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Immunohistochemical and SSCP Analysis of p53 in Malignant Lymphomas

We immunohistochemically investigated Epstein-Barr virus (EBV)-positive and negative 31 malignant lymphomas (MLs) for p53 protein using a monoclonal antibody which is expressed on a wild type and mutant human p53 protein. We evaluated the presence of mutations in exons 5 to 8 of the p53 gene using single-strand confirmation polymorphism analysis. Overexpression of p53 was detected in 13 out of 31 cases (41.9%) of MLs. However, we have documented the presence of structural alterations of the p53 gene in six cases of MLs. The presence of EBV infection in MLs was statistically unrelated to p53 protein overexpression. Excellent correlation was found between p53 immunoreactivity and histologic types of MLs. Even though the reason for discrepancy between p53 gene mutation and p53 protein overexpression remains unclear, p53 protein overexpression may be involved in the process of malignant transformation regardless of EBV infection in MLs.

Key Words : *Protein, p53; Genes, p53; Lymphoma; Herpesvirus 4, human*

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INTRODUCTION

Mutations of the p53 tumor suppressor gene are the most frequent genetic abnormalities detectable in human tumors (1-5). The p53 gene, located on the short arm of chromosome 17, encodes a 53 kDa phosphoprotein capable of binding to DNA and acting as a transcription factor. The normal or wild-type p53 protein is thought to have a role in inhibiting cell proliferation by arresting the cell cycle at the G1-S phase to allow DNA repair to take place (1-5). Alteration or inactivation of p53 by mutation or interaction with oncogene products and tumor viruses may cause depletion of p53 or convert p53 from a tumor suppressor gene into a transforming oncogene (5-7). Molecular studies indicate that mutations in the p53 gene are not generalized in ML, but are specifically associated with lymphoma subtypes, including Burkitt's lymphoma, B cell chronic lymphocytic leukemia, and Richter's syndrome and progression in follicular lymphomas (8, 9). p53 has been localized in Hodgkin's disease and in high-grade or anaplastic malignant lymphomas (MLs) (10-14). EBV has been thought to be one possible etiologic agent in lymphomagenesis (15-18). Previous studies have shown that EBV infection is consistently found in the endemic form of Burkitt's lymphoma and the resulting activation of *c-myc* has been regarded as an essential factor for the progressive growth of the tumor cells (15, 16). Recently, several laboratories found that p53 is mutated in at least 60% of Burkitt's lymphomas (19-20). p53 is therefore one possible candidate for the postulated additional change. Unlike Burkitt's lymphoma, very little is known about the pathogenesis of EBV-positive lymphomas. Therefore, we investigated p53 abnormalities in MLs to correlate with EBV infection.

MATERIALS AND METHODS

The study group comprised formalin-fixed paraffin-embedded lymph node biopsies of 31 MLs and 6 reactive hyperplasias (RHs) including 2 Castleman's diseases and 1 progressive transformation of germinal centers (PTGC). Among the ML cases, EBER in situ hybridization (ISH) positive and negative cases on screening were selected for the study. All of the cases were reviewed histologically and classified by the modified International Working Formulation (6).

Immunohistochemical studies

Immunophenotypic studies were performed on forma-

lin-fixed, paraffin-embedded sections using the monoclonal antibodies that are reactive in routinely processed paraffin-embedded tissues by a streptavidin-alkaline phosphatase method: a panel of monoclonal antibodies against T cells [CD43 (MT-1, BioGenex); CD45RO (UCHL-1, Dakopatts)]; B cells [CD20 (L-26, Dakopatts), MB-2 (BioGenex)]; macrophage [CD68 (Dakopatts)]; and activated T, B, and Reed-Sternberg cells [CD30 (Ki-1, Dakopatts)].

Overexpression of p53 was assessed using the DO7 monoclonal antibody (MoAb) (Novocastra, Newcastle, United Kingdom), which specifically detects human wild type and mutant p53. Because of the half-life of normal p53 is short and the amount of normal p53 expressed is low, the detection of stainable levels of p53 in a tumor cell suggests a p53 mutation. Before the application of the primary antibody, an antigen recovery technique was performed. The deparaffinized slides were placed into 50 mm Tris buffer (pH 7.4), and microwaved for 5 minutes. The DO7 antibody was used at a concentration of 1:50 and applied for 60 minutes. The antibody was detected by means of an alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique, using Fast Red as chromogen. Levamisole was empolyed to inhibit endogenous alkaline phosphatase. The slides were counter-stained with hematoxylin. A case was regarded as p53 positive if greater than 10% of the malignant cells stained with the antibody, although in practice all positive cases had 20% or more of their cells marked.

