Expressions of Nuclear Factor κB, Inducible Nitric Oxide Synthase, and Vascular Endothelial Growth Factor in Adenoid Cystic Carcinoma of Salivary Glands: Correlations with the Angiogenesis and Clinical Outcome

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Abstract

Objective: To evaluate the expressions of nuclear factor κB (NF-κB p65), inducible nitric oxide synthase enzyme (iNOS), and vascular endothelial growth factor (VEGF) in relation to angiogenesis (microvessel density, MVD) and clinical outcomes in adenoid cystic carcinoma (ACC) of salivary glands.

Methods: Immunohistochemical staining was used to quantify the protein expression levels of NF-κB p65, iNOS, and VEGF in 80 surgically resected ACCs and 20 normal salivary tissues. In all cases of ACCs, MVD was evaluated by counting CD34-reactive endothelial cells or endothelial cell clusters.

Results: The nuclear localization of NF-κB p65 was only detected in ACC cells. Both iNOS and VEGF staining activities in ACCs were more significant than those in normal gland tissues (P < 0.01). MVD had significant correlations with NF-κB p65, iNOS, and VEGF expressions (P < 0.01). In three histologic types of ACCs, the NF-κB, iNOS, VEGF expressions, and MVD were significantly higher in solid type than in cribriform and tubular types (P < 0.01). The NF-κB, iNOS, VEGF expressions, and MVD were significantly correlated with clinical stage, tumor size, vascular invasion, recurrence, and metastasis (P < 0.05). Multivariate analysis showed NF-κB, iNOS and VEGF expression, MVD, solid histotype, and perineural invasion had an independent prognostic effect on overall survival.

Conclusion: The expressions of NF-κB p65, iNOS, and VEGF were related with MVD. Clinical outcomes raised the possibility that the overexpression of these cytokines might contribute to tumor angiogenesis and have prognostic value in ACCs.

Adenoid cystic carcinoma (ACC), mostly occurring in the major and minor salivary glands, has some unique characteristics such as slow growth, diffuse invasion, and high incidence of distant metastasis. Although reasons for the invasiveness and aggressive metastatic dissemination of ACCs remain unclear, angiogenesis may be a possible mechanism involved (1).

Angiogenesis, the development of new blood vessels from the preexisting vascular beds, is an essential pathophysiologic event occurring in tumor growth and metastasis. This process is tightly regulated by the actions of angiogenic cytokines released by tumor cells in a paracrine manner, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiopeptin-1, and transforming growth factor-β (1–3). Among various angiogenic factors, the most notable is VEGF, which is a heparin-binding, dimeric polypeptide growth factor that is known to exert its mitogenic activity especially on endothelial cells, and it has been mainly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells (4). Although the process of activation of VEGF is complicated, it has been shown that hypoxia, hypoglycemia, hormones, growth factors, and the deregulation of several genes are responsible for the up-regulation of VEGF expression (5). Recent evidences, furthermore, have indicated that overexpression of nuclear factor κB (NF-κB) and inducible nitric oxide synthase (iNOS) are the key components of the angiogenic cascade, which contribute to VEGF-induced angiogenesis through up-regulation of VEGF in many tumors (6, 7).

NF-κB belongs to the transcription factors family which includes p50, p52, RelA (p65), RelB, c-Rel, v-Rel and dorsal as well as Dif proteins (8). In most cells, homodimeric or heterodimeric complexes of p50 and p65 subunits exist as the main form of NF-κB and remain inactive in the cytoplasm associated with the NF-κB inhibitory protein (I-κB). Activation of NF-κB by cytokines or oxidative stress requires either the degradation of its cytoplasmic inhibitor IκB-α or proteolytic cleavage of p105 (9). Free NF-κB dimers translocate to the nucleus and up-regulate the expression of several target genes encoding cytokines, chemokines, growth factors, cell
adhesion molecules, and some enzymes. They play key roles in embryonic development, lymphoid differentiation, apoptosis, immune and inflammatory responses (10) and development of cancer (11, 12). Activation of NF-κB also up-regulates a number of genes necessary for the angiogenesis of tumors, such as iNOS, cyclooxygenase-2, interleukin-6, interleukin-8, urokinase-type plasminogen activator, and VEGF, which have κB binding sites in their promoter regions (13). In pancreatic cancer, inhibiting NF-κB activation by expressing IκBαM significantly reduced in vivo expression of a major proangiogenic molecule, VEGF, and hence, decreased neoplastic angiogenesis (7).

