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The Immune Signature of CD8⁺CCR7⁺ T Cells in the Peripheral Circulation Associates with Disease Recurrence in Patients with HNSCC

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Abstract

Purpose—Patients with cancer have an increased frequency of circulating apoptosis-sensitive CD8⁺ CCR7^{neg} T cells and few CD8⁺CCR7⁺ T cells versus normal controls. The functional and clinical significance of this imbalance was investigated using peripheral blood of patients with squamous cell carcinoma of the head and neck (HNSCC).

Experimental Design—The frequency of circulating CD8⁺ T cells co-expressing CCR7, CD45RO, CD28, and Annexin V (ANXV) was evaluated in 67 patients and 57 normal controls by flow cytometry. Spearman rank correlations among immunophenotypic profiles were analyzed. Recursive partitioning classified subjects as patients or normal controls based on CD8⁺CCR7⁺ T-cell percentages. Kaplan–Meier plots estimated disease-free survival (DFS).

Results—The CD8⁺CCR7⁺ T-cell frequency was low, whereas that of total CD8⁺CCR7^{neg} and ANXV-binding CD8⁺CCR7^{neg} T cells was higher in patients with HNSCC than in normal controls ($P < 0.001$ – 0.0001). ANXV binding correlated with the absence of CCR7 on CD8⁺ T cells ($P < 0.001$). ANXV binding was negatively correlated with the CD8⁺CD45RO^{neg}CCR7⁺

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No potential conflicts of interest were disclosed.

(T_N) cell frequency ($P < 0.01$) but positively correlated ($P < 0.01$) with that of CD8⁺CD45RO⁺CCR7⁺ (T_{CM}) T cells and of the two CCR7^{neg} subsets (T_{PM} and T_{TD}). In recursive partitioning models, the CD8⁺CCR7⁺ T-cell frequency of 31% distinguished patients from normal controls with 77% to 88% accuracy after cross-validation. In 25 patients tested before any therapy, the CD8⁺CCR7⁺ T-cell frequency of less than 28% predicted disease recurrence within 4 years of definitive therapy ($P < 0.0115$).

Conclusion—The CD8⁺CCR7⁺ T-cell frequency in HNSCC patients' blood tested at diagnosis can discriminate them from normal controls and predicts disease recurrence.

Introduction

One mechanism of tumor evasion in patients with cancer, including those with squamous cell carcinoma of the head and neck (HNSCC), is targeted apoptosis of activated T lymphocytes at the tumor site and in the peripheral circulation (1, 2). The proportion of CD8⁺ T cells undergoing spontaneous apoptosis in the blood of patients with HNSCCs is elevated relative to that in sex- or age-matched normal controls (1). CD8⁺ T cells are more sensitive to apoptosis than CD4⁺ cells, and the tumor-specific CD8⁺ T cells are preferentially targeted for cell death (3–5). In patients with cancer, tumor epitope-specific T cells are eliminated either directly through the Fas/FasL or TRAIL/TRAILR pathways (6, 7) or indirectly through the release of tumor-derived exosomes carrying death receptor ligands (8–10). Antitumor functions of effector CD8⁺ T cells may be also suppressed in cancer through the recruitment of regulatory T cells (11, 12) or myeloid-derived suppressor cells (13).

Earlier studies showed that expression of the chemokine receptor CCR7, which binds CCL19 and CCL21, by CD8⁺ T cells protected them from apoptosis via Akt phosphorylation and increased Bcl-2 expression (9, 14). Although fewer CD8⁺CCR7⁺ than CD8⁺CCR7^{neg} cells bound Annexin V (ANXV), the frequency of circulating CD8⁺CCR7⁺ T cells was significantly reduced in patients with cancer versus normal controls, and CD8⁺CCR7⁺ cells were replaced by an excess of apoptosis-sensitive CD8⁺CCR7^{neg} T cells (14).

