Pakistan J. Zool., pp 1-7, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230803165114

Bioinformatics Analysis of Promoter Methylation and Clinical Significance of Tumor Suppressor Gene *HOXA5* in Oral Cancer

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Article Information
Received 03 August 2023
Revised 25 August 2023
Accepted 04 September 2023
Available online 31 October 2023 (early access)

Authors' Contribution
HZ and CY contributed equally to this work as co-corresponding author as they participated in conceiving the design of the study and collecting and reviewing the data and coordination of project. HW and YL contributed equally to this work as co-first author as they participated in doing literature review, collecting the data and analysis and in preparing the manuscript. JW and WW helped in critical revision and finalizing the manuscript. All authors read, revised, and approved the final manuscript.

Key words
Bioinformatics analysis, Oral cancer, Tumor suppressor genes, Methylation status, Clinical significance, Cancer

ABSTRACT

The *HOXA5* gene is an important tumor suppressor gene, which has the function of regulating cell cycle, proliferation, and differentiation. Down regulation or inactivation of *HOXA5* expression occurs in a variety of malignant tumors such as oral cancer (OC) and breast cancer. The promoter region of *HOXA5* gene is often methylated, and it is of great value to explore the relationship between its methylation status and gene expression level. The objective of this study was to explore the promoter methylation status of tumor suppressor gene *HOXA5* in OC. *HOXA5* mRNA expression was detected by RT-PCR. OC CAL-27, H357, and HSC-3 cells were treated with different concentrations of 5-aza-deoxyeytidine to analyze the inhibitory effect of 5-aza-deoxyeytidine on methylation. The methylation status of *HOXA5* gene promoter was closely related to OC. 5Aza-CdR could induce the expression of *HOXA5* mRNA in OC cells, promote cell apoptosis, and inhibit the growth and spread of tumor cells. The promoter region of *HOXA5* gene is hypermethylated in OC. It was concluded that 5Aza-CdR can inhibit the activity of DNA methyltransferase, restore the expression of *HOXA5* gene, and inhibit the proliferation and spread of cancer cells.

INTRODUCTION

Oral cancer (OC) is a common malignant tumor with an increasing incidence year by year. OC is a highly aggressive cancer, which not only affects the quality of life of patients, but also may be life-threatening. It is meaningful to explore the occurrence and development mechanism of OC (Fan *et al*., 2022). *HOXA5* is a tumor suppressor gene (He *et al*., 2022). The expression of *HOXA5* is closely related to the prognosis of a variety of tumors, and its mechanism of action in tumors has attracted wide attention. Studies have shown that the deletion or abnormal expression of *HOXA5* gene is closely related to the occurrence and development of tumors, suggesting that the abnormality of *HOXA5* gene may be one of the important mechanisms of OC occurrence and development (Liang *et al*., 2022; Lu *et al*., 2021). The mechanism of *HOXA5* gene in OC is still not fully understood, and further studies are needed.

In recent years, more and more studies have shown that some key biomolecules may undergo a series of epigenetic modifications during the occurrence and development of OC, including DNA methylation modification (Wang *et al*., 2023; Wu *et al*., 2022). DNA methylation is an important link in the regulation of gene expression, which can directly affect the expression of genes. The methylation status of *HOXA5* gene promoter is one of the key factors affecting gene expression. Research based on bioinformatics analysis can explore the methylation status of *HOXA5* gene promoter, and further explore its association with OC disease. Studies have shown that there is a significant abnormality in the methylation status of *HOXA5* gene promoter in some

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0030-9923/2023/0001-0001 $ 9.00/0

cancer patients (Liu *et al*., 2020). Methylation can lead to gene silencing or down-regulation of expression, and *HOXA5* is an important tumor suppressor gene that has the function of regulating cell cycle, proliferation, and differentiation. In OC, the expression level of *HOXA5* is significantly reduced. The high level of methylation in the promoter region of *HOXA5* gene can reduce its expression level, thereby promoting the growth of tumor cells and enhancing the degree of malignancy and invasion of cells.

