Original Article

# Evaluation of *NOTCH* family genes' expression and prognostic value in prostate cancer

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**Background:** Abnormal regulation of the *NOTCH* signaling pathway in prostate cancer (PCa) can promote tumorigenesis, progression, and T cell exhaustion. However, there has not been a comprehensive analysis of *NOTCH* family genes (*NOTCH*s) as potential therapeutic targets and prognostic biomarkers for PCa patients.

**Methods:** *NOTCH*s expressions in various types of cancer tissues and normal adjacent tissues in the TIMER and UALCAN database were screened. Immunohistochemistry (IHC) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were applied to validate the expression pattern of *NOTCH*s in clinical samples. The relationships of *NOTCH*s expression and clinicopathologic parameters or disease-free survival (DFS) were evaluated via GEPIA2 and UALCAN. A proteins network was built using STRING and GeneMANIA. Additionally, *NOTCH*s mutation status was analyzed by cBioportal. Finally, we used GDSC and TIMER to investigate *NOTCH* signaling-related drugs and immune cell infiltration.

**Results:** The transcriptional levels of *NOTCH1* and *NOTCH4* in PCa tissues were significantly lower than in normal tissues, which was further validated in clinical patients' tissue samples. Furthermore, *NOTCH1*, *NOTCH3*, and *NOTCH4* expressions in PCa were associated with worse DFS. Interestingly, there was a significant positive correlation between *NOTCH*s and androgen receptor (*AR*), but not with *AR*-related genes (*KLK3* and *TMPRSS2*). Finally, we found that *NOTCH*s expressions were remarkably associated with infiltration of B cells, $CD8^+$/$CD4^+$ T cells, macrophages, neutrophils, and dendritic cells, which indicated that *NOTCH*s mutation status might be a potential therapeutic target for -tinib antineoplastic drugs.

**Conclusions:** The expression and mutation of *NOTCH1–4* in PCa were associated with disease progression, prognosis, immune cell infiltration, and drug sensitivity.

**Keywords:** Bioinformatics; *NOTCH* family genes; immuno-microenvironment; prognosis; prostate cancer (PCa)

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## Introduction

Prostate cancer (PCa) is one of the most prevalent tumors globally. It is predicted that there will be 268,490 newly diagnosed cases and approximately 34,500 deaths from PCa in the USA in 2022 (1). With the progress in sequencing technology, whole-genome sequencing and exome sequencing of numerous organisms have identified diverse somatic mutation patterns and pathway alterations in PCa (2-4). Recent results revealed major frequent mutations in PCa, including the androgen receptor (*AR*), *SPOP*, *CHD1*, and *TP53*, thus dividing patients with different mutations into different prognostic subtypes of PCa (5-7). Carrying different mutations may affect a patient's sensitivity to different treatments, underlining the importance of individualized medication. A study has highlighted hormone resistance dependent or independent on the *AR* signaling pathway in patients with metastatic castration-resistant prostate cancer (CRPC) (8). However, in terms of clinical application, the current findings cannot meet the complex clinical requirements, and lots of drugs designed to overcome hormone resistance are now in stages of clinical trials. There is an urgent need to find the next potential biomarkers for prognosis and reaction indicators for these new therapeutic agents and targeted drugs in PCa patients.

The *NOTCH* family includes four distinct *NOTCH* receptor subtypes, namely *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4*. And for the *NOTCH* family genes, there are five ligands, Jagged1, Jagged2, Delta-like ligand 1 (*DLL1*), *DLL3*, and *DLL4*, which together make up the *NOTCH* signaling pathway (9). *NOTCH* signaling is activated by a ligand binding to receptors on adjacent cells. The binding of the receptor and ligand leads to cleavage of the *NOTCH* gene (10). The cleaved intracellular part translocates to the nucleus, where it cooperates with downstream target genes to influence transcription (11). The *NOTCH* signaling pathway plays a role in maintaining homeostasis, so its changes may cause a series of abnormal responses in the body that then lead to diseases (12).

It is worth noting that *NOTCH* signaling is one of the highest-ranking pathways enriched in PCa patients with a high tumor burden (13). Studies have shown that down-regulated *NOTCH* signaling promotes the invasion and non-anchoring growth of prostate cancer cells, and promote tumor growth and metastasis *in vivo*, but others elucidated that *NOTCH* signaling promotes tumor growth and development by triggering the AKT, FoxM1, and other targets (14-16). The exact role of *NOTCH* signaling in the development of PCa has not been determined because mutation-induced excessive activation of *NOTCH* family genes is multifaceted nature, both promoting and inhibiting cancer development. In addition, some studies have shown that the *NOTCH* signaling pathway can cooperate with some chemotherapy drugs (e.g., docetaxel) or anti-androgen drugs (e.g., enzalutamide) to exert synergism and an anti-tumor effect (17,18).

Although previous studies have partially determined the general expression profile and function of some *NOTCH* genes in PCa, there is not scientific consensus about the global landscape of *NOTCH* family genes in PCa and the feasibility of *NOTCH* family genes as potential therapeutic targets or prognostic biomarkers for PCa patients. Based on existing studies, in this study, we analyzed multiple databases and revealed that NOTCHs expressions in PCa were associated with worse disease-free survival, and there was a significant positive correlation between NOTCHs and androgen receptor. Furthermore, NOTCHs expression in PCa were associated with immune cell infiltration and NOTCHs mutation status might be a potential therapeutic target for -tinib antineoplastic drugs. We hope this could make a further explanation of *NOTCH* family genes' role in PCa based on existing studies and provide a new reference for clinical prognosis and drug selection. We present the following article in accordance with the STREGA reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-281/rc).

## Methods

### *RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay*

As for qRT-PCR, total RNA was extracted from 24 prostate cancer tissues and adjacent normal tissue using the RNeasy reagent (QIAGEN, Shanghai, China) according to the manufacturer's instructions. The ND-2000 Nanodrop system (Thermo Scientific, USA) was used to detect the concentration and purity of RNA, and an A260/280 ratio between 1.8 and 2.0 was considered the premise of acceptable quality. Single-stranded cDNA was generated from 1 μg total RNA in a 20-μL reaction volume using oligodT primers according to the protocol supplied with the Primer Script TM RT Reagent (TaKaRa, Japan). The relative expression levels were measured by qPCR using the ABI 7900HT instrument (Applied Biosystems, USA) in a total volume of 10 μL with the SYBR green

 

detection system (Takara, Japan) and GAPDH was used as an endogenous control. The cDNA was amplified by PCR using the following primers: *NOTCH1* forward primer: 5'-TGGACCAGATTGGGGAGTTC-3', reverse primer: 5'-GCACACTCGTCTGTGTTGAC-3'; *NOTCH2* forward primer: 5'-CCTTCCACTGTGAGTGTCTGA-3', reverse primer: 5'-AGGTAGCATCATTCTGGCAGG-3'; *NOTCH3* forward primer: 5'-CGTGGCTTCTTTCTACTGTGC-3', reverse primer: 5'-CGTTCACCGGATTTGTGTCAC-3; *NOTCH4* forward primer: 5'-TGTGAACGTGATGTCAACGAG-3', reverse primer: 5'-ACAGTCTGGGCCTATGAAACC-3'. GAPDH (internal control) forward primer: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse primer: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

### *Immunohistochemistry (IHC)*

A total of 15 PCa tissues and 6 benign prostate hyperplasia (BPH) tissues were collected from patients undergoing radical prostatectomy or transurethral prostatectomy in The Third Affiliated Hospital of Sun Yat-sen University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study involving human tissue was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University (No. 2019-02-153-01). All patients signed an informed consent form and agreed to the use of the surgically removed tissue. Prostate tissue samples were embedded in paraffin, which was removed with xylene, and then hydrated with ethanol. Next, 0.3% hydrogen peroxide ($H_2O_2$) and citric acid buffer (pH 6.0) were used to block endogenous peroxidase and repair antigen. Antibodies against *NOTCH1* (Proteintech #20687-1-AP), *NOTCH2* (Proteintech #28580-1-AP), *NOTCH3* (Proteintech #55114-1-AP), and *NOTCH4* (Abclonal #A8303) were incubated overnight at 4 ℃. All immunostaining scores were independently confirmed by two pathologists who were unaware of the patient's clinical information. Each pathologist took three photographs for a total of six photos were taken of each sample. Three pictures were randomly selected for final analysis.

### *TIMER*

Tumor Immune Estimation Response (TIMER; https://cistrome.shinyapps.io/timer) is a web server for comprehensive analysis of pan-cancer gene expression patterns and tumor-infiltrating immune cells, which pre-calculates the levels of six tumor-infiltrating immune subgroups (19). In our study, the DiffExp module was used to study the differential expression between tumor and adjacent normal tissues for *NOTCH* family genes across all TCGA (The Cancer Genome Atlas) tumors. The gene module was used to visualize the correlation of *NOTCH* family genes' expression with the level of infiltration of six types of immune cells (B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells) by Spearman correlation in diverse cancer types.

