

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 (PPAR γ) play key roles in SCC growth (11-15). As FABP4 is known to mediate transcription with PPAR γ (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 \pm 16.4 years, mean age \pm 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- μ m

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Table I. Characteristics of the patients with squamous cell carcinoma of the tongue, their histological diagnosis and expression of FABP4 in the tissue specimens.

Case no.	Age (years)	Gender	Differentiation	FABP4 expression	
				Tumor area	Non-tumor area
1	34	Male	Well-differentiated SCC	1	0
2	46	Female	Well-differentiated SCC	2	0
3	57	Male	Well-differentiated SCC	1	1
4	64	Male	Well-differentiated SCC	0	0
5	69	Male	Well-differentiated SCC	0	0
6	71	Female	Well-differentiated SCC	1	0
7	71	Male	Well-differentiated SCC	1	0
8	72	Male	Well-differentiated SCC	2	0
9	78	Female	Well-differentiated SCC	1	0
10	79	Male	Well-differentiated SCC	1	1
11	47	Male	Moderately differentiated SCC	1	0
12	61	Male	Moderately differentiated SCC	1	0
13	61	Female	Moderately differentiated SCC	1	0
14	72	Male	Moderately differentiated SCC	1	0
15	77	Male	Moderately differentiated SCC	1	2
16	83	Female	Moderately differentiated SCC	3	2
17	92	Female	Moderately differentiated SCC	1	0
18	30	Male	Poorly differentiated SCC	2	1
19	33	Male	Poorly differentiated SCC	0	0
20	38	Male	Poorly differentiated SCC	0	0
21	51	Female	Poorly differentiated SCC	0	0
22	52	Male	Poorly differentiated SCC	2	0
23	55	Female	Poorly differentiated SCC	2	0
24	65	Male	Poorly differentiated SCC	1	3
25	67	Male	Poorly differentiated SCC	3	2
26	41	Female	Differentiation unknown SCC	0	0
27	68	Female	Differentiation unknown SCC	2	0

Expression of FABP4 by immunohistochemical staining in the tumor and non-tumor areas is scored and expressed as 0-3.

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).

Cell culture and cell growth assay. We used a human oral SCC cell line (SAS) that was established from tongue SCC (13). Cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37°C under 0.5% CO₂. For the cell growth experiment, cells were trypsinized and replated onto culture dishes (11-15,17,18). SCC cells were counted using a Countess Automated Cell Counter (Invitrogen, Eugene, OR, USA). Inhibition of cell growth was compared with the vehicle-treated controls.

RNA interference approach. The SAS cells were trypsinized and resuspended in DMEM without FBS, and then separated placing ~2x10⁵ 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 CAUUA AUCUGAAAGUACCUUU-3' and antisense,

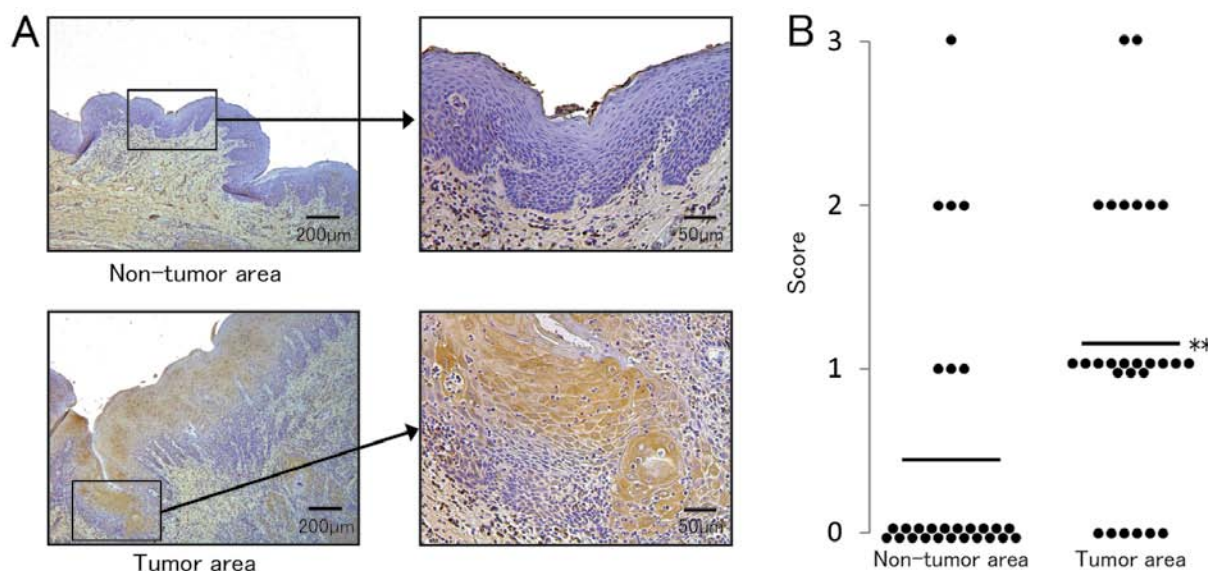


Figure 1. Expression of fatty acid binding protein 4 (FABP4) in tumor cells in tongue squamous cell carcinoma. (A) FABP4 expression in the tumor area (upper panel) of primary tongue squamous cell carcinoma and the non-tumor area (lower panel) from the same tissue section (case 13 in Table I) using immunohistochemical staining. The brown color shows positive FABP4 staining; blue is a counterstain. (B) Comparison of FABP4 expression between tumor and non-tumor areas. The score of the distribution of FABP4 expression is indicated by black circles from the data in Table I. A horizontal line shows the average score. Data indicate the mean \pm SEM from 27 cases. ** $P < 0.01$.

