Abstract. The aim of the present study was to explore and verify the potential mechanism of seminoma progression. Data on 132 RNA-seq and 156 methylation sites from stage II/III and I seminoma specimens were downloaded from The Cancer Genome Atlas database. An initial filter of |fold-change| > 2 and false discovery rate < 0.05 were used to identify differentially expressed genes (DEGs) which were associated with differential methylation site genes; these genes were considered potential candidates for further investigation by survival analysis. Potassium voltage-gated channel subfamily C member 1 (\textit{KCNC1}) expression was verified in seminoma human tissues and three seminoma cell lines. The invasive, proliferative and apoptotic abilities of the human testicular tumor Ntera-2 and normal human testis Hs1.Tes cell lines were assessed following aberrant \textit{KCNC1} expression. \textit{KCNC1} was identified as a DEG, in which hypermethylation inhibited its expression and it was associated with poor overall survival in patients with seminoma. The present results demonstrated that \textit{KCNC1} is negatively correlated with methylation. Due to the abnormal expression of \textit{KCNC1} in seminoma cells, it was suggested that \textit{KCNC1} could be used as a diagnostic indicator and therapeutic target for the progression of seminoma.

Introduction

Testicular germ cell tumors (TGCTs) are the most common solid tumors in men aged 15 to 35 years and account for >90% of primary malignant tumors of the testes (1). TGCTs can be clinically classified into two main categories: Seminoma and non-seminoma. The latter can be further subdivided into embryonic cell carcinoma, choriocarcinoma, yolk sac tumor or teratoma (2). Non-seminomas have a high degree of differentiation but can have multiple morphological phenotypes, since they contain both stem cells and cells that have differentiated to varying degrees towards somatic lineages. Seminomas have a lower level of differentiation and resemble primordial germ cells and/or intratubular germ cell neoplasia of unclassified type cells (3-5). Seminomas can be staged according to the degree of proliferation and metastasis. Stage I seminomas can be completely treated by surgery and additional interventions, such as close follow-up, drug-assisted chemotherapy and adjuvant radiotherapy (6). However, stage II/III seminomas have a lower survival rate following surgery and postoperative radiotherapy and chemotherapy (7). Recent studies have shown that immunotherapy and gene therapy are effective in treating seminomas (8,9). Therefore, exploring the gene expression profile of seminomas is essential to improve the understanding of its oncogenesis and progression.

DNA methylation is an important epigenetic modification that changes genomic expression without altering DNA sequences. DNA methyltransferase 3α (\textit{DNMT3A}), encodes a DNA methyltransferase that is thought to function in methylation. Several studies have shown that DNA methylation plays an important role in the development and progression of tumors, mainly through the changes in DNA methylation patterns and DNA methyltransferase expression levels (10). Evidence has shown that hypermethylation can inhibit the expression of tumor-suppressor genes, thereby promoting tumor progression. For example, in sporadic retinoblastoma (RB), RB tumor-suppressor gene functions may become inactivated due to methylation, with its expression levels reduced to 8%, as compared with unmethylated RB (11,12).

Potassium voltage-gated channel subfamily C member 1 (\textit{KCNC1}) encodes a member of the family of membrane proteins that mediate voltage-dependent potassium ion permeability in excitable membranes (13). The abnormal expression and activity patterns of K+ channels on the surface of cancer cells can drive tumor transformation, malignant progression and metastasis, or drug resistance, through Ca2+ activated IK or BK channels (14,15). Other studies have shown that K+ channels can affect cell proliferation and cell cycle...
progression in breast cancer cells (16). However, K⁺ channels are poorly studied in seminoma, and their role in seminoma progression remains unclear. Previous studies have found that microRNA (miRNA/miR)-199a-3p, as a tumor-suppressing miRNA, negatively regulates the expression of methylation marker DNMT3A and impacts the level of glycometabolism in testicular germ cells, ultimately inhibiting the progression of seminomas (17,18).

In the present study, the methylation sites and RNA-seq data collected from The Cancer Genome Atlas (TCGA) were integrated to identify KCNC1 as a methylation-regulated gene that plays an important role in the progression of malignant seminoma. Furthermore, the expression of KCNC1 in seminoma tissues and cell lines was verified by immunohistochemical staining, western blot analysis and reverse transcription-quantitative (RT-q)PCR. In the present study, we hypothesized that hypermethylation can affect the expression of KCNC1, thereby promoting seminoma progression. The objective was to discover novel target genes for the treatment of seminoma in order to inhibit its progression and improve the prognosis of patients.

