Altersations of the DR5/TRAIL Receptor 2 Gene in Non-Small Cell Lung Cancers

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Abstract

Chromosome 8p21-22 is a frequent site of allelic losses in many types of human tumors, including non-small cell lung cancer (NSCLC). Tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (TRAIL-R2) is a cell-surface receptor involved in cell death signaling. The TRAIL-R2 gene recently has been mapped to chromosome 8p21-22. To explore the possibility that the TRAIL-R2 gene might be the relevant gene to the frequent deletion of 8p21-22 in NSCLC, we have analyzed the entire coding region and all splice sites of 104 NSCLCs. Overall, 11 tumors (10.6%) were found to have TRAIL-R2 gene mutations in the death domain known to be involved in the transduction of an apoptotic signal. Our data indicate that somatic mutation of TRAIL-R2 may play a role in the pathogenesis of some NSCLCs and that the TRAIL-R2 gene is one of the genes relevant to the frequent loss of chromosome 8p21-22 in NSCLC.

Introduction

Apoptosis is a fundamental biochemical cell-death pathway essential for normal tissue homeostasis, cellular differentiation, and development (1). Several members of the TNF family such as TNF-α, Fas ligand, and TRAIL have been shown to induce apoptosis in susceptible cells (1-4). TRAIL can induce apoptosis by interaction with two receptors, referred to as TRAIL-R1 and TRAIL-R2 (2-4). These receptors were found to be widely expressed in normal and neoplastic cells (2-10), but the expression of these proteins does not necessarily predict susceptibility to killing (10). This can reflect the presence of inhibiting mechanisms of TRAIL-induced apoptosis. TRAIL-induced apoptosis can be blocked by several mechanisms, including the expression of decoy receptors for TRAIL (10), the loss of TRAIL receptor expression (5), the overexpression of inhibitory proteins in signal transduction pathways such as FLICE-inhibitory protein (7), and the mutation of the primary structure of TRAIL-R2 (11). Blockade of TRAIL-induced apoptosis may lead to the loss of its apoptotic function and prolong the survival of affected cells.

Dysregulation of normal apoptotic mechanisms provides a growth advantage to cancer cells (1). The apoptotic pathway of Fas, one of the TNF receptor family members, is frequently blocked by several mechanisms in cancer cells, one of which is Fas gene mutation (12, 13). Interestingly, Fas mutations have been detected in several types of human cancers with frequent allelic losses of chromosome 10q24 where this gene resides (12, 13). Therefore, it can be hypothesized that other members of TNF receptor family are mutated in the cancers with frequent allelic losses at the chromosome where each TNF receptor family gene resides.

The TRAIL-R2 gene was mapped to chromosome 8p21-22 (3). Allelic losses of the chromosome 8p21-22 have been reported as a frequent event in several cancers, including lung cancer, breast cancer, colon cancer, prostate cancer, hepatocellular carcinoma, and head and neck cancer (14-19). These data strongly indicate that chromosome 8p21-22 may harbor one or more tumor suppressor genes and suggest that the TRAIL-R2 gene might be one of the candidate tumor suppressor genes in this region. To date, mutations of the TRAIL-R2 gene have been reported in head and neck cancers (11). In NSCLC, frequent allelic losses (~50%) involving the 8p21-22 region have been detected by polymorphic markers (14, 15). However, the mutation of candidate tumor suppressor genes at chromosome 8p21-22 has not yet been reported in NSCLC.

In the present study, to explore the possibility that the TRAIL-R2 gene is the gene relevant to the frequent allelic losses at chromosome 8p21-22 in NSCLC, we analyzed a series of 104 NSCLCs for the detection of somatic mutations of the TRAIL-R2 gene.

Materials and Methods

Tissue Samples. Formalin-fixed and paraffin-embedded archival tissues of human NSCLCs were obtained from 104 surgically treated patients. Histopathological examination of these NSCLCs revealed that these tumors included 49 squamous cell carcinomas and 55 adenocarcinomas. The clinicopathological stage was determined after surgery using standard criteria. The cancers consisted of 30 stage I, 36 stage II, 34 stage III, and 4 stage IV NSCLCs.

Microdissection and DNA Extraction. Malignant cells were selectively procured from H&E-stained slides without any contamination by normal cells, using a 30G12 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (20). We also microdissected infiltrating lymphocytes for corresponding normal DNA from the same slide in all cases. DNA extraction was performed by a modified single-step DNA extraction method, as described previously (20).

