p53 Mutations in Nasal Natural Killer/T-Cell Lymphoma from Mexico

Association with Large Cell Morphology and Advanced Disease

Leticia Quintanilla-Martinez,* Marcus Kremer,*[†]
Gisela Keller,[†] Michaela Nathrath,*
Armando Gamboa-Dominguez,[‡]
Abelardo Meneses,[§] Lourdes Luna-Contreras,[§]
Antonello Cabras,*[†] Heinz Hoefler,*[†]
Alejandro Mohar,[§] and Falko Fend[†]

From the Institutes of Pathology, GSF-National Research Center for Environment and Health, Neuberberg, Germany; and Technical University,[†] Munich, Germany; and the Instituto Nacional de la Nutrición,[‡] and the Instituto Nacional de Cancerología,[§] Mexico City, Mexico*

Nasal NK/T-cell lymphoma is a unique form of lymphoma highly associated with Epstein-Barr virus, and with a characteristic geographic distribution. Recently, we showed that p53 is overexpressed in a high percentage of nasal NK/T-cell lymphomas. The aim of this study was to analyze the status of the p53 gene, and correlate it with the expression of p53 protein and its downstream target, the cyclin-dependent kinase inhibitor p21, in a series of 25 cases of well-characterized nasal NK/T-cell lymphoma from Mexico. The highly conserved exons 5 to 8 of the p53 gene were amplified by polymerase chain reaction and screened for mutations by denaturing high-pressure liquid chromatography. Abnormal polymerase chain reaction products detected by denaturing high-pressure liquid chromatography and additional selected cases were sequenced. In addition, the incidence of loss of heterozygosity at the p53 locus was analyzed in 12 cases. Of the 25 patients, 17 were male and 8 female (M:F ratio, 2.1:1), with a median age of 43 years (range, 21 to 93 years). Morphologically, most of the cases were composed of a mixture of medium-sized cells and large transformed cells (21 cases), and four cases were composed exclusively of large transformed cells. Three different groups determined by p53 gene status and expression of p53 protein were identified: group 1 was p53 +/p53 mutated (five cases, all with p53 missense mutations). Morphologically, three of the five cases were composed of large cells. All five cases revealed overexpression of p53 in the majority of the tumor cells with a mean of 86%. Unexpectedly, three of these cases also showed over-

expression of p21. Four of the five patients presented with clinical stage IVB and died with disease. Group 2 was p53+/p53 wild-type (10 cases). Histologically, nine cases were of the mixed type, and one of the large cell type. The percentage of p53 overexpressing cells was lower than in the previous group with a mean of 23%. p21 was positive in 7 of the 10 cases. Six patients in this group presented with clinical stages I to II and four patients with advanced disease (stage III and IV). Five patients are alive 12 to 120 months later (mean, 24 months), three with no evidence of disease. Group 3 was p53-/p53 wild-type (10 cases). All cases showed mixed cell morphology. p21 was positive in 5 of 10 cases. Four patients presented with clinical stage I to II and six patients with advanced disease. Four patients are alive with no evidence of disease 9 to 60 months later (mean, 10 months). Overall, p53 mutations were present in 24% (5 of 21) of the evaluable cases, all of them overexpressing p53 in the majority of tumor cells. Cases with p53 mutations were associated with large cell morphology ($P = 0.0162$) and presented more often with advanced stage disease. Loss of heterozygosity at chromosome 17p was found only in 2 of the 12 (17%) cases investigated, both cases showed p53 mutations of the remaining allele. P21 overexpression (60% of cases) is frequent in nasal NK/T-cell lymphoma and seems to be independent of p53 gene status. The overexpression of p53 and p21, independent of p53 mutations, although as yet not clear, might be the result of Epstein-Barr virus infection, and warrants further investigation. (*Am J Pathol* 2001, 159:2095-2105)

Nasal natural killer (NK)/T-cell lymphoma is a distinct clinicopathological entity characterized by progressive necrotic lesions associated with frequent invasion and

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Address reprint requests to Leticia Quintanilla-Martinez M.D., Institute of Pathology, GSF-Research Center for Environment and Health, Ingolstädter Landstrasse 1, D-85764, Oberschleissheim, Germany. E-mail: quintanilla-fend@gsf.de.

destruction of blood vessels.^{1,2} Although the midline nasal area is the most common site of presentation, nasal-type NK/T lymphoma may present in diverse extranodal sites such as gastrointestinal tract, skin, testis, liver, and spleen.³ It is a relatively rare disease associated with a poor prognosis and a strong geographic predilection. Most cases are reported from Asia,⁴⁻⁶ Mexico, and South America,^{7,8} and this tumor is extremely rare in the United States and in Europe.⁹

