Scientific Research Report

Expression of FAP in Oral Leukoplakia and Oral Squamous Cell Carcinoma

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ARTICLE INFO

Article history:
Received 16 August 2023
Received in revised form
20 December 2023
Accepted 29 December 2023
Available online 26 January 2024

Key words:
Fibroblast activation protein
Oral leukoplakia
Oral squamous cell carcinoma
Biomarker

ABSTRACT

Objective: This study aimed to investigate the potential of fibroblast activation protein (FAP) as a biomarker in the progression of oral leukoplakia (OLK) carcinogenesis. This was achieved by evaluating FAP expression at different levels of the organisation, namely oral normal mucosa (NM), OLK, and oral squamous cell carcinoma (OSCC).

Materials and methods: Altogether, 88 paraffin-embedded tissue samples were examined, including 55 cases of OLK, 13 cases of OSCC, and 20 cases of NM (control group). An exhaustive investigation was performed to examine FAP expression in NM, OLK, and OSCC tissues via immunohistochemistry (IHC). The relationship between FAP expression and clinical pathologic characteristics was analysed. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot (WB) also proved the expression of FAP in NM, OLK, and OSCC cells. Aberrant FAP expression in OLK and OSCC was explored using in vitro experiments.

Results: Immunohistochemical results showed that high FAP expression was significantly correlated with histopathologic grade ($P = .038$) but not correlated with age, sex, or region ($P = .953$, .622, and .108, respectively). The expression level of FAP in NM tissues (0.15 ± 0.01) was minimal, whereas it was observed in OLK (0.28 ± 0.04) and OSCC (0.39 ± 0.02) tissues with a noticeable increase in expression levels ($P < .001$). The expression level of FAP in OLK with severe abnormal hyperplasia (S-OLK) tissues (0.33 ± 0.04) was significantly higher than in OLK with mild abnormal hyperplasia (MI-OLK, 0.26 ± 0.02) and OLK with moderate abnormal hyperplasia (MO-OLK, 0.28 ± 0.03) tissues ($P < .001$ and $P = .039$, respectively). The results of RT-PCR illustrated that the relative expression of FAP mRNA in OLK cells (2.63 ± 0.62) was higher than in NM cells (0.87 ± 0.14), but lower than in OSCC cells (5.63 ± 1.06; $P = .027$ and .012, respectively). FAP expression was minimal in NM cells (0.78 ± 0.06), modest in OLK cells (1.04 ± 0.06), and significantly elevated in OSCC cells (1.61 ± 0.09) based on the results of WB ($P < .001$).

Conclusions: Significant variations in FAP expression were observed in NM, OLK, and OSCC tissues and cells. These findings revealed that FAP may be a reliable biomarker for the early diagnosis and evaluation of OLK carcinogenesis.

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https://doi.org/10.1016/j.identj.2023.12.011

Introduction

Oral leukoplakia (OLK) is defined as a predominantly white patch or plaque on the oral mucosa that cannot be wiped away and is not clinically or histologically characterised as any other definable disorder.[1] The incidence of malignant transformation was significantly elevated, with an estimated overall rate of 3.5%, ranging from 0.13% to 34.0%.[2] Currently, various treatment modalities are available for the management of OLK, including pharmaceutical interventions, surgical procedures, and laser ablation. Nevertheless, the incidence of OLK recurrence remains high, ranging from 10% to 45%.[3] Oral squamous cell carcinoma (OSCC), the prevailing form of oral cancer, currently lacks a comprehensive understanding of its aetiology and pathophysiology.[4] Numerous clinical studies and animal tests have provided substantial evidence supporting the notion that oral cancer undergoes systematic progression, starting with normal oral mucosa, progressing to aberrant epithelial hyperplasia, and ultimately culminating in carcinoma in situ. The transition from oral precancerous lesions to oral cancer is a complex, chronic, and multistage process.

