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XRCC1 and *ERCC1* variants modify malignant mesothelioma risk: A case–control study

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ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare aggressive tumor associated with asbestos exposure. The possible role of genetic factors has also been suggested and MPM has been associated with single nucleotide polymorphisms (SNPs) of xenobiotic and oxidative metabolism enzymes. We have identified an association of the DNA repair gene *XRCC1* with MPM in the population of Casale Monferrato, a town exposed to high asbestos pollution. To extend this observation we examined 35 SNPs in 15 genes that could be involved in MPM carcinogenicity in 220 MPM patients and 296 controls from two case–control studies conducted in Casale (151 patients, 252 controls) and Turin (69 patients, 44 controls), respectively. Unconditional multivariate logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs). Two DNA repair genes were associated with MPM, i.e. *XRCC1* and *ERCC1*. Considering asbestos-exposed only, the risk increased with the increasing number of *XRCC1*-399Q alleles (Casale: OR = 1.44, 95%CI 1.02–2.03; Casale + Turin: OR = 1.34, 95%CI 0.98–1.84) or *XRCC1* −77T alleles (Casale + Turin: OR = 1.33, 95%CI 0.97–1.81). The *XRCC1*-TGGGGGAACAGA haplotype was significantly associated with MPM (Casale: OR = 1.76, 95%CI 1.04–2.96). Patients heterozygotes for *ERCC1* N118N showed an increased OR in all subjects (OR = 1.66, 95%CI 1.06–2.60) and in asbestos-exposed only (OR = 1.59, 95%CI 1.01–2.50). When the dominant model was considered (i.e. *ERCC1* heterozygotes CT plus homozygotes CC versus homozygotes TT) the risk was statistically significant both in all subjects (OR = 1.61, 95%CI 1.06–2.47) and in asbestos-exposed only (OR = 1.56, 95%CI 1.02–2.40). The combination of *ERCC1* N118N and *XRCC1* R399Q was statistically significant (Casale: OR = 2.02, 95%CI 1.01–4.05; Casale + Turin: OR = 2.39, 95%CI 1.29–4.43). The association of MPM with DNA repair genes support the hypothesis that an increased susceptibility to DNA damage may favour asbestos carcinogenicity.

1. Introduction

Malignant mesothelioma (MM) is a rare and aggressive tumor that arises from mesothelial cells. Most frequent locations are in the order pleura (malignant pleural mesothelioma, MPM) and peritoneum. In Western Europe, 5000 patients die of MM each year. In countries where asbestos use has been discountinued or banned, the incidence of MM is increasing and it is expected to peak in the year 2020 [1]. Asbestos represents the main risk factor for the development of MM, but its mechanisms of carcinogenicity are not fully understood. Asbestos fibers could interfere with the mitotic spindle formation of cells resulting in chromosomal abnormalities [2]. Asbestos can also lead to the generation of reactive oxygen species (ROS) either by iron-catalyzed reactions on the surface of

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the fibers or during frustrated phagocytosis [3]. Asbestos-induced oxidative damage has been clearly demonstrated, both *in vitro* and *in vivo*. Its consequences include DNA single-strand breaks and DNA base modifications [4]. Moreover asbestos fibers may induce signal transduction (i.e. activation of NF-KB pathway [5]) and methylation of gene promoters [6].

According to the Italian Mesothelioma Register, that collects data from over 2500 patients, the median latency period between the time of initial asbestos exposure and the development of MPM was about 45 years [7]. The male:female ratio was approximately 3:1, likely due to occupational exposure [8]. Eighty per cent of MPM patients have a history of asbestos exposure, but only 2% to 10% of the individuals with heavy, prolonged asbestos exposure develop MPM [9]. The combined role of genetics and asbestos exposure in familial aggregation is debated [10–12] and has been discussed in two extensive literature reviews [13,14]. Several genetic association studies addressed the identification of the traits that may predispose to asbestos damage susceptibility and MPM [15]. We were the first group to report an association between *XRCC1* 399Q variant and MPM and to suggest a role of deficient DNA repair in MPM carcinogenesis [16]. In the present paper, we expand the analyses to 35 single nucleotide polymorphisms (SNPs) in 15 genes possibly involved in asbestos carcinogenicity (i.e. DNA repair, redox state control and inflammation). Our data highlight a significant association of MPM with two DNA repair genes, i.e. *XRCC1* and *ERCC1*.

2. Material and methods

2.1. Study subjects

2.1.1. Casale Monferrato panel

We conducted a population based case–control study on MPM within the Local Health Authority (LHA) of Casale Monferrato. This area was characterized by widespread exposure to asbestos in the general population because of an asbestos cement factory that had been active from 1907 to 1986 [17].

The case group included subjects with histologically diagnosed MPM, resident in the LHA of Casale Monferrato, who were recruited between January 2001 and December 2006. Two controls per patient, matched for age (±18 months) and gender, were randomly selected from the local population using the rosters of the LHA of Casale Monferrato. Trained personnel submitted a standard questionnaire to MPM cases and controls to collect information about demographic variables, life-style, occupational history and asbestos exposure [17]. The evaluation of asbestos exposure was conducted blindly by an industrial hygienist (D. Mirabelli) and summarized as: "certain occupational", "probable occupational", "possible occupational", "household exposure", "environmental exposure" and "no evidence of exposure". Exposure was further considered as a binary variable (exposed versus non-exposed). All subjects in the study signed an informed consent form before the interview. Blood samples were collected before therapy in vacutainers with ethylenediaminetetraacetic acid (EDTA) and stored at −20 °C until use.

Ninety-four cases and three hundred controls refused to gave their blood sample. Consequently, the study involved 151 MPM patients and 252 population controls. Table 1 summarizes the main characteristics of patients and controls who participated in the study. All participants were caucasians with the same ethnic origin, evaluated by place of birth.

