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A Common Polymorphism Within MSLN Affects miR-611 Binding Site and Soluble Mesothelin Levels in Healthy People

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Abstract

INTRODUCTION: Soluble mesothelin related peptide (SMRP) was proposed as a promising diagnostic marker for malignant pleural mesothelioma (MPM). In a previous study, we found that rs1057147 within the 3' untranslated region of MSLN gene was associated with SMRP levels. Thus, we aimed to (1) confirm the previous association on a large series of volunteers and (2) test the hypothesis that the SNP could affect microRNA binding sites.

METHODS: The association analysis was verified in 759 subjects. Then, in silico predictions highlighted miR-611 and miR-887 as candidate miRNAs binding to the polymorphic site. Thus, chimeric constructs bearing the alternative alleles (G > A) were assayed alone or in cotransfection with the miRNA mimics, with dual luciferase reporter assay in non-MPM Met-5A cells. The miRNAs were also assayed by western blot analysis for their ability to down-regulate endogenous mesothelin in the MPM Mero-14 cell line.

RESULTS:.We confirmed that, among non-MPM volunteers, GG homozygotes have the lowest SMRP levels. When the genotype is taken into account, the specificity of SMRP as biomarker improves from 79.7% to 85.3%. Dual-luciferase assays showed a significantly lower reporter activity when the vector harbored the G allele as compared to A allele. miR-887 mimic caused a reduced reporter activity of vectors harboring A or G alleles, while miR-611 was effective only on the vector harboring the G allele. Transfection of these miRNAs into Mero-14 cells significantly reduced endogenous MSLN protein.

CONCLUSION:.SMRP performance as diagnostic biomarker improved by considering the genotype rs1057147. This polymorphism most likely affects a binding site for miR-611.

Introduction

Malignant pleural mesothelioma (MPM) is a fatal tumor mainly related to asbestos exposure. According to assessments based on the long latency period between exposure and diagnosis (typically 25–30 years), it is expected that its incidence will reach a peak in years 2015 to 2020 in countries where asbestos was banned too belatedly.[1,2] The mesothelin gene (MSLN) encodes a 70-kDa precursor protein, abundantly expressed in most of MPM patients. The precursor is cleaved into a 31-kDa fraction (the megakaryocyte potentiating factor) and a membrane-bound 40-kDa glycoprotein (mesothelin), thought to be involved in promoting tumor invasion.[3] A soluble form of mesothelin can be found in serum, the so-called SMRP (ie, "Soluble Mesothelin–Related Peptide"). It has not been definitively ascertained whether SMRP is formed following an alternative mRNA splicing or a proteolytic cleavage of the 40-kDa fragment.[4] However, SMRP is currently considered one of the best available biomarkers for diagnosis of MPM patients.[5] Unfortunately, according to a recent meta-analysis, wide interindividual variations with overlaps between MPM and nonaffected subjects limited its performance as marker.[6] Our previous study carried out on 59 healthy workers with a past exposure to asbestos suggested an association between the single nucleotide polymorphism (SNP) rs1057147 G > A within the 3' untranslated region (3'UTR) of the MSLN gene and SMRP levels.[7] This could be ascribed to the fact that the SNP falls within a miRNA binding site, thereby affecting the strength of miRNA::mRNA binding. In other words, rs1057147 could be a putative miRSNP. Several examples suggest that miRSNPs could have important phenotypic consequences. Previous studies found miRSNPs associated with the risk of breast,[8] colorectal,[9] ovarian,[10] and lung[11] cancers. Moreover, they were reported to be involved in chemoresistance,[12] risk of myocardial infarction,[13] and regulation of body height.[14].

In the present work, we analyzed a large sample set and were able to replicate the association between rs1057147 and SMRP levels. Thus, to further evaluate the role of rs1057147 in the regulation of MSLN: (1) in silico predictions were carried out to detect miRNAs having rs1057147 within their putative binding sites, (2) the expression of predicted miRNAs was measured in cell lines, healthy pleural and MPM specimens, and (3) luciferase reporter assays and western blot analyses were performed to verify whether MSLN 3'UTR was an actual target of the miRNAs predicted in (1).

