Clinical Significance of apoptosis-associated speck-like protein containing a caspase recruitment domain in Oral Squamous Cell Carcinoma

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ABSTRACT

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. The correlations among ASC expression, histological differentiation, and apoptosis of OSCCs were also investigated.

Study design: ASC expression was studied using immunohistochemistry in 119 OSCCs patients. The relationships between ASC expression and clinical and pathological parameters of OSCC were statistically analyzed. To test the hypothesis that ASC had a significant role in regulating cell differentiation and apoptosis, the relationships between ASC expression and markers involcrin (IVL) and TdT-mediated dUTP nick end labeling (TUNEL) positive cell number were investigated.

Results: ASC score showed significant correlations with parameters including clinical tumor stage, mode of invasion, and histological differentiation, and had a significant impact on survival of OSCCs in the Cox proportional hazard model. The distribution of ASC well agreed with that of IVL. ASC protein level as well as IVL increased with advancing differentiation of oral epithelial cells. ASC expression was significantly correlated with the TUNEL-positive cell number.

Conclusions: Lower ASC expression was well correlated with clinical and pathological malignancy and, consequently, poor prognosis of OSCC. ASC had a close association with cell differentiation and apoptosis.

KEY WORDS:

Oral cancer, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC),

apoptosis, cell differentiation, prognosis, involucrin (IVL), HaCaT, normal human epithelial keratinocyte (NHEK), cancer pearl

1. INTRODUCTION

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 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 recent widespread use of combination treatment has progressed, the 5-year relative survival for SCC has not decreased significantly over the past 30 years, and the five-year survival rate of less than 60% remains unsatisfactory [3]. The development of molecular markers is an important strategy for improving the diagnosis and assessment of tumor progression and metastasis in OSCC.

Although OSCC is believed to arise through accumulation of genetic and epigenetic alterations impairing the function of tumor-suppressor genes or provoking the functions of oncogenes, the genes associated with OSCC carcinogenesis either directly or indirectly remain unclear. 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 1and proIL-1e for theinflammasome. 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. 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 proapoptotic 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 in 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 adaptor protein ASC and caspase-1 demonstrate increased 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 tumorigenesis [13].

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

2. MATERIALS AND METHODS

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

2.1 Studies in clinical samples

2.1.1 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 median 60.0 months (interquartile range: 9.0-74.0 months). Tissues from their 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 1. As a control, normal oral mucosa were also obtained from 6 volunteers during removal of a lower wisdom tooth after the volunteers provided informed consent.

2.1.2 Expression of ASC and involcrin (IVL)

The expressions of ASC, as well as IVL, a molecule that has been used as a marker of differentiation of keratinocytes, were assessed by immunohistochemical (IHC) study. Tissues embedded in paraffin were cut into 3-μm sections and de-waxed [14]. 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 [15]. We utilized the ASC by using glomeruli of the kidney as a negative control and collecting ducts as a positive control. [16] In IVL, we used human skin for positive and negative controls[17].

In the stained sections, cells with brown cytoplasm were regarded as ASC-positive. Three representative fields of each section (magnification x 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 the modified method of Wei Q et al [18]. Each section was examined by two independent examiners (H. Ko, H. Ai) and there was no disagreement in the final ASC score.

2.1.3 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_20_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 hour 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).

2.1.4 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 grades of histological differentiation 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 obtained as the ratio of TUNEL-positive cells per 1,000 cancer (epithelial) cells[19].

2.1.5 Analysis of clinical and pathological significance of ASC

The relationships between expression of ASC and clinicopathological indices were evaluated by Spearman's rank correlation or Mann-Whitney U-test. Clinicopathological factors including age, gender, primary site, tumor stage, histological differentiation, mode of invasion, and lymphocytic infiltration were collected from patient records. The grade of histological differentiation of OSCCs was determined according to the criteria proposed by the World Health Organization (WHO) (2004). The mode of invasion was classified according to the criteria proposed by Yamamoto and Kohama [20].

The effect of expression of ASC on survival of OSCC patients was also estimated. The primary end point was disease specific survival (DSS) time. Calculation was started at the time of the patient's first visit. All patients were treated/managed according the proposal of NCCN guidelines. The Kaplan-Meier estimates of five-year DSS rate were compared among the three ASC score groups. In addition, Cox's multivariate proportional hazard model was employed to analyze hazards after controlling for the potentially

confounding variables listed above.

Analyses were performed using the StatView software package for Macintosh (SAS Institute, Inc., NC, USA). All p values < 0.05 were considered significant.

2.2 Studies in vitro

2.2.1 Cell culture

Cells of the spontaneously immortalized human keratinocyte cell line, HaCaT, were obtained from Tohoku University. Normal Human Epidermal Keratinocytes, NHEK, were isolated from normal oral mucosa obtained from healthy volunteers. Both the HaCaT cells and NEHK cells were cultured in Keratinocyte Serum Free Media (KSFM, Gibco, Cergy Pontoise, France) already containing 0.09 mM calcium, supplemented with 25 mg／ml Bovine Pituitary Extract (Gibco) and 1.5 ng／ml of recombinant Epidermal Growth Factor (Gibco)[21]. Six cancer cell lines (HSC2, HSC3, SQUU-A, SQUU-B, Ca9-22 and SAS) established from OSCC patients were also used. HSC2, HSC3, Ca9-22 and SAS were obtained from the Japanese Collection of Research Bioresources. SQUU-A and SQUU-B were kindly provided by Kyushu University[22]. All cell lines except SAS were maintained in Eagles' medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) at 37ºC in 95% air/5% CO2. SAS was maintained in DMEM/F12 (Gibco). For all experiments, freshly trypsinized cells were seeded at a density of $4x10^4$ cells／cm^2.

