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A comprehensive multi-omics analysis reveals molecular features associated with cancer via RNA cross-talks in the Notch signaling pathway

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ABSTRACT

The Notch signaling has an important role in multiple cellular processes and is related to carcinogenic process. To understand the potential molecular features of the crucial Notch pathway, a comprehensive multi-omics analysis is performed to explore its contributions in cancer, mainly including analysis of somatic mutation landscape, pan-cancer expression, ncRNA regulation and potential prognostic power. The screened 22 Notch core genes are relative stable in DNA variation. Dynamic expression patterns are associated with the Notch activity, which are mainly regulated by multiple ncRNAs via interactions of ncRNA:mRNA and ceRNA networks. The Notch pathway shows a potential prognostic ability through integrating multi-omics features as well as their targets, and it is correlated with immune infiltration and maybe available drug targets, implying the potential role in individualized treatment. Collectively, all of these findings contribute to exploring crucial role of the key pathway in cancer pathophysiology and gaining mechanistic insights into cross-talks among RNAs and biological pathways, which indicates the possible application of the well-conserved Notch signaling pathway in precision medicine.

1. Introduction

The Notch pathway, one of the main signaling pathways, is an evolutionarily conserved pathway across metazoans and involved in growth and development of organisms. It has an important role in multiple cellular processes, mainly including cell proliferation, differentiation and apoptosis [1]. Increasing evidences have shown that the abnormal activation of Notch signaling pathway may contribute to neoplastic diseases [2], such as pancreatic cancer [3], gastric cancer [4], bladder cancer [5] and non-small cell lung cancer [6]. Notch is crucial to tumorigenesis, which contributes to understanding of cancer pathology and Notch may be a potential therapeutic target [7,8]. It can act as an oncogene or tumor suppressor in diverse tissues or cells, which further promotes proliferation or apoptosis [9,10]. The abnormal activation may cause mutation or amplification of relevant genes, and the dysregulation of Notch always promotes tumor formation and contributes to tumorigenesis [11].

The Notch pathway contains a gene family of transmembrane Notch receptors, and their canonical ligands, negative and positive modifiers and transcription factors [12,13]. Many studies have validated that specific non-coding RNAs (ncRNAs), a series of important regulators, mainly including microRNAs (miRNAs) and their multiple isomiRs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), can perturb the Notch pathway that further contributes to cancer pathology. For example, lncRNA DLEU2 promotes cervical cancer cell proliferation by regulating cell cycle and Notch pathway [14], LINC01783 can facilitate cell proliferation, migration and invasion in non-small cell lung cancer by targeting miR-432-5p to activate the Notch pathway [15], miRNA-34b-5p inhibits proliferation, stemness, migration and invasion of retinoblastoma cells via Notch signaling [16], and miR-200 deficiency promotes lung cancer metastasis by activating Notch signaling in cancer-associated fibroblasts [17]. These interactions or cross-talks between ncRNA and the Notch pathway have critical roles in tumorigenesis. It is important to further understand the

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molecular features of the Notch pathway, especially for the potential cross-talks with ncRNAs, which will greatly contribute to revealing the cancer progression.

Based on the potential role of the Notch pathway in cancer, many studies have focused on the well-conserved signaling pathway. However, the current studies mainly pay attention to the effect of individual or some components in specific cancer type, but a systematic characterization via a pan-cancer analysis is urgent to provide insight into critical roles in multiple processes, especially in human carcinogenic process. Herein, to characterize molecular features of the Notch pathway, we performed an integrative pan-cancer analysis to understand the molecular portrait of the Notch, which would provide knowledge and reference for determining the potential application in cancer diagnosis, prognosis and treatment, especially in anticancer drugs.

2. Materials and methods

2.1. Data resource

To perform multi-omics analysis in cancers, high-throughput sequencing datasets in multiple molecular levels were obtained from The Cancer Genome Atlas (TCGA) and Genome Data Commons (https://gdc.cancer.gov/about-data/publications/pancanatlas) [18] (Table S1). The clinical data, mainly including survival status, cancer stage and grade, survival time, and molecular subtype, were also obtained to access the potential prognostic values in cancer. Other relevant data sources were mentioned in article and summarized in Table S2.

2.2. Screening of core genes in the Notch pathway

Genes in the Notch pathway were mainly obtained in gene set enrichment analysis (GSEA) [19], BioMart [20], AmiGo [21] and public references (Table S3). A total of 177 genes were firstly obtained, and candidate core genes were further screened if a gene could meet at least one of the following conditions: (1) associated with cancer, (2) directly contributed to Notch function and regulatory process, (3) associated with phenotype of Notch family, and mutation or deficiency caused loss of similar phenotype of Notch family. Finally, 50 candidate core genes were collected, mainly including 5 classical ligands and 4 noncanonical ligands, 4 Notch receptors, 11 genes involved in three-step digestion reaction, 1 transcription factor, 8 transcriptional coactivators, 6 transcriptional co-repressors, and 11 positive or negative regulatory factors. These candidate genes were used to classify involved samples. Further, 22 core genes (including ligands, receptors, transcription factor and transcriptional coactivators) were selected to perform further analysis to understand the potential molecular features associated with cancer. Simultaneously, another 40 downstream genes were selected to analyze transcription output of Notch pathway activity.

2.3. Somatic mutation and copy-number alteration analysis

A total of 9,125 patients were collected from different cancer types, and filtering steps were used to eliminate artifacts and avoid false-positive calls [22]. There were 8,407 patients remained to perform further analysis. The mutation frequencies for the core Notch genes were estimated in diverse cancer types using MutSigCV1.4 [23] with a q-value cutoff of 0.25, and the potential driver gene mutations were simultaneously screened. The somatic copy number aberration (SCNA) scores were estimated, and significantly amplified and deleted genes ($q < 0.25$) were screened in each cancer type with GISTIC2.0 [24]. The lollipop charts of specific genes were mainly obtained from cBioPortal [25]. Further, to assess genetic alternation load of the Notch genes, mutation frequency and copy number alteration were calculated in randomly selected gene set in each cancer type ($n = 1000$), and the detailed rank of the Notch genes was used to estimate the potential contributions in tumorigenesis.

2.4. Analysis of differentially expressed profiles of RNAs

To understand the detailed expression patterns of RNAs, mainly including mRNAs, miRNAs/isomiRs and lncRNAs, differentially expression profiles were estimated using DESeq2 [26], and genes were considered to be significantly differentially expressed if $|\log_2\text{FC}| > 1.5$ and $padj < 0.05$. Based on the potential regulatory roles of isomiRs, the screened miRNAs related Notch were queried for the detailed expression patterns from classical miRNA and isomiR levels, respectively. Furthermore, to understand the DNA methylation profiles and potential roles in immune infiltration, the core Notch genes were further queried for methylation changes and associations with immune processes using GSCALite [27].

To understand the potential change of the Notch in cancer, the mRNA-based Notch pathway scores were calculated. Specifically, expression z-scores of genes were firstly estimated, then the average values for 22 core genes were calculated in each individual. To validate the score of the Notch, the absolute values of spearman rank correlation coefficient of all the other genes (20,310) and Notch were calculated. The correlation coefficients of 22 randomly selected other genes were also estimated (repeated 10,000 times), and the median values were simultaneously estimated to present the detailed distribution.

