Lung Cancer (2006) **54**, 109—116

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Promoter methylation of *RASSF1A*, *RARβ* and *DAPK* predict poor prognosis of patients with malignant mesothelioma

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Received 11 May 2006; received in revised form 27 June 2006; accepted 29 June 2006

KEYWORDS
Hypermethylation;
Mesothelioma;
Prognosis;
Methylation-specific PCR;
Molecular marker

Summary Hypermethylation occurs frequently in neoplastic cells and affects tumorigenesis. Malignant mesothelioma is an aggressive cancer developing in the thoracic cavity and patients have a rather bad prognosis. Our goal was to determine epigenetic alterations of a series of genes and to analyse the potential correlation of such changes with overall survival. We have analysed the methylation status of the promoter region of nine genes in serum DNA of mesothelioma patients by a nested methylation-specific PCR. Modest methylation frequencies were detected for *APC1A* (14.3%), *RASSF1A* (19.5%) and *DAPK* (20.0%) while hypermethylation of *E-cadherin* (71.4%) and *FHIT* (78.0%) occurred at a high incidence. Intermediate values were seen for $p16^{INK4a}$ (28.2%), *APC1B* (32.5%), $p14^{ARF}$ (44.2%) and *RARβ* (55.8%). The methylation status of none of the single genes significantly influenced prognosis. In contrast, combining *RARβ* with either *DAPK* or *RASSF1A* showed a significantly shorter overall survival of those patients who had both genes methylated compared to those with only one or no epigenetic alteration ($P = 0.025$ and 0.040, respectively). Similarly, the combination of all three genes revealed a worse prognosis for patients with double or triple methylations compared to the group which had only one or no gene methylated ($P = 0.028$). Our results support the idea that the prognostic value of a combination of epigenetic alterations is superior to the impact of an individual gene alone on overall survival.

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doi:10.1016/j.lungcan.2006.06.017

1. Introduction

Malignant mesothelioma (MM) develops from mesothelial cells which line the pleura or the peritoneal cavity. The majority of reported cases arise after exposure to asbestos and a long latency period between 30 and 40 years. MM is a rather infrequent tumor with an incidence of approximately two per 1,000,000 in females and between 10 and 30 in males [1]. More than 2000 new cases of MM are diagnosed in the United States per year [2] and the peak values are expected in the next decade. MM is an aggressive cancer which progresses mostly locally and causes death due to respiratory failure or intestinal obstruction. At presentation most of the patients have an advanced stage. Only a small fraction of patients is eligible to curative resection. Numerous cytotoxic agents such as cisplatin and gemcitabine have been tested either as single agents or in combination in phase II studies [3—5]. Recently, pemetrexed (ALIMTA) a novel multitargeted antifolate with antineoplastic activity [6] has shown promising effects in clinical trials [7—9] and it has now been approved for the treatment of patients with MM [10]. Despite this progress long-term survivors occur quite rarely and the 5-year survival rate is below 15% [1,11,12].

Hypermethylation is an important mechanism to inactivate tumor suppressor genes. Such epigenetic alterations are thought to play a role in the development and diagnosis of neoplasms including those of the lung [13]. In lung carcinomas several publications have determined the hypermethylation pattern [14—16] and the impact of such alterations was analysed with respect to diagnosis [13,17], prognosis [18,19] or the appearance of secondary lung cancers [20]. In contrast, the molecular biology of MM in that area has received rather little attention and only a limited number of analyses have reported epigenetic alterations in MM. The X-linked recessive overgrowth gene *GPC3* is inactivated in MM tumors and cell lines by methylation [21]. In addition, tumor suppressor genes such as $p16^{INK4a}$ [22,23] and *RASSF1A* [24,25] have been shown to be epigenetically altered in MM. A recent publication investigated the hypermethylation profile of 14 loci in a limited number of MM tumors and cell lines [26] and 12 genes have been tested in a larger series of MM [27].

We have investigated hypermethylation of the promoter region of nine candidate genes in genomic DNA derived from serum of MM patients. We have determined the frequency of methylation of these genes and we analysed the potential prognostic value of the methylation status of individual loci or combinations thereof.