Another angiogenic modulator, nitric oxide (NO), originating from L-arginine by the nitric oxide synthase enzyme, is a short-lived, pleiotropic biomolecule. It is involved in several biological activities closely related with carcinogenesis (14, 15). Compared with the other two isoforms of nitric oxide synthase enzyme (nNOS, eNOS), there is a growing body of evidence suggesting that the output of NO generated by iNOS is higher in malignant tissues and causes elevated tumor growth rates, vascular density, and invasiveness (6). More and more investigations suggest that NO may have a dual pro- and antitumor action depending on local concentration. High concentrations of NO (for example, generated by activated macrophages), might mediate cancer cell apoptosis and the inhibition of cancer growth. Although at (relatively) low concentrations of NO (for example, at concentrations measurable in many different types of clinical cancer samples), tumor growth, proliferation, and angiogenesis are promoted (6). Several in vitro studies directly showed that endogenous NO promoted tumor angiogenesis by up-regulating angiogenic factors, especially VEGF mRNA expression (16). The NO/cyclic guanosine monophosphate pathway is also likely to be important for the activity of VEGF released by tumor cells (17). More recently, some evidence also shows that NO could induce NF-κB activity via the generation of peroxynitrite (ONOO−; ref. 18). NO donors such as sodium nitroprusside and diethylamine NONOate could induce NF-κB activity in the articular chondrocytes (19). Considering the fact that NF-κB is the upstream regulator of iNOS, which induces NO generation, they might make a positive feedback loop that amplifies the downstream responses of angiogenic factors (20).

Tumor angiogenesis not only plays a pivotal role in tumor growth and metastasis, but also acts as a plausible candidate in relation to tumor prognosis. Several studies have found that VEGF, VEGF receptors, and microvessel density (MVD) have a direct correlation with prognosis, node-free intervals, and recurrence-free periods in non–small cell lung cancer (21). In ACCs, Lim et al. (22) showed that high VEGF expression is crucial in tumor progression, hematogenous invasion, metastasis, as well as its prognosis. However, to our knowledge, the expression and relationship of NF-κB, iNOS, and VEGF with angiogenesis in malignant salivary gland tumors, especially ACCs has not been previously studied and reported. The present study first compared the protein levels of NF-κB, iNOS, and VEGF in ACC tumors with normal salivary gland tissues, and their correlation with MVD. The relevance of the expression levels of NF-κB, iNOS, and VEGF with clinicopathologic features and clinical outcome were further evaluated with an aim to decide their roles in the prognosis of ACCs.

Expressions of NF-κB, iNOS, and VEGF in Adenoid Cystic Carcinoma

Patients and samples. Tissue samples of primary ACCs and tumor-free salivary gland tissues around ACCs were retrieved from archival material in the Department of Oral Pathology, Stomatologic School of Wuhan University from 1992 to 2002. Simultaneously, 10% formalin-fixed paraffin-embedded tissue sections, from consenting patients undergoing surgery without pretreatments, were cut and stained with H&E. These slides were reviewed by two consultative pathologists before immunostaining both to confirm the diagnosis and to categorize ACCs into three histologic patterns: cribriform, tubular, and solid. Eighty ACCs (34 cribriform, 29 tubular, and 17 solid) and 20 normal salivary gland tissues that fulfilled the above criteria were employed. The classification of ACC histologic patterns were based on WHO classification. Clinical staging according to the 1997 criteria of the International Union Against Cancer (23) were evaluated by reviewing medical charts and pathologic records. All patients underwent surgical treatment and recurrence after surgery was confirmed by radiograph and pathology biopsy. The follow-up period at the time of analysis was 2 to 14 years (mean, 72 months).

Antibodies and immunohistochemistry. Immunohistochemical studies were done using the following antibodies: monoclonal mouse anti-human NF-κB p65, polyclonal rabbit anti-human iNOS (dilution 1:200 and 1:80, respectively) from Santa Cruz Biotecnology, Inc. (Santa Cruz, CA); monoclonal mouse anti-human VEGF, monoclonal mouse anti-human CD34 (dilution 1:80 and 1:200, respectively) from ZYMED, Ltd. (South San Francisco, CA). SP immunohistochemical test kit purchased from MaXin Ltd. (Fuzhou, China).

