

Original Article

Abnormal methylation of HOXA11 promoter promotes tumor progression in testicular germ cell tumor

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Abstract: Objective: To investigate the methylation of HOXA11 gene promoter in testicular germ cell tumor (GCT). Method: The clinicopathological data of 63 patients with primary testicular GCT who underwent surgery during Apr. 2019 to Mar. 2021, were retrospectively analyzed. Their GCT tissue and paraneoplastic testicular tissue were obtained, and genomic DNA was extracted from both. The methylation of HOXA11 gene promoter region was detected by methylation-specific PCR (MSP). The incidence of HOXA11 methylation in testicular GCT and adjacent tissues was compared, and the connection between methylation level in testicular GCT and clinicopathologic features of patients was statistically analyzed. Testicular GCT cells were treated with methylated transferase inhibitor 5-Aza-dC *in vitro*, and HOXA11 mRNA expression was detected by real-time PCR. Results: The positive rate of HOXA11 promoter methylation in testicular GCT tissues was notably higher than that of paired adjacent tissues ($P<0.05$). The abnormal methylation of HOXA11 gene promoter was correlated with lymph node metastasis and TNM stage in patients ($P<0.05$). HOXA11 mRNA expression in testicular GCT cells treated with 5-Aza-dC was increased ($P<0.05$). Conclusion: Abnormal methylation of HOXA11 gene promoter in testicular germ cell tumor tissue inhibits transcription and expression of HOXA11 gene. The abnormal methylation of HOXA11 promoter region is tightly associated with lymph node metastasis and TNM staging in testicular germ cell tumors.

Keywords: Testicle, germ cell tumor, HOXA11 gene, gene promoter methylation

Introduction

Testicular germ cell tumor (GCT) is the most common solid malignancy in young men aged 15-40. Histopathologically, testicular GCT can be divided into seminoma and non-seminoma [1]. At present, the conventional treatment for testicular GCT includes chemotherapy, radiotherapy and surgical treatment, with radical orchiectomy being the standard surgical approach. Testicle-sparing surgery carries a risk of local recurrence 3 to 5 years after surgery, despite adjuvant radiation or chemotherapy [2]. Despite the high chemosensitivity of testicular GCT, 20-30% of patients exhibit resistance to conventional chemotherapy [3]. In addition, while radiotherapy and chemotherapy, as the most important treatments, can prolong

the survival of patients, they significantly impair fertility [4]. The single or combination treatment can lead to a significant decrease in sperm quality, which brings permanent infertility to the patients [5]. Therefore, understanding the gene regulatory mechanism of testicular GCT to provide schemes for seeking new therapeutic targets has become a critical area of research.

DNA methylation exerts a crucial influence in the pathogenesis of colorectal cancer. Among DNA methylation, tumor suppressor gene methylation has been a hot topic in recent years [6]. Down-regulation of HOXA11 due to abnormal methylation affects the occurrence, progression and prognosis of tumors [7]. For instance, it has been confirmed that abnormal methylation of the HOXA11 gene promoter CpG island

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in ovarian cancer is an independent risk factor for poor prognosis [8]. In malignant glioma, the abnormal low expression of HOXA11 is closely related to radio/chemotherapy resistance and affects patients' survival and prognosis [9]. However, the role of HOXA11 methylation in testicular GCT, particularly its impact on tumor cell biology, remains unexplored. Therefore, we analyzed the methylation of HOXA11 gene promoter and its correlation with clinicopathologic features of patients with testicular GCT.

Materials and methods

Tissue specimens

A total of 63 cases of primary testicular GCT, who underwent surgery in the Institutes of Shanxi Bethune Hospital during Apr. 2019 to Mar. 2021, were included in study. Their testicular GCT tissues and paraneoplastic testicular tissues were obtained. All patients met the diagnostic criteria for testicular GCT and had not received preoperative radiotherapy or chemotherapy. Diagnoses were confirmed through pathologic examination of tumor biopsy or surgical tissue specimen. Adjacent tissues were verified as normal testicular tissue, free of residual GCT tissue, by pathological examination. Patients with cryptorchidism, prostatic disease, neoplastic diseases, urinary tract infections, or with severe heart, liver, kidney, and hematological diseases were excluded. The study was carried out under approval of Ethics Committee of the Institutes of Shanxi Bethune Hospital.

