The p16-cyclin D/Cdk4-pRb Pathway as a Functional Unit Frequently Altered in Melanoma Pathogenesis

Jirina Bartkova, Jiri Lukas, Per Guldberg, Jan Alsner, Alexei F. Kirkin, Jesper Zeuthen, and Jiri Bartek

Division of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen [J. B. J. L., P. G., A. F. K., J. Z., J. B.], and Department of Experimental and Clinical Oncology, Danish Cancer Society DR-8000 Aarhus C. [J. A.], Denmark

ABSTRACT

The p16\(^{ink4a/CDKN2}\) D-type cyclins, their partners Cdk4/Cdk6, and pRb constitute a G\(_1\) regulatory pathway commonly targeted in tumorigenesis. Genetic, immunohistochemical, and functional cell cycle analyses showed abnormalities of this pathway in each of 22 human melanoma cell lines examined. Normal melanocytes and all melanoma lines expressed Cdk4, Cdk6, and cyclins D1 and D3. The tumor suppressors p16\(^{ink4a/CDKN2}\) and pRb were lost in 17 and 4 cases, respectively, due to various genetic mechanisms, including transcriptional block of p16 and nonsense mutations of RBL1. Ectopic expression of p16 prevented S-phase entry of Rb\(^{+/+}\) p16\(^{-}\) but not Rb-deficient melanoma lines. The SK29-MEL-1 cell line harboring an R24C mutation in Cdk4 expressed wild-type pRb and overabundant p16, the latter preventing endogenous Cdk6 but not Cdk4 from associating with cyclin D1. Microinjection of cyclin D1-neutralizing antibody arrested the SK29-MEL-1 cells in G\(_1\), whereas p16 did not, indicating that the cyclin D1/Cdk4-R24C complex is required for G\(_1\) progression, and the resistance of the complex to p16 in vivo. These data strongly support the candidacy of Cdk4 as a novel proto-oncogene, providing further evidence for the p16-cyclin D/Cdk4-pRb pathway as a functional unit, and suggest that deregulation of this checkpoint may represent a common step in the multistep progression of sporadic malignant melanomas.

INTRODUCTION

Recent genetic and biochemical investigations of the molecular mechanisms governing the G\(_1\) to S progression in mammalian cells demonstrated a central role for D-type cyclins and their partner kinases Cdk4 and Cdk6 (1–5). The cell cycle-accelerating function of cyclin D-associated kinases appears to be mediated by neutralizing the growth-restraining ability of pRb\(^{3}\) via its phosphorylation in advanced G\(_1\), a process negatively regulated by p16\(^{ink4a/CDKN2}\) and other Cdk inhibitors (1–6). pRb is itself a tumor suppressor, the unphosphorylated form of which prevents premature S-phase entry by physically sequestering a series of transcription factors, such as E2F, required to activate S-phase genes (4). Phosphorylation of pRb by cyclin D-Cdk4/6 kinases in G\(_1\) appears to release the captured transcription factors, thereby inducing gene expression and metabolic changes leading to onset of DNA replication. The emerging critical role of the p16-cyclin D/Cdk-pRb-E2F pathway in cell cycle regulation is further supported by frequent aberrations of the individual components of this checkpoint mechanism in human tumors (1–8).

Subsets of both sporadic and familial melanoma cases have recently been shown to harbor mutations in two genes, the products of which are involved in G\(_1\) phase control. First, the p16\(^{ink4a/CDKN2/MTS1}\) tumor suppressor gene on chromosome 9p21, frequently lost in a wide range of malignancies (1–14), is deleted or mutated in more than one-half of human sporadic melanomas and cell lines derived therefrom (9, 10, 15), and it appears to be the predisposing mutation in some familial melanoma kindreds (16–19). Second, a small fraction of melanomas harbor a missense mutation (causing an arginine to cysteine change at codon 24, R24C) in the cdk4 gene, potentially activating this positive G\(_1\) regulatory kinase as an oncogene (20, 21).

