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Overexpression of FAM3C is associated with poor prognosis in oral squamous cell carcinoma

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ABSTRACT

Expression of the family with sequence similarity 3 member C (FAM3C) is necessary for the epithelial-mesenchymal transition (EMT). However, the expression level and clinicopathological significance of FAM3C in oral squamous cell carcinoma (OSCC) has not been thoroughly elucidated to date. We performed immunohistochemical staining on human OSCC specimens with FAM3C, co-inhibitory immune checkpoints, EMT markers, and cancer stem cells (CSCs) markers to analyze the expression levels and clinicopathological features of FAM3C in OSCC. There were 210 primary OSCC specimens, 69 oral epithelial dysplasia and 42 normal oral mucosae in our human OSCC tissue microarrays cohort. We observed that FAM3C expression was upregulated in OSCC compared with normal mucosa and epithelial dysplasia ($P < 0.001$). Moreover, patients with higher FAM3C expression levels had a worse prognosis than those with lower expression levels ($P < 0.05$). Also, FAM3C expression was positively correlated with the immune checkpoints PD-L1, VISTA, and B7-H4, the EMT marker Slug and the CSC markers SOX2 and ALDH1. In conclusion, these findings suggested that overexpression of FAM3C in human OSCC may predict a poor prognosis for OSCC patients.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common tumor, accounting for 95% of oral malignant lesions, and this carcinoma is increasing in incidence [1]. In spite of aggressive, site-specific multimodal therapy, the prognosis of patients with OSCC is poor due to recurrence, lymph node metastasis, and resistance to radiotherapy and chemotherapy [2]. Risk factors associated with OSCC included smoking, drinking, human papillomavirus (HPV) infection, and immunosuppressive status, which has emerged in recent years [3]. In contrast to the trend of declining mortality rates for the four major cancers (i.e., lung cancer, breast cancer, prostate cancer and colorectal cancer), the death rate of OSCC has increased slightly over the past two decades [4]. Therefore, a new treatment for OSCC is still urgently needed.

The family with sequence similarity 3 member C (FAM3C), also known as the interleukin-like epithelial-mesenchymal transition inducer (ILEI), is a member of the FAM3 family and was discovered while exploring new cytokines. FAM3C is essential for the epithelial-mesenchymal transition (EMT) and is associated with metastasis in tumor development [5]. Recent studies suggest that FAM3C is overexpressed in colorectal cancer [6] and esophageal cancer [7], and is associated with poor prognosis. It is worth mentioning that FAM3C is a potential biomarker of EMT in rectal cancer [6]. However, the expression and function of FAM3C in OSCC remains to be determined. A more thorough study of the molecular mechanisms of FAM3C may prevent the invasion and progression of OSCC from interfering with EMT and help to identify efficient strategies for the diagnosis and treatment of OSCC patients.

OSCC cells easily invade neighboring tissues or migrate to cervical lymph nodes, which result from EMT [8]. EMT is a process activated by EMT-activating transcription factors (EMT-TFs), such as the SNAIL, TWIST and ZEB families, and plays an key role in malignant tumor progression, invasion and dissemination [9]. In addition, EMT is characterized by the downregulation of epithelial markers, such as E-cadherin, and the acquisition of mesenchymal markers, such as vimentin [10]. Moreover, Slug is a zinc finger transcriptional repressor that can downregulate E-cadherin and plays a crucial role in EMT [11]. A study determined that EMT progression is a key regulator of the cancer stem cell (CSC) phenotype [12]. Aldehyde dehydrogenase 1 (ALDH1) has

