Genetic polymorphisms of *CYP3A4*, *GSTT1*, *GSTM1*, *GSTP1* and *NQO1* and the risk of acquired idiopathic aplastic anemia in Caucasian patients

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Background and Objectives. Various drugs and xenobiotics are involved in the pathogenesis of acquired aplastic anemia. Their harmful potential depends on the amount of exposure to them and on the detoxifying capacity of the recipient. Genetic polymorphisms of some important detoxifying enzymes are associated with low or absent cata-lytic activity of the protein. We assessed whether, in a Caucasian population, low or null activity polymorphisms of CYP3A4, GSTT1, GSTM1, GSTP1 and NQO1 were associated with the risk of developing aplastic anemia and with the response to immunosuppressive therapy.

Design and Methods. In 77 Caucasian patients with aplastic anemia and in 156 normal controls we evaluated the distribution of the following polymorphisms which are associated with low or no activity of the corresponding enzyme: (i)-290 A→G of the *CYP3A4* gene, deletions of (ii) *GSTT1* and (iii) *GSTM1* genes, (iv) 313A→G of the *GSTP1* gene and (v) 609 C→T of the *NQO1* gene.

Results. The distribution of the genotypes of all tested polymorphisms was not different in patients and controls. No differences were seen among the patients when the group was subdivided by age and severity of the disease. Only the *GSTM1* null genotype was significantly more frequent in male patients than in male controls. The frequency of all tested polymorphisms did not differ in patients who did or did not respond to immunosuppressive therapy.

Interpretations and Conclusions. The low/null activity polymorphisms of the detoxifying enzymes CYP3A4, GSTT1, GSTM1, GSTP1 and *NQO1* are not associated with the risk of developing aplastic anemia or to the response to immunosuppressive therapy in Caucasian patients.

Keywords: CYP3A4, GSTs, NQO1, aplastic anemia.

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According to one of the current models, idiopathic aplastic anemia would result from the destruction of hematopoietic cells by an autoimmune attack that represents the final common pathway of different initial triggering events occurring at the level of the stem cell and including genetic changes as well as damage induced by viruses, drugs and chemicals.[1] Numerous drugs and xenobiotics[2-4] have been indirectly implicated in the pathogenesis of idiopathic acquired aplastic anemia. A faulty catabolism of these substances and/or their metabolites might enable them to exert their effects on the stem cell in a more prolonged or intense way thus facilitating, via T-cell activation, damage to the hematopoietic cells and the development of aplastic anemia. Some detoxifying enzymes present genetic polymorphism encoding for unstable, less active or absent protein which may reduce their catalytic activity. Cytochromes (CYP3A4),[5-6] glutathione S-transferases (GSTT1, GSTM1,[7-10] GSTP1[11]) and NAD(P)H: quinone oxidoreductase (NQO1)[12] are implicated in the metabolism of substances both involved in the genesis of aplastic anemia, such as non-steroidal anti-inflammatory drugs,[2,13] benzene and its metabolites, and used in its treatment, such as steroids and cyclosporine A.[5-6]

A single nucleotide polymorphism (-290 A→G) in the 5'-flanking region of the *CYP3A4* gene has been associated with reduced levels of the corresponding protein[14] in Caucasian subjects. *GSTT1* and *GSTM1* genes have a deletion which, in the homozygous state, leads to the absence of the protein (null genotype). The *GSTP1* gene displays a polymor-

phism (313A→G) (NCBI dbSNP: rs 947894) producing an isoleucine to valine substitution at codon 105, this being responsible for a less efficient variant enzyme. The *NQO1* point mutation polymorphism (609 C→T) (NCBI dbSNP: rs1800566) produces a proline to serine substitution that destabilizes and inactivates the protein.[12]

We investigated whether null or reduced activity polymorphisms of the enzymes CYP3A4, GSTT1, GSTM1, GSTP1, and NQO1, which metabolize compounds that may induce aplastic anemia, were associated with the risk of developing idiopathic aplastic anemia in a Caucasian population. Since these enzymes may also be involved in the metabolism of drugs used in the immunosuppressive treatment of aplastic anemia, we also assessed whether the polymorphisms might influence the response to this therapy.

