Combined Serum Mesothelin and Plasma Osteopontin Measurements in Malignant Pleural Mesothelioma

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Introduction: Malignant pleural mesothelioma (MPM) is a lethal tumor related to asbestos exposure. At present, the only instruments for screening and diagnosis are based on radiological tests, posing evident economic and radio-protectionist problems. Some authors are evaluating biological indicators, such as plasma osteopontin (pOPN) and serum soluble mesothelin-related peptides (SMRP). This study aimed to evaluate whether a combination of these two markers could increase sensitivity and specificity in diagnosis of epithelioid MPM.

Methods: We enrolled 93 healthy subjects, 111 individuals with benign respiratory disease (BRD), and 31 patients with MPM, histologically and/or cytologically confirmed. SMRP and pOPN levels were determined using commercially available enzyme-linked immunosorbent assay kits. Though a logistic regression analysis, SMRP and pOPN were combined and translated into a new index, called "combined risk index."

Results: Differences in both SMRP and pOPN mean values between epithelial MPM patients and healthy subjects or BRD patients were statistically significant ($p < 0.0001$), whereas there was no difference in SMRP and pOPN mean values between healthy subjects and BRD patients. The performance in MPM diagnosis resulted improved by the combination of the two markers. The results of our study should be confirmed by a larger scale and, possibly, a multicenter study, which could better take into consideration the influence of some possible confounding factors such as glomerular filtration rate and other blood parameters.

Conclusions: We combined SMRP and pOPN dosages to increase diagnostic accuracy. This study showed for the first time that combined SMRP and pOPN measurements can increase both sensitivity and specificity in terms of combined risk index.

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Malignant mesothelioma is a lethal tumor arising from the serosal covering pleura and, less frequently, the pericardial and peritoneal cavities and from the tunica vaginalis of the testis.

Its relationship to asbestos exposure has been established, but several aspects of its etiopathological mechanism are still under examination. Other factors such as viral agents and/or genetic determinants may also play a role.[1,2]

Italy was an important producer and importer of raw asbestos until it was banned in 1992, and our country is now experiencing severe public health consequences. Until 2004, the national standardized mortality rate in Italy was 3.42 (×100,000 inhabitants) for men.[3] National Standardized Mortality Ratios (MR) are often quite different even within countries with a similar history of asbestos use, which can be explained by both a inhomogeneous development in national surveillance systems and, partially, an intrinsic bias in malignant pleural mesothelioma (MPM) registering according to the International Classification of Disease. In a recent study of Nishikawa et al., the world global median age-adjusted MR was as high as 7.8 deaths/million/yr, whereas in Europe it ranged from 1.9 in Romania up to 30.0 in Netherlands and 31.1 in the United Kingdom. In general, MRs were higher in Northern and Western than in the Eastern and Southern European countries. High MRs were observed in Australia (25.5), New Zealand (20.5), intermediate in Canada (10.3) and USA (9.0), and low or very low in some Asian or Central and Southern American countries. Remarkably, almost all countries with low MRs showed an increasing trend in MPM mortality.[4] The incidence of mesothelioma is expected to increase in many other countries such as Britain,[5] Australia,[6] and the United States,[7] due to a widespread use of asbestos in the past.

Latency time for MPM shows great variability, but a latency period shorter than 10 years is very rare.

Early diagnosis and differential diagnosis are very difficult. Because of the usually advanced stage at presentation, only a minority of patients are eligible for radical surgery[8]; most are candidates for chemotherapy during the course of

their disease. Retrospective studies report median survival of less than 1 year and 5-year survival rates of 1% or less.[9]

Despite this dramatic scenario, at present, the only instruments for screening and early diagnosis are based on radiological tests, with evident economical problems as well as radio-protectionist issues, due to cancer risk added by the use of ionizing radiation-based diagnostic tests (i.e., chest radiography and computed tomography). For this reason, some authors are evaluating biological indicators as screening and early diagnosis markers, such as serum and plasma osteopontin (OPN) and soluble mesothelin-related peptides (SMRP).

OPN is a glycoprotein overexpressed in several human neoplasms such as lung, breast, and colon cancer.[10] OPN modulates cell-matrix interactions; high levels correlate with tumor invasion, progression, and metastasis. Serum OPN (sOPN) levels in patients with MPM have been reported to be higher than in healthy subjects.[11,12] However, a recent study by Park et al.[13] reported that sOPN levels are elevated in subjects with asbestos-related disorders without MPM; these data indicate that sOPN may be influenced by nonmalignant processes. In a relatively small population of workers previously exposed to asbestos, Foddis et al.[14] observed that age, duration of exposure, restrictive respiratory function, and smoking habit could affect the result of sOPN measurement.