Expression of CD45RA/CD45RO and CCR7 on the surface of T cells is generally used as a measure of their differentiation (15, 16). When naïve (T_N) CD8⁺ T cells (CD45RA⁺/RO^{neg}CCR7⁺) interact with antigen-presenting cells (APC) in secondary lymph nodes, they undergo clonal expansion and differentiate into T central memory (T_{CM}) CD45RA^{neg}/RO⁺CCR7⁺ and T peripheral memory (T_{PM}) CD45RA^{neg}/RO⁺CCR7^{neg} cells, also referred to as T effector memory (T_{EM}) cells. The T_{PM} cells migrate to peripheral tissues to mediate effector functions, whereas T_{CM}, which have no or little effector function, home to secondary lymphoid organs and retain the ability to proliferate and differentiate to T_{PM} in response to antigen stimulation (15, 16). The co-stimulatory receptor, CD28, is also used to distinguish subsets of differentiating T cells. Similar to CCR7, it is expressed on T_N and is required for the generation of antigen-specific effector T cells through the interaction with its 2 ligands, B7.1 (CD80) and B7.2 (CD86), on APC. In contrast, antigen-experienced T_{PM} downregulate CD28, and most of the memory T cells, including terminally differentiating T cells (T_{TD}), become CD28^{neg} (17).

Here, all 3 surface markers, CD45RA/RO, CCR7, and CD28, were used to evaluate the frequency of CD8⁺ T_N, T_{CM}, T_{PM}, and T_{TD} cells in the blood of patients with HNSCCs. The propensity of these cells to undergo spontaneous apoptosis was evaluated by ANXV binding. ANXV preferentially binds to phosphatidylserines exposed on the lymphocyte surface, serving as a useful marker of early apoptosis. These immunologic markers were interrogated for their usefulness in discriminating patients with HNSCC from normal

controls. Furthermore, by relating patient outcome after up to 4-year follow-up to the imbalance in CD8⁺CCR7⁺ T cells at the time of diagnosis, we show that the frequency of these T cells in the patients' peripheral blood may serve as the surrogate marker for the disease recurrence.

Materials and Methods

HNSCC patients and healthy volunteers

Samples of venous blood were obtained from 67 patients with HNSCCs seen in the outpatient Otolaryngology Clinic of the University of Pittsburgh Cancer Institute (Pittsburgh, PA) between February 2007 and November 2008. Patients willing to participate in this study were approached by the staff for donating peripheral blood samples. Before phlebotomy, all subjects signed an informed consent approved by the Institutional Review Board (IRB# 96-82). The disease status, treatment history, and the HPV status in the tumor were recorded at the time of the blood draw. The patient cohort included 52 males and 15 females (mean age, 56.6 years). The normal control cohort included 57 age- and sex-matched volunteers (34 males and 13 females; mean age, 53 years). Controls were selected on the basis of criteria specified in the IRB-approved protocol: age ranging from 18 to 80 years at the date of enrollment and no history of cancer at a head and neck site (verified by clinical examination and testimony of clinician or dentist or review of primary medical records). Participants completed an interviewer-administered questionnaire collecting demographic and personal/family cancer history.

Two sequentially enrolled groups of subjects were studied. First, the cohort of 52 patients and 42 volunteers was enrolled and designated as the "study subset." Next, a cohort of 15 patients with HNSCCs and 15 normal controls was designated as the "confirmatory subset" for statistical analyses. Table 1 lists age, sex, and clinicopathologic characteristics of the patients and normal controls in both subsets.

Twenty-nine of 67 patients with HNSCCs had an active disease at the time of phlebotomy: 23 were untreated and the 6 with recurrent disease were previously treated with the standard-of-care therapies. Among 38 patients with no evident disease (NED), 26 had tumor resection and 22 of these also received radiotherapy and/or chemotherapy. None were undergoing treatment at the time of the blood draw. The therapy, if previously given, was terminated from 2 weeks to 7 years before the time of phlebotomy.

Collection of PBCM

Venous blood (20–30mL) was drawn into heparinized tubes. The samples were hand carried to the laboratory and centrifuged on Ficoll-Hypaque gradients. Peripheral blood mononuclear cells (PBMC) were recovered, washed in RPMI medium (Invitrogen), counted in the presence of a trypan blue dye, and used for experiments.

Antibodies and reagents

Anti-human monoclonal antibodies (Abs) with the following specificities were used for flow cytometry: CD3-ECD, CD4-ECD, CD8-PC5, CCR7-PE, CCR7-FITC, CD45RO-ECD, CD28-PE, Fas-FITC, and fluorescein isothiocyanate (FITC)-conjugated ANXV. Abs and their respective isotypes were purchased from Beckman Coulter, except for anti-CCR7-FITC and anti-CCR7-PE (BD Biosciences). All Abs were titrated using normal PBMCs to establish optimal staining dilutions. All cell culture reagents were purchased from Invitrogen.

