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# Somatic Mutations of *Fas* (Apo-1/CD95) Gene in Cutaneous Squamous Cell Carcinoma Arising from a Burn Scar

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**Fas (Apo-1/CD95) is a cell-surface receptor involved in cell death signaling, and recent reports have suggested that defects within the Fas receptor pathway such as *Fas* mutation play an important part in the development and progression of human tumors. Burn scar-related squamous cell carcinoma of skin is a unique subtype of cutaneous squamous cell carcinoma, and tends to be more aggressive in nature than conventional squamous cell carcinoma. The molecular mechanisms underlying the development and progression of burn scar-related squamous cell carcinoma, however, are not clear. In this study, we analyzed the entire coding region and all splice sites of the *Fas* gene for the detection of the somatic mutations in a series of 50 conventional squamous cell carcinomas and 21 burn scar-related squamous cell carcinomas by polymerase chain reaction, single strand conformation polymorphism, and DNA sequencing. We detected mis-sense mutations in three of 21 burn scar-related squamous cell carcinoma**

**(14.3%), whereas no mutation was detected in 50 conventional squamous cell carcinomas. Of the three *Fas* mutations detected in the burn scar-related squamous cell carcinomas, one was found in *Fas* ligand-binding domain, another one was identified in the death domain known to be involved in the transduction of an apoptotic signal, and the other one was found in the transmembrane domain. Our data show that some burn scar-related squamous cell carcinomas have *Fas* gene mutations in important regions for the apoptosis function and suggest that these mutations might be involved in the pathogenesis of burn scar-related squamous cell carcinomas. In addition, our results provide an important clue to understanding the difference between burn scar-related squamous cell carcinoma and conventional squamous cell carcinoma at the molecular level. **Key words:** apoptosis/loss of heterozygosity/skin/squamous cell carcinoma. *J Invest Dermatol* 114:122–126, 1999**

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**I**t is now believed that clonal expansion and tumor growth is the result of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis) (Nagata, 1997). Failure of apoptosis could allow the survival of transformed cells that are prone to undergo further genetic damage and play an important part in the pathogenesis of tumors (Nagata, 1997).

The Fas–Fas ligand (FasL) system has been recognized as a major pathway for the induction of apoptosis in cells and tissues (Nagata, 1997). Fas is a member of the death receptor subfamily of the tumor necrosis factor receptor superfamily (Itoh and Nagata, 1993; Nagata, 1997). Ligation of Fas by either agonistic antibody or by its natural ligand transmits a “death signal” to the target cells,

potentially triggering apoptosis (Trauth *et al*, 1989; Leithäuser *et al*, 1993; Owen-Schaub *et al*, 1994). Fas is widely expressed in normal and neoplastic cells (Leithäuser *et al*, 1993), but the expression of this protein does not necessarily predict susceptibility to killing (Owen-Schaub *et al*, 1994). This can reflect the presence of inhibiting mechanisms of Fas-mediated apoptosis. Fas-mediated apoptosis can be blocked by several mechanisms, including the mutation of the primary structure of Fas (Owen-Schaub *et al*, 1994; Nagata, 1997).

There is mounting evidence that *Fas* gene mutation is involved in the pathogenesis of tumors. Mice bearing the germ line mutation of *Fas* gene (*lpr*) have been reported to have spontaneous development of plasmacytoid tumors (Davidson *et al*, 1998). Lymphomatogenesis driven by the E $\mu$ -*myc* transgene was shown to be markedly accelerated in *lpr* mice compared with wild-type mice, confirming a causal, rather than correlative, role for Fas loss in tumor development (Zörnig *et al*, 1995). Germline mutations of the *Fas* gene in human results in autoimmune lymphoproliferative syndrome, and some these patients have been reported to have malignancies (Drappa *et al*, 1996; Bettinardi *et al*, 1997; Infante *et al*, 1998; Martin *et al*, 1999), including multiple tumor development in one patient (Drappa *et al*, 1996). In cancer patients, somatic mutations of *Fas* gene have been mainly described in lymphoid