5'-AAAGGUACUUUCAGAUUUAUGGUG-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 \pm SEM or \pm 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

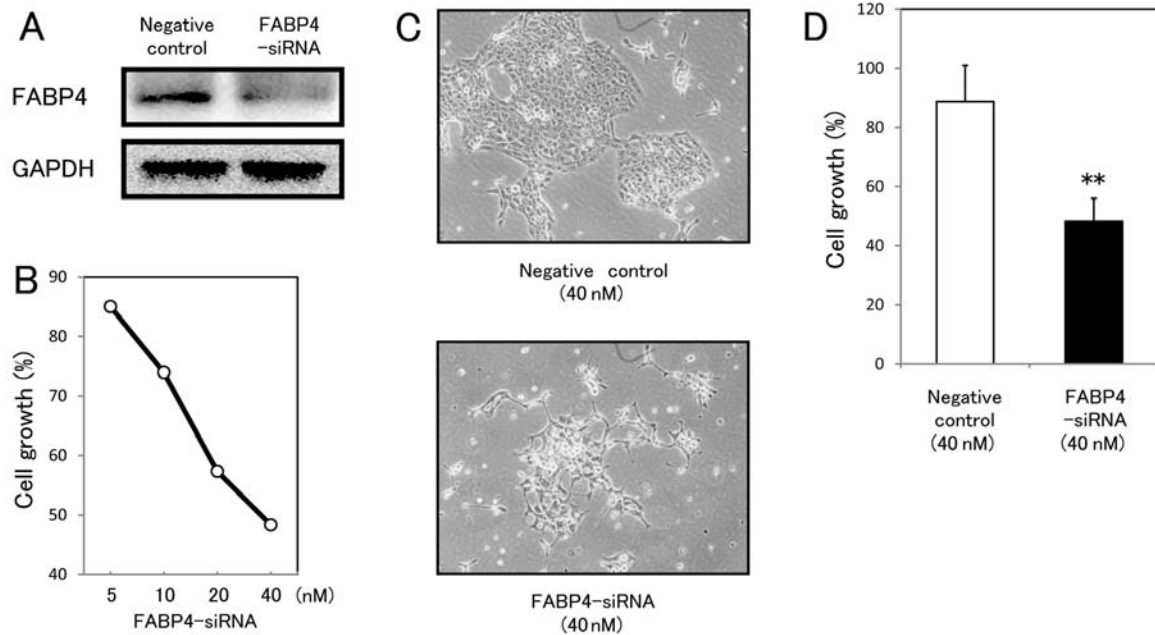


Figure 2. Effect of *FABP4* knockdown on the growth of SCC cells treated with *FABP4*-siRNA. (A) Western blot analysis of *FABP4* expression in SAS cells treated with *FABP4*-specific siRNA (40 nM) or negative control siRNA (40 nM). Samples were collected 12 h after treatment. *GAPDH* was used as the standard for equivalent application. (B) A concentration-dependent effect of *FABP4*-specific siRNA on cell growth. SAS cells were treated with *FABP4*-specific siRNA for 24 h, followed by additional culture for 72 h. Left axis presents the percentage of cell growth compared with vehicle (non-siRNA) control. (C) Typical images and (D) percentage of cell growth of SAS cells transfected with siRNA (40 nM) or negative control siRNA (40 nM) for 24 h and cultured for 72 h, followed by viable cell counting. Each value in D represents the percentage of cell growth compared with the vehicle (non-siRNA) control from six independent experiments. The black column represents the growth of cells transfected with *FABP4*-siRNA; the white column represents the growth of cells transfected with the negative control-siRNA. Error bars represent standard errors. ** $P < 0.01$ vs. negative control.

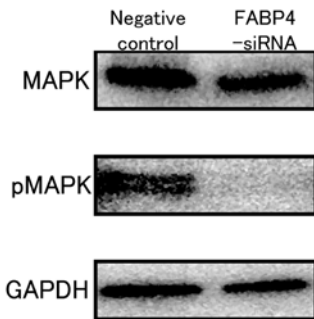


Figure 3. *FABP4*-specific siRNA inhibits expression of mitogen-activated protein kinase (MAPK) and its phosphorylation. A representative western blot analysis shows MAPK and p-MAPK expression in SAS cells. SAS cells were treated with *FABP4*-specific siRNA (40 nM) or negative control siRNA (40 nM). Samples were collected 12 h after the treatment. *GAPDH* was used as a standard for equivalent application.

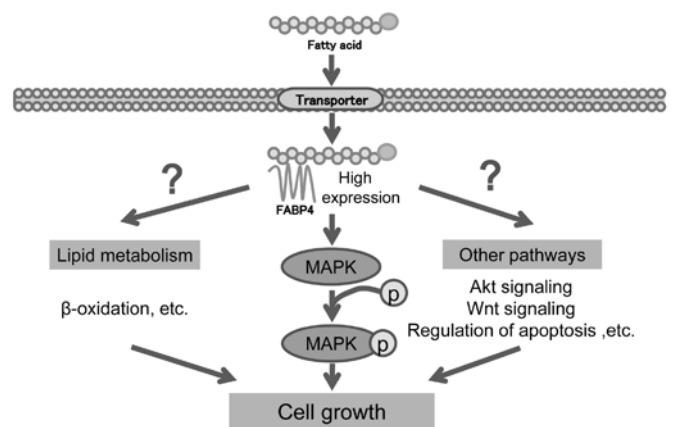


Figure 4. Schematic illustration of possible mechanisms of cell growth inhibition by *FABP4*-specific siRNA.

in the non-tumor area in the same tissue samples. 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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