MATERIALS AND METHODS

Population Description and Genotyping

A total of 371 healthy people, 318 patients affected by benign respiratory diseases (BRD), and 70 MPM patients were recruited at the University Hospital of Pisa in the period 2009 to 2012 as part of an occupational surveillance program on workers previously exposed to asbestos. Guidelines of the surveillance protocol stated that during the first medical examination all subjects underwent clinical tests (ie, chest radiography, spirometry tests, and SMRP evaluation from serum sample) and were asked to answer a detailed questionnaire regarding asbestos exposure, occupational tasks, smoking habits, previous respiratory diseases, and current health status (data summarized in Table 1). According to clinical examination results, those who were negative for all the tests were classified as “healthy subjects.” Subjects designated as BRDs were patients experiencing noncancerous diseases of the lungs, pleura, or airways, described in details in Table 1. All MPM patients were enrolled and tested for SMRP levels at diagnosis, before any treatment. The diagnoses were confirmed by histological and cytological examinations of pleural biopsies collected during thoracoscopy surgery, following the normal clinical routine. The preoperative work-up included an accurate physical and imaging check up, and a positron emission tomography (PET) scan aiming to exclude other synchronous malignancies. Exclusion criteria were a previous treatment of the disease or a suspicion of other neoplastic diseases. Subjects were recruited as a consecutive series of volunteers as soon as they were entered in the surveillance program. The follow up consisted of a routine lung examination and various hematologic exams to be repeated every 3 years for healthy subjects and every 12 months for BRD people. Specific details of these protocols were reported in a previous publication.[15] The study was approved by the institutional ethical committee of the University Hospital of Pisa. All subjects gave written informed consent. Whole blood and serum samples were obtained by venipuncture during the first medical examination and were kept at −80°C without additives until use. DNA was extracted from whole blood samples using EuroGOLD Blood DNA Mini Kit (EuroClone, Pero, Italy). Genotyping of rs1057147 was performed using KASPar PCR SNP genotyping system (KBiosciences, Herts, UK) with a success rate more than 96%. Allele frequencies were in agreement with those reported within the HapMap project for Caucasians (CEU population: G allele 0.78, A allele 0.22) and followed the Hardy-Weinberg equilibrium ($p > 0.05$). Serum SMRP levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Mesomark, Fujirebio Diagnostics, Japan). Serum samples were diluted 1:101 before analysis, using Calibrator A buffer provided by Mesomark kit. All the serum samples were assayed in duplicate randomly, i.e. the genotype information was not known when the ELISA was performed. First, the logistic regression analysis (LR) was used to examine the association between genotype and the risk of MPM. An inheritance dominant model, where AA and GA genotypes were grouped together and compared to the GG homozygotes, was used to assess the raw Odd Ratio (OR) and its 95% confidence interval (CI). Then, to verify the association between rs1057147 genotypes and serum SMRP levels, one-way analysis of variance (ANOVA) was performed, stratified for health status (healthy, BRD, MPM). Then, healthy subjects were grouped with BRD patients (forming a group from now on referred to as “non-MPM group”) and compared with MPM patients. Tukey’s multiple comparison test was performed to assess pairwise differences between the three genotypes within each group. The statistical significance threshold was set at 0.05 and StatGraphics Centurion XVI software (Manugistic, CA) was used for statistical analyses. Receiver operating characteristic (ROC) curves were generated with MedCalc statistical software (version 12.7.2.0, MedCalc Software, Belgium) comparing the non-MPM group versus the MPM group to evaluate SMRP as marker. First, the ROC curves were calculated without taking into account the genotypes. Then, because the association between SMRP level and the polymorphism was observed only among nonMPM subjects, the curves were recalculated using, alternatively, GG, GA, or AA volunteers of the non-MPM group, versus the whole group of MPM patients.

MicroRNA Selection

Specialized algorithms (miRBase,[16] miRanda,[17] TargetScan,[18] PicTar,[19] and Diana-microT)[20] were used to identify putative miRNA-binding sites within the 3'UTR of MSLN. We focused on microRNAs having rs1057147 within their binding sites. We then used RNAhybrid (http://bibiserv. techfak.uni-bielefeld.de/rnahybrid/) to measure the freeenergy (ΔG) of the miRNA::mRNA binding. The effect of the miRSNP was evaluated by calculating the absolute value of the difference between the binding free energies of the two alleles (|ΔΔG|), in analogy to what reported in previous articles.[9,21] MiRNAs showing the highest |ΔΔG| (ie, miR-611 and miR-887) were selected for further investigations through luciferase assay and western blot analysis.

