Cyclin-Dependent Kinase Inhibitory Protein Expression in Human Choroidal Melanoma Tumors

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PURPOSE. Recent studies have demonstrated the close link between oncogenesis and cell cycle machinery. Cyclin-dependent kinase inhibitory proteins (CKIs) have been shown to play a critical role in the regulation of cell cycle progression. Alteration of CKI levels and/or functions could be implicated in cell transformation. The three CKIs—p16, p21, and p27—were investigated in human uveal melanoma tumors, and an attempt was made to correlate their levels with clinicopathologic parameters, as well as to p53 and Ki-67 (Mib-1) protein levels.

METHODS. Immunohistochemistry was performed on 32 formalin-fixed, paraffin-embedded specimens of malignant choroidal melanoma. Immunoblot was performed to confirm the immunochemistry study. Prognostic histologic markers such as cell typing, pigmentation, larger tumor dimension, mitotic figures, nucleolar size, scleral invasion, and optic nerve head invasion were reported.

RESULTS. Nuclear positivity for p16 was observed in 11 tumors (34%) without any association with clinicopathologic parameters. Tumor cells positive for p21 were detected in 12 choroidal melanomas (37%). Unexpectedly, a positive relationship was seen between p21 and scleral invasion (P = 0.008). Nuclear positivity for p27 was observed in nine tumors (28%). An inverse correlation was observed between the number of mitotic figures and p27 immunoreactivity (P = 0.03), as well as between Mib-1 positivity and p27 expression (P = 0.02). Western blot assays of tumor extracts confirmed overexpression of p21 and p27.

CONCLUSIONS. The results suggest that p21 and p27 may be involved in tumorigenesis in choroidal melanoma. (Invest Ophthalmol Vis Sci. 2000;41:2837–2843)

In recent years, there has been increasing interest in understanding the biochemical mechanisms involved in the regulation of the cell cycle and the development of cancer. The orderly progression of cells through the cell cycle depends on a finely tuned balance between the levels of activated cyclins and cyclin-dependent kinases (CDKs), which provide positive growth signals, and kinase inhibitors (CKIs), which suppress the effects.1,2 Two families of mammalian CDK inhibitors have been identified. One family includes p21(WAF1), p27(kip1), and p57(kip2). All these have broad specificity, acting on the different G1 CDK cyclins as well as on the S-phase CDK2 cyclin A complexes. The p21 family also seems to be of major importance to the differentiating cells and has been shown to be induced by both p53-dependent and independent pathways.3–6 Moreover, p21 can inhibit DNA replication by blocking the ability of proliferating cell nuclear antigen (PCNA) to activate DNA polymerase-δ.7,8 The p27 protein has a 42% amino acid homology with p21. The role of p21 and p27 in regulating cell cycle progression in normal and neoplastic cells has not been completely elucidated. However, recent studies have shown that multiple-organ hyperplasia develops in p27-deficient mice, suggesting that this inhibitory protein has anti-proliferative activity in vivo.9,10 Furthermore, reduced expression of p27 has been shown to be a powerful negative prognostic marker in breast, colorectal, and non–small-cell lung carcinomas.11–14 In contrast, overexpression of p21 has been reported in brain and skin cancers.15–17 In any case, mutations in p21 and p27 appear to be surprisingly rare in tumors.18–22 The second and unrelated family of CKIs includes p16(INK4) and p15(INK4B) and associated proteins, which specifically inhibit CDK4 and CDK6. These CKIs have been suggested to be the products of potential antioncogenes. The p15 and p16 genes lie in tandem on human chromosome 9p21, the site of the multiple tumor suppressor (MTS) locus, which is rearranged, deleted, or mutated in many tumor-derived cell lines and in some primary tumors.23–24 A principal characteristic of p16 is that it is often found to be mutated in members of families prone to skin melanoma.25

