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Polimorfismos dos alelos m1 e m2 do gene CYP1A1: Susceptibilidade genética para o cancro do pulmão

CYP1A1 m1 and m2 polymorphisms: genetic susceptibility to lung cancer

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Resumo

O cancro do pulmão é considerado uma doença relacionada com o meio ambiente, consequência da exposição a agentes mutagénicos, nomeadamente os presentes no fumo do tabaco. O gene CYP1A1 codifica a enzima aril hidrocarboneto hidroxilase (AHH), da fase I, do sistema multienzimático do citocromo P450, que desempenha uma função preponderante na bioactivação dos procarcinogénios do tabaco. Dois polimorfismos do CYP1A1, m1 (transição T6235C) e m2 (transição A4889G), estão associados a uma maior actividade enzimática, tendo sido referidos como factores genéticos de susceptibilidade para o cancro do pulmão.

Abstract

Lung cancer is considered an environment-related disease that develops as a consequence of exposure to mutagenic agents, namely those present in tobacco. The CYP1A1 gene codifies the phase I enzyme aryl hydrocarbon hydroxilase (AHH) belonging to the cytochrome P450 system that plays a major role in the bio-activation of tobacco procarcinogenes. Two CYP1A1 polymorphisms, m1 (T6235C transition) and m2 (A4889G transition), are associated with greater enzymatic activity and have been described as genetic susceptibility factors for lung cancer.

The aim of this study was to verify if this association holds true in blood samples of 175 lung cancer pa-

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Este trabalho teve como objectivo verificar esta possível associação em 175 doentes com cancro do pulmão e 217 controlos da Região Centro de Portugal, por RFLP (polimorfismo de comprimento de fragmentos de restrição).
Foi encontrada a seguinte distribuição para as frequências alélicas: 0.12 e 1.14, para os alelos mutados C e G, respectivamente, na população controlo. Os resultados não revelaram significado estatístico quando comparados com a distribuição encontrada na população de doentes. Relativamente à distribuição genotípica, a situação foi semelhante, não se registando significado estatístico, mesmo quando foram considerados genótipos de alto risco. Tal como noutras populações de diferente origem étnica, parece existir desequilíbrio de ligação para ambos os polimorfimos na população-controlo. Concluímos que nesta amostra de população portuguesa os polimorfismos m1 e m2 de CYP1A1 são particularmente raros, parecendo não existir relevância clínica nem associação à susceptibilidade ao cancro do pulmão.

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tients and 217 non-cancer patients from Portugal's midlands region. The samples were studied by restriction fragment length polymorphism (RFLP) assay.
The allelic frequencies of the mutant alleles were 0.12 for allele C and 1.14 for allele G in the control population. The results were not statistically different from those alleles in the patient population. There was also no statistically significant difference in genotype distribution in lung cancer patients and controls even when combining high risk genotypes. In our control sample, as in other populations of different ethnic origin, both polymorphisms also seem to be in linkage disequilibrium. We conclude that in this sample of the Portuguese population, CYP1A1 m1 and m2 polymorphisms are too rare to be of clinical relevance, and do not seem to be associated with susceptibility to lung cancer.

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Key-words: Lung cancer, smoking, cytochrome P450, CYP1A1, linkage disequilibrium.

Introduction

Cancer arises as a result of a complex sequence of mutational events that contributes to the breakdown of regulatory mechanisms, such as cell cycle control, apoptosis and differentiation. Some of the genes involved in these mechanisms, such as TP53, KRAS, or RB1, may become mutated after environmental carcinogenic exposure. Inter-individual variations in the activity of detoxifying enzymes such as cytochrome P450 enzymes, glutathione S-transferase (GSTs) and N-acetyltransferases (NATs) are also important in susceptibility to cancer[1-4]. Genetic polymorphisms explain these functional differences. The search for susceptible genetic profiles is a new and promising field of preventive medicine. Two main approaches have been used: the search for specific candidate genes and genome-wide searches based on microarray technologies. We followed the former, classical approach to search for

genetic polymorphisms associated with susceptibility to sporadic lung cancer.

Lung cancer is the leading worldwide cause of mortality[5,6]. In Portugal it is the third highest cause of death and the leading cause of cancer death. In lung cancer, both exposure to chemical agents, such as polycyclic aromatic hydrocarbons (PAH) in cigarette smoke and nitrosamines and inherited differences in metabolic capacity are thought to play a primary role in carcinogenesis[7,8]. Smoking appears to be responsible for 85% of lung cancer in the Portuguese population[9]. In addition to tobacco compounds, other carcinogens such as alcohol, radon, silica and diesel exhaust particles may also contribute[10,11].

