Integrated Bioinformatic Analysis of DNA Methylation and Immune Infiltration in Endometrial Cancer

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Research Article

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Abstract

Background: Endometrial cancer greatly threatens the health of female. Emerging evidences demonstrated that the DNA methylation and immune infiltration involve in the occurrence and development of endometrial cancer. However, the mechanism and prognosis biomarker of endometrial cancer is unclear. In this study, we attempt to assess the DNA methylation and immune infiltration via bioinformatic analysis.

Methods: The latest RNA-Seq, DNA methylation data and clinical data of endometrial cancer were download from the UCSC Xena dataset. The methylated driven genes were selected out, and then the risk score was obtained using the "*MethylMix*" and "*corrplot*" package. The correction of methylated genes and prognosis values, and the expression of above driven genes were identified by via *survminer* package and *beeswarm* package, respectively. Finally, the role of *VTCN1* gene in immune infiltration was analyzed via *CIBERSORT* package.

Results: In this study, 179 up-regulated genes, and 311 down-regulated genes are identified, which are related to the extracellular matrix organization, cell-cell junction, and cell adhesion molecular binding. Above them, 13 methylated driven genes were selected out. Meanwhile, it's obvious that the expression of *PGR* and *VTCN1* are lower in tumor group than that of in normal group. Conversely, *VTCN1* gene expresses the opposite. high methylation & low expression of *PGR* and *GYPC* are positive correction with the prognosis, while the *VTCN1* has the inverse relationship with the prognosis of endometrial cancer. More importantly, the hypomethylation of *VTCN1* promoter leads to its high expression, which can cause tumor development by inhibiting $CD8^{+}$T cell infiltration.

Conclusions: In total, our study firstly reveals the mechanism of endometrial cancer combining DNA methylation and immune cell infiltration via integrated bioinformatic analysis. Besides, we found the pivotal prognosis biomarker for this disease. Our study may provide the potential targets for the diagnosis and prognosis of endometrial cancer.

Introduction

Endometrial cancer, a second most common female malignancy, greatly threaten the health of female. According to the statistics in 2018, the new cases and deaths is over 380,000 and 89,000 across world regions, respectively [1]. With the increase of obesity and population aging, the incidence of endometrial cancer is increasing [2]. Approximately 70% of endometrial cancers are confined to the uterus when they are diagnosed, which mainly be treated by removing the uterus, posing a better prognosis effect about 75% [3, 4]. However, there are still 10 ~ 15% of endometrial cancers extend beyond the uterus tissues when they are diagnosed. The survival rate is less than 5 ~ 15% [5]. It is highly desirable to develop novel targets for the treatment and intervention of endometria cancer patients.

Cancer is generally considered to be an epigenetic disease caused by abnormal gene expression. The epigenetic alternation plays a critical role in the endometria cancer progress [6, 7]. DNA methylation, a

common epigenetic change, can active or silence some genes to promote or inhibit related signal pathways [8]. Abnormal DNA methylation, including hyper- and hypo-methylation, both can result in the occurrence of disease [9]. The DNA methylation in cancers, such as lung cancer [10], prostate cancer [11], and breast cancer [12], has been widely studied.

With the development of single-cell technology, the role of tumor microenvironment (TME) in endometria cancer, consisting of immune cells, fibroblasts, endothelial cells, inflammatory mediators, and extracellular matrix attracted more and more attention [13, 14]. Immuno-response is an important factor for the prognostic value in endometria cancer [15]. Usually, the increased number of cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells) act as an independently prognosis indicating the better prognosis in endometria cancer [15]. CIBERSORT is broadly performed to explore the abundance of immune cells in normal and tumor tissues [16, 17]. Nowadays, accumulating studies applied the immune cells in TME to explain the mechanism of cancer, such as prostate cancer [18], clear cell renal cell carcinoma [19], and endometria cancer [20]. However, it's rare that to study the mechanism and prognosis biomarkers via DNA methylation and immune cell infiltration.

In this study, TCGA data were downloaded from UCSC Xena dataset. The data were processed via R software. There are five driven methylated genes (*TSPYL5, KLF9, GYPC, VTCN1* and *PGR*). According to their relationship with survival time, 3 genes were screened out (*GYPC, PGR* and *VTCN1*). It demonstrated that the higher expression of *GYPC* and *PGR* exhibited a good prognosis, while the higher expression of *VTCN1* possess a poor prognosis. *VTCN1* (a T cell activation suppressor 1), also known as B7-H4, can regulate T-cell activation in non-small-cell lung cancer [21], hepatocellular carcinoma [22] and prostate cancer [23]. In order to further explore whether *VTCN1* is involved in regulating the occurrence and development of endometrial cancer through immune factors, we further analyzed the level of immune cell infiltration in normal and tumor tissues. It showed that *VTCN1* is indeed negatively correction with $CD8^+$ T cells in endometria cancer, and there is lower T cell infiltration in tumor tissues in high *VTCN1* expression group.