Choroidal melanoma is the most primary malignancy of the eye in adults.26 This tumor poses a serious threat to life, and despite recent advances in treatment, the prognosis for patients with such a neoplasm remains poor. Studies have reported important prognostic factors, such as cell type, tumor size, and location. After enucleation, pathologists may assign a histologic classification by considering the histologic features: scleral or optic nerve invasion, degree of pigmentation, mitotic index, nucleolar size, or Ki-67 expression.27–31 Compared with
human choroidal cell cultures, p21 and p27 have been shown to be downregulated and the p16–CDK4 interaction to be suppressed in choroidal melanoma cell lines.\(^5\)\(^6\) The purpose of this work was to investigate the contribution of CKI expression dysregulation to the genesis of choroidal melanoma and to attempt to correlate their levels with clinicopathologic parameters, as well as to p53 and Ki-67 (Mib-1) protein.

**Materials and Methods**

**Tumor Material**

Archival, paraffin-embedded tumor specimens were obtained from the histopathology department from a series of patients treated in our ophthalmology division between 1996 and 1998. In the authors’ opinion, the methods for securing human tissue were humane and complied with the tenets of the Declaration of Helsinki. There were 32 cases with sufficient tumor tissue for analysis. Five patients underwent proton beam irradiation, and enucleation was performed for neovascular glaucoma in each case. Patients consisted of 19 men and 13 women, whose ages ranged from 34 to 87 with a mean of 65 ± 12.9 years. The right eye was involved in 15 cases and the left eye in 17 cases.

After surgical resection, each tumor was divided into two parts. The first part was frozen in liquid nitrogen and stored at −80°C until used for biochemical studies. The second part was formalin fixed for 24 hours at room temperature and then paraffin embedded for histologic investigation. The specimens were stained with hematoxylin and eosin, and histologic evaluation was performed with a light microscope. Cell type was classified by using the modified Callender’s classification: spindle type (S), mixed type (M), or epithelioid type (E).\(^27\)\(^28\) The largest macroscopic tumor dimension (LTD) was measured from the slides. The degree of pigmentation was noted as previously described. Amelanotic tumors were given a grade of 1, whereas tumors showing melanin pigment in almost every neoplastic cell were given a grade of 4.\(^29\) Mitotic figures were counted in 15 high-power fields with a total magnification of ×400, by using an eyepiece grid, and the number of mitoses was averaged. Mean diameter of the 10 largest nucleoli (MLN) was calculated for each tumor according to previously published methods.\(^30\) Optic nerve head invasion was also analyzed. Scleral invasion was graded as follows: non scleral or innermost layers of sclera (N), within sclera (S), extrascleral extension (E).\(^31\)

**Immunohistochemistry**

Blocks for testing were selected according to the presence of representative tumor tissue and, when available, adjacent normal tissue, such as normal choroid, cornea, retina, and pigment epithelium. The 4-μm paraffin-embedded sections were mounted on silane-coated glass slides. Slides were air dried, heated at 60°C for 1 hour, and deparaffinized and rehydrated with toluene, absolute alcohol, and deionized water. For CKIs, antigen was retrieved by heating in 10 mM citrate buffer (pH 6.0) in a oven. For p53 and Mib-1, retrieval was performed in an autoclave pressure cooker. After a rinse in deionized water, slides were treated to remove endogenous peroxidase with 3% H₂O₂ in methanol-alcohol for 5 minutes and then rinsed. After blocking with preimmune goat or rabbit serum, the sections were incubated for 60 minutes at room temperature with primary antibodies anti-p16 (monoclonal, DCS-501H4; Oncogene Science, Manhasset, NY; polyclonal, anti-p16; PharMingen, San Diego, CA), anti-p21 (monoclonal, EA10; Oncogene; polyclonal, C19; Santa Cruz Biotecchnology, Santa Cruz, CA), anti-p27 (monoclonal, G173-524; PharMingen; polyclonal, C19; monoclonal, SX55G8; Dako, Carpinteria, CA), anti-p53 (monoclonal, DO-7; Dako; monoclonal, Pab 1801; Oncogene) and anti-Ki-67 (Mib-1; Immunotech, Westbrook, ME). Sections were subsequently stained with a biotinylated secondary antibody and a streptavidin-biotin-peroxidase detection kit (Dako), in which diaminobenzidine was used as chromogen. The slides were mounted using a water-miscible mounting medium after counterstaining with Mayer’s hematoxylin.

