

The Potential Roles of CDO1 and JAM3 Methylation in the Occurrence of Nasopharyngeal Carcinoma

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Abstract: *Background:* Nasopharyngeal carcinoma (NPC) is a highly malignant tumor derived from nasopharyngeal epithelial tissue. Due to their strong capacity for hidden lesions and unobvious early symptoms, Given the critical problem for clinical diagnosis, NPC has a high rate of misdiagnosed and missed, which seriously threatens human health. The underlying mechanism of the occurrence and development of nasopharyngeal carcinoma is still not elucidated. In recent years, increasing attention has been paid to the study of some tumor-related genes, especially the epigenetic regulation of genes. *Objective:* To investigate the role of CDO1 and JAM3 gene methylation in the occurrence of nasopharyngeal carcinoma. *Methods:* Methylation-specific PCR and pyrosequencing were used to detect the difference in methylation rates of CDO1 and JAM3 in nasopharyngeal carcinoma and nasopharyngeal chronic inflammation tissues. The abundance of protein expression and mRNA for CDO1 and JAM3 were detected by fluorescence quantitative PCR and Western Blot in nasopharyngeal carcinoma and chronic nasopharyngitis tissues. *Results:* Pyrosequencing indicated that the methylation rates of CDO1 and JAM3 in nasopharyngeal carcinoma tissues were remarkably increased, compared with nasopharyngeal chronic inflammation tissues. Furthermore, the qPCR and Western Blot suggested that the expression of CDO1 and JAM3 in nasopharyngeal carcinoma tissues was significantly lower than that in nasopharyngeal chronic inflammation tissues. *Conclusion:* The methylation of the CpG island in the promoter region of CDO1 and JAM3 genes leads to a decrease in their gene expression, which may be related to the occurrence of nasopharyngeal carcinoma.

Keywords: Nasopharyngeal Carcinoma, Cdo1, Jam3, Methylation

1. Introduction

Nasopharyngeal carcinoma (NPC) is a highly malignant tumor derived from nasopharyngeal epithelial tissue. Due to their strong capacity for hidden lesions and unobvious early symptoms, Given the critical problem for clinical diagnosis, NPC has a high rate of misdiagnosed and missed, which seriously threatens human health. Therefore, how to improve the early diagnosis rate of nasopharyngeal carcinoma patients, giving appropriate treatment, and improving the survival rate of patients is a challenge for otorhinolaryngologists. In addition, the underlying mechanism of the occurrence and development of nasopharyngeal carcinoma is still not elucidated, which may be related to the geographical environment, living habits, genetic inheritance, EB virus

infection, and other factors [1-3]. In recent years, increasing attention has been paid to the study of some tumor-related genes, especially the epigenetic regulation of genes. Epigenetics refers to the heritable changes in gene expression without changes in gene sequence. DNA methylation, As an important epigenetic phenomenon, usually changes in tumor cells, and the highly hypermethylated CpG islands of tumor suppressor genes are linked to gene silencing [4]. In this study, the methylation and expression of tumor suppressor genes CDO1 and JAM3 were detected in nasopharyngeal carcinoma tissues, respectively. Furthermore, the potential roles of gene methylation of CDO1 (Cysteine dioxygenase1) and JAM3 (Junctional adhesion molecules3) in the occurrence of nasopharyngeal carcinoma were revealed.

2. Materials and Methods

2.1. Clinical Data

All tissue samples were collected from patients with suspected nasopharyngeal neoplasm who were admitted to our hospital from May 2021 to April 2022. A biopsy was performed with the consent of the patient. Some tissues were sent for pathology, and some were used in this study. Tissue samples included 10 cases of nasopharyngeal carcinoma and 10 cases of nasopharyngeal chronic inflammation. Among the 10 patients with nasopharyngeal carcinoma, there were 7 males and 3 females. According to the 8th edition of UICC TNM staging criteria, there were 4 cases of T1 stage 2 and T2 stage, 1 case of T3 stage 3 and T4 stage, 6 cases with lymph node metastasis, and 4 cases without lymph node metastasis. All patients had no radiotherapy or chemotherapy before a biopsy.