Detection of methylation-specific PCR (MSP)

PCR-based techniques were used to assess the methylation status. Total DNA was extracted from formalin-fixed and paraffin-embedded primary testicular GCT and normal adjacent samples using the QIAamp DNA FFPE tissue kit (Qiagen, Germany), followed by bisulfite conversion and purification of DNA using EpiTect Bisulfite kit (Qiagen, Germany). After bisulfite modification, unmethylated cytosine (C) residues were preferentially deaminated and converted into uracil (U), which is substituted for thymine (T) residues once upon PCR amplification while methylated C residues remained unmodified. Bisulfite-converted DNA were amplified based on the primers set for methylated

(M) and un-methylated (UM) HOXA11 gene. Methylation primers were designed using Methyl Primer Express v1.0 and were synthesized by Sangon Biotech Company (Shanghai, China). Forward and reverse primers (M): 5'-TTTGGTTTAATTCGTGCGC-3', 5'-ACGAAGCTCCCGCGAAATA-3', with the amplified fragment length of 113 bp. Forward and reverse primers (UM): 5'-TTTAGGTTTAATTTGTGTGT-3', 5'-ACAAA-CTCCTCCACAAAATA-3', with the amplified fragment length of 112 bp. The PCR procedure for methylation status was as follows: initial denaturation at 95°C for 3 minutes, 36 rounds of denaturation at 94°C for 25 s, annealing at 60.1°C for 25 s and extension at 72°C for 15 s, and a final extension at 72°C for 5 minutes. Similar PCR procedure except optimized annealing conditions (54.1°C for 25 s) was applied for the determination of un-methylation status. The quality of PCR product was validated by 2% agarose gel and visualization under UV light.

H&E staining

The paraffin-embedded sections were dewaxed and stained with hematoxylin staining solution (Biyuntian Biotechnology Co., LTD., China) for about 1 minute. After staining, the sections were washed with distilled water to remove the excess staining solution, and then the differentiation operation was performed using 1% hydrochloric acid alcohol. After differentiation, the sections were immersed in tap water for about 15 minutes for bluing treatment. Next, the sections were stained with eosin dye (Biyuntian Biotechnology Co., LTD., China) for 6-7 minutes. After staining, the sections were washed again with distilled water. The sections were placed on the staining rack subjected to a series of dehydration and clarifying steps using xylene and ethanol. Finally, the processed sections were placed on the slide for sealing treatment.

Immunohistochemistry

The protein expression of paraffin-embedded sections was determined by immunohistochemistry. First, xylene was used to dewax the sections, followed by antigen retrieval using a 0.01 mol/L citrate solution (pH 6.0-6.5) in a microwave oven for 15 minutes. After cooling to room temperature, the sections were incubated with 3% H₂O₂ deionized water for 15 min to

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Table 1. Primer sequences

Primer	Sequence
HOXA11 mRNA	Forward primer: 5'-GCAGCAGAGGAGAAGAGGG-3'
	Reverse primer: 5'-GTGGATTGCTGAGTAGTACTG-3'
GAPDH	Forward primer: 5'-CCCATCACCATCTCCAGGAG-3'
	Reverse primer: 5'-CTTCTCCATGGTGGTGAAGACG-3'

block endogenous peroxidase. The sections were then washed with PBS buffer for 10 minutes and incubated with goat serum blocking solution for another 15 minutes. Then, pan-cytokeratin, chromogranin A, CD56, and Ki-67 primary antibodies (Abcam, America) were added for incubation overnight at 4°C. On the second day, after another 10-minute PBS wash, biotin-labeled goat anti-rabbit secondary antibodies and horseradish peroxidase (both from Abcam, USA) were applied for 15 minutes each, with a PBS wash in between. Sections were then developed with DAB working solution (Biyuntian Biotechnology Co., LTD., China) and rinsed with tap water for 3 minutes. Hematoxylin was used for counterstaining, followed by washing. After dehydration, sections were sealed with conventional neutral gum.

