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Advances in Brief

Absent or Low Expression of the ζ Chain in T Cells at the Tumor Site Correlates with Poor Survival in Patients with Oral Carcinoma[1]

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Abstract

Immunohistology for expression of the CD3ζ and CD3ε chains in TIL was performed in 138 paraffin-embedded primary oral squamous cell carcinoma tissues and 10 nontumor, inflammatory lesions. Semiquantitative analysis of the staining intensity for ζ chain expression and number of ζ chain expression-positive cells distinguished tumors with absent or low ζ expression (42 of 132) from those with normal ζ expression (90 of 132). ζ chain expression was inversely correlated with the tumor stage. Survival was significantly lower in patients whose TIL had absent or low ζ expression, controlling for stage ($P = 0.003$) and lymph node status ($P = 0.0005$). The prognostic value of ζ chain was restricted to patients with stage III or IV tumors ($P = 0.003$). The data indicate that absent or decreased ζ expression in TIL combined with tumor stage or nodal status defines a group of patients with oral squamous cell carcinoma who have an extremely poor prognosis.

Introduction

Alterations in the expression of signaling molecules in T cells of patients with cancer have been reported by many investigators (1–5). These alterations, including a decrease in expression or absence of the signal-transducing ζ chain, are thought to be responsible for functional impairments of immune cells at the tumor site and in the peripheral circulation of patients with malignancies (reviewed in Ref. 6). Decreased ζ chain expression has been observed in the circulating T cells of patients with melanoma, renal cell carcinoma, ovarian carcinoma, cervical carcinoma, colon carcinoma, and other cancers (1–9). Absent or low expression of the ζ chain is especially pronounced in TIL[3] (1, 10). This observation and the evidence for a significant decrease or absence of the ζ chain in T cells coincubated with human tumor cells *in vitro* (11) suggest that this alteration may be tumor induced. The mechanism(s) responsible for a decrease in ζ expression are unknown, and various explanations have been advanced, including the possibility that it is an artifact induced by monocytes during isolation of peripheral blood mononuclear cells (12). More recently, decreased expression of the ζ chain has been linked to the process of apoptosis induced in T cells by tumor-derived[4] or other apoptotic signals (11). Overall, however, the biological significance of ζ chain abnormalities has remained unclear.

To address the presence and biological significance of the ζ chain alterations in T cells present in the tumor microenvironment, a retrospective immunohistochemical study was performed on tumor biopsies obtained from 138 patients with oral carcinoma over a period of several years. Semiquantitative evaluation of ζ chain expression in $CD3^+$ T cells at the tumor site established correlations between staining intensity for the ζ chain in TIL and prognosis or patient survival. The results indicated that patients with stage III or IV tumors and absent or low expression of the ζ chain in TIL have extremely poor survival compared to patients with either normal ζ chain expression or early-stage oral SCC.

Materials and Methods

Patients and Specimens. Tissue samples were collected retrospectively from 138 patients with SCC of the oral cavity. Of these, clinical follow-up data were available for 132. The sites of primary tumors were as follows: floor of the mouth, 56 tumors; maxilla including palate, 15 tumors; retromolar trigone, 7 tumors; gingiva of the mandible, 24 tumors; buccal mucosa, 5 tumors; and tongue, 25 tumors. The patients underwent potentially curative tumor resection between 1980 and 1993. No preoperative radio- or chemotherapy had been performed. Among the patients, 101 were male and 31 were female.

The median age was 58 years (range, 36–89 years). Control specimens were obtained from lesions in 10 nontumor patients (3 patients with chronic inflammation of the maxillary sinus; 3 patients with periapical cyst; 3 patients with tonsilitis; and 1 patient with chronic sialadenitis of the parotid gland). Of 138 patients, whose specimens were studied, only 11 had no history of smoking and/or alcohol abuse. All others were in a high-risk group for oral carcinoma.

The biopsy specimens were fixed in 4% formalin, embedded in paraffin, sectioned, and stained with H&E. The TNM staging categories were determined according to the criteria proposed by the International Union Against Cancer. Clinical definitions of the tumor stage were confirmed by histopathology and were as follows: stage I, 30 patients; stage II, 33 patients; stage III, 7 patients; and stage IV, 62 patients. Histopathology indicated that 80 tumors were N_0, 11 were N_1, and 41 were N_2. Tumor size was defined as the largest diameter of the tumor.