The cytochrome P450 is a multi-enzymatic system involved in Phase I metabolism of a wide range of structurally diverse substrates[12]. P450 enzymes insert molecular oxygen, increasing the hydrophility and excretion of substrates from the cell[13]. The enzyme aryl hydrocarbon hydroxilase (AHH), codified by the CYP1A1 gene, belongs to the cytochrome P450 system and is responsible for the activation of PAH such as benzo(a)pyrene and coronene[14]. More specifically, AHH catalyses the transformation of PAH to the BP-7.8-epoxide form which is further oxidised to BP-7.8-dihydrodiol by the epoxide hydrolyse (EH) enzyme. BP-7.8-dihydrodiol is then transformed by AHH to BP-7.8-dihydrodiol-9.10-epoxide[15,16]. PAH metabolites are powerful carcinogens responsible for countless mutations present in squamous cell carcinomas (SCC) of the lung. PAH also work as powerful inducers of CYP1A1 gene product by binding to a citosolic receptor, the transcription factor aryl hydrocarbon receptor that ultimately activates and regulates the expression of the gene[13,17-19].

It has been demonstrated that AHH expression is induced in the bronchial airways of more than 80% of lung cancer patients who are smokers[20], presumably due to the high concentration of PAH and other carcinogens present in tobacco smoke. As a result, the relative levels of CYP1A1 expression in bronchi have been linked to the pathogenesis of tobacco induced lung cancer.

CYP1A1 gene is located in chromosome 15q22–q24 and in addition to the lung it is also expressed in the liver, gastrointestinal tract, brain, lymphocytes and macrophages[21,22]. Four different polymorphisms of CYP1A1 gene, m1, m2, m3 and m4 have been described[23] and the m4 polymorphism has a very low frequency in Caucasian populations. The m1 and m2 polymorphisms are more widely studied not only due to their higher genotype frequency but also their possible involvement in lung carcinogenesis[24]. The m1 polymorphism, involving an *Msp*I restriction site, is a T6235C transition in the 3' non-coding region of the gene, 250 bp downstream from the polyadenylation site[20]. The nucleotide substitution appears to be associated with increasing levels of CYP1A1 expression in response to PAH[25]. The m2 polymorphism is an A4889G transition in exon 7 leading to an isoleucine to a valine exchange and involves a *BsrD*I restriction site[26]. The amino acid exchange lies within the hem-binding region of the protein and has been shown to increase the catalytic activity of the enzyme seven-fold[27]. As AHH activates procarcino genes, individuals with high CYP1A1 gene inducibility or increased enzymatic activity may be more susceptible to the carcinogens of tobacco

smoke and to developing lung cancer. Recent studies have suggested that m2 and m1 polymorphisms are probably involved in linkage disequilibrium[25,28], but this remains a controversial issue.

The present study aimed to evaluate the importance of CYP1A1 polymorphisms as susceptibility factors for lung cancer and to verify the existence of the allelic linkage disequilibrium.

Materials and methods

Cases and controls

The lung cancer cases consisted of 175 patients (130 males and 45 females; average age ± SD = 62.1 ± 10.2 yrs) who were followed in the Pulmonology Department of the Hospital da Universidade de Coimbra, Portugal. Histological diagnosis was performed according to the WHO 2004 classification. The control group included 217 unrelated volunteers (143 males and 74 females; mean age ± SD = 70.8 ± 11.8 yrs) who had no history of cancer and as age and gender have never been shown to influence CYP 450 polymorphisms[29]. All individuals were from Portugal's midlands region and Caucasian. Informed consent from patients, as well as from controls was obtained together with permission from the Ethics Committee.

Methods

DNA was extracted from frozen peripheral blood using the urea DNA extraction technique. DNA concentration was verified using a spectrophotometer (UV-160A-Shimadzu). CYP1A1 m1 and m2 genotypes were characterised using RFLP assay.

For m1 polymorphism, a PCR was carried out in a total volume of 50µl containing 200 ng of DNA, 1x reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 5 % of dimethyl sulfoxide, 0.2 µM of primer forward (5' TAG GAG TCT TGT CTC ATG CCT 3'), 0.2 µM of primer reverse (5' CAG TGA AGA GGT GTA GCC GCT 3'), and 1U Taq DNA Polymerase (Bioline). The samples were amplified using a thermal cycler with an initial denaturation at 94ºC for 5 mins followed by 30 cycles with denaturation at 94ºC for 30 seconds, annealing at 62ºC for 60 seconds and primer extension at 72ºC for 60 seconds, followed by a final extension step at 72ºC for 5 mins. The PCR products were then analysed in a 1% agarose gel with ethidium bromide and visualised using a UV transilluminator. For RFLP assay, digestion was carried out overnight at 37ºC, in a total volume of 15 µl containing 5 µl of PCR product, 1x buffer and 3U *Msp I* In the presence of the 6235C polymorphism, the enzyme *Msp*I, digested the 340 bp PCR product in two bands of 200 and 140 bp. The wild type 6235C form corresponds to the absence of the restriction site.

