A Study of TRAIL Receptors in Squamous Cell Carcinoma of the Head and Neck

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Objective: To determine the potential immediate applicability of tumor necrosis factor–related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and TRAIL-R2, the apoptotic forms of TRAIL-Rs, for preclinical testing.

Design and Setting: Head and neck squamous cell carcinoma (HNSCC) tumors were studied for TRAIL-R1 and TRAIL-R2 expression by immunohistochemical analysis. In addition, matched tumor and peripheral blood DNA samples were screened for 2 known TRAIL-R1 coding single nucleotide polymorphisms (C626G and G422A).

Subjects: Tumor samples taken from 43 patients (37 samples for immunohistochemical analysis and 6 additional ones included for polymorphism analysis).

Main Outcome Measures: The expression of TRAIL-R1 and TRAIL-R2 and the presence of the TRAIL-R1 polymorphisms C626G and G422A.

Results: Fewer than 25% of HNSCC tumor cells expressed TRAIL-R1 and TRAIL-R2. Surrounding tumor-infiltrating polymorphonuclear cells expressed TRAIL-R1 and TRAIL-R2 in 12 (32%) and 14 (38%) of cases, respectively. The TRAIL-R1 polymorphisms C626G and G422A were present in 36 (88%) and 33 (89%) cancer cases, respectively. Compared with control groups from another study, these polymorphism frequencies were statistically significant (P = .01 and .003, respectively).

Conclusions: TRAIL-R expression was detected in less than half of the tumor specimens studied but not in any surrounding normal tissue and was found in a higher frequency on tumor-infiltrating polymorphonuclear cells than on tumor cells. These findings support the idea that the presence of TRAIL-Rs on some HNSCC tumors may make them more susceptible to apoptosis, and they also suggest that TRAIL-R–associated mechanisms may result in immune-modulatory effects on tumor-infiltrating polymorphonuclear cells. Furthermore, the significant association of somatic TRAIL-R1 genetic polymorphisms in this sample of patients with HNSCC suggests a potential association between constitutive TRAIL-R1 polymorphisms and development of HNSCC. Defining TRAIL-R expression and genetic polymorphisms in HNSCC represents the first step in examining TRAIL-related mechanisms for their potential as therapeutic targets.


Loss of Apoptotic Regulation is regarded as a crucial step in carcinogenesis. Apoptosis is regulated by a number of proteins present as receptors on the cell surface, intracytoplasmic inhibitors and intranuclear proteins. p53, a tumor suppressor whose gene is the most commonly altered gene in human cancer, is a nuclear protein that facilitates apoptosis under conditions of irreparable DNA damage. The mechanisms through which p53 activates apoptosis include expression of proteins involved in direct mitochondrial-associated apoptotic events, up-regulation of cell cycle inhibitor p27KIP1, and finally through induction of apoptosis-inducing receptors such as Fas and members of the tumor necrosis factor (TNF)–related apoptosis-inducing ligand receptor (TRAIL-R) family. TRAIL is a member of the TNF family of ligands, which is primarily expressed as a membrane protein but also exists in a soluble form. TRAIL interacts with 5 distinct receptors belonging to the TNF receptor superfamily. Two of these receptors, TRAIL-R1 and TRAIL-R2, contain cytoplasmic death domains, which trigger caspase activation, eventually leading to cell death via apoptosis. TRAIL-R3 (TRAIL-R without an intracellular domain DCR1) and TRAIL-R4 (TRAIL-R with truncated death domain DCR2) are unable to induce apoptosis because they lack functional death domains. It has been shown that overexpression of these “decoy” receptors confers resistance to...
TRAIL-R–mediated apoptosis, suggesting an important regulatory mechanism.\(^5\)\(^,\)\(^7\) The fifth TRAIL-R is a low-affinity soluble receptor named osteoprotegerin, whose role is not entirely clear. Figure 1 illustrates the general mechanisms of the major TNF-R family members, including TRAIL-R1 through TRAIL-R4. Complex interactions exist among the TNF superfamily of receptors and the promotion or inhibition of apoptosis, and these interactions have not been studied comprehensively in head and neck squamous cell carcinoma (HNSCC). The present study focuses on TRAIL-R1 and TRAIL-R2, the apopotic forms of TRAIL-Rs, to determine their potential immediate applicability for preclinical testing.

