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research article

Matrix metalloproteinases polymorphisms as baseline risk predictors in malignant pleural mesothelioma

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Background. Malignant mesothelioma (MM) is a rare disease, linked to asbestos exposure in more than 80% of the cases. Matrix metalloproteinases (MMPs) have been identified as modulators of the tumour microenvironment and carcinogenesis. Polymorphisms of selected MMPs have been studied as potential biomarkers of time to progression (TTP) and overall survival (OS) in MM. The aim of our study was to investigate selected MMP polymorphisms as baseline risk predictors in MM development in combination with other well known risk factors, such as asbestos exposure.

Patients and methods. The study included 236 patients and 161 healthy blood donors as the control group. Ten different polymorphisms in three MMP genes were genotyped using a fluorescence-based competitive allele-specific assay (KASPar): *MMP2* rs243865, rs243849 and rs7201, *MMP9* rs17576, rs17577, rs2250889 and rs20544, and *MMP14* rs1042703, rs1042704 and rs743257. In statistical analyses continuous variables were described using median and range (25%–75%), while frequencies were used to describe categorical variables. Deviation from the Hardy-Weinberg equilibrium (HWE) was assessed using the standard chi-square test. The additive and dominant genetic models were used in statistical analyses. The association of genetic polymorphism with MM risk were examined by logistic regression to calculate odds ratios (ORs) and their 95% confidence intervals (CIs).

Results. Carriers of at least one polymorphic *MMP2* rs243865 allele tended to have a decreased risk for MM (OR = 0.66, 95% CI = 0.44–1.00; P = 0.050). The association was more pronounced in patients with known asbestos exposure: carriers of at least one polymorphic allele had significantly lower MM risk (OR = 0.55, 95% CI = 0.35–0.86; P = 0.009). None of the other tested polymorphisms showed association with the risk of malignant pleural mesothelioma.

Conclusions. The *MMP2* rs243865 polymorphism may have a protective role in malignant pleural mesothelioma development. This finding is even more evident in patients exposed to asbestos, implying a strong gene-environment interaction.

Key words: matrix metalloproteinases; genetic polymorphism; malignant mesothelioma

Introduction

Malignant mesothelioma (MM) is a rare disease, linked to asbestos exposure in more than 80% of the cases. The latency period can last up to thirty years and estimated median survival is 9–12 months. The worldwide incidence of mesothelioma is slowly rising, with approximately 94 000 new cases per year. The most affected areas are parts of Europe, Australia and the USA.[1] The rise in the MM incidence has been noticed in the Slovene population as well. The Slovenian national registry follows the data on MM since 1961. The incidence in 2014 was 37 new cases per year in a population of approximately 2 million.[2]

Several preclinical studies have identified matrix metalloproteinases (MMPs) as modulators of the tumour microenvironment and having an important role in carcinogenesis.[3] MMPs are calcium-dependent, zinc-containing endopeptidases, with three common domains containing the propeptide, catalytic and haemopexin-like C-terminal domain.[4] They are involved in tissue remodelling by interfering with the cell-cell and cell-extracellular matrix interactions. Studies have shown that MMPs, particularly MMP-2 and MMP-9, play a role in tumour angiogenesis, invasion and metastasis.[5] The studies performed thus far show that MMPs and their inhibitory molecules, tissue inhibitors of metalloproteinases (TIMPs), have an important role in proliferation and progression of MM and some other, more frequent malignancies, such as colon and breast cancer. Different MMP genes (*MMP2, MMP9, MMP11, MMP14*) and their expression were studied in mesothelioma tissue as potential prognostic markers.[6] In a previous paper we studied the possible role of single nucleotide polymorphisms (SNPs) as potential markers of treatment response.[7] We identified *MMP9* rs2250889, *MMP9 rs20544, MMP14* rs1042703 as statistically significantly associated with overall survival (OS) in MM. Carriers of the polymorphic *MMP9* rs2250889 and *MMP14* rs1042703 alleles had shorter OS, compared to non-carriers, while carriers of polymorphic *MMP9* rs20544 allele had longer OS.[7]