Immunophenotypic and genotypic studies have shown that nasal NK/T-cell lymphomas, in addition to the almost universal association with Epstein-Barr virus (EBV),^{10,11} express the NK cell marker CD56,¹² and are usually negative for surface CD3 (Leu4), although cytoplasmic CD3 can be detected in paraffin sections. This phenotype, together with the lack of clonal T-cell receptor gene rearrangements, strongly supports the notion that these tumors are derived from NK cells, and not from conventional T cells.¹³

Although our understanding of nasal NK/T-cell lymphoma has increased in the last years, very little is known about the cytogenetic and molecular changes of this disease.¹⁴ This might be because, in part, of the small samples available with frequently extensive necrotic changes, and to the rarity of this entity. In a recent immunohistochemical study, we showed that p53 was overexpressed in a high percentage of cases of nasal NK/T-cell lymphoma from Peru.¹⁵ However, the mutational status of the p53 gene was not determined.

In the present study of a series of nasal NK/T-cell lymphoma from Mexico, we aimed to investigate the frequency of p53 gene alterations [mutations and loss of heterozygosity (LOH)], and to correlate them with the expression of p53 protein, and its downstream target, the cyclin-dependent kinase inhibitor p21, as a surrogate marker of p53 functionality. In addition, we examined the potential prognostic implication of p53 alterations for clinical outcome.

Materials and Methods

Tissue Samples

Thirty-six paraffin-embedded blocks of nasal lymphoma biopsy material from the Instituto Nacional de la Nutrición and the Instituto Nacional de Cancerología, Mexico City, Mexico were studied. Eight cases immunophenotypically corresponded to B-cell non-Hodgkin's lymphomas (NHLs) and three cases showed extensive necrosis with lack of viable tissue and, therefore, were excluded from the series. The remaining 25 cases form the basis of this study. Clinical information was obtained from the patients' medical records. Hematoxylin and eosin-stained slides and immunoperoxidase studies were reviewed in all cases by two of the authors (LQ-M and FF). In addition, cytological features and immunophenotype were recorded. The term NK/T-cell lymphoma is used in this study as defined in the new World Health Organization classification¹⁶ and corresponds to angiocentric lymphoma in the Revised European American Lymphoma

classification (REAL).¹⁷ Cases of lymphoma presenting in nasal or paranasal tissues were included in this study if they showed expression of cytoplasmic CD3ε, cytotoxic granule associated proteins (TIA-1), EBERs, and in most cases CD56.

Immunohistochemistry

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical System, Inc., Tucson, AZ) according to the company's protocols, with slight modifications. After deparaffinization and rehydration, the slides were placed in a microwave pressure cooker in 0.001 mol/L citrate buffer, pH 6.0, containing 0.1% Tween 20, and heated in a microwave oven at maximum power for 30 minutes. The antibody panel used included L26 (CD20; DAKO, Glostrup, Denmark) polyclonal-CD3 (DAKO), CD56 (Novocastra, Newcastle, UK), TIA-1 (Coulter, Miami, FL), the monoclonal antibody DO7 against the wild-type/mutant p53 (DAKO), and p21 (BD Transduction Laboratories, San Diego, CA). Tissues were scored as p53-positive if $\geq 10\%$ of the tumor cells had nuclear staining. Because p21 expression is found only in isolated lymphocytes and in the epithelium of normal tonsils, any staining $\geq 5\%$ was considered positive. A grid ocular objective was used to count 300 cells over three high-power fields ($\times 40$) and the percentage of positive cells was reported as 0 to 100%.

In Situ Hybridization

In situ hybridization for EBV early RNA (EBER) was performed on fixed paraffin-embedded sections as previously described.¹⁵ The *in situ* hybridization procedure was performed under RNase-free conditions. The fluorescein-conjugated EBER peptide nucleic acid probe was obtained from DAKO. A well-characterized EBV+ Hodgkin's lymphoma case was used as positive control.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification of p53 Gene

Genomic DNA was extracted from serial, dewaxed paraffin sections mounted on glass slides. In the majority of cases, depending on the amount and distribution of the tumor infiltrate, neoplastic cells were enriched either by manual microdissection or by laser capture microdissection of hematoxylin-stained slides as previously described.¹⁸ Adjacent sections stained for CD56 or p53 were used to identify the areas with the highest tumor cell content. The tissue fragments were digested with proteinase K overnight at 56°C, and the crude extract was used for PCR.

