Sheddase Activity of Tumor Necrosis Factor-α Converting Enzyme Is Increased and Prognostically Valuable in Head and Neck Cancer

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Abstract

Tumor necrosis factor α converting enzyme (TACE) is a sheddase overexpressed in cancers that generates cancer cell growth and survival factors, and is implicated in carcinogenesis and tumor growth. This indicates that TACE could be a potentially important cancer biomarker. Unexpectedly, TACE expression in cancer tissues does not correlate with cancer stage or invasiveness. Although TACE sheddase activity is a more direct and potentially better indicator of TACE biology and might be a better cancer biomarker than TACE expression, it has not been studied in cancer tissues. In the present study, we developed a reliable specific assay for quantification of TACE sheddase activity, investigated TACE activity and TACE protein expression in head and neck cancer (HNC) tissues, and examined the correlation of the results with HNC clinical stages and likelihood to recur.

Introduction

Prognosis and treatment planning of head and neck cancer (HNC) are mainly based on traditional clinical methods such as the tumor-node-metastasis classification and histopathology. These methods are both subjective and lack sensitivity for cancer aggressiveness, prognosis, and treatment responsiveness. Therefore, new biomarkers are needed to provide clinicians with more precise information on the intrinsic biological properties of HNC. In recent years, several biomarkers, including epidermal growth factor receptor (EGFR), p53, cyclooxygenase-2, and matrix metalloprotease (MMP)-9, have been discovered and have shown both biological relevance and significant ability to determine the aggressiveness and prognosis of HNC (1-4). However, these and other promising biomarkers await clinical validation to be approved for application in clinical practice.

Another potentially important cancer biomarker might be tumor necrosis factor-α (TNF) converting enzyme (TACE, ADAM17 or CD156g). TACE is an integral plasma membrane zinc-dependent metalloprotease and member of the A Disintegrin And Metalloprotease family of molecules. In recent years, several biomarkers, including epidermal growth factor receptor (EGFR), p53, cyclooxygenase-2, and matrix metalloprotease (MMP)-9, have been discovered and have shown both biological relevance and significant ability to determine the aggressiveness and prognosis of HNC (1-4). However, these and other promising biomarkers await clinical validation to be approved for application in clinical practice.

We found that HNC cell lines and tissues contained remarkably higher quantities of TACE activity and TACE protein than normal keratinocytes or oral mucosa. siRNA silencing of TACE resulted in the inhibition of release of the tumorogenic factors amphiregulin and transforming growth factor α, and tumor protective factors tumor necrosis factor receptors from HNC cells. Importantly, TACE activity, but not TACE protein expression, was significantly higher in large, T3/T4, primary tumors relative to small, T1/T2, primary tumors, and especially in primary tumors likely to recur relative to those unlikely to recur. These data show that increased TACE activity is biologically and clinically relevant, and indicate that TACE activity could be a significant biomarker of cancer aggressiveness.
cancer cell proliferation, survival, and tumor growth (11-14). In addition, the TACE-generated soluble TNF and TNF receptors (TNFR) mediate procarcinogenic inflammation and protection of tumor cells from TNF killing, respectively, and are increased in cancer hosts (15-20). Therefore, TACE plays a critical role in malignant transformation and tumorigenesis, and potentially represents a significant new diagnostic/prognostic cancer biomarker as well as a new target for cancer therapy.

Increased expression of TACE mRNA and/or protein has been consistently found in a variety of human cancers (18, 21-27). However, no correlation between increased TACE expression in cancer tissues and cancer clinical stages or aggressiveness has been shown. The quantities and ratios of the two TACE forms, inactive proenzyme and active enzyme, vary in tissues. Because the tumorigenic activity of TACE is mediated by the TACE active form and its sheddase activity, but not by the TACE inactive form (5-7), TACE activity is a better indictor of TACE biology and might be a better biomarker of tumorigenicity and cancer invasiveness than TACE expression. To date, TACE enzymatic activity in cancer tissues has not been examined.

In the present study, we investigated TACE sheddase activity within HNC tissues, and correlated the results with HNC clinical stages and likelihood to recur. Our data show that TACE sheddase activity is increased in HNC and can discriminate cancer from normal tissues, and primary tumors likely to recur from those unlikely to recur, implying that TACE activity could be potentially an important cancer biomarker.