Our study demonstrated that DNA methylation of *GYPC, PGR* and *VTCN1* are closely related to patient prognosis, and *VTCN1* may involve in the occurrence and development of endometrial cancer by inhibiting $CD8^+$ T cell infiltration. The study may contribute to explain the mechanism and provide the prognosis biomarker for endometria cancer.

Methods

Data download and pre-processing

The latest RNA-Seq and DNA methylation data were download from the UCSC Xena dataset (https://xenabrowser.net/datapages/). The data were processed and normalized via R software. The differentially expressed genes (DEGs) were analyzed by the "*limma*" packages. The DEGs were screened out with the criteria |logFC| > 2 and adj-*P*-value < 0.05. Gene Ontology (GO) function and Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed by *org.Hs.eg.db* package. A value of $P < 0.05$ was defined as statistically significant.

Screening for driven genes

Generally, the MethylMix package were used to explore the driven methylated genes. For transcriptomic profiling, differential expression analysis on TCGA RNA-seq data that matched with DNAm profiles. Owing to methylated genes usually negatively regulate their mRNA, it showed 13 driven differentially methylated genes (DEMs), including 2 downregulated (*RP11-469H8.6* and *VTCN1*) and 11 upregulated methylated genes (*KLF9, PGR, DDR2, TSPYL5, FAXDC2, HSPB6, GYPC, CDO1, C8orf88, TMEM132C* and *WT1-AS*).

The risk score of above genes

The clinical data were download from the UCSC Xena dataset. Riskscore were calculated via survival package. The survival curve and heatmap were drew according to the high and low risk score. Subsequently, there are 5 genes that were selected out, including *TSPYL5, KLF9, GYPC, VTCN1* and *PGR*.

The survival analysis

The survival analysis of the high methylated genes and low expressed genes were evaluated by R software.

The evaluation of protein expression

The expression of *GYPC, VTCN1* and *PGR* genes in paired tumor and normal group are analyzed by limma and ggpubr package. The protein expression is analyzed by The Human Protein Altas (https://www.proteinatlas.org/).

Immune cell infiltration

CIBERSORT is a deconvolution algorithm that uses a set of reference gene-expression values (a signature with 547 genes) considered a minimal representation for each cell type. Based on those values, it infers cell type proportions in data from bulk tumor samples with mixed cell types using support vector regression. CIBERSORT can be applied to distinguish 22 human immune cells, including B cells, T cells, NK cells, macrophages, DCs and myeloid subsets, based on the high specificity and sensitivity of gene expression profile. To determine whether there is a correlation between tumor infiltration with immune cells, and immune-related gene expression, the tumor infiltration with six types of immune cells ($CD4^+$ T

cells, $CD8^+$ T cells, B cells, neutrophils, macrophages, mast cell and dendritic cells) was analyzed by CIBERSORT.

The *VTCN1* gene expression

The relative genes of *VTCN1* were selected out via STRING dataset (https://string-db.org/). The GO pathway of those genes was analyzed by Metascape dataset (http://metascape.org/gp/index.html#/main/step1). The expression of *VTCN1* is divided into two group according to the wilcoxTest function. The correction of *VTCN1* gene expression with immunity cells were visualized by *vioplot* package.

Statistical analysis and visualization

The raw data were collated by Practical Extraction and Report Language (Perl, version 5.30.0) and R software (version 4.0.3). The statistical analysis and visualization of the statistical results were completed with R software and Cytoscape (version 3.8.0).

Results

The functional analysis of DEGs

According to the criteria that |logFC| > 2 and adj-*P*-value < 0.05, There are 490 DEGs, among which there are 179 up-regulated genes, and 311 down-regulated genes. The volcano plot was shown in the Fig S1. Secondly, Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed via R software. It shows that endometria cancer is associated with cell cycle, P53 signal pathway, and focal adhesion in KEGG pathway (Fig 1A). GO includes biological process (BP), cellular component (CC) and molecular function (MF). The GO analysis shows that endometria cancer is related to the extracellular matrix organization, cell-cell junction, and cell adhesion molecular binding (Fig 1B).

The methylated driven genes

It is well noted that there is often inverse correlation between DNA methylation and mRNA levels. Then the methylated driven genes are selected out using the "*MethylMix*" and "*corrplot*" package in R software. There are 13 methylated driven genes. They are *WT1-AS, CDO1, RP11-469H8.6, TMEM132C, GYPC, TSPYL5, VTCN1, DDR2, HSPB6, KLF9, C8orf88, FAXDC2* and *PGR.* Among them, the expression of *RP11-469H8.6* and *VTCN1* are upregulated, and other are downregulated. The methylated and expressed heatmap of above 13 DNA are displayed in the Fig 2A and 2B (|R| > 0.3, *P* < 0.05).