TRAIL-R1 and TRAIL-R2 have become recognized as candidate tumor suppressors because their absence and/or mutation could provide a survival advantage for tumor cells. The carcinogenic significance of TRAIL-R1 through TRAIL-R4 has been further suggested by their location on chromosome 8p21-22, a site of frequent allelic loss in head and neck squamous cell carcinoma (HNSCC).\(^8\) In addition, mutations have been identified in the death domain of TRAIL-R2 in both HNSCC\(^9\) and lung cancer.\(^10\)

TRAIL has emerged as an attractive chemotherapeutic candidate because recombinant TRAIL derivatives have been shown to induce apoptosis specifically in tumor cell lines, with the absence of cytotoxic activity in normal cells.\(^11\)\(^,\)\(^12\) In HNSCC cell lines, synergistic apoptotic effects of TRAIL with cisplatin were demonstrated and found to be dependent on activation of caspase 3.\(^3\)\(^,\)\(^13\)

To help elucidate the potential role of TRAIL-R1 and TRAIL-R2 as tumor suppressors, we examined TRAIL-R1 and TRAIL-R2 receptor expression in HNSCC specimens by immunohistochemical analysis. We also investigated the frequencies in our sample population of 2 coding single nucleotide polymorphisms (SNPs) previously identified in the ectodomain region of the TRAIL-R1 gene, C626G and G422A.\(^8\) Though SNPs may or may not have functional significance, the importance of these 2 coding alterations is suggested by resulting amino acid changes in the ligand-binding domains Arg→Thr and His→Arg, respectively. These amino acid changes in the final TRAIL-R protein have the potential to affect TRAIL binding.

**IMMUNOHISTOCHEMICAL STAINING FOR TRAIL-Rs**

Following approval by the institutional review board at the Mount Sinai School of Medicine, New York, NY, 4-µm thick, paraffin-embedded tissue sections from 37 patients' tumors were stained for TRAIL-R1 and TRAIL-R2 using primary goat polyclonal antibodies (anti–TRAIL-R1 from R&D Systems, Minneapolis, Minn, and anti–TRAIL-R2 from Calbiochem, San Diego, Calif). Paraffin sections of normal human occipital cortex were used as positive controls. Sections were deparaffinized and rehydrated with xylene and alcohol. Antigen retrieval was then performed by incubating the slides for 10 minutes in basic antigen retrieval agent (R&D Systems) heated to just boiling. The sections were then immersed in a 0.3% hydrogen peroxide in methanol solution for 30 minutes to block endogenous peroxidase activity and then 1.5% normal blocking serum in phosphate-buffered saline for 10 minutes to reduce nonspecific binding. Sections were then incubated with primary antibody in a humidified chamber at room temperature for 1 hour. Subsequent rinses, addition of biotinylated secondary antibody, and avidin-peroxidase complex for immunohistochemical analysis were carried out using the manufacturer’s protocol (Vector Laboratories, Burlingame, Calif). Diaminobenzidine was used as a chromogen, and light counterstaining was performed with hematoxylin. Tissue sections, which all contained areas of normal tissue and areas of tumor cells with associated immune cell infiltrates, were then jointly examined by 3 of the authors (M.S.T., M.S.B-G., and D.C.D.). The immunohistochemical staining was determined to be grossly positive (if ≥5 cells stained in 3 high-power fields) or negative for the following locations: tumor tissue, surrounding normal tissue, and surrounding tumor-infiltrating polymorphonuclear (TIP) cells. Quantitation of stained cell numbers was not performed because this was a pilot study for antibody staining, detection, and localization in HNSCC. Based on studies in other models, we expected that tumor tissues would stain positive and that surrounding normal tissues would stain negative, although no previous HNSCC tissue studies were found in the literature for comparison. The probability of the staining of immune cells could not be estimated prior to examination. Pairwise statistical analysis of staining results between groups was not performed.

**ANALYSIS OF TRAIL-R1 POLYMORPHIC ALLELES: C626G AND G422A**

Using genomic DNA isolated from matched tumor and normal tissue and peripheral blood of 43 patients (all 37 patients from the immunohistochemical analysis group and 6 additional ones), exons 3 and 4 of TRAIL-R1 were amplified by polymerase chain reaction (PCR). Template DNA, 20 ng, was amplified in a 25 µL reaction containing 1 x PCR buffer, 1.5 µL of 25mM magnesium chloride, 1 unit of Taq polymerase (all from Roche Molecular Systems, Branchburg, NJ), 4 pmol of each primer (previously published sequences\(^8\)), and 0.5 µL of 10mM mixed deoxynucleoside triphosphates. Conditions for PCR were as follows: initial denaturation at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 30 seconds each,
annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, then elongation at 72°C for 5 minutes.

