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SHORT REPORT

Polymorphisms of glutathione-*S*-transferase M1 and manganese superoxide dismutase are associated with the risk of malignant pleural mesothelioma

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Individual response to oxidative stress, due to exposure to asbestos fibres plays a significant role in the malignant pleural mesothelioma (MPM) etiology. The differential impact on MPM risk of polymorphic alleles of glutathione-*S*-transferases (*GSTs*) and manganese superoxide dismutase (*MnSOD*/*SOD2*) genes involved in the defence against oxidative damage has been investigated. Ninety cases of MPM and 395 controls were genotyped using the arrayed-primer extension technique. Logistic regression analysis was applied to assess the predictive role of single nucleotide polymorphisms (SNPs) potentially involved in MPM carcinogenesis after adjustment for potential confounders. An increased risk of MPM was found in subjects bearing a *GSTM1 null* allele (OR = 1.69, 95% CI = 1.04–2.74; p = 0.034), and in those with the Ala/Ala genotypes at codon 16 within *MnSOD* (OR = 3.07, 95% CI = 1.55–6.05; p = 0.001). A stronger effect of *MnSOD* was observed among patients without a clear exposure to asbestos fibres. No effect was found for *GSTA2*, *GSTA4*, *GSTM3*, *GSTP1* and *GSTT1* genes. These findings, if replicated, contribute substantial evidence to the hypothesis that oxidative stress and cellular antireactive oxygen species systems are involved in the pathogenesis and in the natural history of MPM.

Key words: mesothelioma; oxidative stress; superoxide dismutase; polymorphism; genetic glutathione transferase; molecular epidemiology

Malignant pleural mesothelioma (MPM) is an aggressive cancer, generally refractory to therapy and characterized by a poor prognosis. The development of MPM is frequently linked to the inhalation of asbestos fibres, with a long latency period from the beginning of the exposure to the clinical onset of the disease.

Since 1965, when a first familial cluster has been described, the possible role of genetic factors in MPM has been considered.[1] Another familiar cluster has been reported 13-years later,[2] and since then many authors have reported familiar mesothelioma cases, mostly in subjects exposed to asbestos. Recently[3,4] other articles have suggested that susceptibility for mesothelioma may be genetically transmitted and they asserted that *"...mesothelioma appears to have a complex aetiology in which environmental carcinogens (asbestos and erionite), ionizing radiation, viruses, and genetic factors act alone or in concert to cause malignancy...."*

The role of metabolic genes polymorphisms in the aetiology of mesothelioma has been reported in 1995,[5] followed by other positive studies in more recent years.[6,7] A study indicate a possible role of individual susceptibility to mesothelioma in subject with lower asbestos exposure.[8]

The availability of genetic markers of individual susceptibility could help to identify the subjects at higher risk of mesothelioma within a population exposed to asbestos fibres.

Many different mechanisms have been hypothesized for the carcinogenic effect of inhaled asbestos fibres. Among them, oxidative stress, caused by free radicals and reactive oxygen species (ROS), plays a crucial role, either directly or due to the activation of inflammatory cells.[9,10] The effect of the oxidative stress is hindered by molecules which have an antioxidant action, such as the glutathione (GSH), an ubiquitous intracellular thiol present in all tissues including lungs. Its depletion in the lung has been associated with reduced pulmonary function and the increased risk of neoplastic and nonneoplastic diseases.[11] The redox system of GSH consists of primary and secondary antioxidants, including glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-*S*-transferase (GST) and glucose 6-phosphate dehydrogenase (G6PD). The GST family catalyzes the conjugation of reduced GSH to electrophilic centres on a wide variety of substrates. It is represented by several isozymes and many of them are polymorphic in humans.[12]

GSTM1 is highly expressed in the lung and it bears a common polymorphism (the *null* allele) that is present in about 40% of Caucasians in a homozygous form and causes the lack of enzymatic activity.[13] Two studies have shown that the *GSTM1 null*/*null* genotype is associated with an increased risk of MPM in asbestos-exposed individuals, in agreement with the hypothesis that cellular antiROS mechanisms are important in protecting from MPM.[14,15] Other GSTs have been reported to play a role in the MPM pathogenesis.[16]