Recently, our study group investigated the usefulness of plasma OPN (pOPN).[15] Mean pOPN values were significantly higher in MPM patients than in controls and BRD patients, and no statistically significant difference was found comparing the mean value of controls and BRD groups. Moreover, neither clinical status nor smoking habit could affect the result of the pOPN measurement.

Mesothelin is a 40 kDa cell surface glycophosphatidylinositol-anchored protein expressed at a low level by normal mesothelial cells in the pleura, peritoneum, and pericardium. It is highly expressed in pancreatic cancer, ovarian cancer, mesotheliomas, and some other cancers.[16,17] Mesothelin has been proposed for diagnosis and prognosis of epithelioid MPM as an immunohistochemical pleural fluid, and serum marker.[12,18–24]

The clinical limitation of these studies is that serum mesothelin is useful for diagnosis and possibly for monitoring patients but has insufficient sensitivity.[25] The aim of this study was to evaluate whether a combination of the two markers (serum mesothelin and pOPN) could increase sensitivity and specificity in diagnosis of epithelioid MPM.

PATIENTS AND METHODS

Patients and Controls

This study was approved by the ethical committee for pharmaceutical experimentation of Pisa Hospital. All subjects gave written and oral informed consent.

Serum and plasma samples were available from consecutive patients presenting at the University Hospital of Pisa. We studied 93 healthy subjects, 111 individuals with benign respiratory disease, and 31 patients with MPM, histologically and/or cytologically confirmed. All subjects underwent clinical examination, including chest radiography, functional respiratory tests, and in some cases low-dose computerized tomography. According to these results, those who were negative for all the tests were classified as "healthy subjects." Subjects designated as BRDs were patients suffering from one or more of the following diseases: lung asbestosis, asbestos pleuritis, emphysema, lung fibrosis, and pleural fluid. Of the BRDs, 13 had pleural plaques and 48 unspecific lung nodules ($<$10 mm diameter) but no functional impairment or clinical symptoms. Healthy and BRD subjects were recruited within a population of workers previously exposed to asbestos, undergoing a preventive cancer program.

MPM patients were enrolled at the time of diagnosis, before beginning any therapeutic treatment. All MPMs were of epithelioid type, histologically confirmed. Mixed and sarchomatoid mesothelioma were excluded because of the paucity of available cases and the proved lack of association between SMRP and nonepithelioid MPM.[19]

Biomarker Assays

The Human Osteopontin Assay Kit (IBL, Gunma, Japan), a commercially available ELISA (enzyme-linked immunosorbent assay), was used to determine the level of pOPN. Briefly, plasma samples were diluted 1:10 with EIA buffer and 100 μl of blank, and standards and samples were applied in duplicate in a O-17 antibody precoated microwell plate and were incubated for 1 hour at 37°C. The plate was washed eight times and 100 μl of labeled antibody 10A16 was added in each well. After an incubation period of 30 minutes at 4°C, the plate was washed nine times and chromogen was added. The plate was incubated for 30 minutes at room temperature in the dark and Stop solution was added. Adsorbance read at 450 nm was used to quantify the OPN concentration in ng/ml by comparison with the standard curve plotted by Microsoft Excel.

Serum mesothelin concentration was measured using a sandwich-type ELISA, Mesomark (Cisbio International, Gif/Yvette, France), according to instructions. Briefly, patient serum samples were diluted 1:101 with the assay diluent. Next, 100 μl of blank, provided standards, and samples were applied in duplicate in a microwell plate precoated with antibody 4H3. After 1-hour incubation on a shaking plate at room temperature, the wells were washed and antibody OV569-HRP was added for 1 hour. After a second washing step, TMB substrate was added to wells for 15 minutes, and then 100 μl of stop solution was added. Absorbance read at 450 nm was used to quantify the SMRP concentration in nM by comparison of mean of the duplicate measurement with a calibration curve fitted by CourbesRD software (InstallShield Corporation, Inc., France).

Statistics

All data were presented as mean $\pm$ SD. Comparisons between groups were performed using the Mann-Whitney *U* test for unpaired samples. Linear regression analysis was used to determine the correlation between SMRP and pOPN levels. Logistic regression was used to determine the weight given to each marker and then to calculate a specific formula to provide a combined risk index. To estimate whether this