Materials and Methods

Cell Lines. Established HNC cell lines PCI-4A, PCI-4B, PCI-15A, PCI-15B, and PCI-13 were generated in Dr. Theresa Whiteside's laboratory. The “A” and PCI-13 cell lines were derived from primary tumors and “B” cell lines were derived from lymph node metastases. The PCI-13-26 cell clone was generated by limiting dilution of PCI-13 cells, and showed an increased ability to release the TACE-generated tumor-protecting factor soluble TNFR1. Tumor cell lines were cultured in DMEM containing 10% FCS (Life Technologies). We also used three different cell lines of freshly isolated normal adult human keratinocytes (Cambrex Bioscience). Normal adult human keratinocyte cell lines were propagated in KGM serum-free culture medium (Cambrex Bioscience), whereas the experiments with these cell lines were done in DMEM containing 10% FCS.

Tumor Tissues and Patients. Fresh tissue samples of 45 HNC primary tumors, 15 recurrent HNC tumors (R3), 12 lymph node metastases paired with 12 HNC primary tumors, 3 unstaged HNC tumors, and 16 normal oral mucosa tissues were obtained by surgery, snap-frozen in liquid nitrogen, and provided to the investigators by the University of Pittsburgh HNC Specialized Programs of Research Excellence Tissue Core. Dr. Jennifer Hunt, a trained pathologist, selected the tumor tissues for this study. Tumor samples containing >75% cancer cells based on the cytopathology scrape analyses were obtained from the interior of the main tumor mass. Sections of adjacent areas were histopathologically analyzed to confirm the high percentage of tumor cells. Tumor samples with necrosis or extensive inflammatory infiltrates, or paucicellular or fibrotic contents were not used in this study. The tissue samples were maintained in the frozen state at ~80°C until their use in each assessment and were exclusively used in the studies described in the present article. All tissue donors signed the University of Pittsburgh Institutional Review Board (#891206)—approved informed consent. Donors of primary tumor tissues were previously untreated HNC patients. Donors of recurrent tumor tissues were HNC patients previously treated for primary tumors by surgery, radiation, chemotherapy, or a combination, and remained untreated for at least 1 mo before obtaining tumor tissues. Donors of normal tissues were individuals who underwent surgery for sleep apnea or chronic tonsillitis and were otherwise healthy. The primary tumor samples were classified according to the pathologically determined tumor size and lymph node status as T1 or T2 (T1/T2), T3 or T4 (T3/T4), lymph nodes without metastases (N0), and lymph nodes with metastases (N+). They were also classified according to their ability to recur after initial therapy as either primary tumors that did not develop recurrences after therapy (NR1-T1/T2 and NR2-T3/T4) or primary tumors that developed recurrences after therapy (R1-T1/T2 and R2-T3/T4; Table 1). The follow-up of HNC patients after therapy of primary tumors was at least 2 y (9 patients, 2 y; 21 patients, 3 y; 9 patients, 4 y; 18 patients, 5 y; and 6 patients, 6 y; median, 3.3 y; range, 2.0-6 y), which was expected to be sufficient period of time for the possible development of recurrent tumors. All tumors were squamous cell carcinomas of the head and neck (oral cavity, tongue, pharynx, or larynx).

Western Blot. Cells from cultured lines (1 × 10⁶) or tissues (50 mg) were washed twice in cold PBS and then lysed with 50 or 500 μL, respectively, of radioimmunoprecipitation assay cell-lysis buffer (Upstate) containing EDTA-supplemented protease inhibitor cocktail (Roche Applied Science). The protein levels in the tissue lysates were determined by Bradford’s method using the Quick Start Bradford kit (Bio-Rad Laboratories, Inc.). Ten micrograms of the lysate proteins were subjected to 8% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Perkin-Elmer). TACE and β-actin proteins were detected on Western blots using the rabbit polyclonal affinity-purified goat IgG antibodies to human TACE cytoplasmic domain (reactive with both nonactivated and activated affinity-purified goat IgG antibodies to human TACE cytoplasmic domain (reactive with both nonactivated and activated TACE forms) or control nonimmunized goat IgG (R&D Systems). Subsequently, they were

ELISA. ELISA kits for human TACE, TNFR1, TNFR2, TGF-α, and amphiregulin were purchased from R&D Systems. ELISAs were done according to the manufacturer’s instructions. TNFR1, TNFR2, TGF-α, and amphiregulin were measured in cell culture conditioned media obtained by culturing 1 × 10⁶ cells/mL for 24 h. The quantity of TACE protein was measured in cell or tissue lysates that were obtained as described above for the preparation of lysates for Western blotting.

