Frequent *Fas* Gene Mutations in Testicular Germ Cell Tumors

Hitoshi Takayama,* Tetsuya Takakuwa,*
Yuichi Tsujimoto,* Yoichi Tani,[†] Norio Nonomura,[‡]
Akihiko Okuyama,[‡] Shigekazu Nagata,[§] and
Katsuyuki Aozasa*

From the Departments of Pathology,* Urology,[‡] and Genetics,[§]
Osaka University Medical School, Osaka; and the Department of
Immunohistochemistry,[†] DAKO Japan, Kyoto, Japan

The *Fas* (Apo-1/CD95)/*Fas* ligand (L) system is involved in cell death signaling, and has been suggested to be important for the regulation of germ cell apoptosis in the testis. Mutations of the *Fas* gene may result in accumulation of germ cells and thus might contribute to testicular carcinogenesis. The open reading frame of *Fas* cDNA was examined in 24 cases of testicular germ cell tumors (TGCTs), comprised of 19 pure histological type (15 seminomas, 3 embryonal carcinomas, 1 immature teratoma) and 5 mixed-type tumors. Mutations of the *Fas* gene were found in nine (37.5%) of these cases. Each lesion with a homogeneous histological picture was selectively microdissected using a laser capture microdissection method: samples consisted of 18 lesions from seminomas, 7 embryonal carcinomas, 4 immature teratomas, 2 choriocarcinomas, and 1 from a yolk sac tumor. Microdissected genomic DNA was examined to determine which mutations were derived from which kind of histological lesion. Eleven mutations were detected in 10 TGCT lesions from nine cases, but none were found in benign lesions. All were point mutations, and eight missense mutations occurred in exon 9 encoding the core protein of the death domain essential for apoptotic signal transduction. Three were silent mutations. Mutations were found in the seminoma (27.8%) and embryonal carcinoma lesions (62.5%), but none were found in the one yolk sac tumor, two choriocarcinomas, or four immature teratoma lesions. Each seminoma and embryonal carcinoma lesion found in the same case had a different type of *Fas* mutation from the others. Mouse T-cell lymphoma cells transfected with missense mutated genes were resistant to apoptosis induced by anti-*Fas* antibody, indicating these to be loss-of-function mutations. These findings suggested a role of *Fas* gene mutations in the pathogenesis of TGCTs. (*Am J Pathol* 2002, 161:635–641)

Fas receptor is a 45-kd transmembrane protein of the tumor necrosis factor receptor superfamily that can induce programmed cell death (apoptosis) through cross-linkage with *Fas* ligand (*FasL*).^{1,2} Apoptosis mediated by the *Fas*/*FasL* system plays an important role in the normal development of T lymphocytes by elimination of self-reactive lymphocytes.² *Fas* is widely expressed in normal tissues including the thymus and liver, and in activated T and B lymphocytes^{3,4} and neoplastic cells.⁵ In the testis, recent studies demonstrated the expression of *Fas* in germ cells and of *FasL* in Sertoli cells.^{6,7} Pentikäinen and colleagues⁸ suggested that the *Fas*/*FasL* system is important for regulation of germ cell apoptosis in the testis, ie, a *Fas*-mediated pathway might be actively involved in the signaling of germ cell apoptosis and thus partly account for the immune-privileged status of the testis.

Mutations of the *Fas* gene in the death domain lead to loss of its apoptosis-inducing function, a loss-of-function mutation, resulting in accumulation of cells. Recent studies suggested that mutations of the *Fas* gene might contribute to the pathogenesis and development of lymphoid-lineage malignancies.^{9,10} We postulated that mutations of the *Fas* gene might also be involved in carcinogenesis of testicular germ cells. However, there have been no reports suggesting the contribution of *Fas* gene mutations in the development of testicular germ cell tumors (TGCTs).

