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Pooled analysis of NAT2 genotypes as risk factors for asbestos-related malignant mesothelioma

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Abstract

Malignant mesothelioma (MM) is a rare and aggressive tumor of the pleura. The most important causal factor for the development of MM is occupational exposure to asbestos. Different lines of evidence suggest a role of genetic background in MM development, as for other cancers. Two published studies observed an association between MM and *N*-acetyl-transferase 2 (NAT2) polymorphisms. First, a Finnish study observed that the NAT2 slow acetylator phenotype was associated with an increased risk of MM. Conversely, MM risk was higher in Italian subjects carrying the NAT2 fast acetylator genotypes. The conflicting results obtained in Finland and Italy could be ascribed to random chance, considering the small panel of patients and controls in the two studies, but also ethnic or other differences may have been important. To ascertain the role of NAT2 genotype, we performed a study on 252 MM patients and 262 controls recruited in two Northern Italy areas that were characterized by high asbestos exposure, due to intense industrial activities (an asbestos cement factory in Casale Monferrato, mainly shipyards and refineries in Liguria). Unconditional multivariate logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs). NAT2 fast acetylator genotypes showed an increased OR, although not statistically significant, both in asbestos-exposed subjects (OR = 1.47; 95% CI = 0.96–2.26) and in the entire population (OR = 1.38; 95% CI = 0.93–2.04). These results suggest that NAT2 polymorphisms do not exert a strong effect on individual susceptibility to MM.

Keywords: Asbestos; Mesothelioma; *N*-acetyltransferase; Oxidative stress; Single nucleotide polymorphism

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Introduction

The association of asbestos exposure and malignant mesothelioma (MM) is well documented, but the mechanism of action has not been completely clarified (Robinson et al., 2005). Asbestos fibers chronically retained in the lung and the pleura can be carcinogenic as the result of mechanical effects, such as interference with mitotic spindle formation and the segregation of chromosomes, leading to breaks and aberrations (Jensen et al., 1996). A different hypothesis involves the generation of reactive oxygen species (ROS) either by reactions promoted by catalytic iron ions contained in asbestos molecules or by frustrated fagocytes (Chen et al., 1996; Fung et al., 1997). Asbestos-induced oxidative damage has been clearly demonstrated, both in vitro and in vivo: its consequences include DNA single-strand breaks and DNA base modification (Wang et al., 1998). A promotion effect is also suggested by the effect of asbestos exposure on the induction of signal transduction and methylation of gene promoters (Lee and Testa, 1999; Manning et al., 2002; Yang et al., 2006; Zanella et al., 1996; Christensen et al., 2008).

About 80% of MM patients have a history of asbestos exposure (Robinson et al., 2005), but only 5–10% of individuals exposed to high levels actually develop MM and its onset is usually 30–40 years after the first exposure (Bianchi et al., 1997). The combined role of genetics and asbestos exposure, suggested by familial aggregation (Ascoli et al., 1998; Roushdy-Hammady et al., 2001), has been discussed recently in two extensive literature reviews (Ascoli et al., 2007; Ugolini et al., 2008).

Several genetic association studies addressed the identification of the traits that may predispose to asbestos damage susceptibility and MM (reviewed in Neri et al., 2008). Most studies so far have addressed specific hypotheses, under the candidate gene approach. An association of gene polymorphisms leading to defective DNA repair with an increased risk of MM has been explored, for the first time, in the framework of a population-based case–control study conducted in Casale Monferrato, a Northern Italian area subjected to heavy exposure due to an important asbestos cement factory (Dianzani et al., 2006). An association of *XRCC*1-399Q and *XRCC*3-241 T with the risk of MM was observed.