The immunohistochemical results were determined by the Mattern integral method. Staining intensity was scored as 1 (weak), 2 (moderate), or 3 (strong). The proportion of positively stained cells was classified as 0 (0%), 1 (1%-25%), 2 (26%-50%), or 3 (51%-100%). Scores from intensity and proportion were multiplied to classify results as negative (≤ 3), positive (> 3), and strongly positive (> 6). Five random fields were observed under 100 \times magnification, and the average value was taken.

Cell lines

Two testicular GCT cell lines, TCam-2 and I-10 (purchased from American Tissue Culture Preservation Center (ATCC)), were used in this study. Cells were cultured using DMEM medium containing 10% fetal bovine serum (Gibco, America), 100 U/ml sodium penicillin (Gibco, America), and 100 mg/ml streptomycin (Gibco, America). Culture environment was maintained at 37°C in a 5% CO₂ atmosphere with saturated humidity.

Detection of qRT-PCR

HOXA11 mRNA expression in testicular tissues and GCT cells was detected by qRT-PCR. The

two testicular GCT cell lines were cultured in 5-Aza-dC (20 μ mol/L) for 72 h to induce demethylation. After collecting tissues and cells, the total RNA was extracted with Trizol reagent (Gibco, America), and reverse transcribed into cDNA using a reverse transcription kit (Takara, Japan). Primers for HOXA11 and

GAPDH were synthesized by Shanghai Sangon Bioengineering (Shanghai) Co., LTD. The primer sequences are listed in **Table 1**. The PCR reaction conditions included pre-denaturation at 95°C for 1 min, 95°C for 10 s, and 59°C for 30 s, with 50 rounds in total. The experiment included three replicates per sample group. The relative expression of target gene was calculated by $2^{-\Delta\Delta Ct}$ with GAPDH as reference gene.

Detection of cell proliferation by CCK-8

TCam-2 and I-10 cells in logarithmic growth phase were digested with trypsin (Hyclone, America) to prepare cell suspensions. After cell counting, the suspension was transferred into a 96-well plate. 100 μ l suspension was added to each well, with a cell density of 4000 to 5000 cells per well, and then put in an incubator for culture. The cells in demethylated group were added with 20 μ mol/L 5-Aza-dC and cultured for 0, 12, 24, 48, and 72 hours. Each time point had five replicates. 10 μ l CCK-8 solution (Solarbio, China) was added to each well, and subsequently incubated in an incubator at 37°C for another 2 h. The absorbance value of the cells at 450 nm was detected by microplate reader.

Statistical analysis

Data analysis was conducted by Statistical software SPSS 26.0. Measured data were expressed as ($\bar{x} \pm s$) and compared by t test for inter-group comparison or paired t test for intra-group comparison. Counted data were expressed as a rate and compared by χ^2 test. A difference was statistically significant if $P < 0.05$.

Results

Clinical data

The observation group consisted of patients aged between 22 to 51, with a mean age of (28.37 \pm 7.21) years. The cohort included 39

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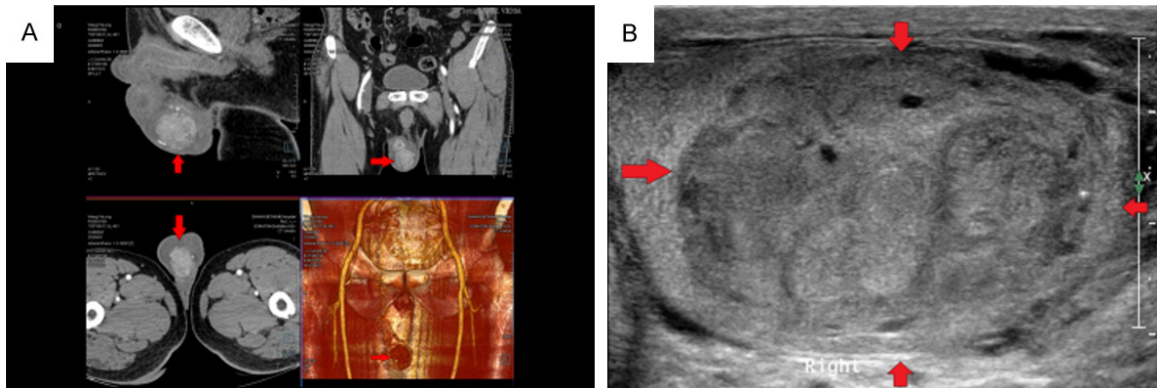


Figure 1. Imaging findings of primary testicular carcinoma. A. Enlarged right testis with high-density calcification and exudate as shown by computed tomography (CT); B. A solid echogenic lesion with well-defined borders and heterogeneous echotexture in the middle and lower parenchyma of the enlarged right testis by ultrasound examination.