### *cBioPortal*

cBioPortal for Cancer Genomics (cBioPortal, http://www.cbioportal.org, version v3.2.11) is an open-access online tool integrating the raw data from large-scale genomic projects including but not limited to TCGA and ICGC (20). In this study, according to the cBioPortal online instructions (https://www.cbioportal.org/tutorials#webinar-1), the website was used for visualization and comparison of genetic alterations of *NOTCH* family genes in varies cancer types, including PCa. Co-occurrence and mutual exclusivity of genetic alterations between each enquired *NOTCH* gene were determined by log2 odds ratio, P value, and *q* value, and results with *q* value <0.05 were considered significant.

### *GEPIA2*

Gene Expression Profiling Interacting Analysis (GEPIA2; http://gepia2.cancer-pku.cn/) is an updated version of GEPIA, which was developed by a Peking University project team and is capable of analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from TCGA and GTEx projects (21). In our study, GEPIA's "Expression DIY" module was used to analyze the differential expression of the *NOTCH* family between tumor and normal tissues. We used the "PRAD" dataset, using GEPIA's "multi-gene comparison" module, to perform a multi-gene comparative analysis of *NOTCH* genes. The P value cutoff value was 0.05. Student's *t*-test was used to generate a P value for expression or pathological staging analysis. The "Survival Analysis" module outputs a Kaplan-Meier curve to show the relationship between *NOTCH* signaling and survival prognosis.

### *UALCAN*

UALCAN (http://ualcan.path.uab.edu/) is a comprehensive,

 

reliable, and interactive cancer omics data analysis network resource. It is built based on TCGA, MET500, and CPTAC and uses JavaScript and CSS to provide high-quality graphics (22). We used the TCGA analysis module UALCAN and selected the prostate cancer analysis module to analyze the correlation between different Gleason scores and *NOTCH* family genes across PCa and normal tissues, as well as in different tumor subsets based on tumor grade, sex, cancer stages and other clinicopathological characteristics.

### *GeneMANIA*

GeneMANIA (http://www.genemania.org) is an online analytical tool that provides information on protein and genetic interactions, gene enrichment and co-expression analysis, and prediction of the function of interesting genes (23). We selected the target species as Homo sapiens on GeneMANIA's home page, and we typed in the target gene lists that we wanted to analyze (i.e., *NOTCH* family genes) and the generated visual genes network was analyzed and predicted according to the physical interactions strength, co-expression relationships, prediction results, co-localization, and other indexes.

### *STRING*

STRING (https://string-db.org/cgi) integrates public data and analyzes protein-protein interactions (PPI), including direct (physical) and indirect (functional) connections (24). We used STRING to analyze the PPI relationships between the different genes screened in the GEO database that were related to the *NOTCH* pathway. In brief, the identified prostate cancer-related targets were input into the STRING interaction database together with the selected Homo sapiens category for visualization of the interaction network. The confidence of the interactive network construction is 0.4–0.7, which is medium. On this basis, the K-means algorithm was used to classify the PPI networks and identify different clustering networks.

### *GDSC*

Genomics of Drug Sensitivity in Cancer (GDSC; https://www.cancerrxgene.org/) is an online resource for therapeutic biomarker discovery in multiple cancer cells (25). In this study, we used the GDSC database to identify potential therapeutic compounds that were sensitive for the *NOTCH1–2* mutation. We then constructed a volcano plot and a scatter diagram to annotate our target of interest and finally, we performed a Mann-Whitney-Wilcoxon (MWW) analysis to explore potential applications.

### *Statistical analysis*

Gene expression data from the TCGA and GTEx databases were analyzed using Student's *t*-test. The correlation analysis was evaluated using Spearman's correlation analysis. Cox regression analyses were used to evaluate prognostic factors. All statistical examinations were performed by database-derived tools. Scatter plots and histograms were generated using GraphPad Prism 8.0. IBM SPSS 21.0 software was used for statistical analysis. The differences between groups were compared using standard Student's *t*-test, paired *t*-test, or Mann-Whitney *U* test. ANOVA analysis or Kruskal-Wallis test was used to compare differences between two or more groups. P values <0.05 were statistically significant.

## Results

### *Abnormal expression of NOTCH family genes in PCa patients*

To determine the differences in the expression of *NOTCH* family genes in tumors and normal tissues, we used the TIMER DifExp module to analyze the mRNA levels of *NOTCH1–4* in various cancer types. Consistent with a remarkable difference of *NOTCH1–4* expression in breast cancer, kidney cancer, or lung cancer, significant depression of *NOTCH1* and *NOTCH4* was observed between PCa tissue and normal tissue (P=1.96e-9 and 2.04e-06, for each). However, *NOTCH2* and *NOTCH3* did not show this expression difference in PCa (P=8.20e-2 and 2.39e-1, respectively) (*Figure 1A*). To further verify the *NOTCH* family genes' expression in PCa, we performed a screening and analysis via the TCGA-based UALCAN database and validated that *NOTCH1* and *NOTCH4* mRNA expression were indeed lower in PCa tissue than in normal tissue (P=1.047e-6 and 5.93e-5, respectively) (*Figure 1B*). IHC of the prostate specimens showed that *NOTCH1*, *NOTCH3*, and *NOTCH4* levels were higher in cancer tissues than in BPH tissues, but no such phenomenon was found for *NOTCH2* (*Figure 1C*). The following RT-qPCR of *NOTCH1–4* expression was carried out. A total of 24 paired samples of PCa tissue and adjacent normal prostate tissue were collected and tested. The RT-qPCR results were

 

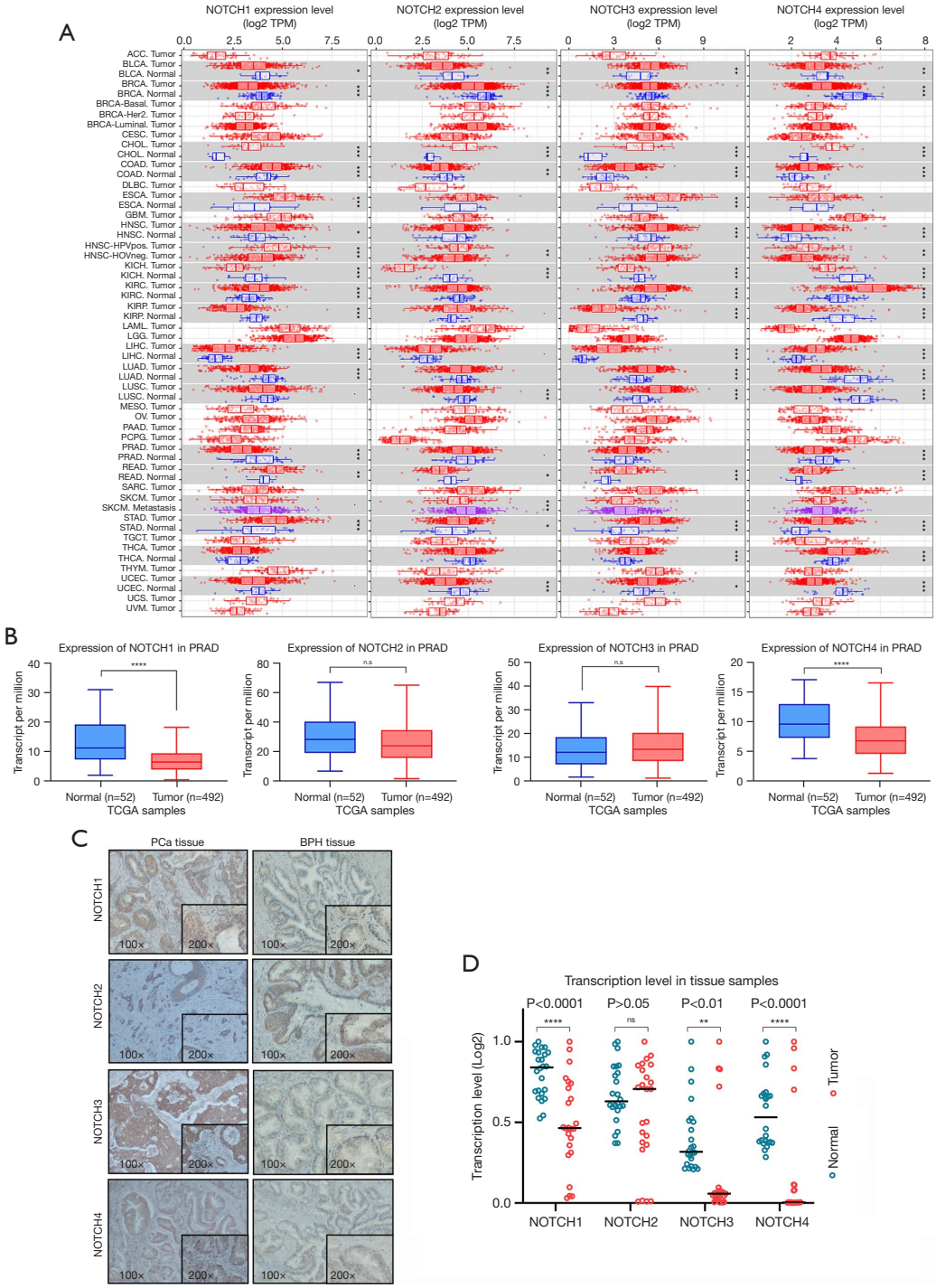


**Figure 1** Abnormal expression of *NOTCH1–4*. (A) Full landscape of *NOTCH1–4* expression profiles in multiple cancer types in the TIMER database. (B) TCGA-based UALCAN database screening and analysis showing a significant difference in the *NOTCH1* and *NOTCH4* expressions between normal tissue and PCa tissue (P=1.047e-6, 1.05e-1, 5.70e-2, 5.93e-5, respectively). (C) IHC staining of *NOTCH1–4* in PCa or BPH tissues respectively [N (tumor) =15, N (BPH) =6]. (D) qRT-PCR results for the mRNA levels of PCa tissues and adjacent normal prostate tissues. (T =24, N =24). *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001. ns, no significant. PRAD, prostate adenocarcinoma. qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; BPH, benign prostate hyperplasia; IHC, immunohistochemistry; PCa, prostate cancer.

