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Frequent Mutations of *Fas* Gene in Thyroid Lymphoma[1]

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ABSTRACT

Fas (Apo-1/CD95) is a cell-surface receptor involved in cell death signaling through binding of Fas ligand. Mutation of the *Fas* gene results in accumulation of lymphoid cells and thus might contribute to lymphomagenesis. Thyroid lymphoma (TL) is supposed to arise from active lymphoid cells formed in the preceding autoimmune chronic lymphocytic thyroiditis (CLTH). We examined the open reading frame of *Fas* cDNA in 11 cases of CLTH and 26 cases of TL. These patients were admitted to the hospital with varying degrees of goiter. All of the CLTH patients were female, with median age of 65 years, and all but five cases of TL were female, with median age of 61 years. Mutations of the *Fas* gene were detected in 3 (27.3%) of 11 cases of CLTH and 17 (65.4%) of 26 of TL. The *Fas* mutations comprised 18 frameshift, 3 missense, and 1 nonsense mutation. Frameshift mutations were caused by insertion of 1 bp (A) at nucleotide 1095 in 10 cases and by lack of exon 8 in 8 cases. The insertion of 1 bp (A) at nucleotide 1095 has never been reported in other kinds of malignancies. Thus, this might be unique in TL and CLTH and might be mutational hotspots in these diseases. All mutations occurred in the cytoplasmic region (death domain) known to be involved in the apoptotic signal transduction and thus could be loss-of-function mutations. These findings suggested that accumulation of lymphoid cells in CLTH with *Fas* mutation provides a basis for development of TL.

INTRODUCTION

Fas (Apo-1/CD95) is a M_r 45,000 membrane protein belonging to the tumor necrosis factor receptor family and mediates programmed cell death (apoptosis) on trimerization induced by cross-linking by FasL[3] (1–3). *Fas* locates on the chromosome 10q24.1 and comprises 9 exons and 8 introns. It consists of 325 amino acids with a single transmembrane domain, including signal peptide. The 80-amino acid portion designated as a death-signaling domain is essential for apoptotic signal transduction (4).

Fas is expressed on the surface of activated T and B lymphocytes, and Fas/FasL-induced apoptosis is important for eliminating autoreactive immature T cells during ontogenesis and for maintaining peripheral lymphocyte homeostasis (3, 5). The *lpr* mice that harbor deleterious mutations in the *Fas* gene show enlargement of lymph nodes and spleen attributable to accumulation of $CD4^-$ $CD8^-$ (double negative) T cells, exhibit B-cell lymphocytosis, and produce large amount of IgG and IgM autoantibodies, including anti-DNA antibodies and rheumatoid factor (6). Children who carry inherited defects in the *Fas* gene exhibit a similar, albeit variable, pattern of phenotypes that have been collectively termed as ALPS (7–13).

B-cell NHL is a particular neoplastic disease in which a malignant clone develops from the immune lymphoid system. Recent study indicated that resistance to Fas-mediated apoptosis is a widespread phenomenon in NHL, allowing the escape of malignant B cells from immune regulation (14, 15). Gronbaek *et al.* (16) reported the rather higher frequency of *Fas* mutations in lymphomas, especially in those with clinical features suggestive of autoimmune disease. Although this finding is interesting and might provide an insight into the mechanism of B-lymphomagenesis, localization and clinical backgrounds of autoimmune disease in each case were quite diverse, whereas the *Fas* gene mutation could not be detected in any cases of lymphoproliferative disorders associated with Sjögren syndrome and type II mixed cryoglobulinemia reported by Bertolo *et al.* (17).

TL is a minor constituent of NHL, accounting for 2.5% of all of the cases of extranodal lymphomas in the series of Freeman *et al.* from North America (18) and 2.2% from Japan (19). However, TL had attracted attention of investigators because of its putative origin from active lymphoid cells in autoimmune lymphocytic thyroiditis, *i.e.*, Hashimoto's thyroiditis or CLTH (20). Follow-up studies confirmed an important role of CLTH in the development of TL (21, 22). Autoimmune disease can be divided into organ-specific and systemic forms, and CLTH is categorized as one of the organ-specific autoimmune disease.

Giordano *et al.* (23) reported that thyrocytes from patients with CLTH expressed both Fas and FasL, suggesting the potential involvement of these molecules in the pathogenesis of CLTH. In the current study, we examined the *Fas* mutations in CLTH and TL to clarify whether *Fas* mutations are involved in the pathogenesis of TL.