TGCTs are the most common solid malignancies in young men between the ages of 20 to 40 years, and have an overall incidence of 2 to 4 per 100,000 in the population.¹¹ Classifications of the TGCT vary widely, but they can be divided into two broad categories: tumors of one histological pattern such as seminomas, embryonal carcinomas, and yolk sac tumors, and tumors showing more than one histological pattern. Seminoma is the most common type of TGCTs, and histologically shows proliferation of tumor cells in small nests that are confined by fibrous septa containing numerous lymphocytes. TGCT lesions frequently co-exist with nonneoplastic lesions or show a variety of histological pictures even in the same patient, which can be recognized accurately only at the microscopic level. Therefore, little information regarding the molecular genetics in each TGCT lesion could be ob-

Accepted for publication April 25, 2002.

Address reprint requests to Katsuyuki Aozasa, Department of Pathology (C3), Osaka University Medical School, Yamada-oka 2-2, Suita, Osaka, Japan 565-0871. E-mail: aozasa@molpath.med.osaka-u.ac.jp.

Table 1. Amplification Primers for Mutation Analysis of the *Fas* Gene

Primer/(exon)	First PCR			Second PCR		
	Sequence (5'→3')	Size of PCR product (bp)	Annealing temperature (°C)	Sequence (5'→3')	Size of PCR product (bp)	Annealing temperature (°C)
cDNA primers						
Full-F	5'-cacttcggaggattgctcaaca-3'	1154	52	5'-tcggaggattgctcaacaacc-3'	1057	46.5
Full-R	5'-tatgtggctcttcagcgcta-3'			5'-ctaattgcataactcagaa-3		
Genomic primers I						
2-F (exon2)	5'-gttgcttacttcagaaatcaataa-3'	249	50	5'-agaaatcaataaaattcttcatgc-3'	237	50
2-R	5'-actgtaatctctggatgtttgt-3'			5'-actgtaatctctggatgtttgt-3'		
9-F1 (exon9)	5'-ggtttcactaatgggaattca-3'	192	50	5'-atttcagactattttctatttctat-3'	156	50
9-R1	5'-cattgtcattcttgatctcatct-3'			5'-cattgtcattcttgatctcatct-3'		
9-F2 (exon9)	5'-cactaagtcaagttaaggct-3'	170	50	5'-cactaagtcaagttaaggct-3'	155	50
9-R2	5'-gatcttaataatgtgtcatcac-3'			5'-gtgtcatcacgcttcttctt-3'		
9-F3 (exon9)	5'-gaaagttcaactgcttcgtaattg-3'	230	50	5'-cttcgtaattggcatcaactcat-3'	217	50
9-R3	5'-ctaattgcataactcagaa-3'			5'-ctaattgcataactcaggaa-3'		
Genomic Primers II						
PA-F (promoter, 1377)	5'-gccctataccatcctccttat-3'	172	55	5'-gccctataccatcctccttat-3'	145	55
PA-R	5'-ctgtcactgcacttaccacc-3'			5'-gtagggtgtgataggctgtct-3'		
PB-F (promoter, -670)	5'-cctcttgaaaataaaaact-3'	242	56	5'-cctcttgaaaataaaaact-3'	228	56
PB-R	5'-tcaactcagagaaagacttgcgg-3'			5'-gacttgcggggcatttgac-3'		
3-F (exon3)	5'-acttcccaccctgttacctg-3'	310	55	5'-acttcccaccctgttacctg-3'	250	55
3-R	5'-acttcccaccctgttacctg-3'			5'-tgtgtgtcaacatagaccac-3'		
7-F (exon7)	5'-tcttagtgtgaaagtattgtctc-3'	223	46	5'-ctacaaggctgagacctgagtt-3'	203	55
7-R	5'-caaatacctaatttctatttt-3'			5'-aggaaagtaacaaaagccaaatc-3'		

PA, Promoter region A containing the polymorphism at nucleotide -1377; PB, promoter region B containing the polymorphism at nucleotide -670.

tained until the development of microdissection techniques.

Laser capture microdissection under direct microscopic visualization enables rapid one-step procurement of selected human cell populations from histological sections. This method made microdissection of selected cells much easier, and thus extensive studies of the lesions became possible. In the present study, mutations of the *Fas* gene were examined in 24 cases of TGCTs. First, the open reading frame of *Fas* cDNA was examined. Subsequently, microdissected genomic DNA from each lesion containing a heterogeneous histological picture was examined to determine which mutations were derived from which kind of histological lesion.