Then, in order to understand expression patterns of the Notch genes, an unsupervised clustering method was used to perform a pan-cancer analysis. To obtain a robust clustering result, 50 relevant genes that could mediate or regulate Notch pathway were selected to estimate z-scores, and then they were performed unsupervised consensus clustering analysis using ConsensusClusterPlus package [28] to identify expression patterns of 50 and 22 core genes using Pearson distance and Ward's method, respectively. According to 80 % of sampling rates ($n = 1000$), clusters 1–10 were obtained, and $k = 6$ was finally selected based on delta area plot. To assess the prognostic values of the clusters, the long-rank test and Kaplan-Meier survival curve were used to compare the overall survival time between the different groups. Moreover, in order to compare the clustering pattern of the Notch pathway and other 9 major biological pathways, the gene set was further summarized. The distribution associated with female reproductive cancers was quantified via the ratio of the size of the second largest cluster and the number of samples in the entire cohort, and the Wilcoxon rank-sum test was used to assess whether there was a statistically significant difference between cancers of female and non-female reproductive systems.

2.5. Function enrichment analysis

In order to understand the potential functional implication for involved genes, functional enrichment analysis was analyzed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 [29]. Simultaneously, involved genes were queried for associations with hallmarks of cancer [30] (https://software.broadinstitute.org/gsea/msigdb/), genes in Cancer Gene Census (CGC) [31] (http://cancer.sanger.ac.uk/census), core essential genes according to the common data of Hart et al. [32], Blomen et al. [33] and Wang et al. [34], oncogenes and tumor suppressor genes [35], and actionable genes [36], which would contribute to further understanding the potential roles of genes in tumorigenesis. Simultaneously, the core Notch genes were also queried for

the potential roles in other KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. In order to avoid random results, all of the relevant results were further performed using randomly selected other genes (n = 1000).

2.6. Interactions of ncRNAs and Notch genes

As a class of important negative regulators, miRNA/isomiR, lncRNA and circRNA, were analyzed to understand the potential cross-talks of ncRNAs with the Notch pathway. The interactions of ncRNA and mRNA were firstly retrieved from starbase [37], and simultaneously involved in experimentally validated interactions in miRTarBasev6.0 [38,39]. Interactions of ncRNA:mRNA with significant negative correlations at least in 3 cancer types were further screened as potential Notch-related ncRNAs. Specifically, based on collected miRNA:mRNA, and relevant isomiR landscapes were analyzed to understand the coding-non-coding RNA regulatory network. Relevant lncRNAs and circRNAs were then obtained according to screened miRNAs, and competing endogenous RNA (ceRNA) networks were constructed to understand the potential interactions of ncRNAs and mRNAs.

2.7. Estimation of prognostic ability and survival analysis

To understand the prognostic ability and potential prognostic value in cancer, the clinical effect of the Notch pathway activity was estimated according to expression levels of NOTCH gene family and RBPJ, Notch scores based on 22 core genes and 40 target genes. Survival analysis was performed to verify whether involved genes have prognostic values in cancer. The log-rank test was used to estimate the potential differences between the groups of high and low expression, and univariate Cox proportional hazard model was used to estimate correlation of the total survival time. A $p < 0.05$ indicated significant difference. Moreover, we also obtained tumor purity data from Genomic Data Commons (GDC) [40] and repeated the above analysis by taking tumor purity as a Cox model covariate.

Further, the 22 core Notch genes were estimated the potential prognostic values using integrative data of mRNA expression, CNV, methylation and miRNA expression data. For obtained DNA methylation data (450 k, 27 k), we only selected the probe with the most significant negative correlation with its mRNA expression in each cancer type. Genes with no significant correlated probes were removed from analysis. Firstly, the missing values were filled with the median value, but sample was removed if it contained missing eigenvalues more than 20 % in cancer. All the features were normalized as mean = 0 and variance = 1. Secondly, the high dimensional data were trained using denoising autoencoder (DAE), and representative features were obtained via constantly updating the weight coefficients. Finally, these obtained simplified features were input univariate COX-PH model to calculate significant relevant features with survival times according to long-rank p values ($p < 0.05$), which were used to estimate cancer risk. The concordance index (C-index) was used to evaluate prediction ability of the model using the Harrell's C statistics [41,42]:

$$C - index = \frac{1}{num} \sum_{i \in \{1 \ldots n | \delta_i = 1\}} \sum_{t_i > t_j} I[r_i > r_j] \quad (1)$$

where r_i and r_j were the predicted risks of patients i and j, t_i and t_j were the actual survival times for corresponding patients, δ indicated that the sample was uncensored or not, *num* showed the number of comparable patient pairs and I[.] was the indicator function. Furthermore, to understand the potential contributions of single molecular level, the prognostic models were also constructed for the single molecular data and different combinations.

2.8. Experimental validation using Western blotting

In order to validate the detailed protein expression patterns and the potential regulation relationship, further experimental validation of NOV and NOTCH1 were performed using Western blotting in cell lines (human MCF-10A, breast cancer lines of MCF-7 and MDA-MB231) that were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS; Shanghai, China). NOV plasmid was constructed and transfected into 3 cell lines (overexpression vector NOV, 1000 ng), and empty vector-transfected cells were simultaneously used as controls. The sequences of NOV siRNA and negative control siRNA were synthesized in Biotend, China, and the sequences of siRNAs were: si-NOV sense, 5′ TAACTGCCCAGCTCCAAGAAdTdT 3′, and antisense, 5′ GAACCCCATACCACAGCTCTdTdT 3′.

Then, the cell lines were transfected with siRNAs (100 nm) for 48 h, and the cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology, Beijing, China) supplemented using protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) at 4 °C for 15 min. The protein concentration was then estimated by bicinchoninic acid (BCA) assay kit (Beyotime), and the protein/lane was detected using SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies against NOTCH1, cleaved-NOTCH1 and GAPDH at 4 °C overnight after blocking with 5 % skim milk for 1–2 h. Then, the blots were incubated using secondary antibodies conjugated to horseradish peroxidase for 1 h after washing with Trisbuffered saline and Tween-20 (TBST) buffer three times (5 min per time). Finally, the protein bands were observed using enhanced chemiluminescence and visualized with a Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). Primary antibodies against NOTCH1 and Cleaved-NOTCH1 (Val1744) were purchased from Cell Signaling Technology (Beverly, MA), and secondary antibodies (goat anti-rabbit) were purchased from Proteintech (Wuhan, China).

2.9. Statistical analysis and network visualization

An unpaired *t* test and the Wilcoxon rank-sum test were used to perform hypothesis testing for the unpaired numeric samples, a trend test was used to understand expression patterns among multiple groups, and other relevant statistical analysis were mentioned in relevant methods. Expression relationships between genes, especially between ncRNAs and mRNAs, were estimated using Pearson or Spearman correlation coefficients to understand their expression patterns. Interaction networks between diverse RNAs were mainly constructed and presented using Cytoscape 3.6.0 [43] to show their multiple relationships. All of these statistical analyses were analyzed using R programming language (version 4.0.5), and venn distributions were performed using a publicly available tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

3. Results

3.1. Somatic alteration landscape of the Notch pathway

A total of 22 Notch core genes were obtained (Fig. 1A), and these genes may contribute to tumorigenesis by regulating biological processes of cell proliferation, differentiation and apoptosis. The overall aberration level was low (Fig. 1 and Table S4), indicating that these genes were relative stable in DNA variation. Of these, NOV and NOTCH2 had higher amplification levels (Fig. 1B and S1). Potential oncogenic driver genes, such as NOTCH2 (1p12)