The survival analysis

Survival analysis was performed with the product-limit method (Kaplan–Meier method). At same time, the log-rank test (Mantel-Cox test) was used to compare the difference in the survival status between the high- and low-risk groups via *survminer* package. Cox model was applied to build a risk model to get the patient's risk value. the coxGene of the risk are *TSPYL5, KLF9, GYPC, VTCN1* and *PGR*. The patient grouping was based on the median value of the risk score (Fig 3A). Obviously, lower risk possesses the better prognosis and higher risk exhibits the poorer prognosis (Fig 3B). The heatmap of *TSPYL5, KLF9, GYPC, VTCN1* and *PGR* expression is shown in Fig 3C. The expression of *PGR*, *GYPC* and *VTCN1* are divided into high expression group and low expression group. The survival curve of above 13 methylated driven genes were drew by survival software. Three genes (*PGR, VTCN1* and *GYPC*) are considered statistically significant ($P < 0.05$). The negative correlation of *PGR, VTCN1* and *GYPC* expression and methylation are displayed in Fig 4A, 4B, 4C. The higher expression group of *PGR* and *GYPC* displays better prognosis, while the lower expression group shows poor prognosis ($P<0.05$) (Fig 4DE). Conversely, the lower expression of *VTCN1* gene display better prognosis (Fig 4F).

The expression of *PGR, GYPC* and *VTCN1*

The expression of *PGR, GYPC* and *VTCN1* were visualized by *beeswarm* package. It's obvious that the expression of *PGR* and *VTCN1* are lower in tumor group than that of in normal group. While *VTCN1* expression is higher in tumor group than that of in normal group (Fig 5A, 5B, 5C). The protein expression in The Human Protein Altas (https://www.proteinatlas.org/) further confirms the results (Fig 5D, 5E, 5F).

The immune score

CIBERSORT is a deconvolution algorithm that uses a set of reference gene-expression values (a signature with 547 genes) considered a minimal representation for each cell type. Based on those values, it infers cell type proportions in data from bulk tumor samples with mixed cell types using support vector regression. In the immunity low group, StromalScore, ImmuneScore, and EstimateScore are -1257.90±283.05, -378.62 ± 401.33, and -1545.68 ± 608.60, respectively; In the immunity high group, StromalScore, ImmuneScore, and EstimateScore are -539.79±1241.98, 930.89±583.08, and 462.35±834.37, respectively. The survival curve based on the above immunity score shows that higher ImmuneScore displays better prognosis ($P<0.05$). While StromalScore and EstimateScore shows no statistical significance ($P>0.05$).

The mechanism of *VTCN1* gene in immune regulation

VTCN1 gene is closely related to immunity. In order to further explore the mechanism of *VTCN1* involved in the endometria cancer. The relative proteins of *VTCN1* are selected via STRING database. They

are *B7RP1, BTLA, CD28, CD80, CD86, CTLA4, ICOSL, IL4, IL6,* and *PDCD1LG2* (Fig 6A). Those genes are mainly involved in Lymphocyte costimulation, regulation of T cell activation, proliferation, B cell activation, immune response-regulating cell surface receptor signaling pathway (Table 1). The percentage of immune cells in normal and tumor tissues are analyzed. The box plot is displayed in Fig 6B according to the StromalScore, ImmuneScore, EstimateScore and Tumorpurity. The box plot of the immunity cell percentage in the two group are analyzed by the *ggpubr* package. It is obvious that B cell native and T cells CD4 memory resting, and M2 macrophagocyte are lower in tumor group than that of normal group. While Tregs and M1 macrophagocyte are higher in tumor group than that of normal group (P<0.05). Likewise, the higher expression of *VTCN1* exhibits positive correlation with T cells CD4 memory resting, while the higher expression of *VTCN1* is negatively with T cells CD8 and T cells CD4 memory activated cells (Fig 6C). The correction of T cells CD8 and VTCN1 is shown in the Fig 6D.

Table 1

The GO analysis of *VTCN1-reatived genes.*

Gene lists	GO analysis
IL4/IL6	Lymphocyte costimulation
B7RP1	Regulation of T cell activation
BTLA	Regulation of T cell proliferation
CD28 CTLA4	Control of immune tolerance by Vasoactive Intestinal peptide
CD80	Cell adhesion molecules (CAMs)
CD86	B cell activation
ICOSL PDCD1LG2	Immune response-regulating cell Surface Receptor signaling pathway

Discussion

Endometria cancer is a lethal female reproductive malignant tumor. The incidence of endometrial cancer is usually second only to cervical cancer in gynecological diseases in China[24]. The average age of onset of endometrial cancer is 63 years old, usually in postmenopausal women, especially obese and diabetic women. Traditional treatments usually include surgical resection, radiotherapy, and chemotherapy. However, It's troublesome for the therapy of patients with advanced endometria cancer, which often have a poor prognosis[25]. Nowadays, the mechanism and prognosis biomarkers of endometria cancer are not unclear.

DNA methylation and immune cell infiltration often participate in the development of various cancers, including gastric cancer [26], clear cell renal cell carcinoma [27], and colorectal cancer [28]. In our study, we find that high methylation & low expression of *PGR* and *GYPC* exist poor prognosis, while low methylation & high expression of *VTCN1* possess a poor prognosis. Besides, the high expression of *VTCN1* in tumor, as an immune regulator, may promote the development of endometria cancer by inhibiting the $CD8^+$ T cell infiltration.