Expression of miR-611 and miR-887 in Cell Lines and MPM and Healthy Tissues

Expression of miR-611 and miR-887 was assessed in Met-5A and Mero-14 cell lines, 14 pleural biopsies from MPM patients and 14 healthy pleural biopsies from non-MPM subjects affected by early-stage lung cancer. These samples were collected from the Cardiothoracic Department of the University Hospital of Pisa and are independent from the genotyped population reported before. MPM biopsies were collected at thoracoscopy before any treatment and diagnoses were confirmed by histological and cytological examinations. Small healthy pleural specimens were collected far from the tumor site. To ascertain that no evidence of lung cancer spread was present, healthy pleural biopsies were eye-inspected by surgeons and always confirmed by a pathologist under hematoxylin-eosin staining and immunohistochemistry evaluation. Specimens were kept in RNAlater (Life Technologies, Carlsbad, CA) and at −20°C until RNA extraction. Total RNA was extracted with Tri-Reagent (Sigma Aldrich, MO). Small noncoding RNAs were also isolated by adding glycogen to isopropanol during the precipitation phase.

MiR-887 expression was analyzed with TaqMan MicroRNA Reverse Transcription kit (Life Technologies) using stem-loop-specific miRNA primers starting from 20 ng of total RNA. Real-time PCR was performed using TaqMan Universal PCR Master Mix II no UNG (Life Technologies) and miR-887 TaqMan MicroRNA Assay (Life Technologies). Analysis of relative miRNA expression was performed using the ΔΔCt method[22] with U6 as endogenous control. A TaqMan microRNA assay was not available formature miR-611, so we analyzed primary miR-611 expression with specific TaqMan Pri-miRNA Assay. IScript cDNA Synthesis Kit (Bio-Rad, CA) was used to retro-transcribe 1 µg of total RNA. Real-time PCR was performed using 5x HOT FIREPol Probe qPCR Mix Plus no ROX (Solis BioDyne, Tartu, Estonia) and pri-miR-611 TaqMan PrimiRNA Assay (Life Technologies). HPRT, TBP, and RPLP0 were used as endogenous controls for normalization after stability analysis through geNorm software.[23] A nonparametric test (Kruskal-Wallis) was used to assess differences between cases and controls.

Cell Lines

Nonmalignant transformed human pleural mesothelial cells24 (Met-5A) were purchased from ATTC (American Type Tissue Collection) and cultured in Medium 199 (Gibco in Life Technologies) supplemented with 10% FBS, 1% pen/ strep, 3 nM epidermal growth factor, 400 nM hydrocortisone, and 870 nM insulin. Human malignant mesothelioma cells (Mero-14) were kindly donated by Istituto Tumori of Genova (National Research Council, Genova, Italy), and maintained in Dulbecco's Modified Eagle Medium (Lonza, MD) with 10% FBS and 1% pen/strep. Both cell lines were grown in 5% CO2 at 37°C in a cell culture incubator.

Allele-Specific Oligonucleotide PCR (ASO-PCR) was performed to determine the genotype of rs1057147 G > A in Mero-14 cell line. DNA was extracted with JETQUICK Blood and Cell Culture DNA Spin Kit (Genomed, Löhne, Germany). ASO-PCR was carried out using the common primer 5'-AGCAGGGTCAGGAAGACCTC-3' and, alternatively, the G allele-specific primer 5'-GCCAGGAGCAGGCACGGGTGG-3' or the A allele-specific primer 5'-GCCAGGAGCAGGCACGGGTGA-3'. GG genotype was identified from ASO-PCR of the Mero-14 cell line.

Plasmid Construction and Dual-Luciferase Assay

The 3′UTR of MSLN (NM_005823.5) bearing the G allele of rs1057147 was cloned downstream the luciferase gene into pmiRGLO vector (Promega, Madison, WI) by GenScript Company (Piscataway, NJ) using SacI and XhoI as restriction enzymes at 5′ and 3′ end, respectively. The c.*69G > A (rs1057147) mutation was created using the QuikChange II Site-Directed Mutagenesis Kits (Agilent, Santa Clara, CA). The sequences of the mutagenic primers were: F = GAGCAGGCACGGGTGATCCCCGTTCCACCC R = GGGTGGAACGGGGATCACCCGTGCCTGCTC. These plasmids will be referred to from now on as "pmiR_G" and "pmiR_A." Met-5A cells were transfected with pmiR_G/_A (400ng) using Polyfect (Qiagen, Hilden, Germany). In another series of experiments, a cotransfection was performed with pmiR_G/_A (350 ng) together with, alternatively, hsamiR-611 or hsa-mir-887 mimics (30 nM) using Attractene (Qiagen). The AllStars Negative Control siRNA (Qiagen) was used as the negative control. Twenty-four hours after transfection, a Dual-Luciferase Reporter Assay (Promega) was performed. Relative luciferase units (RLU) were expressed as mean value of the firefly luciferase/Renilla luciferase ratio of three independent experiments.