Immunohistochemistry. Eight-micrometer-thick cryosections of freshly frozen normal oral mucosa or HNC tumor tissues were prepared. The tissue sections were fixed in ice-cold acetone and stained with unconjugated affinity-purified goat IgG antibodies to human TACE cytoplasmic domain (reactive with both nonactivated and activated TACE forms) or control nonimmunized goat IgG (R&D Systems). Subsequently, they were...
stained with R-phycocerythrin–conjugated affinity-purified F(ab)2 fragment of donkey anti-goat IgG (H+L) antibodies (Jackson ImmunoResearch Laboratories). To visualize the tissue morphology, the same tissue sections were stained with hematoxylin and examined, after immunofluorescence staining images were captured. The tissue sections were examined, and photomicrograph images were generated by a Nikon Eclipse E800 fluorescence microscope equipped with standard filters for rhodamine/phycocerythrin (Ex535/50, DM565, BA610/75) and using a manually set, fixed exposure time. Quantification of TACE expression was done using the MetaMorph software that enabled setting of fluorescence threshold. Pixels (on a 0-255 scale) above their average +1.8x SD of isotype control antibody–stained tissue sections were considered positive and were measured. Data were presented as the number of TACE pixels per cell, which were computed from 549 normal tissue cells and 1,077 HNC tissue cells analyzed, and TACE pixels per area of 0.015 mm2 of tissue section, which were computed from six areas of normal tissue section and eight areas of HNC tissue section analyzed. The number of cells was determined by counting the number of nuclei in defined areas of tissue sections. Negative and positive internal controls were TACE-expressing PCI-13 cells stained with normal goat IgG or anti-TACE antibodies, respectively (Supplementary Fig. S1). To estimate fractions of normal epithelial cells or tumor cells in normal or tumor tissues, respectively, we stained the tissues with anti-cytokeratin antibodies (data not shown).

**TACE Sheddase Activity Assay.** The assay was done using the previously described method (28), modified by us as follows. Briefly, cultured cells (1 x 106) were lysed in 50 μL of lysis buffer, and fresh tissues (50 mg) were homogenized using the tissue grinder system (Fisher Scientific) and lysed in 500 μL of lysis buffer on ice, for 1 h. The lysis buffer was composed of Tris-HCl (50 mmol/L), NaCl (150 mmol/L), SDS (0.1%), sodium deoxycholate (1%), Nonidet P40 (1%) and Triton X-100 (1%), and EDTA-free protease inhibitor cocktail (Roche Applied Science). The lysates were centrifuged at 16,000 g for 20 min, and the supernatants were collected and tested. TACE enzymatic assay was done with the 150 μL mixture of 10 μg lysate proteins, assay buffer [Tris-HCl [50 mmol/L (pH 7.4), NaCl (25 mmol/L), glyc erol (4%)], EDTA-free protease inhibitor cocktail (Roche Applied Science), and 50 μmol/L of TACE-specific fluorophore/quencher-capped end substrate with the amino acid sequence Abz-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Dap(Dnp)-NH2 containing the TACE-specific cleavage site Ala-Val (Peptide International). The enzymatic reaction was induced by the incubation of the lysate substrate mixture at room temperature for 3 h. The fluorescence was measured in a LS55 Luminescence SPECTRUM (Perkin-Elmer) using an excitation wavelength (λex) of 320 nm and an emission wavelength (λem) of 420 nm. Recombinant TACE (rTACE; 1-100 ng), a specific inhibitor of TACE (recombinant tissue inhibitor of metalloproteases-3, rTIMP-3, 100 mmol/L), a specific inhibitor of MMPs (rTIMP-1, 100 mmol/L; R&D Systems), and a general inhibitor of metalloproteases (EDTA, 10 mmol/L) were used to assess the specificity of the TACE sheddase assay. The data are presented as TACE activity units, computed by the following formula: TACE activity units = Experimental sample mean fluorescence intensity – Background sample mean fluorescence intensity.

