Expression of p21 in SV40 large T antigen positive human pleural mesothelioma: relationship with survival

A Baldi, A M Groeger, V Esposito, R Cassandro, G Tonini, T Battista, M P Di Marino, B Vincenzi, M Santini, A Angelini, R Rossielo, F Baldi, M G Paggi

Background: Mesothelioma is the most commonly occurring primary pleural neoplasm. Several studies have documented an increase in the incidence of this malignancy during the last decades. Although the association between asbestos exposure and development of mesothelioma is generally accepted, the exact mechanism of carcinogenesis is unknown. Recently, Simian virus 40 large T antigen (SV40 Tag) expression has been detected in pleural mesothelioma. The ability of SV40 oncoproteins to inactivate p53 and retinoblastoma tumour suppressor proteins has been proposed as an important step in the pathogenesis of human mesothelioma.

Methods: To obtain a better understanding of the molecular mechanisms of the pathogenesis of mesothelioma, the expression of the cell cycle inhibitor p21<sup>WAF1/CIP1</sup> (p21), a downstream target of p53, was evaluated immunohistochemically in a group of 29 mesothelioma specimens already characterised for the presence of SV40 Tag sequences.

Results: Statistical analysis did not reveal any correlation between p21 expression and histopathological type of mesothelioma using the χ<sup>2</sup> test (p=0.577). A significant positive relationship was found between p21 expression level and the patients’ overall survival according to the Kaplan-Meier survival curves and using a log rank test (median difference in survival 7 months, 95% CI 4.8 to 9.9; p<0.001).

Conclusions: Determination of p21 expression bears a prognostic significance in patients affected with mesothelioma, further underlining the role of SV40 in the pathogenesis of malignant pleural mesothelioma.

METHODS

Patients

Tissues from 29 malignant mesothelioma specimens (16 epithelial, six sarcomatoid, and seven mixed mesotheliomas) obtained from open biopsy specimens or pleurectomies were evaluated. All the patients were diagnosed and treated at the Second University of Naples from 1980 to 1993. Survival data were collected from hospital charts and from periodic interviews with patients and their relatives. Two subjects who died of causes other than mesothelioma during the follow up period were excluded from the study. Only in three cases did

<table>
<thead>
<tr>
<th>Variable</th>
<th>No of patients</th>
<th>Median survival (months)</th>
<th>Standard error</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>p21 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10%</td>
<td>15</td>
<td>5.00</td>
<td>0.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;10%</td>
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<td>12.00</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td>0.577</td>
</tr>
<tr>
<td>Epithelial</td>
<td>16</td>
<td>10.00</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>6</td>
<td>5.00</td>
<td>2.45</td>
<td></td>
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<tr>
<td>Mixed</td>
<td>7</td>
<td>8.00</td>
<td>2.62</td>
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</tr>
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</table>
the clinical history of the patients demonstrate a clear exposure to asbestos. The clinical and histopathological data of the patients are listed in table 1.

All the specimens were already characterised for the presence of SV40 sequences. 

**Histological examination**

The formalin fixed, paraffin embedded samples were cut into sections of 5 µm thickness and stained with haematoxylin and eosin. The histological diagnosis was re-examined. In addition, the most representative blocks were selected to be cut into new 5 µm thick sections for immunohistochemical studies.

**Immunohistochemistry**

Sections 5 µm thick were cut from each specimen, mounted on glass, and dried overnight at 37°C. All sections were then deparaffinised in xylene, rehydrated through a graded alcohol series, and washed in phosphate buffered saline (PBS). PBS was used for all subsequent washes and for antibody dilution.

Endogenous peroxidase activity was blocked by 5% hydrogen peroxide. The primary monoclonal antibody for p21 (sc-6246, Santa Cruz Biotechnology, CA, USA) was applied at room temperature for 1 hour at a dilution of 1:100. The optimal working dilution was defined on the basis of a titration experiment. The sections were then immunostained with streptavidin-biotin (Dako, Carpintera, CA, USA) using 3-amino-9-ethylcarbazide (AEC) as the final chromogen and haematoxylin as the nuclear counterstain. Negative controls for each tissue section were prepared by leaving out the primary antibody. A positive control was run with each set of slides. All samples were processed under the same conditions.

**Scoring and quantification of immunoreactivity**

Three observers (AB, RR, and FB) estimated the staining pattern of p21 separately and scored each specimen for the percentage of positive nuclei (<10% and >10% of cells expressing p21). The level of concordance, expressed as the percentage of agreement between the observers, was 90% (27 of 30 specimens). In the remaining specimens the score was obtained after collegial revision and agreement.

**Statistical analyses**

The cumulative probability of death was calculated for each group according to the Kaplan-Meier method and compared using log rank test. To evaluate the influence on survival of p21 expression the population was divided into two groups: <10% and >10% of cells expressing p21. The influence of histological type on survival was evaluated by dividing the specimens into epithelial, sarcomatoid, and mixed types. Correlation between the rate of expression of p21 and histological type was performed using a χ² test, grouping mesotheliomas as epithelial or non-epithelial. There was no need to perform multivariate analysis to estimate the influence of predictive factors. A p value of ≤0.05 in a two tailed test was considered significant. SPSS software Version 9.00 (SPSS, Chicago, IL, USA) was used for statistical analysis.

**RESULTS**

Twenty nine specimens (16 epithelial, six sarcomatoid, and seven mixed mesotheliomas) were evaluated. All were positive for the presence of SV40 sequences. Immunoreactivity for
p21 was found in both normal and neoplastic tissues. p21 immunostaining was always nuclear, with a low to absent background (fig 1). The number of positive cells varied in different specimens (table 1). Fifteen of the 29 specimens had ≤10% positivity for p21 expression; the remaining 14 specimens had a higher percentage of positive cells. No significant correlation was found between p21 expression and histological type using the χ² test (p=0.577). The median survival time was 5 months for the group with ≤10% positive cells and 12 months for those with >10% positive cells (median difference 7.0 months, 95% CI 4.8 to 9.9).

A positive relationship was found between the level of p21 expression and overall survival. To support the validity of these data, survival curves were constructed using Kaplan-Meier survival analysis (fig 2). The cumulative probability of death was calculated for each group according to the Kaplan-Meier method and compared using the log rank test (p<0.001).

**DISCUSSION**

In this study we found p21 protein expressed both in normal and in neoplastic tissue. Immunohistochemical analysis was chosen for this investigation because it has the advantage of allowing visualisation of the staining pattern of each section, enabling non-neoplastic elements such as stromal, endothelial, and inflammatory cells (which always affect nucleic acid based approaches) to be excluded. Moreover, recent studies have shown that p21 protein and RNA expression are strictly correlated.

p21 is generally considered a downstream target of p53. Nevertheless, since a basal level of p21 can be found in p53−/− cells, it appears that p21 can also be induced by pathways independent of p53. The p21 pathway inhibits DNA replication by interacting with PCNA, but it has also been shown that this same inhibitory interaction with p21 does not affect PCNA dependent DNA repair. It has recently been proposed that the SV40-Tag detected in mesothelioma specimens could play a part in the pathogenesis of this malignancy by impairing the function of several tumour suppressor and growth suppressor proteins such as p53 and the RB gene.

Moreover, most mesotheliomas retain mutations involving the CDKN2/ARF locus, impairing the functional unit composed by D-type cyclins, and in neoplastic tissue. Immunohistochemical analysis was chosen for this investigation because it has the advantage of different specimens (table 1). Fifteen of the 29 specimens had ≤10% positivity for p21 expression; the remaining 14 specimens had a higher percentage of positive cells.

**REFERENCES**