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Abbreviations: BSCC, burn scar-related squamous cell carcinoma; CSCC, conventional squamous cell carcinoma; SSCP, single strand conformation polymorphism; FasL, Fas ligand.

tumors, including multiple myelomas (Landowsky *et al*, 1997), childhood T cell lymphoblastic leukemias (Beltinger *et al*, 1998), adult T cell leukemias (Tamiya *et al*, 1998; Maeda *et al*, 1999), and non-Hodgkin's lymphomas (Grønbaek *et al*, 1998). In adult T cell leukemia, the somatic mutation of *Fas* gene was found to contribute to lymph node metastasis of the tumor (Maeda *et al*, 1999). In nonlymphoid tumors, we previously reported *Fas* gene mutations in malignant melanomas (Shin *et al*, 1999), transitional cell carcinomas of urinary bladder (Lee *et al*, 1999a) and non-small cell lung cancers (Lee *et al*, 1999b). These data indicate that resistance against Fas-mediated apoptosis through the *Fas* gene mutation may contribute to the development and progression of human tumors.

There have been many case reports over the years describing the occurrence of cancers in burn scars which are most frequently squamous cell carcinoma (SCC) (Bartle *et al*, 1990). Burn scar-related SCC (BSCC) of skin is more aggressive in nature and carry a poorer prognosis than conventional SCC (CSCC), although the microscopic picture of BSCC is that of typical SCC (Bartle *et al*, 1990). It is believed that the mechanisms underlying the development and progression of BSCC is different from those of CSCC. There have been few data, however, explaining these mechanisms (Bartle *et al*, 1990; Harland *et al*, 1997; Sakatani *et al*, 1998). Because it is possible that *Fas* gene mutation is involved in the pathogenesis of nonlymphoid malignancies, we considered the possibility that *Fas* gene mutation might be involved in the pathogenesis of BSCC. In this study, we performed a polymerase chain reaction (PCR)-based mutational analysis of *Fas* gene in 21 BSCC and 50 CSCC. The data here demonstrate that some BSCC have *Fas* gene mutations and suggest that the *Fas* mutations might be one of the mechanisms involved in the pathogenesis of BSCC.

#### MATERIALS AND METHODS

**Tissue samples and microdissection** Paraffin-embedded tissues were obtained from BSCC (n = 21) and CSCC (n = 50) patients undergoing surgery. Each tumor was graded histologically according to the proportion of differentiated cells (grades 1–4) (Broders, 1921). The CSCC samples consisted of 31 grade 1, 12 grade 2, seven grade 3 and no grade 4 tumors and the BSCC samples consisted of 17 grade 1, four grade 2, no grade 3, and no grade 4 tumors.

Malignant cells were selectively procured from hematoxylin and eosin-stained sections using a 30G1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (Lee *et al*, 1998). We also microdissected infiltrating lymphocytes from the slides and used them for corresponding normal DNA. This microdissection technique used in this study has been proven to be precise and effective for procurement of tumor cells without normal cell contamination (Lee *et al*, 1998). DNA extraction was performed by a modified single-step DNA extraction method, as described previously (Lee *et al*, 1998).