**RNA Interference.** A pool of three human TACE siRNA silencers with the sequences 614 - UUACAAACUCUCAAAAGUGGtt, CCACUUUGGAGAUUGGUAtt; 784 - UUUCACUGAAGACACGACtt, GCUUGUU-CUAGUGAGAAAtt; and 815 - UGUCUUCAUG-GAUCUGGtt, CCAGAUCCUAAGAAGACtt was purchased from Santa Cruz Biotechnology; and individual TACE siRNA with the sequences s13718 - GUGGAUG-GUAAGAACGAAtt, UUCGUUUUUAACUCCCAc; s13719 - GGGCAUCAGCAGACAAUAtt, UAUUGUU-CUCCGGAUCGGCac s13720 - GGAUGUAUUUGAAGCACAUUt, AACUGUUAUCAUUCAACUgt were obtained from Applied Biosystems/Ambion. Control siRNA with the sequence UUCUCGAGUCGUACCGTT, ACUGACACGGUUCCGGAATT was purchased from Santa Cruz Biotechnology. Transduction of PCI-13 cells with siRNA was done using Lipofectamine 2000 (Invitrogen). Briefly, PCI-13 cells were grown in six-well plates to 40% to 50% confluence. One to two hundred picomoles of siRNA solutions were mixed with 10 μL of lipofectamine in 200 μL of Opti-MEM I Reduced Serum Medium (Invitrogen). After 30 min of incubation at room temperature, the siRNA/lipofectamine mixture was diluted with 800 μL of Opti-MEM I Reduced Serum Medium and added to PCI-13 cells. The cells and siRNA/lipofectamine mixture were incubated for 5 h at 37°C, supplemented with 1 mL of DMEM containing 20% FCS, and cultured for 24 to 96 h. The siRNA transfection efficiency was monitored using 6-h co-transfection of PCI-13 cells with TACE siRNA and siGLO Green fluorescent oligonucleotide transfection indicator (Thermo Fisher Scientific Dharmacon), and the cell fluorescence was measured by flow cytometry. The flow cytometry data were analyzed and percentages of transfected cells were determined using the Summit v4.3 software and histogram subtraction tool (Dako Colorado, Inc.).

**Statistics.** The data are reported as means (+SD), and medians, quartiles, and ranges. The statistical significance of differences between the results was tested using a two-sided unpaired Student's t test. The results were considered significantly different when P < 0.05. Correlations between data were assessed using the Pearson's correlation coefficient test. Specificity and sensitivity of the TACE sheddase activity assay or TACE ELISA were assessed using the receiver operating characteristic (ROC) curves. Cutoff was defined as a result which multiplication of sensitivity and specificity gave the highest value. Cutoff was also defined by median. Risk of tumor recurrence was tested using Kaplan-Meier, parametric Cox, and binary logistic regression analyses. The statistical analyses were done using NCSS 2000 Statistical Software.

**Results**

**TACE Is Overexpressed in HNC Cells and Tissues.** To determine whether HNC cells and tissues overexpress TACE, we investigated the level of TACE protein expression in cultured HNC cells and normal adult human keratinocytes, and fresh HNC and normal oral mucosal tissues (Fig. 1). Using Western blot analysis, we consistently detected two characteristic immunoreactive protein...
Table 1. Characteristics and classification of patients and their tumors

<table>
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<td>0</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
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<td>1</td>
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<td>2</td>
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<td>1</td>
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<tr>
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Abbreviations: NR1-T1/T2, T1/T2 primary tumors that did not develop recurrences after initial therapy; R1-T1/T2, T1/T2 primary tumors that developed recurrences after initial therapy; NR2-T3/T4, T3/T4 primary tumors that did not develop recurrences after initial therapy; R2-T3/T4, T3/T4 primary tumors that developed recurrences after initial therapy; R3, recurrent tumors; N0, lymph node without metastases; N+, lymph node with metastases.

bands, corresponding to the 120 kDa immature (inactive) form and 100 kDa mature (active) form of TACE in the blots of lysates of all tested cell lines and tissues, including cultured normal keratinocytes and HNC cell lines, and randomly selected normal oral mucosa and HNC primary tumor tissues (Fig. 1A and B). The levels of both immature and mature TACE forms in HNC cell lines and tissues were found to be highly increased relative to normal cell lines and tissues, respectively. However, the HNC cell lines and tissues varied considerably in their TACE levels. Thus, the HNC cell lines derived from metastases and the PCI-13-26 clone of the PCI-13 cell line, which released increased levels of TACE-dependent soluble TNFR1 (data not shown), contained notably higher levels of both TACE forms than the cell lines derived from primary tumors or the uncloned PCI-13 cell line, respectively.

These findings were confirmed and extended by quantitative measurement of total TACE protein in the lysates of cells and tissues using TACE ELISA (Fig. 1C and D). Therefore, all tested HNC cell lines showed significantly higher total TACE protein levels than normal keratinocytes, and the HNC cell lines derived from metastases and the PCI-13-26 clone contained significantly higher levels of TACE protein than cell lines derived from primary tumors and the PCI-13 cell line, respectively (Fig. 1C). Remarkably, the median (0.44 ng) and range (0.07-1.07 ng) values of TACE protein in HNC tumor tissues showed higher levels than the median (0.17 ng) and range (0.05-0.40 ng) values of TACE protein in normal oral mucosa. The difference between median values was 2.6-fold, and highly statistically significant ($P < 0.0001$; Fig. 1D).

