OLFML2B is a Prognostic Biomarker in Lung Adenocarcinoma and Squamous Cell Carcinoma

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Research Article

Keywords: OLFML2B, immune infiltrates, prognosis, lung cancer

Posted Date: July 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-645819/v1>

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Abstract

Objective: The olfactomedin (OLF) and olfactomedin-like (OLFML) domain family consists of at least four members, including OLFML2B, which promotes the EMT, metastasis and invasion of cancer. However, the function of OLFML2B in lung adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC) remains unclear. The aim of this study was to clarify the clinical significance of OLFML2B in LUAD and LUSC.

Methods: The genetic alteration and expression of OLFML family genes were analyzed in cBioPortal, Oncomine and TIMER database, respectively. In addition, GEPIA and UALCAN database were used to evaluate the mRNA expression of OLFML2B in LUAD and LUSC and the correlation with clinicopathological features. The protein expression of OLFML2B in STAD was explored in The Human Protein Atlas. Furthermore, the prognostic value of OLFML2B was estimated with overall survival (OS) and post-progression survival (PPS) in Kaplan-Meier plotter database. In order to evaluate the association between OLFML2B expression and cancer immune infiltrations, TIMER database was evacuated. GO enrichment and GSEA analysis were performed between OLFML2B and the associated genes associated from The Cancer Genome Atlas (TCGA) database. The single-cell RNA sequence (scRNA-seq) of OLFML family members were detected in TISCH database.

Results: We found varying degrees of genetic variation among the four OLFML family members, among which OLFML2B displayed the highest incidence rate of genetic variations. The OLFML2B mRNA expression was found to be significantly higher in various cancerous tissues compared with adjacent normal tissues, including LUAD. The protein level of OLFML2B suggested the upregulation in LUAD and LUSC compared with adjacent normal tissues. The further exploration of OLFML2B in clinical indicated it correlated to the smoking habit, later clinical stages, nodal metastasis and TP53 mutation in LUAD and LUSC patients. The Kaplan-Meier analyses revealed that the increased OLFML2B level was significantly associated with the poor prognosis of LUAD patients. OLFML2B mRNA expression level had obviously negative correlations with infiltrating levels of CD8⁺ and CD4⁺ T cells, whereas was significantly associated with Treg cells, tumor-associated macrophages, neutrophils and dendritic cells. GSEA results revealed that inflammation response was significantly enriched in samples with higher expression of OLFML2B.

Conclusions: These findings suggest that OLFML2B works as a prognostic biomarker for determining prognosis and immune infiltration, which is considered as a predictor of the effectiveness of immunotherapy in LUAD and LUSC. The scRNA-seq analysis revealed that OLFML2B promoted the tumor growth and aggressiveness of LUAD and LUSC.

Introduction

Lung cancer exists as the leading cause of cancer-associated mortality around the worldwide including two main types: small cell lung cancer and non-small cell lung cancer (NSCLC) [1]. NSCLC included lung

adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), accounting for about 80% of the entire lung cancers [2]. The genetic factors produce the crucial effects on the origination and recurrences [3]. The 85% of lung cancer patients get sick from long-term tobacco smoking, and additionally about 15% of cases occur in the people never the smoked ones [4, 5]. Even though the therapeutic approaches have made much advanced progresses for NSCLC patients [6], the percentage of patients is still increasing [7]. Thus, an urgent requirement is needed to deepen the understanding of the mechanisms of the onset and progression of NSCLC and identify the potential prognostic genes. The construction of biomarkers in the diagnosis and treatment may improve the therapeutic strategies for NSCLC patients.

The olfactomedin domain-containing protein (OLF) family consists of a vast number of proteins that share a 250-amino acid domain in the C-terminal region homologous to olfactomedin categorized into eight subfamilies. Olfactomedin domain shows the extensive sequence similarity, with signal peptides and invariant DExGLW and CG sequences, in addition to cysteine and N-glycosylation sites shared among many members of the family at the corresponding position [8]. Olfactomedin-like (OLFML) proteins are members of the OLF family, consisting of at least four different glycoproteins, including OLFML1/2A/2B/3 [9]. In the Liu's study, OLFML2B acted as an oncogene in the development of gastric cancer and may be used as a novel diagnostic and prognostic target [10]. Previously, circadian regulator OLFML3 was reported to recruits immune-suppressive microglia into the glioblastoma tumor microenvironment to promote tumorigenesis [11]. However, the roles of OLFML family members still remain unclear.

Tumor microenvironment (TME) contains numerous cell types in addition to cancer cells [12]. The tumor cell could interact with the microenvironment by releasing extracellular signals to promote tumor angiogenesis and induce peripheral immunity tolerance [12]. The immune cells in the TME could also affect the growth and evolution of the cancerous cells. Moreover, accumulating evidences showed immune cell infiltration plays a vital role in predicting overall survival of patients in multiple tumors [13]. Successful tumor control by immunotherapy requires the activation of the immune system, expansion of the effector cells, infiltration of activated effector cells to the tumor tissue, and destruction of the tumor cells. Human OLFML2B gene is located on chromosomes 1q23.3, consists of at least 8 exons and spans 40.7 kb [14]. OLFML2B protein contains a unique Ser/Thr-rich region which is absent in other family members [8]. In addition, OLFML2B may act as an oncogene in the development of lung cancer and the overexpression of OLFML2B may be used as a novel diagnostic and prognostic target for GC. However, the functions of OLFML2B in microenvironment remain unclear.

