Mutations of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptor 1 (TRAIL-R1) and Receptor 2 (TRAIL-R2) Genes in Metastatic Breast Cancers

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Abstract

Several lines of evidence suggest that apoptosis dysregulation plays an important role in cancer metastasis. In this study, to explore the possibility that the mutations of death receptors are involved in the metastasis mechanism, we analyzed the death domains of Fas and tumor necrosis factor-related apoptosis-inducing ligand receptor 1 and -2 (TRAIL-R1 and -R2) genes for the detection of somatic mutations in 57 breast cancers with (n = 34) or without (n = 23) metastasis to the regional lymph nodes. We found seven mutations (three TRAIL-R1 and four TRAIL-R2 mutations), and these mutations were detected only in the breast cancers with metastasis. Furthermore, we also analyzed the allelic losses of chromosome 8p21–22, where TRAIL-R1 and R2 reside in the same series of breast cancers, and found that the allelic losses were significantly higher in metastatic breast cancers. We expressed the tumor-derived TRAIL-R1 and TRAIL-R2 mutants in 293 cells and found that apoptosis was suppressed. These data suggest that TRAIL-R1 and R2 genes are relevant to the frequent loss of chromosome 8p21–22 in breast cancer and that the inactivating mutations of TRAIL-R1 and -R2 genes play a role in the metastasis of breast cancer.

Introduction

It is now believed that clonal expansion and tumor growth is the result of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis; Ref. 1). Aberrant cell survival resulting from the inhibition of apoptosis is expected to contribute to the development and progression of cancers, and cancer cells would gain a selective growth advantage by blocking the apoptosis (1). Metastasis is a highly complex process involving the survival of cancer cells, both in the blood stream and within specific organs. Recent findings showed that metastatic cancer cells exhibit a higher resistance to apoptosis compared with their poorly-metastatic counterparts (2), which indicates that metastatic cancer cells may have many more dysregulations in the apoptosis pathway. However, there have been few experimental data linking apoptosis and metastasis (3, 4).

Death factor/death receptor-induced cell death is one of the main apoptosis pathways (1). Death receptors such as Fas, TRAIL-R1 and -R2 transmit a death signal on binding with their natural ligands (death factors; Refs. 1, 5–7). However, many types of cancer cells are resistant to death factor-induced apoptosis (1). Fas ligand and TRAIL-induced apoptosis can be blocked by several mechanisms in cancer cells, one of which is the mutation of the primary structures of Fas and TRAIL-R2 (8–10). Interestingly, Fas and TRAIL-R2 mutations have been detected in several types of human cancers with frequent allelic losses of chromosomes 10q24 and 8p21–22, where Fas and TRAIL-R2 reside, respectively (8–10). These data indicate that chromosomes 10q24 and 8p21–22 may harbor one or more tumor suppressor genes and suggest that Fas and TRAIL-R2 might be the candidate tumor suppressor genes in these loci. Furthermore, because TRAIL-R1 gene also exists in chromosome 8p21–22, it might be another candidate tumor suppressor gene in chromosome 8p21–22 (11). Breast cancer has also shown frequent allelic losses of 10q24 and 8p21–22 (12, 13), but the mutational studies of Fas, TRAIL-R1, and TRAIL-R2 genes in breast cancer have not yet been reported.

There is evidence that death receptor mutation is involved in the pathogenesis of human cancer metastasis (4). In one adult T-cell leukemia patient, Fas gene mutation was detected in metastatic cancer cells but not in primary cancers, and the metastatic cancer cells were resistant to agonistic Fas antibody treatment, but the primary cancer cells were not (4). Therefore, it can be hypothesized that other members of the death receptor are mutated in some cancers and such mutations might be involved in the metastasis mechanisms of the cancers. Also, there are reports that allelic losses of chromosomes 10q24 and 8p21–22 of breast cancer occur during the progression of the tumors (12, 14, 15).

