Original Article

Identification of cystic fibrosis transmembrane conductance regulator as a prognostic marker for juvenile myelomonocytic leukemia via the whole-genome bisulfite sequencing of monozygotic twins and data mining

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Background: Linked deoxyribonucleic acid (DNA) hypermethylation investigations of promoter methylation levels of candidate genes may help to increase the progressiveness and mortality rates of juvenile myelomonocytic leukemia (JMML), which is a unique myelodysplastic/myeloproliferative neoplasm caused by excessive monocyte and granulocyte proliferation in infancy/early childhood. However, the roles of hypermethylation in this malignant disease are uncertain.

Methods: Bone marrow samples from a JMML patient and peripheral blood samples from a healthy monozygotic twin and an unrelated healthy donor were collected with the informed consent of the participant's parents. Whole-genome bisulfite sequencing (WGBS) was then performed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to analyze specific differentially methylated region (DMG) related genes. The target genes were screened with Cytoscape and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), which are gene/protein interaction databases. A data mining platform was used to examine the expression level data of the healthy control and JMML patient tissues in Gene Expression Omnibus data sets, and a survival analysis was performed for all the JMML patients.

Results: The STRING analysis revealed that the red node [i.e., the cystic fibrosis transmembrane conductance regulator (*CFTR*)] was the gene of interest. The gene-expression microarray data set analysis suggested that the *CFTR* expression levels did not differ significantly between the JMML patients and healthy controls (P=0.81). A statistically significant difference was observed in the *CFTR* promoter methylation level but not in the *CFTR* gene body methylation level. The overall survival analysis demonstrated that a high level of *CFTR* expression was associated with a worse survival rate in patients with JMML (P=0.039).

Conclusions: *CFTR* promoter hypermethylation may be a novel biomarker for the diagnosis, monitoring of disease progression, and prognosis of JMML.

Keywords: Bioinformatics analysis; cystic fibrosis transmembrane conductance regulator (*CFTR*); monozygotic twins

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a unique myelodysplastic/myeloproliferative neoplasm caused by the excessive proliferation of monocytes and granulocytes in infancy/early childhood (1). Its clinical manifestations include hepatosplenomegaly, lymphadenopathy, skin rash, leukocytosis, monocytosis, thrombocytopenia, anemia, and respiratory failure. With a global annual incidence of 1.2/1,000,000, JMML accounts for 2–3% of all hematological malignancies. The median age of JMML patients at the time of diagnosis is 2 years, and the male-to-female ratio is 2–3 to 1 (2). Allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the only cure for JMML. However, the low 5-year overall survival rate of 50–60% and the high post-HSCT recurrence rate remain major challenges for allo-HSCT (3). Recurrence is still the main reason for treatment failure in JMML patients.

Approximately 90% of JMML patients harbor germline or somatic mutations in Protein Tyrosine Phosphatase Non-Receptor Type 11 (*PTPN11*), NRAS Proto-Oncogene, GTPase (*NRAS*), KRAS Proto-Oncogene, GTPase (*KRAS*), Cbl Proto-Oncogene (*CBL*), or Neurofibromin 1 (*NF1*) (4). The mutation of these genes can activate the RAS signaling pathway (5). Epigenetics is the study of heritable alterations in gene expression that are not due to changes in the deoxyribonucleic acid (DNA) sequence. Epigenetic modifications alter DNA accessibility and/or the chromatin structure by which the expression levels of certain genes are regulated (6). DNA methylation (DNAm) involves the addition of a methyl group at position C5 in the DNA cytosine ring catalyzed by DNA methyltransferase enzymes (7). As an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine-, the DNAm patterns observed in cancer genomes can be classified as either gene promoter hypormethylated and hypermethylated. CpG islands (CGIs) located in promoter regions in normal human tissues are in a hypomethylated state. However, the promoter hypermethylation of tumor suppressor genes can silence those genes and cause cancer (8). Thus, DNAm is an important aspect to consider when studying human diseases. Whole-genome bisulfite sequencing (WGBS) can detect the precise boundaries between methylated and unmethylated regions at single-base resolution and is considered the gold-standard method for DNAm analysis (9).

A study showed that some genes are hypermethylated or have superhigh methylation in JMML patients. This study also reported 4 hypermethylated genes (i.e., *BMP4*, *CALCA*, *CDKN2B*, and *RARB*) (10). Previous studies have shown an association between activation of the DNA methylation machinery and specific JMML mutational profiles (11). Moreover, numerous studies have suggested that aberrant DNAm is linked to poor outcomes in JMML (10,12,13). However, clinical outcomes were significantly improved by treatment with the DNA-hypomethylating agent azacitidine prior to HSCT in patients with JMML (14). Thus, DNA hypermethylation is a novel independent prognostic factor for the poor prognosis of JMML (15). Identifying promising key genes associated with the progression and prognosis of JMML could lead to a marked improvement in the long-term survival of patients. Previous research has mainly focused on quantitative measurements of DNAm in large cohorts using the Infinium Human Methylation 450 Bead Chip (Illumina), quantitative high-resolution mass spectrometry (Sequenom MassARRAY), or bisulfite conversion and pyrosequencing techniques (13,16). To the best of the authors' knowledge, the relationship between gene methylation and the carcinogenesis of JMML has not been reported. Monozygotic (MZ) twins essentially share identical genomes and early-childhood family environments. An MZ twin study is thus an ideal approach for examining the effects of epigenetic modifications on various diseases. In this study, we conducted the WGBS of twins, a JMML patient and his healthy brother, and data mined from a Gene Expression Omnibus (GEO) data set to explore the causative genes and their potential effects on JMML. We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-381/rc).