The response to ROS also involves other gene products, such as the manganese superoxide dismutase (MnSOD, also reported as *SOD2*), one of the most important antioxidant enzymes in mammalian tissues,[10] induced by asbestos fibres[17] and by inflammatory cytokines.[9,18] MnSOD catalyzes the dismutation of superoxide radicals in the mitochondrion, producing H_2O_2 and oxygen. H_2O_2 may

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be converted into H_2O and O_2 by catalase, or contribute to further generation of ROS by a reaction catalyzed by myeloperoxidase.[18]

MnSOD reactivity is almost absent in nonmalignant human pleural mesothelium and cultured mesothelial cells, but is high in human pleural mesothelioma tissues.[19] Several experimental studies have shown that MnSOD transfection evokes increased resistance of malignant cells to oxidants, cytokines, asbestos fibres and cytotoxic drugs, while a deficiency of this peptide leads to increased oxidant sensitivity and cellular apoptosis.[20,21] The most common polymorphism of MnSOD results in an Alanine (Ala) to Valine (Val) amino acid change at codon 16, producing a conformational change in the protein secondary structure that may impair the transport of the protein into the mitochondria.[22] There is a well-supported evidence that MnSOD plays a role in tumorigenesis acting not only against ROS but also as a tumor suppressor gene in epithelial tumors.[23,24]

The aim of the present study was to investigate the association of the polymorphic genotypes of *SOD2*, which encodes for MnSOD, and of several members of the GST family (namely *GSTM1, GSTM3*, *GSTP1*, *GSTT1*, *GSTA2* and *GSTA4*) with the risk of MPM, given the role of the enzymes in the cellular defence systems against ROS and oxidative stress. The availability of efficient array-based techniques, such as the arrayed-primer extension (APEX),[25,26,20] has allowed the simultaneous analysis of single nucleotide polymorphisms (SNPs) that are potentially relevant to MPM etiology, improving the study efficiency. Ninety cases of MPM and 395 controls were genotyped and the role of asbestos exposure was also evaluated.

Materials and methods

Study population

Patients with MPM diagnosed between March 1996 and August 2003 were recruited from respiratory medicine departments of 3 Northern Italy general hospitals (Genova, Casale Monferrato and La Spezia). All these areas were characterized by the presence of asbestos-associated industrial and shipping activities. All MPM diagnoses were confirmed by cytohistological examination of pleural biopsies obtained through thoracoscopy or thoracotomy. Immunohistochemical analyses including positivity to antibodies to mesothelial-associated antigens cytokeratin, vimentin, HBME-1 and calretinin and negativity to carcinoembryonic antigen was also performed so to complete the diagnostic process. Date and method of diagnosis and histological subtype were obtained from clinical records.

Control subjects were recruited among blood donors and from patients hospitalized for nonneoplastic, nonrespiratory conditions (most of them were admitted for traumatic diseases or for eye diseases). Participating institutions Ethics Committees have approved the study protocol and a written informed consent was obtained from all subjects before enrolment.

Trained personnel has administered a standardized questionnaire to MPM cases and controls. Detailed information was collected on demographic variables, life-style and occupational history. The presence of exposure to asbestos occurring at workplace, in the place of residence or in other circumstances was carefully recorded in all MPM cases and in a sample of the controls. The extent of occupational exposure was assessed according to the job title and the narrative report included in the questionnaires.

A group of experienced epidemiologists and occupational hygienists blindly classified cases and controls according to exposure intensity. Subjects with definite high exposure (mostly shipyard and port workers) were identified first, then all other subjects were pooled for statistical reasons in 1 category defined as "no evidence/low exposure." This latter class included subjects with no acknowledged occupational or environmental exposure, subjects with low probability of exposure and subjects with definite low exposure (*e.g.*, teachers and housewives).

Peripheral blood samples from MPM patients and controls were collected by routine venipuncture with Vacutainers. All samples were coded to ensure a blind analysis and immediately stored at −80°C until use.

Polymorphisms selection

For *GSTA2*, we selected the variation T111S because it falls into the active site and it was suggested to affect the activity of the enzyme.[27] For *GSTM1* and *GSTT1* we selected known and well-characterized polymorphisms consisting in the complete lack of the genes (*i.e.* causing a complete deficiency of enzymatic activity in the homozygous status).[28]

For *GSTA4*, as there are no published variants, we selected 2 SNPs from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/): the first (rs405729) in the 3′ UTR showed the highest heterozygosity among the validated SNPs (to allow the highest statistical power); the second (rs1802061) was the only validated SNPs within the coding region with a determined allele frequency.