Design and Methods

Seventy-seven Caucasian patients, diagnosed as having idiopathic aplastic anemia according to International criteria,[15] entered the study.

Samples were studied after informed consent had been given by the parents of the patients or, whenever eligible, from the patients themselves. Table 1 shows the characteristics of the patients. Their polymorphisms were compared with those observed in 156 Caucasian children (99 [63%] males, 57 [37%] females, median age 4.08, range 0-19.6 years) who were hospitalized in Institutions throughout the Italian territory for conditions (e.g trauma) for which a genetic background can be excluded. Within the population of patients the distribution of the polymorphisms was also analyzed according to response to immunosuppressive treatment.

Table 1. Characteristics of the 77 patients with idiopathic aplastic anemia.

	N° (%)
Age at diagnosis (years)	median 12.6 range1.2-59.0
Sex	
Male	41 (53)
Female	36 (47)
Severity of aplastic anemia°	
not severe	10 (13)
severe	51 (66)
very severe	13 (17)
n.d.	3 (4)
Immunosuppressive therapy according to the EBMT protocol§	59 (77)
Eligible for evaluation 1 year after immunosuppressive therapy	52 (67)
Responders	41 (53)
Non-responders	11 (14)
Bone marrow transplant&	12
Other immunosuppressive therapy (steroids)*	12
Cytogenetics	
Normal	56 (73)
Abnormal@	4 (22)
n.d.	17 (5)

*°The severity was defined according to the criteria of Camitta.[15] Very severe aplastic anemia is defined as a polymorphonuclear cell count (PMN) < 0.2×10⁹/L; §the EBMT Severe Aplastic Anemia Working Party Protocol consists of anti-lymphocyte globulin 1.5 vials/10kg/day, day 1-5; cyclosporine A 5 mg/kg/day, day 1-180; methylprednisolone 2 mg/kg/day i.v. day 1-5 and thereafter tapering off; granulocyte colony-stimulating factor 5 µg/kg/day: ^responders were defined as patients who achieved and maintained transfusion independence of red cells and platelets and PMN>500/m³ at 1 year after starting therapy. The remaining were considered non-responders (n= 11); &in the case of patients who underwent bone marrow transplantation (BMT), pre-transplant samples were utilized;*patients could be counted more than once. i.e. 5 patients who did not respond to two or more cycles of immunosuppressive-therapy then underwent BMT, and 3 patients received other immunosuppressive-therapy (steroid) before undergoing BMT; @pathological cytogenetics: (1) 46 XY 50%/trisomy 1q, monosomy20q, -t1:20 50%; (2) 46 XY, -7,+8 [17]/46XY[7]; (3) 47 XX, +8; (4) 46 XX 70%/47XX, +15 30%. n.d.: not determined.*

Polymorphism analysis

Genotyping was performed on DNA extracted from bone marrow (patients) or peripheral blood (controls) samples. The genotypes for *GSTM1* and *GSTT1* were determined by polymerase chain reaction (PCR) as previously described by Chen *et al.*[16] This assay distinguishes two categories for each *GSTM1* and *GSTT1*. One, *present*, being either homozygous or heterozygous for *GSTM1* or *GSTT1* and the other, *null* as having a homozygous deletion of *GSTM1* or *GSTT1*. The *GSTP1* genotype was analyzed by PCR/restriction fragment length polymorphism (RFLP) studies of codon 105 according to Harries *et al.*[17] This assay distinguishes genotypes homozygous for the wild type allele (AA), heterozygous (AG) and homozygous for the variant allele (GG).

The C609T polymorphism of the *NQO1* gene was analyzed by standard PCR/RFLP as previously described.[18] Based on genotype analysis, wild type individuals (CC) were assigned to the high NQO1 activity category, while those heterozygous (CT) or homozygous (TT) for the C609T polymorphism, were assigned to the low activity category. Genotyping of *CYP3A4* was performed by standard PCR/RFLP as described elsewhere.[19]

Statistical analysis

Descriptive statistics were reported as absolute frequencies and percentages for qualitative data. Comparisons of genotypes between patients and controls, and between responders and non-responders to immunosuppressive treatment was per-

Table 2. Distribution of *CYP3A4*, *NQO1*, *GSTP1*, *GSTM1* and *GSTT1* genotypes between cases and controls.