Methods

General information about the patient

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committees of Nanfang Hospital, Southern Medical University (No. NFEC-2020-203). Before participating in this study, the patient's parents provided written informed consent. The patient was a 6-year-old boy diagnosed with JMML. On first admission, his white blood cell count was 42.79×10^9/L, his monocyte count was 13.48×10^9/L, his platelet count was 21×10^9/L, and his hemoglobin was 21 g/dL. The examination showed that the bone marrow of the patient contained 8% naive

granulocytes and 7% promyelocytes. The cytogenetic analysis showed a normal karyotype (46, XY), while targeted next-generation sequencing revealed an NF1 (S1399fs/R1748X) mutation. A physical examination showed splenomegaly, with a mean spleen size of 7 cm under the costal margin, and hepatomegaly, with a mean liver size of 2 cm under the costal margin. The patient appeared pale and had many café-au-lait spots on his lower limbs and trunk. His older brother was in good health.

Sample preparation

Bone marrow samples of the patient and peripheral blood samples of his older sibling and a healthy donor were collected. Total DNA from the above-mentioned samples was extracted using DNA extraction kits (TIANGEN, Beijing, China) following the manufacturer's protocol.

WGBS

Isolated genomic DNA was sheared into fragments of 100–300 bp in length. The 3' and 5' overhangs were repaired to create blunt ends, and a single "A" was added to the 3' end of the blunt fragment. Methylated adapters were ligated to the A-tailed fragment and bisulfite modified using a ZYMO EZ DNA Methylation-Gold Kit. A final polymerase chain reaction (PCR) amplification step was then performed for library qualification. Only the qualified library with the targeted size range was sent for sequencing.

Genome-wide methylation analysis and gene set enrichment analysis

Global DNAm profiles in all 3 samples were examined. The data were then analyzed by a pairwise comparison to identify the key genes. DNA methylation in eukaryotes occurs on cytosine bases in the context of CG, CHG, and CHH (H = A, C, or T). First, the CG, CHG, and CHH methylation in each sample were analyzed separately to compare the DNAm features. Next, the average DNAm levels of 7 different transcription elements in the genome, including the upstream region, first exon, first intron, internal exon, internal intron, last exon, and downstream region, were analyzed. Next, the differentially methylated regions (DMRs) among the samples were analyzed and compared. We performed a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and a Gene Ontology (GO) enrichment analysis of the DMR-related genes to explore the role of epigenetic variations in pathways and biological processes and identified the overlapping differential signaling pathways. Network an enrichment analysis of the overlapping genes was performed using Cytoscape and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), which are gene/protein interaction databases, to identify the hub genes.

Real-time qPCR

The total ribonucleic acid (RNA) was extracted using TRIzol reagent (Invitrogen) from bone marrow blood samples from 3 JMML patients and peripheral blood samples from 3 healthy donors, and complementary DNA (cDNA) was reverse transcribed using a cDNA synthesis kit (Yeasen, Shanghai, China). The quantitative gene expression analysis was performed in an ABI7500 Real-Time PCR System (Applied Biosystems, California, USA) with a quantitative PCR (qPCR) SYBR Green Master Mix Kit (Yeasen, Shanghai, China). β-actin was used as the housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression levels of the genes of interest. The primers used to amplify cystic fibrosis transmembrane conductance regulator (*CFTR*) and β-actin are listed in Table S1.

Data mining and survival analysis

A data mining platform was used to query the expression level data for the hub genes identified in the healthy controls and the JMML patient samples in the GEO database, and differences between the means were compared by using a 2-tailed Student's *t*-test. For the survival analysis, the samples were divided into 2 groups according to the median expression levels of the hub genes, and the overall survival rates of the 2 groups were compared. A result was deemed to be statistically significant if the P value was <0.05.

Results

The analysis showed that the CHG and CHH methylation levels in the 3 children were similar. However, the distribution of cytosine methylation (mC) and CG methylation (mCG) in the JMML patients differed significantly between the healthy sibling and the healthy control (see *Figure 1*). We also analyzed the average DNAm levels of different transcription elements in the genomes of the 3 subjects and found no significant differences (see *Figure 2*).