In the present study, to explore the possibilities that Fas, TRAIL-R1, and TRAIL-R2 genes are relevant to the frequent allelic losses at chromosomes 10q24 and 8p21–22 in breast cancers and that these potential mutations show different incidences between nonmetastatic and metastatic breast cancers, we analyzed a series of 57 IDCs of the breast for the detection of genetic alteration of the death domains of Fas, TRAIL-R1, and TRAIL-R2 genes.

Materials and Methods

Tissue Samples. Formalin-fixed and paraffin-embedded tissues of human IDC of breast with (n = 34) or without (n = 23) regional lymph node metastasis were obtained from 57 surgically treated patients. The stage was determined according to the American Joint Committee on Cancer (16). The cases with regional lymph node metastasis consisted of 9 stage IIA, 19 stage IIB, 4 stage IIIA, and 2 stage IIB IDCs, and the cases without regional lymph node metastasis consisted of 11 stage I, 10 stage IIA, and 2 stage IIB IDCs.

Microdissection and DNA Extraction. Malignant cells were selectively procured from H&E-stained slides without any normal cell contamination using a 30-gauge ½-inch hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (17). We also microdissected infiltrating lymphocytes for corresponding normal DNA from the same slide in all of the cases. DNA extraction was performed by a modified single-step DNA extraction method, as described previously (17).

SSCP Analysis. Genomic DNA, each from tumor cells or corresponding normal cells was amplified with 3 primer pairs for the Fas gene, 2 primer pairs...
for the TRAIL-R1 gene, and 3 primer pairs for the TRAIL-R2 gene covering the death domain of each gene. The primers for the TRAIL-R1 death domain were designed with the program Oligo (National Biosciences, Plymouth, MN) using sequences obtained from GenBank (accession no. U90875) and the primer sequences were as follows: 5‘-ACCCCACTGAGACTCTAGTCTGTG-3’ and 5‘-CGTGGTTTTGTTAGCCATTTCC-3’ for exon 9A; and 5‘-AATGGAGATCTGTGGTACAGACGC3’ and 5‘-ACCTTCCAGTCCACCAAGCGG-3’ for exon 9B. The primers for the TRAIL-R2 death domain and the Fas death domain were the same primers described by Arai et al. (11) and Lee et al. (9), respectively. Numbering of cDNA of the TRAIL-R1 and TRAIL-R2 codon (GenBank accession no. AB014710-8) was done in respect to the ATG start codon (5, 6). Each PCR reaction was performed under standard conditions in a 10-μl reaction mixture containing 1 μl of template DNA, 0.4 μM each primer, 0.2 mM 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 30 cycles (denaturing for 40 s at 94°C, annealing for 40 s at 59–68°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 for 5 min at 95°C at a 1:1 dilution of sample buffer containing 98% formamide/5 mM NaOH and were loaded onto an SSCP gel (FM C 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 subjected to autoradiography using Kodak X-OMAT film. Complete or partial tandem repeats were carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA) according to the manufacturer’s recommendation.

**LOH Analysis.** Because one biallelic polymorphism at nucleotide position 1322 A/G of the TRAIL-R1 gene (18), three at nucleotide positions 95 T/C (exon 1), 200 T/C (exon 2) and 662 C/T (exon 5) of the TRAIL-R2 gene (11), and two at nucleotide positions −1377 (promoter region) and −670 (promoter region) of the Fas gene are known (10), SSCP analysis at these polymorphic sites was used for the detection of LOH of each gene. The PCR and SSCP conditions of the LOH study were the same as the conditions described above.

LOH analysis of the TRAIL-R1 and TRAIL-R2 genes was also performed using microsatellite markers (D8S258, D8S261, and D8S560) at chromosome 8p21–22. After PCR with these markers, reaction products were then denatured and electrophoresed in 6% polyacrylamide gels containing 7M urea. After electrophoresis, the gels were transferred to 3 MM Whatman paper, dried, and subjected to autoradiography with Kodak X-OMAT film. Complete or nearly complete absence of one allele in tumor DNA of informative cases, as defined by direct visualization, was considered as LOH.

