Clinical significance of apoptosis-associated speck-like protein containing a caspase recruitment domain in oral squamous cell carcinoma

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Objectives. To assess apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) expression in oral squamous cell carcinoma (OSCC) and analyze its clinical and pathological significance.

Study design. ASC expression was studied using immunohistochemistry in 119 OSCCs patients. The relationships between ASC expression and clinical and pathological parameters were statistically analyzed. In addition, the relationships between ASC expression and cell differentiation [IVL (involcrin) expression] and apoptosis (TUNEL [TdT-mediated dUTP nick end labeling] positive cell number) were investigated.

Results. ASC expression showed significant correlations with parameters including clinical tumor stage, mode of invasion, and histological differentiation, and had a significant impact on survival of OSCC. The distribution of ASC correlated well with that of IVL. ASC expression was significantly correlated with the TUNEL-positive cell number.

Conclusions. Lower ASC expression correlates with clinical and pathological malignancy and, consequently, poor prognosis of OSCC. ASC has a close association with cell differentiation and apoptosis. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:799-809)

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is the most common head and neck cancer, with 263,900 new cases and 128,000 deaths resulting from oral cavity cancer (including lip cancer) worldwide in 2008.[1] OSCC ranks as the third most common cancer in developing nations.[2] In spite of improvements in the diagnosis and management of OSCC, long-term survival rates have improved only marginally over the past decade. More specifically, although the use of combination treatment has become more widespread, the 5-year relative survival for OSCC has not decreased significantly over the past 30 years, and the five-year survival rate remains at less than 60%.[3] The development of molecular markers is an important strategy for improving the diagnosis and assessment of the risk of tumor progression and metastasis in OSCC.

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) has been identified as an intracellular protein partitioned into an insoluble cytoskeletal fraction, termed "speck," in human pro-myelocytic leukemia HL-60 cells undergoing apoptosis.[4] Martinon et al.[5] reported that ASC is indispensable for the activation of procaspase 1 and proIL-1beta for the inflammasome. Large proportions of the genes induced by ASC activation are related to transcription (23%), inflammation (21%), or cell death (16%), indicating that ASC is a potent inducer of inflammatory and cell death-related genes.[6] It is also reported that ASC plays pivotal roles in regulating autoinflammatory diseases and cancer. Conway et al.[7] showed that ectopic expression of ASC induced apoptosis and inhibited the survival of human breast cancer cells. They also showed inhibition of ASC gene expression by methylation in human breast cancer, suggesting that ASC is anti-oncogenic through its pro-apoptotic function.[7] Subsequent down-regulation of ASC expression mediated by aberrant methylation was reported in melanoma,[8] colon cancer,[9] and prostate cancer.[10] In autoinflammatory disease, contribution of the inflammasome to tumorigenesis has been reported. IL-1-mediated autoinflammation was demonstrated to contribute to the development and progression of human melanoma.[11] Mice lacking the inflammasome adapter

Statement of Clinical Relevance

ASC is an independent and significant predictor of mortality risk in OSCC. Clinically, ASC expression is a useful marker for assessing prognosis of OSCC. ASC might play roles in regulating cell differentiation and apoptosis of oral epithelial and tumor cells.

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Table I. Specific primer for real-time PCR

Gene	*Sense primer*	*Antisense primer*
β-actin	5′-GGACTTCGAGCAAGAGATGG-3′	5′-GTGGATGCCACAGGACTCCAT-3′
ASC	5′-ACATGGGCCTGCAGGA-3′	5′-GCCACTCAACGAGCAAGAGATGG-3′
IVL	5′-GGGTGGTTATTTATGTTTGGGTGG-3′	5′-GCCAGGTCCAGACATTCAAC-3′

protein ASC and caspase-1 demonstrate improved disease outcome, morbidity, tumorigenesis, and polyp formation by dextran sulfate sodium stimulation, correlating with attenuated levels of IL-1beta and IL-18 in acute and recurring colitis and colitis-associated cancer.[12] IL-18 production downstream of the Nlrp3 inflammasome leads to production and activation of the tumor suppressors and tumorigenesis.[13]

ASC might have an important role in regulating cancer. However, the expression and potential roles of ASC in OSCC have not been investigated. Therefore, we assessed ASC expression in OSCC and analyzed its clinical and pathological significance. In addition, a possible role of ASC in differentiation and apoptosis of OSCC was investigated.