External positive controls included lymphocytes of Burkitt lymphoma for p16 and p27, normal colon for p21, breast carcinoma for p53, and, in situ cervix carcinoma for Mib-1. Internal positive control was observed in adjacent normal tissue. Negative control sections were incubated with normal mouse or rabbit serum instead of primary antibodies.

**Assessment of Immunohistochemical Results**

All scoring and interpretation of immunohistochemical results were performed by a pathologist without knowledge of the clinical history. Cells were considered positive when the nucleus was distinctly stained. On the basis of microscopic observation (×400), the staining pattern of the different proteins was scored. Four nonconsecutive high-power fields were chosen randomly, and 2000 cells were counted. Cells were positive for HMB-45 and S-100 and negative for CD68 excluding bystander cells such as melanin-laden macrophages and lymphocytes. Percentages of positive cells were reported for statistical analysis. For better clarity, cells were graded as negative (0), weakly positive (+), moderate positive (++), and strongly positive (+++).

**Statistical Analysis**

Fischer’s exact test and Spearman’s correlation coefficient were used to determine the relationships between the different variables (SAS system; SAS, Cary, NC). Total population (N = 32) was included for the tests. P < 0.05 was considered significant.

**Western Blot Analysis**

Frozen samples of melanoma tumor tissue (30-g pieces) were homogenized at 4°C (Ultra-Turrax T8 homogenizer; IKA, Cincinnati, OH) three times for 10 seconds in 10 volumes of sample buffer (75 mM Tris-HCl [pH 6.8], 15% [wt/vol], sodium dodecyl sulfate [SDS], 20% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol and 0.001% [wt/vol] bromophenol blue, including a protease inhibitor cocktail). The homogenate was then processed and subjected to a homogenous 10% or 12% SDS-polyacrylamide gel electrophoresis as described.\(^32\) After electrophoresis, the proteins were transferred to nitrocellulose membranes. Choroidal melanocyte cultures (TMC-10) and choroidal melanoma cells (OCM-1) were used as controls.

**Results**

**Histologic Results**

Results are mean ± SD. Histologic cell identification according to Callender’s classification showed three melanomas to be epithelioid-cell type (E), whereas 10 were mixed-cell type (M),
and 19 were spindle-cell type (S). The average LTD was 14.6 ± 5.7 mm. The mean of mitotic figures was 2.6 ± 3.4 cells in division, MLN was 2.8 ± 0.9 μm, and mean pigmentation was 2.34 ± 0.93. Scleral invasion was observed in 15 cases, extrascleral extension in 5, and optic nerve head invasion in 12. No invasion of the lamina cribrosa was observed (Table 1).

CKI Immunoreactivity and Relationship with Clinicopathologic Parameters

Immunohistochemical staining revealed nuclear localization of p16, p21, and p27 (Fig. 1). The same results were obtained with the different antibodies. However, nonspecific staining in cytoplasm and with melanin was also observed for most of the p27-positive melanoma tumors with the monoclonal anti-p27 G173-524 in contrast to the nuclear staining observed with the monoclonal anti-p27 SX53G8 or anti-p27 polyclonal C19 (Fig. 1). No positive CKI staining was observed in any of the negative controls. Because CKIs are thought to act as tumor suppressors by inhibiting cell proliferation, we expected that expression of these proteins may have an inverse correlation with the aggressivity of the tumor. We then analyzed the relationship between CKIs expression and clinicopathologic parameters. Table 2 summarizes these correlations.

