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ORIGINAL ARTICLE

Impact of genetic polymorphisms determining leukocyte/neutrophil count on chemotherapy toxicity

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Neutropenia and infection are major dose-limiting side effects of chemotherapy. The risk of initial infection and subsequent complications are directly related to the depth and duration of neutropenia. Recent genome-wide association studies identified variants in *DARC* and *CXCL2* genes, and in *ORMDL3-GSDMA-CSF3* locus on chromosome 17q21 that influence white blood cell and neutrophil counts in healthy individuals. To investigate whether polymorphisms in these loci in conjunction with chemotherapy may modulate risk of treatment complications, we analyzed 21 SNPs across these genes for an association with chemotherapy-related neutropenia and infection in 286 Caucasian children with acute lymphoblastic leukemia. After correction for multiple testing, *DARC* polymorphism *rs3027012* in 5′-UTR was associated with higher risk of low absolute phagocyte count (APC < 500 and < 1000 cells per microliter, $P = 0.001$ and $P < 0.0005$, respectively) and hospitalization due to febrile neutropenia ($P = 0.002$). Protective effect was instead seen for DARC *rs12075* A to G substitution ($P = 0.004$). The SNP *rs3859192* in the *GSDMA* were associated with hospitalization due to infection ($P = 0.004$); infection was also modulated in the additive manner by the *CXCL2 rs16850408* ($P = 0.002$). This study shows for the first time that the variations in *DARC, GSDMA* and *CXCL2* genes may play a role in the onset of chemotherapy complications.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy accounting for more than 25% of cancer cases. The implementation of multi-agent treatment protocol has remarkably increased the prognosis of children with ALL.[1] Despite this improvement, treatment-related toxicity remains unacceptably high. Chemotherapy-induced neutropenia is the most common hematological toxicity in children with ALL. In addition, febrile neutropenia (FN) remain a frequent cause of hospitalization, and infection is still a significant factor contributing to treatment-related mortality.[2] Increased susceptibility to chemotherapeutic agents through prolonged neutropenia represents a dose-limiting factor in the administration of chemotherapy regimens; 24% of patients treated on the Dana-Farber Cancer Institute (DFCI) ALL Consortium 05-01 protocol required reduction of 6-MP/MTX administration during consolidation due to leukopenia and neutropenia.[3] Furthermore, frequent reductions in chemotherapy dose intensity may compromise disease control and survival,[4] whereas longer duration of neutropenia leads to the increased risk of infection.[5] Severity of neutropenia can be influenced by a variety of factors, including chemotherapy regimen. White blood cell (WBC) and absolute neutrophil counts (ANC) can also be modulated by individual genetic factors. Lower counts are commonly observed in certain ethnic groups, in particular African–American descents[6–8] and to a lower extent in Arabic peninsula populations.[9] Single nucleotide polymorphisms (SNP) in the Duffy antigen receptor for chemokine (*DARC*) were the first to be associated with lower WBC, ANC and monocyte count.[10] *DARC rs2814778* SNP, which influences the expression of the Duffy antigen, has been linked to benign neutropenia in African–Americans.[11,12] The change of the Duffy expression has been suggested to alter the concentrations and distribution of plasmatic and tissue chemokines, thereby regulating neutrophil production and migration.[13,14] The same SNP was also associated with worse outcome in the case of acute lung injury occurring within the setting of critical illnesses.[15] This SNP is rare in Caucasians, but recent genome-wide association study (GWAS)[11] identified SNPs in the *CXCL2* (chemokine ligand 2) gene that were linked to the lower WBC and ANC across different populations including Europeans. CXCL2 is a chemokine implicated in neutrophil mobilization and recruitment through granulocyte colony-stimulating factor (GCSF or CSF-3). Interestingly, SNPs in the *ORMDL3-GSDMA-CSF3* locus were also linked to lower WBC in Europeans.[12,16,17] Clinical consequences of these findings are not yet clear, particularly in situations which may potentiate genetic effect, such as illness or anticancer treatment. Here we sought to investigate whether O*RMDL3-GSDMA-CSF3* locus as well as the *DARC* and *CXCL2* genes in conjunction with chemotherapy may predispose patients to higher risk of treatment-related complications. To test this, we interrogated an association between the polymorphisms in these genes and the onset and frequency of neutropenia and infection in childhood ALL patients.

