

MUTATIONS OF THE p53 GENE AS A PROGNOSTIC FACTOR
IN AGGRESSIVE B-CELL LYMPHOMA

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ABSTRACT

Background Mutations of the p53 gene are associated with a poor prognosis in several types of cancer. We investigated the prognostic importance of p53 mutations in patients with aggressive B-cell lymphoma.

Methods We examined the relation between the presence or absence of a detectable p53 mutation in lymphoma cells and the response to chemotherapy and overall survival in 102 previously untreated patients with aggressive B-cell lymphoma. Mutations of the p53 gene were identified by polymerase-chain-reaction-mediated analysis of single-strand conformation polymorphisms and by direct sequencing.

Results Of 102 cases of aggressive B-cell lymphoma, 22 (22 percent) involved p53 mutations. The rate of complete remission was significantly lower in patients with a tumor carrying a p53 mutation (6 of 22 patients, 27 percent) than in those with the wild-type p53 gene (61 of 80 patients, 76 percent) ($P < 0.001$). Overall survival was significantly lower among patients with p53 mutations than among those with the wild-type p53 gene; the Kaplan-Meier estimates of survival at five years were 16 percent and 64 percent, respectively ($P < 0.001$). Multivariate analysis incorporating prognostic factors from the international prognostic index demonstrated that p53 mutations had independent effects on the rates of complete remission and survival. When we categorized patients according to the international prognostic index, we found no effect of p53 mutations in patients in the groups at high-intermediate and high risk. However, these mutations were significantly associated ($P < 0.001$) with low rates of complete remission (33 percent vs. 91 percent) and survival (27 percent vs. 81 percent at five years) in the groups at low and low-intermediate risk.

Conclusions Mutations of the p53 gene are associated with a poor prognosis in patients with aggressive B-cell lymphoma. (N Engl J Med 1997;337:529-34.)

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COMBINATION chemotherapy has improved the outcome of patients with intermediate-grade or high-grade non-Hodgkin's lymphoma (aggressive lymphoma).¹⁻⁵ Nevertheless, many patients do not have a complete remission or ultimately relapse. If such patients could be identified at diagnosis, they might benefit from strategies other than conventional chemotherapy.

Various factors, such as age,⁶ clinical stage,⁷ the presence or absence of B symptoms,⁷ performance status,⁸ tumor size,⁸ tumor burden,⁹ the number of extranodal sites,¹⁰ the presence or absence of bone marrow involvement,¹¹ the lactate dehydrogenase level,¹² the interleukin-2 receptor level,¹³ and karyotype,¹⁴ have been found to influence the outcome of treatment in non-Hodgkin's lymphoma. Recently, the international prognostic index, which includes age, lactate dehydrogenase level, performance status, stage, and number of extranodal disease sites, was proposed as a way of establishing the prognosis in patients with aggressive non-Hodgkin's lymphoma.¹⁵ Molecular abnormalities, such as overexpression of bcl-2 protein¹⁶ and alteration of the *bcl-6* gene,¹⁷ are also related to prognosis in B-cell lymphoma.

Many oncogenes and tumor-suppressor genes have been associated with various types of cancers. One of the most widely studied of these genes is p53. Allelic loss or mutation of p53 has been detected in tumors of the colon, lung, breast, esophagus, liver, brain, and other organs.¹⁸ The results of several studies support a relation between p53 mutations and the development or progression of tumors.¹⁹⁻²¹ Moreover, p53 mutations have been implicated in drug resistance.^{22,23} Mutation of the p53 gene or an accumulation of p53 protein in tumor cells has been linked to prognosis in several types of cancer.²⁴⁻²⁷ We found p53 mutations in 9 of 48 patients with B-cell lymphoma; 8 of these 9 patients were in clinical stage IV at the time of diagnosis.²⁸ In this study we investigated whether p53 mutations are related to the rates of complete remission and survival after potentially curative chemotherapy for aggressive lymphoma.

