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The hENT1 and DCK genes underlie the decitabine response in patients with myelodysplastic syndrome

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ABSTRACT

Decitabine is approved for the treatment of MDS, but resistance to this agent is common. To determine the mechanisms underlying decitabine resistance, we measured the mRNA expression of metabolism (hENT1, DCK, CDA) and apoptosis (BCL2L10) genes and found that the hENT1 mRNA level was significantly higher in response compared with non-response patients ($P = 0.004$). Furthermore, the DCK level was significantly reduced for relapse ($P = 0.012$) compared with those with continued marrow CR ($P = 0.222$). These findings indicate that the decitabine metabolic pathway affects its therapeutic effects, lower hENT1 expression may induce primary resistance and down-regulated DCK expression may be related to secondary resistance.

1. Introduction

Myelodysplastic syndrome (MDS) comprises a group of biologically and clinically heterogeneous clonal hematopoietic neoplasms characterized by aberrant myeloid differentiation, dysplastic changes, ineffective hematopoiesis and increased genomic instability, which clinically manifest as peripheral blood (PB) cytopenias and variably increased rates of leukemia progression [1]. Epigenetic deregulation (particularly gene hypermethylation and histone deacetylation) plays an important role in the pathogenesis of MDS [2]. The inhibitor of DNA methyltransferases (DNMTs) 2-deoxy-5-azacitidine/decitabine has been approved by the US Food and Drug Administration (FDA) for the treatment of MDS [3,4].

A number of clinical trails have shown that decitabine provides significant clinical benefit for patients with MDS [5–8], but a number of patients do not initially respond (primary resistance), and a majority of patients lose response or progress despite continued therapy (secondary resistance) [9]. Furthermore, the mechanisms underlying decitabine resistance remain unknown. Previous studies indicate that a metabolic pathway is related to primary and secondary resistance [10], and genes related to decitabine metabolism include human equilibrative nucleoside transporter 1 (hENT1), hENT2, deoxycytidine kinase (DCK) and cytidine deaminase (CDA) [11–13]. Moreover, other studies have demonstrated that BCL2L10 is a predictive factor for resistance to the DNA methyltransferase inhibitor azacitidine for MDS and AML patients [14]. In view of this information, we aimed to investigate whether the expression level of these genes could predict the response to decitabine for MDS patients.

2. Materials and methods

2.1. Patients

Adults with a diagnosis of MDS who were referred to Guangdong General Hospital & Guangdong Academy of Medical Science were enrolled in this study according to FAB criteria. 28 newly diagnosed patients with MDS who received decitabine more than 2 cycles of standard treatment (2–23 cycles, median number cycles: 7). A total of 14 patients had an overall response after treatment. 14 patents showed no response. Among the responders, 5 patients achieved more than 8 courses of mCR (continued mCR), but 5 patients relapsed or lost response despite continued therapy. In addition, 3 relapsed AML patients after treatment with decitabine were also included in this study.

2.2. Samples collected

A total of 44 BM and PB samples were collected in this study. 28 samples were from 28 MDS patients(14 responders and 14 non-responders) at diagnosis. 5 samples were from 5 MDS patients with continued mCR.5 samples were from 5 MDS patients at relapse. 6 samples were from 3 AML patients at diagnosis and relapse.

2.3. RNA extraction and cDNA synthesis

White blood cells were collected from the BM and PB samples following red cell lysis and then total RNA was extracted with TRIzol (Invitrogen) from the White

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Table 1
Target gene PCR primer and probe sequences.

Primer and probe	Sequence (5′–3′)
CDA-F	TGCAGGCAAGTCATGAGAGAGT
CDA-R	ACGTACCATCCGGCTTGGT
CDA-P	FAM-TGGCACCAACTGGCCCGTGTACAT-TAMRA
hENT1-F	ACTGTGGTCTTCGAGCACGAT
hENT1-R	GCAGAGGCTGGCGAGGTA
hENT1-P	FAM-TCTTCATGGCTGCCTTTGCCTTCTCC-TAMRA
DCK-F	GGACCCGCATCAAGAAAATC
DCK-R	TTCCCAATCTTCACACAATTGTTT
DCK-P	FAM-CCATCGAAGGGAACATCGCTGCA-TAMRA
BCL2L10-F	GCTGGGATGGCTTTTGTCA
BCL2L10-R	GCCTGGACCAGCTGTTTTCTC
BCL2L10-P	FAM-TCTTCAGGACCCCCTTTCCACTGGC-TAMRA

blood cells according to the manufacturer's recommendations. The RNA quality was analyzed in a 0.8% agarose gel stained with ethidium bromide. The RNA (~1 μg) was synthesized into first single-strand cDNA with random hexamer primers using the Superscript III First Strand Synthesis kit (Invitrogen, Rockville, MD).