2.2. Methods

2.2.1. Methylation-Specific PCR and Pyrosequencing

After grinding and centrifuging, the DNA of each group of cells was extracted by DNA extraction kit, and the sample concentration was determined by NanoDrop ND-2000 spectrophotometer to homogenize the samples. The extracted DNA was transformed with bisulfite, and then the transformed DNA was purified by the Zymo-Spin™ IC purification column. The primers of CDO1 and JAM3 gene methylation and unmethylation primer sequences were synthesized. See References [4, 5] for details. CDO1 primer sequence: F: 5'-TTCGGCGTAGGTTGTTCGTA-3' M-R: 5'-CGCGAAAACGCAACCAAC-3'; U-F: 5'-GTTTTGGTGTGTAGGTTGTTTGTATTG-3'; U-R: 5'-CAACAATCCCACCCCAATC-3'. JAM3 primers: M: F-5'-CGTAGTTAGGGTTGGGATTC-3'R-5, -GAAATCCGACGACTATCCGA-3'; U: -F: 5'-TG TAGTTAGGGTTGGGATTT-3' R: 5'-CAAAATCCAACA ACTATCCA-3'. Four pairs of primers were used to amplify the DNA of each group of cells modified by sulfite, and the annealing temperature was 55 °C. The amplified products were sequenced by pyrophosphate reaction on the sequencer respectively.

2.2.2. Fluorescence Quantitative PCR

The total RNA of the above cells was extracted with a TRIzol reagent. Three specimens were randomly selected to take 1 µl of RNA samples, and 1% agarose gel electrophoresis was performed at 80V × 20min. The 5s rRNA, 18s rRNA and 28s rRNA bands of total RNA were observed by the gel imaging system. If the three bands were complete, it could be proved that the total RNA extraction was complete (see Figure 1). Reverse transcription was performed with reverse transcriptase to form the first strand of DNA (cDNA). Three pairs of primers, CDO1, JAM3, and β-actin, were designed according to the gene sequence in GeneBank, and PCR was performed using cDNA as a template. CDO1 primer: F: 5-TCTCTGTTGGGGTGAAGGAC-3', R: 5'-AGTGAAGGC

TCACAGCAGGT-3'; JAM3 primers: F: 5'-CTGCTGTTTACACAAGGACGACGAC-3', R: 5'-CAGATGCCCAACGTGATCAG-3'; β-actin primers: F: 5'-TCGTCCCAGTTGGTGACGAT-3'. Reaction system: H₂O 6.5 µl, Premix EX Taq™ (Probe qPCR) 2 × 10 µl, Forward primer (10 pmol / µL) 0.5 µl, Reverse Primer (10 pmol / µL) 0.5 µl, cDNA 2 µl, total volume 20 µl. The annealing temperature is 60 °C. The relative expression of the sample = $2^{-\Delta\Delta CT}$. The total protein in the above cells was extracted with the total protein extraction kit (Beijing Pulilai Gene Technology Co., Ltd.) (steps according to the instructions), and the total protein was quantified with the BCA protein quantification kit. Western blotting protein expression was detected by the following steps: SDS-PAGE gel, protein loading, electrophoresis, membrane transfer, blocking, primary antibody incubation, secondary antibody incubation, color development, digital gel image analysis system photography and analysis of optical density value recording results.

