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Roles of Epithelial-Mesenchymal Transition in squamous cell carcinoma of the temporal bone

Hisashi Sugimoto, MD, PhD[1)], Makoto Ito, MD, PhD[1)]*, Miyako Hatano, MD, PhD[1)], Satoru Kondo, MD, PhD[1)], Shioto Suzuki, MD[2)], PhD and Tomokazu Yoshizaki, MD, PhD[1)]

[1)]Department of Otolaryngology-Head and Neck Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

[2)]Molecular and Cellular Pathology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

*Address correspondence and reprint requests to: Dr. Makoto Ito, MD, PhD.

Department of Otolaryngology Head and Neck Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan; Telephone: 76-265-2413; Fax: 76-234-4265; E-mail: makoto@med.kanazawa-u.ac.jp

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Malignant tumor of the temporal bone shows a clinically aggressive course, and prognosis has remained poor despite recent advances in surgical strategies, radiotherapy and chemotherapy. This poor prognosis has been attributed to a complicated anatomy and to its proximity to the dura mater and nearby neural and vascular structures. These important structures are all close to the bone in the external auditory canal (EAC) and middle ear (ME). Bone involvement by the malignant tumor can thus be the key to the prognosis. Many reports have tried to evaluate survival in this aggressive disease, but patient numbers have been limited due to the rarity of this disease.(1-9) We encountered 16 cases of primary squamous cell carcinoma (SCC) of the temporal bone between 2001 and 2006, and evaluated prognostic factors associated with the survival rate for SCC of the temporal bone. As expected, extensive bone erosion correlated with a worse prognosis of SCC of the temporal bone, whereas extensive soft tissue involvement did not correlate with prognosis.(10) We subsequently wondered why extensive bone erosion was associated with poor prognosis. This question led us to suspect that epithelial-mesenchymal transition (EMT) is associated with extensive bone erosion in SCC of the temporal bone.

EMT is a process whereby epithelial cells lose polarity and cell-to-cell adhesions and undergo dramatic remodeling of the cytoskeleton.(11) Concurrent with loss of epithelial cell adhesion and cytoskeletal components, cells undergoing EMT acquire expression of mesenchymal components and gain a migratory phenotype. Wound healing and progression of carcinoma to invasive and metastatic phenotypes also involve localized EMT. EMT was first recognized during embryogenesis in the early 1980s. Accumulating evidence suggests that carcinoma cells activate a dormant EMT program to promote cell migration, invasion, and metastasis.(11-13) To date, no reports

concerning EMT in malignant tumor of the temporal bone appear to have been published. The present study therefore used immunohistochemical methods to analyze expressions of EMT in patients with SCC of the temporal bone and studied the association between EMT and prognosis. We also investigated correlations between EMT and extensive bone erosion.

Transforming growth factor (TGF)-β is a growth factor that has been demonstrated to trigger EMT and to promote the invasive ability of many epithelial cells in vitro.(14) These phenomena suggested a possible role of TGF-β in EMT for SCC of the temporal bone. We therefore also performed immunohistochemical analyses to clarify correlations between TGF-β and EMT in SCC of the temporal bone, and investigated the mechanisms by which EMT might be induced by TGF-β.

MATERIALS AND METHODS

Patients

We studied 16 patients with primary cancer of the EAC and ME for whom pathological materials and adequate medical records and follow-up data were available. A total of 16 patients (7 men, 9 women) underwent treatment at Kanazawa University Hospital (Kanazawa, Japan) between 2001 and 2006. The mean age of patients at first presentation was 64 years (range, 41-83 years). Tumors of the 16 patients were staged according to the revised Pittsburgh staging system (Table 1). They include three T1, two T2, three T3, and eight T4 patients. Thirteen patients were treated with surgical resection. The surgical resection performed most often was lateral temporal bone resection (LTBR). LTBR was performed combined with radiotherapy for tumors that remained lateral to the tympanic membrane. Three patients with T4 disease received