PGR, the receptor of progesterone, widely expresses in the uterus, mammary gland and ovary [29]. In uterine, breast, ovarian and breast cancer, the lower expression of PGR is associated with poor prognosis [30]. But it's controversial whether the expression of PGR serves as the prognostic marker in endometrial cancer. GYPC (glycophorins), is defined as a negative prognostic biomarker in ovarian cancer [31]. But there has no report about the prognostic value of GYPC in endometrial cancer. In our investigation, we first identified PGR and GYPC as a favorable prognostic for endometrial cancer.

VTCN1 (*B7-H4*), belonging to the B7 family, function as a cell surface trans-membranous protein negatively regulating T-cell medicated immune response via interaction with a receptor protein on the surface of T-cell to inhibit T-cell activation and proliferation, cytotoxic factor production [32, 33]. Accumulating studies have showed that *VTCN1* are often overexpressed in tumor tissues of ovarian [34], lung [35] and breast cancers [36]. *Takashi* demonstrated that *VTCN1* is overexpressed in high-risk uterine endometrial cancer and negatively correction with tumor T-cell infiltration [37]. In our study, we find that *VTCN1* is downregulated in tumor tissue via DNA methylation analysis. Besides, we performed the CIBERSORT package to analyze the immune cell distribution in the normal and tumor tissues. Subsequently, the relationship of *VTCN1* expression and immune cell infiltration were analyzed by bioinformatic method. The result indicated that the expression of *VTCN1* is inversely correction with $CD8^+$ T cell infiltration.

Conclusion

In total, our study firstly reveals the mechanism of endometrial cancer combining DNA methylation and immune cell infiltration. The hypomethylation of *VTCN1* promoter leads to its high expression, which can cause tumor development by inhibiting $CD8^+$ T cell infiltration. Furthermore, high methylation & low expression of *PGR* and *GYPC* are positive correction with the prognosis, while the *VTCN1* has the inverse relationship with the prognosis of endometrial cancer. Our study explains the mechanism via immune infiltration and provide the potential targets for the diagnosis and prognosis of endometrial cancer.

Declarations

Acknowledgements

Not applicable.

Author contributions

DFF, DZM and CYX made the design of this study. DFF, DZM, HM did the acquisition and analysis of data. YMQ, YDY, LSY, ZYJ did the interpretation of data. DFF drafted the article. HM, CYX revised this study critically. All authors approved of the version to be published. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets analyzed during the current study are available in the TCGA repository.

Ethics approval and consent to participation

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

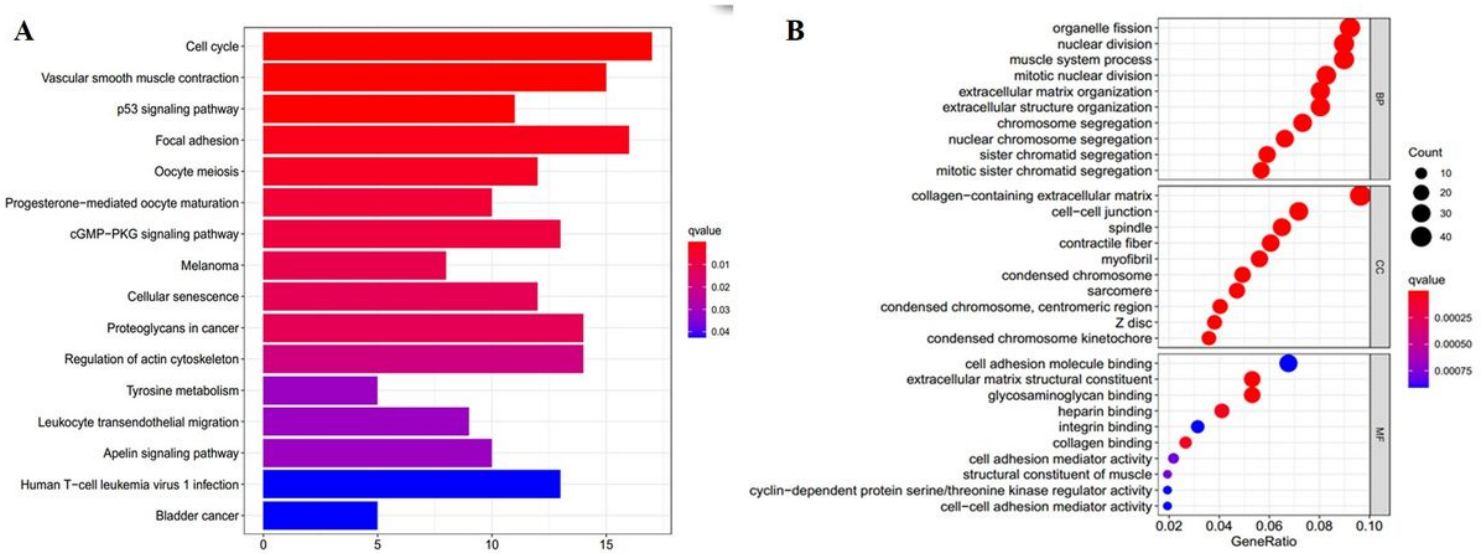

Figure 1

The map of (A) KEGG pathways and (B) GO function.