Abs. The unlabeled mAb to CD3ζ (6B10.2) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The CD3ε mAb and the vimentin mAb were purchased from DAKO (Glostrup, Denmark). Secondary Abs, which were biotinylated and included in the LSAB2 kit from DAKO, were used to perform the immunohistological avidin-biotin technique. In all experiments, isotype Abs purchased from DAKO were used as controls.

Immunohistochemistry. Paraffin sections (5 μm thick) were consecutively cut from each specimen and mounted on electrostatically precharged slides (Superfrost Plus; Menzel-Gläser, Frankfurt, Germany). The sections were deparaffinized and rehydrated. After endogenous peroxidase quenching (0.3% H_2O_2 in PBS for 30 min), antigens were retrieved by boiling the sections in 1 mM EDTA-NaOH solution (pH 8.0) in a microwave oven with the power set at 750 W for three cycles (5 min each). The same number of slides ($n = 20$) was always placed in a rectangular plastic staining jar for microwave treatments. After the completion of the third cycle, slides were allowed to cool at room temperature for 30 min.

Immunohistology was performed by use of an avidin-biotin technique. Optimal working dilutions of the primary Abs were as follows: CD3ζ, 1:800; CD3ε, 1:200; and vimentin, 1:200. The working dilutions were determined in preliminary titration experiments performed with human tonsils. Staining was developed with peroxidase and amino-9-ethylcarbazole. The mAb against vimentin was used as an internal control to ascertain the quality of antigen

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[3] The abbreviations used are: TIL, tumor-infiltrating lymphocytes; Ab, antibody; mAb, monoclonal Ab.

[4] Gastman, B. R., Johnson, D. E., Whiteside, T. L., and Rabinowich, H. Activation of caspase-3 and cleavage of Bcl-2 in tumor-induced apoptosis of T lymphocytes, submitted for publication.

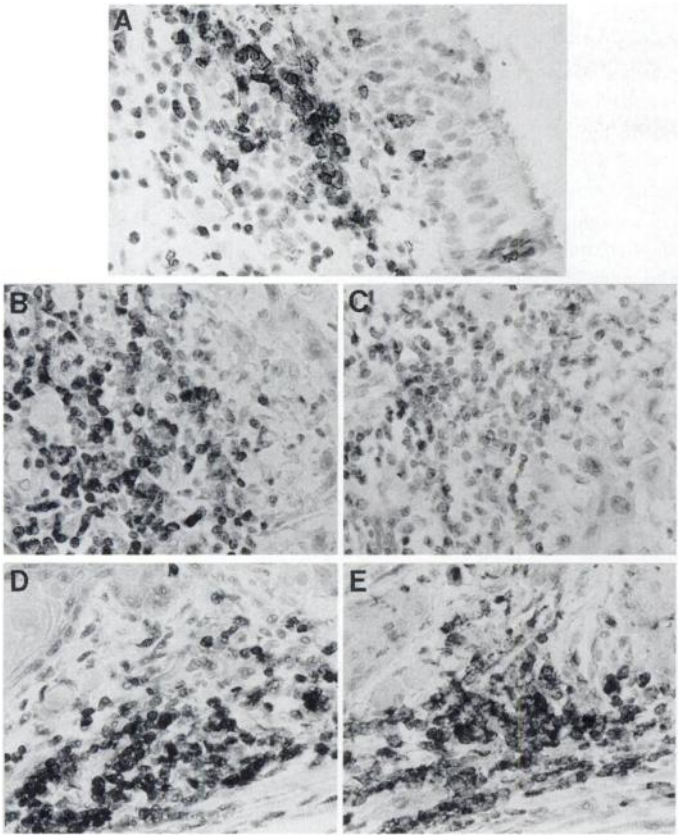

Fig. 1. Immunoperoxidase staining with anti-CD3 ζ mAb of a paraffin section of a chronic inflammatory lesion in the maxillary sinus (*A*, positive control). Note strongly stained T lymphocytes in the connective tissue underlying the epithelium of the maxillary sinus. In *B* and *C*, immunoperoxidase staining of serial paraffin sections of an oral SCC with anti-CD3ε Ab (*B*) and anti-CD3ζ mAb (*C*). Note the near absence of staining for ζ in *C*. In *D* and *E*, serial sections of another oral SCC with normal expression of both ε (*D*) and ζ (*E*) in TIL. Original magnification, ×400.

preservation and uniformity of staining in the formalin-fixed tissue sections (13).