**Site-directed Mutagenesis.** Site-directed mutagenesis was performed using a 5′-to-3′ exchange side-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. To change a base, plasmids that contained either the TRAIL-R1 or TRAIL-R2 gene in pcDNA3.1-HA (Invitrogen, Carlsbad, CA) were used as a template. The sequences of the mutagenized plasmids were confirmed.

**Cell Culture, Transfection, and Apoptosis Assay.** 293 cells were maintained in EMEM (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 1 mM l-glutamine, and antibiotics. 293 cells (10⁶) were plated per well in two-chamber slide and transfected by the Superfect transfection reagent (Qiagen, Valencia, CA) with 1.3 μg of the wild type or mutant gene construct with 0.2 μg of GFP marker plasmid pEGFP (CLONTEC, Palo Alto, CA). Cultured cells were recovered 48 h after transfection, and the percentage of GFP-positive cells with nuclear apoptotic morphology was determined by fixing in 10% methanol for 15 min and staining with 0.1 μg/ml DAPI for 15 min (mean ± SD; n = 4; Fig. 3A).

After 24 h of transfection, cell lysates were prepared from each transfectant followed by Western immunostaining for confirmation of expression.

**Results**

**Mutational Analysis of the Death Domains of Fas, TRAIL-R1, and TRAIL-R2.** Through the microdissection technique, we selectively procured tumor cells from the histological sections of 57 IDCs (Fig. 1). Because most somatic mutations of the Fas and TRAIL-R2 genes in human cancers have been detected in the death domains of these genes (8–10), in this study, we did the mutational analysis of Fas, TRAIL-R1, and TRAIL-R2 genes only in the death domains. Genomic DNA was isolated and analyzed for potential mutations of Fas, TRAIL-R1, and TRAIL-R2 in the death domains, by PCR-SSCP analysis. Enrichment and sequence analysis of aberrantly migrating bands led to the identification of mutations in 7 of the 57 samples (12.3%; Table 1). Three TRAIL-R1 mutations and four TRAIL-R2 mutations were observed, whereas no Fas mutations were detected in the samples. None of the normal samples showed evidence of mutations by SSCP (Fig. 2, A and B), which indicated that the mutations detected in the IDC specimens had risen somatically. As for the relationship between the presence of metastasis and the death receptor mutations, interestingly, all of the 7 mutations were found in IDCs with regional lymph node metastasis (20.5% of the 34 IDCs with lymph node metastasis) but not in IDCs without metastasis (0% of the 23 IDCs without metastasis; Table 1), and statistically, there was a significant difference in mutation frequency of IDC between those with metastasis and those without metastasis (Fisher’s exact test, two tailed, P < 0.05). Three mutations (2 TRAIL-R2 and 1 TRAIL-R1 mutations) were observed both in primary tumors and in metastatic lesions, whereas four mutations (2 TRAIL-R2 and 2 TRAIL-R1 mutations) were observed only in metastatic lesions (Table 1).

All seven mutations identified were missense variants caused by single-nucleotide substitution. All of the three TRAIL-R1 mutations were identified within the death domain coding regions. These mutations affected codons 373, 376, and 402. In contrast to TRAIL-R1, the two TRAIL-R2 mutations (cases 6 and 8) were identified within the death domain, whereas the other two mutations (cases 2 and 9) were detected at the flanking region of the death domain. Two of the four

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missense mutations of \textit{TRAIL-R2} showed an identical Ala to Gly transition at bp 1247 (cases 6 and 8; Table 1; Fig. 2D), resulting in the substitution of Glu to Arg at codon 416. We repeated the experiments three times, including tissue microdissection, PCR, SSCP, and sequencing analyses to ensure the specificity of the results, and found that the data were consistent (data not shown).