Other studies were focused on genes involved in xenobiotic metabolism and in protection from ROS, under the hypothesis that a reduced protection might increase DNA damage and facilitate carcinogenesis. A group of subjects from the Cancer of RESpiratory Tract (CREST) biorepository in Liguria (Northern Italy), where asbestos has been extensively used in port, shipyard and industrial activities, showed an increased risk of MM in subjects bearing a GSTM1 null allele and in those with the Ala/Ala genotypes at codon 16 within MnSOD (Landi et al., 2007).

Controversial results were found regarding MM risk associated to *N*-acetyltransferase 2 (*NAT*2). Hirvonen et al. (1995, 1996) showed an increased risk of MM in Finnish subjects occupationally exposed to asbestos and carrying the *NAT*2 slow acetylator phenotype, whereas Neri et al. (2005) found an increased risk for *NAT*2 fast acetylator in CREST subjects. The latter study also reported an association of MM with microsomal Epoxide Hydrolase (mEH) and found that *mEH* interacted with both *NAT*2 and *GSTM*1 genes according to a multiplicative model. The *GSTM*1 null genotype showed an increased risk in studies from both Finland and Liguria (Neri et al., 2006).

The discrepancies among the studies of Hirvonen and Neri could be due to chance, but the different ethnic origin and habits of the two studied populations, the Finns and the Italians, could have a role. A main drawback of these studies was the low number of cases and controls.

NAT2 phenotypes have been associated with other cancer types, such as bladder cancer (Sanderson et al., 2007; Vineis, 2002): in this case the biological effect is expected to be mediated by exposure to carcinogenic aromatic amines. The carcinogenic mechanism favoured by these SNPs in MM is unclear.

In this paper, we evaluated NAT2 genotypes in two studies conducted in Northern Italy: a panel of 133 cases and 182 controls from the case–control study in Casale Monferrato and 119 patients and 80 controls from Liguria CREST biorepository. The pooled analysis of both datasets was carried on.

Patients and methods

Study population: the Casale Monferrato panel

A population-based case–control study on MM of the pleura was conducted within Casale Monferrato Local Health Authority (LHA) to assess the risk associated with environmental asbestos exposure and the interaction of such exposure with genetic polymorphisms. Asbestos exposure (both occupational and domestic/environmental) was frequent because an asbestos cement factory had been active in Casale from 1907 to 1985 (Magnani et al., 2000, 2001, 2007).

This study involved the cases of MM histologically diagnosed during the study period (from January 2001 to December 2005) in subjects resident in Casale Monferrato LHA. Routine histology included immunohistochemical staining. Histological types of patients are reported in Table 1.

The study design has been reported in detail elsewhere (Dianzani et al., 2006). Briefly, controls were randomly sampled from the local population using the rosters

Table 1. Descriptive analyses of the subjects included in the study

	Casale Monferrato		CREST-Liguria	
	Cases *n* (%)	Controls[a] *n* (%)	Cases *n* (%)	Controls[a] *n* (%)
Gender				
Male	89 (67%)	127 (70%)	100 (84%)	57 (71%)
Female	44 (33%)	55 (30%)	19 (16%)	23 (29%)
Total	133 (100%)	182 (100%)	119 (100%)	80 (100%)
	$p = 0.59$		$p = 0.03$	
Age (mean ± standard deviation)	66.3 ± 11.8	62.8 ± 11.3	68.3 ± 10.3	63.2 ± 13.0
	$p = 0.008$		$p = 0.002$	
Asbestos exposure				
Exposed	115 (86%)	131 (72%)	99 (83%)	40 (50%)
Not exposed	2 (2%)	51 (28%)	20 (17%)	40 (50%)
n.a.	16 (12%)	0	0	0
	$p < 0.0001$		$p < 0.0001$	
Type of asbestos exposure				
Occupational (high)	63 (55%)	70 (54%)	80 (81%)	14 (35%)
Domestic/environmental (low)	52 (45%)	61 (46%)	19 (19%)	26 (65%)
Total	115 (100%)	131 (100%)	99 (100%)	40 (100%)
Histological subtypes				
Epithelioid	94 (71%)		75 (63%)	
Sarcomatous	10 (8%)		8 (7%)	
Mixed	26 (20%)		16 (13%)	
Undefined	3 (2%)		20 (17%)	
Total	133 (100%)		119 (100%)	

