Expression of the membrane-cytoskeletal linker Ezrin in salivary gland adenoid cystic carcinoma

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Objective. The aim of this study was to investigate the relationship between membrane cytoskeleton linker protein Ezrin and CD44v6, iNOS, Ki-67, and clinicopathologic characteristics, and the prognostic significance of Ezrin expression in salivary gland adenoid cystic carcinoma (SACC).

Study design. Immunohistochemistry and reverse-transcription polymerase chain reaction were used to quantify the expression of Ezrin, CD44v6, inducible nitric oxide synthase (iNOS), and Ki-67 in 75 primary SACCs, 25 tumor-free salivary tissues, and 2 SACC cell lines (ACC-M and ACC-2). Survival analysis was performed to find the prognostic significance of Ezrin expression.

Results. Expressions of Ezrin, CD44v6, iNOS, and Ki-67 in SACC tissues, especially with distant metastasis, were significantly higher than in tumor-free tissues. Ezrin mRNA and protein levels in ACC-M cells were significantly higher than in ACC-2 cells. Ezrin, CD44v6, iNOS, and Ki-67 expressions were significantly higher in solid pattern than in cribriform and tubular patterns. Ezrin and its partners, CD44v6, iNOS, and Ki-67, were significantly related to tumor size, clinical stage, perineural and vascular invasion, and recurrence. Furthermore, Ezrin had an independent prognostic effect on overall survival.

Conclusions. The increased expression of Ezrin and its partners, CD44v6, iNOS, and Ki-67, in SACC correlated with histologic patterns, may play a role in distant metastasis, and might indicate poor clinical outcome. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:96-104)

Salivary gland adenoid cystic carcinoma (SACC) is one of the most common epithelial salivary gland carcinomas and is characterized by extensive local tissue infiltration, early development of hematogenous metastasis and poor long-term survival.1-3 The molecular mechanisms for the aggressive invasiveness and distant metastasis remain unclear. Therefore, identification of the key regulatory molecules and signal transduction is crucial for understanding tumor dissemination and for the development of novel interventions.

The identification of Ezrin as a key component in the metastasis of osteosarcoma4 and rhabdomyosarcoma5 is an advance in understanding tumor dissemination. Ezrin is a predominant member of the Ezrin-Radixin-Moesin protein family which was described as a cross-linker between membrane proteins and cytoskeletal actin filaments, through its N-terminal and C-terminal domains, respectively, and is implicated in several important cellular complexes and processes, e.g., in cell adhesion to the extracellular matrix and in cell-cell interactions, and as a conduit for signals between metastasis-associated cell-surface molecules (such as CD44) and signal transduction components.6-9 Additionally, recent studies10,11 showed Ezrin is a negative regulator of Fas-mediated apoptosis both in cancer and normal cells. Evidence from cancer cell lines, animal models, and prospective human studies show that overexpression of Ezrin plays a crucial role in tumor dissemination and indicates poor patient survival.5,12-15

In addition to Ezrin, tumor metastasis–associated molecules CD44v6, inducible nitric oxide synthase (iNOS), and Ki-67 nuclear antigen, as partners of Ezrin, have demonstrated their contributions to tumor development and metastasis in various carcinomas. CD44v6 is related to regulating tumor invasion and metastasis in head and neck malignancies.16 Interestingly, Ezrin directly interacts with the cytoplasmic tail of the CD44 involved in tumor metastasis.9 iNOS, an important component on the angiogenic cascade, contributes to angiogenesis through regulating nitric oxide (NO) production in tumor growth and metastasis.17,18 The precise regulation of NO production in epithelial cells19 by
interaction between iNOS and Ezrin-Radixin-Moesin–binding phosphoprotein 50 (EBP50) suggests some associations between Ezrin and iNOS in angiogenesis. Ki-67 nuclear antigen expresses in all active phases of the cell cycle (G1, S, G2, and mitosis) but is absent in G0; it has been confirmed as a good marker in cell proliferation, as well as in diagnostic and prognostic values for many tumors.²⁰,²¹ Therefore, there is a need for evaluation of these tumor-associated molecular alterations and their correlations with distant metastasis and patient prognosis of SACC.