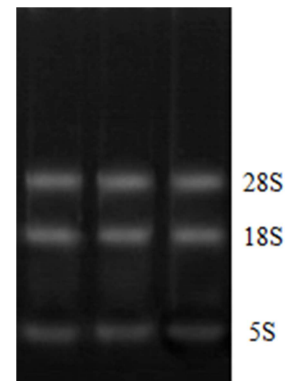


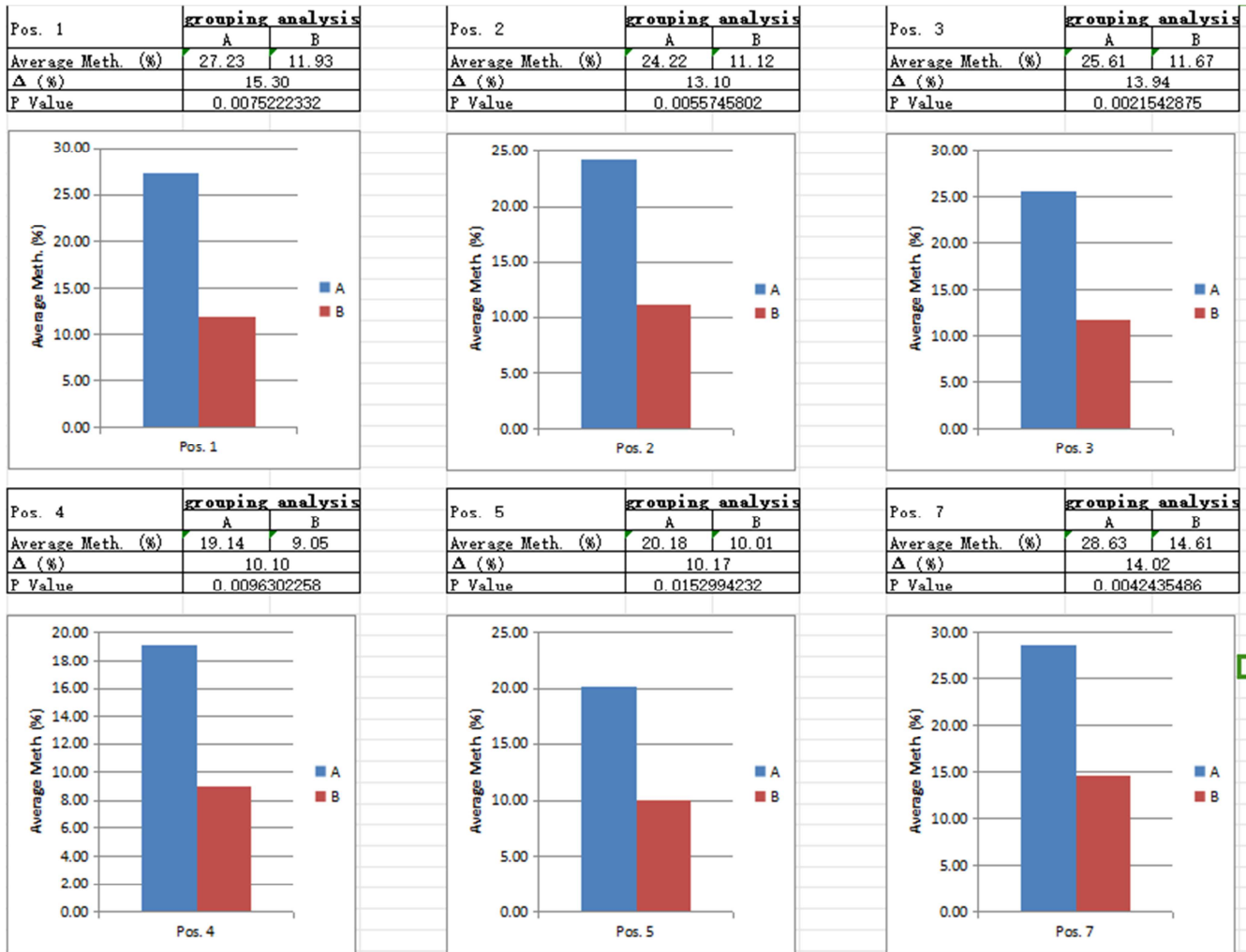
Figure 1. Total RNA electrophoresis map.

The 5s rRNA, 18s rRNA and 28s rRNA bands of total RNA were observed by the gel imaging system.

