entre los dos subgrupos de CEV (18–21,73). En nuestra cohorte, las pacientes con tumores asociados a VPH presentaron mejor supervivencia libre de enfermedad en el análisis univariado y multivariado. A nivel de mortalidad por enfermedad, se objetivó una tendencia a una menor mortalidad en las pacientes con tumores asociados a VPH, que no alcanzó la significación estadística. En tumores de otras localizaciones en los que el VPH también tiene potencial oncogénico, como el cáncer de cuello del útero (74,75) o el de cabeza y cuello (76,77), la asociación a VPH también se ha asociado a mejor pronóstico. Hay autores que sugieren que este fenómeno podría explicarse por una mejor respuesta a la radioterapia de los tumores asociados a VPH (18,19), que ha sido descrita tanto en el CEV (78,79), como en el cáncer de cuello de útero (80) y el cáncer de cabeza y cuello (76).

El estado de los márgenes quirúrgicos (libres o afectos por tumor) y la radicalidad de la cirugía podrían haber tenido un impacto en el riesgo de recidiva (81,82) y, por lo tanto, podrían suponer un sesgo en nuestros resultados. McAlpine *et al*. no identificaron diferencias pronósticas entre tumores asociados e independientes de VPH al realizar un sub-análisis de la cohorte de pacientes tratadas antes de 1995, cuando el tratamiento quirúrgico del CEV era más radical; en cambio, los

tumores independientes de VPH presentaron peor pronóstico en la cohorte de pacientes tratada a partir de 1995, cuando se adoptaron estrategias quirúrgicas más conservadoras (18). En nuestra cohorte de pacientes, la proporción de pacientes que fueron tratadas con vulvectomía radical y escisión local amplia estaba balanceada entre los grupos de estudio, pero las pacientes con tumores asociados a VPH presentaron una mayor tasa de márgenes afectos por el carcinoma invasivo. En el análisis univariado, la presencia de márgenes quirúrgicos afectos por tumor invasivo no se asoció a peor supervivencia libre de enfermedad o supervivencia específica por enfermedad; sin embargo, la presencia de márgenes quirúrgicos afectos por lesión premaligna sí que se asoció a un incremento en el riesgo de recidiva, tanto en el análisis univariado como en el análisis multivariado. Esta asociación ha sido reportada previamente por otros autores (83,84) y subraya la importancia de incluir la totalidad de la lesión premaligna adyacente al tumor dentro de los márgenes quirúrgicos.

Recientemente, algunos autores han propuesto una sub-clasificación de los tumores independientes de VPH en base al estado de p53/*TP53* (*wild-type* o mutado) (19,21,22). Desde un punto de vista morfológico, una elevada proporción de pacientes con CEV independiente de VPH y p53 normal (25%) presentó tumores de tipo verrugoso, un subtipo de CEV que raramente presenta metástasis ganglionares y que se asocia a mejor pronóstico (85–87). Además, las pacientes con CEV independiente de VPH y p53 normal se han asociado a lesiones precursoras tipo VAAD/DEVIL (21,24,88,89). En nuestra cohorte de pacientes, un 30% de los casos de CEV independiente de VPH y p53 normal se asociaron a este tipo de lesión precursora; de hecho, las lesiones precursoras tipo VAAD/DEVIL solo se evidenciaron asociadas a este subtipo de tumores. A nivel molecular, Nooij *et al.*describen que estos tumores con frecuencia presentan mutaciones en *NOTCH1* y *HRAS* (21); posteriormente, Tessier-Cloutier *et al.* reportan que el CEV de tipo verrugoso y sus lesiones precursoras asociadas (DEVIL y VAAD) podrían formar parte del espectro de este subtipo de CEV independiente de VPH p53 *wild-type*, que presenta alteraciones frecuentes en *HRAS* y *PIK3CA*, sugiriendo que este subtipo de CEV podría presentar una vía oncogénica específica (88).