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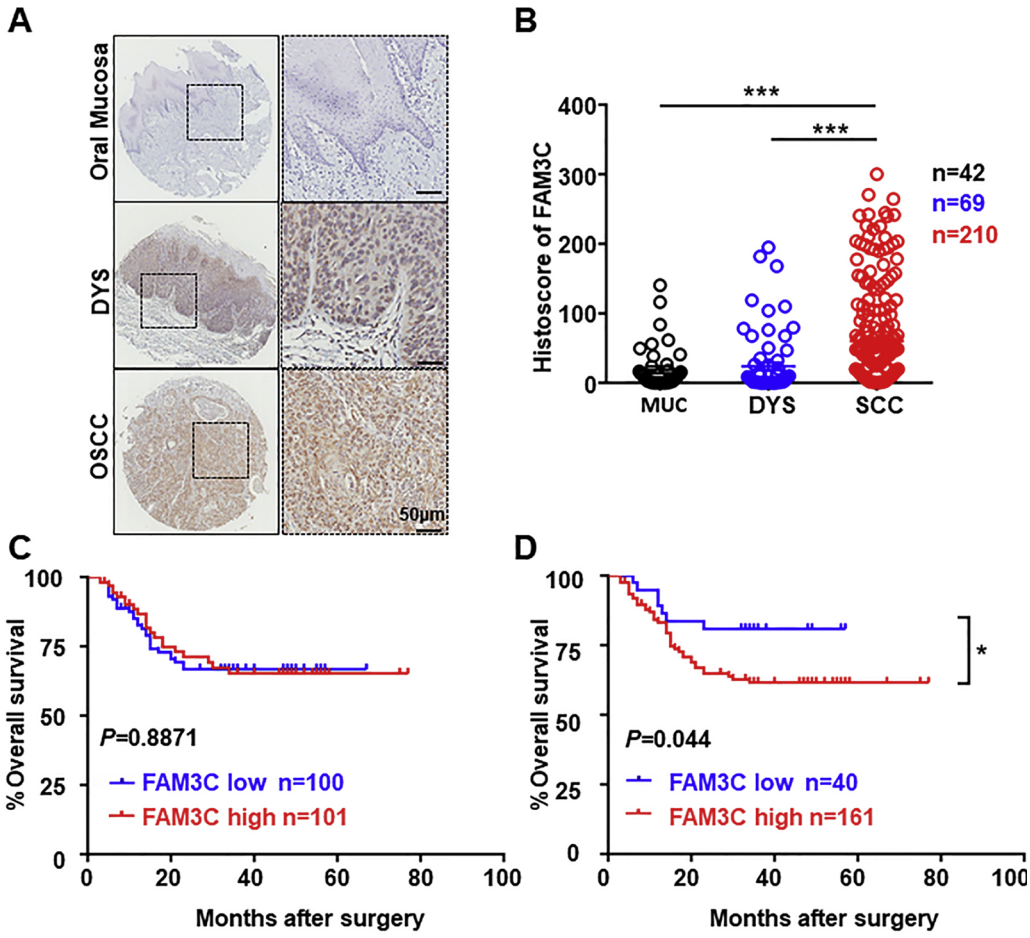

Fig. 1. FAM3C is highly expressed in OSCC and associated with poor prognosis of OSCC. **A:** The immunohistochemical staining of FAM3C in normal oral mucosa and OSCC tissue. Scale bar, 50μm. **B:** Quantitative analysis of immunohistochemical staining of FAM3C in normal mucosa (MUC, $n = 42$), epithelial dysplasia (DYS, $n = 69$) and oral squamous cell carcinoma (OSCC, $n = 210$, $^{***}P < 0.001$). **C:** When the median value (histoscore = 24.05364) was used as the cut-off point, the Kaplan-Meier curve shows that the expression of FAM3C is not related to OSCC prognosis ($P = 0.8871$). **D:** The Kaplan-Meier curve shows that the high expression of FAM3C is correlated with the poor prognosis of OSCC when the best cut-off was used (histoscore = 4.193, $P = 0.044$).

been used as prospective CSC-specific marker and transcription factor SOX2 which is indispensable for stemness maintenance of CSCs [13]. In addition, PD-L1 [14], VISTA [15], and B7-H4 [16] are overexpressed in many cancers and are associated with prognosis.

In this study, we took advantage of human tissue microarrays and immunohistochemistry to research the expression of FAM3C in OSCC. Furthermore, we probed the relationships among FAM3C, clinical parameters, and prognosis. At the same time, we also explored the relationship between FAM3C expression and PD-L1, VISTA, B7-H4, Slug, and SOX2, as well as ALDH1, expression in OSCC.

2. Materials and methods

2.1. Ethical statement

Full ethical approval was granted by the Medical Ethics Committee of Hospital of Stomatology Wuhan University (PI: Zhi-Jun Sun; 2014LUNSHENZI06). Informed consent was obtained from each patient.

2.2. Tissue microarrays

In order to study the expression level of FAM3C in OSCC, we constructed tissue microarrays (TMA) by collecting tumor tissue samples from surgically resected oral cancer specimens from the Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology Wuhan University. There were 210 primary OSCC specimens [15], 42 normal oral mucosae (MUC), 69 oral epithelial dysplasia (DYS), 25 recurrent OSCC patients, 15 OSCC patients who had received pre-surgical radiotherapy and 20 OSCC patients who had received pre-surgical TPF (cisplatin, docetaxel and fluorouracil) inductive chemotherapy in our human OSCC tissue microarrays (T12-412-TMA2, T15-411, T17-790). The TNM classification of the OSCC patients was classified based on the guidelines of the International Union against Cancer (8th edition)) [17], and the histology grade was determined by the World Health Organization guideline [18]. The clinicopathological characteristics of the cohort (T17-790) are shown in Supplementary Table. We obtained informed consent from each patient. Detailed patients' information, including name, age, gender, TNM classification, smoking and drinking history, lymph node status, histological grade, and follow-up data, were accessible from the electronic medical records.