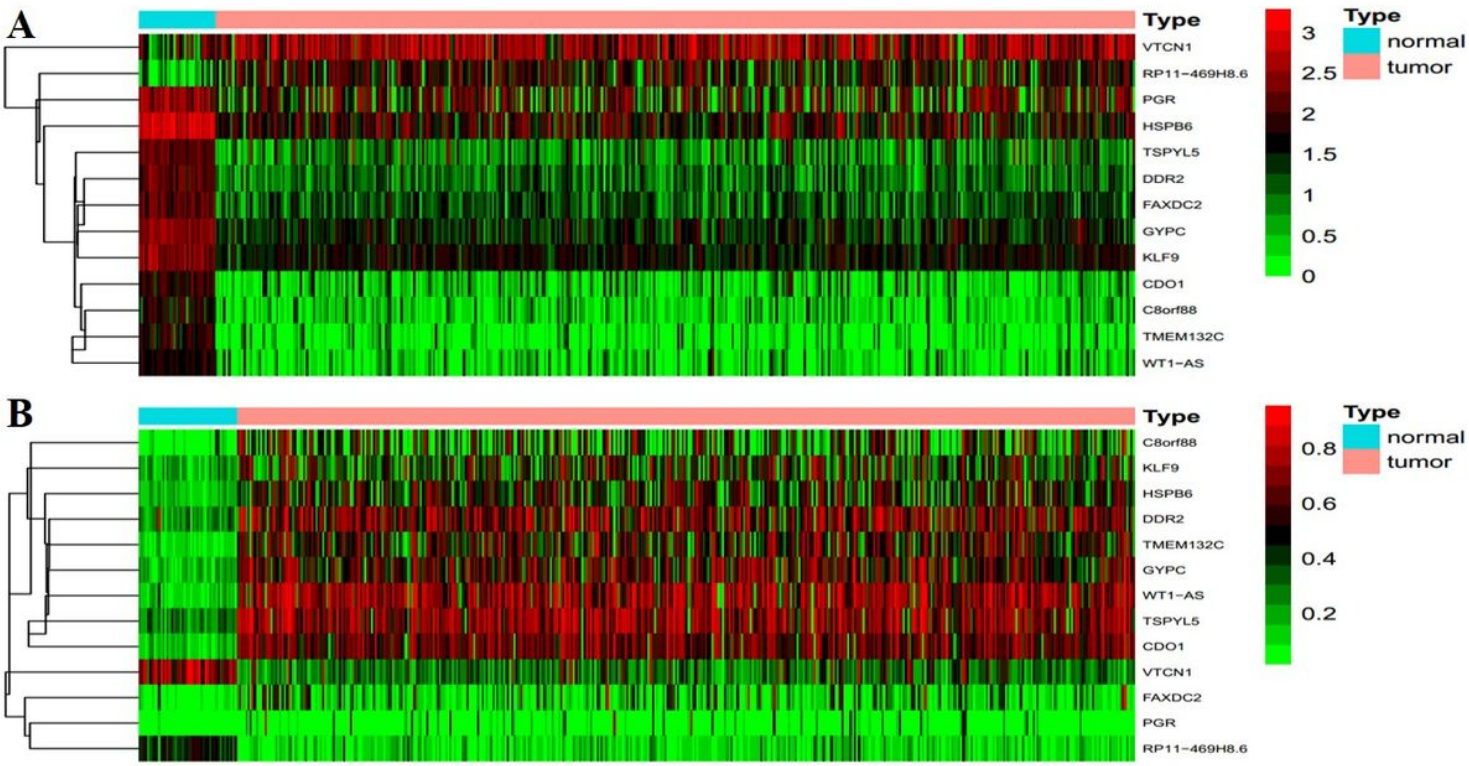

Figure 2

(A) The heatmap and (B) expressed heatmap of methylated driven genes.