Detection of EBV genome in formalin-fixed paraffin-embedded tissues

In situ hybridization studies

To detect EBV nuclear RNA transcripts, in situ hybridization studies (ISH) were performed with a fluorescein-conjugated oligonucleotide probe, EBER (Y017, Dakopatts, Denmark). Briefly, 10 μm thick sections cut from paraffin blocks of formalin-fixed tissues were mounted on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma, St.Louis, MO, U.S.A). After deparaffinization and rehydration, the sections were digested with proteinase K (Sigma) at a concentration of 3 μg/ml for 30 min at 37℃, washed in water, and dehydrated in gradient ethanol. After complete drying of the slides, one drop of FITC-conjugated EBERs was applied and incubated for 2 hours at 37℃. After washing twice in Tris-buffered saline (TBS) containing 0.1% Triton X-100, the slides were incubated for 30 min at 37℃ with rabbit F(ab') anti-FITC antibody conjugated with alkaline phosphatase (1:50, Dakopatts). After washing in TBS, the slides were incubated in NBT/BCIP (1:50, Dakopatts) diluted in 0.1 M TBS containing 0.1 M NaCl and 50 mM $MgCl_2$, pH 9.0. Dark purple discoloration of the nuclei was interpreted as a positive signal (Fig. 1).

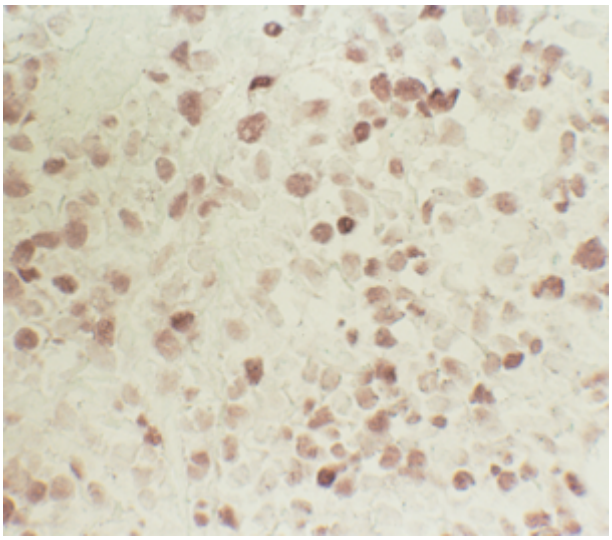

Fig. 1. EBER in situ hybridization of diffuse large cell lymphoma showing positive signals in the nuclei of neoplastic cells (×400).

Polymerase chain reaction (PCR) amplification of EBV genome

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using a QIAamp tissue kit (Qiagen, Germany) as described in protocol. For PCR amplification of EBV genome, 20 base primers were designed to amplify 138 bp segment in the EBNA-1 region and were designated as EBNA1-PF: 5'-TGA TAA CCA TGG ACG AGG AC-3' and EBNA1-PR: 5'-GCA GCC AAT GCA ACT TGG AC-3'. 1 μl of genomic DNA was added to 19 μl of PreMix™-TOP (K2016, Bioneer) PCR amplification kit. After being denatured at 95℃ for 3 min, samples were subjected to 35 cycles of amplification (30 sec at 94℃, 1 min at 58℃, 2 min at 72℃), and a final extension at 72℃ for 15 min. Known EBV negative and positive DNAs from lymphoma patients were used as controls.