The highly conserved exons 5 to 8 of the p53 gene, including the intron/exon boundaries, were amplified by PCR. The primers used are listed in Table 1. The integrity of the DNA was assessed by amplification of a 268-bp fragment of the β -globin gene. The quality and correct size of the PCR products were checked on agarose gels.

Table 1. P53 Primers and PCR Conditions

Exon	Forward primer sequence	Reverse primer sequence	Amplicon length (bp)	Annealing-temperature (°C)
5	ATC TGT TCA CTT GTG CCC TG	AAC CAG CCC TGT CGT CTC TC	200	60
6	AGG GTC CCC AGG CCT CTG AT	CAC CCT TAA CCC CTC CTC CC	199	62
7	CCA AGG CGC ACT GGC CTC ATC	CAG AGG CTG GGG CAC AGC AGG	207	56
8	TTC CTT ACT GCC TCT TGC TT	TGT CCT GCT TGC TTA CCT CG	217	56

Denaturing High-Pressure Liquid Chromatography (DHPLC)

Mutational screening was performed according to the method described by Oefner and Underhill¹⁹ on an automated DHPLC analysis system (Transgenomic, Omaha, NE). The PCR products were denatured for 4 minutes at 94°C and cooled to room temperature at a rate of 1°C/minute. Three to 15 μ l of PCR product were applied to a preheated reverse-phase column (DNA-Sep, Transgenomic). Elution of DNA was performed in a linear acetonitrile gradient. The temperature for optimal resolution of heteroduplex and homoduplex DNA detection was determined by analyzing the melting behavior of each PCR fragment while the temperature was increased at 1°C steps beginning at 50 to 55°C until the fragment was completely melted. The optimal temperatures for the identification of mutations was established for each of the four examined exons using a set of test DNA samples with known mutations. The overall sensitivity of this method for the detection of p53 mutations in exons 5 to 8 is in the range of 95%. A detailed discussion of this technique is given in a separate report.²⁰ A mutation can be detected reliably, if ~10 to 20% of the PCR product shows the mutation. In cases with a high tumor cell content, DHPLC was performed both with and without admixture of wild-type PCR product of the corresponding exon to ensure detection of mutations, because absence of wild-type DNA as a result of LOH at the p53 locus, could potentially lead to a false-negative result.

Direct Sequencing

Automated fluorescent sequencing was performed with the BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) and ABI Prism 377 automated sequencer (PE Applied Biosystems). All chromatograms were evaluated independently by two observers (GK and FF). Comparison with the wild-type sequences was performed with DNASIS 2.6 software (Hitachi Software Engineering, Yokohama, Japan).

LOH Analysis

Normal and neoplastic tissues were obtained by laser capture microdissection of hematoxylin-stained slides as described above. The primer sequences of the microsatellite marker TP53 used in this study target a highly informative dinucleotide repeat polymorphism.²¹ PCR amplification was performed using a TC1 thermal cycler (Perkin Elmer) under standard conditions in a final vol-

ume of 15 μ l containing 2 μ l of template DNA, 1.5 μ l of 10 $\times$ PCR buffer (15 mmol/L MgCl₂, 0.5 mol/L KCl, 0.1 mol/L Tris-HCl, pH 9.0), 1.25 mmol/L dNTPs, 20 pmol of each primer oligonucleotide (forward primer is fluorescently labeled), and 1.5 U of *Taq* polymerase (Amersham Pharmacia). PCR cycles of denaturation, primer annealing, and extension were performed at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds for 35 cycles. Reaction products were separated and detected using an automated sequencer (ABI 377) and analyzed with the ABI Prism GeneScan software. LOH was defined as a reduction of one allele in tumor tissue by at least 50% when compared to the corresponding allele in normal tissue. Homozygous cases were considered to be not informative.

Statistical Analysis

The significance of the association of p53 mutations with morphology and clinical stage at presentation and the significance of overall survival and clinical stage was assessed using Fisher's exact test.

