Alterations of Fas (APO-1/CD95) Gene in Transitional Cell Carcinomas of Urinary Bladder

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Abstract

Fas (APO-1/CD95) is a cell-surface receptor involved in cell death signaling. The key role of the Fas system in negative growth regulation has been studied mostly within the immune system, and somatic mutations of Fas in cancer patients have been described solely in lymphoid-lineage malignancies. We analyzed somatic mutations and loss of heterozygosity of Fas gene in 43 transitional cell carcinomas of urinary bladder. Overall, 12 tumors (28%) were found to have Fas mutations, including 11 missense mutations and 1 frameshift mutation. Ten of the 12 mutations were located in the death domain known to be involved in the transduction of an apoptotic signal, and 8 of these 10 mutations showed an identical G to A transition at bp 993, indicating a potential hotspot in bladder cancers. Three of eight (38%) informative tumors carrying Fas mutations showed LOH at polymorphic sites in the promoter region. This is the first report on the Fas gene mutations in nonlymphoid malignancies, and our data suggest that alterations of the Fas gene might lead to the loss of its apoptotic function and contribute to the pathogenesis of some bladder cancers.

Introduction

The Fas-Fas ligand (FasL) system has been recognized as a major pathway for the induction of apoptosis in cells and tissues. Fas is a member of the death receptor subfamily of the tumor necrosis factor receptor superfamily. Fas has three cysteine-rich extracellular domains and an intracellular death domain essential for signaling. Ligation of Fas by either agonistic antibody or by its natural ligand transmits a “death signal” to the target cells, potentially triggering apoptosis. Fas is widely expressed in normal and neoplastic cells (3), but the expression of this protein does not necessarily predict susceptibility to killing (4–9). This can reflect the presence of inhibiting mechanisms of Fas-mediated apoptosis. Fas-mediated apoptosis can be blocked by several mechanisms, including the production of soluble Fas (4), the overexpression of inhibitory proteins in signal transduction pathways such as Fas-associated phosphatase-1 (5) and FLICE-inhibitory protein (6), and the mutation of the primary structure of Fas (8–15).

The consequences of the Fas gene mutations have been well demonstrated in germ-line mutation models of this gene (12–16). Mice bearing the Fas gene mutation (lpr) have an abnormality of mature T-cell deletion in the peripheral tissues, resulting in lymphadenopathy, splenomegaly, and systemic autoimmune disease (12). Germ-line mutations of the Fas gene in humans also result in ALPS, which is characterized by an increase in double-negative T-cells and profound lymphadenopathy (13–16), as observed in lpr mice. Most of the Fas mutations in ALPS, of which the majority were point mutations in the death domain, were heterozygous and showed a dominant-negative phenotype (13, 14). Interestingly, the lpr mice have been reported to have spontaneous development of plasmacytoid tumors (17), and some ALPS patients have been reported to have malignancies (14, 15), including multiple tumor development in one patient (14). Although it is not clear whether the tumors that occurred in ALPS patients arose as a result of Fas mutations, it is conceivable that Fas mutation might have influences on tumor development in these patients.

The key role of the Fas system in negative growth regulation has been studied mostly within the immune system, and the mutations of Fas gene in cancer patients have been described solely in lymphoid-lineage malignancies, including childhood T-cell lymphoblastic leukemias (8), adult T-cell leukemias (9), multiple myelomas (10), and non-Hodgkin’s lymphomas (11). Therefore, resistance against Fas-mediated apoptosis may lead to a longer survival of affected tumor cells and might contribute to tumorigenesis of these lymphoid-lineage malignancies.

There is mounting evidence that disruption of the Fas system is not uncommon in nonlymphoid malignancies as well (4, 7). To date, however, somatic mutations of the Fas gene, one of the possible mechanisms that mediate the disruption of Fas system, have not yet been reported in nonlymphoid malignancies, including TCC of urinary bladder. In addition, a previous LOH study has suggested that loss of one or more tumor suppressor genes at chromosome 10q24.1–24.3 may be involved in the development of bladder TCC (18). One of the candidate genes in this region is Fas, which is located at chromosome 10q24.1. In the present study, to characterize the potential apoptosis-resistant pathway of the Fas system in human bladder TCC, we analyzed a series of 43 bladder TCCs for the detection of somatic mutations of the Fas gene.