2.1.2. Turin panel

We also conducted a hospital-based case–control study on MPM in the Turin area. The case group included subjects with a histologically confirmed diagnosis of MPM, admitted to the chest surgery units of the San Giovanni Battista (Turin) or the San Luigi Gonzaga (Orbassano) hospitals between January 2004 and October 2008. All cases were resident in Turin or in the province of Turin at the time of diagnosis. After case identification, control was chosen among inpatients within the same hospital (mostly in the general medicine or urology units) of the corresponding case, and had to live in Turin or in the province of Turin at the time of admission and were not affected by neoplastic or respiratory conditions. All study subjects were Caucasians. Patients and controls were interviewed using the same questionnaire described above. Asbestos exposure was evaluated as for the Casale group. All subjects in the study signed an informed consent form. Blood samples were collected and stored as reported above.

We had planned to select one control per patient matched by age (±24 months) and gender, but no eligible control was found for 25 cases. Thus, the study involved 69 MPM patients and 44 controls. Table 1 summarizes the main characteristics of patients and controls who participated in the study.

2.2. SNP study

2.2.1. SNP selection

SNPs were chosen because some have a significant effect on the transcript (i.e. amino acid substitution or possible splice defect) and/or have been associated with certain cancer types and/or deficient DNA repair [18–25].

In detail, 10 out of 15 genes (14 SNPs) are involved in the repair of DNA damage. Our working hypothesis is that the presence of polymorphic variants could reduce the efficiency of the DNA repair machinery. The unrepaired DNA damage caused by asbestos exposure would result in mutagenesis and cancer.

The *SEP15* gene (one SNP) was analyzed because the encoded selenoprotein is downregulated in 60% MPM cell lines [26].

Moreover, we evaluated two genes (two SNPs) involved in the control of the cellular redox state (*SOD2*, *GPX1*). In this case, our working hypothesis was that a reduced protection from ROS could predispose mesothelial cells to neoplastic transformation.

Our study included also the *OPN* gene (three SNPs) that encodes an inflammatory cytokine, since a higher susceptibility to inflammation might be a risk factor for mesothelioma. High plasma OPN levels have been considered a marker of MPM [27].

For haplotype analyses we studied nine SNPs in *XRCC1* and six SNPs in *ERCC1* and its adjacent gene *RAI*: these SNPs were reported as tag SNPs by Haploview v4.1 on HapMap CEU panel, release #24.

2.2.2. SNP analysis

Genomic DNA was isolated and purified from peripheral blood lymphocytes using QIAamp® DNA Blood Maxi Kit (QIAGEN).

We used different genotyping approaches to analyze SNPs involved in the study (Table 2). Most of the polymorphisms (rs1799782, rs3213247, rs12973352, rs2854496, 2307174, rs2023614, rs1799778, rs3213356, rs3213371, rs3213403, rs1799796, rs1799793, rs11615, rs2298881, rs3212948, rs3212965, rs3136820, rs12917, rs3626, rs10412761, rs4803817, rs6966) were genotyped with the PCR-based fluorescence 5′ exonuclease assay (TaqMan® Genotyping Master Mix, Applied Biosystems). The other polymorphisms (rs25487, rs3213245, rs861539, rs13181, rs1052133, rs1805794, rs101028, rs7687316, rs45594140, rs9138, rs1799725, rs1050450, rs1136410) were genotyped with the SNaPshot assay (SNaPshot® Multiplex Kit, Applied Biosystems) that uses a multiplex primer extension technique and primers of different length to analyze at the same time up to eight/ten SNPs.

Table 1
Characteristics of cases and controls in the study.

	Casale Monferrato		Turin[a]	
	Cases (%)	Controls (%)	Cases (%)	Controls (%)
Eligible	245 (100%)	552 (100%)	201 (100%)	44 (100%)
Blood sample given	151 (62%)	252 (46%)	69 (34%)	44 (100%)
Blood sample refused[a]	94 (38%)	300 (54%)	132 (66%)	0
Samples included in the analyses	151 (100%)	252 (100%)	69 (100%)	44 (100%)
Gender				
Males	101 (67%)	173 (69%)	48 (70%)	34 (77%)
Females	50 (33%)	79 (31%)	21 (30%)	10 (23%)
Age (mean ± standard deviation)	66.8 ± 11.5	61.7 ± 11.1	68.9 ± 8.4	68 ± 8.7
Histology				
Epithelioid	102 (68%)		48 (70%)	
Sarcomatous	14 (9%)		8 (12%)	
Mixed	31 (21%)		10 (14%)	
Undefined	2 (1%)		3 (4%)	
Missing	2 (1%)		0	
Asbestos exposure				
Occupational	71 (47%)	111 (44%)	54 (78%)	18 (41%)
Domestic	26 (17%)	24 (10%)	4 (6%)	2 (5%)
Environmental	34 (23%)	59 (23%)	9 (13%)	5 (11%)
Not exposed	2 (1%)	58 (23%)	1 (1.5%)	19 (43%)
Undefined	18 (12%)	0	1 (1.5%)	0

[a] Turin study: no eligible control refused blood sample, but 132 cases had confirmation of diagnosis after discharge and had no further admission to the recruiting hospitals.

Table 2
SNPs analyzed in this study.

Gene	Chromosome	Function[a]	rs	Variants
XRCC1	19q13.2	BER	25487	R399Q
			3213245	−77T>C
			1799782	R194W
			3213247	IVS2 G>T
			12973352	IVS2 A>G
			2854496	IVS2 A>G
			2307174	E50E
			2023614	IVS3 C>G
			1799778	IVS3 A>C
			3213356	IVS4 A>G
			3213371	IVS10 C>G
			3213403	3′UTR A>G
XRCC3	14q32.3	DSBR/HR	861539	T241 M
			1799796	IVS5 A>G
ERCC2	19q13.3	NER	13181	K751Q
			1799793	D312N
OGG1	3p26.2	BER	1052133	S326C
ERCC1	19q13.2	NER	11615	N118N
			2298881	IVS1 G>A
			3212948	IVS3 C>G
			3212965	IVS5 C>T
APEX	14q11.2	BER	3136820	D148E
MGMT	10q26	DRR	12917	L84F
PCNA	20p12	BER	3626	3′UTR G > C
NBS1	8q21	DSBR	1805794	E185Q
SEP15	1p31	Selenoprotein	101028	3′ UTR G>A
OPN	4q21	Inflammatory	7687316	-156delG
		Cytokine	45594140	-66T>C
			9138	+1239A>C
SOD2	6q25	Mn-Superoxide	1799725	A16 V
		Dismutase	(4880)	
GPX1	3p21.3	Glutathione Peroxidase 1	1050450	P198L
PARP (ADPRT)	1q41	BER	1136410	V762A
RAI (PPP1R13L)	19q13.2	Protein	10412761	5′UTR A>G
		phosphatase 1	4803817	IVS1 A>G
		(inhibitor)	6966	EX13 A > T

[a] BER, base excision repair; NER, nucleotide excision repair; DSBR, double-strand break repair; HR, homologous recombination; DRR, direct reversion repair.

