

FABP7 inhibits proliferation and invasion abilities of cutaneous squamous cell carcinoma cells via the Notch signaling pathway

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Abstract. Cutaneous squamous cell carcinoma (CSCC) is one of the most common non-melanoma skin cancers worldwide. Fatty acid-binding protein 7 (FABP7) has been reported to be involved in the occurrence, development, metastasis and prognosis of various tumors. In addition, downregulated FABP7 expression was demonstrated in cutaneous malignant melanoma in a previous study. Therefore, we speculated that FABP7 may be a biomarker for CSCC diagnosis. The aim of the present study was to determine the molecular mechanism underlying the effects of FABP7 in CSCC, which may provide a new diagnostic biomarker or treatment target for CSCC. Reverse transcription-PCR, western blotting and immunohistochemistry assays were performed to detect the expression levels of FABP7 in CSCC tissues and cells. Overexpression of FABP7 was achieved in A431 and colo-16 cell lines by transfection with an overexpression vector (oeFABP7). Cell proliferation, colony formation, migration and invasion were detected by Cell Counting Kit-8, crystal violet, scratch and Transwell assays, respectively. Following FABP7 overexpression, western blotting was used to determine the expression levels of proliferation-, invasion- and Notch pathway-associated proteins, including Snail, N-cadherin, Twist, matrix metalloproteinase (MMP)-2, MMP-7, Notch 1 and Notch 3. In addition a CSCC model in nude mice was constructed. Immunohistochemistry was used to determine the expression levels of FABP7, Ki67, Notch 1 and Notch 3. It was demonstrated that FABP7 expression levels were significantly reduced in human CSCC tissues and cells compared with normal samples. Overexpression of FABP7

inhibited the proliferation, invasion and migration abilities of A431 and colo-16 cells compared with those in the negative control group. In addition, transfection with oeFABP7 reduced the expression levels of proliferation-, invasion- and Notch pathway-associated proteins compared with those in the negative control group. Overexpression of FABP7 also reduced the growth of CSCC tumors *in vivo* and inhibited the expression of Ki67, Notch 1 and Notch 3. Therefore, the results of the present study suggested that FABP7 may inhibit the proliferation and invasion of CSCC cells via the Notch signaling pathway.

Introduction

Cutaneous squamous cell carcinoma (CSCC) is common among elderly individuals and severely impacts the life quality, as well as poses a threat to their lives (1,2). CSCC is characterized by small nodules on the surface of the skin, which gradually become larger and harder to form cauliflower-like hyperplasia (3). A number of patients experience ulceration in the lesions, which gradually increases, accompanied by symptoms such as pus, bleeding and foul odor (4). In China, CSCC is the most common skin malignant tumor, and its incidence accounts for 78-90.9% of dermatological malignancies (5). However, if the early diagnosis and radical resection can be performed, the surgical effects are satisfactory and the cure rate is high, with potential complete remission. Therefore, it is necessary to explore early clinical biomarkers of CSCC and determine the associated molecular mechanisms.

Previous studies have demonstrated that abnormal gene expression can affect cell proliferation, invasion and migration processes, and thus affect the occurrence of tumors (6). Gene therapy introduces exogenous genes with certain functions into cells to treat squamous cell carcinoma (SCC). The metastatic ability of tumor cells is associated with their ability to induce proteases, as well as degrade the extracellular matrix and the basement membrane (7). Matrix metalloproteinases (MMPs) are a group of proteases that degrade almost all extracellular matrix and basement membrane components (8). Therefore, gene therapy by antisense MMP transfection and inhibition of MMP secretion in malignant tumor cells may be an effective treatment option. In addition, various genes have been identified to serve crucial roles in CSCC. For example, Zaravinos *et al* (9) demonstrated that downregulated Raf-1 kinase inhibitor protein mRNA levels and abnormal BRAF

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signaling pathway were involved in the pathogenesis of CSCC. Another study in cancer cells has also reported that microRNA-21 targets grainyhead-like transcription factor 3 to upregulate the expression of PTEN and prevent SCC (10). Although various genes have been researched, further research is needed in the clinical application of gene therapy, especially for patients with grade III and IV CSCC who present with a high degree of invasion, high recurrence and metastasis rates.

The role of fatty acid-binding protein 7 (FABP7) in tumor tissues is a controversial issue (11). In a previous study, overexpression of FABP7 was demonstrated to promote cell proliferation and predict poor prognosis of clear cell renal cell carcinoma (12). FABP7 is associated melanoma cell proliferation via modulation of Wnt/ β -Catenin signaling (13). Currently, only a few studies have reported the exact function of FABP7 in cutaneous malignant melanoma (12,14,15). Thus, the present study hypothesized that FABP7 may be a diagnostic biomarker of CSCC, and aimed to determine the detailed mechanism underlying the potential effects of FABP7 in CSCC.

Notch signaling pathway is involved in regulating the development of cutaneous tissues (16). Functional genes in Notch pathway including NCOR2, NCSTN, and MAML2 predict survival of patients with cutaneous melanoma (17). Thereby, the mechanism of Notch signaling pathway was also researched in this study. In this study, we speculated that FABP7 might be a biomarker for CSCC diagnosis. The present study aims to determine the molecular mechanism underlying the effects of FABP7 in CSCC, which may provide a new diagnostic biomarker or treatment target for CSCC.