Many studies investigated the role of *MMP* polymorphisms in the baseline genetic risk for common diseases and tumours, however, the role of *MMP* polymorphisms was found to be conflicting in different diseases. In a large nested case-control study investigating skin cancer risk, *MMP9* Arg668Gln polymorphism has been associated with a decreased risk of squamous cell skin cancer (SCC).[8]

The opposite effect was observed in T-cell acute lymphoblastic leukaemia (T-ALL), where *MMP2* rs243865 and *MMP9* rs3918242 polymorphisms were associated with an increased risk of T-ALL.[9]

The data from the literature, linking *MMP* polymorphisms with tumour risk and the statistically significant associations between the selected *MMP2*, *MMP9* and *MMP14* SNPs and time to progression (TTP) and OS in MM, led us to further investigate their potential role in baseline genetic risk of MM development. Our aim was to investigate selected *MMP* polymorphisms as baseline risk predictors in MM development in combination with other well known risk factors, such as asbestos exposure.

Patients and methods

Patients

Patients with histologically confirmed pleural or peritoneal mesothelioma diagnosed and treated between 2007 and 2016 were included in this retrospective study. Patients were diagnosed mostly at the University Clinic Golnik and at the Department of Thoracic Surgery of the University Medical Centre Ljubljana. Patients were treated and followed-up at the Institute of Oncology Ljubljana, Slovenia.

Most patients included in the study were also participating in previous studies on pharmacogenomics of MM treatment, conducted at the Institute of Oncology Ljubljana, Slovenia. Some of the patients were also included in the clinical trial AGILI (Trial registration ID: NCT01281800).[10]

Clinical characteristics at diagnosis were obtained from medical records or assessed during clinical interview. Regarding asbestos exposure, patients were divided in two groups: patients with no known asbestos exposure and patients with known occupational or environmental exposure.

The control group consisted of 161 unrelated healthy Slovenian blood donors, aged 49 to 65.

The study was approved by the Slovenian National Medical Ethics Committee and was carried out according to the Declaration of Helsinki.

DNA extraction and genotyping

Genomic DNA was extracted from frozen whole-blood samples collected at the inclusion in any of the above mentioned studies using the Qiagen FlexiGene Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

Ten different polymorphisms in three *MMP* genes were genotyped: *MMP2* rs243865, rs243849 and rs7201, *MMP9* rs17576, rs17577, rs2250889 and rs20544, and *MMP14* rs1042703, rs1042704 and rs743257. Predicted function of these polymorphisms was assessed using SNP Function Prediction tools.[11]

The genotyping of all the SNPs was carried out using a fluorescence-based competitive allele-specific assay (KASPar), according to the manufacturer's instructions (LGC Genomics, UK).

For all investigated polymorphisms, 15% of samples were genotyped in duplicates. Genotyping quality control criteria included 100% duplicate call rate and 90% SNP-wise call rate.

TABLE 1. Patients' characteristics (N = 236)

Characteristic		N (%)
Gender	Male	174 (73.7)
	Female	62 (26.3)
Age	Median (25%-75%)	66 (58-72)
Stage	I	18 (7.6)
	II	60 (25.4)
	III	70 (29.7)
	IV	67 (28.4)
	Peritoneal	20 (8.5)
	Not determined	1 (0.4)
Histological type	Epitheloid	169 (71.6)
	Biphasic	27 (11.4)
	Sarcomatoid	26 (11.0)
	Not characterized	14 (5.9)
ECOG performance status	0	15 (6.4)
	1	114 (48.3)
	2	92 (39.0)
	3	15 (6.4)
Metastases	No	206 (87.3)
	Yes	30 (12.7)
Asbestos exposure	Not exposed	61 (26.5) [6]
	Exposed	169 (73.5)
Smoking	No	123 (57.7)
	Yes	106 (46.3)

Numbers in square brackets denote the number of patients with missing data.
ECOG = Eastern Cooperative Oncology Group