chemo-radiation therapy alone because their tumors showed extensive invasion of dura matter and major vessels. Six patients in the T4 group and all patients in the T3 group showed extensive bone involvement, including T4 with bone erosion of the deep temporal bone and T3 with apparent bone erosion of the external auditory canal and/or a tumor involving the middle ear cavity. Classification of the tumors was based on CT and MRI analysis. The degree of bone involvement is described in detail below (the numbers correspond to the patient numbers shown in Table 2): 6, erosion of the osseous external auditory canal (full thickness). 7, erosion of the osseous external auditory canal (full thickness) 8, erosion of the osseous external auditory canal (full thickness) and a tumor involving the middle ear cavity. 9, bone erosion of the deep temporal bone involving dura. 12 erosion of carotid canal , involvement of the temporal bone and mandibula, erosion of the osseous external auditory canal (full thickness) 13, erosion of carotid canal and the jugular foramen. erosion of the osseous external auditory canal (full thickness) 14, bone erosion of the deep temporal bone ,involving dura and the middle wall of the middle ear ,evidence of facial paresis. 15, bone erosion of the deep temporal bone, involving dura and evidence of facial paresis. 16, erosion of dura and the carotid canal. erosion of the osseous external auditory canal (full thickness)

All medical records were retrospectively reviewed for collection and evaluation of data regarding age, sex, presenting symptoms, findings from computed tomography (CT), TNM status at the time of operation, type and extent of operation, histological diagnosis, adjunctive therapy (postoperative radiotherapy, chemotherapy), sequelae, and treatment outcomes. The period from symptom onset to diagnosis was unknown for four patients (1, 3, 6, 14); one month for four patients (2, 7, 9, 13), two months for three patients (5, 11, 12), three months for two patients (10, 16), five months for one patient

(4), and seven months for two patients (8, 14).

Single immunostaining of TGF-β and vimentin

The various protein expressions were visualized using an indirect immunoperoxidase technique. Paraffin-embedded blocks of specimens were cut into 5-μm-thick serial sections. After deparaffinization, sections were treated with methanol containing 3% hydrogen peroxide for 10 min. After blocking the endogenous peroxidase, sections were incubated in protein block solution (Dako Cytomation, Glostrup, Denmark) for 30 min, then incubated overnight at 4°C with each primary antibody. We applied the following primary antibodies: vimentin (NeoMarkers Ab-2 V9; Thermo Scientific,(USA), TGF-β1 v (SANTA CRUZ BIOTECHNOLOGY inc, Europe) The dilutions used were each 1:100. The sections were next incubated for 1 h at room temperature with goat anti-mouse immunoglobulins (Igs), which were conjugated to peroxidase-labeled polymer (Envision; Dako Cytomation). We used 3,3'-diaminobenzidine tetrahydrochloride as the chromogen, followed by light counterstaining with hematoxylin. Negative controls were evaluated by substituting the primary antibody with similarly diluted non-immunized mouse serum.

Dual-fluorescent immunostaining of TGF-β and vimentin

SCC in temporal bone specimens expressing both TGF-β and vimentin were used for dual-fluorescent immunostaining, to clarify the location of cancer cells expressing vimentin. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 min.

Specimens were incubated with antibodies to TGF-β and vimentin (the same

antibodies used in single immunostaining) overnight at 4°C. The reaction product was visualized with fluorescent goat anti-mouse and anti-rabbit IgG antibodies (1:500; Molecular Probes, Eugene, OR, USA). Specimens were counterstained using Prolong Gold antifade reagent with DAPI (Invitrogen Molecular Probes, USA) and were observed under a microscope (ECLIPSE E1000M; Nikon, Japan). No positive staining was obtained when primary antibodies were omitted or replaced with normal mouse serum in the negative controls of staining procedures.

Evaluation of specimens

For each of the immunohistochemical markers studied, the number of immunoreactive cells was calculated using an image analysis system (ACT-1C for DXM1200C; Nikon). Specimens were evaluated independently by two of the authors that were blinded to the clinical data, and were then reviewed by the other authors. Two examiners each selected four representative fields, with each field containing more than 200 tumor cells. Both the number of immunoreactive cells and the total number of tumor cells were counted, and calculated expression scores. At least 800 tumor cells were counted and evaluated by each examiner.

Data analysis

Survival rates were estimated using Kaplan-Meier methods, and outcomes were analyzed using Windows personal computers and PASW Statistics 18 (SPSS Japan Inc, IBM company). Log-rank testing for two related samples was used to compare means of the paired variables, with values of $P<.05$ considered significant. The increased vimentin expression score in patients with extensive bone involvement was examined

using the Mann-Whitney U test, with values of P<.05 considered significant.