Fibroblast activation protein (FAP) is a type II transmembrane serine hydrolase.[5] The human FAP gene is located on chromosome 2q23. It contains 760 amino acid residues and is composed of a short cytoplasmic tail containing only 6 amino acids, a single transmembrane domain of 20 amino acids, and an extracellular domain of 734 amino acids.[6] Dipeptidase activity[7] and collagenase activity[8] are present in the human FAP, which can degrade dipeptides and gelatin in vivo. FAP is significantly upregulated in pathologic conditions associated with inflammation and cellular fibrosis such as liver cirrhosis, pulmonary fibrosis, osteoarthritis, and rheumatoid arthritis.[9] FAP is preferentially expressed in stromal fibroblasts in the majority of epithelial tumours, such as ovarian, lung, gastric, and breast cancers.[10-15] However, it is uncommon to observe FAP in regular human tissues.[9] FAP is abundant in tumour-associated fibroblasts and contributes to various aspects of tumour progression, including tumour cell growth, multiplication, metastasis, and immunologic inhibition.[16] FAP is overexpressed in cancer-associated fibroblasts (CAFs) and recognised as its main marker.[17] CAFs play a crucial role in the tumour microenvironment (TME). Their presence is vital for promoting multiple processes associated with tumour progression. These processes include expedited tumour growth, heightened proliferation, augmented invasion and metastasis, remodelling of the extracellular matrix, and induction of epithelial-mesenchymal transition.[18,19]

OLK is a pathologic disorder with malignant potential that requires continuous surveillance. In clinical practice, there is a prevailing belief that the reversal of OLK accompanied by mild or moderate epithelial dysplasia can be achieved by the implementation of suitable therapeutic interventions. Consequently, timely identification and intervention of OLK, exhibiting a high propensity for malignant progression, are crucial for impeding its carcinogenic process.[20] Therefore, the primary objective of this study was to evaluate the expression of FAP in oral normal mucosa (NM), OLK, and OSCC and investigate the potential of FAP as a biomarker for the early detection and diagnosis of OLK carcinogenesis. Our study was conducted primarily using immunohistochemistry (IHC), reverse transcription-polymerase chain reaction (RT-PCR), and western blot (WB) techniques.

Materials and methods

Patients and specimens

This study included 55 cases of OLK and 13 cases of OSCC, which were diagnosed by histopathologic evaluation in the Shanghai Ninth People's Hospital, and 20 cases of NM that were included in the control group, between 2020 and 2022. The OSCC tissues used in this study evolved from OLK. According to the World Health Organization's histologic diagnosis and classification criteria of precancerous lesions, OLK was further categorised into mild abnormal hyperplasia (MI-OLK, n = 24), moderate abnormal hyperplasia (MO-OLK, n = 21), and severe abnormal hyperplasia (S-OLK, n = 10). The patients in the experimental group comprised 36 men and 32 women in the age range of 26 to 79 years, with an average age of 52.5 years.

The NM specimens used in this study were obtained from the surplus normal mucosa excised during maxillofacial surgery. The control group was matched to the experimental group by age and sex. The inclusion criteria for this study were as follows: (1) patients who received a first-time diagnosis or did not exhibit any systemic illnesses or other immunologic disorders and (2) patients diagnosed with OSCC who did not have any medical history of radiotherapy, chemotherapy, or any other form of cancer treatment prior to the surgery. The exclusion criteria were (1) patients who presented with other mucosal disorders in the oral cavity or malignant tumours in other anatomic regions as well as those who used hormonal and immunologic pharmaceutical agents and (2) patients exposed to any chemical carcinogens, including cigarettes, betel nuts, or alcohol.

The study was approved by the Shanghai Ninth People's Hospital Ethical Committee after obtaining informed consent from all patients (IRB: SH9H-2023-T7-1). Patients were well informed that the specimens we procured consisted of surgically excised tissues, which did not have any adverse impact on the process of postoperative recovery or increase the patient's susceptibility to infection. Furthermore, the acquisition of these samples posed no discernible risk or discomfort to the patients. The experiments involved in this study were carried out in accordance with the relevant regulations. The authors obtained consent from the research participants to publish relevant data. The data supporting the findings of this study are available upon request to the corresponding author.

IHC

IHC is an experimental technique used to locate and qualitatively and semi-quantitatively detect antigens in human and animal tissues through antibody staining. It has high specificity and sensitivity. IHC was used to detect FAP expression and assess the protein expression levels of FAP during the progression of OLK carcinogenesis in NM, OLK, and OSCC

tissues. By directly observing FAP expression in these tissues, we determined whether FAP was involved in the occurrence of these diseases and whether it was a biomarker in the progression of OLK carcinogenesis. The present study also aimed to investigate the association between FAP expression and clinicopathologic characteristics of individuals diagnosed with OLK and OSCC.

