The Immunoexpression of Visfatin in Oral Carcinogenesis from Korean Patients

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Visfatin is a pro-inflammatory cytokine, which is thought to play a central role in systemic inflammation and the pathogenesis of obesity related diseases. Only a few studies investigated the effect of visfatin on human cancers. Furthermore, there have been no studies on the association between the expression of visfatin in OSCC tissue and its effect on OSCC patients. Hence, the present study analyzed the expression of visfatin in OSCC from Korean patients. Immunohistochemistry for visfatin was performed using 12 normal oral mucosas (NOM), 16 oral leukoplakias (with/without dysplasia), and 58 OSCC patients samples. Immunoreactivity was semi-quantitatively scored and the correlation between the expression of visfatin and clinicopathological parameters of OSCC patients was analyzed. The immunohistochemical analysis demonstrated that the expression level of visfatin increased in OSCC alone (p<0.05). Moreover, the immunoexpression score of visfatin was significantly correlated with TNM stage of OSCC patients. Our findings suggested that visfatin can play a certain role in the pathogenesis of OSCC. In addition, visfatin was associated with the tumor progression of OSCC patients and may act as independent biomarker of OSCC.

Key words: Oral Squamous Cell Carcinoma, Visfatin, Adipocytokine, Tumor Progression, Biomarker

1. INTRODUCTION

Cancer is one of the leading causes of death in Korea1). Oral squamous cell carcinoma (OSCC) is the most common cancer in oral cavity and more than 650,000 cases are diagnosed annually worldwide2,3). It occurs through multistep carcinogenesis, composed of a spectrum from leukoplakia to infiltrative OSCC4). Multistep carcinogenesis
involves continuous genetic and cellular changes in its individual step). However, the detailed mechanism of multistep carcinogenesis is not fully understood yet.

Adipose tissue has come to be regarded as a kind of endocrine organ. Adipokines are secreted by adipose tissue and have endocrine and paracrine functions in various tissues. They act as a balancing agent in glucose metabolism, lipid metabolism, insulin signaling, and inflammation. In addition, accumulating evidence indicates that adipokines can play a major role in cancer development and recurrence, and thus contribute to fatalities. It has been reported that obesity is associated with increased risk prevalence of several cancers including breast, endometrium, colon, prostate, ovarian and pancreatic cancers.

Visfatin, a highly conserved 52-kDa protein, has been reported as a nicotinamide phosphoribosyl-transferase (NAMPT), later redescribed as pre-B-cell colony-enhancing factor (PBEF). It is a novel adipokine that plays various physiological roles, including immune cell signaling, insulin mimetic effects, and regulation of the NAD synthesis. Recent data indicated that overexpressed visfatin is correlated with tumor progression and clinical outcome of various human cancers. The dysregulation of visfatin is likely to be closely associated with cancer development and therapy; however, the detailed mechanism of visfatin in OSCC is poorly understood.

The objective of this study is to investigate the expression pattern of visfatin by performing immunohistochemistry analysis in normal oral mucosa (NOM), leukoplakia and OSCCs. In addition, we assessed the relationships between immunohistochemical scores of visfatin and clinicopathological parameters to find out whether visfatin can be a putative predictor of outcome. We also determined the pathological role of visfatin protein expression using tissues from patients with leukoplakia and OSCC.

II. MATERIALS AND METHODS

1. Patients and tissue specimens

A total of 86 tissue specimens were selected containing 12 NOM, 16 leukoplakia (with/without dysplasia), and 58 OSCC. It was collected from the Department of Pathology, School of Medicine, Pusan National University dating between January 1996 to December 2007. The samples of NOM were obtained from adult patients with no pathologic lesions in Pusan National University Dental Hospital, which were obtained from the third molar removal. Tissue specimens were fixed in 10% neutral-buffered formalin overnight and paraffin-embedded.

2. Immunohistochemical stain for visfatin

In brief, the paraffin blocks of NOM, leukoplakia, and OSCC were prepared, and thin sections (4-5 μm) from selected areas were deparaffinized. One section was served for routine hematoxylin-eosin staining and others were used in immunohistochemical stain. Immunostaining procedure was performed according to the manufacturer’s instructions of SuperPictureTM 3rd Gen IHC Detection kit (Invitrogen). In brief, after antigen retrieval for visfatin by boiling the slides for 15 min in the citrate buffer (pH 6.0, Invitrogen), slides were cooled for 25 min at room temperature (RT), according to the manufacturer’s instructions. Then, the slides were washed thoroughly in PBS (pH 7.4) for 5 min three times each and incubated with H2O2 solution (Invitrogen) for 20 min at RT in a humid chamber. The primary antibody against visfatin protein (diluted 1:100, Cell Signaling Technology, Beverly, MA, USA) was applied on the slides and incubated overnight at 4°C. Then, horseradish peroxidase-labeled secondary antibody was applied in SuperPictureTM 3rd Gen IHC Detection kit according to the manufacturer’s instructions.
The slides were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with Mayer’s hematoxylin, mounted using Malinol through dehydration and transparency. Then, the slides were thoroughly observed under light microscopy (Motic Instrument Inc., HongKong, China). For negative control, the staining was performed using PBS solution instead of primary antibody, and the same procedure was conducted with breast cancer specimen as a positive control.