Paraffin-embedded, multiple tissue sections of 4 μm from the 80 diseased and 20 normal salivary glands were placed on positively charged slides and then heated at 50°C for 40 minutes. Deparaffinization of all sections was done through a series of xylene baths for 15 minutes each, with dehydration using graded alcohols for 6 minutes each and finally into water. To block endogenous peroxidase activity, sections were placed in 3% hydrogen peroxide in absolute alcohol for 15 minutes and treated with a boiling solution of freshly prepared citrate buffer (pH 6.0), and for 2 minutes in a pressure cooker for antigen retrieval. Following treatment with a working dilution of normal goat serum for 15 minutes, sections were incubated with primary antibodies for 1 hour at 37°C, then for 20 minutes with biotinylated anti-mouse or rabbit immunoglobulin G antibody (working dilution), followed by staining with avidin-biotin peroxidase complex (DAKO, Carpinteria, CA); monoclonal mouse anti-human VEGF, monoclonal mouse anti-human CD34 (dilution 1:80 and 1:200, respectively) from ZYMED, Ltd. (South San Francisco, CA). SP immunohistochemical test kit purchased from MaXin Ltd. (Fuzhou, China).

Negative controls were treated in the same product but omitting the primary antibodies. Positive controls were oral squamous cell carcinoma slides known to have positive staining for NF-κB p65, iNOS, VEGF, and CD34.

Evaluation of staining. Sections were blindly evaluated by two investigators in an effort to provide a consensus on staining patterns by light microscopy (Olympus, Tokyo, Japan). NF-κB staining was localized within the cell cytoplasm and/or nucleus, and the nuclear localization of NF-κB p65 was categorized as positive expression. The rate of the nuclear localization of p65 was calculated according to the recommendation of Nakayama et al. (24): by counting positive nuclear staining NF-κB from total cancer cells and calculating the percentage. Each case was counted on every 10 randomly selected high-power fields (×200). iNOS and VEGF staining were assessed according to the methods described by Hara et al. (25) with minor modifications. Each case was rated according to a score that added a scale of intensity of staining (viewed at a magnification of ×400) to the area of staining seen (at a magnification of ×400). The intensity of staining was on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as...
follows: 0, no staining of cells in any microscopic fields; 1+, <25% of tissue stained positive; 2+, between 25% and 50% stained positive; 3+, between 50% and 75% stained positive; 4+, >75% stained positive. The minimum score when summed (extension + intensity) was therefore 0 and the maximum, 7. The combined staining score (extension + intensity) ≤3 was considered as negative staining (low staining); a score between 4 and 5 was considered as moderate staining; between 6 and 7 was strong staining.

MVD was evaluated according to the method described by Weidner et al. (26). The entire tumor section was scanned at low magnification (×40) to find the area that showed the most intense neovascularization, i.e., the highest density of brown-stained CD34-positive cells (hotspot). The five most highly vascularized hotspots in each case were selected in a ×200 field. Any immunoreactive endothelial cell or endothelial cell cluster, which was clearly separated from adjacent microvessels, was considered as a single countable microvessel. Vessel lumens, although usually present, were not necessary for a structure to be defined as a microvessel. Results regarding angiogenesis were expressed as the highest number of microvessels identified in the hotspot within a microscopic field.

**Statistical analysis.** Statistical analysis was done by using SPSS (version 11.5). The Spearman rank correlation coefficient test and linear tendency test were applied for the correlation among the expression of NF-κB, iNOS, and VEGF and MVD. The correlation

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**Fig. 1.** NF-κB p65 expressed in cribriform (A), tubular (B), and solid (C) pattern of ACCs and the tubular cells of normal salivary gland (dark arrow; D); iNOS expressed in cribriform (E), tubular (F), solid (G) pattern of ACCs and the tubular cells of normal salivary gland (dark arrow) (H); VEGF expressed in cribriform (I), tubular (J), solid (K) pattern of ACCs and the tubular cells of normal salivary gland (dark arrow; L; original magnification, ×400).

**Fig. 2.** Tumor nest with perineural invasion (dark arrow) showed more intense iNOS staining than the tumor nest without perineural invasion (hollow arrow; original magnification, ×400).
between those four factors and three histopathologic types was analyzed by the ANOVA test and Student-Newman-Keuls test. The homogeneity-of-variance test was also done. Significant differences between the expression of those four factors and clinical variables, including gender, age, tumor size, site, clinical stage, perineural and vascular invasion, tumor recurrence, and distant metastasis were compared by the χ² test or Fisher's exact probability test when appropriate. Survival rates were obtained by the Kaplan-Meier method, and the differences were compared by log-rank tests. The deaths caused by ACCs were considered as outcomes; the deaths by other causes were censored and the missing values were replaced by series mean method. A univariate analysis with Cox proportional hazards model was used to determine each identified prognostic factor; multivariate analysis with Cox proportional hazards model was used to explore combined effects. A difference of \( P < 0.05 \) was considered significant.