\textbf{Allelic Status.} For LOH analysis, we examined the allelic status of \textit{TRAIL-R1} and \textit{TRAIL-R2} using intragenic polymorphic markers (1 for \textit{TRAIL-R1} and 3 for \textit{TRAIL-R2}), and three microsatellite markers at chromosome 8p21–22. Overall, 53 of the 57 cases analyzed were informative for at least one of the seven markers and 25 (45.2%) of the 53 informative cases showed LOH with one or more markers. Five (22.7%) of 22 informative cases without metastasis and 20 (64.5%) of 31 informative cases with metastasis showed LOH with at least one marker (Fig. 2E). There was a significant difference in LOH frequencies between IDCs with metastasis and without metastasis (Fisher’s exact test, two tailed, \( P < 0.01 \)). Among the 20 IDCs with metastasis with LOH, 12 cases showed LOH in both primary and metastatic lesions, and 8 cases showed LOH in metastatic lesions only. All of the seven cases with \textit{TRAIL-R1} or \textit{TRAIL-R2} mutations were heterozygous for at least one marker, and four (57%) of them showed evidence of allelic loss in the 8p21–22 microsatellite markers or intragenic polymorphic markers of each gene (Table 1). Interestingly, case 6 showed LOH and mutation in the metastatic lesion only.

The polymorphism (Lys 441 Arg) in the death domain of \textit{TRAIL-R1} has been reported to inhibit \textit{TRAIL-R1}-mediated cell killing in a dominant-negative fashion (18). Four cases (two nonmetastatic and two metastatic) were Lys/Arg heterozygous, and one case was Arg/Arg homozygous.

For LOH analysis of the \textit{Fas} gene, we examined the allelic status of \textit{Fas} with two intragenic polymorphic markers. Overall, 37 of 57 cases were informative for at least one of the markers and 5 (13.5%) of 37 informative cases showed LOH with at least one marker. As for the relationship between metastasis and LOH of \textit{Fas}, all five tumors with LOH were IDCs with metastases, but it was not statistically significant (Fisher’s exact test, two tailed, \( P = 0.05 \)).

\textbf{Abrogation of Apoptosis Activities by \textit{TRAIL-R1} and \textit{TRAIL-R2} Mutations.} To determine whether the mutant forms of \textit{TRAIL-R1} and \textit{TRAIL-R2} are functionally defective, we generated \textit{TRAIL-R2 Mutations.}
TRAIL-R1 and TRAIL-R2 mammalian expression constructs containing the mutations found in this study by using site-directed mutagenesis. On transfection into 293 cells together with GFP reporter construct, we found that all of the TRAIL-R1 and TRAIL-R2 mutants found in the breast cancers showed significant defects in apoptosis function (Fig. 3B).

**Discussion**

The aim of the current study was two-fold. First, we tried to address whether human breast cancer has inactivating mutations of the death receptor genes, TRAIL-R1, TRAIL-R2, and Fas. If so, the second aim was to address whether there is a significant difference in these mutation frequencies between IDCs with and without metastasis. We found that IDCs of breast had TRAIL-R1 and TRAIL-R2 mutations, and the metastatic IDCs had higher mutation frequencies of these genes than nonmetastatic IDCs. Transfection study revealed that the breast cancer-derived TRAIL-R1 and TRAIL-R2 mutants showed a loss of apoptotic function. Furthermore, the LOH frequency of 8p21–22, where TRAIL-R1 and TRAIL-R2 reside, was significantly higher in metastatic IDCs than in nonmetastatic IDCs. These data suggest that TRAIL-R1 and TRAIL-R2 mutations are involved in the pathogenesis of breast cancer, and that these mutations play an important role in the metastasis of human breast cancers.

During the metastatic process, cancer cells must undergo a step-by-step sequence of events and are subjected to intense selection pressures such that the majority die (19). To be metastasized, cancer cells must escape from the immune attack from lymphoid cells. It has been known that most breast cancer cells express TRAIL-R1 and TRAIL-R2 (20). Because TRAIL is widely expressed in human tissues, including lymphoid cells (21), metastatic breast cancer cells may have TRAIL-resistance mechanisms such as TRAIL-R1 and TRAIL-R2 mutations to escape TRAIL-induced apoptosis. In this study, among the seven mutations detected, one mutation of TRAIL-R1 and two mutations of TRAIL-R2 were observed both in primary tumors and in...
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metastatic foci, but the remaining four mutations (two mutations of TRAIL-R1 and two mutations of TRAIL-R2), observed in only metastatic lesions indicate that these mutations play a role in the progression or metastasis of breast cancers.