In vivo experiment⁴ shows that the increased expression of Ezrin in cancer cells is necessary for the development of pulmonary metastasis of osteosarcoma, which suggests that Ezrin may also play a role in distant metastasis, especially in pulmonary metastasis of SACC. In our previous study,²² we demonstrated that Ezrin was related to proliferation and invasion of SACC. We also showed the correlations among the expressions of Ezrin, iNOS, and Ki-67 nuclear antigen in pleomorphic adenoma of salivary gland, which suggested that these alterations promoted tumor proliferation and malignant transformation.²³ However, little research has been devoted to studies of the role of Ezrin and its partners, CD44v6, iNOS, and Ki-67 nuclear antigen, in SACC development, and to the best of our knowledge there are no studies on correlations of Ezrin expression in prognostic and clinical parameters of SACC. The present study compared the expression levels of Ezrin, CD44v6, iNOS, and Ki-67 nuclear antigen in SACC tissues with tumor-free salivary gland tissues and their correlation with distant metastasis. The relevance of Ezrin to clinicopathologic features and clinical outcome was further evaluated with an aim to decide its role in the prognosis of SACC.

MATERIAL AND METHODS

Tissue sampling and cell lines

Seventy-five primary SACCs and 25 tumor-free salivary gland tissues were retrieved from archival material in the Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, from January 1995 to January 2005. All of the patients recruited into the present study did not receive any other treatment before or after operation. The slides were reviewed by a consultant pathologist blinded to the clinical data before immunostaining both to confirm the diagnosis and to categorize SACCs into 3 histologic patterns based on the new World Health Organization criteria.²⁴ Clinical stages were categorized based on TNM classification.²⁵ All of the selected patients had been followed 52-138 months (99.37 ± 19.88 mo) until their death, whereby survival, local recurrence, and the occurrence of distant metastasis were recorded. The cutoff for clinical follow-up was January 2010. The clinicopathologic characteristics are summarized in Table I.

In addition, 2 SACC tumor tissues and the adjacent normal salivary gland tissues, ≥2 cm distal to tumor margins, were snap-frozen in liquid nitrogen and were evaluated by reverse-transcription polymerase chain reaction (RT-PCR) recently. ACC-M and ACC-2 cell lines, obtained from the Department of Oral and Maxillofacial Surgery, Ninth People’s Hospital, Shanghai Jiao Tong University, School of Medicine, have been characterized and studied previously.²⁶ They were maintained in RPMI1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in 5% CO₂.

Antibodies and immunohistochemistry

Antibodies against Ezrin, CD44v6, Ki-67 nuclear antigen (mouse monoclonal), and iNOS (rabbit monoclonal) were from Neomarker, Fremont, CA, USA. Streptavidin peroxidase test kit was from Zhongshan Biotechnology, Beijing, China.

Immunohistochemical studies in 75 SACC specimens and 25 tumor-free tissues were done according to the method described by the manufacturer’s instructions and our previous study.²³ Negative control samples were treated in the same product but omitting the primary antibodies, and the positive control samples were mammary carcinomas known to have positive staining for Ezrin, CD44v6, iNOS, and Ki-67 nuclear antigen.

The expression of Ezrin protein in ACC-2 and ACC-M cells was detected according to the methods described by our previous study on tongue squamous cell carcinoma. Stained cells were analyzed and quantified by using an IMS image analysis system (Shanghai Shenteng information Technology Co., Shanghai, China). The integral optical density was calculated according to the formula: intensity × area × K-area coefficient. The mean intensity for each series of samples was considered to reflect the intensity of Ezrin protein expression.

Immunohistochemical evaluation

Ezrin, CD44v6, and iNOS stainings were assessed and rated basing on the rate of positive tumor cells and the intensity of stain according to methods described by Zhang et al.²⁸ The number of tumor cells stained was evaluated as follows: 0, <10% of cells stained in the microscopic fields; 1, 10%-25% of cells stained positive; 2, 25%-50% stained positive; 3, 50%-75% stained positive; and 4, >75% stained positive. The intensity of staining was on the following scale: 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The combined score was between 0 and 7. A score
Table 1. Correlation among clinicopathologic status and the expression of Ezrin, CD44v6, inducible nitric oxide synthase (iNOS), and Ki-67 nuclear antigen in adenoid cystic carcinoma patients