3. Results

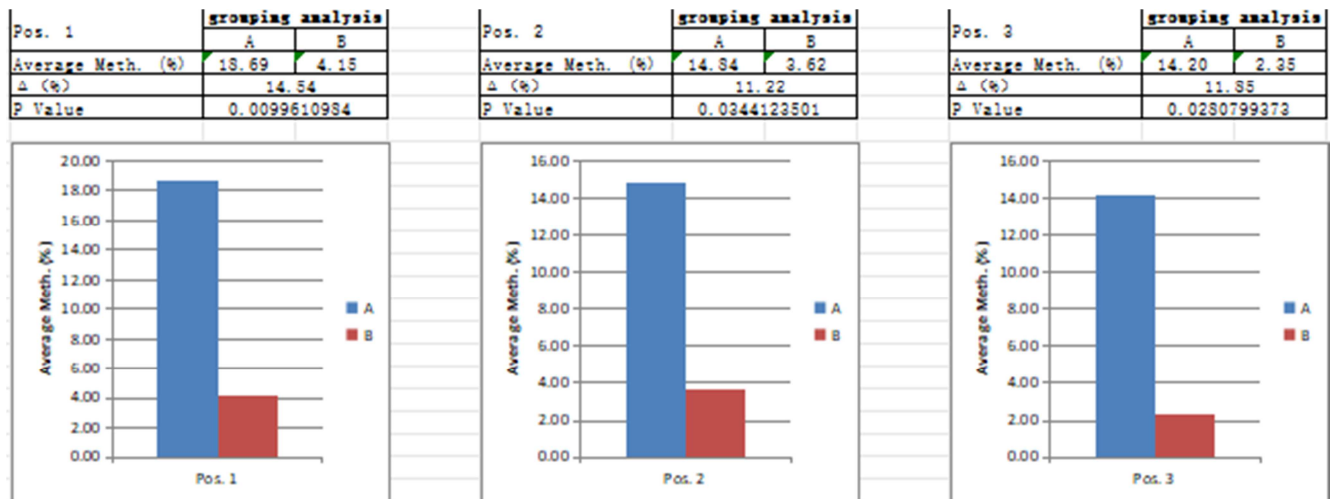
3.1. Pyrosequencing

The results showed that the gene CDO1 had four transcripts, one transcription initiation site, one CpG island in the promoter region, and seven CpG sites. The peak value of the sixth site deviated during the sequencing process, and the detection failed. The remaining six sites correctly detected the methylation rate of CDO1 in each group of tissues. The methylation rate of the 6 CpG sites in the nasopharyngeal carcinoma group was significantly higher than that in the nasopharyngeal chronic inflammation group (see Figure 2), and the difference was statistically significant. Meanwhile, the JAM3 gene has 2 transcripts, 1 transcription initiation site, 1 CpG island in the promoter region, and 9 CpG sites. The methylation rate of each site in the nasopharyngeal carcinoma group was significantly higher than that in the nasopharyngeal chronic inflammation group (see Figure 3), and the difference was statistically significant.



A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group. Pos6 detection failed, so there is no result for Pos6 in the figure

Figure 2. The methylation rate of the eight CpG sites in the promoter region of the CDO1 gene.





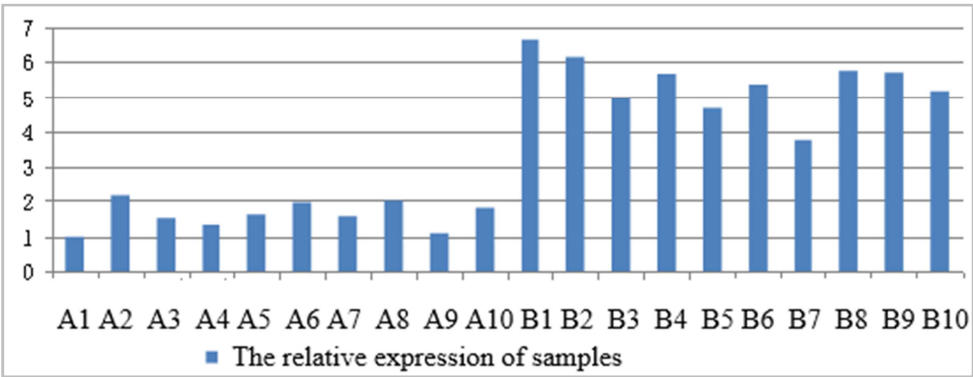
A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group.

