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Gene methylation in pleural mesothelioma: Correlations with clinico-pathological features and patient's follow-up

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Summary Methylation of tumor suppressor genes is among the most frequent alterations in patients with malignant pleural mesothelioma (MPM). The aim of this study was to analyze the promoter methylation status of four tumor suppressor genes, $p15^{INK4B}$, $p16^{INK4A}$, RASSF1A and NORE1A in MPM. Samples of 79 MPM patients were analyzed using a methylation-specific PCR method. Associations between methylation status, clinico-pathological parameters (including proliferation index) and overall survival (OS) were examined. The analysis documented methylation in 30 cases (38%). The methylation frequency for individual genes was 19% for $p15^{INK4B}$ ($n=15$), 11.4% for $p16^{INK4A}$ ($n=9$), 20.2% for RASSF1A ($n=16$) and 5.1% for Nore1A ($n=4$). In the whole series methylation was associated to an increased proliferation index ($P=0.05$). In patients treated with extrapleural pneumonectomy, methylated MPM showed a trend to a poorer OS in comparison to unmethylated cases (median OS 16 months vs. 35 months, $P=0.06$, HR = 2.01, 95% CI 0.95–4.30). In the overall population, methylation did not correlate to patient outcome but a trend to an improved survival was detectable in ummethylated MPM treated with extrapleural pneumonectomy. This result suggests the need to select homogeneously treated and staged patients with MPM to address whether their methylation profile may impact on patient's survival.

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1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor with poor prognosis. Although uncommon, its incidence is rising in the world, peaking in the United States in 2000 and expected to further rise in the next 10—15 years in Europe. Annually, 2500 new cases are reported in the United States and 5000 in Europe [1]. Therapy for MPM remains controversial, with little agreement regarding standards of care. A minority of patients is eligible for radical surgery [2]; encouraging results have been reported with multimodality approach including extrapleural pneumonectomy (EPP), adjuvant chemotherapy and hemithoracic radiotherapy [3], even though with significant morbidity and mortality. MPM is poorly responsive to chemotherapy and radiation therapy; recently, the novel multitargeted antifolate pemetrexed was shown to have activity both as single agent [4] and in combination with platinum compounds [5,6]. Despite this, long-term survivors are rare and the 5-year survival rate is still below 15% [1]. Therefore, there is a pressing need for new treatment modalities [7].

The molecular pathogenesis of MPM appears to involve a still poorly understood combination of exposure to environmental (asbestos) and somatic genetic alterations; the role of infectious agents (SV40) remains debated and controversial [8,9]. The most frequent alterations reported in MPM are deletions at specific sites in chromosomes 1, 3p, 9p, 6q and 22 [10,11], and the methylation of distinct tumor suppressor genes [12—14]. Cell culture experiments have shown that DNA methylation increases upon immortalization and transformation of human mesothelial cells [12,15], and demethylating agents were been reported to have some activity in MPM [16—18].

Two putative tumor suppressor genes, $p15^{INK4B}$ and $p16^{INK4A}$, have been mapped to 9p21 and were found frequently methylated in a large number of cancers [19]. Both genes encode structurally and functionally similar cyclin-dependent-kinase (CDK) inhibitors, which act on CDK4 and CDK6, preventing the formation of cyclinD/CDK4/CDK6 complexes and the G1-phase cell-cycle progression [20]. Homozygous deletion of $p16^{INK4A}$ was reported as a common occurrence in MPM cell lines (85%), but significantly less common in fresh tumors (22%) [21]. Other authors reported co-deletion of $p15^{INK4B}$ and $p16^{INK4A}$ in 72% of tumor samples [22]. In primary tumor tissue, absence of $p16^{INK4A}$ immunodetection frequently occurred in MPMs, exceeding $p16^{INK4A}$ deletion [23]. Although the 9p21 locus is commonly deleted in MPM, $p16^{INK4A}$ was also found hypermethylated in 5—19% of cases [13,14,24], while $p15^{INK4B}$ methylation status has not been previously investigated in MPM.

Two other putative tumor suppressor genes, RASSF1A and NORE1A, acting in concert in the pro-apoptotic pathway of RAS signalling, are located at regions frequently deleted in MPM (3p21 and 1q32.1, respectively) [10,11]. NORE1A and RASSF1A have been recently found methylated in different tumor cell lines and in several cancers [25,26]. It has been suggested that RASSF1A methylation is one of the more common aberration so far identified in human cancers and that the loss of the functional protein may promote the development of many human tumors [27]. While NORE1A is rarely methylated (3%) in MPM [15], recent reports have suggested RASSF1A as frequently methylated (32%) [13,15], preferentially in the presence of SV40 DNA sequences [12,15].

In the present study the methylation status of $p15^{INK4B}$, $p16^{INK4A}$, RASSF1A and NORE1A was evaluated in tissue samples of 79 MPM patients; methylation was correlated to patient clinico-pathological features (gender and age, tumor histological type, proliferation index) and to patient's survival.

2. Materials and methods

2.1. Patients and samples

Seventy-nine consecutive MPM patients who underwent surgical procedures at Istituto Clinico Humanitas of Milan from 1997 to 2005 have been analyzed in this study. Their characteristics are reported in Table 1. Forty-one patients (52%) were radically resected with EPP, 32 patients underwent partial pleurectomy/decortication (40%); in six cases (8%) only a diagnostic biopsy was valuable for study. All patients received EPP within the context of a multimodality treatment program including post-operative chemotherapy and radiotherapy. Tissue samples representative of the tumors were routinely fixed in 10% buffered neutral formalin and processed for conventional histological examination. Standard 2 μm-thick sections from all routinely processed tissue samples were stained with hematoxylin—eosin and examined by light microscopy. MPM histotype was established according to the WHO classification of lung and pleural tumors [28]. The amount of tumor cells in tissue samples for molecular analysis was equal or exceeded 50% of the sample.

2.2. Methylation analysis

2.2.1. DNA extraction

DNA was isolated from five sections (5 μm thick) of each paraffin-embedded MPM. After xylene deparaffinization, all samples were treated by digestion with proteinase K (Finzyme, Espoo, Finland), followed by standard phenol-

Table 1 Patient characteristics ($N = 79$)

Variable	No. (%)
Age (median range)	61 (35—86)
Gender	
Female	24 (30)
Male	55 (70)
Histology	
Epithelial	63 (80)
Mixed	13 (16)
Sarcomatous	3 (4)
Surgery	
EPP	41 (52)
PD	32 (40)
None	6 (8)

EPP: extrapleural pneumonectomy; PD: partial pleurectomy/decortication.