**Results**

**Immunohistochemical staining of nuclear factor κB, inducible nitric oxide synthase, vascular endothelial growth factor, and CD34.** NF-κB p65 was detected in the cytoplasm of most tumor cells, but was only detected in the nucleus of some tumor cells. The mean rate of NF-κB p65 nuclear staining detected in 80 ACC specimens was 12.5 ± 4.58% (mean ± SD). Activated NF-κB p65 were detected in some but not all tumor cells. In the tubular type of ACC, ductal lining cells had more nuclear staining than the outer layer (Fig. 1B). The cytoplasm of tubular cells in normal salivary glands, fibroblast, smooth muscle cells, stromal macrophages, and neutrophils had a slight staining of NF-κB p65, whereas there was no staining in the acinar cells of normal salivary glands (Fig. 1D). Nuclear staining of NF-κB p65 could not be detected in all of the normal cases. It was evident that the rate of NF-κB p65 nuclear localization in ACCs was significantly higher than that in normal salivary glands.

Immunopositivities for iNOS and VEGF were confined to the cytoplasm in ACC cells (Fig. 1). The iNOS-specific proteins showed moderate to strong staining in 78 of 80 ACC specimens, and the mean score was 5.34 ± 1.35 (mean ± SD). VEGF proteins showed moderate to strong staining in 61 of 80 ACC specimens, and the mean score was 4.76 ± 1.70 (mean ± SD). It is also evident that both iNOS and VEGF had higher immunoreactivity in the inner layer of cells of the tubular pattern than in the outer layer of cells of the tubular pattern (Fig. 1F and J). In solid type, the cells located in the outer area of the tumor nest showed higher expressions levels of both iNOS and VEGF (Fig. 1G and K). We also found that the tumor nest with perineural invasion showed more intense iNOS staining than the tumor nest without perineural invasion (Fig. 2). iNOS and VEGF were also faintly distributed in some salivary ducts of 20 normal cases, whereas unnoticed in parenchyma. There were three cases with moderate or strong iNOS staining and four cases with moderate or strong VEGF staining in 20 normal salivary gland tissues, and the mean scores were 2.05 ± 1.28 and 2.15 ± 1.35, separately. There is a highly significant difference between staining of iNOS and VEGF in the tumor and in the normal gland tissues (\( P < 0.01 \); Table 1).

Vascular endothelial cell or endothelial cell cluster around the tumors was evident by anti-CD34 antibody (Fig. 3A-C), and the mean value of MVD was 47.07 ± 13.44. The scores from solid, tubular, and cribriform types were 58.07 ± 9.80, 46.46 ± 11.85, and 41.95 ± 13.31, respectively. The microvessels around the solid tumor nest were much denser than that around the cribriform and tubular tumor nest.

**The relationships among expression of nuclear factor κB, inducible nitric oxide synthase, and vascular endothelial growth factor and microvessel density.** To evaluate whether there was any link between the MVD and angiogenesis-related factors (NF-κB, iNOS, and VEGF), and the correlation among NF-κB, iNOS, and VEGF in ACCs, the Spearman correlation analysis was done to quantitate the degree of linear association between two variables. The individual levels of NF-κB, iNOS, and VEGF were all significantly correlated to MVD (\( P < 0.01 \); Fig. 4). Expression of NF-κB, iNOS, and VEGF was also significantly correlated at \( P = 0.01 \) (Fig. 5).

### Table 1. The difference of iNOS and VEGF expression between ACC and NSG

|        | Low (0-3) | Moderate and high (4-7) | \( P \)  |
|--------|-----------|-------------------------|--------|
| iNOS   | ACC 80    | 8                       | 72     | 0.000*    |
|        | NSG 20    | 17                      | 3      |           |
| VEGF   | ACC 80    | 19                      | 61     | 0.000*    |
|        | NSG 20    | 16                      | 4      |           |

*Fisher’s exact test, \( P < 0.01 \).

NOTE: NSG, normal salivary glands.

**Fig. 3.** MVD around the tumor nest in cribriform (A), tubular (B), and solid (C) pattern of ACCs (original magnification, ×400).
Correlation of nuclear factor \( \kappa B \), inducible nitric oxide synthase, vascular endothelial growth factor, and microvessel density expression with histologic types of adenoid cystic carcinoma.

Among the three histologic types of salivary gland ACC, the expression of NF-\( \kappa B \), iNOS, VEGF, and MVD was significantly higher in solid type than those in cribriform and tubular types \((P < 0.01)\), whereas no significant difference was found between cribriform and tubular types (Table 2).

