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The effect of granulocyte colony stimulating factor receptor gene missense single nucleotide polymorphisms on peripheral blood stem cell enrichment

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ABSTRACT

Granulocyte-colony stimulating factor (G-CSF) has become the most effective agent supporting hematopoietic stem cell transplantation (HSCT). The cognate interaction between G-CSF and its specific receptor, G-CSFR, induces the mobilization of HSCs and increases their pool in the peripheral blood. G-CSFR has a highly conserved structure which may be functionally modulated by the presence of missense single nucleotide polymorphisms (SNPs). In this study, we asked whether the missense SNPs in G-CSFR could affect the response to G-CSF in HSCT patients and donors. Here, for the first time, G-CSFR missense SNPs were screened and minor allele frequencies were determined in a specific population with Turkish racial background. Five (rs3917991, rs3918001, rs3918018, rs3918019, and rs146617729) out of 16 missense SNPs screened were determined with minor allele frequencies lower than 0.04. Subsequent association analyses indicated potential impact of rs3918001, rs3918018, and rs3918019 minor alleles on peripheral blood $CD34^+$ cell enrichment. Although their frequency is rather low, certain missense SNPs, especially which are placed in the conserved regions of G-CSFR may possess the capacity to influence the response to G-CSF treatment.

1. Introduction

G-CSF, a hematopoietic growth factor, has become the most commonly used agent for mobilizing the hematopoietic stem and progenitor cells (HSPCs) for clinical transplantation [1–3]. Under physiological conditions, the $CD34^+$ HSPCs constitute less than 0.05% of the white blood cells (WBCs) [4,5]. Upon subcutaneous administration of G-CSF, the amount of $CD34^+$ cells can considerably increase, peaking between the days 4 and 7. For a successful hematopoietic reconstitution with fast hematopoietic recovery and low incidence of graft-versus-host disease, high numbers of HSPCs must be transferred [2–4]. However, the amount of HSPCs mobilized by G-CSF tends to vary across individuals. In addition, a significant proportion of individuals (up to 5%) may not respond to G-CSF or fails to mobilize sufficient number of cells. Poor mobilization can occur in 25% of patients with hematological malignancies and 10–20% in healthy donors [3,6,7].

The receptor for G-CSF, G-CSFR, is a member of Type-I cytokine/hematopoietin receptor family characterized with four highly conserved cysteine residues and a tryptophan–serine repeat (WSXWS) located in the cytokine receptor homology (CRH) region of the extracellular domain [8,9]. Alternative splicing of G-CSFR mRNA produces five isoforms differing only in the transmembrane or intracellular domains. The 'isoform A' (regarded as the native isoform) is the most widely distributed and highly expressed G-CSFR isoform. Yet, the physiological role of other isoforms remains not very well defined [10,11].

Human G-CSFR gene is mapped to the chromosome 1p32-35. It contains 17 exons encoding an 812-813 amino acid-long transmembrane protein [8,12]. There are certain mutations and a polymorphism in the G-CSFR gene which have been reported to perturb signaling and increase susceptibility to certain myeloid cell disorders such as severe congenital neutropenia (SCN), myeloid dysplastic syndrome (MDS), and acute myeloid leukemia (AML). Most of these genetic alterations are on the intracellular domain of the receptor carrying three conserved motifs required for transduction of proliferation and differentiation signals [8,13,14].

Here, we hypothesized that the missense SNPs in the G-CSFR gene may be responsible for the G-CSF response variations in HSPC

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enrichment. Therefore, we determined the frequency of sixteen SNPs in Turkish HSPC transplantation donors and recipients treated with G-CSF. Additionally, association analyses were performed with the PBSC enrichment data between minor and major allele carriers.

2. Materials and methods

2.1. Study subjects and peripheral blood stem cell collection data

This study was conducted on the archived samples of hematopoietic stem cell transplantation (HSCT) patients or healthy donors who undergone therapy in Hacettepe University Oncology Hospital, Stem Cell Transplantation Unit between May 2001 and August 2011. The study group included 54 healthy donors and 249 HSCT patients treated with recombinant G-CSF analog (10 μg/kg/day; Roche, Milan, Italy). The summary of patient and healthy donor data is given in Table 1. The absence of JAK2-V617F mutation (JAK2 MutaScreen Assay, Ipsogen, Luminy Biotech, Marseille, France) was confirmed in the study subjects.

The first day of G-CSF administration was accepted as "day 0" and PBSC collection was started on "day 5 or 6" for the healthy donors and on "day 5–10" for the patients as decided by the specialist in the bone marrow transplantation unit according to the rise in leukocyte number. PBSC collection was continued on every other day until a minimum of 5×10^6 $CD34^+$ cells/kg obtained.