Eleven (34%) of the 32 melanomas were considered p16 positive, but no correlation to cell type, LTD, scleral or optic nerve head invasion, mitotic figures, or MLN was observed. The percentage of p16-positive nuclei ranged from 1% to 29% (mean positive cells, 7%).

P21-positive tumor cells were detected in 12 choroidal melanomas (37%). The percentage of p21-positive nuclei ranged from 1% to 14% (mean positive cells, 7%). Unexpectedly, a positive relationship was seen between p21 and scleral invasion (P = 0.008). The incidence of positive cases was significantly higher in tumors invading the sclera. However, no correlation between the depth of scleral invasion and the number of p21-positive nuclei was detected (data not shown). No correlation between p21 detection and irradiation was observed.

Nine tumors (28%) were considered p27 positive. The percentage of positive nuclei ranged from 1% to 8% (mean positive cells, 5%). Cells positive for p27 were found in four of the five cases treated with proton beam therapy. No correlation between p27 positivity and parameters such as cell type, LTD, MLN, or scleral or optic nerve head invasion was observed. A significant negative association, however, was found...
between this number and the number of mitotic figures \( (P = 0.03) \).

**Relationship between CKI Immunoreactivity and Ki-67 and p53 Accumulation**

The relationship between CKI expression and abnormal accumulation of p53 protein was also analyzed. Of the 32 tumors, only 4 showed p53 nuclear staining and in two cases, cell type was epithelioid (E; Table 1). The correlation between p53 overexpression and the expression of p21 was investigated, and no significant correlation between active p53 and p21 was found \( (P = 0.13) \). Moreover, no correlation between p53 detection and irradiation was observed.

In the whole sample, the mean number of Ki-67–positive cells was 7.7\% (SD). Positive correlation was detected between Ki-67 immunostaining and mitotic figures \( (P = 0.001) \). The relationship between CKI expression and the proliferative activity mediated by Ki-67 was examined. An inverse correlation between p27 and Ki-67 \( (P = 0.02) \) was found. The incidence of cells strongly positive for Ki-67 was higher in p27-negative tumors than in p27-positive tumors. Moreover after irradiation, only the p27-negative tumors showed moderate levels of Mib-1, whereas the p27-positive tumors after irradiation expressed no or weak levels. No other significant correlations were detected.

**Western Blot Analysis**

Western blot analysis was used to confirm overexpression of the proteins studied (Fig. 2). As expected, high levels of p27 and p21 were detected in lysates of tumor samples that exhib-

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**Table 2. Correlation of Tumor Parameters with CKI Expression**

<table>
<thead>
<tr>
<th>Histologic features</th>
<th>p16</th>
<th>p21</th>
<th>p27</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTD</td>
<td>0.58</td>
<td>0.57</td>
<td>0.92</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>0.49</td>
<td>0.17</td>
<td>0.91</td>
</tr>
<tr>
<td>MLN</td>
<td>0.95</td>
<td>0.33</td>
<td>0.45</td>
</tr>
<tr>
<td>Mitotic figures</td>
<td>0.42</td>
<td>0.96</td>
<td>0.03*</td>
</tr>
<tr>
<td>Mib-1</td>
<td>0.22</td>
<td>0.16</td>
<td>0.02*</td>
</tr>
<tr>
<td>Cell type</td>
<td>0.99</td>
<td>0.08</td>
<td>0.58</td>
</tr>
<tr>
<td>Scleral invasion or extrascleral extension</td>
<td>0.99</td>
<td>0.008</td>
<td>0.99</td>
</tr>
<tr>
<td>Optic nerve head invasion</td>
<td>0.99</td>
<td>0.45</td>
<td>0.67</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
</tbody>
</table>

MLN, mean of the ten largest nucleoli; LTD, largest macroscopic tumor dimension.
* Inverse relationship.
modulates the expression of genes important for DNA repair, was detected. Increased p21 expression may be explained as relationship with growth fraction, cell type, or tumor location.