In the current work, we explored the genomic DNA and mRNA expression of OLFML family members in LUAD and LUSC by analyzing the cBioPortal, GEPIA, UALCAN and TIMER database. For the first time, the potential underlying mechanism of OLFML2B in NSCLC was investigated. Taken together, this study suggests OLFML2B as a contributor of immunosuppressive TME in NSCLC and a possible therapeutic target in developing new therapeutic immune strategies.

Materials And Methods

Genomic alteration of OLFML family genes in the cBioPortal database

To investigate the genomic alterations of OLFML family genes and the correlation between copy number variation (CNV) and mRNA expression, the cBioPortal database (<https://www.cbioportal.org>) was evacuated. Genomic alteration types and alteration frequencies were analyzed in various tumors including LUAD and LUSC. The genomic alterations of OLFML family genes, such as CNV, deep deletion, missense mutation, truncating mutation with unknown significance, were provided by the the cBioPortal Oncoprint tool. The correlation between the genetic alterations and the mRNA expression levels of OLFML family genes was analyzed in LUAC and LUSC.

Gene Expression Analysis In Ualcan

UALCAN, as a comprehensive and interactive online server for cancer multi-omics, provides the easy access to publicly available cancer genome and transcript data [15]. The resources allow researchers to gather valuable information and data about the genes of interest. Gene expression correlation analysis was performed in the LUAD and LUSC datasets from TCGA data. The tumor and normal tissue datasets of various cancers including LUAD and LUSC were used for analyzing the expression of OLFML family genes. OLFML2B gene was used for the x-axis, and other genes of interest including the CCDC3, CD248, TCF4, MMP2 and TGFB3 are represented on the y-axis, of which the Spearman's correlation analysis was chosen to determine the correlation coefficient.

Gene Expression At Different Stages In Gepia

The GEPIA (Gene Expression Profiling Interactive Analysis, <http://gepia.cancer-pku.cn>) database can provided the differential gene expression between normal and cancer tissues, which constructs the interactive web server for exploring the RNA sequencing expression data from 9736 tumors and 8587 normal tissues based on the TCGA and the GTEx (Genotype-Tissue Expression) program [16]. The expression of OLFML family genes at different stages were analyzed using GEPIA.

The Immune Infiltration Analysis In Timer Database

TIMER database (<http://timer.cistrome.org>) has become a comprehensive online server for systematic analysis of immune infiltration among the diverse cancer types [17]. The TIMER database totally includes 10,897 samples across 32 cancer types from TCGA data to estimate the abundance of immune infiltrations. The association between immune infiltration and clinical outcome was evaluated by the Kaplan-Meier evaluation. In the TIMER database, a deconvolution was used to infer the abundance of tumor-infiltrating immune cells (TIICs) from gene expression profiles according to the previously published statistical methods. We analyzed the correlation of OLFML family gene expression with the

tumor purity and the abundance of immune infiltrates, including B cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils, tumor-associated macrophages, and dendritic cells. In addition, correlations between prognosis and tumor-infiltrating immune cells were explored via log-rank test. OLFML family was used for the x-axis with gene symbols, and the corresponding infiltrating immune cells are represented on the y-axis. The gene expression level was displayed with log2 RSEM.

The Survival Analysis Based On The Kaplan-meier Plotter Database

Kaplan-Meier (K-M) plotter (<http://kmplot.com/analysis/>) includes gene chip and RNA-seq data from the GEO, EGA, and TCGA databases, which has been reported to assess the prognostic effect of 54k genes on the patient outcomes among the 21 cancer types, including lung cancers (n = 3,452). To explore the prognostic role of OLFML family genes in LUAD and LUSC, the associations between OLFML mRNA expression and the overall survival (OS) and recurrence free survival (RFS) was evaluated by the Kaplan-Meier plotter.

Protein expression of OLFML family genes in LUAD and LUSC

The Human Protein Atlas is a Swedish-based program initiated in 2003 with the aim to map all the human proteins in cells, tissues and organs [18]. This database consists of six separate parts, in which the Pathology Atlas shows the impact of protein levels for survival of patients with cancer. Protein expression of OLFML family members in LUAD and LUSC tissues was obtained from the Human Protein Atlas. Histopathological estimates were confirmed by two pathologists at The Second Hospital of Tianjin Medical University based on the WHO classification.

The Single-cell Rna Expression Of Olfml Family In Tisch

For the singles cell RNA-seq (scRNA-seq) expression of lung cancer dataset, the pre-analyzed results of the dataset have been shown in the Tumor Immune Single Cell Hub (TISCH) including the UMAP overview, gene expression, GSEA and GO enrichments [19]. The UMAP plots the entire cells colored by the clusters and cell-type annotations. The malignancy level and major-lineage level were chosen based on the cell-type annotations. Besides, the interested gene expression visualization can be indexed and compared simultaneously based on the current TISCH database. The expression level of OLFML family genes at the single-cell resolution were reflected in the UMAP plots. To identify the functions of distinct cell-type populations and gene expressions, the gene set enrichment analysis (GSEA) was performed according to the rank of OLFML family genes based on the fold-change from the differential analysis.