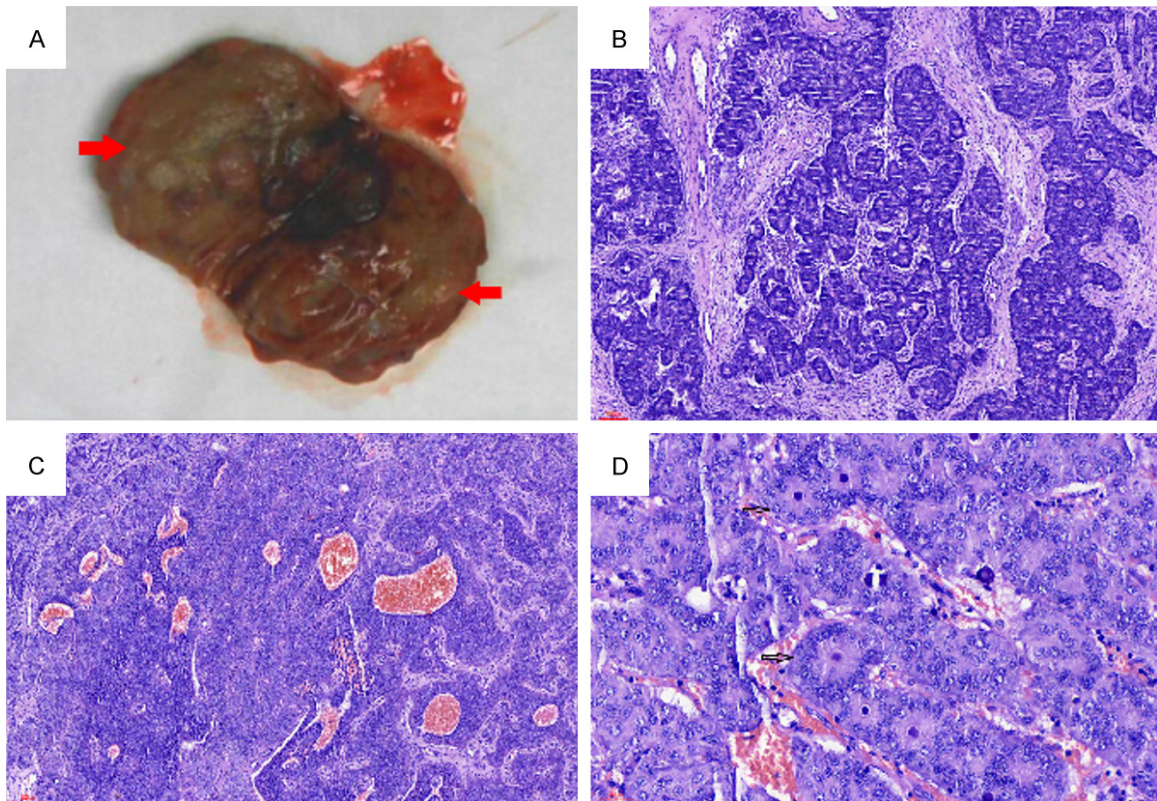


Figure 2. Gross observation and H&E staining of primary testicular carcinoma. A. 3.7 cm × 3.0 cm × 2.5 cm well-circumscribed tumor with gray-yellow color, firm and medium texture and hemorrhagic areas; B. A mixed insular, acinar, and trabecular pattern separated by delicate fibroconnective stroma in carcinoid tumor (magnification, ×100); C. Neoplastic cells were relatively homogeneous and medium in size, solid or pseudo-adenoid, arranged in string-like trabecular adenoid clustering and chrysanthemum-like (magnification, ×100); D. The cytoplasm of the cells was rich and eosinophilic and the nucleus was round or oval with granular chromatin concentrated along the nuclear membrane (magnification, ×400).

cases of seminoma and 24 cases of non-seminoma; 35 cases in stage I, 21 cases in stage II, and 7 cases in stage III; 33 cases of left testis and 30 cases of right testis.