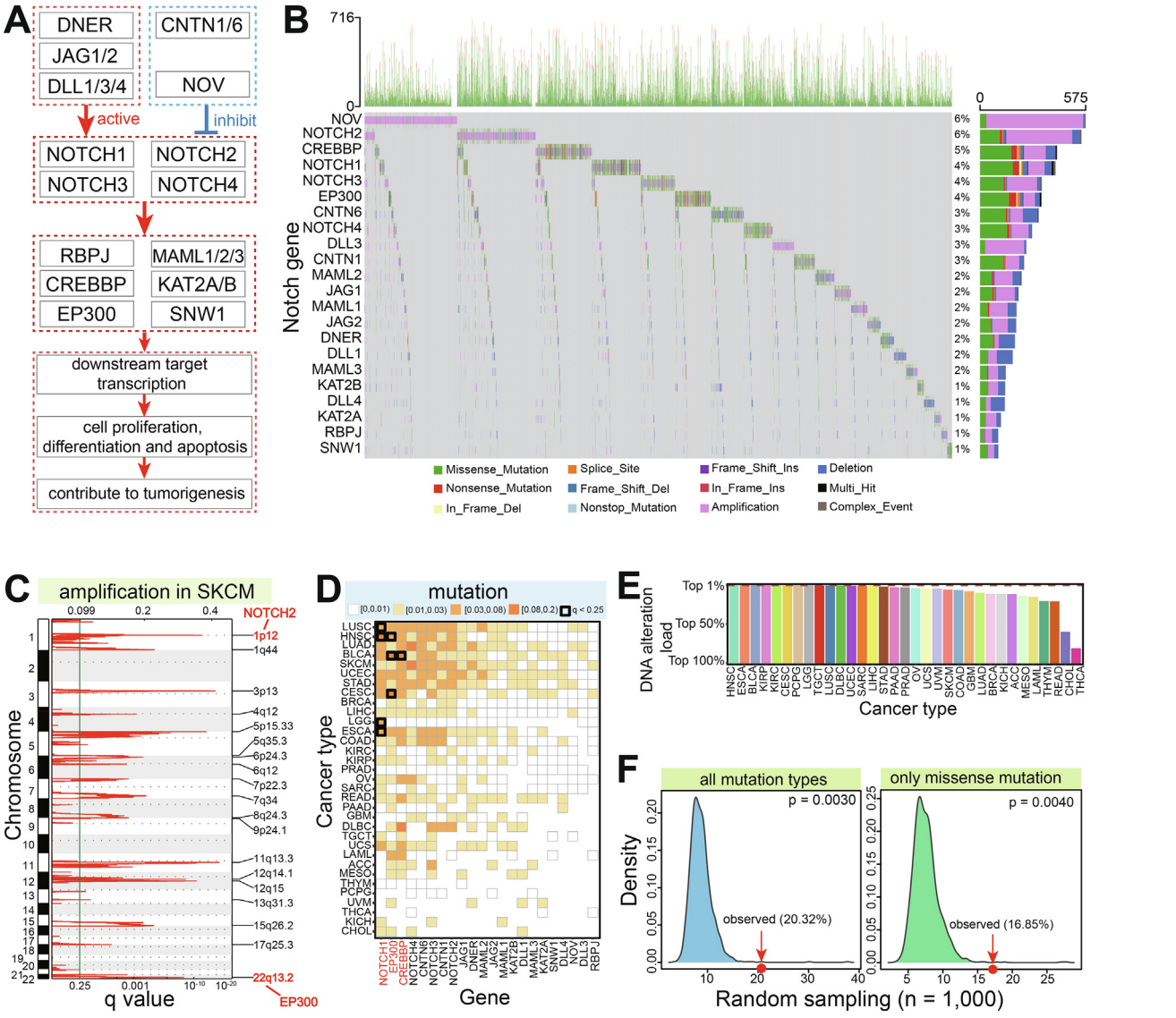

Fig. 1. Somatic DNA alteration landscape of the Notch pathway.

and EP300 (22q13.2), were frequently amplified genomic regions in some samples of SKCM (Fig. 1C). Regarding the mutational profile, higher mutation frequencies could be found in several cancer types (Fig. 1D). The Notch genes indicated a trend of higher mutation frequency ($p = 0.0030$ for all mutation types, $p = 0.0040$ for missense mutation). Several core genes, NOTCH1, EP300 and CREBBP (Fig. 2A), showed significant higher mutation frequencies in some cancers, and they were prone to involve in mutation events than other randomly selected 22 gene sets (Fig. S2). Indeed, mutations in NOTCH1, EP300 and CREBBP may contribute to some diseases [44–47], implicating the potential contributions in some cancers.

Moreover, 5 core genes (CREBBP, EP300, MAML2, NOTCH1 and NOTCH2) were associated with cancer (CGC), and some genes were characterized as core essential genes or tumor suppressor genes (Fig. 2B). These results were not random comparing with other randomly selected 22 genes (Fig. 2C and Fig. S3A). Further, 10 genes (45.45 %) were identified with a role in hallmarks of cancer, especially in insensitivity to antigrowth signals and self-sufficiency in growth signals (Fig. 2D and 2E). RBPJ and NOTCH1 were found in 3 hallmarks of cancer, indicating their roles in tumorigenesis (Fig. 2F, $p = 0.0140$). These core genes were also found with potential roles in other KEGG pathways, especially in dorso ventral axis formation (Fig. 2G), and EP300 and CREBBP were detected in multiple KEGG pathways. These findings supported that these Notch genes were prone to have an important role in tumorigenesis, which provided references for the potential application in anticancer drug design and further cancer treatment.

3.2. Pan-cancer expression patterns of the Notch pathway

To understand the detailed activity of the Notch pathway, activity scores were estimated. We found that the Notch scores showed diverse distributions across cancers ($p < 2.2e{-}16$), and score in LIHC was the lowest, while it was the highest in LGG (Fig. 3A). Dynamic expression patterns could be found in different tissues (Fig. 3B, S3B and S3C). These expression patterns, especially for cancer-specific expression, may imply their multiple roles in diverse cancer types. In BRCA, Notch genes showed diverse expression levels (Fig. 3C), and DLL3 was not dominantly enriched compared to other genes. Of these, NOV, also termed CCN3, its expression in human prostate cancer is directly suppressed by the androgen receptor [48], and it had an inhibition role for NOTCH (Fig. 1A). Our previous study showed that NOV can promote EMT