The death receptor subfamily of the tumor necrosis factor receptor family are characterized by the presence of a cytoplasmic death domain, which functions to initiate the intracellular apoptotic signaling cascade (1, 5–7). Binding of TRAIL to TRAIL-R1 and TRAIL-R2 induces trimerization of TRAIL-R1 and TRAIL-R2, and FADD/MORT-1 binds to the trimerized TRAIL-R1 and TRAIL-R2 death domains (7). Then, FADD/MORT-1 acts as an adaptor molecule by recruiting caspase 8, which initiates a proteolytic cascade involving other caspases that eventually leads to cell death by apoptosis (7). In our study, the three TRAIL-R1 mutations were detected within the death domain. Two of them (Ala 420 Val and Pro 376 Leu) seemed to be hemizygous mutations without allelic deletion (Table 1). Therefore, in these cases, it is possible that the hemizygous mutated TRAIL-R1 protein(s) may bind with other normal TRAIL-R1 protein(s) to construct a structurally abnormal TRAIL-R1-trimer, which might have a defect in binding to the adaptor protein. In contrast, all of the four cases with TRAIL-R2 mutations showed LOH at the intragenic markers and/or the microsatellite markers (Table 1), indicating potential biallelic inactivation of the TRAIL-R2 gene in these cases. In addition, transfection studies of these mutants showed that TRAIL-R2 Q416R and TRAIL-R2 G426R were less effective in cell death induction than were wild type. The two cases (cases 6 and 8) with TRAIL-R2 mutations in the death domain seem to show biallelic inactivation of TRAIL-R2 genes. Although the other two TRAIL-R2 mutations were detected outside death domain, they showed a loss of apoptotic function when they were expressed in 293 cells.

In addition to the TRAIL-R1 mutations, four breast cancer samples showed polymorphism (Lys 441 Arg) in the death domain of TRAIL-R1 (data not shown), which was reported to block TRAIL-induced apoptosis (18). However, the role of this polymorphism in breast cancer tumorigenesis remains unknown, because this polymorphism was reported to be present in 20% of the normal population (18). To evaluate the potential role of this polymorphism in breast cancer pathogenesis, additional studies on the relationship between the incidence of breast cancer and this polymorphism in a large population are required.

Recent study showed that TRAIL expression was suppressed by anchorage in breast cancer cells, and that anchorage also decreased the susceptibility of the breast cancer cells to TRAIL-induced apoptosis, suggesting that TRAIL-dependent apoptosis may play a role in anoikis in breast epithelial cells (22). Therefore, the mutants detected in this study may inhibit TRAIL-dependent anoikis and influence metastasis in breast cancer cells.

Previous studies showed that most breast cancer cell lines were resistant to Fas-mediated apoptosis (23). Because breast cancers showed frequent LOH at 10q24, where the Fas gene resides (13), we hypothesized that Fas mutation is responsible for the Fas resistance of breast cancers at least in part. However, our data suggest that Fas mutation is not responsible for the Fas resistance of breast cancer and that another gene besides Fas may be the relevant gene for LOH at chromosome 10q24 in breast cancer.

Despite increased awareness of breast cancer and improved methods for early detection, many breast cancer patients die of disseminated breast cancer (24). In the present study, we found somatic mutations of TRAIL-R1 and TRAIL-R2 in breast cancer metastasis. These findings are the first data linking TRAIL receptor gene mutations and cancer metastasis. It is conceivable that TRAIL-R1 and TRAIL-R2 gene mutations could be used as a tumor marker for metastasis in the future. There are many types of tumors with LOH at 8p21–22 (13). Clearly, therefore, studies are now needed that attempt to find the potential TRAIL-R1 and TRAIL-R2 mutations in metastatic lesions of other cancers.

References

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