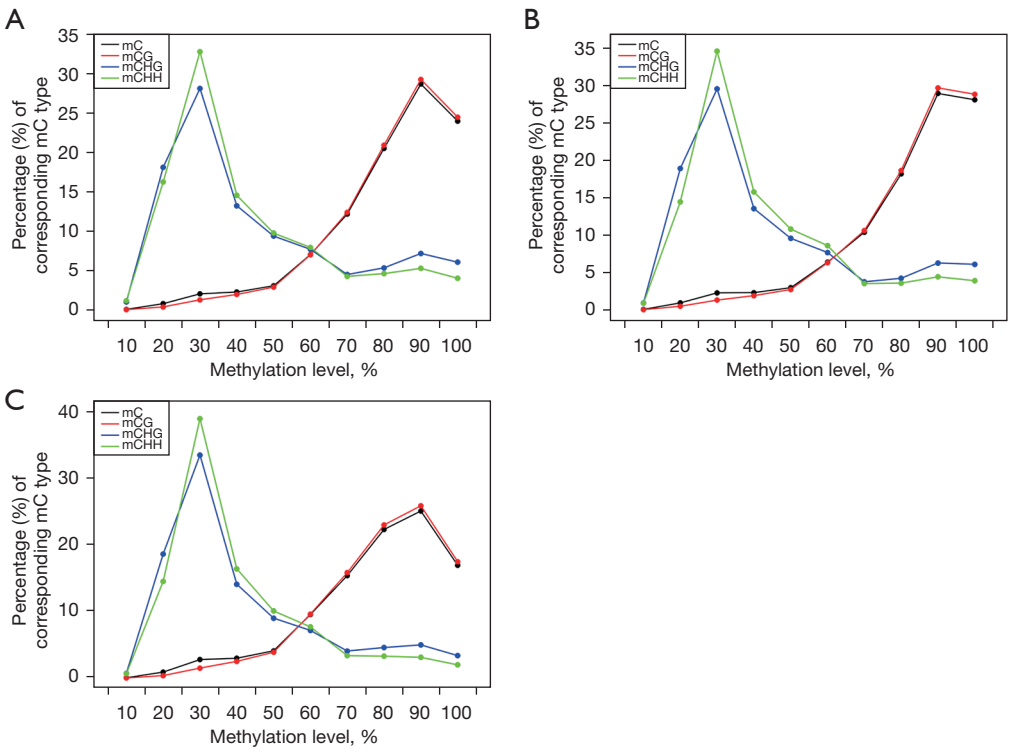

Figure 1 Schematic representations of the cytosine methylation levels in each subject, as determined by WGBS. The x-axis shows the methylation level. (A) Cytosine methylation levels in the healthy control. (B) Cytosine methylation levels in the healthy control MZ twin. (C) Cytosine methylation levels in the patient. WGBS, whole-genome bisulfite sequencing; MZ, monozygotic; C, cytosine; G, guanine; CHG, where H is non-G nucleotides.

A KEGG pathway enrichment analysis and GO functional annotation analysis were performed on the DMR-associated genes in the 3 subjects. The DMR-related genes in the subjects had high consistency in functional clustering (P>0.05; see *Figure 3*). Interestingly, we found no significant differences between the heathy controls and the patient in the genes, but the results of KEGG pathway enrichment analysis showed significant differences in multiple signaling pathways in the promoter region (P<0.01; see *Figure 4* and https://cdn.amegroups.cn/static/public/tp-22-381-1.xlsx, https://cdn.amegroups.cn/static/public/tp-22-381-2.xlsx). As the Venn diagram analysis shows, 57 pathways, including the RAS signaling pathway and the pathways associated with cancer, were significantly enriched and overlapped in the 3 subjects (see *Figure 5* and https://cdn.amegroups.cn/static/public/tp-22-381-3.xlsx).

We performed a gene set enrichment analysis followed by network visualization of the gene data in the 57 overlapping pathways using the Cytoscape and STRING databases. The nodes represent the indicated genes, and the colored nodes represent the genes enriched in certain signaling pathways. The gene set enrichment analysis revealed that the node in red (i.e., CFTR) was the hub gene (see *Figure 6*).

qPCR was used for validation

The relative expression levels of the target genes were verified in the 3 selected JMML patients and 3 healthy children's bone marrow or peripheral blood. The leukemia group showed slightly higher expression levels of CFTR; however, the difference was not statistically significant (P=0.22; see *Figure 7*).

Data mining and statistical analysis

Publicly available data from the GEO database (https://www.ncbi.nlm.nih.gov/gds/) were mined. We removed batch effects between the 2 cohorts using the ComBat function in the sva package (17). The mean values between the patients and controls were compared using 2-tailed *t*-tests. As the

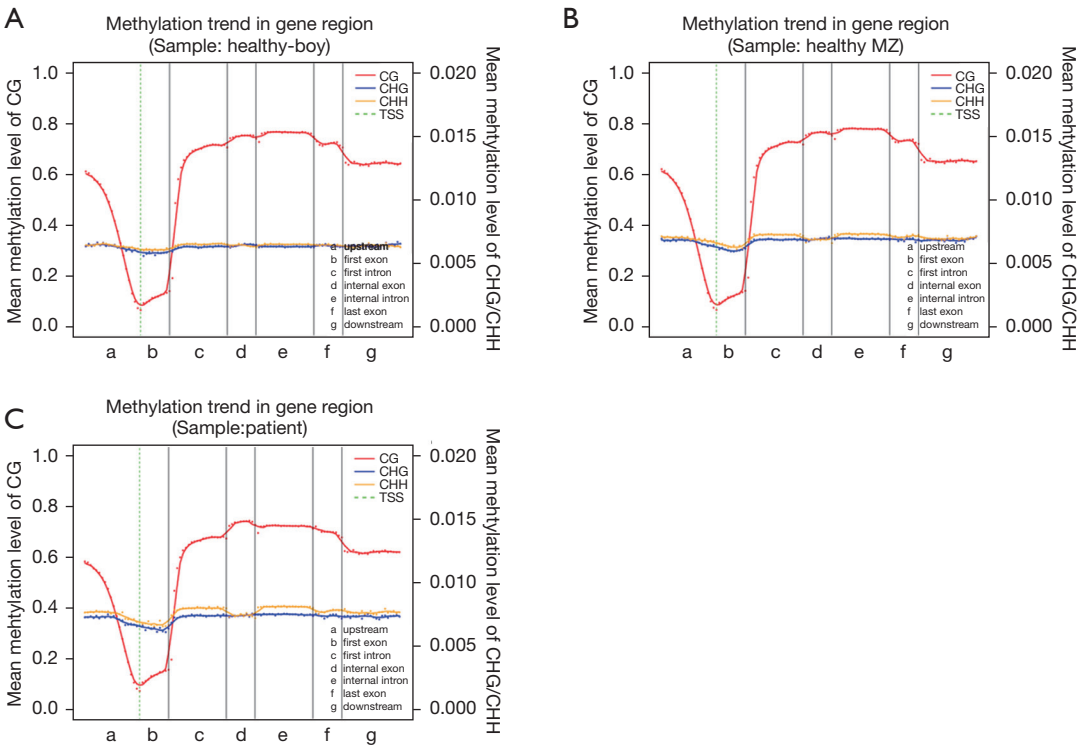

Figure 2 Methylation trends in gene regions. (A) Methylation trends in the healthy control. (B) Methylation trends in the healthy control MZ twin. (C) Methylation trends in the patient. MZ, monozygotic; C, cytosine; G, guanine; CHG, where H is non-G nucleotides.