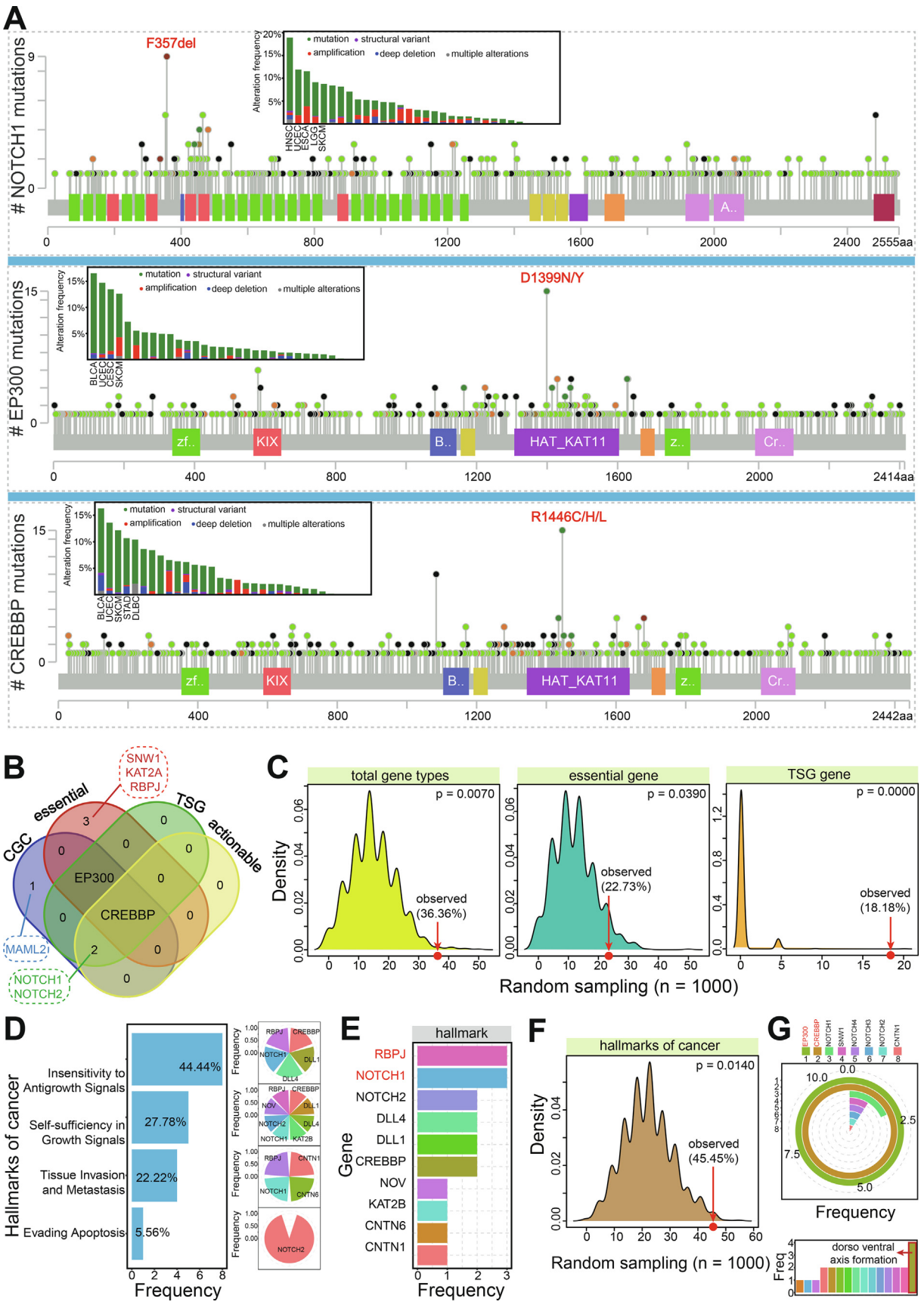

Fig. 2. Examples of dominant mutation sites and functional enrichment analysis.

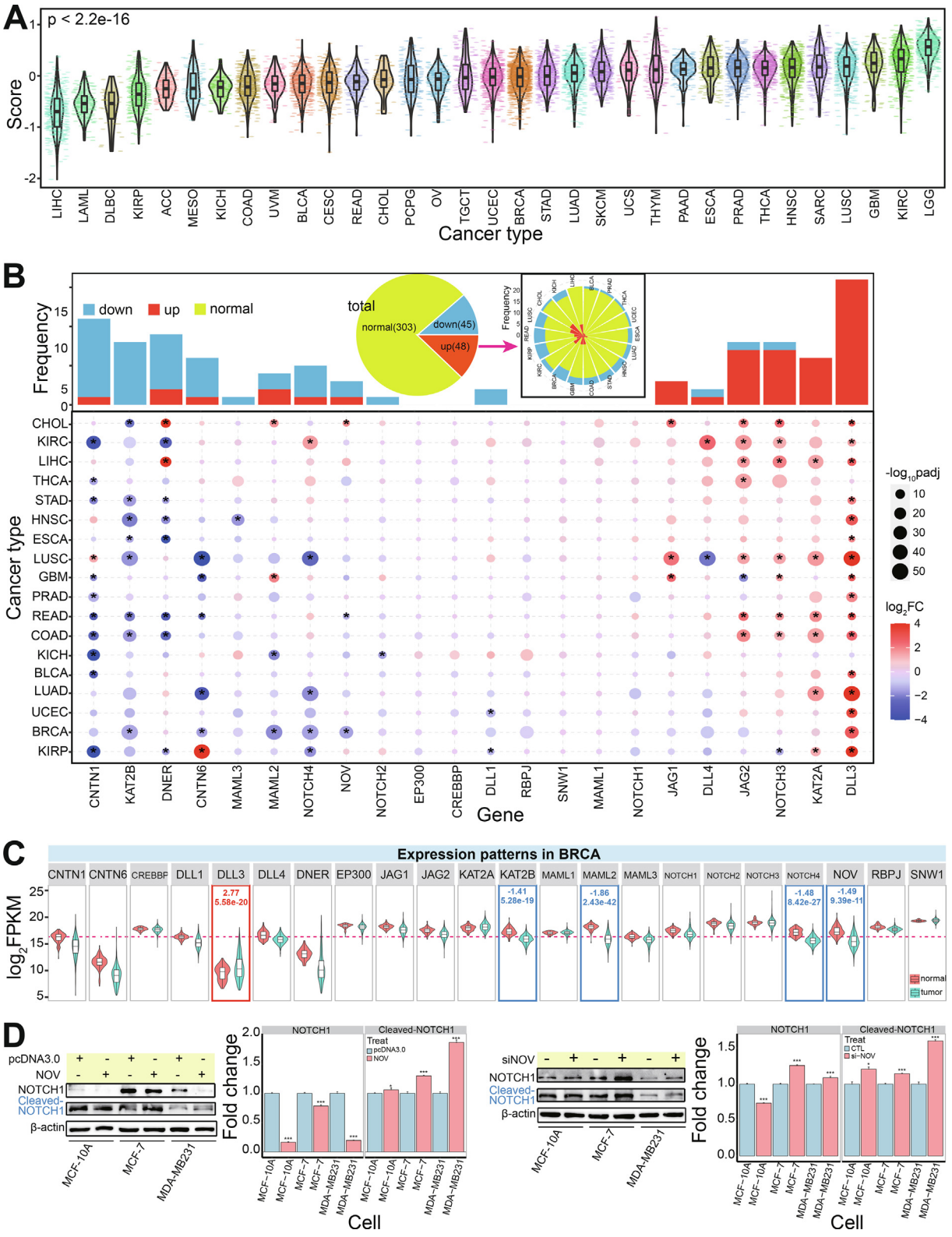

Fig. 3. Gene expression pattern of the Notch core genes.

and increase migration and invasion of cholangiocarcinoma cells [49]. NOV was significantly down-regulated via transcriptome analysis in BRCA and READ, while it was specifically up-regulated in CHOL. Then, we chose NOV and NOTCH1 to perform experimental validation in human breast epithelial cells MCF-10A and breast cancer cells (MCF-7 and MDA-MB231). The western blotting results showed that overexpression of NOV significantly inhibited the expression of NOTCH1 protein ($p < 0.05$), but significantly induced the expression of cleaved NOTCH1 protein ($p < 0.05$) (Fig. 3D). After si-NOV, the expression of NOTCH1 protein was inhibited in MCF-10A cells ($p < 0.05$), but it was significantly promoted in breast cancer cells. Similar to si-NOV, the expression of

cleaved NOTCH1 protein was significantly induced in all cells ($p < 0.05$). These results suggested that NOV played a negative regulation role in downstream target genes by negatively regulating NOTCH1.

In addition, in order to obtain a global view of expression pattern, expression analysis was performed using 50 Notch genes containing the 22 core genes and 28 related genes. A total of 6 robust clusters were constructed (Figs. S3D, S3E and 4A), and most samples in specific cancer always fell into one cluster. Strikingly, samples in several cancers, mainly including 3 female cancers (BRCA, CESC and UCS) and LUSC, were split into 2 or 3 clusters, especially in clusters C1, C2, C3 and C4. The consensus clustering analysis was also performed in other 9 major pathways, and we found that it was significantly higher in the female samples than that in other non-female samples ($p = 0.0170$, Fig. 4B). Moreover, clusters of the Notch pathway may be also associated with cancer prognosis, implying their potential roles in prognosis and further cancer treatment. The total survival probability showed significant differences among diverse clusters ($p < 0.0001$ for 6 clusters, Fig. 4C), and cluster 6 (a PRAD-specific cluster) indicated survival advantage than other clusters. In female cancers, the total survival probability also showed significant difference among different clusters ($p < 0.0001$ for 4 clusters), implying that these subtypes had potential prognostic values.