Results

Patient Characteristics and Histological Findings

Clinical data and histology are summarized in Table 2. Of the 25 patients, 17 were male and 8 female (M: F ratio, 2.1:1), with a median age of 43 years (range, 21 to 93 years). The most common presenting symptom was nasal obstruction. In 16 cases the tumor sample was from the nasal cavity, in 5 cases from the nasopharynx, and in 4 cases from the palate. Patients were treated with combined radiation and chemotherapy modalities in 15 cases, chemotherapy alone in 4 cases, and radiation alone in 1 case. Eleven patients presented with early stage disease (stage I to II), and 14 patients with advanced disease (stage III to IV). Thirteen patients died with disease; 1 patient declined treatment because of advanced disease, 2 patients died before treatment could be started, and 10 patients died despite treatment. Two of these patients initially had a complete response (cases 7 and 16) and died with recurrence of the disease at 16 and 8 months after the initial diagnosis, respectively. Nine patients are alive; seven with no evidence of disease, and two patients with disease. Three patients were lost to follow-up, one during the treatment period, and two patients (cases 2 and 11), because the treatment was declined.

Table 2. Clinicopathological Features from 25 Cases of Nasal NK/T-Cell Lymphomas from Mexico

Case	Age	Sex	Tumor sample	Histology	Stage	Treatment	Follow-up	
Group 1. p53+/p53 mutated								
1	42	F	Nasal cavity	Mixed	IVB	CT	DwD	3 months
2	93	F	Nasal cavity	Large cell	IIB	Declined	LFU	
3	57	F	Nasal cavity	Large cell	IVB	Declined	DwD	<1 month
4	36	F	Nasopharynx	Large cell	IVB	***	DwD	<1 month
5	36	M	Nasal cavity	Mixed	IVB	CT	DwD	<1 month
Group 2. p53+/p53 wild type								
6	57	M	Palate	Mixed	IIIB	CT+RT	DwD	10 months
7	30	F	Nasal cavity	Mixed	IB	CT+RT	DwR	16 months
8	49	M	Nasal cavity	Mixed	IIB	CT+RT	ANED	16 months
9	21	F	Palate	Mixed	IIB	CT+RT	ANED	12 months
10	66	F	Nasopharynx	Mixed	IIIB	CT+RT	AwD	24 months
11	62	M	Nasal cavity	Mixed	IIB	Declined	LFU	
12	65	M	Nasal cavity	Large cell	IIA	CT+RT	ANED	4 years
13	54	M	Nasal cavity	Mixed	IVB	CT+RT	DwD	3 months
14	22	M	Nasal cavity	Mixed	IVB	***	DwD	<1 month
15	61	M	Nasal cavity	Mixed	IA	CT+RT	AwD§	10 years
Group 3. P53−/p53 wild type								
16	32	M	Palate	Mixed	IIA	CT+RT	DwR	8 months
17	38	M	Nasopharynx	Mixed	IA	CT+RT	ANED	10 months
18	23	M	Nasopharynx	Mixed	IIIA	CT+RT	ANED	5 years
19	28	F	Nasal cavity	Mixed	IIIB	CT+RT	DwD	4 months
20	43	M	Nasal cavity	Mixed	IVB	CT	LFU	
21	60	M	Palate	Mixed	IIB	CT+RT	ANED	9 months
22	53	M	Nasal cavity	Mixed	IB	CT+RT	ANED	9 months
23	36	M	Nasopharynx	Mixed	IIIB	RT	DwD	8 months
24	74	M	Nasal cavity	Mixed	IVB	CT	DwD	<1 month
25	41	M	Nasal cavity	Mixed	IVB	CT+RT	DwD	3 months

***Died before treatment could be started.

CT, chemotherapy; RT, radiotherapy; DwD, died with disease; LFU, lost to follow-up; AwD, alive with disease; ANED, alive no evidence of disease; DwR, died with recurrence of the disease; AwD§, the patient recurred locally after 10 years.

Histologically, the lymphoid infiltrate was either diffuse or patchy with angioinvasion identified in some of the cases. Karyorrhexis was usually prominent, and focal or confluent necrosis was frequently observed. The cytological spectrum differed from case to case, however, most of the cases were composed of a mixture of medium-sized cells and large transformed cells (21 cases) (Figure 1A). Cytological atypia characterized by nuclear irregularities and clumped chromatin were often identified. Inflammatory cells including neutrophils, eosinophils, plasma cells, and histiocytes, frequently were intermingled with the tumor cells. In the remaining four cases, the tumor was composed almost exclusively of large transformed cells with irregular nuclei, clumped chromatin and often prominent nucleoli (Figure 1B). The mitotic activity was high and the inflammatory component was less prominent.