Southern blot analysis of PCR amplified samples

PCR amplified products were visualized with UV light as a discrete single band of 138 bp by staining with ethidium bromide after agarose gel electrophoresis. The PCR products were transferred to Hybond N+ membranes (Amersham, Buckinghamshire, England) by Southern blotting. Membranes underwent 5h of prehybridization at 43℃ with a solution containing 5X SSPE (1X SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 0.1% sodium dodecyl sulfate). An 18 bp EBNA1 oligonucleotide probe (EBNA1P) was labelled

Table 1. Primers used for amplification of the p53 gene

Amplified fragment	Primers		PCR product
	Name	Sequence	
Exon 5	P55	5'-CTCTTCCTGCAGTACTCCCCTGC	211 bp
	P53	5'-GCCCCAGCTGCTCACCATCGCTA	
Exon 6	P65	5'-GATTGCTCTTAGGTCTGGCCCCTC	185 bp
	P63	5'-GGCCACTGACAACCACCCTTAACC	
Exon 7	P75	5'-GTGTTGTCTCCTAGGTTGGCTCTG	139 bp
	P73	5'-CAAGTGGCTCCTGACCTGGAGTC	
Exon 8	P85	5'-ACCTGATTTCCTTACTGCCTCTGGC	200 bp
	P83	5'-GTCCTGCTTGCTTACCTCGCTTAGT	

with ^{32}P γATP using a 5'oligonucleotide end labelling kit (Amersham). The oligonucleotide probe was designed and designated as EBNA1P: 5'-TCA GGA TCA GGG CCA AAA-3'. The ^{32}P-labelled probes (100 pmol) were hybridized to the blots for 12 hrs at 50℃, washed under stringent conditions and exposed to Agfa X-ray films overnight.

Detection of p53 mutations by single strand confirmation polymorphism (SSCP) analysis

SSCP analysis for p53 was accomplished using the human p53 amplification kit (K4120, Bioneer, Korea) and amplified exons 5, 6, 7, 8 of the p53 gene (Table 1). The PCR was performed on an automated heat-block (DNA Thermal-Cycler, Hybaid). 1 μl of genomic DNA and 19 μl of distilled water was added to PreMix™-TOP (K4120, Bioneer, Korea) PCR amplification kit, in a final volume of 20 μl. After being denatured at 94℃ for 5 min, samples were subjected to 35 cycles of denaturation (65℃ for exon 5 and 62℃ for exons 6, 7, 8), and a final extension at 72℃ for 5 min. SSCP analysis of PCR products was performed by silver staining. Briefly, samples (4 μl of PCR product and 14 μl of 5X formamide dye) were heated at 95℃ for 5 min, chilled on ice, and immediately loaded onto a 8% polyacrylamide gel containing 10% glycerol. Gels were run at 9 watts for 15 hrs at room temperature. The gels were fixed in 10% acetic acid for 20 to 30 min, and washed in distilled water. Silver staining was performed with staining solution containing formaldehyde (1.5 ml/L) using the silver staining system (C-1010, Bioneer, Korea) as described in protocol.

Statistical analysis

Data was analyzed by Chi-square tests. P values were estimated by Pearson Chi-square.

RESULTS

A total of 31 MLs and 6 reactive lymph nodes were examined. The summarized results of the patient's clinicopathologic features, immunophenotypes, EBV status,

Table 2. Clinicopathologic features of patients and results of p53 immunostaining and SSCP analysis in Epstein-Barr virus negative lymphomas

Case No.	Age/Sex	Stage	Primary sites	Histology	Phenotype	p53 immunoreactivity	SSCP
1	28/M	IE	Nasal cavity	Diffuse mixed	T	−	−
2	57/M	IV	Lymph node	Diffuse mixed	T	−	−
3	13/M	?	Lymph node	Diffuse mixed	T	++	−
4	35/M	II	Skin	Diffuse mixed	T	−	−
5	64/M	III	LN	Diffuse mixed	B	−	−
6	71/M	IE	Esophagus	Small	B	−	−
7	59/F	IIIE	Stomach	Diffuse large	T	+	−
8	68/F	II	Small intestine	Diffuse large	B	−	−
9	59/F	IV	Ileum	Immunoblastic	T	+++	+(exon 5)
10	33/M	II	Ileum	Immunoblastic	B	+++	−
11	4/M	?	Lymph node	Burkitt's	B	+++	−
12	42/M	IE	Ileum	Burkitt's	B	+++	+(exon 6)
13	15/M	IV	Lymph node	Lymphoblast	T	+	−
14	64/M	IE	Stomach	Diffuse mixed	B	−	−