 

essentially in accordance with the results from the online databases, *NOTCH1*, *NOTCH3*, and *NOTCH4* showed a significant downtrend of transcription level in tumor tissues compared with normal tissues, but there was little difference of *NOTCH2* mRNA level between tumor and normal tissues (*Figure 1D*). These results indicated that the suppression of the *NOTCH* family genes in PCa tissues may be related to tumorigenesis.

### *Upregulation of NOTCH family genes correlated with Gleason score and patients survival in PCa*

Our analysis showed a significantly lower expression of *NOTCH* family genes in PCa tissue than in normal tissue, so we decided to analyze the *NOTCH* family genes' transcription level alterations during PCa progression. Interestingly, the expression and clinical correlation analysis via the UALCAN database also revealed that the transcription levels of *NOTCH3* and *NOTCH4* were significantly enhanced with an increase of Gleason score. Compared with the patients in the Gleason score 6–8 groups, both *NOTCH3* and *NOTCH4* displayed the highest transcription levels in the Gleason score 9 group (*Figure 2A*). The positive correlation between *NOTCH3–4* expression and Gleason score might indicate a promoting role of *NOTCH* signaling in PCa.

Furthermore, to evaluate the role of *NOTCH* family genes' expression in the prognosis of PCa patients, we used GEPIA to assess the correlation between *NOTCH1–4* expression and clinical outcomes. A survival map, which represents the survival contribution of *NOTCH1–4* in multiple cancer types, showed that *NOTCH3* and *NOTCH4* may affect prognosis in PCa patients (*Figure 2B*). The disease-free survival (DFS) curve shown in *Figure 2C* illustrates that patients with higher transcription levels of *NOTCH1*, *NOTCH3* and *NOTCH4* had significantly poorer DFS (P=4.8e-2, 5.6e-3 and 2.9e-2, respectively). At the same time, an overall survival (OS) analysis implied that *NOTCH1–4* transcription did not affect the OS of PCa patients (P=9.3e-1, 3.3e-1, 9.9e-1, and 8.4e-1, respectively, *Figure 2D*). In summary, *NOTCH1*, *NOTCH3*, and *NOTCH4* perhaps worsen prostate cancer outlook.

### *Co-expression and interaction analysis of NOTCH family genes in PCa*

The correlation heat map showed the internal relationships among *NOTCH1–4* and the interaction between *NOTCH1–4* and *NOTCH*-related genes (*Figure 3A*). As the plot shows, there was a significant moderate to high correlation among *NOTCH1–4* co-expressions. The association between *NOTCH1–4* and the classical *NOTCH* downstream genes (i.e., *DLL1*, *DLL3*, *JAG1*, *HES1*) was also verified as a generally positive correlation. Because it has been reported that there is an interaction between the *NOTCH* and *AR* signaling pathways (26,27), we screened the interaction among *NOTCH1–4*, *AR*, and *AR*-related genes (*KLK3* and *TMPRSS2*). Interestingly, *NOTCH1–4* and *AR* were observed to have a significant positive correlation, whereas *AR*-related *KLK3* and *TMPRSS2* were negatively correlated to *NOTCH1–4* at the transcription level (*Figure 3A*). All the correlations of the genes were plotted into a scatterplot using the GEPIA database, and the significant results are presented in Figures S1,S2. We presume that it is the crosstalk between *NOTCH* signaling and *AR* that leads to the Gleason score increasing and the poor prognosis of PCa patients.

Next, to explore the potential mechanism of *NOTCH* family genes' involvement in cancer, we used GeneMANIA and STRING to construct *NOTCH* family genes-related PPI networks. As shown in *Figure 3B,3C*, *NOTCH1–4* had a strong physical interaction with *FURIN* and this correlation was further confirmed by co-expression analysis through GEPIA (Figure S3). *FURIN* has been reported to play a critical role in both inflammation and tumorigenesis (28,29). These results suggested that *NOTCH* genes might be involved in tumor promotion and inflammation, and then accelerate tumor progression through *AR* signaling and *FURIN*.

### *Effect of NOTCH family genes' mutations on tumor-related biological pathways in PCa*

We conducted a comprehensive analysis to uncover the molecular characteristics and genetic alteration of *NOTCH* family genes using the online dataset cBioportal. The results showed that *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4* had 7%, 8%, 5%, and 5% mutational frequency in the queried PCa samples, respectively. (*Figure 4A*). Among these mutations, Shallow Deletion and Amplification accounted for the highest proportion (Figure S4). Mutations are always accompanied by alteration of some other genes. The *NOTCH*-mutation-related differentially expressed genes (DEGs) in PCa were collected and the top 10 most significant DEGs of *NOTCH1–4* are shown in *Figure 4B*. At the same time, the common genes among *NOTCH1–4*

 