Materials and methods

Patient samples and cell lines. A total of 34 punch biopsies and 34 non-lesion epithelial skin tissue samples were obtained from patients with CSCC who underwent surgery between January 2018 and January 2019 at Shanghai Skin Disease Hospital, Tongji University School of Medicine (Shanghai, China). All patients and their families were informed about the study; written informed consent was obtained from all patients, and the study was performed according to the Declaration of Helsinki. Ethics approval for the study was provided by the Ethics Committee of the Shanghai Skin Disease Hospital, Tongji University School of Medicine (Shanghai, China; approval no. SSDH10561). All tissue samples were snap-frozen in liquid nitrogen and stored at -70°C until further use for mRNA isolation and immunohistochemistry (IHC).

Human CSCC cell lines A431, colo-16 and SCL-1 and the immortalized human normal keratinocyte cell line HaCaT were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). When the cells reached 70–80% confluency, they were trypsinized at room temperature for 3 min and passaged.

Transfection and grouping. The pLV-puro lentiviral vector (Thermo Fisher Scientific, Inc.) was digested with the *AgeI* enzyme, and the digested product was identified by 1% agarose gel electrophoresis. According to the FABP7 sequence in GenBank (Gene ID: 2173), the amplification primers of

FABP7 were designed and transferred via PCR using the PCR amplification kit (mlbio Co., Ltd.). The following primer sequences were used: 5'-ATCGGATCCATGFTFFAFFCT TTCTGT-3' (upstream) and 5'-ATAGGATCCATGAGGACT CTCAGCAC-3' (downstream). The PCR product contained an *AgeI* cleavage site sticky end (ACCGGT) at both ends, which was directly ligated into the downstream of the digested lentiviral expression vector CMV promoter by ligase reaction. The reaction conditions were as follows: Pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 3 min, and a final extension at 68°C for 10 min. The PCR product was detected by 1% agarose gel electrophoresis. The purified ligation product was transferred to fresh bacterial competent cells (JMI09), and the cells were cultured in LB liquid medium at 37°C for 45 min. The transformed competent cells were transferred to LB agar medium containing 20 mmol/l MgSO_4 and ampicillin solution (100 mg/ml), and cultured at 37°C for 16 h. Positive clones were identified by PCR using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Inc.) and sent to the Synbio technologies Co. Ltd. or sequencing, and the vector with the correct expression sequence of recombinant FABP7 gene was selected. The following primer sequences were used: 5'-GTCAAGCTT CTAAGTTTGTCTCCATCC-3' (upstream) and 5'-ATCAAG CTTCCCGACCAGGAACATTTT-3' (downstream). The FABP7 overexpression plasmid and the two lentiviral helper plasmids in the lentiviral packaging system were extracted using the plasmid extraction kit (Qiagen, Inc.). 293T cells were seeded at a density of 6×10^6 cells/ml in a 15-cm culture dish, cultured at 37°C with 5% CO_2 to 70–80% confluence, and the lentivirus plasmids were co-transfected into 293T cells using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C for 6 h. The supernatant of 293T cells transfected for 72 h was collected, centrifuged at $4,000 \times g$ for 10 min at 4°C to remove cell debris, filtered, centrifuged at $7,000 \times g$ for 5 min at 4°C , resuspended in ice-cold PBS to detect the titer, and stored in at -80°C . Based on the transfection, the cells were divided into FABP7 overexpression (oe-FABP7) and negative control (NC) groups. The CSCC cell lines (A431 and colo-16) at a density of 6×10^5 cells/well in a 6-well culture plate were infected with the FABP7 with a multiplicity of infection of 20, and the FABP7 gene expression sequence carried by the lentivirus was integrated into the cell to obtain stable overexpression. A431 and colo-16 cells were transfected with lentivirus/medium at a ratio of 1:50. Stable cell lines were selected by puromycin (Sigma-Aldrich, Merck KGaA) at $5 \mu\text{g/ml}$ for 2 weeks. The lentivirus vectors contained enhanced green fluorescent protein (eGFP).

Reverse transcription-quantitative (RT-q)PCR assay. RT-qPCR was used to detect the expression levels of FABP7 in tissues and cells before and after transfection. The tissue samples were ground, and total RNA was extracted with TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and chloroform, precipitated with isopropanol, washed with 75% ethanol, dried, dissolved in a diethyl pyrocarbonate-containing solution and stored at -80°C until use. PCR amplification was performed for total RNA using the PCR amplification kit (Takara Bio, Inc.). For cells, total RNA was extracted using the

Tripure Isolation reagent (Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. The A260/A280 ratio was determined using a DU-800 UV spectrophotometer (Beckman Coulter, Inc.) to determine RNA purity. A total of 1 μ g RNA was collected from each sample to synthesize the first strand of cDNA using the M-MuLV First-strand cDNA Synthesis kit (Shanghai Shenggong Bioengineering Technology Service Co., Ltd.) according to the manufacturer's instructions in a 20- μ l reaction system. The reaction was terminated at 70°C for 10 min, and the system was placed on ice for subsequent experiments. mRNA expression levels were detected by qPCR. qPCR was subsequently performed using the SYBR GREEN RT-PCR kit (Qiagen GmbH). The following thermocycling conditions were used for qPCR: 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 60°C for 15 sec, extension at 72°C for 20 sec, a total of 40 cycles. Quantitative molecular detection Real-Time PCR (BioRad-CFX system; Bio-Rad Laboratories, Inc.) was used. GAPDH was used as internal reference, and the gene-specific primers were as follows: FABP7 forward, 5'-TCAGGAAGGCGGCAAAGTGGT-3' and reverse, 5'-CATAACAGCGAACAGCAACGACATC-3'; and GAPDH forward, 5'-TCCCTGAGCTGAACGGGAAG-3' and reverse, 5'-GGAGGAGTGGGTGTCGCTGT-3'. Data were analyzed using the $2^{-\Delta\Delta C_q}$ method (18).