Immunohistochemical Findings and in Situ Hybridization

The results of the immunophenotypic studies and the EBV analysis are summarized in Table 3. All cases expressed CD3ε. The neoplastic cells showed CD56 expression in 20 of 25 cases (80%). TIA-1, which is a well-characterized cytotoxic molecule primarily restricted to cytotoxic T cells and NK cells, was strongly positive in the cytoplasm of the tumor cells in all cases. Overexpression of p53 was observed in >10% of the tumor cells in 15 of the 25 cases (60%). In 9 of these 15 cases the

percentage of p53+ cells ranged between 14% and 45%, the intensity of the expression varied from cell to cell in any given case. In the remaining six cases, p53 was strongly positive in >50% of the tumor cells. Overexpression of p21 was observed in 15 cases (60%). All cases showed labeling for EBERs in a large proportion of viable, neoplastic cells.

Mutational Analysis of the p53 Gene

The results of the mutational analysis are summarized in Table 3. Successful DNA amplification was obtained in 21 of the 25 cases. In 9 of 21 cases the mutational screening of exons 5 to 8 of the p53 gene showed an abnormal (five cases) or questionable (four cases) DHPLC pattern in at least one of the analyzed exons (Figure 2; A, B, and C). All PCR products showing an abnormal or questionable DHPLC pattern were subjected to direct sequencing of both DNA strands. In all five cases with abnormal DHPLC curve, missense mutations leading to amino acid substitutions were detected (one each in exon 5 and 6, and three in exon 7) (Figure 2C). In contrast, in none of the four questionable cases were p53 mutations found (Figure 2B). In addition, all four exons from the cases with a high level of p53 staining without abnormalities of the DHPLC pattern (cases 8, 9, 11, and 12), and further randomly chosen PCR products were sequenced. In none of these cases were p53 mutations identified.

Figure 1. A: Nasal lymphoma with angioinvasion. The neoplastic cells infiltrate the wall of a medium-sized vessel. The neoplastic infiltration is composed of a mixture of medium-sized cells and large transformed cells intermingled with rare eosinophils and plasma cells. **B:** In contrast to the previous example, the neoplastic population is composed of large transformed cells with cytological atypia, nuclear irregularities, and clumped chromatin. Note the presence of rare small lymphocytes (arrows). H&E; original magnifications, $\times 400$.

Correlation between p53 Gene Status, and p53 and p21 Protein Expression

Three different groups determined by the expression of p53 protein and p53 gene status were identified (Figure 3; A to D). Group 1 contained the five cases with p53 missense mutations. All five cases revealed strong nuclear overexpression of p53 in the majority of the tumor cells with a mean of 86% (range, 53 to 90%) (Figure 3A). Unexpectedly, three of these cases also showed overexpression of p21 (Figure 3B). Group 2 included the 10 cases that showed overexpression of p53, but retained wild-type (wt) configuration of the p53 gene in the nine amplifiable cases. The percentage of p53-positive cells was lower than in the previous group with a mean of 23% (range, 14 to 52%). Only one case in this group showed p53 positivity in $>50\%$ of the tumor cells. Although this case revealed a normal DHPLC elution curve, direct sequencing of exons 5 to 8 was performed to corroborate the presumable wt status of the p53 gene. P21 was positive in 7 of the 10 cases including the case with high p53 expression (Figure 3C). Group 3 included 10 cases with low or null expression of p53. All seven amplifiable cases in this group showed a wt p53 gene. P21 was

overexpressed in 5 of the 10 cases. In two cases (cases 16 and 20) the expression of p21 was relatively high in comparison with the other tumors (Figure 3D).

Correlation between p53 Gene Status/Expression, Clinicopathological Characteristics, and Outcome

The tumor morphology and clinical characteristics of the patients were analyzed in the three different groups described above (Table 2). Group 1 included five cases of mutated p53+/p53. Histologically, three cases were composed of large transformed cells and two cases were composed of a mixture of medium-sized cells and large cells. Four patients in this group presented with advanced disease (Stage IVB) and died shortly after diagnosis. The fifth patient presented with stage IIB, declined treatment and was lost to follow-up. Group 2 included 10 cases of p53+/p53 wild type. Histologically, nine cases were composed of a mixture of medium and large-sized cells, and one case was composed predominantly of large cells. Five patients are alive 12 to 120 months later (mean, 24 months), three with no evidence of disease, and two with disease. The three patients that are alive with no evidence of disease presented clinically with stage IIA-B. Case 15 presented with stage IA and recurred locally (stage IA) after 10 years of complete remission. He is currently undergoing chemotherapy. Four patients died with disease, three of them had presented with advanced disease. Case 7 presented with stage IB and initially had a complete remission; however, 16 months later recurred and died with disease shortly thereafter. Case 11 presented with stage IIB, the patient refused treatment and was lost to follow-up. Group 3 included 10 cases of p53-/p53 wild type. Histologically, all cases in this group were composed of a mixed population of medium-sized and large cells. Four patients are alive with no evidence of disease 9 to 60 months later (mean, 10 months), three of these patients presented clinically with stages IA-B and IIB. Five patients died with disease, four of them presented with advanced disease. Case 16 presented with clinical stage IIA and had initially a complete remission, however, recurred 8 months later with disease in lung and liver and died with disease. Case 20 presented with clinical stage IVB and was lost to follow-up during the treatment period.

