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Frequent polymorphic changes but not mutations of TRAIL receptors DR4 and DR5 in mantle cell lymphoma and other B-cell lymphoid neoplasms

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Background and Objectives. Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 have been mapped to chromosome 8p21-22, a region frequently deleted in different lymphoid neoplasms.

Design and Methods. To investigate the potential alterations of these genes in lymphoid neoplasms, we examined the presence of gene mutations in exons 3, 4, and 9 in 69 cases with mantle cell lymphoma (MCL), 16 with chronic lymphocytic leukemia (CLL), 12 with follicular lymphomas (FL) and 17 with large B-cell-lymphomas (DLBCL), as well as in 4 lymphoid cell lines carrying the t(11;14) translocation, and 91 healthy blood donors.

Results. Three CLL and three MCL cases had 8p deletions. Two nucleotide changes in or near the intron 3 splice consensus sequence and a silent change were found. These rare changes were also present in the germ-line of the patients. The DR4 death domain A1322G polymorphism was significantly more frequent in MCL [odds ratio (OR) = 5.9; 95% confidence interval (CI), 1.92-18.1] and CLL (OR = 4.5; CI, 1.18-17) patients than in a sex and age-adjusted healthy population. In contrast, the DR4 exon 4 C626G polymorphism was associated with a significant overall decreased risk for MCL (OR = 0.3; CI, 0.12-0.8). No mutations or cancer-associated polymorphic changes were found in DR5 domains.

Interpretation and Conclusions. These findings indicate that mutations of DR4 and DR5 are uncommon in lymphoid neoplasms but DR4 polymorphic alleles may contribute to the pathogenesis of these malignancies.

Key words: TRAIL receptors, mantle cell lymphoma, DR4, DR5, polymorphisms.

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The tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a cytokine involved in the modulation of cell survival in different cell models.[1-6] Similarly to other tumor necrosis factor (TNF) family members, TRAIL mediates apoptosis by binding to two receptors, DR4 and DR5, with a common structural organization characterized by an extracellular cysteine-rich domain required for ligand binding and an intracellular death domain essential for the apoptotic signal transduction.[7-13] Like the homologous FAS, TRAIL receptors seem to participate in the regulation of different lymphoid cell populations, in particular by preventing the expansion of autoreactive lymphocytes and the development of autoimmune disorders. Inactivation of the coding for these receptors may interfere with the normal mechanisms of cell survival facilitating the accumulation of abnormal lymphoid cell clones and eventually the development of lymphoid neoplasms. Apoptosis-related TNF receptors have been found to be inactivated in several human neoplasms and cancer cell lines. Germ-line mutations of FAS cause an autoimmune lymphoproliferative syndrome and an increased risk of lymphoid malignancies.[14,15] In addition, somatic mutations of FAS have been detected in human tumors, including lymphoid neoplasms.[16] Similarly, mutations of the DR4 and DR5 TRAIL receptors have been found in different types of solid tumors and occasional cases of non-Hodgkin´s lymphoma (NHL).[7,17-22] Most of these mutations occur in the functional death and ligand binding domains and changes in other regions of the receptors are extremely rare. In addition to somatic mutations, several polymorphisms of regulatory regions or functional domains of FAS and TRAIL receptors have been frequently asso-

ciated with different types of human tumors.[6,19,24,25] Some of these polymorphic variants seem to impair the normal functional mechanism of the receptors and may facilitate the development or expansion of aberrant cell clones. Thus, polymorphic changes in the FAS promoter region have been associated with cervical squamous cell carcinoma and acute myeloid leukemia, whereas increased frequencies of several polymorphisms of the TRAIL receptor DR4 death and ligand domains have been detected in human ovarian, bladder, lung, head and neck, and gastric carcinomas, suggesting that these polymorphic variants may be associated with a predisposition to common malignancies.[6,23-25]

Mantle cell lymphoma (MCL) is an aggressive lymphoid neoplasm characterized by the t(11;14) translocation with deregulation of the cyclin D1 gene.[26] The response of this tumor to conventional chemotherapeutic treatments is particularly poor with frequent relapses and short survival. The reasons for this drug resistance are not well known. TRAIL receptors DR4 and DR5 have been mapped to chromosome 8p21-22,[27,28] a region frequently lost in MCL (7-57%) and chronic lymphocytic leukemia (CLL) (7%).[29-31] Interestingly, 8p deletions have been associated with clinical progression and cell transformation in CLL, a lymphoid neoplasm in which failure of apoptotic mechanisms seems to play a crucial pathogenic role.[31] To determine the potential role of inactivation of DR4 and DR5 TRAIL receptors in MCL and other lymphoid neoplasms we examined the presence of mutations and polymorphic variants in the ligand binding and death domains of these genes in a large series of patients with tumors and in the healthy population.

Design and Methods

Case selection

Tumor specimens from 114 patients with non-Hodgkin's lymphoid neoplasms were obtained from the Department of Pathology of the Hospital Clinic, University of Barcelona, and the Department of Pathology, University of Würzburg, on the basis of the availability of frozen samples for molecular studies. The patients had given their informed consent and the study was approved by the hospital review board. The types of tumor were MCL (n=69), CLL (n=16), follicular lymphoma (FL) (n=12), and diffuse large B-cell lymphomas (DLBCL) (n=17). Twenty-eight tumors had been analyzed by comparative genomic hybridization (CGH), and 3 cases each of CLL and MCL showed losses of chromosome 8p region. We also studied REC-1, Granta-519, JVM-2, and NCEB-1 cell lines carrying the t(11;14) translocation and samples from 91 healthy randomly selected blood donors. Data on age and sex were available for all included subjects.