Evaluation of Stained Sections. All immunostained sections were examined by light microscopy by one of us (T. E. R.). Only those sections that showed a uniform and intense vimentin staining in cells of the mesenchymal origin were included in the study. To evaluate CD3ζ expression in TIL, at least 10 high-power fields were randomly selected for microscopic examinations at the border of the tumor and within the peritumoral tissue. The staining intensity was assessed with coded slides. Only two groups were distinguished in respect to staining intensity: group 1, normal CD3ζ expression; and group 2, reduced or absent CD3ζ expression. Staining intensity in TIL was judged relative to lymphocytes adjacent to normal squamous epithelium within the same specimen and relative to lymphocytes in the nontumor tissues used as controls, which were stained and examined in parallel. In addition, the number of positively stained cells was determined, to provide a semiquantitative estimation of CD3ζ expression in TIL. Cells were counted in five high-power (×400) fields with an ocular grid. The number of CD3ζ+ cells was compared to the number of CD3ε+ cells. In cases in which CD3ζ+ cells were <50% of CD3ε+ cells, the staining was judged to be reduced (group 2).

Statistical Analysis. Survival data were analyzed using proportional hazards regression and Kaplan-Meier survival estimation. Nodal status as an outcome was analyzed using logistic regression. Categorical associations were evaluated using Fisher exact tests. Where appropriate, sparse categories were combined. For this reason, the four stages were collapsed into stage I–II and stage III–IV. Because only 7 patients had stage III disease, it cannot be determined whether stage III resembles stage IV or stage I–II.

Results

Patient Follow-up Data. Of the group of 132 patients with oral SCC whose tumor specimens were included in the study, 60 had died and 72 were alive as of July 1998. Median follow-up time was 41 months. Estimated median survival was 78 months.

ζ Chain Expression in Tumor or Control Tissues. Using expression of vimentin as a measure of antigen preservation in formalin-fixed, paraffin-embedded SCC tissues, it was possible to confirm that all tissues examined were uniformly positive (data not shown). This was an essential control step because many antigens undergo denaturation during fixation, embedding, or microwave treatment and become nonreactive with specific mAbs (13). Staining of nontumor tissues containing inflammatory infiltrates was another essential control that we included. This staining showed that T lymphocytes in all 10 inflammatory lesions had strong expression of the ζ chain (Fig. 1*A*), which was comparable in intensity to CD3ε staining in the serial section of the same tissue (data not shown). In contrast, a large proportion (42 of 132) of oral SCCs showed absent or low expression of the ζ chain in TIL, as compared to staining for CD3ε in the same specimen (Fig. 1, *B* and *C*) or to ζ staining in control sections (Fig. 1*A*). The distinction between normal ζ expression (Fig. 1*E*, group 1) and "low" ζ expression (Fig. 1*C*, group 2) was based on two separate criteria: (a) visual comparison of experimental and control sections and (b) the number of ζ T cells in serial sections stained for CD3ζ or CD3ε. In 9 of the tumors, no ζ expression was detected, and it was judged to be low in 33 SCC tissues in comparison to control specimens. Because of the small number of samples ($n = 9$) in the "absent" category (designated 0), it was combined with the low category (designated 1) in subsequent analyses.

Association of ζ Chain Expression with the Tumor Stage. The relationship between advanced tumor stage and low ζ expression was strong (odds ratio = 3.3, $P = 0.003$). Absent/low expression of the ζ chain was observed in only 19% of the stage I–II tumors, but it was observed in 43% of stage III–IV tumors (Table 1).

Association between ζ Chain Expression and Tumor Size, Nodal Involvement, or Distant Metastases. Primary tumor size (T), the presence and type of tumor spread to the local lymph nodes (N), and the presence or absence of distant metastases (M) are all important staging and prognostic factors in oral SCC. Our data indicated that low ζ expression was associated with lymph node involvement (odds ratio = 3.4; $P = 0.002$), worse primary tumor status (odds ratio = 3.8; $P = 0.0009$), and the presence of distant metastases (odds ratio = infinite; $P = 0.03$).

To clarify what role ζ expression might play in the process of tumor progression, the prediction of nodal involvement by ζ expression, controlling for actual tumor dimension, was investigated. It was found that low/no ζ expression increases the risk of nodal involvement by an odds ratio of 1.68 ($P = 0.01$).

