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Original Article

CCND1 G870A polymorphism is associated with toxicity of methotrexate in childhood acute lymphoblastic leukemia

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Abstract: CCND1 plays a key role in cell cycle progression and may cause methotrexate (MTX) resistance, as well as its cytotoxicity. *CCND1* 870A variant allele is associated with altered transcripts of this gene. We hypothesized that this polymorphism may contribute to the elimination rate and hepatotoxicity of MTX in childhood acute lymphoblastic leukemia (ALL). We genotyped the *CCND1* G870A polymorphism in 125 childhood ALL patients treated with HDMTX. We found no notable associations between G870A polymorphism and the risk of delayed MTX elimination. However, this polymorphism was significantly associated with an increased risk of MTX hepatotoxicity [adjusted odds ratio (OR) = 4.44, 95% confidence interval (CI) = 1.35-14.63 for AG versus GG and adjusted OR = 6.39, 95% CI = 1.82-22.43 for AA versus GG]. Our results indicated that the *CCND1* G870A polymorphism may be involved in the hepatotoxicity of MTX and act as a biological marker.

Keywords: *CCND1*, polymorphism, MTX toxicity, childhood acute lymphoblastic leukemia

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignant disorder in childhood and the incidence is increasing worldwide. This hematological malignancy was speculated to arise by a combination of the toxic substance exposure and genetic susceptibility [1]. Recently remarkable advances have been made in chemotherapy and have resulted in an expected cure rate more than 80% for childhood ALL [2]. High-dose methotrexate (HDMTX) in prevention of extramedullary leukemia has been an important mean in the chemotherapy of childhood ALL and has significantly reduced the recurrence rate of ALL children, but a considerable part of patients experienced a variety of side effects such as delayed excretion of MTX, skin and mucous membrane damage, serious hepatotoxicity [3]. Clinical progression and some treatment response are shown to be strongly associated with different genotypes at particular loci [4].

Cyclin D1 (CCND1), a member of the D-type cyclin family, is an essential regulator of cell cycle progression. The activity of CCND1 is maximum during G1 phase and it is associated with CDK4 and CDK6 in the mid to late G1 phase; therefore, it is one of the major cyclins involved in this transition [5]. The expressed D-type cyclins form a holoenzyme complex with either cyclin-dependent kinase (cdk) 4 or 6 and then phosphorylate retinoblastoma protein (pRb) after being activated by cdk-activating kinase. Phosphorylated pRb releases transcription factors such as E2F which then activates the transcription of genes whose products are required for entry into S phase of the cell cycle [6, 7]. Over expression of CCND1 disrupts normal cell cycle process and possibly promote the development and progression of cancers, including childhood ALL [5]. Besides, CCND1 can regulate DHFR protein level through increased E2F and thus lead to resistance to methotrexate (MTX) or interfere with the cytotoxicity of MTX [7], which is an important component of chemotherapy of childhood ALL.

Table 1. Characteristics of the study subjects

Patient Characteristics	N (%)
Total number	125
Mean age	
≤ 5	73 (58.4)
> 5	52 (41.6)
Gender	
Male	71 (56.8)
Female	54 (43.2)
Immunophenotype	
B-phenotype ALL	107 (85.6)
T-ALL	18 (14.4)
Risk group	
Low-risk	68 (54.4)
Medium-risk	17 (13.6)
High-risk	40 (32.0)
CCND1 G870A	
GG	33 (26.4)
AG	60 (48.0)
AA	32 (25.6)

In 1995, Betticher and colleagues identified a single nucleotide polymorphism (G870A) in exon 4 of cyclin D1 gene (*CCND1*), which increases alternative splicing. The variant nucleotide interferes with splicing from exon 4 to exon 5 because of its unique localization within a conserved splice donor region and the A allele can produce more altered transcripts due to the altered splicing. Although both the normal and the altered transcripts encode a protein that contains amino acids thought to be responsible for the cyclin D1 function, the protein encoded by the altered transcript may have a longer half-life [8]. Therefore, it has been suggested that DNA damage in cells from subjects with the A allele may bypass the G1/S checkpoint more easily than damage in cells not carrying the polymorphism [9]. Studies have also shown that subjects with the AA genotype had a higher risk of childhood ALL and poorer outcome than those with the other two genotypes [10, 11]. A possible explanation for the 870A allele associated poor prognosis of childhood ALL might be related to the response to MTX treatment.