DNA extraction and polymerase chain reaction

High molecular weight DNA was extracted from frozen samples using the standard proteinase K/RNAse treatment and phenol-chloroform extraction. DNA from samples from the healthy blood donors was extracted using the Qiagen DNA extraction kit (Qiagen, GmbH, Germany). DNA from normal tissues or granulocytes from a subset of patients was extracted to examine whether the changes detected in the tumor DNA were present in the germ-line. Polymerase chain reaction (PCR) was performed in a total volume of 50 μL using 100 ng genomic DNA. The primers used for the amplification of the death and ligand binding domains of DR4 and DR5 TRAIL receptors are described in Table 1. DR4 and DR5 exon 9, coding for the death domain, was amplified in two overlapping fragments A and B including the intronic splicing sites and a portion of introns 8 and 9. Similarly, PCR fragments for exon 3 and 4 also included the splicing sites and adjacent intronic regions.

Amplifications were performed using a touchdown PCR (step-down PCR). Conditions were: 95°C for 5 min, three cycles with denaturation at 95°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 1 min, three cycles with annealing at 61°C, three cycles with annealing at 59°C, and 25 cycles with annealing at 58°C. The PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide, and band sizes were determined. All the amplifications were carried out in a Peltier PTC-225 Thermal Cycler (MJ Research).

WAVE detection system for DNA sequence changes

All amplified products were screened for the presence of sequence changes using the WAVE system (Transgenomic, Omaha, NE, USA). This system consists of ion-paired, denaturing high performance liquid chromatography (DHPLC) that analyzes the formation of heteroduplexes using different melting temperatures. The two sequences to be compared (wild type controls and tumor samples) are denatured and reannealed prior to DHPLC analysis to allow the formation of the original homoduplexes and possible mismatched heteroduplexes due to the presence of variations in the sequence. These heteroduplexes are thermally less stable and show reduced retention on the chromatographic separation matrix generating distinctly different peak profiles. In order to identify all the potential heteroduplexes, the chromatography was performed at three different melting temperatures. The normal and tumoral PCR products were mixed (2:3 ratio) and denatured at 94°C for 5 min followed by a 30-min ramp to 24°C (0.1°C/s). The denatured and reannealed samples were run on the WAVE instrument at different melting temperatures depending on the PCR products being analyzed. The gradient

Table 1. Primers for PCR and sequencing.

Identification letter	*Fragment*	*Primer (Tm °C)*	*Length*
TRAIL receptor DR4			
A	DR4-exon9-A F	5'-CCCAACTCATCTGGCTGTCT-3' (54.3)	
B	DR4-exon9-A R	5-TGCATGTCTCTCTTCCATCC-3' (52.9	273 bp
C	DR4-exon 9-B F	5'-ATGAAATGGGTCAACAAAACTG-3' (52.4)	
D	DR4-exon 9-B R	5'-ACACCTAAGAGGAAACCTCTGG-3' (52.3)	206 bp
E	DR4-exon 3 F	5'-TTGGCTTTTCTCTCCCTTCC-3' (54.3)	
F	DR4-exon 3 R	5'-GCCCCTCACTCCACCTCT-3' (54.4)	181 bp
G	DR4-exon 4 F	5'-AGGTCAAGGGACACGTCAGG-3'(58.5)	
H	DR4-exon 4 R	5'-GCTTCTGTGGTTTCTTTGAGG-3' (52.7)	219 bp
TRAIL receptor DR5			
I	DR5-exon 9-A F	5'-CCAACTCACCTGGCTGTCTC-3' (54.9)	
J	DR5-exon 9-A R	5'-AGCGTCTCCAAGGCATCC-3' (55.4)	263 bp
K	DR5-exon 9-B F	5'-CACGATGCTGATAAAGTGGG-3'(53)	
L	DR5-exon 9-B R	5'-GGTCTGACTTCCTGAAGAGA-3'(58.5)	202 bp
M	DR5-exon 3 F	5'-TTCTGGGAATCCTGTGGCAT-3' (56.6)	
N	DR5-exon 3 R	5'-CCCCGCATTCCACCTTTA-3' (55.7)	208 bp
O	DR5-exon 4 F	5'-TTCCCAAAACCTTATGCTCTG-3' (52.8)	
P	DR5-exon 4 R	5'-GGGGTTCCATGGAGCTACTG-3' (55.6)	183 bp
Generation of positive controls			
Q	DR4-exon 9-mut-F	5'-CAGCATTGCTTACAAGGCAT-3'(52.7)	
R	DR4-exon 9-cut-R	5'-ACCCAGCTCTGATGCTGTTC-3'(54.4)	
S	DR5-exon 9-mut-F	5'-CCTCAGCATTAGCCACCTTT-3'(53)	
T	DR5-exon 9-cut-R	5'-CAGTGCTTCGATGACTTTGC-3' (53.6)	
Effect at expression level of mutants of TRAIL receptor DR4			
U	DR4-exon3+4 F	5'-GGAGCCTGTAACCGGTGCAC-3' (58.9)	
V	DR4-exon3+4 R	5'-CTTCCGGCACATCTCAGCAG-3' (58.2)	
W	DR4-exon3+intron3 F	5'-CAGCTTGTAAATCAGCCTGGAGT-3' (52.9)	
X	DR4-exon3+intron3 R	5'-CCCTCTTGGAGCTGATAGTTTG-3' (53.8)	

for each PCR product was calculated using WAVEMaker software.

Generation of mutant positive controls

In order to have a positive control of nucleotide changes to use in the screening for gene mutations by the WAVE system we generated mutants for exon 9 of DR4 and DR5 by PCR technology (Figure 1). We adapted a previously described protocol for the introduction of point mutations in the middle region of a target.[32] Briefly, exon 9 of DR4 and DR5 was amplified in two partially overlapping fragments. The longer fragment (fragment B) covered most of the sequence except a small 5' region. The shorter fragment (fragment A) covered the 5' sequence, including the sequence missing from fragment B, and carried a single nucleotide substitution introduced by primer design. DR4 fragment A was amplified with primers Q, carrying the mutation, and A (Table 1). DR4 fragment B was amplified with primers R and B. The primers used to amplify DR5 fragment A were S, carrying the mutation, and I whereas the primers for DR5 fragment B were T and J (Table 1). Amplifications were performed using the touchdown PCR (step-down PCR) described above. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide and purified using a GenElute PCR Clean-Up Kit (Sigma). The purified A and B fragments were mixed 1:1 and amplified with a touchdown PCR to generate a full exon 9 fragment that would contain a point mutation in the middle region. First, the PCR mix, containing fragments A and B but no primers, was subjected to an extension step consisting of 95°C for 5 min, 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min. The primers A and B for the DR4 A mutant and I and J for the DR5 A mutant were added afterwards and then the PCR was performed. The mutants were electrophoresed, purified, and sequenced to confirm the nucleotide changes introduced.