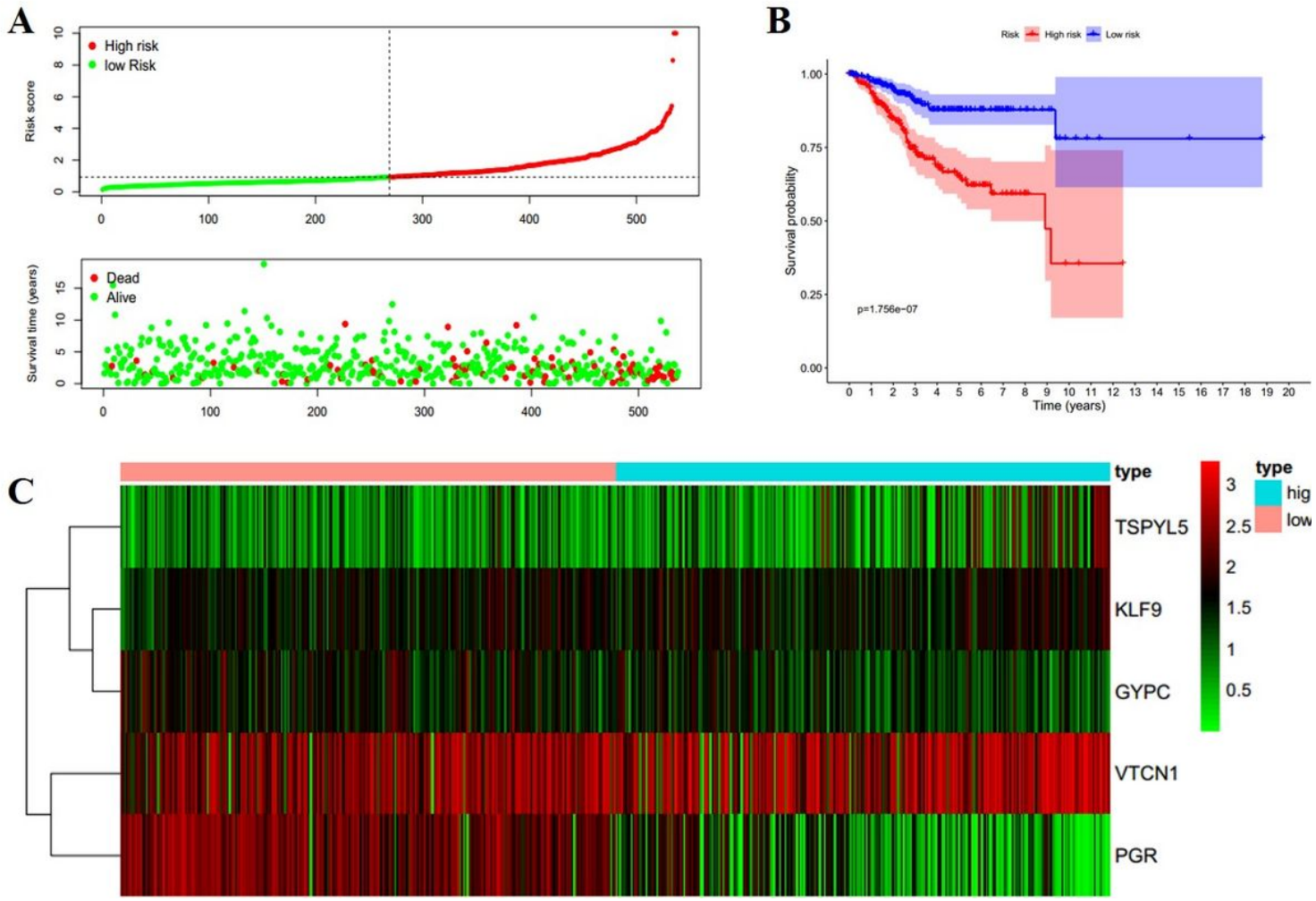

Figure 3

(A) The risk score of survival time; (B) The survival analysis of high-risk score and low risk; (C) The expressed heatmap of TSPYL5, KLF9, GYPC, VTCN1 and PGR according to the risk score.