Paraffin tissue microarray sections of 4 μm thick were baked for approximately 30 minutes, deparaffinised in xylene, and rehydrated through a graded alcohol series. Antigen repair was performed in a microwave with a temperature of 100 °C. The endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide in a wet box, followed by dropwise addition of 3% BSA to block the nonspecific antigen at 37 °C for half an hour. The slices were incubated with a primary antibody against FAP (1:150 dilution, ab207178, Abcam) at 4 °C overnight, followed by goat anti-rabbit HRP secondary antibody at 37 °C for half an hour the next day. Tissue sections were stained with DAB and nuclei were stained with haematoxylin, dehydrated, fixed in xylene, and sealed with neutral gum. Finally, the stained samples were observed microscopically to analyse the results.

RT-PCR

Cell line and source

The study utilised 3 distinct and adherent cell lines, namely human oral keratinocyte (HOK), human dysplastic oral keratinocyte (DOK), and human oral squamous cell carcinoma-15 (SCC-15). These cell lines were selected to reflect the 3 stages of OLK carcinogenesis and purchased from Shenzhen Huatuo Cell Biology. HOK cells were cultured in a medium with 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. DOK cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% animal blood ware and 1% penicillin-streptomycin. SCC-15 cells were cultured in DMEM, 10% FBS, and 1% penicillin-streptomycin. The cells were cultivated in an incubator maintained at a temperature of 37 °C and a carbon dioxide concentration of 5%.

RT-PCR

RT-PCR is an in vitro technique used to amplify DNA fragments, which can amplify specific DNA fragments in large quantities in a short time. It can be used to investigate the expression and variations of specific genes during disease progression. It was used to quantitatively assess the FAP mRNA levels in HOK, DOK, and SCC-15 cells. Using RT-PCR, we explored whether obvious differences in FAP expression occurred between OLK and OSCC cells at the genetic level, thereby gaining a deeper understanding of its participation in the occurrence and development of OLK carcinogenesis.

Total RNA was extracted using Eastep Super Total RNA. Following quality inspection, the M5 Super qPCR RT kit (Mei5 Biotechnology, Co.) was used to reverse-transcribe RNA into cDNA. The primer pairs were presented: FAP, forward was 5'-GAAGTGCCTGTTCCAGCAATGATAG-3' and reverse was 5'-TTAGCCACTGCAAACATACTCGTTC; β-actin, forward was 5'-CACCATTGGCAATGAGCGGTTC-3' and reverse was 5'-AGGTCTTTGCGGATGTCCACGT-3'. $2^{\wedge -\triangle\triangle Ct}$ method was applied to estimate relative gene expression levels; β-actin was chosen as the reference gene.

$$\Delta Ct = Ct(FAP) - Ct(\beta - actin)$$

$$\Delta\Delta Ct = \Delta Ct(DOK \text{ or } SCC - 15) - \Delta Ct(HOK)$$

$$2^{\wedge -\Delta\Delta Ct} = 2^{\wedge (\Delta Ct(HOK) - \Delta Ct(DOK \text{ or } SCC - 15))}$$

WB

WB is a technique used to detect the expression levels and types of proteins in cells or tissue samples. It can detect multiple proteins simultaneously and has a high resolution and specificity for proteins. This was used to evaluate the expression of FAP in NM, OLK, and OSCC cells at the protein level. We also demonstrated the changes in FAP levels under various conditions by detecting differences in protein expression in HOK, DOK, and SCC-15 cells. The use of WB helped to further determine whether it can serve as a biomarker to predict OLK carcinogenesis.

After extracting proteins from cells using RIPA lysate, the proteins were quantified using the BCA protein quantitation kit, and samples were prepared after loading, electrophoresis, membrane transfer, and skim milk blocking buffer. The primary antibody was incubated for 12 hours, and then the film was washed, followed by secondary antibody incubation, and the colour was developed with ECL developer solution after the film washing.

Statistical analysis

All images from the tissue samples were analysed using ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation). This study utilised IBM SPSS Statistics 26.0 (SPSS, Inc.) for the data analysis. Descriptive statistics were expressed by mean ± standard deviation. Pearson's chi-square test was used to analyse the relationship between clinicopathologic characteristics and FAP expression. The Kolmogorov–Smirnov test and Shapiro–Wilk test were accessed for normal distribution. Differences between groups were analysed by the Kruskal–Wallis H test, followed by Dunn post hoc test for IHC data, Welch analysis of variance (ANOVA) and Dunnett T3 post hoc test for the RT-PCR data, and one-way ANOVA followed by Tukey post hoc test for the WB data. Statistically significant differences were considered at $P < .05$.

