Original Article

Downregulation of olfactomedin 4 expression contributes to tumorigenesis of non-small cell lung cancer

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Abstract

Background and Aim: Olfactomedin 4 (OLFM4), a member of the 'sense of smell' mediated olfactomedin-related protein family, confers resistance to glycoprotein apoptosis. This study aims to examine the correlation between OLFM4 expression and clinicopathologic data in non-small cell lung cancer (NSCLC), including the prognosis of patients.

Methods: Ninety-eight NSCLC patients from 2001 to 2013 were included in the study. OLFM4 expression was compared between lung cancer tissues and adjacent non-tumor tissues. In total, 98 and 27 specimens were used for immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (RT-PCR), respectively. The association of OLFM4 with clinicopathological parameters was evaluated using Pearson's correlation. Overall survival (OS) was evaluated by Kaplan–Meier survival analysis.

Results: IHC and RT-PCR analyses demonstrated low expression of OLFM4 in the cancer tissues ($P < 0.05$). High OLFM4 expression was positively correlated with peritumor intravascular cancer emboli ($P = 0.013$) but not associated with other clinical features, such as age, gender, tumor size, NSCLC subtype, or lymph node status ($P > 0.05$). Kaplan–Meier survival curves showed that the OS rate was not significantly associated with OLFM4 expression ($P = 0.927$).

Conclusion: High OLFM4 expression could be a potential protective factor but not a prognostic factor for the tumorigenesis of NSCLC.

Key words: Immunohistochemistry, non-small cell lung cancer, olfactomedin 4, quantitative real-time polymerase chain reaction

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As per World Health Organization estimation, in 2025, lung cancer deaths will reach 1 million in China, which would then be the top lung cancer country in the world.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, with an annual increase in its incidence. However, the progress in treatment is limited. The 5-year overall survival (OS) rate was 15%, with tumor metastasis as the main cause of death.[1] The best treatment phase is also unknown because of the current lack of means for the early diagnosis of lung cancer. Improving the prognosis of lung cancer in patients is crucial for developing a new and effective method for the early detection of lung cancer. Common tumor markers such as carcinoembryonic antigen, squamous cell carcinoma (SCC) antigen, cytokeratin fragment 21-1, and neuron-specific enolase enzyme are often used to diagnose lung cancer.[2,3] Each of these tumor markers has limited sensitivity and specificity for detecting lung cancer; therefore, combined detection of these markers could be beneficial. However, tumor markers can be increased by many benign lesions, such as benign tumor, inflammation, and degenerative diseases. Thus, further exploration of efficient and specific markers of lung cancer, particularly those for early diagnosis, is extremely important.

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Olfactomedin 4 (OLFM4, also known as hGC-1[4] and GW112[5]), confers resistance to glycoprotein apoptosis. This protein has been newly discovered in recent years to be expressed in various human normal tissues, such as in the bone marrow, stomach, small intestine, colon,[6] prostate, and pancreas.[4,5,7] However, the function of OLFM4 and its association with clinical outcomes in NSCLC remains unclear. This

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is the first study to examine the function of OLFM4 using immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (RT-PCR) at the same time in several patients. This study aims to investigate the correlation between OLFM4 expression and clinicopathologic features, including survival rate, in Chinese NSCLC patients.

MATERIALS AND METHODS

Patients and tissue sample collection

We obtained archived formalin-fixed and paraffin-embedded (FFPE) tumor tissues from 98 NSCLC patients who underwent surgery between January 2001 and December 2013 at the Affiliated Hospital of Guangdong Medical College in China. Patients with complete clinical data who underwent any form of preoperative chemotherapy and/or radiation therapy were excluded. Furthermore, none of the patients enrolled in this study suffered from any other type of cancers. Each patient with NSCLC was classified on the basis of the tumor-node-metastasis classification (TNM) of the International Union Against Cancer.[8] This study protocol was approved by the ethics committee of the Affiliated Hospital of Guangdong Medical College and a written informed consent was obtained from all subjects involved in this study.