No significant differences in the frequency of the main clinical parameters, ie, B symptoms (100% *versus* 80% *versus* 70%), advanced disease (stage III and IV; 80% *versus* 40% *versus* 60%), and overall survival (0% *versus* 56% *versus* 44%) was observed between groups 1, 2, and 3. Nevertheless, it is of clinical interest that four of five (80%) patients in group 1 presented with stage IVB and died with disease. Furthermore, morphologically, three of five cases in group 1 were composed of large transformed cells (60%), in contrast to only one case in groups 2 and 3 (5%). Comparison between morphology and the presence of p53 mutations showed that nasal NK/T-cell lymphomas with large cell morphology were more likely to be associated with p53 mutations ($P = 0.0162$).

Table 3. Immunophenotype, *in Situ* Hybridization and Mutation Analyses of the p53 Gene in 25 Cases of Nasal NK/T-Cell Lymphoma

Case	CD3	TIA-1	CD56	p21	p53	EBER	p53 Mutation			
							Exon	Codon	Nucleotide	Amino acid
Group 1. p53+/p53 mutated										
1	+	+	+	25%	53%	+	7	258	GAA-AAA	Glu → Lys
2	+	+	+	<5%	89%	+	5	134	TTT-CTT	Phe → Leu
3	+	+	+	<5%	86%	+	7	242	TGC-CGC	Cys → Arg
4	+	+	+	18%	52%	+	7	226	GGC-GAC	Gly → Asp
5	+	+	+	22%	90%	+	6	218	GTG-GAG	Val → Glu
Group 2. p53+/p53 wild type										
6	+	+	+	15%	26%	+	WT*			
7	+	+	+	32%	14%	+	WT‡			
8	+	+	+	38%	45%	+	WT†			
9	+	+	+	<5%	29%	+	WT†			
10	+	+	neg	<5%	16%	+	WT‡			
11	+	+	neg	35%	52%	+	WT†			
12	+	+	+	20%	30%	+	WT†			
13	+	+	+	<5%	14%	+	NA			
14	+	+	neg	12%	17%	+	WT‡			
15	+	+	+	35%	20%	+	WT‡			
Group 3. P53-/p53 wild type										
16	+	+	neg	41%	<5%	+	WT†			
17	+	+	+	35%	<5%	+	WT‡			
18	+	+	+	<5%	<5%	+	NA			
19	+	+	+	27%	<5%	+	WT*			
20	+	+	neg	43%	<5%	+	WT‡			
21	+	+	+	<5%	<5%	+	NA			
22	+	+	+	<5%	<5%	+	NA			
23	+	+	+	<5%	<5%	+	WT*			
24	+	+	+	25%	<5%	+	WT*			
25	+	+	+	<5%	<5%	+	WT‡			

NA, DNA not amplifiable; WT, wild type.

*The DHPLC pattern was suspicious for mutation, the sequence analysis was normal.

†The DHPLC pattern and sequence analysis were normal.

‡The DHPLC pattern was normal, no sequence analysis was performed.

LOH Analysis

Despite the use of laser capture microdissection, only 12 cases rendered sufficient normal tissue for LOH analysis. Three cases corresponded to group 1 (cases 1 to 3), six to group 2 (cases 6, 9, 10, 13 to 15), and three to group 3 (cases 16, 17, and 20). Analysis of informative loci showed LOH in 2 of 12 cases (17%), both of which were associated with p53 mutations of the remaining allele (cases 1 and 3) (Figure 4). In one case with p53 mutation in exon 5, no LOH was identified (case 2). One case in group 3 (case 17) was noninformative.

Discussion

Recently, we reported that p53 was expressed in a high percentage of nasal NK/T-cell lymphomas from Peru.¹⁵ In this study of nasal NK/T-cell lymphomas from Mexico, we extended our analysis to the genetic level. p53 mutations were identified only in a minority of cases (24%), and were restricted to tumors with high levels of p53 overexpression. Interestingly, cases with p53 mutations were associated with large cell morphology ($P = 0.0162$) and presented more often with advanced stage disease.