TABLE 2. Variant allele characteristics, frequencies and agreement with HWE

Gene	SNP	SNP characteristics	Variant allele frequency	P_{HWE}
MMP2	rs243865	c.-1306C>T	0.24	0.165
	rs243849	c.999C>T, p.Asp333=	0.14	0.798
	rs7201	c.*260A>C	0.41	0.441
MMP9	rs17576	c.836A>G, p.Gln279Arg	0.36	0.785
	rs2250889	c.836A>G, p.Gln279Arg	0.05	0.535
	rs17577	c.2003G>A, p.Arg668Gln	0.15	0.096
	rs20544	c.*3C>T	0.44	0.445
MMP14	rs1042703	c.22T>C, p.Pro8Ser	0.26	0.164
	rs1042704	c.817G>A, p.Asp273Asn	0.20	0.830
	rs743257	c.*83C>T	0.50	0.519

HWE = Hardy-Weinberg equilibrium; SNP = single nucleotide polymorphism

Statistical analyses

Continuous and categorical variables were described using median and range (25%-75%) and frequencies, respectively. Deviation from the Hardy-Weinberg equilibrium (HWE) was assessed using the standard chi-square test. The additive and dominant genetic models were used in statistical analyses. The associations of genetic polymorphisms with MM risk were examined by logistic regression to calculate odds ratios (ORs) and their 95% confidence intervals (CIs).

All statistical analyses were carried out by IBM SPSS Statistics, version 21.0 (IBM Corporation, Armonk, NY, USA). Haplotypes were reconstructed and analysed using Thesias software, version 3.1. The most frequent haplotype was used as the reference. All statistical tests were two sided and the level of significance was set to P = 0.05. Due to the exploratory nature of the study, no adjustments for multiple comparisons were used.

Results

Patient characteristics

In total, we included 236 patients with MM and 161 healthy blood donors as a control group. Clinical characteristics of patients are summarized in Table 1. Among controls, 125 (77.6%) were male and 36 (22.4%) were female. Median age was 55 (52–58.5) years. There were no significant differences between cases and controls regarding gender (P = 0.375), however, controls were significantly younger than MM patients (P < 0.001).

Genotyping analysis

Variant allele frequencies for investigated SNPs are presented in Table 2. The distributions of all the investigated SNPs in the control group were in agreement with the Hardy-Weinberg equilibrium.

Duplicate call rate was 100% for all SNPs. With the exception of one SNP that had a call rate of 92%, all SNPs had a call rate above 97%.

Genotype frequencies for cases and controls are presented in Table 3. Carriers of at least one polymorphic *MMP2* rs243865 allele tended to have a decreased risk for MM (OR = 0.66, 95% CI = 0.44-1.00; P = 0.050). The association was more pronounced in patients with known asbestos exposure: carriers of at least one polymorphic allele had significantly lower MM risk (OR = 0.55, 95% CI = 0.35–0.86; P = 0.009). As the number of homozygotes for poly-

TABLE 3. The association of investigated SNPs with risk for malignant mesothelioma