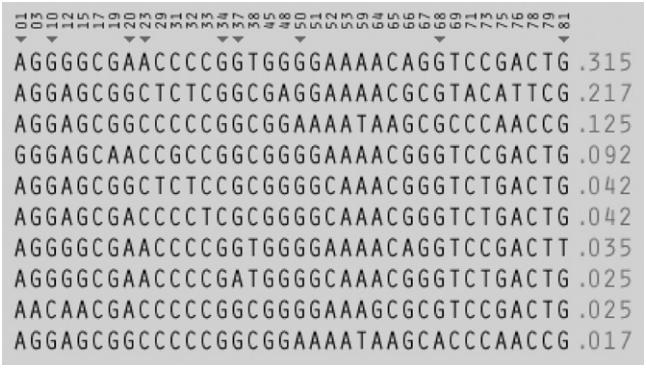

Fig. 1. *XRCC1* tag SNPs (TAG SNPs are loci that capture most of the genetic variation in a region and can be used in association studies to reduce the number of SNPs needed to detect LD-based association between a trait of interest and a region of the genome.) identified using Haploview v4.1. 01: rs3213403; 10: rs3213371; 20: rs3213356; 23: rs1799778; 34: rs2023614; 37: rs2307174; 50: rs2854496; 68: rs12973352; 81: rs3213247.

2.2.3. Quality control

For each SNP, samples with the three genotypes (i.e. homozygous for the wild-type sequence, heterozygous and homozygous for rarer sequence) were directly sequenced and used as internal control in each analysis. Ten per cent of samples were randomly sequenced. Concordance was 100% for all comparisons.

2.3. Sequencing

We sequenced the seventeen exons and exon–intron boundaries of *XRCC1* in ten wild-type homozygous, in ten heterozygous and in ten variant homozygous for SNP R399Q using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.4. Haplotype analysis

By using Haploview v4.1 [28] and the HapMap-CEU panel genotype data (release #24) we identified nine different SNPs as *XRCC1* tag-SNPs and six different SNPs as *ERCC1 RAI* tag-SNPs (Figs. 1 and 2, respectively).

The haplotypes of *XRCC1* and *ERCC1 RAI* were inferred by SHEsis software platform [29]. Haplotype frequency and corresponding standard deviation was obtained from genotype data of each polymorphism with unknown genetic phase, using maximum likelihood estimation. The analyses were performed using HAPLO.STATS software package developed using the R language. Odds ratios (ORs) and 95% confidence intervals (95%CIs) were calculated after estimating the number of patients and controls for each haplotype, considering the most frequent haplotype as reference. These analyses were carried out both in the overall sample and in the subgroup of subjects with asbestos exposure. All analyses were adjusted for age and gender.

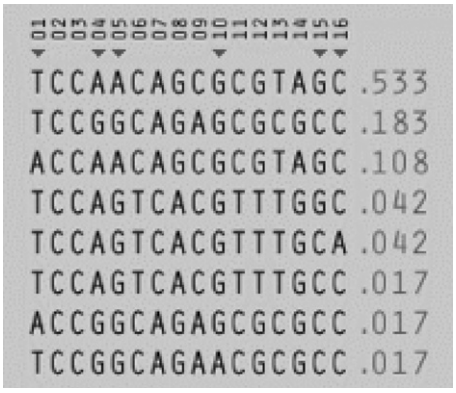

Fig. 2. *ERCC1* RAI tag SNPS identified using Haploview v4.1. 01: RAI rs6966; 04: RAI rs4803817; 05: RAI rs10412761; 10: *ERCC1* rs3212965; 15: *ERCC1* rs3212948; 16: *ERCC1* rs2298881.

2.5. XRCC1 expression

Seventy-one samples of normal pleura were obtained from donors that were subjected to thoracoscopy for a condition different from MPM and signed an informed consent.

Total RNA was extracted from frozen, normal pleural tissues by RNeasy® Plus Mini Kit (QIAGEN) according to the manufacturer's protocol.

cDNA was retrotranscribed from 500 ng of total RNA using random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems).

The levels of mRNA were measured by quantitative real-time PCR (qPCR).

Relative gene expression quantification for *XRCC1* and the reference gene *UBC* was carried out in triplicate, with TaqMan® chemistry using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primers and probes used to quantify the transcripts of *XRCC1* and *UBC* were Taqman gene expression assays (Applied Biosystems).

After initial denaturation at 95 °C for 10 min, the reaction proceeded with 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To assess variation between experiments, a standard cDNA was included in each plate. The analyses were carried out using the comparative C_T method [30]. The threshold cycle (C_T), is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). C_T levels are inversely proportional to the amount of mRNA in the sample (i.e. the lower C_T level the greater the amount of mRNA in the sample).

C_T level was determined for each gene and the relative expression of *XRCC1* was then estimated by calculating the dC_T value, defined as the difference in the C_T value for the target gene (*XRCC1*) and the reference gene (*UBC*).