IHC assay. The specimens were fixed in 4% neutral formalin solution at 4°C overnight, embedded in paraffin and cut into 4 μ m slices, and then placed in the center of a slide and heated in an oven at 69°C overnight. The Pv-9000 polymer detection system (Guangzhou Dingguo biology Co. Ltd.) was used to detect the protein expression of FABP7, Ki67, Notch 1 and Notch 3 according to the manufacturer's instructions. The tissue sections were treated with xylene at room temperature twice for 5 min and washed with 95% ethanol four times. The samples were subsequently rinsed with tap water, placed in a hydrogen peroxide solution at room temperature for 10 min. After cooling, the sample was washed in PBS. The primary antibody (FABP7; cat. no. ab32423; 5 μ g/ml; 1:800; Abcam) was added to the samples, incubated at 37°C for 1 h and rinsed with PBS. Subsequently, the secondary antibody (cat. no. ab150077; 1:1,000; Abcam) was added to the sample and incubated for 20 min at 37°C. The 3,3'-diaminobenzidine (DAB) coloring solution was added, and the sample was stained with hematoxylin at room temperature for 3 min. Hydrochloric acid alcohol was used to remove the excess hematoxylin, and 1% ammonia water solution was used for bluing. The sample was dehydrated, made transparent by xylene and sealed. Protein expression was calculated with the positive staining cells under a light microscope (Olympus Corporation, Tokyo Japan, magnification, x40-400) in five randomly selected visual fields. The images were analyzed using Photoshop (Adobe Systems, Inc.).

Database prediction. The STRING database (cn.string-db.org/) was used to predict FABP7 associated signal pathways according to the previous study (19).

Western blotting. Western blotting was used to determine the protein expression levels of FABP7 in CSCC tissues and cells before and after transfection, as well as to detect the expression of proliferation-, invasion- and Notch

pathway-associated proteins. The primary antibodies used were as follows: Anti-FABP7 (cat. no. ab253552); Rabbit monoclonal (EPR3821) anti-proliferating cell nuclear antigen (PCNA; cat. no. ab92552); rabbit monoclonal (EPR21043) anti-SNAIL (cat. no. ab216347); rabbit monoclonal (EPR1791-4) anti-N-cadherin (cat. no. ab202030); mouse monoclonal (10E4E6) anti-Twist (cat. no. ab175430); rabbit monoclonal (EPR1184) anti-MMP2 (cat. no. ab92526); rabbit monoclonal (EPR17888-71) anti-MMP7 (cat. no. ab207299); rabbit anti-activated Notch1 (cat. no. ab8925); and rabbit polyclonal anti-NOTCH3 (cat. no. ab23426) and anti-GAPDH (cat. no. ab8245). All primary antibodies were purchased from Abcam and used at 1:1,000 dilution. Extraction of total cellular proteins and western blotting were performed as previously described (20). A total 40 μ g protein/lane was added to a 10% polyacrylamide gel, separated by electrophoresis and electroporated to a PVDF membrane. The transferred PVDF membrane was rinsed 3 times with 0.1% TBS-Tween-20 (TBST) and blocked with a 0.5% skim milk powder blocking solution at 37°C for 1 h. The membrane was placed into a working solution containing the primary antibodies and gently shake overnight at 4°C. The next day, the samples were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Color development was performed at 4°C, avoiding light using a DAB Chromogenic kit (mlbio Co., Ltd.), according to the manufacturer's instructions. The ImageQuant LAS 4000 mini (General Electric Company) was used to calculate the relative protein expression levels. Western Blotting quantified using Quantity One Software (ChemIDoc XRS, Bio-Rad Laboratories, Inc.).

Cell counting kit-8 (CCK-8) assay. A431 and colo-16 cells were seeded in 6-well plates at a density of 2×10^4 . Following 24-h culture, the cells were infected with the empty vector control or FABP7 overexpression virus. The cells were harvested at 96 h post-infection and inoculated in 96-well plates at a density of 2,000 cells/well. Subsequently, at 12, 24, 48 and 72 h, 10 μ l CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added and incubated at 37°C for 2 h. A microplate reader was used to measure the absorbance at 450 nm, and cell proliferation curves were plotted.

Colony formation assay. A431 and colo-16 cells infected with the empty vector control or FABP7 overexpression virus for 96 h were inoculated into 6-well plates at a density of 200 cells/well and cultured in the 37°C with 5% CO₂ for 14 days. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, washed twice with PBS, stained with Giemsa at room temperature for 20 min, washed three times with deionized water and cloned, photographed and counted. Colonies were then counted under an invert microscope (Leica, Gerny). Only colonies >32 cells were scored.

Cell scratch assay. A431 and colo-16 cells were incubated and treated by FABP7 overexpression transfection. A total of 5×10^5 cells/well (A431 and colo-16) were inoculated and cultured overnight. The next day, a 200- μ l pipette tip for was