To assess the role of alterations within the cyclin D/Cdk-p16-pRb pathway in human melanoma pathogenesis in a broader perspective, we have now examined all the key components of this G\(_1\) checkpoint mechanism by a combination of genetic, immunohistochemical, and functional approaches in a series of novel melanoma cell lines and, where possible, in the corresponding tumor specimens. Our data show that in all cases examined, at least one of the cell cycle regulators involved in the above pathway is aberrant, consistent with the emerging concept of the cyclin D-Cdk-p16-pRb interplay as a candidate obligatory target in oncogenesis (1–5). In addition, our results from functional analyses extend the evidence for this biochemical pathway as a functional unit, including direct demonstration of the in vivo consequences of the R24C mutation for protein-protein interactions and G\(_1\) regulatory role of Cdk4.

MATERIALS AND METHODS

Cell Culture. The establishment and properties of melanoma cell lines FM3, FM6, FM9, FM28, FM45, FM48, FM60, FM55\(_{p}\), FM55\(_{m}\), and FM55\(_{w}\) were described previously (22); the last three lines were established from invasive primary and two s.c. metastatic lesions, respectively, removed from the same patient. The additional melanoma cell lines FM39, FM57, FM58, FM62, FM66, FM70, FM74, FM76, FM77, and FM79 were further established in our laboratory using similar methods. The melanoma cell lines BLM (23) and SK29-MEL-1 (20) were kindly provided by Dr. G. J. Adema (University Hospital, Nijmegen, the Netherlands) and Dr. Pierre van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). Normal cultured melanocytes (NHEM2486) and melanocyte culture medium were purchased from Clonetics (San Diego, CA), and melanocytes were cultured according to the supplier’s instructions. Melanoma cell lines were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. All FM melanoma lines were used at early passages (passages 3–6) corresponding to a total of 5 weeks to 3 months in culture, depending on the cell line. The control human cell lines MCF-7 and BT549 (both breast cancer) and U-2-OS (osteosarcoma) were cultured as described previously (24).

Nucleic Acid Analyses. Detection of homozygous deletions in the p16\(^{ink4a/CDKN2}\) gene was performed by PCR analysis of genomic DNA, using previously described conditions and primers (16, 25). RT-PCR for p16 expression was performed as described previously (25). Double-stranded PCR products were directly sequenced using the Thermo Sequenase sequencing kit (Amersham) and a 3P-labeled amplification primer as sequencing primer. In the search for the RBL1 gene mutations, genomic DNA was isolated, and the RBL1 promoter, the 27 exons including intron sequences, and the polyadenylinc acid signal region were all analyzed by direct sequencing of PCR products. Primers were selected essentially as described by Hogg et al. (26) and Lohman et al. (27). PCR was performed using 50 ng of DNA in a 50-pi volume of 1X GeneAmp PCR buffer (Perkin-Elmer Cetus), 0.2 mm dNTP (Pharmacia), 1 unit of AmpliTaq (Perkin-Elmer Cetus), and 25 pmol of each primer (Pharmacia). The amplification conditions included an initial cycle of 10 min at 94°C, 3 min at the annealing temperature (55 and 58°C for exons 6 and 14, respectively), and 3 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 5475

Received 6/1/96; accepted 9/30/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Danish Cancer Society and the Danish Eye Research Foundation.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: RT, reverse transcription; MAb, monoclonal antibody; Rb, retinoblastoma; pRb, Rb protein; Cdk, cyclin-dependent kinase; DGGE, denaturing gradient gel electrophoresis; GST, glutathione S-transferase; BrdUrd, bromodeoxyuridine.