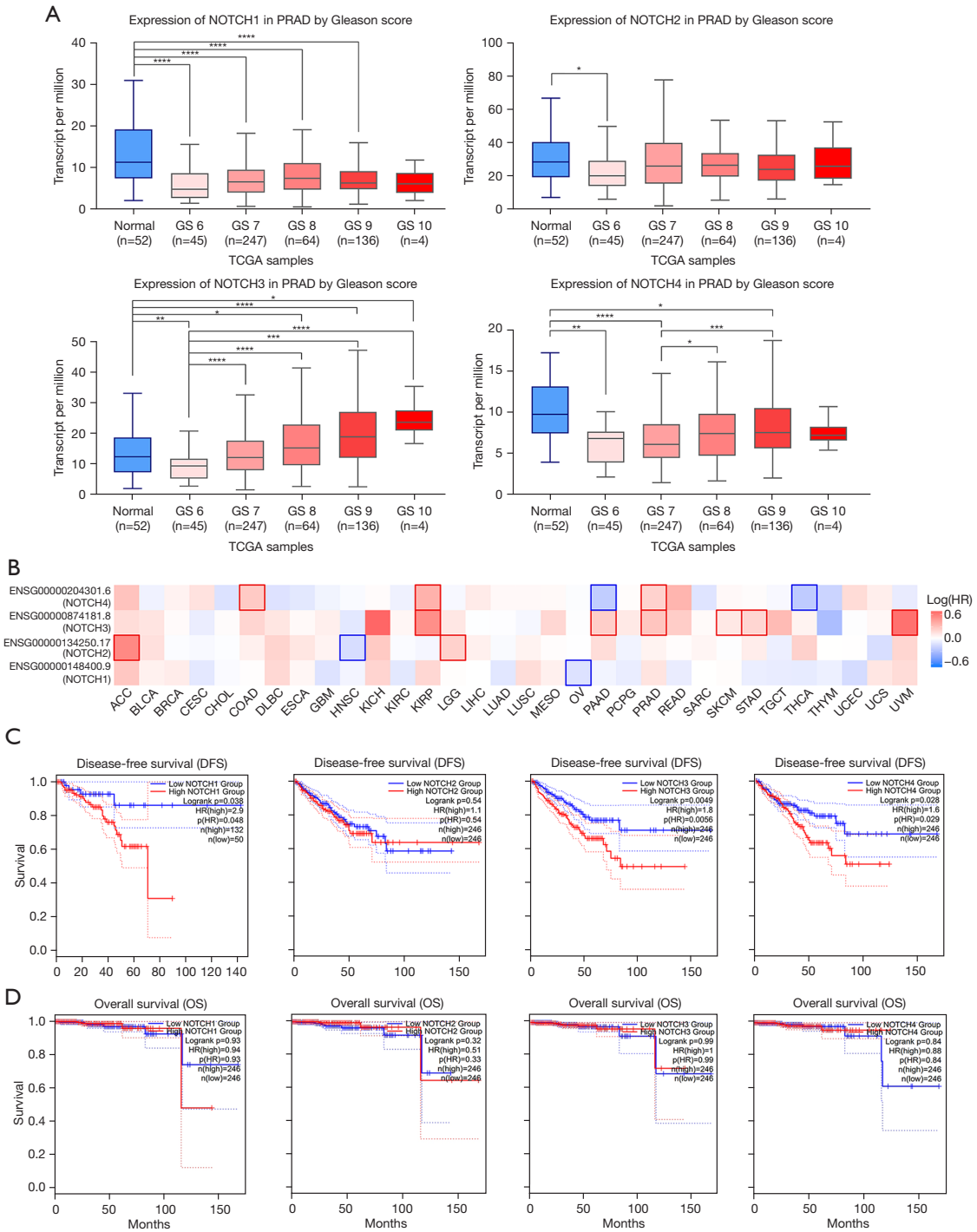


**Figure 2** Correlation between *NOTCH1–4* expression and Gleason score or prognosis in PCa patients. (A) UALCAN database analysis showing that the transcriptional level of *NOTCH3* and *NOTCH4* is significantly enhanced with increasing Gleason score. (B) The Mantel–Cox test with GEPIA2 showing that the expression of *NOTCH3* and *NOTCH4* exhibited a positive correlation with prognosis in PCa patients. (C) Cox regression analysis showing that patients in the low *NOTCH1*, *3* and *4* groups had a significant better disease-free survival (DFS) than those in the high group (P=4.8e-2, 5.6e-3, and 2.0e-2, respectively). (D) Cox regression analysis showing that there was no significant difference in overall survival (OS) between high and low NOTCH family genes' expression. *, P<0.05, **, P<0.01, ***, P<0.001 and ****, P<0.0001. GS, Gleason score; PRAD, prostate adenocarcinoma; PCa, prostate cancer.

 

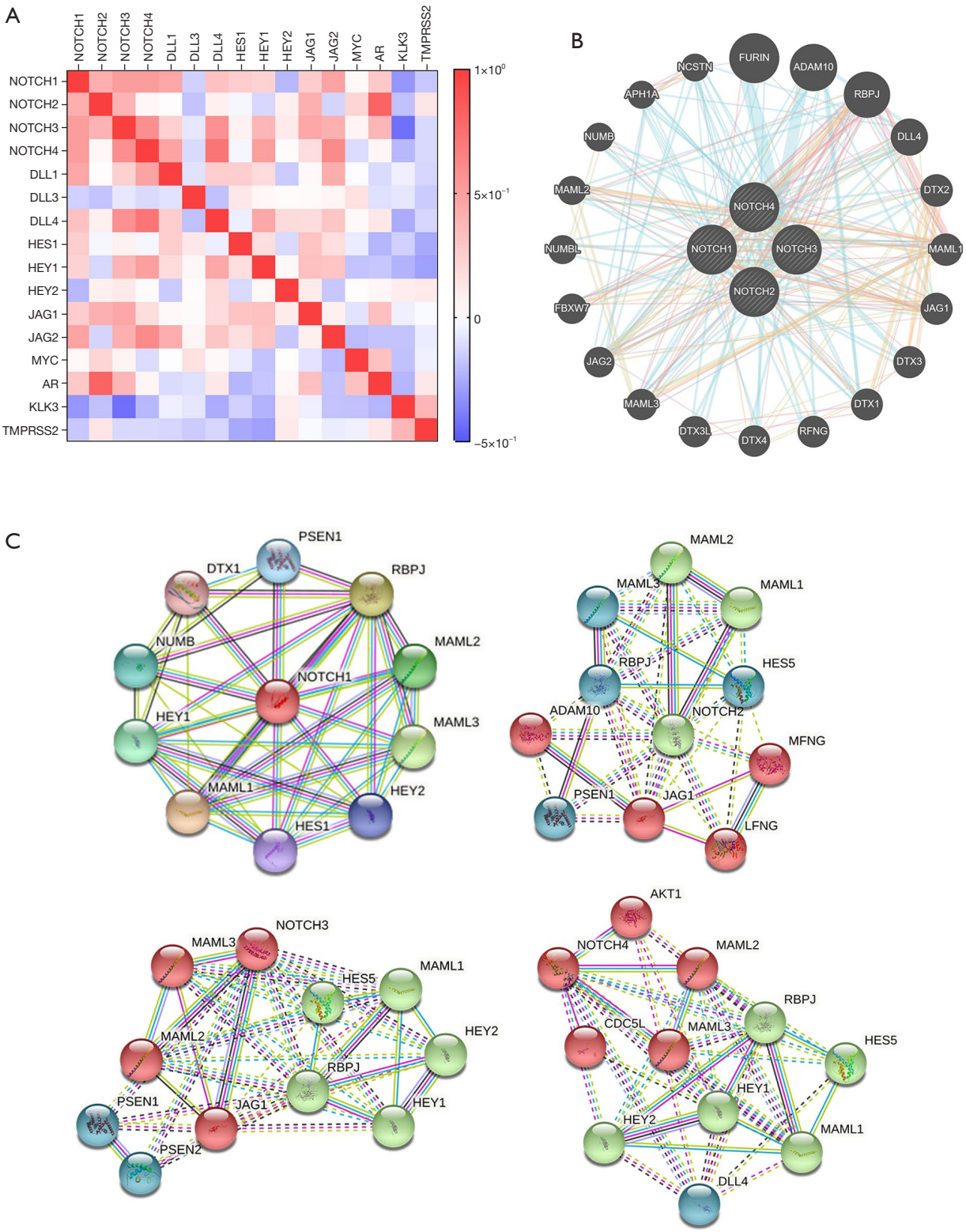


**Figure 3** Neighbor gene network and interaction analyses of *NOTCH1–4* in PCa patients. (A) Correlation heat map of *NOTCH* family genes and *NOTCH*-related genes in PCa patients. (B,C) Protein-protein interaction networks of differently expressed *NOTCH* family genes. PCa, prostate cancer.

 

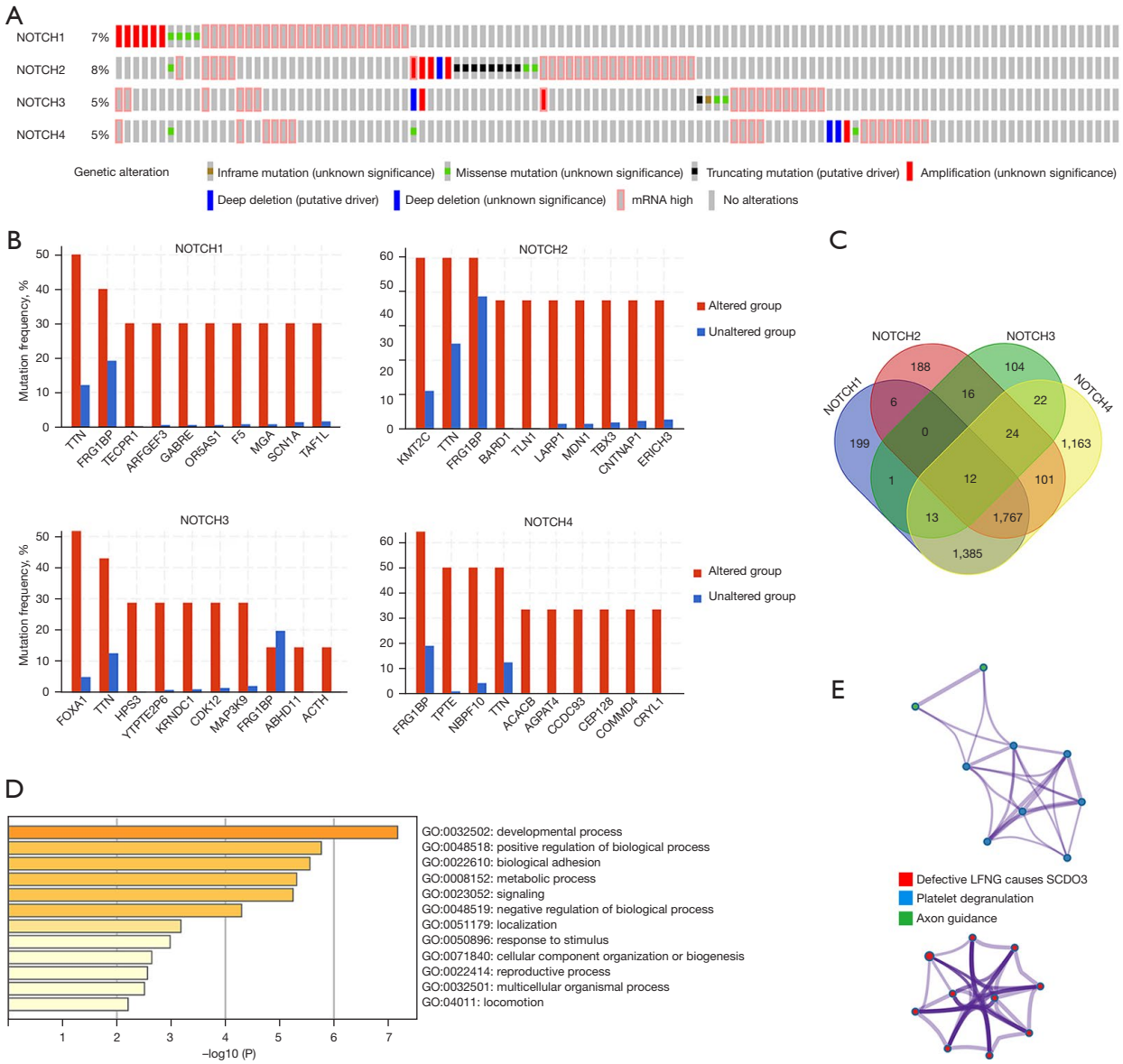


**Figure 4** Characterization of genetic alterations and mutations of *NOTCH1–4*. (A) Mutational frequencies of *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4* in PCa were 7%, 8%, 5%, and 5%, respectively. (B) Top 10 most significant mutation-related genes of *NOTCH1–4*. (C) Wayne chart showing the shared mutation-related genes of *NOTCH1–4*. (D) Bar graph showing the top 20 results from the GO enrichment analysis. (E) GO enrichment analysis showing the gene networks. GO, gene ontology; PCa, prostate cancer.