PATIENTS AND METHODS

Cases. Thyroid specimens were collected from 26 patients with TL and 11 with CLTH. They were admitted with varying degrees of goiter to Kuma Hospital (Kobe City, Japan) during the period 1995–1998. Results of studies on microsatellite instability and k-*ras* and *p53* mutations in a part of TL and CLTH cases were previously reported (24). All of the CLTH patients were female, and all but 5 cases of TL were female patients. The age of the patients on admission ranged from 45 to 85 years (median, 65 years) in TL and 52 to 75 years (median, 61 years) in CLTH. All patients underwent surgery including total, partial thyroidectomy, or open biopsy, and the histological specimens were fixed in 10% formalin and routinely processed for paraffin-embedding. Samples in all of the cases were snap-frozen with or without OCT compound at −150°C and stored at −80°C until use. Criteria for the diagnosis of CLTH included increased consistency of the thyroid gland, occasional hypothyroidism, high level of thyroid-stimulating hormone, low ^{123}I uptake, and the presence of antimicrosomal and/or antithyroglobulin antibodies in the serum. Histological findings of the CLTH included lymphocytic infiltration, usually forming lymphoid follicles with germinal centers, varying degrees of fibrosis, and oxyphilic change or squamous metaplasia of epithelial cells of the thyroid follicles. Lymphoma cells in all of the cases showed a B-cell immunophenotype, *i.e.*, $CD20^+$ and/or $MB\text{-}1^+$, $CD3^-$, or $CD45RO^-$. TL were classified according to the revised European-American Classification for Lymphoid Neoplasms (REAL; Ref. 25); diffuse large B-cell lymphoma in 10 cases, follicle center cell lymphoma in 8, and marginal zone B-cell lymphoma of the extranodal type (MALT) in 8. In the majority of cases with TL, the presence of lymphoid follicles with a germinal center could be confirmed, indicating the preexisting CLTH.

Isolation of Total RNA, Reverse Transcription-PCR, and Detection of Mutations. Tissue samples from TL and CLTH were homogenized, and total RNA was extracted in the presence of TRIzol reagent (Life Technologies, Inc.,

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[3] The abbreviations used are: FasL, Fas ligand; NHL, non-Hodgkin's lymphoma; ALPS, autoimmune lymphoproliferative syndrome; TL, thyroid lymphoma; CLTH, chronic lymphocytic thyroiditis; MALT, mucosa-associated lymphoid tissue.

Table 1 *Oligonucleotide primers used for PCR reactions*

	Primer sequence	Ta (°C)[a]	PCR products (bp)[b]
cDNA primers			
Fas full	5′-CACTTCGGAGGATTGCTCAACA-3′	54	1.163 (170–1336)
	5′-TATGTTGGCTCTTCAGCGCTA-3′		
FAS I	5′-ATGCTGGGCATCTGGACCCT-3′	49	669 (195–863)
	5′-ATTTATTGCCACTGTTTCAGG-3′		
FAS II	5′-TGCAAAGAGGAAGGATC-3′	49	517 (687–1203)
	5′-TCTAGACCAAGCTTTGGATTTC-3′		
Genomic primers			
8-F1	5′-TATTTTTATTTGTCTTTCTCTGCTTCC-3′	52	137
8-R1	5′-TTTACTCTGAAATTGGCCTATTAC-3′		
8-F2	5′-ATTAAGGAAAAATTAGAAGTTCACATT-3′	52	217
8-R2	5′-ATCCCATAATATGTCACTGAAA-3′		

[a] Ta, annealing temperature.
[b] Range of nucleotides in parentheses.