Immunohistochemistry

Paraffin sections (3 μm) were dewaxed in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was completed in citrate solution (10 mmol/L, pH 6.0) at 100°C for 30 min. Sections were cooled down and incubated in 0.3% H_2O_2 solution for 15 min to block the endogenous peroxidase activity. The sections were rinsed thrice with phosphate-buffered saline (PBS; pH 7.4) for 5 min each and then incubated with OLFM4 antibody (1:200 dilution, ab85046, Abcam, Eugene, OR, USA) in a humid chamber at 4°C overnight. Negative controls were prepared without primary antibody. After rinsing thrice with PBS (pH 7.4) for 5 min each, the sections were incubated with secondary antibody (Dako REAL™ EnVision™/HRP, Rabbit/Mouse, Dako, Glostrup, Denmark) at room temperature for 30 min. After washing with PBS, the sections were stained with 3,3′-diaminobenzidine, counterstained with hematoxylin, and then differentiated with 0.1% hydrochloric acid alcohol. Finally, the sections were dehydrated, cleared, and mounted.[9]

Ribonucleic acid extraction and real-time RT-PCR

Total ribonucleic acid (RNA) from paraffin-embedded samples was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was conducted according to standard methods as previously described.[10] The primers selected for OLFM4[11] (Shanghai ShengGong Biological Engineering Co., Ltd., China) were as follows: Forward 5′-TAACCTGACCACCAACACGA-3′ and reverse 5′-TCCATTCTCATCCACAGCAA-3′. Expression data were normalized to the geometric mean of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[12] (Shanghai ShengGong Biological Engineering Co., Ltd., China; forward: 5′-TGGCACCCAGCACAATGAA-3′ and reverse: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′) to control the variation in expression levels. Fold changes were calculated according to the $2^{-\Delta\Delta Ct}$ method where $^{\Delta}Ct$ = ([Ct of OLFM4] − [Ct of GAPDH]) represents the threshold cycle for each transcript.

Evaluation of OLFM4 positive staining

To quantify OLFM4 protein expression, the extent and intensity of immunoreactivity were assessed and scored independently by two pathologists who were blinded to the patients' clinical data. The pathologists used a light microscope (Nikon E400, Japan) at magnifications of ×200 and ×400 with a computer-based interface. On the basis of the percentage of OLFM4-positive cells in each microscopic field of view, the extent of staining was categorized as follows: 0 (<10% positive cells), 1 (10-30% positive cells), 2 (31-50% positive cells), 3 (51-70% positive cells), and 4 (>71% positive cells). The staining intensity in the cytoplasm was also evaluated on a scale of 0 to 3 as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive).[9] Multiplying the scores for extent and intensity, we labeled group 0 as negative and the other groups as positive.

Statistical analysis of data

Differences in OLFM4 expression between NSCLC tissues and adjacent non-tumor tissues were analyzed by nonparametric Mann–Whitney *U* test. Correlations between OLFM4 expression and clinical parameters were calculated using Pearson's Chi-square test. Survival time was calculated from the first day of diagnosis to the date of last follow-up or death. Survival curves were analyzed using Kaplan–Meier curves, and the difference in survival rate was examined using the log-rank test. All statistical analyses were conducted using SPSS (Statistic Package for Social Science), version 13.0 (SPSS, Chicago, IL, USA). Statistical significance was considered at $P < 0.05$.

RESULTS

The expression of OLFM4 in the NSCLC and paired adjacent non-tumor tissues ($n = 98$) was analyzed to investigate whether or not the OLFM4 protein is dysregulated in NSCLC (from OLFM4 protein expression in the cytoplasm) [Figure 1]. The expression level of OLFM4 was significantly lower in the NSCLC tissues than in the adjacent non-tumor tissues ($P < 0.005$) [Table 1]. We conducted RT-PCR using FFPE tissues (randomly selected 27 cases from the 98 patients) to determine

the messenger RNA (mRNA) level of OLFM4. The mRNA expression level of OLFM4 was significantly lower in the NSCLC tissues than in the adjacent non-tumor tissues ($P < 0.05$) [Figure 2]. The results of RT-PCR were consistent with those of immunohistochemistry.

The clinicopathological features of 98 NSCLC cases are summarized in Table 2. We investigated the association between OLFM4 protein expression and clinicopathological characteristics of NSCLC. OLFM4 expression in NSCLC lesions was significantly correlated with peritumor intravascular cancer emboli ($P = 0.013$) but not with other clinical parameters, such as patient age, gender, tumor subtype, size, lymph node metastasis location, and TNM stage ($P > 0.05$) [Table 2].

No significant difference in survival rate was detected between the NSCLC patients from the OLFM4-positive expression group and OLFM4-negative group ($P = 0.927$) [Figure 3].