Table 3. Clinicopathologic features of patients and results of p53 immunostaining and SSCP analysis in Epstein-Barr virus positive lymphomas

Case No.	Age/Sex	Stage	Primary sites	Histology	Phenotype	p53 immunoreactivity	SSCP
15	33/M	IE	Nasal cavity	Diffuse mixed	T	−	−
16	54/M	IV	Nasopharynx	Diffuse mixed	T	−	−
17	32/M	IE	Jejunum	Diffuse mixed	T	+	−
18	68/F	IV	Lymph node	Diffuse mixed	T	+	−
19	2/M	IV	Lymph node	Diffuse mixed	T	−	+(exon 7)
20	58/M	IIIE	Skin	Diffuse mixed	T	−	−
21	7/F	III	Lymph node	Diffuse mixed	T	−	−
22	51/M	II	Lymph node	Diffuse large	B	−	+(exon 6)
23	32/M	II	Lymph node	Immunoblastic	B	−	−
24	45/M	II	Stomach	Diffuse large	T	−	+(exon 7)
25	30/F	IE	Skin	Diffuse large	T	+	−
26	52/F	III	Nasal cavity	Diffuse large	T	+	−
27	42/M	I	Lymph node	Diffuse large	B	++	−
28	42/F	IV	Terminal ileum	Diffuse large	B	−	−
29	58/M	IV	Lymph node	Burkitt's	B	++	−
30	31/M	IV	Lymph node	Follicular large	B	−	−
31	48/M	IE	Eyeball	Small cleaved	B	−	+(exon 5)

and p53 protein expression are shown in Tables 2 and 3. The patients consisted of 23 males and 8 females with a median age of 41.8 years (range, 2-71 years). The primary biopsy sites were lymph node in 13 cases, gastrointestinal tract in 10 cases (esophagus 1, stomach 3, terminal ileum 5, jejunum 1), nasal cavity in 3 cases, nasopharynx in 1 case, skin in 3 cases, and eyeball in one case. A T cell origin was likely in 17 cases, and a B cell origin in 14. Clinical stages of the MLs were distributed as follows: stage 1 in 9 cases, stage II in 7 cases, stage III in 5 cases, stage IV in 8 cases, and unknown in 2 cases.

Results of EBER in situ hybridization and Southern blot analysis

In this study, EBER ISH and/or EBV DNA PCR with Southern blot analysis and autoradiography positive cases were counted as EBV positive cases. EBER ISH was performed in 33/37 (28/31 MLs and 5/6 RHs) cases studied. Among these, 7/28 MLs and 1/5 RHs were EBER ISH positive cases. 2/33 EBER ISH positive cases (2/28 MLs and 0/5 RHs) failed to reveal EBV DNA sequences on autoradiography.

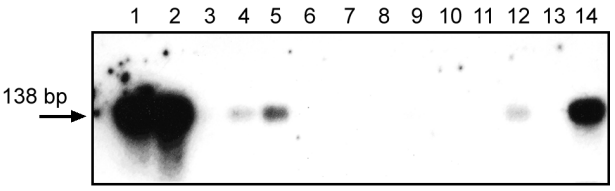

Fig. 2. Southern blot hybridization of EBNA1 PCR products probed with γP^{32} dATP-labelled EBNA1 probe.

11/33 EBER negative cases (8/28 MLs and 3/5 RHs) turned out to be EBV DNA positive with Southern blot analysis (Fig. 2). EBV bands were detected only after Southern blot hybridization in 10/37 cases (5/31 MLs and 5/6 RHs). In summary, among the 31 cases of MLs, 17 cases were EBV positive and 14 were EBV negative. In view of the primary sites, EBV was detected in 3/4 nasopharyngeal lymphomas (75%), 8/13 lymph nodes (61.5 %), 1/3 skin (33.3%), and 3/10 gastrointestinal tract (30%).