MATERIALS AND METHODS

This study was approved by the Committee on Medical Research of Shinshu University.

Studies in clinical samples

Subjects. This retrospective study included 119 consecutive patients with previously untreated OSCC who were diagnosed at the Department of Dentistry and Oral Surgery, Shinshu University School of Medicine between 1990 and 2005. They consisted of 72 men and 47 women averaging 65.6 years of age (range, 27-91 years). The follow-up time was a median of 60 months (interquartile range: 9-74 months). Tissues from the primary tumor were collected during biopsy or surgical resection of the tumor after patients signed the informed consent form approved by the Institution Review Committee. Characteristics of the subjects are summarized in Table II. As controls, normal oral mucosa was also obtained from 6 volunteers during removal of a lower wisdom tooth after the volunteers provided informed consent.

Expression of ASC and involcrin (IVL). The expression of ASC, as well as IVL, was assessed by immunohistochemical (IHC) study. IVL was employed as a marker of cell differentiation of OSCCs.[14,15] Tissues embedded in paraffin were cut into 3-μm sections and de-waxed.[16] The specimens were treated with mouse anti-human ASC antibody diluted 1:400 and mouse anti-human IVL: SY5 (Sigma−Aldrich, Tokyo, Japan) antibody diluted 1:500, and then incubated with Envision System (Dako, Glostrup, Denmark). Anti-human ASC antibody was kindly provided by Dr. Junji Sagara.[17] We used glomeruli of the kidney as a negative control and collecting ducts as a positive control.[18] In IVL, we used human skin epithelium for positive and subcutaneous tissue for negative controls.[19]

In the stained sections, cells with brown cytoplasm were regarded as ASC-positive. Three representative fields of each section (magnification ×60) were selected and captured by digital imaging. The percentage of ASC-positive cells in carcinoma (epithelial) cells was calculated in the printed image (300 dot per inch). We classified the sections into three grades (ASC score 1; positive cells ≤33%, score 2; 34%-66%, and score 3; 67%-100%) according to the modified method of Wei et al.[20] Each section was examined by two independent examiners (H. Ko and H. Ai) and there was no disagreement in the final ASC score.

Expression of ASC and IVL in immunofluorescence study. Ten specimens that showed strong and significant staining for ASC and IVL in IHC were randomly selected and subjected to immunofluorescence double staining for ASC and IVL. De-paraffined and rehydrated sections were rinsed and endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2 and 1% fetal calf serum (Sigma−Aldrich), respectively. The sections were incubated overnight at 4 °C with mouse anti-human ASC antibody-labeled by Cy3 (HiLyte Fluor 555 Labeling kit; Doujindo, Kumamoto, Japan). Sections incubated with mouse anti-human IVL antibody were then incubated for 1 h at room temperature with Alxa 488 diluted 1:400 (Amersham Biosciences, Amersham, UK). Finally, tissue sections were photographed with a confocal-laser scanning microscope (Leica TCS SP2 AOBS, Leica Microsystems, Tokyo, Japan).

Assessment of apoptosis. IHC staining for TdT-mediated dUTP nick end labeling (TUNEL) was employed to assess apoptosis. Three tissue samples each were randomly selected from different histologic grades of OSCC (well, moderate, and poorly differentiated OSCC). As a control, normal oral mucosa ($n = 4$) samples were obtained from consenting patients. Apoptotic cells were detected using an In Situ Apoptosis Detection Kit following the manufacturer's protocol (Takara, Ohtsu, Shiga, Japan). The apoptotic index (AI) was calculated as the ratio of TUNEL-positive cells per 1000 cancer (epithelial) cells.[21]