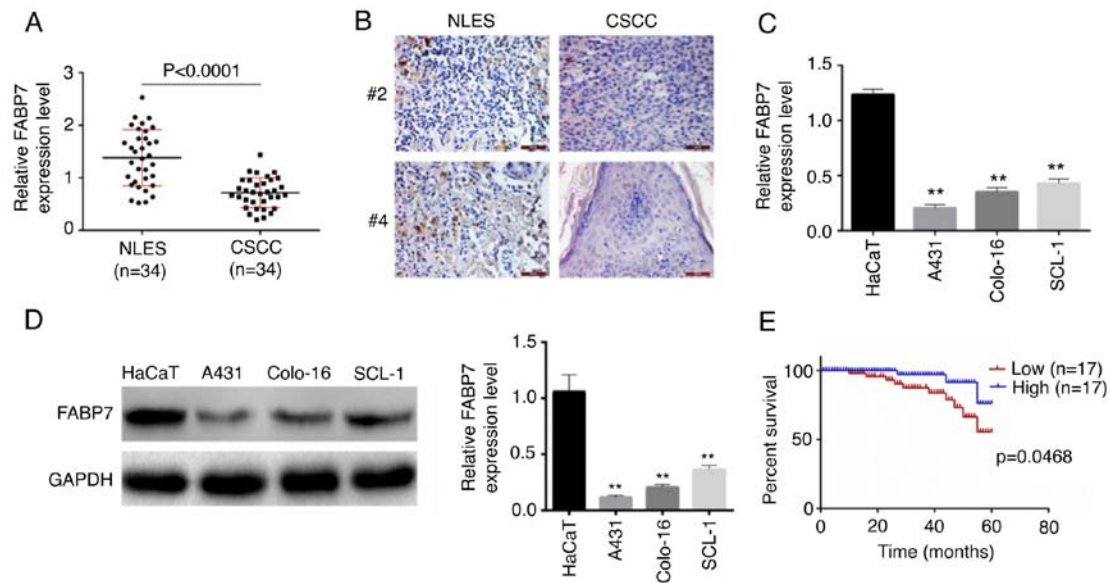


Figure 1. FABP7 is expressed at low levels in CSCC tissues and cell lines. (A) RT-qPCR was used to detect the expression levels of FABP7 in the punch biopsies from CSCC and NLES samples. (B) The FABP7 protein expression was detected by immunohistochemistry assay (Scale bar, 50 μm). ‘#2’ and ‘#4’ refer to the patient ID. (C) The expression levels of FABP7 in human CSCC cell lines A431, colo-16 and SCL-1 and immortalized human normal keratinocyte cell line HaCaT were detected by RT-qPCR. (D) The protein expression levels of FABP7 in human CSCC and HaCaT cells were detected by western blotting. (E) Kaplan-Meier survival curves of patients in the FABP7 high- and low-expression groups. ** $P < 0.01$ vs. HaCaT. FABP7, fatty acid-binding protein 7; CSCC, cutaneous squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR; NLES, non-lesion epithelial skin.

used to create a wound. The cells were washed three times with PBS, the cells in the wound were removed, and 2 ml cell culture medium containing 2% fetal bovine serum was added. The cells were placed in a 37°C incubator with 5% CO_2 and cultured for 24 h. Images were captured at 0 and 24 h, and the width of wound was measured.

Transwell invasion assay. A431 and colo-16 cells were diluted in serum-free DMEM medium and inoculated into the upper chambers of Transwell inserts at $\sim 2 \times 10^5$ cells/well. The lower chamber was filled with DMEM containing 10% serum, and the cells were incubated at 37°C for 24 h. The upper chamber was removed and washed with PBS, fixed with 10% methanol for 20 min and stained with 0.1% crystal violet at room temperature for 20 min. Following three rinses with PBS, the cells on the upper surface of the membrane were scraped with a cotton swab. The number of cells that migrated to the lower surface of the membrane was counted under an inverted microscope (Leica Microsystems GmbH; x200 magnification), and the experiment was repeated three times.

Tumor formation experiment. All animal experiments were performed in accordance with the principles and procedures approved by the Animal Experimentation Ethics Committee of the Shanghai Skin Disease Hospital (Approval number: AN12047J), Tongji University School of Medicine. A total of 12 specific pathogen-free-Balb/c nude male mice (age, 6-8 weeks; weight, 18-22 g) were purchased from Beijing Weitong Lihua Experimental Animal Co., Ltd. and housed in a periodically UV-sterilized environment at 22°C and 50% humidity. The mice had free access to water and food. Animal health and behavior were monitored daily. The mice were divided into NC (n=6) and oe-FABP7 (n=6) groups. A431 cells at the logarithmic phase were transfected, the concentration

of the cell suspension was adjusted using serum-free DMEM medium, and the cells (2×10^6) were subcutaneously inoculated into the left flanks of the mice for modeling. Notable tumor growth at the injection site was used to verify successful modeling. The tumor diameters were measured every 7 days by a vernier caliper, and the volume was calculated as follows: $V = 0.52 \times \text{length} \times \text{width}^2$. The mice were sacrificed at 35 days post-modeling by anesthesia with sodium pentobarbital (50 mg/kg) and rapid cervical dislocation. The maximum tumour volume was 1.5 mm^3 . No animals were found dead during the experiment.

Statistical analysis. SPSS 10.0 statistical software (SPSS, Inc.) was used for all statistical analyses. All experiments were repeated three times for statistical analysis. The data are presented as the mean \pm SD of ≥ 3 replicates. Unpaired two-tailed Student's t-test was performed to compare the data between the two groups. The χ^2 test was used to compare the differences in categorical variables between two groups. One-way ANOVA was used to determine the difference between >2 groups, while a t-test was used to analyze differences between two groups. Kaplan-Meier curves and Cox regression analysis were used for survival and multivariate analysis respectively, which were used to determine the associations between the biomarker expression and patient survival. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FABP7 is expressed at low levels in CSCC tissues and cell lines. RT-qPCR was used to determine the expression levels of FABP7 in the punch biopsies from CSCC (n=34) and non-lesion epithelial skin (n=34) samples. As presented in Fig. 1A, the expression levels of FABP7 were significantly