Uno de los hallazgos principales del segundo trabajo fue en relación al impacto pronóstico de esta clasificación en tres subgrupos del CEV (asociado a VPH, independiente de VPH p53 normal e

independiente de VPH p53 alterado): las pacientes con CEV independiente de VPH p53 normal presentaron un pronóstico intermedio, con una elevada tasa de recidiva, pero tendencia a una menor mortalidad por enfermedad que los tumores independientes de VPH con p53 alterado. Estos resultados contrastan con los descritos por Nooij, que describen una menor supervivencia libre de recidiva de los tumores p53 alterado en comparación con los p53 normal, sin hallar diferencias significativas entre los tres grupos en la supervivencia global (21). Woeber *et al.* realizan una comparación entre CEV p16 positivo/p53 normal, p16 negativo/p53 normal y p16 negativo/p53 alterado, y describen diferencias significativas en las tasas de recurrencia y muerte (de menor a mayor, en ambos casos: CEV p16 positivo/p53 normal, p16 negativo/p53 normal y p16 negativo/p53 alterado). En el análisis multivariado, los autores encontraron un riesgo significativamente aumentado en la supervivencia libre de enfermedad de los tumores p53 alterados en comparación con los p53 normales (90). En 2020, Kortekaas *et al.* también evaluaron las diferencias pronósticas entre los tres subtipos de CEV, hallando diferencias significativas en el análisis multivariado únicamente en el riesgo de recurrencia de los tumores independientes de VPH con p53 alterado (19). En un meta-analísis publicado en 2018 (91), se describe un mayor riesgo de mortalidad en las pacientes con p53 mutada (en comparación con p53 *wild-type*, sin tener en cuenta el *status* VPH). Así pues, existe evidencia sobre las diferencias clínicas y pronósticas del CEV asociado a VPH, independiente de VPH con p53 alterado e independiente de VPH con p53 normal; sin embargo, son necesarios más estudios para acabar de caracterizar este último subtipo de CEV. También son necesarios estudios prospectivos para evaluar si las diferencias clínicas y en el pronóstico de estos grupos podrían justificar cambios en las pautas de tratamiento y seguimiento del CEV, adaptándolas a cada grupo.

Las principales fortalezas de los dos estudios de investigación original de la presente tesis doctoral son el gran tamaño muestral incluido, el hecho de que en todas las pacientes incluidas se haya realizado la determinación de VPH y las tinciones inmunohistoquímicas para p16 y p53, así como la información clínica detallada y el largo tiempo de seguimiento disponible. Las principales limitaciones de ambos estudios son su naturaleza retrospectiva, la posibilidad de sesgo de selección al ser unicéntricos y la heterogeneidad de las estrategias terapéuticas empleadas en relación al extenso periodo de inclusión. Así pues, todas las pacientes fueron tratadas de acuerdo

con los protocolos asistenciales de nuestro centro, que fueron cambiando a lo largo del tiempo de estudio, lo que podría haber alterado los resultados. Sin embargo, la proporción de pacientes en cada periodo de tiempo estaba balanceada entre los grupos de estudio (en ambos estudios). Finalmente, debido a la baja prevalencia y baja mortalidad del CEV, la cohorte de pacientes estudiadas probablemente no sea suficientemente grande como para confirmar los hallazgos que apuntan a diferencias en mortalidad específica por enfermedad entre los grupos de estudio.

5.3. Caracterización molecular del carcinoma escamoso de vulva

La revisión sobre el panorama molecular del CEV, incluido como estudio preliminar en esta tesis doctoral, pretendía obtener una visión detallada del estado actual de este campo, con el objetivo de contextualizar molecularmente los dos estudios anteriores y planificar un trabajo de *whole exome sequencing* en CEV que se está desarrollando actualmente por nuestro grupo.