Our present study, based on previous observation on the association between *CCND1* polymorphism and clinical response to MTX treatment [11, 12], hypothesized that the G870A variation may be related to serum MTX concentration and incidence of toxicity after MTX administration in childhood ALL patients.

Material and methods

Study population

The recruitment of most of subjects has been described previously [13]. Briefly, 125 childhood ALL patients diagnosed in Nanjing Children's Hospital Affiliated to Nanjing Medical University were enrolled in this study. All the patients had been diagnosed by morphology, immunology, cytogentic and molecular biology. Blood samples were taken from each patient after informed consent was obtained from the parents. Chemotherapeutic protocol used was Suggested Diagnosis and Treatment of Children with ALL, published by the Society of Pediatrics, Chinese Medical Association in 2006, which has been documented [14]. After induction and consolidation treatment, all the 125 patients reached complete remission (CR) and then methotrexate was administrated in a high dose of 5.0 g/m^2 (for high-risk ALL and medium-risk ALL) or 3.0 g/m^2 (for low-risk ALL), and lasted for 24 hours. Hydration and alkalization was given at the same time. Leucovorin rescue treatment was used to eliminate the MTX toxicity in 48 hours after MTX treatment. Given that HDMTX exposure may induce acquired MTX resistance and thus interfere with our investigation of the association between genetic variation and excretion or toxicity of MTX, we only collected laboratory data described above after the first course of HDMTX treatment of each patient. The research protocol was approved by the Medical Ethics Committee of Nanjing Children' Hospital affiliated to Nanjing Medical University.

Genotyping, plasma MTX concentration and hepatotoxicity assessment

Genotyping of *CCND1* G870A was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers, lengths, and restriction enzymes have been described previously [15]. The genotype analysis was done by two persons independently in a blind fashion. 10% of the samples were randomly selected for repeated genotyping and the results were 100% concordant.

Table 2. CCND1 G870A polymorphism and their associations with risk of delayed MTX elimination

	Without Delay	Delay	Adjusted OR (95% CI)†	*P**
Total	67	58		
GG	16 (23.9%)	17 (29.3%)	1	0.688
AG	32 (47.8%)	28 (48.3%)	0.86 (0.36-2.03)	
AA	19 (28.3%)	13 (22.4%)	0.64 (0.24-1.74)	
870AG/AA	51 (76.1%)	41 (70.7%)	0.78 (0.35-1.76)	
A allele	0.52	0.47		0.378

†Adjusted for age and gender in logistic regression model. *Two-sided χ^2 test for either genotype distributions or allele frequencies between subjects with and without delayed MTX elimination.

Table 3. CCND1 G870A polymorphism and their associations with risk of MTX-related hepatotoxicity

	Without hepatotoxicity	Hepatotoxicity	Adjusted OR (95% CI)†	*P**
Total	84	41		
GG	29 (34.5%)	4 (9.8%)	1	0.008
AG	38 (45.2%)	22 (53.6%)	4.44 (1.35-14.63)	
AA	17 (20.3%)	15 (36.6%)	6.39 (1.82-22.43)	
AG/AA	55 (65.5%)	37 (90.2%)	5.10 (1.64-15.90)	
A allele	0.43	0.63		0.003

†Adjusted for age and gender in logistic regression model. *Two-sided χ^2 test for either genotype distributions or allele frequencies between subjects with and without MTX-related hepatotoxicity.

We measured plasma MTX concentrations of each subject at two time points (44 and 68 hours after the start of the MTX administration). It was defined as delayed elimination if plasma MTX level was higher than 1 μmol/L at 44 hours or 0.1 μmol/L at 68 hours. Assessment of hepatotoxicity was based on laboratory tests values of liver enzymes (alanine aminotrasferase and aspartate aminotrasferase) which were evaluated 10 days after HDMTX was given. Hepatotoxicity was graded according to Common Terminology Criteria for Adverse Events of the National Cancer Institute (CTCAE 4.0).