SNP	Genotype	Controls N (%)	Cases N (%)	OR (95% CI)	P	Cases exposed to asbestos N (%)	OR (95% CI)	P
MMP2 rs243865	CC	90 (55.9)	155 (65.7)	Ref.		118 (69.8)	Ref.	
	CT	65 (40.4)	77 (32.6)	0.69 (0.45-1.05)	0.081	48 (28.4)	0.56 (0.35-0.89)	**0.015**
	TT	6 (3.7)	4 (1.7)	0.39 (0.11-1.41)	0.150	3 (1.8)	0.38 (0.09-1.57)	0.181
	CT+TT	71 (44.1)	81 (34.3)	0.66 (0.44-1.00)	**0.050**	51 (30.2)	0.55 (0.35-0.86)	**0.009**
MMP2 rs243849	CC	108 (75.0) [17]	163 (71.5) [8]	Ref.		116 (71.2) [6]	Ref.	
	CT	33 (22.9)	57 (25.0)	1.14 (0.70-1.87)	0.592	42 (25.8)	1.18 (0.70-2.00)	0.527
	TT	3 (2.1)	8 (3.5)	1.77 (0.46-6.81)	0.408	5 (3.1)	1.55 (0.36-6.65)	0.554
	CT+TT	36 (25.0)	65 (28.5)	1.20 (0.74-1.92)	0.459	47 (28.8)	1.22 (0.73-2.02)	0.451
MMP2 rs7201	AA	56 (35.9) [5]	78 (33.5) [3]	Ref.		63 (37.5) [1]	Ref.	
	AC	71 (45.5)	114 (48.9)	1.15 (0.73-1.81)	0.539	78 (46.4)	0.98 (0.60-1.58)	0.923
	CC	29 (18.6)	41 (17.6)	1.02 (0.56-1.82)	0.960	27 (16.1)	0.83 (0.44-1.56)	0.560
	AC+CC	100 (64.1)	155 (66.5)	1.11 (0.73-1.70)	0.622	105 (62.5)	0.93 (0.59-1.47)	0.765
MMP9 rs17576	AA	64 (40.3) [2]	100 (42.9) [3]	Ref.		74 (44.3) [2]	Ref.	
	AG	75 (47.2)	114 (48.9)	0.97 (0.63-1.49)	0.900	79 (47.3)	0.91 (0.57-1.44)	0.691
	GG	20 (12.6)	19 (8.2)	0.61 (0.30-1.23)	0.165	14 (8.4)	0.61 (0.28-1.30)	0.196
	AG+GG	95 (59.8)	133 (57.1)	0.90 (0.59-1.35)	0.599	93 (55.7)	0.85 (0.55-1.31)	0.458
MMP9 rs2250889	GG	146 (90.7)	212 (90.2) [1]	Ref.		152 (89.9)	Ref.	
	GA	15 (9.3)	23 (9.8)	1.06 (0.53-2.09)	0.876	17 (10.1)	1.09 (0.52-2.26)	0.820
MMP9 rs17577	GG	113 (70.2)	169 (72.8) [4]	Ref.		119 (71.3) [2]	Ref.	
	GA	47 (29.2)	60 (25.9)	0.85 (0.54-1.34)	0.490	45 (26.9)	0.91 (0.56-1.47)	0.699
	AA	1 (0.6)	3 (1.3)	2.01 (0.21-19.53)	0.549	3 (1.8)	2.85 (0.29-27.79)	0.368
	GA+AA	48 (29.8)	63 (27.2)	0.88 (0.56-1.37)	0.565	48 (28.7)	0.95 (0.59-1.53)	0.831
MMP9 rs20544	CC	33 (20.6) [1]	38 (16.3) [3]	Ref.		29 (17.4) [2]	Ref.	
	CT	74 (46.3)	121 (51.9)	1.42 (0.82-2.46)	0.210	82 (49.1)	1.26 (0.70-2.27)	0.441
	TT	53 (33.1)	74 (31.8)	1.21 (0.68-2.18)	0.518	56 (33.5)	1.20 (0.64-2.25)	0.563
	CT+TT	127 (79.4)	195 (83.7)	1.33 (0.79-2.24)	0.275	138 (82.6)	1.24 (0.71-2.15)	0.453
MMP14 rs1042703	TT	90 (57.0) [3]	147 (63.4) [4]	Ref.		109 (65.7) [3]	Ref.	
	TC	54 (34.2)	67 (28.9)	0.76 (0.49-1.18)	0.225	44 (26.5)	0.67 (0.41-1.09)	0.110
	CC	14 (8.9)	18 (7.8)	0.79 (0.37-1.66)	0.530	13 (7.8)	0.77 (0.34-1.71)	0.518
	TC+CC	68 (43.0)	85 (36.6)	0.77 (0.51-1.16)	0.204	57 (34.3)	0.69 (0.44-1.08)	0.108
MMP14 rs1042704	GG	103 (64.0)	160 (68.1) [1]	Ref.		113 (66.9)	Ref.	
	GA	51 (31.7)	64 (27.2)	0.81 (0.52-1.26)	0.346	47 (27.8)	0.84 (0.52-1.35)	0.475
	AA	7 (4.3)	11 (4.7)	1.01 (0.38-2.69)	0.982	9 (5.3)	1.17 (0.42-3.26)	0.761
	GA+AA	58 (36.0)	75 (31.9)	0.83 (0.55-1.27)	0.395	56 (33.1)	0.88 (0.56-1.39)	0.581
MMP14 rs743257	CC	40 (26.0) [7]	59 (25.1) [1]	Ref.		41 (24.4) [1]	Ref.	
	CT	73 (47.4)	104 (44.3)	0.97 (0.59-1.59)	0.892	76 (45.2)	1.02 (0.59-1.75)	0.955
	TT	41 (26.6)	72 (30.6)	1.19 (0.68-2.07)	0.538	51 (30.4)	1.21 (0.67-2.21)	0.526
	CT+TT	114 (74.0)	176 (74.9)	1.05 (0.66-1.67)	0.848	127 (75.6)	1.09 (0.66-1.80)	0.746