MATERIALS AND METHODS

Patient population

The study population, described previously,[18,19] consisted of 286 Caucasian children diagnosed with ALL at the Sainte-Justine University Health Center (SJUHC), Montreal, between January 1989 and July 2005. The consecutively

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accrued patients for whom the DNA and clinical data were available underwent treatment with the DFCI ALL Consortium protocols DFCI 87-01, 91-01, 95-01 or 00-01.[20] The study is approved by Ethics Committee of SJUHC and all participants signed informed consent prior the enrollment to the study.

SNPs selection

SNPs in *DARC* and *CSF3* genes were selected from the 1000 Genomes database [http://1000genomes.org] based on their minor allele frequency (MAF) $\geqslant 5\%$ in Caucasians. TagSNPs were then selected by pairwise linkage disequilibrium (LD) r^2 statistics[21] implemented in Haploview among SNPs spanning ~2 kb upstream and downstream of the gene. Eleven tagSNPs were identified in *DARC* and five in *CSF3* gene (Supplementary Figures 1a and 1b, respectively). Additionally, SNPs identified through GWAS, as associated with WBC in the *ORMDL3-GSDMA*[16,18] and *CXCL2*[11] loci, were also analyzed. These included two SNPs in *CXCL2* locus (*rs9131* and *rs16850498*)[11] (Supplementary Figure 1c), whereas among five GWAS hits[16,17] two (*rs4794822* and *rs8078723*) were in full LD (r2 = 1) with tagSNP in *CSF3* (rs1042658), and three remaining (*rs17609240*, *rs3894194*, *rs3859192*) were included in the analysis (Supplementary Figure 1d). Genotypes were obtained using genotyping platforms at McGill University and Genome Quebec Innovation Center or by allele-specific oligonucleotide hybridization as previously described[22] (Supplementary Table 1). SNPs are presented as major-to-minor allele substitutions and were all in Hardy–Weinberg equilibrium.

Outcome measures

Clinical outcomes were defined as neutropenic or infectious complication and included episodes of febrile neutropenia, low absolute phagocyte count (APC), proven infections and hospitalization due to infection and neutropenia. The data were collected during consolidation and maintenance treatment through medical chart reviews. The occurrence of these episodes (at least one vs none) and their severity, assessed by the frequency of each of them in a total number of chemotherapy cycles, or by the length of hospitalization per total length of treatment, were assessed through association analysis.

The APC was calculated as the sum of the absolute neutrophil count (ANC) and absolute monocyte count (AMC); APC $< 0.5 \times 10^9$ cells per liter and APC $< 1 \times 10^9$ cells per liter are both used in DFCI protocols as a threshold for either a delay in treatment or dose modification, and were both also used in the analysis. Infections were graded using National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 3.0 http://www.ctep.cancer.gov. Higher grades (grades 3 and 4) were considered; grade 3 assumes infection that requires intravenous administration of antibiotics (with or without hospitalization), and grade 4 an infection that causes sepsis or death. Hospitalization due to infection (viral, bacterial and fungal) was defined based on clinical and/or microbiological evidence of infection, but without neutropenia that required hospitalization and the use of oral or parenteral antibiotics. Hospitalization due to FN included children with leukemia who presented with fever and neutropenia and are hospitalized for parenteral antibiotics with and without documented infection.

Statistics

Associations of three genotypes with binary clinical outcomes were evaluated using χ^2 test; *P*-values for all associations are given in Supplementary Table 2. A false discovery rate (FDR) described in Storey *et al.*[23] was used to adjust for multiple testing (including all polymorphisms and all outcomes studied). The cutoff *P*-value, when controlling FDR at 15% was 0.01. Odds ratio (OR) with 95% confidence interval (CI) for SNPs that sustained correction for multiple testing was calculated in comparison to major allele homozygotes (Table 1). On the basis of the frequency distribution, ORs were also estimated according to the appropriate genetic model. The same model was then used to assess an association between associated genotypes and the severity of outcomes (frequency of APC reduction, frequency of infectious complications and the length of hospitalization) using Mann–Whitney test (Figure 1). Multivariate analysis was performed by logistic regression and included beside genotypes associated in univariate analysis, age, sex, risk group, protocol and type of corticosteroid (prednisone or dexamethasone) received. SNPs that correlated with chemotherapy complications were also analyzed for an association with event-free- and overall survival (EFS and OS) by Kaplan–Meier analysis.

All analyses were performed by SPSS statistical package (Chicago, ILm USA), version 20.0.