box plot shows (see *Figure 8A*), the CFTR expression levels did not differ significantly between the JMML patients and healthy donors (GEO: GSE71935–JMML =38, normal =9; GEO: GSE71449–JMML =44, normal =7; P=0.81). However, according to the median gene expression levels in the microarray analysis, we divided patients into low- and high-expression subgroups. The Kaplan-Meier curve for the 82 well-defined JMML patients enrolled in the database revealed that higher CFTR expression was linked to lower survival probability (P=0.019; see *Figure 8B*). A comparison of the different prognosis subgroups of the JMML patients with the healthy controls showed that the expression of CFTR was significantly elevated in the poor prognosis group (P=0.0049; see *Figure 8C*). Conversely, patients with a lower expression level of *CFTR* may had a relatively better prognosis (P=0.00097; see *Figure 8D*).

Discussion

JMML is a clonal myeloproliferative/myelodysplastic neoplasia. Approximately 35% of JMML patients who receive HSCT relapse within 5 years, indicating the strong need for a better understanding of the molecular mechanisms of JMML (3). Our study identified the important role of DNAm in the development and progression of JMML. A total of 57 overlapping signaling pathways, including RAS, were identified as target pathways that were involved in the pathogenesis of JMML. Some studies have described candidate genes that are transcriptionally regulated by methylation in JMML (10,12); however, this study is the first to show that *CFTR* gene methylation is significantly correlated with JMML. A total of 5 signaling pathways (i.e., the Cyclic Adenosine monophosphate (cAMP) signaling, AMP-activated protein kinase (AMPK) signaling, bile secretion, gastric acid secretion, and pancreatic secretion pathways) were identified in this study (see https://cdn.amegroups.cn/static/public/tp-22-381-4.xlsx). Due to the limited sample size, no significant difference between the JMML patients and healthy children was found in relation to the expression of *CFTR*. However, a survival analysis of a larger cohort indicated that patients with high expression levels of *CFTR*

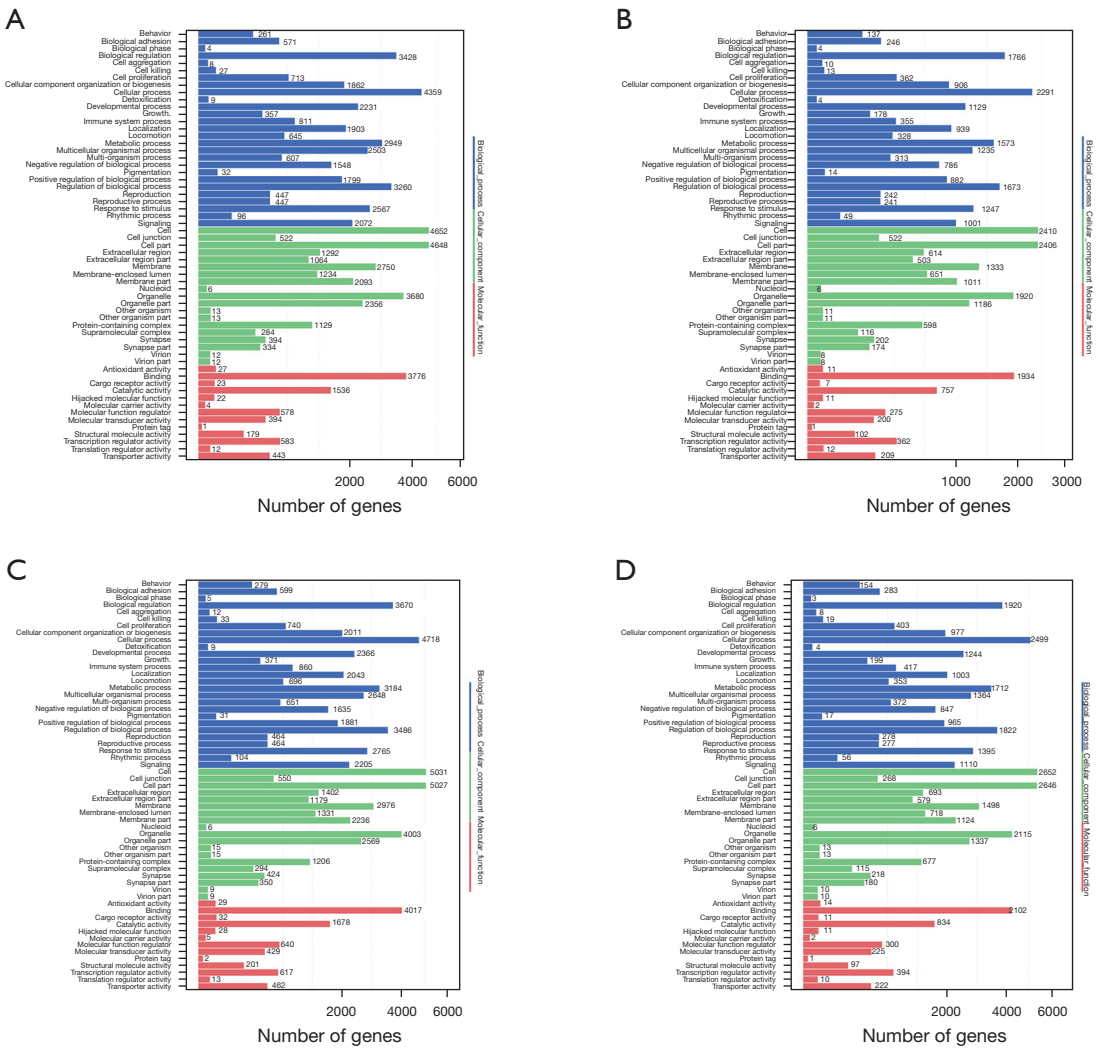

Figure 3 GO enrichment analysis of DMR-associated genes. (A,B) Expression of DMR-related genes and promoters between the twins. (C,D) Expression of DMR-related genes and promoters between the JMML patient and the healthy controls. GO, Gene Ontology; DMR, differentially methylated regions; JMML, juvenile myelomonocytic leukemia.

may exhibit a poorer prognosis than patients with low expression levels of *CFTR*.