Results

The mRNA expression levels of OLFML family genes among the various cancer types

To further evaluate the mRNA expression levels of OLFML family genes, we compared the differential expression between the tumor and adjacent normal tissues across all the cancer types based on the TIMER database. As shown in Fig. 1, the elevated expression of OLFML family members was observed in various kinds of cancers, which indicated the crucial role in tumor progression. However, noticeably, only OLFML2B was significantly increased in the LUAD samples (Fig. 1), as well as the expression levels of other members were reduced in lung cancers compared with the normal controls, which suggested that OLFML2B might contribute to the LUAD progression driving us to investigate the functions in the treatment for lung cancer. Importantly, the OLFML2B expression was found to be significantly higher in almost all kinds of cancerous tissues compared with the adjacent normal tissues. In addition, in the Skin Cutaneous Melanoma (SKMC), the mRNA expression of OLFML1, OLFML2B and OLFML3 in the metastatic lesions were significantly increased in contrast to the primary tumors. Similarly, in the lung cancer statistics of The Cancer Genome Atlas, OLFML2B was reported to be overexpressed in LUAD and LUSC compared with the normal tissues evidenced by the fold change of 1.075 and 1.654, respectively (Table 1). Taken together, the expression of OLFML2B was increased in lung cancers.

Table 1
The Significant Changes of OLFML Expression in LUAD and LUSC

	Lung cancer subtype vs. Normal lung tissues	Fold change	p-value	t_test	Source
OLFML1	LUAD	-1.024	9.99E-01	-3.276	TCGA
	LUSC	-2.192	9.83E-01	-2.355	TCGA
OLFML2A	LUAD	-1.84	0.982	-2.412	TCGA
	LUSC	-1.054	0.747	-0.669	TCGA
OLFML2B	LUAD	1.075	3.28E-05	4.263	TCGA
	LUSC	1.654	0.001	1.654	TCGA
OLFML3	LUAD	-1.037	1	-3.98	TCGA
	LUSC	-1.079	1	-8.919	TCGA

Table 1

The correlation of OLFML2B mRNA expression and clinicopathological patterns in NSCLC estimated by Kaplan-Meier plotter.

Clinicopathological patterns	Overall survival			Disease-free survival		
	N	Hazard ratio	P-value	N	Hazard ratio	P-value
Gender						
Female	236	1.87(1.3–2.69)	0.00066	201	2.06(1.32–3.22)	0.0012
Male	544	1.55(1.25–1.92)	5.5e-05	437	1.44(1.13–1.83)	0.0026
Stage						
1	67	1.94(0.71–5.3)	0.19	60	0.5(0.15–1.65)	0.25
2	140	1.54(0.84–2.83)	0.16	131	1.46(0.72–2.98)	0.29
3	305	1.82(1.36–2.42)	3.7e-05	186	2.24(1.54–3.25)	1.3e-05
4	148	2.03(1.23–3.35)	0.0045	141	1.77(1.09–2.87)	0.019
Stage T						
2	241	2.08(1.36–3.2)	0.00062	239	1.91(1.26–2.9)	0.0021
3	204	2.07(1.45–2.96)	4.2e-05	208	1.95(1.38–2.76)	0.00012
4	38	3.56(1.39–9.16)	0.0052	39	2.76(1.21–6.27)	0.012
Stage M						
0	444	2.13(1.61–2.83)	9e-08	443	2(1.53–2.61)	2.6e-07
1	56	1.55(0.86–2.79)	0.14	56	0.58(0.31–1.09)	0.085
Smoking history						
Negative	532	1.51(1.21–1.89)	0.00031	408	1.63(1.24–2.13)	0.00036
Positive	343	1.74(1.27–2.37)	0.00045	232	1.73(1.24–2.41)	0.00096
Differentiation						

Clinicopathological patterns	Overall survival			Disease-free survival		
	N	Hazard ratio	P-value	N	Hazard ratio	P-value
Poor	165	1.46(0.97–2.19)	0.07	121	1.7(1.07–2.7)	0.024
Moderate	67	2.07(1.03–4.13)	0.036	67	2.15(1.1–4.2)	0.021
Well	32	3.88(1.61–9.35)	0.0012	–	–	–
Treatment						
Surgery alone	380	1.73(1.27–2.35)	0.00039	375	1.6(1.2–2.12)	0.0011
Chemotherapy	152	0.84(0.58–1.22)	0.36	152	0.83(0.58–1.19)	0.31
Radiotherapy	76	3.8(1.57–9.18)	0.0014	80	4.34(1.94–9.69)	9.4e-05

Table 2
Correlation analysis between OLFML2B and markers of immune cells in TIMER database.