DNA sequencing

PCR products showing abnormal chromatogram profiles in the WAVE analysis were sequenced. The amplified products were purified using a GenElute PCR Clean-Up Kit (Sigma) and sequenced using cycle sequencing

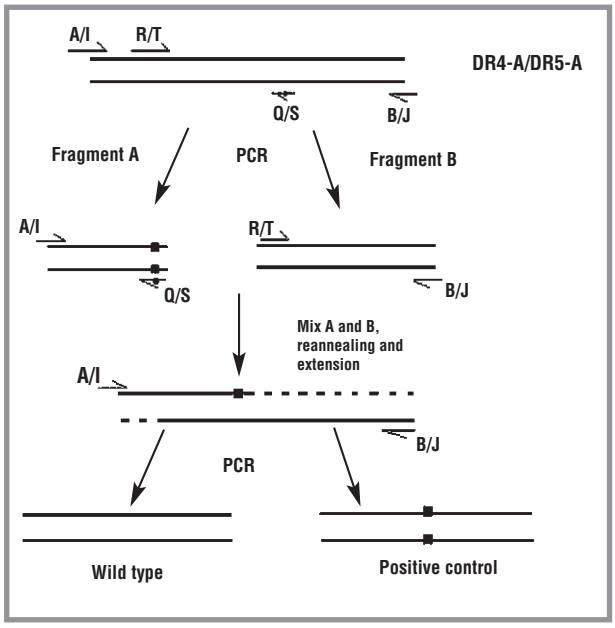

Figure 1. Generation of WAVE mutant positive controls. DR4 and DR5 exon 9 were amplified in two partially overlapping fragments: fragment A, which was the shorter of the two and carried the mutation (by amplification with mutated primer Q in DR4 or S in DR5, Table 1), and fragment B. Purified A and B PCR products were mixed 1:1 and subjected to a reannealing and extension step prior to amplification. The PCR products generated contained a point mutation in the middle region of exon 9.

with BigDye Terminator chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were run on an Abi Prism ABI-3100 automated sequencer. All nucleotide changes were confirmed by sequencing both strands.

RNA extraction and reverse transcription PCR

Total RNA was obtained from frozen tissues using guanidine isothiocyanate extraction and cesium chloride gradient centrifugation. For cDNA synthesis, 1 μg of RNA and Taqman Reverse Transcriptions reagents (including Multiscribe™ reverse transcriptase and random hexamers) were used, as described by the manufacturer (Applied Biosystems). To examine the presence of the nucleotide change G1405T (Stop469Leu) of DR4 exon 9 in a sample for which only RNA was available, the coding region was amplified using primers C and D as described above. The potential effect at the RNA level of the two nucleotide changes detected in the TRAIL-R1/DR4 exon 3-intron 3 splicing region was studied by amplifying exon 3 and exon 4 coding regions near the exon-intron boundaries with primers U (exon 3) and V (exon 4); and exon 3-intron 3 with the primers W (including the splicing site nucleotide change) and X (intron 3).

Immunohistochemistry

DR4 protein expression was examined immunohistochemically on formalin-fixed, paraffin-embedded tissues using a murine monoclonal antibody (HS101) (Alexis Biochemicals, UK), standard antigen retrieval protocols, the Envision detection system (Dakocytomation, Carpinteria, CA, USA) and the automated immunostainer TechMate 500 Plus (Dako).

Statistical analyses

Differences in allele and genotype frequencies were determined among controls using the χ^2 test. Allele frequencies in the control group were evaluated for the Hardy-Weinberg equilibrium. The *p* value among controls for the A1322G polymorphism was 0.6, for the C626G it was 0.5 and for the G422A was 0.6. To test the hypothesis of an association between the different polymorphisms and lymphoid malignancies, multivariate methods were used based on logistic regression analysis. Cases were divided into groups and polytomous logistic regression was used comparing each group of cases with the whole set of controls. Odds ratios (OR) and 95% confidence intervals were calculated for each age (in tertiles) and sex adjusted group. Homozygosity for the more frequent allele among controls was set as the reference class for polymorphisms. The linear trend of the OR was tested using the categorized variable as quantitative after assigning a linear score to each ordered category.

Results

DR4 death domain

TRAIL receptor DR4 death domain is necessary for the transduction of the apoptotic signal and is encoded by exon 9. The majority of the DR4 mutations described in human tumors, including several types of lymphomas, have been detected in this region.[7;17-22] The analysis of the 69 cases of MCL showed an anomalous pattern of DNA mobility in 24 (34.8%) of them (Figure 2). All cases showed an identical mobility profile, suggesting that it could correspond to a polymorphic variant. Sequencing analysis revealed that this anomalous pattern was due to the known polymorphism, A1322G, resulting in the conversion of the lysine at codon 441 to arginine. Only one of these 24 cases was homozygous for this variant. This polymorphism has been previously described in several cancer cell lines, human tumors, and in 2 of 10 (20%) healthy volunteers. This change seems to make the DR4 receptor less sensitive to the TRAIL signal.[6] To examine whether this polymorphism was more frequent in patients with MCL than in the healthy population we examined 91 DNA samples from normal controls. The A1322G polymorphism was signif-