Surface staining and flow cytometry

PBMCs were washed twice in staining buffer (0.1% w/v bovine serum albumin and 0.1% w/v NaN₃) and stained for cell surface markers as described (11). Briefly, cells were incubated with relevant Abs for 20 minutes at room temperature in the dark, washed twice, and fixed in 1% (v/v) paraformaldehyde (PFA) in PBS. Four-color flow cytometry was conducted using a Beckman Coulter flow cytometer. The gate was set on lymphocytes, and at least 10⁵ cells were collected for analysis. Data were analyzed using Coulter EXPO 32v1.2 analysis software.

ANXV-binding assay

ANXV binding to PBMCs was measured by flow cytometry as previously described (14). Briefly, after staining with Abs specific for surface markers, cells were incubated with FITC-conjugated ANXV for 15 minutes on ice. Staining with 7-aminoactinomycin D (7-AAD) was conducted to identify dead versus live cells and to discriminate between nonapoptotic live cells (ANXV^{neg}/7AAD^{neg}), early apoptotic cells (ANXV⁺/7AAD^{neg}), and late apoptotic or necrotic cells (ANXV⁺7AAD⁺; ref. 18). Routinely, fewer than 10% of cells were 7-AAD⁺, both in patients and normal controls. The cells were analyzed within 30 minutes of staining.

Absolute lymphocyte counts

A single-platform technique, tetraONE System (Beckman Coulter), based on four-color flow cytometry in the presence of counting beads was used. The identification of lymphocytes by expression of bright CD45 and low side scatter signals was followed by the identification of T-cell subsets based on the expression of CD3, CD4, and CD8, as described previously (19). The sample acquisition and analysis were conducted on the EPICS CL flow cytometer equipped with a fully automated software. The number of cells (or cell subsets) per microliter was obtained by calculating the number of cells counted $\times$ concentration of beads/number of beads counted.

Statistical analysis

Differences in percentages and counts of lymphocyte subsets between patients and normal controls were age-adjusted by linear regression models after suitable data transformations. If age adjustment was unnecessary, differences were tested with the *t* test or the Wilcoxon test. The associations among lymphocyte subsets and categorical patient clinical characteristics (i.e., site of disease, type of treatment) were tested with the *t* test or Wilcoxon–Mann–Whitney test for 2-group differences or the Kruskal–Wallis test for 3 or more groups. Tests of trend with ordinally scaled endpoints such as T and N stages were conducted with Jonckheere–Terpstra test. Linear regression models were compared among patient subgroups by conducting simultaneous tests of the equality of regression slopes and intercepts. Residuals were examined for conformity to distributional assumptions. Recursive partitioning models were used to classify patients by the combination of lymphocyte phenotypes. Predictive classes from recursive partitioning models were evaluated with leave-one-out cross-validation. Longitudinal follow-up was conducted for all active disease patients whose blood draw was obtained just before oncologic therapy. These patients were followed for evidence of disease recurrence or a second primary tumor of the upper aerodigestive tract for up to 4 years. The frequency of baseline CD8⁺CCR7⁺ T cells was tested for association with disease-free survival (DFS) using proportional hazards regression and the log-rank test.

Results

CCR7⁺ T-cell frequency and absolute numbers in the blood of HNSCC patients

Although CD8⁺ CCR7^{neg} T cells are significantly more sensitive to spontaneous apoptosis than their CCR7⁺ counterparts (14), it is the CD8⁺CCR7⁺ T-cell subset that is contracted in the circulation of patients with HNSCCs. Not only percentages but also absolute numbers of CD8⁺ CCR7⁺ and CD4⁺CCR7⁺ T cells were decreased ($P < 0.0001$ for both) in patients relative to normal control (data not shown). Using new subject cohorts, we confirmed that apoptosis of circulating CD8⁺ T cells was higher ($P < 0.0005$) in patients with HNSCCs than in normal controls (Supplementary Fig. S1A). As expected, ANXV⁺ cells were predominantly CCR7^{neg} in patients and normal controls. However, in patients, more CD8⁺CCR7^{neg} T cells were ANXV⁺ than in normal controls (Supplementary Fig. S1A). Using immunohistochemistry, we have found CD8⁺CCR7⁺ T cells in tumor tissues (Supplementary Fig. S2), which confirms their potential to migrate to tumor sites.

