

Mutations of the *p53* Gene in Nasal NK/T-Cell Lymphoma

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SUMMARY: Mutations of the *p53* tumor suppressor gene are reported in various kinds of malignancies including lymphomas. However, *p53* gene mutations in nasal NK/T-cell lymphoma have not been reported because most parts of tumors are necrotic and a small amount of living tumor tissues is available for the molecular study. Expression and mutations of the *p53* gene were examined in the paraffin-embedded specimens of the nasal lesions from 42 Chinese (Beijing and Chengdu) and Japanese (Okinawa and Osaka) patients with nasal NK/T-cell lymphoma by the immunohistochemistry and single strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) amplified products followed by direct sequencing. Thirty single-nucleotide substitution mutations were observed in 20 of 42 cases (47.6%). Among the 30 mutations, 18 were missense (mainly G:C to A:T transitions), 9 were silent, and 1 was a nonsense mutation. The remaining 2 mutations involved intron 5 and exon 5 terminal points. Abnormal expression of the *p53* protein was also observed in 19 of 42 (45.2%) cases. The incidence was significantly (4-fold) higher in the cases of Osaka than those in other areas, although the incidence of *p53* mutations in the cases of Osaka was one-half to one-third of those in the other three areas. The results may suggest some racial, environmental, or lifestyle differences in the cause of nasal tumorigenesis. (*Lab Invest* 2000, 80:493–499).

Nasal lymphoma frequently shows a polymorphous pattern of proliferation, consisting of large atypical cells, small lymphocytes, plasma cells, benign looking macrophages occasionally showing with phagocytic figures, neutrophils, and eosinophils much less frequently. Thus the term polymorphic reticulosis (PR) was proposed for this condition (Eichel et al, 1966). PR constitutes lethal midline granuloma (LMG), which is characterized by necrotic and granulomatous lesions mainly affecting the nasal cavity. Malignant lymphoma with monomorphous proliferation and Wegener's granulomatosis also show clinical features of LMG (Kassel et al, 1969). Majority of nasal lymphoma, especially PR, was recently categorized as NK/T cell lymphoma (Jaffe et al, 1996). Through immunophenotypic and genotypic studies, we have shown that PR is an NK cell lymphoma (Ohsawa et al, 1999). Epidemiologic studies of PR revealed the clustering of patients in the east Asian countries (Aozasa et al, 1989) and Epstein-Barr virus (EBV) association (Harabuchi et al, 1990; Tomita et al, 1995), however little is known about mechanisms for development of PR.

p53 is a well-known tumor suppressor gene that causes cells with damaged DNA to arrest at the G1 phase of cell cycle or stimulating expression of the *bax* gene, the protein that promotes apoptosis (Levine et al, 1991). In a wide variety of human cancers, *p53* gene mutations have been detected mainly in exons 5 through 8 (Hollstein et al, 1991). High incidence of malignant lymphoma in the *p53* knockout mice has been reported (Donehower et al, 1992), suggesting an important role of *p53* gene mutations in lymphomagenesis. The mutated *p53* gene encodes mutant *p53* protein, which has a much longer half-life time than that of wild type *p53* protein, thus accumulating in the cytoplasm in an amount sufficient for immunohistochemical detection. Previous immunohistochemical study showed that *p53* is overexpressed in a high percentage of cases with nasal NK/T-cell lymphoma (Quintanilla-Martinez et al, 1998). These findings suggest that the *p53* gene mutations might be frequent in nasal NK/T-cell lymphoma. In this study, we analyzed the mutations of the *p53* gene in a series of 42 cases of nasal lymphomas of PR type collected from China and Japan by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct sequencing.