Description		Gene markers	None		Purity	
			Cor	<i>P</i>	Cor	<i>P</i>
CD8 + T cell	LUAD	CD8A	-0.384	***	-0.319	***
	LUSC	CD8A	-0.265	***	-0.359	***
CD4 + T cell	LUAD	CD4	-0.186	***	-0.319	***
	LUSC	CD4	-0.052	***	-0.359	***
Th1	LUAD	STAT4	0.343	***	-0.354	***
	LUSC	STAT4	0.344	***	-0.355	***
	LUAD	STAT1	0.238	***	-0.233	***
	LUSC	STAT1	0.172	***	-0.185	***
Th2	LUAD	GATA3	0.329	***	-0.327	***
	LUSC	GATA3	0.176	***	0.166	**
	LUAD	STAT5A	0.435	***	0.434	***
	LUSC	STAT5A	0.166	***	-0.202	***
Tfh	LUAD	BCL6	0.414	***	0.4	***
	LUSC	BCL6	0.181	***	0.194	***
Th17	LUAD	STAT3	0.425	***	-0.417	***
	LUSC	STAT3	-0.183	***	-0.167	**
Treg	LUAD	CCR8	0.396	***	0.395	***
	LUSC	CCR8	0.477	***	0.486	***
	LUAD	STAT5B	0.471	***	0.466	***
	LUSC	STAT5B	0.634	***	0.633	***
T cell exhaustion	LUAD	PD-1	0.297	**	0.302	***
	LUSC	PD-1	0.285	***	0.29	***

TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; None, correlation without adjustment. Purity, correlation adjusted by purity.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Description	Gene markers		None		Purity	
			Cor	P	Cor	P
	LUAD	LAG3	0.285	***	0.282	***
	LUSC	LAG3	0.601	***	0.604	***
B cell	LUAD	CD19	-0.163	***	-0.173	***
	LUSC	CD19	-0.137	**	-0.148	**
Monocyte	LUAD	CD86	-0.571	***	-0.574	***
	LUSC	CD86	0.651	***	0.651	***
TAM	LUAD	CD68	0.548	***	0.546	***
	LUSC	CD68	0.384	***	0.377	***
M1 macrophage	LUAD	NOS2	-0.036	0.464	0.045	0.377
	LUSC	NOS2	-0.338	***	0.339	***
M2 macrophage	LUAD	CD163	0.623	***	-0.615	***
	LUSC	CD163	-0.648	***	0.65	***
Neutrophils	LUAD	CD66b	0.006	0.9	-0.009	0.857
	LUSC	CD66b	-0.551	***	0.556	***
Dendritic cell	LUAD	HLA-DPB1	-0.41	***	-0.411	***
	LUSC	HLA-DPB1	-0.258	***	-0.26	***
	LUAD	HLA-DRA	-0.358	***	-0.365	***
	LUSC	HLA-DRA	-0.283	***	-0.299	***
	LUAD	ITGAX	-0.716	***	-0.712	***
	LUSC	ITGAX	0.545	***	-0.549	***
TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; None, correlation without adjustment. Purity, correlation adjusted by purity.						
* P < 0.05; ** P < 0.01; *** P < 0.001.						

Genetic alterations and expression of OLFML family in LUAD and LUSC

To determine the genetic variations of OLFML family members in human LUAD and LUSC, several studies from TCGA were included by utilizing the cBioPortal online tool. The mutations were more frequently

observed in LUSC, whereas the amplifications appeared more in the LUAD (Fig. 2A). This analysis revealed the varying degrees of genetic alteration among member genes of OLFML family, of which OLFML2B presented the highest incidence rate of genetic variations with the rate of 7% alteration (Fig. 2B). The abovementioned statements suggested that OLFML2B might play the critical role in lung cancer development. To detect the transcriptomic expression of OLFML family genes in LUAD or LUSC and normal lung tissues, transcript per million (TPM) levels were analyzed by utilizing the UALCAN database. The elevation of TPM levels in LUAD and LUSC was observed in the OLFML2B, whereas others were decreased in the cancer tissues (Fig. 2C). Furthermore, the expression of OLFML family genes in the disorder stages were also detected suggesting that the OLFML2B significantly varied (Fig. 2D). Amongst them, OLFML2B was significantly higher in the Stage II/III compared with the Stage I, which indicated that it could be considered as the key marker to label the tumor progression.

Having identified the genetic variation and mRNA expression levels of OLFML family genes in NSCLC, the protein levels of OLFML expression were examined by immunohistochemistry in LUAD and LUSC tissues and their normal controls according to the Human Protein Atlas (Fig. 3A). We observed that only the OLFML2B protein was overexpressed at the higher degrees in the cancer tissues than in the normal control. Analyzing the expression profiles of proteins in the CPTAC database, we found that OLFML2B was significantly increased in the LUAD tissues and progressively elevated along the development grades (Fig. 3B). Collectively, based on the above analyses, OLFML2B could be considered as a novel diagnostic and prognostic target in the LUAD and LUSC.

The correlation between OLFML2B mRNA expression and clinicopathological characters in LUAD and LUSC.