This article aimed to explore the promoter methylation status of tumor suppressor gene *HOXA5* in OC and its correlation with the occurrence and development of OC, providing new plans and ideas for OC. The present study analyzed the change pattern of *HOXA5* gene promoter methylation status and its clinical significance, and discussed the regulatory mechanism of *HOXA5* gene in OC, providing new theoretical support and guidance for the prevention and treatment of OC. This article provides a reference for the study of gene promoter methylation status in other types of cancers. It is innovative and has important clinical prospects in OC.

MATERIALS AND METHODS

Experimental materials

Oral cancer (OC) cells CAL-27, H357, HSC-3 (ATCC, Virginia, USA); Oral tissues (Department of Pathology, Department of Stomatology, The First Affiliated Hospital of Yangtze University, Jingzhou, 434000, Hubei Province, China); GAPDH quantitative standard (Shanghai Shenyou Biotechnology Co., LTD., Shanghai, China); DMEM low glucose medium (SenBeiJia Biological Technology Co., Ltd., Jiangsu, China); TRIzol (Shanghai Kanglong Biological Technology Co., LTD., Shanghai, China); Chloroform (Hebei Crovell Biotechnology Co., LTD., Hebei, China). Isopropanol (Shanghai Yuanye Biotechnology Co., LTD., Shanghai, China); 5-aza-deoxyeytidine (Shanghai Rhawn Chemical Technology Co., LTD., Shanghai, China); Taq enzyme (Hebei Sanshi Biotechnology Co., LTD., Hebei, China).

Experimental instruments

Electric thermostatic water bath SYG-2-4 (Tianjin Teste Instrument Co., Ltd., Tianjin, China); High-speed refrigerated centrifuge H1750R (Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsham, China); Medical real-time PCR instrument Archimed X4 (Kunpeng (Xuzhou) Scientific Instrument Co., Ltd., Xuzhou, China); Electrophoresis instrument (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China); SuPerMax 3100 Multifunction Microplate Reader (Shanghai Flash Biotechnology Co., Ltd., Shanghai, China).

Experimental methods

HOXA5 mRNA expression detected by RT-PCR: Total RNA was extracted from CAL-27, H357, and HSC-3 cells, 1 mL TRIzol was added, left for 10 min, and 200μL chloroform was added. It was centrifugated at 13,000 RPM at 4°C for 15 min, the aqueous phase was aspirated, and isopropanol was put. The supernatant and ethanol were discarded after centrifugation, and the concentration was measured to pack after drying. The extracted RNA was converted into single-stranded cDNA, and M-MLV reverse transcriptase combined with random hexaplex primers was used for reverse transcription. The *HOXA5* cDNA in the sample and the PCR mixture containing fluorescent probes in the reaction system were added into the reaction wells, and the negative control, positive control, and standard curve were prepared. The reaction mixture was put into the reaction wells and then transferred to a real-time fluorescent PCR instrument for amplification. The fluorescent signal released during the PCR reaction was monitored in real time, and the fluorescence value of multiple amplification reactions was recorded. The CT value obtained after the reaction was analyzed, and the fluorescence intensity value was compared with the standard curve to calculate the relative expression of gene mRNA. Table I displays the primer sequences used for the RT-PCR assay.

RT-PCR reaction system of *HOXA5* gene: Double distilled water 11.2μL, 10× reaction buffer 2.5μL, MgCI 1.5μL, dNTP 0.5μL, *HOXA5* upstream primer 2.0μL, *HOXA5* downstream primer 2.0μL, *HOXA5* probe 1.0μL, HotstarTaq 0.3 μL, template 4.0μL, and a total of 25μL. The PCR reaction conditions were as follows: Hot start at 95°C for 15 min, then denaturation at 94°C for 30 s, 60°C for 1 min, a total of 50 cycles, ending at 4°C.

Table I. Primer sequences used for RT-PCR assay.