Then, 18 µL of exon 3 PCR product was incubated in a 25 µL reaction with 2 units of FokI, 0.1 mg/mL of bovine serum albumin, and 1 × NEB buffer No. 4 (New England Biolabs, Boston, Mass) at 37°C for 90 minutes. Similarly, 18 µL of exon 4 PCR product was incubated in a 25 µL reaction with 10 units of DraIII, 0.1 mg/mL of bovine serum albumin, and 1 × NEB buffer No. 3 (New England Biolabs, Boston, Mass) at 37°C for 90 minutes. All products were then electroforetically separated on a 2% agarose gel. Samples representative of wild-type and homozygous or heterozygous polymorphisms were then purified using the QIAquick PCR purification kit (Qiagen, Valencia, Calif), and confirmatory sequencing was performed.

χ² Analysis was performed to compare the rate of these 2 polymorphisms in our cancer patient group to a previously published control group.⁶

RESULTS

IMMUNOHISTOCHEMICAL STAINING FOR TRAIL-Rs

Immunohistochemical expression of either TRAIL-R1 or TRAIL-R2 was uncommon in our HNSCC samples; less than 25% of carcinoma specimens stained positive for these receptors (see Figure 2 and the following tabulation that summarizes the immunohistochemical results; data are given as number [percentage] of positive tumor samples [n=37]).

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<th>n</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>TRAIL-R1</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>TRAIL-R2</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>TRAIL-R1 TIP cells</td>
<td>14</td>
<td>38</td>
</tr>
<tr>
<td>TRAIL-R2 TIP cells</td>
<td>12</td>
<td>32</td>
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However, none of the surrounding normal tissue stained positive, thus supporting previous observations that the apoptotic R1 and R2 forms of TRAIL-Rs are expressed in cancer cells but not in normal cells.¹¹,¹²

Tumor-infiltrating polymorphonuclear cells stained positive for TRAIL-R1 and TRAIL-R2 in 12 (32%) and 14 (38%) cases, respectively (see Figure 3 and the previous tabulation). A few tumors (n=4; 11%) expressed both TRAIL-R subtypes and also contained TIP cells expressing TRAIL-R. In the absence of tumor staining, 8 (22%) to 10 (27%) patients had positive TIP staining results (see the following tabulation that gives the relationship of tumor and TIP cell expression of TRAIL-Rs; data are given as number [percentage] of patients [n=37]).
Tumor/H11545 TIP Cells

Tumor/H11545 TIP Cells− Tumor−/ TIP Cells


T-lymphocytes were not observed to have positive staining results with either TRAIL-R1 or TRAIL-R2. Of note, positive controls using normal human occipital cortex tissue demonstrated specific, consistently positive staining using this antibody (data not shown).

ANALYSIS OF TRAIL-R1 POLYMORPHIC ALLELES: C626G AND G422A

Single nucleotide polymorphisms are single nucleotide changes that may or may not have functional relevance. These single base pair (bp) changes result in differences in restriction enzyme digestion products; the DNA alteration results in either the formation of a new restriction enzyme site or the elimination of a previously existing one. The C626G alteration eliminates a unique DraIII restriction site in exon 4 of TRAIL-R1. The wild-type exon 4 PCR product, which is 220 bp long, normally yields 2 smaller fragments (164 and 56 bp) when digested with DraIII. However, only the uncut 220-bp product is seen if there is a homozygous C626G alteration. In heterozygous samples, 3 bands (220, 164, and 56 bp) are produced. Screening for SNPs in tumor DNA was clearly readable in 41 of 43 cases and revealed either heterozygous or homozygous alteration for C626G (Figure 4) in 36 cases (88%).

The G422A alteration, by contrast, creates a unique FokI restriction site in exon 3 of TRAIL-R1. The wild-type PCR product for exon 3 is 230 bp long and is not digested by FokI. A homozygously altered G422A, however, yields 2 smaller fragments (160 and 70 bp) on incubation with FokI. In heterozygous samples, 3 bands (230, 160, and 70 bp) result. Screening for SNPs in tumor DNA was clearly readable in 37 of 43 cases and revealed either heterozygous or homozygous alteration for G422A (data not shown) in 89% of cases.

Representative samples of the homozygously and heterozygously altered alleles and wild-type alleles were positively confirmed by direct DNA sequencing (Figure 5).

In all patient tumor DNA samples tested, identical sequence alterations were present in the paired nontumor DNA, demonstrating that all polymorphisms were germline. When compared using $\chi^2$ analysis against allele frequencies of a healthy control population from an independent study, the rates of polymorphisms in our patients were significantly higher for both C626G ($P=0.01$) and G422A ($P=0.003$) alleles (see the following tabulation that gives frequency of C626G and G422A; data are given as number/total number [percentage] of tumor samples clearly readable after repeated experiments [$n=43$]).