Materials and Methods

Patients

Twenty-four patients who underwent orchiectomy under the diagnosis of TGCT were selected for the present study: they were admitted to Osaka University Hospital during the period 1996 to 2000. None of the 24 patients received preoperative chemotherapy or radiation therapy. The age of patients ranged from 23 to 61 years (median, 32 years). Pathological stage based on the TNM system (The Union Internationale Contre le Cancer)¹² was stage T1 in all cases. Histological specimens were fixed in 10% formalin, and routinely processed for paraffin embedding. Histological sections cut at a thickness of 4 μ m were stained with hematoxylin and eosin, and were reviewed by three of the authors (HT, YT, KA) for diagnosis. Tumors with one histological pattern (pure type)

were found in 19 cases (seminoma, cases 1 to 15; embryonal carcinoma, cases 16 to 18; immature teratoma, case 19), and tumors showing more than one histological pattern (mixed type) in 5 cases; 4 cases had embryonal carcinoma together with seminoma and immature teratoma in case 20, immature teratoma in case 21, seminoma and choriocarcinoma in cases 22 and 23. One case (case 24) had yolk sac tumor and immature teratoma. A part of each sample from each case was snap-frozen with or without OCT compound at -150°C and stored at -80°C until use.

Detection of Mutations and Loss of Heterozygosity (LOH)

Total RNA was extracted from the fresh samples using Trizol reagent (Life Technologies, Inc., Grand Island, NY) and reverse-transcribed by random hexamer priming. cDNAs were subjected to a first round of polymerase chain reaction (PCR) for 10 cycles with the cDNA primers (Table 1) followed by a second round of PCR for 35 cycles with 0.1% first-round PCR products as the template, denaturation for 30 seconds at 95°C, annealing for 30 seconds at various temperatures, and extension for 30 seconds at 72°C. Then, PCR products were cloned in pCR2.1-TOPO (Invitrogen, Carlsbad, CA). To control for potential PCR errors, 12 clones were sequenced. When common mutations were found in more than two clones, we regarded them as potential mutations responsible for the tumor.

As TGCTs showed various histological features in the same case and contained nonneoplastic tissues,

Figure 1. Seminoma and embryonal carcinoma lesions (**A**) in representative cases were microdissected (**D**). Note successful resection of seminoma (**black arrow**) and embryonal carcinoma (**white arrow**). High-power view of seminoma (**B**) and embryonal carcinoma lesion (**C**). H&E; original magnifications: $\times 40$ (**A** and **D**); $\times 100$ (**B** and **C**).

genomic DNA extracted from the paraffin-embedded samples by microdissection were examined to specify which mutations were derived from which kind of histological lesion. Each lesion with a homogeneous histological picture was selectively microdissected as described previously (Figure 1).¹³ The numbers of microdissected lesions were as follows: 18 seminomas, 7 embryonal carcinomas, 4 immature teratomas, 2 choriocarcinomas, and 1 yolk sac tumor. Genomic DNA was subjected to first-round PCR for 10 cycles with the genomic primers listed in Table 1 (genomic primers I) followed by second PCR for 35 cycles with 0.1% of first-round PCR products as the template, denaturation for 30 seconds at 95°C, annealing for 30 seconds at various temperatures, and extension for 30 seconds at 72°C. Subcloning was performed as described above, and 12 clones were sequenced per lesion. When common mutations were found among two methods using cDNA and genomic DNA as the template, we finally regarded them as defi-

nite. Frequencies of mutation-positive clones ranged from 33.3 to 91.7%, and mutations were found in more than 75% of clones in 3 of 10 mutational lesions (Table 2).

LOH was examined at four sites of known polymorphisms, ie, at nucleotides -1377, -670 (promoter region), 416 (exon 3), and 836 (exon 7). DNA was amplified using four sets of genomic primers (genomic primers II; Table 1) flanking the four polymorphic sites. Polymorphisms at -1377, 416, and 836 were examined by direct sequencing, and that at position -670 was examined by restriction fragment length polymorphism by digestion with *Mva*I (Fermentas, Vilnius, Lithuania).¹⁴

Immunohistochemistry

Immunohistochemical analysis of paraffin-embedded sections was performed using the avidin-biotin-peroxidase complex method. For detection of Fas protein,