3. Evaluation of immunohistochemical stain

For the evaluation of visfatin immunoreactivity, 5 non-overlapping fields were randomly selected for every NOM, leukoplakia and OSCC slides. Then, each field was photographed using light microscopy with a digital camera (Motic Instrument Inc., HongKong, China, ×100). The stained slides were evaluated and scored by two oral pathologists independently.

The expression levels of visfatin were assessed by semi-quantitative manner. The scores of selected fields were classified into five groups: >75% positive-stained cells (point 4), 50%-75% positive-stained cells (point 3), 25%-50% positive-stained cells (point 2), 1-24% positive-stained cells (point 1) and no stained cells (point 0).

4. Statistical Analysis

All data were presented as mean ± standard deviation (S.D.). The normality of distribution of our data was assessed by Kolmogorov-Smirnov test. Because our data did not show normal distribution, Kruskal-Wallis test and Mann-Whitney test were performed to evaluate the differences of visfatin immunoreactivity score between NOM, hyperplastic leukoplakia, leukoplakia with dysplasia and OSCC. Furthermore, the relationships among variables including visfatin and clinicopathologic parameters of OSCC patients were analyzed by the Spearman Rank Correlation Coefficiency using Windows PASW (Predictive Analytics SoftWare) version 21.0 (SPSS Inc, Chicago, IL, USA). A p-value of less than 0.05 was considered as statistically significant.

III. RESULTS

1. Immunohistochemical stain for visfatin

The immunoreactivity of visfatin protein was scarcely observed in the basal cell layer and the lower spinous layer of the epithelium within NOM, hyperplastic leukoplakia and leukoplakia with dysplasia (Fig. 1A, 1B and 1C). On the contrary, visfatin immunoreactivity was found in most cases of OSCC tissue and revealed intense immunoreactivity throughout OSCC tissue. The immunoreactivity of visfatin was observed in the cytoplasm as well as nuclei of OSCC cells (Fig. 1D). High expression of visfatin was observed in 4.0% of normal oral mucosa, 10.0% in hyperplastic leukoplakia, 16.7% in leukoplakia with dysplasia, and 65.6% in OSCC (38/58).

Visfatin expression of hyperplastic leukoplakia and leukoplakia with dysplasia did not show significantly different from that of NOM. However, the immunoreactivity of visfatin in OSCC tissue significantly increased compared with those of NOM, hyperplastic leukoplakia and leukoplakia with dysplasia (p<0.05) (Fig. 2 and Table 1).

2. Correlation between visfatin and the clinicopathological parameters of OSCC patients

To evaluate the association between the visfatin score and clinicopathological parameters of OSCC patients, Spearman’s Rank Correlation Coefficient was calculated. There was a significant correlation between the immunoreactivity of visfatin and TNM stage (r=-0.477, p<0.000). However, there was no significant correlation between the
Figure 1. Visfatin immunoexpression in NOM (A), hyperplastic leukoplakia (B), leukoplakia with dysplasia (C) and OSCC (D). Visfatin immunohistochemistry, X100

Figure 2. The difference of visfatin immunoexpression between NOM, hyperplastic leukoplakia, leukoplakia with dysplasia and OSCC.
Table 1. Visfatin immunoreactivity in NOM, hyperplastic leukoplakia, leukoplakia with dysplasia and OSCC.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Immunoreactivity of visfatin (mean±S.D.)</th>
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<tbody>
<tr>
<td>NOM</td>
<td>12</td>
<td>0.35±0.65</td>
</tr>
<tr>
<td>hyperplastic leukoplakia</td>
<td>10</td>
<td>0.56±0.51</td>
</tr>
<tr>
<td>leukoplakia with dysplasia</td>
<td>6</td>
<td>0.40±0.52</td>
</tr>
<tr>
<td>OSCC</td>
<td>58</td>
<td>2.42±1.47</td>
</tr>
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</table>

*S.D.: standard deviation

Visfatin can be considered a pro-inflammatory, immune-modulating, and apoptosis inhibiting cytokine. It is essential for preserving a proper intracellular NAD level, thereby resulting in the increase of cell proliferation and playing a central role in survival under genotoxic stress. Ninomiya et al. suggested that visfatin stimulation strongly induced the proliferation of various hepatocellular carcinoma cell lines and might act as a growth factor in the cells. The elevated expression of visfatin triggered the induction of the sirtuin family, silent information regulator (SIRT) 1 to SIRT7. Menissen et al. reported that SIRT1 suppressed cellular senescence in cells and inhibited c-Myc induced apoptosis. The cells with visfatin knockdown were very sensitive to genotoxic stress and had a tendency to increase apoptosis. In addition, several researches indicated that visfatin evoked cell proliferation through the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and p38 pathways.