Materials and methods

Data sourcing and analysis. RNA-seq data from 26 patients with stage I seminoma and 106 patients with stage II/III seminoma were downloaded from The Cancer Genome Atlas (TCGA) database https://www.cancer.gov/tcga (19). GDCRNATools (version 1.10.1) (20) in the R package (version 4.0.3) (https://cran.r-project.org/) was used to analyze differentially expressed genes (DEGs). |Fold-change| >2 and false discovery rate (FDR) <0.05 were set as the screening thresholds. Differential methylation sites of 156 seminoma specimens in the TCGA database were analyzed using ELMER software package (version 2.14.0) (21). A total of 162 differential hypermethylation sites between stage II/III and stage I seminomas were obtained, and the regulated genes around these methylation sites were then confirmed. The genes associated with differential hypermethylation sites were cross-referenced with the DEGs, and correlation analysis was performed. A total of 14 methylation-DEGs were obtained and survival analysis was performed. The results confirmed that KCNC1, a gene regulated by hypermethylation, affected the overall survival of seminoma patients. Correlation analysis between KCNC1 and methylation and demethylation markers was performed using the GEPIA online tool (http://gepia.cancer-pku.cn/) (22).

KCNC1 gene expression analysis. Public data were analyzed using eBioPortal (http://www.ebioportal.org/) and GEPIA. The expression of KCNC1 in the normal tissue was compared to that in TGCTs, as well as in metastatic and non-metastatic TGCTs. All data originated from the TGCT dataset of TCGA. Disease-free survival analysis based on KCNC1 expression was obtained from GEPIA.

Cell culture and immunohistochemical staining. Tissue specimens (collected from 2015 to 2019 from patients 30-40 years of age) were obtained from the Pathology Department of The Second Hospital of Tianjin Medical University, Tianjin, China. The use of tumor tissue for immunohistochemistry was approved by the Second Hospital of Tianjin Medical University Ethics Committee (Tianjin, China). The family members of the patients were informed that the tumor tissue removed during surgery would be used for further scientific research, and the approval of the family members of the patients and their informed consent was obtained.

Immunohistochemical staining of formalin-fixed, paraffin-embedded seminoma and adjacent non-tumor tissues was performed. These specimens were preserved by our hospital, and were approved by a pathologist prior to use. Tissues were classified according to normal testicular tissue, localized seminoma and metastatic seminoma (3 cases per group). A total of 9 tissue sections from the following pathological groups were prepared: normal, stage I and stage II/III. Immunohistochemical staining using 0.4-µm thick sections was performed using a KCNC1 antibody (rabbit; dilution, 1:500; Abcam) and stained with hematoxylin. The staining was performed according to normal immunohistochemical procedures. The human Ntera-2 testicular tumor (NT2), normal human testis Hs1.Tes (HT) and Tcam-2 cell lines (human testicular seminoma cells) were purchased from the American Type Culture Collection. They were cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin.

Western blot analysis and RT-qPCR. Protease inhibitors (Beyotime Institute of Biotechnology) were mixed with total protein extraction reagent (Beijing Solarbio Science & Technology Co., Ltd.) at a ratio of 1:100, and then mixed with cultured cells. The protein concentration was determined using BCA. Protein samples were mixed with loading buffer and loaded onto a 10% SDS-PAGE for western blot analysis. The protein expression levels of KCNC1, DNMT3A and GAPDH, as well as epithelial-mesenchymal transition (EMT)-related markers and apoptotic markers were detected by western blot analysis. The antibodies used are listed in Table I. The primary antibody was incubated at 4°C overnight, the secondary antibody was incubated at room temperature for 1 h, and ECL (Biochannel Co., Ltd.) was used for exposure.

Total RNA was extracted using TRizol® reagent (Thermo Fisher Scientific, Inc.), and the Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) was used for reverse transcription. FastStart Universal SYBR-Green Master Mix (Hoffmann-La Roche Ltd.) was used for RT-qPCR following the manufacturer's instructions (Table II). GAPDH mRNA expression was used as an endogenous control for normalization in each sample. The specific steps are as follows: reaction temperature and reaction time: Pre denaturation at 94°C for 3 min, denaturation for 0.5 min at 94°C, annealing at 58°C for 0.5 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The melting curve and amplification curve of qPCR were observed. The experimental data were calculated using the 2^(-ΔΔCt) formula (23).