Table 2. *Fas* Gene Mutations in Testicular Germ Cell Tumors

Case	Histological diagnosis	Lesions with <i>Fas</i> mutation	Mutation				Frequency of clones with <i>Fas</i> mutations among 12 clones(%)
			Site	Nucleotide changes	Codon	Amino acid	
Pure type							
3	SE	SE	Exon 9	A1039T	266	His/Leu	66.6
			Exon 9	A1077G	279	Ile/Val	
10	SE	SE	Exon 2	T297C	19	Leu/Leu	91.7
16	SE	SE	Exon 9	A1054T	271	Lys/Met	41.7
17	EC	EC	Exon 9	A1069G	276	Asp/Gly	66.6
18	EC	EC	Exon 9	A1144G	302	Ile/Val	75.0
Mixed type							
20	SE, EC, IT	SE	Exon 9	A871G	210	Asp/Gly	58.3
21	EC, IT	EC	Exon 9	A1004G	254	Thr/Thr	33.3
22	SE, EC, CC	EC	Exon 9	T950C	236	Asn/Asn	50.0
		SE	Exon 9	A958G	239	Asn/Ser	75.0
23	SE, EC, CC	EC	Exon 9	C904G	221	Ala/Gly	58.3

SE; seminoma; EC; embryonal carcinoma; IT; immature teratoma; CC; choriocarcinoma.

mouse anti-human *Fas* antibody (4B4-B3) that recognizes the extracellular domain of *Fas* was prepared by Dr. S. Nagata (unpublished data). The histological sections were deparaffinized and rinsed in three changes of phosphate-buffered saline. Then, the antibody diluted to 1:200 was applied at 37°C for 45 minutes.

Plasmid Construction, Transfection, and Cell-Killing Assay

The mouse T-cell lymphoma cell line WR19L (ATCC TIB52) was grown in RPMI 1640 medium containing 10% fetal calf serum. The 1.1-kb *EcoRI-XbaI* fragment containing the full-length of human *Fas* cDNA with and without point mutations was transferred into the mammalian expression plasmid pEF-BOS-EX.¹⁵ The WR19L cells were transfected with the plasmid by electroporation as described previously.¹⁶ After selection with 0.9 mg/ml of G-418, transformants expressing the mutated *Fas* were identified by fluorescence-activated cell sorting analysis using mouse anti-human *Fas* monoclonal antibody (CH-11; MBL, Nagoya Japan) and fluorescein isothiocyanate-conjugated anti-mouse Ig antibody (Capel, Aurora, OH). The cell-killing assay was performed in 96-well microtiter plates as described previously.¹⁶ Briefly, 2.5×10^4 transformed cells (100 μ l) were incubated at 37°C for 16 hours with various concentrations (3 ng/ml to 2 μ g/ml) of anti-*Fas* antibody. Dead and viable WR19L cell transformants were distinguished by staining with trypan blue.

Results

Fas Gene Mutations

Total RNA extracted from the fresh tumor samples was examined to specify which cases had *Fas* gene mutations. Mutations of the *Fas* gene were detected in 9 of 24 cases (37.5%); 4 of 5 cases (80.0%) of the mixed type, which was significantly higher than the incidence in the pure type (5 of 19 cases; 26.3%) ($P < 0.05$ by Fisher's

probability test). Among pure types, two of three cases with embryonal carcinoma, 3 of 15 with seminoma, and none of the immature teratomas showed *Fas* gene mutations. Differences in the frequency between each pure type were not significant. All were point mutations, and eight missense mutations were detected in exon 9, which encodes the death domain region of the *Fas* receptor.² Three silent mutations were detected in exons 2 and 9. G to A transition was the most common pattern of *Fas* gene mutation (6 of 11; 54.5%), followed by C to T transition (two mutations), and A to T transversion (two mutations).

As TGCTs showed various histological features in the same case and contained nonneoplastic tissues, DNA extracted from the paraffin-embedded samples by microdissection was examined to specify which mutations were derived from which kind of histological lesions. A total of 11 mutations were detected in 10 lesions from nine cases (Table 2). Nonneoplastic lesions adjoining TGCTs never showed mutations of the *Fas* gene. In one seminoma lesion (case 3), there were double mutations with different types of nucleotide substitutions in the same exon. In the mixed type, mutations were detected in some lesions but not in other lesions of the same case.