Western Blot

Mero-14, MPM cell line expressing high levels of MSLN, were transfected at 60% to 80% confluence with either hsa-miR-611 or hsa-miR-887 mimics, at 5 nM and 30 nM, using Hiperfect reagent (Qiagen). AllStars Negative Control siRNA (Qiagen) was used as control.

Forty-eight hours after transfection, cells were lysed in 100 µl of ice-cold cell lysis buffer. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes, that were then incubated at 4°C overnight with primary anti-mesothelin (Mesothelin antibody G-1, Santa Cruz, TX; 1:3000) or anti-ß-actin (Anti-Actin, Clone C4, Millipore, MA; 1:5000) antibodies. Then, IgG-HRP Santa Cruz (1:6000) was used as secondary antibody for 1 h at room temperature. Detection was achieved using an ECL kit (Perkin Elmer, MA) according to manufacturer's instructions, and densitometry results were analyzed with Image J software (NIH, Bethesda, MD). For quantitative analysis, the signal intensity of each band was normalized with ß-actin densitometry values. Three independent experiments were performed and the results were analyzed with analysis of variance. See also the Supplemental Materials and Methods

RESULTS

rs1057147 Is Associated with SMRP Levels and the Two Alleles Perform Differently in a Dual-Luciferase Reporter Assay

No association was found between polymorphism rs1057147 and risk of MPM, when cases and controls were compared for their genotypes (dominant model, GA + AA vs GG genotypes; OR_{ass} = 0.91; 95% CI = 0.53–1.55; P_{ass} = 0.72). On the contrary, as expected, the group of MPM patients showed a mean level of serum SMRP of 3.58nM (± 0.49, standard error), significantly elevated (ANOVA, $p < 0.0001$) when compared with the groups of healthy (0.94±0.03) or BRD (1.04±0.03) subjects.

When the SMRP levels were analyzed in relation to genotypes, no association was found among MPM patients. In fact, the mean level of SMRP in patients with GG genotype (3.10±0.40, n = 49) was similar to that of patients with GA (3.64±0.82, n = 18) or AA genotype (2.15±0.59, n = 3), and the difference was not statistically significant (ANOVA, p = 0.658). However, interestingly, the association was found in the group of non-MPM subjects. In both healthy and BRD groups, the GG genotype was associated with the lowest levels of SMRP (0.82±0.03, n = 242 and 0.96±0.04, n = 226, respectively). Within each group, the mean SMRP levels measured in GA (1.06±0.05, n = 119, for healthy; 1.26±0.08, n = 79, for BRD) and AA (2.35±0.5, n = 10 for healthy; 1.22±0.21, n = 13, for BRD) genotypes were increased and was statistically significant, when

compared to the GG genotype. Thus, the analyses were repeated considering healthy and BRD subjects as a unique group (the non-MPM group) and a trend of increasing levels of SMRP (GG, 0.89±0.02, n = 468; GA, 1.14±0.04, n = 198; and AA, 1.71±0.27, n = 23) is observed in relation to the number of A alleles (ANOVA, $p < 0.0001$). The results of this analysis are reported in Figure 1, together with the Tukey's test pairwise comparison. When SMRP was evaluated as a biomarker, by comparing MPM versus non-MPM regardless of the genotype information, the ROC curves showed an AUC of 0.860 (95% CI = 0.833–0.884). The Youden's J index (0.554) pointed at the SMRP cutoff value of 1.28 nM, resulting in a sensitivity of 75.7% and a specificity of 79.7%. At a cutoff value of 1 nM (as one suggested in previous works),7,25 the sensitivity rose to 87.1%, but the specificity dropped to 63.9%. Since SMRP levels were not affected by genotype in MPM group, all patients were used as diseased group in generating ROC curves, while non-MPM subjects were stratified by genotype. As shown in Table 2, cutoff values, and alternatively, sensitivities or specificities were genotype-dependent. The lowest rates of false positives were obtained among non-MPM subjects with GG genotype, where Youden's J index rose to 0.61 (at 1.28 nM); the AUC to 0.888; and the specificity to 85.3%. An increasing number of A alleles led to corresponding decreasing specificities (at 1.28 nM they were 70.7% for GA and 43.5% for AA genotypes).
Then, the influence of the SNP of interest on MSLN regulation at a post-transcriptional level was evaluated in nonMPM Met-5A cells through a luciferase reporter assay without the use of miRNA mimics. RLU was significantly reduced to about 70% (± 6%, $p < 0.0001$) when the reporter was chimerised with the 3'UTR bearing the G allele, as compared to the construct with the A allele.

