Genetic polymorphism of CYP2D6, GSTM1 and NAT2 and susceptibility to haematological neoplasias

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Xenobiotic-metabolizing enzymes constitute an important line of defence against a variety of carcinogens. Many are polymorphic, constituting the basis for the wide inter-individual variation in metabolic capacity and possibly a source of variation in the susceptibility to chemical-induced carcinogenesis. The aim of this study was to determine the existence of any association between the main genetic polymorphisms of cytochrome P450 2D6 (CYP2D6), glutathione S-transferase M1 (GSTM1) and N-acetyltransferase 2 (NAT2) and an altered risk for haematological neoplasias. A total of 160 patients and 128 controls were genotyped by means of PCR–RFLP-based assays. Mutated alleles comprising CYP2D6*4, GSTM1*0, NAT2*5A, *5B, *5C, *6 and *7 were analysed along with the wild-type alleles. The results showed a higher frequency of CYP2D6 extensive metabolizers carrying two functional alleles in the leukaemia group, when compared with controls (76.6 versus 57.0%, P = 0.008). No differences were found in the case of Hodgkin and non-Hodgkin lymphomas. Analysis of the GSTM1 and NAT2 polymorphisms failed to show an association with any of the neoplasias, although a near significant increase in fast acetylators was also found in the leukaemia group (50.0 versus 35.9%, P = 0.06). The results suggest an association of extensive metabolism with an increased risk for leukaemia, possibly by an increase in the metabolic activation of chemical carcinogens or linkage to another cancer-causing gene. Opposite findings presented in other studies may reflect geographical differences in the type of environmental carcinogens to which different populations are exposed.

Introduction

Xenobiotic-metabolizing enzymes constitute one of the first lines of defence against environmental chemicals and are present in all higher organisms. A number of enzymatic systems, covering a vast range of substrates, have evolved as an adaptive response to environmental aggression and include the cytochrome P450s, the glutathione S-transferases and the N-acetyltransferases (1). Many of these enzymes are genetically polymorphic and are responsible for wide inter-individual variation in drug metabolism (2). This variation may also result in different patterns of susceptibility to the effects of carcinogenic substances and several studies have attempted to link genetic polymorphisms of xenobiotic-metabolizing enzymes [most commonly of cytochrome P450 2D6 (CYP2D6), glutathione S-transferase M1 (GSTM1) and N-acetyltransferase 2 (NAT2)] with an altered risk for some cancers, particularly those with an important environmental contribution, such as lung, bladder and colon cancer (for reviews see refs 3,4).

CYP2D6 (debrisoquine hydroxylase) is one of the most thoroughly studied enzymes since lack of its activity is the basis for adverse events occurring during therapy with some drugs (5). About 5–10% of Caucasians have inactivating mutations in both alleles of the CYP2D6 gene (22q13.1) and have been designated poor metabolizers (PM) (6). The most frequent inactivating mutation among Caucasians is the splice site G1934A transition (CYP2D6*4 allele) that causes a truncated protein (7). Other rarer alleles have been described and correlated with variable metabolic activity (8–10).

The glutathione S-transferases (GSTs) have been classified into four classes of cytosolic enzymes (GSTA, M, P and T) and a micosomal enzyme, with further subdivisions (11). The GSTM1 locus (1p13) has at least three allelic variants, GSTM1*A and *B, which differ by a single base in exon 7, and GSTM1*0, in which the entire gene is deleted. Individuals homozygous for GSTM1*0 (GSTM1 null genotype) express no protein and represent ~50% of most Caucasian populations (12). GSTM1 metabolizes a wide range of toxic and mutagenic compounds, including some arising from phase I metabolism by cytochrome P450s.

The N-acetyltransferases (NATs) are responsible for the acetylation of amino, hydroxyl and sulphhydryl groups of many compounds, including a large number of arylamine carcinogens (13). The well-known bimodal distribution in the rate of elimination of isoniazid (an antituberculosis drug) has been the basis for the classic division of patients into fast and slow acetylators. The NAT locus (8p22) comprises two functional genes (NAT1 and NAT2) and a pseudogene (NATP) (14). NAT2 has long been known to be responsible for the inter-individual variation in acetylation rate and several alleles have been described (15,16). The most frequent NAT2 alleles consist of the wild-type fast allele NAT2*A and five slow alleles NAT2*A5A, *5B, *5C, *6 and *7 (6). The presence of at least one wild-type allele confers a fast acetylator phenotype, whereas two mutant alleles are required for the slow acetylator phenotype.

Many xenobiotic-metabolizing enzymes act sequentially in the disposal of toxic substrates. Drugs or carcinogens can be activated into more toxic forms by cytochrome P450s (phase I metabolism) and then conjugated by GSTs or NATs (phase II metabolism) to increase the solubility of the product in such a way as to facilitate its excretion (17,18).