Results

IHC

Based on the professional diagnoses of the pathologists, all tissue samples were divided into 3 main groups: NM (n = 20), OLK (n = 55), and OSCC (n = 13). The OLK group was further divided into the MI-OLK (n = 24), MO-OLK (n = 21), and S-OLK (n = 10) groups. FAP is mainly located in the basal and spinous layers of the epithelium and mesenchyme. The expression level of FAP was significantly associated with histopathologic

Table – The statistical relationships between clinicopathologic characteristics and FAP expression of patients.

Clinical variable	FAP expression		χ^2	P value*
	+(%)	++(%)		
Age, y				
<60	24 (60)	17 (60.7)	0.004	.953
≥60	16 (40)	11 (39.3)		
Sex				
Male	19 (47.5)	15 (53.6)	0.243	.622
Female	21 (52.5)	13 (46.4)		
Region				
Buccal mucosa	15 (37.5)	5 (17.9)	8.161	.108
Lingual mucosa	20 (50)	21 (75)		
Gingival mucosa	1 (2.5)	1 (3.6)		
Palatal mucosa	2 (5)	0		
Maxillary and mandibular mucosa	2 (5)	0		
NA	0	1 (3.6)		
Histopathologic grade				
Mild abnormal hyperplasia	17 (42.5)	7 (25)	8.494	.038
Moderate abnormal hyperplasia	15 (37.5)	6 (21.4)		
Severe abnormal hyperplasia	4 (10)	6 (21.4)		
OSCC	4 (10)	9 (32.1)		

* Chi-square was used for data analysis.FAP, fibroblast activation protein; NA, not available; OSCC, oral squamous cell carcinoma.

grade ($P < .05$) but not with age, sex, or sampling region ($P > .05$; Table). The average optical density (AOD) values of FAP in NM, OLK, and OSCC were 0.15 ± 0.01 (95% confidence interval [CI], 0.15–0.16), 0.28 ± 0.04 (95% CI, 0.27–0.29), and 0.39 ± 0.02 (95% CI, 0.38–0.41), respectively, and the remaining pairwise comparisons amongst the 3 groups were statistically significant ($P < .001$). The expression level of FAP was distinctly higher in the OLK group than in the NM group but lower than in the OSCC group (Figure 1). The AOD values of FAP in MI-OLK, MO-OLK, and S-OLK, respectively, were 0.26 ± 0.02 (95% CI, 0.25–0.27), 0.28 ± 0.03 (95% CI, 0.27–0.29), and 0.33 ± 0.04 (95% CI, 0.30–0.36), the differences were statistically significant ($P < .05$, $P < .001$). In the OLK group, the expression level of FAP was lower in the MI-OLK and MO-OLK groups than in the S-OLK group (Figure 2).

RT-PCR

The results of RT-PCR showed that the FAP mRNA content in HOK, DOK, and SCC cells separately was 0.87 ± 0.14 (95% CI, 0.65–1.09), 2.63 ± 0.62 (95% CI, 1.65–3.62), and 5.63 ± 1.06 (95% CI, 3.94–7.31), respectively. In DOK and SCC cells, the expression level of FAP mRNA was approximately 2 and 5 times higher than that in HOK cells, respectively, and the results were statistically different ($P < .05$, $P < .01$; Figure 3).

WB

The protein expression levels of β-actin in 3 cell types showed a high degree of similarity. The protein expression values of FAP in HOK, DOK, and SCC cells were found to be 0.78 ± 0.06 (95% CI, 0.63–0.94), 1.04 ± 0.06 (95% CI, 0.90–1.18), and 1.61 ± 0.09 (95% CI, 1.40–1.83). They were significantly upregulated in DOK and SCC cells compared with that of HOK cells ($P < .01$, $P < .001$; Figure 4).

Discussion

The heterogeneous OLK has the potential to transform into cancer, with a prevalence ranging from 0.13% to 17.5%[21] or higher in certain regions. Most OSCC cases are not diagnosed in the early stages; therefore, prompt intervention for OLK is a crucial preventive strategy for malignant transformation. The major issue pertaining to OLK therapies is the effective inhibition and reversal of aberrant epithelial hyperplasia. Nevertheless, the diagnosis and prevention of malignant transformation of OLK are accompanied by many ambiguities.[22,23] Finding universally approved and conclusive advanced therapeutic interventions has become the most important factor in controlling this process. Because of its high sensitivity and accuracy in molecular diagnosis, it has been widely applied in the following areas: early diagnosis and targeted therapy of tumours, sensitive diagnosis of infectious diseases, and detection of genetic diseases. Hence, we identified a biomarker for determining the malignant transformation of OLK using molecular diagnosis.