Table 1: Analysis of OLFM4 expression in NSCLC and normal tissues

OLFM4 expression	Cancer, *n* (%)	Normal, *n* (%)	χ^2	*P* value*
Negative	42 (42.86)	3 (3.06)	21.94	<0.005
Positive	56 (57.14)	95 (96.94)		

OLFM4: Olfactomedin 4, NSCLC: Non-small cell lung cancer. *P<0.05 was considered significant. Analysis of data was done by using Pearson's χ^2 test

DISCUSSION

OLFM4 expression was first observed in the extracellular matrix of the olfactory nerve epithelial cell of bullfrog. No homology was observed between the amino acid sequence and other proteins.[13,14] OLFM4 expression was detected in several tumors, including gastric cancer,[15,16] pancreatic cancer,[7] colon cancer,[6] breast cancer, lung cancer,[17] cervical cancer,[18] endometrial carcinoma,[19] and prostate cancer.[20] However, the function of OLM4 in tumorigenesis remains controversial. Luo *et al.,*[15] found that the positive expression rate of OLFM4 protein is significantly higher in gastric cancer tissues than in their corresponding normal gastric mucosa tissues. Oue *et al.,*[16] confirmed the high gene expression of OLFM4 in gastric cancer patients using the hybridization and series analysis method. Yu *et al*.,[18] showed that the gene expression level of OLFM4 increases in normal cervical tissue, cervical intraepithelial neoplasia, and cervical cancer with increasing severity in cervical lesions. Duan *et al.,*[19] observed that the protein expression level of OLFM4 increases in normal endometrium, endometrial atypical hyperplasia, and endometrial cancer with significant differences. However, Chen *et al.,*[20] confirmed that OLFM4 exerts an antitumor effect on prostate cancer. They determined that OLFM4 is highly expressed in normal prostate tissues and cells, moderately expressed in the benign hyperplasia of prostate tissues, and decreased or lacking in prostate cancer tissues and cells.