Uno de los principales hallazgos de este primer articulo es que la evidencia sobre las características moleculares del CEV es escasa y muy heterogénea, tanto en cuanto a metodología como a resultados. Así pues, la mayoría de estudios que integran la revisión presentan un tamaño muestral limitado y analizan un número limitado de genes. Los dos estudios en los que se realizó *whole exome sequencing* (92,93) no alcanzan las 50 muestras de CEV en total, mientras que el mayor estudio basado en *next generation sequencing* explora 406 genes (50), menos del 2% de la cobertura del genoma de cualquier estudio con *whole exome sequencing,* lo que podría haber limitado el hallazgo de perfiles moleculares sólidos. Metodológicamente, los 14 estudios incluidos son muy heterogéneos: en el tipo de muestras incluidas (algunos solo incluían casos de CEV, otros también lesiones premalignas, otros CEV recurrente), el tipo de alteraciones estudiadas (mutaciones somáticas, *copy number alterations*, o ambas) y el tipo de análisis molecular realizado (*next generation sequencing* con paneles de genes, *whole exome sequencing*, espectometría de masas Sanger, *Multiplex Ligation-dependent Probe Amplification*), lo que dificulta la comparativa entre ellos. Sin embargo, aunque sus resultados se tengan que interpretar con cautela, la revisión aporta información relevante sobre las alteraciones genómicas más estudiadas y más

frecuentemente identificadas en las pacientes con CEV. Además, la revisión permite contextualizar y entender las dos vías de carcinogénesis del CEV (asociada e independiente de VPH).

En primer lugar, es importante destacar las diferencias moleculares entre los tumores asociados e independientes de VPH. Mientras algunas series sugieren que los tumores independientes de VPH tienen una mayor carga mutacional (21,93), otras sugieren que la carga mutacional no difiere entre tumores asociados e independientes de VPH (50,92). Previsiblemente, son varias las series de casos que identifican *TP53* como el gen más comúnmente alterado en los tumores independientes de VPH (21,50,92–96). Williams *et al.* (50) describen que la mayoría de mutaciones identificadas en la vía de *PI3K/AKT/mTOR* se identificaron en los tumores asociados a VPH. Cabe destacar que la principal limitación de los estudios incluidos es la variabilidad en las estrategias empleadas para realizar la clasificación del CEV asociado e independiente de VPH: la tinción inmunohistoquímica para p16 solo se ha usado en la mitad de los estudios incluidos en la revisión (incluyendo los dos estudios basados en *whole exome sequencing* y en la cohorte más grande de *next generation sequencing*), mientras que la combinación de p16 y la PCR para VPH se ha empleado en menos de la mitad de los estudios, por lo que los resultados deben interpretarse con cautela.

Se identificaron 6 trabajos en los que se analizaron molecularmente las lesiones precursoras de CEV (21,24,51,88,97,98). Esta información resulta de gran utilidad en el estudio de las vías de carcinogénesis del CEV. Varios autores describen una mayor frecuencia de mutaciones somáticas en las lesiones precursoras no asociadas a VPH que en las lesiones precursoras asociadas a VPH (21,51,88). Ello podría explicar el elevado potencial oncogénico de dVIN, a pesar de ser un tipo de lesión histológicamente bien diferenciada (51). Sin embargo, Swarts *et al.* describen un mayor número de *copy number alterations* en las lesiones precursoras asociadas a VPH que en las lesiones independientes de VPH. De forma particular, identifican que las ganancias en el cromosoma 1 constituyen un factor de riesgo de progresión de HSIL a CEV asociado a VPH (98).