Signals from p21. This result may also be explained by alteration of p21 stoichiometry. Increased p21 expression has been reported in uveal melanoma, Coupland et al. reported positive tumor reactivity of p21 is correlated to invasion of the tumor. In carcinomas of the colon, ovary, and stomach and in skin melanoma, high immunoreactivity of p21 is correlated to invasion of the tumor. In uveal melanoma, Coupland et al. found 45% of cases (mean positive cells, 12%) to be p16 positive but with no clinicopathologic correlation. The variable expression of p16 observed in uveal melanoma in the present study parallels to some extent the findings of the previous study, which reported chromosomal abnormalities involving the p16 gene.

In our specimens, 37% of the choroidal melanoma expressed p21. In immunohistochemistry regardless of histologic type. Because p21 negatively regulates cell cycle progression and transient expression of p21 in tumor cells results in the inhibition of cell proliferation, we expected an inverse correlation between p21 expression and proliferative activity or malignant behavior in choroidal melanoma. We found in the present study, however, that it was more complicated. The expression of p21 showed a positive correlation with scleral invasion rather than an inverse correlation, but in choroidal melanoma, only extrasceral extension has previously been shown to be a clinicopathologic parameter. In carcinomas of the colon, ovary, and stomach and in skin melanoma, high immunoreactivity of p21 is correlated to invasion of the tumor. In uveal melanoma, Coupland et al. reported positive tumor cells for p21 in 65% of cases (mean positive cells, 8%), but no relationship with growth fraction, cell type, or tumor location was detected. Increased p21 expression may be explained as a corollary of tumor cells’ having escaped terminal differentiation and growth arrest by becoming resistant to inhibitory signals from p21. This result may also be explained by alteration of p21 stoichiometry.

The p53 gene encodes a nuclear protein that binds to and modulates the expression of genes important for DNA repair, cell division, and cell death by apoptosis. At least half of all malignant tumors have mutations or rearrangements of both copies of the p53 gene. In many human cancers, positive immunohistochemical staining for p53 protein is associated with p53 gene sequence abnormalities, and p21 has been shown to be induced through both p53-dependent and -independent pathways. Although significant correlation between p21 and p53 has been previously reported in pancreatic cancer, breast cancer, and uveal melanoma, our data do not confirm such observations. On the contrary, we report conflicting results with regard to the levels of p53 observed by Western blot analysis or immunohistochemistry. This discrepancy has been described in other cell types and could be accounted for by differing fixation techniques or differing antibodies. Immunohistochemistry often fails to provide quantitative data and is not reliable under detection thresholds. In contrast, Western blot analysis analyzes total extracts that include proteins from melanoma cells and also proteins from lymphocytes and vascular cells. As a consequence, Western blot analysis seems to be more sensitive, although less specific, than immunohistochemical studies.

Concerning p16, Reed et al. observed loss of p16 expression in metastatic skin melanomas compared with other melanocytic lesions. Talve et al. showed that all benign skin lesions are p16 positive, and the extent of invasion according to the Clark index was significantly higher in p16-negative than in p16-positive tumors. In uveal melanoma, Coupland et al. found 45% of cases (mean positive cells, 12%) to be p16 positive but with no clinicopathologic correlation. The variable expression of p16 observed in uveal melanoma in the present study parallels to some extent the findings of the previous study, which reported chromosomal abnormalities involving the p16 gene.

In our study, 34% of the samples were considered p16 positive, but no correlation with clinicopathologic parameters was observed. In contrast to cutaneous lesions, benign choroidal tumors are difficult to obtain. Choroidal nevi are present in 6.5% of the population, and observation of such nevi in enucleated eye tissue is a rare event. Thus, we also performed immunohistochemical staining in adjacent structures, such as the choroid, which contained normal melanocytes. But uveal stroma also contained many fibroblasts, vascular endothelial cells, pericytes, ganglion cells, and macrophages. It is difficult in standard microscopy to differentiate melanocytes from other cell types. However, in a few specimens we observed weak p16 staining in the choroid (data not shown). Tam et al. showed that the expression level of p16 in serum-stimulated fibroblasts oscillates as much as fourfold during the cell cycle, peaking at the G1/S transition with lowest expression in G0 quiescent cells. Immunohistochemical analysis cannot distinguish between absolute loss of expression and the reduction of protein levels to extremely low and undetectable levels. Because normal melanocytes do not generally proliferate, the weak expression or absence of p16 in normal choroid may correspond to G0 cells such as normal melanocytes.