5475

Downloaded from cancerres.aacrjournals.org on July 20, 2017. © 1996 American Association for Cancer Research.
annealing temperature, and 1 min at 72°C. In the last cycle, the polymerization step was extended by 10 min. Biotinylated PCR products were sequenced in both directions as described by the manufacturers (Dynabeads M280, Dynal AS, and ABI Prism Sequenase Terminase, Perkin-Elmer Cetus) on an ABI 373 sequencer and compared to the wild-type RB1 (GenBank accession number L11910). To scan for mutations in reverse-transcribed cdk4 mRNA, we established an assay based on PCR, GC-clamping, and DGGE (28). Primers for amplification of a 337-bp fragment covering codons 1-78 were: CCGTCCGCAGCCCGCTCGCCCGCCGC- GGGCTCGCCGGTCGGTAGGGCTC and CGTACGTTGGAGTGGGC. The theoretical melting map (29) for the amplification product displays a two-domain structure, which is considered optimal for resolution of mutations. PCR amplification was performed with the GeneAmp PCR System 9600 (Perkin-Elmer Cetus) in 25-μl reaction volumes containing 10 mm Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.02% gelatin, 5% DMSO, 200 μM each dNTP, 0.8 μM each primer, 500 ng of DNA, and 0.8 unit of Tag DNA polymerase. The amplification protocol consisted of 40 rounds of thermal cycling (94°C for 10 s, 62°C for 10 s, and 72°C for 10 s) and a final incubation step at 72°C for 5 min. Ten μl of the PCR products were loaded onto a 6% polyacrylamide gel containing a gradient of urea and formamide from 35 to 70%, followed by electrophoresis at 160 V for 5 h in 1 X TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA) kept at a constant temperature of 58°C. For quantitative analyses, [α-32P]dATP was included in the PCR, and the DGGE gel was dried and inspected using storage phosphor technology (Molecular Dynamics).

**Immunochemical Methods.** Mouse Mabs DCS-6, DCS-11 (30, 31), and 5D4 (donated by Dr. M. Seto, Aichi Cancer Center, Nagoya, Japan) to cyclin D1; MAB DCS-5 to cyclin D2 (32); MAb DCS-20 (33) and DCS-22 (34) to cyclin D3; MAb 245 to pRb (donated by Dr. W.-H. Lee, University of Texas Health Science Center, San Antonio, TX); and MAb DCS-50 to p16 (35) were used as hybridoma tissue culture supernatants or IgG affinity purified on protein A. Rabbit antisera to Cdk4 and Cdk6 were from Santa Cruz Biotechnology (Santa Cruz, CA) and donated by Dr. G. Peters (Imperial Cancer Research Fund, London, United Kingdom), respectively, and the mouse antibody against the CD20 membrane marker was from Becton Dickinson. The p16 cDNA in pGEX-2TK (Pharmacia) was used in Escherichia coli BL21 (DE3) pLysS to express the GST-p16 fusion protein and as pCMV-p16 vector in human cells (35). The immunoprecipitation, immunoblotting, and immunofluorescence methods were described previously (30, 31, 36). Immunohistochemical staining of pRb and cyclin D1 on formalin-fixed, paraffin-embedded sections was performed essentially as described (37), except that a microwave unmasking step (10-20 min in 0.01 M citrate buffer, pH 6.0) was used instead of the trypsinization of the sections. The results were evaluated by an experienced dermatopathologist.

**Gene and Protein Transfer.** Cells synchronized in mitosis by nocodazole block and “shake-off” procedure (30) or in G1 by lovastatin treatment for 36 h (36) were microinjected with either the affinity-purified GST-p16 wild-type fusion protein (2 mg/ml), affinity-purified Mab DCS-6 to cyclin D1 (6 mg/ml), or control mouse immunoglobulin as described previously (35), using the AIS microinjection apparatus (Zeiss). Alternatively, cells blocked in mitosis by nocodazole were cotransfected via a modified calcium phosphate method with 25 μg of p16 plus 5 μg of CD20, or a control vector plus the CD20 expression plasmid DNAs as described previously (38). Cell cycle progression of the cells released from the lovastatin block by removal of the drug and addition of fresh medium supplemented with 10% FCS and mevalonate (36) was evaluated by combined in situ double immunofluorescence analysis of BrdUrd incorporation and p16, CD20, or immunoglobulin content as described (31, 35). Each experiment was performed 2-4 times to verify reproducibility.