lower in CSCC tissues compared with those in the normal epithelial samples ($P<0.0001$). Similar results were obtained by immunohistochemistry (Fig. 1B). In addition, the expression levels of FABP7 in human CSCC cell lines A431, colo-16 and SCL-1, and the immortalized human normal keratinocyte cell line HaCaT, were detected by RT-qPCR (Fig. 1C) and western blotting (Fig. 1D). The results demonstrated that FABP7 levels were significantly lower in A431, colo-16 and SCL-1 cell lines compared with those in HaCaT cells ($P<0.01$). Based on RT-qPCR results, the median value (0.78) of FABP7 expression level of all patients was chosen as threshold, 34 patients with CSCC were divided into FABP7 high- and low-expression groups. Patients in the FABP7 low-expression group exhibited a lower survival rate compared with those in the FABP7 high expression group (Fig. 1E). In addition, the clinicopathological characteristics of the patients were recorded and presented in Table I. The age and sex were not different between the two groups. However, the patients in the FABP7 low-expression group presented with a higher Tumor-Node-Metastasis stage based on TNM stage (21), histological grade and degree of differentiation, compared with those in the FABP7 high-expression group. Based on the Cox multivariate analysis, the age and sex between two groups were exponent of B [exp (B)] >1 , although no significant differences were identified. Notably, the expression levels of FABP7 were exp (B) <1 and $P<0.05$, indicating that it may be a suitable prognostic factor for cutaneous squamous cell carcinoma (Table II).

FABP7 overexpression inhibits A431 and colo-16 cell proliferation, invasion and migration abilities. RT-qPCR and western blot assays were used to detect the transfection efficiency of oe-FABP7 in A431 and colo-16 cells. Following transfection, the levels of FABP7 were significantly overexpressed in both A431 and colo-16 cell lines compared with those in the NC-transfected cells ($P<0.01$; Fig. 2A and B). Following a 72-h transfection, the optical density in the oe-FABP7 groups was significantly lower compared with that in the control groups, which confirmed that cell viability was inhibited ($P<0.01$; Fig. 2C). In addition, the numbers of colonies formed by cells transfected with the oe-FABP7 vector were significantly lower compared with those in the corresponding control groups ($P<0.01$; Fig. 2D). Similarly, the relative wound width in oe-FABP7 group was larger than control ($P<0.01$; Fig. 2E). The results of the Transwell invasion assay confirmed that invasion was inhibited in oe-FABP7 groups compared with the NC-transfected cells ($P<0.01$; Fig. 2F).

FABP7 overexpression inhibits cell proliferation, invasion and the Notch signaling pathway. PCNA, Snail, N-cadherin, Twist, MMP-2 and MMP-7 are crucial proteins associated with cell proliferation and invasion (22). Following transfection, these proteins were detected by western blotting. The expression levels of all these proliferation- and invasion-associated proteins (PCNA, Snail, n-Cad, Twist, MMP-2 and MMP-7) were lower in the oe-FABP7 groups compared with those in the NC groups ($P<0.01$; Fig. 3A). Based on the STRING database, FABP7 is associated with the Kyoto Encyclopedia of Genes and Genomes Notch pathway. Notably, overexpression of FABP7 inhibited the expression levels of Notch 1 and Notch

Table I. Association between FABP7 expression levels and patient clinicopathological characteristics in cutaneous squamous cell carcinoma.

Characteristics	Patients, n	FABP7 expression, n		P-value
		High	Low	
Total	34	17	17	
Age, years				0.241
<45	13	8	5	
≥ 45	21	9	12	
Sex				0.500
Female	15	8	7	
Male	19	9	10	
TNM Stage				0.042
I-II	16	11	5	
III-IV	18	6	12	
Histological grade				0.015
I-II	16	12	4	
III-IV	18	5	13	
Differentiation				0.040
High	14	10	4	
Low	20	7	13	

FABP7, fatty acid-binding protein 7; TNM, Tumor-Node-Metastasis.

3 compared with those in the NC groups ($P<0.01$; Fig. 3B). Therefore, FABP7 overexpression may inhibit the proliferation, invasion and migration of A431 and colo-16 cells.

FABP7 overexpression inhibits CSCC growth in vivo. At 35 days post-inoculation, the volume and weight of mouse xenograft tumors were significantly lower in the oe-FABP7 group compared with those in the control group ($P<0.01$; Fig. 4A and B), suggesting that tumor growth was inhibited by oe-FABP7 *in vivo*. RT-qPCR was used to detect FABP7 expression in the tumor tissues; the results demonstrated that the levels of FABP7 were decreased *in vivo* in the FABP7 overexpression group compared with those in the NC group ($P<0.01$; Fig. 4C). Immunohistochemistry was used to detect the expression of FABP7, Ki67, Notch 1 and Notch 3. The results demonstrated that overexpression of FABP7 inhibited the protein expression levels of Notch 1 and Notch 3 compared with those in the control group (Fig. 4D).

Discussion

In order to determine the molecular mechanism underlying the effects of FABP7 in CSCC, FABP7 overexpression models *in vivo* and *in vitro* were constructed, and cell viability, invasion and the Notch pathway signaling pathway were analyzed in the present study. The results demonstrated that FABP7 expression levels were significantly lower in human CSCC tissues and cells compared with those in non-tumor

Table II. Cox regression analysis for multivariate analysis.

Variable	B	SE	Wald	df	P-value	Exp (B)	95% CI for Exp (B)
Age	0.120	0.675	0.031	1	0.859	1.127	0.300-4.234
Sex	0.968	0.588	2.706	1	0.100	2.633	0.831-8.342
FABP7 expression	-1.802	0.754	5.710	1	0.017	0.165	0.038-0.723

FABP7, fatty acid-binding protein 7; B, model coefficient; SE, coefficient standard error; Wald, Wald test value; df, degree of freedom; Exp (B), HR value (odds ratio); 95% CI for Exp (B), confidence interval of Exp (B).