3.3. Related small RNA regulation in the Notch pathway

Based on the widespread regulation roles, miRNAs were used to track their potential regulation associated with Notch genes. Except for CNTN6 and NOTCH4, other genes were detected multiple related miRNAs that may negatively regulate Notch. Co-expression analysis screened 1,173 miRNA:mRNA pairs with significant negative correlations in cancers (Fig. 4D), and LGG was found with the most miRNA:mRNA interactions, followed by TGCT and THYM (Fig. S4). NOTCH2 was detected with the most interactions, and miRNA-mRNA interaction network showed that NOTCH2 had a hub role in coding-non-coding RNA regulatory network (Fig. 4F). Although complex miRNA:mRNA interactions exited in multiple cancers based on their biological and expression correlations, it is also important to discuss the interactions between mRNAs and isomiRs that have been validated with important roles in the coding-non-coding RNA regulatory network [50–52]. We selected three cancer types (LUSC, KIRP and CHOL) as examples to understand the detailed interactions of isomiRs and Notch genes because they have more dysregulated genes than other cancers. A total of 289 gene pairs (containing 11 Notch genes and 224 isomiRs from 69 miRNA loci) were detected, and all of involved RNAs were detected abundant enrichment levels (Fig. S4D and S4E). IsomiRs in several miRNA loci were negatively associated with target Notch genes (Figs. 5 and S5), and these isomiRs showed diverse enrichment levels as well as their diverged sequences. The multiple interactions from the isomiR levels may contribute to the plasticity of gene regulation. Homologous miRNA loci may show inconsistent dysregulation patterns (Fig. 5D), although they had sequence similarities and potential functional relationships. Most isomiRs had consistent seed sequences (nucleotides 2–8), but several isomiRs were involved in novel functional sequences. Specifically, an isomiRs in miR-30a-5p locus was involved in seed shifting event, but it still could regulate target mRNA of JAG2 (Fig. S5E). Consistent isomiR expression profiles in miR-30a-5p were obtained in different cancers (Figs. 5E and S5G), indicating that these expression patterns were always stable in different tissues. Indeed, isomiR with shifted seed sequence may be involved in gaining new targets or losing targets that further perturbed the coding-non-coding interaction network [50,53]. These diverse and flexible isomiRs complicated the regulatory network, which would contribute to further exploring the interesting small RNA world, especially for the potential interactions among diverse RNAs.

3.4. ncRNA-mRNA interactions via competing endogenous RNA network

Although miRNAs/isomiRs had been found with the negative regulation in the Notch pathway, the coding-non-coding RNA regulatory network would be more complex because existence of interaction with lncRNAs, especially via ceRNA network. Herein, to understand the potential interaction among different RNAs, lncRNAs were screened based on obtained 36 miRNAs associated with the Notch. We finally obtained 59 related lncRNAs that could be as miRNA sponges, and a total of 17 pairs of miRNA:lncRNA were collected based on their negative expression correlations ($R < -0.20$ and $FDR < 0.05$, Table S5 and Fig. 6A), including 12 miRNAs and 12 lncRNAs. These lncRNAs were abundantly expressed, and some of them showed abnormal expression in some cancers (Figs. 6A and S6A). Of these, PVT1 was significantly up-regulated in 12 cancers and SNHG4 was found in 7 cancers, while both AGAP11 and MEG3 showed diverged expression patterns across cancers.

Constructed RNA network showed that lncRNAs could as miRNA sponges to disturb Notch gene expression (Fig. 6B and C), and the coding-non-coding RNA interactions were more complex than we thought, particularly the potential competition and/or collaboration among multiple isomiRs. The ceRNA network could show the complex cross-talks among diverse RNAs, implying the potential balance between miRNA/isomiR:lncRNA and miRNA/isomiR:mRNA interactions. Furthermore, the similar interaction was also found among miRNA, mRNA and circRNA (Fig. 6D), indicating that the interactions among RNAs might contribute to precisely regulating target Notch genes. The cross-talks among RNAs may perturb gene expression and subsequent biological process, which would largely contribute to the Notch activity and roles in tumorigenesis.

3.5. Methylation and immune analysis of the Notch genes

A total 12 Notch genes were found different degrees of methylation (Fig. S6). Of these, NOTCH4, CREBBP and CNTN6 were found with higher methylation levels in many cancers, and DLBC was detected with higher methylation level. To understand whether these Notch genes had potential role in immune infiltration, the associations between immune cells' infiltrates and single-nucleotide variant (SNV) mutants were evaluated using the Immune Cell Abundance Identifier (ImmuCellAI) algorithm. We found that significant correlations could be found in some cancers (Figs. 6E, S7A and S7B), and the associations implied that these Notch genes had a role in immune infiltration. Notch signaling is an important modulator of T cell-mediated immune responses [54,55], and it contributes to the generation and maintenance of hematopoietic stem cells, lymphocyte development, and several immune responses [56]. Further, gene set variance analysis (GSVA) scores of the Notch genes indicated the potential differences between tumor and normal samples in some cancers (Fig. 6F). The interactions between the Notch genes and pathway presented the complex correlations (Fig. S7C and S7D), implying the potential roles in multiple biological pathways that could contribute to the pathophysiological process.

3.6. Prognostic ability of the Notch pathway

In order to estimate the clinical impact of abnormally expressed Notch genes, especially for the potential correlations between pathway activity and the patients' overall survival times, we firstly

