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The *miR-1206* microRNA variant is associated with methotrexate-induced oral mucositis in pediatric acute lymphoblastic leukemia

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Five-year survival rates of pediatric acute lymphoblastic leukemia (ALL) have reached 90% in the developed countries. However, toxicity because of methotrexate (MTX) occurs frequently. Variety in the occurrence of toxicity is partly determined by single nucleotide polymorphisms (SNPs) in coding regions. Recently, five SNPs in non-coding pre-microRNAs and microRNA processing (miRNA) genes were identified in association with MTX-induced oral mucositis. This study aimed to replicate the association of these miRNA variants in relation to MTX-induced oral mucositis in a prospective childhood ALL cohort. Three out of five SNPs with a minor allele frequency more than 0.15 [CCR4-NOT transcription complex (*CNOT4*) rs3812265, *miR-1206* rs2114358, *miR-2053* rs10505168] were analyzed in 117 pediatric ALL patients treated with 5 g/m^2 MTX (DCOG ALL-10). Oral mucositis was defined as grade more than or equal to 3 according to the National Cancer Institute criteria. rs2114358 in *miR-1206* was associated with oral mucositis [odds ratio (OR): 3.6; 95% confidence interval (CI): 1.1–11.5], whereas we did not confirm the association of *CNOT4* rs3812265 (OR: 0.69; 95% CI: 0.27–1.80) and *miR*-2053 rs10505168 (OR: 2.50; 95% CI: 0.76–8.24). Our results replicate the association between rs2114358 in *miR-1206* and MTX-induced oral mucositis in childhood ALL. Genetic variation in *miR-1206* has potential as a novel biomarker to predict MTX-induced toxicity. *Pharmacogenetics and Genomics* 27:303–306

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Introduction

Acute lymphoblastic leukemia (ALL) is the most frequently occurring pediatric cancer, accounting for 20–25% of all malignancies. Treatment outcome has improved markedly, with 5-year survival rates exceeding 90% [1]. However, patients often suffer from toxicity of chemotherapeutic drugs such as methotrexate (MTX). Identifying predictors of the adverse effects of MTX would be valuable to select patients who can benefit from personalized therapy strategies [2].

Previously, we showed that oral mucositis occurs in 20% of patients in a prospective study of children with ALL treated with 5 g/m^2 MTX [3]. As the frequency and severity of toxicity vary among children, the influence of genetic variation on treatment-related toxicity in pediatric ALL has been addressed [4–6]. Most studies focused on coding regions, which correspond only to about 1.5% of the entire genome. Recently, awareness was raised of the important regulatory functions of non-coding regions such as miRNAs [7,8].

A recent retrospective study was the first to assess 118 non-coding single nucleotide polymorphisms (SNPs) in pre-miRNAs and miRNA processing genes in a cohort of 152 pediatric precursor B-ALL patients [9]. Five SNPs were associated with the development of MTX-induced oral mucositis, including three SNPs in genes of the RNA-induced silencing complex and two in pre-miRNAs. In the current prospective study, we aimed to replicate the

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role of these miRNA-related SNPs in MTX-induced oral mucositis in a prospective well-documented cohort of Dutch children with ALL.

Patients and methods

Patients

Children (1–19 years) with ALL treated according to the standard-risk and medium-risk arms of the Dutch Childhood Oncology Group ALL-10 protocol were eligible for this study (Fig. 1). Written Informed consent was obtained from all patients and their parents. The study was approved by the Medical Ethical Committee (MEC-2005-358). A description of the MTX treatment protocol has been reported previously [3] (Supplementary Fig. 1, Supplemental digital content 1, *http://links.lww.com/FPC/B222*). Data on developing MTX-induced oral mucositis were prospectively collected according to The National Cancer Institute (NCI) [10] Common Terminology Criteria for Adverse Events, v.3.0 score system (Supplementary Table 1, Supplemental digital content 2, *http://links.lww.com/FPC/B223*). Relevant clinical oral mucositis was defined as NCI grade more than or equal to 3.