Statistical analyses

Chi-square test was performed to compare the differences in frequency distributions of genotypes of the CCND1 G870A polymorphism between patients with and without delayed elimination, as well as hepatotoxicity. We used unconditional univariate and multivariate logistic regression analyses to obtain the crude and adjusted odds ratios (ORs) for estimating the potential association between G870A variation and delayed elimination of MTX or its toxicity. Non-parametric test was performed to evaluate the correlation between the plasma MTX levels and genotypes of the *CCND1* G870A polymorphism. All statistical tests were two-sided at a significance level of 0.05 and were analyzed using the SAS software (version 9.1.3; SAS Institute, Cary, NC, USA) unless otherwise indicated.

Results

Patient characteristics

Among the 125 patients we recruited in our study, 71 were male and 54 were female. The mean patient age was 5 years. Of the 125 childhood ALL patients, subjects with B-phenotype ALL were in the majority (85.6%). Furthermore, 68 patients were in the low-risk group (54.4%) while patients with medium-risk and high-risk ALL were 17 (13.6%) and 40 (32.0%) respectively. The basic demographic characteristics of these subjects were described in **Table 1**.

Association between CCND1 G870A polymorphism and plasma concentration of MTX

The *CCND1* G870A genotype frequencies and their associations with delayed elimination of MTX are shown in **Table 2**. There are no significant associations between G870A polymorphism and the probability of delayed elimination of MTX (P = 0.688). The variant an allele had a protective effect of delayed MTX clearance, though the difference was not significant (adjusted OR = 0.86, 95% CI = 0.36-2.03 for AG and adjusted OR = 0.64, 95% CI = 0.24-1.74 for AA, compared with GG genotype). In further stratification analysis, we find no obvious relation between this variation and the incidence of delayed MTX elimination whether in subgroup administrated with MTX in a dose of 5.0 g/m^2 (high-risk or medium-risk ALL) or 3.0 g/m^2 (low-risk ALL) (data not shown).

Table 4. Stratification analyses of the *CCND1* G870A polymorphism and their associations with risk of MTX-related hepatotoxicity among high-risk and medium-risk ALL children

	Without hepatotoxicity	Hepatotoxicity	Adjusted OR (95% CI)†	*P**
Total	34	23		
870GG	14 (41.1%)	2 (8.7%)	1	
870AG	11 (32.4%)	11 (47.8%)	8.21 (1.38-48.76)	0.027
870AA	9 (26.5%)	10 (43.5%)	8.81 (1.45-53.59)	
870AG/AA	20 (58.9%)	21 (91.3%)	7.88 (1.53-40.57)	
A allele	0.43	0.67		0.013

†Adjusted for age and gender in logistic regression model. *Two-sided χ^2 test for either genotype distributions or allele frequencies between subjects with and without MTX-related hepatotoxicity.

Table 5. Stratification analyses of the *CCND1* G870A polymorphism and their associations with risk of MTX-related hepatotoxicity among low-risk ALL children

	Without hepatotoxicity	Hepatotoxicity	Adjusted OR (95% CI)†	*P**
Total	50	18		
870GG	15 (30.0%)	2 (11.1%)	1	
870AG	27 (54.0%)	11 (61.1%)	3.07 (0.59-15.60)	0.227
870AA	8 (16.0%)	5 (27.8%)	5.00 (0.66-37.77)	
870AG/AA	35 (70.0%)	16 (88.9%)	3.40 (0.69-16.78)	
A allele	0.43	0.58		0.124

†Adjusted for age and gender in logistic regression model. *Two-sided χ^2 test for either genotype distributions or allele frequencies between subjects with and without MTX-related hepatotoxicity.