CFTR, which is critical for carcinogenesis, was identified as one of the hub genes (18). CFTR is a glycoprotein with 1480 amino acids that belongs to the family of Adenosine triphosphate binding cassette (ABC) transporters (15). ABC transporters are frequently overexpressed in metastatic cancers, contributing to chemoresistance (19). Additionally, CFTR is a cAMP-regulated chloride channel that contains 2 nucleotide-binding domains (NBDs) (i.e., NBD1, and NBD2), and a cytosolic region called the R domain in addition to 12 transmembrane helices (15). A function of CFTR is to transport Cl– and HCO_{3-}, and it also regulates other ion channels (Na+, K+, Ca_{2+}, and other Cl– channels) (20-22). Additionally, CFTR also has roles in osmoregulation, membrane potential maintenance, lipid homeostasis, cell polarity, the metabolism of glucose and other substrates, oxidative stress, inflammation, mucus production, microbiome alterations, pH regulation, cell motility, autophagy, mitochondrial dysfunction, apoptosis, cell polarity, cell-cell contact, stem cell function, and cellular immune responses (23-33). Some studies have shown multiple associations between CFTR and cancer; however, its expression levels vary between different types of tumors. DNAm is the major epigenetic approach for gene regulation, and the destruction of DNAm is related to

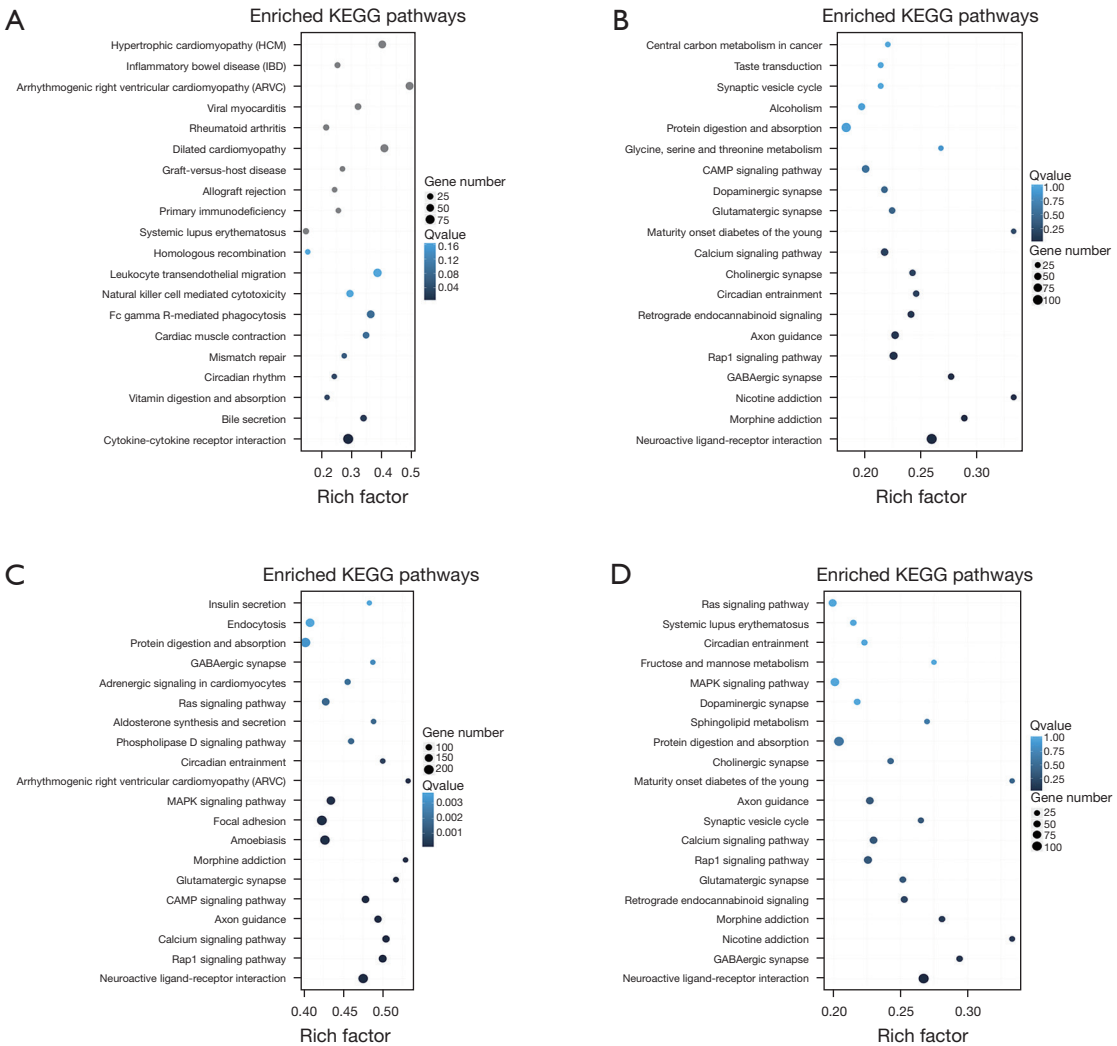

Figure 4 Bubble diagram of KEGG enrichment analysis results. (A,B) Pathway enrichment analysis of DMR-associated genes and relevant promoters between the twins. (C,D) Pathway enrichment analysis of DMR-associated genes and relevant promoters between the JMML patient and the healthy controls. KEGG, Kyoto Encyclopedia of Genes and Genomes; DMR, differentially methylated regions; JMML, juvenile myelomonocytic leukemia.