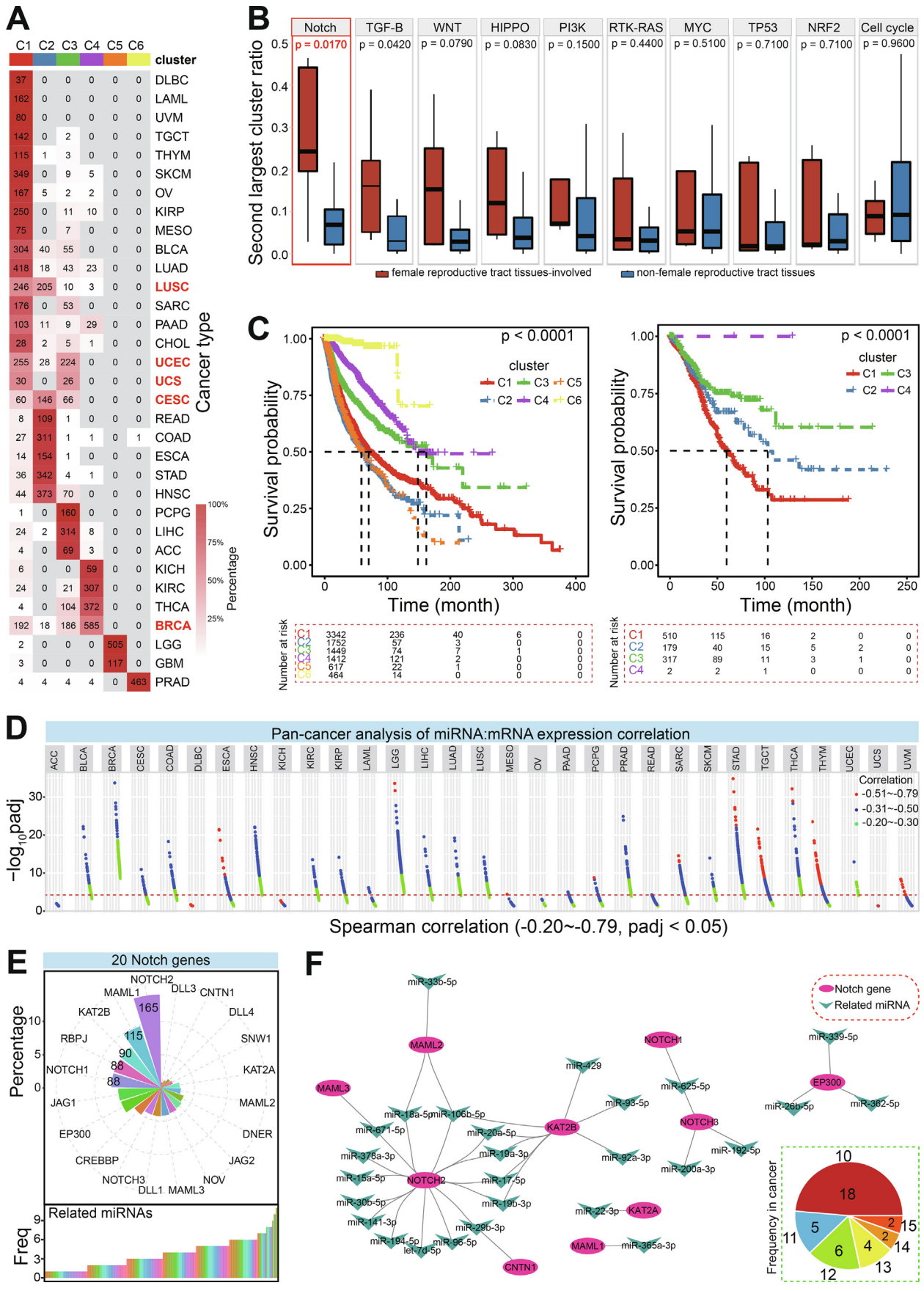

Fig. 4. Clustering analysis and screening related miRNAs.

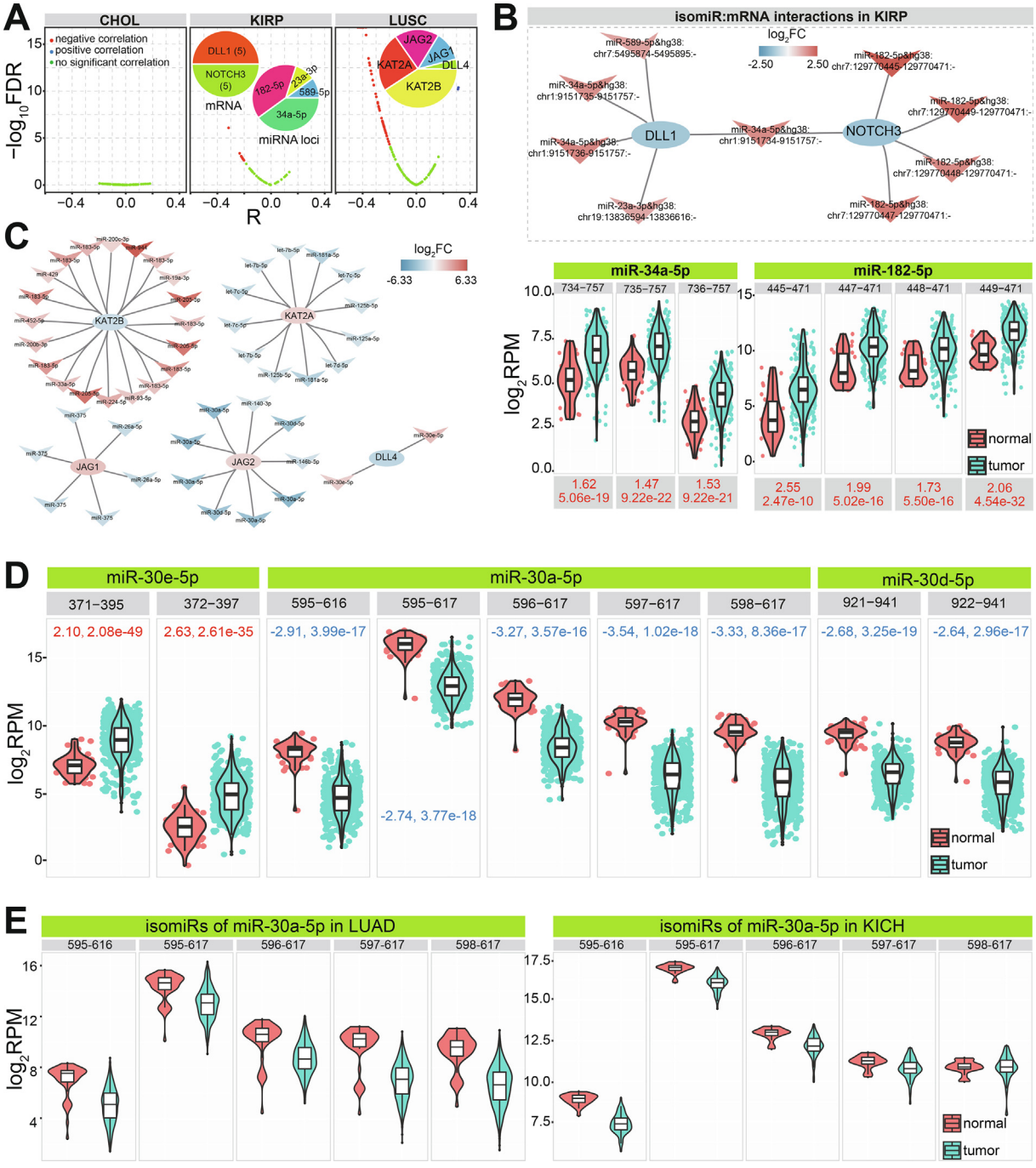

Fig. 5. Interactions of multiple isomiRs and target Notch genes.

screened 40 downstream target genes to perform survival analysis. Target genes might be prognostic marker in some cancers (Fig. 7A and B), and many targets showed abnormal expression patterns as well as the Notch genes (Fig. S7E). Indeed, the Notch genes could play roles as oncogenes or tumor suppressor genes, which mainly depended on the specific cancer type. According to scores of Notch gene expression, significant correlations could be detected between target expression and survival time (Fig. 7B), implying that these target mRNAs could be a robust index to track the activity of the Notch pathway. Prognostic power was also detected in some cancers based on the Notch genes via integrative analysis of mRNA expression, CNV, methylation and related miRNA regulators (Figs. 7C and S8A). According to the top 20 representative characteristics using denoising autoencoder (DAE), we found significant difference of survival time between high-risk and low-risk groups in 15 cancer types, and only 2 cancers (LAML and OV) were not detected potential prognostic values. The significant association of the Notch genes and cancer prognosis also indicated that these Notch genes had an important role in the occurrence and development of cancers, implying their roles as potential prognostic markers. Compared with the integrative analysis of multi-omics data, the single molecular level and their multiple combinations also showed potential prognostic values in some cancers (Table 1, Table 2 and Fig. S8). Of these, mRNA expression level had a larger proportion containing significant survival advantages, followed by methylation and CVN levels. The significant survival advantages in

