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Research article

Long non-coding RNA TTTY14 promotes cell proliferation and functions as a prognostic biomarker in testicular germ cell tumor

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ABSTRACT

Testicular germ cell tumors (TGCTs) commonly occur in males between the ages of 15 and 34, accounting for 98% of testicular malignancies. Long non-coding RNAs (LncRNAs) have been reported to play important roles in TGCT proliferation, invasion, and functioned as prognostic biomarkers. Testis-specific transcript, Y-linked 14 (TTTY14), a long non-coding RNA localized on Chr Y q11.222, has been found to be a potential prognostic biomarker for laryngeal squamous cell carcinoma, gastric cancer, and osteosarcoma. The biological role of TTTY14 in TGCT is not well understood. In this study, we aim to clarify the biological role of TTTY14 in TGCT, as well as its role in TGCT survival prognosis and immunotherapy efficacy prediction through the deep mining of public data combined with the verification of cell biological experiments. We found that high TTTY14 expression was a poor survival prognostic factor in TGCT patients and the expression of TTTY14 might be regulated by copy number variation and DNA methylation. TTTY14 knockdown significantly inhibited the proliferation of TGCT in vitro. TTTY14 expression was positively correlated with immune cell dysfunction, and significantly negatively correlated with B cells, $CD8^+$ T cells, and macrophages, suggesting that TTTY14 may also affect the drug sensitivity by regulating the tumor immune microenvironment. In conclusion, we revealed that lncRNA TTTY14 was a novel oncogene and a biomarker in TGCT. TTTY14 may influence the drugs sensitivity through regulating the tumor immune microenvironment.

1. Introduction

Testicular germ cell tumors (TGCTs) commonly occur in males between the ages of 15 and 34, accounting for 98% of testicular malignancies [1]. Over the past decade, the incidence of TGCTs has increased by about 3% per year on average. TGCTs can be divided

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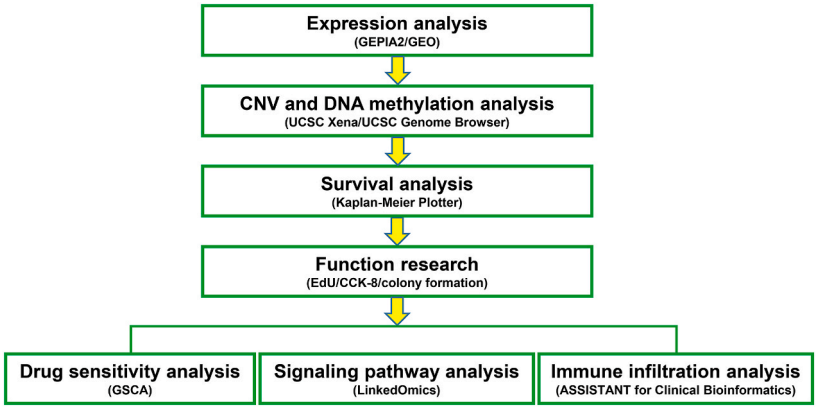

Fig. 1. The flowchart presents research methodology.

into: seminoma (seminoma, SE) and non-seminoma (non-seminoma, NS) [2]. Among them, 60.6% of TGCTs are SE, which is a transformed germ cell homologous tumor, and is similar to primordial germ cells and gonocytes; 38.8% of TGCTs are NS, which is a heterologous tumor [3]. NS may occur in various differentiation stages of undifferentiated cells or highly differentiated cells [4]. It has been reported that SE can be converted to NS by reprogramming or activating pluripotency genes [5]. Prognosis of TGCT is typically dismal, with disease progression despite high-dose salvage chemotherapy treatment [6].

Studies have shown that TGCTs are caused by abnormal expression of genes that related to proliferation, pluripotency, and epigenetics. Our previous study found that several long non-coding RNAs (LncRNAs), including lncRNAs RFPL3S, LINC00313, and LINC00467, played important roles in TGCT proliferation, invasion, and functioned as prognostic biomarkers [7–9]. This indicates that long non-coding RNAs play an important role in the occurrence and development of TGCT.

Testis-specific transcript, Y-linked 14 (TTTY14) is a long non-coding RNA localized on chromosome Y and at q11.222. TTTY14 has been found to be a potential prognostic biomarker for laryngeal squamous cell carcinoma, gastric cancer, and osteosarcoma [10–12]. However, as its name suggests that as a long noncoding RNA initially thought to be testis-specific, the biological role of TTTY14 in TGCT is not well understood. Through the deep mining of public data combined with the verification of cell biological experiments, this study clarified the function of TTTY14 in TGCT, as well as its role in TGCT survival prognosis and immunotherapy efficacy prediction.

2. Materials and methods

2.1. Ethics statement

This study did not require ethical approval, because it did not involve human participants. This study analyzed the public data from The Cancer Genome Atlas (TCGA) and performed cell biological experiments in NCCIT (CRL-2073) cells.

2.2. Data sources

The cohort data and DNA methylation data of TGCT in TCGA was downloaded from UCSC XENA [13]. The data of GSE3218 (including 17 cases seminomas, 42 cases non-seminomas germ cell tumors, and 5 cases normal testis specimens) with lncRNA expression data that contains TTTY14 was chosen and downloaded from GEO database [14]. The research methodology was shown as flowchart (Fig. 1).