mutation-related DEGs were identified (*Figure 4C*, *Table 1*). Armed with this data, GO enrichment analysis and the network of enriched terms were performed to explore the pathway(s) affected by *NOTCH1–4* mutations. As shown in *Figure 4D*,*4E* and *Table 2*, the functions of *NOTCH* signaling and its adjacent genes are mainly enriched in the cell physiology and tumor formation signaling pathways. In brief, all the results summarized the characteristics of *NOTCH1–4* mutation status in PCa and indicated that *NOTCH1–4* mutations may participate in the development

 

**Table 1** Common differentially expressed genes among *NOTCH1–4* mutations

| # | Symbol | Description | Category |
|---|---|---|---|
| 1 | TLCD3B | TLC domain containing 3B | Protein coding |
| 2 | DDI1 | DNA damage inducible 1 homolog 1 | Protein coding |
| 3 | CRYL1 | Crystallin lambda 1 | Protein coding |
| 4 | SLC25A23 | Solute carrier family 25 member 23 | Protein coding |
| 5 | TNK2 | Tyrosine kinase non receptor 2 | Protein coding |
| 6 | DDIT4 | DNA damage inducible transcript 4 | Protein coding |
| 7 | NDNF | Neuron derived neurotrophic factor | Protein coding |
| 8 | IL31RA | Interleukin 31 receptor A | Protein coding |
| 9 | TXNDC16 | Thioredoxin domain containing 16 | Protein coding |
| 10 | MLLT1 | MLLT1 super elongation complex Subunit | Protein coding |
| 11 | FGD1 | FYVE, RhoGEF and PH DOMAIN Containing 1 | Protein coding |
| 12 | KIF20A | Kinesin family member 20A | Protein coding |

**Table 2** Top 3 clusters with their representative enriched terms

| GO | Category | Description | Count | % | Log10 (P) | Log10 (q) |
|---|---|---|---|---|---|---|
| R-HAS-5083630 | Reactome gene sets | Defective LFNG causes SCDO3 | 4 | 10.81 | –10.89 | –6.54 |
| GO:0002576 | GO biological processes | platelet degranulation | 3 | 8.11 | –3.18 | –0.63 |
| R-HAS-422475 | Reactome gene sets | Axon guidance | 4 | 10.81 | –2.23 | 0.00 |

GO, Gene Ontology.

of PCa.

### *NOTCH family genes' mutations and target drug sensitivity*

To determine the potential role of antitumor agents, we used the GDSC database to identify potentially sensitive and selective agents for patients with and without mutations of *NOTCH1* and *NOTCH2* (because of the absence of *NOTCH3* and *NOTCH4* data in the GDSC database). GDSC screening results showed a large number of candidate drugs targeting *NOTCH1–2* mutations. In both individual screenings, the overwhelming majority of *NOTCH1* mutation-targeted drugs were -tinib antineoplastic drugs, such as BMS-754807, linsitinib, saracatinib, and erlotinib, which had sensitivity differences between *NOTCH1* mutation and wild-type groups, and cells with *NOTCH1* mutations were more sensitive to these four drugs than wild-type ones. *NOTCH2* mutation seemed to act as an entospletinib desensitizer (*Figure 5A*). The drug-sensitivity scatterplot is shown in *Figure 5B* and the details are shown in *Table 3*. It is worth noting that most of the *NOTCH1–2* mutation-related drugs belong to the family of -tinib drugs, which gives us a hint as to how we can focus on the application of -tinib antineoplastic drugs in the treatment of PCa with different *NOTCH* mutations.

### *Relationship of NOTCH family genes' expression to lymph node metastasis and immune cell infiltration in PCa*

It has been reported that *NOTCH* family genes can participate in tissue inflammation and tumor lymphocyte infiltration, and thus may change the tumor's response to immune-related treatments and the prognosis of patients (30,31). Firstly, we assessed the correlation between *NOTCH1–4* expression and lymph node metastasis status. The results indicated that patients with N1 stage exhibited a higher *NOTCH3* expression than those with N0 stage

 

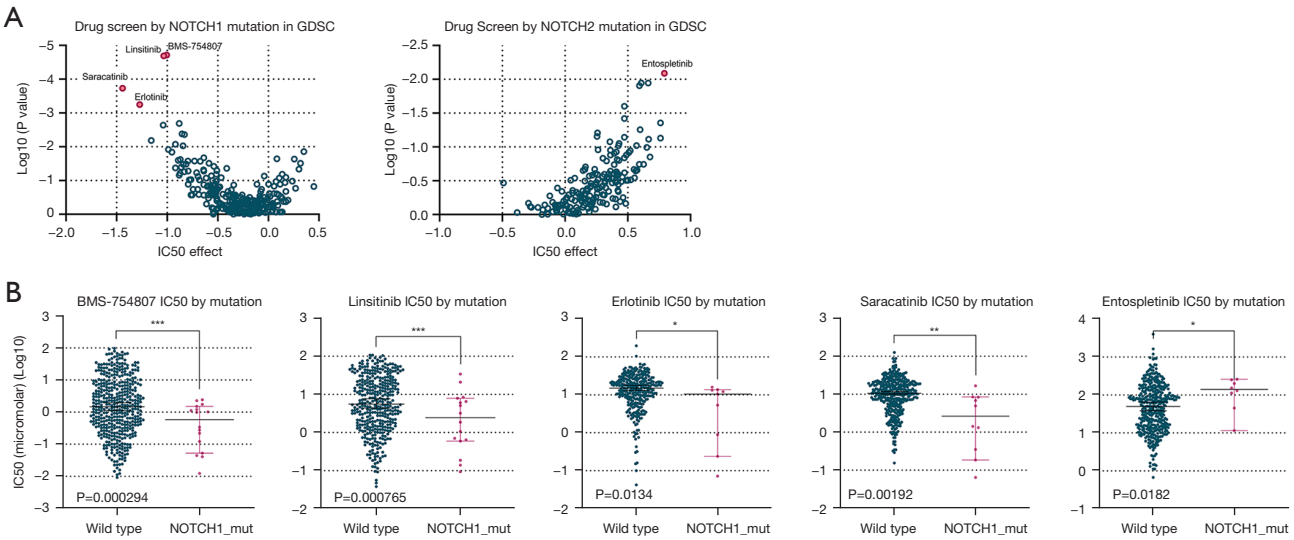


**Figure 5** *NOTCH1–4* mutations and drug sensitivity. (A) Volcano plots showing that multiple cancer cell types with the *NOTCH1* mutation were sensitively targeted by BMS-754807, linsitinib, saracatinib and erlotinib (left-hand panel). Tumor cells with the *NOTCH2* mutation showed potential antagonism against entospletinib (right-hand panel). (B) Scatter plots showing that tumor cells were significantly suppressed by the use of BMS-754807, linsitinib, saracatinib, erlotinib and entospletinib in the *NOTCH1* mutation group compared with the wild-type group (P<0.05 for all). *, P<0.05, **, P<0.01, and ***, P<0.001. IC50, half maximal inhibitory concentration; GDSC, genomics of drug sensitivity in cancer; Mut, mutation.

**Table 3** *NOTCH* mutation-related drug-sensitivity analyzed using ANOVA

| Gene_mutation | Drug | Drug target | Effect size | P value | FDR (%) | Tissue |
|---|---|---|---|---|---|---|
| *NOTCH1*_mut | BMS-754807 | IGF1R, IR | 0.792 | 0.00817 | 1.16 | PCa |
| *NOTCH1*_mut | Linsitinib | IGF1R | 0.606 | 0.0112 | 1.35 | PCa |
| *NOTCH1*_mut | Erlotinib | ABL, SRC | 0.661 | 0.0113 | 5.1 | PCa |
| *NOTCH1*_mut | Saracatinib | EGFR | 0.593 | 0.0122 | 15.5 | PCa |
| *NOTCH2*_mut | Entospletinib | SYK | 0.792 | 0.00817 | 43.2 | PCa |

FDR, false discovery rate; Mut, mutation; PCa, prostate cancer.