**RESULTS**

**Abundance and Localization of D cyclins, CDK4/6, p16, and pRb.** Immunoblotting analyses of the p16 and pRb tumor suppressors in total lysates of exponentially proliferating normal human melanocytes showed moderate expression of either protein, in contrast to frequent lack of p16 or absence of pRb found in melanoma cell lines examined in parallel (Fig. 1A; Table 1). The comparison of the two sets of immunoblotting results revealed an inverse correlation between the presence of p16 and pRb among the melanoma lines, and the absence of pRb was accompanied by elevated amounts of p16 (Fig. 1A). Of the 22 melanoma cell lines examined, 4 lacked pRb, and 17 showed undetectable levels of the p16 protein (Table 1). The only cell line with both pRb and p16 clearly detectable by immunoblotting was SK29-MEL-1 (Fig. 1A; Table 1). The spectrum of detectable D-type cyclins included cyclins D1 and D3, analogous in both normal melanocytes and all the melanoma lines investigated (Fig. 1B; Table 1). There was some variation in the abundance of cyclins D1 and D3 from cell line to cell line, with the pRb-deficient lines usually expressing low levels of cyclin D1. In contrast, no obvious correlation was seen between the pRb status and the abundance of Cdk4 or Cdk6 proteins that were detectable in all cell types examined (Fig. 1B; Table 1).
Table 1. Aberrations in the cyclin D-Cdk-p16-pRb pathway in human melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pRb/RB1 gene</th>
<th>p16/INK4a/CDKN2a protein</th>
<th>Cdk4 protein</th>
<th>Cyclin proteins</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>Cdk6 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM55p</td>
<td>/mut</td>
<td>++/wt</td>
<td>++/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM55M1</td>
<td>/mut</td>
<td>++/wt</td>
<td>++/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM55M2</td>
<td>/mut</td>
<td>++/wt</td>
<td>++/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM39</td>
<td>-/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM45</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM43</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM28</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM57</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM49</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM60</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM70</td>
<td>+/ND</td>
<td>-/del</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM58</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM79</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM9</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>BLM</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>FM74</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>SK29-MEL-1</td>
<td>+/ND</td>
<td>-/t-block</td>
<td>+/wt</td>
<td>±</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Normal melanocytes</td>
<td>+/ND</td>
<td>+/ND</td>
<td>+/ND</td>
<td>+/ND</td>
<td>+/ND</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a pRb was examined by immunoblotting and immunostaining with concordant results. -, no detectable protein; +, clearly detectable nuclear protein. The RB1 gene was sequenced in the FM55p, FM55M1, and FM55M2 cells, and two hemizygous mutations resulting in stop codons were identified (mut); ND, not determined.

b The p16 protein was overabundant (+ +), detectable (+), or undetectable (−) by immunoblotting and immunofluorescence; the gene appeared wild-type (wt), homozygously deleted (del), mutant (mut), or not expressed (t-block) as judged from PCR and/or RT-PCR analyses.

c Cdk4 was detectable by immunoblotting and immunofluorescence (+); the gene showed no mutations within codons 1–78 by RT-PCR/DGGE analysis (wt), except for the R24C mutation in SK29-MEL-1 cells.

d D-type cyclins were clearly detectable (+), weakly expressed (+), or undetectable (−) by immunoblotting and immunofluorescence.

e Cdk6 was detectable (+) by immunoblotting.

The aberrations detected in Rb, p16, and Cdk4 are shown in bold type.

To independently verify the immunoblotting data and to examine the subcellular localization of the G1 regulatory proteins, an immunofluorescence analysis of exponential cultures of each cell type in our panel was carried out using specific antibodies to pRb, p16, cyclin D1, cyclin D2, cyclin D3, and Cdk4. In normal melanocytes, pRb was nuclear; the immunostaining intensity varied moderately from cell to cell (Fig. 2A); the p16 signal was relatively weak and mainly localized to the nuclei but was also detectable in the cytoplasm (Fig. 2B); cyclin D2 was undetectable, in contrast to cyclins D1 and D3, the predominantly nuclear staining of which was extremely variable among the exponentially growing cells (Fig. 2C); and Cdk4 showed granular nuclear staining (Fig. 2D). Among the melanoma cell lines, the immunofluorescence results correlated very well with the immunoblotting data in terms of the presence versus lack of detectable pRb or p16 (see Fig. 3, A–D, and Fig. 4 for examples), pRb being localized exclusively in the nuclei, whereas p16 was localized in both nuclei and cytoplasm. Cyclin D2 was generally undetectable or showed only extremely weak staining in rare cells (not shown), whereas cyclins D1 and D3 displayed highly variable nuclear staining, with cyclin D3 generally detectable in a higher proportion of cells than cyclin D1 (e.g., compare panels E–H of Fig. 3). Cdk4 immunostaining was nuclear with variable intensity, but unlike D cyclins it was detectable in all cells of the exponentially growing melanoma cell populations (Fig. 3, I and J).