**Single strand conformation polymorphism (SSCP) analysis for mutation and loss of heterozygosity (LOH)** Genomic DNA each from normal lymphocytes or tumor cells was amplified with the primer pairs of *Fas* gene described in our previous studies (Lee *et al*, 1999a, b; Shin *et al*, 1999). The primers were designed with the program Oligo (National Biosciences, Plymouth, MN) using sequences obtained from GenBank (accession number M67454) and cover the entire coding region and parts of the promoter region of *Fas* gene (Lee *et al*, 1999a, b; Shin *et al*, 1999). Each PCR reaction was performed under standard conditions in a 10  $\mu$ l reaction mixture containing 1  $\mu$ l of template DNA, 0.5  $\mu$ M of each primer, 0.2  $\mu$ M of each deoxynucleotide triphosphate, 1.5 mM MgCl<sub>2</sub>, 0.4 units of Taq polymerase, 0.5  $\mu$ Ci of [<sup>32</sup>P]dCTP (Amersham, Buckinghamshire, U.K.), and 1  $\mu$ l of 10 $\times$  buffer. The reaction mixture was denatured for 1 min at 94°C and incubated for 40 cycles (denaturing for 40 s at 94°C, annealing for 40 s at 49–60°C, and extending for 40 s at 72°C). Final extension was continued for 5 min at 72°C. After amplification, PCR products were denatured 5 min at 95°C at a 1:1 dilution of sample buffer containing 98% formamide/5 mmol per l NaOH and were loaded on to a SSCP gel (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT) with 10% glycerol. After electrophoresis, the gels were transferred to 3 mm Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY). For the detection of mutations, DNAs showing mobility shifts were

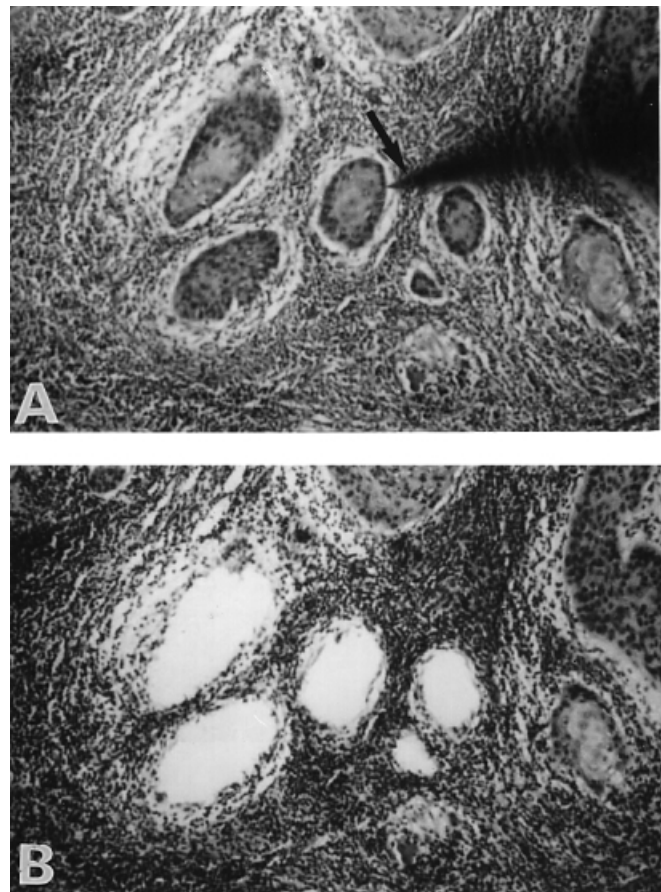
cut out from the dried gel, and re-amplified for 35 cycles using the same primer set. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's recommendation.

Because it has been known that four bi-allelic polymorphisms at positions -1377 (promoter region), -670 (promoter region), 416 (exon 3), and 836 (exon 7) are located in *Fas* gene (Fiucci and Ruberti, 1994; Huang *et al*, 1997), SSCP analysis at these polymorphic sites was used for the detection of LOH as well as for the detection of mutations. The PCR and SSCP conditions of LOH study were the same with the condition described above. Complete or nearly complete absence of one allele in tumor DNA of informative cases, as defined by direct visualization, was considered as LOH.