<table>
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<tr>
<th>Characteristic</th>
<th>n</th>
<th>Ezrin Mean ± SD</th>
<th>P value</th>
<th>CD44v6 Mean ± SD</th>
<th>P value</th>
<th>iNOS Mean ± SD</th>
<th>P value</th>
<th>Ki-67 PI Mean ± SD</th>
<th>P value</th>
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<tr>
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<td>4.41 ± 1.43</td>
<td>.081</td>
<td>4.68 ± 1.28</td>
<td>.837</td>
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<td></td>
<td>34</td>
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<td>4.38 ± 1.43</td>
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<td>4.69 ± 1.10</td>
<td>.906</td>
<td>61.72 ± 13.97</td>
<td>.072</td>
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<td>4.72 ± 1.09</td>
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<td>.233</td>
<td>4.36 ± 1.44</td>
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<td>4.64 ± 1.05</td>
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<td>&gt;4 cm</td>
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<td>5.62 ± .92</td>
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<td>5.19 ± 1.21</td>
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<td>5.05 ± 1.24</td>
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<td>32</td>
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<td>.000</td>
<td>4.25 ± 1.22</td>
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<td>4.31 ± .97</td>
<td>.000</td>
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<td>4.56 ± .97</td>
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<td>5.75 ± .86</td>
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<td>5.14 ± 1.21</td>
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<td>.000</td>
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<td>4.44 ± 1.05</td>
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<td>4.40 ± 1.04</td>
<td>.001</td>
<td>61.72 ± 12.54</td>
<td>.000</td>
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<td>Positive</td>
<td>28</td>
<td>5.79 ± .83</td>
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<td>5.39 ± 1.07</td>
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<td>4.46 ± 1.27</td>
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<td>4.52 ± 1.09</td>
<td>.035</td>
<td>62.18 ± 13.31</td>
<td>.041</td>
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<td>Death</td>
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<td></td>
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<td>5.21 ± .99</td>
<td></td>
<td>69.08 ± 14.01</td>
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_Pi_, proliferation index.

of ≤3 was considered to be low expression, 4 or 5 moderate expression, and 6 or 7 strong staining.

The nuclear localization of Ki-67 nuclear antigen was considered to be positive expression. This study used Ki-67 proliferation index (Ki-67 PI) as evaluation of Ki-67 expression, according to the method described by our previous study.23

Reagents and RT-PCR analysis

PCR primers for Ezrin, CD44v6, iNOS, and the internal control β-actin were designed using Olig5 software and were synthesized by the Shanghai Biosynthesis Co., China. Ezrin: forward (F) 5’-GGC GTG GGA TCA GAT AAA GA-3’, reverse (R) 5’-AGT GAT GCC CTT CTC CTC AT-3’. CD44v6: F 5’-CCC TGC TAC CAG AGA CCA AGA C-3’, R 5’-GCA GGT GCC TTG TCT CAT CAG C-3’; iNOS: F 5’-GGA GCC AGC TCT GCA TTA TC-3’, R 5’-TTT TGT CTC CAA GGG ACC AG-3’; β-actin: F 5’-GAT GAG ATT GCC ATG GCT TT-3’, R 5’-CTC AAG TTG GGG GAC AAA AA-3’.

According to the manufacturer’s instructions, total RNAs from SACC tissues and the adjacent normal tissues, as well as from ACC-2 and ACC-M cells were extracted by using the Trizol Reagent (Invitrogen, Carlsbad, CA). The RT and PCR reagents were from Takara, Shiga, Japan. Amplified PCR products were electrophoresed in 1.5% agarose gels (Gene Tech, South San Francisco, CA) and visualized under ultraviolet illumination by ethidium bromide staining. The intensities of bands were measured by computerized image analysis. All experiments were carried out 3 times: each time, the reading was taken in triplicate and the mean and standard deviations were calculated.
Statistical analysis was carried out by using SPSS for Windows version 13.0. Values are presented as mean ± SD. Results were analyzed with one-way analysis of variance test, Spearman order correlation analysis, Kaplan-Meier survival curves by log-rank test, and Cox proportional hazards model. *P < .05 was considered to be statistically significant.

RESULTS
Expression and correlationship of Ezrin, CD44v6, iNOS, and Ki-67 nuclear antigen

Immunopositivities for Ezrin, CD44v6, iNOS, and Ki-67 nuclear antigen were confined to different cell localizations (Fig. 1). For tumor-free normal salivary gland tissues, faint staining was found in ductal cells. In the SACC specimens, Ezrin and iNOS proteins showed moderate to strong cytoplasmic staining, and CD44v6 immunostaining was strongly detected in cytomembrane in some but not all tumor cells. Ki-67 nuclear antigen was detected in nuclei of tumor cells with brownish staining, and Ki-67 PI is presented in Table I.