The p53 gene is the most frequently mutated gene in human cancer, and the prototype of checkpoint regulator.²² However, p53 gene mutations are relatively rare in

NHL. In a large study of B- and T-cell NHL, only Burkitt's lymphoma and progressed B-cell chronic lymphocytic leukemia (Richter's transformation) were associated with p53 mutations.²³ Among peripheral T-cell lymphomas, with the exception of adult T-cell leukemia/lymphoma,²⁴ p53 mutations are found in <10% of the cases.²⁵ In comparison, we found a relatively high incidence of p53 mutations in nasal NK/T-cell lymphoma from Mexico (24%). Accordingly, a high incidence of p53 mutations (47.6%) was also found in a recent study of nasal NK/T-cell lymphomas from Japan and China.²⁶ The incidence of p53 mutations observed in that study varied depending on the geographic origin of the patients, suggesting that the development of p53 mutations is influenced by geographic, environmental, or ethnic differences. The mutational pattern observed in the five cases in our study is similar to previous findings in NHL.^{23,27} All mutations corresponded to missense mutations, with a predominance of transitions over transversions. However, none of our mutations involved a CpG dinucleotide or any of the predominant mutational hot spots. In contrast to our series, Li and colleagues²⁶ observed a relatively high percentage of silent mutations, as well as an absence of p53 expression in some cases with missense mutations. The reasons for these differences are not clear.

As a screening method for the detection of p53 mutations we used DHPLC, a relatively new technique based

Figure 2. Mutational analysis of p53 in nasal NK/T-cell lymphomas from Mexico by DHPLC. **A:** A normal DHPLC melting curve of any given exon, suggestive of wild-type p53. **B:** A questionable DHPLC melting curve, suspicious in this case of a p53 mutation in exon 6. The sequence analysis was normal. The red arrow highlights the area where an equivocal second peak is seen. **C:** Abnormal DHPLC pattern strongly suggestive of a p53 mutation (red arrow). The sequence analysis confirmed a mutation in exon 7, codon 242 (case 3).

on the different melting characteristics of hetero- and homoduplex forms between wild-type and mutated DNA fragments.¹⁹ Until now, the method has mainly been used to detect germline mutations in various inherited diseases for which a high degree of sensitivity ranging from 95 to 100% has been reported.^{28–31} Compared to currently used conventional screening techniques including single-strand conformation polymorphism and denaturing gradient gel electrophoresis, DHPLC is an easy and reliable screening method that significantly reduces the screening time for large numbers of tumors. Its sensitivity, in the range of 95%, compares favorably with these more labor-intensive techniques. All mutations in this study confirmed by sequencing had shown a clearly abnormal DHPLC pattern. None of the PCR products with questionable (four cases) or normal DHPLC elution curves were found to have a mutation. Although we cannot completely exclude that mutations outside of the examined exons 5

to 8 were responsible for p53 overexpression in some of the cases of group 2, we consider this unlikely. Hematological neoplasms, in general, show a very low rate of mutations outside the conserved domain, and when present, these are frequently nonsense mutations not leading to p53 protein accumulation.²⁷

In an effort to see whether the overexpressed p53 protein in cases of nasal NK/T-cell lymphoma with retention of wild-type p53 gene is functional, we studied the level of expression of its downstream target, the cyclin-dependent kinase inhibitor p21.²² Previous studies have shown that the expression of p21 is a very good surrogate marker for the status of the p53 gene.^{32,33} In cases with overexpression of wild-type p53, p21 protein is usually detectable, whereas the absence of p21 correlates with the presence of missense p53 mutations. To our surprise, even though most of the cases in group 2 (p53+/p53 wild-type) showed expression of p21, as ex-

Figure 4. LOH analysis. Electropherogram of normal/tumor pair in cases 1 and 3 (left and right side, respectively). The p53 informative locus is shown. The y axis represents the peak height in fluorescence units. **Top:** Amplification from normal tissue. The green peaks represent the heterozygous alleles. **Bottom:** Amplification from tumor tissue, the red arrows mark the lost allele.