**Immunohistochemistry** Antibody for human Fas (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect Fas on tissue sections. Immunohistochemical procedures were performed as described previously (Lee *et al*, 1999a, b; Shin *et al*, 1999). Tumors were interpreted as positive for Fas by immunohistochemistry when at least weak to moderate cytoplasmic staining was seen in greater than 30% of the neoplastic cells. The immunostaining with each antibody was judged to be antibody-specific by several criteria, including: (i) use of normal rabbit serum at the same dilution produced no consistent immunostaining of any cells; (ii) intensity of the signal diminished as the dilution of the antibody was increased; and (iii) preincubating the antibody with blocking peptides abrogated the positive immunostaining. The results were reviewed independently by three pathologists.

#### RESULTS

***Fas* gene mutations** Through the microdissection technique, we successfully procured tumor cells from histologic sections of 71



**Figure 1. Microdissection of BSCC.** (A) Malignant cells are arranged in irregularly shaped nests in the dermis. The needle tip (arrow) is attached to a tumor cell nest. (B) Tumor cell nest was dissected leaving large holes behind. Original magnification,  $\times$  150.

**Table I. Demographic data and *Fas* gene mutations of the BSCC**

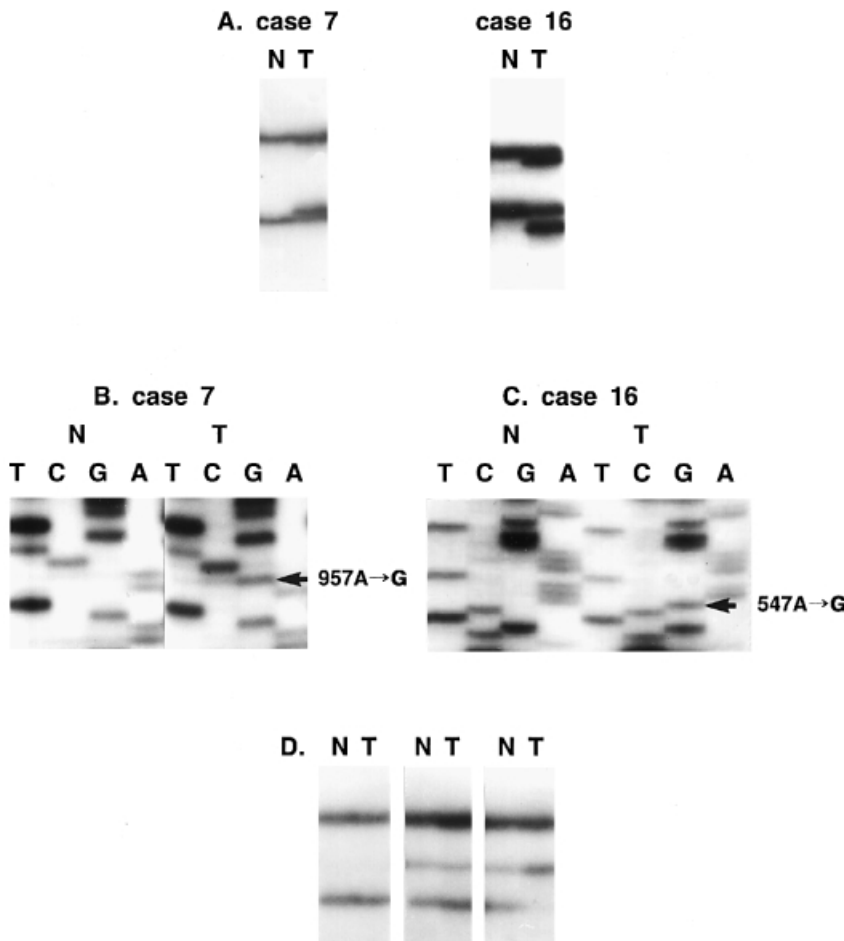
No.	Age (sex)	Lag time <sup>a</sup>	Location	Metastasis <sup>b</sup>	Fas expression	Cytologic grade <sup>c</sup>	LOH analysis <sup>d</sup>				Exon (codon)	Nucleotide change	Predictive effect
							PA	PB	3	7			
1	56 (M)	5 y	Thigh	Negative	Positive	1	Het	Het	NI	NI			
2	62 (M)	59 y	Thigh	Negative	Positive	2	Het	Het	NI	NI			
3	41 (M)	31 y	Thigh	Negative	Positive	1	LOH	Het	NI	NI			
4	56 (M)	17 y	Leg	Negative	Negative	1	NI	NI	NI	NI			
5	69 (M)	64 y	Foot	Negative	Positive	1	Het	LOH	NI	NI			
6	44 (M)	37 y	Forearm	Negative	Positive	1	NI	NI	NI	NI			
7	37 (M)	20 y	Leg	Positive	Positive	1	LOH	LOH	NI	NI	9 (239)	957 A to G	Asn to Asp
8	57 (F)	53 y	Thigh	Negative	Negative	1	NI	NI	NI	NI			
9	73 (F)	65 y	Leg	Negative	Positive	1	NI	Het	NI	NI			
10	41 (F)	36 y	Forearm	Negative	Negative	2	NI	Het	NI	NI			
11	42 (M)	36 y	Thigh	Positive	Positive	1	NI	Het	NI	NI			
12	39 (M)	10 y	Leg	Negative	Positive	2	Het	NI	NI	Het			
13	28 (M)	14 y	Leg	Positive	Positive	2	Het	LOH	NI	LOH	6 (162)	726 T to C	Cys to Arg
14	28 (M)	25 y	Foot	Negative	Negative	1	NI	NI	NI	NI			
15	64 (M)	43 y	Forearm	Negative	Positive	1	NI	NI	NI	NI			
16	41 (M)	34 y	Leg	Negative	Positive	1	Het	Het	NI	NI	4 (102)	547 A to G	Asn to Ser
17	43 (M)	3 y	Foot	Positive	Negative	1	NI	NI	NI	NI			
18	56 (M)	34 y	Forearm	Negative	Positive	1	NI	NI	NI	NI			
19	74 (M)	40 y	Hand	Negative	Positive	1	Het	Het	NI	NI			
20	24 (F)	22 y	Forearm	Positive	Positive	1	NI	NI	NI	NI			
21	25 (M)	22 y	Leg	Positive	Positive	1	NI	NI	NI	NI			