The CD8⁺CCR7⁺ T-cell frequency and that of ANXV⁺CD8⁺ T cells in the blood of patients or normal controls did not correlate (Supplementary Fig. S1B). In fact, ANXV binding positively correlated with the absence of CCR7 on CD8⁺ T cells ($P < 0.001$). Patients with HNSCCs with active disease or NED had a lower frequency of CD8⁺CCR7⁺ T cells and a higher percentage of ANXV binding CD8⁺ T cells relative to normal controls (Supplementary Fig. S1B) and could be distinguished from normal control by these 2 parameters, which did not, however, correlate with each other. These correlative data imply that the low frequency of CD8⁺CCR7⁺ cells and increased ANXV binding to CD8⁺CCR7^{neg} cells may be independently regulated.

T-cell differentiation and apoptosis

A possibility was considered that ANXV binding to CD8⁺ T cells could be related to their maturation stage in patients. Therefore, ANXV binding to 4 lymphocyte differentiation subsets, defined by CCR7, CD45RO/RA, and/or CD28 expression (Fig. 1A), was studied by multiparameter flow cytometry. Figure 1 shows that the higher percentages of CD8⁺CCR7^{neg} T cells in patients with HNSCCs than in normal controls resulted from the accumulation of terminally differentiating (T_{TD}) T cells ($P < 0.0001$). In contrast, naïve (T_N) cells were reduced in frequency versus normal controls ($P < 0.0001$; Fig. 1B). The frequency of T_{PM} cells and T_{CM} cells was comparable in patients and normal controls. Patients with active disease or NED had a similar frequency of these T-cell subsets (data not shown).

In patients with HNSCCs, the T_{TD} cells contained the highest percentage of ANXV⁺ cells (Fig. 1C), whereas the T_{CM} and T_N subsets contained fewer ANXV⁺ T cells, albeit still higher than those in normal controls. Figure 1D illustrates ANXV binding to T cells in each of the 4 subsets in representative normal controls and HNSCC specimens. CD8⁺CCR7^{neg} T cells, that is, the T_{TD} and T_{PM} subsets, contained the bulk of ANXV⁺ T cells in patients and normal controls. These data clearly link ANXV binding to the T-cell maturation stage but do not explain a loss of CD8⁺CCR7⁺ T cells.

A distinct “immune signature” of HNSCC patients

To better define phenotypic characteristics of CD8⁺ T cell subsets in the blood of patients and normal controls, multicolor flow cytometry was conducted using 20 marker combinations. The data from patients and normal controls were combined to compute Spearman correlation coefficients for all 20 phenotypes. In a heatmap (Fig. 2), the CCR7^{neg} T cells, T_{PM} and T_{TD}, positively correlated with ANXV binding (red/brown), whereas among the CCR7⁺ subsets, only T_{CM} showed a weak positive correlation. T_N cells were negatively correlated with ANXV binding (green). This suggested that T_{PM} and T_{TD} cells

are more likely to undergo apoptosis whereas T_N may be protected. In the T_{CM} subset, this protection was less evident. The heatmap positively correlated circulating $CD8^+CCR7^{neg}$ to ANXV binding T cells in patients and normal controls.

As the low frequency of $CD8^+CCR7^+$ T cells and the high frequency of $CD8^+ANXV^+$ T cells were consistently observed in patients with HNSCCs, we asked which of these characteristics would better serve as an “immune signature” for the patient population. In unsupervised hierarchical cluster analysis (Fig. 3A), including 23 normal controls and 44 patients with HNSCCs (20 active disease and 24 NED), 2 distinct clusters of interest were identified: the “control” cluster comprised predominantly normal controls (green) and a “patient” cluster comprised active disease or NED patients. These 2 clusters were respectively characterized by high and low percentages of $CD8^+ANXV^+$ T cells. The “control” and “patient” clusters also differed with respect to CCR7 expression in $CD8^+$ T cells and, especially, in the percentage of T_N T cells (Fig. 3A). The “control” cluster contained only 6 patients with HNSCCs who, according to the clustering algorithm, were more similar to controls than to other patients. Importantly, at the time of analysis, 2 of 6 patients were NED following curative therapy. Thus, CCR7 expression and ANXV binding could discriminate patients from normal controls.

