Experimental and Molecular Pathology 93 (2012) 154–161

Contents lists available at SciVerse ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Aberrant expression of cell cycle regulatory genes predicts overall and disease free survival in malignant pleural mesothelioma patients

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A R T I C L E I N F O

Article history:
Received 2 January 2012
and in revised form 5 March 2012
Available online 6 April 2012

Keywords:
Malignant pleural mesothelioma
Prognosis
p16
p14
p53
p21^{WAF}
p27
Rb

A B S T R A C T

Background: Malignant pleural mesothelioma (MPM) is a highly aggressive disease with a generally poor prognosis. Since escape from cell cycle checkpoint control is common in several solid tumors, the present study was performed to evaluate the role of some cell cycle regulatory genes in the development and progression of MPM.

Patients and methods: Aberrant expression of $p14^{ARF}$, $p16^{INK4A}$, $p21^{waf}$, $p27^{KIP}$, *p53*, *mdm2* and *Rb* was assessed in 55 MPM cases from Egypt using immunohistochemistry and PCR techniques. Results were correlated with clinico-pathological prognostic factors, overall and disease free survival (OS&DFS).

Results: Altered expression of $p14^{ARF}$, $p16^{INK4A}$, $p21^{waf}$, $p27^{KIP1}$, *Rb*, *p53* and *mdm2* proteins was detected in 50.9%, 54.5%, 53.3%, 61.8%, 53.3%, 58.2%, and 50.8% of cases, respectively. SV40 infection significantly correlated with $p14^{ARF}$, 16^{INK4A}, $p27^{kip1}$ and *Rb* aberrations ($p=0.014$, $p=0.02$, $p=0.01$, $p=-0.01$). Asbestos exposure significantly correlated with *p53*, $p21^{waf}$ and *mdm2* aberrations ($p=0.001$, $p=0.03$, $p=0.02$). On multivariate analysis PS≥2, $p27^{KIP1}$ and *Rb* aberrations were independent prognostic factors for OS ($p=0.016$, $p=0.011$, $p=0.003$) whereas on tumor recurrence, $p27^{KIP1}$ and *Rb* aberrations were independent prognostic factors for DFS ($p=0.002$, $p=0.03$, $p=0.01$).

Conclusions: MPM is a complex disease characterized by multiple genetic aberrations; some of them involve cell cycle regulatory genes. $p14^{ARF}$, $p16^{INK4A}$, *Rb* and $p27^{KIP1}$ seem to be involved in SV40-associated MPM whereas *mdm2*, *p53* and $p21^{WAF}$ are related to asbestos exposure. In addition to recurrence and PS, only $p27^{KIP1}$ and *Rb* could be used as molecular prognostic markers in MPM.

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor, which is resistant to conventional methods of treatment. Since the TNM staging system is of limited prognostic value in patients with MPM this has lead to alternative prognostic scoring systems such as those developed by the CALGB and EORTC. Although these systems can be useful in clinical trials, unfortunately there are only a small proportion of patients who respond well to therapy and have prolonged survival (Curran et al., 1998; Herndon et al., 1998).

The identification of new molecular prognostic factors holds promise to further help in counseling and management of MPM patients, possibly via better categorization of patients and identification of new target therapies (Kumar and Kratzke, 2005; Røe et al., 2009). Since uncontrolled cell growth and proliferation, mainly through loss of control at the cell cycle check points, are the hallmarks of cancer, understanding genetic aberrations affecting the cell cycle control is considered of utmost importance in cancer management. The cell cycle is governed by a family of *cyclins*, *cyclin dependent* kinases (*CDKs*) and their inhibitors (*CDKIs*) through activating and inactivating phosphorylation events (Carbone et al., 2002; Sherr, 1996). *CDK* mediated phosphorylation is inhibited by *CDKIs* such as $p14^{ARF}$, *p15*, $p16^{INK4A}$, *p18*, *p19*, $p21^{WAF}$, and $p27^{KIP1}$ (Kumar and Kratzke, 2005; Sherr, 1996).