<sup>a</sup>Bum to cancer diagnosis.

<sup>b</sup>Metastasis to regional lymph node at the time of diagnosis.

<sup>c</sup>Graded histologically according to the proportion of differentiated cells (grade 1–4) by the classification of Broders (22).

<sup>d</sup>PA, the polymorphism in promoter of *Fas*; PB, the polymorphism in promoter of *Fas*; 3, the polymorphism in exon 3 of *Fas*; 7, the polymorphism in exon 7 of *Fas*. LOH, loss of heterozygosity; NI, not informative; HET, retention of heterozygosity



**Figure 2. Mutations and deletions of *Fas* gene in cutaneous SCC.** SSCP (A, D) and sequencing analysis (B, C) of DNA from tumors (lane T) and normal tissues (lane N). (A) Part of exon 9 was amplified using primer set 9A. SSCP of DNA from tumor (T) of case 7 (left) and case 16 (right) show wild-type bands and additional aberrant bands as compared with SSCP of DNA from the corresponding normal lymphocytes (N). (B) Sequencing analysis from aberrant band of case 7. There is an A–G transition at nucleotide 957 (arrow) in tumor tissue as compared with normal tissue. (C) Sequencing analysis from aberrant band of case 16. There is an A–G transition at nucleotide 547 (arrow) in tumor tissue as compared with normal tissue. (D) Detection of allelic loss by amplifying a region encompassing the bi-allelic polymorphism, –1377, in the *Fas* promoter with primer PA. Representative SSCP show “not informative” (left), “retention of heterozygosity” (middle), and “loss of heterozygosity” (right). Right: Loss of bands was observed in DNA from tumor cells (T) compared with the SSCP from normal cells (N).