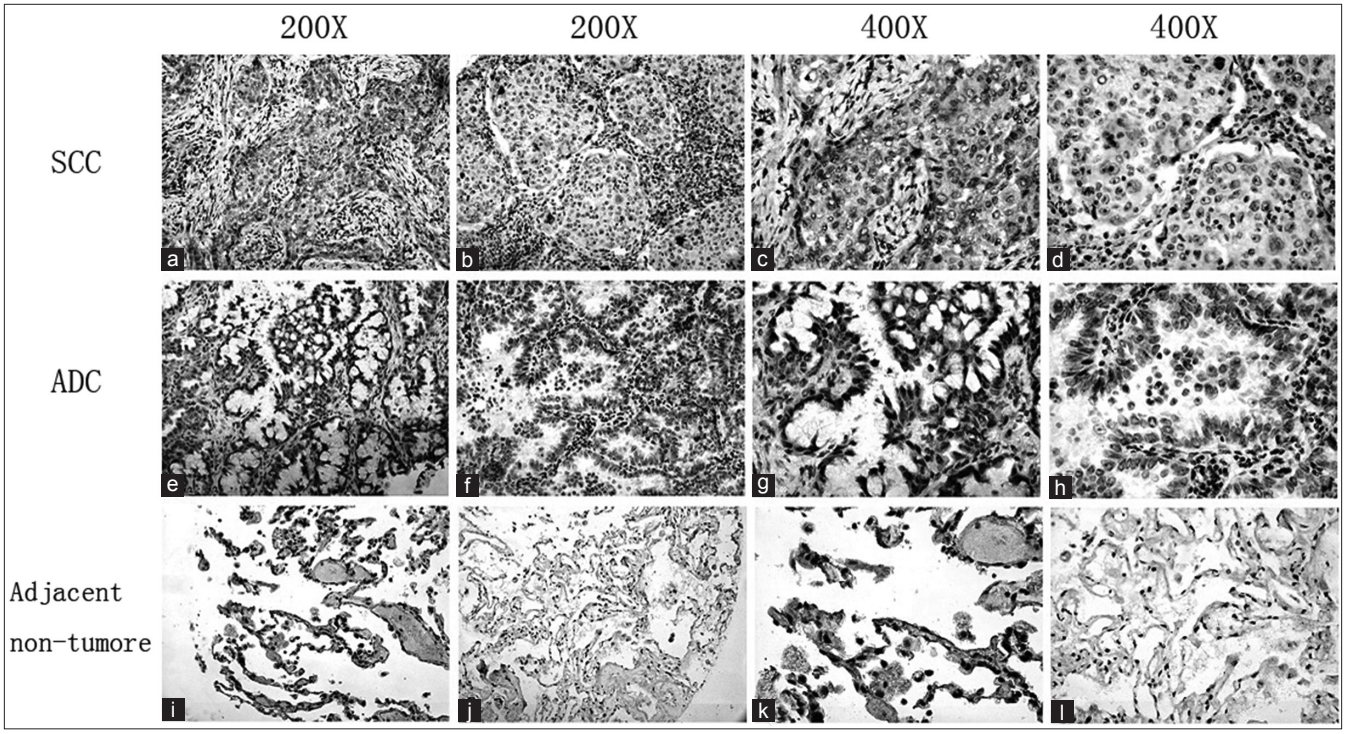

Figure 1: Immunohistochemical expression of Olfactomedin 4 in squamous cell carcinoma (SCC), adenocarcinoma (ADC), and adjacent non-tumor tissues and in the cytoplasm. Positive staining (a, c, e, g, i, and k) signal of OLFM4 was observed in SCC, ADC, and adjacent non-tumor tissues at two magnifications (×200 and ×400). Negative staining (b, d, f, h, j, and l) was observed in SCC, ADC, and adjacent non-tumor tissues at two magnifications (×200 and ×400)

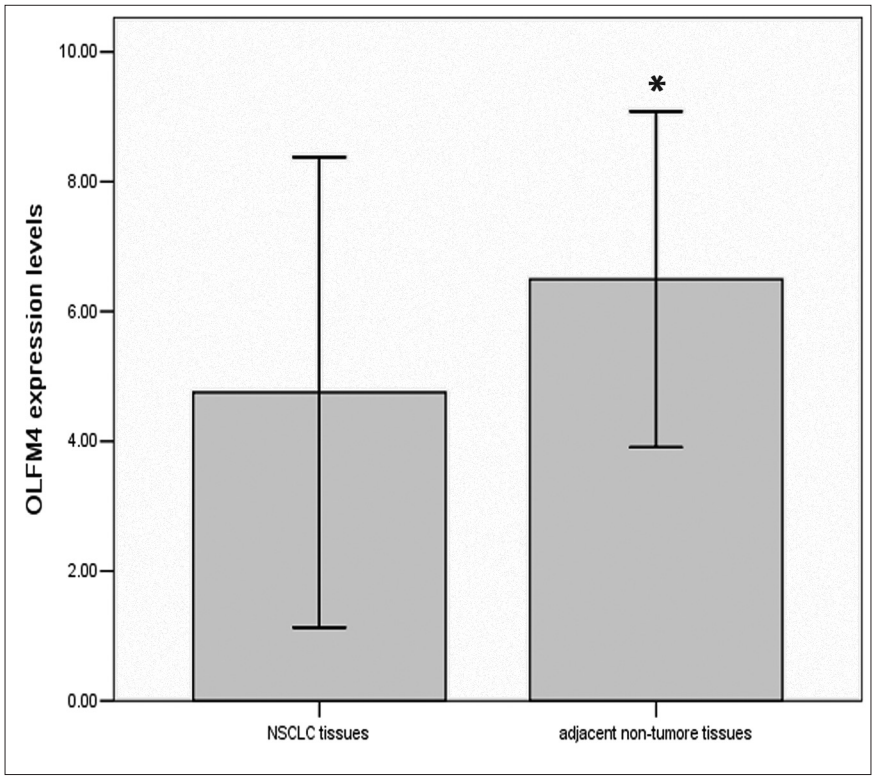

Figure 2: Real-time polymerase chain reaction (RT-PCR) analysis of olfactomedin 4 (OLFM4) expression in 27 paired non-small cell lung cancer (NSCLC) and adjacent non-tumor tissues. The median expression level was used as the cutoff. Low expression of OLFM4 in 27 patients was classified as having a $2^{-\Delta\Delta Ct}$ value < 1.0. High expression of OLFM4 in 27 patients was classified as having a $2^{-\Delta\Delta Ct}$ value > 1.0. The messenger ribonucleic acid (mRNA) expression of OLFM4 was significantly higher in adjacent non-tumor tissues than in NSCLC tissues (*P = 0.038)