In order to investigate the alteration of ASC and IVL levels according to cell differentiation, NHEK and

HaCaT cells were stimulated and induced to differentiate by calcium, and the ASC and IVL expressions were evaluated. NHEK cells and HaCaT cells were allowed to adhere for 24 hours in normal KSFM (0.09 mM calcium) and the medium was then changed to KSFM containing a high concentration of calcium (1.2 mM calcium). Samples were harvested before and 24 and 48 hours after the medium change [23].

2.2.2 Expression of ASC and IVL in cultured cells

The ASC and IVL levels in cultured cells were analyzed by Western blot analysis and real-time PCR.

In Western blotting, 10μg total protein was separated on 12.5% SDS-PAGE gel. Dilutions of primary antibodies were IVL: SY5 (1:2000), ASC (1:5000) and mouse monoclonal anti-human ß-actin (1:5000; Sigma-Aldrich). All blots were developed by Immobilon™ Western (Millipore Corporation, Billerica, MA, USA), following the manufacturer's protocol.

In Real-time PCR, total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (100ng) was reverse-transcribed with PrimeScript® RT reagent Kit (Takara). The resulting cDNAs were analyzed using the Thermal Cycler Dice Real Time System (Takara, Ohtsu, Shiga, Japan) and SYBR® Premix Ex Taq™ II (Takara). The reaction mixture containing cDNA, SYBR green and primer mix (300 nm) was placed in each well of a 96-well plate. The reaction conditions were set at 95 ºC for 3 minutes followed by 40 cycles at 95 ºC for 15 seconds and 60 ºC for 30 seconds [23]. Primer sequences are provided in Table 1[23],.

3. RESULTS

3.1 ASC expression in normal oral epithelium and OSCC

In normal oral epithelium, strong immunoreactivity for ASC was seen in the cells in the granular cell layer. Weak immunoreactivity was seen in the cells in the prickle cell layer (Fig.1A, B). In the well-differentiated OSCC, immunoreactivity for ASC was observed in the cytoplasm of cancer cells in the outer layers of cancer pearls with weak activity in their centers (Fig.1C). In moderately-differentiated OSCC, weak immunoreactivity for ASC was seen in the cancer pearls (Fig.1D). In poor-differentiated OSCC, expression of ASC was hardly detected (Fig.1E).

The ASC scores in each layer of normal oral epithelium are shown in Figure 1F. ASC scores were 1.02 ± 0.14 (mean ± standard deviation) in the basal, 1.93 ± 0.84 in the prickle, 2.87 ± 0.34 in the granular, and 2.17 ± 1.13 in the cornified cell layers. The ASC-positive cell number increased according to the cell differentiation, but decreased in cells with adenucleus. On the other hand, in OSCCs, the ASC score was 1.55 ± 0.69 in poorly-differentiated, 2.00 ± 0.59 in moderately-differentiated, and 2.73 ± 0.45 in well-differentiated OSCC. There was a significant correlation between the ASC score and histological differentiation grade of OSCC (Spearman's rank correlation, $r=0.62$, $p<0.01$).

3.2 Correlation between ASC score and clinicopathological parameters

The relationships between ASC score and various clinicopathological parameters are summarized in Table 2. ASC score showed a significant correlation with primary site, T-classification, clinical stage, mode of invasion, histopathological differentiation, and lymphocytic infiltration. ASC score decreased with

advancing clinical and pathological malignancy grades.

3.3 Clinical impact of ASC expression on survival of OSCC patients

The Kaplan-Meier estimates of DSS with different grades of ASC score are shown in Figure 2. The survival rate was higher in patients with ASC score 3, followed by those with score 2 and those with score 1. The survival rates among those patient groups were statistically significant (Log-rank test, $p<0.01$).

The results of multivariate analysis using the Cox proportional hazards model are shown in Table 3. ASC score was given priority in the analysis, and pathological differentiation and mode of invasion were not included in the model because they confounded the ASC score; both differentiation and mode of invasion showed a statistically significant correlation with ASC score, as shown in Table 4. The results of multivariate analysis showed that both the ASC score and clinical stage had a statistically significant impact on DSS ($P<0.05$). Higher ASC score was related with better DSS.

3.4 Correlation of ASC and IVL expression

IHC and immunofluorescence staining for both ASC and IVL were carried out in normal mucosa and OSCC tissue (Figure 3). The expression patterns of ASC and IVL were similar in the IHC staining (Figure 3A vs. 3B and 3G vs. 3H). Localization of ASC coincided with the expression of IVL in both normal and OSCC tissue. In the results of the immunofluorescence study, the distribution of ASC agreed well with that of IVL (Figure 3C vs. 3D, 3E vs. 3F in normal and 3I vs. 3J, 3K vs. 3L in OSCC).

3.5 ASC expression and apoptosis

The association between ASC expression and apoptosis was evaluated. Apoptotic cells were detected using

TUNEL staining. In normal oral mucosa, TUNEL-positive cells were localized only in the cornified cell layer (Fig.4C). In OSCC, TUNEL-positive cells were scattered in the cancer pearls of well-differentiated OSCC and sparsely in the moderately-differentiated OSCC (Figure 4G and 4K). If the distribution of TUNEL positive cells was compared with that of ASC positive cells, TUNEL positive cells existed closest to ASC positive cells and, especially in OSCC tissue, TUNEL positive cancer cells were surrounded by the ASC positive cancer cells (Fig. 4C vs. 4A, 4G vs. 4E, and 4K vs. 4I).