Gene	Primer	Sequence
HOXA5	Upstream primer	5' - AACTCATTTTGCGGTCGCTAT- 3'
	Downstream primer	5' - TCCCTGAATTGCTCGCTCAC- 3'
GAPDH	Upstream primer	5' - AACTCATTTTGCGGTCGCTAT- 3'
	Downstream primer	5' - AACTCATTTTGCGGTCGCTAT- 3'

GAPDH gene RT-PCR reaction system: Double distilled water 13.2μL, 10 × reaction buffer 2.5μL, MgCI 3.5μL, dNTP 0.5μL, GAPDH upstream primer 1.0 μL, GAPDH downstream primer 1.0 μL, GAPDH probe 1.0μL, Taq enzyme 0.3μL, template 2.0μL, a total of 25μL. The PCR conditions were denaturation at 95℃ for 5 min, followed by denaturation at 94℃ for 30 s, 60℃ for 1 min, and 45 cycles to 4℃.

CAL-27, H357 and HSC-3 cells treated with 5-aza-deoxyeytidine: The cells in log phase in plates with 6 wells cultured with 2 mL drug-free DMEM low-glucose medium. After 24 h, the cells were cultured in 5-aza-deoxyeytidine containing 0.01μM, 0.05μM, 0.1μM, 0.5μM, 1.0μM, and 5.0μM. DNA and mRNA were extracted and used for MSP and RT-PCR.

Statistical methods

Excel 2016 was adopted to record and summarize the data. SPSS 20.0 was adopted for data statistics and analysis. Mean±S.D ($\bar{x}$±s) was adopted for measurement data, *t* test was adopted. $P<0.05$ was considered statistically significant.

RESULTS

The results for *HOXA5* mRNA expression levels in OC cells are presented in Figure 1. Expression levels of *HOXA5* mRNA in OC cells suggested CAL-27, H357, and HSC-3 with methylation in the promoter region of the *HOXA5* gene lacked *HOXA5* mRNA expression. The results for GAPDH gene detection by RT-PCR in OC cells are presented in Figure 2. RT-PCR of GAPDH gene in OC cells revealed that CAL-27 had the lowest expression of GAPDH gene and H357 had the highest expression.

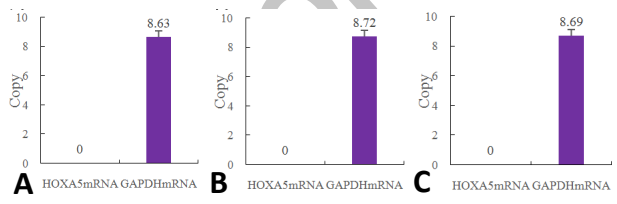

Fig. 1. Analysis of *HOXA5* mRNA expression levels in OC cells. A, CAL-27; B, H357; C, HSC-3

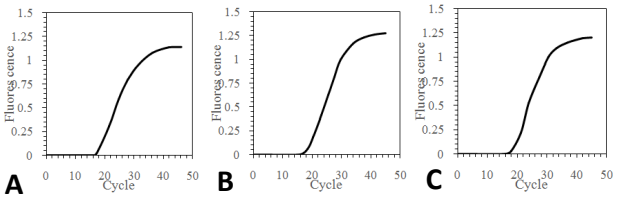

Fig. 2. Real-time time RT-PCR detection of GAPDH gene in OC cells. A, CAL-27; B, H357; C, HSC-3.

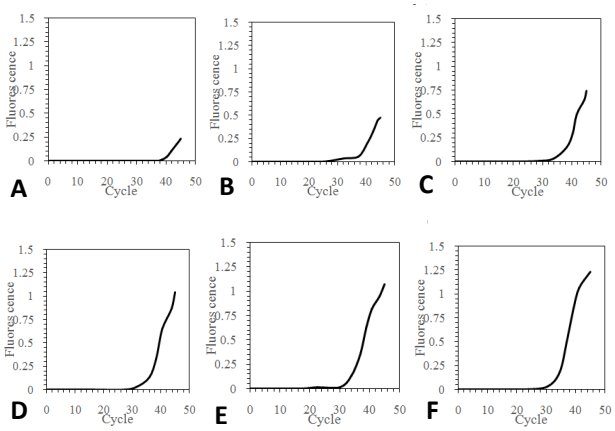

Fig. 3. Induction of *HOXA5* mRNA expression in OC cells by different concentrations of 5Aza-CdR.
A, concentration = 0.01; B, concentration = 0.05; C, concentration = 0.1; D, concentration = 0.5; E, concentration = 1; F, concentration = 5.