To determine which cells in normal oral mucosa and HNC tumors express TACE, we performed fluorescence- and light-microscopic analyses of the tissue sections following staining with fluorescent anti-TACE antibodies and H&E, respectively. We found that only a small proportion of the cell populations in normal oral mucosa, including the tiny tiers of basal layer and suprabasal layer cells of the stratified squamous epithelium lining and rare fibroblasts in the lamina propria, were weakly stained with anti-TACE antibodies (Fig. 1E and F). In sharp contrast, in HNC tumors, all cancer cells and especially those present around the blood vessels were strongly stained with anti-TACE antibodies, sharply contrasting with the unstained cancer stroma consisting of normal connective tissue (Fig. 1G and H) and the large majority of normal oral mucosal epithelial and lamina propria cells (Fig. 1E and F).

Fluorometric analyses showed that HNC tissue cells expressed significantly higher levels of TACE than normal epithelial tissue cells (Supplementary Fig. S2). These findings confirm that normal oral mucosa express low levels of TACE, whereas HNC tumors express high levels of TACE, and show that the source of TACE in normal oral mucosa is mostly epithelial basal layer cells, whereas in HNC tissues, the source is exclusively cancer cells.

**TACE Sheddase Activity Assay.** The levels of TACE mature (active) form did not consistently correspond to the levels of TACE immature (inactive) form in Western blots of either cultured HNC cells or HNC tissues (Fig. 1A and B), indicating that TACE sheddase activity might be a better marker of TACE function in tumor tissues than TACE protein expression. However, although the activity of matrix metalloproteinases in tumor tissues has been correlated with an aggressive tumor phenotype (4), TACE sheddase activity has not been investigated previously in tumor tissues. To study TACE sheddase activity in HNC tissues, we modified a previously described TACE sheddase activity assay (28). The modified assay was based on the peptide substrate containing the TACE-specific TNF cleavage site Ala-Val. It reproducibly measured TACE sheddase activity in the solutions of rTACE or HNC cell lysates, and showed strong linear correlations between the quantities of rTACE or HNC cell lysates and the levels of sheddase activity (Fig. 2A and B). In addition, both rTACE and HNC cell-derived sheddase activities were blocked by the TACE-specific inhibitor TIMP-3 or the general metalloprotease inhibitor EDTA, but not by the specific inhibitor of MMPs TIMP-1 (Fig. 2C). TACE siRNA treatment consistently induced in PCI-13 cells similar and profound decreases in TACE protein expression and TACE activity (experiment in Fig. 2D and E; 86% inhibition of both TACE protein and TACE activity; means of 10 experiments: TACE protein, 74% inhibition; TACE activity, 70% inhibition). Therefore, in our experimental system, the modified TACE sheddase activity assay was specific for TACE and was robust. The assay measured TACE sheddase activity in nanograms of recombinant TACE and micrograms of cell lysates, and thus was highly sensitive. It ably discriminated the amounts of TACE sheddase activity in ranges of low, intermediate, and high concentrations of recombinant TACE and cell lysates, and was highly precise. It was done in 96-well plates, using multichannel pipettes, which enabled high throughput testing of a large number of samples in a short period of time, and was easy to perform. In addition, the assay showed low intraexperimental and interexperimental variation, and was reproducible.

**TACE Sheddase Activity Is Increased in HNC.** Using the modified TACE sheddase assay, we determined that the lysates of all tested HNC cell lines had significantly higher TACE activity than that of normal keratinocytes, and the lysates of cell lines derived from HNC metastases and the PCI-13-26 clone had significantly higher TACE sheddase activity than that of cell lines derived from normal keratinocytes and HNC primary tumor tissues.
from primary tumors or the uncloned PCI-13 cell line, respectively (Fig. 3A). These data were similar to those of total TACE protein expression assessed by ELISA (Fig. 1C). The median (17.9) and range (3.2-57.8) values of TACE activity units in HNC tissues were substantially higher than the median (5.2) and range (0.2-18.1) values of TACE activity units in normal oral mucosa tissues (Fig. 3B). The difference between the medians of TACE activity units in HNC tissues were substantially higher than the median (5.2) and range (0.2-18.1) values of TACE activity units in normal oral mucosa tissues (Fig. 3B). The difference between the medians