Current studies propose that visfatin has to do with the development and progression of a number of human cancers. However, the biologic mechanisms involving visfatin in OSCC are not yet clear. It has been reported that the expression of the visfatin protein in prostate cancer, breast cancer, malignant lymphoma and bladder cancers.

3. Correlation between visfatin and NF-κB p65 in OSCC patients

The Spearman’s Rank Correlation Coefficient between visfatin and NF-κB p65 was calculated as 0.559 (p<0.000).

IV. DISCUSSION

This is the first study to demonstrate high visfatin expression in OSCC tissue and the clinical correlation between clinicopathological factors of OSCC patients. Our immunohistochemical analysis did not identify any significant difference between NOM, hyperplastic leukoplakia, and leukoplakia with dysplasia, whereas there was a significantly increased expression of visfatin protein in OSCC tissue (p<0.05). In addition, the visfatin expression showed significant correlation with TNM stage. Further quantitative studies are needed to assess the importance of this observation.

The Spearman correlation coefficient of clinicopathological parameters in OSCC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spearman correlation coefficient</th>
<th>P value</th>
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<tr>
<td>TNM stage vs. visfatin immunoreexpression</td>
<td>-0.477</td>
<td>0.000</td>
</tr>
<tr>
<td>TNM stage vs. alcoholic assumption</td>
<td>0.202</td>
<td>0.009</td>
</tr>
<tr>
<td>TNM stage vs. survival period</td>
<td>-0.437</td>
<td>0.000</td>
</tr>
<tr>
<td>Lymph node metastasis vs. survival period</td>
<td>-0.476</td>
<td>0.000</td>
</tr>
</tbody>
</table>
cancer is significantly higher than that of normal tissue^17,19-21). The role of visfatin in the development and progression of cancers can be summarized as follows: first, visfatin can directly induce the secretion of inflammatory cytokines, such as IL-6 and SIRT6^10,22). SIRT6 is regarded as a link between inflammatory response and cancer promotion^22). Second, visfatin can enhance cancer cell survival ability and increase the risk of cancer development^7). This mechanism acts via the pathways of ERK1/2, p38, and SDF-1 and effectively increases NF-κB and AP-1, eventually leading to cell survival and migration^23). Third, visfatin may protect cancer cells from cytotoxic damage from reactive oxygen species. Therefore, visfatin can protect cancer cells from apoptosis and allow the cells to survive and proliferate^24).

Our results indicated that immune-expression of visfatin increased in OSCC tissue and showed significant correlation with the TNM stage. Previous reports showed that visfatin expression was associated with the progression of gastric cancer and colorectal cancer and suggested that it was a useful biomarker for these cancers^25,26). Our findings showed a similarity to the results of the aforementioned reports and that visfatin is considered a promising biomarker of OSCC progression.

Lastly, we reported that NF-κB p65 increased in oral multistep carcinogenesis, and that the expression of NF-κB p65 was associated with clinocopathological factors in OSCC patients. In the present study, the Spearman correlation coefficient between visfatin and NF-κB p65 was calculated as 0.559 (p<0.000). Fan et al. described that nuclear NF-κB p65 was activated by visfatin over time in atheroma^18). Visfatin alone induced the activation of IκBα and increased the NF-κB transcription into the nucleus^27) and upregulated the NF-κB p65 DNA binding activity in human keratinocytes^10). The main function of NF-κB p65 is involved with the regulation of cell proliferation and cell survival. The proteins of NF-κB pathway play a central role in the escape from the apoptosis of various human cancer cells, including oral cancer. In addition, it has been reported that the activation of NF-κB can be associated with the development and progression of OSCC^12). Therefore, we speculated that visfatin activated the transcriptional activity of NF-κB p65 in OSCC tissue. Moreover, the author proposed that the enhancement of these factors can be associated with the clinicopathological factors of OSCC patients, including recurrence, TNM stage, and lymph node metastasis. These findings may have implication for developing target for OSCC treatment.

Taken together, our result demonstrated that visfatin expression significantly increased in OSCC tissue, compared to leukoplakia and NOM, suggesting visfatin was expressed in late stage in oral carcinogenesis. In addition, the immunoexpression of visfatin appeared to be associated with the TNM stage, and NF-κB p65 expression. The overexpression of visfatin protein may play a particular role in oral carcinogenesis, and, thus, the detection of visfatin may be considered a biomarker of cancer progression of OSCC. The possible role of visfatin in oral carcinogenesis should be investigated with larger cohorts.

V. REFERENCES

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