Published in final edited form as:

Head Neck. 2013 March ; 35(3): 388–398. doi:10.1002/hed.22968.

Suppression of natural killer-cell and dendritic-cell apoptotic tumoricidal activity in patients with head and neck cancer

Dejan Baskic, MD, PhD^{1,2,3}, Lazar Vujanovic, PhD^{2,4}, Nebojsa Arsenijevic, MD, PhD³, Theresa L. Whiteside, PhD^{1,5,6}, Eugene N. Myers, MD⁶, and Nikola L. Vujanovic, MD, PhD^{1,3,5,*}

¹Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania

²University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania

³University of Kragujevac Department of Microbiology and Immunology, Kragujevac, Serbia

⁴Department of Medicine, Kragujevac, Serbia

⁵Department of Immunology, Kragujevac, Serbia

⁶Department of Otolaryngology, Kragujevac, Serbia

Abstract

Background—Natural killer (NK) cells and dendritic cells (DCs) mediate tumor cell apoptosis using tumor necrosis factor superfamily ligands (TNFSFLs). This cytotoxicity is an important anticancer immune defense mechanism.

Methods—We examined TNFSFL expression and apoptotic tumoricidal activity (ATA) of purified NK cells and DCs, and peripheral blood mononuclear leukocytes (PBMLs) of healthy individuals and patients with head and neck cancer (HNC) before and after cancer ablation.

Results—PBMLs, NK cells and DCs, but not NK-cell/DC-depleted PBMLs, expressed multiple TNFSFLs and mediated ATA. Both TNFSFL expression and ATA were suppressed in tumor-bearing, and restored in tumor-ablated patients with (HNC) Soluble TNF superfamily receptors (solTNFSFRs) were increasingly bound by PBNLs of tumor-bearing HNC patients. Dissociation of solTNFSFR led to more pronounced increases in TNFSFL expression and ATA of PBMLs of patients with HNC than healthy individuals.

Conclusion—NK-cell and DC TNFSFL expression and ATA are suppressed in patients with HNC. This suppression is tumor-dependent and possibly mediated by solTNFSFRs.

Keywords

TNF superfamily ligands; TNF superfamily receptors; innate immunity; cancer immunosuppression

INTRODUCTION

Natural killer (NK) cells and dendritic cells (DCs) are major effector cells of the innate immune system that rapidly recognize and eliminate microbial pathogens and abnormal

cells, and induce and regulate adaptive immune functions.¹⁻⁴ An essential common feature of NK cells and DCs is potent tumoricidal activity.⁵⁻⁹ Freshly isolated resting NK cells express the cell necrosis-mediating perforin in the lysosomal granules and the proapoptotic (apoptosis-mediating) transmembrane tumor necrosis factor superfamily ligands (TNFSFLs) tumor necrosis factor (TNF), lymphotoxin (LT) $\alpha 1\beta 2$, and FasL on the plasma membrane. Using these functionally different cytotoxic molecules, NK cells mediate 2 distinct mechanisms of cancer cell killing, the secretory/necrotic and nonsecretory/apoptotic killing, respectively.^{5,6} In contrast to NK cells, DCs do not express the cell necrosis-inducing molecule, but express on their plasma membrane the TNFSFL TNF, LT $\alpha 1\beta 2$, FasL, and TNF-related apoptosis-inducing ligand (TRAIL), and mediate only nonsecretory/apoptotic killing of cancer cells.^{7,8} The NK-cell necrotic cytotoxic mechanism is triggered by a balanced interaction of the killer cell inhibitory and activating receptors with the target cell major histocompatibility complex (MHC) class I molecules and MHC-like ligands, respectively. The ligand-receptor interactions lead to polarized secretion of NK-cell lysosomal granules and perforin release into effector-target cell contact area, insertion of perforin, and pore formation in target-cell plasma membrane. Subsequently, calcium influx, swelling, and lysis of target cells occur. The necrotic tumoricidal activity efficiently mediates lysis of only rare leukemia cell targets,^{1,5} and has a limited and selective anticancer function. The apoptotic cytotoxic mechanism is mediated by the simultaneous engagements of multiple NK-cell and DC proapoptotic plasma-membrane transmembrane TNFSFLs with the corresponding target-cell tumor necrosis factor superfamily receptors (TNFSFRs). It efficiently induces apoptosis in virtually all types of malignant cells, and likely has a broad anticancer function. Therefore, the apoptotic tumoricidal activity may represent an important anticancer immune surveillance mechanism, which impairment might be required for cancer development and progression.⁵⁻⁹

Published studies indicate that NK cells and DCs of cancer hosts are functionally suppressed. Thus, peripheral blood NK cells of patients with cancer undergo spontaneous apoptosis and have low necrotic tumoricidal activity.¹⁰⁻¹² Similarly, DCs of tumor-bearing animals and patients display severe phenotypic and functional abnormalities,^{13,14} including immature phenotype lacking CD80 costimulatory molecule and CD83 maturation marker, as well as decreased abilities to secrete interleukin-12 and prime type-1 T-cell response.¹⁵⁻²¹ Soluble TNFSFRs, which are capable of binding to and downregulating TNFSFLs (mediators of apoptotic tumoricidal activity), have been found to be produced at high levels by cancer cells and increased in peripheral blood of patients with cancer.²² The increased solTNFSFRs could suppress apoptotic tumoricidal activity. However, no published information is available on the TNFSFL-mediated apoptotic tumoricidal activity and its potential downregulation in patients with cancer.

In the present study, we show that in peripheral blood of not only healthy individuals but also patients with HNC NK cells and DCs are the main immune cells that inherently express on plasma membrane multiple proapoptotic transmembrane TNFSFLs and mediate apoptotic tumoricidal activity. The expression of TNFSFLs and apoptotic tumoricidal activity are suppressed in tumor-bearing, restored in a proportion of tumor-ablated, and the suppressions correlate with clinical stages and aggressiveness of cancer in patients with HNC, indicating that these suppressions are tumor-dependent. Our data also suggest that a cause of the immune suppression could be solTNFSFRs.