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**Figure 1.** HNC expresses increased levels of TACE protein. A. Western blot of lysates of cultured normal keratinocytes (keratinocytes) and HNC cell lines (PCI-4A, PCI-4B, PCI-15A, PCI-15B, PCI-13, and PCI-13-26). B. Western blot of lysates of normal oral mucosa (normal tissues, lanes 1-4) and HNC tissues (HNC tissues, lanes 5-8). Western blot was done using anti-TACE (top rows) and anti–β-actin (bottom rows) antibodies. C. TACE ELISA of lysates of cultured normal keratinocytes (tested three different lines) and HNC cell lines. Columns, mean from three experiments of TACE nanograms per 10 micrograms of total cell lysate proteins (TACE ng/10 μg); bars, SD. *, significant increases in TACE expression in HNC cell lines relative to normal keratinocytes; **, significant increases of TACE in HNC metastatic cell lines and PCI-13-26 clone compared with primary tumor cell lines and PCI-13 cell line, respectively. D. TACE ELISA of lysates of fresh normal oral mucosa tissues (normal) and fresh HNC tissues (HNC). Data are from one of three assessments of the tissue samples, each resulting in similar results. Results are presented as medians, quartiles, and ranges of TACE nanograms per 10 μg of tissue lysate total proteins (TACE ng/10 μg). E to H. Immunohistochemistry. In normal mucosa, low level of TACE is selectively expressed in epithelial basal layer and suprabasal layer cells and in rare lamina propria fibroblasts (E-F). In HNC tissues, high level of TACE is selectively expressed in cancer cells (G-H). H&E (E and G). Light microscopy photomicrographs and fluorescent anti-TACE antibody (F and H). Fluorescence microscopy photomicrographs staining. The presented photomicrographs were taken using ×125 magnification. ep, epithelium; lp, lamina propria; cc, cancer cells; ts, tumor stroma; bv, blood vessel; the bars are 100 μm long.
was 3.4-fold, and highly statistically significant \( (P < 0.0001; \text{Fig. 3B}) \). In addition, the specific TACE activity per nanogram of TACE protein showed significantly higher levels \( (P = 0.0046; \text{Fig. 3C}) \) in HNC tissues relative to normal tissues. TACE ELISA discriminated HNC from normal tissues with 90.3% sensitivity and 75.0% specificity, based on the ROC differentiation area of 89.7% and optimal cutoff of 0.24 TACE ng \( (P < 0.0001) \).
Similarly, the TACE sheddase activity assay discriminated HNC from normal tissue with 77.8% sensitivity and 93.7% specificity, resulting from the ROC differentiation area of 91.6% and optimal cutoff of 10.9 TACE activity units \( (P < 0.0001) \). Based on its larger ROC differentiation area, it could be predicted that the TACE activity assay would better discriminate HNC from normal tissue than the TACE ELISA.

These findings show that HNC cells and tissues have not only highly increased TACE protein expression, but also a significantly and more consistently higher TACE sheddase activity relative to normal cells and tissues.

**Increased TACE in HNC Is Biologically Relevant.** To determine the biological relevance of increased expression and activity of TACE in HNC, we examined the effect of TACE silencing by TACE siRNA on the shedding of TACE-dependent cancer growth factors amphiregulin and TGF-\( \alpha \), and cancer protecting factors TNFR1 and TNFR2 by HNC cells. The siRNA transfection was done with a consistently high efficiency of 78% to 88%, as determined by PCI-13 cell cotransfection with TACE siRNA and siGLO fluorescent probe and flow cytometry (Supplementary Fig. S3). The silencing of TACE (Fig. 2D and E) resulted in a significant reduction of shedding of amphiregulin (84%; Fig. 4A), TGF-\( \alpha \) (36%; Fig. 4B), TNFR1 (84%; Fig. 4C), and TNFR2 (78%, Fig. 4D). These findings indicate that shedding of tumorogenic and tumor protective molecules is a function of TACE in HNC cells.