2.6. Statistical analysis

To evaluate the hypothesis of association between genetic polymorphisms and MPM, unconditional multivariate logistic regression method was used. ORs and 95%CIs were calculated for each genotype compared to the reference group, that is the homozygous for the more frequent allele among controls. Each SNP was considered in separate analyses as both a categorical variable (i.e. wild-type homozygous, heterozygous and variant homozygous) and a continuous variable (number of mutated alleles). Genotypes were also divided into two categories (risk versus non-risk genotypes) on the basis of literature (i.e. prior knowledge of the functional significance of the variants and existing epidemiological evidence) and of our data. In particular, *XRCC1* 399Q (rs25487) was considered a risk factor under a dominant model [16,31], whereas −77C (rs3213245) was considered a protective factor under a dominant model (our data).

For the analysis of the combination of *XRCC1* R399Q and *ERCC1* N118N polymorphisms, subjects were categorized according to the cumulative number of variant alleles.

The analyses were carried out both in overall sample and in the subgroup of subjects with asbestos exposure. All analyses were adjusted by age, gender and asbestos exposure for overall sample, and by age and gender for the asbestos-exposed subjects. The analyses were performed using SAS v.8.01 and the SNPassoc software package developed using R language [32].

Since several of the SNPs that were studied in this work were the object of two previous papers [33,34], we also carried out a meta-analysis. Gemignani et al. [33] used two panels of controls: panel 1 included individuals whose asbestos exposure was known, whereas panel 2 included blood donors whose asbestos exposure had not been evaluated. We performed our meta-analysis using panel 1 only, since these controls were more similar to our control group, and had detailed information about asbestos exposure. Meta-analysis was carried out using a variance based (fixed effects) method. Study weights were proportional to the inverse variance of the OR, estimated from the confidence interval [35].

As the SNPs considered in the study were selected in accordance with a well defined hypothesis supported by previous observations, we did not apply a correction for multiple testing. Instead, we estimated the false-positive report probability (FPRP) using the Bayesian approach proposed by Wacholder et al. [36]. This method requires the estimation (from previous biochemical or molecular information and/or results from meta-analysis) of the prior probabilities that the specific SNPs are associated with the disease under study. We gave a high prior probability (0.2) when (a) the biological plausibility was high, and (b) the existing epidemiologic evidence of association with cancer was fair; a prior probability of 0.1 when biological plausibility was high but the prior epidemiological evidence was poor; a prior probability of 0.01 when both were poor [22]. The available epidemiologic evidence did not allow higher prior probabilities [19].

Analysis of variance and *t*-test were used to determine differences in the *XRCC1* mRNA relative expression levels (normalized to *UBC* levels) between subjects carrying different genotypes for *XRCC1* −77T>C.

3. Results

3.1. Subject characteristics

The main information on the cases and controls are reported in Table 1. The Casale Monferrato panel included 151 MPM patients and 252 population controls. Participation was not the same among cases and controls. Overall 151/245 cases and 252/552 controls in the Casale Monferrato panel agreed to donate a blood sample. That proportion was influenced by gender (in controls more often men accepted and women refused -$p < 0.05$-, no gender differences were observed in cases) and age (in both cases and controls mean age for participating subjects was lower than for refusers, this difference was statistically significant -$p < 0.05$- in controls). These figures were expected given the fact that blood sampling is an annoying procedure and it is less accepted in elderly women and in absence of an health related need (as shown by a lower participation among home interviewed controls compared to hospital admitted cases). No differences were observed by residence, thus participation was not associated to social pressure or exposure related feelings. As far as to our knowledge, none of the variables considered and influencing participation is associated to the analyzed SNPs and therefore differential participation cannot pose a confounding threat in the present study [37]. Almost all subjects were exposed to asbestos, only 23% of controls and 1% of cases were not exposed (12% of cases had an undefined asbestos exposure). The main asbestos exposure was occupational both in cases (47%) and in controls (44%). Environmental asbestos exposure in the area accounts for the very large proportion of exposed [38].

The Turin panel, including 69 MPM patients and 44 controls, derives from a hospital-based case–control study still under way. The imbalance between the number of cases and controls, thus, is due to the fact that controls are recruited after cases, so that for part of the MPM patients a suitable control had not been included yet at the time of analysis. Almost all cases were exposed to asbestos. Only 1.5% were not exposed and 1.5% had an undefined asbestos exposure. The 56% of controls were exposed to asbestos. The main asbestos exposure was occupational both in cases (78%) and in controls (41%).

3.2. SNP analysis

We analyzed 35 SNPs in 15 genes (10 DNA repair genes, 1 selenoprotein, 2 redox state genes, 1 inflammatory gene and 1 phosphatase gene), listed in Table 2. All polymorphisms were in Hardy–Weinberg equilibrium both in cases and controls, except *ERCC2* rs13181 in Casale Monferrato controls, *ERCC2* rs1799793 and *OPN* rs7687316 in Turin controls. This may be due to the small size of Turin panel or to chance.

In Casale Monferrato panel, 18 MPM patients were excluded from statistical analyses because of an undefined asbestos exposure. For the same reason one patient was excluded from Turin panel. Data for *XRCC1* R399Q and *XRCC1* −77T>C are not available in one control of Turin panel.

First statistical analyses were carried out considering separately Casale Monferrato (133 MPM patients and 252 population controls) and Turin (68 MPM patients and 44 controls), then the panels were pooled (201 MPM patients and 296 controls). Moreover statistical analyses were performed in the subgroup of asbestos-exposed subjects (Casale: 131 MPM patients and 194 controls; Turin: 67 MPM patients and 25 controls; Casale + Turin: 198 MPM cases and 218 controls). Overall data are included in Supplementary Table S1, S2 and S3. The ORs for the statistically significant associations are listed in Tables 3 and 4. In Casale Monferrato panel (Table 3), when we considered only asbestos-exposed subjects, the risk of MPM increased with the increasing number of *XRCC1*-399Q alleles and results are statistically significant (OR = 1.44, 95%CI 1.02–2.03). When the dominant model was considered (i.e. *XRCC1* RQ heterozygotes plus QQ homozygotes versus RR homozygotes) the association with MPM was borderline significant (OR = 1.50, 95%CI 0.98–2.28). When *XRCC1* rs1799778 was considered as a continuous variable the risk was statistically significant (OR = 1.43, 95%CI 1.02–2.01). This change is located in intron 3. A possible functional role is not reported.