Rockville, MD). Those from normal leukocytes of the patients were not available. Five μg of total RNA were reverse-transcribed by random hexamer priming, and the indicated fragments were amplified by 35 cycles in a thermocycler (Model 9700 thermocycler; Applied Biosystems, Foster City, CA). The primers were selected to amplify the *Fas* open reading frame (Fas full) or to amplify two segments named Fas I and II (Table 1). PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Santa Clarita, CA) and cloned in the pCR 2.1-TOPO (Invitrogen, San Diego, CA). To control potential PCR error, 8 to 12 clones from three different PCR reactions (Fas full, Fas I, and Fas II) were sequenced individually. When common mutations were found in more than two PCR results, we regarded them as definite mutations. Frequency of mutations among clones ranged from 12.5 to 77.3%. Sequencing was performed by the dideoxy chain termination method using the DNA sequencing kit (Applied Biosystems). The samples were analyzed by the Genetic Analyzer (ABI PRISM 310′; Applied Biosystems). Ampli *Taq* Gold DNA polymerase (Applied Biosystems) and platinum pfx DNA polymerase (Life Technologies) were used for amplification of the Fas I and II segments and the Fas full segment, respectively.

Detection of Mutations in the Exon/Intron Boundary of Exon 8. Genomic DNA extracted from the frozen tissues were subjected to PCR with the oligonucleotide primers, 8F1 and 8R1 or 8F2 and 8R2 (Table 1), which flank exon 8. PCR products were cloned in the pCR 2.1-TOPO (Invitrogen). Eight to 12 clones were sequenced.

Immunohistochemical Detection of Fas Protein. Immunohistochemical study on the paraffin sections was carried out using the avidin-biotin-peroxidase complex method. For detection of Fas protein, mouse monoclonal anti-human Fas antibody (4B4-B3), which recognizes the extracellular domain of Fas, was prepared by S. Nagata.[4]

RESULTS

Mutation of the *Fas* Gene. Mutations of the *Fas* gene were detected in 3 (27.3%) of 11 cases of CLTH and 17 (65.4%) of 26 of TL (Table 2). Detection frequency was significantly higher in TL than in CLTH cases by Fisher's exact probability test ($P < 0.05$). Mutations were more frequently found in cases with marginal zone B-cell lymphoma and follicle center cell lymphoma, although the difference in frequency among each histological type of lymphoma was not significant. All cases had mutations in the death domain. Two cases (cases 2 and 16) had two different mutations (Table 3).

Frameshift Mutations. The *Fas* gene has a 6-(A) tract from nucleotide 1088 to 1094 of the *Fas* cDNA sequence (Ref. 26; GenBank accession no. M67454). Nine cases of TL showed insertion of 1 bp (A) at nucleotide 1095 that resulted in a frame shift. As a result, a stop codon was introduced at residue 303. No cases had mutations in a 7-(T) tract from nucleotide 591 to 597 of the *Fas* coding sequence. Six cases of TL showed a 25-bp deletion from nucleotide 846 to 870, which corresponded to exon 8. Lack of exon 8 also result in a frame shift, which generated a stop codon at residue 221. As a result, the whole intracytoplasmic death domain was deleted.

Mutations in the acceptor splice site of *Fas* intron 7 or in the donor spice site of exon 8 may cause the splice variant transcripts to lack exon 8. To examine this hypothesis, genomic DNA from the patients was amplified using primers flanking exon 8, cloned, and then sequenced. One case (case 39) had a transition of the invariable T at position +2 of the donor splice site of intron 8, which resulted in exon skipping. Three cases (cases 2, 3, and 60) had mutations in the consensus sequence of the acceptor splice site of intron 7 and one (cases 8) had the donor splice site of intron 8, although it is not certain whether these mutations cause exon-skipping or not (data not shown).

Point Mutations. Three of the point mutations in TL were missense ones, which caused substitutions of nonconserved amino acids. All mutations were detected in exon 9, which encodes the death domain region of the Fas receptor. Two different transversions (G to A and G to C) at position 972, one causing the substitution of Asp with Asn and the other Asp with His at codon 244, were found in two TL cases, suggesting that this site might also represent a mutational hotspot. One mutation introduced premature termination signals at codon 216 within the death domain.

In CLTH cases, two showed a 25-bp deletion from nucleotide 846 to 870, which corresponded to exon 8. Genomic DNA from the same cases (cases 18 and 25) also had mutations located in the splice-site consensus sequences of exon 8, although it is not certain whether these mutations really cause exon-skipping (data not shown). One case showed insertion of 1 bp (A) at nucleotide 1095.

Immunohistochemistry. Fas protein was expressed in the lymphoma cells and in the infiltrating reactive lymphoid cells, including germinal center cells in 15 (58%) of 26 TL cases and in the infiltrating lymphoid cells in 5 (45%) of 11 CLTH cases. There were no prominent differences in the positive rate of lymphoma cells among each histological type. Expression of Fas protein was more frequent in the lymphoma cases with the mutated *Fas* gene, 14 (70%) of 20 cases, than in the nonmutated cases, 6 (35%) of 17 cases ($P < 0.05$, Table 4).