When *Fas* gene mutations were evaluated by histological type, 5 (27.8%) of 18 lesions with seminoma and 5 (62.5%) of 8 lesions with embryonal carcinoma showed mutation, but the yolk sac tumor, choriocarcinomas, and immature teratomas did not show mutations.

Allelic Status of the *Fas* Gene

The *Fas* gene is known to have four polymorphic sites, ie, at nucleotides -1377, -670 (promoter region), 416 (exon 3), and 836 (exon 7). LOH in the TGCTs was examined in cases with proven heterozygous alleles in the nonneoplastic tissues. Heterozygosity was confirmed in cases 3, 5, 12, 21 (-670) and case 22 (exon 7). Of these five informative cases, three (cases 3, 5, 12) with seminoma showed LOH in the promoter region (-670) (data not shown). Case 3 had *Fas* gene mutations within

Figure 2. Cytolytic effect of anti-Fas antibody on WR19L transformant clones. **A:** Time course of survival of WR19L cells expressing wild-type human Fas receptor. Transformants incubated with 2 $\mu\text{g/ml}$ (▲) or 100 ng/ml (×) of anti-Fas antibody died gradually until 4 to 5 hours. As a control, WR19L cells without transfection were incubated with 2 $\mu\text{g/ml}$ of anti-Fas antibody, and all cells survived (◆). Viable cell counts were determined by the trypan blue exclusion method, and the percentage of viable cells was plotted. **B:** The mouse WR19L cell line (◆) and its transformant clones expressing wild-type human Fas receptor (▲) were incubated with various concentrations of anti-Fas antibody at 37°C for 16 hours. As WR19L cells did not express human Fas receptor, these cells were resistant to apoptosis. **C:** The mouse WR19L cell line expressing recombinant human Fas protein with missense mutations found in cases 3, 16, 17, 18, 20, 22, 23, or without (wild type) point mutations were incubated with 100 ng/ml (shaded bar) and 2 $\mu\text{g/ml}$ (white bar) of anti-Fas antibody at 37°C for 16 hours. Clones expressing Fas with missense mutations were resistant to apoptosis.

exon 9. In case 5, Fas protein was not detected by immunohistochemistry.

Immunohistochemistry

Whether the TGCTs expressed Fas protein was examined immunohistochemically. Positive cells showed intracytoplasmic punctate staining. The staining in each case was distinct, ie, almost all cells were positive in positive cases and negative in negative cases. Expression of Fas protein was found in all lesions except for one seminoma lesion (case 5) that showed LOH in the promoter region (−670) (data not shown).

Apoptotic Signal Transduction by Mutant Fas Receptor

Eight missense mutations in exon 9 caused substitutions of nonconserved amino acids. To examine whether these mutations abolished apoptotic signal transduction by Fas, these mutant Fas gene cDNAs were prepared, introduced into a mammalian expression vector, and expressed in WR19L cells (mouse T-cell lymphoma cells).

Fluorescence-activated cell sorting analysis of stable transformants using anti-Fas monoclonal antibody indicated that more than 80% of G-418-resistant transformant clones expressed human Fas on the cell surface. The levels of Fas protein expression among the positive clones were similar (data not shown). The ability of the mutated Fas to transduce apoptotic signals was then examined by treatment of the transformants with agonistic anti-Fas antibody. To exclude clonal variations of Fas expression, two independent clones were chosen for each mutant. WR19L cells without transfection were not killed at all even after incubation with 2 $\mu\text{g/ml}$ of anti-Fas antibody, whereas the WR19L cells expressing the wild-type Fas receptor were killed within 6 hours by incubation with the anti-Fas antibody (Figure 2A). The transformant clones expressing wild-type Fas were killed by anti-Fas antibody in a dose-dependent manner (Figure 2B). On the other hand, clones expressing Fas with missense mutations were resistant to apoptosis induced by the anti-Fas antibody even at 2 $\mu\text{g/ml}$ (Figure 2C). These findings indicated that the eight missense mutations found in TGCTs were loss-of-function mutations.