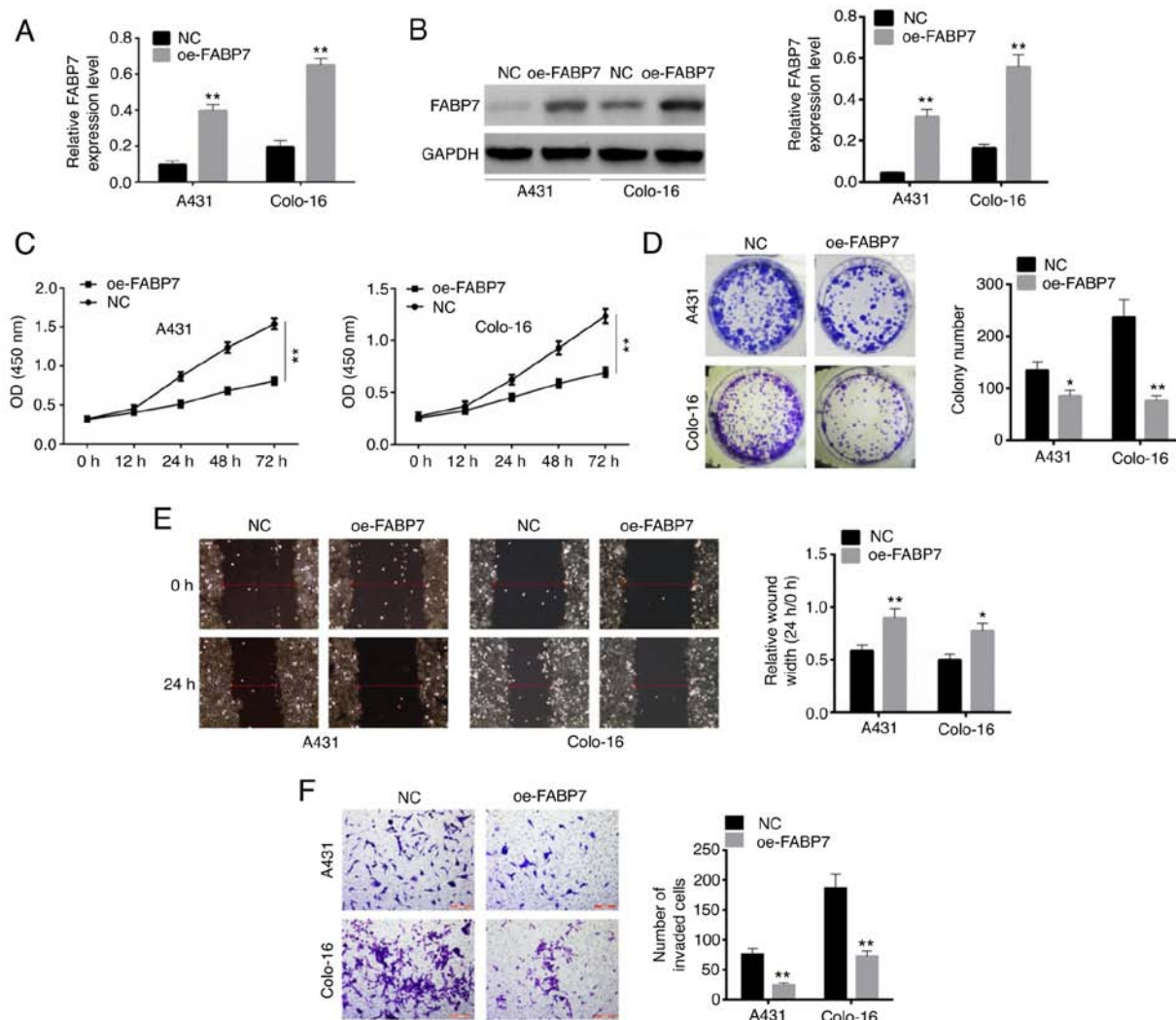


Figure 2. FABP7 overexpression inhibits A431 and colo-16 cell viability, invasion and migration abilities. (A) Reverse transcription-quantitative PCR was used to validate the transfection of oe-FABP7. (B) Western blotting confirmed the successful transfection of oe-FABP7. (C) Cell Counting Kit-8 assay was used to detect the viability following transfection. (D) The numbers of colonies formed by oe-FABP7-transfected cells were significantly lower compared with those than control groups. (E) Cell scratch assay was used to determine the migration of cells following transfection. (F) Transwell invasion assay was used to detect the cell invasion capacity (Scale bar, 200 μ m). * P <0.05 and ** P <0.01 vs. NC. FABP7, fatty acid-binding protein 7; oe, overexpression vector; NC, negative control; OD, optical density.

tissues and cells. Transfection with oe-FABP7 inhibited the viability, invasion and migration of A431 and colo-16 cells compared with those observed in the control cells. In addition, overexpression of FABP7 reduced the expression levels of proteins associated with cell proliferation, invasion and the Notch signaling pathway compared with those in the NC

groups. FABP7 overexpression reduced the growth of CSCC tumors *in vivo*, and inhibited the expression levels of Ki67, Notch 1 and Notch 3 compared with those in the control group.