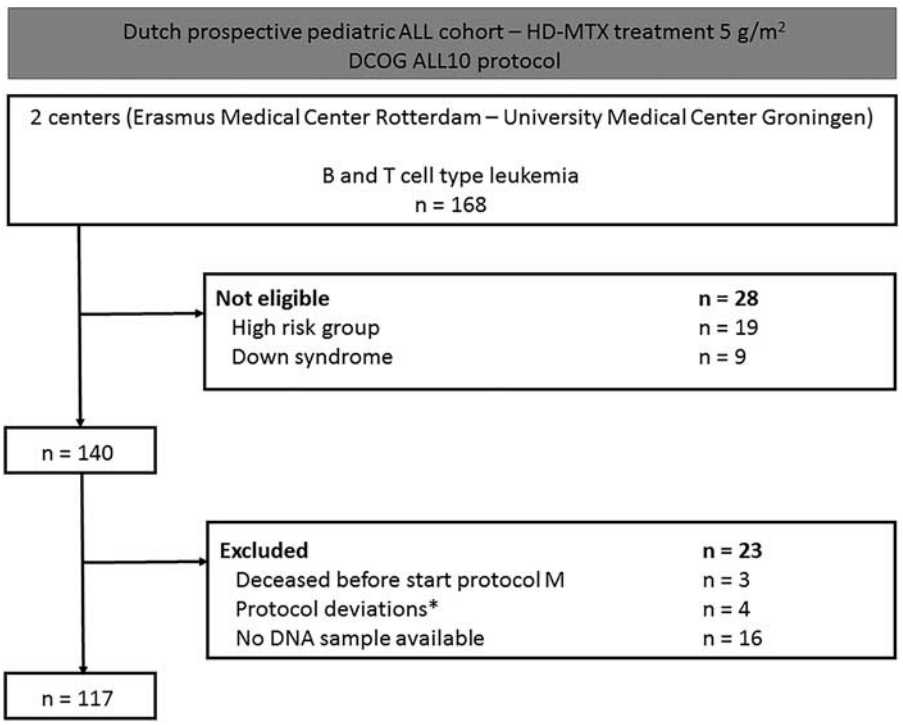
Genetic analysis

Out of the five previously described SNPs in miRNA function, three SNPs with a minor allele frequency more than 0.15 were considered for analysis, which made statistical analysis feasible in our cohort: CCR4-NOT transcription complex (*CNOT4*) (rs3812265), *miR-1206* (rs2114358), and *miR-2053* (rs10505168) [9]. Genomic DNA was extracted from peripheral blood before the start of MTX therapy in patients in remission using the MagnaPure Compact Nucleic Acid isolation kit (Roche Molecular Biochemicals, Almere, the Netherlands). Genotyping was performed using allele-specific PCR for rs3812265 (*CNOT4*) and a Taqman assay for rs2114358 (*miR-1206*) and rs10505168 (*miR-2053*) (Supplementary Table 2, Supplemental digital content 3, *http://links.lww.com/FPC/B224*).

Statistical analysis

CNOT4 (rs3812265) was studied in a dominant model (wild-type vs. heterozygote + variant); *miR-1206* (rs2114358) and *miR-2053* (rs10505168) were studied in a recessive model (wild-type + heterozygote vs. variant) using a χ^2-test based on power using SPSS Statistics, version 20.0.0.1 (SPSS Inc., Chicago, Illinois, USA). Univariate logistic regression was used to calculate odds ratio's and 95% confidence intervals. Multiple logistic regression analysis was used to examine the possible confounding effect of sex and age. Results were considered statistically significant when the *P*-value was less than 0.05.

Fig. 1



Flowchart of patient inclusion. ALL, acute lymphoblastic leukemia; HD-MTX, high-dose methotrexate; DCOG, Dutch Childhood Oncology Group; SNP, single-nucleotide polymorphism. *One patient had neurological damage before the start of HD-MTX treatment, one patient was transferred to another hospital, one patient had an adjusted protocol because of a SPINKS mutation, and one patient was initially treated otherwise because of another diagnosis. High-risk patients were excluded as they received a different treatment regimen with concomitant drugs. Patients with Down's syndrome were excluded as they received lower doses of MTX.

In-silico analysis

To investigate the in-silico impact of the SNPs on miRNA structure, the RNAfold web tool (*http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi*) was used [11]. This tool calculates the minimum free energy of secondary structures and the energy change ($\Delta\Delta G$) of the hairpin structure of the miRNAs.

Results

Baseline characteristics

A total of 117 pediatric ALL patients were included in the study (Fig. 1). Baseline characteristics are summarized in Supplementary Table 3 (Supplemental digital content 4, *http://links.lww.com/FPC/B225*). MTX-induced oral mucositis was observed in 18.8% of patients ($n = 22$).

Genotyping results

All three SNPs were in Hardy–Weinberg equilibrium, Supplementary Table 4 (Supplemental digital content 5, *http://links.lww.com/FPC/B226*). Univariate analysis showed that the homozygous GG genotype of rs2114358 (*miR-1206*) was associated significantly with an increased risk of MTX-induced oral mucositis (odds ratio: 3.6; 95% confidence interval: 1.1–11.5; $P = 0.024$) (Table 1). The SNPs rs3812265 in *CNOT4* and rs10505168 in *miR-2053* were not significantly associated with oral mucositis (Table 1). Age and sex did not affect these associations significantly.

In silico analysis rs2114358 miR-1206

In silico analysis predicted that the substitution of the G allele for an A allele in rs2114358 of *miR-1206* induced an energy change ($\Delta\Delta G$) of 1.8 kcal/mol (from -35.7 to -33.9 kcal/mol). This allelic change might induce a change in the secondary structure of mature *miR-1206* (Fig. 2).