Materials and Methods

Tissue Samples. Paraffin-embedded tissues of human bladder TCC were obtained from 43 surgically treated patients. The tumors were graded according to criteria recommended by the WHO (19) and staged according to the Tumor-Node-Metastasis classification (20). They consisted of 1 T a , 2 TIS, 8 T 1 , 16 T 2 , 14 T 3 , and 2 T 4 tumors. grade 1 were 20, grade 2 were 15, and grade 3 were 15.

Microdissection. Malignant cells were selectively procured from H&E-stained sections using a 30G1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (21). We also microdissected infiltrating lymphocytes for corresponding normal DNA from the same slide in all cases. This microdissection technique used in

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4 The abbreviations used are: FasL, Fas ligand; ALP, autoimmune lymphoproliferative syndrome; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.
this study has been proven to be precise and effective for the procurement of tumor cells without normal cell contamination (21).

SSCP Analysis for Mutation and LOH. Genomic DNA from normal lymphocytes or tumor cells was amplified with primer pairs covering the entire coding region and parts of the promoter region of the Fas gene (Table 1). Oligonucleotide primers were designed with the program Oligo (National Biosciences, Plymouth, MN) using sequences obtained from GenBank (accession no. M67454). Each PCR reaction was performed under standard conditions in a 10-μl reaction mixture containing 1 μl of template DNA, 0.5 mM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM MgCl₂, 0.4 unit of Taq polymerase, 0.5 μCi of [³²P]dCTP (Amersham, Buckinghamshire, United Kingdom), and 1 μl of 10× 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 variable temperatures as described in Table 1, 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/l NaOH and were loaded onto 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 reamplified 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 polymorphisms at positions –1377 (promoter region), –670 (promoter region), 416 (exon 3), and 836 (exon 7) are located in the promoter region, 670 (promoter region), 416 (exon 3), and 836 (exon 7) are

<table>
<thead>
<tr>
<th>Primer(s) (site of PCR)</th>
<th>Sequence</th>
<th>Size of PCR product (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-F (promoter)</td>
<td>5′-ctcattcctattatcaccattcttt-3′</td>
<td>124</td>
<td>60</td>
</tr>
<tr>
<td>PA-R</td>
<td>5′-gttggctattcactatctt-3′</td>
<td>145</td>
<td>58</td>
</tr>
<tr>
<td>PB-R</td>
<td>5′-agccttgctatcaggtaggt-3′</td>
<td>171</td>
<td>61</td>
</tr>
<tr>
<td>1-F (exon 1)</td>
<td>5′-ccgagctcgctccgagt-3′</td>
<td>192</td>
<td>56</td>
</tr>
<tr>
<td>1-R</td>
<td>5′-actccctgctctccgagtaa-3′</td>
<td>249</td>
<td>53</td>
</tr>
<tr>
<td>2-F (exon 2)</td>
<td>5′-gttgctatcactatctt-3′</td>
<td>171</td>
<td>57</td>
</tr>
<tr>
<td>2-R</td>
<td>5′-actcctgcactctatgt-3′</td>
<td>228</td>
<td>55</td>
</tr>
<tr>
<td>3A-F (exon 3)</td>
<td>5′-gctcgctatcactatgt-3′</td>
<td>180</td>
<td>53</td>
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<tr>
<td>3B-R (exon 3)</td>
<td>5′-gttgctatcactatctt-3′</td>
<td>164</td>
<td>55</td>
</tr>
<tr>
<td>4-F (exon 4)</td>
<td>5′-ctcattcctattatcaccattcttt-3′</td>
<td>117</td>
<td>53</td>
</tr>
<tr>
<td>4-R</td>
<td>5′-ctcaaacagctcgagctgtt-3′</td>
<td>175</td>
<td>49</td>
</tr>
<tr>
<td>5-F (exon 5)</td>
<td>5′-ccgagctaattcttcaccacg-3′</td>
<td>191</td>
<td>50</td>
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</table>

F, forward primer; R, reverse primer.

Results

Through the microdissection technique, we successfully procured tumor cells from paraffin-embedded histological sections of 43 TCCs, as shown in Fig. 1. 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 12 of the samples (28%; Fig. 2, A–C; Table 2). None of the normal samples showed evidence of mutations by SSCP (Fig. 2, A–C), indicating that the mutations detected in the TCC specimens had risen somatically.