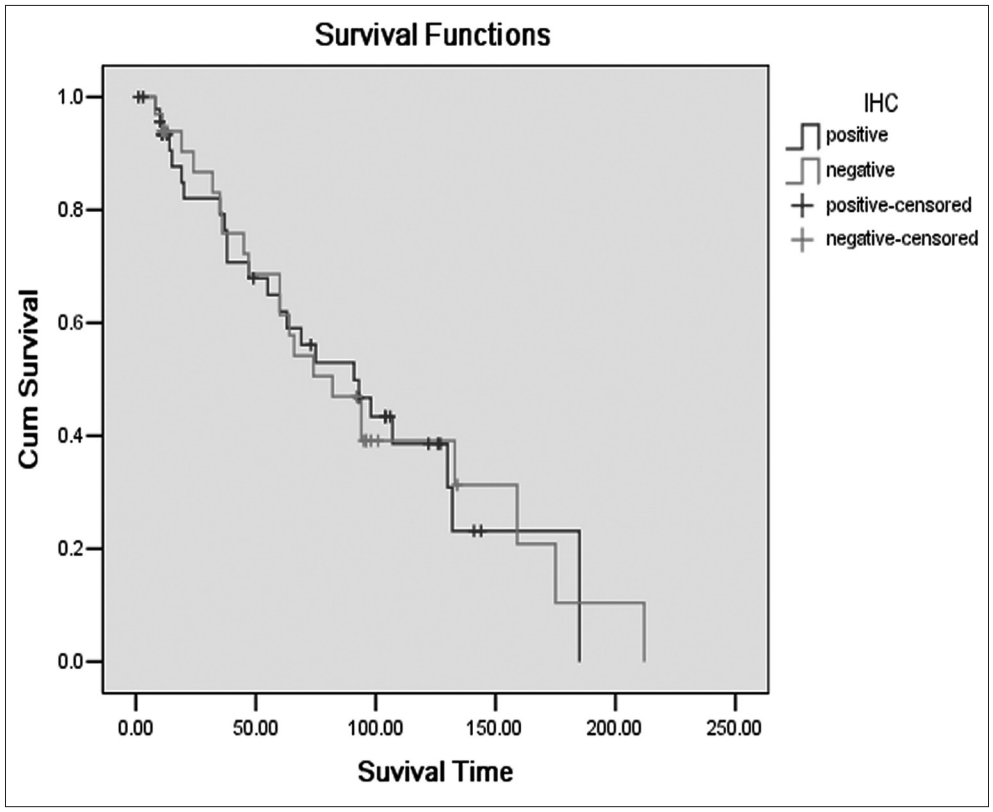

Figure 3: Kaplan–Meier survival analysis of non-small cell lung cancer (NSCLC) patients. No significant difference in survival rate was detected between the NSCLC patients from the OLFM4-positive expression group and olfactomedin 4-negative group (P = 0.927)

Table 2: Association of OLFM4 with the clinicopathological parameters

Characteristics	*n*	OLFM4 expression (%)		χ^2 value	*P* value*
		Negative	Positive		
Age (years)				0.014	0.906
<63	48	20 (48.8)	28 (49.1)		
≥63	50	21 (51.2)	29 (50.9)		
Gender				0.340	0.560
Male	70	28 (68.3)	42 (73.7)		
Female	28	13 (31.7)	15 (26.3)		
NSCLC subtype				0.788	0.674
ADC	55	22 (53.7)	33 (58.9)		
SCC	18	7 (17.1)	11 (19.6)		
Others	24	12 (29.3)	12 (21.4)		
Size				0.011	0.917
≤7cm	76	31 (79.5)	45 (80.4)		
>7cm	19	8 (20.5)	11 (19.6)		
Lymph node metastasis				0.087	0.769
Without	48	19 (48.7)	29 (51.8)		
With	47	20 (51.3)	27 (48.2)		
Distant metastasis				0.014	0.905
Without	81	34 (89.5)	47 (88.7)		
With	10	4 (10.5)	6 (11.3)		
TNM stage				2.850	0.241
I	43	21 (52.5)	22 (40.0)		
II	10	2 (5.0)	8 (14.5)		
III/IV	42	17 (42.5)	25 (45.5)		
Peritumor intravascular cancer emboli				6.173	0.013
Negative	71	26 (66.7)	45 (88.2)		
Positive	19	13 (33.3)	6 (11.8)		