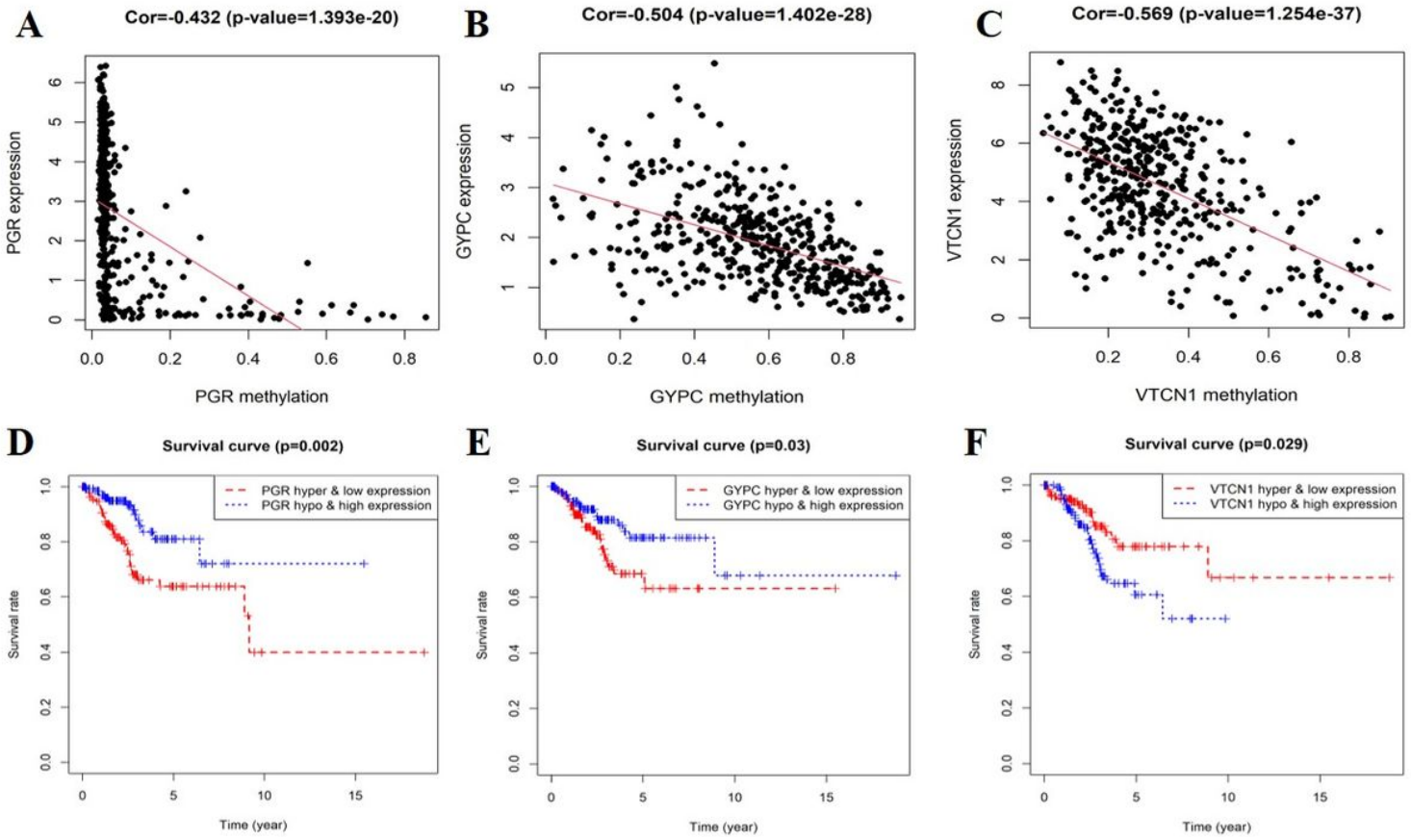

Figure 4

The correction of gene expression and methylation (A) PGR, (B) GYPC, and (C) VTCN1. The survival curve of gene hyper methylation & low expression, or hypo methylation & high expression (D) PGR, (E) GYPC, and (F) VTCN1.