MSLN Is a Candidate Target of miR-611 and miR-887

To understand the biological mechanisms underneath the observed association, we exploited the polymorphism to evaluate possible miRNAs regulating MSLN expression.
Thus, in silico predictions were performed to detect putative miRNAs having the miRSNP within their binding site. The predictions of miRNAs and the difference of the binding free energies between G and A alleles are reported in Table 3. Various miRNAs were predicted to have very low binding free energies to MSLN 3'UTR, however the binding sites for miR-887 and miR-611 were predicted as the most affected by the nucleotide variation ($|\Delta\Delta G|$ of 6.7 and 6.6 kcal/mol, respectively). The predicted miRNA::mRNA interactions are depicted as follows (miRSNP in bold, seed region underlined):

caGUCUGGGGCUCCCCAGGAGCG (miR-611)
  | | | : | | | : | | | | | | | | :
agCAGG–CACGGGTGGTCCCCGT
ggagcCCUACCGCGGGCAAGug (miR-887)
      | | : | | | : | | | | | | |
ggcacGGGTGGTCCCCGTTCca

Thus, the effect of these miRNAs in relation to the genotype was evaluated in further experiments. Initially, real-time PCRs were performed on pri-miR-611 and on miR-887 to verify whether they were actually expressed. MiR-611 was expressed in both cell lines, whereas miR-887 was detected only in Mero14 cells. In tissues, these miRNAs were expressed in both normal and malignant tissues. When the Shapiro-Wilk normality test was performed (as appropriate since the sample size was quite small), we rejected the hypothesis of a normal distribution of the expression level of pri-miR-611 and miR-887 ($p < 0.0001$ for both). The Kruskal-Wallis nonparametric test was subsequently performed and showed no statistically significant

difference between normal and MPM groups (pri-miR-611 p = 0.63 and miR-887 p = 0.78). Moreover, Mood's median test was also performed, resulting in p = 1 for both microRNAs.

Then, to ascertain whether miRNAs could directly target MSLN, a luciferase reporter assay was performed on Met-5A cells, by cotransfecting either pmiR_G/_A with, alternatively, miR-611 or miR-887 mimics. As shown in Figure 2, RLU of pmiR_G construct was reduced to 80% and 70% in the presence of either miR-611 or miR-887, respectively. On the other hand, the vector pmiR_A was sensitive only to miR-887 (RLU was 70% of that measured in negative controls), whereas the cotransfection with miR-611 did not elicit any change in the RLU, as compared to the negative control. We further evaluated the ability of the miRNAs to target MSLN by performing an ectopic transfection of miR-611 or miR-887 in a model of MSLN overexpression, using the MPM cell line Mero-14, in three independent experiments. As shown in Figure 3, transfection of 5nM of miR-611 caused a reduction of MSLN protein expression. The measured level was 76% of that obtained following the treatment with the negative control (the level was 86% for miR-887). At a miRNA mimic concentration of 30nM, miR-887 also caused a reduced expression to 64% (the level was 66% for miR-611). Although the pairwise comparisons did not reach the statistical significance for both miRNA mimics at 5nM (p = 0.116 for miR-611, and p = 0.378 for miR-887), the significance was reached at 30nM for miR-611 (p = 0.016), and it was approached for miR-887 (p = 0.077). However, the reduction of protein expression was statistically significant when considering the effect or the treatments overall (p = 0.0022 and p = 0.037, for miR-611 and miR-887, respectively).