Small interfering RNA (siRNA) transfection and plasmid construction. Following the manufacturer's instructions,
siRNA and negative control siRNA (Suzhou GenePharma Co., Ltd.) were transfected into HT cells with X-treme gene siRNA transfection reagent (Suzhou GenePharma Co., Ltd.). NT2 cells were transduced with a lentivirus encoding KCNC1 overexpression or control plasmid (Beijing Syngentech Co., Ltd.). The knockdown of KCNC1 expression following siRNA transfection was verified by RT-qPCR and western blot analysis. The siRNA sequence used was 5'-CCG GGC CCG TCA TCG TGA ACA ATT TCT CGA GAA ATT GTT CAC GAT GAC GGG CTT TTT G-3'. The negative control sequence used was: 5'-GTT CTC CGA ACG TGT CAC GTT TCA AGA GAA CGT GAC ACG TTC GGA GAA CTT TTT TG-3'. Six hours after siRNA transfection, the transfection medium was removed and the new medium was added.

Transwell and Cell Counting Kit (CCK)-8 cell viability assays. In the Transwell assay, 2-3,000 transfected NT2 and HT cells were transferred to the upper Transwell chamber. RPMI-1640 medium (200 µl) was added to the upper chamber and full medium (600 µl) to the lower chamber. Then, 1 day later, DAPI staining was used to observe cell membrane permeability under a fluorescence microscope.

HT and NT2 cells were inoculated in a 96-well culture plate. When the cells reached 30-50% confluence, lv-KCNC1 and KCNC1-siRNA or negative control was used for transfection. At 24, 48 and 72 h after transfection, CCK-8 solution was added to 96-well plates at a ratio of 1:9. The optical density value at a 450 nm wavelength was measured by a microplate reader.

Flow cytometry. Flow cytometry was performed 48 h after the transfection of NT2 and HT cells. A total of 1x10^6 transfected NT2 and HT cells were resuspended in 500 µl PI/RNase staining solution (Sungene Biotech) 15 min before flow cytometry, and Annexin V-FITC/PI kit (US Everbright, Inc.) was used for cell apoptosis detection.

Dot blot analysis. Genomic DNA was extracted from NT2 and HT cells, using a DNA isolation kit, following the manufacturer's instructions (Qiagen AB). DNA samples were dropped on the corresponding spots of the nitrocellulose membrane soaked in sodium citrate. The nitrocellulose membrane was baked in the oven at 80°C for 1 h, and then exposed to ultraviolet light for 30-45 min for sealing. Finally, the nitrocellulose membrane was incubated with a 5-nc and anti-5-hmc antibody (dilution, 1:1,000; Abcam) in a refrigerator at 4°C overnight.

Statistical analysis. All experiments were carried out at least three times. All data are expressed as the mean ± standard deviation. ANOVA was performed to evaluate the difference of three or more groups by Turkey post hoc test, and the statistical analysis was carried out by using GraphPad Prism 8.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference. SPSS version 22 was used for statistical analysis (IBM, Corp.).

Results
KCNC1 participates in the malignancy and prognosis of seminomas. RNA-seq data were collected from TCGA-TGCT datasets. |Fold-change| >2 and FDR <0.05 were set as the screening thresholds. A total of 864 DEGs were identified, with 410 upregulated and 456 downregulated genes (Fig. 1A). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analysis was performed (Fig. 1B and C). The two major signaling pathways associated with the DEGs were found to be ‘drug metabolism-cytochrome P450’ and ‘metabolism of...
xenobiotics by cytochrome P450. The average methylation level of stage II/III seminomas was higher than that of stage I seminomas, with 162 elevated methylation sites in stage II/III seminomas (Fig. 2A). A total of 20 genes in close proximity to the differential methylation sites were selected, and their correlation with the DEGs was assessed. A total of 14 differentially methylated sites and DEGs were paired up (Fig. 2B). Pearson's correlation analysis was carried out at the level of paired methylation and gene expression. Survival analysis of 14 methylation-DEGs revealed that only KCNC1 expression affected disease-free survival in patients with seminomas (Fig. 2C and D; Table III). These results indicate methylation-DEG gene KCNC1 expression in stage II/III seminomas is significantly lower than that in stage I seminomas and lower KCNC1 expression reduces disease-free survival in patients with seminomas.

High KCNC1 expression of mRNA is found in normal tissues and localized seminomas. KCNC1 gene expression across all tumor samples and paired normal tissues was analyzed using the GEPIA online tool. The expression of KCNC1 in glioblastoma multiforme (GBM), brain lower grade glioma (LGG) and TGCTs was significantly lower than that in normal tissues (Fig. 3A and B). The expression of KCNC1 in localized seminoma was also analyzed by cBioportal, which was significantly higher than that in metastatic seminoma (Fig. 3C). In addition, there was no mutation of DNMT3A in seminoma at present by analyzing of cBioportal. These results further confirmed the expression of KCNC1 in different stages of seminoma.