To discriminate between active disease and NED patients within the “patient” cluster based on phenotypic signatures, we constructed a heatmap where the subject classes normal controls, active disease, and NED were grouped together (Fig. 3B). We found that the immunologic markers did not clearly separate active disease from NED patients, although they discriminated patients from normal controls.

Recursive partitioning of HNSCC patients based on phenotypes

To determine whether the frequency of $CD8^+ANXV^+$ T cells discriminates patients from normal controls better than that of $CD8^+CCR7^+$ T cells, we used recursive partitioning methods. A series of decision tree algorithms was created to generate a simple set of rules for classifying the subjects. Initially, using the combination of only the frequency of the above listed phenotypes within the T_{TD} subset, we partitioned subjects into normal controls or patients with the 88% cross-validated accuracy (Fig. 4A). To validate the accuracy of the prediction models experimentally, we applied the same classification rules to the consecutively enrolled, independent confirmatory subsets of 15 normal controls and 15 patients with HNSCCs who were comparable in age, sex, and clinicopathologic parameters to the original cohort (Table 1). Furthermore, the cohorts had a similar mean apoptosis sensitivity within the cell subsets used for discrimination (Supplementary Table S1). This verification confirmed that the predictive model using only 2 phenotype profiles (as in Fig. 4A) correctly discriminated patients and controls with cross-validated accuracy of 93% (Supplementary Table S2). If CD28 is substituted for CCR7, the predictive model showed discrimination accuracy of 90% (Supplementary Table S2). Whether using the frequency of $ANXV^+CD8^+$ T cells with the T_{PM} phenotype or the T_{TD} phenotype as a differentiating factor, patients and normal controls were correctly classified (Supplementary Table S2). These results suggest that the predictive models based on ANXV binding to $CD8^+$ T cells have very high discriminating accuracy. However, measurements of ANXV binding to $CD8^+$ T cells require freshly harvested (2 hours) specimens and are influenced by the time elapsed from phlebotomy. In routine immune monitoring, this is an impediment. Therefore, we asked whether the use of only one phenotype, $CD8^+CCR7^+$, could discriminate patients from normal controls. Using recursive partitioning and the $CD8^+CCR7^+$ T-cell frequency of 31% determined by the algorithm, we showed that this phenotype discriminates patients from controls with 77% to 85% accuracy (Fig. 4B). In addition, to test whether the patients' HPV status influenced the frequency of $CD8^+CCR7^+$ T cells, the data available for 12

HPV^{neg} and 6 HPV⁺ patients were compared. No significant difference in the frequency of CD8⁺CCR7⁺ T cells was observed between these 2 cohorts ($P = 0.7974$).

Frequency of circulating CD8⁺CCR7⁺ T cells and the tumor recurrence

As the CD8⁺CCR7⁺ phenotype accurately discriminated patients from normal controls, we asked whether the frequency of circulating CD8⁺CCR7⁺ T cells in patients with HNSCCs at diagnosis and before any curative therapy could serve as a prognostic marker for disease recurrence. Among our training set of 43 patients, 25 had active disease and donated blood just before definitive therapy with surgery, chemotherapy, radiation, or chemoradiotherapy. All were treated with curative intent and were followed for up to 4 years for disease recurrence by the standard clinical criteria.

In this cohort of 25 patients, 12 were treated with surgery and 13 were nonsurgically treated. At the median follow-up for NED patients of 25 months (range, 9–43 months), there were 11 recurrences of HNSCCs and 1 new primary of the upper aerodigestive tract (lung). The median recurrence-free survival (RFS) has not been reached. This is typical for our institution, where the median RFS occurs around 30 to 36 months. Patients with a higher percentage of CD8⁺CCR7⁺ T cells before therapy had a decreased risk of disease recurrence. A proportional hazards model estimated the HR of 0.51 [95% confidence interval (CI), 0.27–0.96] for each 10% increase in the baseline frequency value for CD8⁺CCR7⁺ T cells. A recursive partitioning model found a stable cutoff of 28% CD8⁺CCR7⁺ to discriminate patients by their risk of disease recurrence regardless of therapy subsequently received. A Kaplan–Meier plot of DFS by $\pm 28\%$ is shown in Fig. 5. This discriminator identified a subgroup of 10 low-risk patients with high ($>28\%$) CD8⁺CCR7⁺ cells. Only one patient in this subgroup has recurred (log-rank, $P = 0.0115$ with leave-one-out cross validation). The data indicate that the CD8⁺CCR7⁺ phenotype may have a significant predictive value in identifying patients at high risk of tumor recurrence.