<table>
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<th>Tumor / TIP Cells</th>
<th>Tumor / TIP Cells</th>
<th>Tumor / TIP Cells</th>
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Figure 4. Single nucleotide polymorphisms analysis of tumor necrosis factor–related apoptosis-inducing ligand receptor 1 exon 4 for C626G. The C→G alteration causes the loss of a DraIII restriction site, thus resulting in a single, nondigested band of 220 base pairs when homozygously (HOM) altered. Heterozygous (HET) samples demonstrate incomplete digestion, seen as 3 separate bands. WT indicates wild-type.

Figure 5. DNA sequencing chromograms confirming wild-type, homozygously altered, and heterozygous versions of tumor necrosis factor–related apoptosis-inducing ligand receptor 1, base position 626. Arrows indicate base of interest that shows alterations.

C626G

<table>
<thead>
<tr>
<th></th>
<th>Cancer patients</th>
<th>Control group</th>
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<tr>
<td>Cancer patients</td>
<td>36/41 [88]</td>
<td>29/48 [60]</td>
</tr>
<tr>
<td>Control group</td>
<td>33/37 [89]</td>
<td>31/48 [65]</td>
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We find a low frequency of apoptotic TRAIL-R expression detectable by immunohistochemical analysis in HNSCC tumor cells, indicating the need for further study before exploiting the TRAIL system as a therapeutic tar-
get. More samples showed positive TRAIL-R staining results in TIP cells compared with tumor cells. The expression of TRAIL-R in TIP cells suggests that in the presence of TRAIL ligand, binding may induce apoptosis of infiltrating polymorphonuclear cells, allowing evasion of the immune response by tumor cells. Certain HNSCC tumor cells that secrete TRAIL ligand or a protein that similarly activates TRAIL receptors may thus directly exert a host immunomodulatory effect via the TRAIL-R pathway.

Although the exact impact of TRAIL-induced apoptosis of TIP cells on tumor behavior is unknown, it is possible that circulating TRAIL ligand activates apoptosis of TIP cells and other immune cells within the HNSCC tumor environment. A similar scheme has been demonstrated in the spontaneous apoptosis of T lymphocytes in patients with HNSCC, whereby another TNF receptor superfamily member, the Fas/FasL pair, is involved in cytotoxicity of T lymphocytes, both in patients with active tumors and also those rendered free of clinically active tumor. In another model, Secchiero et al demonstrated a regulatory role for TRAIL on myeloid differentiation in HL-60 cells, an acute myeloid leukemia line. TRAIL induced rapid apoptosis of HL-60 cells as well as maturation of the remaining normal monocytic cells. A separate study by this group demonstrated TRAIL-induced up-regulation of cyclooxygenase 1 activity and prostaglandin E2 production in HL-60 cells. Recent studies have specifically investigated associations between TRAIL and neutrophil apoptosis. Renshaw et al found that TRAIL ligand as well as TRAIL-R2 and TRAIL-R3 were detected at both the messenger RNA and protein levels within neutrophils. Furthermore, human neutrophil apoptosis was accelerated by a cross-linked form of TRAIL in vitro. Whiteside has demonstrated the immunomodulatory effects of the Fas-FasL activation in the environment of HNSCC. The potential for TRAIL-mediated cytotoxic activity directed against the host immune system is suggested by our immunohistochemical findings and warrants further investigation. Studies of TRAIL secretion by HNSCC tumor cells will be informative to test this hypothesis.

The role of TRAIL-R1 as a tumor suppressor gene is suggested by the SNP and sequencing data from our cancer patients. Specifically, SNPs coding for a nonfunctional receptor in tumor cells would permit cancer growth even in the presence of TRAIL. In the analysis of TRAIL-R1 for 2 specific polymorphisms, significant differences exist between patients with HNSCC and normal controls (C626G [P = .01] and G422A [P = .003]). These polymorphisms, when present, were found in mismatched tumor DNA and peripheral blood, indicating that they are germline alterations. Genetic polymorphisms in TRAIL-R1 may predict a resistance to TRAIL-induced apoptosis and may signify an increased relative risk for development of HNSCC. A major caveat to these data are that age and ethnic differences can confound the comparison of the cancer and control groups. To determine the significance of the observed SNP frequency in this HNSCC population, it would be valuable to study a control population that is specifically ethnically matched to our cancer patients.