2.4. Quantitative PCR (Q-PCR)

Quantification of gene transcript numbers in the BM and PB samples was performed by quantitative reverse-transcription polymerase chain reaction using 5′nuclease (TaqMan) assays with the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of the fluorescent emission after the cleavage of sequence-specific probes by the nuclease activity of Taq polymerase. The internal control gene was ABL, and ABL transcript quantitative PCR primers and probe sequences were obtained from the Europe Against Cancer Program [15]. Target gene PCR primers and probe sequences were designed by Express software and synthesized by Invitrogen (Table 1).

To create a standard curve, cDNA from a normal individual was amplified by PCR using ABL primers designed for Q-PCR. PCR products were cloned into the pMD18-T vector (Takara, Dalian, China). Selected plasmid clones were sequenced for insert confirmation. After subsequent bulk production, the plasmids were extracted using the Takara MiniBEST Plasmid Purification Kit Ver. 2.0 (Takara, Dalian, China) according to the manufacturer's instructions, and they were quantified by spectrophotometer. The copy number for 1 μg was estimated according to the molecular weight of the vector and insert. The plasmid was serially diluted with EASY Dilution (Takara, Dalian, China). Five successive dilutions (1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 copies/μl) were prepared. The corresponding standard curve generated a slope of 3.32–3.40. The amplification efficiency of the target genes was close to that of the ABL reference gene, thus, they shared a set of standards.

Serial dilutions of the plasmids at known concentrations were tested by Q-PCR. Q-PCR reactions for the cDNA samples, DNA standards, and water as a negative control (NTC) were conducted in a total volume of 20 μL including 10 μL 2× TaqMan Universal Master Mix (Applied Biosystems, Branchburg, New Jersey, USA), 300 nM of each primer and 200 nM probe. The thermal cycler parameters were as follows: 2 min at 50 °C, 10 min at 95 °C and 45 cycles of 95 °C for 15 s and 62 °C for 1 m. Standard curve extrapolation for copy number determination was performed for the target genes and internal control gene ABL. Sample data were normalized by dividing the number of copies of target gene transcripts by the number of copies of ABL transcripts. All PCR assays were performed in duplicate and reported as means.

3. Statistical analyses

Statistical analyses were performed using SPSS version 11.5 for Windows. Statistical differences between different groups were analyzed by nonparametric tests. The Mann–Whitney *U* test was used to compare the difference of genes expression between two groups. The difference of gene expression at diagnosis vs relapse or mCR was compared by Wilcoxon Signed Ranks Test. *P* values less than or equal to 0.05 were considered statistically significant.

4. Results

In this study, we investigated the mechanisms of primary and secondary resistance to decitabine. Thus, we examined the expression level of different metabolism genes in MDS patients who received decitabine and compared that of responders and non-responders for primary resistance. For secondary resistance, we analyzed the expression level of different genes in patients who lost response or progressed and continued mCR while on hypomethylating agent-based therapy. The patient characteristics are described in Tables 2 and 3.

Table 2
Characteristics of patients with primary resistance.

	Responder	Non-responder
Sex, *n*		
Male	10	12
Female	4	2
Age, *n*		
>60	6	6
<60	8	8
IPSS, *n*		
Low	0	0
Intermediate-1	6	6
Intermediate-2	7	5
High	1	3

4.1. The hENT1 expression level is a potential predictive indicator for primary resistance in patients with MDS who received decitabine

As a first step toward identification of the transport mechanisms underlying the therapeutic activity of decitabine, we analyzed the expression profile of nucleoside transporters in bone marrow or peripheral blood from 28 MDS patients. Further analysis was performed in responders and non-responders. The results indicated that the hENT1 mRNA level in the non-response group was significantly lower than that in the response group for patients with MDS ($P = 0.004$, Fig. 1). Similarly, we further examined the expression level of DCK, CDA and BCL2L10 and found that they did not affect the response rate ($P_{DCK} = 0.687$; $P_{CDA} = 0.201$; $P_{BCL2L10} = 0.201$, Fig. 1).

4.2. The relationship between the related genes expression and gender

DCK, CDA, hENT1 and BCL2L10 expression level of males and females were 96.70 ± 36.3 vs 128.72 ± 36.3 ($P = 0.157$), 2048.69 ± 1254.57 vs 1679.65 ± 1555.03 ($P = 0.295$), 191.91 ± 66.68 vs 217.75 ± 94.38 ($P = 0.295$) and 1.19 ± 1.21 VS 1.45 ± 0.75 ($P = 0.176$) respectively. The results showed that gender does not influence all of the genes related to metabolism.

4.3. The DCK expression level is a potential predictive indicator for secondary resistance for MDS and AML patients who received decitabine

As a second step toward identifying the transport mechanisms underlying the therapeutic activity of decitabine, we used quantitative real-time PCR to measure the mRNA expression level of genes related to DAC metabolism in bone marrow or peripheral blood from patients with recurrence at diagnosis and relapse. The data

Table 3
Characteristics of patients with secondary resistance.

	Relapse	Continued CR
Sex, *n*		
Male	6	4
Female	2	1
Age, *n*		
>60	5	2
<60	3	3
Diagnosis, *n*		
MDS	5	5
AML	3	0