[a]In both Casale and Liguria participation was higher for cases than controls and, for the latter among younger subjects. In Liguria a difference was also observed for the two sexes. In both groups the expected association of mesothelioma with asbestos exposure was observed. All analyses were adjusted for these factors.

made available from the LHA of Casale Monferrato and including all the residents in the area. Cases and controls were matched by sex and age (1-year-interval). In order to increase the study power in the age classes when mesothelioma incidence is lower, two sampling ratios were used: two controls per case if the case was older than 60 or four controls per case if the case was younger than 60 years.

Informations about cases and controls (lifelong occupational, residential history) were collected with a standard questionnaire (Magnani et al., 2000). An informed consent statement was signed by all participants before the interview. Blood samples were collected from 133 MM patients and 182 controls out of the 185 and 418 eligible.

The information about asbestos exposure, collected by the questionnaires, was evaluated blindly by an industrial hygienist (Dr. Mirabelli) and summarized as: ''certain occupational'', ''probable occupational'', ''possible occupational'', ''household occupational'', ''environmental occupational'' and ''no evidence of exposure''. Exposure was further summarized as a binary variable (exposed versus non-exposed).

Study population: the Liguria panel from CREST biorepository

The Cancer of RESpiratory Tract (CREST) biobank was established in 1996 within the National Cancer Research Institute of Genoa. The main objective of this initiative was to support the conduction of multi-centric molecular epidemiology and translational studies of lung cancer and MM to investigate biological mechanisms and to develop tools and strategies for primary and secondary prevention of respiratory tract cancer, with a special focus on MM (Ugolini et al., manuscript in preparation). The establishment of specialized biorepositories linked to extensive databases of clinical and epidemiological information is an efficient approach to address emerging priorities in cancer research.

Biological specimens are collected from incident cases recruited in pneumology departments of major general hospitals located in Liguria region. MM incidence is particularly high here, because of the high concentration of asbestos related industries, including an extensive shipyard activity (Gennaro et al., 2005). Controls are

healthy subjects (blood donors, recreational associations) and controls hospitalized for non-neoplastic, non-respiratory conditions (mostly traumatic diseases or eye diseases). In addition, patients hospitalized for non-neoplastic respiratory conditions, mostly Chronic Obstructive Pulmonary Disease (COPD) and asbestosis, were recruited, but were not included in the present study.

Subjects are recruited after active search, and before contributing any kind of biological sample they are requested to sign an informed consent.

An extensive questionnaire is administered by trained personnel, including socio-demographic data, residence history, current and past occupations or hobbies in which the subject may have been in contact with asbestos, lifestyle information (tobacco smoke and diet), medical history and family history of cancer in first degree relatives. Questionnaires are then processed and valid data are stored in the CREST database.

Peripheral blood samples are collected with coded vacutainers by routine venipuncture. Several different biological samples are preserved: whole blood, plasma, serum, lymphocytes and, whenever possible, pleural fluid and bioptic or surgery cancer tissue samples. Collection, transportation, and storage are performed according international standards. The overall number of subjects recruited as of January 2008 is 1600 (including 210 MM and 440 lung cancer). The bank includes a total of 10,055 sample aliquots.

Genotyping analysis: the Casale Monferrato panel

Human blood samples (6 ml) were collected into EDTA-vacutainer and stored at −20 °C. Genomic DNA was extracted from peripheral blood lymphocytes using QIAamp® DNA Blood Maxi Kit (QIAGEN).

