Increased frequency of wild-type arylamine-\(N\)-acetyltransferase allele NAT2*4 homozygotes in Portuguese patients with colorectal cancer

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Here we report that colorectal cancer patients show a markedly higher frequency (3-fold) of wild-type NAT2*4 allele homozygotes than the control population. However, a marked difference in NAT2*4/NAT2*4 genotype frequency associated with the patients gender was observed pointing to a male-specific effect of this genotype as a risk factor in colon cancer. The arylamine-\(N\)-acetyltransferase (E.C. 2.3.1.5.) NAT2, a phase II detoxification enzyme, has been implicated in procarcinogen activation, namely from food contained arylamines, cigarette smoking, as well as environmental amines of various types. NAT2 is encoded by a polymorphic gene presenting several allelic variants encoding partially inactive enzymes expressed in human liver and colon. Epidemiological studies based on phenotype determination have long indicated the importance of the NAT2 active phenotype as a susceptibility factor in colorectal cancer. In the present study we investigated the NAT2 allelic frequencies and genotype distribution in a group of 114 unrelated colorectal cancer patients, in parallel with 201 healthy Portuguese subjects. We first demonstrate that the frequency of the wild-type NAT2*4 allele in the Portuguese sample population (23.4%) does not significantly differ from the values described for other Europeans. Besides the 3-fold higher frequency of NAT2*4 homozygotes found in colorectal cancer subjects, the NAT2*4/NAT2*5A compound genotype, known to determine a faster acetylator phenotype than other heterozygotic combinations, also increased by the same order of magnitude. These two genotypes represent 32% of the patients population versus 11% of the healthy controls. Taken together, our results strongly indicate that NAT2 genotype, particularly NAT2*4 allele zygosity, constitutes an individual susceptibility trait associated with sporadic colorectal cancer development, probably due to the local dietary habits in Portugal.

Introduction

NAT2, an arylamine-\(N\)-acetyltransferase (E.C. 2.3.1.5.), is one of the main enzymes that participates in the bioconversion of heterocyclic amines into electrophilic nitrogen ions, which are important ultimate carcinogens that are directly implicated in tumour initiation processes (1,2). NAT2, expressed at high levels in the liver, is encoded by a polymorphic gene presenting several nucleotide substitutions, all of them resulting in partially inactive enzymes (3). Consequently, the presence of the different alleles in each individual genome produces a broad range of metabolic phenotypes that vary from the fully active homozygous rapid metabolizers to the less active allele homozygous slow metabolizers. Although no strict limits can be established between NAT2 phenotype groups, essentially three types have been defined, the rapid, the slow and the intermediate metabolizers, the latter being heterozygous and bearing one wild-type allele (4).

NAT2 acetylation is considered a potential risk factor in the aetiology of certain multifactorial malignancies upon heavy exposure to environmental chemicals, including cigarette smoking. In bladder cancer, co-segregation has been found with the slow acetylator phenotype (5,6), while in lung cancer patients, a significant overrepresentation of rapid acetylators was reported (7). Epidemiological studies based on phenotype determination have long indicated the importance of acetylation as a susceptibility factor in colorectal cancer (8–11). Consistently, colon carcinogenesis has been related to the mutagenic properties of heterocyclic amines, compounds that are structurally similar to the well-known colon carcinogen 3,2’-dimethyl-4-aminobiphenyl (DMABP*), where NAT2 catalysis results in \(N\)-hydroxy-DMABP and subsequent DNA binding (12). Conversely, an increased risk in breast cancer was described among NAT2 slow acetylators who are post-menopausal cigarette-smoking women, illustrating heterogeneity in response to carcinogenic exposure (13).