Association between ζ Chain Expression and Patient Survival. Absent or low ζ expression was associated with poor survival of patients with oral SCC, even after data were controlled for tumor stage (Table 2; hazard ratio = 1.6, $P = 0.0003$). The interaction term was significant as well (Table 2 and Fig. 2*A*), indicating that the prognostic value of ζ expression is greater in more advanced disease. The data presented in Fig. 2*A* strongly suggest that the concurrent presence of

Table 1 *Association between ζ chain expression in TILs and tumor stage in human oral SCC*

	Tumor stage				
ζ chain expression[a]	I	II	III	IV	Total specimens
Absent/low	7	5	1	29	42 (32)[b]
Normal	23	27	6	34	90 (68)
Total	30	32	7	63	132 (100)

[a] Expression of the ζ chain in TILs was defined microscopically, as described in "Materials and Methods."

[b] Numbers in parentheses represent percentages.

Table 2 *Association between ζ chain expression in TIL tumor stage and survival in patients with oral SCC*

Model term	Estimated hazard ratio	*P* (two-sided)
Stage[a]	1.59	0.00140
ζ[b]	1.61	0.00034
Stage* ζ[c]	1.54	0.01800
High stage, low ζ[d]	2.28	3.4×10^{-9}

[a] Stages 1 and 2 *versus* 3 and 4, controlling for ζ.
[b] None or low *versus* normal ζ chain expression, controlling for the tumor stage.
[c] Interaction term.
[d] Weakest link model: high stage and low ζ *versus* all other combinations.

advanced disease with absent/low ζ expression in TIL predicts a high hazard of death. The best fit was represented by a "weakest link" model (Table 2), in which patients with both stage III–IV disease and low/absent ζ expression were contrasted with all other patients (hazard ratio = 2.3; $P = 3 \times 10^{-9}$).

Because nodal involvement has prognostic significance in oral SCC, we also determined patient survival by ζ expression in TIL after controlling for the presence or absence of regional lymph node involvement. As shown in Fig. 2*B*, patients with positive nodes and absent/low ζ expression in TIL had significantly shorter survival than patients with either negative nodes or normal ζ expression. Also, absent or low ζ expression in TIL indicated a poor prognosis for all patients with positive lymph nodes, regardless of the extent of nodal involvement (data not shown).

Among patients with stage I or II disease and patients with negative lymph nodes, ζ chain expression had no predictive value for survival. Surprisingly, among patients with normal ζ chain expression, neither tumor stage nor nodal involvement predicted survival (Fig. 2).

Discussion

The presence, functional significance and mechanisms responsible for the ζ chain abnormalities in immune cells of patients with cancer have been the subject of a considerable controversy (reviewed in Ref. 6). The initial observation of this phenomenon was made by Mizoguchi and colleagues (14) in tumor-bearing mice, and then this phenomenon was observed by these investigators and others in TIL and peripheral blood T and natural killer cells of patients with cancer (1–5). These abnormalities consist of variably decreased but often detectable expression of the ζ protein relative to its expression in T cells of healthy donors. Quantitative measures of the ζ protein indicate that its loss is greater in tumor-associated T cells than it is in circulating T cells and its loss is also greater in immune cells of hosts with advanced disease than it is in those with early disease (1, 10). Because mRNA for ζ is normally expressed in these cells, modifications of the ζ chain appear to be a posttranslational event (10, 15). However, normal ζ expression has been reported in many established tumor models in mice (16) and in T cells of some patients with cancer (17). Furthermore, the mechanism(s) responsible for degradation of the ζ chain in lymphocytes of tumor-bearing hosts remain undefined and have been attributed to the tumor-derived factors, macrophage-derived enzymes, or products, such as H_2O_2, activation-induced apoptosis, and others (11, 18, 19). Biological consequences of the decreased ζ expression in immune cells may be profound because of the key role of this protein in T-cell receptor or FcγRIII signaling (14, 20). Immune effector cells with ζ abnormalities have altered cytokine profiles, as noted by many investigators (*e.g.*, Refs. 5 and 21), and are functionally impaired (10, 19). Their antitumor functions may be compromised (22). To date, however, only one report exists, in patients with metastatic melanoma, demonstrating that absent or decreased ζ expression in peripheral blood T cells is associated with poor prognosis and significantly shorter survival (2).