2.3. Immunohistochemistry

Tissue arrays were stained with the following antibodies: FAM3C (Cell Signaling Technology, 1:500), PD-L1 (Cell Signaling Technology, 1:100), VISTA (Cell Signaling Technology, 1:400), B7-H4 (Cell Signaling Technology, 1:800), Slug (Cell Signaling Technology, 1:200), SOX2 (Cell Signaling Technology, 1:300) and ALDH1(Cell Signaling Technology,1:800). Specific immunohistochemical processes, such as deparaffinization, dehydration, antigen retrieval, combination of primary antibody and secondary antibody and DAB staining, are available as supplementary information. Positive slide and negative slides were set at this experiment, the positive slide and negative slide both fabricated by paraffin-embedded tissues.

2.4. Scoring system

First, we scanned the TMA with Aperio ScanScope CS scanner (Vista, CA, USA). Next, we quantified the member, nuclear, or pixel

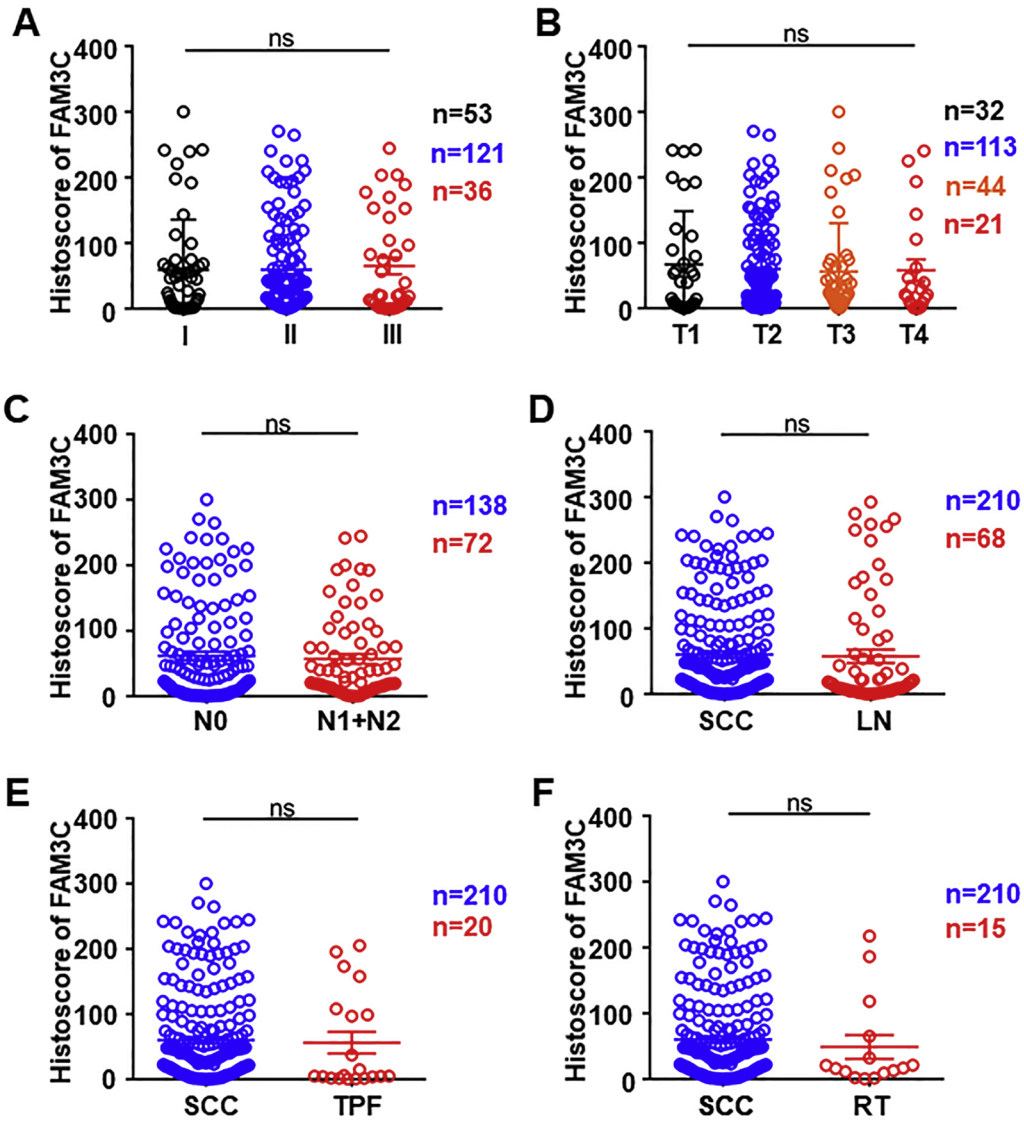

Fig. 2. Clinicopathological significance of FAM3C **A:** The expression level of FAM3C in different OSCC grades (I = 53, II = 121, III = 36, $P > 0.05$). **B:** Quantitative analysis of the expression level of FAM3C in four tumor sizes (T1 = 32, T2 = 113, T3 = 44, T4 = 21, $P > 0.05$). **C:** Quantitative analysis of the expression level of FAM3C in different lymph node states (N0 = 138, N1 + N2 = 72, $P > 0.05$). **D:** Quantitative analysis of the expression level of FAM3C in primary OSCC and metastatic lymph nodes (OSCC = 210, LN = 68, $P > 0.05$). **E:** Quantitative analysis of different expression level of FAM3C in primary OSCC and OSCC after TPF (OSCC = 210, TPF = 20, $P > 0.05$). **F:** Quantitative analysis of different expression levels of FAM3C in primary OSCC and OSCC after RT (OSCC = 210, RT = 15, $P > 0.05$).

immunohistochemical staining with Aperio Quantification software algorithms as previous described [19]. The calculation of intensity is based on the color of each pixel. The weakly positive, moderately positive and strongly positive slides were defined and set up by Aperio Scanscope standard slices. An area of concern was sorted, scanning and quantifying, then histoscore of pixel quantification was calculated using the following formula: (1 × the percentage of weakly positive staining) + (2 × the