RESULTS

Vimentin was expressed on temporal bone tumor cells

Medical records were reviewed retrospectively for all of the 16 patients with primary SCC of the EAC (n=13) and ME (n=3) (Table 2). Tumor cells clearly showed an all-or-nothing pattern for expression of vimentin (Fig. 1), with all expression scores either >10% or <1%. Outcomes of vimentin staining in tumor cells were classified as either positive (expression score >10%; n=9) or negative (expression score <1%; n=7). Patients showing vimentin-positive tumor cells were regarded as EMT-positive, as gain of vimentin has been reported as a hallmark of EMT for several types of cancer.

Correlation of Vimentin staining in temporal bone SCC with prognosis

Figure 2 shows Kaplan-Meier survival curves for disease-specific survival (DSS) in patients with and without EMT. No significant differences were seen using log-rank testing.

Vimentin expression correlated significantly with extensive bone involvement

We tried to analyze correlations between the vimentin expression score and bone invasion. Interestingly, a significant increase in the vimentin expression score was seen in patients with extensive bone involvement (P<.05) (Fig. 3).

TGF-β1-positive cells were seen in the same section of the vimentin-positive areas

Figure 4 shows double staining of TGF-β1 and vimentin in temporal bone tumor cells.

TGF-β1-positive cells were seen in the same areas as vimentin-positive cells, but were not totally co-localized. We examined 9 vimentin-positive cases using the same methods and revealing the same findings.

DISCUSSION

In the process of embryonic development, epithelial cells often lose their epithelial features and present with a mesenchymal phenotype, a phenomenon known as EMT. This process is important during fetal development, wound repair and tissue remodeling, allowing epithelial cells to acquire fibroblast-like properties, reducing intracellular adhesion and increasing motility. EMT may be a mechanism by which epithelial cells acquire the ability to migrate, and this process is normally under tight control. Several recent studies have suggested that EMT might be involved in the invasion and metastasis of malignant tumors, and whether EMT plays a role in metastatic behaviors remains unclear.

This study tried to investigate the role of EMT in SCC of the temporal bone. First, we studied associations between EMT and prognosis. Immunohistochemical analysis demonstrated that gain of vimentin in tumor cells tended to correlated with poor clinical outcomes for SCC of the temporal bone (Fig. 2), but no significant differences were identified. Our previous study showed that extensive bone involvement significantly correlated with worse prognosis (10) We therefore examined the correlation between bone involvement and EMT. Interestingly, the present study showed a significant increase in vimentin expression among patients with extensive bone involvement ($p<0.05$) (Fig. 3). We were unable to show a direct correlation between EMT and prognosis, but this may be due to the rarity of the disease, with only 16 cases in this

study. Investigation of more cases may thus confirm a direct correlation.

On the other hand, TGF-β1 has been demonstrated to trigger EMT in vitro, and is overexpressed in many malignant human tumors, including SCC. Overexpression of TGF-β1 at the early stages of carcinogenesis provides tumor-suppressive effects primarily via growth inhibition, whereas TGF-β1 overexpression at late stages of carcinogenesis promotes tumor progression, metastasis, and EMT, potentially via loss of adhesion molecules, angiogenesis, proteinase activation, and immune suppression.(15)

In SCC of the temporal bone, EMT is expected to be induced by TGF-β1**.** Bone is known to include abundant TGF-β1 and the EAC and ME are bony structures. TGF-β1 released from destroyed bone after SCC invasion may thus induce EMT in temporal bone. This theory is consistent with the correlation we identified between bone erosion and EMT. In the present study, a large difference in five-year survival rate was observed between early-stage (T1 and T2; five-year survival rate, 80%) and late-stage (T3 and T4; 45%) tumors, indicating that late-stage primary squamous cell carcinoma of the temporal bone follows an extremely aggressive course. This may be related to the aforementioned increase in tumor malignancy caused by the release of TGFβ1 due to bone involvement as well as to the associated induction of EMT. In addition, in this study, double-staining of EMT-positive SCC cells demonstrated that TGF-β-positive areas occurred in the same areas showing vimentin gains, although exact colocalization was not identified. This may indicate that TGF-β1 is released from the tumor cells themselves. This phenomenon implies that in progressive SCC of the temporal bone, TGF-β1 that is both released from destroyed bone and secreted from the tumor cells themselves would induce EMT. However, the exact mechanisms underlying the

induction of EMT remain yet to be elucidated.