The data of Ezrin, CD44v6, iNOS, and Ki-67 expression are presented in Table I. There were 3 cases with moderate Ezrin staining, 1 case with moderate CD44v6 staining, 3 cases with moderate to strong iNOS staining in 25 tumor-free normal salivary gland tissues. Sixty-nine of 75 SACC specimens were scored with moderate to strong Ezrin staining, CD44v6 was detected with moderate to strong positive staining in 60 specimens, and iNOS in 64. It was evident that immunostaining of Ezrin, CD44v6, iNOS, and Ki-67 in SACC tissues was significant higher than in tumor-free salivary gland tissues (*P = .000). Furthermore, higher levels of Ezrin, CD44v6, and iNOS mRNA were detected in the SACC tissues compared with the adjacent normal tissues (*P values .001, .002, .009, respectively; Fig. 2).
In addition, we analyzed the relationship among the 4 molecules and found that the individual expressions of CD44v6, iNOS, and Ki-67 all significantly correlated with Ezrin\( (r = .714, P = .000; r = .627, P = .000; r = .615, P = .000; \) respectively; Table I), and Ki-67 was also significantly correlated with iNOS\( (r = .703, P = .000; \) Table I).
Fig. 4. Kaplan-Meier survival curves for salivary gland adenoid cystic carcinoma (SACC) patients plotted on Ezrin, CD44v6, inducible nitric oxide synthase (iNOS), and Ki-67 nuclear antigen expressions, tumor size, distant metastasis, and histologic pattern. Kaplan-Meier curves with log-rank tests showed statistical difference in survival between patients with high (immuno-
Relationship of Ezrin and its partners, CD44v6, iNOS, and Ki-67 nuclear antigen in distant metastasis and other clinicopathologic characteristics

In this study, we found Ezrin immunostaining scores in SACC with distant metastasis averaged 5.79 ± .83, which was significant higher than the average score of 4.47 ± .88 in SACC without distant metastasis (P = .000; Table I). Furthermore, Ezrin mRNA and protein expression levels in ACC-M cells with high lung metastasis potentials were significant higher than in ACC-2 cells (P values .01 and .000, respectively; Fig. 3). No significant association was observed between Ezrin expression and the patients’ gender, age, or tumor location. However, tumors with advanced clinical stages, tumor size >4 cm, perineural and vascular invasion, or local recurrence expressed higher levels of Ezrin (Table I). The association of several other clinicopathologic characteristics with CD44v6, iNOS, and Ki-67 is presented in Table I.

Among the three histologic patterns of SACC, Ezrin, CD44v6, iNOS, and Ki-67 were significantly higher in the solid pattern than in cribriform and tubular patterns (P = .000; Fig. 1, Table I), whereas no significant difference was found between cribriform and tubular patterns. Interestingly, we found that tubular lining cells and luminal walls had more iNOS stainings than the outer layer cells. In contrast, CD44v6 stainings were stronger in outer layer cells than lining cells of SACC tissues, which indicated that the different distributions might relate to different functions (Fig. 1).

Survival analysis

To further evaluate Ezrin and clinical outcome in SACC, we analyzed the association of Ezrin-immunopositive stainings with survival of postoperative patients. Figure 4 shows survival curves analyzed by the Kaplan-Meier method (log-rank test). Patients with low and moderate Ezrin expression survived significantly longer than those with high Ezrin expression (P = .002; Fig. 4, A). In addition, high CD44v6, iNOS, and Ki-67 immunopositivities prognosis worse survival (P values .006, .027, and .037, respectively; Fig. 4, B-D), but the correlation was not as notable as that with Ezrin. Patient survival was also associated with tumor size (P = .000; Fig. 4, E), distant metastasis (P = .000; Fig. 4, F), and histologic patterns (P = .000; Fig. 4, G). Multivariate analysis (Cox proportional hazards model) showed that Ezrin expression, independent of other clinicopathologic covariates, can be used as an independent prognostic factor for SACC (hazard ratio 2.897; P = .003).