percentage of moderately positive staining) + (3 × the percentage of strongly positive staining) [20], and the formula total intensity/total cell number was used to assess the histoscore of pixel quantification. Hierarchical clustering was performed using Cluster 3.0 with the average linkage based on Pearson's correlation, visualization was performed using the Java TreeView 1.0.5.

2.5. Statistical Analysis

We used GraphPad Prism 7 for Windows to analyze data. Student's *t*-test was applied to analyze the data between two experimental groups and one-way ANOVA followed by Tukey's multiple comparisons test was used to contradistinguish additional groups of immunostaining in each group. Overall survival difference was evaluated by Kaplan-Meier analysis and Log-rank test. We used median and best cut-off as nodes to analyze the differences in survival rate. The survival rate analysis was limited to 201 patients because 9 people were lost during follow-up. As previously reported, the best cut-off value was found on the Cutoff Finder [21]. Two-tailed Person statistics was utilized for the correlation between FAM3C and PD-L1, VISTA, B7-H4, Slug, SOX2, and ALDH1. *P*-values less than or equal to 0.05 were considered to be statistically significant.

3. Results

3.1. Overexpression of FAM3C is associated with poor prognosis in OSCC

In order to study the role of FAM3C in OSCC, we searched the publicly available cancer microarray database Oncomine® [dataset] [22] and found that compared to the normal mucosa, *FAM3C* mRNA was highly expressed in OSCC (P = 4.35E-4, Supplementary Fig. S1A). In Toruner Head-Neck gene expression profiling dataset [23], *FAM3C* mRNA was highly expressed in the oral cavity squamous cell carcinoma compared with a normal counterpart (P = 5.56E-7, FC = 4.341, Supplementary Fig. S1B). Also, in Ginos Head-Neck gene expression profiling dataset [24], the expression level of *FAM3C* mRNA in head and neck squamous cell carcinoma was higher than normal mucosa (P = 3.27E-10, FC = 3.194, Supplementary Fig. S1C). Furthermore, in Pyeon Multi-cancer gene expression profiling dataset [25], compared to normal mucosa, *FAM3C* mRNA was overexpressed in tongue carcinoma (P = 4.25E-6, FC = 3.193, Supplementary Fig. S2 A), oral cavity