CONCLUSION

This study showed significant correlations in EMT with extensive bone involvement. The results suggest that EMT correlates with poor prognosis for SCC of the temporal bone, and that this correlation is an indirect relationship resulting from the promotion of bone invasion by EMT. In addition, investigation of EMT in biopsy specimens before treatment may help in the estimation of bone involvement and prognosis. Such analyses might help in drafting better strategies.

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Fig1 Immunohistochemical staining of vimentin in squamous cell carcinoma of the temporal bone. A) Including vimentin-positive tumor cells and B) All cells are negative for vimentin.

Fig2 Kaplan-Meier survival curves of disease-specific survival for patients with or without EMT. Statistically did not show significant increase.

Fig3 A statistically significant increase of_vimentin expression score was seen in patients with extensive bone involvement ($p<0.05$)

Fig4 Double staining of TGFβ1 and vimentin in temporal bone tumor cells. TGFβ1 positive cells were seen in the same section of the vimentin positive areas.

Table 1. University of Pittsburgh staging system (Moody's modified) for squamous cell carcinoma of the external auditory canal

T status

T1: tumor limited to the external auditory canal without bony erosion or evidence of soft tissue involvement

T2: tumor with limited external auditory canal bony erosion (not full-thickness), or limited (<0.5 cm) soft tissue involvement

T3: tumor eroding the osseous external auditory canal (full-thickness) with limited (<0.5 cm) soft tissue involvement, or tumor involving middle ear and/or mastoid

T4: tumor eroding the cochlea, petrous apex, medial wall of middle ear, carotid canal, jugular foramen, or dura, or with extensive (≥0.5 cm) soft tissue involvement, such as involvement of temporomandibular joint or styloid process, or evidence of facial paresis

N status

Lymph node involvement is a poor prognostic sign; any node involvement should automatically be considered as advanced stage, ie, T1N1 = stage III and T2, 3, 4 N1 = stage IV

M status

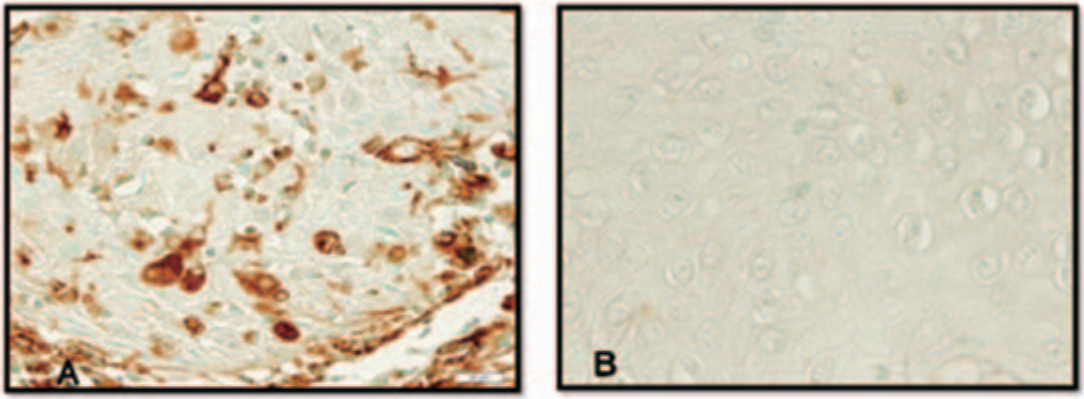
Distant metastases indicate a very poor prognosis and should be considered as stage IV disease