**Increased TACE Activity Is Associated with HNC Recurrence.** The increased TACE activity in cancer tissues might contribute to growth and survival of cancer cells and therefore growth and recurrence of HNC tumors. We tested this possibility by correlating TACE protein expression and TACE sheddase activity with size, likelihood to recur (Fig. 5A and B), and lymph node metastases of HNC primary tumors. We found that both TACE protein and TACE sheddase activity levels were higher in T3/T4 \( (A2) \) than in T1/T2 \( (A1) \) primary tumors. However, the differences were more prominent and only statistically significant for TACE activity \( (P = 0.05) \), but not for TACE protein expression \( (P = 0.14) \). T1/T2-T3/T4 primary tumors that developed recurrences after initial therapy \( (R1-2) \) showed significantly increased levels of TACE sheddase activity \( (P = 0.0001) \), but not of TACE protein \( (P = 0.15) \) relative to T1/T2-T3/T4 primary tumors that did not develop recurrences \( (NR1-2) \). Recurrent tumors \( (R3) \) had significantly higher TACE protein and more...
prominently higher TACE activity levels than T1/T2 (A1) and NR1-2 primary tumors (TACE protein, Fig. 5A: \( P = 0.0058 \) and \( P = 0.026 \); TACE activity, Fig. 5B: \( P < 0.0001 \) and \( P = 0.0008 \), respectively). In addition, TACE sheddase activity, but not TACE protein expression, was significantly higher in R3 than in T3/T4 (A2) primary tumors (TACE protein, Fig. 5A: \( P = 0.21 \); TACE activity, Fig. 5B: \( P = 0.042 \)). In sharp contrast, neither TACE protein nor TACE sheddase activity differed in R1 versus R2, and in R1 or R2 versus R3 tumors; and in NR1 versus NR2 primary tumors indicating their biological similarity. Kaplan-Meier hazard assessment of tumor recurrence showed that the risk of tumor recurrence was significantly greater (\( P < 0.0001 \)) in HNC patients whose primary tumors had >17.5 TACE activity units per 100 μg of tissue lysate protein than in patients whose primary tumors had <17.5 (Fig. 5C). Moreover, Cox regression analysis showed without cutoff that the risk of tumor recurrence was dependent in HNC patients on the levels of TACE activity in primary tumors (\( P = 0.004 \); odds ratio, 1.068; confidence interval, 1.021-1.117; and increase in recurrence risk of 6.8% for the increase of TACE activity units by 1). It also appeared that primary tumors with higher TACE activity had a higher likelihood of earlier recurrence than those having lower TACE activity. ROC curve analysis, using 83.4% differentiation area and optimal cutoff of 17.5 TACE activity units, showed that the TACE sheddase activity assay was able to discriminate R1-2 from NR1-2 primary tumors with the 92.3% sensitivity and 77.4% specificity (\( P < 0.0001 \)). The cutoff and related sensitivity and specificity defined by ROC curve were very similar to those defined by the more commonly used median-based method (17.1%, 92.9%, and 67.7%, respectively).

Fifty-two percent of NR1-2 primary HNC tumors, which had lower TACE activity and did not recur, were associated with lymph node metastases. In contrast, 71% of R1-2 primary HNC tumors, which had higher TACE activity and recurred after initial treatment, were associated with lymph node metastases. These data show that more R1-2 primary tumors than NR1-2 primary tumors are associated with both increased TACE activity and lymph node metastases. In the context of our findings that the metastasis-originated HNC cells have increased TACE activity relative to the primary tumor-originated HNC cells, the results indicate that the increases in TACE activity in primary tumors may be related to lymph node metastases. However, Kaplan-Meier hazard analysis showed that, in sharp contrast to TACE activity, lymph node status (\( P = 0.219 \)), but not TACE protein levels (\( P = 0.075 \)) and size (\( P = 0.152 \)) of primary tumors, were not risk factors of tumor recurrence. Similarly, multiparametric Cox regression analysis showed that only TACE sheddase activity (\( P = 0.001 \); odds ratio, 1.110; confidence interval, 1.042-1.182, increase in recurrence risk of 11% for the increase of TACE activity units by 1), but not TACE protein level (\( P = 0.389 \); odds ratio, 0.324; confidence interval, 0.265-0.4203), size (\( P = 0.390 \); odds ratio, 0.773; confidence interval, 0.430-1.390), and/or lymph node status (\( P = 0.152 \); odds ratio, 2.476; confidence interval, 0.716-8.566) of

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Figure 4. Silencing TACE in HNC cells leads to decreased production of TACE-dependent cancer growth (A and B) and protecting (C and D) factors. TACE siRNA transduction induces suppression of PCI-13 HNC cell ability to release the cancer growth factors amphiregulin (AR; A) and TGF-α (B) and the cancer protecting factors TNFR1 (C) and TNFR2 (D). Forty-eight hours after TACE siRNA transduction into PCI-13 cells, the cell culture conditioned media were assessed by ELISA for the presence of soluble amphiregulin, TGF-α, TNFR1, and TNFR2. Data are from a representative experiment of two to five similar experiments done. Columns, means of triplicates of ng of amphiregulin, TGF-α, TNFR1, or TNFR2 per 1 mL of cell culture conditioned media of 10⁶ PCI-13 HNC cells (ng/mL /10⁶ cells); bars, SD. *, significant decrease of a soluble product of PCI-13 cells following TACE siRNA treatment relative to control siRNA treatment.
primary tumors, was risk factor of tumor recurrence. These findings indicate that TACE activity is an independent prognostic factor that excludes other tested factors. These novel observations show the biological and clinical association of TACE sheddase activity and, to a lesser extent, TACE protein expression in HNC, and indicate that increased TACE activation could play a role in HNC recurrence, and could be potentially a discriminative biomarker of the likelihood of HNC tumors to recur after initial treatment.