Figure 3. The methylation rate of the eight CpG sites in the promoter region of the JAM3 gene.

3.2. Real-Time Quantitative PCR

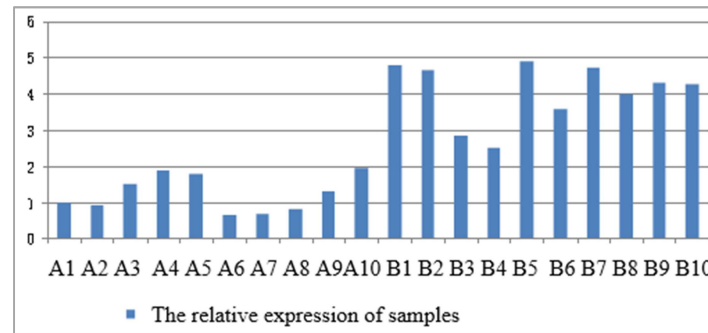
The expression of CDO1 and JAM3 mRNA was detected by fluorescence quantitative PCR, which was expressed as relative expression of single sample. The relative expression of CDO1 mRNA in nasopharyngeal carcinoma tissues (1.604 ± 0.390) was significantly lower than that in chronic nasopharyngitis tissues

(5.41 ± 0.80), and the difference was statistically significant (see Figure 4), $t=13.52$, $P < 0.05$. The relative expression of JAM3 mRNA in nasopharyngeal carcinoma tissues (1.258 ± 0.503) was significantly lower than that in chronic nasopharyngitis tissues (4.062 ± 0.841), and the difference was statistically significant (see Figure 5), $t=9.05$, $P < 0.05$.



A: Nasopharyngeal carcinoma tissues; B: Chronic nasopharyngitis tissues

Figure 4. The relative expression of CDO1 mRNA.



A: Nasopharyngeal carcinoma tissues; B: Chronic nasopharyngitis tissues.

Figure 5. The relative expression of JAM3 mRNA.

3.3. Detection of Protein Expression by Western Blot

The expression of CDO1 and JAM3 protein in nasopharyngeal carcinoma and chronic nasopharyngitis tissues was detected by Western Blot. The average optical density value of the CDO1 protein band in the nasopharyngeal carcinoma group (864.02 ± 394.81) was significantly lower than that in the nasopharyngitis group (1124.95 ± 376.39). The difference was significant (see Figure 6), $t=3.264$, $P < 0.05$. The average optical density value of the JAM3 protein band in the nasopharyngeal carcinoma group (375.36 ± 132.86) was significantly lower than that in the nasopharyngitis group (642.64 ± 126.45) (see Figure 7), $t=4.608$, $P < 0.05$.

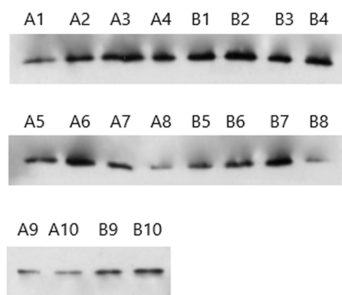


Figure 6. The expression of CDO1 protein in the nasopharyngeal cancer group and the nasopharyngitis group.

A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group.

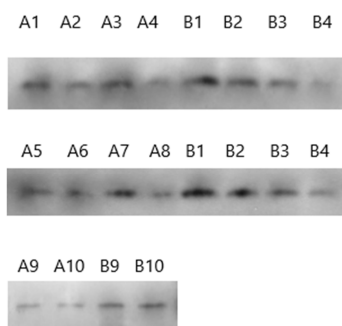


Figure 7. The expression of JAM3 protein in the nasopharyngeal cancer group and the nasopharyngitis group.