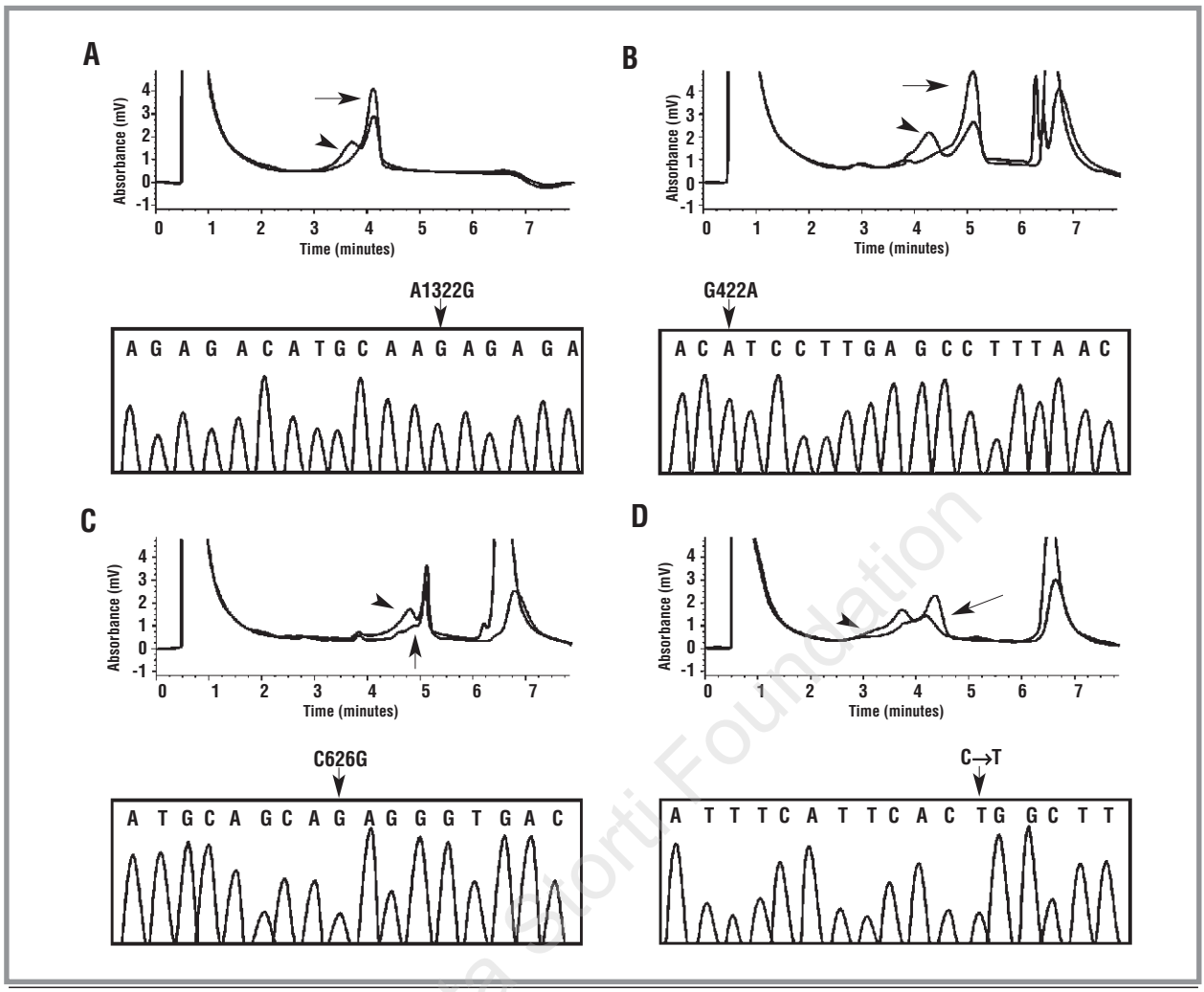

Figure 2. Polymorphisms in TRAIL receptors DR4 and DR5. Panels A, B, C and D represent the chromatogram (upper panel) and sequence (lower panel) of different DR4 and DR5 polymorphisms: (A) A1322G in the death domain of DR4; (B) G422A in the extracellular domain of DR4; (C) C626G in the extracellular domain of DR4; (D) intronic polymorphism C→T at -21 intron 3 of DR5. Arrow: normal, arrowhead: polymorphism.

icantly more common among patients with MCL, being present in 24 of 69 tumor samples (34.8%), as compared to in 16 of the 91 (17.6%) control samples. In MCL, the age- and sex-adjusted odds ratio (OR) for the presence of the G allele was 5.9 (95%CI=1.92-18.1); 5.2 (95% CI=1.64-16.1) in heterozygosis and 24.7 (95% CI=1.04-587.6) for the homozygous variant of the altered allele (Table 2).

To determine the possible presence of DR4 death domain mutations and the incidence of the A1322G polymorphism in other lymphoid neoplasms, the whole DR4 exon 9 was screened in 16 CLL, three of them with 8p deletions, 12 FL, 17 DLBCL and the four human lymphoid cell lines carrying the t(11;14) translocation. No mutations were detected in any of these cases. However, the A1322G polymorphism was observed in 6 (37.5%) CLL cases, which was a significantly higher incidence than that in the controls (OR=4.5, 95% CI = 1.18-17). The polymorphism was identified in only in 1 (8.3%) FL and 3 (17.6%) DLBCL with an incidence similar to that in the normal population (Table 2). None of the cell lines analyzed had the polymorphic variant. Two samples from normal controls showed an abnormal pattern in their WAVE chromatograms, different from the characteristic A1322G profile (Figure 3). Cycle sequencing revealed a heterozygous alteration at nucleotide 1405 changing a guanine to thymine (G1405T) which leads to a substitution of the normal 469 stop codon for a leucine. This modification would encode for a putative DR4 protein variant with 14 additional amino acids. Intriguingly, one of these two normal individuals had a sister with acute myeloid leukemia who also shared this alteration in heterozygosity. This change was not detected in any of the 114 lymphoid tumors.

Table 2. Allele frequency of A1322G in non-Hodgkin´s lymphomas and odds ratio in relation to frequencies in controls.