Correlation between nuclear factor \( \kappa B \), inducible nitric oxide synthase, vascular endothelial growth factor, and microvessel density expression and clinicopathologic factors.

Table 3 shows the association of several clinicopathologic factors with NF-\( \kappa B \), iNOS, and VEGF and MVD expression. NF-\( \kappa B \) expression was significantly correlated with some of the clinicopathologic factors examined, such as tumor size, tumor-node-metastasis (TNM) clinical stage, perineural invasion, vascular invasion, recurrence, and distant metastasis \((P < 0.05)\). iNOS expression was low in 8 cases (10%), moderate in 29 cases (36.3%), and high in 43 cases (53.7%). Its expression was correlated with tumor size, TNM clinical stage, perineural invasion, vascular invasion, recurrence, and distant metastasis \((P < 0.05)\).

The immunoreactivity of VEGF was low in 19 cases (23.8%), moderate in 24 cases (30%), and high in 37 cases (46.2%), and was also correlated with tumor size, clinical stage, vascular invasion, recurrence, and distant metastasis \((P < 0.05)\). Cases with larger tumor size or advanced clinical stages showed more intense iNOS staining. Tumors with higher iNOS expression tended to have deeper vascular invasion, more frequent recurrence, and distant metastasis. Expression of NF-\( \kappa B \), iNOS, and VEGF did not correlate with patients' gender, age, and tumor sites. Expression of VEGF also was not related with perineural invasion.

![Fig. 4. Correlation between NF-\( \kappa B \) p65, iNOS, VEGF expressions and MVD.](image1)

Spearman correlation and linear regression were used to determine the relationship among the different proteins tested. MVD correlated positively with nuclear NF-\( \kappa B \) p65 (A), iNOS (B), and VEGF (C).

![Fig. 5. Correlation among the expressions of NF-\( \kappa B \) p65, iNOS, and VEGF.](image2)

Spearman correlation and linear regression were used to determine the relationship among the different proteins tested. There were significant correlations among NF-\( \kappa B \), iNOS, and VEGF at \(P = 0.01\) (A, B, and C).
A MVD value of ≥48 (the median value) was considered high. MVD was low in 38 cases (47.5%) and high in 42 cases (52.5%), and was correlated with tumor size, TNM clinical stage, perineural invasion, vascular invasion, recurrence, and distant metastasis (P < 0.05). The correlation between MVD and gender, age or tumor location was not found.

Survival analysis. Survival curves were calculated for total 80 cases. Four patients (5.0%) were lost during the following up; at the end of the study, 23 patients (28.8%) had died of ACCs, 2 patients (2.5%) had died of unrelated causes, and 51 patients (63.7%) were still alive; 40 patients (50%) showed recurrence of the disease, 29 patients (36.2%) showed distant metastasis (with 28 cases to lungs and 1 case to bone). The mean follow-up period was 72.72 ± 31.94 months; 63 patients (78.8%) had a follow-up until death or for >5 years. Using univariate analysis (Cox’s proportional hazards model), the following variables were found significantly associated with a worse prognosis, such as histologic types, perineural invasion, vascular invasion, recurrence, metastasis, NF-κB, iNOS, and VEGF expression and MVD (P < 0.05; Table 4). Figure 6 showed survival curves calculated by the Kaplan-Meier method and analyzed using the log-rank test. The results showed that the survival rates of patients with high NF-κB, iNOS, VEGF, and MVD expression were significantly worse than that of patients with low or moderate NF-κB, iNOS, VEGF, and MVD expression. Multivariate analysis (Cox’s proportional hazards model) showed that NF-κB, iNOS, VEGF expression, MVD, histotypes, and perineural invasion had an independent prognostic effect on overall survival (P < 0.05; Table 5).

Discussion

ACCs are characterized by extensive, insidious invasion, and extension along basement membrane–rich tissues, such as perineural and vascular sheaths. It has great potential for local recurrence and late distant metastasis and poor long-term survival. In this study, we differentiated the immunohistochemical expression of NF-κB, iNOS, and VEGF in 80 cases of ACCs and 20 cases of normal salivary glands. We also compared their expression with the tumor angiogenesis, clinicopathologic factors, and survival data of the patients.