Aromatic amines, such as pyridines, quinoxalines and quinolines, are formed in cooked fish and meat, as well as in peptide pyrolysates (14–16). In the liver cell, these compounds are \(N\)-hydroxylated by inducible P450 enzymes, giving rise to phase II enzyme substrates such as acetyltransferases. \(O\)-Acetylation catalyzed by NAT2*4 (17) results in reactive electrophiles that bind covalently to DNA giving rise to mutation and potential tumour initiation (1). The liver expressed NAT2 determines the blood levels of these ultimate carcinogens, which particularly affect gut tissues where the enzyme is also expressed (18). However, further characterization of the NAT2 genotype in colorectal cancer patients has not shown a direct correlation with the phenotype data that indicate rapid acetylation as a potential trait of susceptibility to colorectal cancer. Such results were found in studies on a small sample population of mixed African- and Caucasian-Americans (19), in the United Kingdom (20,21) and in Japan (22).

It is known that NAT2 allele relative frequencies may vary between different anthropological groups (23,24). The proportion of slow and rapid acetylators has long been observed to widely differ in populations from different geographic areas and anthropological origins. The frequency of slow acetylators has been found to range between 90% in Moroccans and 5% in Canadian Eskimos. In Europe, it is generally admitted that rapid and slow phenotypes are equally represented (25), although a relative variation between different population groups cannot be excluded as indicated by previous data (26).
Consequently, NAT2 genotyping should be carried out in each different population group to allow a better evaluation of relative allele frequencies as potential individual susceptibility traits in sporadic cancers. Moreover, the degree of exposure to food-borne procarcinogenic arylamines depends on the particular lifestyle of the individual, which is considered to determine an important environmental risk factor, namely in gut malignancies (27). Of particular relevance are the dietary habits, which may significantly vary in the different geographic areas and countries.

In the present work, we investigated the NAT2 allelic frequencies and genotype distribution in a group of 201 healthy Portuguese subjects, in parallel with 114 unrelated colorectal cancer patients. The results strongly support the idea that the NAT2 genotype constitutes an important individual trait that contributes to the multifactorial aetiology of sporadic gut malignancies, probably in association with local nutritional habits characteristic of this Southern Europe area.

Materials and methods

Subjects

All experimental protocols were approved by the Ethical Boards for clinical trials of the Centro de Saúde de Oeiras and the Instituto Português de Oncologia. The cases were 114 unrelated colon cancer patients from the Lisbon area or South/Central Portugal, average age: 64.24 years (SD = 10.99) and admitted to the Gastroenterology Unit of the Instituto Português de Oncologia in Lisbon during the period May 1994 to May 1996. Diagnosis was confirmed by flexible sigmoidoscopy and histopathological analysis. The group consisted of 72 men (aged 37–86, average: 65.17 years, SD = 10.77) and 42 women (38–77 years old, average age: 61.66, SD = 10.90).

The control group consisted of 201 subjects, average age 46.36 years (SD = 19.58), who were recruited from regular medical check-up at the local Health Centre (Centro de Saúde de Oeiras), and who did not present with a clinical history of cancer. Personal data including family origin and birthplace show that the cohort studied of these permanent residents in the Lisbon area represented a fair sampling of the overall population from the same geographical area as the pathological cases.

NAT2 genotyping

Peripheral blood (3–5 ml) was collected in sterile K3 EDTA tubes, upon informed consent, for genomic DNA extraction.

DNA was isolated from leukocytes by Nonidet P40/Tween 20/proteinase K treatment (28). PCR amplification of a 547-bp long fragment of the NAT2 gene coding exon was performed in a Stratagene Robocycler by using the 5′ primer, 5′ GGT GGG TCT GGA AGC TCC TC 3′ and the 3′ primer 5′ TTT GGT GAT ACA TAC ACA AGG G 3′. Thirty-four cycles were performed consisting of 94°C (30 s) for denaturation, 59°C (30 s) for primer annealing and 72°C (30 s) for primer extension (21), using Promega DNA Taq polymerase. The PCR product encompasses the polymorphic loci Kpn I, Taq I, Dde I and Bam HI (positions 481, 590, 803 and 857) characteristic of the different NAT2 alleles to be screened. Restriction enzyme digestions were performed according to the standard protocols of the supplier (Stratagene). Plasmid pSK (Stratagene) was included in the Bam HI and Kpn I restriction media as an internal control for complete digestion and to prevent misinterpretation of results. Restriction products were electrophoresed in 8% polyacrylamide gels and each individual genotype determined from the restriction fragment patterns.