2. Materials and methods

2.1. Patients and sample collection

The study included 43 patients with histologically confirmed MM who were treated between February 2003 and July 2004 at the Thoraxklinik am Universitätsklinikum Heidelberg, Germany. All patients were enrolled into a clinical phase III study and received treatment with pemetrexed (ALIMTA) alone or in combination with platinum compounds. The median time from diagnosis to start of treatment was 5.8 months. Prior to the onset of chemotherapy venous blood (10 ml) was collected into sterile vacutainer tubes containing clot activator. A limited number of blood samples was obtained from fourteen healthy control persons. Serum was obtained after centrifugation at 3000 rpm for 10 min and stored in aliquots at −80 °C until further use. The procedure was acknowledged by the local ethical committee and each patient signed an informed consent for the molecular assessment.

2.2. DNA extraction and chemical modification

Genomic DNA was extracted from 500 μl serum using the QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA was subjected to sodium bisulfite treatment for 16 h using the CpGenome DNA modification kit (Intergen, Oxford, UK) according the manufacturer's recommendation. This treatment deaminates unmethylated cytosines into uracil but does not affect 5-methyl cytosines. Modified DNA was resuspended in 30 μl TE buffer and stage 1 MSP analyses were performed the same day.

2.3. Methylation-specific PCR (MSP)

Hypermethylation of the promoter region was detected by a two-stage nested MSP which increases the sensitivity to detect hypermethylation more than 50-fold [28] and this approach yielded a much better reproducibility than a single PCR. The repetition of MSP experiments revealed a sturdiness of approximately 85%. Two microlitres of modified DNA were amplified in stage 1 MSP using primers which recognize the bisulfite-modified template but do not discriminate between methylated and unmethylated alleles. The conditions for stage 1 PCR were: 95 °C for 10 min, denaturation at 95 °C for 8 s, annealing for 5 s at 52 °C (*APC1B*), 53 °C (*APC1A*, $p14^{ARF}$), 56 °C (*DAPK*, *E-cadherin*, *FHIT*, $p16^{INK4a}$), 57 °C (*RARβ*) or 60 °C (*RASSF1A*) and synthesis at 72 °C for 15 s for 50 cycles using the following primers: *APC1A* forward AGG GTT AGG TAG GTT GTG, reverse ACC AAT ACA ACC ACA TAT C; *APC1B* forward TGT TTA GGT AGT AAT GGT TTA, reverse ACA ATA CCT AAA AAC AAC ATC; *DAPK* forward GGA GTG TGA GGA GGA TAG T, reverse CAC AAC TAA AAA ATA AAT AAA AAA C; *E-cadherin* forward TTA GTA ATT TTA GGT TAG AGG, reverse TTA ACT AAA AAT TCA CCT ACC; *FHIT* forward GAG GTA AGT TTA AGT GGA A, reverse ATC CCA CCC TAA AAC CTC; $p14^{ARF}$ forward TGT AGT TAA GGG GGT AGG AGT, reverse CTC CTC AAT AAC ATC AAC AC; $p16^{INK4a}$ forward GAA GAA AGA GGA GGG GTT GG, reverse CTA CAA ACC CTC TAC CCA CC; *RARβ* forward GTT GTT TGA GGA TTG GGA TGT, reverse TAC CAT TTT CCA AAC TTA CTC; *RASSF1A* forward GGA GGG AAG GAA GGG TAA GG, reverse CAA CTC AAT AAA CTC AAA CTC CC. Products were diluted 1:50 and 2 μl were subjected to stage 2 PCR using primers selective for the methylated or unmethylated genotype as published for *APC1A* [29], *APC1B* [29], *DAPK* [14], *E-cadherin* [14], *FHIT* [30], $p14^{ARF}$ [31], $p16^{INK4a}$ [14], *RARβ* [14] and *RASSF1A* [32]. Conditions for stage 2 PCR were: 95 °C for 10 min, denaturation at 95 °C for 8 s, annealing for 5 s at 60 °C (*APC1B*, *RARβ*, *RASSF1A*), 62 °C (*APC1A*, *E-cadherin*), 64 °C (*DAPK*, $p14^{ARF}$), 65 °C ($p16^{INK4a}$) or 68 °C (*FHIT*) and synthesis at 72 °C for 10 s