Apoptosis indices (AI) of different differentiations of OSCC are shown in Figure 5A. AI was 0.57±0.40 in well-differentiated, 0.22±0.23 in moderately-differentiated, and 0.13±0.02 in poorly-differentiated OSCC. There was a significant correlation between AI and histological differentiation (Spearman's rank correlation test, $r=0.74$, $p<0.05$). If the association between ASC score and AI was assessed, there was a significant correlation between them (Spearman's rank correlation test, $r=0.74$, $p<0.01$). Cell apoptosis increased with increasing expression of ASC (Fig.5A-B).

3.6 ASC and IVL levels in oral cancer cell lines and normal keratinocytes.

Expression levels of ASC and IVL in six established cell lines of OSCC (SAS, Ca9-22, HSC2, HSC3, SQUU-A and SQUU-B) and normal keratinocytes (HaCaT cell and NHEK) were assessed by western blotting (Fig. 6A). ASC was observed in almost all cell lines. However, the expression was stronger in HaCaT and NHEK than in the cancer cell lines. As for IVL, it was detected in NHEK and HaCaT, but not in OSCC cell lines. The results of real time PCR showed higher mRNA expression of both ASC and IVL in normal cell lines (NHEK and HaCaT) than in OSCC cell lines (Fig.6B and 6C).

3.7 The alteration of ASC and IVL level according to cell differentiation

NHEK and HaCaT cells were stimulated and induced to differentiate by calcium, and the ASC and IVL expressions were evaluated. In the assessment by western-blotting, increased protein levels of ASC as well as IVL were suggested at 24 hours and 48 hours after induction of cell differentiation in both cell lines (Fig. 7A and 7C). Additionally, ASC- and IVL-mRNA expressions were quantified by real time PCR. Both ASC and IVL mRNA levels increased after the stimulation by calcium. Forty-eight hours after the induction, the mRNA level became 2.38-fold in ASC and 2.49-fold in IVL in NEHK cells (Fig.7B). In HaCat cells, the levels of mRNA were 3.34-fold in ASC and 2.7-fold in IVL at 48 hours after the medium change.

4. Discussion

In this paper, we studied ASC expression in OSCCs. We believe this is the first report to assess ASC expression in OSCCs.

We demonstrated strong immunoreactivity for ASC in the OSCC cells in the outer layer of cancer pearls and weak immunoreactivity in the cancer pearls. In normal oral epithelium samples, strong immunoreactivity for ASC was seen in the cells in the granular cell layer and weak immunoreactivity was seen in the cells in the granular and pickle cell layers. Masumoto et al. studied ASC expression in normal epithelium of the colon and skin and reported that ASC expression was detected in the epithelial surface and in some differentiated functional cells for which proliferation is regulated[24]. The expression patterns of ASC were associated with differentiation of the squamous epithelium in the skin, tonsil, and mucosal epithelium

of the intestine. ASC expressed in the epithelial cells located in the upper region of the colon mucosa rather than in deeper regions of the human normal colon mucosa. Our results were compatible with these findings.

Few studies have assessed ASC expression in cancer tissues. In human lung cancer, Ota et al. reported that bronchioloalveolar adenocarcinoma and low malignant adenocarcinoma showed similar ASC expression levels to normal lung parenchyma[25], while high malignant small cell carcinoma had less ASC expression[25]. In the results of our study, immunoreactivity for ASC was observed in the cancer cells of the cancer pearls, while expression of ASC was hardly detected in the poorly differentiated SCC. There was a significant correlation between the ASC expression and histological differentiation of OSCC. These results suggested that ASC tends to appear in less malignant and less-differentiated cancer cells.

IVL is known as a differentiation marker of epithelial tissue[23]. In clinical study, ASC and IVL co-localized well in the normal epithelial and OSCC tissues. ASC expression was associated with cell differentiation of oral epithelial and OSCC cells.

The results of in vitro studies also showed that ASC as well as IVL increased after the induction of cell differentiation in cultured oral epithelial cells. HaCaT and NHEK cell lines are known to be induced to differentiate by using Ca^{2+} [23]. In this study, the protein and mRNA levels of ASC and IVL increased when these cell lines were differentiated by stimulation using Ca^{2+}. It was reported that ASC expression was detected in the epithelial surface and in some differentiated functional cells for which proliferation is regulated.[24] Stefan K et al. reported that ASC regulates normal cell proliferation and is decreased in skin cancer[26]. These results suggested that ASC has possible roles in regulating oral epithelial cell

differentiation.

In contrat, in the assessment of ASC and IVL levels in the cultured cells, OSCC cell lines had smaller amounts of ASC and IVL than normal keratinized cells. The OSSC cell lines used in this study had different cell differentiation profiles. HSC2 and SQUU-A have the ability to form well-differentiated OSCC in nude mice[22]. SQUU-A has CK13 which is a differentiation marker of normal epithelia, while SQUU-B has the ability to form poorly-differentiated OSCC in nude mice[22]. The origins of SAS and HSC3 were poorly-differentiated SCC[27] and that of Ca9-22 was well-differentiated OSCC. We think that the relatively undifferentiated nature of these OSCC cell lines might be responsible for the lower levels of ASC as well as IVL. However, in the results of this study, SQUU-A and HSC2 had relatively high mRNA expressions of both ASC and IVL compared with SAS, Ca9-22, SQUU-B, and HSC3, although the differences were not statistically significant. The cell lines that were related with well-differentiated OSCC showed higher level of ASC and IVL.