(P<0.05). However, there were no significant differences of *NOTCH1*, *NOTCH2* and *NOTCH4* expression between patients with N0 and N1 stages (*Figure 6A*). Furthermore, we used the TIMER database to comprehensively explore the correlation between the expression differences of *NOTCH1–4* and immune cell infiltration. As shown in *Figure 6B*, there was a significant correlation between *NOTCH* signaling and lymphocyte infiltration, especially $CD8^+$ T cells and $CD4^+$ T cells, which are the key components of tumor immunity. Because it has been reported that there is a significant impact of immune infiltration in the prognosis of various cancer types (32-34), the results indicated that the expression of *NOTCH* family genes was closely related to the degree of immune infiltration, which then affects the treatment and prognosis in PCa patients.

## Discussion

The incidence rate of PCa is one of the highest among the male malignant neoplasms. The clinical manifestations of PCa are heterogeneous. Conventional treatment strategies include reducing the tumor burden and/or testosterone level through radiotherapy, surgery, and/or androgen

 

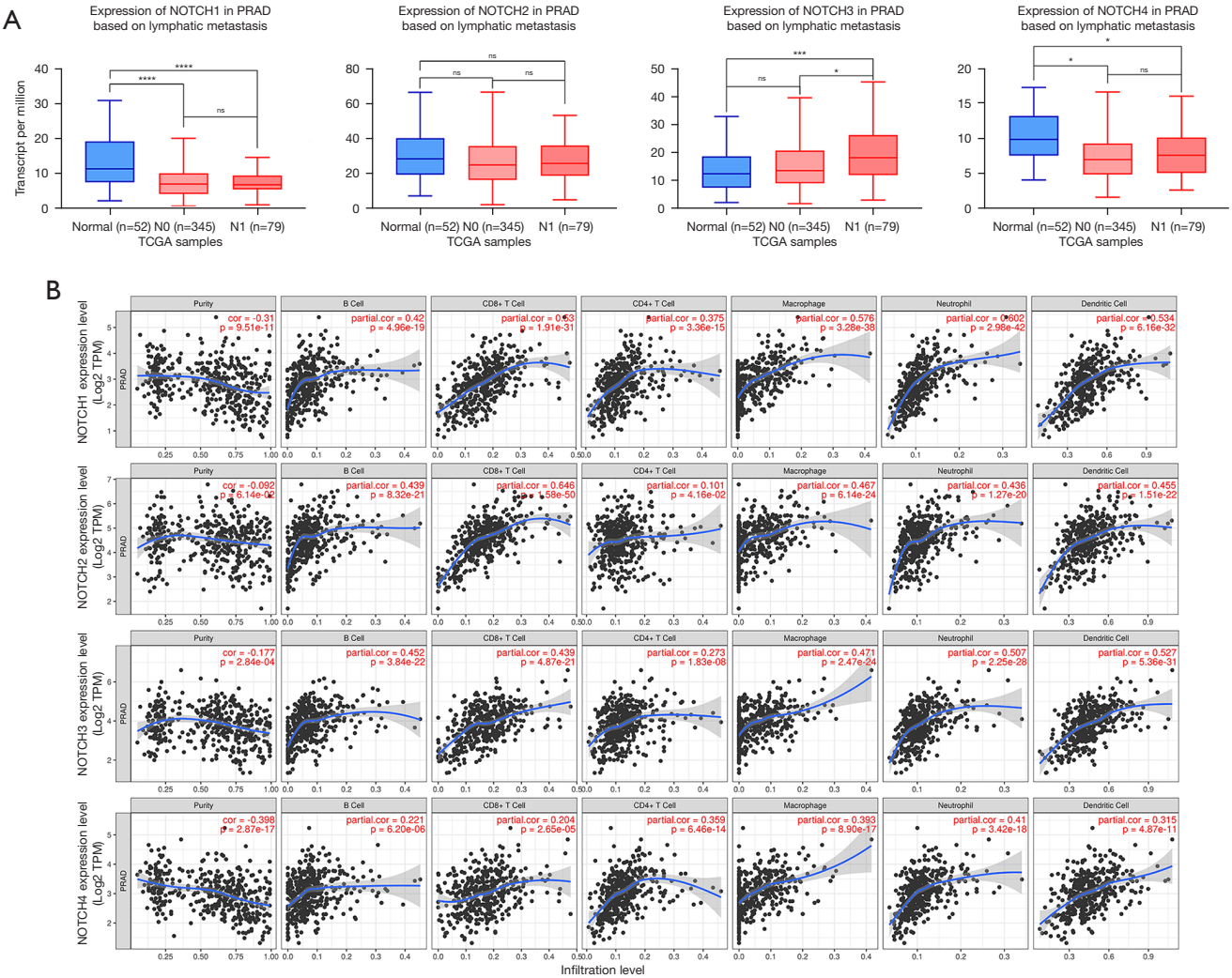


**Figure 6** Correlation between differently expressed *NOTCH1–4* and lymphatic metastasis or immune cell infiltration. (A) TIMER database analysis showing that patients with N1 stage had significant higher expression of *NOTCH3* than those with N0 stage (P<0.05, N0 = no lymphatic metastasis, N1 = lymphatic metastasis). (B) Associations between *NOTCH1–4* and lymphocytes infiltration in PCa patients. *, P<0.05, ***, P<0.001 and ****, P<0.0001. ns, no significant. PCa, prostate cancer; PRAD, prostate adenocarcinoma; TIMER, tumor immune estimation resource.

deprivation. However, PCa inevitably progresses to CRPC, which has limited treatment options and a grim prognosis. It is a matter of the utmost urgency to find new therapeutic targets for PCa. Recently, the presence of lymphocytic infiltrates in PCa tissue suggested an immune mechanism, and thus, targeted immunotherapy is an attractive option for PCa.

This research aimed to expound a joint viewpoint on the potential applications of *NOTCH* genes as prognostic biomarkers and as molecular drug targets for PCa patients. The tantalizing thing is that *NOTCH* signaling is paradoxical, being both a tumor inhibitor and promotor (35,36). Correspondingly, our multidimensional analysis found versatility of *NOTCH* signaling in PCa. Specifically, the transcriptional level of *NOTCH1* and *NOTCH4* was significantly lower in PCa tissue than in normal tissue. Based on this, *NOTCH* signaling might be considered a tumor suppressor. However, the analysis from a clinically perspective found that the expression trend of *NOTCH3* and *NOTCH4* increased as the Gleason score rose. A high

 

expression of *NOTCH1*, *NOTCH3*, and *NOTCH4* appears to be the cause of the poor prognosis of PCa patients; therefore, the role of *NOTCH* signaling is much more complicated than simply either being beneficial or harmful in PCa. Two plausible hypotheses may explain this: one, as the main factor in the internal environment, protein plays a pivotal role in homeostasis and functioning. Changes in the transcription level are not a proxy for the physiological process of cells. So, analysis limited to transcription is challenging to depict the overall picture. *NOTCH* proteins detection should be done in further studies. Another possible reason is that there were more samples in the Gleason score ≤7 groups than in the Gleason score >7 groups. Also, a lower mRNA expression of *NOTCH* in the Gleason score ≤7 groups may further drag down the overall *NOTCH* transcription levels; therefore, some further research based on larger samples is required. It is expected that the role of *NOTCH* family genes in PCa will be better defined with a clearer understanding of the different microenvironments and subtypes with differences in biological characteristics.

Next, we investigated the internal relationship among *NOTCH* family genes. The moderate-to-high correlations among *NOTCH1–4* were evident, and imply a cooperative role of *NOTCH* family genes working together as a contribution to PCa progression. In addition, it is reported that selective inhibition of *NOTCH1* attenuates PCa cell growth in the castrated scenario (37). Some studies have claimed that as a synergistic inhibitor of *AR*, the *NOTCH* signaling downstream target gene Hey1 can inhibit the activation of *AR* and the expression of *AR*-related genes (27,38), although *AR* activation could also inhibit *NOTCH* signaling activation in turn (39). Contrary to previous studies, our data demonstrated that *NOTCH1–4* had a positive correlation to *AR* expression and negatively regulated *KLK3* and *TMPRSS2*. We speculate that the *NOTCH* family genes perhaps have the ability to prevent *AR* nuclear translocation and inhibit DNA binding, by which it impedes *AR*-mediated transcription. The loss of coordination between *NOTCH* signaling and *AR* signaling might alter tumor cells' characteristics. These complicated correlations between the expression of *NOTCH* family genes and *AR*/*AR*-related genes may also result in a higher Gleason score in PCa patients and worse prognosis, and further research still needs to be done.

