Expression of fatty acid binding protein 4 is involved in the cell growth of oral squamous cell carcinoma

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Abstract. Fatty acid binding proteins (FABPs) are a family of small and highly conserved lipid chaperone molecules with highly varied functions. Among them, fatty acid binding protein 4 (FABP4, also known as aP2) is highly expressed by adipocytes, macrophages and dendritic cells. Although the role of FABP4 in cancer is still unclear, it has been reported to be highly expressed by human tumors such as ovarian and bladder cancers. In the present study, we investigated the expression and role of FABP4 in oral squamous cell carcinoma (SCC) and its expression in oral SCC tissues. Immunohistochemical staining revealed that FABP4 expression in the tumor tissue was much higher than that in the non-tumor area of the same specimen. In the in vitro studies, an FABP4-knockdown SCC cell line (established through FABP4-specific siRNA) showed inhibited growth, and inhibited expression and activation of mitogen-activated protein kinase (MAPK). These results indicate that expression of FABP4 plays an important role in the cell growth of oral SCC through the MAPK pathway.

Introduction

Oral squamous cell carcinoma (SCC) is a major neoplasm of the oral cavity with an increasing rate of incidence (1-3). The optimal therapy for early oral SCC is surgery, but the overall survival rate has exhibited only a slight change (1-3). Therefore, more effective therapies for oral SCC are needed.

Fatty acid binding proteins (FABPs) are a family of small and highly conserved lipid chaperone molecules that bind long-chain fatty acids and other hydrophobic ligands. Their functions are wide ranging (4-6). Among them, fatty acid binding protein 4 (FABP4, also known as aP2) is highly expressed in adipocytes, macrophages and dendritic cells (5,7). As a result of its distribution, FABP4 is the most extensively researched FABP in endocrinology and metabolomics. FABP4 affects metabolic syndrome progression; FABP4-deficient mice were found to have reduced hyperinsulinemia and insulin resistance in obesity (7,8) and showed protection from atherosclerosis (9).

However, little is known concerning the role of FABP4 in cancer, including oral SCC. Recently, Nieman et al. (10) reported that adipocytes promote ovarian cancer metastasis and tumor cell growth by providing energy mediated by FABP4. Therefore, increased FABP4 expression may affect the growth of various tumor types. Our research group also reported that molecules controlled by peroxisome proliferator-activated receptor γ (PPARγ) play key roles in SCC growth (11-15). As FABP4 is known to mediate transcription with PPARγ (4,16), we hypothesized that FABP4 may regulate SCC growth. Therefore, in the present study, we investigated FABP4 expression and its effects on SCC of the tongue.

Materials and methods

Tissue samples. All clinical studies were approved by the Ethics Committee of Osaka University Dental Hospital, Osaka. Twenty-seven SCC specimens from resected tongue tissue were obtained at the Osaka University Dental Hospital during 1986-2008 after patient informed consent (Table I). Patients received no preoperative therapy, including chemotherapy and irradiation therapy. The age range of the patients was 30-92 years (61.6±16.4 years, mean age ± SD); 17 patients were men and 10 were women.

Antibodies. The anti-FABP4 polyclonal antibody was obtained from Bioss Inc. (Woburn, MA, USA). Antibodies against p44/42MAPK and the phosphorylated p44/42MAPK antibody were from Cell Signaling Technology (Beverly, MA, USA).

Immunohistochemical staining and evaluation of FABP4 expression. FABP4 expression in tissues was detected by an anti-FABP4 antibody using standard immunohistochemical techniques (12-15). Formalin-fixed and paraffin-embedded continuous sections were selected and sliced into 5-µm
sections. Briefly, incubation with an anti-FABP4 polyclonal antibody was performed at 4°C for 16 h; sections were then washed. After applying the secondary antibody, the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used with a 3,3'-diaminobenzidine substrate kit, according to the manufacturer's instructions. The staining endpoint was determined when the standard tissue sections were constantly stained at the intensity as previously described (12,17).

The intensity of the immunohistochemical staining with the anti-FABP4 antibody was evaluated by scoring according to four groups: 0, <10%; 1, 10-20%; 2, >20-50%; and 3, >50% of the cells exhibiting cytoplasmic staining (12,17). To confirm the reproducibility, the anti-FABP4 immunohistochemical staining was re-evaluated by a pathologist who was unaware of the original assessment. Non-tumor areas were selected as comparatively normal areas separated from the tumor areas by an appropriate distance and confirmed by the pathologist (14,15).

**RNA interference approach.** The SAS cells were trypsinized and resuspended in DMEM without FBS, and then separated placing ~2x10^5 cells in each dish. The *FABP4*-specific siRNA (Stealth siRNA) was purchased from Invitrogen Japan (Tokyo, Japan). We purchased three sequences and performed preparatory experiments to determine the most effective sequence. The sequences of the selected *FABP4*-siRNA were: sense, 5'-CAC CAUUAACUCUGAAAGUACCUUU-3' and antisense,
5'-AAAGGUACUUUCAGAUUUAAUGGUG-3'. For transfection, FABP4-siRNA or a negative control (Stealth RNAi negative control duplex; Invitrogen Japan) solution was added to the DMEM containing Lipofectamine RNAiMax (Invitrogen Japan) and incubated for 20 min at room temperature to create the transfection mixture. The transfection mixture was then added to the cells at the indicated final siRNA concentrations. Following 24 h of transfection, the medium was replaced by DMEM containing 10% FBS, at which time viable cells were counted using a Countess Automated Cell Counter. Cell growth was expressed as a percentage of the vehicle-treated control growth.