During the past 2 years, a large number of studies have examined the diagnostic and prognostic significance of p27 expression in various tumors. Nearly all the studies reported decreased p27 expression in more aggressive tumors. Expression of p27 is reported to be an independent prognostic factor and to be potentially useful in the diagnosis of a broad spectrum of tumors. In vitro studies have shown that p27 is overexpressed in normal melanocytes. In skin melanomas,
patients with tumors with fewer than 5% p27-staining cells have a significantly higher risk of early relapse of disease than do those with moderate or high levels. Moreover, in human breast, colorectal, gastric, and esophageal cancers or in malignant lymphomas, low p27 expression is associated with a poor prognosis. In the present study, we observed nuclear staining of p27 in 28% of the tumors, but the mean number of positive cells was low (3%). In Western blot analysis, overexpression was confirmed, but also in lysates from negatively stained tumors, suggesting a better sensitivity of biochemical analysis compared with immunohistochemistry.

Expression of P27 was found to be inversely related to the level of Ki-67 antigen. The Ki-67 antigen detected with Mib-1 antibody is a marker of cell proliferation and is expressed in all stages of the cell cycle except G0. We found positive correlation between Ki-67 immunostaining and mitotic figures. An inverse relationship between p27 and Ki-67 staining has been described in parathyroid and thyroid tumors, in non-small-cell lung cancer, and in adrenocortical neoplasms. We also found a significant negative correlation suggesting that p27 may be useful in assessing the biologic potential marker of low-grade malignancy in choroidal melanoma.

In agreement with previous considerations concerning p27 immunostaining, we thought that distinct nuclear immunoreactivity could be a sign of p27 positivity. Although distinct nuclear immunoreactivity for p27 was observed in nine tumors, some tumors demonstrated additional cytoplasmic positivity when the monoclonal antibody G173-524 was applied. This cytoplasmic staining was probably due to a non-specific binding of the protein, because the other anti-p27 antibodies used showed only nuclear staining. However, cytoplasmic staining may indicate that the protein is no longer able to bind to the nucleus or to the CDK2 complex. This may indicate that these tumors harbor a p27 gene mutation, although mutations in this gene appear to be surprisingly rare in tumors. More than 500 tumors have been examined for p27 mutations, and fewer than 5 have shown specific mutations. Alternatively, truncation of p27 has been reported recently to alter CDK2 binding and nuclear localization.

Induction of p21 or stabilization of p53 has been reported in many cell types after irradiation. Expression of p27 has also been shown to be increased after x-irradiation in testicular lysates. In uveal melanoma, overexpression of p53 has been detected in tumors previously treated by telecobalt or ruthenium irradiation. As in our study, Coupland et al. reported no association between p53 or p21 expression and irradiation in uveal melanoma. Moreover, in our samples positive p27 cells were found in four of the five specimens from patients treated with proton beam therapy, suggesting an induction of p27 after irradiation. In these samples, no or weak staining of Ki-67 were found. A negligible Ki-67 index is considered to be a reliable indicator for radiation-induced loss of proliferative potential. Thus p27 may also be useful in assessing the biologic potential of choroidal melanoma after irradiation as a low potential marker of malignancy, but such cases are not sufficient for statistical analysis. This hypothesis must be clarified with a larger series of patients.

To better understand the significance of CKI positivity in immunohistochemistry, prospective studies including relapse of the disease and date of death should be performed to assess the influence on survival. Further analysis should provide new insights into the mechanisms underlying the molecular changes leading to cellular differentiation or tumorgenesis in choroidal melanoma.

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