Numbers in square brackets denote the number of patients with missing data. Significant values are printed in bold.
CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism

TABLE 4. The association of haplotypes with frequencies above 5% for investigated genes with risk for malignant mesotjelioma in patients with asbestos exposure

Gene	Haplotype	Estimated frequency	OR (95% CI)	P
MMP2	CCA	0.377	Ref.	
	CCC	0.272	1.14 (0.77 - 1.68)	0.518
	CTA	0.144	1.14 (0.70 - 1.85)	0.599
	TCC	0.144	0.77 (0.48 - 1.25)	0.291
	TCA	0.056	0.59 (0.26 - 1.38)	0.223
MMP9	ACGT	0.572	Ref.	
	GCGC	0.204	0.86 (0.59 - 1.26)	0.440
	GCAC	0.137	0.81 (0.52 - 1.26)	0.353
MMP14	TGC	0.338	Ref.	
	TGT	0.267	1.39 (0.94 - 2.06)	0.103
	CGT	0.125	0.85 (0.52 - 1.37)	0.494
	TAT	0.110	1.23 (0.71 - 2.12)	0.461
	CGC	0.080	1.33 (0.71 - 2.46)	0.371

The single nucleotide polymorphisms are ordered from the 5'- to 3'-end as follows: *MMP2*:rs243865, rs243849, rs7201; *MMP9*:rs17576, rs17577, rs2250889, rs20544; *MMP14*:rs1042703, rs1042704, rs743257.

CI = confidence interval; OR = odds ratio

morphic allele was low, we only observed a significant association with decreased MM risk for heterozygotes in the additive model (Table 3).

In haplotype analysis, no significant associations with MM risk were observed, even when asbestos exposure was taken into account (Table 4). Nevertheless, haplotypes that included the polymorphic *MMP2* rs243865 allele had slightly lower risk, consistent with single SNP analysis, but the association did not reach statistical significance.

Discussion

This study investigated the influence of *MMP2*, *MMP9* and *MMP14* gene polymorphisms on baseline risk for MM in comparison with healthy control subjects. Carriers of *MMP2* rs243865 CT or CT/TT genotypes had significantly decreased risk for developing MM in comparison with CC homozygous genotype, especially in patients with known asbestos exposure.

MMP2 rs243865 (c.-1306C>T) is a promoter polymorphism and our prior *in silico* analysis has shown that it may influence binding of transcription factors and may alter chromatin states.[7] The data on whether the *MMP2* rs243865 T allele has a protective function or if it contributes to higher risk for cancer, is somewhat conflicting for different malignancies.

MMP polymorphisms have been extensively studied in many different common and rarer malignancies.[12,13] The most attractive and most significant *MMPs* in risk assessment studies were *MMP2*, *MMP9* and *MMP3* polymorphisms. *MMP2* rs243865, that was associated with modified cancer risk in our study, had the greatest influence on cancer risk in general. In accordance with our study, two meta-analysis presented the results showing that *MMP2* rs243865 polymorphism had a protective role in lung cancer susceptibility in both dominant and recessive models, which is consistent with our results. Seventeen studies were included in the meta-analysis and reported that the *MMP2* rs2438651 polymorphism had a protective role only in the Asian population.[12,13]

Considering that lung cancer is the most common thoracic malignancy, these results can be parallel to a less common thoracic malignancy such as MM. However, *MMP* polymorphisms in other common malignancies in the Asian population have been frequently studied. A meta-analysis of 12 publications studying urinary (renal and bladder) cancers showed a lower risk for bladder cancer with the T allele of *MMP2* rs243865 in Asian patients but not in the Caucasian population.[14]