A fragment of 559 bp of NAT2 gene was amplified by PCR. The PCR reactions were performed in a total volume of 25 µl containing 100 ng genomic DNA, 1 U Taq polymerase (PE Applied Biosystems) in 1X Buffer (Tris-HCl, KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 µM each primers (NAT2F 5′-TGACGGCAGGAATTACATTGTC-3′ and NAT2R 5′-ACACAAGGGTTTATTTTGTTCC-3′).

The NAT2 genotypes were determined using a SNaPshot technique, a multiplex primer extension that allow to study at the same time until eight SNPs using primers with increasing length (primer SNP NAT2 M2 5′-ATTTACGCTTGAACCTC-3′, primer SNP NAT2 M1 5′-AAAAAAAAGAGAGGAATCTGGTAC-3′, primer SNP NAT2 M3 5′-AAAAAAAAAAAAAAAC-CCAAACCTGGTGATG-3′).

From these genotypes we derived NAT2 alleles and their associated phenotypes (slow and fast acetylator) according with the NAT2 nomenclature database (www.louisville.edu/medschool/pharmacology/NAT.html) (Hein et al., 2000a, b).

For the determination of acetylator status, we classified as fast acetylator phenotypes those genotypes possessing at least one NAT2*4 allele (the NAT2*4 allele has been designated as the wild-type human allele) (Hein et al., 2000a, b). We classified as slow acetylator phenotypes those genotypes possessing two variant alleles. It should be noted that some authors consider also an intermediate phenotype, that includes genotypes heterozygous for the NAT2*4 allele.

Genotyping analysis: the Liguria panel from CREST biorepository

Blood samples (3 ml) were obtained by venipuncture, collected in vacutainer containing sodium citrate and stored at −80 °C until use. All samples were coded to ensure a blind analysis. DNA was extracted from peripheral blood lymphocytes and genotyping analyses were performed on all the subjects.

Subjects have been genotyped for NAT2 polymorphism by RFLP-PCR firstly (Neri et al., 2005). Subsequently, most of the same patients plus a number of newly recruited subjects have been genotyped as described in Landi et al. (2007), by a microarray technique (APEX) that was capable to give information on hundreds of SNPs, including NAT2.

With RFLP-PCR method, three slow acetylator alleles (*NAT*2*5, *NAT*2*6 and *NAT*2*7) were identified using primers 5′-TGACGGCAGGAATTACATTGTC and 3′-ACACAAGGGTTTATTTTGTTCC in the PCR. Aliquots of the PCR products were incubated with restriction enzymes *Kpn* I, *Taq* I and *Bam* HI to distinguish the slow acetylator alleles from the wild type *NAT*2*4 allele (Neri et al., 2005).

With the APEX method, the polymorphisms were analysed all at once for a given sample by a microarray technique based on the arrayed-primer extension (APEX) principle. APEX consists of a sequencing reaction primed by an oligonucleotide anchored with its 5′ end to a glass slide and terminating just one nucleotide before the polymorphic site. Details on this technique were reported extensively in several previous publications and are not described here for brevity (Guo et al., 1994; Landi et al., 2003, 2005; Metspalu and Shumaker, 1999). For each polymorphism, the flanking sequences and their related APEX-probes are available elsewhere (Gemignani et al., 2002). Five-prime (C-12) aminolinker oligonucleotides were synthesized by Sigma Genosys (Sigma-Genosys Ltd., Cambridge, UK) and spotted onto silanized slides (Rothman, 1990; Tõnisson et al., 2002). Slides were imaged by a Genorama-003 four-color detector, equipped with Genorama image analysis software (Asper Biotech, Tartu, Estonia).

Fluorescence intensities at each position were converted automatically into base calls by the software, under the supervision of trained personnel. In case of more than one signal present on a given position, only the main signal was considered if the intensity of the weaker signal was lower than 10% of the main signal.