Results

The fragment length patterns characteristic of each endonuclease digestion for NAT2 allele identification are shown in Figure 1. This protocol does not discriminate allelic variants NAT2*6A from B and NAT2*7A from B, encoding decreased activity transferers, nor genotype NAT2*4/NAT2*5B from genotype NAT2*5A/NAT2*5C. In such cases NAT2*4/NAT2*5B is assumed considering the expected rarity of NAT2*5A/NAT2*5C genotype (Dr Dale Smith, pers. comm.).

The results presented in Table I show that the Portuguese random population is characterized by a frequency of the wild-type NAT2*4 allele of 23.4%. The most common forms of this gene are NAT2*5B (36.6%) and NAT2*6 (29.4%), both encoding partially inactive enzymes. Alleles NAT2*5A, NAT2*5C and NAT2*7 were found at relatively low frequencies, representing 6.2%, 3.7% and 0.7% respectively. These values do not significantly differ from the NAT2 allele distribution described for other European populations (4,29).

In colon cancer patients (Table I) a significantly different pattern of NAT2 allele distribution was observed, the wild-type NAT2*4 being the most frequent variant present in 38.6% of the population, a value that corresponds to 1.5 times the frequency in the healthy controls. A comparable relative increase (1.9-fold) was detected for the relatively less abundant NAT2*5A, while NAT2*6 was 2-fold less abundant in the patients group.

Individual NAT2 genotypes presented in Table II reveal a striking increase of NAT2*4 homozygotes among the colon cancer patients, representing a total of 19.3% of this population, versus 6.5% of the healthy subjects ($\chi^2 = 12.125$, P < 0.01, OR = 3.46). A marked difference in the NAT2*4/NAT2*4 genotype frequency associated with the patients gender was observed in the affected population. This genotype represented 22.9% of the men and 9.8% of the women with colorectal cancer.
that this is known to determine the less active metabolizer phenotype among NAT2*4 heterozygotes (4).

Although a larger number of control subjects (n = 201) than colon cancer subjects (n = 114) were genotyped, they were on average 20 years younger than the cases. The possibility that this control group could contain potential cases and bias our conclusions regarding the increased frequency of NAT2*4 homozygotes in the colorectal cancer population is ruled out by further comparison with a larger control Portuguese population (manuscript in preparation). Actually, the analysis of an elderly subgroup population of 98 subjects aged over 55 (average: 67.89 years, SD = 8.41) showed 2.04% of NAT2*4 homozygotes, 17.35% of NAT2*4/NAT2*5B and 3.06% of NAT2*4/NAT2*5A genotypes. The increase in NAT2*4 homozygotes, associated with colon cancer here reported is confirmed, since a 10-fold higher frequency of NAT2*4 homozygotes is found in the patients population when compared with the elderly subgroup population.

The marked difference in allele frequency by gender points to a male-specific effect of NAT2*4/NAT2*4 genotype as a risk factor in colon cancer. Actually, in the genotyped groups, a slight non-statistically significant increase of NAT2*4 homozygous was found in female colon cancer patients (χ² = 0.41, P = 0.521, OR = 1.51), while a marked overrepresentation of this genotype was detected in men (χ² = 13.23, P < 0.001; OR = 7.48). Interestingly, a similar situation was recently described in a lung cancer study (7), in which only the males exhibited a statistically significant increase of the NAT2*4/NAT2*4 genotype associated with this malignancy. NAT2*4 heterozygous genotypes did not appear to constitute risk factors either in men (χ² = 0.09, P = 0.771; OR = 0.86) or in women (χ² = 2.27, P = 0.132; OR = 1.81). However, the gender specific analysis of relative frequencies of inactivant allele genotypes reflected the uneven distribution of NAT2*4 homozygous in the colorectal cancer patient population, suggesting that recessive genotypes may constitute a protective trait for this pathology in men (χ² = 4.17, P < 0.05; OR = 0.53) but not in women (χ² = 3.35, P = 0.067; OR = 0.49). The nature of this uneven distribution should be further investigated, in order to define the potential importance of hormonal status and environmental exposures in carcinogenesis.