2.3. Bioinformatic analysis

GEPIA2 (http://gepia2.cancer-pku.cn/#index) [15] was used to analyze the expression of TTTY14 in various tumors. The TGCT dataset GEPIA used is also based on the UCSC Xena project. TCGA Pan-Cancer dataset was used to analyze the expression level of TTTY14 across various cancer types. The expression of TTTY14 in this database were normalized and presented using log2(TPM+ 1). The Kaplan-Meier Plotter was used to analyze the relationship between TTTY14 and survival prognosis of TGCT patients [16]. All analyses were grouped using the best cut-off values for the site based on TTTY14 expression. LinkedOmics was used to analyze co-expression gene and Gene Set Enrichment Analysis (GSEA) based on TCGA TGCT cohort data, and the default parameters were used in this online tool [17]. UCSC XENA was used to analyze the correlation between TTTY14 and DNA methylation and copy number, and the relationship between TTTY14 copy number and prognosis of TGCT patients [13]. The online tool can plot heat maps of samples and TTTY14 copy number levels, expression levels, and methylation levels, and can sort these data from high to low for easy display. log2 (norm_count+1) was used for normalization and display of TTTY14 expression values, beta for TTTY14 methylation, and log2 (tumor/normal) for TTTY14 copy values. TTTY14 copy values were displayed using log2(tumor/normal). ASSISTANT for Clinical

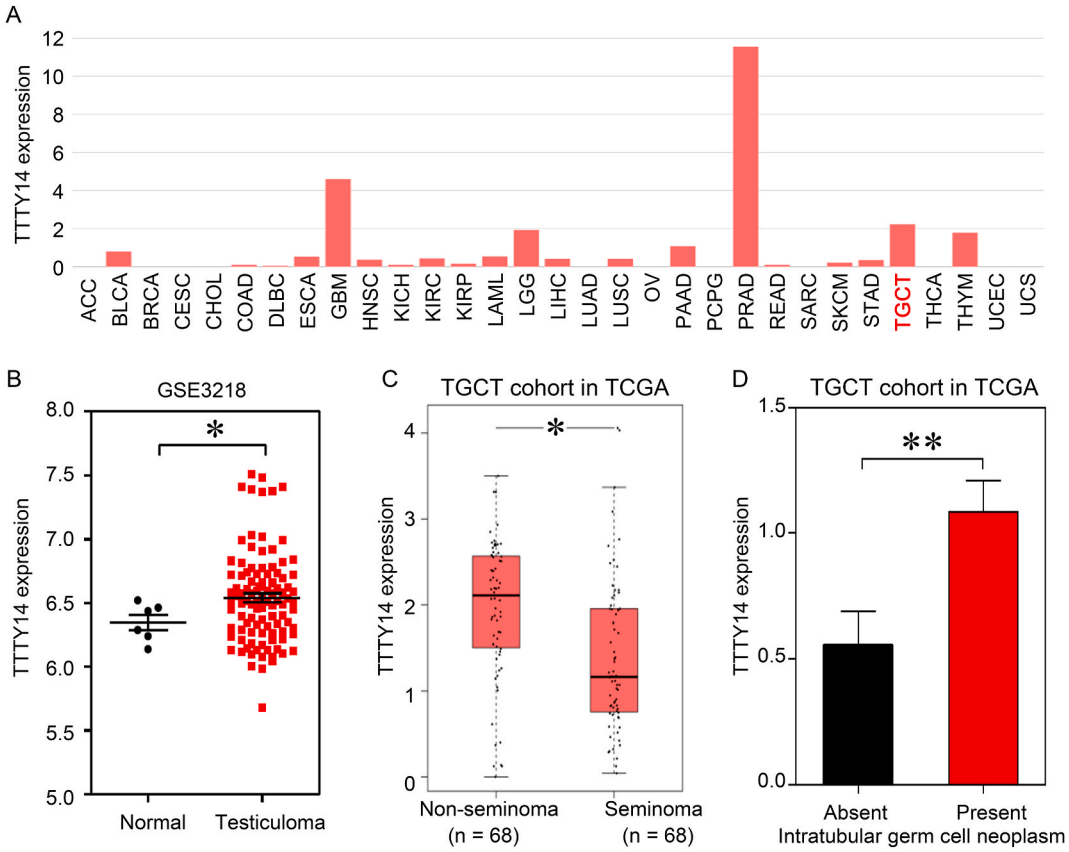

Fig. 2. Expression of Testis-specific transcript, Y-linked 14 (TTTY14). (A) GEPIA was used to analyze the expression of TTTY14 in various tumors. TTTY14 is highly expressed in GBM, PRAD and TGCT. The normalization method is Transcripts Per Million (TPM). The scale of y-axis is log2 (TPM+1). (B) The expression of TTTY14 in normal testis samples and testicular tumor samples was analyzed using the GSE3218 dataset in the GEO database. (C) The expression of TTTY14 in non-seminoma and seminoma was analyzed by GEPIA. (D) The TGCT cohort data was downloaded from the UCSC XENA database to analyze the expression of TTTY14 in intratubular germ cell tumors. *P $<$ 0.05, **P $<$ 0.01.

Bioinformatics tool was used to analyze the relationship between TTTY14 expression and the abundance of various immune cells in TGCT samples based on TIMER algorithm and Tumor Immune Dysfunction and Exclusion (TIDE) algorithm [18]. Using Gene Set Cancer Analysis (GSCA) online tool to analyze the correlation between TTTY14 expression and the sensitivity of Genomic s of Drug Sensitivity in Cancer (GDSC) drugs and the sensitivity of cancer therapeutics response portal (CTRP) drugs in TGCT [19].