Typical cases

The typical case was a 48-year-old male patient who presented in May 2020 with local testicu-

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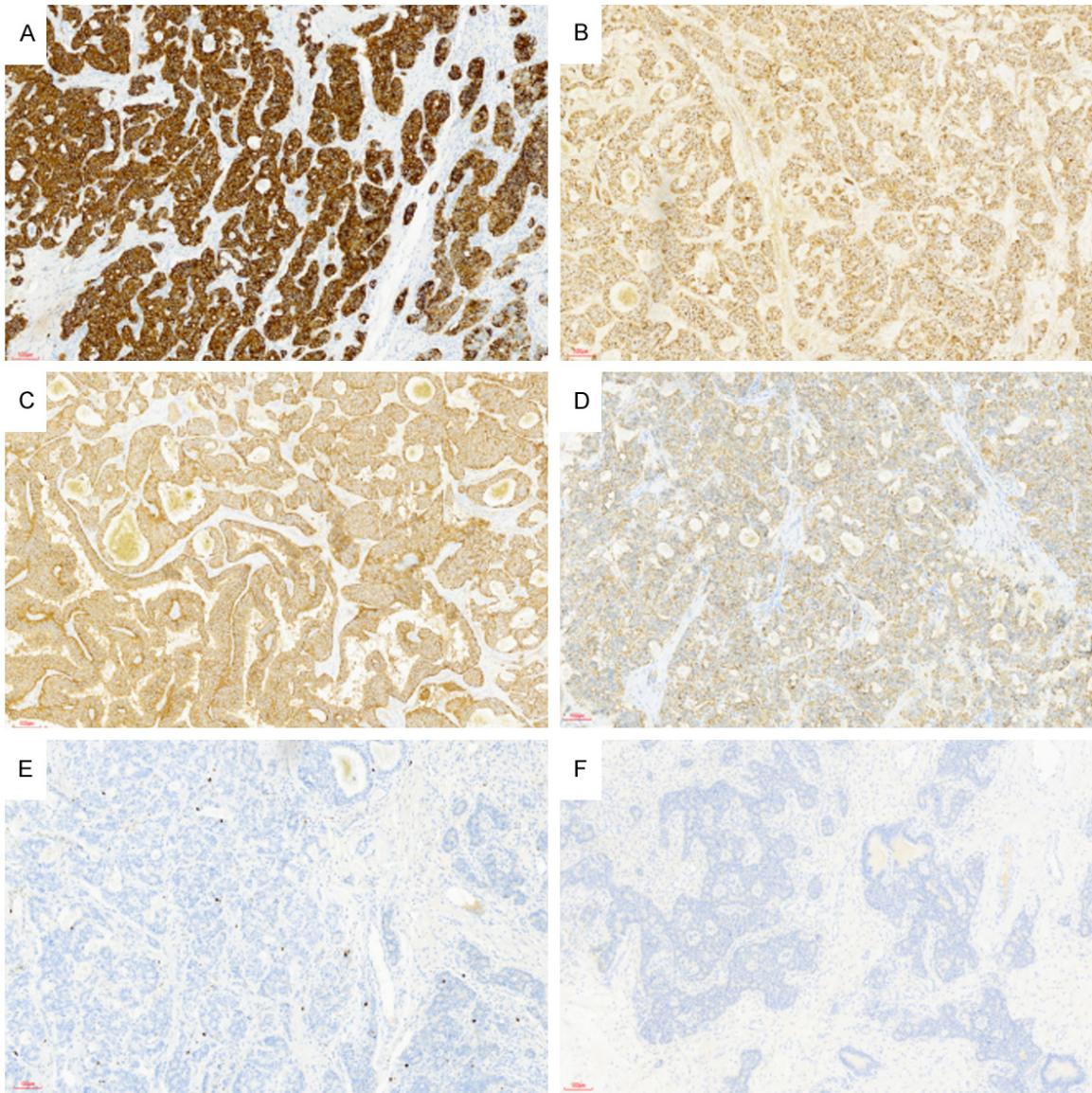