For CYP1A1 m2, the PCR was carried out in a total volume 50 µl containing 200 ng of DNA, 1x reaction buffer, 1.5mM of $MgCl_2$, 200 µM of dNTPs, 5% of dimethyl sulfoxide, 0.2 µM of primer forward 5' CAG ACC AGG TAG ACA GAG 3', 0,2 µM of primer reverse 5' GTC CAC CCT CTT AAG CTC T 3' and 1U Taq DNA Polymerase (Bioline). The PCR protocol was carried out in the same thermal cycler with an initial denaturation at 94ºC for 5 mins followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 61ºC for 60 seconds and primer extension at 72ºC

for 60 seconds. The PCR products were analysed in a 1% agarose gel with ethidium bromide and visualised using a UV transilluminator. For RFLP assay, digestion was carried out overnight at 65ºC in a total volume of 15 µl containing 5 µl of PCR product, 1x buffer and 4U of *BsrD*I. In the presence of the 4889G polymorphism, the enzyme *BsrD*I, digested the 350 bp PCR product in two bands of 232 and 128 bp. The 4889A wild type polymorphism corresponds to the absence of the restriction site.

Statistical analysis

The odds ratio (OR) with the corresponding 95% confidence interval (CI) and Pearson chi-square test of independence (χ^2) were used in data analysis. The level of significance was set at $p<0.05$.

Results

The histological diagnosis included squamous cell carcinoma, adenocarcinoma and small cell carcinoma. Table I shows the frequency of the histological types studied. Table II depicts the allelic frequencies in the tumoral and control populations. There were no statistically significant differences in the allelic frequencies in the tumoral and control group. The distribution of genotype frequencies for both polymorphisms is shown in Table III. As it is rare to find patients homozygous for the variant alleles C and G, homozygous and heterozygous were analysed together. In the patient group there were only 3 individuals homozygous for C allele and 2 homozygous for G allele and in the control group there were only 4 homozygous for C allele and 1 for G allele. There were also no statistically significant differences between the two groups. That were shown to be in the Hardy-Weinberg equilibrium ($p>0.05$). We also analysed the frequency of individuals simultaneously homozygous for both polymorphisms associated with lower enzymatic activity (TT and GG), expecting they would be represented higher in the control group, but no significant difference was found between patients and controls. Fig. 1 illustrates the analysis of genotypes using RFLP assays.

Table I – Distribution of histological diagnosis in patients

Histological type	No. of patients
Adenocarcinoma	59 (34%)
Squamous cell carcinoma	53 (30%)
Small-cell carcinoma	33 (19%)
Others*	30 (17%)

* Others = pleomorphic carcinoma, neuroendocrine carcinoma adenosquamous carcinoma, non-small-cell carcinoma and giant cell carcinoma

Table II – Distribution of allelic frequencies in the two groups

Alleles		Patients	Controls	OR 95% CI	p (χ^2)
m1	T	0.90	0.88	1.3 (0.7-2.4)	0.964(0.002)
	C	0.10	0.12		
m2	A	0.89	0.86	1.3 (0.7-2.4)	0.950(0.004)
	G	0.11	0.14		

Table III – Distribution of genotype frequencies

		Patients No. (%)	Controls No. (%)	OR (95% CI)	χ^2 p
m1	T/T	142 (81.2%)	161 (74.2%)	1.5 (0.9-2.4)	0.103
	T/C and C/C	33 (18.8%)	56 (25.8%)		
m2	A/A	137 (78.3%)	168 (77.4%)	1.1 (0.7-1.7)	0.838
	G/A and G/G	38 (21.7%)	49 (22.6%)		
Total		175	217		