Association of CCND1 G870A polymorphism with MTX hepatotoxicity

Table 3 shows the frequency distributions of G870A genotypes among patients with and without hepatotoxicity. There were 41 subjects suffering from CTCAE grade1 or greater hepatotoxicity. When the wild genotype GG was used as a reference, multivariate logistic regression analysis indicated that individuals carrying the variant An allele have a higher rates of hepatotoxicity (adjusted OR = 4.44, 95% CI = 1.35-14.63 for AG versus GG and adjusted OR = 6.39, 95% CI = 1.82-22.43 for AA versus GG, respectively). The combined genotypes AG/AA were also related with an obvious increased risk of toxic events (adjusted OR = 5.10, 95% CI = 1.64-15.90). Furthermore, the frequency of variant an allele were significantly different among patients with and without hepatotoxicity (P = 0.003). Similar result was also found in stratification analysis, that is, in high-risk and medium-risk ALL patients, we found a significant association of G870A polymorphism with increased risk of hepatotoxicity (as shown in **Table 4**, adjusted OR = 8.21, 95% CI = 1.38-48.76 for AG and adjusted OR = 8.81, 95% CI = 1.45-53.59 for AA). However, in low-risk ALL group, we didn't find any association between this polymorphism and the risk of MTX-related hepatotoxicity (**Table 5**).

Discussion

In the present study, we investigated whether there exists an influence of *CCND1* G870A polymorphism on plasma MTX levels and incidence of MTX-related hepatotoxicity. We observed that compared with the wild genotype GG carriers, individuals with AA genotype had a pronounced lower 44 h MTX concentration, but the differences of genotype frequency between subjects with and without delayed MTX clearance were not significant. Furthermore, we found a significant increased risk of MTX-related hepatotoxicity in patients carrying variant an allele, compared with the 870GG genotype.

Until now, more than 50 years have passed after MTX was first introduced in the clinic [16]. It has been widely used in the treatment of a number of solid, hematological malignancies including childhood ALL. High-dose methotrexate (HDMTX) refers to infused MTX in doses of more than 1 g/m^2 [17]. The use of HDMTX has shown great benefit in the treatment of childhood ALL and the prevention of extramedullary leukemia (i.e., central nervous system leukemia and testicular leukemia) [18, 19]. The advantage of MTX in the prevention of extramedullary leukemia is that it can effectively penetrate across the blood-brain barrier and blood-testis barrier. Pitman et al. have shown that HDMTX (3-7.5 g/m^2) is capable of reaching high CSF concentrations [20]. The primary target of MTX is the enzyme dihydrofolate reductase (DHFR), which can catalyze the reduction

of folate and 7, 8-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate [7]. Once inside the cell, MTX will bind and acts as an inhibitor of DHFR. This inhibition results in depletion of biological active tetrahydrofolate forms and thus the transference of carbon group in the reaction that synthesize pyrimidine and purine will be blocked. This will lead to the impairment of nucleic acid synthesis and the resulting cellular death [21]. Many proteins involved in the folate metabolic pathway (i.e. MTHFR, RFC, TS) may contribute to MTX cytotoxicity and clinical elimination.

Cyclin D1, as an important protein regulating progression through the G1 phase of the cell cycle, is also involved in folate metabolic pathway and has been shown to influence cellular response to MTX [22]. An increased level of Cyclin D1 will exert a positive effect on the phosphorylation of the retinoblastoma protein (pRb) and thus mediates functional inactivation of the pRb [21, 22]. This will subsequently lead to increased level of E2F, which can promote transcription of genes encoding folate metabolic enzymes, such as DHFR [23]. Studies have demonstrated that increased DHFR is a major mechanism of resistance to MTX [24] and that tetrahydrofolate (THF), which is regenerated from dihydrofolate by DHFR are important for MTX cytotoxicity [21, 25]. This may also indicated that CCND1 will influence the clearance of MTX and MTX-associated toxicity partly through the regulation of DHFR and THF.