a variety of diseases (34). The downregulation of the *CFTR* gene by promoter methylation has been demonstrated in various cancer types, including lung cancer (35), liver cancer (36), and head and neck cancer (37). However, *CFTR* is overexpressed in other cancers, such as ovarian cancer (38). This discrepancy indicates that the *CFTR* gene may act as both a proto-oncogene and an anti-oncogene.

The dual role of *CFTR* may be linked to its complex gene expression pattern, and the interaction of its promoter with intronic enhancers may coordinate gene transcription (39,40). In addition to being expressed in actively proliferating epithelial cells, *CFTR* is also widely expressed in immune cells of the blood system and exerts biological functions, and its anion transport and regulation characteristics are the same as those of epithelial cells (41,42). The epigenetic regulation of *CFTR* has been discovered in other solid tumors, but very few studies of hematologic malignancies have been reported. Indeed, until 2017, only 1 study had shown that *CFTR* acts as an oncogene in Ph+ acute lymphoblastic leukemia (Ph+ALL) (43), and the expression level of the *CFTR* in Ph+ALL patients was found to be higher than that

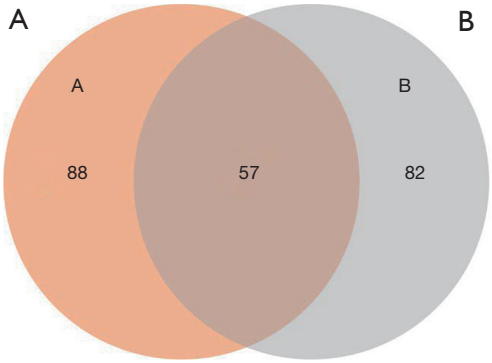

Figure 5 Venn diagram showing the overlap among the 3 subjects. (A) Pathway enrichment analysis of the differentially expressed genes between the twins (light color). (B) Pathway enrichment analysis of the differentially expressed genes between JMML patients and healthy controls. Pathway enrichment analysis of the overlapping differentially expressed genes. JMML, juvenile myelomonocytic leukemia.

of healthy controls in another study (44). However, the relationship between *CFTR* and the development of JMML remains unclear.

In this study, consistent with similar results for other solid tumors, we found that the promoter of *CFTR* was hypermethylated in JMML patients. The CFTR promoter is a "housekeeping" type promoter rich in CpG. In addition to promoter methylation, hypoxia-responsive elements, intronic enhancers, and insulator elements that functionally interact with promoters in a cell-type-specific manner can also partially control the spatial regulation of CpG CFTR expression (45). Generally, DNA promoter methylation and gene expression are negatively correlated, but the high expression levels of some genes can be maintained even when their promoter regions are methylated (46). Further, promoter methylation status has been shown to be correlated with prognosis; for example, the hypermethylation of *CFTR* was found to be associated with an unfavorable survival rate in patients with prostate cancer (47).

Based on the role of *CFTR* in other tumors and the results of our methylation analysis, it is reasonable to assume that *CFTR* is an important JMML-associated gene. Thus, we downloaded microarray data sets and clinical follow-up data from the GEO database. The Kaplan-Meier curves revealed that high *CFTR* expression was associated with inferior prognosis in JMML, suggesting that *CFTR* and JMML may have a close relationship. However, this possibility requires further exploration.

Autophagy has emerged as an effective escape mechanism for promoting tolerance to chemotherapeutic drugs, ultimately leading to poor clinical outcomes (48). The constitutive activation of the RAS signaling pathway, the main pathogenic mechanism of JMML, is closely related to autophagy. Inhibition of KRAS→RAF→MEK→ERK signaling triggers autophagy, protecting cancer cells from the cytotoxic effects of RAS signaling pathway inhibition (49). However, high expression of CFTR may lead to autophagy and resistance to chemotherapeutic drugs in cancer cells (50). In terms of treatment efficiency, there is currently no type of chemotherapy that can lead to long-term remission in JMML patients.

Studies have shown that *CFTR* expression is also upregulated in ovarian cancer (37). The Ras-MAPK/Erk-ETS1/CFTR1 axis was found to be upregulated in ovarian cancer cell lines. This upregulation may increase the proliferation and invasion and reduce the drug absorption and apoptosis of tumor cells, ultimately resulting in chemotherapeutic resistance (51). *ETS1*, a main downstream effector in the Ras/ERK signaling pathway, is generally considered a transcriptional activator and is commonly hyperactivated in cancer. More specifically, the Ras-MAPK signaling pathway can be activated by *ETS1* via a dual functional role (52). Increased *ETS1* expression in ovarian cancer was found to be associated with a poor prognosis (53), and *ETS1* can regulate the expression of *CFTR* by binding to its promoter regions (50). These findings suggest that *CFTR* is associated with the ETS1 and Ras signaling pathways. Additionally, the overexpression of *CFTR* in serous ovarian cancer can activate the c-Src signaling pathways (54), which cooperate with the RAS signaling pathway to promote tumorigenesis (55). Despite this evidence, more studies need to be conducted to demonstrate the direct relationship between the overexpression of *CFTR* and the activation of the RAS/ERK signaling pathway.