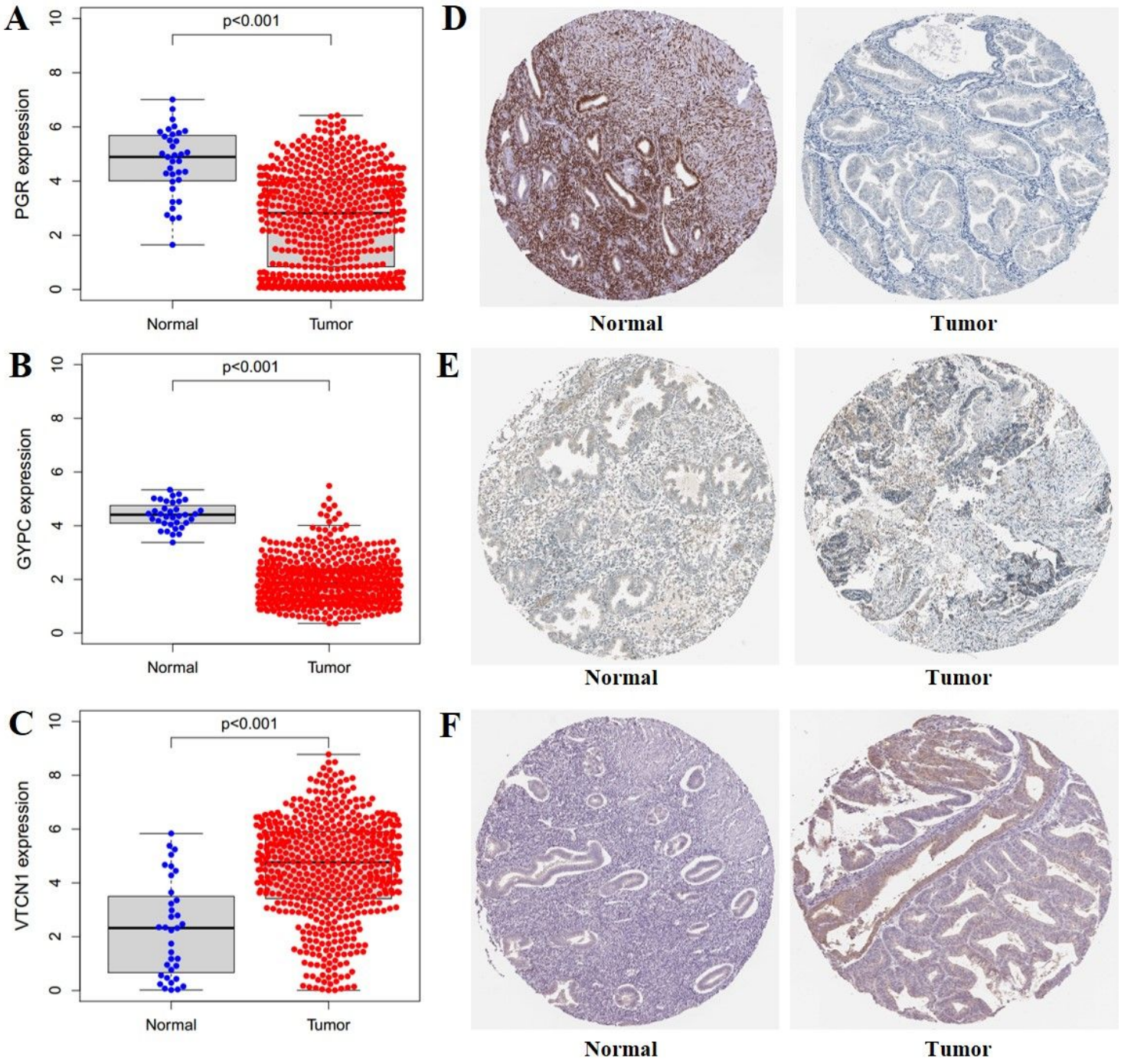

Figure 5

The expression of (A) PGR, (B) GYPC and (C) VTCN1 in normal and tumor tissues.

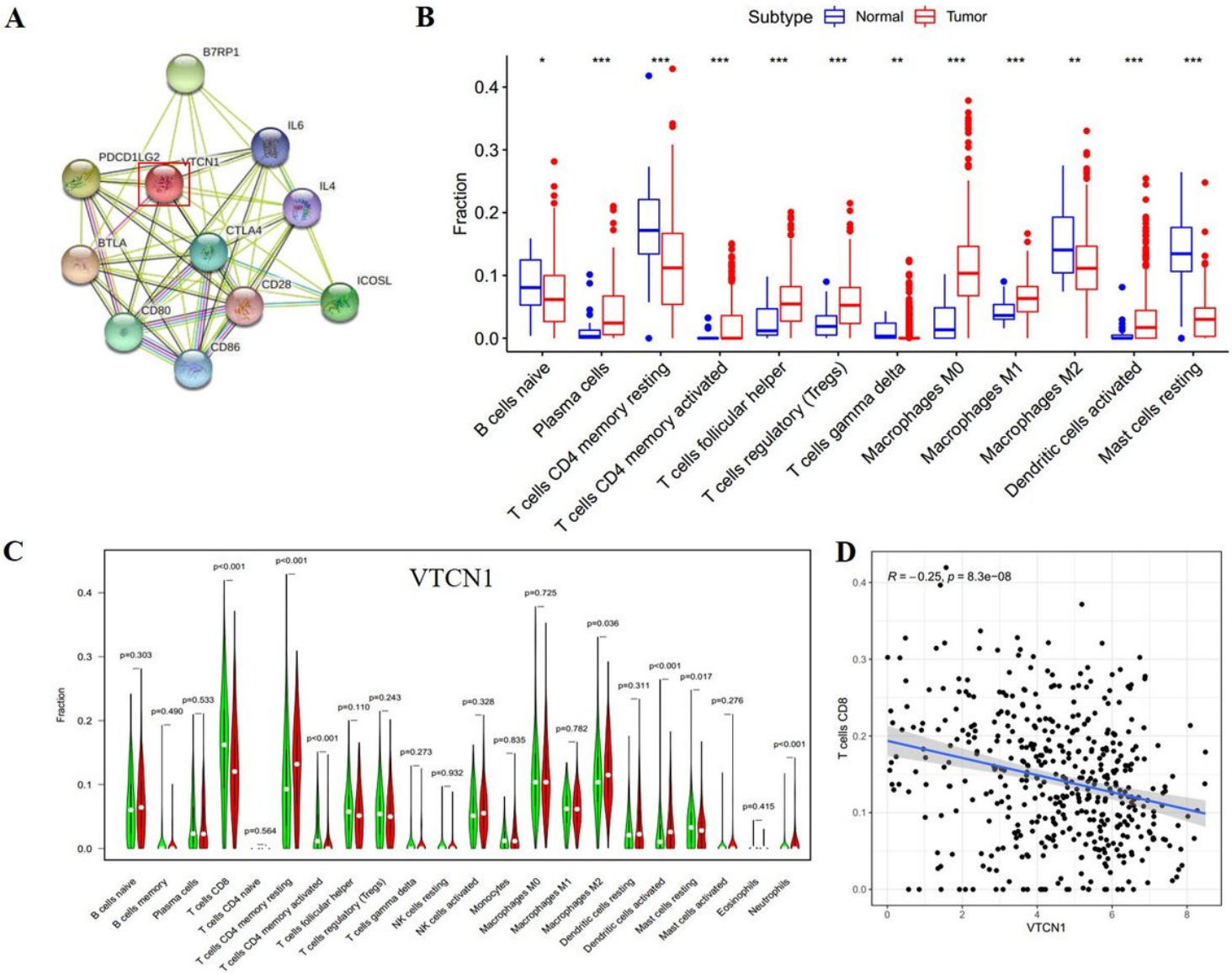

Figure 6

(A) The related proteins of VTCN1; (B) The box plot of the immunity cell percentage in the normal and tumor group; (C) The violin plot of VTCN1 expression in immunity cell; (D) The correction of T cells CD8 and VTCN1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.pdf