There are some reports about the relationship between ASC and apoptosis. ASC was originally identified as a protein that forms specks in HL-60 human leukemia cells treated with chemotherapeutic agents[28]. ASC is required to translocate Bax to the mitochondria, which releases cytochrome c and in the activation of caspase-9, -2 and -3, resulted in apoptosis through a p53-Bax network in a breast cancer cell line, osteosarcoma cell line and colorectal cancer cell line[15]. In the results of this study, there was a significant correlation between ASC expression and the apoptotic (TUNEL-positive) cell numbers. Apoptosis (TUNEL positive cell) was more frequently observed in the well-differentiated OSCC than moderately- or

poorly-differentiated OSCC that showed higher expression levels of ASC. It was reported that ASC is a tumor suppressor and is silenced in many human cancers.[29] In carcinogenesis of the skin, ASC acts as a tumor-suppressor in keratinocytes[9, 26]. In melanoma, colorectal cancer, and prostate cancer, silencing of ASC caused by DNA methylation resulted in resistance to a kind of apoptosis [8, 9, 10]. These results suggested that ASC appeared in the cells where apoptosis was underway.

ASC positive cells were present close to TUNEL positive cells but they did not overlap. In the OSCC tissues, especially in well-differentiated OSCC, TUNEL-positive cells were scattered in the cancer pearls and were surrounded by ASC-positive cancer cells. When assessed in the normal oral epithelium, TUNEL-positive cells localized in the cornified cell layer where enucleation was underway, while ASC was positive in the granular and prickle cell layers. It is well known that keratinocytes differentiate from the basal cell layer to the prickle, to the granular, and finally to the cornified layer. Also in the OSCC tissues, cancer cells in the cancer pearl are thought to differentiate toward the center of the cancer pearl. The cells in the cornified layer, and also those in the center of the cancer pearls, are thought to fall into apoptosis. If differentiation of the oral epithelial/cancer cells was considered, ASC appeared in the proapoptotic cells next to the apoptotic cells. ASC appeared prior to apoptosis.

The results of this study showed that the expression level of ASC correlated well with clinical and pathological parameters of OSCC. In addition, a multivariate analysis using the Cox proportional hazard model demonstrated that the ASC expression level was a significant independent predictor for prognosis of OSCC; The higher the ASC expression, the better the prognosis. No previous study has assessed the

correlation between ASC expression and mortality of any cancer. This is the first report to demonstrate the clinical importance of ASC expression in the diagnosis and treatment of cancer. As suggested above, ASC expression was correlated with apoptosis of OSCCs. It has been reported that cancers with a high capacity to induce apoptosis have a better prognosis[30]. ASC had a close association with cell differentiation and apoptosis. It could be suggested that ASC may play roles in regulating cell differentiation and apoptosis in OSCCs. Expression of ASC induced apoptosis and might impede the survival of OSCC cells. In addition, it has also been reported that ASC links the formation of inflammasome and caspases[30,31]. ASC was thought to relate to the chemosensitivity of tumor cells and inflammation.[32]

In conclusion, the results of this study demonstrated that lower ASC expression was well correlated with clinical and pathological malignancy and, consequently, poor prognosis of OSCCs. It was also shown that ASC expression had a close association with cell differentiation and apoptosis in normal oral mucosa and OSCCs by in vivo and in vitro study. ASC may play roles in regulating cell differentiation and apoptosis of normal oral mucosa and OSCCs. Expression of ASC induced apoptosis and might inhibit the survival of OSCC cells.

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Figure1

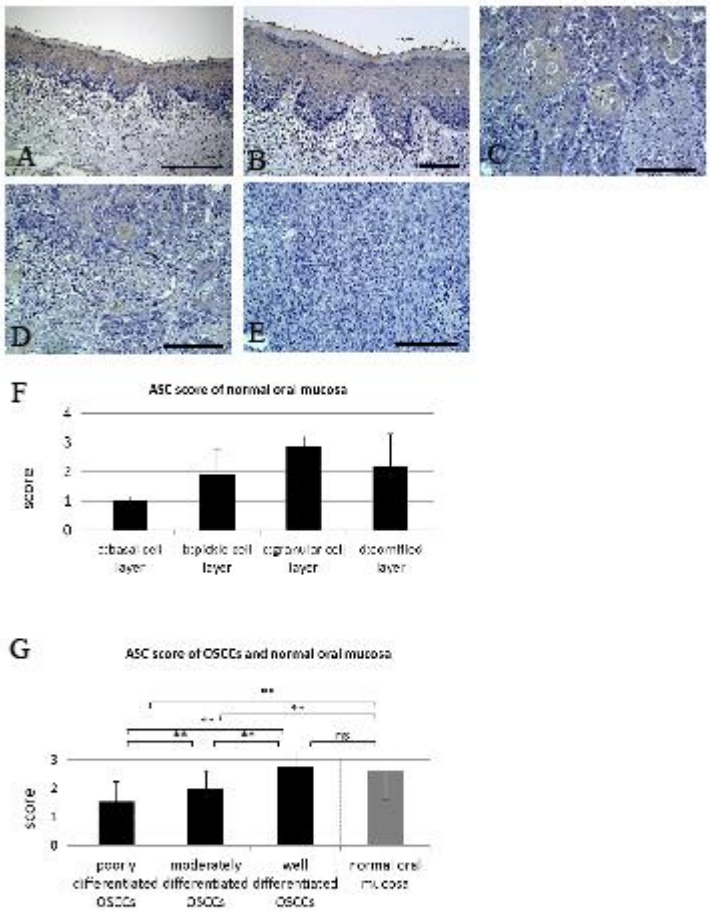

Figure 1. Expression of ASC in normal oral mucosa and OSCC immunohistochemical staining.