The most important cell cycle inhibitors ($p14^{ARF}$, *p15p* and 16^{INK4A}), which are commonly targeted in many cancers are located at the short arm of chromosome 9 (9p21 locus) (Niehans et al., 1999). Previous studies showed that $p16^{INK4A}$ loss is common in MPM due to silencing of the $p16^{INK4A}$ gene via hypermethylation. This loss was found to be significantly associated with prolonged survival. Therefore $p16^{INK4A}$ could be used as a prognostic marker in MPM (Wong et al., 2002). Similarly, the loss of the $p14^{ARF}$ gene product was reported in MPM patients in some reports. $p14^{ARF}$ plays an important role in cell cycle

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0014-4800/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2012.04.001

control through promoting apoptosis and causing tumor suppression. This function is mainly achieved through binding to and degradation of *mdm2* thus, neutralizing the action of *mdm2* and stabilizing the *p53* (Yang et al., 2000). This seems to be the main mechanism contributing to loss of function of the *p53* in MPM, since MPM harbor relatively few mutations of the *p53* whereas overexpression is usually high (Zekri et al., 2007).

On the other hand, only few studies reported differential expression of $p21^{WAF}$ and $p27^{KIP1}$ in MPM patients, which was associated with the pathogenesis of MPM and also with reduced survival rates (Baldi et al., 2002; Bongiovanni et al., 2001; Isik et al., 2001).

The present study aimed to evaluate the role of multiple interrelated cell cycle regulatory genes ($p14^{ARF}$, $p16^{INK4A}$, $p21^{WAF}$, $p27^{kip1}$, *p53*, *mdm2*, and *Rb*) in the development and progression of MPM. The possible prognostic value of these biomarkers was also assessed via correlation with standard clinico-pathological prognostic factors of MPM and with survival rates.

Methods

Patients

This prospective study included 55 cases of pathologically confirmed MPM who attended the National Cancer Institute (NCI) clinics during the period from January 2005 to December 2007. Tumor tissues and five milliliter blood samples were freshly obtained from the patients at the operation room. Tumor tissues were divided into two pieces; the first piece was immediately snap-frozen and preserved in −70 °C to be used in molecular studies, while the second piece was put in 10% neutral buffered formalin and embedded in paraffin for routine histopathological examination and immunohistochemistry (IHC). All cases were diagnosed according to the World Health Organization (WHO) criteria based on microscopic examination of hematoxylin and eosin-stained sections combined with IHC using the routine diagnostic panel (calretinin or keratin 5/6, EMA, and viamentin) (Churg et al., 2004). Only cases with more than 75% tumor cells in microscopically examined sections were included in the study. Twenty normal lung tissue samples were also included in the study as a control. A written consent was obtained from all patients prior to enrollment in the study, and the ethical committee of the NCI approved the protocol which was in accordance with the ethical guidelines of the 2004 Declaration of Helsinki.

Clinical workup and treatment protocols

According to the IMIG staging classification (Van Ruth et al., 2003), 23 (41.8%) cases were diagnosed as having advanced disease stage (III and IV) and 32 cases (58.2%) were diagnosed as early stage (I and II). Forty seven cases were operable and 8 were inoperable at presentation. The 8 inoperable patients received a platinum based chemotherapy with or without radiotherapy. Out of the 47 operable cases, 6 were subjected to extrapleural pneumonectomy only, and 41 received multimodality treatment (33 patients received postoperative chemotherapy only and 8 received postoperative chemo- and radiotherapy).