Low KCNC1 expression is associated with malignant seminomas. Immunohistochemical analysis showed that the expression of KCNC1 protein was the highest in normal
tissues, significantly higher than that in non-metastatic and metastatic seminoma tissues (Fig. 4A). KCNC1 expression was further verified in three cell lines. The expression of KCNC1 was the highest in the normal HT cell line and the lowest in the metastatic NT2 cell line (Fig. 3B and C). The results above indicated that KCNC1 is negatively correlated with the malignancy of seminoma, and as a tumor-suppressor gene, KCNC1 plays an important role in tumor progression.

Silencing and overexpression of KCNC1 alters seminoma cell invasion and metastasis, respectively. To determine whether KCNC1 plays a functional role in seminoma cells, KCNC1-specific siRNAs were transiently transfected into HT cells, and a lentivirus encoding KCNC1 into NT2 cells. Changes in the KCNC1 mRNA and protein expression were confirmed by RT-qPCR and western blot analysis (Fig. 5A and B). The expression of EMT-related markers was verified following knockdown and overexpression of KCNC1 in the HT and NT2 cells by western blot analysis. The expression of vimentin, ZEB1 and N-cadherin was significantly altered, which means that the metastatic ability of the KCNC1-overexpressing NT2 cells and KCNC1-silenced HT cells were altered accordingly (Fig. 5C). A Transwell invasion assay showed that the invasion ability of HT cells was significantly enhanced following KCNC1 knockdown. However, overexpression of KCNC1 attenuated the invasion ability of NT2 cells (Fig. 5D). Fig. 5E shows the quantification of invaded cells. The invasion ability of tumor cells allows them to metastasize to distant organs and thus increases its malignancy.

Changes in the apoptosis and proliferation of seminoma cells are observed following the aberrant expression of KCNC1. CCK-8 proliferation assay showed that KCNC1 knockout in HT cells and overexpression in NT2 cells significantly increased and decreased cell proliferation at 48, 72 and 96 h, respectively (Fig. 6A). The apoptosis-related markers caspase-3 and Bax (associated with the promotion of apoptosis) and Bcl-2 (associated with the inhibition of apoptosis) expression levels also changed accordingly (Fig. 6B). The flow cytometry results demonstrated that adjustments in KCNC1 induced an apparent change in the number of early- (Annexin V signal only) and late- (Annexin V plus PI signal) apoptotic cells. Annexin V/PI staining (Fig. 6C) and quantitative processing were performed as shown in Fig. 6D. KCNC1 knockdown in HT
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Table III. Disease-free survival of the differentially methylated genes.

| Gene        | P-value |
|-------------|---------|
| KCNC1       | 0.025a  |
| KIAA0513    | 0.24    |
| LRMP        | 0.88    |
| PTPRC       | 0.53    |
| FMN2        | 0.16    |
| FMO3        | 0.44    |
| PIK3CG      | 0.50    |
| KIAA0513    | 0.24    |
| SLC18A2     | 0.43    |
| TUBB8       | 0.43    |
| SNORD37     | 0.88    |
| NKPD1       | 0.15    |
| BLK         | 0.27    |

*Only KCNC1 expression affected disease-free survival in patients with seminomas. KCNC1, potassium voltage-gated channel subfamily C member 1.

Discussion

The presence of seminoma poses a serious threat to the health of men aged 15-35 years. Its biology and treatment
remain an active area of research worldwide. For early-stage seminoma, surgical treatment can achieve a curative effect. However, when tumors progress and metastasize, treatment options are limited and patient prognosis is poor. Therefore,
Figure 6. Changes in the apoptosis and proliferation of human testis Hs1.Tes (HT) and Ntera-2 testicular tumor (NT2) cells were observed following respective treatments. (A) The CCK-8 assay was used to indicate the proliferation of human testis Hs1.Tes (HT) and Ntera-2 testicular tumor (NT2) cells (*P<0.05). (B) Apoptosis-related markers (Bcl-2, caspase-3 and Bax) were confirmed by western blot analysis following the overexpression and silencing of KCNC1. (C) The apoptosis of human testis Hs1.Tes (HT) and Ntera-2 testicular tumor (NT2) cells following KCNC1 knockdown and overexpression was analyzed by flow cytometry. (D) Quantification of C (*P<0.01). KCNC1, potassium voltage-gated channel subfamily C member 1.