For *GSTP1*, we selected 2 SNPs (I105V and A114V) that were studied extensively and are suspected to affect the function of the enzyme.[29] For *GSTM3*, we selected a commonly studied genetic variation (rs1799735, a 3 bp deletion within the intron 6, also known as *GSTM3*B*).[30] Eventually, for MnSOD we selected SNP V16A (rs1799725), the genetic variation with both the most complete information on the biological function and the highest allele frequency.[22]

Genotyping

Firstly, Genomic DNAs were amplified to enrich the fragments carrying the SNPs by using specific primer pairs. The PCR sequences primers and the protocol for the PCR amplifications were given in previous articles.[25,26] PCRs were performed with 50 μM dUTP and 150 μM of dTTP and 200 μM of dATP, dCTP and dGTP, so to allow PCR product fragmentation (see later). PCR products were pooled and purified-concentrated using Microcon MY30 columns, following the provider instruction (Millipore, Billerica, MA). The 15 μL eluate from the column were treated with 1 U uracil *N*-glycosylase (UNG, Epicentre Technologies, Madison, WI) and 1 U shrimp alkaline phosphatase (sAP, Amersham Biosciences, Milwaukee, WI). The mixture was then incubated at 37°C for 1.5 hr and at 95°C for 30 min. DNA with abasic sites is labile and it is denatured and fragmented at 95°C, whereas UNG and sAP are inactivated. APEX is a classical single-base extension reaction occurring on a solid substrate rather than in solution. APEX consists of a sequencing reaction primed by an oligonucleotide anchored with its 5′ end to a glass slide and terminating just 1 nucleotide before the polymorphic site. A DNA polymerase extends the oligonucleotide by adding 1 fluorescently labelled dideoxy-nucleotide (ddNTP) complementary to the variant base. The reading of the incorporated fluorescence identifies the base in the target sequence. Since both sense and antisense strands are sequenced, 2 probes were designed for each polymorphism. Five-prime (C-12) aminolinker oligonucleotides were synthesized by Sigma Genosys (Sigma-Genosys, Cambridge, UK) and spotted onto silanized slides.[31] For APEX reaction, fragmented PCR products were incubated onto the slides together with the fluorescently labelled ddNTPs (4 × 50 pmol), 10× buffer, and 4 U of ThermoSequenase (Amersham Biosciences, Amersham, UK). All the details of the experimental protocol, including primer and probe sequences, were reported in previous articles.[25,26] Previously, APEX was used under different conditions showing to be a cost-effective and reliable technique of genotyping.[25,26] *GSTM1* and *GSTT1* polymorphisms genotyping was conducted through PCR followed by agarose gel, as specified in detail elsewhere.[32]

To ensure quality control, we followed several strategies: (*i*) DNA samples from case patients and control subjects were randomly distributed, and all genotyping was conducted by personnel who was blinded to the case-control status of the DNA sample; (*ii*) each APEX oligonucleotide was spotted in replicate; (*iii*) each SNP was analyzed independently, by genotyping both the sense and the antisense strands of the DNA (in case of disagreement the base call was discarded); (*iv*) on the corners of the micro-array, in-

ternal positive controls allowed to verify that the intensities of the 4 channels (A, C, T, G) were equilibrated; (*v*) base-calls were carried out by the surveillance of 3 independent trained operators; discordant results were rechecked, and, in case of disagreement, were discarded; (*vi*) DNA samples from individuals of known genotypes were added to ensure the validity of the genotyping; (*vii*) we randomly selected 10% of the study subjects (*i.e.*, both case patients and control subjects) and reanalyzed their DNA samples for each polymorphism. Because of some failure in the genotyping, not all the samples were analyzed for all the polymorphisms. Samples were repeated to increase the call rate, however some of the samples had a limiting amount of DNA and for rs1802061 within *GSTA4* we could not raise over the 63%.

TABLE I – SELECTED CHARACTERISTICS OF MPM CASES AND CONTROLS

	Cases, *n* (%)	Controls *n* (%)
Gender		
Men	74 (82.2)	217 (54.9)
Women	16 (17.8)	178 (45.1)
Age (years)		
≤58	19 (21.1)	107 (27.1)
59–67	25 (27.8)	103 (26.1)
68–75	21 (23.3)	79 (20.0)
≥76	25 (27.8)	106 (26.8)
Histology		
Epithelioid	52 (57.8)	
Sarcomatous	9 (10.0)	
Mixed	11 (12.2)	
Desmoplastic	3 (3.3)	
NOS[1]	15 (16.7)	
Total	90 (100.0)	395 (100.0)

[1]Not otherwise specified.