DISCUSSION

In the present study, several evidences suggested that rs1057147 within the 3'UTR of the MSLN gene could be a putative miRSNP and that miR-611 and, to a lesser extent, miR-887 might be involved in this process. First, a genotype-dependent variation in the levels of SMRP was found in vivo. Although this association was already suggested by our preliminary work,7 the higher number of recruited volunteers allowed to exclude any chance finding obtained previously and to build more robust bases for further analyses. Second, the genotype-dependent levels of SMRP paralleled the results obtained in vitro on non-MPM Met-5A cells, that is, the G allele was associated with a low reporter activity. These experiments allowed also to shed some lights on the underneath mechanisms. In fact, the two variant 3'UTRs were likely differentially regulated by the endogenous miRNAs (no miRNA mimics were cotransfected with pmiR vectors). Following this idea, miR-611 was one of the best candidates, since it scored highly in in silico predictions and it was found expressed in healthy pleura and in Met-5A cells. The role of miR-611 was further corroborated when miRNA mimics were cotransfected in Met-5A together with the reporter vectors. MiRNA selection was based on the predicted effect of the polymorphism on miRNA::mRNA binding energy. Although other miRNAs (for example, miR-608) were predicted as the best candidates in terms of ΔG by more than one algorithm, they could unlikely explain the effect of the SNP in vivo according to the |ΔΔG| calculation. MiR611 and miR-887 showed the highest |ΔΔG|, thus they were selected for functional analysis based on luciferase assay and western blot. Actually, in a cell-based reporter assay, miR-611 mimic was active only on G allele. Furthermore, when the miR-611 mimic was assayed on Mero-14 cells, a reduced MSLN expression was induced, and this is consistent with the fact that the cell line has a GG genotype. MiR-887 was effective on the constructs carrying A or G alleles to a similar extent. This finding could be explained by the fact that the polymorphism, unlike for miR-611, falls peripheral to the seed region. Thus, RNAhybrid algorithm could have better predicted the effect of the nucleotide change when it occurs within the seed region, whereas it could have over-emphasized the effect when the change is located more towards the 3' end. On Mero-14 cells, miR887 mimic at 30 nM was able to reduce the amount of MSLN to a similar extent to what observed for miR-611, in agreement to the results of the gene reporter assays. At 5 nM, miR-887 seemed less effective, likely reflecting differences in endogenous processing of the two miRNAs (e.g., miRNAs could have different strength of

binding to their targets or they could be incorporated into RISC with a different efficiency). In summary, the fact that both miRNAs were found expressed in mesothelium and that in vitro assays yielded coherent results in different cellular systems further corroborated the idea that miR-611 and miR-887 are involved in the regulation of MSLN. To our knowledge, this is the first study providing evidences for a functional role of the miRSNP rs1057147 elicited through interactions with miRNAs in normal mesothelium. However, the lack of differential expression of the miRNAs between specimens from MPM patients and controls suggests that miR-611 and miR-887 are unlikely to play a role in the aberrant overexpression of MSLN in MPM. Thus, it could be hypothesized that the steep increase of SMRP associated with the malignancy has not to be ascribed to the deregulation of miR611 and miR-887 but to other, still unknown, mechanisms. Considering the effect of the miRSNP, different sensitivities and specificities were found when SMRP was employed as biomarker. The inclusion of the genotype in the calculation of ROC curves leads to a slightly improved performance of the marker, with the lowest rate of false positives, in GG homozygotes, implying that high levels of SMRP could be more alarming for people carrying this genotype. We acknowledge that the present study could bear potential weaknesses. For example, the A allele is relatively rare, therefore the number of AA-homozygotes is not adequate for robust statistical analyses, especially in the sub-groups. Indeed, for this reason we preferred to analyze BRD and healthy volunteers as a unique group. Moreover, in the present study other relevant information potentially linked to SMRP levels could not be ascertained, such as, for example, the individual rate of glomerular filtration (GFR).[26] Likely, the inclusion of these parameters could further improve the sensitivity and specificity of SMRP as biomarker for screening subjects previously exposed to asbestos.