NAT2 phenotype studies in colorectal cancer have indicated the prevalence of rapid acetylators among patient populations (8–11). These observations are in agreement with the hypothesized mechanism of food-contained arylamine activation by a NAT2-mediated O-acetyltransferase in the liver and colon (12,18,30). Accordingly, the slow acylator phenotype should decrease the generation of critical intracellular concentrations of such ultimate carcinogens and consequent tumourigenesis upon environmental exposure to precursors.

The results found in the present work reveal a markedly increased frequency of the allele encoding the fully active enzyme, particularly of NAT2*4 homozygotes, among the patient cohort that entirely fit the above model, and are consistent with the phenotype-based studies that demonstrate an excess of rapid acetylators among colon cancer patients. However, similar genotyping studies by Rodriguez et al. (19) on a small ethnically mixed population, 25% African and 75% Caucasian Americans, did not show significant differences in the NAT2 allelic frequencies in association with colon cancer. Also in Japanese (22) as well as in Scottish population samples (21) no increase in the frequency of the fully active NAT2

### Table II. NAT2 genotype frequencies in control and colon cancer populations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 201)</th>
<th>Colon cancer (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT2<em>4/NAT2</em>4</td>
<td>13 (0.065; 0.35–0.108)</td>
<td>22 (0.193; 0.121–0.265)</td>
</tr>
<tr>
<td>NAT2<em>4/NAT2</em>5A</td>
<td>9 (0.045; 0.021–0.083)</td>
<td>14 (0.123; 0.063–0.183)</td>
</tr>
<tr>
<td>NAT2<em>4/NAT2</em>5B</td>
<td>41 (0.204; 0.148–0.260)</td>
<td>22 (0.193; 0.121–0.265)</td>
</tr>
<tr>
<td>NAT2<em>4/NAT2</em>5C</td>
<td>2 (0.010; 0.001–0.036)</td>
<td>4 (0.035; 0.010–0.088)</td>
</tr>
<tr>
<td>NAT2<em>4/NAT2</em>6</td>
<td>16 (0.080; 0.046–0.126)</td>
<td>4 (0.035; 0.010–0.088)</td>
</tr>
<tr>
<td>Total</td>
<td>68 (0.338; 0.273–0.404)</td>
<td>44 (0.368; 0.297–0.475)</td>
</tr>
<tr>
<td>NAT2<em>5A/NAT2</em>5A</td>
<td>3 (0.015; 0.003–0.043)</td>
<td>3 (0.026; 0.005–0.075)</td>
</tr>
<tr>
<td>NAT2<em>5A/NAT2</em>5B</td>
<td>1 (0.005; 0.0002–0.027)</td>
<td>2 (0.018; 0.002–0.062)</td>
</tr>
<tr>
<td>NAT2<em>5A/NAT2</em>6</td>
<td>8 (0.040; 0.017–0.077)</td>
<td>4 (0.035; 0.001–0.088)</td>
</tr>
<tr>
<td>NAT2<em>5A/NAT2</em>7</td>
<td>1 (0.009; 0.0002–0.027)</td>
<td>0 (0.000)</td>
</tr>
<tr>
<td>NAT2<em>5B/NAT2</em>5B</td>
<td>29 (0.144; 0.095–0.193)</td>
<td>18 (0.158; 0.091–0.225)</td>
</tr>
<tr>
<td>NAT2<em>5B/NAT2</em>6</td>
<td>46 (0.229; 0.171–0.287)</td>
<td>8 (0.070; 0.031–0.134)</td>
</tr>
<tr>
<td>NAT2<em>5C/NAT2</em>5C</td>
<td>2 (0.010; 0.001–0.036)</td>
<td>1 (0.009; 0.0002–0.048)</td>
</tr>
<tr>
<td>NAT2<em>5C/NAT2</em>6</td>
<td>8 (0.040; 0.017–0.077)</td>
<td>1 (0.009; 0.0002–0.048)</td>
</tr>
<tr>
<td>Total</td>
<td>68 (0.338; 0.273–0.404)</td>
<td>44 (0.368; 0.297–0.475)</td>
</tr>
<tr>
<td>NAT2<em>6/NAT2</em>6</td>
<td>19 (0.095; 0.058–0.144)</td>
<td>7 (0.061; 0.025–0.122)</td>
</tr>
<tr>
<td>NAT2<em>6/NAT2</em>7</td>
<td>2 (0.010; 0.001–0.036)</td>
<td>0 (0.000)</td>
</tr>
<tr>
<td>Total</td>
<td>120 (0.597; 0.529–0.665)</td>
<td>48 (0.421; 0.330–0.512)</td>
</tr>
</tbody>
</table>