FABP7 is a protein-coding gene involved in various diseases and cell functions. For example, FABP7 has

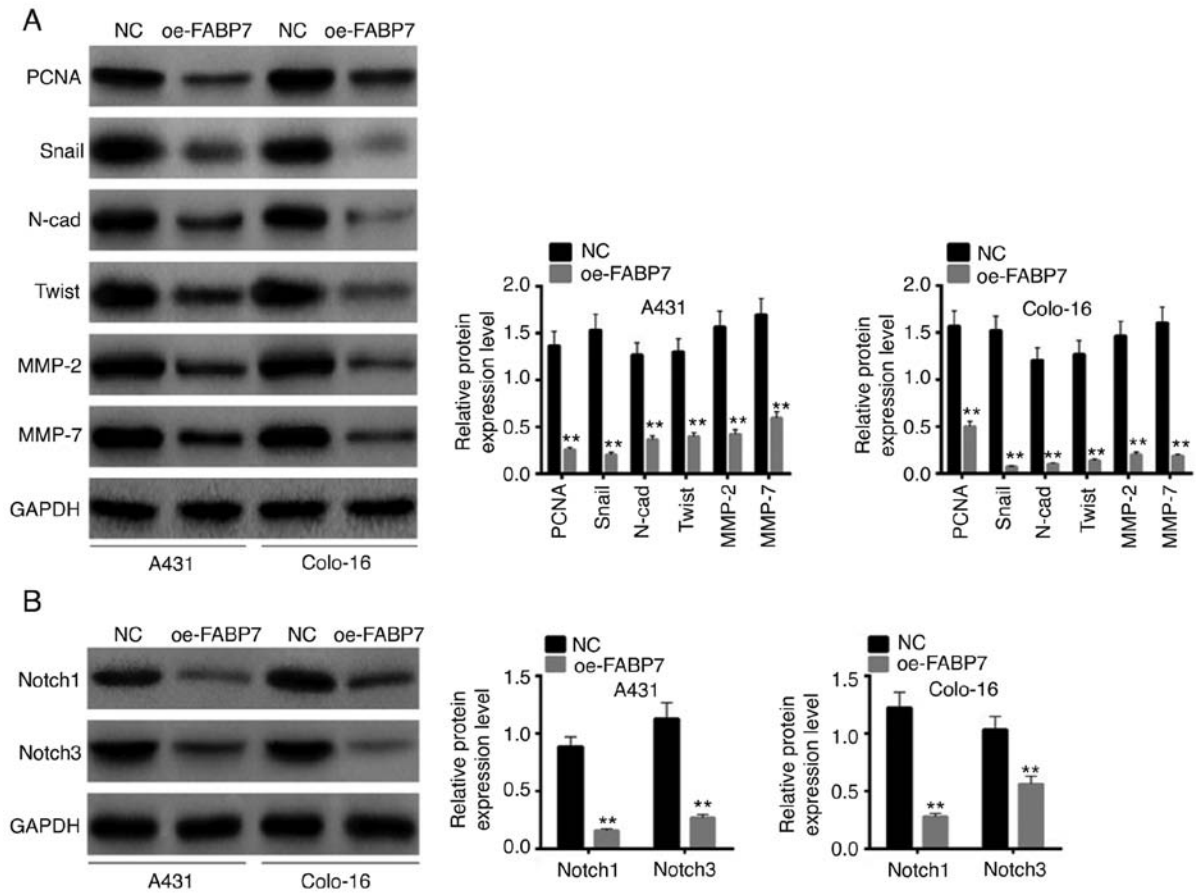


Figure 3. Effects of FABP7 overexpression on proliferation-, invasion- and Notch pathway-associated proteins. (A) The expression levels of PCNA, Snail, N-cad, Twist, MMP-2 and MMP-7 were detected by western blotting. (B) Notch pathway-related proteins were detected by western blotting. ** $P < 0.01$ vs. NC. FABP7, fatty acid-binding protein 7; oe, overexpression vector; NC, negative control; PCNA, proliferating cell nuclear antigen; N-cad, N-cadherin; MMP, matrix metalloproteinase.