Malignant transformation of OLK accompanied by certain changes may be a key factor in predicting its cancerisation. Proven factors promoting OLK carcinogenesis include sex (females have a higher cancer rate), persistent existence, nonsmoking, locations of frequent occurrence (the mouth floor or ventral tongue), heterogeneous lesions, concurrent *Candida albicans* infections, and abnormal tissue hyperplasia in pathology.[21,24-26] Angiogenesis facilitates the provision of nutritional resources necessary for the growth and migration of tumours and plays a pivotal role in OLK carcinogenesis. Folkman et al[27] found that neovascularisation occurred when the islets of transgenic mice developed early tumours. In the experiment of "observation and study on angiogenesis during the evolution of hamster cheek pouch cancer," it was found that during the formation and carcinogenesis of OLK, the values of vascular density and vascular area density continued to rise, showing great angiogenic activity.[28]

FAP is one of the surface antigens of tumour-associated fibroblasts (TAFs) in the TME that participates in extracellular matrix (ECM) degradation and angiogenesis. FAP has enzymatic properties such as collagenase and dipeptidyl peptidase activities. These enzymatic activities enable the FAP to cleave gelatin and dipeptidyl peptidases in the matrix. Consequently, this enzymatic action contributes to the destruction of the ECM, facilitating the separation, invasion, and metastasis of tumour cells from their initial location.

The primary role of FAP is to facilitate the proliferation, migration, and invasion of tumours, which can be attributed to the following 4 factors.

1. Degradation and remodelling of the extracellular matrix (ECM): Tumour cells are dependent on adhesion proteins present in the ECM for attachment. The enzymatic activity of FAP leads to hydrolysis of the ECM, facilitating the

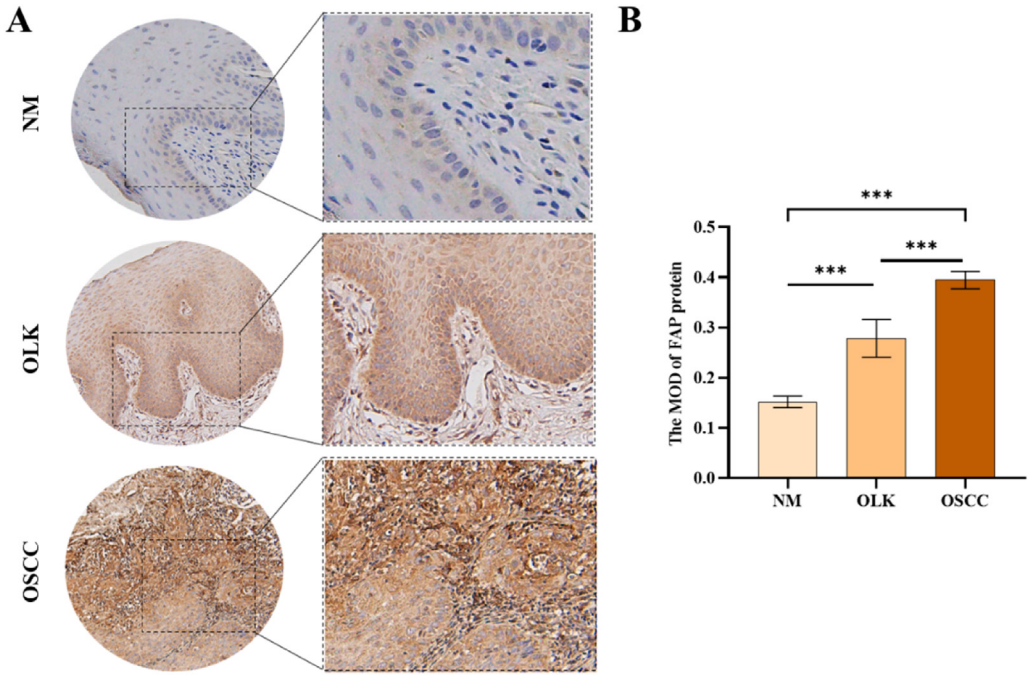

Fig. 1 – Immunohistochemistry expression levels of fibroblast activation protein (FAP) in oral normal mucosa (NM; n = 20), oral leukoplakia (OLK; n = 55), and oral squamous cell carcinoma (OSCC; n = 13) tissues: A, FAP immunohistochemical staining was increased in OLK and OSCC tissues. Left side = 100 μm, right side = 50 μm. B, The average optical density (AOD) of FAP was quantitatively assessed using ImageJ software. Data were presented as mean ± SD. The intercomparison of the 3 groups revealed significant differences, with a sequential increase observed in the AOD of FAP ($P_{NM\ vs\ OLK} < .001$, $P_{NM\ vs\ OSCC} < .001$ and $P_{OLK\ vs\ OSCC} < .001$). ***P < .001 analysed by Kruskal–Wallis H test, followed by Dunn post hoc analysis.