A) Immunohistochemistry of normal oral mucosa. B) Magnification of inset of A. C) Well-differentiated OSCC tissue. D) Moderately-differentiated OSCC tissue. E) Poorly-differentiated OSCC tissue. A, B, D, E; scale bar = 100 μm. C; scale bar = 25 μm. Arrows show ASC staining. F) ASC score of normal oral mucosa. G) ASC score of OSCCs and normal oral mucosa. Tested by Mann-Whitney's U test.

Figure2

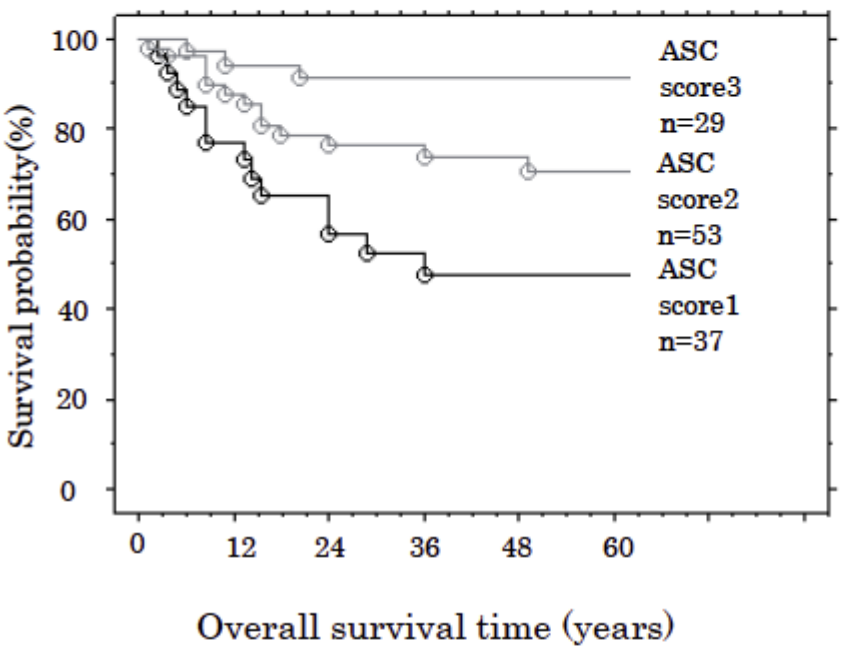

Figure 2.

Kaplan-Meier survival curves of patients with OSCCs according to expression score of ASC. The survival

curves were analyzed by the log-rank test (p =0.0009).

Figure3

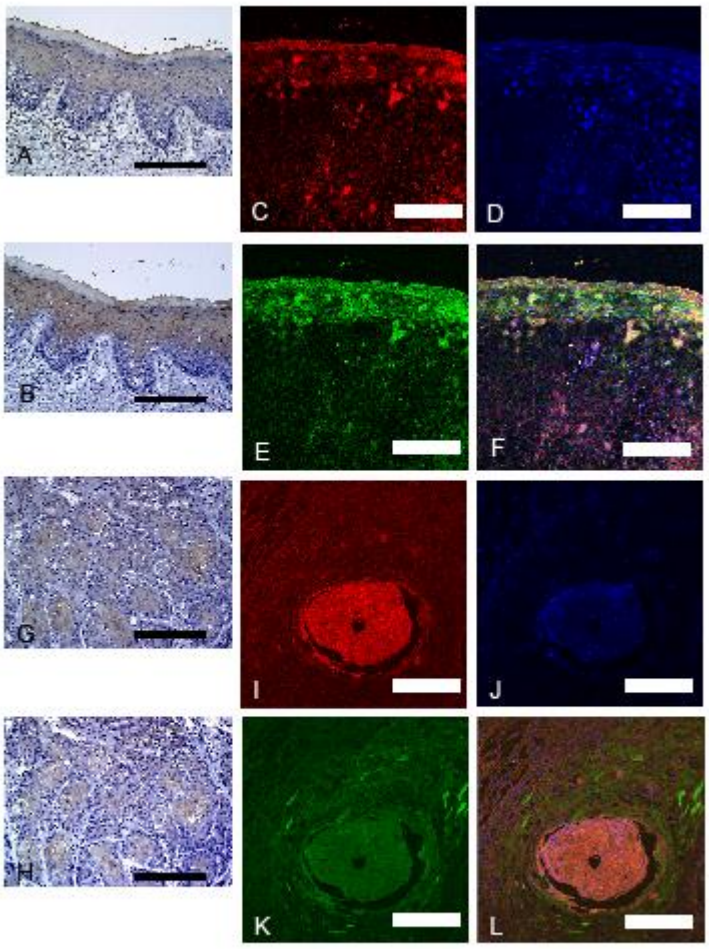

Figure 3. Co-localization of ASC and IVL expressions in normal oral mucosa (A-F) and well-differentiated OSCC (G-L)

A, B) Immunohistochemistry of normal oral mucosa

Arrows show expression of ASC (A) and expression of IVL (B).