OLFM4: Olfactomedin 4, NSCLC: Non-small cell lung cancer, ADC: Adenocarcinoma, SCC: Squamous cell carcinoma, TMN: Tumor-node-metastasis classification. *P<0.05 was considered significant. OLFM4 expression in NSCLC lesions was significantly correlated with peritumor intravascular cancer emboli (P=0.013)

To the best of our knowledge, no study has ever evaluated OLFM4 expression in human NSCLC in the Chinese population. The present study is the first to investigate the relationship between OLFM4 and OS. Therefore, we investigated the expression pattern of OLFM4 in Chinese NSCLC patients to evaluate its potential clinical relevance. Our results showed that the protein expression of OLFM4 was significantly lower in the NSCLC tissues than in the adjacent non-tumor lung tissues ($P < 0.05$). This finding is consistent with prostate cancer research results.[10,21]

Studies have shown that the expression and function of OLFM4 genes differ during different tumor stages. Some studies[20,22] reported that OLFM4 inhibits cell growth, whereas in contrast, other studies[5,23] revealed that OLFM4 promotes cell growth. The above results demonstrate that the effect of OLFM4 on cell proliferation and apoptosis is tissue specific; hence, similar to p21, OLFM4 can inhibit cell growth and survival in some circumstances.[24] Studies have also shown[25] that OLFM4 can regulate immune function. These two functions may explain the contradicting results of this study on comparison with other cancer research. OLFM4 may have different functions in different tumors. As such, we should conduct further experiments to confirm the specific mechanism of action of OLFM4.

Correlation analysis of OLFM4 and clinical characteristics demonstrated no significant relationship between OLFM4 staining and clinical parameters, such as patient age, gender, tumor subtype, size, lymph node metastasis location, and TNM stage in NSCLC. However, OLFM4 expression was significantly correlated with peritumor intravascular cancer emboli. These results indicated the function of OLFM4 in promoting the tumorigenicity of human NSCLC. No significant difference in survival rate was detected between the NSCLC patients from the OLFM4-positive expression group and OLFM4-negative group ($P = 0.927$). This result indicates that OLFM4 is not a prognostic factor for NSCLC.

Limitations of the study

In the present study, OLFM4 is detected only from the organization level, not in serum and cellular levels. As such, we cannot definitely conclude whether the OLFM4 expression in cancerous tissues is high or low. Further research should be conducted by different groups to determine the mechanism of OLFM4.

CONCLUSION

We examined the protein and gene expression levels of OLFM4 using IHC and RT-PCR. Our results showed that the expression level of OLFM4, a candidate tumor suppressor gene for NSCLC, frequently decreased in NSCLC. This should be further verified using serological and cytological analyses. In future, studies should be conducted to investigate the contribution of the molecular mechanisms of OLFM4 in the carcinogenesis of NSCLC and other tumors. These mechanisms include the cellular localization and crosstalk of OLFM4 with other OLFM4 family members. These studies will also help characterize the diagnostic and therapeutic potentials of OLFM4 for NSCLC and other tumors.

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REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013;63:11-30.
2. Rastel D, Ramaioli A, Cornillie F, Thirion B. CYFRA 21-1, a sensitive and specific new tumor marker for squamous cell lung cancer. Report of the first European multicentre evaluation. Eur J Cancer 1994;30A: 601-6.
3. Wieskopf B, Demangent C, Purohit A, Stenger R, Gries P, Kreisman H, *et al*. CYFRA 21-1 as a biologic marker of non-small celllung cancer: Evaluation of sensitivity,