SSCP Analysis for Mutation and LOH. Genomic DNA from normal lymphocytes or tumor cells was amplified with 15 primer pairs covering the entire coding region (nine exons) of the TRAIL-R2 gene, which were the same primers described by Ariai et al. (21). Numbering of the cDNA of TRAIL-R2 was done in respect to the ATG start codon according to Ariai et al. (GenBank accession no. AB014710-8; Ref. 21). Each PCR reaction was performed under standard conditions in a 10-μl reaction mixture containing 1 μl of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM MgCl2, 0.4 units of Taq polymerase, 0.5 μCi of [32P]dCTP (Amer sham, Buckinghamshire, United Kingdom), and 1 μl of 10× buffer. The reaction mixture was denatured for 1 min at 94°C and incubated for 30 cycles (denaturing for 40 s at 94°C, annealing for 40 s at 59–68°C, and extension 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 in a 1:1 dilution of sample buffer containing 98% formamide-5 mM NaOH and were loaded onto
a SSCP gel (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT) with 10% glycerol. Samples were electrophoresed at 8 W at room temperature overnight. 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 30 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 biallelic polymorphisms are located in the TRAIL-R2 gene at nucleotide positions 95 T/C (exon 1), 200 T/C (exon 2), 572 C/T (exon 5), and 662 C/T (exon 5; Ref. 21), 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 the LOH study were the same as 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.

Results

Mutations. Through the microdissection technique, we selectively procured tumor cells from histological sections of 104 NSCLCs (Fig. 1). Genomic DNA was isolated and analyzed for potential mutations in all nine exons and splice sites of the TRAIL-R2 gene by PCR-SSCP analysis. Enrichment and direct sequence analysis of aberrantly migrating bands led to the identification of mutations in 11 of the samples (10.6%; Table 1; Fig. 2). The majority of the mutations (10 of 11) were detected in exon 9, which encodes the death domain region of TRAIL-R2 (21), and the remaining mutation was detected in intron 8. The mutations consisted of eight missense mutations, one nonsense mutation, one splice-site mutation, and one silent mutation (Table 1). None of the corresponding normal samples showed evidence of mutations by SSCP (Fig. 2, A and B), indicating that the mutations detected in the NSCLC specimens had arisen somatically.

Three of the eight missense mutations (cases 24, 44, and 55) showed an identical C-to-T transition at bp 1087 in unrelated patients, and this mutation would result in the substitution of Leu to Phe at codon 363 (Table 1). The remaining missense mutations affected codons 353, 355, 367, 415, and 436. The nonsense mutation showed a point mutation introducing premature termination signal at codon 354 (Fig. 2, A and C). The splice-site mutation affected position −1 of the consensus sequence of the acceptor splice site of intron 8. 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).

As for the relationship between the histological types and the TRAIL-R2 gene mutations, 8 of the 11 mutations (73%) were detected in adenocarcinomas, but the relationship was not statistically significant (Fisher’s exact test, two tails, P > 0.05). The 11 mutations detected were 3 stage I, 4 stage II, and 4 stage III tumors (Table 1).

Allelic Status. For LOH analysis, 12, 35, 3, and 1 NSCLC cases were informative with regard to the polymorphic loci in nucleotide positions 95, 200, 572, and 662, respectively. Overall, 48 of 104 cases (46%) were informative for at least one of the four polymorphisms, and 18 of 48 (37%) informative cases showed LOH with one or more polymorphic loci. Four of 12 (33%), 12 of 34 (35%), 2 of 3 (67%), and 0 of 1 (0%) informative cases showed LOH with the polymorphisms at nucleotides 95, 200, 572, and 662, respectively.

Among the 11 cases with the TRAIL-R2 mutations, 5 were heterozygous for one of the polymorphisms, and 2 of them (40%) showed evidence of allelic loss (Table 1). Interestingly, in 2 of the 11 mutations (cases 24 and 42), SSCP patterns at the mutation sites (exon 9) showed only aberrant bands of the mutant allele without those of the wild-type allele (Fig. 2B), and sequencing analysis also revealed only mutation sequences without wild-type ones, indicating either a homozygous mutation or a hemizygous mutation with allelic loss (Fig. 2D). In contrast, the other nine cases showed that the bands of the wild-type allele were present together with aberrant bands of the mutant allele (Fig. 2A). The bands of the wild-type alleles in the latter eight cases could result from contamination by normal tissue, but we used a micrometrically precise, microdissection technique using a micromanipulator (Fig. 1; Ref. 20), and all LOH findings in the present study showed almost complete absence of signals in deleted alleles of tumor DNA (Fig. 2E), indicating that the microdissected tumor samples were nearly devoid of contamination by normal tissue. Therefore, the SSCP analysis of the four intragenic polymorphisms, and the SSCP patterns at mutation sites and sequencing analysis suggest that the second allele had also been altered in 4 of 11 patients carrying TRAIL-R2 mutations and that the wild-type alleles were retained in the other 7 cases. Sixteen of 43 (37%) informative cases in which no TRAIL-R2 mutations had been detected showed evidence of allelic loss.