Figure 7. Association between KCNC1 and methylation in seminoma cells. (A and B) mRNA and protein expression of KCNC1 and DNMT3A in three types of seminoma cells (*P<0.05, **P<0.01). (C) Correlation analysis of KCNC1, DNMT3A/DNMT3b and TET/TET2. (D) Dot blot analysis was used to detect changes in the methylation level after altering KCNC1 expression.
novel and more effective therapeutic targets for seminoma are urgently required. Public databases, such as TCGA and Gene Expression Omnibus databases, contain a wealth of valuable transcriptome sequencing data. Fully utilizing these public databases provides a more in-depth understanding of the biomarkers and therapeutic targets of seminoma, as well as the mechanisms underlying their development and progression. In the present study, the RNA-seq data from TCGA-TGCT dataset were analyzed, to screen for DEGs between stage II/III and stage I seminomas. Methylation data of seminoma specimens was also analyzed using the Elmer package. Corresponding methylation-regulated DEGs were thus obtained, and a new seminoma-related gene, KCNC1, was identified. Immunohistochemical staining, western blot analysis and RT-qPCR confirmed the expression of KCNC1 in seminoma tissues and cells. The results showed that hypermethylation could inhibit the expression of KCNC1, promoting seminoma progression and adversely affecting the disease-free survival of seminoma patients. Following the aberrant expression of KCNC1 in HT and NT2 cells, their invasion, metastasis and proliferation abilities were significantly altered, which influenced the progression of seminoma malignancy. This suggested that KCNC1 can be used as a potential clinical therapeutic target, and that the overexpression of KCNC1 can effectively inhibit the progression of seminoma.

Normal body fluid volume, osmotic pressure and electrolyte content are crucial to maintaining a normal metabolism, stable internal environment and normal function of various organs. When tumors occur, the tumor cells and surrounding environment create the tumor microenvironment (TME) (25). In the TME, the opening and exchange of ion channels on the surface of tumor cells also change accordingly, which has a certain impact on the activity, invasion and proliferation of tumor cells, and plays a role in the occurrence and development of tumors (24,26). The K+ channel on the plasma membrane is involved in several cellular processes, including cell proliferation, migration, invasion and apoptosis. KCNC1 is a subunit of the Kv3 potassium channel (27). Voltage-gated K+ channels are critically involved in the proliferation of tumor cells. Moreover, in certain cells, the inhibition of the K+ channels has been shown to be beneficial to apoptosis, whereas the activation of the K+ channel can prevent apoptosis (28). It was found herein that hypermethylation can regulate the expression of KCNC1, and then affect the proliferation, invasion and metastasis of seminoma cells. By changing the expression of KCNC1, the metastasis ability of the seminoma cell line was significantly altered, which was mainly reflected in the level of EMT-related markers. At present, research on the associated mechanism has not been elucidated, and no relevant literature is available. Further research would therefore be beneficial.

In conclusion, the present study revealed that KCNC1 is associated with seminoma progression and is regulated by methylation. The abnormal expression of KCNC1 may alter the number of K+ channels on the surface of cancer cells, potentially promoting tumor transformation, malignant progression and metastasis. Based on the present findings, this may be a potential mechanism of seminoma progression, and overexpression of KCNC1 may be an innovative strategy for the treatment of seminomas. The mechanism of KCNC1 remains unclear. The present study demonstrated that the expression of KCNC1 can affect the expression of DNMT3A/DNMT3B and TET1/TET2, and then change the methylation level of seminoma cells. Therefore, it needs to be explored whether KCNC1 can affect the expression of a promoter by regulating their promoter sequence by double luciferase reporter gene detection experiment and Chip experiment. In addition, the effects of DNMT3A on the methylation of the promoter lesion of KCNC1 is also a research direction worthy of further study, because at present, the regulation mode between many genes is bidirectional. If we can confirm that DNMT3A regulates the promoter of KCNC1, it will deepen our understanding of the function of KCNC1 in the progression of seminoma. Only through further study of the mechanism is it possible to fully identify the important role of KCNC1 in the progression of seminoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

SC conducted the project development, data collection, data analysis, and manuscript writing. LX carried out the data analysis. HP performed the data collection. ZW assisted in the experimental procedures. JB conducted the project development, and coordinated financial support. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The use of tumor tissue for immunohistochemistry was approved by the Second Hospital of Tianjin Medical University Ethics Committee (Tianjin, China). The family members of the patients were informed that the tumor tissue removed during surgery would be used for further scientific research, and the approval of the family members of the patients and their informed consent was obtained.

Ethics approval and consent to participate

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.
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