Figure 3. Immunohistochemical staining of testicular carcinoma. A. Neoplastic cells were positive for Pan-cytokeratin (magnification, $\times 100$); B. positive for chromogranin A (magnification, $\times 100$); C. positive for Syn (magnification, $\times 100$); D. positive for CD56 (magnification, $\times 100$); and E. Ki-67 proliferation index was about 2%. F. Neoplastic cells were negative for alpha-fetoprotein (magnification, $\times 100$).

lar swelling, pain, and redness of the scrotum. The CT showed an irregular shape of right testicle, 3.86 cm \times 2.73 cm in size, with high-density calcification, and an exudate around a linear low-density area (Figure 1A). Ultrasonography of the patient's urinary system revealed a solid echogenic lesion of 4.1 cm \times 2.4 cm in size with well-defined boundaries and an inhomogeneous echogenic structure in the middle and lower parenchyma of the right testis (Figure 1B).

Gross examination showed a nodular tumor, measuring 3.7 cm \times 3.0 cm \times 2.5 cm, with a gray-yellow color, firm and medium texture with local bleeding was observed (Figure 2A). Microscopically, the tumor exhibited a mixed insular, acinar and trabecular pattern, separated by delicate fibro-connective stroma in carcinoid tumor (Figure 2B). Neoplastic cells were homogeneous and medium-sized, displaying solid or pseudo-adenoid arrangements in string-like trabecular adenoid clusters and

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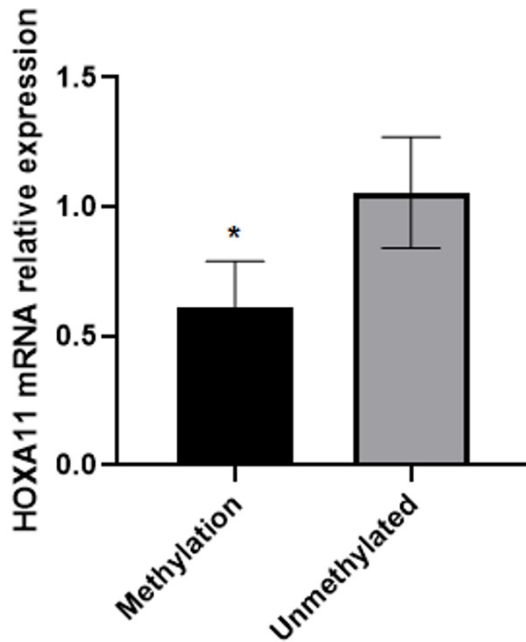


Figure 4. Comparison of HOXA11 mRNA expression in testicular tumor with different HOXA11 gene promoter methylation status. Note: Compared to unmethylated tissues, * $P < 0.05$.

chrysanthemum-like formations (**Figure 2C**). The cytoplasm of the cells was rich and eosinophilic, and the nuclei were round or oval with granular chromatin obviously concentrated along the nuclear membrane, without obvious atypia (**Figure 2D**).

We detected three groups of protein as auxiliary diagnosis biomarkers for the testicular carcinoma using immunohistochemistry staining. The biomarkers for differentiating benign tumors from malignant tumors, including AE1/AE3, CgA, Syn, and CD56, were all positive (**Figure 3A-D**). The proportion of cells with positive Ki-67 (Ki-67) was approximately 2% or lower (**Figure 3E**). Markers including AFP (**Figure 3F**), vimentin, PLAP, CD30, CK7, EMA, α -inhibin, S-100 protein, and CD34 were all negative. The immunohistochemical staining findings confirmed the diagnosis of testicular carcinoma.

Abnormal methylation of HOXA11 in testicular GCT

The methylation of CpG island of HOXA11 gene promoter in testicular GCT tissues and adjacent tissues was detected by Methylmion Specific PCR (MSP). HOXA11 gene methylation

was found in 47 (74.60%) of 63 testicular GCT tissues. HOXA11 gene methylation was detected in 11 cases (17.46%) of 63 normal adjacent tissues. The methylation rate of HOXA11 gene in testicular GCT tissues was higher than that of adjacent tissues ($\chi^2=41.404$, $P < 0.05$).