Results

Histological and Immunohistochemical Findings

Histologically, varying degrees of necrosis were found in the upper respiratory lesions. Diffuse proliferation of large atypical mono- or multinucleated cells was ob-

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served with various numbers of lymphocytes, plasma cells, and macrophages, giving a polymorphous appearance. An angiocentric pattern of proliferation was occasionally observed. Histologic grade based on the criteria described by Lipford et al (1988) was Grade II in 17 cases and Grade III in 25 cases. Percentage of large atypical cells among infiltrating cells was more than 30% and 80% in Grade II and III lesions, respectively. Immunohistochemically, tumor cells were CD20⁻, TIA-1⁺, CD56⁺, CD3⁺, or CD43⁺ (Fig. 1).

Expression and Mutation of the p53 Gene

Positive immuno-reactivity for DO-7, suggestive of abnormal expression or stabilization of p53 protein, was found in 19 of 42 (45.2%) cases (Table 1). The incidence of abnormal p53 expression was 4-fold higher in the cases in Osaka than those in Okinawa and 2-fold higher than those in China (Table 2).

By the direct sequencing of SSCP products, 30 single-nucleotide substitution mutations were detected in 20 of 42 cases: 13 cases had a single mutation, 5 had two mutations, 1 had three mutations, and 1 had four mutations (Fig. 2) (Table 1). With the exception of 2 mutations involving the intron 5 and exon 5 terminal point (codon 187), 18 were missense mutations leading to amino acid substitutions, 1 was a nonsense mutation, and 9 were silent mutations re-

sulting in no amino acid changes. No specific mutations were observed, although G:C to A:T transition was predominant (19 lesions), followed by A:T to G:C (3 lesions) transition. For mutation frequency by histologic grade, 10 of 17 cases (58.8%) in Grade II and 10 of 25 cases (40%) in Grade III showed p53 mutations.

There were no significant differences in the incidence of p53 mutation among the cases in the four areas. However, the incidence in the cases in Osaka was one-half to one-third of those in other areas, although the incidence of abnormal p53 expression was very high in the cases in Osaka (Table 2). The results suggest the difference in the cause of nasal lymphomagenesis between the cases in Osaka and the three other areas, Beijing, Chengdu, and Okinawa. Histologic grade of the cases that stained positive for p53, but were negative by SSCP, was Grade II in 3 cases and Grade III in 8.

EBV

The proliferating cells in 8 of 10 cases showed positive signals in the nucleus. Percentage of positive cells per 1000 cells counted under high power fields ranged from 11% to 64% (mean, 35%).

Discussion

Pathological characteristics of 42 nasal lymphomas, ie, varying degrees of necrotic change in the upper respiratory tract, and the polymorphous pattern of proliferation were identical with those in LMG of PR type (Eichel et al, 1966; Kassel et al, 1969), which is now classified as an NK/T- or NK-cell lymphoma (Jaffe et al, 1996). Indeed immunohistochemical findings of the present 42 cases were in agreement with those of NK/T- or NK-cell lymphoma, ie, TIA-1⁺, CD56⁺, CD3⁺, or CD43⁺.

In the non-nasal and ordinary lymphomas, p53 mutations were rare; none of 43 cases in the United States (Gaidano et al, 1991) and 8 of 48 (17%) in Japan (Ichikawa et al, 1992) had mutations. Meanwhile it was reported that aggressive high-grade B-cell non-Hodgkin's lymphoma (NHL) had approximately 30% incidence of p53 mutations, whereas its indolent counterpart rarely had incidence (Lo Coco et al, 1993). Among T-cell lymphomas, frequency of p53 gene mutations was reported to be less than 10%. Conversely frequency of p53 mutations was high in the specific category of lymphomas; greater than 50% in Burkitt's lymphoma (Villuendas et al, 1993) and approximately 70% in cases with pyothorax-associated lymphoma (Hongyo et al, 1998). Both types of lymphoma are known to be EBV-associated. The present study on NK/T-cell lymphoma revealed a high frequency (47.6%) of p53 gene mutations. NK/T-cell lymphoma is also EBV-associated (Harabuchi et al, 1990; Tomita et al, 1995). There have been no reports on p53 gene mutations in NK/T-cell lymphoma by using the PCR-SSCP method followed by direct sequencing. This must be due to availability of a small