White blood cell (WBC), total mononuclear cell (MNC) counts and viability of the cells in the apheresis product were routinely determined. The amount of PBSCs was calculated after determination of the percentage $CD34^+$ cells by flow cytometry. Briefly, the cells were labeled with mouse anti-human CD34 (clone 8G12; Becton Dickinson, San Jose, CA, USA) or mouse IgG1κ isotype control (clone 40×, Becton Dickinson) monoclonal antibodies and the analysis was performed on an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA). The total amount of $CD34^+$ cells/kg was calculated by using the formula: [(Dilution factor × WBC count/ml × total collection volume)/weight (kg)] × $CD34^+$% cells. The archived apheresis samples and patients' data were used with the permission of Hacettepe University Local Ethics Committee (Approval No. FON09/32).

2.2. Determination of SNPs by PCR-RFLP

Genomic DNA isolation from the apheresis products stored in liquid nitrogen was performed with QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Fourteen regions of the G-CSFR gene (NG_016270.1) reported to potentially contain 16 missense SNPs were amplified with PCR using forward and reverse primers designed (Supplementary Table 1).

Table 1
Summary of the subjects' data enrolled in the study.

Study groups	Gender		Age (mean ± SD)	
	Male	Female	Male	Female
Non-Hodgkin's lymphoma	50	28	44 ± 13	38 ± 15
Multiple myeloma	54	27	55 ± 10	54 ± 10
Hodgkin's lymphoma	29	24	31 ± 14	31 ± 10
Germ cell cancer	13	2	32 ± 10	34 ± 18
Acute myeloid leukemia	5	6	48 ± 16	35 ± 11
Other disorders[a]	6	5	38 ± 13	29 ± 16
Healthy donor	26	28	N.A.	N.A.
Total	183	120		

N.A., Not available.
[a] Other disorders: rhabdomyosarcoma, nasopharyngeal cancer, primary amyloidosis, neuroblastoma, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and mantle-cell lymphoma (MCL).

PCRs were carried out in a reaction mixture containing 1× PCR buffer, 0.2 μM primer oligonucleotides, 0.2 μM dNTP mix, 2.5 mM $MgCl_2$ and 0.05 U/μl DNA polymerase (DyNAzyme II, Finnzymes, Espoo, Finland) using the cycling conditions; 30″ at 94 °C, 30″ at specific annealing temperatures for each primer pair, 30″ at 72 °C (GeneAmp PCR system 9700, Applied Biosystems, CA, USA).

PCR products were digested with restriction endonucleases (New England Biolabs, Hitchin, UK or Fermentas International, Vilnius, Lithuania). The list of restriction enzymes, size of PCR products and the size of post-digestion DNA fragments expected according to minor and major alleles are given in Supplementary Table 1. PCR-RFLP products were resolved on 2% agarose or 20% polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized under UV light (*Kodak Gel Logic* 1500 Imaging System, Carestream Health, Rochester, NY, USA).

2.3. DNA sequence analysis

The validation of determined minor alleles was performed by DNA sequencing. The amplified sequences were analyzed by automatic DNA sequencing on forward strands using the sense primers (Supplementary Table 1) (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems, CA, USA). The comparative analysis of the nucleotide and translated amino acid sequence was carried out using the NCBI – BLAST (National Center for Biotechnology Information, Basic Local Alignment Search Tool, USA) network server.

2.4. Statistical and prediction analyses

All minor alleles determined were subjected to functional interference prediction analyses performed with two online available programs, the Sort Intolerant from Tolerant (SIFT) [15] and the Polymorphism Phenotyping v2 (PolyPhen-2), running based on the sequence homology, and the phylogenetic and structural properties of a given polymorphic sequence. The median and interquartile range (IQR) of each group was analyzed by using the SPSS software (version 13.0, IBM, Chicago, IL, USA). Where required for multiple testing in statistical analysis, corrections were performed according to Bonferroni method.

3. Results

By using the publicly available SNP and genomic DNA sequence databases, primer oligonucleotides were designed and appropriate restriction endonucleases were selected for the restriction fragment length polymorphism (RFLP) analysis of 16 missense SNPs located in the human G-CSFR gene (Supplementary Fig. 1A). Following the RFLP analysis, confirmation of the minor allele genotypes was performed with DNA sequencing (Supplementary Fig. 1B).

In HSCT patients and donors ($N = 303$), out of 16 missense SNPs analyzed, only five (rs3917991, rs3918001, rs3918018, rs3918019, and rs146617729) were detected with minor alleles (Table 2). Haplotype frequencies were calculated by using the data obtained from the total study group. The probability of any of these 5 minor alleles SNPs to be carried was 0.014. Accordingly, the most common SNPs rs3917991 and rs3918018 were determined with the minor allele frequencies 1.65% and 0.99%, respectively. rs3918018 was the only SNP identified with a minor allele homozygous genotype. There was no significant difference between the minor allele frequencies of patient and healthy donor groups. In addition, minor alleles of missense SNPs in G-CSFR were randomly distributed within sub-groups of the patients diagnosed with different

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