MATERIALS AND METHODS

Patients with HNC and healthy individuals

Fifty-one newly diagnosed patients with HNC were enrolled in this study before anticancer therapy (Table 1). They were 23 to 87 years old and had histologically diagnosed head and

neck squamous cell carcinoma. Twenty of these patients were examined both before and 2 months after surgical ablation of cancer. These patients did not have any additional anticancer therapy during the 2-month period after surgery or before the second testing. All patients were clinically followed for 45 to 78 months (mean, 63.6; median, 64.0). Thirty-one healthy individuals, 50 to 78 years old, were recruited as controls. All the investigated subjects signed the University of Pittsburgh Review Board–approved informed consent and were fully informed about their examination.

Reagents and kits

The following reagents were used in this study: unconjugated mouse antibodies to extracellular private domains of human TNF, TRAIL (R&D Systems, Minneapolis, MN), and FasL (Caltag Laboratories, Philadelphia, PA); fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies to human CD3, CD56, CD19 (BD Pharmingen, San Diego, CA), and CD14 (Caltag Laboratories); CyCr-conjugated mouse antibody to human HLA-DR; Cy5 and phycoerythrin (PE)-conjugated mouse antibody to human CD56; corresponding unconjugated and conjugated non-reactive isotype control monoclonal antibodies (BD Pharmingen); biotin-conjugated goat anti-mouse immunoglobulin antibodies; PE-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA); and magnet-activating cell sorting human DC and NK cell isolation kits (Miltenyi Biotec, Auburn, CA).

Isolation and storage of peripheral blood mononuclear leukocytes

Peripheral blood mononuclear leukocytes (PBMLs) were isolated from 30 to 50 mL of heparinized blood of patients with HNC and healthy individuals using Ficoll-Hypaque density gradient centrifugation, suspended in a solution of 10% dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA) and 90% fetal calf serum (FCS; Life Technologies, Grand Island, NY), and stored in liquid nitrogen.

Purification of dendritic cells and natural killer cells

DCs and NK cells were purified from PBMLs as >92% pure populations of CD3[−]CD14[−]CD19[−]CD56[−] HLA-DR⁺CD11c⁺CD141^{bright} and CD3[−]CD14[−]CD19[−] HLA-DR[−]CD56⁺ cells, respectively, by a 2-step method using magnet-activating cell sorting isolation kits (Miltenyi Biotec) as follows. First, PBMLs were labeled with magnetic beads coated with anti-CD304, anti-CD141, and anti-CD11c monoclonal antibodies, and positively selected using a magnetizing column. Cells retained in the magnetizing column were highly enriched DCs. Second, NK cells were purified from the remaining unlabeled cells. These cells were labeled with biotin-conjugated antibodies specific for T cells, B cells, DCs, monocytes, granulocytes and erythrocytes, and magnetic beads coated with anti-biotin antibody, and negatively selected using a magnetizing column.

Purified DCs, NK cells, and DC/NK cell-depleted PBMLs (T cells, B cells, and monocytes) were tested for purity by 2-color flow cytometry using FITC-conjugated anti-CD3, anti-CD56, anti-CD14 and anti-CD19, and CyCr-conjugated anti-MHC class II (DC panel), and FITC-conjugated anti-CD3 and PE-conjugated anti-CD56 antibodies (NK cell panel). DC/ NK cell depleted PBMLs consistently contained <1% DCs or NK cells.

Tumor cell lines

Human K562 myeloid leukemia and PCI-13 HNC cell lines were cultured in Roswell Park Memorial Institute 1640 and Dulbecco's modified Eagle's media, respectively, supplemented with 10% FCS (Life Technologies). K562 cells were grown as single-cell suspensions and used in experiments when they were in the log phase of growth. PCI-13

cells were grown as adherent cells and used in experiments when they were 70% confluent. Both cell lines were mycoplasma free.

Cytotoxicity assays

Perforin-mediated necrotic tumoricidal activity of NK cells was measured using the standard cell necrosis-specific 4h ^{51}Cr release cytotoxicity assay against K562 cell targets, as previously described.⁵ TNFSFL-mediated apoptotic tumoricidal activity of NK cells and DCs was measured using the apoptosis-specific 2h methyl thiazolyl-tetrazolium (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 1h [^3H]thymidine release assays against TNFSFL-mediated apoptotic tumoricidal activity susceptible and perforin-mediated necrotic tumoricidal activity resistant PCI-13 HNC cell targets, as previously described.^{5,7} Data are presented as lytic unit (LU)_{20/10⁷} effector cells that are calculated from means of triplicate percentages of killing obtained in 30:1, 10:1, 3:1, and 1:1 effector to target cell ratios.

Release of soluble tumor necrosis factor superfamily receptors from peripheral blood mononuclear leukocytes

PBMLs (5×10^7) were washed twice in ice-cold 1× phosphate-balanced salt solution (PBS), resuspended in 200 μL of ice-cold 5× PBS, and incubated for 10 seconds. PBMLs were immediately pelleted by 15-second centrifugation at 800 g. One hundred μL of cell-free supernatants were collected, mixed with 400 μL of distilled water, and used to quantify the released solTNFSFRs. The pelleted PBMLs were immediately resuspended and washed in 50 mL ice-cold Roswell Park Memorial Institute 1640 medium and assessed for the expression of TNFSFLs and apoptotic tumoricidal activity.

Measurement of soluble tumor necrosis factor superfamily receptors

Cell-free supernatants derived from 5× PBS-treated PBMLs were assessed for the presence and quantity of TNFR1, TRAILR2, and Fas using specific enzyme-linked immunosorbent assay (Biosource, Sunnyvale, CA; and R&D Systems).