Discussion

TRAIL harbors potential as a cancer therapeutic agent because it has been known to efficiently kill many tumor cell lines but not normal cells (7, 8, 10). In vivo, systemically administered TRAIL is not only tumoricidal in mice but is also nontoxic (22, 23). However, because approximately one-third of tumor cell lines tested to date have been reported to be resistant to TRAIL-induced apoptosis (5–9), studies on the underlying mechanisms of the TRAIL resistance in cancer has been required. TRAIL resistance is known to be mediated

![Fig. 1. Microdissection of adenocarcinoma of the lung. A, malignant cells are arranged in irregularly shaped glands in the lung tissue. The needle tip (arrow) is attached to a tumor cell gland. B, tumor cell glands were dissected, leaving large holes behind. Original magnification, ×100.](image-url)
by several mechanisms (5, 7, 10, 11). The level of FLICE-inhibitory protein expression was reported to be correlated with the TRAIL resistance in some cancer cells (7). A recent report also indicated that the loss of TRAIL-R2 protein expression and the shift of cell-surface TRAIL-R2 expression to the cytoplasm are related to TRAIL resistance in cancer cells (5). The decoy receptor for TRAIL had been considered a main regulator of TRAIL resistance, but it is not likely to play an important role in this resistance because no correlation between TRAIL resistance and expression of the decoy receptor has been identified (2, 5, 7). In this study, we have systemically examined the TRAIL-R2 gene and documented somatic mutations in 11 of 104 NSCLCs (10.6%). These findings suggest that TRAIL-R2 mutations may be one of the important mechanisms underlying the TRAIL resistance in NSCLCs.

Although functional studies have not yet been performed, some of the mutations identified in the present study are likely to disrupt or alter the normal function of TRAIL-R2. One of the mutations (case 5) is predicted to cause premature termination of protein synthesis, and hence resembles typical loss-of-function mutations. Mutations at positions 1–1 of the consensus sequence of the acceptor site of an intron have been shown to cause cryptic splice site utilization and exon skipping in various human disease genes (24). Thus, it is possible that the splice-site mutation observed in intron 8 may result in abnormal splicing of TRAIL-R2 mRNA. To date, TRAIL-R2 gene mutations have been reported in two cases of head and neck cancer, and these mutations were detected in exon 9, which encodes for death domain (11). The death domain is evolutionarily highly conserved and is shown to be necessary for the transduction of an apoptotic signal (1–4). In the current study, all 10 TRAIL-R2 mutations in the coding sequences were identified in this conserved area, suggesting that the mutations might disrupt TRAIL-induced death signaling.

Binding of TRAIL to TRAIL-R2 induces trimerization of TRAIL-R2, and FADD/MORT-1 binds to the trimerized TRAIL-R2 cytoplasmic region (death domain; Ref. 4). FADD/MORT-1 then acts as an adapter molecule by recruiting caspase 8 and caspase 10, which initiate a proteolytic cascade involving other caspases that eventually lead to cell death by apoptosis (4). In our study, seven TRAIL-R2 gene mutations seemed to be hemizygous mutations without allelic deletion (Table 1). Therefore, in these cases it is possible that the hemizygously mutated TRAIL-R2 protein(s) may bind with other normal TRAIL-R2 protein(s) to construct a structurally abnormal TRAIL-R2-trimer, which might have a defect in binding to the adapter protein (dominant negative mutation). In contrast, cases 40 and 44 showed LOH at one of the intragenic polymorphic sites (Table 1), and cases 24 and 42 showed only aberrant bands of the mutant allele without those of the wild-type allele on SSCP of exon 9 (Fig. 2B), indicating potential biallelic inactivation of the TRAIL-R2 gene in these cases. This biallelic inactivation of the TRAIL-R2 gene may also lead to abnormal construction of TRAIL-R2-trimer, but the functional difference between monoallelic and biallelic inactivation of TRAIL-R2 gene alterations in the tumorigenesis of NSCLC remains unknown at this stage.