2.4. Cell culture and siRNA transfection

NCCIT (CRL-2073, NCCIT was established by Shinichi Teshima (National Cancer Institute, Tokyo, Japan) in 1985 from a mediastinal mixed germ cell tumor.) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in RPMI-1640 media plus 10% fetal bovine serum (FBS) and 1% penicillin at 37 °C in 5% CO_2. For knockdown TTTY14, 10×10^5 NCCIT cells were seeded in a 6-well plate and added with 20 nM TTTY14 siRNA or negative control (NC) siRNA that mixed with Lip3000 for 6 h. The medium was refreshed and the cells were cultured for 48 h. To validate the specificity and efficiency of the knockdown, qRT-PCR was performed to detect the expression of TTTY14 after transfection. The transfected cells were used for further analysis. The TTTY14 siRNA sequences and non-target negative control (NC) was synthesized by Ribobio (Guangzhou, China).

2.4.1. Quantitative real time PCR (qRT-PCR)

qRT-PCR was accomplished as our previous described [20]. After siRNA transfection, NCCIT cells were used for total RNA by TRIzol reagent (Invitrogen). A First Strand cDNA Synthesis Kit (Roche, NJ, USA) was used to reverse transcribe RNA into cDNA. qPCR was accomplished on a Roche real-time PCR detection system (LightCycler480, Roche, USA). The following primers were utilized: TTTY14, forward: 5′- CCGTCCAGTCTTCGAATCCC-3′; reverse: 5′- GCATCTTTCTCCCAGTCAAAGC-3′; ACTB, forward: 5-CTGAGGATGC-GAGGTTCTGCTTG-3, reverse: 5-GTCACCGGAGTCCATCACGAT-3. ACTB was used as an internal control. The PCR primers were designed in-house. The specificity of primer was determined based on the melted curve.

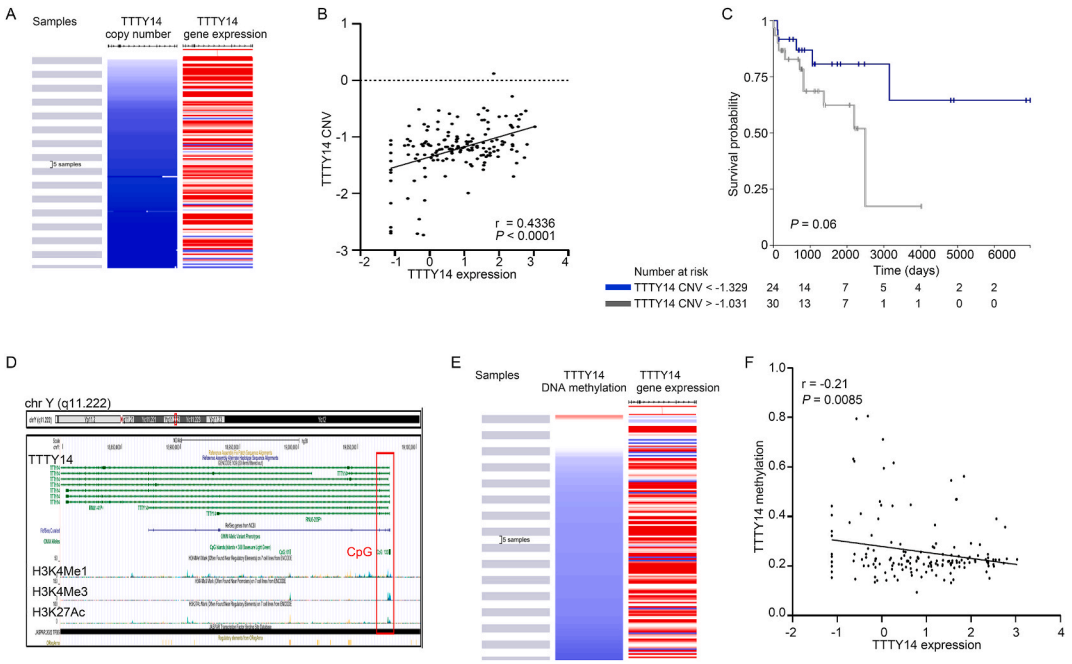

Fig. 3. The association between TTTY14 expression, methylation, and copy number in TCGA TGCT cohort. The UCSC XENA database was used to download the methylation data, expression data, and prognosis data of TTTY14 from the TCGA TGCT cohort. (A–B) The copy number was significantly positively correlated with TTTY14 expression. The copy number column was sorted from smallest to largest values. The redder the color, the larger the value; the bluer the value, smaller the value. (C) Correlation between TTTY14 copy number and overall survival of TGCT cohort from TCGA. Patients with high TTTY14 copy number had lower survival probability. (D) The CpG sites of TTTY14 were showed. (E–F) TTTY14 methylation level was significantly negatively correlated with its expression. The DNA methylation column was sorted from smallest to largest values. The redder the color, the larger the value; the bluer the value, smaller the value. CNV, copy number various. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Cell counting kit 8 (CCK-8) assay

CCK-8 assay was performed as our previous described [21]. CCK-8 kit was used for determining the cell viability of NCCIT cells after siRNA transfection according to the instructions.

2.6. Edu staining

A Cell-Light EdU Apollo In Vitro Kit (C10310-1, RiboBio, Guangzhou, China) was used for analyzing cell proliferation according to the instructions. Briefly, after siRNA transfection, 4×10^3 NCCIT cells were seeded in 96-well plates and cultured for 24 h. 100 μL of 50 μM EdU medium was add to each well and incubated for 2 h. After cells were washed with PBS 1–2 times, 50 μL of 4% paraformaldehyde was added to each well and incubated at room temperature for 30 min. Then, 50 μL of 2 mg/mL glycine was added to each well and incubated for 5 min on a shaker. After incubation with 0.5% TritonX-100 for 10 min, 100 μL of 1 × Apollo staining reaction solution was added to each well and incubated in the dark at room temperature for 30 min on a shaker. The cells were stained with a 1 × Hoechst 33,342 reaction solution for 30 min. Immediately after the staining was completed, the observation was performed, and the ratio of the number of Edu-positive cells was calculated. The Edu-positive rate was obtained by dividing the number of Edu-positive cells by the number of Hoechst-positive cells in the same well.