pected, three of five cases with missense p53 mutations (group 1), as well as 5 of 10 cases without p53 expression also showed high expression of p21. This finding indicates that p21 is up-regulated independent of p53 gene status. An interesting, but still speculative possibility is that EBV might be responsible for, or contributes to p21 nuclear accumulation. Accordingly, in the study of Villuendas and colleagues³² a tendency for EBV-positive cases to harbor high levels of both p53 and p21 was found, indicating that EBV could be involved in the nuclear accumulation of both proteins in NHL. In nasopharyngeal carcinoma, another EBV-associated malignancy, p53 gene mutations are rarely identified despite the frequent detection of p53 overexpression, again suggesting a role for EBV in the p53 accumulation in EBV-associated neoplasias.³⁴ A further support for the notion that EBV itself, rather than the neoplastic transformation, is responsible for p53 overexpression in tumors with wild-type p53 is the up-regulation of p53 in EBV-infected, nonneoplastic B-cells in infectious mononucleosis,³⁵ and in angioimmunoblastic T-cell NHL.³⁶ The mechanism(s) through which EBV could potentially achieve accumulation of p53 and p21 is still unresolved. For p53, several hypotheses have been proposed, including p53 binding to EBV nuclear antigen 5 (EBNA-5)³⁷ or ZEBRA protein,^{38,39} and up-regulation of p53 through induction of nuclear factor kappa B (NF- κ B) by EBNA-2 and LMP1.⁴⁰

In accordance with the tumor suppressor gene model, mutation of one allele is often accompanied by deletion of the remaining allele, although this loss is not a requisite. In this study, of the 12 cases analyzed, only 2 cases (17%) showed LOH on chromosome 17p13, both of which showed p53 mutations of the remaining allele. A recent LOH study of different NK-cell neoplasias from China⁴¹ showed that LOH at chromosome 17p13 occurred only in nasal NK/T-cell lymphoma (four of nine, 44%), with a higher incidence at presentation (three of six) than at relapse (one of three). However, in addition to

the small number of cases, the clinical stage of the patients at presentation was not mentioned, nor was a mutational analysis of p53 performed in that study.

Although the number of cases in our study is too small to allow any firm conclusion to be drawn, it is important to mention that four of the five patients whose tumors revealed p53 mutations, presented with advanced disease and died soon after diagnosis. Secondly, seven of the nine patients that presented with clinical stages I and II from whom follow-up was available, are alive, six with no evidence of disease. The seventh patient had recently a local recurrence after 10 years of complete remission. In contrast, 11 of 13 patients with stages III and IV are dead, some of them despite combined treatment. (overall survival, 78% versus 15%; $P = 0.0059$) The high percentage of cases with advanced stages (III and IV) in this study, most probably reflects the fact that many patients in Mexico seek medical help late in their disease allowing it to take its natural course. Nevertheless, our data suggest that similar to other NHL subtypes,^{23,42,43} p53 mutations in nasal NK/T-cell lymphoma are associated with tumor progression and late-stage disease. The outcome of the patients in this series is in agreement with other studies that have identified clinical stage as probably the most important prognostic parameter.⁴⁴⁻⁵¹ Although patients in stages I and II seem to have a good response to combined treatment, the relapse rate in extranodal sites, especially in those patients treated only with radiotherapy, is still relatively high and confers a poor prognosis.^{46,52,53}

Interestingly, three of the five cases with p53 mutations revealed large cell morphology, in contrast to only 1 of the 20 cases without p53 mutations (60% versus 5%, $P = 0.0162$). To our knowledge, there are no studies so far that have analyzed the possible relationship of morphology and molecular changes and/or prognosis in nasal NK/T-cell lymphoma. Our study indicates that large cell

Figure 3. Immunohistochemical study of p53 and p21 in nasal NK/T-cell lymphomas from Mexico with p53 missense mutations (A and B) and wild-type p53 gene (C and D). **A:** Nasal lymphoma with strong nuclear overexpression of p53 in the majority of tumor cells and negative p21 expression (case 3, group I). **B:** In contrast to the previous example, note the positive nuclear expression of p21 in some tumor cells (case 5, group I). **C:** Nasal lymphoma positive for p53 with variable nuclear intensity and p21 expression (case 11, group 2). **D:** Nasal lymphoma with p53 expression in <5% of tumor cells and high expression of p21 (case 17, group 3). Immunoperoxidase staining, original magnifications, $\times 400$.

morphology is associated with the presence of p53 mutation in this neoplasm.

In summary, p53 mutations in nasal NK/T-cell lymphoma from Mexico are present in 24% of the cases and show a good correlation with high overexpression of p53. LOH at chromosome 17p13 was found in 2 of 12 (17%) cases investigated, and associated with mutations of the remaining allele. p53 mutations are associated with large cell morphology and advanced disease, and thus, might be involved in tumor progression. So far, clinical stage seems to be the most important prognostic indicator. The overexpression of p53 and p21 independent of p53 gene status, although as yet not clear, might be the result of EBV infection and an important mechanism of viral pathogenesis.

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