In summary, the combined immunoblotting and immunofluorescence analyses of a series of melanoma cell lines frequently revealed abnormalities of the p16 and, less commonly, deficiencies of the pRb tumor suppressor proteins, in addition to a general lack of cyclin D2 and a common, albeit variable, expression of cyclins D1 and D3 and their partner kinases Cdk4 and Cdk6.

Because our finding of the absence of pRb in a subset of the melanoma cell lines was unexpected, we examined paraffin tissue sections from 18 of the 22 original melanoma specimens matching 18 of the 22 used to establish the cell lines, by immunoperoxidase staining. The presence versus lack of detectable pRb or p16 (see Fig. 3, A–D, and Fig. 4 for examples), pRb being localized exclusively in the nuclei, whereas p16 was localized in both nuclei and cytoplasm. Cyclin D2 was generally undetectable or showed only extremely weak staining in rare cells (not shown), whereas cyclins D1 and D3 displayed highly variable nuclear staining, with cyclin D3 generally detectable in a higher proportion of cells than cyclin D1 (e.g., compare panels E–H of Fig. 3). Cdk4 immunostaining was nuclear with variable intensity, but unlike D cyclins it was detectable in all cells of the exponentially growing melanoma cell populations (Fig. 3, I and J).
Genetic Analyses of RBL, p16<sup>ink4a/CDKN2</sup>, and Cdk4. Because our study of the proteins participating in the G<sub>1</sub> control pathway revealed the common lack of the p16 or pRb tumor suppressor proteins in the melanoma cell lines, we next examined the genetic basis for these aberrations. Homozygous deletions of the p16<sup>ink4a/CDKN2</sup> gene were detected in 12 of the cell lines by PCR analysis of genomic DNA (Fig. 6A). In all cases, the deletions included all three exons of the gene, and RT-PCR analyses in these cell lines yielded no detectable products (Fig. 6, B and C). In the remaining cell lines, a product was obtained by PCR analysis of reverse-transcribed mRNA, using primers located in exons 2 and 3, respectively. Two of the cell lines, BLM and FM74, showed aberrantly sized products (Fig. 6B), indicating the presence of defects in pre-mRNA splicing. Direct sequencing of the PCR products from the BLM genomic DNA disclosed a G—→A transition at the first nucleotide of the splice donor site of intron 2 (data not shown), and sequence analysis of the aberrantly sized

Although p16 status could not be investigated by this approach due to the lack of an antibody suitable for staining of archival formalin-fixed tissues, the immunohistochemistry generally indicated a good correlation with the above analysis of the melanoma cell lines and confirmed that the lack of pRb expression seen in the cell lines occurred already at the stage of the original tumor rather than during the in vitro culture period.
RT-PCR product of BLM revealed that the IVS2nt1g→a mutation results in the utilization of a cryptic splice site located in exon 2, 75 bp upstream from the normal donor splice site. The gene defect responsible for aberrant pre-mRNA splicing in FM74 cells has not been identified. Three additional cell lines without homozygous dele-

![Fig. 6. Nucleic acid analyses of the p16<sup>INK4a</sup>/CDKN2 and Rb status in melanoma cell lines. A, detection of p16<sup>INK4a</sup>/CDKN2 homozygous deletions by PCR amplification of exon 2 from genomic DNA. B, RT-PCR analysis of p16 expression, using primers for exons 2 and 3. C, RT-PCR analysis of p16 expression, using primers for exons 1 and 2. The names of cell lines are indicated on the top: M, 100-bp ladder; C, positive control. D, sequence analysis of Rb-1 alleles from FM55<sub>M1</sub> cell line shows heterozygous point mutations at position 596 in exon 6 (converting codon 199 from TTA/leucine to a TAA stop codon; left panel) and at position 1363 in exon 14 (converting codon 455 from CGA/arginine to a TGA stop codon; right panel).](image)