2.6.1. Colony formation assay

After siRNA transfection, 200 NCCIT cells were inoculated into a 6-well plate containing 2 mL of medium, and were gently rotated to disperse the cells evenly, and then placed in a cell incubator for about 2 weeks. When visible clones appeared in the dish, the culture was terminated. The colonies were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet. The colonies were observed and counted under a microscope.

2.7. Statistical analysis

Cell biology experimental data were obtained from at least three independent experiments. Statistical analysis was performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). Differences between the two groups were assessed by Student's t-test. A P value < 0.05 indicates a statistically significant difference.

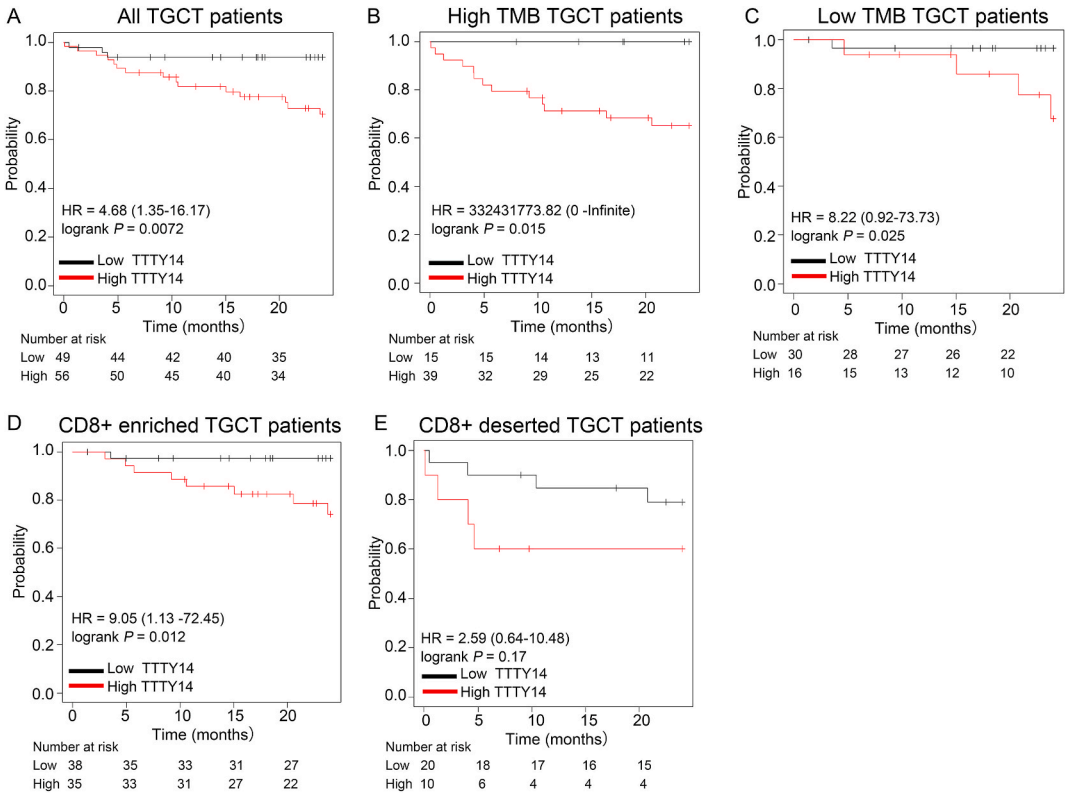

Fig. 4. Relationship between TTTY14 and survival prognosis of TGCT patients. Based on TCGA TGCT data, the Kaplan-Meier Plotter online tool was used to analyze relationship between TTTY14 expression and two-year survival in TGCT patients. (A) The relationship between TTTY14 expression and two-year survival in all TGCT patients was analyzed. (B) The relationship between TTTY14 expression and two-year survival in TGCT patients with high tumor mutational burden (TMB) was analyzed. (C) The relationship between TTTY14 expression and two-year survival in TGCT patients with low TMB was analyzed. (D) The relationship between TTTY14 expression and two-year survival in $CD8^+$ T cell-enriched TGCT patients was analyzed. (E) The relationship between TTTY14 expression and two-year survival in patients with TGCT $CD8^+$ T cell-deserted was analyzed.

3. Results

3.1. The expression of TTTY14 in TGCT

Using the online tool GEPIA, we analyzed the expression of TTTY14 in different tumors. TTTY14 expressed in a variety of tumors, with the highest expression in prostate cancer (PRAD) and high expression in TGCT (Fig. 2A). Because TTTY14 was a testis-specific transcript, we further analyzed the expression pattern of TTTY14 in TGCT. In the GSE3218 dataset, TTTY14 expression was significantly increased in testicular tumors compared with normal tissues (Fig. 2B). GEPIA analysis found that the expression of TTTY14 was higher in non-seminomas than in seminomas (Fig. 2C). The appearance of intratubular germ cell neoplasm is a malignant behavioral phenotype of testicular tumors that exhibits a higher proliferative capacity. We downloaded the TGCT cohort data from the TCGA using the UCSC XENA database and found that the expression of TTTY14 in TGCTs with intratubular germ cell neoplasm was significantly higher than that in TGCTs without intratubular germ cell neoplasm (Fig. 2D), suggesting that high TTTY14 expression is a key promoter gene of TGCT malignant proliferation.