Polymorphism	*Genotype*	*Patients with aplastic anemia N° (%)*	*Controls N° (%)*	*p*
CYP3A4-290A→G	AA (wild type)	65 (88)	135 (92)	
	AG (low activity)	6 (8)	5 (8)	0.18
	GG (low activity)	3 (4)	4 (3)	
NQO1 609 C→T	CC (wild type)	49 (64)	90 (62)	
	CT (low activity)	25 (33)	48 (33)	0.62
	TT (low activity)	2 (3)	8 (5)	
GSTP1 313 A→G	AA (wild type)	40 (53)	77 (50)	
	AG (low activity)	33 (44)	63 (41)	0.25
	GG (low activity)	2 (3)	13 (9)	
GSTM1	M present	33 (43)	77 (50)	
	M null	44 (57)	76 (50)	0.29
GSTT1	T present	64 (83)	118 (82)	
	T null	13 (17)	26 (18)	0.69
GSTM1/GSTT	M null/T null	8 (10)	13 (8)	0.61
Other combinations of GSTM1 and GSTT1			69 (90)	143 (92)

For technical reasons it was not possible to analyze all the genotypes in all the eligible patients.

formed by means of the χ^2 test or the Fisher's exact test in the case of expected frequencies less than 5. All the statistical tests were two sided; a p value <0.05 was considered as statistically significant. The statistical package *Stata* (STATA, release 7, Stata Corporation, College Station, TX, USA) was used to perform all the analyses.

Results

The frequencies of *CYP3A4*, *GSTT1*, *GSTM1*, *GSTP1* and *NQO1* polymorphisms were very similar (Table 2) in the patients and in the controls. Analysis by severity of the disease,[15] age and gender, did not show differences between patients and controls for any of the polymorphisms except for the *GSTM1* null genotype that was significantly (p=0.012) more frequent in aplastic males (70.7%) than in controls male (47.4%).

Regarding the response to treatment (Table 3) there was no significant difference in the frequency of any of the analyzed polymorphisms between the group of the patients responding to immunosuppression and the group not responding.

The frequency of *CYP3A4*, *GSTT1*, *GSTM1*, *GSTP1* and *NQO1* mutated/null genotypes in our control population overlapped those reported for Caucasians in the literature.[20-24] This was also true for the *GSTT1* and *GSTM1* null genotypes when the subjects analyzed were divided by gender.[25]

Discussion

This study is one of the largest conducted in the setting of idiopathic aplastic anemia in Caucasian populations both regarding the sample size and the number of the tested polymorphisms. Our data indicate that genetic polymorphisms of the detoxifying enzymes GSTT1, GSTM1, GSTP1, CYP3A4 and NQO1 are not associated with the risk of developing idiopathic aplastic anemia, suggesting that these polymorphisms do not play a major role in the pathogenesis of idiopathic aplastic anemia in Caucasian patients.

Whereas two former studies on *GSTM1*[26-27] in Caucasian subjects were in agreement with ours, another study, performed on a Korean population,[28] showed a significant association between *GSTM1* and *GSTT1* null genotypes and the risk of aplastic anemia. It is noteworthy that the *GSTT1* and *GSTM1* null genotypes are more frequent in the normal Korean population (*GSTT1* 45.3 %, *GSTM1* 60%) than in our Caucasian controls (*GSTT1* 18%, *GSTM1* 50%). This reflects genetic variation between the two races and is in keeping with the three-fold increased frequency of aplastic anemia in the Far East.

The greater frequency of the *GSTM1* null genotype in male patients with aplastic anemia than in normal male subjects has no obvious explanation. The *GSTM1* null genotype is reported not to be influenced by sex in Caucasians[21] and, when analyzed according to gender, the distributions of the *GSTM1* genotypes in our controls matched those in other normal Caucasian control groups.[21] We cannot exclude that this difference has occurred by chance.