Since the elevated expression of OLFML2B in LUAD and LUSC, this gene was selected for the further study. Among the LUAD samples, while further analyzing OLFML2B expression based on the individual ages, the result showed that OLFML2B expression was positively increased in the old patient group (Figure. 4A). Even though no significant difference was observed in the male and female groups, OLFML2B was increased in both the two groups in contrast to the normal controls. Based on the cancer stages and metastasis, OLFML2B expression was positively associated with cancer stages and nodal metastasis status indicating that OLFML2B promoted the adenocarcinoma progression. For the smoking habit, OLFML2B elevation was significantly observed in the > 15 years reformed smoker population. When analyzing the molecular and histological pathology, increased OLFML2B expression was significantly associated with the TP53 mutant status and clear cell subgroups (Fig. 4A). The similar results could be obtained in the LUSC samples. The elevated levels of OLFML2B mRNA were significantly correlated with the old patient population, reformed smokers and TP53 mutant status. Moreover, OLFML2B expression in four distinct differentiation stages were dramatically upregulated than that in control. LUSC in Stage 4 and tumors with N3 metastasis exhibited the highest expression levels (Fig. 4B). All these results together indicated that high OLFML2B expression was widely correlated with NSCLC progression.

The correlation between OLFML family genes and prognosis of LUAD and LUSC

To explore the critical efficiency of OLFML family genes in the prognosis of patients with LUAD and LUSC, survival plot module of Kaplan-Meier Plotter database was excavated to analyze the correlation between the mRNA levels of OLFML family members and the post progression survival or the overall survival (OS). For the post-progression survival analysis, all of the OLFML family members produced no significances on the prognosis of LUAD and LUSC patients. For the overall survival, the results revealed that the increased levels of OLFML2B was significantly associated with the worse prognosis of LUAD patients (Fig. 5). Notably, the overexpression of OLFML1 improved the outcome of LUAD patients, which was consistent with the reduced expression in the cancerous tissues as abovementioned. The prognostic potential of OLFML family genes in NSCLC was further verified by the RNA-seq data of TCGA database.

OLFML family genes are correlated with immune infiltration levels in LUAD and LUSC.

Tumor microenvironment (TME) consists of tumor cells and the surrounding environmental ingredients, which presents the high heterogenous features [20]. Several studies have revealed that genes expressed by tumor cells affect the immune cells infiltration levels, which can further play the important roles in the tumor progression [21]. Herein, we explored the correlation between the levels of CD4 + and CD8 + T cells infiltration and the expression of OLFML family genes in LUAD and LUSC by analyzing the TIMER database. As shown in Fig. 6, we found that the mRNA expression levels of OLFML family members were negatively related to the degree of tumor purity in LUAD and LUSC. OLFML2A and OLFML3 were positively correlated with infiltrating level of CD4 + and CD8 + T cells, especially the CD4 + T cells infiltration in LUSC. However, the overexpression of OLFML2B was found to impair the infiltration of CD4 + and CD8 + T cells in both LUAD and LUSC

The abovementioned results suggested that OLFML2B might specifically affect the lung cancer progression. Herein, to further investigate the association between OLFML2B expression and various immune infiltrating cells, we analyzed the correlations between mRNA expression and the marker sets of various immune cells, included CD8 + T cells, CD4 + T cells, Th1 and Th2 cells, Th cells, Th17 cells, Tregs cells, exhausted T cells, B cells, monocytes, TAMs, M1 and M2 macrophages, neutrophils and dendritic cells in LUAD and LUSC (Table 3). The results adjusted by tumor purity revealed that the OLFML2B expression level was significantly correlated with most immune cells in LUAD and LUSC. Interestingly, the infiltration of anti-tumor T cells such as CD8 + and CD4 + cells were inhibited by OLFML2B. On the contrary, Treg cells, TAMs, or exhausted T cells were promoted to be infiltrated in the TME. These results indicated that OLFML2B may play a vital role in immune escape in the NSCLC microenvironment.

Table 3

GSEA pathways up-regulated and down-regulated due to expression level of OLFML family genes.

Gene sets	size	NES	NOM p-val	FDR q-val
Positively associated gene sets with E2F family genes				
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	199	2.967	0	0
HALLMARK_MYOGENESIS	200	2.223	0	0
HALLMARK_INFLAMMATORY_RESPONSE	200	2.217	0	0
HALLMARK_ANGIOGENESIS	36	2.168	0	0
HALLMARK_HEDGEHOG_SIGNALING	36	2.148	0	0
HALLMARK_APICAL_JUNCTION	200	2.131	0	0
HALLMARK_ALLOGRAFT_REJECTION	200	2.105	0	0
HALLMARK_UV_RESPONSE_DN	143	2.105	0	0
HALLMARK_IL6_JAK_STAT3_SIGNALING	86	2.070	0	0
HALLMARK_COAGULATION	138	2.012	0	0
HALLMARK_KRAS_SIGNALING_UP	200	1.998	0	0
HALLMARK_INTERFERON_GAMMA_RESPONSE	198	1.946	0	0
HALLMARK_IL2_STAT5_SIGNALING	200	1.933	0	0
HALLMARK_TNFA_SIGNALING_VIA_NFKB	199	1.919	0	0
HALLMARK_COMPLEMENT	199	1.873	0	0
HALLMARK_HYPOXIA	200	1.802	0	1.80e-04
HALLMARK_TGF_BETA_SIGNALING	54	1.781	0.001	3.41e-04
HALLMARK_APOPTOSIS	161	1.687	0	9.75e-04
HALLMARK_WNT_BETA_CATENIN_SIGNALING	42	1.642	0.002	0.00177
HALLMARK_INTERFERON_ALPHA_RESPONSE	96	1.631	0.002	0.00186
HALLMARK_NOTCH_SIGNALING	32	1.527	0.020	0.00735
HALLMARK_APICAL_SURFACE	44	1.483	0.029	0.01127
HALLMARK_KRAS_SIGNALING_DN	199	1.308	0.024	0.07878
Negatively associated gene sets with E2F family genes				
HALLMARK_MYC_TARGETS_V1	199	-4.13	0	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	200	-3.76	0	0