Nooij *et al.* describen alteraciones frecuentes en *TP53*, *NOTCH1* y *HRAS* en las lesiones premalignas de CEV, especialmente en las independientes de VPH (21). Zieba *et al.* y Tessier-Cloutier *et al.* también describen *TP53* como el gen más comúnmente alterado en las lesiones precursoras de CEV (51,88), seguido por *CDKN2A* (51). Los autores sugieren que estas alteraciones podrían ser

acontecimientos tempranos en la carcinogénesis del CEV (21,51,88). Cabe destacar que las mutaciones en *NOTCH1* y *HRAS*, descritas por Nooij, se identificaron con frecuencia en ausencia de mutaciones de *TP53*, por lo que podría tratarse de *drivers* de carcinogénesis por una vía independiente de *TP53*, como se ha comentado con anterioridad (21). De forma similar, Tessier-Cloutier *et al*. no identificaron alteraciones en *TP53* asociadas a lesiones tipo DEVIL y VAAD, que mostraron alteraciones en *PIK3CA* y/o *HRAS* (88). En uno de los trabajos incluidos en la revisión, se analiza también el perfil mutacional del liquen simple, en el que no identificaron prácticamente alteraciones moleculares, en concordancia con otros trabajos previos (49,51). Hay autores que sugieren otras causas que promueven la progresión del liquen simple, como las epigenéticas o factores inmunes (99–101).

El papel pronóstico de las alteraciones genómicas no se evaluó en todos los estudios y se limita a las alteraciones identificadas de forma recurrente. Más que identificar el significado pronóstico de alteraciones individuales, la mayoría de estudios identifican diferencias pronósticas entre combinaciones de mutaciones. Las co-mutaciones asociadas a peor pronóstico fueron las de *TP53* con *HRAS*, *CDKN2A* o *PIK3CA* (21,88,94). Este hallazgo es concordante con los resultados reportados por varios, en los que los tumores independientes de VPH p53 mutados se asociaron a peor pronóstico (18–21,73). De forma similar, en el segundo artículo de la presente tesis doctoral, se identificó una tendencia a una mayor mortalidad de los tumores p53 mutados, aunque no alcanzó la significación estadística.

Aunque no era el objetivo de los trabajos incluidos, el hallazgo de alteraciones moleculares asociadas al CEV abre nuevos horizontes en cuanto al uso de terapias dirigidas en el CEV. Varios estudios reportan mutaciones en la vía de *PI3K/AKT/mTOR*. Una revisión sistemática reciente (102) describe esta vía como la más comúnmente alterada en el CEV. Varios agentes de la vía *PI3K/AKT/mTOR*, como *PIK3CA,PTEN, KMT2* o *FBXW7* han sido descritos en la *Drug Gene Interaction database* como *targets* de fármacos conocidos (103). Por ejemplo, Williams *et al.* sugieren que las pacientes con CEV y alteraciones en *KMT2* podrían beneficiarse de tratamientos con inhibidores de aurora kinasa (50), como se ha propuesto recientemente en las pacientes con tumores de cuello de útero y de cabeza y cuello (104). Los mismos autores (50) identifican amplificaciones en *EGFR* en el 11% del CEV independientes de VPH, lo que confiere plausibilidad

biológica a un posible tratamiento con Cetuximab (105,106). Un ensayo clínico de fase II que evalúa el rol de *Erlotinib* (inhibidor de tirosin kinasa anti-EGFR) en CEV muestra un perfil de seguridad aceptable con una respuesta clínica significativa (27% de pacientes mostraron respuesta parcial y 40% enfermedad estable), aunque las tasas de respuesta sostenida fueron limitadas (9).

Así pues, aunque el nivel de evidencia de los estudios que evalúan el perfil molecular del CEV es limitado, se han descrito alteraciones moleculares en el CEV y sus lesiones precursoras que contribuyen al conocimiento de sus vías de carcinogénesis, especialmente de la vía independiente de VPH. Hallazgos recientes también sugieren la existencia de una tercera vía (minoritaria) de carcinogénesis, independiente de VPH y de p53. Algunas de las alteraciones moleculares identificadas parecen tener implicaciones en el pronóstico y, además, abren nuevos horizontes en el uso terapias dirigidas para el tratamiento del CEV.