Effect of HOXA11 methylation on mRNA expression of HOXA11 in testicular GCT tissue

The relative expression level of HOXA11 mRNA in methylated testicular GCT tissues was significantly lower than that of non-methylated GCT tissues ($P < 0.05$) (**Figure 4**).

Relationship between HOXA11 promoter methylation and clinicopathologic features

The methylation of HOXA11 gene promoter in testicular GCT tissues was closely related to lymph node metastasis and stage (all $P < 0.05$). Specifically, the positive rate of HOXA11 gene promoter methylation was higher in cases with lymph node metastases and in those with clinical stage II to III, as shown in **Table 2**.

5-Aza-dC effect on HOXA11 mRNA expression

After 72 h of 5-Aza-dC treatment, mRNA/expression of HOXA11 was seen with a substantial increase in testicular GCT cells compared to untreated ($P < 0.05$) (**Figure 5**).

Effects of 5-Aza-dC on proliferation

After being treated with 5-Aza-dC for 24, 48 and 72 hours, cell proliferation activity of TCam-2 and I-10 cells was apparently inhibited as compared to the control group ($P < 0.05$) (**Figure 6**).

Discussion

Genetic changes in gene function have been the focus of research so far. Those changes that occur without alterations in DNA sequence, known as epigenetic changes. This is of great significance for studying the occurrence and progression of tumors [10-12]. Different epigenetic mechanisms, such as DNA methylation, histone modifications, and gene silencing by miRNA, play integral roles in regulating gene expression, either activating or repressing genes synergistically or through separate pathways [13-15]. Abnormal DNA methylation,

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Table 2. Relationship between HOXA11 promoter methylation and clinicopathologic features of patients

Group	Number of Cases	Methylation (n, %)	χ^2	P
Age				
<30 years old	34	25 (73.53)	0.007	0.936
≥30 years old	29	22 (75.86)		
The pathologic types				
Seminoma	39	30 (76.92)	0.291	0.590
Non-seminomatous tumor	24	17 (70.83)		
Lymph node metastasis				
Yes	15	15 (100.00)	5.058	0.025
No	48	32 (66.67)		
Clinical staging				
Stage I	32	20 (62.50)	10.127	0.002
Stage II-III	28	27 (96.43)		

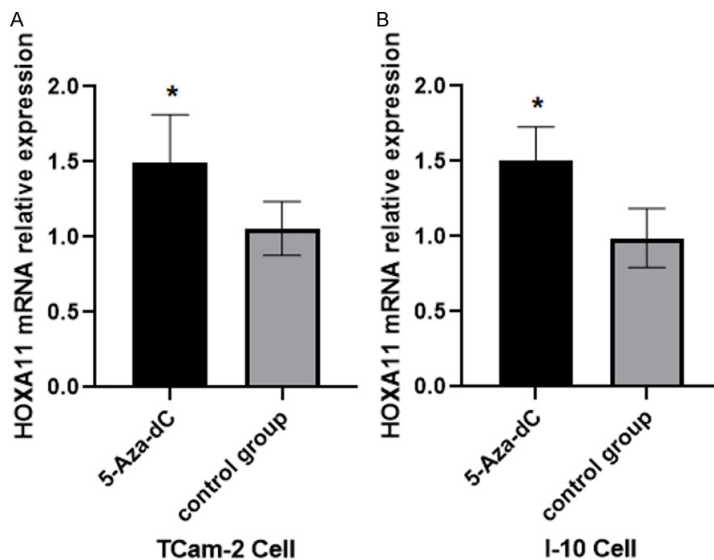


Figure 5. Effect of 5-Aza-dC treatment on HOXA11 mRNA expression in testicular GCT cells. A. Relative expression of HOXA11 mRNA increased significantly in TCam-2 cells treated with 5-Aza-dC; B. Relative expression of HOXA11 mRNA in I-10 cells treated with 5-Aza-dC significantly increased. Note: Compared to control cells, * $P < 0.05$.

especially hypomethylation of oncogenes and tumor suppressor genes, is an important mechanism of tumorigenesis and development. Tumor suppressor genes inhibit gene transcription and protein expression due to epigenetic modifications from excessive methylation, contributing to the progression of malignant tumors [16-19]. Abnormal methylation of tumor suppressor genes is widespread in numerous malignant tumors. Current studies show that methylation of some tumor-related genes can

serve as a biomarker for the diagnosis and targeted therapy of malignant tumors [20, 21].