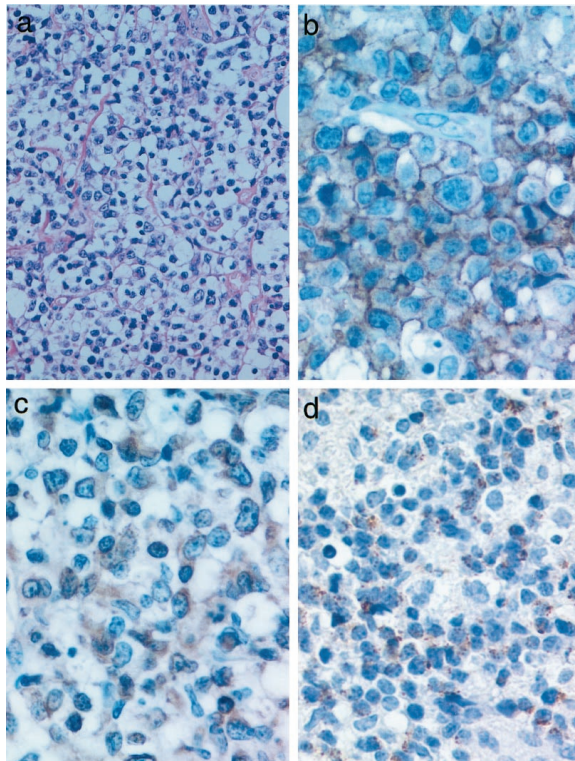


Figure 1.

Nasal NK/T-cell lymphoma, Grade II. a, Rather polymorphous pattern of proliferation of large to medium sized lymphoid cells admixed with other inflammatory cells (hematoxylin and eosin [HE] $\times 300$). b, Tumor cells were stained positive with CD56 (ABC $\times 600$); c, CD3 (ABC $\times 500$); and d, TIA-1 (ABC $\times 400$).

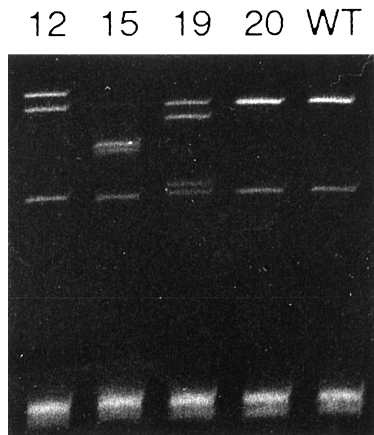
Table 1. Mutations and Overexpression of p53 gene in 42 Cases of Nasal NK/T-Cell Lymphoma from China and Japan