METHODS**Patients and Chemotherapy**

We studied 102 consecutive patients with untreated intermediate-grade or high-grade B-cell lymphoma. Patients with lymphoblastic lymphoma or small-noncleaved-cell lymphoma were

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excluded. The lymphomas were classified as aggressive B-cell lymphomas according to the Working Formulation.²⁹ Chemotherapy was initiated between September 1988 and October 1994 at the Nagoya University School of Medicine. Data on 48 of these 102 patients were published previously.²⁸ The median follow-up period was 40.1 months, and the maximum was 9.5 years. Fifty-six of the 102 patients were alive after 8 to 114 months of follow-up (median, 4.1 years), and the other 46 patients died 1 to 55 months after diagnosis (median, 12 months). The patients were observed until July 30, 1995, or until death. All pathological specimens were reviewed by experienced hematopathologists.

Disease stage was determined for all patients according to the Ann Arbor classification system.⁷ The evaluation included a complete history taking and physical examination; chest roentgenography; bone marrow aspiration and biopsy; computed tomography of the chest, abdomen, and pelvis; blood-cell and differential counts; and routine blood-chemistry tests. Laparotomies were not performed for purposes of staging.

Patients with clinical stage I or II disease were included only if they had bulky disease or extensive extranodal lesions not readily covered by a radiotherapy field. Patients with primary gastrointestinal or other extranodal lesions and noncontinuous nodal involvement were considered to have stage IIE disease and were included in this investigation. Patients with a history of severe cardiac, renal, pulmonary, or hepatic disease were excluded. Tumor volume was assessed by standard methods developed at the M.D. Anderson Hospital, Houston.⁹

All patients were treated with potentially curative combination chemotherapy regimens containing doxorubicin: 44 patients received cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP),³⁰ 33 received CHOP plus bleomycin (CHOP-B),³¹ and 25 received vincristine, cyclophosphamide (Endoxan), prednisone, doxorubicin, and methotrexate (VEPA-M).³² These regimens were of standard dose intensity as defined by Fisher et al.⁵

All the patients were reevaluated when they had completed chemotherapy, then every 3 months for 24 months, and subsequently every 6 months. Reevaluation included physical examination, blood-cell and differential counts, blood-chemistry tests, and computed tomography of the chest, abdomen, and pelvis.

Complete remission was defined as an absence of clinical evidence of active tumor for at least four weeks after treatment or, in patients with a residual radiographic mass and no other evidence of disease, for at least three months after treatment.³³ Partial remission was defined as a decrease of 50 percent or more in the sum of the products of the maximal perpendicular diameters of all measured lesions that was maintained for at least four weeks. Treatment failure was defined as the absence of complete or partial remission. Patients with complete remission at the end of treatment received no further therapy. Patients with partial remission or treatment failure received combinations of radiation and salvage chemotherapy.

DNA Samples

DNA samples from the cell lines CEM (mutations in the p53 gene at codons 175 and 248³⁴), HUT78 (mutation at codon 196³⁴), and SW480 (mutations at codons 273 and 309¹⁹) were used as positive controls for polymerase-chain-reaction-mediated analysis of single-strand conformation polymorphisms (PCR-SSCP). Molt-4 was used as a negative control (no mutations detected between codons 135 and 296³⁴).

Preparation of DNA, PCR-SSCP Analysis, and Direct Sequencing

After informed consent had been obtained from the patients, DNA samples were extracted from biopsy specimens taken for diagnosis from lymph-node tumors or extranodal tumors, according to the method described by Ichikawa et al.²⁸ DNA was extracted with phenol and chloroform, precipitated with ethanol, and resuspended in sterile TE buffer (10 mM TRIS [pH 8.0] and

1 mM EDTA) for storage. DNA samples were stored in areas physically separate from those in which the PCR products were manipulated.

PCR-SSCP analysis was performed as described previously.³⁵ Briefly, genomic DNA corresponding to exons 5 to 9 of p53, which contain regions that are highly conserved and are the sites of frequent mutations in various cancer cells,¹⁸ was amplified by PCR. The oligonucleotide primers have been described previously.²⁸ The amplified PCR products were separated by denaturation into single strands, which were resolved by electrophoresis under non-denaturing conditions according to their sequence-dependent three-dimensional conformation. A single nucleotide change can be readily detected as an electrophoretic mobility shift.

Portions of the tissue used for DNA analysis were also subjected to histologic and surface-marker analyses by standard immunohistochemical methods.