Considering the role in detoxification played by these enzymes, the existence of common polymorphisms and previous reports of their association with some cancers (3,4), and considering the possibility of an environmental contribution

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to haematological malignancies, we aimed at determining whether any association exists between the main genetic polymorphisms of CYP2D6, GSTM1, NAT2 and an altered susceptibility to haematological neoplasias.

Materials and methods

Subjects

A group of 160 unrelated Portuguese patients with haematological neoplasias was studied (84 males, 76 females, mean age ± SD 49.9 ± 21.1). This group was divided according to the type of neoplasia into acute lymphocytic leukemia (51 cases), acute myeloid leukemia (13 cases), acute myeloid leukaemia (18 cases), chronic myeloid leukaemia (11 cases), non-Hodgkin lymphoma (71 cases) and Hodgkin lymphoma (25 cases). All patients were undergoing chemotherapy, on an outpatient basis, at the University Hospital of Coimbra, Portugal. A control group of 128 unrelated Portuguese Caucasian volunteers (56 males, 72 females, mean age ± SD 30.8 ± 14.2) was also studied. Controls were recruited among members of hospital and faculty staff and students and were considered healthy with no history of cancer or other chronic diseases. All subjects were from the same ethnic group. Apart from a few minority groups, the Portuguese are virtually an ethnically homogeneous population, genetically related to other Iberian populations (19). Age differences between cases and controls were not considered to influence the study since previous data showed no correlation between genotype and age (20,21). All subjects gave their informed consent. Ten millilitre venous blood samples drawn into EDTA were obtained from each subject and genomic DNA was isolated by conventional methods (22).

CYP2D6 genotyping

A PCR–RFLP assay was used for the detection of the G1934A mutation responsible for the poor metabolizer allele CYP2D6*4, as described by Gough et al. (7). Amplification was carried out in a 50 µl reaction volume containing 150 ng of primer 5′-GCTGGCACACAACTCCG-3′, 150 ng of primer 5′-AAATCTGCTTTCTCCGAGGC-3′, 1 U of Taq DNA polymerase, 200 µM each of dNTP, 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris–HCl (pH 9.0), 5% DMSO and 100–200 ng of DNA. After an initial denaturation at 94°C for 5 min, 30 cycles were performed consisting of annealing at 60°C for 1 min, extension at 72°C for 2 min and denaturation at 94°C for 1 min. This yielded a 334 bp fragment. Digestion of 20 µl of the PCR product was carried out with 5 U of the restriction enzyme BstNI. Fragments were then separated on an 8% polyacrylamide gel and stained with ethidium bromide.

The frequency of individuals presenting with a combination of the homozygous extensive metabolizer and fast acetylator genotypes were also determined and shown to be significantly increased in the leukaemia group compared with controls [43.8 versus 20.3%, P = 0.0007, odds ratio 3.05 (95% CI 1.58–5.88)].

Discussion

The results showed a higher frequency of CYP2D6 homozygous extensive metabolizers in the leukemia group when compared with the control group [76.6 versus 57.0%, P = 0.008, odds ratio 2.46 (95% CI 1.25–4.84)]. An increase in the wild-type allele frequency was also observed in this group [85.2 versus 75.4%, P = 0.028, odds ratio 1.87 (95% CI 1.06–3.29)]. No significant differences were observed in the lymphoma groups.

Frequencies of NAT2 fast and slow acetylators in the control group were 35.9 and 64.1%, respectively. No significant differences were observed in any neoplasia, although the frequency of fast acetylators in the leukaemia group showed an increase approaching statistical significance [50.0 versus 35.9%, P = 0.06, odds ratio 1.78 (95% CI 0.97–3.28)]. The GSTM1 null genotype was present in 57.8% of controls and did not differ significantly from patients.

The frequency of individuals presenting with a combination of the homozygous extensive metabolizer and fast acetylator genotypes were also determined and shown to be significantly increased in the leukaemia group compared with controls [43.8 versus 20.3%, P = 0.0007, odds ratio 3.05 (95% CI 1.58–5.88)].

NAT2 genotyping

Analysis of the six most common NAT2 alleles was based on the assay described by Smith et al. (24), which is >95% predictive of phenotype. Aliquots of 200–400 ng of DNA were amplified in a 100 µl reaction volume containing 250 ng of primer 5′-TGTGGTGATACATACACAAGGG-3′, 2 U of Taq DNA polymerase, 200 µM each dNTP, 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris–HCl (pH 9.0) and 5% DMSO. After an initial denaturation at 94°C for 5 min, 34 cycles were performed consisting of annealing at 52°C for 1 min, extension at 72°C for 1 min and denaturation at 94°C for 1 min. Products were run on a 2% agarose gel and stained with ethidium bromide. P1 and P2 can anneal either to the GSTM1 or the GSTM4 gene if they give rise to a 157 bp fragment which serves as an internal control of the PCR reaction. P3 anneals only to the GSTM1 gene and, together with P1, amplifies a 230 bp fragment which is specific for the GSTM1 gene. This method does not distinguish heterozygotes from wild-type homozygotes.