C-F) Immunofluorescence study of normal mucosa. C) ASC (Red, Cy3). D) nuclei (Blue). E) IVL (Green,

Alexa488) and F) merge

G, H) Immunohistochemistry of OSCC. Arrows show expression of G) ASC and expression of H) IVL.

I-L) Immunofluorescence study of OSCC. I) ASC, J) nuclei, K) IVL and L) merge

A-L; scale bar = 100 μm.

Figure4

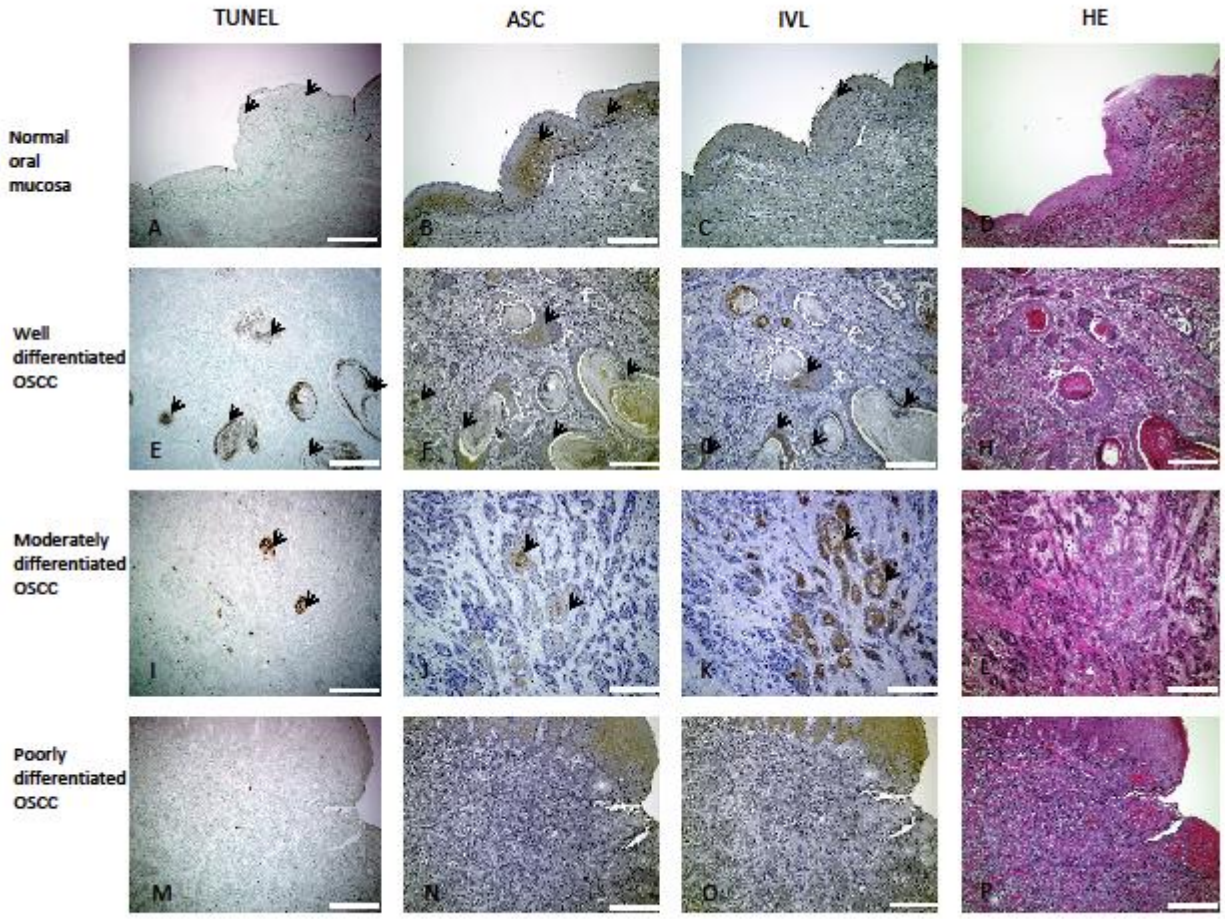

Figure 4. Apoptosis positive tissues in normal oral mucosa and OSCCs were surrounded by ASC high-expression tissues.

A-D) Immunohistochemistry of normal oral mucosa. A) Immunohistochemistry of ASC. B) Immunohistochemistry of IVL. C) Apoptosis detected by in situ Apoptosis Detection Kit. D) H-E staining. E-H) Well-differentiated OSCC. E) Immunohistochemistry of ASC. F) Immunohistochemistry of IVL. G) Apoptosis detected by in situ Apoptosis Detection Kit. H) H-E staining. G

I-L) Moderately-differentiated OSCC. I) Immunohistochemistry of ASC. J) Immunohistochemistry of IVL. K) Apoptosis detected by in situ Apoptosis Detection Kit. L) H-E staining. M-P) Poorly-differentiated OSCC. M) Immunohistochemistry of ASC. N) Immunohistochemistry of IVL. O) Apoptosis detected by in situ Apoptosis Detection Kit. P) H-E staining Figures are representative tissues. A-L; scale bar = 100 μm.