We further analyzed the reasons for the high expression of TTTY14 in TGCT. We found that the copy number was significantly positively correlated with TTTY14 expression (Fig. 3A–B). And Patients with high TTTY14 copy number had lower survival probability (Fig. 3C). In addition, multiple CpG sites were found at the 3′ end of TTTY14. We analyzed the methylation status of TTTY14 from the TGCT cohort data, and found that TTTY14 methylation level was significantly negatively correlated with its expression (Fig. 3D–F). These results suggest that increased copy number and reduced methylation level are the main reasons of TTTY14 highly expressed in TGCT.

3.2. The association between TTTY14 and survival of TGCT patients

Based on the TCGA TGCT data, we continued to analyze the relationship between TTTY14 expression and survival of TGCT patients using the Kaplan-Meier Plotter online tool. The overall survival rate of TGCT patients with high TTTY14 expression was significantly lower than that of patients with low TTTY14 expression (Fig. 4A). We then analyzed in depth the relationship between TTTY14

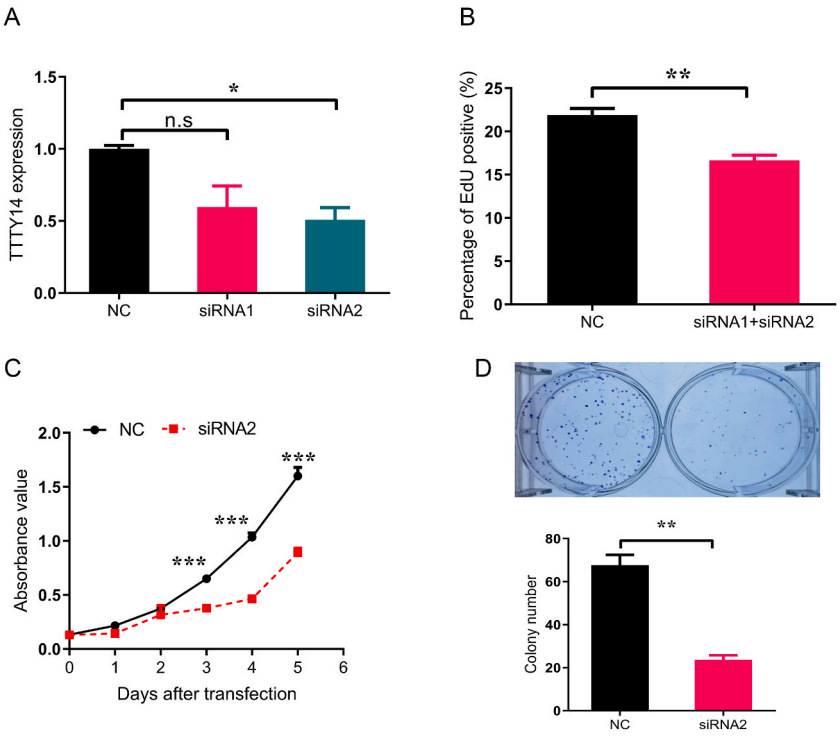

Fig. 5. Knockdown of TTTY14 inhibits NCCIT cell proliferation. (A) NCCIT cells were transfected with TTTY14 siRNA, and the expression of TTTY14 was detected by qPCR. (B) Edu staining was used to measure the proliferation of NCCIT cells transfected with TTTY14 siRNA. (C) CCK8 assay for cell viability of NCCIT cells transfected with TTTY14 siRNA. (D) Colony formation assay was used to detect the cell cloning ability of NCCIT cells transfected with TTTY14 siRNA. *P < 0.05, **P < 0.01, ***P < 0.001.

expression and two-year survival in patients with different molecules pathological types of TGCT. We found that high TTTY14 expression was a poor survival prognostic factor in both high and low tumor mutational burden (TMB) TGCT patients (Fig. 4B and C). In addition, to analyze the relationship between TTTY14 and $CD8^+$ T, we divided TGCT patients into $CD8^+$ T cell-enriched TGCT patients and $CD8^+$ T cell-deserted TGCT patients. We found that the survival rate of patients with high TTTY14 was lower than that of patients with low expression of TTTY14 in these two groups, but it was only statistically significant in patients with in $CD8^+$ T cell enriched TGCT patients (Fig. 4D and E), suggesting that TTTY14 may be closely related to TGCT immunotherapy.

3.3. *Knockdown of TTTY14 inhibits the proliferation of TGCT cells*

To verify the function of TTTY14, we knocked down TTTY14 in the TGCT cell line NCCIT cells, and detected the proliferation ability of the cells by Edu staining, CCK8 and colony formation assays. siRNA transfection reduced the expression of TTTY14, especially the siRNA 2 interfering sequence (Fig. 5A), so we chose siRNA 2 for subsequent experiments. Edu staining results showed that knockdown of TTTY14 significantly reduced the percentage of Edu-positive cells (Fig. 5B), inhibited the viability of NCCIT cells (Fig. 5C), and decreased the ability to colony formation (Fig. 5D). These results indicate that TTTY14 is an oncogene that promotes the malignant proliferation of TGCT. We further analyzed the correlation between TTTY14 expression and the sensitivity of Genomics of Drug Sensitivity in Cancer (GDSC) drugs (top 30) in TGCT, and the correlation between TTTY14 expression and the sensitivity of cancer therapeutics response portal (CTRP) using the GSCA online tool Drugs (top 30) in TGCT. The results showed that TTTY14 expression was negatively correlated with the sensitivity of most tumor therapy drugs (Fig. 6A–B). Based on the cohort data of *anti*-PD1, we found that the overall survival rate and progression-free survival rate of high TTTY14 expression group in TGCT patients treated with *anti*-PD1 were significantly lower than those of low TTTY14 expression group (Fig. 6C). Furthermore, we used LinkedOmics to analyze the co-expressed genes, and the downstream pathway of TTTY14 and found that TTTY14 were associated with proliferation and immune pathways, including regulation of epithelial cell differentiation, organ growth, T cell receptor signaling pathway, and primary immunodeficiency (Fig. 7A–C, Table S1). These results suggest that TTTY14 may be a predictive marker of immunotherapy outcome in TGCT patients.