Results of p53 Immunohistochemical staining

Using an arbitary threshold of 10% positive staining, which has been used by other authors, overexpression of p53 was detected in 13 out of 31 cases (41.9%). In all cases antigen was localized to the nucleus of neoplastic lymphoid cells, and was finely granular and variable in intensity. Cases of p53 positive were arbitrarily divided into three groups according to the number of p53 positive cells and p53 immunoreactivity. Weak positive cases contained about 30% p53 immunoreactive cells, moderately positive 30 to 60%, and strongly positive 60 to 100%. Immunoreactivity was strong in four of the 12 p53 positive cases, moderate in three cases, and weak in six cases. There were significant correlations between p53 immunoreactivity and histologic subtypes of MLs. Diffuse strong positive immunoreactivity was the most constant finding in high grade MLs; positive immunoreactivity was found in 2/3 immunoblastic lymphomas and in 2/3

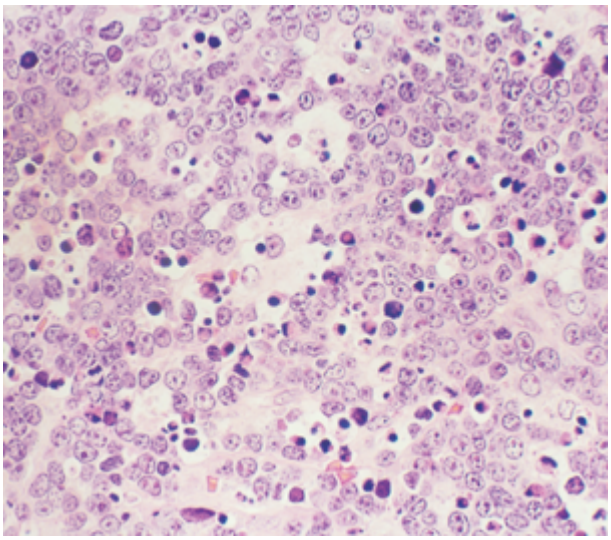

Fig. 3. Light microscopic findings of Burkitt's lymphoma (Case No. 12, H&E; ×400).

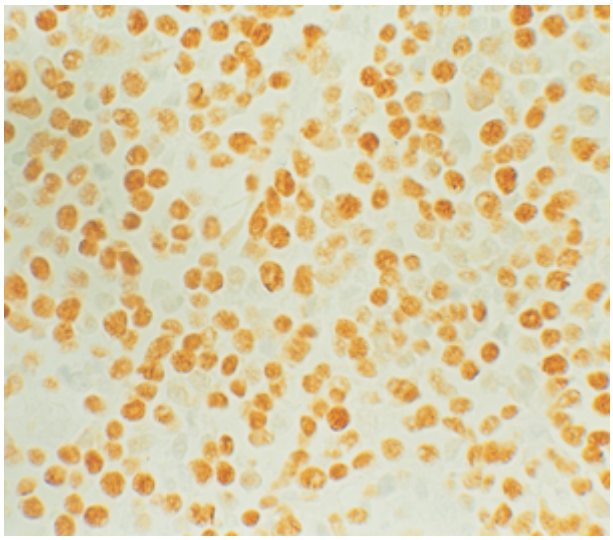

Fig. 4. Immunoperoxidase staining with anti-p53 monoclonal antibody showing diffuse strong nuclear immunoreactivity for the p53 protein (Case No. 12, ×400).

Burkitt's lymphomas (Fig. 3, 4). Moderate staining was detected in 1/11 diffuse large cell types, 1/13 diffuse mixed cell types, and 1/3 Burkitt's lymphomas. The remaining weak positive cases go as follows: 3 were diffuse large cell types, 2 diffuse mixed cell types, and one lymphoblastic type. Low grade lymphomas were p53 negative. Six of the 13 cases which showed p53 immunoreactivity were EBV positive and the remaining seven cases were EBV negative. However, only six out of the 13 p53-immunoreactive NHLs had abnormally migrating bands. Among these cases, 4 cases were EBV positive and 2 cases were EBV negative. Two out of the six cases exhibiting abnormally migrating bands displayed diffuse strong positive p53 immunoreactivity and the remaining four cases showed negative p53 immunoreactivity. Two immunoblastic lymphomas and two Burkitt's lymphomas which showed diffuse strong positive p53 immunoreactivity were all EBV negative. p53-positive cells were present in 5/14 B-cell (35.7%) and 8/17 T-cell (47.1%) NHLs. p53 protein was found in 44.4% (4/9), 16.7% (1/6), 40% (2/5), and 44.4% (4/9) of lymphomas in stage I, II, III, and IV respectively.