Fig. 6. Nucleic acid analyses of the p16<sup>INK4a</sup>/CDKN2 and Rb status in melanoma cell lines. A, detection of p16<sup>INK4a</sup>/CDKN2 homozygous deletions by PCR amplification of exon 2 from genomic DNA. B, RT-PCR analysis of p16 expression, using primers for exons 2 and 3. C, RT-PCR analysis of p16 expression, using primers for exons 1 and 2. The names of cell lines are indicated on the top: M, 100-bp ladder; C, positive control. D, sequence analysis of Rb-1 alleles from FM55<sub>M1</sub> cell line shows heterozygous point mutations at position 596 in exon 6 (converting codon 199 from TTA/leucine to a TAA stop codon; left panel) and at position 1363 in exon 14 (converting codon 455 from CGA/arginine to a TGA stop codon; right panel).
detected the mutation in the SK29-MEL-1 cell line used in the study by Wölfe et al. (20) and revealed a 3-fold excess of the mutant over the wild-type mRNA [Fig. 7A; consistent with the amplification of the former predicted by Wölfe et al. (20)] but failed to detect any cdk4 aberrations in the remaining melanoma cell lines (Table 1).

**Functional Consequences of Aberrant Cdk4, p16, or pRb for G1 Control.** The candidacy of Cdk4 as a proto-oncogene was significantly strengthened by the reports of the R24C mutation in melanomas (20, 21), and the evidence from *in vitro* experiments that the baculovirus-expressed Cdk4-R24C was much more resistant to inhibition by p16 than the wild-type Cdk4 protein (20). On the other hand, there has been no report as to what are the biochemical and/or biological consequences of the presence of such a Cdk4 mutant protein in living mammalian cells. To address this issue, we first asked what is the composition and relative abundance of the protein detected the mutation in the SK29-MEL-1 cell line used in the study by Wölfe et al. (20) and revealed a 3-fold excess of the mutant over the wild-type mRNA [Fig. 7A; consistent with the amplification of the former predicted by Wölfe et al. (20)] but failed to detect any cdk4 aberrations in the remaining melanoma cell lines (Table 1).

**Functional Consequences of Aberrant Cdk4, p16, or pRb for G1 Control.** The candidacy of Cdk4 as a proto-oncogene was significantly strengthened by the reports of the R24C mutation in melanomas (20, 21), and the evidence from *in vitro* experiments that the baculovirus-expressed Cdk4-R24C was much more resistant to inhibition by p16 than the wild-type Cdk4 protein (20). On the other hand, there has been no report as to what are the biochemical and/or biological consequences of the presence of such a Cdk4 mutant protein in living mammalian cells. To address this issue, we first asked what is the composition and relative abundance of the protein detected the mutation in the SK29-MEL-1 cell line used in the study by Wölfe et al. (20) and revealed a 3-fold excess of the mutant over the wild-type mRNA [Fig. 7A; consistent with the amplification of the former predicted by Wölfe et al. (20)] but failed to detect any cdk4 aberrations in the remaining melanoma cell lines (Table 1).

**Functional Consequences of Aberrant Cdk4, p16, or pRb for G1 Control.** The candidacy of Cdk4 as a proto-oncogene was significantly strengthened by the reports of the R24C mutation in melanomas (20, 21), and the evidence from *in vitro* experiments that the baculovirus-expressed Cdk4-R24C was much more resistant to inhibition by p16 than the wild-type Cdk4 protein (20). On the other hand, there has been no report as to what are the biochemical and/or biological consequences of the presence of such a Cdk4 mutant protein in living mammalian cells. To address this issue, we first asked what is the composition and relative abundance of the protein detected the mutation in the SK29-MEL-1 cell line used in the study by Wölfe et al. (20) and revealed a 3-fold excess of the mutant over the wild-type mRNA [Fig. 7A; consistent with the amplification of the former predicted by Wölfe et al. (20)] but failed to detect any cdk4 aberrations in the remaining melanoma cell lines (Table 1).

**DISCUSSION**

This study represents the most broadly based analysis of the p16-cyclin D-Cdk-pRb pathway in human melanomas performed to date, and the results provide several novel pieces of evidence to support the emerging significant role of subverting this G1 checkpoint mechanism in human melanoma pathogenesis.