RESULTS

Polymorphisms that sustained correction for multiple testing were retained for further analysis; details are depicted in Table 1. The summary of association analysis for all polymorphisms is provided in Supplementary Table 2. Two SNPs in the *DARC* gene (*rs12075* and *rs3027012*) were associated with hospitalization due to FN. The protective effect in additive manner was seen for *rs12075* A to G substitution; risk decreased with the number of G alleles (OR = 0.6, 95% CI = 0.4–0.8 $P = 0.004$, Table 1). The minor T allele of *rs3027012* was in contrast associated with the higher risk of FN-related hospitalization (OR = 2.2, 95% CI = 1.3–3.5, $P = 0.002$). The same allele conferred higher risk of APC either below 500 cellsper microliter (OR = 2.4, 95% CI = 1.5–4.0, $P = 0.001$) or 1000 cells per microliter (OR = 14.4, 95% CI = 1.9–108.3, $P < 0.0005$). Carriers had longer hospitalization due to FN ($P = 0.002$, Figure 1a) and had higher number of cycles, with APC reduction compared to patients without this allele ($P \leqslant 0.002$, Figures 1b and c).

In *CXCL2* locus, the minor *rs16850408* A allele correlated in additive manner with the increased risk of high grade infection (OR = 1.7, 95% CI = 1.2–2.4, $P = 0.002$, Table 1). The analysis of *ORMDL3-GSDMA-CSF3* revealed that the carriers of minor T allele of *rs3859192* in *GSDMA* gene had higher risk of hospitalization due to the infection (OR = 2.2, 95% CI = 1.3–3.7, $P = 0.004$, Table 1) and longer hospitalization time ($P = 0.009$, Figure 1d).

All SNPs remain significant in logistic regression analysis when analyzed individually with non-genetic factors. When both *rs12075* and *rs3027012* SNPs associated with FN-related hospitalization were entered in the multivariate model, only *rs3027012* remained significant, suggesting its major influence or correlation between two of them. Indeed, when their combined effect was analyzed, highest risk (OR = 3.2, 95% CI = 1.2–6.3) was noted for *rs3027012* TT/CT on the background of non-protective *rs12075* AA genotype (Figure 2) None of the SNPs associated with neutropenia or infection influenced significantly EFS or OS probability.

DISCUSSION

Two polymorphisms (*rs12075* and *rs3027012)* in the *DARC* gene were found associated with FN-related hospitalization. The *rs12075* lowered the risk of FN-related hospitalization in additive manner. This SNP leads to Gly42Asp replacement differentiating Duffy Fya and Fyb antigens and has been associated with WBC counts in the subjects of African ancestry.[12] The association was also noted in Europeans, but did not reach genome-wide significance level.[12] Because erythrocyte Darc may act both to limit free chemokine activity and to present chemokine to key local environments when needed, genetic variation may have significant implications.[24] The *rs12075* was studied in relation to a number of phenotypes and it was reported to influence serum levels of monocyte chemoattractant protein-1 (MCP-1) levels, a chemokine that is central to the inflammatory process. Circulating MCP-1 is bound by the Darc and its concentrations are elevated in sepsis, autoimmune diseases and cancer. This polymorphism was also reported to influence the IL8 concentrations, the chemokine sequestration ability and the metastatic potential in breast cancer.[25–29] The most notable effect of *DARC* polymorphisms seen in our study was that of *rs3027012*. The minor T allele was associated with higher risk of hospitalization due to FN and remarkably higher risk of APC reduction. Almost all patients that were carriers of minor T allele had APC below 1×10^9cells per liter during at least one chemotherapy cycle. There was also an association with the severity of these complications; T allele carriers had higher number of cycles with APC reduction and longer hospitalization periods. The risk of FN seems to be highest

Table 1. Association of genetic polymorphisms in *DARC, CXCL2* and *GSDMA* with neutropenic and infectious complications during childhood ALL treatment