Conversely, the high expression of *CFTR* is positively correlated with nuclear factor kappa beta (NF-κB) activation (56). Activated NF-κB binds to the src homology 2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2) promoter, leading to increased SHP2 expression, which enhances the activation of the RAS/RAF/MEK/ERK pathway (57). The constitutive activation of the RAS pathway is the main pathogenic mechanism of JMML (4,5). Conversely, microRNA -150-5p, a small non-coding RNA, was discovered to play roles in both cancer and autoimmune disease (58,59). *CFTR*-mediated

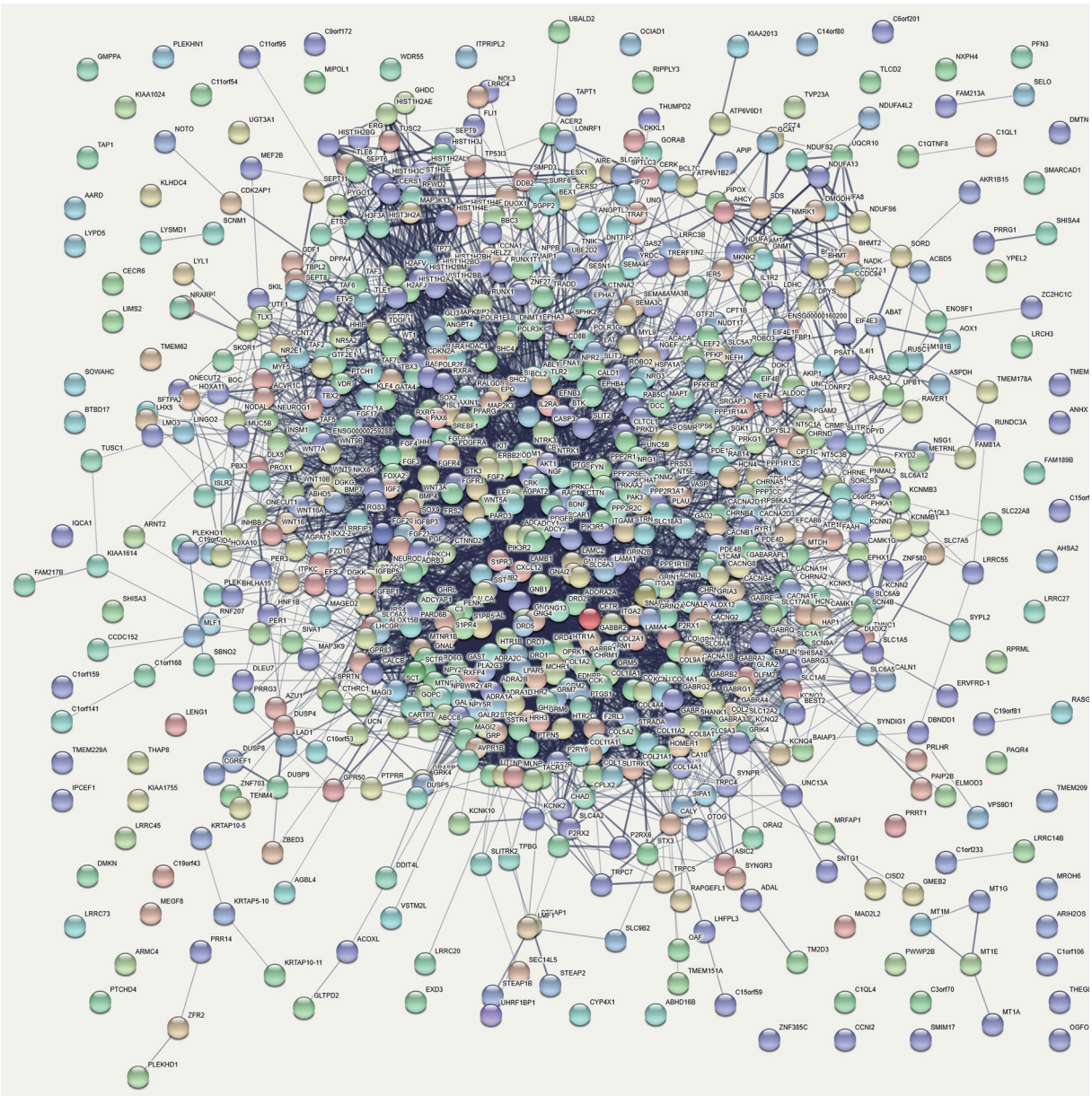

Figure 6 Network visualization based on pathway enrichment analysis with Cytoscape and STRING. STRING, Search Tool for the Retrieval of Interacting Genes/Proteins.

HCO_{3-} influx can activate soluble adenylate cyclase (sAC), which in turn activates protein kinase A (PKA)-dependent NF-κB signaling (60). Activated NF-κB1 interacts with miR-150-5p, which negatively regulates the miR-150-5p expression level (61). The downregulation of miR-150-5p leads to the phosphorylation of *STAT5b* and activates *KRAS*, *NRAS*, *NF1*, and *PTPN11* in JMML (62). This observation suggests that microRNAs might function as intermediate links between *CFTR* and JMML pathogenesis/disease progression. Thus, we speculate that *CFTR* and the pathogenesis of JMML may be closely connected via RAS pathway regulation. Further investigations urgently need to be conducted to identify the mechanisms underlying this relationship. Subsequent research work will focus on genome-wide methylation analysis in expanded samples of JMML patients, and functional verification at the cellular level.