All of the above discussed publications present the *MMP2* rs243865 T allele as having a somewhat protective role in cancer. There are publications that suggest the opposite effect of the T allele in *MMP2* rs243865. A control based study that included a Caucasian population investigated six different polymorphisms in *MMPs* and *TIMPs* in bladder cancer patients. They concluded that the combined genotype carrying *MMP2* rs243865 allele T with *MMP9* rs3918242 allele T was found to increase bladder cancer risk.[15] These results are the opposite to the previously mentioned Asian based metaanalysis.[14]

According to the db SNP and HapMap data on rs243865 frequency in genetically different populations, the C allele is more common in Caucasian populations. That can perhaps contribute to the different results in different studied populations.[16]

Nevertheless, all of the cited studies find that *MMP2* rs243865 could play a role as a risk factor in a variety of different malignancies. With regard to the *MMP2* rs243865, T allele containing genotypes seem to have a protective role in predominantly thoracic malignancies, such as lung cancer and MM.

Genome Wide Associated Study (GWAS) of 759 subjects in the Northern Italian population investigated 15 different SNPs in several genes, and

one of them was *MMP14* rs2236304. Almost all of these SNPs had either a significant positive (higher risk after asbestos exposure) or negative (lower risk after asbestos exposure) interaction with asbestos exposure, even after statistical corrections (Bonferroni) had been applied. But, the study has some limitations, such asthe small sample size, the age unbalanced control group and the possible rare genetics variants that could have been excluded from the GWAS statistical analysis.[17]

The *MMP2* rs243865, T allele genotypes seem to have a protective role in predominantly thoracic malignancies, such as lung cancer and MM. Moreover, thoracic malignancies are also well known to have a strong environmental component (eg. smoking, asbestos exposure).[18] The gene-environment interactions have been studied extensively in MM. The study that investigated the role of microsomal epoxide hydrolase *(mEH)*, glutathione S-transferases (*GSTM1*, *GSTT1)*, N-acetytransferase 2 (*NAT2)*, and cytochrome P1A1 (*CYP1A1)* genotypes concluded that the presence of synergisms between genotypes, i.e., *mEH* and *NAT2*, *mEH* and *GSTM,* and *NAT2* and *GSTM1* combined with the interaction observed with exposure to asbestos, suggests the presence of gene-environment and gene–gene interactions in the development of MM.[19]

Our results suggest a combined effect of asbestos exposure and *MMP2* rs243865. Gene-environment interactions in asbestos related diseases have been previously studied in enzymes such as catalase (CAT), superoxide dismutase (SOD 2, SOD3) and inducible nitric oxide synthase (iNOS), which are part of the enzymatic defence system against reactive oxygen species (ROS). Besides gene-gene interactions between *MnSOD* Ala -9Val and *CAT* -262 C>T polymorphisms as well as *iNOS* and *CAT* -262 C>T polymorphisms and the risk of asbestosis, gene-environment interactions were also reported. A strong interaction was reported between *GSTM1*-null polymorphism and smoking, *iNOS* (CCTTT)n polymorphism and smoking, and between *iNOS* (CCTTT)n polymorphism and cumulative asbestos exposure, suggesting that interactions between different genotypes, genotypes and smoking, and between genotypes and asbestos exposure have an important influence on the development of asbestosis.[20] These studies on asbestosis suggest, that gene-environment interactions should be investigated also in other asbestos related diseases, including MM, since asbestos exposure is a proven environmental risk factor in MM.

Despite some limitations of our study, such as a small sample size and a control group that was not appropriately age balanced, low rate of patient asbestos exposure and lacking this data of the control group, our results reached statistical significance and showed that there could be a genetic predisposition of certain MMP SNPs for MM and that there is a potential gene-environment interaction between MMP SNPs and asbestos that is a major risk factor for MM.

In conclusion, our data suggests that *MMP2* rs243865 polymorphism may have a protective role in malignant pleural mesothelioma. This finding is even more pronounced in patients exposed to asbestos, implying a strong gene-environment interaction.

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