Statistical analysis

χ2 tests were used to examine differences of allele and genotype frequencies between patients and controls. When group size was <40 or when expected values in contingency tables were less than five, Fisher’s exact test was used. Possible interactions between genotypes shown to be significantly different in patients and controls were also studied and combined frequencies analysed by the same procedure.

Results

Allele and genotype frequencies of CYP2D6, GSTM1 and NAT2 are presented in Tables I, II and III, respectively.

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Table I. CYP2D6 genotype and allele frequencies in patients and controls (%)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 128)</th>
<th>All neoplasias (n = 160)</th>
<th>All leukaemias (n = 64)</th>
<th>ALL (n = 22)</th>
<th>CLL (n = 13)</th>
<th>AML (n = 18)</th>
<th>CML (n = 11)</th>
<th>NHL (n = 71)</th>
<th>HL (n = 25)</th>
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<tbody>
<tr>
<td>EM</td>
<td>57.0</td>
<td>71.3*</td>
<td>76.5b</td>
<td>77.3</td>
<td>83.3</td>
<td>63.6</td>
<td>64.8</td>
<td>76.0</td>
<td></td>
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<tr>
<td>HEM</td>
<td>36.7</td>
<td>23.1</td>
<td>17.2</td>
<td>18.2</td>
<td>7.7</td>
<td>16.7</td>
<td>27.3</td>
<td>29.6</td>
<td>20.0</td>
</tr>
<tr>
<td>PM</td>
<td>6.3</td>
<td>5.6</td>
<td>6.3</td>
<td>4.5</td>
<td>15.4</td>
<td>0</td>
<td>9.1</td>
<td>5.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Allele

| wt       | 75.4               | 82.8c                    | 85.2d                   | 86.4         | 80.8        | 91.7        | 77.3        | 79.6        | 86.0        |
| m        | 24.6               | 17.2                     | 14.8                    | 13.6         | 19.2        | 8.3         | 27.7        | 20.4        | 14.0        |

ALL, acute lymphocytic leukaemia; CLL, chronic lymphocytic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; NHL, non-Hodgkin lymphoma; HL, Hodgkin lymphoma.

EM, homozygous extensive metabolizer (two functional alleles); HEM, heterozygous extensive metabolizer (one functional allele); PM, poor metabolizer (no functional alleles); wt, wild-type; m, mutant (CYP2D6*4).

*Statistically significant relative to controls, \( P \leq 0.012 \).

**Statistically significant relative to controls, \( P \leq 0.008 \).

***Statistically significant relative to controls, \( P \leq 0.028 \).

Table II. GSTM1 genotype frequencies observed in patients and controls (%)

<table>
<thead>
<tr>
<th>GSTM1*a</th>
<th>Controls (n = 128)</th>
<th>All neoplasias (n = 160)</th>
<th>All leukaemias (n = 64)</th>
<th>ALL (n = 22)</th>
<th>CLL (n = 13)</th>
<th>AML (n = 18)</th>
<th>CML (n = 11)</th>
<th>NHL (n = 71)</th>
<th>HL (n = 25)</th>
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<tr>
<td>42.2</td>
<td>42.5</td>
<td>40.6</td>
<td>36.4</td>
<td>61.5</td>
<td>44.4</td>
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<td>43.7</td>
<td>44.0</td>
<td></td>
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<tr>
<td>57.8</td>
<td>57.5</td>
<td>59.4</td>
<td>63.6</td>
<td>38.5</td>
<td>55.6</td>
<td>81.8</td>
<td>56.3</td>
<td>56.0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations as in Table I.

*a Gene present (includes both homozygotes and heterozygotes).

Table III. NAT2 genotype and allele frequencies observed in patients and controls (%)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 128)</th>
<th>All neoplasias (n = 160)</th>
<th>All leukaemias (n = 64)</th>
<th>ALL (n = 22)</th>
<th>CLL (n = 13)</th>
<th>AML (n = 18)</th>
<th>CML (n = 11)</th>
<th>NHL (n = 71)</th>
<th>HL (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast/Fast</td>
<td>6.2</td>
<td>5.6</td>
<td>9.4</td>
<td>0</td>
<td>23.1</td>
<td>11.1</td>
<td>9.1</td>
<td>1.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Fast/Slow</td>
<td>29.7</td>
<td>36.9</td>
<td>40.6</td>
<td>40.9</td>
<td>30.8</td>
<td>50.0</td>
<td>36.4</td>
<td>38.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Slow/Slow</td>
<td>64.1</td>
<td>57.5</td>
<td>50.0*</td>
<td>59.1</td>
<td>46.1</td>
<td>38.9</td>
<td>54.5</td>
<td>60.6</td>
<td>68.0</td>
</tr>
</tbody>
</table>