*CI (95%).

Discussion

The results presented herein demonstrate that colorectal cancer patients show a markedly higher frequency (3-fold) of the wild-type NAT2*4 homozygous genotype than the corresponding Portuguese control population. When NAT2*4 homozygotes and heterozygotes are grouped together, as both have been considered to define rapid metabolizers in former phenotyping studies, the difference found between the two populations still shows a significantly higher frequency (1.5-fold) of the wild-type allele carriers within the patient group. Although the relative frequency of total NAT2*4 heterozygous genotypes does not show significant difference as a whole, a strikingly higher representation (3-fold) of the NAT2*4/NAT2*5A genotype was found in the patient population, in which they represent 12.3%. According to genotype and phenotype correlation studies, such a compound genotype determines a more rapid acetylator phenotype than NAT2*4 heterozygous genotypes bearing NAT2*6, NAT2*7 and NAT2*13 alleles (4). Conversely, NAT2*4/NAT2*5B and NAT2*4/NAT2*5C heterozygotes do not show significant variation, while the NAT2*4/NAT2*6 genotype is less represented in the patient cohort. In spite of its relative rarity, it is interesting to note...
allele has been detected in colon cancer patients. The discrepancy with the results here described for a Portuguese population in the assessment of genetic risk traits for this multifactorial disease, may derive from different characteristics of each anthropological group as studied up to now, including not only specific NAT2 genotype profiles but also important differences in the degree of exposure to environmental procarcinogens. A wide variation in NAT2 allele distribution in different populations is illustrated by the above studies, which have shown the wild-type allele to be present in ~90% of the Japanese population (22), while it is found in ~30% of Scottish subjects (21).

The potential role of NAT2 activity in the generation of carcinogenic derivatives, particularly affecting gut tissues, should be related to individual exposure to procarcinogens, namely to food contained arylamines. The importance of the interplay between the individual genetic profile and environmental chemical injury is well illustrated by the fact that the Japanese population, characterized by a particularly high frequency of wild-type NAT2 alleles, shows a reduced incidence of colon cancer (26), a situation that has been recently reported to be changing in parallel with changes in local dietary habits (22), as well as migration of Japanese subjects.

In Portugal, besides the long established importance of broiled and fried fish in the local diet, the level of exposure to food arylamines has been significantly increasing as consumption of grilled and fried meat has more than doubled (251%) in the last two decades (31). Considering the high penetrance of the NAT2*4 homozygotes found with colorectal cancer cases, such local dietary habits could be contributing to the 210% higher incidence of this malignancy registered in Portugal along the same period (31).

Arylamine carcinogen activation depends on a complex array of different metabolic pathways, particularly those involving the participation of CYP1A2 and NAT1 as well as NAT2 (14,15,17). However, the results presented herein clearly indicate polymorphism of NAT2 as an individual trait that significantly contributes to sporadic colorectal cancer susceptibility and/or development associated with the wild-type NAT2*4 allelic zygosity.

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Wild-type arylamine-N-acetyltransferase allele NAT2*4 homozygotes


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