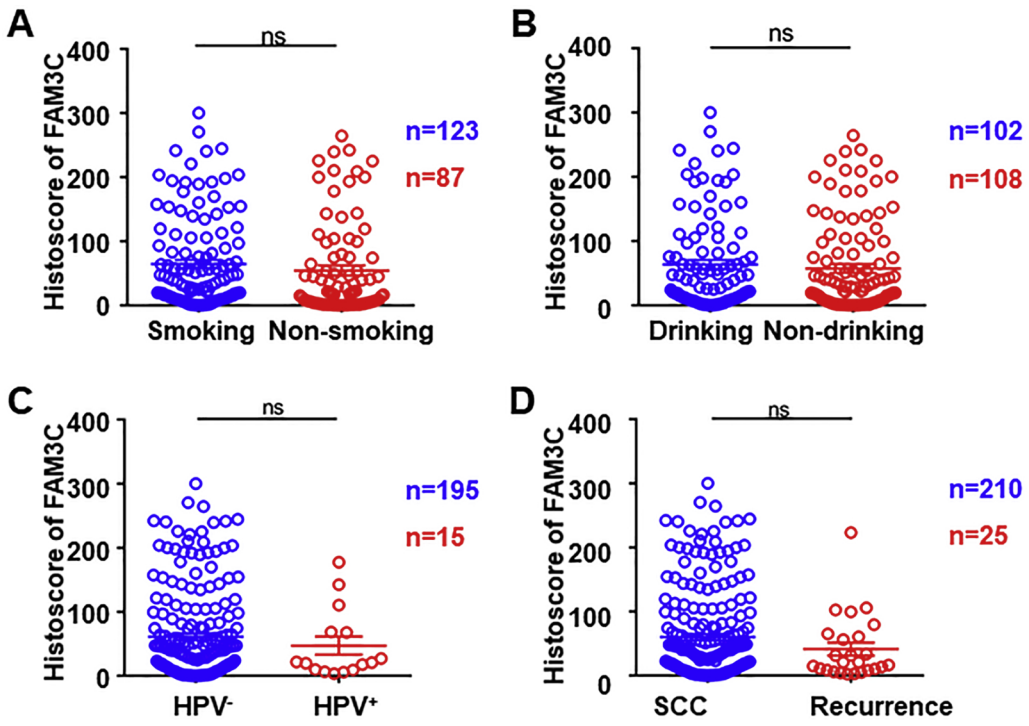

Fig. 3. Relationship between the expression level of FAM3C and risk factors. **A:** Quantitative analysis of FAM3C expression between smoking OSCC patients and non-smoking OSCC patients (Smoking = 123, Non-smoking = 87, $P > 0.05$). **B:** Quantitative analysis of FAM3C expression between drinking OSCC patients and non-drinking OSCC patients (Drinking = 102, Non-drinking = 108, $P > 0.05$). **C:** Quantitative analysis FAM3C expression between HPV^- OSCC patients and HPV^+ OSCC patients (HPV^- = 195, HPV^+ = 15, $P > 0.05$). **D:** Quantitative analysis of FAM3C expression between primary OSCC and recurrence (OSCC = 210, Recurrence = 25, $P > 0.05$).

carcinoma (P = 8.65E-4, FC = 3.695, Supplementary Fig. S2B), oropharyngeal carcinoma (P = 3.83E-4, FC = 3.196, Supplementary Fig. S2C) and floor of the mouth carcinoma (P = 4.29E-4, FC = 3.690, Supplementary Fig. S2D). Based on the above findings, we used immunohistochemistry to study and quantitatively analyze the expression of FAM3C in OSCC and found that the expression of FAM3C in OSCC (n = 210) was remarkably higher than that in normal mucosae (n = 42, $P < 0.001$) and epithelial dysplasia (n = 69, $P < 0.001$, Fig. 1A and B). In addition, we also took advantage of Kaplan-Meier survival analysis to analyze the correlation between expression level of FAM3C and the prognosis of patients with OSCC, as shown in Fig. 1C and D. When the median value was used as the cut-off point (cut-off = 25.72004), the expression of FAM3C was not related to the prognosis (P = 0.8871); however, when the best cut-off (cut-off = 4.193) was utilized, overexpression of FAM3C is associated with poor overall survival of OSCC patients (P = 0.044). Moreover, we inquired the TCGA database, and draw a conclusion that the expression of FAM3C was correlated with the disease-free survival when we compare the top 45% and low 55% (P = 0.049, Supplementary Fig. S3).

3.2. Clinicopathological significance of FAM3C and its relationship with risk factors

There was no remarkable difference in the expression of FAM3C among three different pathological grades of primary OSCC (I, II and III, $P > 0.05$, Fig. 2A) and four different tumor sizes (T1, T2, T3 and T4, $P > 0.05$, Fig. 2B) in OSCC. In addition, the expression of FAM3C was not correlated with lymph node states in primary OSCC patients (N0 vs N1 + N2, $P > 0.05$, Fig. 2C). In addition, there was no statistical difference in the expression of FAM3C between primary OSCC and metastatic lymph nodes (OSCC vs LN, $P > 0.05$, Fig. 2D). Moreover, no significant differences were observed for FAM3C expression between primary OSCC and patients who received preoperative chemotherapy (TPF, $P > 0.05$, Fig. 2E) or radiotherapy (RT, $P > 0.05$, Fig. 2F). Furthermore, the expression of FAM3C was not related to smoking (Smoking vs Non-smoking, $P > 0.05$, Fig. 3A), alcohol consumption (Drinking vs Non-drinking, $P > 0.05$, Fig. 3B), HPV infection (HPV^- vs HPV^+, $P > 0.05$, Fig. 3C) and recurrence (OSCC vs Recurrence, $P > 0.05$, Fig. 3D).