A small area of the PCR-SSCP gel corresponding to the position of bands with or without a mobility shift was cut out, and single-stranded DNA was eluted from the dried gel as described previously.³⁶ The eluted sample was subjected to asymmetric amplification by PCR, and amplified products were subjected to sequencing by dideoxy termination.³⁷

Statistical Analysis

The associations of p53 mutations with clinical characteristics and with the response to chemotherapy were analyzed by the chi-square test with two-way tables.³⁸ The means were compared by two-sample t-tests. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Survival was plotted according to the method of Kaplan and Meier.³⁹ The statistical significance of the differences among curves was determined by the generalized Wilcoxon test. The factors affecting complete remission and survival were assessed by a multivariate logistic-regression analysis⁴⁰ and a multivariate regression analysis according to the Cox proportional-hazards regression model,⁴¹ respectively. A P value of less than 0.05 was considered to indicate statistical significance. All calculations were performed with SAS software, version 6.10 (SAS Institute, Cary, N.C.).

RESULTS

Mutations of the p53 Gene

Twenty-three p53 mutations were identified in 22 of the 102 patients by PCR-SSCP analysis and direct sequencing. Nine of these 23 mutations had been previously found in a group of 48 patients with aggressive lymphoma.²⁸ These mutations included 5 in exon 5, 6 in exon 6, 11 in exon 7, and 1 in intron 5, and they were predicted to result in amino acid substitutions or deletions, truncated proteins, or abnormal splicing (Table 1).

Characteristics of the Patients

Table 2 shows the characteristics of the 102 patients, grouped according to the presence or absence of p53 mutations. Patients with tumors in which a p53 mutation was detected were older (mean age, 65 years; $P = 0.001$), had a more advanced clinical stage ($P = 0.04$), and had higher lactate dehydrogenase levels ($P = 0.01$) than patients without a p53 mutation. These two groups did not differ significantly in other characteristics (sex, histologic subtype, constitutional symptoms, presence or absence of bulky disease, bone marrow involvement, or extranodal dis-

TABLE 1. MUTATIONS IN THE p53 GENE IN 22 PATIENTS WITH AGGRESSIVE B-CELL LYMPHOMA.

PATIENT NO.*	LYMPHOMA TYPE†	MUTATION				CLINICAL STAGE
		CODON	EXON	NUCLEOTIDE	AMINO ACID	
1	DL	257	7	CTG→CCG	Leu→Pro	IV
2	DL	248	7	CGG→TGG	Arg→Trp	IV
3	DL	148	5	GAT→GAA	Asn→Glu	IV
4	DL	254	7	ATC→AAC	Ile→Asn	IV
5	DL	194	6	CTT→CGT	Leu→Arg	III
6	DL	196	6	CGA→TGA	Arg→Stop	IV
7	DL	237	7	ATG→AGG	Met→Arg	II
8	DL	251	7	ATC→AGC	Ile→Ser	III
9	DL	248	7	CGG→CAG	Arg→Gln	IV
10	DL	179	5	CAT→GAT	His→Asp	III
11	DL	234	7	TAC→TGC	Tyr→Cys	II
12	DL	Acceptor‡		A→C		IV
13	DL	248	7	CGG→CAG	Arg→Gln	III
14	DL	141	5	TGC→TAC	Cys→Tyr	II
15	DSC	244	7	GGC→GCC	Gly→Ala	IV
16	DSC	216–218	6	GTG deletion	Val deletion	IV
17	DSC	250	7	CCC→AAC	Pro→Asn	IV
18	DSC	220	6	TAT→AAT	Tyr→Asn	IV
19	DM	216–218	6	GTG deletion	Val deletion	IV
20	DM	159	5	GCC→CCC	Ala→Pro	III
21	DM	211	6	ACT→AAT	Thr→Asn	IV
22	FL	187	5	GGT→AGT	Gly→Ser	III
		254	7	ATC→AGC	Ile→Ser	

*Data on Patients 1, 2, 3, 4, 15, 16, 17, 19, and 22 have been published previously.²⁸

†Classification is based on the Working Formulation²⁹: DL denotes diffuse, large-cell; DSC diffuse, small-cleaved-cell; DM diffuse, mixed; and FL follicular, large-cell.