Cases	*Polymorphism*	*No. of cases*	*%*	*O.R.*[1]	*95% CI*	*Trend test*	*p value*[2]
Control population		91					
	A/A	75	82.4				
	A/G	15	16.5				
	G/G	1	1.1				
	A/G + G/G	16	17.6				
MCL		69				10.6	0.0011
	A/A	45	65.2	1	-		
	A/G	23	33.3	5.2	(1.64-16.1)		
	G/G	1	1.5	24.7	(1.04-587.6)		
	A/G + G/G	24	34.8	5.9	(1.92-18.1)		
CLL		16				5.9	0.0148
	A/A	10	62.5	1			
	A/G	5	31.	3.7	(0.92-14.9)		
	G/G	1	6.3	22.6	(1.03-495.9)		
	A/G + G/G	6	37.5	4.5	(1.18- 17)		
FL		12				0.2	0.6985
	A/A	11	91.7	1			
	A/G	1	8.3	0.7	(0.07-6.2)		
	G/G	0	0	0			
	A/G + G/G	1	8.3	0.7	(0.07-6.7)		
DLBCL		17				0.03	0.8625
	A/A	14	82.3	1			
	A/G	3	17.6	1.3	(0.29-5.6)		
	G/G	0	0	0			
	A/G + G/G	3	17.6	1.3	(0.29-5.5)		

[1]*OR: odds ratio; OR are adjusted for sex and age in tertiles;* [2]*p-value for test of linear trend.*

DR4 ligand-binding domain

A mutational analysis of exons 3 and 4 of TRAIL receptor DR4 was performed in the same series of MCL and other lymphoid neoplasms described above. These exons codify for the principal elements of the extracellular cysteine-rich domain of the receptor required for TRAIL binding. Sequence alterations in this region may lead to deficient ligand binding and affect the downstream apoptotic signaling.[23]

Screening the genomic DNA revealed an abnormal chromatogram of the exon 3 fragment in two cases of typical MCL and one case of CLL. Sequencing analysis of these cases demonstrated three different nucleotide changes in the tumors (Figure 3). One MCL had a change in the splice site conserved sequence AGGT at the exon 3-intron 3 boundary, changing a guanine to cytosine at position +1 of intron 3. The second MCL had a change near the splicing site at position +6 of intron 3, also changing a guanine to cytosine. The CLL showed a silent change at nucleotide 465 changing an adenine for a cytosine (Thr155Thr). All three cases were heterozygous for the altered allele. These changes were also detected in the germ-line of the patients but in none of the 91 healthy controls. To analyze the potential effect of these changes at the RNA level, cDNA from the two cases of MCL was generated and amplified with two different sets of primers. The first set consisted in primers which amplified exon 3 and exon 4 coding regions near the exon-intron boundaries and the second set consisted in a forward primer that carried the mutation corresponding to each case, and a reverse primer which annealed to a near region in intron 3 (Table 1). Genomic DNA from the cases was used as the positive control. No anomalous transcripts were detected in these lymphomas suggesting that these changes may not be functionally relevant.

In addition to these distinct chromatograms, two anomalous profiles were detected in more than one case suggesting that they could correspond to polymorphic variants (Figure 2). Sequencing analysis revealed two previously described polymorphisms: G422A and C626G at exon 3 and 4, respectively.[23] These changes result in a substitution of a histidine for arginine and an arginine for threonine, respectively. These two polymorphic variants seem to co-segregate homozygously in patients with lung, head and neck, and gastric cancer, in whom their incidence is significantly higher than in healthy controls.[23] To determine whether these polymorphisms were differentially represented in patients with lymphoid neoplasms we also screened a series of 91 healthy