Phenotype	*Gene*	*SNP*	*Subst.*	*Gen.*	*Complications* N *(%)*		*OR (95% CI)*	P	*Gen.*	*Complications* N*(%)*		*OR (95% CI)*	P
					+	–				+	–		
FN	DARC	rs12075	A > G	AA	61 (45.5)	47 (31.8)	1					0.6 (0,4–0,8)[a]	0.004
				AG	60 (44.8)	71 (48)	0.7 (0.4–1.1)	0.1					
				GG	13 (9.7)	30 (20.3)	0.3 (0.2–0.7)	0.004					
		rs3027012	C > T	CC	74 (54.0)	107 (71.8)	1		CC	74 (54)	107 (71.8)	1	
				CT	56 (40.9)	38 (25.5)	2.1 (1.3–3.5)	0.004	CT/TT	63 (46)	42 (28.2)	2.2 (1.3–3.5)[d]	0.002
				TT	7 (5.1)	4 (2.7)	2.5 (0.7–9)	0.1					
APC < 0.5		rs3027012	C > T	CC	78 (53.4)	103 (73.6)	1		CC	78 (53.4)	103 (73.6)	1	
				CT	61 (41.8)	33 (23.6)	2.4 (1.5–4.1)	0.0007	CT/TT	68 (46.6)	37 (26.4)	2.4 (1.5–4)[d]	0.001
				TT	7 (4.8)	4 (2.9)	2.3 (0.7–8.2)	0.2					
APC < 1		rs3027012	C > T	CC	159 (60.5)	22 (95.7)	1		CC	159 (60.5)	22 (95.7)	1	
				CT	94 (35.7)	0 (0.0)	—	0.02	CT/TT	104 (39.5)	1 (4.3)	14.4 (1.9–108.3)[d]	< 0.0005
				TT	10 (3.8)	1 (4.3)	1.4 (0.2–1.3)	0.3					
Infection grade 3/4	CXCL2	rs16850408	C > A	CC	36 (26.1)	57 (39.3)	1					1.7 (1.2–2.4)[a]	0.002
				CA	69 (50.0)	71 (49.0)	1.6 (1–2,8)	0.07					
				AA	33 (23.9)	17 (11.7)	3.1 (1.5–6.3)	0.002					
Infection hospit.	GSDMA	rs3859192	C > T	CC	25 (22.1)	65 (38.2)	1		CC	25 (22.1)	65 (38.2)	1	0.004
				CT	67 (59.3)	74 (43.5)	2.4 (1.3–4.2)	0.003	CT/TT	88 (77.9)	105 (61.8)	2.2 (1.3–3.7)[d]	
				TT	21 (18.6)	31 (18.2)	1.8 (0.9–3.6)	0.1					

Abbreviations: APC, absolute phagocyte count; CI, confidence interval; FN, hospitalization due to febrile neutropenia; Gen, genotype; infection hospit, hospitalization due to high-grade infection; OR, odds ratio; Subst, substitution. OR is estimated for heterozygous and homozygous individuals in comparison to major allele homozygotes and for appropriate genetic model (dominant[d] or additive[a], given on the right-hand side of the table). All SNPs are presented as a substitution from major to minor allele. Frequencies of genotypes (summing to 100%) are calculated for each group, defined as presence (+) or absence (−) of indicated complication. APC 0.5 and 1×10^9 cells per liter.

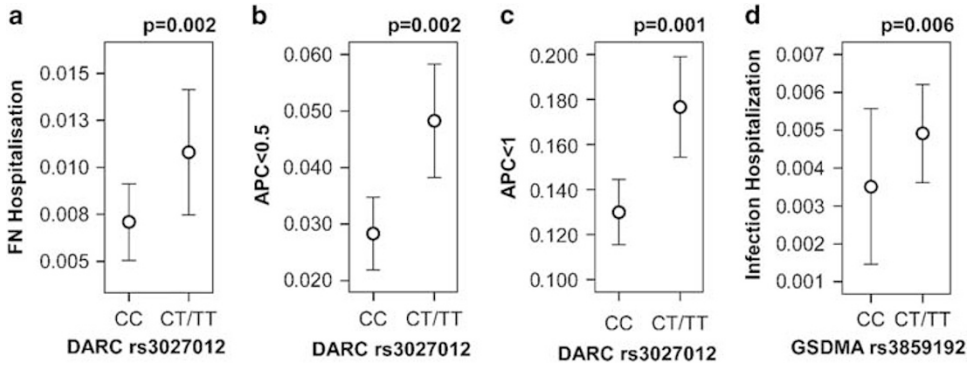

Figure 1. *DARC* and *GSDMA* gene genotype in relation to the frequency of neutropenia and duration of hospitalization. DARC rs3027012 is plotted against hospitalization due to febrile neutropenia (a), APC below 0.5×109 cells l^{-1} (b) and 1×109 cells l^{-1} (c). Relation between GSDMA rs3859192 and hospitalization due to infection (d). Average frequency (number of episodes per total number of cycles) and average length of hospitalization with 95% CI are given for individuals with and without indicated genotypes. *P*-value for the difference between groups, obtained by Mann–Whitney test is indicated on the plots. APC, absolute phagocyte count, FN, febrile neutropenia.