Regions	Cases No	Age	Sex	Site	Histologic grade	p53 Expression	p53 mutation				
							Exon	Codon	Nucleotide	Amino acid	
China											
Beijing	1	53	M	paranasal	III	—	none ^a				
	2	47	M	nose	III	—	7	246	ATG→GTG	Met→Val	
	3	22	M	nasopharynx	II	+	7	245	GGC→AGC	Gly→Ser	
							8	276	GCC→ACC	Ala→Thr	
	4	34	M	tonsilla	II	—	none				
	5	57	M	paranasal	II	+	none				
	6	67	M	nose	III	—	none				
	7	18	M	trachea	II	—	5	144	CAG→CTG	Gln→Leu	
	8	31	M	nose	III	—	5	ND ^b			
	9	28	M	nose	II	+	7	251	ATC→AGC	Ile→Ser	
	10	39	M	nose	II	+	7	251	ATC→AGC	Ile→Ser	
	11	40	M	nose	III	—	6	204	GAG→GAA	Glu→Glu	
							intron 5		third position		
									G→A		
	12	42	M	nose	II	—	5	142	CCT→CTT	Pro→Leu	
	13	32	M	nose	II	+	6	193	CAT→TAT	His→Leu	
	Chengdu	14	33	M	nasopharynx	II	+	none			
		15	24	M	paranasal	III	+	5	151	CCC→CTC	Pro→Leu
		16	79	M	nose	III	—	5	151	CCC→TCC	Pro→Ser
							7	245	GGC→AGC	Gly→Ser	
							8	273	CGT→CAT	Arg→His	
17		60	M	nose	III	+	none				
18		48	M	nose	III	—	none				
19		38	M	nose	III	—	5	162	ATC→GTC	Ile→Val	
							8	269	AGC→AAC	Ser→Asp	
Japan											
Okinawa	20	45	M	nose	II	—	none				
	21	64	F	nose	II	—	5	3' terminal G→A			
	22	39	M	nose	III	—	6	203	GTG→GAG	Val→Term	
							7	244	GGC→GAC	Gly→Asp	
	23	60	M	nose	III	—	none				
	24	53	M	nose	II	—	5	159	GCC→GCT	Ala→Ala	
	25	69	F	nose	II	—	8	273	CGT→CAT	Arg→His	
	26	34	M	nose	III	—	none				
	27	51	M	oral	II	—	none				
	28	71	F	nose	II	—	none				
	29	62	M	nose	III	+	none				
	30	64	M	nose	III	—	8	285	GAG→AAG	Glu→Lys	
	31	44	F	nose	II	—	6	193	ATG→ACG	Met→Thr	
							8	294	GAG→GAA	Glu→Glu	
	32	37	M	nose	III	+	5	149	TCC→TCT	Ser→Ser	
							5	154	GGC→GGT	Gly→Gly	
							5	161	GCC→GCT	Ala→Ala	
							8	276	GCC→GCT	Ala→Ala	
	Osaka	33	57	M	nose	III	+	none			
		34	78	F	nose	III	+	6	206	TTG TTA	Leu→Leu
		35	71	M	nose	II	+	none			
		36	60	M	nose	III	+	6	193	CAT GAT	His→Asp
37		71	M	nose	III	+	none				
38		48	M	nose	III	+	none				
39		57	M	nose	III	+	none				
40		52	M	nose	III	—	none				
41		50	F	oral	III	+	none				
42		49	M	nose	III	+	none				

^a no mutation; ^b not determined.

Table 2. Overexpression and Mutations of *p53* Gene in the 4 Different Areas

Regions	No. of Cases	<i>p53</i> overexpression	<i>p53</i> mutation
Beijing	14	6 (42.9%)	8 (57.1%)
Chengdu	5	2 (40.0%)	3 (60.0%)
Okinawa	14	3 (21.4%)	7 (50%)
Osaka	9	8 (88.9%) ^a	2 (22.2%)
Total	42	19 (45.2)	20 (47.6)

^a $p < 0.01$ versus Okinawa by Fisher's exact test.**Figure 2.**

Nonradioactive single strand conformation polymorphism (cold SSCP) analysis of *p53* exon 5 from representative cases with nasal lymphoma. Aberrant migration patterns compared with normal wild type (WT) bands are seen in cases 12, 15, and 19 and with point mutation at codons 142, 151, and 162, respectively.

amount of samples with necrotic change from PR lesions.

The ability to find a mutation is partially dependent upon the percentage of tumor cells. Based on the criteria proposed by Lipford et al (1988), our cases had Grade II or III histology, composing more than 30% of large atypical cells. Nasal NK/T-cell lymphoma is one of the EBV-associated lymphomas, and the percentage of EBV genome-containing cells was more than 10% (mean, 35%) in our cases. We previously reported that the PCR-SSCP method used in this study could detect the alterations of the *p53* gene in 3% cells of population (Hongyo et al, 1993). Therefore *p53* mutations could be detected in all of our cases if they occurred.

Transitions at the CpG dinucleotides site were the predominant pattern of substitutions in NHL cases, and G:C to T:A transversion is uncommon (Hollstein et al, 1991). In our series of NK/T-cell lymphoma, more than 70% of substitutions were transitions, however the CpG site was involved in only three lesions. The predominance of transition mutations (G:C to A:T) in our series of NK/T-cell lymphoma suggests that some "endogenous" mutagens act in lymphomagenesis. The transition pattern of the *p53* mutation is known to be more susceptible to spontaneous genetic instability than transversion. Meanwhile 75% of the cases in the current series had at least one mutation that

changed an amino acid, which might have provided the selection pressure for expansion.