DISCUSSION

In this study, we detected overexpression of Ezrin, CD44v6, iNOS, and Ki-67 nuclear antigen in SACC tissues and related immunostaining with histologic pattern. In vitro, expression levels of Ezrin mRNA and protein in ACC-M cells were significantly higher than in ACC-2 cells. We identified overexpression of Ezrin and related it to distant metastasis and shortened survival of SACC patients. This was determined to be a prognostic factor independent of other clinicopathologic factors. In addition, we supposed that expression of Ezrin, CD44v6, and iNOS in ductal cells might play a role in secretion and potentially in the morphogenesis of neoplasms, as indicated in previous studies.29-31

The present study confirmed that overexpression of Ezrin related to distant metastasis and poor outcome of SACC patients. Significant correlations between Ezrin expression and invasiveness as well as metastasis have been shown in several common cancers.4,5,12,13,32 The exact mechanism of Ezrin’s contribution to tumor invasion and metastasis remains to be elucidated. As a cross-linker between membrane proteins and the cytoskeleton, and participating in signal-transduction pathways, functional activation of Ezrin upon threonine and tyrosine phosphorylation can mediate many changes in the metastasis-associated cell-surface signals and intracellular signaling cascade and, ultimately, in the nucleus that confer the metastatic capability in tumor cells.8,9,33 It has been shown that Ezrin is necessary for several signaling pathways important to tumor dissemination, including mitogen-activated protein kinase, protein kinase B, protein kinase C, PI3K, Rho, transforming growth factor β, CD44, and others.4,5,9,12,13,34 In addition, Ezrin has been reported to associate with micro-RNA and c-Myc in invasion and metastasis of cancer cells.14,35

CD44 is confirmed to mediate cell-cell and cell-matrix interactions and is known to induce intracellular signaling.36 Ezrin directly interacts with the cytoplasmic tail of the CD44, which has been reported in tumor
metastasis. In agreement with these functions of Ezrin and CD44, we observed that Ezrin was associated with CD44v6 expression which correlated with distant metastasis, histologic pattern, perineural and vascular invasion, and clinical stage of SACC in the present study. We also found that Ezrin was strongly expressed in some tumor cells and that CD44v6 staining was stronger in nonluminal cells than in luminal cells, which indicated that the tumor-associated markers might not absolutely exist in all tumor cells. In addition, some studies suggest that Ezrin, together with CD44, is involved in Fas-mediated apoptosis both in Jurkat cells and normal lymphocytes, which may or may not be involved in metastasis-associated apoptosis resistance.

It was reported that NO production induced by vascular endothelial growth factor in endothelial cell was attenuated by silencing Ezrin through down-regulating a series of signaling transductions, which indicated that NOS is functionally linked to Ezrin in regulating angiogenesis. A strong and significant correlation was also observed between Ezrin expression and iNOS in SACC in the present study, which suggested that they might also play a role in tumor angiogenesis. Glynne et al. reported that the association between iNOS and EBP50 regulated precise NO production in epithelial cells, which might play an important role in angiogenesis. Interestingly, the biologic functions of Ezrin and iNOS may interconnect in tumor angiogenesis, because iNOS expressions are associated with Ezrin and were correlated with malignant proliferation of salivary gland pleomorphic adenoma in our previous study.

In the present study, Ki-67 was associated with Ezrin expression in SACC. This also correlated with histologic pattern, distant metastasis, and poor prognosis, as noted in an earlier study. In the present study, Ki-67 was expressed strongly in solid components of SACC tissues, which may explain a worse prognosis in the solid pattern. A recent study in osteosarcoma shows that the nuclear localization of phosphorylated Ezrin Tyr354 suggests its possible role as a nuclear factor. These results suggest that the intracellular signaling cascade mediated by Ezrin in the nucleus may be associated with Ki-67 nuclear antigen in SACC.

In conclusion, the present study demonstrated that Ezrin expression is significantly associated with histologic pattern, tumor diameter, distant metastasis, clinical stage, and poor survival time. Further in vitro and in vivo experiments are encouraged to address the exact mechanism. However, these results expand the understanding of local invasiveness and distant metastasis in SACC, which suggests that Ezrin and its partners might represent potential targets for future anticancer therapeutic strategies in SACC.

The authors thank the Department of Oral and Maxillofacial Surgery, Ninth People’s Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China, for providing the ACC-2 and ACC-M cell lines.

REFERENCES


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