If the observed SNP variants result in effective differences in receptor function or expression, one could hypothesize that certain HNSCC tumor cells evade toxic effects from TRAIL/TRAIL-R interactions through decreased expression of functional receptors. Expression of TRAIL ligand by tumor cells that are insensitive to its cytotoxic effects could result in death to surrounding TIP cells, thus providing a tumor survival advantage. Generally, immunohistochemical receptor staining is not an effective method for correlation with mildly altered receptor forms. In the present study, all of the tumor samples that stained positive for TRAIL-R1 had both G422A and C626G alterations, and 12 (86%) of 14 tumor samples that stained positive for TIP cells had both alterations as well. It is likely that the antibodies used against TRAIL-R1 are not likely to be specific enough to be affected by a single- or 2-base genomic alteration at the protein level and thus cannot distinguish between functional and non-functional receptors. Further in vitro correlation between genome and receptor function will be required to validate this hypothesis.

TRAIL-Rs and their expression are likely influenced by the levels, expression, and phenotype of p53 in HNSCC. Specifically, p53 has been confirmed to upregulate expression of apoptotic TRAIL-R1 and TRAIL-R2 through the presence of p53 binding sites on the transcription sites for TRAIL-R1 and TRAIL-R2. Of note, up-regulation of decoy receptor expression also occurs through p53-dependent mechanisms. Because approximately 40% of HNSCC express p53, it is possible that the levels of TRAIL-R1 and TRAIL-R2 observed in this study are a direct result of aberrant p53 signaling. Another hypothesis is that individual p53 mutations may be determining different ratios of proapoptotic TRAIL-Rs to decoy receptors expressed on individual cells, ultimately affecting the response of the cell to exogenous TRAIL ligand. We did not investigate the p53 status of tumors in the present study, but it is likely that at least half express mutant p53, which may be affecting levels of TRAIL-R expression. Furthermore, we did not study the expression of decoy receptors, but these could also determine functional responses to TRAIL ligand exposure.

The loss of proapoptotic TRAIL-R may be a direct phenotypic response to mutation of p53 and may occur as a natural progression in carcinogenesis. In many models, the activity of the Fas-FasL death activity as well as the TRAIL/TRAIL-R activity is seen to change as tumor progression occurs. Comparative studies of Fas and FasL expression revealed down-regulation of Fas and up-regulation of FasL expression during melanoma progression. The Fas apoptotic pathway is intact in early- and intermediate-stage melanomas but is often impaired in highly metastatic late-phase melanomas. Many malignancies, including melanomas, demonstrate decreased TRAIL activities, resulting in decreased TRAIL-dependent apoptosis with tumor progression. In this context, it is possible that late-stage tumors may no longer be sensitive to TRAIL, and that low expression of apoptotic TRAIL-R is not significant.

Exploiting therapeutic possibilities for TRAIL/TRAIL-R interactions in HNSCC will likely require a 2-pronged approach that includes overexpression or up-regulation of the proapoptotic wild-type receptors as well as introduction...
of exogenous TRAIL. One method of maximizing TRAIL-induced apoptosis might include repair of mutant p53 by transduction of a wild-type p53, which improves apoptosis in HNCC treatment when introduced through gene therapy.22-26 Direct overexpression of wild-type TRAIL-R1 and R2 are also inducible via gene therapy and allow for bypass of p53-defective mechanisms present in HNCC. Studies in human lung cancer cell lines demonstrate promising results with combined additive effect on apoptosis using combination TRAIL and p53 gene transfer.27 Reinstatement of apoptotic competence in cancer cells through overexpression of wild-type TRAIL-R may improve sensitivity to exogenous or autocrine secretion of TRAIL. Functional studies in HNCC cell lines will help to determine answers to these questions.

The present findings suggest a role for TRAIL ligand as an immunosuppressive agent within an environment where tumor cells may be insensitive to TRAIL-induced cytotoxicity. Genetic polymorphisms in exons coding for TRAIL-R1 receptors are consistent with another study4 and may predict a resistance to TRAIL-induced apoptosis with an increased relative risk for development of HNCC.

Submitted for Publication: May 4, 2004; final revision received January 4, 2005; accepted February 2, 2005.

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Acknowledgment: We acknowledge Michael J. Fisher and Wafik S. El-Deiry for providing TRAIL-R sequences and primers; Cathy Miselen for extensive technical support; Alexis Boneparth for assistance in immunohistochemical staining; and James Godbold, PhD, Director, MSSM Biostatistics Shared Resources Facility for Cancer Research, for advice in statistical analysis.

REFERENCES