3.4. *Relationship between TTTY14 and immune cells and immune checkpoint molecules*

The above results have suggested that TTTY14 is closely related to the immunotherapy of TGCT. Based on the TCGA TGCT cohort data, we utilized the ASSISTANT for Clinical Bioinformatics tool to analyze the relationship between TTTY14 expression and the

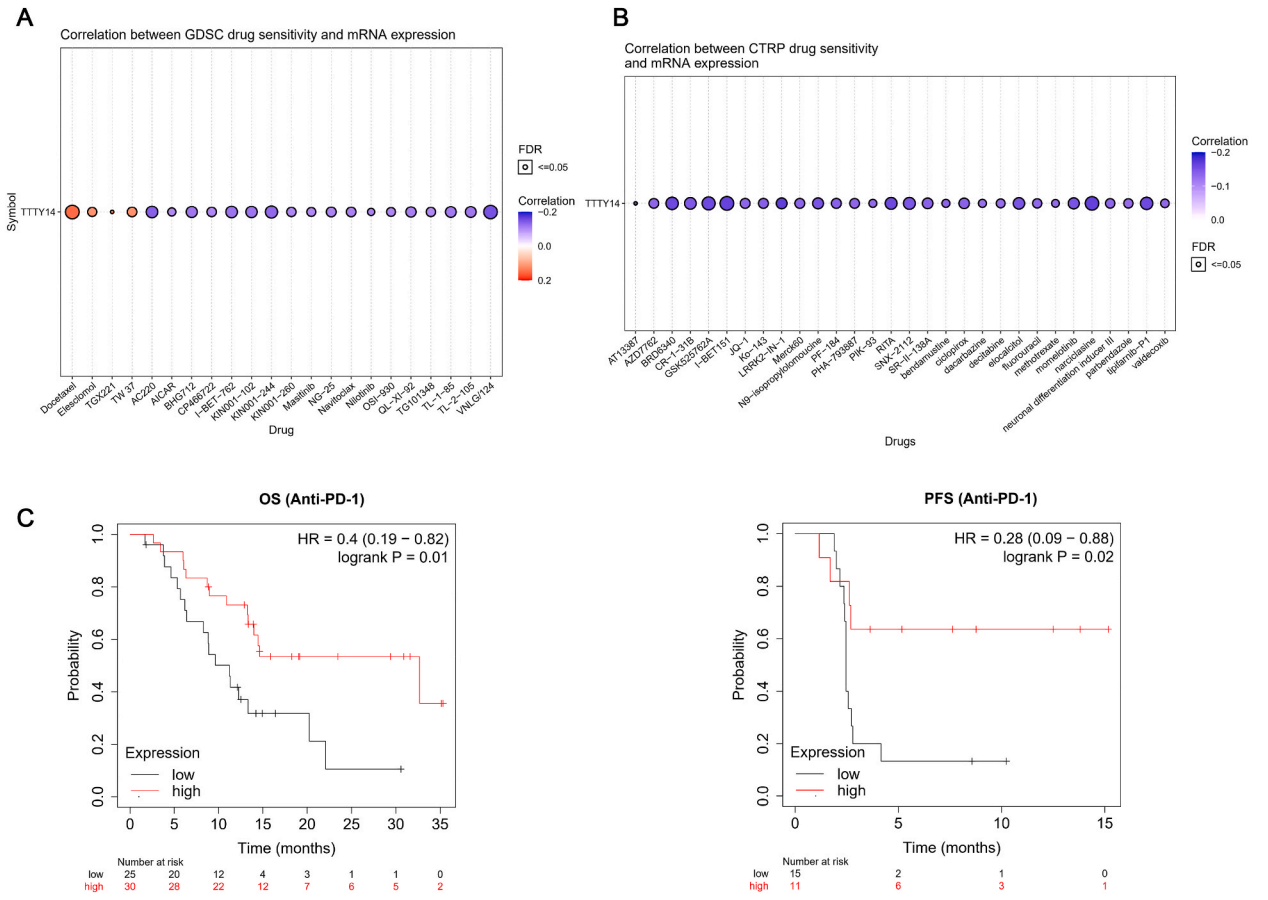

Fig. 6. The correlation between TTTY14 and antitumor drug sensitivity. GSCA online tool was used to analyze the correlation between TTTY14 expression and the sensitivity of Genomics of Drug Sensitivity in Cancer (GDSC) drugs (top 30) in TGCT (A), and the correlation between TTTY14 expression and the sensitivity of cancer therapeutics response portal (CTRP) drugs (top 30) in TGCT (B). (C) Based on *anti*-PD1 cohort data, the Kaplan-Meier Plotter online tool was used to analyze the correlation between TTTY14 expression and the immune checkpoint inhibitors. Relationship between overall survival and progression-free survival in this cohort of patients was shown. High expression of TTTY14 is a predictive marker for poor outcome of *anti*-PD1immunotherapy in TGCT patients.