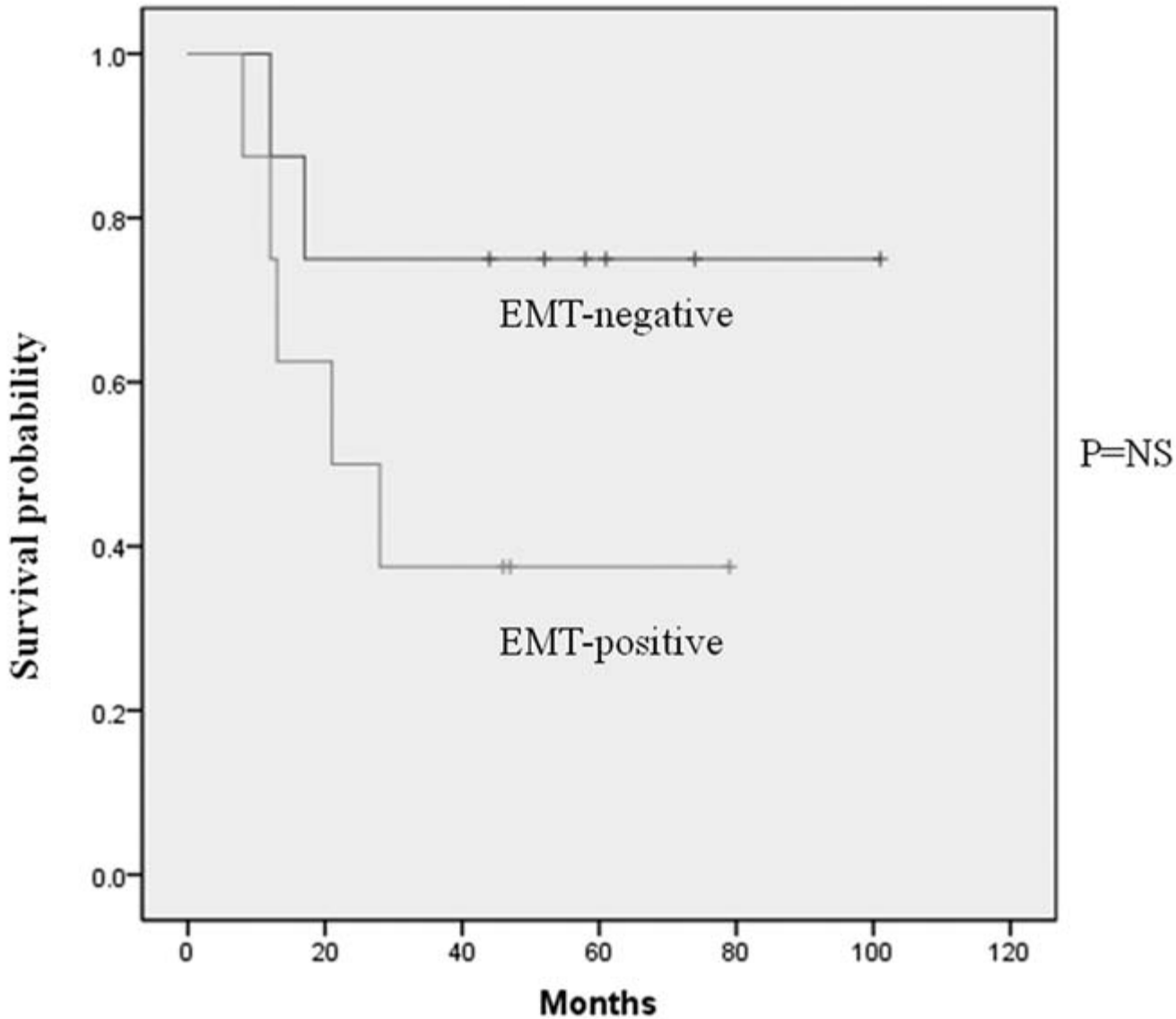
In the absence of metastastic lymph nodes or distant metastases, T status of the tumor defines the clinical stage