Discussion

The chemokine receptor, CCR7, plays a key role in migration of naïve and memory T cells as well as mature dendritic cells (DC) to lymph nodes (LN) through binding to its ligands expressed on high endothelial venules and in T-cell zones within secondary lymphoid organs (20). CCR7 also contributes to tolerance induction: the CCR7-dependent contact of T cells and DCs within LNs is essential for the induction of peripheral tolerance and the regulation of the immune response by Treg (20). In addition, CCR7 plays a role in establishing the organization of thymic architecture and in the negative selection of self-reactive T cells (21).

Besides its functional importance for the development of immunity and tolerance, CCR7 expression on tumor cells has been positively correlated with the presence of nodal metastases and thus a poor prognosis in different types of cancer, including HNSCCs (22–24). The involvement of chemokine receptors and their ligands in tumor cell survival has been reported (23, 25). For example, CCR7 signaling protected HNSCC cells from cisplatin-induced apoptosis by engaging the Akt-dependent pathway (24). Antiapoptotic effects of CCR7 were also observed in mature DCs, where CCR7-mediated protection involved activation of the PI3K/Akt pathway and NF- κ B translocation to the nucleus (26). Similar survival mechanism is present in CD8⁺CCR7⁺ T cells but not in CD8⁺CCR7^{neg} T cells, as previously reported (14).

In patients with HNSCCs, the frequency of apoptosis-resistant CD8⁺CCR7⁺ T cells is strikingly reduced, and these cells are replaced by the subset of CD8⁺CCR7^{neg} T_{PM} and T_{TD} cells, which are highly sensitive to apoptosis. Our results showing that these CD8⁺ T-cell

subsets are expanded in patients with HNSCCs contradict other *in vitro* and *in vivo* studies, in which memory T cells were found to be more resistant to apoptosis than T_N (27–29). These earlier studies were done in normal controls, where the relative sensitivity/resistance to apoptosis may be regulated by a differential replicative capacity of T-cell subsets. As T_N and T_{CM} cells have a high proliferation rate, whereas T_{PM} and T_{TD} replicate poorly if at all, to maintain cellular homeostasis, the former undergo apoptosis at a higher rate than the latter (28). However, in patients with cancer, the normal pattern of memory CD8⁺ T-cell formation, differentiation and death are altered. Chronic exposure to tumor-associated antigens tends to rapidly drive T_N to T_{TD} and ultimately to death, resulting in the exhaustion of effector cells (30). For example, repeated *in vitro* stimulation of gp100-specific CD8⁺ T cells resulted in a progressive loss of effector functions and proliferation resulting in the conversion of curative to noncurative antitumor responses (31). Also, a preferential death of CD8⁺ T_{CM} and CD8⁺ T_{EM} has been reported under oxidative stress, a condition common in cancer (32). Thus, in cancer, chronic antigenic stimulation and oxidative stress drive the rapid recruitment and turnover of differentiating T cells and culminate in their accelerated death.

While the shift in CCR7 expression on T cells in cancer could be rationally explained by their differentiation status and rapid turnover, its clinical significance remains unclear (33). Nevertheless, it has been suggested that the rapid demise of CD8⁺ effector T cells in cancer contributes to poor antitumor responses often seen in patients with HNSCCs (1–3). An alternative possibility is that CD8⁺CCR7⁺ effector T cells migrate to the tumor and their depletion from the periphery corresponds with the accumulation *in situ*.

Correale and colleagues reported that the tumor infiltration by CCR7⁺ T cells was predictive of favorable outcome in patients with advanced colon carcinoma enrolled in the GOLFIG-2 phase III trial (34). A high content of CD8⁺CCR7⁺ tumor-infiltrating lymphocytes (TIL) in tumor samples obtained at baseline, that is, before any therapy, significantly correlated with a prolonged overall survival (OS) and prolonged DFS after front-line chemotherapy (34). This suggests that CD8⁺CCR7⁺ T cells accumulating at tumor sites play a critical role in immunosurveillance and thus patients' survival, as proposed by Zitvogel and colleagues (35). It also provides an independent confirmation for the potential prognostic importance CD8⁺CCR7⁺ T cell infiltrations in patients with cancer at diagnosis.