Angiogenesis, or the formation of new blood vessels, is an essential component in tumor cell survival, which promulgates tumor metastasis. Newly formed blood vessels provide increased availability of essential oxygen and nutrients to the burgeoning tumor to permit expansion beyond an avascular mass of 1 to 2 mm³ (27). Extensive evidence shows that VEGF released by tumor cells plays a pivotal role in stimulating endothelial cell proliferation and migration, as well as enhancing vascular permeability (28). In our research, we found that the protein level of VEGF was significantly higher in ACCs than in normal salivary gland and it positively correlated to MVD in ACCs. Our results show that VEGF released by ACC cells may play an important role in stimulating endothelial cell proliferation.

NO seems to play dual roles in the cell signaling for both cell proliferation and death, depending on the different physiologic and pathologic conditions such as the site of production and the local concentration (6). NO has been suggested to play a role in the production of salivary amylase (29), and assists protein secretion via an interaction with vasoactive intestinal peptide (30). Our results agreed with a recent study which observed that iNOS expressed in the normal salivary ducts but not in acini (31). The presence of NO in normal human salivary glands was thought to have an antibacterial effect (32).

Although initial findings suggested that the immune cell–generated NO is cytostatic or cytotoxic for tumor cells, later findings have shown that the relatively low concentration of NO contributes to tumor angiogenesis by up-regulating VEGF (6). Using a murine breast cancer model, it was shown that iNOS-induced NO is a key mediator of C3L5 to induce tumor angiogenesis, and the antitumor effects of t-NAME (the inhibitor of iNOS) are partly mediated by reducing tumor angiogenesis (33). In our study, the expressions of iNOS and VEGF in ACCs were much higher than those in normal salivary glands.

### Table 2. Correlation between expression of NF-κB p65, iNOS, VEGF, and MVD histopathologic types

| Histotype | n  | Mean (SD) |
|-----------|----|-----------|
| **NF-κB p65** |    |           |
| C         | 34 | 11.03 (3.37) |
| T         | 29 | 11.96 (3.27) |
| S         | 17 | 16.95 (6.34) |
| **VEGF**   |    |           |
| C         | 34 | 4.15 (1.54)  |
| T         | 29 | 4.76 (1.84)  |
| S         | 17 | 6.00 (1.00)  |
| **iNOS**   |    |           |
| C         | 34 | 4.85 (1.37)  |
| T         | 29 | 5.28 (1.16)  |
| S         | 17 | 6.41 (1.00)  |
| **MVD**    |    |           |
| C         | 34 | 4.195 (13.31) |
| T         | 29 | 46.42 (11.85) |
| S         | 17 | 58.07 (9.80) |

Abbreviations: S, solid type; T, tubular type; C, cribriform type.

*P < 0.01 by Student-Newman-Keul's test.

+P < 0.01 by ANOVA test.
iNOS and VEGF expressions were significantly correlated. Their expressions were also correlated significantly with MVD in the stroma of ACCs. The findings are in agreement with previous studies of iNOS expression in human breast carcinoma (14) and non–small cell lung carcinoma (15). Several studies showed that a positive correlation was found between NO synthase and tumor angiogenesis in head and neck cancer (34). Research has also shown that a positive feedback loop exists between iNOS and VEGF expression. It was shown that VEGF binded to high-affinity tyrosine kinase receptors on vascular endothelial cells, which leads to the release of NO through the activation of both eNOS and iNOS (35). The interactive pathway between iNOS and VEGF may provide a novel area for future research in salivary gland tumors.

In both the laboratory and clinical studies, it has been shown that iNOS induces angiogenesis, which in turn aids tumor growth, invasion, and metastasis (6). Kostourou et al. (36) reported that the overexpression of dimethylarginine dimethylaminohydrolase, which metabolized the endogenous inhibitors of NO synthesis asymmetric dimethylarginine and N-monomethyl-L-arginine, results in increased neovascularization of C6 gliomas in vivo with enhanced growth rate. Our study found that the tumor nest with perineural invasion showed more intense iNOS staining than the tumor nest

Table 3. Correlation between NF-κB p65, iNOS, VEGF, and MVD expression and clinicopathologic features