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TABLE 1. Characteristics of Volunteers, All Exexposed to Asbestos

	Healthy	BRD	MPM
Sex			
Male	97.8% (363)	98.4% (313)	85.7% (60)
Female	2.2% (8)	1.6% (5)	14.3% (10)
Age (y)			
Average	58.9 ± 13.5	65.8 ± 10.7	70.5 ± 10.8
Median	60	66	71
Smoking			
Smokers	16.2% (60)	16.2% (51)	8.3% (6)
Exsmokers	39.9% (148)	54.0% (172)	58.3% (41)
Nonsmokers	43.9% (163)	29.8% (95)	33.4% (23)
Asbestos exposure (years)			
Average	15.6 ± 11.5	20.0 ± 10.7	23.2 ± 19.1
Median	15	20	25
Diagnosis			
		[a]Pleural 56.5% (180)	eMPM 60.0% (42)
		[b]Lung 35% (111)	sMPM 21.4% (15)
		[c]Airways 8.5% (27)	bMPM 18.6% (13)
Allele frequency			
G allele	0.81	0.84	0.83
A allele	0.19	0.17	0.17
Total	371	318	70

Subjects exposed to passive cigarette smoke are grouped with nonsmokers. Average is given with its standard deviation. All the percentage are given on total. Absolute numbers are given in brackets.

[a] Pleural diseases include: pleural plaques 30.8% (98), pleural thickening 20.1% (64), pleural effusion 1.9% (6), pleuritis 3.7% (12).

[b] Lung diseases include emphysema 10.1% (32), lung fibrosis 4.1% (13), nodules 15.1% (48), asbestosis 5.7% (18).

[c] Airways diseases correspond to bronchiectasis 8.5% (27).

[e]MPM, epithelioid malignant pleural mesothelioma; sMPM, sarcomatoid malignant pleural mesothelioma; bMPM, biphasic mesothelioma

TABLE 2. Cutoff Values, Specificities, and Sensitivities According to ROC Curves Generated with non-MPM Group Stratified by Genotype and MPM as a Whole

GG Genotype			
Estimated Specificity at Fixed Sensitivity			
Sensitivity	**Specificity**	**95% CI**	**SMRP**
80.0	77.4	65.6–86.7	>1.123
90.0	67.7	54.7–76.7	>0.973
95.0	57.2	17.3–68.8	>0.822
97.5	54.7	15.2–65.9	>0.804
Estimated sensitivity at fixed specificity			
Specificity	**Sensitivity**	**95% CI**	**SMRP**
80.0	78.6	67.1–87.1	>1.180
90.0	61.4	45.8–71.4	>1.500
95.0	51.4	37.1–62.9	>1.854
97.5	44.3	31.4–55.7	>2.300
GA genotype			
Estimated specificity at fixed sensitivity			
Sensitivity	**Specificity**	**95% CI**	**SMRP**
80.0	62.1	46.2–72.2	> 1.123
90.0	48.0	25.8–60.6	> 0.970
95.0	30.8	7.1–49.5	> 0.817
97.5	30.3	6.6–50.7	> 0.802
Estimated sensitivity at fixed specificity			
Specificity	**Sensitivity**	**95% CI**	**SMRP**
80.0	62.9	50.0–74.3	> 1.487
90.0	50.0	37.1–60.0	> 1.935
95.0	45.7	31.5–55.7	> 2.108
97.5	38.6	20.0–48.6	> 2.793
AA genotype			
Estimated specificity at fixed sensitivity			
Sensitivity	**Specificity**	**95% CI**	**SMRP**
80.0	43.5	20.3–60.9	> 1.123
90.0	30.4	7.4–52.2	> 0.971
95.0	17.4	0.0–30.4	> 0.817
97.5	17.4	1.8–34.8	> 0.776
Estimated sensitivity at fixed specificity			
Specificity	**Sensitivity**	**95% CI**	**SMRP**
80.0	38.6	18.5–58.6	> 2.918
90.0	30.0	8.6–45.7	> 3.408
95.0	27.1	8.6–42.9	> 3.823
97.5	14.3	0.0–27.1	> 5.487

TABLE 3. Prediction of miRNAs Targeting 3'UTR of MSLN mRNA in Correspondence of rs1057147 (G > A)