‡This term denotes the splicing acceptor of intron 5.

case, Eastern Cooperative Oncology Group performance status, tumor burden, and treatment).

There was no significant difference in the proportions of patients with p53 mutations between the group at low or low-intermediate risk and the group at high-intermediate or high risk according to the international prognostic index (P=0.12). Of the 22 patients with a p53 mutation, 12 were in the two lower-risk groups (low or low-intermediate risk).

Prognostic Value of a p53 Mutation

The rate of complete remission differed significantly between the groups with and without mutations. Six of the 22 patients with a p53 mutation (27 percent) had a complete remission, as compared with 61 of the 80 patients with a wild-type p53 gene (76 percent, P<0.001) (Table 2). Among the patients with a partial remission, none of the 10 patients with a p53 mutation and 5 of the 10 patients with a wild-type p53 gene responded to further therapy.

The estimate of survival at five years for all 102

TABLE 2. CLINICAL CHARACTERISTICS AND RESPONSIVENESS TO CHEMOTHERAPY OF 102 PATIENTS WITH AGGRESSIVE B-CELL LYMPHOMA.

CHARACTERISTIC	PATIENTS WITH p53 MUTATIONS	PATIENTS WITH WILD-TYPE p53	P VALUE
Patients — no. (%)	22 (22)	80 (78)	
Mean age — yr	65	53	0.001
Sex — no. (%)			0.48
Male	13 (59)	56 (70)	
Female	9 (41)	24 (30)	
Histologic subtype — no. (%)*			0.92
Diffuse large-cell or large-cell (G or H)	13 (59)	46 (58)	
Diffuse, small-cleaved-cell immunoblastic (E)	4 (18)	15 (19)	
Diffuse, mixed (F)	4 (18)	12 (15)	
Follicular, large-cell (D)	1 (5)	7 (9)	
B symptoms — no. (%)			0.23
Absent	8 (36)	43 (54)	
Present	14 (64)	37 (46)	
Clinical stage — no. (%)			0.04
I or II	3 (14)	31 (39)	
III or IV	19 (86)	49 (61)	
Bulky disease — no. (%)			0.90
Yes	7 (32)	22 (28)	
No	15 (68)	58 (72)	
Bone marrow involvement — no. (%)			0.59
Yes	8 (36)	22 (28)	
No	14 (64)	58 (72)	
Extranodal disease — no. (%)			0.45
Yes	15 (68)	45 (56)	
No	7 (32)	35 (44)	
ECOG performance status — no. (%)†			0.28
0–1	13 (59)	59 (74)	
2–4	9 (41)	21 (26)	
Lactate dehydrogenase level — no. (%)			0.01
Normal	8 (36)	55 (69)	
Elevated	14 (64)	25 (31)	
Tumor burden — no. (%)			0.54
Low	15 (68)	62 (78)	
High	7 (32)	18 (22)	
Treatment — no. (%)‡			0.23
CHOP	13 (59)	31 (39)	
CHOP-B	5 (23)	28 (35)	
VEPA-M	4 (18)	21 (26)	
Complete remission — no. (%)	6 (27)	61 (76)	<0.001

*Subtypes are defined according to the Working Formulation.²⁹

†ECOG denotes Eastern Cooperative Oncology Group.

‡The treatment regimens are defined in the Methods section.

patients was 55 percent; 46 patients died, most of them (40 patients) of lymphoma. There was a significant difference in overall survival between the groups with and without mutations. For patients without a p53 mutation, the Kaplan–Meier estimate of survival at five years was 64 percent, whereas for patients with a p53 mutation it was 16 percent (P<0.001) (Fig. 1).