In reactive lymph nodes and low grade lymphomas, p53 immunoreactivity was either absent or present only in rare, activated cells in the follicular and interfollicular zones.

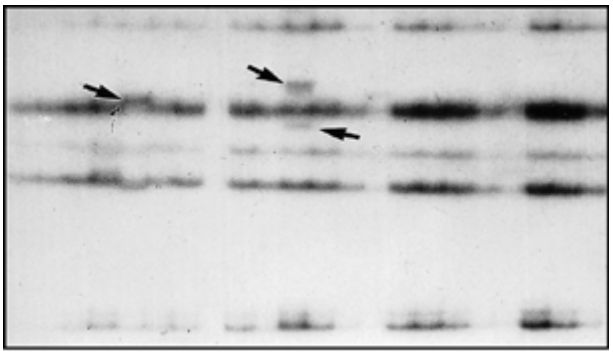

Fig. 5. PCR-SSCP analysis performed for p53 exon 5, where arrows indicate the appearance of abnormal migrating bands in exon 5.

Results of PCR-SSCP analysis of p53

An abnormal migration pattern suggesting the presence of mutation was identified in six out of the 31 (19.4%) MLs. Case 9 and 31 had an abnormal migration pattern in exon 5 (Fig. 5). Case 12 and Case 22 showed the appearance of abnormally migrating bands in exon 6 and Case 19 and Case 24 in exon 7.

DISCUSSION

This study has examined p53 overexpression in EBV-positive and EBV-negative lymphomas using a monoclonal antibody which is expressed on wild type and mutant human p53 protein. Previous studies have shown that 31-61% of MLs (mean 45.8%) overexpress p53 (10, 11, 13, 21-23). Significant p53 overexpression has been found in high-grade B-cell MLs as well as in ALCL (8, 10, 11, 13, 14). Therefore, it was thought that mutation in p53 is a late genetic event in the progession of lymphomas. Recently, there has been growing evidence sug-

gesting that p53 protein overexpression may be related to rapid cell proliferation in tumors that lack a demonstrable mutation (14, 24). In this study, overexpression of p53 was detected in 13 out of 31 cases (41.9%) of MLs. Excellent correlation was found between p53 expression and histologic types or grades as previously reported ($P < 0.05$) (8, 10, 11, 13). Diffuse strong positive p53 immunoreactivity was identified only in high-grade MLs (5/7 cases), especially in the B-cell type. The histologic types of these lymphomas were immunoblastic lymphomas and Burkitt's lymphomas. On the other hand, single scattered p53-positive cells were detected infrequently in low-grade lymphomas and benign reactive lymph nodes.

Despite the large number of cases with p53 protein overexpression, only six cases displayed abnormal migrating bands suggesting mutations by structural analysis of exons 5 to 8 that encode the most conserved portions of the gene where the vast majority of mutations have been documented using SSCP analysis. This is a highly sensitive assay that is capable of detecting point mutations as well as larger deletions (25). Two out of the six cases with p53 mutations were found to have overexpression of the p53 protein and were EBV negative. However, five of the nonmutated cases demonstrated comparable levels and intensities of staining.

p53 protein expression has been studied in a wide variety of tumors (2-4, 26-30). Point mutations in the p53 gene are associated with the stabilization of the protein and lead to detectable overexpression of the p53 gene product by immunohistochemistry (33, 34). But authors have found discrepancies between the frequency of cases overexpressing p53 protein detected by immunohistochemical methods and the frequency of those actually containing gene mutations (14, 24, 26-32).

The relatively low frequency of mutation in this study confirms results previously reported by Matsushima et al. (24) who examined 34 post-thymic T cell lymphomas and found only three mutations although they showed overexpression of the p53 protein in 50% of cases, where in most, a structural alterations could not be detected.

Discrepancy between p53 gene mutation and p53 overexpression has been increasingly recognized in malignant lymphomas and in solid tumors (26-32).

Scarpa et al. (35) found missense mutations by direct sequencing of PCR amplified DNA in 24 of 34 pancreatic adenocarcinomas. However, only nine of these showed immunoreactivity with antibody PAb1801, a commonly used monoclonal antibody that recognizes an epitope shared by wild-type and mutant p53 protein. In this same study, 12 cases not showing p53 mutation did show focal nuclear immunoreactivity with PAb1801.