| Variable          | NF-κB p65 | iNOS | VEGF | MVD |
|-------------------|-----------|------|------|-----|
| Age               | <13%      | >13% | <13% | >13%|
| <50               | 30        | 17   | 0.793| 34  |
| >50               | 33        | 14   | 0.084| 35  |
| Gender            | Male      | 34   | 21   | 0.602| 36  |
| Female            | 46        | 13   | 5    | 15  |
| Size              | <2 cm     | 25   | 23   | 0.001*| 37  |
| >2 cm             | 55        | 26   | 12   | 4   |
| Site              | Major     | 34   | 12   | 0.962| 38  |
| Minor             | 46        | 16   | 4    | 21  |
| TNM stage         | T1+T2     | 42   | 35   | 0.001*| 39  |
| T3+T4             | 38        | 21   | 2    | 24  |
| Perineural invasion| Negative | 49   | 38   | 0.003*| 40  |
| Positive          | 31        | 17   | 4    | 6   |
| Vascular invasion | Negative | 54   | 47   | 0.000*| 41  |
| Positive          | 26        | 21   | 1    | 4   |
| Recurrence        | Negative | 40   | 35   | 0.000*| 42  |
| Positive          | 20        | 23   | 4    | 7   |
| Metastasis        | Negative | 51   | 41   | 0.000*| 43  |
| Positive          | 29        | 18   | 3    | 4   |

| Abbreviations: N, negative; P, positive.

Table 4. Prognostic factors by univariate analysis (Cox’s proportional hazards model)

| Variables                     | Hazard ratio (95% confidence interval) |
|-------------------------------|----------------------------------------|
| Age (≤50/≥50)                 | 1.23 (0.53-2.87)                       |
| Gender (M/F)                  | 0.74 (0.32-1.73)                       |
| Size (<2/≥2 cm)               | 2.97 (1.00-8.80)                       |
| Site (major/minor)            | 0.96 (0.41-2.27)                       |
| Histotypes (solid, tubular, and cribriform) | 0.30 (0.13-0.70) |
| TNM stage (T1+T2/T3+T4)       | 2.56 (1.00-6.59)                       |
| Perineural invasion (N/P)     | 2.26 (1.11-4.64)                       |
| Vascular invasion (N/P)       | 7.18 (2.79-18.47)                      |
| Recurrence (N/P)              | 62.56 (2.00-1,962.16)                  |
| Metastasis (N/P)              | 160.25 (3.47-7,391.75)                 |

*P < 0.05 by χ² test.
without perineural invasion, which indicates that the aberrant expression of iNOS may be correlated with perineural spread. NF-κB belonging to the transcription factors family exists in most cells as homodimeric or heterodimeric complexes of p50 and p65 subunits and remains inactive in the cytoplasm of cells associated with the NF-κB inhibitory protein (IκB). Once triggered and activated, freed NF-κB in the heterodimer of the p65 and p50 subunits translocates from the cytoplasm into the nucleus and binds to the specific sequence in the promoter of target genes. A series of evidence supports the notion that NF-κB plays a key role in the malignant behavior of tumor. In the current study, we found that the nuclear staining of NF-κB p65 could only be found in 12.5 ± 4.58% of ACC cells, although the cytoplasm of luminal cells had a slight staining of NF-κB p65 in normal salivary gland. NF-κB p65 has been previously reported in prostate cancer (11), laryngeal squamous cell carcinoma (37), pancreatic cancer (7), breast cancer (12), and so on. Immunohistochemical study showed that the nuclear localization of p65 was detected in 5.6% of the counted cells in OSCC tissue and in 0% of cells in normal epithelium (24). In Lessard et al.’s study (11), the percentage of p65 nuclear staining in pancreatic cancer cells was mainly <30%. Our results consist with these studies that NF-κB seemed to be activated in some but not all tumor cells. Because the nuclear localization of NF-κB is believed to be equivalent to NF-κB activation, we hypothesize that NF-κB was activated in some ACC cells, which may trigger the transcription of NF-κB-dependent genes and regulate the expression of corresponding proteins.

In the neovascularization of several neoplasms, research has shown that iNOS and VEGF are the potent angiogenic factors which are regulated by NF-κB (38, 39). In our study, it was found that the nuclear staining rate of NF-κB p65 was significantly correlated with iNOS and VEGF protein levels in ACCs. The correlation between NF-κB p65 nuclear localization rate and MVD was also highly significant.

| Variables                      | Hazard ratio (95% confidence interval) | P     |
|--------------------------------|----------------------------------------|-------|
| Histotypes (solid/tubular and cribriform) | 0.16 (0.00-0.14)                       | 0.000 |
| Perineural invasion (N/P)      | 22.55 (2.96-172.01)                    | 0.003 |
| Vascular invasion (N/P)        | 2.56 (0.76-8.63)                       | 0.129 |
| Recurrence (N/P)               | 11.74 (0.00-6.35E+70)                  | 0.976 |
| Metastasis (N/P)               | 405844.0 (0.00-4.10E+69)               | 0.864 |
| NF-κB (<13%/>13%)              | 6.24 (1.01-38.53)                      | 0.049 |
| iNOS (0-3/4-5/6-7)             | 0.12 (0.04-1.01)                       | 0.047 |
| VEGF (0-3/4-5/6-7)             | 9.96 (1.17-84.43)                      | 0.035 |
| MVD (<48/>48)                  | 0.04 (0.00-0.46)                       | 0.010 |