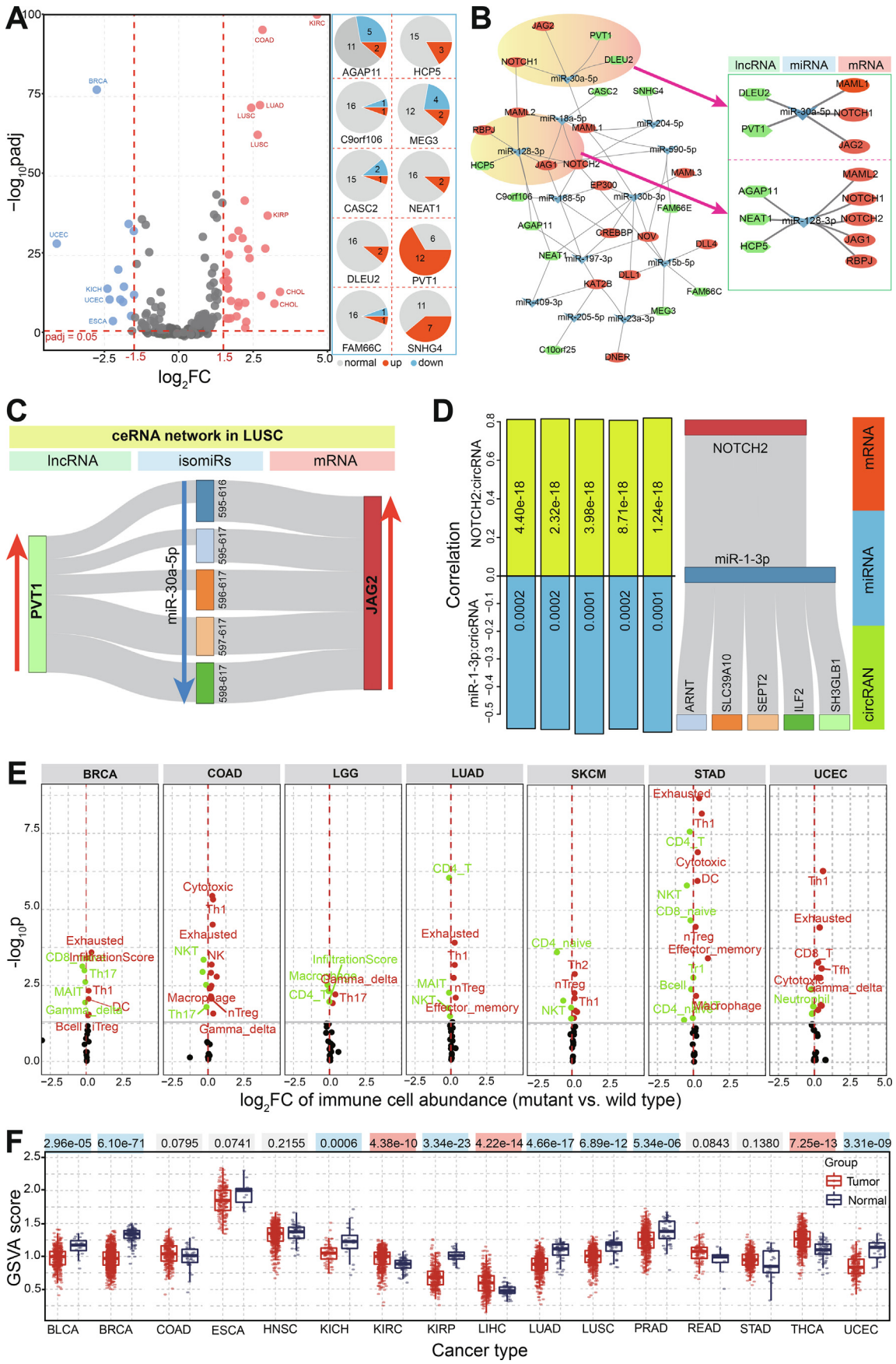

Fig. 6. CeRNA network of diverse RNAs and association of Notch genes with immune infiltration.

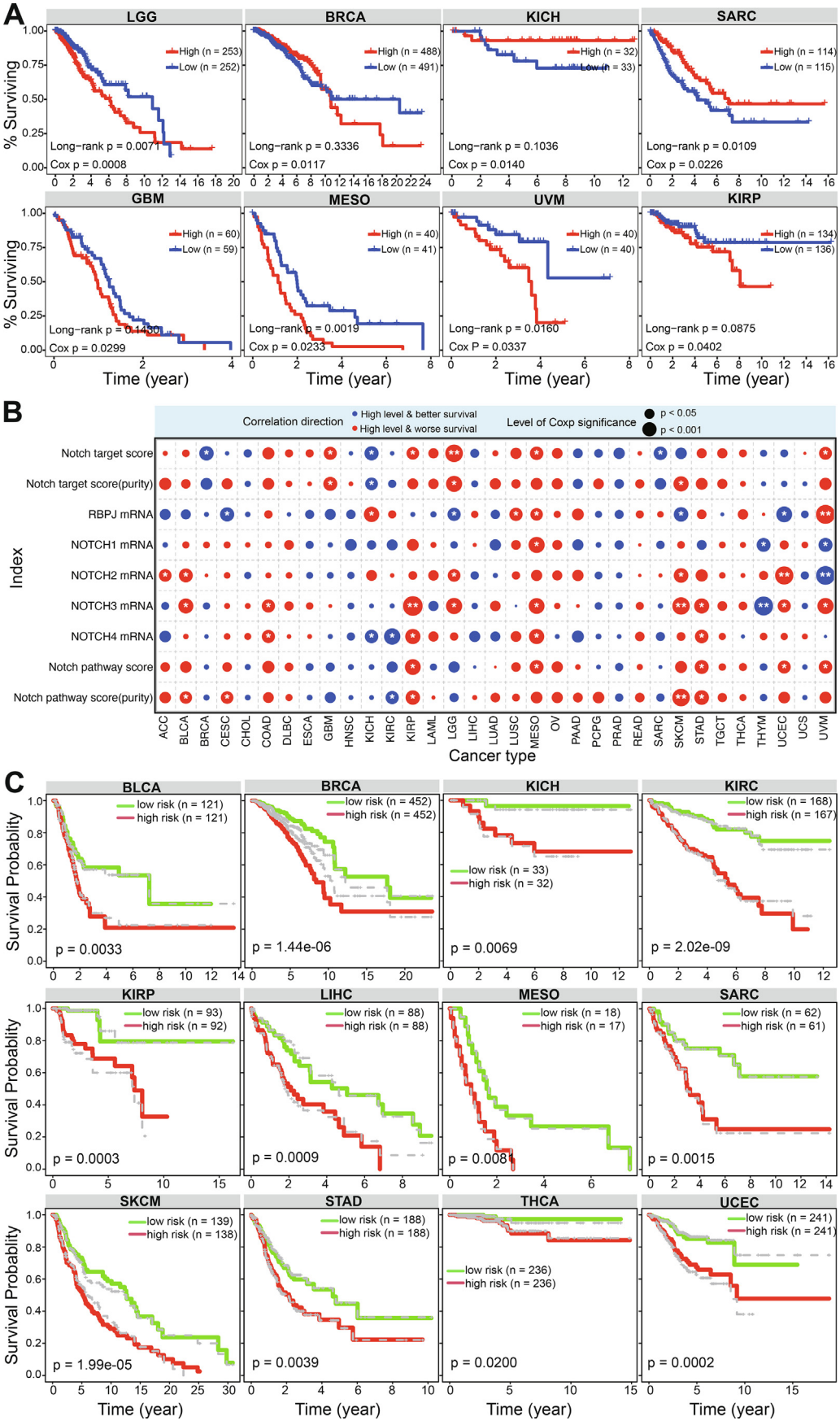

Fig. 7. Prognostic power of the Notch pathway.

Table 1
The C-index of the cross-validations and tests in 17 cancers.