Flow cytometry analysis of plasma membrane-bound tumor necrosis factor superfamily ligands

PBMLs ($2 \times 10^6/\text{mL}$) were suspended in ice-cold fluorescence activated cell sorter (FACS) buffer consisting of PBS supplemented with 1% FCS and 0.1% sodium azide. One hundred μL aliquots of the cell suspension were incubated in the absence or presence of unlabeled primary anti-TNF, anti-FasL, anti-TRAIL, or isotype control antibodies on ice for 60 minutes. The cells were then washed twice in FACS buffer and incubated in the presence of the biotinylated goat anti-mouse immunoglobulin antibodies on ice for 30 minutes. The cells were washed, incubated in the presence of PE-conjugated streptavidin on ice for 30 minutes, and washed again. To demonstrate the expression of TNFSFLs on NK cells and DCs, after the labeling of TNFSFLs, PBMLs were incubated in the presence of anti-CD3-FITC and anti-CD56-Cy5, or anti-HLA-DR-CyCr and FITC-conjugated anti-CD3, anti-CD56, anti-CD19 and anti-CD14 antibodies, respectively, on ice for 30 minutes. Finally, the cells were washed twice in FACS buffer, resuspended and fixed in 1% (w/v) paraformaldehyde solution in PBS, and analyzed by flow cytometry, as previously described.^{6,8}

Statistical analyses

Data were statistically evaluated using the IBM SPSS commercial program package (version 10.0; SPSS, Chicago, IL). The data are reported as individual values, means and medians, quartiles, and ranges. Statistical significances of data were assessed using unpaired and paired *t* tests. The results were considered significantly different when $p < .05$.

RESULTS

Natural killer and dendritic cells are major mediators of apoptotic tumoricidal activity in peripheral blood

We have previously discovered in peripheral blood of healthy individuals that freshly isolated resting NK cells and DCs, but not resting T cells, B cells, and monocytes, express on plasma membrane multiple TNFSFLs and mediate via these ligands apoptotic tumoricidal activity (ATA) against virtually all types of cancer cells, including cultured and freshly isolated HNC cells.⁵⁻⁹ However, activated T cells, B cells, and monocytes also can express multiple TNFSFLs and mediate ATA (unpublished data). The activated immune cells could be present in cancer hosts. We examined whether, in peripheral blood of patients with HNC, ATA is mediated only by NK cells and DCs, or also by some other immune cells. First, we comparatively tested, in 5 independent experiments, cytotoxic activity of whole PBMLs, purified NK cells (>95% CD3⁺CD56⁺), and DCs (>92% lineage marker-MHC class II⁺ or CD11c⁺), and NK cell and DC-depleted PBMLs (<1% of CD3⁺CD56⁺ and lineage marker-MHC class II⁺ or CD11c⁺) of healthy individuals and patients with HNC using the apoptosis specific 2h MTT assay against PCI-13 HNC cell targets. We consistently found that unfractionated PBMLs (Figure 1A) and purified NK cells and DCs (Figure 1B) of both healthy individuals and tumor-bearing patients with HNC mediated significant killing of PCI-13 targets in 2h MTT assays. Purified DCs mediated 2- to 3.5-fold higher killing of tumor cells than purified NK cells (Figure 1B), possibly because DCs kill tumor cells via TNF, FasL, LT- α 1 β 2, and TRAIL, while NK cells do so via TNF, FasL, and LT- α 1 β 2.^{6,8} In sharp contrast to the significant ATA of unfractionated PBMLs and purified NK cells and DCs of both healthy individuals and patients with HNC, NK cell- and DC-depleted PBMLs did not mediate ATA (Figure 1A). In addition, purified NK cells and DCs mediated this activity significantly better than whole PBMLs ($p = .01$ and $p = .001$, respectively). These findings confirm that NK cells and DCs are potent mediators of ATA against cancer cells, and newly demonstrate that NK cells and DCs are the main mediators of this activity among PBMLs not only in healthy individuals but also in patients with HNC. We also found that ATA of purified DCs was significantly suppressed, whereas the activity of PBMLs and purified NK cells showed a trend of suppression in patients with HNC relative to healthy individuals (Figure 1A, B). These data indicate that NK cell-DC ATA is suppressed in patients with HNC and can be investigated using unfractionated PBMLs as effector cells.

Apoptotic tumoricidal activity of peripheral blood mononuclear leukocytes is suppressed in patients with HNC

To determine whether PBML ATA mediated by NK cells and DCs against HNC cell targets was suppressed in patients with HNC, we compared cytotoxic activities of unfractionated PBMLs of healthy individuals (group C) and newly diagnosed, primary tumor-bearing, untreated patients with HNC (group PB) in the apoptosis specific 2h MTT ($n = 28$) and 1h [³H]thymidine release ($n = 7$) cytotoxicity assays against PCI-13 HNC cell targets, and in the cell necrosis-specific 4h ⁵¹Cr release NK cell cytotoxicity assays ($n = 17$) against K562 cell targets (Figure 2). In the apoptosis-specific assays, PBMLs of patients with HNC were significantly less cytotoxic than PBMLs of healthy individuals (Figure 2A; MTT: $p < .0001$; Figure 2B; [³H]thymidine release: $p = .045$). Similarly, in the cell necrosis-specific assays, PBMLs of patients with HNC were significantly less cytotoxic than PBMLs of healthy individuals (Figure 2C; ⁵¹Cr release: $p = .012$). These findings newly demonstrate the suppression of NK cell-DC ATA mechanism and confirm the suppression of NK cell necrotic tumoricidal mechanism¹² in tumor-bearing patients with HNC.

Suppression of apoptotic tumoricidal activity of peripheral blood mononuclear leukocytes is cancer dependent

To assess whether the suppression of NK cell-DC-mediated ATA and NK cell necrotic tumoricidal activity in patients with HNC are induced by or dependent on cancer, we tested these cytotoxic activities of PBMLs obtained from patients with HNC before (group PB), and 2 months after complete surgical ablation of cancer (group PA), and compared that with healthy individuals (group C; Figure 2). Using the apoptosis-specific 2h MTT (Figure 2MTT) and 1h [³H]thymidine release (Figure 2 3HTdR) assays against PCI-13 HNC cell targets, and cell necrosis-specific 4h ⁵¹Cr release assay against K562 cell targets (Figure 2 51Cr), we observed that surgical ablation of cancer led to the significant recovery of PBML ATA in the majority of tested patients with HNC (PB vs PA: MTT, $p = .028$; [³H]thymidine release, $p = .038$), which resulted in the increases of their ATA to similar levels to that of healthy individuals (PA vs C: MTT, $p = .496$; [³H]thymidine release, $p = .191$). In sharp contrast, after surgical ablation of the tumor, these patients did not show significant changes of PBML necrotic tumoricidal activity (⁵¹Cr release: PB vs PA, $p = .149$; PA vs C, $p = .0018$). These data indicate that, in patients with HNC, the suppression of NK cell-DC-mediated ATA is tumor dependent, whereas the suppression of NK cell necrotic tumoricidal activity is mostly tumor independent.