Leahy et al. (28) also found a lack of correlation between SSCP and immunohistochemistry in the detection of p53 abnormalities. Without microwave antigen retrieval, the concordance of results between SSCP and immunohistochemistry was 58 percent. With microwave treatment, the concordance of results was 53 percent. They found that SSCP was positive and immunohistochemistry was negative in 12 cases, both with and without antigen retrieval. SSCP was negative and immunohistochemistry was positive in 16 cases without, and in 19 cases with microwave antigen retrieval. This may be due to a number of biological or methodological reasons.

In those cases where SSCP was positive and immunohistochemistry was negative, the underlying molecular lesion may be a point mutation producing a stop codon, thus truncating the protein product and making nuclear accumulation impossible. Some missense point mutations may not stabilize the p53 protein sufficiently for cellular accumulation. Some forms of mutation, particularly deletions that cause frame shifts, are known to lead to undetectable protein product, and it has been observed that in such cases, immunohistochemical staining can also underestimate the true frequency of mutation (24). Another possibility that has to be considered is that the antigen is heat sensitive which means if could have been lost in some cases during processing and fixation (31).

Where SSCP was negative and immunohistochemistry was positive, a mutation may be present in an exon other than exons 5-8 examined here. In a comprehensive study of mutations occurring in the p53 locus, an esimated 6% were found to exist outside of exons 5 to 9 (5). Mutations within introns may affect RNA splice sites and produce an abnormal p53 protein. A mutation in an upstream transcription regulator outside the p53 gene could signal excess synthesis of a normal protein (36).

Intratumoral heterogeneity may also account for some of the discrepancies between immunohistochemistry and SSCP (37). Samples may be enriched for tumor cell content by microdissecting the tumor from tissue sections (38), or by sorting cells on the basis of DNA content (tumor aneuploidy) (39).

It is also possible that in some instances, immunopositivity for p53 protein may be the result of stabilization of the gene product by mechanisms other than point mutation or by alteration of the normal degradation process (6, 7, 40, 41). The p53 protein is known to bind to viral proteins such as the E6 protein of oncogenic strains of HPV (40, 41) or cellular proteins such as p90, the product of mdm2 oncogene (6).

EBV, which has also been implicated in lymphomagenesis, has been shown to bind to p53 (42). Lee et al. (23) reported that EBER positive lymphomas showed a higher frequency of p53 positivity than EBER negative lymphomas, although the difference was not statistically significant. Therefore, we looked for evidence of EBV

infection by EBER ISH and/or PCR, and a found lack of correlation between EBV infection and p53 immunoreactivity. Our results correspond to previous studies which have shown no statistically significant correlation between EBV infection and p53 protein overexpression (12, 43). Spruck et al. (44) noted that 15 nasopharyngeal carcinomas (NPC) and 4 xenografts that did not contain p53 mutations all harbored EBV in their genomes, while the 3 NPC cell lines containing mutated p53 alleles tested negative for EBV. Their results parallel those for cervical carcinoma cell lines and tumors in which p53 abnormalities are correlated with an absence of human papilloma virus infection (45, 46). However, unlike human papilloma virus, associations between EBV proteins and p53 or other tumor suppressor gene products remain to be demonstrated. It is worth noting that EBV nuclear antigen-2 contains regions of amino acid sequence homology to human papillomavirus E7, adenovirus E1A, and simian virus 40 large-T antigens in the functional domains for growth transformation (47). EBV has shown to be associated with cases of Burkitt's lymphoma (16, 17), but these tumors are frequently mutated in p53 regardless of the status of EBV infection (8, 48). In this study, all three cases of Burkitt's lymphoma showed strong p53 immunoreactivity and two of them were EBV negative.

To summarize, overexpression of p53 was detected in 13 out of the 31 cases (41.9%) of MLs. However, we have documented the presence of structural alterations in the p53 gene in six cases of these MLs. The presence of EBV infection in MLs were statistically unrelated to p53 protein overexpression. Excellent correlation was found between p53 protein expression and histologic types of MLs. Even though the reason for discrepancy between p53 gene mutation and p53 protein overexpression remains unclear, it is suggested that p53 protein overexpression may be involved in the process of malignant transformation regardless of the status of EBV infection in MLs.

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