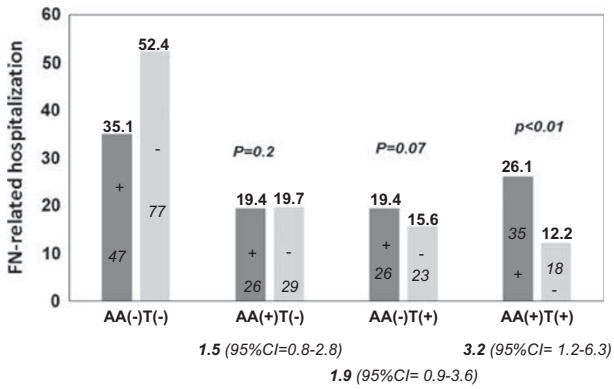

Figure 2. Frequency of hospitalization due to febrile neutropenia in relation to combined effect of *DARC* variations. Hospitalization due to FN is dichotomized as (+) or absence (−) of at least one FN episode. Risk genotypes are AA for *rs12075* (absence of protective G allele) and presence of T allele (CT/TT) for *rs3027012*; − and +, absence and presence of risk genotype(s), respectively. OR with 95% CI and *P*-values are indicated for each genotype combination, estimated relative to the absence of both risk alleles.

on the background of non-protective *rs12075* AA genotype. The *rs3027012* SNP had so far not been investigated, yet it is located in the 5′-UTR of *DARC* gene and is predicted to affect several transcription factors binding sites. Among those is the site for Nkx5-2 homeodomain transcription factor involved in Wnt signaling; some of the Wnt ligands have been shown to influence neutrophil recruitment and inflammatory response.[30,31] Among other *DARC* polymorphisms, *rs2814778,* which has been associated with benign neutropenia in African–Americans, has influenced outcome in patients with lung injury in this population,[17] as well as susceptibility to asthma and atopy, susceptibility to HIV and clinical course in infected patients,[32–34] suggesting that *DARC* polymorphisms might have clinical consequences under particular 'environmental' influences (for example, disease, treatment or cellular environment). Indeed, it was recently suggested that although there is no substantial differences in neutrophil function between patient with and without benign neutropenia, subtle differences could be unmasked in the setting of critical illnesses.[13]

Among polymorphisms in the *ORMDL3-GSDMA-CSF3* locus, which were identified through GWAS to influence WBC count in Europeans,[12,16] one SNP (*rs3859192* located in *GSDMA* gene) correlated with higher risk of infection-related hospitalization and longer hospitalization time. Other groups have linked *ORMDL3-GSDMA* region to neutrophil count levels,[35] inflammatory disorders including asthma, Crohn's disease and type 1 diabetes,[36–38] *ORMDL3* and *GSDMA* mRNA levels, and IL-17 secretion.[39] The function of this locus is not clear and its association with WBC and neutrophil counts was thought to be related in part to the proximity of *CSF3* gene, which stimulates proliferation, differentiation and survival of neutrophil precursors.[40] However, no clear association of *CSF3* polymorphisms with WBC was reported in Europeans.[17] In our study, *CSF3* polymorphisms (at least those analyzed) did not correlate with treatment-related complications.

Among polymorphisms of CXCL2 locus that influenced WBC count levels at the genome-wide significance levels,[11] *rs16850408* was associated in additive manner with an increased risk of high-grade infection in patients with childhood ALL. CXCL2 binds to chemokine receptor promoting rapid release of neutrophils from the bone marrow, thereby elevating blood neutrophil counts during infection or during CSF-induced neutrophil mobilization.[41] A repeat polymorphism (*−665(AC)n*) located in the *CXCL2* promoter has been previously reported to contribute to the development of severe sepsis in post-surgical patients.[42]

In conclusion, this is the first study to our knowledge to show that variants in *DARC, GSDM* and *CXCL2* loci may influence onset and severity of chemotherapy-related neutropenia and infection in children with ALL. The role of these polymorphisms should be evaluated in other populations, and should also be confirmed, though replication and prospective studies in order to better define the feasibility and necessity to adapt chemotherapy or adapt the degree of antibiotic prophylaxis regimen tailored to these SNPs. Notable effect of *rs3027012* in the 5′-UTR of *DARC* gene, as shown by an association with several studied phenotypes, warrants further attention through functional studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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