A polymorphism in *CCND1*, G870A variation has been investigated worldwide. Studies have demonstrated that the 870A allele is associated with the alternative splicing of the CCND1 transcript. This transcript will result in protein CCND1b, which is a variant of CCND1 with a longer half-life resembling CCND1 over-expression [9]. To date, no study has focused on *CCND1* polymorphism and elimination rates of MTX. In this study, we investigated whether there exists an association between G870A genotypes and plasma MTX levels. However, we found that 870A allele was not associated with the risk of delayed elimination of MTX. Because ALL patients were administrated with MTX in different doses (5.0 g/m^2 for children with high-risk or medium-risk ALL and 3.0 g/m^2 for children with low-risk ALL), we conducted a stratification analysis. All the 125 subjects were categorized according to the dose of MTX they administrated. However, we still find no significant associations between G870A polymorphism and risk of delayed elimination of MTX. We further analyzed the association between the plasma MTX level and G870A polymorphism, and the result showed that compared with carriers of GG genotype, subjects with AA genotype have a significant lower concentration of MTX. However, we did not detect the concurrent change in serum creatinine clearance level to evaluate altered renal function; therefore, we could not precisely explain the difference of serum MTX levels between GG and AA carriers. But studies have also demonstrated the association of MTX level with polymorphisms of folate metabolic genes [26, 27]. Although we reported the relation between 44 h serum MTX concentration and G870A polymorphism, we did not find a significant protective effect on the incidence of MTX delayed elimination of variant an allele. Further studies with a larger sample size should be conducted to validate our findings.

Relations between clinical response and systematic toxicity after MTX treatment and gene variation in the folate metabolic pathway have been detected many times [12, 26, 28, 29]. Among them, most studies have focused on MTHFR C677T and RFC G80A and suggested that these polymorphisms contribute to MTX therapy-related toxicity or outcome of childhood ALL. Only two studies genotyped *CCND1* G870A polymorphism. Irina Costea et al. observed that individuals with *CCND1* 870AA genotype had lower rates of leukopenia, thrombocytopenia and liver toxicity. They also found significant lower rates of these toxicities in individuals with a combination of the *CCND1* 870AA and MTHFR 677 TT/CT genotypes [30]. This observation may be explained by the variant isoform of CCND1 with a longer half-life and the correlated higher level of DHFR. However, Dulucq et al. did not find any association between toxicity parameters investigated and the increasing number of event-predisposing genotypes, including DHFR haplotype*1, *CCND1* 870AA and TS 3R3R. But they thought this was most likely due to the fact that in these 3 at-risk genotypes, only the CCND1, but not TS and DHFR, was individually associated with a lower frequency of toxic events [12].

On the contrary, in our study, subjects with 870AG and 870AA genotype have significant higher rates of MTX-related hepatotoxicity. We

also find a statistically significant relation between the hepatotoxicity and the combined genotypes AG/AA, which suggested that 870A allele may be a risk factor of toxic events. Our subgroup analysis showed that the association of G870A polymorphism and an increased risk of MTX-related hepatotoxicity was more pronounced in children administrated with MTX in a dose of 5.0 g/m^2 (high-risk ALL and medium-risk ALL). This discrepancy may be due to different genetic backgrounds and population-specific differences. Furthermore, there may be some other confounding factors which will interfere with our present investigation, such as some unknown gene variations in folate metabolic pathway. At the same time, the fact can not be ignored that our chemotherapy regimen is not identical to other research institutions [12, 30] and some chemotherapeutic drugs we used may also influence the evaluation of MTX-related hepatotoxicity.

One limitation of this study is that we failed to obtain data about coinstantaneously altered renal function so we can not give an exact explanation about the association between G870A polymorphism and elimination of serum MTX. Although the mechanism underlying this association has not been clarified, our present result suggested that genotyping of *CCND1* could be useful in the clinical prediction of MTX-related hepatotoxicity. Second, our sample size was relatively small, so it leads to a larger span of 95% CI. Thus, the association we report now should be verified in a larger population. Third, in the present study, we only explored two clinical indexes (MTX concentration and serum amino transferase). In further studies, other toxicities such as oral mucositis and vomiting should be observed.

Conclusion

Our data suggested that *CCND1* G870A polymorphism may contribute to the clearance of MTX and may be a genetic marker for the prediction of MTX-associated hepatotoxicity. Studies involving ethnically diverse populations and with a larger sample size should be conducted to verify our findings.

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Disclosure of conflict of interest

None.

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