Table II. Analysis of *HOXA5* mRNA expression levels in oral cancer cells induced by different concentrations of 5Aza-CdR.

Group	Concentration (μM)					
	0.01	0.05	0.1	0.5	1	5
CAL-27	0.21	0.37	1.08	4.78	5.82	7.64
H357	0.25	0.42	1.12	4.89	5.93	7.79
HSC-3	0.23	0.38	1.10	4.82	5.87	7.68

Figure 3 presents the induction of *HOXA5* mRNA expression in OC cells at different concentrations of 5Aza-CdR. 5Aza-CdR can induce the expression of *HOXA5* mRNA in OC cells, and with the increase of 5Aza-CdR concentration, the induction of *HOXA5* mRNA expression in OC cells was more obvious.

Table II presents the levels of *HOXA5* mRNA expression in OC cells induced by different concentrations of 5Aza-CdR. With increasing concentrations of 5Aza-CdR, 5Aza-CdR induced increased *HOXA5* mRNA expression in the three OC cells.

Figure 4 shows the comparison of methylation between OC and oral ulcer patients. In OC patients, 27 cases were methylated, 3 cases were not methylated, while none of the patients with oral ulcer were methylated.

DISCUSSION

HOXA5 is a tumor suppressor gene. In the pathological process of OC, the abnormal expression of *HOXA5* has a close relationship with a variety of clinicopathological

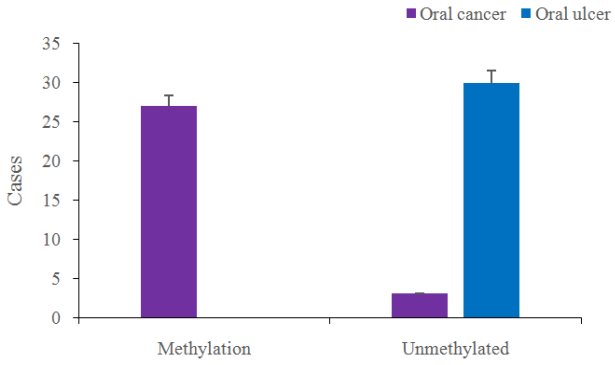

Fig. 4. Comparative analysis of methylation in patients with OC and oral ulcer.

features, including tumor malignancy, clinical stage, and prognosis (Kim *et al*., 2021; Yaiche *et al*., 2021). Studies have shown that the inhibitory effect of *HOXA5* on OC is mainly achieved by controlling cell growth, cell cycle and apoptosis, which is closely related to a series of signaling pathways and molecular mechanisms in the occurrence and development of OC (Pai *et al*., 2022). *HOXA5* has an obvious inhibitory effect on OC by regulating cell growth and proliferation. Studies have shown that when *HOXA5* expression is too low or the gene is deleted, tumor cell growth and proliferation are enhanced, which can lead to rapid growth and high malignancy of OC (Liang *et al*., 2021). *HOXA5* can also regulate the sexual and asexual division process of cancer cells and affect their growth and proliferation ability. Maintaining the stability of *HOXA5* gene expression is crucial for the inhibition of OC. *HOXA5* regulates the apoptotic process of OC cells, which is another important anticancer mechanism in OC (Xiong *et al*., 2022; Wan and Zheng, 2021). *HOXA5* induces apoptosis of OC cells by regulating apoptosis-related proteins and their signaling pathways, and further inhibits tumor growth and spread. The loss and abnormal expression of *HOXA5* can down-regulate the expression of apoptosis-related proteins and reduce the sensitivity of cells, thereby promoting and maintaining the development of OC (Moorthy *et al*., 2023). The control of *HOXA5* on OC is also manifested in the regulation of cell cycle such as impaired cell meiosis and DNA repair. *HOXA5* can target different cytokines and regulatory molecules at different stages of the cell cycle and play different roles (Yang *et al*., 2021).