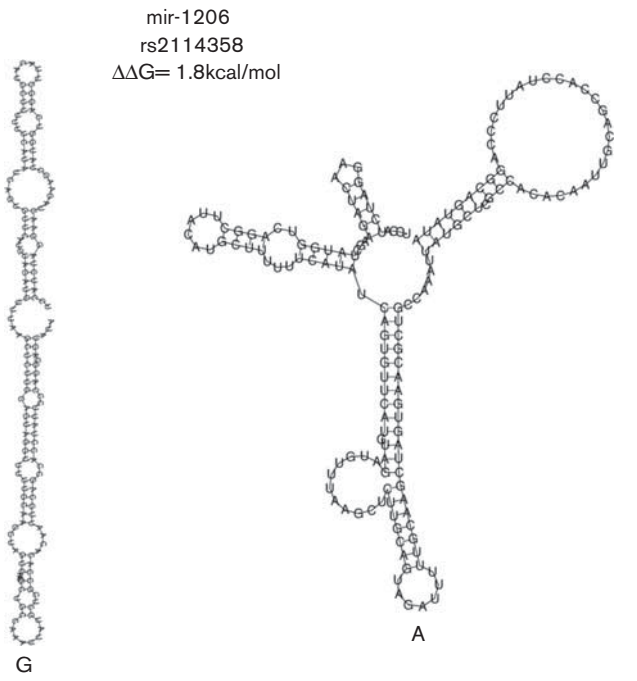
Discussion

In the present work, we replicate the association of rs2114358 in *miR-1206* with the development of MTX-induced oral mucositis in a prospective Dutch cohort of pediatric ALL patients. In our study, the likelihood of developing MTX-induced oral mucositis was 3.6-fold increased in carriers of the GG genotype.

In the previously reported retrospective cohort, the *miR-1206* GG genotype (rs2114358) was associated with a 4.6-fold increased likelihood of developing MTX-induced oral mucositis NCI grade more than or equal to 2 [9]. Except for this previous retrospective study, no information is available in the literature on the role of this germline SNP in toxicity. One study showed that rs2114358 in *miR-1206* affects the expression of mature miR-1206 [12]. However, this result was not consistent throughout different cell types and cell lines.

Our *in silico* analysis showed that the allelic change from G to A induces a positive energy change ($\Delta\Delta G = 1.8$ kcal/mol). It has been suggested that positive energy changes transform the miRNA hairpin from stable to unstable status and a decreased structure stability may reduce the mature

Fig. 2



In-silico analysis predicts that the substitution of the G allele (ancestral allele) for an A allele in rs2114358 of mir-1206 induced an energy change ($\Delta\Delta G$) of 1.8 kcal/mol (from -35.7 to -33.9 kcal/mol). This allelic change also induced a change in the secondary structure of mature *mir-1206*.

Table 1 Association between single nucleotide polymorphisms in pre-miRNAs and miRNA processing genes and mucositis (NCI ≥ 3)

Genes	SNPs	Genotype	No mucositis [*n* (%)]	Mucositis [*n* (%)]	*P*-value	OR	95% CI
CNOT4	rs3812265	CC	52 (79)	14 (21)	0.448	0.69	0.27–1.80
		CT/TT	43 (84)	8 (16)			
miR-1206	rs2114358	AA/AG	86 (84)	16 (16)	**0.024***	3.58	1.12–11.46
		GG	9 (60)	6 (40)			
miR-2053	rs10505168	TT/TC	85 (83)	17 (17)	0.123	2.50	0.76–8.24
		CC	10 (67)	5 (33)			

CI, confidence interval; *CNOT4*, CCR4-NOT transcription complex; NCI, National Cancer Institute; OR, odds ratio; SNP, single nucleotide polymorphism.
*Bold *P*- value < 0.05.

miRNA product [13]. The G allele, which is associated with the development of MTX-induced oral mucositis in our study, is the ancestral allele. The incidence of the A allele has been increasing over time, suggesting a possible advantage in evolution. The in-silico analysis suggests that the G allele carriers have a more stable mature *miR-1206* product, which possibly affects gene expression levels of target genes involved in MTX metabolism, leading to an increased risk of developing oral mucositis.

Of the three analyzed SNPs that were associated with MTX-induced oral mucositis in a previous study, we replicated the association of rs2114358 in *miR-1206*. However, after correction for multiple comparisons, the result did not remain statistically significant ($P = 0.05/3 = 0.017$). This is most likely because of the fact that our study numbers are relatively small. However, this SNP has potential as novel biomarker in future prediction models as this is the second study that shows an effect of rs2114358 in *miR-1206*.

Some differences exist between the Spanish cohort and our cohort. First, our study focused on clinically relevant toxicity (NCI ≥ 3), whereas the previously reported study used lower cut-off points (NCI ≥ 2). Second, the Dutch protocol used four doses of 5 g/m^2 MTX with leucovorin rescue 42 h after MTX infusion, whereas in the Spanish protocols, three doses of MTX at 3 or 5 g/m^2 and leucovorin rescue 36 h after the start of the infusion were administered.

Conclusion

We replicate the finding that rs2114358 in *miR-1206* is associated with the development of clinically relevant MTX-induced oral mucositis. Therefore, this genotype may be relevant for clinical practice. Further functional studies and larger studies are required to validate these results.

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Conflicts of interest

There are no conflicts of interest.

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