Expression of tumor necrosis factor superfamily ligands on natural killer cells and dendritic cells is decreased in tumor-bearing and restored in tumor-ablated HNC patients

Next, we investigated the mechanism of suppression of NK cell-DC-mediated ATA in patients with HNC. As NK cell-DC ATA is mediated by multiple proapoptotic transmembrane TNFSFLs^{6,8} and suppressed in tumor-bearing and restored in tumor-ablated patients, we investigated whether the tumor dependent modulation of NK cell-DC TNFSFLs could be a cause of suppression of NK cell-DC-mediated ATA in patients with HNC. We examined the expression of TNF, FasL, and TRAIL on plasma membrane of NK cells, DCs, and/or PBMLs of patients with HNC before and/or after surgical ablation of primary tumors, and compared with that of healthy individuals. First, using 3-color flow cytometry of PBMLs, we examined the expression of the ligands on CD3⁺CD56⁺ NK cells and lineage marker-MHC class II⁺ DCs in unfractionated populations of PBMLs of 5 healthy individuals and 5 tumor-bearing patients with HNC. We found that not only in healthy individuals but also in patients with HNC, NK cells expressed TNF and FasL (Figure 3A, B), but not TRAIL (data not shown), while DCs expressed TNF, TRAIL, and FasL (Figure 3C–E). In contrast, the rest of the PBMLs did not express detectable TNFSFLs. These findings confirm in healthy individuals^{6,8} and newly shown in patients with HNC that, among PBMLs, NK cells and DCs are the main cells expressing these 3 TNFSFLs. In addition, we found indications that the expression levels of TNF and FasL on NK cells, and TNF, TRAIL, and FasL on DCs were decreased in tumor-bearing patients with HNC (Figure 3A–E).

Subsequently, we sought to demonstrate that the expression of TNFSFLs on PBMLs is suppressed in patients with HNC and the suppression is tumor dependent. Because, among freshly isolated PBMLs of both healthy individuals and patients with HNC, NK cells and DCs are the main cells expressing multiple TNFSFLs, a simple and highly sensitive single-color flow cytometry of PBMLs was further used to measure the expression and changes of NK cell-DC TNFSFLs. We examined the expression of TNF, TRAIL, and FasL on PBMLs of 13 healthy individuals (average percent: NK cells = 8.9, DCs = 3.3) and 13 patients with HNC before (average percent: NK cells = 10.2, DCs = 2.7) and 12 of these patients after (average percent: NK cells = 9.4, DCs = 4.4) surgical ablation of primary tumors. We found that a small fraction of PBMLs expressed all 3 TNFSFLs, and that the expressions of ligands were substantial in healthy individuals, decreased in tumor-bearing patients, and restored in tumor-ablated patients with HNC to the levels of healthy individuals (Figure 3F–H). The

expressions of TNF and FasL were significantly decreased, and the expression of TRAIL showed a trend of decrease in tumor-bearing patients with HNC relative to healthy individuals (Figure 4I; TNF: 10/13; $p = .019$; FasL: 11/13; $p = .0013$; and TRAIL: 7/13). In sharp contrast, the expression of FasL was significantly increased (9/12; $p = .041$), and the expressions of TNF and TRAIL showed trends of increases (7/12 and 6/12, respectively) in patients with HNC after surgical ablation of cancer (Figure 3J). These findings show that the expression of ATA-mediating proapoptotic TNFSFLs on plasma membrane of NK cells and DCs is suppressed in patients with HNC and the suppression is tumor dependent.

Soluble tumor necrosis factor superfamily receptors downregulate expression of tumor necrosis factor superfamily ligands and apoptotic tumoricidal activity of peripheral blood mononuclear leukocytes more prominently in patients with HNC than in healthy individuals

Soluble TNFSFRs (solTNFSFRs) are considered to be important regulators of TNFSFLs. They can bind to and downregulate TNFSFLs.²² This is supported by our previous findings that recombinant soluble TNFR1, Fas, and/or TRAILR2 efficiently inhibit ATA of human peripheral blood NK cells and DCs.^{6,8} Although TNFSFRs are produced as transmembrane molecules, they can also be released as soluble molecules by a process termed “ectodomain shedding.”²³ Both normal and cancer cells produce both transmembrane and solTNFSFRs. However, cancer cells produce larger quantities²³ and growing tumors are increasing sources of solTNFSFRs and their potential TNFSFL downregulating activity in cancer hosts. The experimental evidences supporting the notion of the physiological and pathophysiological regulation of TNFSFLs by their soluble receptors are largely missing. Here, we attempted to obtain supports for the role of naturally produced solTNFSFRs in physiological and cancer-dependent regulations of TNFSFLs. Plasma levels of solTNFSFRs have been found to be relatively high in healthy individuals, and enhanced in a tumor-dependent fashion in cancer hosts.^{24–27, unpublished data} Plasma solTNFSFRs could bind to and regulate NK cell-DC proapoptotic transmembrane TNFSFLs in a concentration-dependent fashion. Therefore, we examined whether PBMLs of healthy individuals and patients with HNC bound pertinently different quantities of solTNFSFRs, and whether the bound solTNFSFRs appropriately modulated the expression of TNFSFLs on plasma membrane and ATA of PBMLs. We found that, after their treatment with hypertonic PBS, PBMLs of both healthy individuals and tumor-bearing patients with HNC released significant amounts of soluble TNFR1 (range, 86–438 pg/5 × 10⁷ PBMLs), TRAILR2 (range, 54–141 pg/5 × 10⁷ PBMLs), and Fas (range, 159–887 pg/5 × 10⁷ PBMLs). Higher quantities of the soluble receptors were released by PBMLs of patients with HNC than PBMLs of healthy individuals (TNFR2: means = 218 vs 160 pg; TRAILR2: means = 87 vs 67 pg; Fas: means = 510 vs 370 pg, respectively; Figure 4A). In addition, the treatment with hypertonic PBS resulted in more consistent and higher increases in TNFSFL expression on PBMLs and PBML-mediated ATA of patients with HNC than healthy individuals (Supplemental Figure 1; TNF: 6/6, average 587 mean fluorescence intensity [MFI] vs 4/6, average 18 MFI; TRAIL: 5/6, average 94 MFI vs 4/6, average 44 MFI; FasL: 5/6, average 229 MFI vs 4/6, average 5 MFI; MTT: 5/5, average 292 LU, $p < .05$ vs 5/5, average 280 LU, $p < .05$, respectively). The increases occurred in PBMLs of patients with HNC at significantly higher levels than in PBMLs of healthy individuals (Figure 5B, C; TNF: $p = .004$; TRAIL: $p = .018$; MTT: $p = .018$). These findings provide first indications that naturally produced solTNFSFRs could regulate the expression and functions of TNFSFLs under physiological and pathophysiological conditions. They also suggest that the increases in amounts of solTNFSFRs bound to transmembrane TNFSFLs of NK cells and DCs could be a cause of the downregulations of PBML TNFSFLs expression and ATA in patients with HNC.