Haplotypes were reconstructed using the software PHASE (Stephens and Donnelly, 2003) and a global test of hypothesis for the gene was carried out, followed by contrasts for specific haplotypes. Haplotypes with frequency below 5% were pooled, to reduce the degrees of freedom. *N*-acetyl transferase 2 genotypes were grouped according to Wikman and co-workers (Wikman et al., 2001). The haplotypes *4, *12, and *13 were all considered associated with a 'fast' acetylator phenotype, whereas the haplotypes *5, *6, and *7 were considered as 'slow' acetylators. Thus, subjects were grouped according to their status as 'fast acetylators' when they carried two 'fast' alleles or when they carried one 'fast' and one allele 'slow' allele, and 'slow acetylators' when they carried two 'slow' alleles.

Quality control measures

DNA samples from case patients and control subjects were randomly distributed, and all genotyping was conducted by personnel who were blinded to the case–control status of the DNA sample. DNA samples from individuals of known genotypes were added to ensure the validity of the genotyping.

The Casale panel of patients and controls (in total 315 subjects) has been subjected to SNaPshot analysis. Sixty of these (20%) have been also analysed with a sequencing technique (ABI Prism 3100).

The Liguria panel (199 subjects) had been analysed by using a custom microarray based on the APEX technology. Moreover, several strategies were followed: (1) each APEX oligonucleotide was spotted in replicate, (2) each SNP was analysed independently, by genotyping both the sense and the anti-sense strands of the DNA (in case of disagreement the base call was discharged), (3) on the corners of the microarray, internal positive controls allowed to verify that the intensities of the four channels (A, C, T, G) were equilibrated, (4) base-calls were carried out by the surveillance of three independent trained operators; discordant results were re-checked, and, in case of disagreement, were discharged, (5) 10% of the study subjects were randomly selected (i.e., both case patients and control subjects) and re-analysed blindly for each polymorphism.

Statistical analysis

Odds Ratio (ORs) and 95% confidence interval (95% CI) were estimated using multivariate unconditional logistic regression. Analyses focused on phenotypes and asbestos exposure and were adjusted by age and gender.

The statistical analyses were conducted separately for Casale Monferrato and Liguria panel and the data of individual studies were pooled together, including the provenience of the samples, in the logistic model. SAS Software (Release 8.2, by SAS Institute Inc., Cary, NC, USA) was used to perform all statistical analyses.

Results

Table 1 summarizes the main information on the patients and controls who participated in the study. Distribution of phenotypes by cases and controls is reported in Table 2.

In the Casale Monferrato panel NAT2 fast acetylators showed a non significantly increased OR for MM compared to slow acetylators, both in asbestos-exposed subjects (OR = 1.45; 95% CI = 0.86–2.44) and in the entire population (OR = 1.34; 95% CI = 0.80–2.23). No trend in OR was observed when the fast and intermediate acetylators were analysed separately (in asbestos exposed subjects: intermediate vs. slow OR = 1.49; 95% CI = 0.86–2.60; fast vs. slow OR = 1.27; 95% CI = 0.47–3.38). None of the results reached statistical significance.

Similarly, in the Liguria panel the OR was higher for subjects bearing the fast acetylator genotypes than for slow acetylators, both in asbestos-exposed subjects (OR = 1.66; 95% CI = 0.74–3.71) and in the entire population (OR = 1.54; 95% CI = 0.82–2.88), without reaching statistical significance.

When we considered the two populations together, we observed a non significant OR increase for MM, both in asbestos-exposed subjects (OR = 1.47; 95% CI = 0.96–2.26) and in the entire population (OR = 1.38; 95% CI = 0.93–2.04).

Discussion

N-acetyl-transferases (NAT1 and NAT2) enzymes are involved in the activation of the carcinogenetic effects of aromatic and heterocyclic amines. They catalyze both *O*-acetylation, leading to activation, and *N*-acetylation, leading to deactivation of these amines (Hein et al., 2000a, b). Since they are polymorphic in humans and are represented by many variants characterised by a different activity, their role in cancer susceptibility has been postulated for many years.