migration of cells to distant locations and the invasion of adjacent healthy tissues. In contrast, FAP can directly accelerate the tumour cell cycle and enhance cell proliferation via intracellular signalling pathways.[29]

2. The process of tumour microvascular development: The angiogenic impact of FAP can be categorised into 2 distinct pathways. One of the substrates of human FAP is neuropeptide Y. Neuropeptide Y and its receptors stimulate of vascular smooth muscle proliferation and migration and play significant roles in the promotion of angiogenesis. Matrix metalloproteinase 9 (MMP-9) is responsible for the angiogenic characteristics observed in tumours exhibiting FAP expression. FAP and MMP-9 are co-expressed to actively contribute to angiogenesis.[9]
3. Epithelial-mesenchymal transition (EMT): EMT facilitates fibroblast activation and enhances FAP expression. EMT is a biological phenomenon characterised by the loss of epithelial cell traits and the acquisition of mesenchymal fibroblastlike cell properties.[30] Numerous studies have demonstrated the transformation process of damaged epithelial cells generating fibroblasts. These fibroblasts subsequently produce a range of inflammatory signals and ECM molecules that are regulated by inflammatory cells and fibroblasts, leading to EMT. Chronic inflammatory responses can cause structural damage to tissues, disrupt the milieu of affected tissues, and ultimately result in tissue abnormalities.[31]
4. Immunosuppression: Tumour cells can hinder the TME. However, when FAP (+) cells are no longer present, the immune response inside the tumour is reestablished. This suggests that FAP overexpression contributes to the immunosuppressive nature of the TME.[32]

In vitro investigations[33] have shown that siRNA-mediated FAP knockout decreased the invasiveness of OSCC cells. In addition, FAP is an independent prognostic factor in patients with OSCC. There was an inverse relationship between the level of FAP expression and patient survival time; higher FAP expression levels were associated with shorter survival times. The interaction between the tumour and the TME is mediated by FAP. An effective antitumour effect can be achieved through the direct inhibition of FAP activity or the clearance of FAP-positive cells.

In the present study, we investigated FAP expression in NM, OLK, and OSCC tissues and cells. These results unequivocally indicated an increase in FAP expression in the progression of OLK carcinogenesis. The probability of OLK carcinogenesis increased with the degree of epithelial dysplasia. FAP expression was observed in MI-OLK and exhibited a gradual increase in positivity in MO-OLK, S-OLK, and OSCC. This observation indicated that the upregulation of FAP expression occurred during the initial phase of OLK carcinogenesis and that the level of expression is associated with the severity of epithelial dysplasia. The anticipated favourable connection between FAP expression and the probability of OLK carcinogenesis offers a significant foundation for the early diagnosis of OLK with malignant potential. Hence, we