To assess the response to chemotherapy, stepwise multivariate analysis by logistic regression was performed, to adjust for prognostic factors in the international prognostic index. The estimated relative risk of failure to achieve a complete remission for pa-

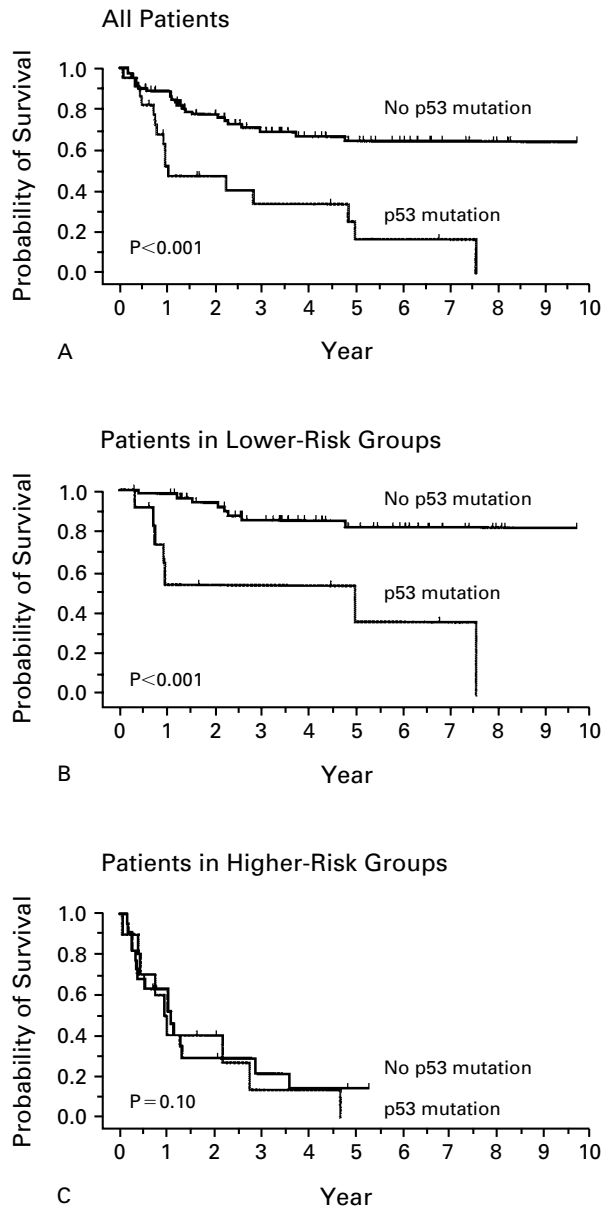


Figure 1. Survival of Patients with Aggressive B-Cell Lymphoma with and without p53 Mutations.

Panel A shows the survival of all patients. On average, the 80 patients without p53 mutations survived significantly longer than the 22 patients with p53 mutations. Panel B shows the survival of the patients in the groups at low and low-intermediate risk. On average, the 58 patients without p53 mutations survived significantly longer than the 12 patients with p53 mutations. Panel C shows the survival of the patients in the groups at high and high-intermediate risk. There was no significant difference in average survival time between the 22 patients without p53 mutations and the 10 patients with p53 mutations.

tients with a p53 mutation was 14.2 (95 percent confidence interval, 3.1 to 65.1; $P < 0.001$) (Table 3). The presence of a p53 mutation had an independent effect on the rate of complete remission.

We used Cox's proportional-hazards regression model to assess survival, adjusting for prognostic factors in the international prognostic index (Table 4). The estimated relative risk of death for patients with a p53 mutation was 3.7 (95 percent confidence interval, 1.7 to 8.0; $P = 0.001$). The p53 mutation was an independent prognostic factor.

We also evaluated the importance of the p53 mutation in patients classified according to the international prognostic index. In the groups at low-intermediate and low risk, patients with a p53 mutation had a complete-remission rate of 33 percent (4 of 12 patients) and a five-year survival of 27 percent, whereas patients with wild-type p53 had a complete-remission rate of 91 percent (53 of 58 patients) ($P < 0.001$) and a five-year survival of 81 percent ($P < 0.001$) (Fig. 1). However, in the groups at high-intermediate and high risk, there was no significant difference between patients with a p53 mutation and those with wild-type p53 (Fig. 1).

DISCUSSION

We found that a p53 mutation in the cells of aggressive B-cell lymphoma predicts a poor response to chemotherapy and short survival. Such mutations were also associated with other predictors of poor outcome in aggressive B-cell lymphoma (older age, advanced clinical stage, and elevated lactate dehydrogenase values), but multivariate analyses showed that the influence of a p53 mutation was independent of these well-established prognostic factors.