Suppressions of apoptotic tumoricidal activity and expression of tumor necrosis factor superfamily ligands of peripheral blood mononuclear leukocytes are associated with cancer burden and likelihood of recurrence

Our findings of the tumor-dependent suppression of PBML ATA and TNFSFL expression in patients with HNC suggest that these suppressions could reflect cancer burden and aggressiveness and could be predictive of cancer outcome. We tested this possibility by correlating PBML ATA and TNFSFL expression with clinical stages (primary tumor size and lymph node metastases) and cancer recurrence in patients with HNC (Figure 5A–D). We also correlated PBML-mediated ATA of patients with HNC before surgical ablation of primary tumors with cancer-caused death (dead of disease) and survival without clinical evidence of disease (no evidence of disease; Figure 5E). A trend of increased suppression of PBML ATA (Figure 5A) and a significantly increased suppression of TNFSFL expression ($p = .043$; Figure 5B) were observed in tumor-bearing patients with HNC having large (T2–T4) primary tumors relative to those having small (T1) primary tumors. Similarly, a significantly increased suppression of PBML ATA (Figure 5A; $p = .009$) and a trend of increased suppression of TNFSFL expression (Figure 5B) were found in tumor-bearing patients with HNC having lymph node metastases relative to those without lymph node metastases. Patients with HNC who developed recurrences had a significantly increased suppression of PBML TNFSFL expression before (Figure 5C; $p = .002$) and a trend of increased PBML ATA suppression after (Figure 5D) surgical ablation of primary tumors relative to patients with HNC who did not develop recurrence. In addition, PBML-mediated ATA before surgical ablation of primary tumors showed a trend of increased suppression in patients with HNC who ultimately died of cancer relative to those who survived cancer-free (Figure 5E). These findings suggest that NK cell-DC ATA and TNFSFL expression in patients with HNC could be biologically significant and potentially clinically relevant.

DISCUSSION

We have previously defined a robust innate anticancer cytotoxic mechanism mediated by healthy individual peripheral blood NK cells and DCs via multiple transmembrane TNFSFLs.^{5–9} The cytotoxic activity has been defined and measured by several cell apoptosis-specific assays, including: (1) 2h MTT, DiOC₆ or rhodamine 123 staining of mitochondria; (2) 1h [³H]thymidine release, terminal deoxynucleotidyl transferase dUTP nick end labeling, or DNA laddering of the fragmented DNA; (3) transmission electron microscopy; (4) annexin V binding to the plasma membrane externalized phosphatidylserine; (5) nuclear matrix protein (N μ MA) release; and (6) caspase activation assessment. In sharp contrast, the cytotoxic mechanism could not be detected by the cell necrosis specific assays 4h ⁵¹Cr release or propidium iodide staining.^{5,7} The cytotoxic mechanism has been therefore considered as apoptotic. It induces efficiently (in low effector to target cell ratios) high levels of cell deaths in large variety of cancer cell types, including HNC cultured and fresh tumor cells, and in normal proliferating vasculature endothelial cells, but not in other normal cells.^{5,7} Therefore, this tumoricidal activity seems to be an essential anticancer defense mechanism, which might be capable of effectively protecting organisms from cancer development and growth. Our previous studies have also demonstrated that healthy individual freshly isolated peripheral blood resting T cells, B cells, and monocytes do not mediate this cytotoxic mechanism.⁵ The present study shows that, in peripheral blood of not only healthy individuals but also of patients with HNC, this cytotoxic mechanism is mediated by NK cells and DCs, but not by other PBMLs such as resting T cells, B cells, and monocytes; and is suppressed in patients with HNC in a cancer-dependent manner. The suppression of the anticancer cytotoxic mechanism is enhanced in patients with HNC with the advanced disease and aggressive cancer, capable of recurrence, and causing death. The study also provides indications that this immunosuppression could

be mediated by increased binding of solTNFSFRs to NK cell and DC transmembrane TNFSFLs.

The conventional NK cell necrotic tumoricidal activity represents another potent anticancer innate immune function and a significant part of the anticancer immune surveillance mechanism.^{1,28–30} However, the necrotic tumoricidal activity has relatively low efficiency and is capable of killing only rare leukemia cell types. Therefore, it could only protect against rare hematological malignancies. As transmembrane TNFSFL-mediated ATA kills a wide range of both solid tumor and leukemia cell types with a high efficiency, it is likely the major anticancer immunosurveillance mechanism that protects against most malignancies.^{5,7}

Our present study indicates that not only transmembrane TNFSFL-mediated NK cell-DC ATA, but also perforin-mediated NK cell necrotic tumoricidal activity are suppressed in patients with cancer. However, although the suppression of ATA seems to be cancer-dependent and induced by growing cancer and/or related changes in cancer host, the suppression of necrotic tumoricidal activity seems to be cancer-independent and, perhaps, constitutive or induced by environmental factors such as smoking, alcohol consumption, and human papillomavirus infection, to which patients with HNC are commonly exposed.