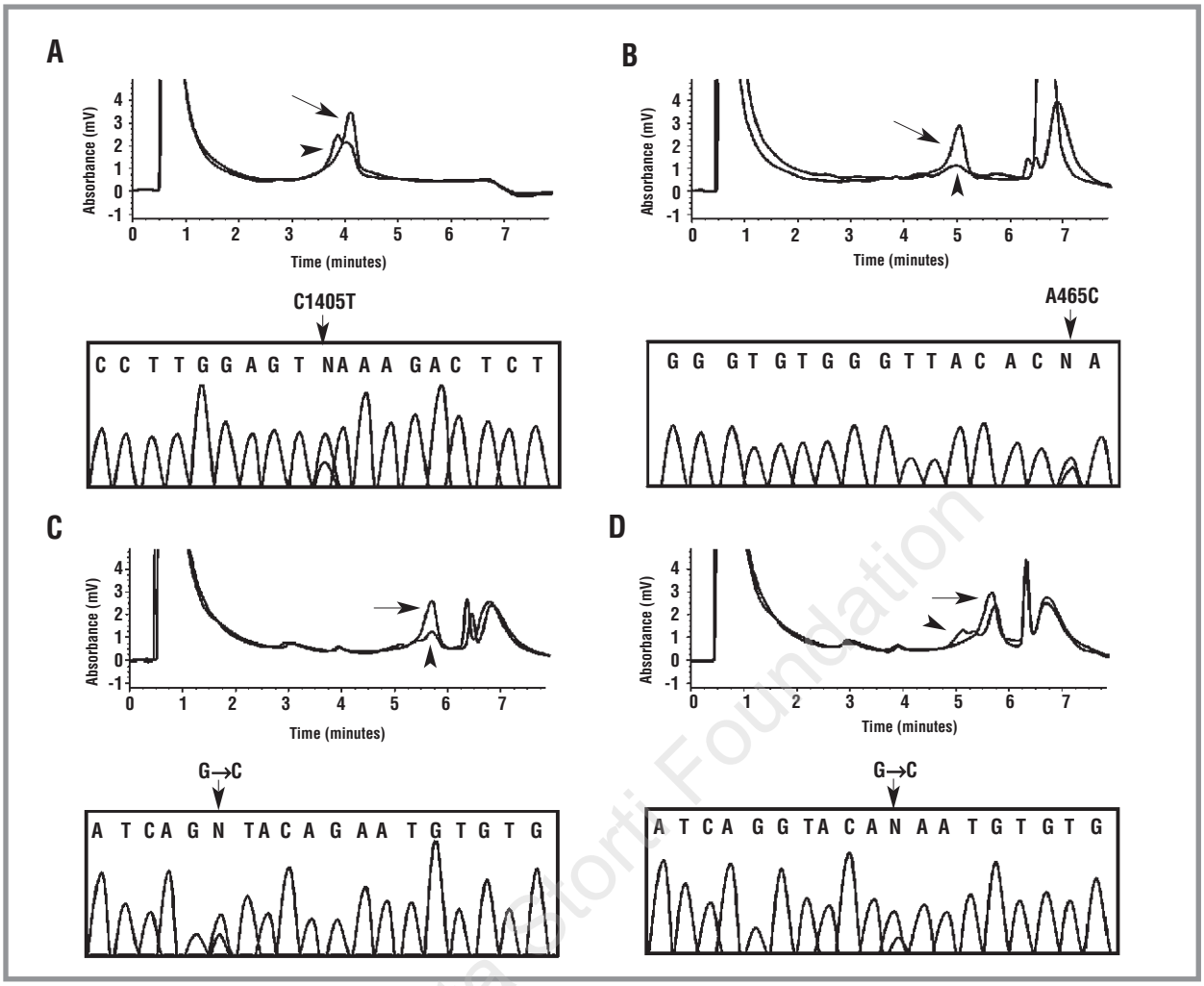

Figure 3. Nucleotide changes in TRAIL receptor DR4. Panels A, B, C and D represent the chromatograms (upper panels) and sequences (lower panels) of different nucleotide changes: (A) G1405T in the death domain; (B) silent mutation A465C at exon 3, in the extracellular domain; (C) G→C at position +1 of the splicing donor site of intron 3; (D) G→C at position +6 of the splicing donor site of intron 3. Arrow: normal, arrowhead: nucleotide change.

controls. The distribution of the CC, CG, and GG alleles of exon 4 C626G polymorphism in the different lymphoma types is shown in Table 3. The frequency of the alleles was similar in CLL, FL, DLBCL and the control population after adjustment for age and sex. However, the presence of the G allele was associated with an overall significant protective effect in MCL compared to in the controls (OR=0.3, 95%CI=0.12-0.8). The G422A variant was also observed at the same allelic frequency in all types of lymphoid tumors and controls (data not shown). Interestingly, a significant association between the homozygous A allele of this polymorphism and the homozygous G allele of codon 626 was observed in MCL since 7 of the 14 MCL patients (50%) homozygous for the 422 AA allele were also homozygous for the 626 GG allele whereas this association was only detected in 3 of the 27 (11%) controls homozygous for the G422A polymorphism (p=0.04).

To determine whether these polymorphic variants were associated with different DR4 protein expression we examined 12 cases of MCL by immunohistochemistry: 6 had the A1322G variant, 8 the C626G variant and 9 the G422A variant. The intensity of DR4 expression was similar in all cases, indicating that DR4 is expressed in all MCL independently of the polymorphic variant.

DR5 death and ligand-binding domains

The TRAIL receptor DR5 death and ligand-binding domains were similarly examined in the 114 lymphoid neoplasms and 91 healthy controls. No mutations or polymorphic variants were detected in the coding regions or splicing sites of exons 3, 4 or 9, of any of the tumors or controls analyzed. Only a common nucleotide change (T for a C) in intron 2, at position -21 from the start of exon 3, was observed in most of the cases studied. The

Table 3. Allele frequency of C626G in non-Hodgkin´s lymphomas and odds ratio in relation to frequencies in controls.

Cases	*Polymorphism*	*No. of cases*	*%*	*O.R.*[1]	*95% CI*	*Trend test*	*p value*[2]
Control population	91						
	C/C	26	28.6				
	C/G	49	53.9				
	G/G	16	17.6				
	C/G + G/G	65	71.4				
MCL		69				3.5	0.0633
	C/C	34	49.3	1	–		
	C/G	17	24.6	0.4	(0.12-1.1)		
	G/G	18	26.1	0.4	(0.12-1.1)		
	C/G + G/G	35	50.7	0.3	(0.12-0.8)		
CLL		12				2.4	0.1198
	C/C	2	16.7	1	–		
	C/G	2	16.7	0.6	(0.08-4.9)		
	G/G	8	66.7	3.6	(0.62-20.8)		
	C/G + G/G	10	83.3	1.6	(0.31-8.6)		
FL		12				0.5	0.4884
	C/C	5	41.7	1	–		
	C/G	3	25.0	0.5	(0.09-2.5)		
	G/G	4	33.3	0.6	(0.12-2.8)		
	C/G + G/G	7	58.3	0.5	(0.12-1.8)		
DLBCL		17				2.1	0.1454
	C/C	5	29.4	1	–		
	C/G	1	5.9	0	–		
	G/G	11	64.7	2.4	(0.66-9.0)		
	C/G + G/G	12	70.6	0.8	(0.24-2.7)		

[1]*OR: odds ratio; OR are adjusted for sex and age in tertiles;* [2]*p-value for test of linear trend.*

frequencies ranged from 77% to 100% in different lymphoma groups, and 86% in normal controls indicating that this must be the most common DR5 sequence in the control population (Figure 2). No significant differences were observed between the different lymphoid tumors and the healthy population.