Table 2 *Distribution of Fas mutations according to the histological subtype*

Histology	Mutated cases[a]
CLTH	3/11 (27)[b]
TL	17/26 (65)
Diffuse large B-cell lymphoma	5/10 (50)
Low-grade MALToma[c]	6/8 (75)
Follicle center cell lymphoma	6/8 (75)

[a] Percentage in parentheses.
[b] CLTH *versus* TL, $P < 0.05$; Fisher's exact probability test.
[c] MALT, mucosa-associated lymphoid tissue.

[4] S. Nagata, unpublished data.

Table 3 *Fas mutations in TL*

Case	Histology[a]	Nucleotide change	Localization	Codon	Predicted effect	Frequencies of mutation positive clones (%)
18	CLTH	846–870 del (exon 8)	Exon8	202–210	Frameshift	42.1
25	CLTH	846 –870 del (exon 8)	Exon8	202–210	Frameshift	12.5
2	DLBL	846–870 del (exon 8)	Exon8	202–210	Frameshift	12.5
3	DLBL	846–870 del (exon 8)	Exon8	202–210	Frameshift	18.8
9	DLBL	846–870 del (exon 8)	Exon8	202–210	Frameshift	12.5
8	MALT	846–870 del (exon 8)	Exon8	202–210	Frameshift	50.0
39	MALT	846–870 del (exon 8)	Exon8	202–210	Frameshift	40.0
60	MALT	846–870 del (exon 8)	Exon8	202–210	Frameshift	12.5
42	MALT	T890A	Exon9	216	Tyr→Stop	33.3
19	FCL	A961T	Exon9	240	Glu→Val	45.5
7	FCL	G972A	Exon9	244	Asp→Asn	77.3
16	FCL	G972C	Exon9	244	Asp→His	73.3
46	CLTH	1095 ins A	Exon9	285	Frameshift	43.8
2	DLBL	1095 ins A	Exon9	285	Frameshift	25.0
23	DLBL	1095 ins A	Exon9	285	Frameshift	18.8
34	DLBL	1095 ins A	Exon9	285	Frameshift	12.5
11	MALT	1095 ins A	Exon9	285	Frameshift	18.8
12	MALT	1095 ins A	Exon9	285	Frameshift	37.5
5	FCL	1095 ins A	Exon9	285	Frameshift	12.5
14	FCL	1095 ins A	Exon9	285	Frameshift	25.0
16	FCL	1095 ins A	Exon9	285	Frameshift	26.7
66	FCL	1095 ins A	Exon9	285	Frameshift	31.3

[a] DLBL, diffuse large B-cell lymphoma; FCL, follicule center cell lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma.

DISCUSSION

Fas mutations have been reported in the lymphoid malignancies: ~10% of cases with multiple myeloma (27) and 11% with sporadic NHL (16). In the current study, mutations of the *Fas* gene were detected in 3 (27.3%) of 11 cases of CLTH and 17 (65.4%) of 26 of TL, showing a much higher frequency in these diseases than that in the previous study on the nonthyroid malignancies. In the thyroid tissues, resistance to Fas-induced cell death might be an important step in cellular accumulation, because FasL was shown to be constitutively expressed in thyrocytes from normal and CLTH thyroid tissues (23). Therefore, lymphoid cells with *Fas* mutations might be allowed to escape from apoptosis induced by Fas-FasL interactions between thyrocytes, infiltrated T cells, and B lymphocytes. Because TL is supposed to arise from active lymphoid cells formed in CLTH, accumulation of *Fas*-mutated lymphoid cells in CLTH lesions might make a basis for development of TL.

The *Fas* mutations in the current series comprised 18 frameshifts, which were caused by a 1-bp insertion in 10 cases (9 TL and 1 CLTH cases) and by lack of exon 8 in eight cases. Mutations in all of the cases occurred in the death domain known to be involved in the apoptotic signal transduction; thus, the mutations in our cases should result in resistance of lymphoid cells to apoptosis. In the previous studies on lymphoid malignancies (16, 27–29) as well as ALPS (7–13, 30), the majority of mutations were located in the death domain, indicating that Fas-induced apoptosis also could be altered by mutations in the *Fas* genes in these cases, although several studies showed relatively rare occurrence of *Fas* mutations in lymphoproliferative diseases (17, 31, 32).