abundance of various immune cells in TGCT samples. The analysis results showed that patients with high TTTY14 expression had lower the abundance of B cells, $CD8^+$ T cells, and macrophages and higher the abundance of $CD4^+$ T cells. There was no significant difference in the abundance of neutrophils and dendritic cells (Fig. 8A). Using the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm, it was found that TTTY14 expression was positively correlated with immune cell dysfunction (Fig. 8B). Analysis of the correlation between TTTY14 and the abundance of various immune cells found that the expression of TTTY14 was significantly negatively correlated with B cells, $CD8^+$ T cells, and macrophages, and significantly positively correlated with $CD4^+$ T cells (Fig. 8C). We used the GEPIA database to analyze the correlation between TTTY14 and three immune checkpoint molecules, CD274, CTLA4 and HAVCR2, and found a significant positive correlation between TTTY14 and CD274, CTLA4 and HAVCR2 (Fig. 8D).

4. Discussion

About 1%–2% of the genes in the human genome encode proteins, and the remaining 98% of the encoded products are called noncoding RNAs (ncRNAs), including lncRNAs [22]. Unlike other non-coding RNAs such as microRNAs (miRNAs), lncRNAs can control gene expression at various levels (epigenetic regulation, transcriptional regulation, and post-transcriptional regulation) [23]. LncRNAs play a role in physiological and pathological processes such as genetic imprinting, apoptosis, tumorigenesis, and epigenetic modification, and are involved in the regulation of spermatogenesis, maturation, and malignant transformation [24–26]. For example, LNC00467 promoted the invasion of TGCT cells by controlling AKT phosphorylation [7]. TTTY14 exhibited a bimodal pattern of expression characterized by low expression in samples from fertile patients and high expression in samples from infertile patients [27]. Although it has been found that TTTY14 may differentially expressed in some tumors, but its specific function and mechanism of action are still unclear. In this study, we conducted in-depth mining and cell biology verification through high-throughput data from public databases, and demonstrated that TTTY14 knockdown significantly inhibited the proliferation of TGCT in vitro, and the expression of

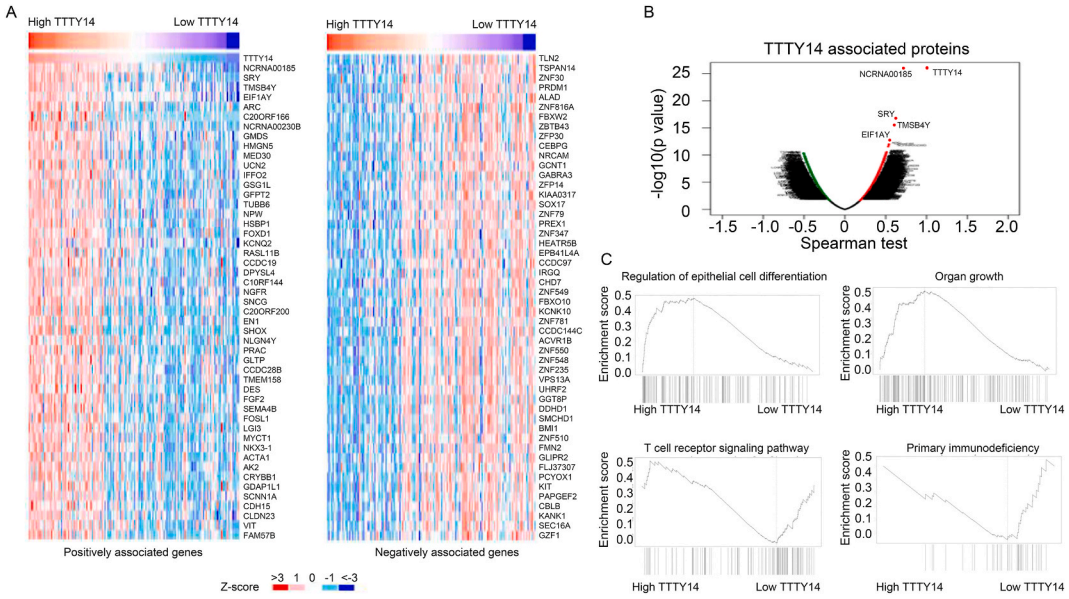

Fig. 7. The TTTY14-associated genes, proteins, and pathways. Based on the TCGA TGCT expression profile data, LinkedOmics was used to analyze the positively associated genes and negatively associated genes of TTTY14 (A), and the associated proteins of TTTY14 (B). (C) GSEA enrichment was performed to analyze the associated pathway of TTTY14.

TTTY14 was regulated by copy number variation and DNA methylation. We found that TTTY14 was highly expressed in intratubular germ cell neoplasm (IGCN). IGCN can be regarded as a dedifferentiated form of intratubular germ cell neoplasia of unclassified type (IGCNU), which then evolves into an aggressive germ cell tumor [28]. Indeed, TTTY14 is expressed in normal testis, and physiological cell proliferation is present in early human germ cells, such as spermatogonia. Since no human germ cell lines were available, we could not experimentally determine the pro-cellular proliferative role of TTTY14 in normal germ cells. However, our data show that TTTY14 promotes testicular tumor cell proliferation with a significant increase in clonogenesis of testicular tumor cells under pathological conditions, suggesting that the stemness of tumor cells may also become stronger with proliferation. In contrast, the proliferation of normal germ cells is not accompanied by an increase in cell stemness. Therefore, we believe that the role of TTTY14 in physiological cell proliferation and pathological cell proliferation is different, and that TTTY14 promotes malignant transformation of cells under pathological conditions. Thus, our results suggested that TTTY14 is a key gene that promotes the malignant biological behavior of TGCT.