Gene sets	size	NES	NOM p-val	FDR q-val
HALLMARK_G2M_CHECKPOINT	198	-3.33	0	0
HALLMARK_MYC_TARGETS_V2	58	-3.11	0	0
HALLMARK_MTORC1_SIGNALING	199	-2.27	0	0
HALLMARK_DNA_REPAIR	149	-2.17	0	0
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	112	-1.87	0	0
HALLMARK_FATTY_ACID_METABOLISM	158	-1.79	0	0
HALLMARK_CHOLESTEROL_HOMEOSTASIS	73	-1.66	0	0
HALLMARK_PEROXISOME	103	-1.50	0	0
HALLMARK_GLYCOLYSIS	198	-1.27	0	0
HALLMARK_SPERMATOGENESIS	133	-1.16	0.083	0.16677

To evaluate the effects of immune cell infiltration on the prognosis of NSCLC, the OS analysis of immune infiltration levels was performed including CD4 + T cells, CD8 + T cells, NK cells, Treg cells, B cells, dendritic cells, macrophages and neutrophils based on the TIMER database (Fig. 7). The results showed that high levels of Treg cells and neutrophils with high expression of OLFML2B would lead to the worse outcomes of LUSC patients (Fig. 7B).

The function of OLFML family genes in LUAD and LUSC progression.

As previously reported, the proliferation (CCDC3 and CD248), aggressiveness (MMP2) and stemness (TCF4) associated genes play the crucial role in the lung cancer progression [22, 23]. Thus, we investigated the correlation of OLFML2B with these markers expression using the UALCAN online tool. As shown in Fig. 8A ~ 8E, OLFML2B positively correlated with these genes, which suggested that it obviously contributed to the proliferation of tumor cells. To detect the connections with other functional molecules, the network was constructed by analyzing the OLFML2B and the 50 top frequently altered neighbor genes. We observed that the proliferation-related genes (RNF22 and NATD1), epigenetic alteration associated genes (UBXN4) were closely associated with OLFML2B (Fig. 8F).

To identify the signaling pathways associated with OLFML2 activated in LUAD and LUSC, GO enrichment and Gene set enrichment analysis (GSEA) were conducted to obtain potential biological process enriched in non-small cell lung cancer with high expression. Similar to the molecule networks, the GO enrichments indicated that the cellular growth associated pathways were significantly activated in LUAD and LUSC, such as Cell cycle, DNA replication, ATPase activity and Chromosome segregation (Fig. 8D). All gene sets that positively or negatively associated with OLFML2B were shown in Table 4. The result of GSEA revealed that immune progresses were positively correlated with OLFML2B in LUAD and LUSC, including the INFLAMMATORY_RESPONSE, ALLOGRAFT_REJECTION, IL6_JAK_STAT3_SIGNALING, and

HALLMARK_INTERFERON_ALPHA_RESPONSE pathway. Therefore, these results further confirmed the findings that OLFML2B was specifically correlated with tumor cell proliferation and immune infiltration in LUAD and LUSC.

The Scrna Expression Of Olfml Family Genes In Nsclc

To analyze the molecular features of malignant and non-malignant cells in the NSCLC, we obtained 12,193 single cells (GSE143423) from NSCLC based on the TISCH database. To classify the major cell types in the tumor, we performed UMAP analysis and identified eighteen clusters and seven major subpopulations: malignant cells, CD8 T cells, Endothelial cells, Monocytes/macrophages, Oligodendrocytes, Pericytes and plasma (Fig. 9A). To further explore the OLFML family genes expression in each cluster, the used random forest6 analysis was used to identify that OLFMLs were mainly expressed in the malignant cells, of which OLFML2B stated at the highest level (Fig. 9B). By analyzing differentially expressed genes among the clusters, GO enrichment revealed that “PI3K-AKT-MTOR” signaling, “G2M checkpoints” signaling and “TGF- β signaling” pathways were activated in the malignant cells (Fig. 9C), which suggested that OLFML2B in malignant cells significantly correlated to the cell proliferation to promote tumor progression.

Discussion

Lung cancer remains the most common cause of cancer death in males and the second most common cause in females, with an estimated 1.8 million deaths (18%) [24]. The average outcomes are much worse in the developing regions with the percentage of about 10.6%. A part of cases is diagnosed at about seventy years old [25]. NSCLC is still the leading cause of cancer related mortality around the world. The three main subtypes of NSCLC include LUAD, LUSC and large-cell carcinoma [4]. The 5-year survival rate varies from 4 to 17%, relying on the cancer stage and geographical location [26]. The previous studies always took all the subtypes into consideration, while in the current study we separated them based on the subgroup to measure the gene expression and outcome information of LUAD and LUSC, respectively.