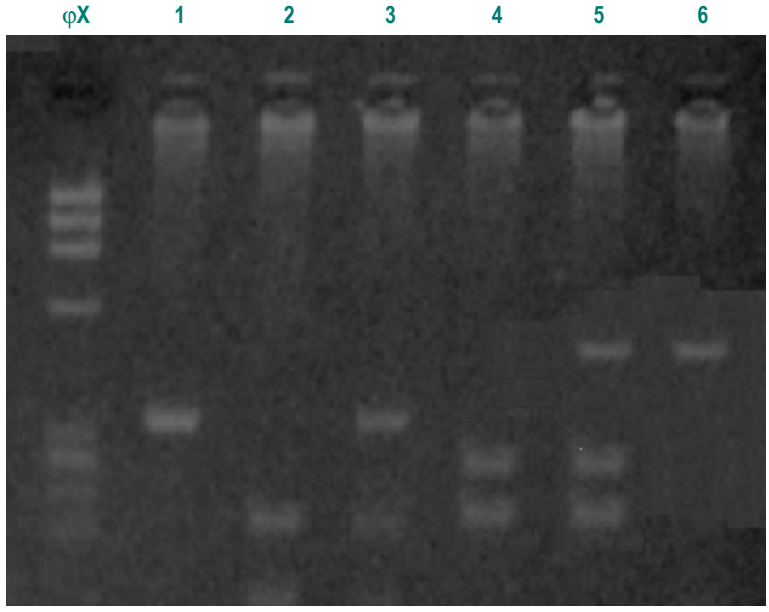

Fig. 1 – Different fragments from Msp I and Bsr DI digestions. φX molecular weight; 1- m1:TT, homozygous wild type (340bp); 2- m1: homozygous CC (200bp and 140bp fragments); 3- m1: heterozygous TC (340bp, 200bp and140bp fragments); 4- m2: AA, homozygous wild type (350bp fragment); 5-m2: heterozygous AG, (350bp, 232bp and 118bp fragments); 6 –m2: GG, homozygous double mutated (232bp,118bp fragments)

Among the different histological types of lung cancer, as squamous cell carcinoma is supposed to be more frequently associated with environmental toxic exposure, we analysed any difference between the distribution of the genotypes in the different types of carcinomas (Table IV). No statistically significant difference was found. Nevertheless, we found a small statistical significance (p=0.049) when we compared the allelic distribution for m2 allele in adenocarcinoma and small-cell carcinoma, and when we considered the small and squamous cell association and the other histological types (p=0.058). Although our samples included very few homozygous genotypes, we analysed if there was any suggestion

Table IV – Distribution of genotypes in different histological types of lung cancer

Genotypes / Histological types	m1		p (χ^2)	m2		p (χ^2)
	TT	TC/CC		AA	AG/GG	
Adenocarcinoma	42 (0.74)	15 (0.26)	0.380	42 (0.74)	15 (0.26)	0.180
Small-cell carcinoma	28 (0.85)	5 (0.15)		30 (0.91)	3 (0.09)	
Squamous cell carcinoma	39 (0.85)	7 (0.15)		37 (0.80)	9 (0.20)	
Others*	33 (0.85)	6 (0.15)		28 (0.72)	11 (0.28)	

* Others = pleiomorphic carcinoma, neuroendocrine carcinoma, adenosquamous carcinoma, non-small-cell carcinoma, giant cell carcinoma

Table V – Linkage disequilibrium analysis in the control population

Genotypes	m 2 - AA	m2 - AG	m2 - GG	p(x2)
m1 - TT	134 (84*)	27 (35*)	0 (4*)[1]	
m1 – TC	31 (39*)	21 (35*)	0 (6*)[1]	0.001 (15.968)
m1 - CC	3 (5*)[1]	0 (6*)[1]	1 (3*)[1]	

* Expected number of individuals for the genotype
[1] Considering the null value the correlation wasn't tested

of allele linkage disequilibrium in the control population for the higher risk genotypes. The results shown in Table V, where the number of observed and expected individuals is compared for each combined genotype, support the existence of linkage disequilibrium.

Discussion

The aim of this work was to define a correlation between the two most widely studied CYP1A1 gene polymorphisms, m1 allele (T6235C transition) and m2 allele (A4889G transition) and susceptibility to lung cancer. The allele and genotype frequencies found in our population samples were quite similar to those described in other Caucasian populations[30-33]. The low frequency of the high-risk genotypes in Caucasian populations complicates data analysis. Only in populations of Asian origin do the frequencies of C and G alleles and of correspondent homozygous genotypes have higher frequencies. For example, frequency for the m1 heterozygous is 0.171 in our lung cancer population is half of the frequency found in the Chinese population (0.395). We also describe a frequency of 0.017 for m1 homozygous, while in the Mongol population the frequency is 0.228[17].