The present analysis includes the panel of patients and controls evaluated in our previous paper [16]. If we exclude these subjects the OR associated with *XRCC1* -399Q in asbestos exposed individuals becomes 1.25 (95%CI 0.76–2.06), based on 55 patients and 109 controls.

Data from Turin panel never reached statistical significance when considered separately, probably due to the small sample. However, even if not significant, the retrieved information was always consistent with that of Casale panel alone.

Considering the Casale Monferrato and Turin panels together (Table 4), in asbestos-exposed individuals the association between *XRCC1* -399Q and MPM decreased and became only borderline statistically significant (OR = 1.34, 95%CI 0.98–1.84). *XRCC1* −77C instead gave some indication of a protective effect with an OR = 0.52 (95%CI 0.27–1.01); though not statistically significant, this SNP suggests a protective effect of the variant allele also when it is considered as a continuous variable (OR = 0.75, 95%CI 0.55–1.03) and when the dominant model was considered (i.e. *XRCC1* TC heterozygotes plus TT homozygotes versus CC homozygotes (OR = 0.58, 95%CI 0.32–1.05).

The haplotype analysis performed by Haploview v4.1 on *XRCC1* R399Q and *XRCC1* −77T>C showed that they were in linkage disequilibrium (LD) ($D' = 0.94$; $r^2 = 0.27$).

To ascertain whether the effect of the reported association was due to a further variant in LD with the studied SNPs we then sequenced the coding and promoter region of *XRCC1*. Several doc-

Table 3
Odds ratios (ORs) and 95% confidence intervals (95%CIs) from multivariate unconditional logistic regression adjusted by age, gender and asbestos exposure for all subjects, and by age and gender for the asbestos-exposed subjects in the Casale Monferrato panel.

Gene SNP (rs) Variants	Cases (%)[a]	Controls (%) All subjects	OR (95%CI)	Cases (%) Asbestos – exposed only	Controls (%)	OR (95%CI)
XRCC1 R399Q (rs25487)						
RR	54 (41%)	114 (45%)	1.00 (ref.)	52 (40%)	93 (48%)	1.00 (ref.)
RQ	59 (44%)	113 (45%)	1.34 (0.82–2.19)	59 (45%)	83 (43%)	1.45 (0.89–2.38)
QQ	20 (15%)	25 (10%)	1.88 (0.90–3.92)	20 (15%)	18 (9%)	2.05 (0.97–4.35)
Continuous variable	133 (34.5%)	252 (65.5%)	1.36 (0.97–1.91)	131 (40%)	194 (60%)	**1.44 (1.02–2.03)**
XRCC1 −77T>C (rs3213245)						
TT	49 (37%)	86 (34%)	1.00 (ref.)	49 (37%)	63 (33%)	1.00 (ref.)
TC	67 (50%)	127 (51%)	0.86 (0.52–1.42)	66 (51%)	101 (52%)	0.83 (0.50–1.38)
CC	17 (13%)	39 (15%)	0.69 (0.33–1.44)	16 (12%)	30 (15%)	0.62 (0.29–1.30)
Continuous variable	133 (34.5%)	252 (65.5%)	0.84 (0.59–1.18)	131 (40%)	194 (60%)	0.80 (0.56–1.13)
XRCC1 IVS3 A>C (rs1799778)						
AA	54 (40%)	114 (45%)	1.00 (ref.)	52 (40%)	93 (48%)	1.00 (ref.)
AC	58 (44%)	112 (44%)	1.34 (0.82–2.19)	58 (44%)	82 (42%)	1.45 (0.88–2.39)
CC	21 (16%)	26 (10%)	1.85 (0.90–3.81)	21 (16%)	19 (10%)	2.02 (0.97–4.21)
Continuous variable	133 (34.5%)	252 (65.5%)	1.36 (0.97–1.89)	131 (40%)	194 (60%)	**1.43 (1.02–2.01)**
ERCC1 N118N (rs11615)						
TT	43 (32%)	99 (39%)	1.00 (ref.)	43 (33%)	78 (40%)	1.00 (ref.)
TC	71 (53%)	119 (47%)	1.49 (0.90–2.44)	69 (53%)	91 (47%)	1.42 (0.86–2.34)
CC	19 (14%)	34 (14%)	1.38 (0.67–2.82)	19 (14%)	25 (13%)	1.39 (0.68–2.88)
Continuous variable	133 (34.5%)	252 (65.5%)	1.24 (0.89–1.74)	133 (40%)	194 (60%)	1.24 (0.88–1.74)

Boldfaced letters: statistically significant.
[a] In Casale Monferrato panel, 18 MPM patients were excluded from statistical analyses because of an undefined asbestos exposure.

umented changes were identified, but none characterized only *XRCC1* 399Q or −77C patients.

An association was found also for *ERCC1* N118N. Heterozygotes (versus wild-type) showed an increased OR both in all subjects (OR = 1.66, 95%CI 1.06–2.60) and in asbestos-exposed only (OR = 1.59, 95%CI 1.01–2.50). Homozygotes CC did not show an association with MPM (probably due to the small size of the group). When the dominant model was considered (i.e. *ERCC1* heterozygotes CT plus homozygotes CC versus homozygotes TT) the risk to develop MPM conferred by the variant allele C was statistically significant both in all subjects (OR = 1.61, 95%CI 1.06–2.47) and in asbestos-exposed only (OR = 1.56, 95%CI 1.02–2.40).

Meta-analysis was performed for those SNPs that were studied either by Gemignani et al. [33] or Landi et al. [34] and in this study. We evaluated also the heterogeneity between these studies and we found that our study and Gemignani et al. were homogeneous, whereas our study and Landi et al. were heterogeneous. Consequently, we here presented only data of meta-analysis with Gemignani et al. A total of 252 patients and 356 controls were included in this meta-analysis (Table 5 and Table S4). Asbestos exposure could not be considered. Interestingly, *APEX* D148E and *ERCC1* N118N showed a statistically significant association with MPM (*APEX* EE homozygotes OR = 1.72, 95%CI 1.02–2.91; *ERCC1* 118N heterozygotes OR = 1.51, 95%CI 1–2.26).