Statistical analysis

Logistic regression modelling was applied to assess the predictive role of the SNPs on the disease outcome,[33] after adjustments for gender and age. Odds ratio point estimates (ORs) were calculated assess the magnitude of the associations between disease outcome and genetic endpoint. For each OR, asymptotic 95% confidence intervals (95% CI) were computed. Model adequacy was checked by plotting residual, leverage and influence measures as diagnostic quantities.

Concerning multiple comparisons, we assessed the probability to obtain a false positive result by applying the method reported by Wacholder *et al*.[34] We estimated the true OR according to the results of our study using the most likely interval between 2.00 and 3.00. Assuming that the *a priori* probability to observe an association of the selected SNPs with mesothelioma varied between 0.25 and 0.10, we defined as noteworthy and commented only those results whose false positive reporting probability was less than 0.20.

Results

The study groups main characteristics are summarized in Table I. The most evident difference is the higher proportion of males among MPM patients, which are also slightly older than reference subjects. For these reasons all risk estimates are adjusted for gender and age.

TABLE II – ODD RATIOS (OR), 95% CONFIDENCE INTERVAL (CI) AND *p* VALUE OF THE ASSOCIATION BETWEEN GENETIC POLYMORPHISMS AND MPM

	Cases	Controls	OR[1]	95% CI	*p*
GSTA2-T111S					
Homozygotes Thr/Thr	20	126	1		
Heterozygotes Thr/Ser	28	136	1.33	0.70–2.52	0.384
Homozygotes Ser/Ser	12	65	1.23	0.55–2.74	0.613
GSTA4 rs1802061					
Homozygotes common allele	54	292	1		
Heterozygotes	3	30	0.62	0.18–2015	0.447
Heterozygotes rarer allele	0	2	–	–	–
GSTA4 rs405729					
Homozygotes common allele	26	111	1		
Heterozygotes	39	192	0.99	0.56–1.76	0.985
Homozygotes	12	75	0.77	0.36–1.66	0.506
GSTM1					
Functional	37	199	1		
Null	52	180	1.69	1.04–2.74	0.034
GSTM1 a/b					
Homozygotes a/a	24	116	4		
Heterozygotes a/b	5	19	1.15	0.38–3.51	0.807
Homozygotes a/b	8	64	0.62	0.26–1.49	0.286
GSTM3 Del(3 bp)					
Homozygotes common allele	38	203	1		
Heterozygotes	26	98	1.41	0.80–2.48	0.236
Homozygotes Del/Del	2	14	0.75	0.16–3.52	0.712
GSTP1 I105V					
Homozygotes Ile/Ile	32	154	1		
Heterozygotes Ile/Val	37	159	1.14	0.67–1.95	0.625
Homozygotes Val/Val	12	41	1.41	0.65–3.06	0.378
GSTP1 A114V					
Homozygotes Ala/Ala	80	353	1		
Heterozygotes Ala/Val	7	36	0.89	0.36–2.16	0.792
Homozygotes Val/Val	1	2	3.30	0.25–42.7	0.361
GSTT1					
Functional	71	317	1		
Null	17	70	1.31	0.71–2.43	0.391
SOD2 V16A					
Homozygotes Val/Val	16	98	1		
Heterozygotes Ala/Val	27	170	0.99	0.50–1.96	0.968
Homozygotes Ala/Ala	37	81	3.07	1.55–6.05	0.001

[1]ORs adjusted by gender and age (Logistic regression).

The genotypes main effects included in the analysis are reported in Table II. A statistically significant association was observed between the *GSTM1 null* allele and the presence of MPM (OR = 1.69, 95% CI = 1.04–2.74; $p = 0.034$). Similarly, subjects with the Ala/Ala genotype at codon 16 within MnSOD were found at increased risk with a high statistical significance (OR = 3.07 in the recessive model, 95% CI = 1.55–6.05; $p = 0.001$). No evident effects were found for *GSTA2*, *GSTA4*, *GSTM3*, *GSTP1* and *GSTT1* genes.