The International Non-Hodgkin's Lymphoma Prognostic Factors Project reported that after combination chemotherapy, patients at low risk had a rate of complete remission of 87 percent, whereas those at low-intermediate risk had a complete-remission rate of 67 percent.¹⁵ We did not find any effect of p53 mutations in patients belonging to the high-intermediate-risk or high-risk group. However, these mutations were significantly associated with a low rate of complete remission and poor survival in patients with lymphomas who were classified as being at low or low-intermediate risk.

Some chemotherapeutic agents and radiation induce apoptosis by a mechanism that requires the p53 protein, and in animal models there is a strong correlation between the p53 status of a tumor and the response of the tumor to treatment.²² Wilson et al.⁴² suggested that mutation of p53 may be an important cause of drug resistance in relapsed or refractory non-Hodgkin's lymphoma. Chin et al.²³ demonstrated that a mutant p53 protein can transactivate the multidrug-resistance gene 1 (*MDR1*). These observations are consistent with the poor re-

TABLE 3. LOGISTIC-REGRESSION MODEL FOR COMPLETE REMISSION WITH FACTORS IDENTIFIED BY THE INTERNATIONAL PROGNOSTIC INDEX.

FACTOR	RELATIVE RISK (95% CONFIDENCE INTERVAL)*	
	UNIVARIATE	MULTIVARIATE
Mutation of p53 gene	8.6 (2.9–25.0)	14.2 (3.1–65.1)
Extranodal disease at >1 site	7.4 (2.6–21.4)	8.8 (2.0–26.2)
ECOG performance status 2–4†	9.7 (3.6–25.6)	7.2 (1.9–39.6)
Lactate dehydrogenase >250 IU/liter	7.6 (3.0–19.0)	3.4 (1.0–11.9)
Stage III or IV disease	6.3 (1.9–19.7)	2.6 (0.6–12.0)
Age >60 yr	1.5 (0.6–3.3)	0.5 (0.1–1.8)

*Relative risks are for failure to achieve complete remission.
†ECOG denotes Eastern Cooperative Oncology Group.

TABLE 4. COX'S PROPORTIONAL-HAZARDS REGRESSION MODEL FOR OVERALL SURVIVAL WITH FACTORS IDENTIFIED BY THE INTERNATIONAL PROGNOSTIC INDEX.

FACTOR	RELATIVE RISK (95% CONFIDENCE INTERVAL)*	
	UNIVARIATE	MULTIVARIATE
Lactate dehydrogenase >250 IU/liter	11.0 (5.2–23.3)	11.4 (4.5–29.0)
Mutation of p53 gene	3.5 (1.9–6.7)	3.7 (1.7–8.0)
ECOG performance status 2–4†	4.4 (2.3–8.2)	3.1 (1.5–6.5)
Extranodal disease at >1 site	2.7 (1.3–5.4)	2.7 (1.1–6.4)
Stage III or IV disease	3.1 (1.3–7.4)	1.0 (0.4–2.8)
Age >60 yr	2.0 (1.1–3.7)	1.0 (0.5–2.0)

*Relative risks are for death.
†ECOG denotes Eastern Cooperative Oncology Group.

mission rate in patients with aggressive B-cell lymphomas in which there was a p53 mutation.

Recent studies have shown that alterations of several genes containing a p53-binding site may have the same consequences as intragenic p53 mutations.⁴³ Lowe et al.²² suggested that dysfunction of the p53-dependent pathway to apoptosis contributes to the cross-resistance of tumor cells to anticancer agents. Thus, alterations in this pathway may explain why some patients with B-cell lymphomas without p53 mutation respond poorly to treatment.

Recent investigations of lung cancer,²⁴ breast cancer,^{25,26} and bladder cancer²⁷ have indicated that p53

mutations or accumulation of p53 protein correlates with poor prognosis. Our results are consistent with these data.

More than 90 percent of mutations of p53 occur in exons 5 to 8 (conserved domains II to IV), and most analysis has focused on this region.^{18,20,44-46} We cannot entirely exclude the possibility of p53 gene mutations located outside conserved domains. It is unlikely that such mutations influenced our results, however, because these cases are rare.^{18,20,21}

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