XRCC1 R399Q was not associated to MPM in this meta-analysis (Table S4).

3.3. The combination of ERCC1 N118N and XRCC1 R399Q polymorphisms

In order to evaluate the joint effect of *ERCC1* and *XRCC1* polymorphisms, we divided subjects into four groups. We defined *ERCC1* TT genotype and *XRCC1* GG genotypes as reference group. The group with at least one variant allele for both polymorphisms

Table 4
Odds ratios (ORs) and 95% confidence intervals (95%CIs) from multivariate unconditional logistic regression adjusted by age, gender, panel and asbestos exposure for all subjects, and by age, gender and panel for the asbestos-exposed subjects in the Casale Monferrato and Turin panel together.

Gene SNP (rs) Variants	Cases[a]	Controls (%) All subjects	OR (95%CI)	Cases (%) Asbestos – exposed only	Controls (%)	OR (95%CI)
XRCC1 R399Q (rs25487)						
RR	85 (42%)	133 (45%)	1.00 (ref.)	82 (41%)	105 (48%)	1.00 (ref.)
RQ	90 (45%)	131 (44%)	1.34 (0.87–2.07)	90 (46%)	92 (42%)	1.46 (0.94–2.27)
QQ	26 (13%)	31 (11%)	1.50 (0.76–2.95)	26 (13%)	21 (10%)	1.67 (0.83–3.34)
Continuous variable	201 (40.5%)	295 (59.5%)	1.26 (0.93–1.71)	198 (47.5%)	218 (52.5%)	1.34 (0.98–1.84)
XRCC1 −77T>C (rs3213245)						
TT	75 (37%)	104 (35%)	1.00 (ref.)	75 (38%)	70 (32%)	1.00 (ref.)
TC	99 (49%)	144 (49%)	0.89 (0.57–1.39)	98 (49%)	111 (51%)	0.85 (0.54–1.34)
CC	27 (14%)	47 (16%)	0.63 (0.33–1.20)	25 (13%)	37 (17%)	**0.52 (0.27–1.01)**
Continuous variable	201 (40.5%)	295 (59.5%)	0.82 (0.60–1.10)	198 (47.5%)	218 (52.5%)	**0.75 (0.55–1.03)**
ERCC1 N118N (rs11615)						
TT	64 (32%)	117 (40%)	1.00 (ref.)	64 (32%)	90 (41%)	1.00 (ref.)
TC	103 (51%)	137 (46%)	**1.66 (1.06–2.60)**	100 (51%)	99 (45%)	**1.59 (1.01–2.50)**
CC	34 (17%)	42 (14%)	1.47 (0.79–2.73)	34 (17%)	30 (14%)	1.49 (0.79–2.79)
Continuous variable	201 (40.5%)	296 (59.5%)	1.30 (0.96–1.74)	198 (47.5%)	219 (52.5%)	1.29 (0.96–1.75)

Boldfaced letters: statistically significant.
[a] In Casale Monferrato panel, 18 MPM patients were excluded from statistical analyses because of an undefined asbestos exposure. Data for *XRCC1* IVS3 A>C (rs 1799778) are not available for Turin panel.

Table 5
Meta-analysis of our data and Gemignani' data [33].

Gene SNP (rs)	OR	95%CI	p (heterog)
APEX D148E (rs3136820)			
Homozygous			
Study			
Casale Monf.to	1.63	0.85–3.1	0.779
Gemignani et al. 2009	1.91	0.78–4.66	
Pool	**1.72**	**1.02–2.91**	
Heterozygous			
Study			
Casale Monf.to	1.04	0.63–1.73	0.739
Gemignani et al. 2009	1.2	0.61–2.52	
Pool	1.09	0.73–1.64	
ERCC1 N118N (rs11615)			
Homozygous			
Study			
CasaleMonf.to	1.38	0.67–2.82	0.932
Gemignani et al.	1.45	0.6–3.52	
Pool	1.41	0.80–2.46	
Heterozygous			
Study			
Casale Monf.to	1.49	0.9–2.44	0.939
Gemignani et al.	1.54	0.78–3.03	
Pool	**1.51**	**1.00–2.26**	

Boldfaced letters: statistically significant.

showed a statistically significant association with MPM in asbestos exposed subjects both in Casale Monferrato panel (OR = 2.02, 95%CI 1.01–4.05) and in Casale Monferrato and Turin pooled (OR = 2.39, 95%CI 1.29–4.43) (Table 6).

Indexes of sinergy, measured as departure from additive interaction [39], were not statistically significant.

3.4. Haplotype analysis

The *XRCC1* haplotypes were characterized by nine tag SNPs (identified by Haploview v4.1 on HapMap CEU panel, release #24) and three other SNPs that we analyzed previously (rs3213245, rs1799782, rs25487). The pairwise linkage disequilibrium (D' and r^2 values) between *XRCC1* genetic markers in the controls group of the Casale Monferrato panel is shown in Fig. S1.

We estimated haplotype frequencies for cases and controls by the SHEsis software platform. We considered only haplotypes with a frequency >3%. Results are reported in Table 7. The *XRCC1*-TGGGGGAACAGA haplotype was more represented in cases than in controls. Among the subjects exposed to asbestos (131 cases, 194 controls) that haplotype was significantly associated with an increased risk to develop MPM (OR = 1.76, 95%CI 1.04–2.96).

We analyzed also the haplotypes that encompassed *ERCC1* and the adjacent gene *RAI*. Overall we examined six tag SNPs (identified by Haploview v4.1) and a further SNP of the region (i.e. rs11615). No haplotype was found significantly associated (data not shown).

Table 7
XRCC1 haplotype analysis: frequencies in cases and controls and OR (95%CI) adjusted for age and gender.