MicroRNA	ΔG G allele (kcal/mol)	ΔG A allele (kcal/mol)	|ΔΔG| G-A (kcal/mol)	miRNA Prediction Tool
miR-887	−31.6	−24.9	6.7	miRBase
miR-611	−30.5	−23.9	6.6	miRBase
miR-3144-5p	−22.0	−16.2	5.8	miRanda, TargetScan
miR-342-5p	−29.3	−25.3	4.0	miRanda, TargetScan
miR-3191-3p	−31.0	−27.5	3.5	miRanda
miR-4664-5p	−27.7	−25.0	2.7	TargetScan
miR-608	−38.3	−35.8	2.5	miRanda, TargetScan, miRBase
miR-3170	−19.3	−16.9	2.4	miRanda, TargetScan
miR-363*	−23.3	−24.2	0.9	miRanda, miRBase
miR-542-5p	−29.9	−30.5	0.6	miRanda, miRBase
miR-1293	−28.5	−29.0	0.5	miRanda
miR-635	−19.2	−18.9	0.3	miRanda, TargetScan
miR-4651	−27.8	−27.7	0.1	TargetScan
miR-3190-5p	−19.9	−19.9	0	miRanda
miR-3165	−22.5	−22.5	0	miRanda
miR-4260	−25.7	−25.7	0	miRanda

Binding free-energy (ΔG), calculated with RNAhybrid and expressed in kcal/mol, is reported for 3'UTR bearing alternatively G or A allele. |ΔΔG| represent the difference between ΔGs with G allele and ΔGs with A allele, in absolute values.

FIGURE 1. Association between rs1057147 and SMRP levels in healthy + BRD (non-MPM group) and MPM subjects. p Values were calculated following ANOVA test. Asterisks show a statistical significance (p < 0.05) in Tukey's test for pairwise differences (GG genotype was used as reference for GA and AA genotypes). The columns represent mean values, the bars show standard error of the mean (SEM).

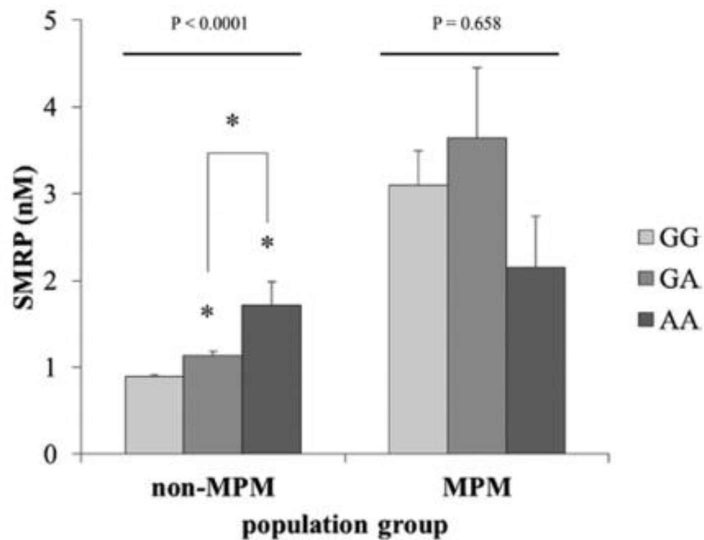

FIGURE 2. Effect of cotransfection of pmiR_G/_A with miR-611 or miR-887. RLU obtained in cotransfection of vectors with negative control is reported as 100% and used as reference for statistical evaluation. Asterisks show a statistical significance (p < 0.05). The columns represent mean values, the bars show standard error of the mean (SEM).

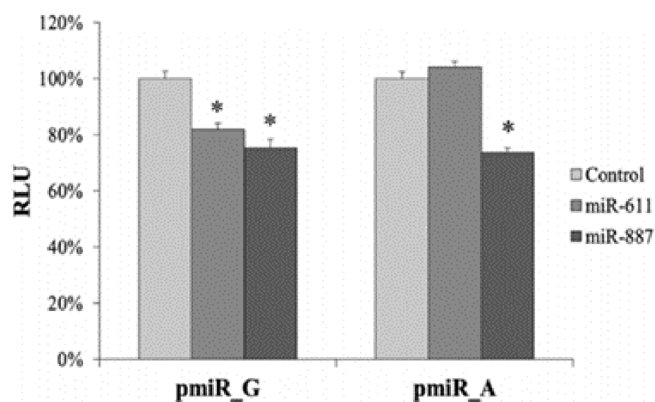

FIGURE 3. Western blot of MSLN protein in Mero-14 MPM cell line transfected with 5 or 30nM of miR-611 or miR-887 mimics. Quantification after internal normalization with β-actin is displayed in bottom panel; MSLN obtained after transfection with negative control is reported as 100%. Asterisks show a statistical significance ($p < 0.05$) in the pairwise comparison with negative control. The columns represent mean values, the bars show standard error of the mean (SEM).