Fas Gene Mutations. The majority (11 of 12) of the mutations were missense variants (Fig. 2, D and F; Table 2). Nine of the missense mutations were detected in exon 9, which encodes the death domain region of Fas (2). Eight of the nine missense mutations in exon 9 showed an identical G to A transition at bp 993 (cases 15, 20, 24, 32, 33, 38, 40, and 42; Table 2; Fig. 2D) in unrelated patients, and this mutation would result in the substitution of Val to Ile at codon 251. The remaining missense mutation within the death domain affected codon 237 (Table 2). In exon 6, which encodes the transmembrane domain of membrane-bound Fas (1, 2), two missense mutations were identified (Fig. 2F; Table 2), and these two mutations were an identical T to C transition at bp 726 and affected codon 162. One nonmissense mutation (case 18) was a 1-bp insertion (Fig. 2E), causing a frameshift and the introduction of a stop codon at residue 245.

Because mutation patterns in exon 9 were identical in eight cases (Table 2), we repeated 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).

As for the relationship between the tumor stage and the Fas gene mutations, all mutations except one case (case 23) were identified in muscle-invasive TCC (T2), but the correlation was not statistically significant (Fisher’s exact test, two-tailed; P > 0.05).

Allelic Status. Because missense mutations in the death domain of Fas in patients with ALPS have been suggested to affect receptor function in a dominant-negative fashion (13, 14), we examined the allelic status of Fas in tumors carrying missense mutations. In TCCs analyzed, heterozygosity rates of the two polymorphic markers in exons 3 and 7 (primers 3A and 7) were too low for LOH study, whereas 26 of 43 (61%) cases showed heterozygosity, with one or both of the two polymorphic markers in the promoter region (primers PA and PB). Among the 12 cases with the Fas mutations, 8 were heterozygous for one or more polymorphism(s), and 3 of them (38%) showed evidence of allelic loss (Table 2; Fig. 2G). In cases with no Fas mutations, 5 of 18 (28%) informative TCCs showed evidence of allelic loss.

Fas and FasL Expression. We demonstrated Fas and FasL expressions in the TCCs by immunohistochemistry. The TCCs showed immunoreactivity for Fas in 41 of 43 cases (95%). The two Fas-negative TCCs were a grade 2/stage T2 tumor and a grade 3/stage T3 tumor. FasL was expressed in all TCCs analyzed (100%). Fas and FasL immunostainings, when present, were cytoplasmic and along the cell membranes; nuclei were clearly negative (data not shown). All TCCs with the Fas gene mutations coexpressed Fas and FasL (Table 2). Immunoreactivity (Fas and FasL), grade, and stage of the TCCs with Fas mutations or allelic deletions are summarized in Table 2.

Discussion

The aim of this study was to detect Fas gene alterations, one of the possible mechanisms that may mediate Fas resistance in bladder TCC in vivo. Although we do not actually know whether TCC is resistant to Fas-mediated apoptosis in vivo, there have been some data that support the idea that TCC may be resistant to Fas-mediated apoptosis. For example, some TCC cell lines were reported to have resistance to Fas-mediated apoptosis, despite expressing Fas (7). In addition, most of the human TCCs analyzed in the present study (95%), expressed both Fas and FasL, suggesting that these tumors may be resistant to Fas-mediated apoptosis in vivo to escape autocrine suicide.

Previous identification of frequent LOH at chromosome 10q in TCC has suggested the presence of putative tumor suppressor genes in this area (18). Recently, Cairns et al. (25) examined PTEN/MMAC1, a tumor suppressor gene mapping to 10q23.3, and found that this gene is occasionally mutated in primary TCCs. In the present study, we have systematically examined the Fas gene on 10q24.1 and documented somatic mutations in 12 of 43 (28%) TCCs. Furthermore, loss of the remaining allele in three of eight informative cases with Fas mutation was also identified (Table 2). Therefore, the presence of Fas gene mutations suggested that this gene, together with PTEN/MMAC1, is likely to be one of the candidate tumor suppressor genes relevant to TCC.