Allele

| *4      | 21.1               | 24.1                     | 29.7                    | 20.5         | 38.5        | 36.1        | 27.3        | 20.4        | 20.0        |
| *5A     | 3.1                | 1.9                      | 1.6                     | 4.5          | 0          | 0           | 0           | 2.8         | 0           |
| *5B     | 37.9               | 42.2                     | 38.3                    | 40.9         | 34.6        | 38.9        | 36.4        | 45.1        | 44.0        |
| *5C     | 2.3                | 1.3                      | 0.8                     | 0            | 3.8         | 0           | 0           | 1.4         | 2.0         |
| *6      | 32.8               | 29.1                     | 29.7                    | 34.1         | 23.1        | 25.0        | 36.5        | 28.2        | 30.0        |
| *7      | 2.7                | 1.6                      | 0                       | 0            | 0           | 0           | 2.1         | 2.1         | 4.0         |

Abbreviations as in Table I.


*\( P \leq 0.06 \).

Other inactivating mutations may exist, however, studies indicate that this mutation occurs in ~80% of Caucasian PMs (7). In our case, this would mean that the true proportion of PMs in the leukaemia group would be 7.9% (6.3% \( \times 80\% \)), meaning misclassification of one (1.6%) individual. This would not have modified our results substantially, even if we assumed that no change occurred in the control group.

Interestingly, a previous study reported an increase in PMs among leukaemia patients (27). It was hypothesized that PM individuals were at increased risk for this neoplasia because of the impaired detoxification of an unidentified chemical. This apparent discrepancy in results, which has also been reported for lung cancer (28), could reflect geographical differences in environmental exposure to carcinogens.

At this point we cannot rule out the possibility that the apparent increase in homozygotes is due to loss of heterozygosity of peripheral leukocytes used for DNA extraction. Regarding this last point, it is interesting to note that in our study there was also a decrease in the frequency of CYP2D6 heterozygotes among leukaemia patients (the same occurred in the previously mentioned study; 27). Allele loss by cells used for genetic analysis could be a source of bias, since the genotyping assay does not distinguish between the homozygous and any hemizygous state. Further studies are required to clarify this point, for example by using other cells as the source of genomic DNA.

Another explanation for the findings would be the existence of linkage disequilibrium of the CYP2D6 locus with other neighbouring genes involved in carcinogenesis.

We also investigated whether other fully active genotoxin-
metabolizing enzymes were over-represented in our patients by analysing the polymorphisms of the GSTM1 and NAT2 genes, which have frequently been associated with predisposition to other neoplasias (3,4). GSTM1 analysis failed to reveal any association with the neoplasias considered in this study. As far as NAT2 analysis is concerned, no significant difference in fast and slow acetylator frequencies was detected between patients and controls, however, there was a near significant increase in fast acetylators in the leukaemia group \( P = 0.06 \), odds ratio 1.78 (95% CI 0.97–3.28)], thus confirming the overall tendency for higher metabolic activity in this group. There have also been reports of associations between fast acetylation and lung (29) and colon cancer, including a recent study with Portuguese patients (30), although it is unclear whether they share common causative factors with the cancers in the present study.

In order to assess the existence of any interaction between the overexpressed genotypes, we calculated the frequency of the simultaneous presence of homozygous EM and fast acetylator genotypes and found a clear increase in the leukaemia group \( P = 0.0007 \); odds ratio 3.05 (95% CI 1.58–5.88)], which suggests a synergistic effect of both genotypes.

In the light of these results, one cannot ignore the fact that all the patients were recruited from a chemotherapy centre. Patients with higher metabolic activity might dispose of cytostatic drugs at a higher rate than that appropriate for adequate remission therapy, thus requiring longer periods of treatment (31). If this is so, then at any given time one would expect to find a higher proportion of active metabolizers among patients undergoing chemotherapy, constituting a source of selection bias. However, this is unlikely to have influenced the results since patients were enrolled in the study before initiating the chemotherapy cycles. Further studies are also underway to assess the influence of these phenotypes on treatment efficacy.

In conclusion, our findings seem to suggest an influence of genetic polymorphisms of xenobiotic-metabolizing enzymes, particularly CYP2D6 polymorphisms, on the susceptibility to some haematological neoplasias, possibly by an increase in the metabolic activation of chemical carcinogens or linkage to another cancer-causing gene. Contradictory data presented in other similar studies may imply a different pattern of carcinogen metabolism or linkage to some haematological neoplasias, possibly by an increase in genetic polymorphisms of xenobiotic-metabolizing enzymes, thus confirming the clinical relevance and why do they exist? Am. J. Hum. Genet., 60, 265–271.


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