6 | Conclusiones

1. Un tercio de las lesiones intraepiteliales asociadas a los carcinomas escamosos de vulva independientes del virus del papiloma humano corresponden a lesiones con características morfológicas de lesión escamosa intraepitelial de alto grado (estudio número 1).
2. Las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano (VPH) con lesión precursora escamosa intraepitelial de alto grado-*like* presentan características clínicas (edad, asociación con liquen escleroso y liquen simple crónico) superponibles a las del resto de las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano, y diferentes de las de las pacientes con carcinoma de vulva asociado al virus. Ello confirma la estrecha relación etiopatogénica de este subtipo de tumores con el resto de carcinomas escamosos de vulva independientes del virus del papiloma humano. Sin embargo, estas pacientes se diagnostican en fases más iniciales (menor tamaño y profundidad de la invasión) que el resto de carcinomas escamosos de vulva independientes del virus, de forma similar a lo que ocurre con los tumores asociados al virus, lo que indica que la lesión intraepitelial es fácilmente visible en la exploración clínica (estudio número 1).
3. Los carcinomas escamosos de vulva independientes del virus del papiloma humano con lesión precursora escamosa intraepitelial de alto grado-*like,* al igual que el resto de carcinomas de vulva independientes del virus, son negativos para p16 y con frecuencia presentan patrones anormales de expresión de p53. Sin embargo, muestran características histológicas semejantes a los carcinomas asociados al virus del papiloma humano, ya que con frecuencia presentan morfología basaloide o condilomatosa (estudio número 1).
4. Las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano con lesión precursora escamosa intraepitelial de alto grado-*like* muestran una importante tendencia a la recidiva, por lo que se debería valorar la posibilidad de realizar un seguimiento más estrecho tras la cirugía. A pesar de ello, no muestran diferencias en cuanto a supervivencia respecto al resto de pacientes con carcinoma escamoso de vulva (estudio número 1).

5. La detección molecular del virus del papiloma humano y la determinación inmunohistoquímica de p16 y p53, permiten clasificar de forma fiable a los carcinomas escamosos de vulva, y diferenciar con precisión a las lesiones intraepiteliales y neoplasias invasoras que, por sus características histológicas, podrían diagnosticarse de forma errónea (estudios números 1 y 2).
6. Las mujeres con carcinoma escamoso de vulva asociado al virus del papiloma humano presentan características clínicas diferentes de las de las mujeres con carcinoma escamoso de vulva independiente del virus del papiloma humano: se diagnostican en mujeres al menos 10 años más jóvenes que los carcinomas independientes del virus y no muestran asociación con lesiones inflamatorias de la vulva (estudio número 2).
7. El carcinoma escamoso de vulva asociado al virus del papiloma humano presenta características histológicas e inmunohistoquímicas diferentes al carcinoma escamoso de vulva independiente del virus del papiloma humano: histológicamente son basaloides o condilomatosos, se desarrollan sobre lesiones escamosas intraepiteliales de alto grado, y muy raramente muestran anormalidad de p53, mientras que los carcinomas independientes del virus histológicamente son queratinizantes, se desarrollan sobre lesiones de tipo neoplasia vulvar intraepitelial diferenciada, y con gran frecuencia muestran anormalidad de p53 (estudio número 2).
8. Las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano presentan mayor riesgo de recidiva que las pacientes con carcinoma escamoso de vulva asociado al virus del papiloma humano, así como tendencia a una mayor mortalidad relacionada con la enfermedad, aunque las diferencias en mortalidad no fueron estadísticamente significativas.
9. Las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano y p53 normal presentan un mayor riesgo de recidiva que las pacientes con carcinoma escamoso de vulva independiente del virus del papiloma humano y p53 anormal, sin que ello implique un mayor riesgo de mortalidad en este subgrupo de pacientes (estudio número 2).

10. Los resultados de nuestro estudio apoyan la utilidad de la nueva clasificación del carcinoma escamoso de vulva de la OMS, basada en sus características etiopatogénicas. Adicionalmente, nuestros resultados aportan evidencia en favor de una subdivisión de los tumores independientes del virus de papiloma humano según el estado de p53 (estudio número 2).

7 | Bibliografía

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