Discussion

DR4 and DR5 TRAIL receptors have been mapped to chromosome 8p21-22 bands.[10,33] This genetic region is frequently deleted in different human tumors suggesting that it may harbor a potential tumor suppressor gene. Inactivating mutations of DR4 and DR5 receptors have been associated with loss of heterozygosity in some tumors, indicating that these genes may be potential targets of 8p deletions.[18,20] DR4 and DR5 mutations have been observed in head and neck squamous cell carcinomas, non-small cell lung carcinomas, breast and gastric cancers, and non-Hodgkin´s lymphomas. Most of these mutations were detected in the death domain and intronic sequences of the genes and consisted of missense alterations and less frequently non-sense, microdeletions, splice-site, and silent mutations.[7,17-22,34,35]

In our study, we detected three nucleotide changes in the DR4 extracellular domain of two patients with MCL and one with CLL. These changes involved position +1 of the intron 3 splice-site donor consensus sequence and position +6 of the same intron in the two cases of MCL respectively, and a silent mutation in codon 155 in the CLL. These three changes were in heterozygosis and were not detected in any of the control samples examined. However, they were also present in the germ-line of the patients and the changes in the splicing sites were not associated with anomalous transcripts, suggesting that they may be rare polymorphisms with no apparent functional significance. Deletions of chromosome 8p were found in 6 tumors (3 CLL and 3 MCL) but none of these cases had mutations in the DR4 extracellular domain. This lack of inactivating mutations in our study is in contrast with the only previous report on non-Hodgkin's lymphomas in a Korean population in which 7% of the tumors showed potential inactivating mutations in the death domain of DR4 or DR5 genes.[17] This difference may be due in part to the spectrum of tumors examined since the previous study included only 3 MCL, 7 CLL, and 4 FL whereas mutations were detected in 5 of 46 (11%) DLBCL, 2 of 23 (9%) MALT lymphomas and 1 of 14 (7%) peripheral T-cell lymphomas. However, a similar differ-

ence in the incidence of TRAIL receptor mutations in Korean and Western populations has been observed in solid tumors. Thus, DR4 or DR5 mutations were noted in 11% of non-small cell lung cancers (NSCLC) and 30% of metastasic breast carcinomas in the Korean population, but in only 3% of head and neck carcinomas, and in none of a large series of NSCLC and breast tumors in Western patients, suggesting a possible population-based difference in the mutation pattern of these genes.[18-21,36]

Although DR4 and DR5 inactivating mutations were not detected in our series, two polymorphic changes in the DR4 death and ligand binding domains were more frequently detected in different types of lymphoid neoplasms than in the normal control population. The DR4 death domain A1322G polymorphism was present in 37.5% and 34.8% of CLL and MCL patients, respectively, but in only 17.6% of the control population. These differences were statistically significant when compared to a sex- and age-adjusted healthy population, suggesting that this polymorphism may be associated with an increased risk of developing these tumors. The allelic frequencies in FL and DLBCL were similar to those in the normal controls. Transfection experiments in ovarian and bladder cancer cell lines have shown that this polymorphism has a dominant negative effect in DR4-mediated killing, rendering cells more resistant to TRAIL.[6] Although this polymorphism may not alone justify complete resistance to TRAIL, its dominant negative effect *in vitro* may explain its heterozygous predominance and suggest a certain contribution to apoptotic resistance facilitating the development of CLL and MCL neoplastic clones.[6,19]

Two additional polymorphisms in the DR4 ligand-binding domain, G422A and C626G, have been associated in homozygosis with an increased risk of different solid tumors. Thus, G422A was found to be associated with gastric, lung, and head and neck cancers whereas C626G was found to be overrepresented in lung, and head and neck tumors.[23] In contrast, the C626G polymorphism had a protective effect in patients with bladder cancer. The DR4 extracellular domain was not examined in the previous study of NHL and, therefore, no information on the distribution of these polymorphisms in lymphoid neoplasms is available.[17] In our study, the frequency of the G422A allele was similar in all types of lymphomas and healthy controls. However, the C626G variant was associated with a significant protective effect for MCL. In urinary bladder carcinomas, the protective effect of this polymorphism was mainly associated with young, light smokers suggesting an association between the development of the tumor, the DR4 polymorphism, and environmental exposure.[37] Epidemiological risk factors in MCL have not been studied and, therefore, the possible relationship of this association and an environmental exposure in such patients is not known. The GG and AA homozygous variants of the 626 and 422 polymorphisms co-segregated in 44-48% of patients with head and neck, lung and gastric carcinomas.[23] Interestingly, the frequency of being homozygous for both alleles was also significantly higher in MCL patients than in controls, suggesting that this association may be common in different types of tumors. The functional effect of these polymorphisms is currently unknown. However, their location flanking the receptor ligand interface regions suggest that sequence changes may alter binding of the ligand to the receptor and apoptotic signaling.

Since TRAIL has a selective action, being more apoptotic to tumor cells than to normal cells, it has been suggested that it could be used as a new therapeutic strategy.[38,39] However, the sensitivity of different tumors to TRAIL-induced apoptosis is variable and the determinants of the different responses are not well known. Primary CLL cells and other hematologic malignancies may be resistant to the TRAIL effect.[13,40] The causes of this resistance appear to be complex, involving different factors such as variable levels of expression of the receptor and other elements of the downstream pathway.[13] The rare presence of inactivating mutations in DR4 and DR5 genes in our series of lymphoid neoplasms, including 6 cases with 8p deletions, indicate that these alterations are not relevant cause of TRAIL resistance. However, the relatively high frequency of several DR4 polymorphisms with a potential functional effect in MCL and CLL suggests that polymorphic variants may play a role in TRAIL resistance in such neoplasms. In addition, the significantly different frequencies of these polymorphic variants in tumors compared with in the normal population suggests that they may also act as genetic modulators in the development of certain malignancies.

VF and PJ examined the presence of mutations and polymorphisms in all cases and healthy controls. SB and IS analyzed the chromosomal alterations of these tumors by comparative genomic hybridization. EG and SS performed the statistical analysis. DC and EM selected all the patients with CLL, some of those with MCL, and the healthy controls. GO and EC reviewed all the pathology samples of the lymphomas. VF and PJ drafted the manuscript and EC wrote the final version. EC, PJ and DC designed the whole study. All authors critically reviewed the final version of the manuscript. We thank Iracema Nayach, Montse Sanchez and Laura Pla for their excellent technical assistance, and David Escuderos for his skilfull help in the making of the figures.