Haplotype[a]	Cases (%)[b]	Controls (%)[b]	OR (95%CI)
TGGGGGAACAGA	87 (33.2%)	104 (26.9%)	1 (ref.)
CGGAGGCGCGGA	**40 (15.3%)**	**75 (19.4%)**	**0.57 (0.34–0.96)**
CGGGGGCGCGGA	58 (22.1%)	81 (20.8%)	0.84 (0.53–1.34)
TGGGGGCATGGG	16 (6.1%)	33 (8.5%)	0.61 (0.31–1.19)
TGGGGGCACGCA	14 (5.3%)	28 (7.2%)	0.58 (0.28–1.2)
TGGGGCCACGGA	13 (5%)	20 (5.1%)	0.71 (0.32–1.60)
TGGGGGCACGGA	11 (4.2%)	17 (4.3%)	0.84 (0.35–2.04)
TTGGGGAACAGA	9 (3.4%)	12 (3.1%)	0.94 (0.36–2.48)

Boldfaced letters: statistically significant.

[a] The haplotype is defined as the allele present at position −77 (T>C, rs3213245), IVS2 (G>T, rs3213247), IVS2 (A>G, rs129763352), IVS2 (A>G, rs2854496), codon 50 (G>A, rs2307174), IVS3 (A>C, rs2023614), IVS3 (A>C, rs1799778), IVS4 (A>G, rs3213356), codon 194 (C>T, rs1799782), codon 399 (G>A, rs25487), IVS10 (C>G, rs3213371), +816 (A>G, rs3213403).

[b] Haplotype frequencies analyzed by SHEsis software platform. Haplotypes with frequencies <3% in both cases and controls are dropped.

3.5. FPRP

FPRP was estimated for significant results pertaining *XRCC1* R399Q, *XRCC1* −77T>C, *XRCC1* IVS3, *ERCC1* N118N and *XRCC1* haplotype. Data are reported in supplementary Table S5.

3.6. Expression analysis

Since *XRCC1* −77T>C (rs3213245) is located into the gene promoter and affects a Sp1 binding site, we evaluated mRNA expression levels from seventy-one normal pleural tissues. All the samples were genotyped for *XRCC1* −77T>C and the analyses were performed considering CC genotype as reference group. When we compared the different genotypes (i.e. 29 TT, 27 CT, 15 CC), for variation in gene expression level, the difference was not statistically significant ($p = 0.3$).

4. Discussion

Our group was the first one to identify an association between MPM and a DNA repair gene, *XRCC1*, on the basis of an a priori hypothesis on the mechanism of damage from asbestos fibers. Our study suggested that a decreased response to DNA damage may favour asbestos carcinogenicity [16]. More recently, by using a custom-made SNP microarray another Italian group studied 50 SNPs in xenobiotic and oxidative metabolism enzymes (XME) genes and 76 SNPs in genes involved in genome stability (GS) in a panel that included 119 MPM patients and two groups of controls (104

Table 6
The combination of *ERCC1* N118N and *XRCC1* R339Q polymorphisms.

XRCC1 combined with *ERCC1* genotypes	Cases (%)	Controls (%) All subjects	OR (95%CI)	Cases (%) Asbestos –exposed only	Controls (%)	OR (95%CI)
Casale Monferrato panel						
GG + TT	20 (13%)	46 (18%)	1.00 (ref.)	19 (15%)	37 (19%)	1.00 (ref.)
GG + CC GG + CT	43 (29%)	68 (27%)	1.24 (0.60–2.54)	33 (25%)	56 (29%)	1.12 (0.54–2.32)
AA + TT GA + TT	27 (18%)	53 (21%)	1.19 (0.55–2.58)	24 (18%)	41 (21%)	1.21 (0.56–2.62)
AA + CT AA + CC GA + CC GA + CT	61 (40%)	85 (34%)	1.97 (0.99–3.92)	55 (42%)	60 (31%)	**2.02 (1.01–4.05)**
Casale Monferrato + Turin panels						
GG + TT	30 (14%)	59 (20%)	1.00 (ref.)	29 (14%)	47 (21%)	1.00 (ref.)
GG + CC GG + CT	65 (30%)	74 (25%)	1.92 (1.02–3.64)	53 (27%)	58 (27%)	1.76 (0.92–3.34)
AA + TT GA + TT	38 (17%)	58 (20%)	1.65 (0.83–3.29)	35 (18%)	43 (20%)	1.68 (0.84–3.38)
AA + CT AA + CC GA + CC GA + CT	87 (39%)	104 (35%)	**2.31 (1.25–4.25)**	81 (41%)	70 (32%)	**2.39 (1.29–4.43)**

Boldfaced letters: statistically significant.

with and 695 without documented asbestos exposure). Statistical analysis showed a significant association with the nucleotidic change 282C>T within *NAT2* [33], reinforcing existing evidences that genetic polymorphisms are possibly involved in the etiology of MPM [40]. That observation is difficult to explain biologically because the mechanism by which NAT2 may influence asbestos carcinogenicity is not clear. Some of the studied DNA repair genes revealed significant associations, but most of them disappeared after Bonferroni correction for multiple analyses. A further study from the same group reported an association with *SOD2* 16A [34].

To extend previous observation we investigated the association of 35 SNPs with MPM in a panel of 220 Italian patients and 296 controls whose asbestos exposure had been accurately evaluated. The largest portion of patients and controls were from Casale Monferrato, a town whose population experienced widespread, non-occupational exposure to asbestos, additional to that of workers employed in the local asbestos-cement factory. It is expected that this situation should reveal genetic susceptibility by increasing penetrance.

Only two genes were found significantly associated with MPM, i.e. *XRCC1* and *ERCC1*, both involved in DNA repair.

Although our study design has some limitations (e.g. low response rate and selection of patients) the *XRCC1* R399Q, *XRCC1* IVS3 and *XRCC1* haplotype associations showed an acceptable level of false reports with a prior probability of 0.25 and an expected OR of 1.5. However, the *XRCC1* −77T>C and *ERCC1* N118N associations showed a low level of false reports only with an expected OR of 2.0 (power = 97%).