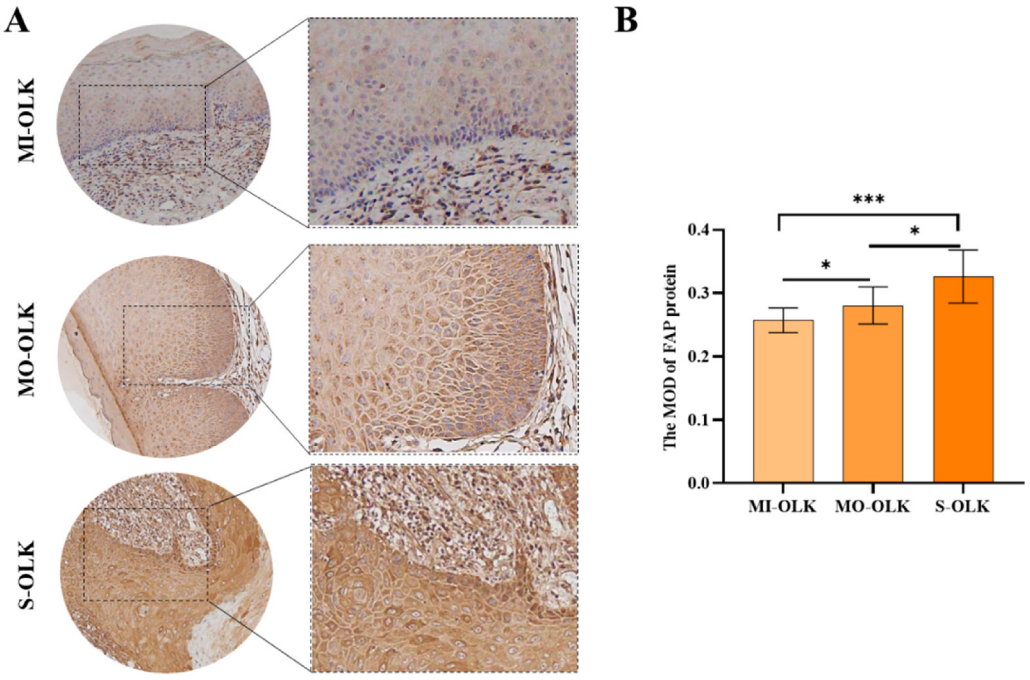

Fig. 2 – Immunohistochemistry expression levels of fibroblast activation protein (FAP) in oral leukoplakia (OLK) tissues, including OLK with mild abnormal hyperplasia (MI-OLK; n = 24), OLK with moderate abnormal hyperplasia (MO-OLK; n = 21), and OLK with severe abnormal hyperplasia (S-OLK; n = 10) tissues: A, FAP immunohistochemical staining was strengthened in the MO-OLK and S-OLK groups. Left side = 100 μm, right side = 50 μm. B, The semiquantitative evaluation of FAP levels in the OLK groups was performed by assessing the average optical density (AOD) using the ImageJ software, which linked with the extent of aberrant epithelial development. Data were presented as mean ± SD. The distinction between any 2 groups was evident, especially between MI-OLK and S-OLK ($P_{MI\text{-}OLK\ vs\ MO\text{-}OLK}$ = .047, $P_{MI\text{-}OLK\ vs\ S\text{-}OLK}$ < .001 and $P_{MO\text{-}OLK\ vs\ S\text{-}OLK}$ = .039). *P < .05, ***P < .001 analysed by Kruskal–Wallis H test, followed by Dunn post hoc analysis.

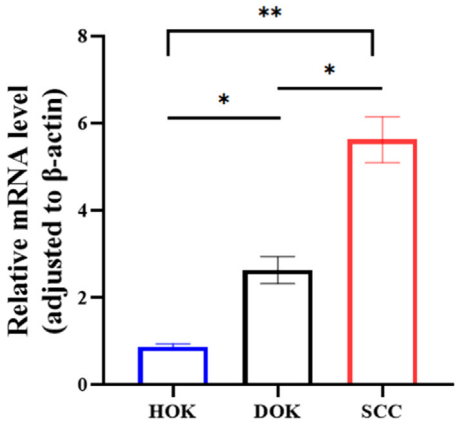

Fig. 3 – The expression levels of fibroblast activation protein (FAP) mRNA in human oral keratinocyte (HOK), human dysplastic oral keratinocyte (DOK), and human oral squamous cell carcinoma (SCC) cells: The reverse transcription-polymerase chain reaction findings were normalised to β-actin expression and shown as the mean of 3 independent experiments (n = 3). Data were presented as mean ± SD. Significant differences were observed through the intercomparison of 3 groups ($P_{HOK\ vs\ DOK}$ = .027, $P_{HOK\ vs\ SCC}$ = .007 and $P_{DOK\ vs\ SCC}$ = .012), Compared to HOK cells, there was slight increase in DOK cells and a considerable increase in SCC cells. *P < .05, **P < .01 analysed by Welch analysis of variance, followed by Dunnett T3 post hoc analysis.

suggested that FAP could be used as a biomarker for discerning the malignant transformation of OLK.