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References

1. Baetu TM, Hiscott J. On the TRAIL to apoptosis. Cytokine Growth Factor Rev 2002;13:199-207.
2. Daniel PT, Wieder T, Sturm I, Schulze-Osthoff K. The kiss of death: promises and failures of death receptors and ligands in cancer therapy. Leukemia 2001;15:1022-32.
3. Sheikh MS, Fornace AJ Jr. Death and decoy receptors and p53-mediated apoptosis. Leukemia 2000;14:1509-13.
4. Fesik SW. Insights into programmed cell death through structural biology. Cell 2000;103:273-82.
5. Degli-Esposti M. To die or not to die--the quest of the TRAIL receptors. J Leukoc Biol 1999;65:535-42.
6. Kim K, Fisher MJ, Xu SQ, El Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 2000;6:335-46.
7. Arai T, Akiyama Y, Okabe S, Saito K, Iwai T, Yuasa Y. Genomic organization and mutation analyses of the DR5/TRAIL receptor 2 gene in colorectal carcinomas. Cancer Lett 1998;133:197-204.
8. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al. The receptor for the cytotoxic ligand TRAIL. Science 1997; 276:111-3.
9. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997;277:818-21.
10. MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J Biol Chem 1997; 272: 25417-20.
11. Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997; 16: 5386-97.
12. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell 1999;4:563-71.
13. MacFarlane M, Harper N, Snowden RT, Dyer MJ, Barnett GA, Pringle JH, et al. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. Oncogene 2002;21:6809-18.
14. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science 1995;268:1347-9.
15. Straus SE, Jaffe ES, Puck JM, Dale JK, Elkon KB, Rosen-Wolff A, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood 2001;98:194-0.
16. Gronbaek K, Straten PT, Ralfkiaer E, Ahrenkiel V, Andersen MK, Hansen NE, et al. Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. Blood 1998;92:3018-24.
17. Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY, et al. Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. Oncogene 2001;20:399-403.
18. Shin MS, Kim HS, Lee SH, Park WS, Kim SY, Park JY, et al. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. Cancer Res 2001; 61: 4942-6.
19. Seitz S, Wassmuth P, Fischer J, Nothnagel A, Jandrig B, Schlag PM, et al. Mutation analysis and mRNA expression of TRAIL-receptors in human breast cancer. Int J Cancer 2002;102:117-28.
20. Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY, et al. Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. Cancer Res 1999; 59: 5683-6.
21. Pai SI, Wu GS, Ozoren N, Wu L, Jen J, Sidransky D, et al. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. Cancer Res 1998; 58:3513-8.
22. Park WS, Lee JH, Shin MS, Park JY, Kim HS, Kim YS, et al. Inactivating mutations of KILLER/DR5 gene in gastric cancers. Gastroenterology 2001;121:1219-25.
23. Fisher MJ, Virmani AK, Wu L, Aplenc R, Harper JC, Powell SM, et al. Nucleotide substitution in the ectodomain of TRAIL-receptor DR4 is associated with lung cancer and head and neck cancer. Clin Cancer Res 2001;7:1688-97.
24. Sibley K, Rollinson S, Allan JM, Smith AG, Law GR, Roddam PL, et al. Functional FAS promoter polymorphisms are associated with increased risk of acute myeloid leukemia. Cancer Res 2003;63:4327-30.
25. Lai HC, Sytwu HK, Sun CA, Yu MH, Yu CP, Liu HS, et al. Single nucleotide polymorphism at Fas promoter is associated with cervical carcinogenesis. Int J Cancer 2003; 103:221-5.
26. Campo E, Raffeld M, Jaffe ES. Mantle-cell lymphoma. Semin Hematol 1999;36:115-27.
27. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, et al. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J Exp Med 1997; 186:1165-70.
28. Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997; 16: 5386-97.
29. Martinez-Climent JA, Vizcarra E, Sanchez D, Blesa D, Marugan I, Benet I, et al. Loss of a novel tumor suppressor gene locus at chromosome 8p is associated with leukemic mantle cell lymphoma. Blood 2001; 98:3479-82.
30. Bea S, Ribas M, Hernandez JM, Bosch F, Pinyol M, Hernandez L, et al. Increased number of chromosomal imbalances and high-level DNA amplifications in mantle cell lymphoma are associated with blastoid variants. Blood 1999;93:4365-74.
31. Bea S, Lopez-Guillermo A, Ribas M, Puig X, Pinyol M, Carrio A, et al. Genetic imbalances in progressed B-cell chronic lymphocytic leukemia and transformed large-cell lymphoma (Richter's syndrome). Am J Pathol 2002;161:957-68.
32. Meetei AR, Rao MR. Generation of multiple site-specific mutations by polymerase chain reaction. Methods Mol Biol 2002;182:95-102.
33. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol 1997; 7: 1003-6.
34. Ozoren N, El Deiry WS. Cell surface Death Receptor signaling in normal and cancer cells. Semin Cancer Biol 2003;13:135-47.
35. McDonald ER III, Chui PC, Martelli PF, Dicker DT, El Deiry WS. Death domain mutagenesis of KILLER/DR5 reveals residues critical for apoptotic signaling. J Biol Chem 2001;276:14939-45.
36. Wu WG, Soria JC, Wang L, Kemp BL, Mao L.TRAIL-R2 is not correlated with p53 status and is rarely mutated in non-small cell lung cancer. Anticancer Res 2000; 20:4525-9.
37. Hazra A, Chamberlain RM, Grossman HB, Zhu Y, Spitz MR, Wu X. Death receptor 4 and bladder cancer risk. Cancer Res 2003; 63:1157-9.
38. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999; 104:155-62.
39. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 1999;5:157-63.
40. Snell V, Clodi K, Zhao S, Goodwin R, Thomas EK, Morris SW, et al. Activity of TNF-related apoptosis-inducing ligand (TRAIL) in haematological malignancies. Br J Haematol 1997;99:618-24.