The methylation status of the promoter region of *HOXA5* gene is an important regulatory mechanism affecting its gene expression. It is closely correlated with the occurrence, prognosis, and treatment response of OC (Gao *et al*., 2022). In OC tissues, its level was clearly superior as against normal oral mucosa tissues (Wang *et al*., 2021). This hypermethylation state can lead to the decrease of *HOXA5* gene expression and the weakening of tumor suppressor effect, thus promoting the occurrence and development of OC (Holzman *et al*., 2021). In clinical practice, the detection of its methylation status can be used as an effective OC risk assessment index to help early identify high-risk groups of OC and take early intervention measures (Jin *et al*., 2023). Studies have shown that in OC patients, the hypermethylation of *HOXA5* gene promoter region has the effect on prediction (Wang *et al*., 2022). When OC patients receive radiotherapy, chemotherapy, and other treatments, its hypermethylation status may lead to a weakened response to treatment, thereby affecting the therapeutic outcome (Liang *et al*., 2023; Porras *et al*., 2022). In the treatment and follow-up of OC, the detection of the methylation status also has important clinical significance. Through drug intervention and other therapeutic means, the methylated region can be reversed, to improve the expression level of *HOXA5* gene and enhance its anticancer effect, which has great potential for the prevention and treatment of OC (Roux *et al*., 2022). Its methylation status has important clinical significance in OC occurrence, prognosis, treatment response, and potential treatment options (Han *et al*., 2021). Through in-depth study of the methylation status and regulatory mechanism of *HOXA5* gene, new strategies and methods can be provided for the management and treatment of OC, thereby improving the prognosis of OC patients.

Padam *et al*. (2022) found that *HOXA5* gene has a potential role in the development of OC, and they are of great value in various cellular processes including proliferation, invasion, migration, epithelial-mesenchymal transition, and metastasis. Padam *et al*. (2021) identified transcription factor binding sites (TFBS) in the *HOXA5* gene promoter and elucidated the comprehensive interaction between transcription factor/gene and *HOXA5*. They found that the predicted TFBS in the *HOXA5* gene promoter played a role in transcriptional regulation by regulating the activity of target genes. The TF gene interaction is essential for understanding OC pathogenesis. Rodini *et al*. (2012) analyzed the expression profile of homeobox genes in oral squamous cell carcinoma (OSCC). It was found that *HOXA5*, HOXD10, and HOXD11 showed higher expression in OSCC samples, and patients with lower *HOXA5* expression had a worse prognosis. Rodrigues *et al*. (2021) explored the DNA copy number and methylation characteristics of homeobox genes *HOXA5*, *HOXA7, HOXA9, HOXB5, HOXB13, HOXC12, HOXC13, HOXD10, HOXD11, IRX4,* and *ZHX1*, and correlated them with clinicopathological parameters and overall survival. *HOXA5*, *HOXB5,* and *HOXD10* were found to be

amplified in surgical margins, whereas *HOXA9, HOXB13,* and *IRX4* were amplified in OSCC. This article explored the methylation pattern and clinical significance of *HOXA5* gene promoter region in OC. 5Aza-CdR can induce the expression of *HOXA5* mRNA in OC cells and inhibit the methylation of *HOXA5*.

The most important limitation in this study is that other biological effects of the region, such as the relationship between cell proliferation and apoptosis, are not analyzed, which needs to be further studied and verified.

CONCLUSION

HOXA5 is a tumor suppressor gene. There is a clear methylation status of the promoter region of *HOXA5* gene in OC cells, which leads to the down-regulation of the expression level of *HOXA5* gene, thereby inhibiting its role as a tumor suppressor gene. 5Aza-CdR is a DNA methyltransferase inhibitor, which can inhibit DNA methyltransferase, thereby inhibiting DNA methylation modification. Treatment with 5Aza-CdR can reduce the methylation modification in this region, leading to the restoration of the promoter region of the *HOXA5* gene to the unmethylated state and the reinitiation of *HOXA5* gene expression.

ACKNOWLEDGEMENT

The auhtors are grateful for the support from The First Affiliated Hospital of Yangtze University.

Funding

Not applicable.

IRB approval

This study was approved by the Advanced Studies Research Board of the First People's Hospital of Jingzhou, Jingzhou, China.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of the First People's Hospital of Jingzhou, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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