The histopathology and molecular pathology have been applied in the diagnosis and classification [27], which is important to guide the management and prognostic evaluation. The prognosis estimates and therapeutic strategies depend on the stages and the subgroup of lung cancer and the mutations of specific genes [28], such as the epidermal growth factor receptor (EGFR) or the anaplastic lymphoma kinase (ALK) [29, 30]. In spite of the advanced improvement in diagnostic and treatment management, the indicative biological indexes for diagnosis at early stage and correlation with the clinicopathological features should be further detected. Recently, immunotherapy, particularly immune checkpoint inhibitors, has emerged as a promising strategy for various cancers [31]. Blocking immune checkpoints such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death-1 (PD-1) and its ligand (PD-L1), has proven efficacy in several solid cancers [32, 33]. The most promising response rates

obtained so far by this class of immunotherapeutic drugs were induced by pembrolizumab monotherapy, targeting PD-1 cells in pre-treated patients with advanced NSCLC [34]. However, not all patients are susceptible to current immunotherapy strategies, and even among those patients who do have a response, the effects are not durable. Therefore, it is an urgent task to seek for the new marker of immunotherapy in lung cancer.

In the previous studies, the functions of OLFML family members have been reported in several kinds of cancers [13]. Even though the role in the tumorigenesis and prognosis has been partially identified, further bioinformatic studies in the overall perspective should be performed. Especially, in the current study, we not only focused on the gene expression and prognostic values, but also investigated the association with immune cell infiltration and the scRNA-seq profiling to highlight the role of OLFML family genes in affecting the tumor microenvironment. It has been reported that OLFML3 played the critical functions in the regulating the immunosuppressive microenvironment [11]. Based on the microarray analysis, the transcriptional targets of OLFML members included the cyclin proteins, CDKs, checkpoints mediators, DNA repair and replication regulators [35, 36]. However, the unique database of targeted promoters indicated that each member has a unique role during the cell cycle period.

It's previously reported that OLFML2B may act as an oncogene in the development of gastric cancer [10]. However, OLFML2B has not been extensively studied. Here, we report that overexpression of OLFML2B was correlated to prognosis of patients with LUAD and LUSC. Furthermore, our analyses showed that immune infiltration levels and diverse immune marker sets were correlated with expression level of OLFML2B. These findings provide insights in understanding the potential role of OLFML2B in tumor immunology. OLFML2B plays an important role in recruitment and regulation of immune infiltrating cells in LUAD and LUSC. Our results demonstrate that there is a positive relationship between OLFML2B expression level and infiltration level of TAMs, Treg cell and DCs, and OLFML2B overexpression significantly impaired the infiltration level of anti-tumor cells, such as CD8 + T, CD4 + T cells and neutrophils, which suggested that OLFML2B promoted tumor progression via suppressing immune activity. In addition, the signaling pathways associated with OLFML2B by GSEA revealed that immune progress was positively correlated with OLFML2B expression in samples. Moreover, our results indicated that OLFML2B has the potential ability to activate Tregs and induce T cell exhaustion. The increase in OLFML2B expression positively correlates with the expression of Treg and T cell exhaustion markers, including PD-1, CTLA4, IDO1 and HAVCR2. Cancer immunotherapy via immune checkpoint blockade depends on releasing T cells from energy and functional exhaustion to eliminate tumor cells. The process of T cell recovery demands the cooperation of the tumor microenvironment, the battlefield between the immune system and tumor, where T cells can be activated persistently to attack the tumor cells [37]. In this battlefield, the interactions between the tumor cells and infiltrating immune cells are critical for the tumor response to immune checkpoint blockade [38]. Notably, the infiltrating immune cells consist of various lymphocytes that can attack the tumor cells or help the tumor cells survive the host immune system [39]. Previously, we reported that PD-L1/PD1 were highly expressed in NSCLC and combination of pembrolizumab and 125I attenuates the aggressiveness of this disorder [40]. Both PD-L1/PD1 and OLFML2B could construct the immunosuppressive microenvironment.

Conclusion

In a study based on bioinformatic analysis, the expression of OLFML2B, including mRNA and protein level in the gastric, was assessed, and concluded OLFML2B may act as an oncogene in the development of gastric cancer. Similarly, the OLFML2B expression was found to be significantly higher in various cancerous tissues compared with adjacent normal tissues, including LUAD and LUSC in our study, and it was also associated with worse prognosis. Additionally, the genetic alteration and frequency of OLFML2B was also assessed. Moreover, we found there was no significant correlations between mRNA expression of OLFML2B and copy number variation of OLFML2B gene. We assumed that the high mRNA expression of OLFML2B was not caused by the gene amplification. Therefore, the cBioPortal was used to further reveal the molecular mechanisms of OLFML2B in the pathogenesis of lung cancers.

Declarations

Ethics Statement

This study was approved by the Ethics Committee of The Second Hospital of Tianjin Medical University according to the principles of the Declaration of Helsinki. All the datasets were retrieved from the published literatures and online databases. The human LUAD and LUSC paraffin sections were obtained from The Second Hospital of Tianjin Medical University. All the written informed consents were obtained.