Mutational analysis of the *p53* gene has been confined principally to exons 5 through 8, because 90% of the mutations in human tumors occurred in this region (Hollstein et al, 1991). In NHL, the predominant site for mutations did not present in previous reports (Adamson et al, 1995; Ichikawa et al, 1992). In our cases with NK/T-cell lymphoma, exon 5 is the most common site for mutations, ie, 13 of 30 (43%) mutations occurred in exon 5. Some carcinogens might cause mutation in specific codons, as was observed in the mutation of codon 249 in lung and liver cancers induced by irradiation (Anderson et al, 1995; Taylor et al, 1994). Relatively restricted distribution of mutational spots were found in our cases; codon 273, one of the so-called mutational "hot spots" (Hollstein et al, 1991), was involved in 2 cases, and codons 151, 193, and 251 were involved in 2 or 3 cases. Distribution of hot spots in *p53* mutations might reflect the function of specific mutant alleles being selected for promoting cell proliferation or the nature and activity of mutagens in different tissue fashion.

Alteration of the *p53* gene results in accumulation of the *p53* mutant protein due to its longer half-life time than that of wild type. Thus immunohistochemical detection of the *p53* protein could be performed in various kinds of malignancies under supposition that *p53* gene mutations might occur frequently in these tumors. On the nasal lymphomas, Quintanilla-Martines et al (1998) reported that the *p53* protein was overexpressed in 22 of 29 cases (76%) examined. In our cases with nasal NK/T-cell lymphomas, 19 of 42 cases (45%) overexpressed the *p53* protein. Our study showed a discordance between *p53* protein overexpression and the presence of mutations: 11 of 19 cases (58%) overexpressing the *p53* protein had no mutations. This kind of discordance had been reported in many types of tumors including lymphomas (Adamson et al, 1995; Martinez et al, 1997), and reasons for this were variously speculated. Recently a link between accumulation of a wild type *p53* protein and EBV has been postulated in the nasopharyngeal carcinoma (Effert et al, 1992). Nasal NK/T-cell lymphoma is also an EBV-associated disease (Harabuchi et al, 1990; Tomita et al, 1995). Accumulation of a wild type *p53* protein was also reported in the acquired immunodeficiency syndrome-related lymphomas (Nakamura et al, 1993). EBV was studied only in 10 of the current cases, therefore we could not evaluate the relationship between abnormal *p53* expression and EBV.

Functional inactivation of *p53* through interaction of the wild type gene product with various viral products such as SV 40 large T antigen, adenovirus E1B, human papilloma virus (HPV) E6 protein, or cellular protein MDM2 was reported (Capoulade et al, 1998). In HPV-positive tumors, binding of the E6 protein obviates the need for *p53* mutations in the genesis of such tumors, but this association was not noted in EBV infection and Hodgkin's disease (Niedobitek et al, 1993). However, EBV immediate-early protein, BZLF1, which is

Table 3. p53 Mutations in Malignant Lymphomas

Lymphomas	Histology	EBV+/total cases (%)	Mutations/ Total cases (%)	G:C to A:T transitions/ total substitutions (%)	G:C to A:T transitions at CpG sites/total substitutions (%)	G:C to A:T transitions at dipyrimidine sites/total substitutions (%)	Reference
Pyothorax-associated Lymphoma	Diffuse immunoblastic	21/21 (100)	14/21 (67) ^{a,b}	12/13 (92)	2/13 (15)	10/13 (77) ^c	Hongyo et al, 1998
Burkitt Lymphoma	Burkitt type	2/4 (50)	3/4 (75)	1/3 (33)	1/3 (33)	1/3 (33)	Gaidano et al, 1991
	Burkitt type	ND ^d	9/27 (33)	5/11 (45)	4/11 (36)	2/11 (18)	
AIDS-Related Lymphoma	Diffuse large cell	22/34 (65)	1/34 (3)	1/1 (100)	0/1 (0)	1/1 (100)	
	Small non-cleaved cell	10/24 (42)	10/27 (37)	8/10 (80)	5/10 (50)	5/10 (50)	
Mucosa-Associated Lymphoid Tissue Lymphoma	B-cell type	ND	21/75 (28)	6/21 (29)	4/21 (19)	3/21 (14)	

^a one deletion.