3.3. Expression level of FAM3C was statistically correlated with PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1

The immunohistochemical staining of FAM3C, PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1 in OSCC is shown in Fig. 4A. Next, we used hierarchical clustering analysis (Fig. 4B) to find that FAM3C, PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1 had similar expression trends in OSCC. Furthermore, we observed that the expression of FAM3C and B7-H4 was most similar. In addition, Spearman's rank correlation test indicated that the expression level of FAM3C was statistically associated with PD-L1 (P = 0.0091, r = 0.2412, Fig. 5A), VISTA (P = 0.0024, r = 0.2794, Fig. 5B), B7-H4 (P = 0.0024, r = 0.2794, Fig. 5C), Slug (P = 0.0001, r = 0.347, Fig. 5D), SOX2 (P = 0.0326, r = 0.1986, Fig. 5E) and ALDH1 (P = 0.0004, r = 0.3229, Fig. 5F).

4. Discussion

In this study, we demonstrated that FAM3C was overexpressed in OSCC compared with normal oral mucosa and epithelial dysplasia. In addition, we found that patients with higher FAM3C expression had poorer prognosis than patients with lower FAM3C expression. In addition, we observed that the expression of FAM3C was statistically related with PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1. Our data indicated that FAM3C might be used as an effective prognostic indicator of OSCC patients.

FAM3C can promote metastasis and angiogenesis, which may result in tumor diffuseness and its translation has been improved during EMT [26]. FAM3C, as a member of the FAM3 family that also includes FAM3 A, FAM3B, FAM3C and FAM3D, was predicted to have the traditional four-helix bundle cytokines; however, FAM3C has a novel structural class of signaling molecule [27]. FAM3C is overexpressed in various tumors, and its altered subcellular localization is closely related to a patient's poor prognosis, and covalent FAM3C self-assembly plays a critical role in EMT induction, tumor proliferation and metastasis [28]. Furthermore, FAM3C also cooperates with Ras to cause TGF-β-independent EMT during liver carcinoma progression [29]. Similarly, our