The immune system in general, and NK cells and DCs in particular, are suppressed in cancer hosts.^{10–19} The cancer-related immunosuppression is probably an important mechanism of cancer escape from immune surveillance. Multiple mechanisms of the immunosuppression have been defined.^{31–35} Cancer cells and normal cells also produce solTNFSFRs, which can bind to and mask the expression and block the activity of TNFSFLs.^{23,36} Here, we provide the indications that this immunoregulatory mechanism could be operative in both healthy individuals and patients with HNC and could be enhanced in patients with HNC. Due to different quantities of solTNFSFRs in peripheral blood of healthy individuals and cancer hosts, the mechanism could differently down-regulate transmembrane TNFSFL-mediated NK cell-DC ATA, and prevent damage of normal cells and enable cancer escape from immune control, respectively. This is supported by findings that soluble TNFR1, TRAILR2, and/or Fas are present at substantial and increased concentrations in peripheral blood plasma^{24–27, unpublished data} and in association with PBMLs of healthy individuals and tumor-bearing patients with HNC, respectively. In addition, NK cell-DC ATA and transmembrane TNFSFL expression are decreased in tumor-bearing patients with HNC and restored to the levels of healthy individuals by dissociation of solTNFSFRs from PBML plasma membrane.

Generation of solTNFSFRs is mediated by the enzymatic cleavage of transmembrane TNFSFRs via sheddase activity of ectoenzymes such as tumor necrosis factor- α converting enzyme (TACE).³⁶ It is well known that TACE cleaves transmembrane TNFR1 and TNFR2 and produces their soluble forms.^{23,36} Similar mechanisms mediate the production of soluble TRAILR2 and Fas.²² Cancer cells have increased TACE sheddase activity and consequently produce increased amounts of solTNFSFR leading to increases of solTNFSFR levels in peripheral blood of cancer hosts^{23,24–27} (data not shown). Therefore, the increased levels of solTNFSFRs in the blood plasma and in association with PBMLs of cancer hosts, and resulting cancer-dependent suppressions of NK cell-DC expression of trans-membrane TNFSFLs and their ATA might be caused by the increased sheddase activity of TACE and probably some other similar enzymes in cancer cells, and consequently increased release of solTNFSFRs.

Our findings of the association of PBML suppressions of transmembrane TNFSFL expression and ATA with cancer burden and recurrence in patients with HNC indicate the potential biologic and clinical relevance of the tumor-induced immunosuppression. These findings parallel our recently published findings that the increase in TACE sheddase activity

in cancer tissues of patients with HNC is associated with cancer burden and recurrence too.²³ This further supports the possibility that increased sheddase activity of TACE and perhaps some other similar enzymes in cancer tissues could be the cause of the increased release of solTNFSFRs by tumor tissues and subsequently of the increased immunosuppression mediated by solTNFSFRs in patients with HNC.

In conclusion, we demonstrate for the first time that transmembrane TNFSFL-mediated NK cell-DC ATA is suppressed in patients with HNC in a cancer-dependent manner. We also provide indications that this suppression could be mediated by solTNFSFRs, which are increased in the plasma of patients with cancer. The presented findings and considerations indicate potential biologic and clinical relevance of transmembrane TNFSFL-mediated ATA and its solTNFSFR-mediated regulation, and they further support the possibility that this innate function is an important anticancer immune surveillance mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grant sponsor: This study was supported by research funding from the National Institute of Health grants I-PO DE13059, RO1 DE14775, RO1 DE17150, the University of Pittsburgh Cancer Institute, and the Henry L. Hillman Foundation to N. L. Vujanovic, and P30CA047904 to UPCI Flow Cytometry Facility.

We thank Jennifer Tan for technical assistance.

References

1. Trinchieri G. Biology of natural killer cells. *Adv Immunol.* 1989; 47:187–376. [PubMed: 2683611]
2. Becknell B, Caligiuri MA. Natural killer cells in innate immunity and cancer. *J Immunother.* 2008; 31:685–692. [PubMed: 18779751]
3. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998; 392:245–252. [PubMed: 9521319]
4. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol.* 2003; 3:984–993. [PubMed: 14647480]
5. Vujanovic NL, Nagashima S, Herberman RB, Whiteside TL. Nonsecretory apoptotic killing by human NK cells. *J Immunol.* 1996; 157:1117–1126. [PubMed: 8757616]
6. Kashii Y, Giorda R, Herberman RB, Whiteside TL, Vujanovic NL. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. *J Immunol.* 1999; 163:5358–5366. [PubMed: 10553060]
7. Janjic BM, Lu G, Pimenov A, Whiteside TL, Storkus WJ, Vujanovic NL. Innate direct anticancer effector function of human immature dendritic cells. I. Involvement of an apoptosis-inducing pathway. *J Immunol.* 2002; 168:1823–1830. [PubMed: 11823515]
8. Lu G, Janjic BM, Janjic J, Whiteside TL, Storkus WJ, Vujanovic NL. Innate direct anticancer effector function of human immature dendritic cells. II. Role of TNF, lymphotoxin-alpha(1)beta(2), Fas ligand, and TNF-related apoptosis-inducing ligand. *J Immunol.* 2002; 168:1831–1839. [PubMed: 11823516]
9. Vujanovic NL. Role of TNF family ligands in antitumor activity of natural killer cells. *Int Rev Immunol.* 2001; 20:415–437. [PubMed: 11878511]
10. Melioli G, Semino C, Margarino G, et al. Expansion of natural killer cells in patients with HNC: detection of “noninhibitory” (activating) killer Ig-like receptors on circulating natural killer cells. *Head Neck.* 2003; 25:297–305. [PubMed: 12658734]
11. Bauernhofer T, Kuss I, Henderson B, Baum AS, Whiteside TL. Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer. *Eur J Immunol.* 2003; 33:119–124. [PubMed: 12594840]