Cancer	Validation	Test	Test (95 % CI)
BLCA	0.556	0.552	0.529–0.615
BRCA	0.644	0.633	0.570–0.695
HNSC	0.585	0.581	0.539–0.623
KICH	0.738	0.738	0.595–0.882
KIRC	0.753	0.715	0.658–0.773
KIRP	0.824	0.863	0.783–0.944
LAML	0.590	0.594	0.527–0.660
LGG	0.771	0.750	0.697–0.804
LIHC	0.641	0.603	0.531–0.676
LUAD	0.612	0.598	0.547–0.650
MESO	0.803	0.717	0.623–0.811
OV	0.609	0.592	0.527–0.657
SARC	0.754	0.643	0.550–0.736
SKCM	0.589	0.603	0.554–0.652
STAD	0.603	0.591	0.540–0.642
THCA	0.760	0.780	0.640–0.920
UCEC	0.705	0.705	0.644–0.766

Table 2
The contribution of data type for cancer outcome evaluation from the final model.

Single molecular level	C-index	Integrative data	C-index
mRNA	0.650	**multiple data**	**0.679**
miRNA	0.635	exclude mRNA	0.648
methylation	0.677	exclude miRNA	0.670
CNV	0.636	exclude methylation	0.671
		exclude CNV	0.662

Omics data show integrative analysis of mRNA, miRNA, methylation and CNV levels. Exclude mRNA shows integrative analysis of multiple molecular levels without mRNA.

single molecular level showed close correlations of the Notch genes and pathophysiological processes, which further verified their crucial roles in tumorigenesis.

Furthermore, the core Notch genes might be potential drug targets (Fig. S9A-S9B), indicating their roles in precision medicine. The Notch genes were also associated with multiple pathways in cancers, mainly including negative correlations of apoptosis and cell cycle, and positive correlations of RAS-MAPK and RTK (Fig. S9C), implying the complex interactions and multiple roles *in vivo* that may largely contribute to tumorigenesis.

4. Discussion

The Notch signaling pathway, a key evolutionary conserved pathway, required by multiple biological processes, is critical for the normal biological processes, mainly including cellular proliferation, differentiation, development, homeostasis and survival [1,57]. Deregulation of the Notch pathway may cause abnormal biological processes that may be associated with progression of diverse human diseases, and the Notch greatly contributes to understanding pathophysiological processes of cancer [3,58,59]. For example, DLL3 regulates the migration and invasion of small cell lung cancer by modulating Snail [60], and it may be an emerging target in small cell lung cancer [61]; upregulated CNTN1 is associated with lymph node metastasis and poor prognosis of colorectal cancer, and it may be a potential biomarker [62]; NOTCH4 can regulate colorectal cancer proliferation, invasiveness, and determines clinical outcome of patients [63], and its inhibition can reduce BCSC activity [64]. Overexpression of NOTCH3 is oncogenic associated with diverse cancers [65–67]. Many Notch genes have important roles in tumorigenesis via abnormal expression patterns, which would provide potential targets for further cancer diagnosis, prognosis and treatment. Based on the broad involvement in diverse cancers, Notch would be potential targets in cancer treatment. It is crucial to reveal the detailed mechanism within the tumor microenvironment that can be used to predict whether Notch-targeting therapies are effective and to further identify novel druggable targets [7].

Herein, to understand the molecular characterization of the Notch pathway and further discuss its potential application in precision medicine, we perform a comprehensive pan-cancer analysis via integrating multiple molecular levels based on the screened 22 core genes. These core genes are relative stable in DNA variation despite they are prone to involve higher mutation frequency than other randomly selected genes. Some are identified as cancer-associated genes, which validates the critical roles of the Notch genes in tumorigenesis. The Notch genes are also involved in hallmarks of cancer and other biological pathways [68,69], and the cross-talks among diverse pathways indicate the multiple roles of the Notch that are quite crucial to ensure the normal biological process. Deregulation expression of the Notch genes can further disturb activity of the pathway that is pivotal in cancer [70,71], and the abnormal expression largely contributes to tumorigenesis. The Notch is also associated with immune infiltration, indicating a role in immunological processes [55,72]. These significant deregulated Notch genes may be potential targets for cancer diagnosis, prognosis and further cancer treatment, implicating its potential value in precision medicine.

The Notch pathway can be regulated by ncRNAs (mainly including miRNAs/isomiRs, lncRNAs and circRNAs) that have been widely accepted as a class of important regulatory molecules in gene expression. A series of miRNAs are identified as negative regulators of the Notch, and the coding-non-coding RNA network is more complex if multiple isomiRs are involved. IsomiRs have expression and length heterogeneities, and some 5′ isomiRs with novel functional regions may perturb the Notch-related regulatory network despite it is unclear whether these short sequences have potential cooperative or competitive interactions [53,73]. The isoform-mediated regulatory network rewiring may directly or indirectly disturb the Notch expression that further influence the pathway activity and related biological processes. In addition, the coding-non-coding RNA network can also be perturbed by lncRNAs and circRNAs via acting as miRNA sponges, implicating that isomiR-mediated ceRNA network may be critical in the Notch pathway. Indeed, ncRNAs have been widely considered as crucial RNAs in cancer biology [74–76], and ncRNA-based therapeutics have provided a new insight for cancer treatment that can facilitate better design of personalized therapeutics. More studies should focus on the detailed interaction mechanism among diverse RNAs and caused multiple cross-talks among diverse biological pathways, which may be quite important to explore the mechanisms of tumorigenesis and metastasis.

As a key pathway, the Notch signaling has been believed with a role in cancer prognosis due to function of oncogenic or oncosuppressive [77,78], and it can be a candidate prognostic marker and potential drug target for an effective therapeutic intervention. Herein, significant survival advantage can be found for the screened targets of Notch that may be a potential marker in cancer prognosis, implicating the potential regulatory roles of the Notch via activation or inhibition processes. Similarly, the core Notch genes are also detected strong prognostic power via an integrative analysis of multi-omics data. Integrated characteristics from DNA to RNA levels, especially involving in ncRNA regulators of the Notch, provide more potential cross-talks of multiple molecule levels that may influence subsequent biological processes. Moreover, the significant survival advantage is also validated from single molecular level, and the core Notch gene set demonstrates a robust prognostic signature for cancers. The prognostic value of the Notch pathway in cancer further broadens the potential application in personalized medicine.

Taken together, based on an integrative analysis of multi-omics data, the core Notch genes show specific molecular features. Diverse ncRNAs largely contribute to dynamic expression of the Notch, and related RNAs may also be potential targets for further cancer treatment. The coding-non-coding RNA regulatory network is more complex than we originally thought, especially involving in multiple cross-talks of various RNAs and pathways. The core Notch genes indicate a robust prognostic indicator as well as their targets. These findings help us to reveal tumor-suppressive/oncogenic role of the Notch signaling, gain mechanistic insights into cross-talks among diverse RNAs and multiple pathways, which will contribute to exploring therapeutic viewpoint of the key Notch pathway.

Abbreviation lists in Figures

ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LIHC, liver hepatocellular carcinoma; LGG, brain Lower grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, Mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; TSG, tumor suppressor gene; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. Supporting data can also be found in the Supplement files.

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CRediT authorship contribution statement

Li Guo: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition, Resources, Supervision. **Sunjing Li:** Methodology, Formal analysis, Investigation, Writing – review & editing, Resources. **Xiaoqiang Yan:** Formal analysis, Investigation. **Lulu Shen:** Formal analysis, Investigation. **Daoliang Xia:** Formal analysis, Investigation. **Yiqi Xiong:** Formal analysis, Investigation. **Yuyang Dou:** Formal analysis, Investigation. **Lan Mi:** Formal analysis, Investigation. **Yujie Ren:** Formal analysis, Investigation. **Yangyang Xiang:** Formal analysis, Investigation. **Dekang Ren:** Formal analysis, Investigation. **Jun Wang:** Methodology. **Tingming Liang:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Resources.

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.

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Appendix A. Supplementary data

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