Figure5

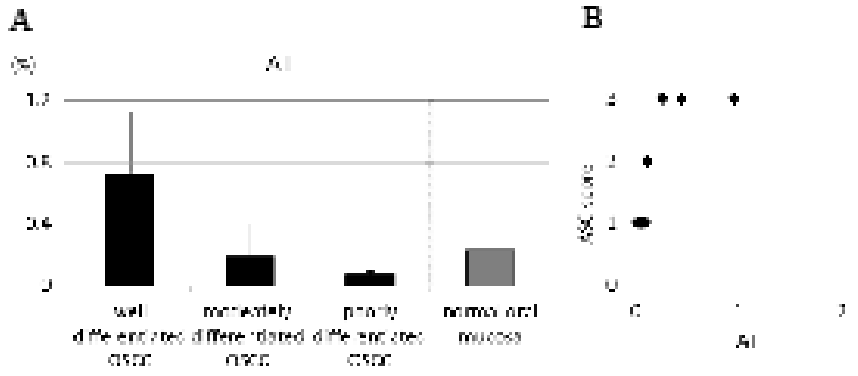
A
B

Figure 5. Apoptosis index: AI and ASC scores of normal mucosa and OSCCs.

A) Apoptosis index: AI of normal oral mucosa, well-, moderately- and poorly-differentiated OSCC. B) Distribution map of ASC score and AI of OSCCs.

Figure6

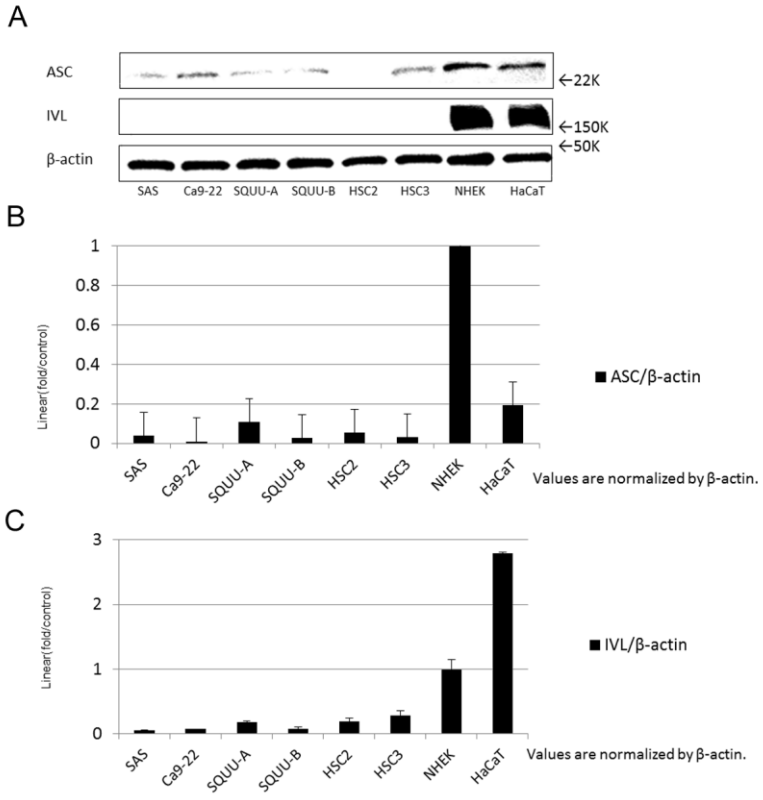

Figure 6. Western blot and real-time PCR analysis of ASC and IVL in OSCC cell lines and normal keratinocytes.

Western blot and real-time PCR analysis were performed on 6 OSCC cell lines and 2 normal keratinocyte

cell lines with antibodies and primers against ASC, IVL and ß-actin.

A: Western blot analysis. This figure is representative of three independent experiments.

B: Real time PCR analysis. Values for ASC are normalized by hree ind

C: Real time PCR analysis. Values for IVL are normalized by ß-actin.

Data are expressed as the mean ± SD of three independent experiments.

Figure7

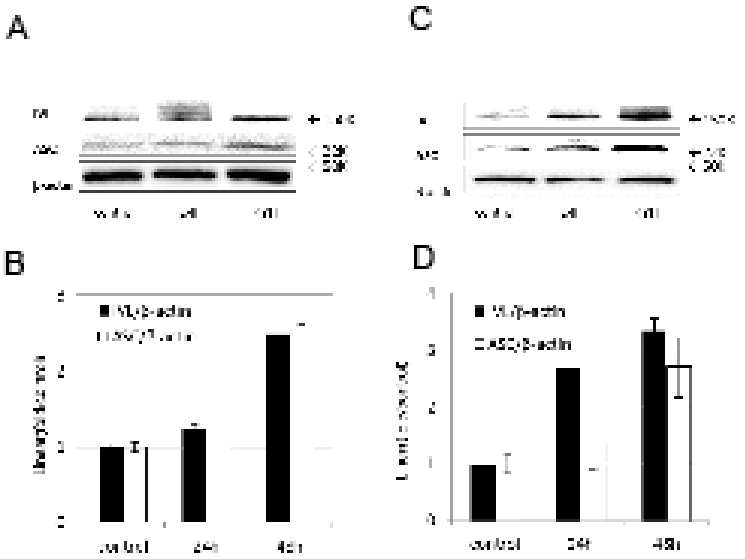

Figure 7. Alteration of protein and mRNA levels of IVL and ASC induced by Calcium-differentiation.

Western blot and real-time PCR of ASC and IVL in NHEK and HaCaT.

A: Western blot analysis of IVL and ASC in NHEK after the differentiation by Calcium.