We also explored if *FURIN* was the most pertinent interaction factor to *NOTCH* family genes, and identified a positive correlation between *FURIN* and *NOTCH1–3* at the transcriptional level. *FURIN* is a widely expressed calcium-dependent protease, and plays a critical role in embryogenesis, as well as catalyzing the maturation of a large number of different pro-protein substrates among which are even some protease systems that regulate diseases. *FURIN* expression is enhanced in a variety of cancer types, and its activity promotes many cancer-related processes, such as cell proliferation, migration and invasion, and vascularization (40,41). To our knowledge, no study to date has linked up *NOTCH* signaling and *FURIN* in PCa. Thus, our results could be the foundation for further investigation.

Finally, current therapies targeting the *NOTCH* signaling pathway mainly focus on the chemical or biological inhibition of *NOTCH* family genes (42). However, the development of new drugs has the problems of high cost, long development cycle, and difficulty to adapt to the fast-changing patients' demand. Here, a new route of drug screening based on *NOTCH1–4* mutation status was discussed to broaden the treatment regimen. Despite the missing data for *NOTCH3–4* in the GDSC database, the analysis targeting *NOTCH1* and *NOTCH2* mutations shed light on the role of -tinib antineoplastic drugs in PCa patients. The -tinib antitumor drugs are a new class of biologically targeted cancer drugs, and research has highlighted their role in PCa (43). Our results suggested that the *NOTCH* genes' mutation status is a clue for the use of -tinib antitumor drugs in PCa patients. Intriguingly, research has shown that -tinib drugs affect the development of murine PCa by activating antitumor innate immunity (44,45). Consistent with the results above, tumor-infiltrated lymphocyte analysis revealed the relationship between the *NOTCH* family genes and classical immune cells (B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages) infiltration. To sum up, full integration of *NOTCH* mutation status, -tinib drugs and tumor immune cell infiltration could lead to a bright prospect in the therapeutic management of PCa patients.

Inevitably, there are still some limitations of our study. First, analysis at the transcriptional level alone does not provide a complete landscape of organism function. Another independent cohort study and *in vitro* or *in vivo* follow-up may be necessary to verify our results.

## Conclusions

We revealed a significant difference in *NOTCH* family genes' expression between normal prostate tissue and PCa tissue, and the expression had a positive correlation with

 

Gleason score, DFS, lymph node metastasis, and immune cell infiltration of PCa patients. *NOTCH* signaling might be involved in tumor promotion and inflammation, and then accelerate tumor progression by inducing *AR* signaling and *FURIN*. Patients with *NOTCH* family gene mutations might be sensitive to -tinib antineoplastic drugs. We hope that our findings give a new perspective on designing new immunotherapy agents, as well as helping clinicians select appropriate drugs and prognostic biomarkers for PCa patients.

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## Footnote

*Reporting Checklist:* The authors have completed the STREGA reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-22-281/rc

*Data Sharing Statement:* Available at https://tau.amegroups.com/article/view/10.21037/tau-22-281/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-281/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University (No. 2019-02-153-01) and informed consent was given by all the patients.



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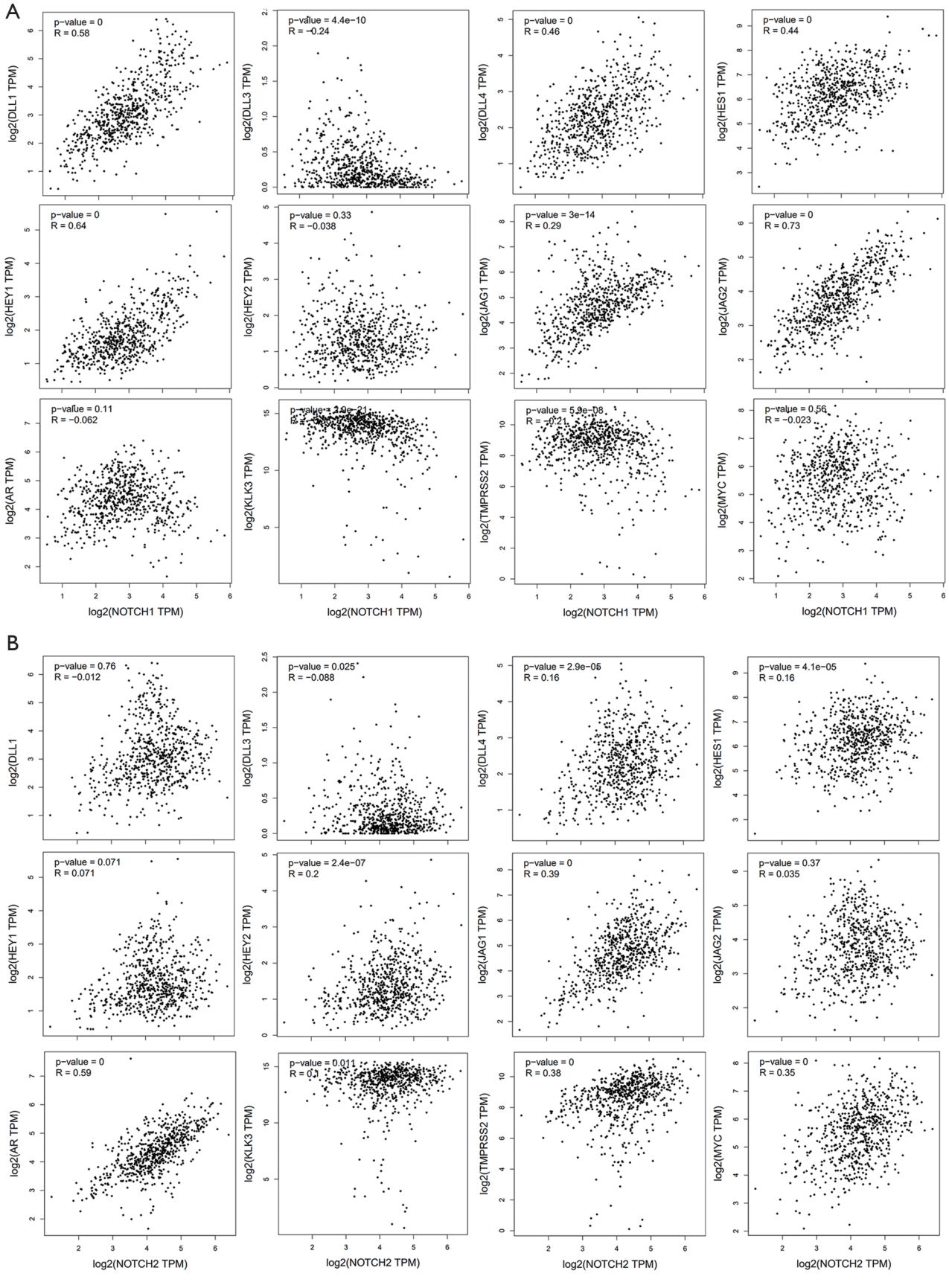


**Figure S1** Correlation between *NOTCH1* (A) or *NOTCH2* (B) and *NOTCH*-related genes and the correlation between *NOTCH1* or *NOTCH2* and AR signaling pathway genes plotted as scatter diagrams of correlation. P<0.05 was considered statistically significant. AR, androgen receptor; TPM, transcription per million.


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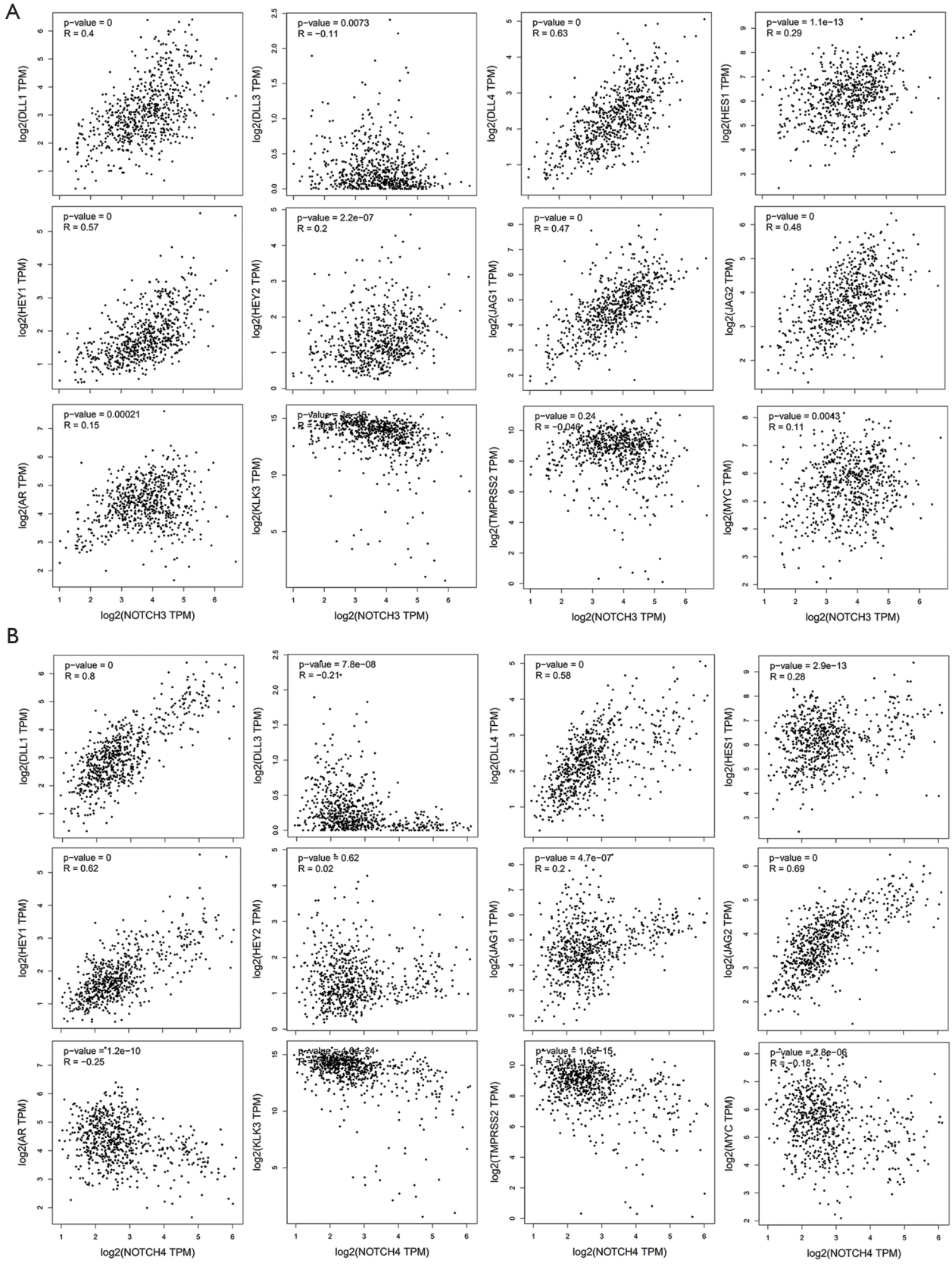