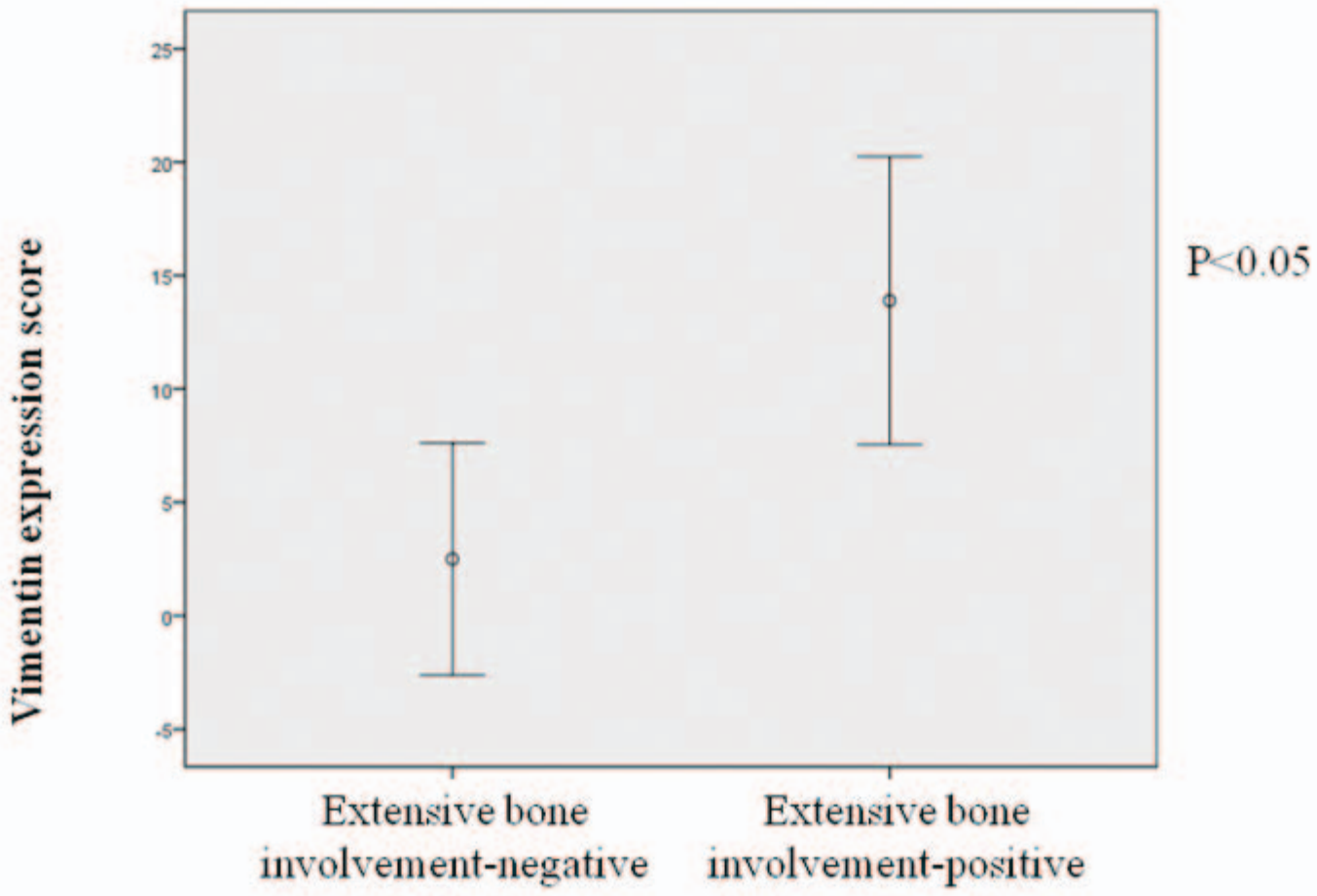
Table 2. Summary of 16 patients with squamous cell carcinoma of the temporal bone

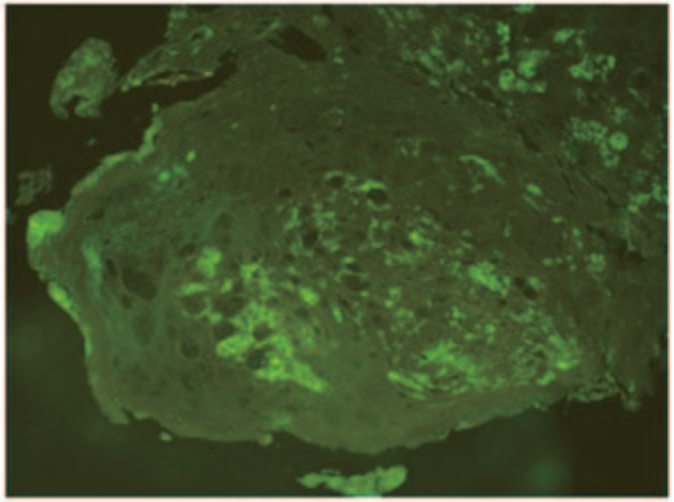
Patient No	Location	Age/Sex	Stage	Extensive bone involvement	Treatment	Outcome	Follow-up(months)	EMT
1	EAC	79/M	T1N0	No	S＋XRT	ND	87	－
2	EAC	77/F	T1N0	No	S	ND	44	－
3	EAC	74/F	T1N0	No	S＋XRT	ND	38	－
4	EAC	49/M	T2N0	No	S＋XRT	ND	30	－
5	EAC	55/F	T2N0	No	S＋XRT	DOD	17	－
6	EAC	69/M	T3N0	Yes	S＋XRT	ND	65	＋
7	EAC	63/M	T3N0	Yes	S＋XRT	ND	33	＋
8	EAC	70/M	T3N0	Yes	S＋XRT	DOD	28	＋
9	ME	72/F	T4N0	Yes	S	ND	60	＋
10	EAC	39/M	T4N0	No	S＋XRT	ND	47	－
11	EAC	50/F	T4N0	No	S＋XRT	ND	32	＋
12	EAC	60/F	T4N1	Yes	S＋XRT	DOD	21	＋
13	EAC	41/F	T4N0	Yes	S＋XRT	DOD	13	＋
14	ME	83/F	T4N0	Yes	XRT	DOD	12	－
15	ME	65/M	T4N0	Yes	XRT	DOD	12	＋
16	EAC	78/F	T4N0	Yes	XRT	DOD	8	＋