Cells were collected and immunoblotted for IVL, ASC and ed and immunoblottedC in NHEK after the differentiationmM) to high (1.2 mM) calcium concentration medium.

This figure is representative of 4 independent experiments.

B: Real time analysis of IVL and ASC in NHEK after the differentiation by Calcium. mRNA levels of IVL, ASC and IVL and ASC in NHEK after the differenconditions. Values were normalized by L and ASC in are expressed in fold versus the control value. Data are expressed as the mean fold ntiation by Calcium. mRNAs. .

C: Western blot analysis of IVL and ASC in HaCaT after differentiation by $Ca2^{+}$.

Conditions were the same as for (A).

D: Real time analysis of IVL and ASC in HaCaT after differentiation by $Ca2^{+}$.

Conditions were the same as for (B).

Table 1. 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'

Table 2. Relationship between ASC score and clinicopathological factors in univarite analysis

ASC score	3	2	1	P value
Age (yrs)				0.5083 †
＞65	23 (19.3)	20 (16.8)	6 (5.0)	
≦65	37 (31.1)	27 (22.7)	6 (5.0)	
Gender				0.5301*
Male	34 (28.6)	31 (26.1)	7 (5.9)	
Female	26 (21.8)	16 (13.4)	5 (4.2)	
Primary site				<0.05*
tongue	29 (24.4)	21 (17.6)	3 (2.5)	
gum	14(11.8)	7(5.9)	2 (1.7)	
buccal	8 (5.0)	8 (6.7)	2 (1.7)	
floor of mouth	4 (3.4)	6 (5.0)	2 (1.7)	
others	5 (4.2)	5 (4.2)	3 (2.5)	
T classification				＜0.01 †
T1	12 (10.1)	6 (5.0)	2 (1.7)	
T2	28 (23.5)	14 (11.8)	1 (0.8)	
T3	6 (5.0)	7 (5.9)	3 (2.5)	
T4	14 (11.8)	20 (16.8)	6 (5.0)	
N classification				0.0531 †
N0	41 (34.5)	24 (20.2)	6 (5.0)	
N1	8 (6.7)	6 (5.0)	0 (0)	
N2a	8 (6.7)	16 (13.4)	3 (2.5)	
N2b	3 (2.5)	1 (0.8)	2 (1.7)	
Stage classification				＜0.001 †
Ⅰ	12 (10.1)	6 (5.0)	2 (1.7)	
Ⅱ	21 (17.6)	10 (8.4)	1 (0.8)	
Ⅲ	7 (5.9)	9 (7.6)	2 (1.7)	
Ⅳ	20 (16.8)	22 (18.5)	7 (5.9)	
Mode of invasion				＜0.01 †
Y-K2	26 (21.8)	22 (18.5)	1 (0.8)	
Y-K3	7 (5.9)	14 (11.8)	12 (10.1)	
Y-K4c	2 (1.7)	14 (11.8)	15 (12.6)	
Y-K4d	2 (1.7)	3 (2.5)	1 (0.8)	
Differentiation				＜0.01 †
Well	53 (44.5)	20 (16.8)	0 (0)	
Moderate	6 (5.0)	23 (19.3)	6 (5.0)	
Poor	1 (0.8)	4 (3.4)	6 (5.0)	
Lymphocytic infiltration				＜0.01 †
Low	18 (15.1)	23 (19.3)	10 (8.4)	
Moderate	26 (21.8)	13 (10.9)	2 (1.7)	
Marked	16 (13.4)	11 (9.2)	0 (0)	

tested by*,kruskal-wallis test, † spearman rank correlation

Table 3. The correlation coefficient among the parameters

	ASC score	Gender	Age	T-classification	N-classification	Stage	Pathological grade	Mode of invasion	Lymphocytic infiltration
ASC score	1.000	-.066	-.057	-.265	-.171	-.240	.599	-.431	.269
Gender	-.066	1.000	-.351	.025	.140	.113	.013	.158	-.142
Age	-.057	-.351	1.000	.120	.075	.129	.086	-.246	.162
T-classification	-.265	.025	.120	1.000	.552	.893	-.104	.189	-.201
N-classification	-.171	.140	.075	.522	1.000	.680	-.160	.263	-.067
Stage	-.240	.113	.129	.893	.680	1.000	-.128	.186	-.194
Pathological grade	.599	.013	.086	-.104	-.160	-.128	1.000	-.370	.236
Mode of invasion	-.431	.158	-.246	.189	.263	.186	-.370	1.000	-.320
Lymphocytic infiltration	.269	-.142	.162	-.201	-.067	-.194	.236	-.320	1.000

Table 4. Cox proportional-hazards regression model predicting disease-specific survival

		P-value	hazard-ratio(95%C.I.)
ASC score(1/2/3)		.0135	
	3		1.000
	2	.0866	3.118(.849-11.454)
	1	.0048	6.895(1.803-26.356)
Gender(M/F)		.6317	.881(.344-1.911)
Age		.1764	1.739(.780-3.877)
Clinical stage		.0261	1.593(1.057-2.2402)
Lymphocytic infiltration		.5188	.846(.846-1.406)
Primary site		.7394	
	Buccal		1.000
	Mouth floor	.5159	1.865(.283-12.341)
	Tongue	.3931	1.956(.419-9.130)
	Gum	.2837	2.465(.473-12.839)
	Others	.8948	1.139(.165-7.846)

likelihood ratio test=0.0031

C.I.:Confidence interval