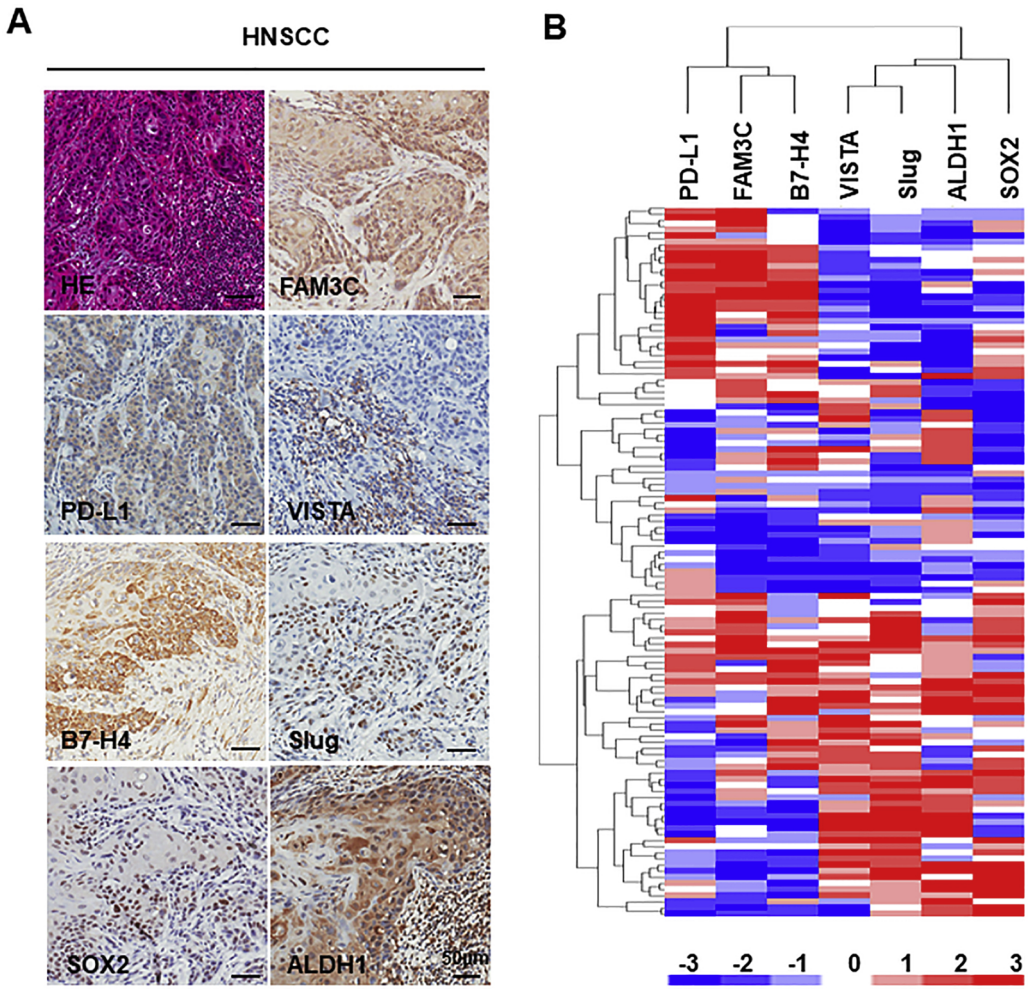

Fig. 4. Expression of FAM3C was related to PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1. **A:** Representative immunohistochemical staining of FAM3C, PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1 in OSCC. Scale bar, 50μm. **B:** Hierarchical clustering depicts the correlation of FAM3C, PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1 in OSCC.

study observed that FAM3C was highly expressed in OSCC; meanwhile, overexpression of FAM3C was closely associated with poor prognosis in OSCC patients. However, in our study, FAM3C expression was independent of the clinicopathological parameters and risk factors, which indicated FAM3C was a common indicator in OSCC. To interest, according to the report that FAM3B was down-regulated in primary OSCC samples [30], this is exactly the opposite of the expression of FAM3C in this study. A reasonable explanation might be that the different structures of FAM3B and FAM3C indicating that they might interact with a similar class but different subtype of binding partners [27].

It has been shown that EMT plays a crucial role in the progress of OSCC, which has been included in Yamamoto-Kohama classification [31]. The expression of EMT markers can be detected at the invasive front of OSCC, which suggested that high expression of EMT markers has a prognostic significance in OSCC [8]. Slug, as a marker of EMT, causes tumor metastasis by maintaining the mesenchymal phenotype and is an indicator of poor overall survival in esophageal squamous cell carcinoma(ESCC) patients [32] and colorectal cancer patients [11]. In our study, we observed that the expression level of FAM3C was correlated with Slug, which may demonstrate that FAM3C also promotes EMT in OSCC and is correlated with poor prognosis as in ESCC [7] and colorectal cancer [6]. Although we did not find any correlation between FAM3C with invasion and metastasis of OSCC, further research is needed. The possible reason for this lack of correlation is that our study contains a small OSCC cohort.

CSCs, which can control cancer hierarchy, harbor stem cell-like properties that are closely related to tumorigenesis and resistance to treatment [33]. Recent studies have shown that overexpression of SOX2 can effectively increase the invasion and metastasis of OSCC cells and was associated with poor survival outcome of OSCC patients. Meanwhile, SOX2 silencing can effectively reduce drug resistance and enhance the sensitivity of radiotherapy [34]. Moreover, overexpression of SOX2 and ALDH1 was associated with lymph node metastasis in OSCC [13]. Interestingly, the knockdown of Slug results in decreased ALDH1 expression and inhibited CSC properties, which may indicate EMT has is involved in the generation of CSCs [35]. In this study, FAM3C was not only statistically related to Slug but also closely related to SOX2 and ALDH1; this finding suggests that the overexpression of FAM3C results in poor prognosis in OSCC patients, possibly because FAM3C is involved in the EMT process and the regulation of CSCs in human OSCC.

Checkpoint blockade immunotherapies targeting T-cell co-inhibitory signaling pathways are being extensively studied and are redefining cancer therapy. Overexpression of the negative immune checkpoints PD-L1 [36], VISTA [15], and B7-H4 [16] is associated with poor prognosis in many tumors. A recent study showed that overexpression of PD-L1 on tumor cells was correlated with EMT status in adenocarcinoma of the lung [37]. Also, a negative checkpoint control protein, VISTA, was involved in the regulation of T cell activation, cell de/differentiation and the inflammatory process, which mimics those associated with EMT [38]. Furthermore, recent studies displayed that B7-H4 could be a novel CSCs marker and prognostic indicator for ESCC [16]. In this study, we observed that the expression levels of FAM3C