**Figure S2** TCORRELATION between *NOTCH3* (A) or *NOTCH4* (B) and *NOTCH*-related genes and the correlation between *NOTCH3–4* and *NOTCH* signaling and *AR* signaling pathway genes plotted as scatter diagrams of correlation. P<0.05 was considered statistically significant. *AR*, androgen receptor; R, Pearson correlation coefficient; TPM, transcription per million.


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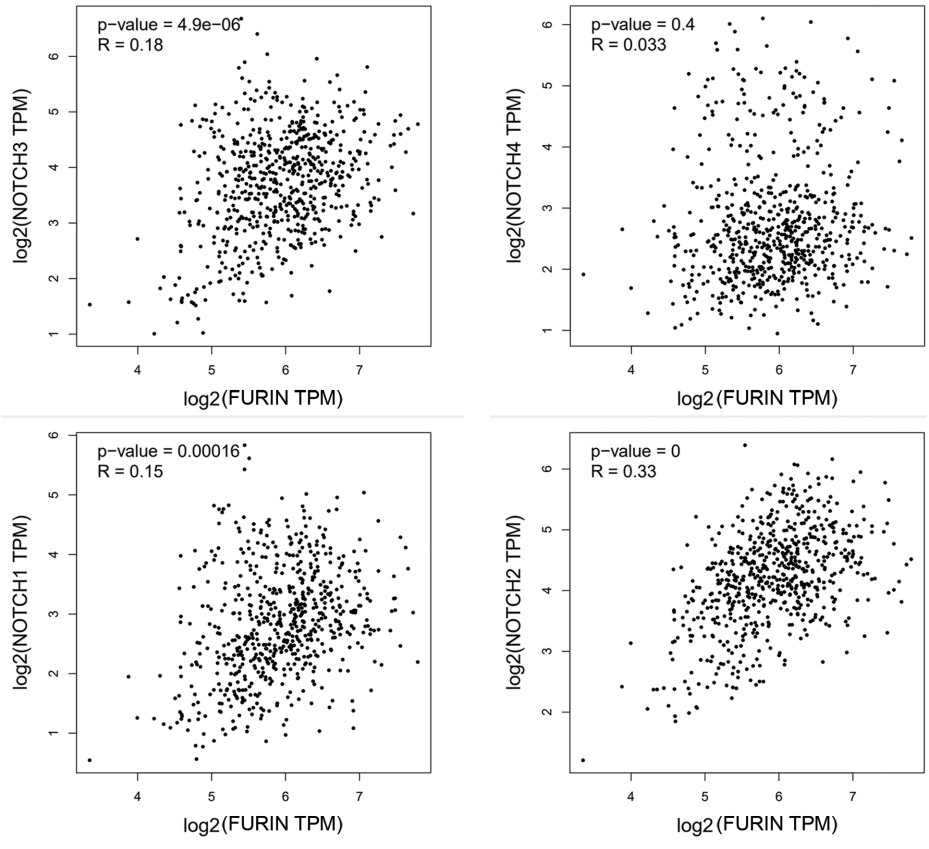


**Figure S3** Analysis of the transcriptional correlation of *NOTCH1–4* and *FURIN* in prostate cancer (P values shown in the plots; P<0.05 considered statistically significant). TPM, transcription per million.


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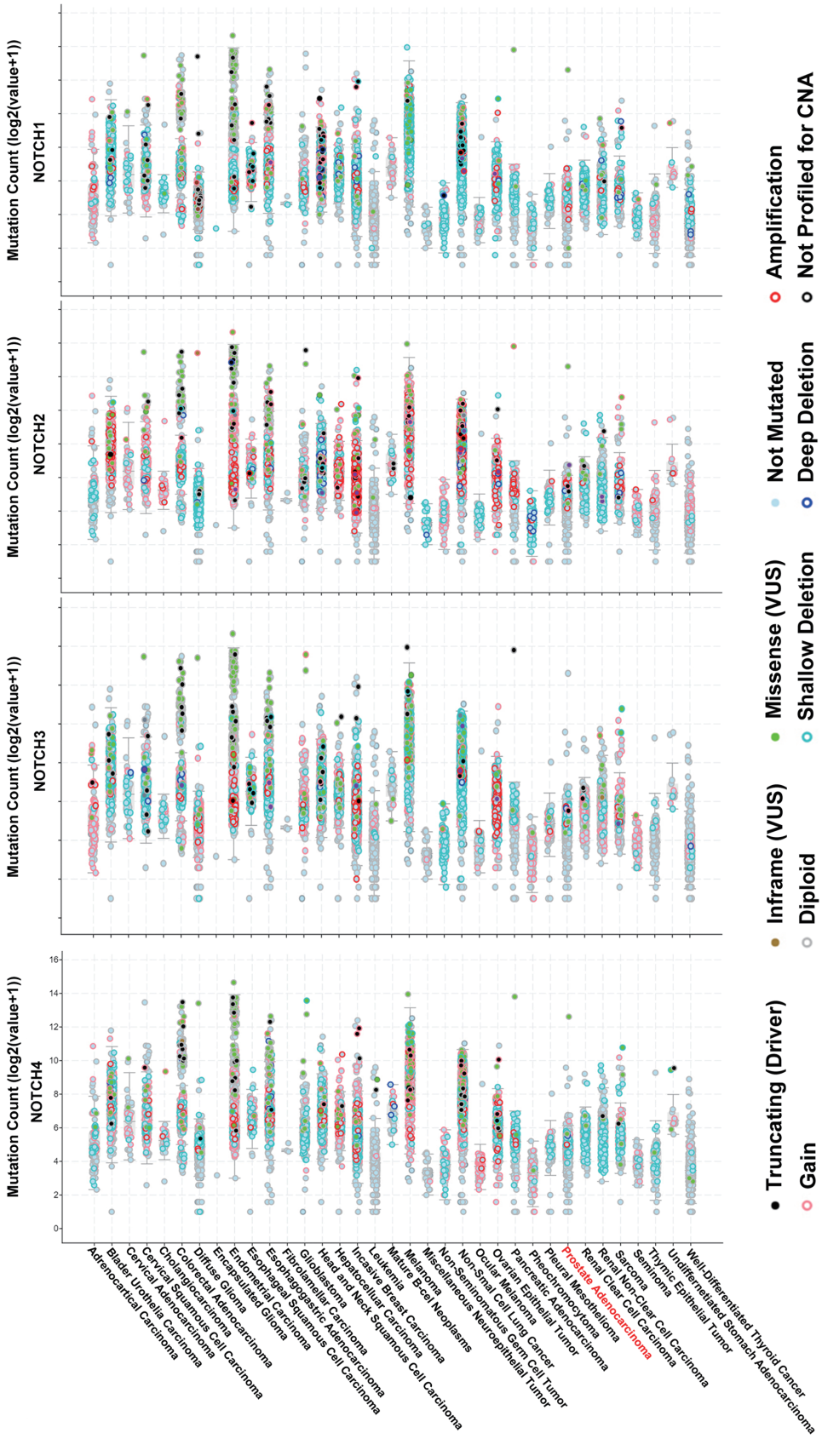


**Figure S4** Characterization of genetic alterations and mutation maps of *NOTCH1–4* in multiple cancer types. VUS, variant of uncertain significance.


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