12. Mickel RA, Kessler DJ, Taylor JM, Lichtenstein A. Natural killer cell cytotoxicity in the peripheral blood, cervical lymph nodes, and tumor of HNC patients. *Cancer Res.* 1988; 48:5017–5022. [PubMed: 3409231]
13. Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer.* 1997; 73:309–316. [PubMed: 9359474]
14. Mohty M, Jarrossay D, Lafage-Pochitaloff M, et al. Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. *Blood.* 2001; 98:3750–3756. [PubMed: 11739182]
15. Gabrilovich DI, Nadaf S, Corak J, Berzofsky JA, Carbone DP. Dendritic cells in antitumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. *Cell Immunol.* 1996; 170:111–119. [PubMed: 8665591]
16. Gabrilovich DI, Ciernik IF, Carbone DP. Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. *Cell Immunol.* 1996; 170:101–110. [PubMed: 8665590]
17. Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res.* 1997; 3:483–490. [PubMed: 9815709]
18. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res.* 2000; 6:1755–1766. [PubMed: 10815894]
19. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol.* 2001; 166:678–689. [PubMed: 11123353]
20. Orsini E, Guarini A, Chiaretti S, Mauro FR, Foa R. The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. *Cancer Res.* 2003; 63:4497–4506. [PubMed: 12907623]
21. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol Immunother.* 2002; 51:293–298. [PubMed: 12111117]
22. Smith CA, Farrah T, Goodwin RG. The TNF receptors superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell.* 1994; 76:959–962. [PubMed: 8137429]
23. Ge L, Baskic D, Basse P, et al. Sheddase activity of tumor necrosis factor- α converting enzyme is increase and prognostically valuable in HNC. *Cancer Epidemiol Biomarkers Prev.* 2009; 18:2913–2922. [PubMed: 19843672]
24. Gatanaga T, Hwang CD, Kohr W, et al. Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. *Proc Natl Acad Sci U S A.* 1990; 87:8781–8784. [PubMed: 2174164]
25. Shibata M, Takekawa M, Amano S. Increased serum concentrations of soluble tumor necrosis factor receptor I in noncachectic and cachectic patients with advanced gastric and colorectal cancer. *Surg Today.* 1998; 28:884–888. [PubMed: 9744395]
26. Kaminska J, Nowacki MP, Kowalska M, et al. Clinical significance of serum cytokine measurements in untreated colorectal cancer patients: soluble tumor necrosis factor receptor type I—an independent prognostic factor. *Tumour Biol.* 2005; 26:186–194. [PubMed: 16006772]
27. Aderka D, Englemann H, Hornik V, et al. Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients. *Cancer Res.* 1991; 51:5602–5607. [PubMed: 1655258]
28. Vujanovic NL, Basse P, Herberman RB, Whiteside TL. Antitumor functions of natural killer cells and control of metastases. *Methods.* 1996; 9:394–408. [PubMed: 8812692]
29. Whiteside TL, Herberman RB. The role of natural killer cells in immune surveillance of cancer. *Curr Opin Immunol.* 1995; 7:704–710. [PubMed: 8573315]
30. Chikamatsu K, Reichert TE, Kashii Y, et al. Immunotherapy with effector cells and IL-2 of lymph node metastases of human squamous-cell carcinoma of the head and neck established in nude mice. *Int J Cancer.* 1999; 82:532–537. [PubMed: 10404067]

31. Menetrier-Caux C, Montmain G, Dieu MC, et al. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood*. 1998; 92:4778–4791. [PubMed: 9845545]
32. Kiertscher SM, Luo J, Dubinett SM, Roth MD. Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J Immunol*. 2000; 164:1269–1276. [PubMed: 10640740]
33. Rice SQ, Crane IJ, Scully C, Prime SS. Production of a suppressor of lymphocyte proliferation by two human oral carcinoma cell lines. *Scand J Immunol*. 1992; 36:443–452. [PubMed: 1387728]
34. Young MR, Wright MA, Lozano Y, Matthews JP, Benefield J, Prechel MM. Mechanisms of immune suppression in patients with HNC: influence on the immune infiltrate of the cancer. *Int J Cancer*. 1996; 67:333–338. [PubMed: 8707405]
35. Bronte V, Mocellin S. Suppressive influences in the immune response to cancer. *J Immunother*. 2009; 32:1–11. [PubMed: 19307988]
36. Black RA. Tumor necrosis factor-alpha converting enzyme. *Int J Biochem Cell Biol*. 2002; 34:1–5. [PubMed: 11733179]

FIGURE 1.

Natural killer (NK) cells and dendritic cells (DCs) are major apoptotic tumoricidal activity (ATA)-mediating effector cells in peripheral blood. Five experiments were performed, each with a different pair of healthy individual (control [C]), and primary tumor-bearing HNC patient (P) PBMLs. (A) Unfractionated PBMLs, NK cell- and DC-depleted PBMLs (depleted PBMLs, <1% NK cells or DCs), and (B) purified NK cells (NK cells, >95%) and DCs (>92%) were tested for ATA using apoptosis-specific 2h MTT cytotoxicity assay against PCI-13 HNC cell targets. Data are medians, quartiles, and ranges of lytic units (LUs)₂₀/10⁷ effector cells (A, B). The *p* values are statistical significances of differences between Control and Patient data.

FIGURE 2.

PBML-mediated ATA is suppressed in tumor-bearing patients with HNC and the suppression is alleviated by surgical ablation of primary tumors. PBMLs of healthy individuals (controls [C]), tumor-bearing patients with HNC (patients before therapy [PB]) and tumor-ablated patients with HNC (patients after tumor ablation [PA]) were tested using apoptosis-specific 2h MTT and 1h [³H]thymidine release (³HTdR) assays against PCI-13 HNC cell targets, and cell necrosis specific 4h ⁵¹Cr release assays against K562 cell targets. Data are medians, quartiles, and ranges of LU₂₀/10⁷ effector cells (PBMLs). The *p* values are statistical significances of differences of C vs PB, PB vs PA, and C vs PA data.

FIGURE 3.