A: The nasopharyngeal cancer group; B: The chronic nasopharyngeal inflammatory group.

4. Discussion

Cysteine dioxygenase (CDO) is an iron-containing enzyme with non-heme structure, which is involved in the conversion of cysteine to inorganic sulfate and plays an irreplaceable role in taurine biosynthesis. For CDO, there are two subtypes, CDO1 and CDO2. CDO1 is considered to be an important tumor suppressor gene, which affects cytosine metabolism, leading to the production of reactive oxygen species (ROS) and the decrease of cell viability and growth ability. Previous studies have found that CDO1 is closely related to the occurrence and development of colon cancer, esophageal cancer, breast cancer, lung cancer, and other tumors [6-8]. According to Brait's study, promoter methylation is the main way of CDO1 gene expression inactivation in a variety of tumor tissues [4]. Yang found that methylated cysteine dioxygenase-1 gene promoter in the serum is a potential biomarker for hepatitis B virus-related hepatocellular carcinoma [9].

Junctional adhesion molecules (JAMs) belong to a subfamily of immunoglobulins, which can directly affect the tight junction between epithelial cells and endothelial cells. presently, junctional adhesion molecule 3 (JAM3) has attracted much attention. As an intercellular link in adhesion and migration and restores the epithelial phenotype of tumor cells, Hajjari found that the expression of JAM3 decreased in gastric adenocarcinoma tissues [10]. Zhou et al. found that the inactivation of JAM3 in a variety of tumor tissues is due to methylation of the promoter region gene [6]. Chen found that decreased junctional adhesion molecule 3 expression induces reactive oxygen species production and apoptosis in trophoblasts. [11]

The results of methylation-specific PCR and pyrosequencing in this study showed that the methylation rate of six CpG sites in the CPG island of the CDO1 gene promoter region in nasopharyngeal carcinoma tissues was significantly higher than that in nasopharyngeal chronic inflammation tissues. Moreover, the consistent results also indicated that the methylation rates of 9 CpG sites in the CpG island of the JAM3 gene promoter region were significantly higher than those in nasopharyngeal chronic inflammation tissues. Real-time quantitative PCR results revealed that the relative expression levels of CDO1 and JAM3 in

nasopharyngeal carcinoma tissues were significantly lower than those in nasopharyngeal chronic inflammation tissues. Beyond this, the results of Western Blot protein detection demonstrated that the expression of CDO1 and JAM3 in nasopharyngeal carcinoma tissues was significantly lower than that in nasopharyngeal chronic inflammation tissues. These results indicate that the decreased expression of CDO1 and JAM3 is related to the methylation of CpG islands in the promoter region of CDO1 and JAM3 genes. The methylation of CDO1 and JAM3 leads to the decrease in their expression, which may be an important factor in the occurrence of nasopharyngeal carcinoma. Our previous studies have found that the inactivation of death-related protein kinase genes in laryngeal cancer and nasopharyngeal carcinoma is related to the hypermethylation of the gene promoter region. After demethylation with 5-aza-2-deoxycytidine, the death-related protein kinase gene is reactivated, and the growth, proliferation, and invasion of cancer cells are inhibited [12, 13]. Recent studies have found that genomic hypomethylation also plays an important role in the activation of tumor-related genes. Our recent study also found that the high expression of the uPA gene in nasopharyngeal carcinoma is related to its gene hypomethylation [14]. Based on these research results, we boldly infer that the expression of genes can be changed by changing the methylation status of tumor-related genes, thereby regulating tumor growth, invasion, and metastasis.

In conclusion, the methylation of the CpG island in the gene promoter region can lead to the decrease of CDO1 and JAM3 expression, which may be related to the occurrence of nasopharyngeal carcinoma. Whether it can be used as an early detection index of nasopharyngeal carcinoma needs further study.

Conflicts of Interest

The authors report no conflict of interest.

Acknowledgements

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