Our study had certain limitations, primarily encompassing 3 key features. First, the relatively small sample size employed in this investigation may have introduced bias and compromised the accuracy of the experimental findings. The effectiveness of statistical inference may decrease when the sample size is small, thereby affecting the accuracy of clinical decision-making. In addition, costs and time consumption may increase when using small sample sizes in clinical trials. Second, this study examined only a single antibody. A single antibody can only recognise and bind to specific antigens, which limits its application and may not provide sufficient insights for clinical research. The preparation and use costs of a single antibody may be high, which may limit its use in clinical or large-scale applications and increase the economic burden on patients. The simultaneous release of many indicators has been implicated in tumour development. The sensitivity and specificity of a single antibody may not be sufficient to detect low-abundance antigens. Utilising multiple markers for screening can compensate for the inherent limitations of relying on a single index, thereby enhancing the sensitivity, specificity, and accuracy of clinical diagnosis.[34,35] Finally, 3 in vivo and in vitro experiments were performed to investigate the expression of FAP in OLK and OSCC, without exploring the specific molecular mechanism of FAP in the progression of OLK carcinogenesis. Thus, it is impossible to gain a deeper understanding of molecular

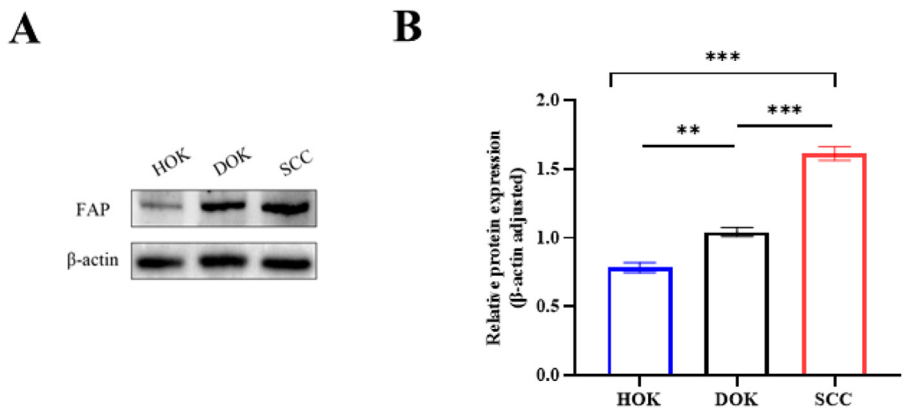

Fig. 4 – Western blot (WB) analysis of fibroblast activation protein (FAP) expression levels in human oral keratinocyte (HOK), human dysplastic oral keratinocyte (DOK), and human oral squamous cell carcinoma (SCC) cells: A, The expression levels of FAP upregulated in DOK and SCC cells. B, The β-actin was used as the loading control for the blot. Densitometry evaluations of the WB were depicted using a bar graph. The results were expressed as the mean ± SD of 3 separate experiments (n = 3). The comparative analysis of relative protein expression indicated they were increased in DOK and SCC cells ($P_{HOK\ vs\ DOK} = .009$, $P_{HOK\ vs\ SCC} < .001$ and $P_{DOK\ vs\ SCC} < .001$). $^{**}P < .01$, $^{***}P < .001$ analysed by 1-way analysis of variance followed by Tukey post hoc analysis.

interactions and their specific mechanisms of action within cells. This limits our understanding of the biological processes and disease mechanisms, leading to difficulties in clinical applications. Therefore, it is imperative to enhance the present investigation by augmenting the sample size and increasing the testing indices. This would enable a more comprehensive understanding of the significance of FAP in OLK and OSCC.

Conclusions

Notable variations were observed in the protein expression and mRNA amplification levels of FAP in NM, OLK, and OSCC. There is speculation on the potential significance of FAP in OLK and its involvement in the initiation and progression of OSCC. In conclusion, FAP has the potential to serve as a biomarker for early diagnosis and intervention in the clinical setting.

Conflict of interest

None disclosed.

Acknowledgements

We sincerely thank those who helped with the experiments and funding the projects. We are thankful to teachers for their guidance in conducting and concluding the experiment. We want to express our gratitude to those people who have helped us in solving problems. Finally, we would like to express our gratitude for the successful implementation and funding received for this experiment.

Author contributions

Ran Li and Rongrong Zhang participated in the experimental process, analysed the data, and wrote the main manuscript. All the authors were involved in writing the manuscript. All the authors reviewed the final article format and agreed to publish the manuscript. Ran Li and Rongrong Zhang contributed equally.

Funding

This work was supported by the Shanxi Province Basic Research Program (20210302123311), the Research Project Supported by Shanxi Scholarship Council of China (2021-087), the Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province (20220020), the Science and Technology Innovation Programs of Higher Education Institutions in Shanxi (2022L174), the Science and Technology Innovation Leader and Key Talent Team Project of Shanxi Province (202204051002034), and the 2023 Shanxi Provincial Higher Education General Teaching Reform and Innovation Project (J20230540).

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