To test the possibility that a loss of CCR7 expression on CD8⁺ T cells in the periphery might be important for cancer prognosis, we simultaneously evaluated 2 phenotypic characteristics of these cells: ANXV binding and CCR7 expression. Aside from discriminating patients with cancer from normal controls based on ANXV binding to CD8⁺ cells, the mere presence or absence of CCR7 on the surface of CD8⁺ T cells was shown to be sufficient for discrimination of patients from normal controls and also to serve as a useful surrogate marker of DFS. In a small cohort of patients whose blood was sampled for lymphocyte phenotyping before any therapy, the frequency of CD8⁺CCR7⁺ T cells appears to accurately predict who is at high risk of recurrence regardless of the type of definitive therapy received. Given a small number of patients involved in the study, this is a surprising and unexpected finding. This is also the first report of surrogate immune marker measured in the blood with a promising clinical application in HNSCCs. The disease recurrence after definitive curative therapy is a major clinical problem in this and other malignancies. The ability to predict recurrence at the time of diagnosis before any therapy with a simple flow cytometry–based blood test offers a possibility for the selection of more aggressive therapy for patients in the high-recurrence cohort. The predictive value of this test will need to be confirmed in much larger studies involving a careful patient follow-up over an extended period of several years and its relationship to existing predictive markers such as smoking

and HPV status analyzed. Nevertheless, the assay is simple and reliable, uses peripheral blood, and is required only at one time point, before curative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

In contrast to normal subjects, patients with squamous cell carcinoma of the head and neck (HNSCC) tested at diagnosis have a low frequency of circulating CD8⁺CCR7⁺ T cells. The low frequency of circulating CD8⁺CCR7⁺ at diagnosis discriminates HNSCCs from normal subjects. Potentially, this paucity of CD8⁺ CCR7⁺ T cells could reflect their extravasation into tissues or a cancer-associated deficit in naïve or central memory CCR7⁺ T-cell subsets. The low frequency of circulating CD8⁺CCR7⁺ T cells is a significant risk factor for disease recurrence. After up to 4 years of follow-up, disease-free survival (DFS) was found to be significantly shorter ($P < 0.0115$) for patients with fewer than 28% of circulating CD8⁺CCR7⁺ T cells than in those with more than 28% of CD8⁺CCR7⁺ T cells at diagnosis. Therefore, CD8⁺CCR7⁺ T cells might play a role in cancer control, and a simple blood test for immune markers at diagnosis may have a prognostic value for predicting disease recurrence in HNSCCs.

Figure 1.

The frequency of differentiating T lymphocytes and ANXV binding in the circulation of patients with HNSCCs and normal controls (NC). A, using flow cytometry, PBMCs can be separated into 4 subsets: T_N , T_{CM} , T_{PM} , and T_{TD} cells based on the differential surface expression of CD45RO and CCR7 or CD28 markers. B, the relative frequency of T_N , T_{CM} , T_{PM} , and T_{TD} CD8⁺ T cells in normal controls and patients with HNSCCs. C, the relative percentages of ANXV⁺ T cells within each of the 4 subsets in normal controls and patients with HNSCCs. B and C, the data are mean values SD for 52 patients and 32 normal controls (*, $P < 0.0001$ compared with normal controls). D, dot plots and histograms show relative percentages of T_N , T_{CM} , T_{PM} , and T_{TD} CD8⁺ T cells and their ANXV binding in PBMCs of normal controls and a patients with HNSCCs.

Figure 2.

A heatmap of Spearman correlation coefficients for all CD8⁺ T-cell phenotypes in patients with HNSCCs and normal controls (NC). The 20 distinct CD8⁺ T-cell phenotypes were defined using multiparameter flow cytometry. The percentage values for each phenotype were scaled to their own mean and SD, so that they could be compared with one another. Positive correlations with ANXV binding (red/red brown) are seen for CD8⁺CCR7^{neg} T-cell subsets (T_{TD} and T_{PM}) and the CD8⁺T_{CM} subset. Negative correlations (light or dark green) are seen for T_N cells. Correlations with an absolute value more than 0.3 are associated with $P < 0.01$.