Discussion

Apoptosis induced via the Fas/FasL system could prevent the development of TGCTs, and therefore escape from apoptosis through *Fas* gene mutations might be involved in testicular carcinogenesis. Previous studies showed that ~10% of cases with lymphoid malignancies including multiple myeloma⁹ and sporadic non-Hodgkin's lymphoma¹⁰ had *Fas* gene mutations. *Fas* gene mutations in lung cancer¹⁷ and urinary bladder cancer¹⁸ were reported to be found in 7.7% and 28% of cases, respectively. The present study showed a relatively high frequency of *Fas* gene mutations in cases with TGCT (37.5%).

In the present cases with TGCTs, all but one of the mutations were detected in exon 9, which encodes the death domain region of the Fas receptor,² but mutational hot spots were not found. None of the cases showed insertion of 1 bp (A) at nucleotide 1095 or a 1-bp deletion at nucleotide 597, which were frequently observed in lymphoid malignancies.¹⁹

Clones expressing Fas with missense mutations of the *Fas* gene were resistant to apoptosis induced by the anti-FAS antibody, indicating that the missense mutations found in TGCTs were loss-of-function mutations. As a result, cells with mutant *Fas* genes accumulate, which might provide a basis for development of TGCTs. Nuclear magnetic resonance spectroscopy revealed that the death domain was localized to residues 202 to 319 and the core protein to 210 to 305.²⁰ The region consisting of the 15 carboxy-terminal residues of Fas (residues 305 to 319) was reported to play a negative regulatory role for apoptosis.³ Indeed, cells transfected with the *Fas* gene with missense mutations in the core region of the death domain were resistant to apoptosis in the current series.

The frequency of mutation-positive clones was more than 75% in three cases (cases 10, 18, 22); these cases must have carried homozygous mutations. Another one case with a positive clone frequency of 67% showed LOH. Functions of Fas protein might be completely abolished in these four cases. The remaining five cases with positive clone frequencies ranging from 33 to 67% did not show LOH, and thus might carry heterozygous mutations. As Fas must trimerize for signal transduction, molecules bearing a mutation in the death domain might behave in a dominant-negative manner. Indeed, some patients with autoimmune lymphoproliferative disease, who show lymphadenopathy, hepatosplenomegaly, and hypergammaglobulinemia within the first 2 years of life, have been found to carry heterozygous mutations of the *Fas* gene.²¹

Using the laser capture microdissection method, we specified the mutations in each histological lesion of TGCT. Mutations of the *Fas* gene were detected in neoplastic lesions, but not in nonneoplastic or normal tissues. It is interesting that the mutations were found in the seminoma and embryonal carcinoma lesions, but not in the yolk sac tumor, choriocarcinomas, or immature teratomas. Especially, five of eight (62.5%) lesions with embryonal carcinoma showed mutations. The germ cells might progress directly to seminoma or to embryonal carcinoma.

Embryonal carcinoma cells are regarded as totipotent, and might progress along the extra-embryonic or trophoblastic route to form yolk sac tumors and choriocarcinomas, or along the embryonic route to form teratomas. Taken together, the present findings suggested that the loss-of-function mutations of the *Fas* gene might underlie the development and/or maintenance of TGCTs in the early stages of germ cell differentiation.

Fifteen of 24 cases (62.5%) had wild-type *Fas* gene. Further studies are required to determine whether alterations in downstream components of the same pathway of Fas-mediated apoptosis, such as FADD/MORT1,^{23,24} caspase 8,^{25,26} and FLICE-inhibitory proteins,²⁷ cause resistance to apoptosis in cases with the wild-type *Fas* gene.

In conclusion, the results of the present study suggested that *Fas* gene mutations play a role in the pathogenesis of TGCTs.