NK cells and DCs are the main expressers of multiple proapoptotic transmembrane TNFSFs among peripheral PBMLs, and PBML TNFSF expression is decreased in tumor-bearing and restored in tumor-ablated patients with HNC. NK cells (A–B), DCs (C–E), and PBMLs (F–H) of healthy individuals (controls [C] and patients with HNC (patients before [PB] and/or patients after [PA] surgical ablation of primary tumors) were examined for expression of TNFSFs on plasma membrane using flow cytometry. PBMLs were stained with fluorochrome-conjugated antibodies specific to TNF, TRAIL, and FasL, and NK cell and DC markers, or only with fluorochrome-conjugated antibodies specific for TNFSFs and analyzed using 3-color (NK cells and DCs) (A–E) or single-color (PBMLs) (F–H) flow

cytometry, respectively. Representative flow cytometry histograms of NK cells ($n = 5$), DCs ($n = 5$), and PBMLs ($n = 13$) are presented. They show cell-surface expression of TNF and FasL on NK cells (A–B); and TNF, TRAIL, and FasL on DCs (C–E) and unsorted PBMLs (F–H) of a healthy individual C, and PB and/or PA surgical ablation of tumors. Data are mean fluorescence intensity (MFI) obtained with isotype-matched nonreactive control antibodies (isotype, open histograms) and specific antibodies (TNF, FasL, and TRAIL, field histograms). (I, J) Data are medians, quartiles, and ranges of percentage of MFI suppression of PB relative to C, and percentage of MFI increase of PA relative to PB PBML TNFSFL expression, respectively. Percentage of MFI suppression was determined using the formula $\text{PB-C/C} \times 100$, where PB and C were MFI of TNF, TRAIL and FasL of tumor-bearing PB treatment and C PBMLs, respectively. Percentage of MFI increase was determined using the formula $\text{PA-PB/PA} \times 100$, where PA and PB were MFI of TNF, TRAIL and FasL of PBMLs of PB and PA surgical ablation of primary tumor, respectively. The numbers in (I) show the frequency of the suppression in tested patients. The numbers in (J) show the frequency of the increase in tested patients. The p values are statistical significances of differences between MFI of PB and C (I), and MFI of PB and PA (J).

FIGURE 4.

PBMLs of healthy individuals and patients with HNC bind different quantities of solTNFSFRs, which dissociation leads to corresponding increases in transmembrane TNFSFLs expression and ATA. Randomly paired PBMLs of healthy individuals (controls [C]) and tumor-bearing patients (P) with HNC were treated with $5 \times$ PBS, in order to release solTNFSFRs bound to their transmembrane TNFSFLs. After this treatment, (A) solTNFR1, TRAILR2 and Fas were assessed in PBML supernatants using ELISAs. In addition, before and after this treatment, (B) cell surface expression of TNF, TRAIL, and FasL on PBMLs was examined using single-color flow cytometry; and (C) PBML-mediated ATA was tested using 2h MTT assays against PCI-13 HNC cell targets. (A) Data are medians, quartiles, and ranges of picograms per mL (pg/mL) of soluble TNFR1, TRAILR2, and Fas released from 5×10^7 PBMLs obtained from healthy individuals (controls [C]) and patients (P) with HNC.

(B, C) Data are the percentage of MFI increase of TNF, TRAIL, and FasL expression and percentage of cytotoxicity increase, respectively, of C and P PBMLs after treatment with 5× PBS. Percentage of MFI increase and percentage of cytotoxicity increase were determined using the following formula $\frac{AT-BT}{AT} \times 100$, where AT and BT were MFI of TNFSFLs and $LU_{S20}/10^7$ of C and P PBMLs after treatment (AT) and before treatment (BT), respectively. The original data for this calculation are presented in Supplementary Figure 1. Lines connecting C and P individual data indicate control-patient pairs in different experiments. The horizontal bars represent means. The *p* values are statistical significances of data differences between C and P. Numbers in figures show the frequency of treatment-induced higher increases of TNFSFL expression and ATA in patient PBMLs relative to experimentally paired C PBMLs.

FIGURE 5.

Suppressions of PBML ATA and transmembrane TNFSFL expression are associated with tumor burden, lymph node metastases, and likelihood to recur. Data of patients with HNC PBML ATA and combined mean fluorescence intensity (MFI) of TNF, TRAIL, and FasL were classified according to clinical stages of the disease: (A) PBML ATA and (B) PBML TNFSFL MFI in tumor-bearing patients with HNC having T1 or T2 to T4 classification primary tumors, and absence (LN-) or presence (LN+) of lymph node metastases; (C) PBML TNFSFL MFI in tumor-bearing patients with HNC who did not develop (NR) or developed (R) recurrences after surgical ablation of primary tumors; (D) percentage of cytotoxicity changes of PBML ATA, which was determined using the formula $PA-PB/PA \times 100$, where PA and PB were MTT LU₂₀/10⁷ PBMLs of PA and PB primary tumor ablation, respectively, who did not develop (NR) or developed (R) recurrences after the treatment; and (E) PBML ATA in patients with who survived disease-free (no evidence of disease [NOD]) or died from cancer (dead of disease [DOD]) 4 to 6 years after their initial therapy. The results are presented as medians, quartiles, and ranges. The *p* values represent statistical significances of differences of data. LN, lymph node.

TABLE 1

Characteristics and classification of patients and their tumors.

Stage of disease	Age, y	No. of men	No. of women	Total
NR-T1-N0	39–75	5	5	10
NR-T1-N+	42–81	1	1	2
R-T1-N0	47–82	3	1	4
R-T1-N+	52–76	1	1	2
NR-T2/T4-N0	42–80	9	2	11
NR-T2/T4-N+	23–87	5	3	8
R-T2/T4-N0	54	1	0	1
R-T2/T4-N+	50–82	6	1	7
Unclassified	64–73	4	2	6
Total	23–87	35	16	51

Abbreviations: NR-T1, T1 classification primary tumor that did not develop recurrences after initial therapy; R-T1, T1 classification primary tumor that developed recurrences after initial therapy; NR-T2/T4, T2, T3, and T4 classification primary tumor that did not develop recurrences after initial therapy; R-T2/T4, T2, T3, and T4 classification primary tumors that developed recurrences after initial therapy; N0, lymph node without metastases; N+, lymph node with metastases.