SCC, including 21 BSCC and 50 CBSCC, as shown in **Fig 1(A, B)**. Genomic DNA was isolated and analyzed for potential mutations in all nine exons of the *Fas* gene by PCR-SSCP analysis. Enrichment and direct sequence analysis of aberrantly migrating bands led to the identification of mutations in three of 21 BSCC (14.3%) (**Table I** and **Fig 2A**), but no mutation was identified in 50 CSCC. None of the normal samples showed evidence of mutations by SSCP (**Fig 2A**), indicating the mutations detected in the BSCC specimens had arisen somatically.

All three mutations identified were mis-sense variants (**Fig 2B, C** and **Table I**). One mutation was detected in exon 9 which encodes the death domain region of the Fas (Itoh and Nagata, 1993). This mutation (case 7) affects codon 239. Another mutation (case 16) was identified in exon 4 and would result in the substitution of Asn to Ser at codon 102. The other mutation (case 13) observed in exon 6, affects codon 162. We repeated the experiments three times, including tissue microdissection, PCR, SSCP, and sequencing analysis to ensure the specificity of the results, and found that the data were consistent (data not shown). The demographic data and *Fas* mutation data in 21 BSCC are summarized in **Table I**.

**Allelic status** Because mis-sense mutations in the death domain of *Fas* in patients with autoimmune lymphoproliferative syndrome have been suggested to affect receptor function in a dominant-negative fashion (Drappa *et al*, 1996; Bettinardi *et al*, 1997; Infante *et al*, 1998; Martin *et al*, 1999), we examined the allelic status of *Fas* in the BSCC and the CSCC. Overall, 12 of 21 BSCC (57%) and 30 of 50 CSCC (60%) were informative for at least one of the four polymorphic markers, and four of 12 informative BSCC (33%) and three of 30 informative CSCC (10%) showed LOH with one or more markers (**Table I**).

Of the three BSCC with the *Fas* gene mutations, two case (cases 7 and 13) showed LOH with the markers (**Fig 2D** and **Table I**). The other case (case 16) with the *Fas* mutation was heterozygous for the markers PA and PB, but did not show any LOH for these markers (**Table I**).

**Expression of Fas protein** We demonstrated Fas expressions in the BSCC and CBSCC by immunohistochemistry. The BSCC and CSCC showed immunoreactivity for Fas in 16 of 21 cases (76%), and in 40 of 50 cases (80%), respectively. As for the relationship between histologic grade and Fas immunoreactivity, the 16 Fas-positive BSCC consisted of 13 grade 1 and three grade 2 tumors, and 40 Fas-positive CSCC consisted of 26 grade 1, 10 grade 2, and four grade 3 tumors. Fas immunostaining, when present, was cytoplasmic and along the cell membranes; nuclei were clearly negative (data not shown). All BSCC with the *Fas* gene mutations expressed Fas (**Table I**).

## DISCUSSION

In order to explore genetic basis for the differences between CSCC and BSCC, we analyzed somatic mutations of the *Fas* gene in these tumors. We detected three somatic mutations in BSCC, one in FasL binding domain, another one in the death domain, and the other one in transmembrane domain (**Table I**). There were no *Fas* gene mutations in 50 CSCC, however. These findings, together with the recent demonstration of a similar frequency of *Fas* mutations in other human malignancies, indicate that *Fas* mutation is one of the mechanisms that mediate Fas resistance in BSCC.

Various postulates have been put forward to explain the development of cancer in burn scar and its aggressive biologic behavior, but there are few data to corroborate these suggestions (Bartle *et al*, 1990; Harland *et al*, 1997; Sakatani *et al*, 1998). Recently, Sakatani *et al* (1998) reported somatic mutation of *p53* gene in BSCC, which may be one of the underlying mechanisms in the pathogenesis of BSCC. Because *p53* gene mutation is thought to be important in tumorigenesis of CSCC as well (Brash *et al*, 1991), however, there may be an additional mechanism by which the pathogenesis in BSCC is differentiated when compared with CSCC. We detected three mutations in the *Fas* gene that were

exclusively observed in BSCC. These results suggest that mutant Fas may be involved in the process of development or progression of BSCC.