Figure 3. Hierarchical clustering of the 20 phenotypes in patients with HNSCCs and normal controls (NC). Clustering is based on the distance applied to subjects (rows) and the 20 phenotypes (columns) defined by flow cytometry. Percentages of positive cells were expressed as standardized values with a mean of 0 ± 1 SD. A color bar on left depicts subject groups as normal control (green), HNSCCs with active disease (yellow), and HNSCCs with NED (olive). A, unsupervised clustering revealed 2 distinct clusters: a high ANX-binding patient cluster and a low ANX-binding normal control cluster. The patient cluster also has a uniformly lower CCR7 expression and a higher percentage of CCR7^{neg} T cells than the normal control cluster. B, a heatmap of subjects (rows) and phenotypes (columns), where

the subject classes normal control, active disease, and NED were grouped together. Patients with active disease do not differ from NED patients according to their CCR7 phenotypes or ANX binding. The identities of all subjects were defined by different colors in the vertical bar on the left.

Figure 4.

Recursive partitioning models used to classify subjects as either patients with HNSCCs or normal controls. A, the subjects were first partitioned on the basis of the frequency of ANXV binding to the T_{TD} T-cell subset and then further partitioned on the basis of the frequency of CD8⁺CCR7⁺ T cells. This classified subjects as patients or normal controls with a 100% accuracy. After leave-one-out cross-validation, this value dropped to 88%. B, partitioning was conducted on the basis of only on the frequency of CD8⁺CCR7⁺ T cells. A recursive partitioning model found the best split for the CD8⁺CCR7⁺ frequency at 31%. This phenotype alone classified subjects as patients or normal controls with an 85% accuracy. After leave-one-out cross-validation, this value dropped to 77%.

Figure 5.

Risk classification of 25 patients with HNSCCs tested for the frequency of circulating CD8⁺CCR7⁺ T cells before any therapy. A recursive partitioning model found the best split of the CD8⁺CCR7⁺ frequency at 28% for these patients (insert). Patients with the frequency of CD8⁺CCR7⁺ cells below this value were classified as high risk and those with the frequency above 28% as low risk. Kaplan–Meier plots for patients in each risk group are shown. Among 9 patients in the low-risk group, only one has recurred ($P = 0.0115$).

Table 1

Clinicopathologic characteristics of the patients with HNSCCs and normal controls in the study subset (left) and the confirmatory subset (right)

Characteristic	Patients (n = 52)	Normal controls (n = 32)	Patients (n = 15)	Normal controls (n = 15)
Age (years)				
Mean (range)	59 (32–80)	52 (22–77)	60 (27–78)	54 (27–70)
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
Sex				
Male	40 (77)	22 (69)	12 (80)	12 (80)
Female	12 (23)	10 (31)	3 (20)	3 (20)
Disease status at blood draw				
NED	30 (58)		8 (53)	
Active disease	22 (42)		7 (47)	
Primary	17 (61)		6 (93)	
Recurrence	5 (29)		1 (7)	
Primary T stage				
1	16 (31)		5 (36)	
2	17 (33)		5 (36)	
3	8 (15)		2 (14)	
4	7 (14)		2 (14)	
Unknown	4 (8)			
Primary N stage				
0	23 (44)		5 (26)	
1	9 (17)		2 (14)	
2	14 (27)		7 (50)	
3	2 (4)			
Unknown	4 (8)			
Primary tumor site				
Oral cavity	25 (48)		7 (50)	
Larynx	16 (31)		5 (36)	
Pharynx	7 (14)		2 (14)	
Nasal	2 (4)			
Maxilla	1 (2)			
Unknown	1 (2)			
Therapy	52		15	
Surgery				
Yes	32 (61)		8 (57)	
No	19 (37)		6 (43)	
Unknown	1 (2)		1	
Radiation therapy				
Yes	22 (42)		7 (50)	
No	23 (44)		7 (50)	

Characteristic	Patients (n = 52)	Normal controls (n = 32)	Patients (n = 15)	Normal controls (n = 15)
Unknown	7 (14)		1	
Chemotherapy				
Yes	15 (29)		7 (50)	
No	29 (56)		7 (50)	
Unknown	8 (15)		1	