Western blot analysis. Adherent or suspended cells were washed in PBS, and the cell extracts were prepared by lysing the cells in lysis buffer. Proteins were separated by electrophoresis using 10% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Detection of proteins was performed with each polyclonal antibody and visualized using an ECL detection kit (Amersham, London, UK) following the manufacturer’s recommended procedure.

Statistical analysis. Results are expressed as the means ± SEM or ± SD. Statistical comparisons were carried out using the Student’s t-test or the Scheffé’s method after analysis of variance. P<0.05 was considered to indicate a statistically significant result.

Results

Tongue SCC tissues express FABP4. We stained tongue SCC tissues using the FABP4-specific antibody. Within single tumor specimens, the non-tumor areas were unstained (Fig. 1A, upper panel), whereas tumor areas showed positive FABP4 staining (Fig. 1A, lower panel). According to the scoring as described above, FABP4 expression between the non-tumor and the tumor area differed significantly (Fig. 1B), and FABP4 was expressed in the tumor areas, but not in the normal tissues.

FABP4-specific siRNA suppresses the growth of tongue SCC. Treatment with FABP4-siRNA markedly decreased FABP4 protein levels in the SAS cells (Fig. 2A), and suppressed SAS cell growth in a concentration-dependent manner (Fig. 2B). Inhibition of SCC growth was also visibly altered (Fig. 2C), and significantly differed between the SAS controls and the FABP4-knockdown SAS cells (Fig. 2D).

FABP4-specific siRNA inhibits expression and phosphorylation of mitogen-activated protein kinase (MAPK). To investigate the mechanisms involved in the growth inhibition induced by suppression of FABP4, we analyzed a type of MAPK, serine/threonine protein kinases. Since they affect cell proliferation, survival and differentiation, aberrant MAPK cascades contribute to cancer and other diseases (19-21). Therefore, we studied the effects of FABP4 knockdown on MAPK expression and phosphorylation. Western blot analysis showed decreased phosphorylated MAPK (pMAPK; Fig. 3, middle panel). Notably, MAPK expression itself was also regulated by FABP4 knockdown (Fig. 3, upper panel).

Discussion

FABP4 expression has been reported in various types of tumors such as ovarian and bladder cancers (10,22), and FABP5 (E-FAPB) has been found in oral SCC (23,24). However, the expression and exact role of FABP4 in oral SCC have not been widely investigated.

In the present study, using an immunohistochemical approach, we consistently found significantly higher expression of FABP4 protein in the tumor area of tongue SCC than
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Therefore, FABP4 expression in tumors may affect SCC cell growth. In fact, we showed that suppression of FABP4 protein by the FABP4-specific siRNA clearly inhibited the growth of SCC cell lines. These results clearly indicate the important role of FABP4 in SCC growth.

FABPs actively facilitate the transport of lipids to specific cellular compartments, including lipid droplets for storage; endoplasmic reticulum for signaling, trafficking and membrane synthesis; mitochondria or peroxisome for oxidation; cytosolic or other enzymes for activity regulation; nuclei for lipid-mediated transcriptional regulation; or even outside the cell for autocrine or paracrine signaling. Among the FABPs, FABP4 is highly expressed in adipocytes, macrophages and dendritic cells and affects these cells in various manners (5). In cancer cells, FABP4 transports energy by carrying fatty acids, encouraging metastasis and tumor cell growth (10). Yet, FABP4 performs other roles in tumor growth, through its various functions. Therefore, we studied the role of FABP4 in the MAPK pathway and the mechanisms of growth inhibition induced by FABP4 suppression.

As the MAPK pathway helps to mediate cell proliferation and cancer growth, it has been widely studied as a potential target for cancer therapy (19-21). In fact, our present research showed decreased MAPK expression and phosphorylation 12 h following treatment with FABP4-specific siRNA.
which indicates that FABP4 affects cell growth through the MAPK pathway. Notably, expression of MAPK itself was also suppressed by FABP4 knockdown; FABP4 may affect transcription of MAPK, which implies a complex role for FABP4 in tumor growth. Inhibition of the MAPK pathway may be one of the several mechanisms through which FABP4 mediates tumor growth (Fig. 4). Further investigation is warranted.

In the present study, FABP4 expression in the tumor tissues was not correlated with age, gender, histological tumor differentiation or survival rate of the cases. Of our 27 cases, 12 had neck lymph node metastasis, all 12 of whom showed FABP4 expression in lymph nodes, similar to that in the primary tumors (data not shown). Thus, FABP4 expression may affect metastasis to neck lymph nodes; this role merits further investigation.

In summary, we demonstrated FABP4 expression in human tongue SCC tissues and cultured SCC cells. Our results suggest an important role for FABP4 in SCC growth and indicate that FABP4 is a potential target for the therapy of oral SCC.

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References