EAC, external auditory canal; ME, middle ear; S, surgery; XRT, radiation therapy; ND, no desease ; DOD, dead of disease

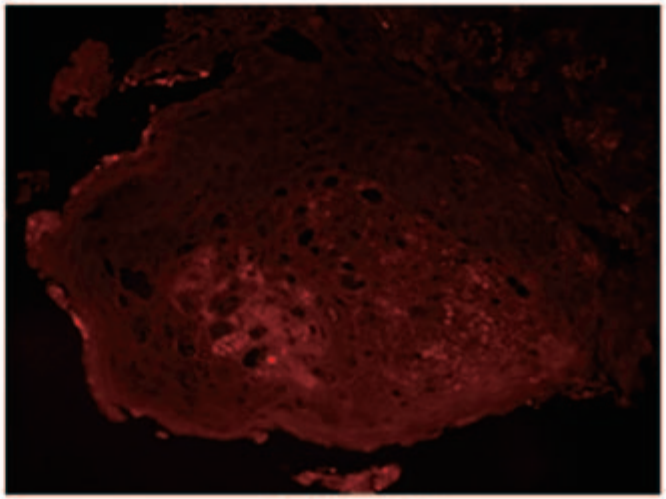

A
B


Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
EMT-negative
EMT-positive
P=NS
0
20
40
60
80
100
120
Months

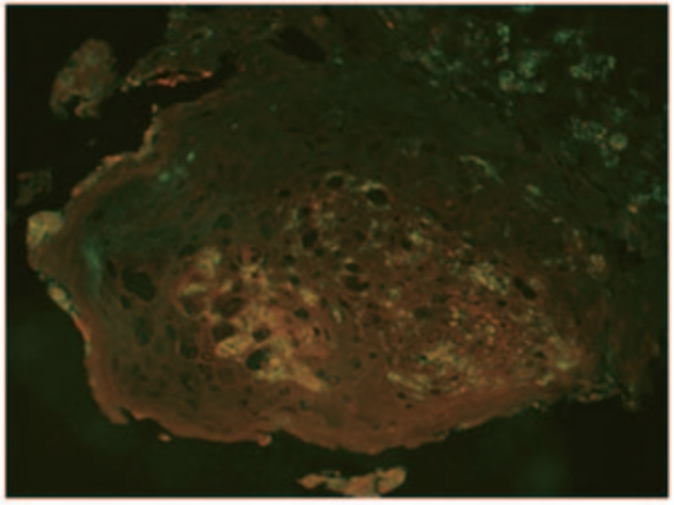

Vimentin expression score
25
20
15
10
5
0
-5
Extensive bone involvement-negative
Extensive bone involvement-positive
P<0.05



TGF-β1



Vimentin



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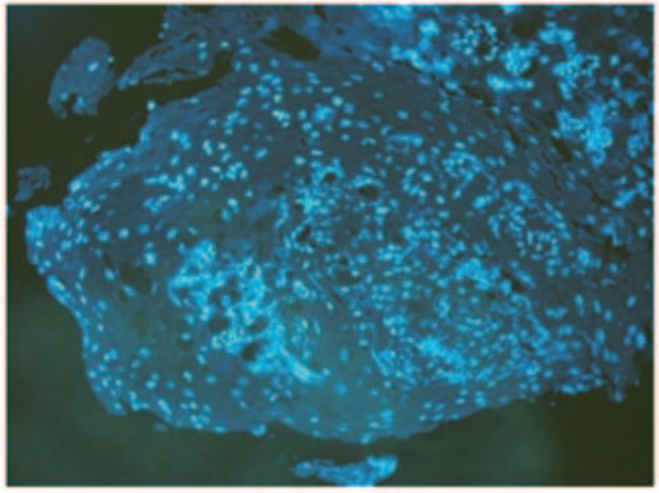

Nuclei