In particular, NAT1 and NAT2 polymorphisms have been involved in the development of bladder, colorectal, breast, head and neck, lung and prostate cancer, often with controversial results. For example, the slow acetylator phenotype has been involved in the development of bladder cancer, while the fast acetylator

Table 2. Association of NAT2 phenotypes and asbestos-related pleural malignant mesothelioma in subjects from Casale Monferrato, from Liguria CREST biorepository and pooled

Study group	NAT2 phenotype	All subjects			Asbestos-exposed subjects		
		Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
Casale Monf.	Slow acetylator	70	108	1.0 (ref.)	60	81	1.0 (ref.)
	Fast acetylator	63	74	1.34 (0.80–2.23)	55	50	1.45 (0.86–2.44)
CREST Liguria	Slow acetylator	48	43	1.0 (ref.)	39	20	1.0 (ref.)
	Fast acetylator	71	37	1.54 (0.82–2.88)	60	20	1.66 (0.74–3.71)
Pooled[a]	Slow acetylator	118	151	1.0 (ref.)	99	101	1.0 (ref.)
	Fast acetylator	134	111	1.38 (0.93–2.04)	115	70	1.47 (0.96–2.26)

Odds Ratio (ORs) and 95% confidence interval (95% CI) from multivariate unconditional logistic regression adjusted by age and gender.
[a]Adjusted for age, gender and study centre.

phenotype has been associated with colon cancer (Hein, 2002). This discrepancy may be due to the different pathogenesis of the two cancers. In fact, NAT2 deactivates the aromatic amines, whose accumulation in the bladder is carcinogenic; but the same enzyme activates the heterocyclic amines, that are involved in colon cancer. Thus, a reduction of NAT2 activity favours bladder cancer, while it may exert a protective effect in the case of colon cancer. Opposite results obtained on the same cancer type are more difficult to explain, such as those reported for lung cancer (Hein et al., 2000a, b) and also for MM.

NAT2 genotypes have been among the first polymorphisms implicated in MM development, but the results were contradictory: the fast acetylator phenotype was reported as a risk factor in Italians and a protective factor in Finns (Hirvonen et al., 1995; Neri et al., 2005). The very low number of cases and controls used in both these studies and the heterogeneity of those results prompted us to further investigate the association in an independent set of cases and controls. We have thus analysed NAT2 genotypes in a panel of patients and controls from Casale Monferrato. Additional subjects from the CREST biorepository in Liguria were also genotyped, and we finally performed a pooled analysis of these data.

This procedure allowed us to obtain the largest number of MM cases ever studied in a molecular epidemiology study. Results from the two Northern Italian areas were in agreement, and after re-analysis NAT2 fast acetylators showed an increased MM risk, although statistical significance was not reached.

Our data show that NAT2 genotypes do not have a strong effect on MM susceptibility, although it is possible that increased numbers of cases and controls would allow our data to reach statistical significance. Moreover, the mechanism that would explain a role for NAT2 in MM development is unclear. Asbestos is not a NAT2 substrate, neither arylamine or heterocyclic amine exposure has ever been associated with MM development. It has been postulated that NAT2 may be involved in carcinogenesis through folate metabolism: NAT1 and NAT2 can acetylate *para*-aminobenzoyl-L-glutamate, a folate catabolite (Wang et al., 2005).

The association between acetylator phenotypes and risk of MM was hypothesized to be mediated by the acetylation step in the interconversion of polyamines (Hirvonen et al., 1995; Seiler and Bolkenius, 1985). It was shown that asbestos fibers are able to induce ornithine decarboxylase enzyme activity resulting in stimulated polyamine synthesis and increased cell proliferation (Marsh and Mossman, 1991). However, at the moment there are not evidences supporting a direct role of *NAT2* in the metabolism of polyamines.

In conclusion, the hypothesis of a role of an association of NAT2 polymorphisms in MM susceptibility may require further investigation.

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