Although functional studies have not yet been performed, the mutations identified in this study are likely to disrupt or alter the normal function of Fas. To date, loss-of-function mutations of *Fas* have been identified in the promoter, exon 2, exon 3, exon 4, exon 6, exon 7, exon 8, and exon 9 (Watanabe-Fukunaga *et al*, 1992; Drappa *et al*, 1996; Bettinardi *et al*, 1997, 1998; Infante *et al*, 1998; Tamiya *et al*, 1998; Maeda *et al*, 1999; Martin *et al*, 1999). Most of the mutations, however, have been detected in exon 9 which encodes the death domain of Fas (Itoh and Nagata, 1993). The death domain is evolutionarily highly conserved and has been shown to be necessary and sufficient for the transduction of an apoptotic signal (Nagata, 1997). In this study, one mutation (Asn 239 Asp) was identified in this conserved area, suggesting that the mutation might disrupt death signaling. Another mutation (Asn 102 Ser) was found in exon 4 (**Table I**), which encodes part of FasL-binding domain of Fas (Starling *et al*, 1998). This mutation might impair the Fas pathway through inappropriate binding with its ligand. The other mutation (Cys 162 Arg) was found in exon 6, which encodes the transmembrane domain of Fas protein, but the functional significance of this mutation remains unknown at this stage.

The *lpr<sup>g</sup>* mice have a mis-sense mutation in exon 9 of *Fas*, which completely abolishes the signal transduction activities of Fas (Watanabe-Fukunaga *et al*, 1992). The human version of the *lpr<sup>g</sup>* mutation would result in amino-acid substitution at codon 238 (Watanabe-Fukunaga *et al*, 1992; Landowsky *et al*, 1997), which is close to the mutation identified in this study (amino acid substitution at codon 239). These data suggest that this area may be a potential hotspot in the *Fas* coding sequence.

Most of the patients with autoimmune lymphoproliferative syndrome carry a heterozygous mutation in the *Fas* gene (Drappa *et al*, 1996; Bettinardi *et al*, 1997; Infante *et al*, 1998; Martin *et al*, 1999). In these patients, the affected Fas protein seemed to work in a dominant-negative fashion, and T lymphocytes from these patients did not die upon activation. In our study, one *Fas* mutation (case 16) seemed to be a hemizygous mutation without allelic deletion (**Table I**). In contrast, cases 7 and 13 showed evidence of alterations of both alleles (a mis-sense mutation and an allelic deletion), indicating potential bi-allelic inactivation of the *Fas* gene. The functional difference between mono-allelic and bi-allelic inactivations of *Fas* gene in the tumorigenesis of BSCC, however, remains unknown at this stage.

We observed Fas protein expression in 76% of BSCC. In the BSCC which were not shown to express Fas protein, loss or downregulation of the protein may be another way to avoid Fas-mediated apoptosis. The *Fas* gene mutations in three BSCC, which showed Fas expression by immunohistochemistry, may be involved in the mechanisms of Fas resistance of those tumors. The presence of Fas-resistance mechanisms in the remaining BSCC without Fas mutations remains to be studied.

Despite the small number of cases, the mutations in the death domain and ligand-binding domain of the *Fas* gene observed in BSCC suggest that mutant Fas may play an important part in the pathogenesis of BSCC, probably through protecting the tumor cells from host immune attack by FasL-bearing lymphocytes. Additional studies in a large patient population and examining the BSCC samples according to the progression, however, will be needed to verify these initial observations, and further identification of the role of apoptosis dysregulation in human tumorigenesis will certainly broaden our understanding of pathogenesis of not only BSCC but also other tumors deserving consideration.

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