Although functional studies have not yet been performed, part of the mutations identified in the present study are likely to disrupt or alter the normal function of Fas. One of the mutations (case 18) is predicted to cause premature termination of protein synthesis, and
Table 2 Mutations and LOH of Fas gene in bladder TCCs

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Grade and stage</th>
<th>Immunohistochemistry</th>
<th>LOH analysis*</th>
<th>Codon</th>
<th>Mutation site</th>
<th>Nucleotide change</th>
<th>Predictive effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>II, T3a</td>
<td>Positive Positive</td>
<td>PA PB 3</td>
<td>7</td>
<td>ENOX 6</td>
<td>726T to C</td>
<td>Cys--&gt;Arg</td>
</tr>
<tr>
<td>28</td>
<td>III, T3a</td>
<td>Positive Positive</td>
<td>NI LOH NI</td>
<td>162</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>15</td>
<td>II, T3a</td>
<td>Positive Positive</td>
<td>--</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>20</td>
<td>III, T3a</td>
<td>Positive Positive</td>
<td>--</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>24</td>
<td>II, T3a</td>
<td>Positive Positive</td>
<td>--</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>32</td>
<td>II, T3b</td>
<td>Positive Positive</td>
<td>--</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>33</td>
<td>III, T3b</td>
<td>Positive Positive</td>
<td>--</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
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<tr>
<td>38</td>
<td>III, T2b</td>
<td>Positive Positive</td>
<td>LOH --</td>
<td>251</td>
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<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>40</td>
<td>II, T3b</td>
<td>Positive Positive</td>
<td>LOH LOH</td>
<td>251</td>
<td>Exon 9</td>
<td>993G to A</td>
<td>Val--ile</td>
</tr>
<tr>
<td>42</td>
<td>II, T3a</td>
<td>Positive Positive</td>
<td>LOH LOH</td>
<td>237</td>
<td>Exon 9</td>
<td>950T to G</td>
<td>Asp--&gt;lys</td>
</tr>
<tr>
<td>43</td>
<td>III, T3a</td>
<td>Positive Positive</td>
<td>LOH LOH NI</td>
<td>244</td>
<td>Exon 9</td>
<td>973 insertion C</td>
<td>Stop at codon 245</td>
</tr>
</tbody>
</table>

* NI, not informative (homozygosity); –, retention of heterozygosity.

hence resemble typical loss-of-function mutations. The remaining 11 mutations were missense variants and were detected within the region encoding the death domain and the transmembrane domain of Fas. The death domain is evolutionarily highly conserved and is shown to be necessary and sufficient for the transduction of an apoptotic signal (1, 2). Thus, nine missense mutations identified in this conserved area might disrupt death signaling. Two mutations were identified in the region encoding the transmembrane domain, but the functional significance of this mutation remains unknown at this stage.

In multiple myeloma, Landowski et al. (10) identified an identical point mutation in two different patients, which generates an amino acid substitution at 253. Also, mutations of codon 248 and 256 of Fas were identified in non-Hodgkin’s lymphomas (11). Furthermore, two ALPS patients were reported to have 2-bp deletion that generates an unrelated amino acid sequence beginning at residue 254 (16). In bladder TCC, eight missense mutations, which would generate amino acid substitution at residues 251 (Table 2), are adjacent to the Fas mutations described above. Furthermore, the eight missense mutations were an identical mutation that occurred in unrelated patients, suggesting that this position may represent a mutational hotspot in the Fas coding sequence.

Most of the patients with ALPS carry a heterozygous mutation in the Fas gene (13–16). 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 (13–16). In TCC, five Fas mutations were consistently associated with retention of the wild-type allele (Table 2). This finding is in line with the previous observation in ALPS. In contrast, some ALPS patients have been reported to be associated with the ALPS phenotype only in the lymphoid system (12–16). In bladder TCC, three cases with mutations also showed biallelic alteration patterns of Fas, i.e., missense mutation of one allele and loss of the other allele. The functional difference of two mutation patterns in the pathogenesis of TCC remains to be clarified.

Several lines of evidence suggest that the loss of Fas function can enhance lymphoid tumor development. For example, lymphomato genesis driven by the Eμ-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 (26). Spontaneous development of B-cell lymphoid tumors in lpr mice also indicated that Fas gene mutation plays a key role in tumorigenesis (17). Moreover, somatic Fas gene mutations in human cancers have been found exclusively in lymphoid malignancies (8–11). These are well correlated with the facts that ALPS patients and lpr mice have shown phenotypical abnormalities only in the lymphoid system (12–16). However, we were able to find Fas gene mutations in bladder TCC, one of nonlymphoid malignancies, which can extend the concept of loss of Fas function to the pathogenesis of nonlymphoid malignancies as well, and it is possible that Fas gene mutations may occur widely in nonlymphoid malignancies. Clearly, therefore, studies are now needed that attempt to find the potential Fas gene mutations in other nonlymphoid malignancies.

References


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