Immunohistochemistry

Five micron thick sections were cut from each MPM and normal lung tissue samples onto positive-charged slides to assess the expression of $p14^{ARF}$, $p16^{INK4A}$, $p21^{waf}$, $p27^{KIP1}$, *p53*, *mdm2* and *Rb* proteins. The standard streptavidirin–biotin-peroxidase detection technique was performed as previously described (Zekri et al., 2007) using the following antibodies: $p14^{ARF}$ (C-22 Santa Cruz Biotechnology 1:300), $p16^{INK4A}$ (6H12, Novocastra Lab, CA, USA,1:40), $p21^{WAF}$ (SX118, Dako Corporation, Carpenteria, CA, USA, 1:50), $p27^{KIP1}$ (SX53G8, DAKO, Dako Corporation, Carpenteria, CA, USA, 1:50), *p53* (DO-7, 1:25 dilution), *mdm2* (IF2, Oncogene, Boston, MA) and *Rb* (*Rb1*, 1:80, DAKO). The antigen retrieval method was performed with microwave pretreatment in 0.01 M citrate buffer (pH 6.0) and manufacturer's protocols were followed for all procedures. The primary antibody was applied and incubated overnight at 4 °C in a humidified chamber and after 3 washes in PBS, the secondary antibody and the avidin–biotin complex (ABC) were added to slides. Diaminobenzidine (DAB) was used as a chromogen and Mayer's hematoxylin as a counterstain. To evaluate the specificity of the antibodies, known positive and negative control tissues were used. Assessment of all markers' expression was based on a nuclear staining pattern, however in case of *mdm2* and *p53*, cytoplasmic expression was also considered. Overexpression of *p53* and *mdm2* was considered with >10% positive cells. Cases were scored as follows: 0 (no immunostaining); +1: ≥10% but <25% positive cells; +2: ≥25% but <50% positive cells; and +3: ≥50% positive cells. Loss of $p16^{INK4A}$, $p14^{ARF}$, $p21^{waf}$ and $p27^{KIP1}$ and heterogeneous expression of *Rb* was defined as staining in <50% of the cells. Complete loss of *Rb* expression was also reported (Cheng et al., 1993; Giannoudis and Herrington, 2000; Khaled et al., 2009; Masumoto et al., 2003; Ordonez, 2006; Zandecki et al., 1995).

Molecular studies

High molecular weight DNA was extracted from fresh tumor and normal tissue samples as well as from the peripheral blood of the patients according to standard protocols. Detection of DNA promoter methylation (PM) and homozygous deletion (HZD; defined as absence of the PCR product from the tumor DNA compared to a strong product from the paired constitutional normal DNA sample from the patient) of $p14^{ARF}$, $p16^{INK4A}$, $p21^{waf}$, $p27^{KIP1}$ and *Rb* was done using methylation specific PCR (MSP) and semi-quantitative differential PCR as previously described (Mattar et al., 2004; Nakamura et al., 2001; Zandecki et al., 1995). To assess for HZD we used primers covering the 1β of the $p14^{ARF}$ with *GAPDH* as a reference gene and exon 1α for $p16^{INK4A}$ with β-*actin* as a reference gene (Table 1).

Statistical methods

Numerical data were expressed as mean ± Standard Deviation (SD), median, minimum and maximum. Qualitative data were expressed as frequency and percentage. Chi square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann–Whitney test. The Kaplan–Meier method was used to calculate OS,

Table 1
Primer sequences of the studied markers.

Marker	Primer sequences	Fragment size
p14	(M): 5-GTGTTAAAGGGCGGCGTAGC-3	200
	5-AAAACCCTCACTCGCGACGA-3	
	(UM): 5-TTTTTGGTGTTARAAGGGTGGTGTAGT-3	205
	5-CACAAAAACCCTCACTCACAACAA-3	
p16	(M): 5-TTATTAGAGGGTGGGGCGGATCGC-3	150
	5-CCACCTAAATCGACCTCCGACCG-3	
	(UM):5-TTATTAGAGGGTGGGGTGGATTGT-3	151
	5-CCACCTAAATCAACCTCCAACCA-3	
$p21^{waf}$ (M)	(M): 5-TTGGGCGCGGATTCGTC-3	184
	5-CTA AAC CGC CGACCC GA-3	
	(UM):5-TTAGTTTTTTGTGGAGTTG-3	184
	5-CTC AAC TCT AAA CCA CCA A-3	
$p27^{kip1}$	(M): 5-AAG AGG CGAGTT AGC GT-3	159
	5-AAG AGG CGAGTT AGC GT-3	
	(UM): 5-ATG GAA GAG GTG AGTTAG T-3	155
	5-AAA ACC CCA ATT AAA AAC A-3	
Rb intron 1	(M):5-GGGAGTTTCGCGGACGTGAC-3	172
	5-ACGTCGAAACACGCCCCG-3	
	(UM):5-GGGAGTTTTGTGGATGTGAT-3	172
	5-ACATCAAAACACACCCCA-3	

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