In this retrospective study, we demonstrate for the first time that decreased ζ chain expression in TIL has a significant impact on survival of patients with oral SCC. This is an important finding, which provides a rationale for considering ζ expression as a potential prognostic biomarker, at least in SCC. Together with the association of low ζ with poor survival reported by Zea and collaborators (2) for metastatic melanoma, our results suggest that the level of ζ in immune cells might prove to have broad relevance in human cancer. Our data showed that normal expression of the T-cell receptor-associated ζ chain in TIL was associated with a lower disease stage, less nodal involvement, and absence of distant metastases in patients with oral SCC. In patients with advanced-stage oral SCC, normal ζ expression predicted much better survival. Absent/low ζ expression was not simply a proxy for other risk factors but had independent prognostic value. In fact, poor survival seemed to require both advanced stage and low ζ expression, and in the presence of normal ζ expression, the prognostic significance of stage and nodal involvement were greatly attenuated. Whether absent/low ζ expression is a result of progressive disease or contributes to its progression or both remains unclear.

Studies in our laboratory indicate that degradation of the ζ protein in TIL or circulating T cells in patients with cancer is linked to tumor-induced apoptosis of effector cells. We have demonstrated that tumor-induced degradation of ζ and ϵ chains in T cells can be blocked

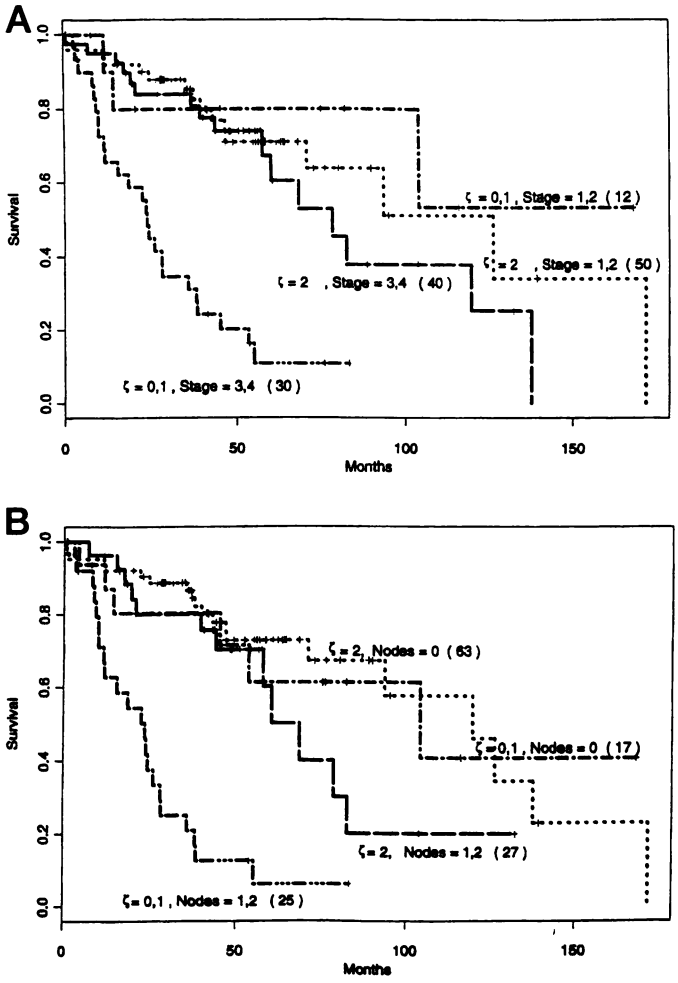

Fig. 2. *A*, Kaplan-Meier plots presenting survival of patients with oral SCC by ζ chain expression in TIL and by tumor stage. *Numbers in parentheses*, numbers of patients in each category (low ζ, stage I–II; normal ζ, stage I–II; low ζ, stage III–IV; and normal ζ, stage III–IV). *B*, Kaplan-Meier plots presenting survival of patients with oral SCC by lymph node involvement and low or normal ζ chain expression in TIL.

by specific inhibitors of caspases (11). More recent evidence indicates that the ζ chain itself may be a substrate for caspase-mediated cleavage.[5] These and other observations in our and other laboratories suggest that degradation of the key signaling molecules, like the ζ chain or nuclear factor κB, might result from proteolytic cleavage initiated as a part of tumor-induced apoptosis (23). The tumor microenvironment seems to play a key role in inducing and sustaining apoptosis of lymphocytes, especially those that accumulate at the tumor site (6, 10). Functional defects and impaired antitumor responses of effector cells at the tumor site might be a consequence of ongoing apoptosis. It is, therefore, no surprise that defects in ζ expression are biologically significant and relate to prognosis and survival in patients with cancer. Further studies are warranted to dissect the mechanisms of ζ chain defects in view of the data reported here and those indicating that reversal of ζ abnormalities occurs in patients who are responders to biological therapies (22).

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