The results described in Table III analyse the association between the two polymorphisms m1 and m2 and the risk of lung cancer. Though no statistically significant difference could be found (p>0.05), the unexpected fact is that the homozygous genotype for the wild genotypes was more frequent in patients, especially for m1 polymorphism (OR=1.5; 95% CI 0.9-2.4). It is likely that results would be different with more extended samples including more homozygous for the mutant high-risk genotypes. We also found no association between the major histological types (Table IV). Nevertheless when we analysed the allelic distribution of m2 polymorphism, we found a small statistical significance in terms of adenocarcinoma and small-cell carcinoma (p=0.049) and also between small and squamous cells and the other histological types (p=0.058).

In Asian populations, where the mutant alleles reach higher frequencies, CYP1A1 m1 and m2 polymorphisms have been widely described as genetic susceptibility factors for environmentally associated lung cancer for both squamous cell and adenocarcinoma[20,34,35]. An association between the presence of at least one copy of the mutant m1 allele and increased risk for squamous cell carcinoma (OR=2.4; 95% CI of 1.2-4.7) was found in more mixed populations, including Japanese, Hawaiian and Caucasians by Le Marchand *et al.*[36]. No statistically significant difference was found for the mutant m2 allele in terms of lung cancer in general and for other histological types. In Caucasian populations, results remain controversial and many other authors also could not find any association[30,32,33]. Other investigators studied both CYP1A1 polymor-

phism and Phase II enzyme polymorphisms, known to influence excretion of genotoxic compounds. Montserrat Garcia-Closas *et al.*[31] found that while neither the CYP1A1 *Msp*I heterozygous genotype alone nor the GSTM1 null genotype alone were associated with a significant increase in lung cancer risk, both genetic traits are associated with a twofold increase in risk (95% CI 1.0-3.4).

The inconsistency of results remains when the association between genotypes, the dose level of tobacco exposure and susceptibility to lung cancer is studied. For instance, Nakachi *et al.*[37] showed that individuals with the m1 susceptible genotype were at a remarkably higher risk at a low dose level of cigarette smoke (OR=7.31; 95% CI 2.13-25.12) and that the difference in susceptibility between genotypes was reduced at high dose levels. On the contrary, Montserrat Garcia-Closas *et al.*[31] could not find enough evidence for a substantial modification of the effect of tobacco pack-years on lung cancer risk by the CYP1A1 m1 and GSTM1 genotypes. In a pooled analysis, Le Marchand *et al.*[27] found a trend to an overrepresentation of m1 polymorphism in lung cancer patients, especially in non-smokers and in women.

In multifactorial cancer, the risk conferred by these polymorphisms, generally called SNPs (from single nucleotide polymorphisms), is typically small to moderate, with small odds ratios generally with wide confidence intervals. Results are also often conflicting. One cause for these discrepancies is the sample size. As these are low penetrance polymorphisms, each one having little influence on the overall risk, large population studies are needed. But even within populations of thousands of individuals, results often remain inconclusive. Recently, microarray technology has allowed a simultaneous study of multiple polymorphisms and hopefully it will allow the discovery of genetic profiles for disease predisposition[38], even though these technologies are very expensive, demand huge population samples and are very prone to statistical errors.

The possibility of allelic linkage disequilibrium for m1 and m2 genotypes has been suggested but never confirmed. In our control sample we also found a suggestion of the linkage ($p< 0.001$), explaining the similarity of results for the two alleles. Linkage disequilibrium has been proved to be associated with evolutionary mechanisms that select functionally important loci[39].

In the future some other important issues must be studied. An important point is the relation between smoking and CYP1A1 polymorphisms. The time of exposure, the number of cigarettes and the age at starting smoking are important to confirm the relation between the polymorphisms and the increasing susceptibility to developing lung cancer. These issues are still under debate and controversial results have been published. The relationship between Phase I enzymes such as CYP1A1 and Phase II enzymes such as GSTM1 is another important parameter for understanding the influence of the activation and detoxification of carcinogens and the development of lung cancer. The study of other regulator genes such as GSTM1, CYP2D6 and NAT2 and their association with the presence of mutations in the tumour suppressor gene TP53 is already in course and will allow a better understanding of lung cancer carcinogenesis. Future studies should also include environmental exposure to other toxins, as well as diet factors such as alcohol.

In conclusion, we did not find any statistically significant difference in allelic and genotype frequency in the populations studied. Apparently there is no association between CYP1A1 m1 and m2 polymorphisms and susceptibility to lung cancer. Nevertheless, the p values found between squamous and small-cell and the other histological types and also for adenocarcinoma and small-cell confirm the controversial results involving these studies and suggest that the population should be extended to obtain unequivocal results. As the statistical analysis suggests the existence of allele linkage disequilibrium in the Portuguese population, the simultaneous study of the two alleles in future investigations seem unnecessary.

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