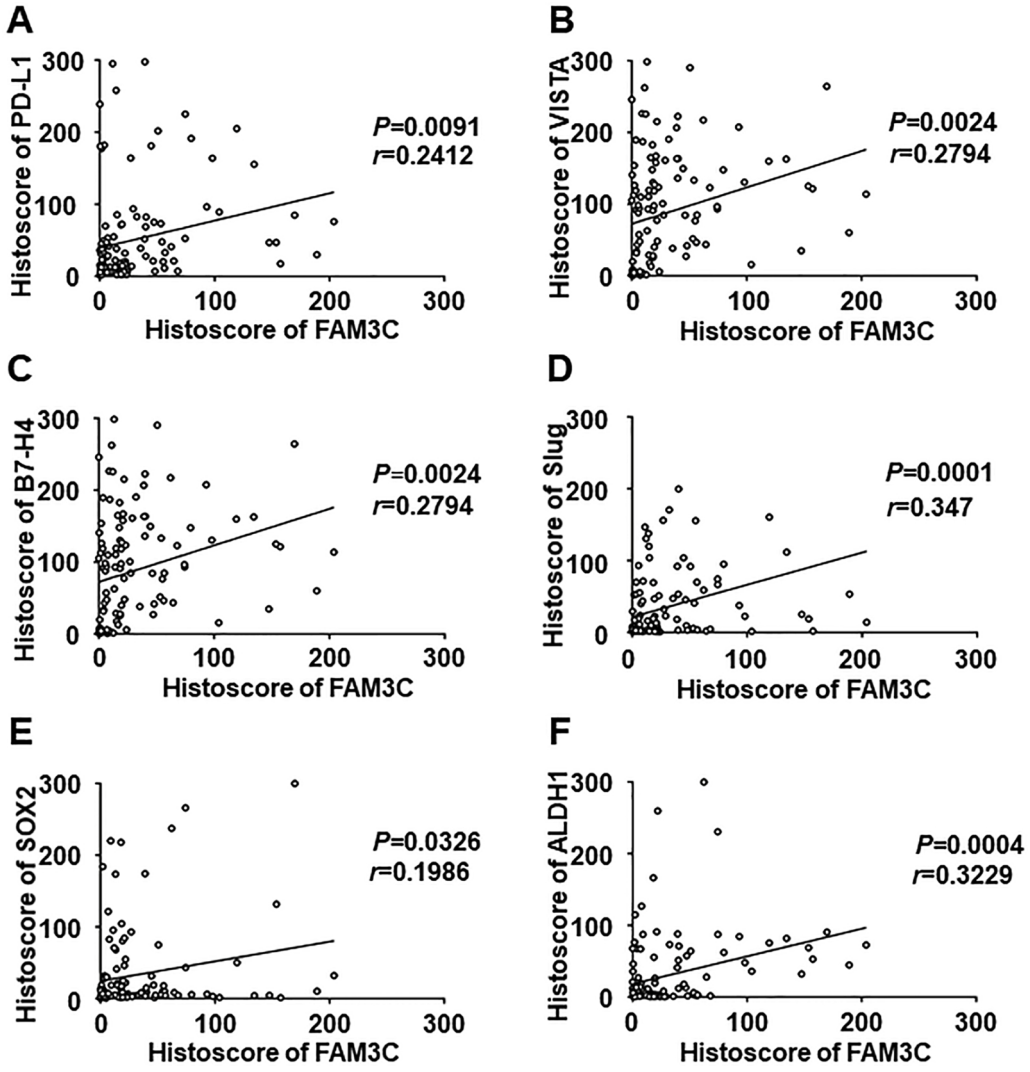

Fig. 5. Spearman rank correlation coefficient test and linear tendency test presented that FAM3C was closely associated with PD-L1($P = 0.0091$, $r = 0.2412$, **A**), VISTA ($P = 0.0024$, $r = 0.2794$, **B**), B7-H4 ($P = 0.0024$, $r = 0.2794$, **C**), Slug ($P = 0.0001$, $r = 0.347$, **D**), and SOX2 ($P = 0.0326$, $r = 0.1986$, **E**), as well as ALDH1 ($P = 0.0004$, $r = 0.3229$, **F**).

and PD-L1, VISTA, and B7-H4 were statistically related. In conclusion, it is further demonstrated that FAM3C may be similar to PD-L1, VISTA and B7-H4 and closely related to EMT and CSCs in OSCC; however, the molecular mechanism for these associations warrants further research.

5. Conclusion

In summary, in this study, we determined that the FAM3C is over-expressed in OSCC and can serve as an indicator of poor prognosis. In addition, the statistical correlation between FAM3C and PD-L1, VISTA, B7-H4, Slug, SOX2 and ALDH1 may indicate that FAM3C plays a critical role in EMT and the regulation of CSCs in OSCC. However, functional studies are warranted in future research.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.01.019.

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