To evaluate the interaction between polymorphisms that resulted significantly associated with MPM and asbestos exposure MPM cases were categorized in 2 groups according to asbestos exposure.

The OR of the *GSTM1 null* genotype was increased of about the same level both in high or low exposure groups (data not shown). Also among the patients with the homozygous variant *MnSOD* (Ala/Ala), the OR appeared to be significantly increased in both groups, but with a very high OR in the group with no evidence/low exposure ($n = 9$; OR = 10.72, 95% CI 1.33–86.68).

This OR is not significantly different from the overall OR, likely because of the low number of subjects with low asbestos exposure, but the very high risk observed among the hypothetical more susceptible individuals is suggestive and deserves further investigations.

Discussion

This is the largest study ever conducted to evaluate the impact on the risk of MPM of polymorphisms of genes involved in the response to oxidative stress. Some MnSOD and GSTM1 polymorphisms, particularly homozygote Ala/Ala *SOD2* and the *GSTM1 null* allele showed an association with the risk of MPM. Several other *GST* family isozymes, investigated for the first time, resulted not associated with MPM.

MPM originates from the mesothelial cells and is strongly associated to asbestos fibres exposure.[35] Free radicals, H_2O_2, and ROS generated by exposures to asbestos fibres directly or indirectly appear to be very important in the pathogenesis of mesothelioma and other asbestos-related lung diseases.[17] MnSOD is a superoxide radical-scavenging enzyme, catalyzing the transformation of the superoxide anion O_2^- into hydrogen peroxide (H_2O_2), an important ROS. Asbestos fibres exert cytotoxic effects on human mesothelial cells (HMC) *via* oxidative stress and it was previously shown that only HMC that survive following this oxidative stress undergo full transformation.[36] Therefore, it is conceivable that under the selective effect of asbestos fibres most of the mesothelial cells undergo cell death and only cells highly expressing MnSOD survive, originating resistant clones. The importance of MnSOD in the development of MPM is also stressed by the clear observation that the MnSOD expression is very low in healthy human pleural mesothelium and high in human malignant mesothelioma.[37]

Other studies associated the Ala16Val polymorphism with the risk of breast,[38] prostate[39] and bladder cancers.[40] Previous studies evaluated also the MnSOD status in relation to asbestos exposures, but no statistically significant interaction was found for the risk of lung cancer.[41] Only 1 study investigated before the role of MnSOD polymorphism for the risk of mesothelioma, however the number of patients (20 MPM) was limited and no significant association was found.[42]

In this study *GSTM1* null allele also appeared to be a risk factor for MPM. This is in agreement with previous findings obtained on a smaller study group from Finland[14] and on a partially overlapping Italian study group.[15] Since *GSTM1* is one of the antioxidant enzymes expressed within the lung,[41] these findings are consistent with the hypothesis that oxidative stress, following exposure to asbestos, might be modulated by *GSTM1* polymorphism. In our study, the combination of *GSTM1 null* and *MnSOD* Ala/Ala contributed to increase the risk both at high and low exposure. Finally, it should be noted that we observed an effect of the genotype for MnSOD also among patients without a clear exposure to asbestos fibres, a group poorly studied in the literature.

All the 15 MPM patients in our study with no or low asbestos exposure had at least a null allele in the *GSTM1* or Val16 *MnSOD* genes. These observations confirm the urgency of studying individual susceptibility factors among people without apparent exposure or with a low indirect exposure (*i.e.* workers wives or children).

There are 2 most important features that usually limit the validity of association studies on genotype. The first is the small size of subgroups. Even though our study is among the largest on MPM, the rarity of this disease did not allow the statistical analysis to reach a satisfying statistical power, but it did not prevent us from highlighting the role of 2 important genes. The second and more subtle difficulty is the control of false positive findings, which assumes a major importance when arrayed assays, with multiple polymorphisms, are used. We adopted the approach described by Wacholder *et al.*[34] which provides a quantitative estimate of the reliability of the risk estimates. In our study the probability of reporting false positive results for most important findings—based on the *a priori* assumption reported in the statistical methods—was always below 20%.

In conclusion, the findings presented here contribute further evidence to the hypothesis that polymorphisms in MnSOD, likely involving the oxidative stress and the cellular anti-ROS systems, may play a role in the pathogenesis of MPM, indicating that future studies on this topic are warranted.

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