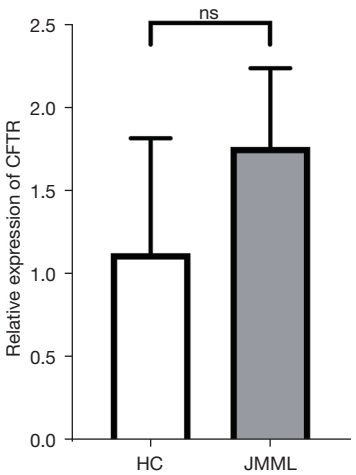

Figure 7 Validation of RNA-seq data by RT-qPCR. Relative expression level of CFTR determined by RT-qPCR. HC, healthy control; JMML, juvenile myelomonocytic leukemia; CFTR, cystic fibrosis transmembrane conductance regulator; RT-qPCR, real-time quantitative polymerase chain reaction; ns, no significance.

This was a preliminary laboratory study; thus, further investigation of *CFTR* function both *in vivo* and *in vitro* are required, and more investigations are needed to identify the mechanisms of *CFTR* in JMML development and progression. In future clinical research, to evaluate the relationship between CFTR promoter methylation and the clinical prognosis of JMML, we will increase the number of cases to dynamically monitor any changes in *CFTR* promoter methylation in JMML patients during treatment. Due to restrictions related to our sample size, our research lacked genome-wide methylation data and the corresponding gene expression profiling data from a large sample. In the next study, the sample size needs to be increased to elucidate this mechanism through a more thorough experimental analysis.

In conclusion, this study was the first to show that the promoter of *CFTR* is hypermethylated in JMML. This was a preliminary laboratory study but combined with the clinical data demonstrated in the study, we are of the view

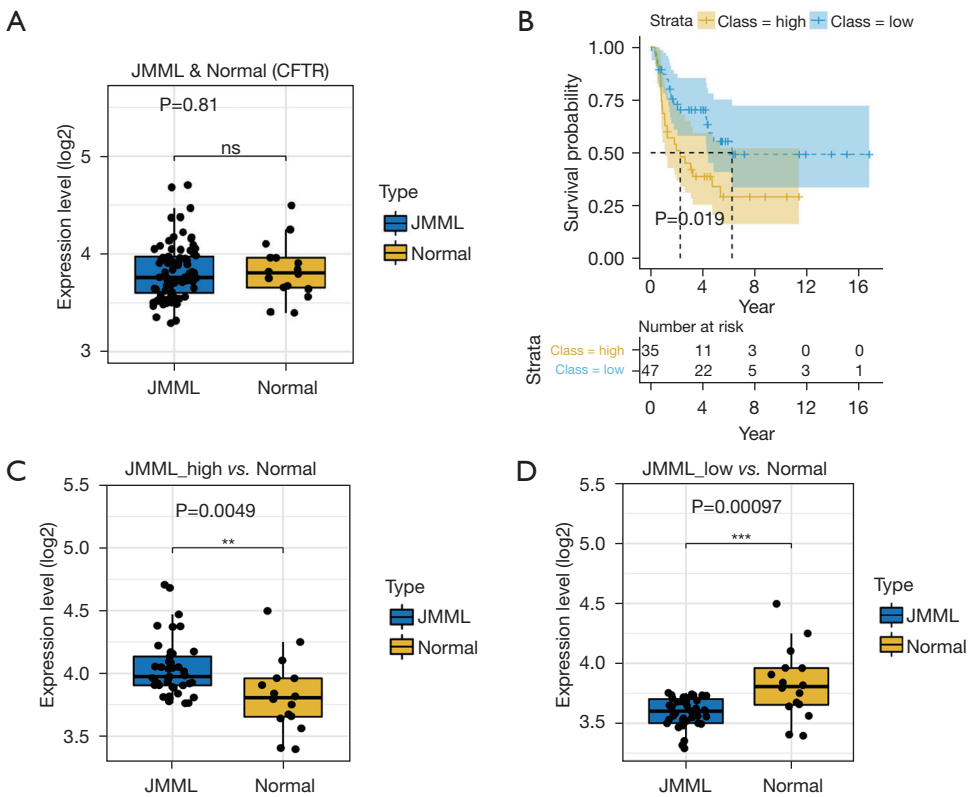

Figure 8 Prognostic value of CFTR for long-term survival in patients with JMML. (A) Differential expression of CFTR between normal (n=16) and JMML (n=82) samples. (B) Kaplan-Meier analysis of overall survival in JMML patients. (C,D) Differential expression of CFTR between the normal (n=16) and the high (n=35) and low (n=47) expression subgroups of samples. **, P<0.01. ***, P<0.001. JMML, juvenile myelomonocytic leukemia; CFTR, cystic fibrosis transmembrane conductance regulator. ns, no significance.

that *CFTR* could be a promising diagnostic, therapeutic, and prognostic biomarker for JMML. However, further investigations of CFTR function both *in vivo* and *in vitro* are required, and more investigations are needed to reveal the role of the *CFTR* gene in JMML development and progression. In future clinical research, to deepen the knowledge of the relationship between *CFTR* promoter methylation and the clinical prognosis of JMML, the authors will increase the sample size to dynamically monitor the changes in *CFTR* promoter methylation in JMML patients during treatment. Genome-wide methylation data of more cases will also be taken into consideration to elucidate the matter.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-381/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-381/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-381/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 Medical Ethics Committees of Nanfang Hospital, Southern Medical University (No. NFEC-2020-203). Before participating in this study, the patient's parents provided written informed consent.

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Supplementary

Table S1 The oligonucleotide primer sequences for RT-qPCR

Gene	Up/down	Primer sequence
Human CFTR	Up	GCCTGGCACCATTAAAGAAA
	Down	GTGTGATTCCACCTTCTCCAA
β-actin	Up	GAGCACAGAGCCTCGCCTTT
	Down	ACATGCCGGAGCCGTTGTC

p: forward primer; Down: reverse primer.

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