With the development of high-throughput genome sequencing technology and the advancement of bioinformatics, studies found that cancer immune-related lncRNAs may play an important role in various stages of tumorigenesis, development, and transformation [29]. Tumor cells can be specifically recognized and eliminated by specific immune cells, indicating that the health of the immune regulatory system is crucial for the regulation of tumor growth [30]. In this study, we found that high TTTY14 expression was associated with decreased number of B cells, $CD8^+$ T cells, and macrophages, suggesting that TTTY14 may affect the tumor immune microenvironment. The relationship between TTTY14 and TGCT immunotherapy need further research in future.

In conclusion, we revealed that lncRNA TTTY14 was a novel oncogene and a biomarker in TGCT. TTTY14 may influence the tumor immune microenvironment and affect the sensitivity of TGCT drugs. However, we have yet to prove that TTTY14 is the cause of these observations. Further research is needed to fully understand the mechanisms by which TTTY14 may be involved in tumorigenesis, immune dysfunction, and drug sensitivity in TGCT.

Author contribution statement

Jian Cao; Lvjun Liu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Lei Xue: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Yanwei Luo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Zhizhong Liu; Jie Guo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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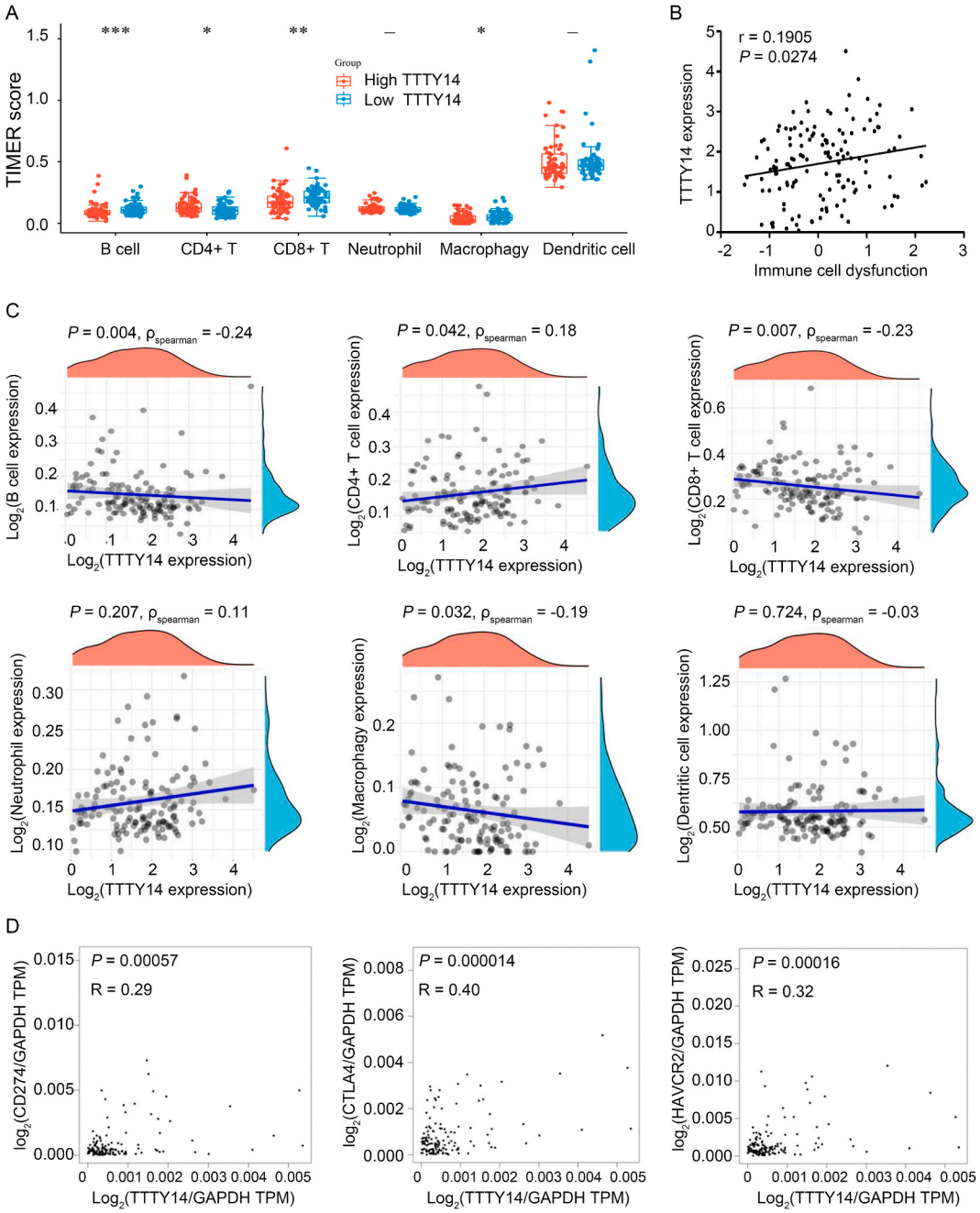

Fig. 8. Relationship between TTTY14 expression, immune cells and immune checkpoint molecules. Based on the TCGA TGCT cohort data, the ASSISTANT for Clinical Bioinformatics tool was used to analyze the abundance of various immune cells in TGCT samples (A), the correlation between TTTY14 expression and immune dysregulation score (B), and the correlation between TTTY14 and the abundance of various immune cells (C). (D) The correlation between TTTY14 and three immune checkpoint molecules CD274, CTLA4 and HAVCR2 was analyzed using the GEPIA database.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e16082.

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