specificity and prognostic role. Chest 1995;108:163-9.
4. Zhang J, Liu WL, Tang DC, Chen L, Wang M, Pack SD, *et al*. Identification and characterization of a novel member of olfactomedin-related protein family, hGC-1, expressed during myeloid lineage development. Gene 2002;283:83-93.
5. Zhang X, Huang Q, Yang Z, Li Y, Li CY. GW112, a novel antiapoptotic protein that promotes tumor growth. Cancer Res 2004;64:2474-81.
6. Shinozaki S, Nakamura T, Iimura M, Kato Y, Iizuka B, Kobayashi M, *et al*. Upregulation of Reg 1alpha and GW112 in the epithelium of inflamed colonic mucosa. Gut 2001;48:623-9.
7. Kobayashi D, Koshida S, Moriai R, Tsuji N, Watanabe N. Olfactomedin 4 promotes S-phase transition in proliferation of pancreatic cancer cells. Cancer Sci 2007;98:334-40.
8. Goldstraw P. In: International Association for the Study of Lung Cancer. Staging Manual in Thoracic Oncology. Orange Park: Editorial Rx Press; 2009.
9. Besson D, Pavageau AH, Valo I, Bourreau A, Bélanger A, Eymerit-Morin C, *et al*. A quantitative proteomic approach of the different stages of colorectal cancer establishes OLFM4 as a new nonmetastatic tumor marker. Mol Cell Proteomics 2011;10.
10. Liao WT, Wang X, Xu LH, Kong QL, Yu CP, Li MZ, *et al*. Centromere protein H is a novel prognostic marker for human non-small cell lung cancer progression and overall patient survival. Cancer 2009;115:1507-17.
11. National center for biological information (www.ncbi.nlm.nih.gov). US: National library of medicine [NCBI reference sequence: NM-006418.4]. Available from: http://www.ncbi.nlm.nih.gov/nuccore/NM_006418.4. [Last accessed on 2014 Jun 01].
12. National center for biological information (www.ncbi.nlm.nih.gov). US: National library of medicine [GenBank: M28424.1]. Available from: http://www.ncbi.nlm.nih.gov/nuccore/M28424.1. [Last accessed on 2014 Jun 01].
13. Snyder DA, Rivers AM, Yokoe H, Menco BP, Anholt RR. Olfactomedin: Purification, characterization, and localization of a novel olfactory glycoprotein. Biochemistry 1991;30:9143-53.
14. Yokoe H, Anholt RR. Molecular cloning ofolfactomedin, an extracellular matrixprotein specific to olfactory neuroepithelium. Proc Natl Acad Sci USA 1993;90:4655-9.
15. Luo Z, Zhang Q, Zhao Z, Li B, Chen J, Wang Y. OLFM4 is associated with lymph node metastasis and poor prognosis in patients with gastric. J Cancer Res Oncol 2011;137:1713-20.
16. Oue N, Aung PP, Mitani Y, Kuniyasu H, Nakayama H, Yasui W. Genes involved in invasion and metastasis of gastric cancer identified by array-based hybridization and serial analysis of gene expression. Oncology 2005;69:17-22.
17. Koshida S, Kobayashi D, Moriai R, Tsuji N, Watanabe N. Specific overexpression of OLFM4 (GW112/HGC-1) mRNA in colon, breast and lung cancer tissues detected using quantitative analysis. Cancer Sci 2007;98:315-20.
18. Yu L, He M, Yang Z, Chen G, Li M, Wang L, *et al*. Olfactomedin 4 is a marker for progression of cervical neoplasia. Int J Gynecol Cancer 2011;21:367-72.
19. Duan C, Liu X, Liang S, Yang Z, Xia M, Wang L, *et al*. Oestrogen receptor-mediated expression of olfactomedin 4 regulates the progression of endometrial adenocarcinoma. J Cell Mol Med 2014;18:863-74.
20. Chen L, Li H, Liu W, Zhu J, Zhao X, Wright E, *et al*. Olfactomedin 4 suppresses prostate cancer cell growth and metastasis via negative interaction with cathepsin D and SDF-1. Carcinogenesis 2011;32:986-94.
21. Li H, Rodriguez-Canales J, Liu W, Zhu J, Hanson JC, Pack S, *et al*. Deletion of the olfactomedin 4 gene is associated with progression of human prostate cancer. Am J Pathol 2013;183:1329-38.
22. Park KS, Kim KK, Piao ZH, Kim MK, Lee HJ, Kim YC, *et al*. Olfactomedin 4 suppresses tumor growth and metastasis of mouse melanoma cells through downregulation of integrin and MMP genes. Mol Cells 2012;34:555-61.
23. Liu W, Lee HW, Liu Y, Wang R, Rodgers GP. Olfactomedin 4 is a novel target gene of retinoic acids and 5-aza-2′-deoxycytidine involved in human myeloid leukemia cell growth, differentiation, and apoptosis. Blood 2010;116:4938-47.
24. AsadaM, Yamada T, Ichijo H, Delia D, Miyazono K, Fukumuro K, *et al*. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. EMBO J 1999;18:1223-34.
25. Liu W, Yan M, Liu Y, Wang R, Li C, Deng C, *et al*. Olfactomedin 4 down-regulates innate immunity against *Helicobacter pylori* infection. Proc Natl Acad Sci U S A 2010;107:11056-61.

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