References

1. Suda T, Takahashi T, Goldstein P, Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993, 75:1169–1178
2. Nagata S: Apoptosis by death factor. *Cell* 1997, 88:355–365
3. Itoh N, Nagata S: A novel protein domain required for apoptosis. *J Biol Chem* 1993, 268:10932–10937
4. Watanabe-Fukunaga R, Brannan C, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S: The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J Immunol* 1992, 148:1274–1279
5. Leithäuser F, Dhein J, Mechttersheimer G, Koretz K, Bründlerlein S, Henne C, Schmidt A, Debatin KM, Krammer PH, Möller P: Constitutive and induced expression of APO-1, a new member of the new growth factor/tumor necrosis receptor superfamily, in normal and neoplastic cells. *Lab Invest* 1993, 69:415–429
6. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC: A role for CD95 ligand in preventing graft rejection. *Nature* 1995, 377:630–632
7. Lee J, Richburg JH, Younkin SC, Boekelheide K: The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 1997, 138:2081–2088
8. Pentikäinen V, Erkkilä K, Dunkel L: Fas regulates germ cell apoptosis in the human testis in vitro. *Am J Physiol* 1999, 276:E310–E316
9. Landowski TH, Qu N, Buyuksal I, Painter S, Dalton WS: Mutations in the Fas antigen in patients with multiple myeloma. *Blood* 1997, 90:4266–4270
10. Grønbaeck K, Straten PT, Ralfkiaer E, Ahrenkiel V, Andersen MK, Hansen NE, Zeuthen J, Hou-Jensen K, Guldberg P: Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. *Blood* 1998, 92:3018–3024
11. Nizas P, Champion AE, Fox M: Germ cell tumors of testis: prognostic factors and results. *Eur Urol* 1990, 18:242–247
12. Mostofi FK: Comparison of various clinical and pathological classifications of tumors of testes. *Semin Oncol* 1979, 18:26–30
13. Takayama H, Takakuwa T, Dong Z, Nonomura N, Okuyama A, Nagata S, Aozasa A: Fas gene mutations in prostatic intraepithelial neoplasia and concurrent carcinoma: analysis of laser capture microdissected specimens. *Lab Invest* 2001, 81:283–288
14. Huang QR, Morris D, Manollos N: Identification and characterization of polymorphisms in the promoter region of the human Apo-1/Fas (CD95) gene. *Mol Immunol* 1997, 34:577–582
15. Mizushima S, Nagata S: pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 1990, 18:5322
16. Itoh N, Yonehara S, Ishii M, Yonehara S, Mizushima M, Sameshima A, Hase A, Seto Y, Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991, 66:233–243

17. Lee SH, Shin MS, Park WS, Kim SY, Kim HS, Han JY, Park GY, Dong SM, Pi JH, Kim CH, Kim SH, Lee JY, Yoo NJ: Alterations of Fas (Apo/CD95) gene in non-small cell lung cancer. *Oncogene* 1999, 18:1754–1760
18. Lee SH, Shin MS, Park WS, Kim SY, Kim HS, Han JY, Park GY, Dong SM, Pi JH, Kim CH, Kim SH, Lee JY, Yoo NJ: Alterations of Fas (Apo/CD95) gene in transitional cell carcinoma of urinary bladder. *Cancer Res* 1999, 59:3068–3072
19. Takakuwa T, Dong Z, Takayama H, Matsuzuka F, Nagata S, Aozasa A: Frequent mutations of Fas gene in thyroid lymphoma. *Cancer Res* 2001, 61:1382–1385
20. Huang B, Eberstadt M, Olejniczak ET, Meadows RP, Fesik SW: NMR structure and mutagenesis of the Fas (Apo-1/CD95) death domain. *Nature* 1996, 384:638–641
21. Straus SE, Jaffe ES, Puck JM, Dale JK, Elkon KB, Rösen-Wolff A, Peters AM, Sneller MC, Hallahan CW, Wang J, Fischer RE, Jackson CM, Lin AY, Baumler C, Siegert E, Marx A, Vaishnaw AK, Grodzicky T, Fleisher TA, Lenardo MJ: The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. *Blood* 2001, 98: 194–200
22. Mostofi FK: Pathology of germ cell tumors of testis: a progress report. *Cancer* 1980, 45:1735–1754
23. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995, 81:505–512
24. Boldin MP, Goncharov TM, Goltsev Y, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor induced cell death. *Cell* 1996, 85:803–815
25. Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D: A novel protein that interacts with the death domain of Fas/Apo 1 contains a sequence motif related to the death domain. *J Biol Chem* 1995, 270:7795–7798
26. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996, 85:817–827
27. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J: Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997, 386: 517–521