Discussion

HNC is a life-threatening malignancy with a 50% mortality rate, which affects >25,000 persons per year in the United States (29). HNC is characterized by destructive/mutilating local growth and tissue invasion, and frequent development of regional lymph node metastases, but rare development of distant metastases. The major medical problem in HNC is local recurrence that occurs following the initial therapy of primary tumors (30). Reliable identification of aggressive tumors would be of a great importance for prognosis and effective treatment of HNC as well as other cancers. Recent studies have suggested that increased quantity and/or activity of EGFR and MMP-9 in cancer tissues and plasma might be significant biomarkers of shortened relapse-free survival in HNC patients. However, no validated biomarkers are currently available to discriminate aggressive from nonaggressive tumors (1-4).

Our study shows that tissue levels of both TACE sheddase activity and TACE protein are highly increased in HNC and can distinguish HNC from normal oral mucosa. More importantly, this study also shows that increased TACE sheddase activity in primary tumors indicates a significantly increased risk of cancer recurrence. The increased TACE activity is also a specific and sensitive biomarker that can discriminate between primary tumors that will recur after initial treatment and primary tumors that will not recur. The Kaplan-Meier analysis and Cox multivariate analysis indicated that only TACE sheddase activity assay, but not TACE ELISA, tumor size, or lymph node status of primary tumors, could significantly predict the risk of HNC recurrence. These findings suggest that TACE sheddase activity is a promising new cancer biomarker that could be developed into a reliable and clinically useful marker for accurate early discrimination of aggressive from nonaggressive HNC, and perhaps other cancers. Such a biomarker would enable clinicians to make an early precise prediction of the outcome of cancer, and timely choose and apply the most appropriate treatment for a given patient.

The increased TACE sheddase activity in cancer tissues reflects the cancer ability to release increased levels of cancer growth and protective factors and, consequently, the increased ability of cancer to grow and recur. The increased EGFR expression in cancer tissues indicates the cancer increased ability to respond to TACE-generated cancer growth factors, and consequently, the cancer increased ability to grow. The increased MMP-9 activity reflects the increased cancer ability to cleave extracellular matrix components and, consequently, the increased ability of cancer to invade surrounding tissues and generate metastases. Although it is unknown at this time which of these cancer biomarkers will prove to be the most clinically useful for cancer prognosis, they are all highly promising and might be complementary. Thus, the individual

![Figure 5.](image)

Figure 5. Highly increased TACE sheddase activity in primary HNC tumors is associated with their potential to recur. Results of HNC tissues presented in Fig. 1D (TACE ELISA) and Fig. 3B (TACE sheddase activity) were classified according to the pathologic stages. Levels of TACE protein (A) or TACE sheddase activity (B) were compared in all T1/T2 primary tumors (A1), all T3/T4 primary tumors (A2), T1/T2-T3/T4 primary tumors that did not recur after initial therapy (NR1-2), T1/T2-T3/T4 primary tumors that recurred after initial therapy (R1-2), and recurrent tumors (R3). The results are presented as medians, quartiles, and ranges of TACE ng and TACE activity units per 10 μg of total tissue proteins (A, TACE ng/10 μg; B, TACE activity units/10 μg, respectively. Statistical significance of data differences is shown. C, Kaplan-Meier probability analysis of tumor recurrence shows the cumulative time-dependent appearance of recurrent tumors following treatment of primary tumors containing <17.5 (broken line) or >17.5 (continuous line) TACE activity units per 10 μg of HNC tissue lysate proteins. P value in C is based on the presented Kaplan-Meier curve data.
and, perhaps more effectively, simultaneous analyses of two or more of these biomarkers might lead to the development of reliable prognostic tests for HNC, and possibly other epithelial cancers. Large, prospective clinical studies of the multiple biomarkers on the same groups of cancer patients using multivariate regression analyses will be needed to determine these possibilities (31).

TACE sheddase activity assay is a high-throughput, highly sensitive, and highly specific assay that can be easily scalable for assessment of large numbers of clinical samples. The assay could be done with relatively small quantities of tissue samples (i.e., 50 mg) that can be obtained by surgical or core-needle biopsy of tumors. Therefore, the assay could be applicable in the large-scale, routine clinical testing of tumor samples.

In summary, our study is the first to investigate TACE sheddase activity in cancer tissues and to show increased TACE sheddase activity in HNC relative to normal tissues, and high increases of TACE sheddase activity in primary HNC tumors likely to recur relative to those unlikely to recur. These novel findings could lead to the validation of TACE sheddase activity as a clinically valuable cancer biomarker, to discriminate aggressive from nonaggressive cancer, and to identify patients with aggressive cancer who may benefit from therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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