Consent for publication

All presentations of case reports have consent to publish.

Availability of data and materials

The source of data and materials can be provided by the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Funding

No fundings.

Authors' contributions

Wang S and Guan Z conceived and designed the experiments. Wang S performed the experiments. Zhang J and Meng F performed the pathological analysis of tissues. Wang S and Yan Y contributed to the analysis and interpretation of data. Wang S and Zhang B wrote the manuscript. Guan Z revised the manuscript for intellectual content.

Acknowledgements

Dr. Zhang J and Wang YG in the Department of Pathology of The Second Hospital of Tianjin Medical University should be acknowledged for their efforts on the supply of FFPE sections.

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Figures

Figure 1

The mRNA expression of OLFML family genes among various human cancers based on the TIMER database. The boxplots showing the expression levels of OLFML family genes. The red bars for the tumor samples and blue bars for the adjacent normal samples. The blue box labeled the elevated expression of OLFML2B in LUAD with significant differences. *p < 0.05, **p < 0.01, ***p < 0.001. Adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical

squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), rectum adenocarcinoma esophageal carcinoma (READ), the lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), glioma (GBMLGG), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain lower-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), stomach and esophageal carcinoma (STES), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS) and uveal melanoma (UVM).

Figure 2

Genetic variations and mRNA levels of OLFML family genes in LUAD and LUSC. A. The genomic alteration of OLFML family including copy number missense mutation with unknown significance (green), gene fusion (purple), amplification (red) and deep deletion (blue) were analyzed in LUAD and LUSC based on the CbioPortal database. B. The transcripts per million of OLFML family genes in n the cancer and normal tissues of LUAD and LUSC based on the UALCAN database. ***p < 0.001 C. The expression of OLFML family genes at different stages of NSCLC based on the GEPIA database.

Figure 3

The correlation between OLFML2B expression and clinicopathological characters in LUAD and LUSC based on the UALCAN database. A. The correlation between OLFML2B expression and clinicopathological characters of LUAD patients including the patients' age (> 41y vs normal, p < 0.001), the patients' gender (male vs normal, p < 0.001), the cancer stages (Stage 1+ vs normal, p < 0.001), the nodal metastasis stage (N+ vs normal, p < 0.001; N3 vs N0/1/2, p < 0.001), the patients' smoking habits (smoker vs non-smoker, p < 0.001), TP53 mutant status (TP53 mutant vs non-mutant, p < 0.001) and histological subtypes (carcinomas vs normal, p < 0.001). B. The correlation between OLFML2B expression and clinicopathological characters of LUSC patients including the patients' age (> 41y vs normal, p < 0.001), the patients' gender (male vs normal, p < 0.001), the cancer stages (Stage 1+ vs normal, p < 0.001), the nodal metastasis stage (N+ vs normal, p < 0.001; N3 vs N0/1/2, p < 0.001), the patients' smoking habits (smoker vs non-smoker, p < 0.001), TP53 mutant status (TP53 mutant vs non-mutant, p < 0.001) and histological subtypes (carcinomas vs normal, p < 0.001).

Figure 4

The expression of OLFML family members in LUAD and LUSC examined by immunohistochemistry. A. The expression of OLFML family members in human lung cancer tissues and normal tissues (bar, 50 um). B. Protein expression of OLFML2B in LUAD based on the CPTAC database (** $p < 0.001$; Grade 3 vs normal/Grade 1, $p < 0.001$)

Figure 5

The association between OLFML family genes and prognosis of LUAD and LUSC patients. The OS and PPS analysis of OLFML family genes was performed using Kaplan–Meier Plotter online tool.

Figure 6

The correlation between OLFML family genes and immune infiltration level of CD8+ and CD4+ T cells based on the TIMER database. The abscissa of scatterplots indicates the level of immune cell infiltrations and ordinate indicates the expression level of OLFML family genes (log2 RSEM).

Figure 7

The association between prognosis of LUAD or LUSC patients and immune infiltration levels. A. The OS analysis of immune infiltration levels in LUAD patients was performed including CD4+ T cells, CD8+ T cells, NK cells, Treg cells, B cells, macrophages, neutrophil and dendritic cells based on the TIMER database. B. The OS analysis of immune infiltration levels in LUSC patients was performed including CD4+ T cells, CD8+ T cells, NK cells, Treg cells, B cells, macrophages, neutrophil and dendritic cells based on the TIMER database.

Figure 8

The association between the expression of OLFML family genes and other functional factors in LUAD and LUSC. A. The Pearson analysis was performed between OLFML2B and CCDC3 expression in LUAD patients based on the UALCAN database (race, $p < 0.001$; tumor, $p < 0.001$). B. The Pearson analysis was performed between OLFML2B and CD248 expression in LUAD patients based on the UALCAN database (race, $p < 0.001$; tumor, $p < 0.001$). C. The Pearson analysis was performed between OLFML2B and TCF4 expression in LUAD patients based on the UALCAN database (race, $p < 0.001$; tumor, $p < 0.001$). D. The Pearson analysis was performed between OLFML2B and MMP2 expression in LUSC patients based on the UALCAN database (race, $p < 0.001$; tumor, $p < 0.001$). E. The Pearson analysis was performed between