A dosage effect, suggestive of a dominant model, was shown for *XRCC1* 399Q.

The meta-analysis with the data on *XRCC1* and *ERCC1* by Gemignani et al. [33], regardless of asbestos exposure, confirmed and extended our results. *XRCC1* R399Q, evaluated on all individuals independently from asbestos exposure, is not associated to MPM, like in the Casale Monferrato and Turin serie. Thus, *XRCC1* R399Q seems to be a risk factor only for asbestos-exposed subjects. Conversely, the meta-analysis confirmed our data and showed that *ERCC1* N118N is a risk factor per se.

Haplotypes for both *XRCC1* and *ERCC1* were also evaluated, but only the *XRCC1*-TGGGGGAACAGA haplotype was found statistically significative, being more represented in cases than in controls. Our data seem to favour the hypothesis that the effect of the contiguous genes *XRCC1* and *ERCC1* is not due to LD.

Sequencing of the promoter and coding region of *XRCC1* in patients and controls did not reveal another DNA change in LD with the R399Q that could functionally explain the association. Thus the association should be due to the studied SNPs or to other SNPs located in the non-sequenced regions.

Both *XRCC1* R399Q and *ERCC1* N118N have been found associated with cancer in different studies [41–47]. The combination of *ERCC1* and *XRCC1* polymorphisms better predicts clinical outcome to oxaliplatin-based chemotherapy in metastatic colorectal cancer [48]. However, little is known about the functional effect of these SNPs.

Epidemiological association studies, performed on different cancer types, have often been inconclusive or contradictory. The *XRCC1* -399Q variant has been associated with lung cancer [41], breast cancer [42,43], colorectal cancer [44], prostate cancer [45], but not with bladder cancer [49].

Functional studies pointed out that *XRCC1* -399Q may be associated with increased DNA damage measured by aflatoxin B1-DNA adducts, glycophorin A (GPA) variant frequency [50], sister chomatid exchange and polyphenol DNA adducts [51]. Moreover, carriers of the 399Q variant showed a significant increase in chromosomal deletions in whole-blood cells [52].

Differently, the 399R and 399Q equally complement both the single-strand break repair defect and the sensitivity to methyl methanesulfonate (MMS) *in vitro* in *XRCC1*-deficient CHO cells, suggesting that the 399Q variant retained a substantial level of function in those experimental conditions [53].

Interestingly, R399Q is located in the BRCT1 domain, i.e. the poly (ADP-ribose) polymerase I (PARP) interaction site. The three-dimensional structure of the wild-type *XRCC1*-BRCT1 domain is characterized by four α helices and three β sheets. The predicted structure of the variant 399Q protein shows the absence of three α helices and two β sheets. This suggests that 399Q may induce a significant conformational change in the BRCT domain. Since α helices are involved in protein–protein interactions, it is possible that the 399Q variant modifies the scaffold function of *XRCC1* [54].

Moreover, the *XRCC1* haplotype that is associated to MPM includes another SNP that may have functional relevance. The −77T>C SNP is located in the promoter region of *XRCC1* within the core of a predicted Sp1-binding sequence. Sp1 is a zinc-finger transcription factor that may act as a transcriptional repressor or activator, depending on the context. Using several non-pleural cell lines Hao et al. [55] demonstrated that the −77C allele has an high affinity to Sp1, but is associated with a reduced transcriptional activity *in vitro*. They concluded that Sp1 acts as a transcriptional repressor of *XRCC1* expression and that the reduced *XRCC1* expression due to the C allele may be the reason of its association with lung [56,57] and gastric cancer [58].

On the contrary, our results show that −77C plays a protective role for MPM. Since −77T and 399Q lie on the same at-risk haplotype our hypothesis was that the reduced Sp1 affinity due to the −77T allele may cause an increased expression of the 399Q variant protein leading to an impaired function, such as abnormal interactions with other DNA repair protein of the BER complex.

However, no data are available for expression of *XRCC1* in pleural tissues relative to specific *XRCC1* genotypes. To ascertain our hypothesis we analyzed *XRCC1* expression in genotyped healthy subjects using real-time PCR. Our data do not suggest an effect of this SNP on *XRCC1* expression on pleural tissues. However, we evaluated expression in pleural tissue as a whole, not specifically in mesothelial primary cell cultures.

Controversial epidemiological association studies are also evident for *ERCC1* N118N. This SNP has been associated with response to treatment of lung cancer [46], but not with colorectal cancer [59]. On the other hand, a study of some authors of this paper showed that not only the *ERCC1* N118N, but also *ERCC1* haplotypes modify bladder cancer risk [47].

The *ERCC1* gene and the *XRCC1* gene are located close one another (approximately 2 Mb apart). Hoeijmakers et al. [60] found that *ERCC1* 3′-terminus overlapped with the 3′-end of another gene, designated *ASE1*. This exceptional type of gene overlap was conserved in mouse and even in the yeast *ERCC1* homolog, *RAD10*, suggesting an important biologic function of this region (19q13.2–q13.3). Besides, haplotypes in this region have been found associated with increased cancer risk [61–65]. Although we have found an association between MPM and a *XRCC1* haplotype and a cumulative of *XRCC1* and *ERCC1* variants, we have not observed an association with *ERCC1* haplotypes. The biological mechanisms that explain the association of these SNPs with MPM are still obscure.

Interestingly, our meta-analysis shows that also another DNA repair gene, *APEX*, is associated to MPM using the codominant model. APEX is part of BER, as *XRCC1* [66]. More data are needed to ascertain this association.

In conclusion, this study shows an association between two DNA repair genes and MPM and strengthen the hypothesis of a role for genetic risk factors in asbestos carcinogenicity. It should be stressed that these risks are one-two orders of magnitude lower than the

risk caused by asbestos exposure itself (OR of 50–100 for asbestos versus 1–2 for genetic factors).

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Conflicts of interest of statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2011.01.001.

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