^b $p = 0.05$ by 2 test with Yates' correction and/or Fisher's exact tests versus other kinds of lymphomas or cancers except squamous cell carcinoma of skin and radon-associated lung cancer.

^c $p = 0.05$ except basal cell carcinoma and squamous cell carcinoma of the skin and AIDS-related lymphoma. Statistical analysis were not done for the samples with less than four cases.

^d ND, not done.

highly expressed in immunodeficiency syndrome-related lymphoma, can interact with *p53* and inhibit its function (Zhang et al, 1994). EBNA-5, another EBV encoded protein necessary for transformation of infected B cells, can also form complexes with both wild type and mutant *p53* protein (Szekely et al, 1993). Taken together, it is possible that EBV gene products could indirectly suppress *p53* function, thus resulting in overproduction and accumulation of wild type *p53* as a compensating function. The lowest incidence of *p53* gene mutations and the highest incidence of *p53* protein expression in the Osaka cases in our series suggest the different causes for nasal lymphomagenesis including racial, environmental, or lifestyle causes.

In conclusion, mutations of the *p53* gene are frequent in nasal NK/T-cell lymphoma with rather restricted sites for mutations. These findings give an insight on the lymphomagenesis of nasal NK/T-cell lymphoma and also give some suggestions for its treatment.

Materials and Methods

Forty-two cases with PR, 19 from China and 23 from Japan, were selected for the current study: they were admitted to hospitals during the period of 1986 to 1997 (Table 1). All patients presented with the necrotic and granulomatous lesions in the upper respiratory tract, which were biopsied for histologic diagnosis before treatment. Age of patients ranged from 18 to 79 years (median, 49.5) with a male to female ratio (M/F) of 6:1. There were differences in the distribution of age

and sex between Chinese and Japanese patients: in China, a median age of 42 years and all men, and in Japan, a median age of 53 years and M/F ratio of 2.8:1. The nasal cavity and paranasal sinuses were the most common sites (37 cases) for involvement followed by naso-/oropharynx (3 cases), tonsil (1 case), and trachea (1 case). Histologic specimens were fixed in 10% formalin and routinely processed for paraffin-embedding. All of the paraffin blocks were gathered from Osaka University, and 3 μ m sections were cut and stored at 4° C before staining with hematoxylin-eosin and immunohistochemical procedures at the same time. Histologic slides were reviewed by two of the authors (LT, KA) for diagnosis. To show the number of the proliferating cells in the lesions, histologic grade was determined based on the criteria described by Lipford et al (1988).

Immunohistochemistry

Immunohistochemical study of the paraffin sections was carried out using the ABC method. The primary antibodies used in the study, their suppliers, and dilutions were as follows: CD3 (1:100; Dakopatts, Glostrup, Denmark), MT-1(CD43) (1:50; Bioscience, Emmenbrucke, Switzerland), Mx-PanB (CD20) (1:200; Kyowa Medex, Tokyo, Japan), 123C3 (CD56) (1:40; Zymed, South San Francisco, California), ZH7 (CD16) (1:200; Novocastra, Newcastle, United Kingdom), and TIA-1(1:500; Coulter, Hialeah, Florida). The alkaline phosphatase-anti-alkaline-phosphatase (APAAP) method was used in *p53* protein detection with use of monoclonal anti-human *p53* protein (DO-7) (Dako-

patts) diluted at 1:10 as the primary antibody. When CD3, CD56, and DO-7 were used as primary antibody, sections were treated in a microwave oven for 15 minutes in 10 mM citrate buffer (10 mM citrate monohydrate in distilled water, pH 6.0) for antigen retrieval. Cases with more than 10% of tumor cells positive for DO-7 were regarded as positive.