Abbreviations: N, negative; P, positive.
Okamoto et al. (40) showed that the inhibitors of the transcription factors NF-κB completely prevented the advanced glycation end product–induced up-regulation of VEGF mRNAs and the subsequent increase in DNA synthesis in endothelial cells. The authors suggested that NF-κB activation might be involved in advanced glycation end product–elicited angiogenesis through overproduction of autocrine VEGF proteins.

There is a possibility of cross-talk between the NF-κB and iNOS systems with synergistic effects in tumor angiogenesis. NF-κB activity is essential for iNOS gene transcription. Chantome et al. (41) reported that activated NF-κB p65 subunit was responsible for increased iNOS gene transcription and NO production in the EMT-6 mouse mammary cancer cell line exposed to either interleukin-1β or lipopolysaccharide. However, recent researches indicated that NO also played a contradictory role in NF-κB activation, depending on the experimental conditions. The iNOS activity inhibits NF-κB activation in T cells (42) and the NO donors such as sodium nitroprusside and nitrosoglutathione inhibits NF-κB activity in a mouse embryonic carcinoma (43). In contrast, increasing evidence shows that the NF-κB activity and NO generation as a result of iNOS can make a positive regulatory loop. NO donors such as sodium nitroprusside and diethylamine NONOate induce NF-κB activity in rat macrophage cells (44) via the protein kinase C and mitogen-activated protein kinase pathways. Further researches are required to elucidate whether the positive feedback loop between iNOS and NF-κB exists in ACCs.

ACC is a malignant tumor of salivary gland origin, which constitutes about 10% of all neoplasms of the salivary gland. The growth patterns can be categorized into three types: solid, tubular, and cribriform. The solid phase is known to be much more aggressive than the other two varieties (45). Lim et al. (22) reported that no correlation was found between histologic types of ACC and VEGF expression. However, they only investigated 15 cases of ACCs, which may not be enough to draw a definitive conclusion. Our study showed that NF-κB, iNOS, VEGF expression, and MVD were significantly higher in solid type than those in tubular and cribriform types. To a certain extent, our results may be an explanation for the more malignant behavior of solid type than the other two types.

The identification of a specific factor for predicting clinical outcome in the case of salivary gland carcinoma would be helpful for selecting more effective treatments. However, there are few reports of the correlation between clinicopathologic factors and NF-κB, iNOS, VEGF, and MVD expression in ACCs. Lim et al. (22) reported that VEGF was correlated with most of the factors such as size, age, lymph node metastasis, clinical stage, perineural and vascular invasion, recurrence, and survival. Our study suggests that NF-κB, iNOS, VEGF expression, and MVD had significant correlations with the clinicopathologic factors, such as tumor size, clinical stage, vascular invasion, recurrence, and distant metastasis. Although except for VEGF, the expression of NF-κB, iNOS, and MVD were all correlated to the perineural invasion, there was a tendency towards higher VEGF staining with more cases with perineural invasion.

Univariate survival analysis showed that histologic types, perineural and vascular invasion, recurrence, metastasis, the expression of NF-κB, iNOS, and VEGF and MVD were important prognostic markers in ACCs. From multivariate survival analysis, it was shown that NF-κB nuclear localization rate, the expression level of iNOS, and VEGF and MVD were associated with a shorter survival period and was determined to be the most important independent prognostic indicators. The results suggest that NF-κB, iNOS, VEGF and MVD may play an important role in the progression of ACCs. High NF-κB, iNOS, VEGF and MVD level in ACCs would warrant a more radical mode of therapy and represent important therapeutic targets. Although several factors indicate a poor prognosis for ACCs, including a solid histologic pattern, recurrent disease, and distant metastasis (46), our study showed that solid histologic type and perineural invasion seemed to be the important clinicopathologic factors involved with shorter survival. Further studies would be needed to elucidate the relationship between those clinicopathologic factors and survival.

Taken together, this study suggests that, in ACCs, the aberrant activation of NF-κB, iNOS, and VEGF and MVD are related to malignancy. The possibility that they may contribute to tumor angiogenesis and have prognostic values should be raised.

Acknowledgments

We thank Yuan Li and Shichun Xiong for their technical assistance.

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