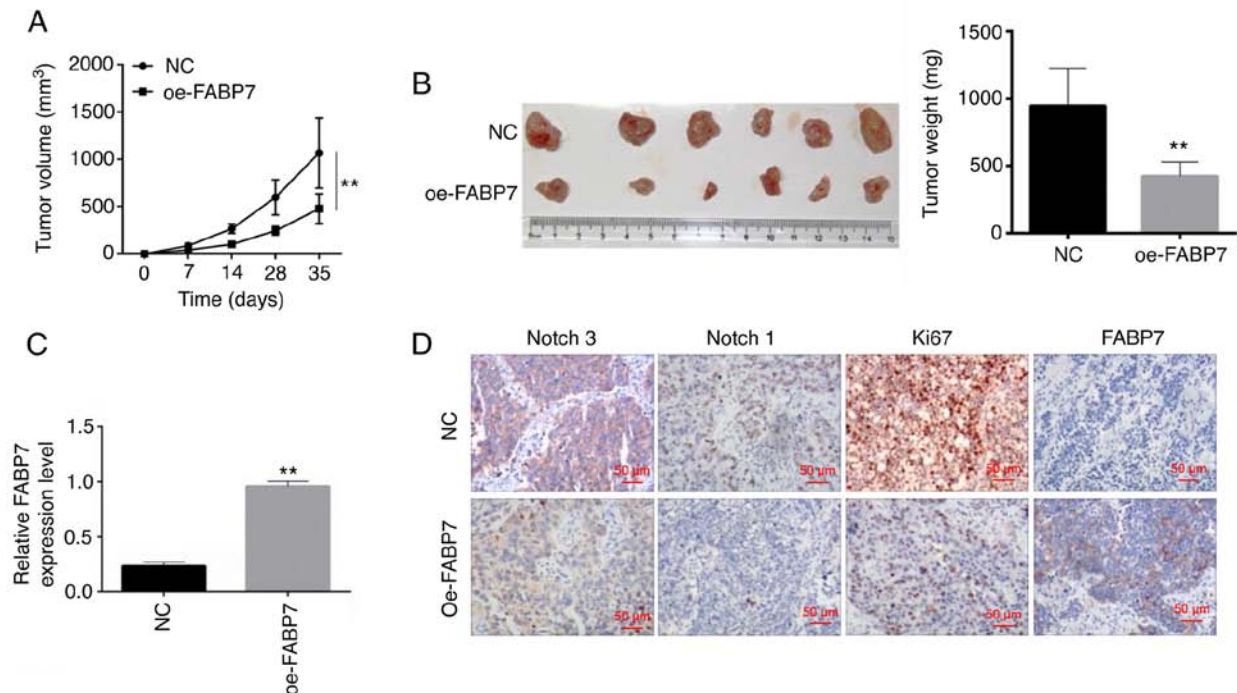


Figure 4. FABP7 overexpression inhibits cutaneous squamous cell carcinoma growth in vivo. (A) Tumor growth curve in nude mice. (B) The tumor was photographed and weighed at 35 days post-inoculation. (C) Reverse transcription-quantitative PCR was used to detect FABP7 expression levels in tumor tissues. (D) Immunohistochemistry was used to detect the protein expression levels of FABP7, Ki67, Notch 1 and Notch 3 (Scale bar, 50 μm). ** $P < 0.01$ vs. NC. FABP7, fatty acid-binding protein 7; oe, overexpression vector; NC, negative control.

been reported to be associated with optic nerve glioma and neoplasm (23). The peroxisome proliferator-activated receptor (PPAR) signaling pathway serves an important role in rat brain astrocytes (24); Nijsten *et al* (25) have reported that PPAR α , PPAR β and PPAR γ are abnormally expressed in CSCC samples and associated with immunoreactivity. PPAR β / δ KO mice displayed increased inflammation in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (26). Notably, Slipicevic *et al* (11) demonstrated that FABP7 enhances the invasion and proliferation of melanoma cells via the ERK and MAPK pathways, and this pathway is crucial for the pathogenesis of cutaneous disease (27). For instance, microRNA-148a inhibits the MAPK pathway and suppresses the development of CSCC (28). In addition, high mobility group box 1 has also been demonstrated to participate in the MAPK signaling pathway and regulate the metastasis of CSCC (29). Various long non-coding RNAs or microRNAs (miRs) induce the expression of ERK and regulate the proliferation of CSCC cells including long non-coding RNA PICSAR, miR-124 and miR-214 (30,31). Therefore, we hypothesized that FABP7 may inhibit the viability, invasion and migration of CSCC cells via the PPAR and MAPK/ERK1/2 pathways.

Overexpression of FABP7 was demonstrated to inhibit the expression of proliferation- and invasion-associated proteins (PCNA, Snail, N-cadherin, Twist, MMP-2 and MMP-7) as well as Notch pathway-related proteins (Notch 1 and Notch 3) in the present study. Invasion and migration of tumor cells are the main features of malignant tumors and the primary cause of malignant tumor-associated death (32). The occurrence of invasion and migration involves complex signaling pathways, such as MAPK and PI3K signaling pathways in tumor cells and the microenvironment in which they were located. Activation and interaction of these signaling pathways are involved in tumor survival and growth (33). A previous study reported that PCNA is expressed at high levels in CSCC, which is associated with grade (34). In addition, Gong *et al* (35) reported that The positivity rates of Snail expression were 0% in normal cervical tissues, 32.0% in CIN tissues, and 66.2% in CSCC tissues. Furthermore, Co-expression of HIF-1 α , TWIST and Snail in primary tumors of patients with head and neck cancers correlated with worst prognosis (36,37). Similarly, Ahmed Haji Omar *et al* (38) demonstrated that MMPs participate in the initiation, metastasis, invasion and evasiveness of CSCC. In addition, the Notch pathway-associated proteins are important genes with high mutation rate in primary CSCC (39). Thus, overexpression of FABP7 may inhibit the proliferation and invasion of CSCC cells by regulating the Notch signaling pathway.

Although the molecular mechanism of FABP7 was studied in cells and nude mice, the present study had certain limitations. First, the number of cases included in the current study was small, and the statistical analysis of experimental results was limited. In addition, due to the experimental conditions, only human CSCC cell lines (A431, colo-16, and SCL-1) and immortalized human normal keratinocyte cell line (HaCaT) were used in the present study. The reliability of these data needs to be further verified. Second, the experiments were not blinded. Blind experiments will be performed in future studies.

Third, the target gene may be regulated by various genes, creating a complicated interaction network. The present study only focused on the Notch pathway, proliferation and invasion. Finally, the functions of proliferation and invasion-associated proteins and Notch pathway related proteins were obtained from previous studies, but were not verified in the present study. The aforementioned limitations will be addressed in our future studies. For instance, a multi-center, double-blind, large-sample study will be designed and conducted. Additional cell lines and animal models will be used to verify the results, and other genes and associated microRNAs and pathways will be studied to construct a regulatory network and identify potential targets for CSCC diagnosis and treatment.

In conclusion, the results of the present study demonstrated that FABP7 inhibited the proliferation and invasion of CSCC cells by participating in the Notch signaling pathway, and may be a diagnostic biomarker or treatment target for CSCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZS, YZ and XW designed the study. ZS, YG and DZ performed the experiments. GZ and YZ analyzed the data. ZS, YZ and XW wrote the manuscript. All authors read and approved the final manuscript, and confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Shanghai Skin Disease Hospital, Tongji University School of Medicine (approval no. SSDH-IEC-SG-029-3.1; Shanghai, China). Written informed consent was obtained from all patients. All animal experiments were performed in accordance with the principles and procedures approved by the Animal Experimentation Ethics Committee of the Shanghai Skin Disease Hospital, Tongji University School of Medicine (Approval number: SSDH-AEEC-SG-331-2.1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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