Our *GSTT1* data do not agree with findings in Caucasian North American,[26] German[27] and Korean[28] populations that showed an association between *GSTT1* deletion and aplastic anemia. Whereas in the Koreans a genetic difference may account for the discrepancy, there is no obvious explanation for the diversity with other Caucasian patients since the frequency of null *GSTT1* genotype in our normal controls overlaps that observed in other Caucasian subjects among whom genetic variations of glutathione S-transferases are reported to be very slight.[21]

Table 3. Distribution of CYP3A4, NQO1, GSTP1, GSTM1 and GSTT1 in 52 patients with aplastic anemia eligible for response evaluation 1 year after immunosuppressive therapy divided into those responding (n=41) and those not responding (n=11) to this therapy.

Polymorphism	*Genotype*	*Responders N° (%)*	*Non-responders N° (%)*	*p*
CYP3A4 -290A→G	AA (wild type)	36 (90)	9 (90)	0.86
	AG +GG (low activity)	3+1 (10)	1(10)	
NQO1 609 C→T	CC (wild type)	24 (60)	6 (55)	0.66
	CT + TT (low activity)	14+2 (40)	5 (45)	
GSTP1 313 A→G	AA (wild type)	19 (46)	7 (64)	0.51
	AG+GG (low activity)	20+2 (54)	4 (36)	
GSTM1	M present	15 (37)	5 (45)	0.60
	M null	26 (63)	6 (55)	
GSTT1	T present	33 (80)	9 (82)	0.92
	T null	8 (20)	2 (18)	
GSTM1/GSTT1	M null/ T null	4 (10)	1 (9)	1
	Other combinations of GSTM1 and GSTT1	37 (90)	10 (91)	

For technical reasons it was not possible to analyze all the genotypes in all the eligible patients.

It is worth noting that in the cases of *GSTM1* and *GSTT1*, the null polymorphism is a gene deletion that causes total absence of the enzyme. Thus the lack of correlation of the above polymorphisms with aplastic anemia also indicates that the absence of these detoxifying systems does not affect the risk of developing this disease in Caucasian subjects.

None of the tested polymorphisms was associated with differences in response to immunosuppressive therapy. This suggests that they do not influence the outcome of patients with aplastic anemia receiving such treatment. It is likely that factors other than metabolic genes, such as the number of residual stem cells or the pressure of the activated immune system on the hematopoietic compartment, may have a more important influence on the outcome of the disease after immunosuppression. Since our study focused only on the role of metabolic polymorphisms, toxic exposure was not investigated. Indeed, very scanty data are available in this respect on our patients. However the lack of association between metabolic polymorphisms and aplastic anemia suggests that toxic exposure is one of the etiological factors of this disease which is worth further investigation.

In summary our study is against the tested metabolic polymorphisms having an important role in the risk of developing aplastic anemia. This does not mean that detoxifying systems do not affect the risk of developing this disease at all. Other enzymes interacting with drugs and toxic agents involved in stem cell damage may be involved. Moreover, since GSTP1, CYP3A and NQO1 activity may be influenced by substrates, inducers and inhibitors,[29-32] it is possible that low activity polymorphisms may not always faithfully reflect the true *in vivo* enzymatic effect. Other factors, such as the characteristics of the exposure to toxics, may turn out to play a more relevant role in the multifactorial pathogenesis of aplastic anemia.

CD conceived and designed the study, analyzed and interpreted the data, wrote the manuscript, revised it critically and finally approved it; JS collected the data of the patients, participated to the interpretation of the data, revised the paper critically and and finally approved it; AB, participated in the design of the study, revised the paper critically and finally approved it; DL participated in the

interpretation of the data, revised the paper critically and finally approved it; SV participated in the interpretation of the data, revised the paper critically and finally approved it; API participated in the design of the study , revised the paper critically and finally approved it; FB carried out the statistical analysis, participated to the interpretation of the data, revised the paper critically and finally approved it; AL participated in the design of the study, revised the paper critically and finally approved it; GM participated in the interpretation of the data, revised the paper critically and finally approved it; UR participated in the interpretation of the data, revised the paper critically and finally approved it; ML participated in the design of the study, carried out the molecular analysis of the samples, participated to the interpretation of the data, revised the paper critically and finally approved it.

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