Previous LOH and karyotypic studies have suggested that a putative tumor suppressor gene at chromosome 8p21-22 may be involved in the development of NSCLC (14, 15). Several candidate tumor suppressor genes of this region have been identified (25, 26). The platelet-derived growth factor-receptor-B-like tumor suppressor gene has been isolated from a region commonly deleted in NSCLCs and

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**Table 1** Mutation and LOH of the TRAIL-R2 gene in NSCLCs

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Histological type</th>
<th>Stage</th>
<th>LOH analysis</th>
<th>SSCP patterns at mutation sites</th>
<th>Mutation sites</th>
<th>Nucleotide change (predicted amino acid change)</th>
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<td>5</td>
<td>Squamous</td>
<td>III</td>
<td>NI</td>
<td>NI</td>
<td>M+W</td>
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<td>NI</td>
<td>M+W</td>
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</tr>
<tr>
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<td>NI</td>
<td>NI</td>
<td>M</td>
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<tr>
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<td>II</td>
<td>NI</td>
<td>NI</td>
<td>M+W</td>
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<tr>
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<td>42</td>
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<td>NI</td>
<td>M</td>
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<td>LOH</td>
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<td>M+W</td>
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<td>NI</td>
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<td>NI</td>
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<td>M+W</td>
<td>Exon 9 (1058 C to T: 355 Ser to Phe)</td>
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<tr>
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<td>III</td>
<td>NI</td>
<td>NI</td>
<td>M+W</td>
<td>Exon 9 (1020 G to A: no change)</td>
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</tbody>
</table>

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**Fig. 2** Mutations and deletions of the TRAIL-R2 gene in NSCLCs. SSCP (A, B, and E) and sequencing analysis (C and D) of DNA from tumors (Lane T) and normal tissues (Lane N). A, part of exon 9 was amplified. SSCP of DNA from tumor (T) of case 5 shows wild-type bands and additional aberrant bands (arrow) when compared with SSCP from normal lymphocytes (N). B, part of exon 9 was amplified. SSCP of DNA from tumor (T) of case 42 shows only two aberrant bands (arrow) without any wild-type bands when compared with SSCP from normal lymphocytes (N). C, sequencing analysis from aberrant band of case 5. There is a G-to-A transition at nucleotide 1062 (arrow) in tumor tissue compared with normal tissue. D, sequencing analysis from aberrant band of case 42. There is a G-to-A transition at nucleotide 1063 (arrow) in tumor tissue compared with normal tissue. E, detection of allelic loss by amplification of a region encompassing the biallelic polymorphism at nucleotide 95 in exon 1. Representative SSCP show "not informative" (left), "retention of heterozygosity" (middle), and LOH (right).
hepatocellular and colorectal carcinomas (25). However, the screening of 107 tumors, including 31 NSCLCs, led to the identification of a single truncating mutation in a colon cancer (25). Mutation of the N33 gene, another candidate gene in this region, has not yet been reported in human cancers (26). Whether TRAIL-R2 is such a tumor suppressor is unknown at this time, but its function as a death receptor would be consistent with the tumor suppressor function. Therefore, the demonstration of TRAIL-R2 gene mutations in NSCLCs in this study suggest that TRAIL-R2 may be one of the tumor suppressor genes at chromosome 8p21-22 and that TRAIL-R2 gene mutations are involved in the development of NSCLCs. Furthermore, TRAIL-R1, another member of the TNF receptor family, was also found to be located at chromosome 8p21 (3). TRAIL-R1 exhibits a similar expression pattern and high sequence homology to TRAIL-R2 (3). Because TRAIL-R1 also behaves as a death receptor and has a signal transduction pathway similar to that of TRAIL-R2 (3, 4), TRAIL-R1 might be another candidate gene in the chromosome 8p21 region. Our study showed that approximately one-third of the NSCLCs showed LOH without any TRAIL-R2 mutations, indicating that alterations of other genes on chromosome 8p in addition to TRAIL-R2 may be also involved in the pathogenesis of NSCLCs. Clearly therefore, studies are now needed that attempt to find another gene alterations at this region, including TRAIL-R1.

In summary, we have studied the mutation of the TRAIL-R2 gene at 8p21-22 in NSCLCs and have found somatic mutations of this gene in 10.6% of the NSCLCs. Although functional studies have not been performed, it is possible that the affected cancer cells might have longer life span through the inactivation of TRAIL-induced apoptosis. Because many other cancers are known to have frequent LOH in this area, our results together with the earlier report of TRAIL-R2 mutations in head and neck cancers suggest the possibilities that the TRAIL-R2 gene mutation might be involved in the pathogenesis of many other human cancers.

References

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