Studies have shown that abnormal HOX gene expression is tied to the occurrence and progression of malignant tumors [22]. HOXA11, located on chromosome 7p, is a key member of the HOX gene. Multiple studies have found that aberrant methylation of HOXA11 gene is prevalent in renal, ovarian, colon, breast, lung, and gastric cancers. Abnormal methylation leads to the suppression of HOXA11 gene expression and promotes the development of tumors [23-26]. In this study, the methylation status of HOXA11 gene in testicular GCT tissues and normal adjacent tissues were detected with MSP. The results revealed that the positive rate of HOXA11

methylation in testicular GCT tissues was higher than that in adjacent testicular tissues, and methylation status in testicular GCT tissues was correlated with the malignant biological behaviors such as lymph node metastasis and clinical stage. Meanwhile, HOXA11 gene expression was downregulated in testicular GCT tissues with positive HOXA11 methylation. The results are consistent with findings from other scholars [27, 28], suggesting that abnormal methylation of HOXA11 in testicular GCT

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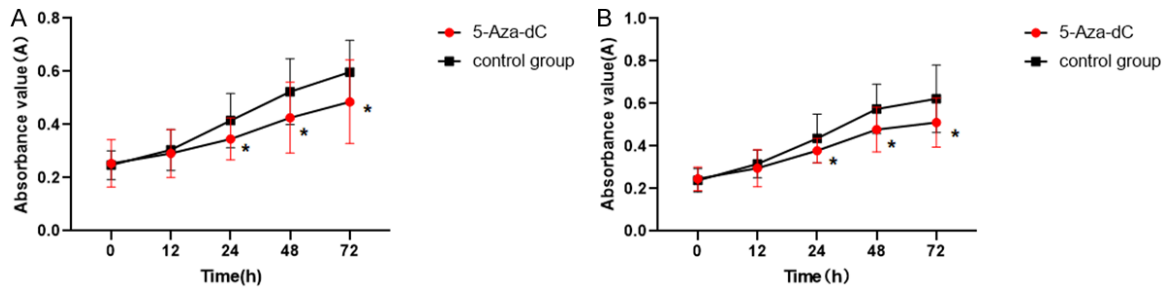


Figure 6. Effect of 5-Aza-dC treatment on proliferation of testicular GCT cells. A. Proliferation activity of TCam-2 cells was decreased after treatment with 5-Aza-dC; B. Proliferation activity of I-10 cells was decreased after treatment with 5-Aza-dC. Note: Compared to control cells, * $P < 0.05$.

may inhibit HOXA11 gene expression, thus promoting the occurrence and development of tumors.

In addition, we treated TCam-2 and I-10 testicular GCT cells with 5-Aza-dC. The results showed that after 72 h of treatment, the relative expression of HOXA11 mRNA was much higher than that of untreated cells. Besides, the proliferation activity of TCam-2 and I-10 cells treated with 5-Aza-dC was lower than that of the control group at 24, 48 and 72 h after treatment. This is consistent with our hypothesis that methyltransferase inhibitor treatment reduces the abnormal methylation level of HOXA11 and promotes HOXA11 gene expression, thereby inhibiting proliferation of testicular GCT cells.

In addition, due to the relatively small sample size included in this study, and since the impact of HOXA11 gene promoter methylation on the prognosis of testicular GCT patients was not analyzed, further in-depth investigation will be conducted to obtain more reliable research conclusions.

In summary, the abnormal methylation of HOXA11 gene promoter significantly inhibits the transcription and expression of HOXA11 in testicular GCT cells and tumor tissues. The abnormal methylation of the HOXA11 promoter region is closely related to lymph node metastasis and TNM staging in testicular germ cell tumor.

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Disclosure of conflict of interest

None.

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