DNA Extraction and PCR for p53 Gene

DNA for PCR amplification was extracted using chelation resin. Three 10- μ m thick paraffin sections were cut, transferred into sterile distilled water containing 20% chelating resin iminodiacetic acid (Sigma, St. Louis, Missouri), and boiled for 30 minutes. After centrifugation, the supernatant was transferred to a sterile 500 μ l tube and stored at -20° C. The PCR primer pairs for the amplification of the *p53* gene exons 5 through 8 were: (a) 5' -GTACTCCCCTGCCCTCAACA-3' and 5' -CTCACC-ATCGCTATCTGAGCA-3' for exon 5; (b) 5' -TTGCT-CTTAGGTCTGGCCCC-3' and 5' -CAGACCTCAGGC-GGCTCATA-3' for exon 6; (c) 5' -TAGGTTGGCTCTG-ACTGTACC-3' and 5' -TGACCTGGAGTCTCCAGT-GT-3' for exon 7; and (d) 5' -AGTGGTAATCTACTG-GGACGG-3' and 5' -ACCTCGCTTAGTGCTCCCTG-3' for exon 8.

Hot start PCR was performed as follows: 45 cycles of denaturation at 95° C for 30 seconds; annealing at 58° C, 62° C, 60° C, and 60° C for 30 seconds for exon 5, 6, 7, and 8, respectively; extension at 72° C for 1 minute, and final extension at 72° C for 7 minutes. Paraffin blocks containing no sample were cut and used as negative controls throughout the procedures. The amplified products were subjected to electrophoresis in 1.5% agarose gel containing 2 μ g/ml ethidium bromide in TBE buffer. After electrophoresis, the gels were examined under an ultraviolet light transilluminator.

SSCP

Nonradioactive SSCP was performed as previously reported (Hongyo et al, 1995). Twenty microliters of reaction mixtures containing 5 μ l of PCR product (20–200 ng of DNA), 0.2 μ l of 1M methylmercury hydroxide, 3 μ l of loading buffer (15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol), and TBE buffer were heated to 90° C for 4 minutes and put on ice and then electrophoresed in 18% polyacrylamide TBE gel at 300 volts, while maintaining the temperature at 35° C for exon 5, 5° C for exon 6, and 25° C for exons 7 and 8. The gels were stained with 0.5 μ g/ml ethidium bromide in TBE buffer for 20 minutes at room temperature. The bands migrated apart from that of wild type were determined as SSCP positive. The bands possibly mutated by SSCP were extracted from the gels and amplified by 25 cycles of PCR to enrich the mutated alleles.

Direct Sequencing

Sequencing was carried out on PCR products of SSCP positive cases. To purify single or double

stranded PCR products with a range of 100 bp, the PCR products were processed using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, California) according to the manufacturer's protocol. Sequencing was performed by the dideoxy chain termination method using the Big Dye Terminator cycle-sequencing kit (Perkin-Elmer Corporation, Foster City, California). The same primers were used as for PCR. Cycle sequencing was performed following the protocol, ie, 30 cycles of denaturation (95° C, 20 seconds), annealing (54° C, 30 seconds), and extension (72° C, 3 minutes). After ethanol precipitation, the samples were analyzed by the Genetic Analyzer (ABI Prism 310, Perkin-Elmer Corporation). The PCR-SSCP analysis and sequencing of the possible positive cases were repeated three times to rule out the contamination and artifacts.

In Situ Hybridization for EBV

EBV RNA in situ hybridization (ISH) was performed in 10 cases as previously described (Weiss et al, 1991). As a positive control, the Raji cell line was used. Hodgkin's disease of mixed cellularity type with the EBV genome was also included as positive control. As negative controls, the hybridizing mixture was used with (a) sense probe and (b) antisense probe after RNase (Sigma) treatment.

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