One base insertion within a polyadenine tract was found in 9 TL and one CLTH cases. The wild-type *Fas* gene has a 6-(A) tract from nucleotide 1088 to 1094, but these mutated cases had 7-(A) tract by insertion of 1 bp (A) at nucleotide 1095. The same kind of mutation has never been reported in other kinds of malignancies, including multiple myeloma (27), sporadic NHL (16), and adult T-cell leukemia (28, 29) as well as ALPS (7–13, 30). Meanwhile, the mononucleotide tract in the coding sequence is a mutational hotspot in many kinds of genes, such as transforming growth factor β receptor type II gene (33) and insulin-like growth factor receptor type II gene (34). These findings suggested that the insertion of 1 bp (A) at nucleotide 1095 was unique in TL and CLTH and might be a mutational hotspot in these diseases. A 1-bp insertion at nucleotide 1095 results in a frame shift at codon 285 and introduces a stop codon at residue 303. Because this site is quite close to the terminal site, whether this mutation would cause loss of function is equivocal, although Ito *et al.* (4) demonstrated that the 130-amino acid portion from 175 to 304 in the cytoplasmic region of the human *Fas* gene is essential for the Fas-antigen triggered apoptotic signal transduction.

Table 4 *Fas protein expression according to Fas gene mutations*

Fas mutations histology	Fas-positive cases[a]
Positive	14/20 (70)[b]
CLTH	2/3 (67)
TL	12/17 (71)
Negative	6/17 (35)
CLTH	3/8 (38)
TH	3/9 (30)

[a] Values in parentheses are percentages.
[b] Positive versus negative, $P < 0.05$; Fisher's exact probability test.

Six TL and 2 CLTH cases lacked exon 8 as splicing variants of the *Fas* gene. These variants have been reported in the apoptosis-resistant clone derived from human T-cell lymphoma cell line HUT78 (35) and in cases of NHL that developed in two patients, one with CLTH and Sjögren syndrome and the other with rheumatoid arthritis (16). These variants also are known to interfere with Fas-mediated apoptosis signaling in a dominant-negative fashion. Recently, two studies showed that deletion of exon 8 was a loss-of-function mutation (13, 14). Thus, this kind of splicing variant might be characteristically found in lymphoid cells involved in systemic and organ-specific autoimmune diseases and in developing lymphoma from them.

Most of the mutations identified in the current cases are likely to disrupt or alter the normal structure and/or function of Fas. Mutations in 19 cases are predicted to cause frameshifts (10 cases), aberrant RNA splicing (8 cases), and premature termination (1 case) and thus are judged as typical loss-of-function mutations. Previous studies showed that the genetic defects resulting in the production of a truncated form of proteins might be able to confer a dominant-negative effect (9, 13, 14). The remaining mutations were missense variants that resulted in substitutions of nonconservative amino acid. Three missense mutations (cases 7, 16, and 19) within the region encoding the Fas death domain affected the codons that are highly conserved in evolution (4). Furthermore, alteration of codon 244, found in cases 7 and 16, has been reported in ALPS (7–14) and NHL

(16). This mutation caused the reduced trimerization of Fas induced by cross-linking of FasL and its binding to FADD/MORT1, which is essential for the apoptotic signal transduction (36).

There were no prominent differences in the histological and clinical findings between the *Fas*-mutated and nonmutated cases. Meanwhile, Fas protein was more frequently expressed in the *Fas*-mutated cases (65% of cases) than in the nonmutated cases. Whether alterations in the expression and/or function of components situated downstream of the same pathway of Fas-mediated apoptosis, such as FADD/MORT1 (37, 38), Caspase 8 (39, 40), and FLICE-inhibitory proteins (41), cause resistance to the apoptosis in cases with the wild-type *Fas* gene or not will be subject to future studies.

In conclusion, the results of our study provide direct evidence that the Fas-mediated apoptotic pathway is abrogated in 65.4% of TL and 27.3% of CLTH cases. These findings suggested that accumulation of lymphoid cells in CLTH with the *Fas* mutation provide a basis for development of TL.

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