The first important conclusion from our data is that at least one component of the above pathway is aberrant in each of the 22 human melanoma cell lines examined. None of the previous studies on melanomas could reach such a conclusion, partly due to a selective examination of just one component rather than multiple genes/proteins involved in the G1 control (10, 11, 15, 40–44), partly due to only very recent discoveries of some of the aberrations, such as the transcriptional block of the p16ink4a/CDKN2 gene (12, 13), which have not been adequately studied in melanomas before (15, 40–42, 44, 45). In fact, one of the novel findings in the present study is the identification of a subset of cases in which the p16 tumor suppressor gene appears to be transcriptionally silenced (3 of 22 cell lines), suggesting that this is an alternative way, apart from the deletions or mutations described before (10, 11, 15–19, 40–42), to eliminate the p16 inhibitor in human melanomas. A critical role of p16 in preventing oncogenic transformation has recently been confirmed by an increased tumor incidence in mice genetically lacking the Ink4a locus, and a potential involvement of p16 in melanocyte function was suggested by a lighter coat pigmentation cosegregating with the Ink4a allele (46). Thus, the chromosome region around this locus may be genetically very active in melanocyte differentiation, thereby potentially contributing to its increased fragility in the melanocyte lineage. Whereas the frequent lack of the p16 gene/protein was to be expected based on previous studies, somewhat surprising is our finding of a subset of cases lacking pRb, a result confirmed at the gene and protein levels and verified on the original biopsy material. Although RBJ gene defects are not commonly detected among melanoma cell lines (43), their involvement in melanoma pathogenesis is suggested by several recent clinical studies demonstrating that Rb patients and their relatives have a higher frequency of atypical melanocytic nevi and an increased risk of melanoma and that the incidence of melanomas is higher in survivors of the hereditary rather than the sporadic form of Rb (47). Taken together, these data warrant further studies to elucidate the role of RBJ abnormalities in melanomagenesis.

To judge the significance of our present data for melanoma as a disease, it is crucial to consider two issues. First, the limitation of this study to invasive and metastatic melanomas does not allow any conclusion as to whether the observed aberrations occurred early or late relative to progression of the disease. This question remains to be clarified, but some data showed that at least the p16 expression is commonly preserved in nevi and *in situ* lesions (15), suggesting that its loss may contribute to progression, rather than initiation of sporadic melanoma development. Second, the possibility of *in vitro,*
Fig. 8. Effects of exogenous p16 or cyclin DI-neutralizing antibody upon G1→S transition of human melanoma cell lines. A and B, the SK29-MEL-1 cells were microinjected with the GST-p16 protein together with the normal IgG as a marker for successfully injected cells (A, Texas red channel). Most of the injected cells progressed into S phase as revealed by BrdUrd incorporation (arrowheads in B, FITC channel). C and D, the SK29-MEL-1 cells microinjected with the MAb DCS-6 to cyclin DI (immunoglobulin-positive in C, Texas red channel), were prevented from entering S phase (note the absence of BrdUrd staining in cells marked by arrowheads in D, FITC channel). E, graph showing data from a representative microinjection experiment with synchronized SK29-MEL-1 cells, documenting the significant inhibitory effect upon S phase entry of the cyclin DI-neutralizing antibody (DCS-6), in contrast to little if any effect of the GST-p16 protein, as compared with noninjected cells or those microinjected by control IgG. In control MCF-7 cells synchronized and microinjected in parallel, GST-16 caused a G1 block at least as efficiently as the DCS-6. F, graphs summarizing inhibition of S phase entry (% of BrdUrd-incorporating cells) in various melanoma cells by exogenous wild-type p16 expressed from a transfected CMV-pl6 plasmid. The rate of S-phase entry upon transfection of the control plasmid was assessed 24 h poststimulation by BrdUrd incorporation (taken as 100%) and compared with parallel cells transfected by the p16 expression plasmid. Note the correlation of G1 arrest by p16 with the presence of functional pRb. Columns, mean values from three independent experiments; bars, SD.
Last but not least, our results also strengthen the notion of the p16-cyclin D-Cdk-pRb pathway as a functional unit and a single, complex target of oncogenic alterations. The data relevant to this point include the observation that the aberrations of pRb, p16, and Cdk4 occurred in mutually exclusive subsets of the melanoma lines, a result generally regarded as evidence for participation of the corresponding gene products in a common biochemical mechanism (1–5, 48–52). The mutual interdependence of these regulators is further supported by the lack of any cell cycle inhibition via p16 transfection into the Rb-deficient FM535P cells, consistent with analogous results in other cell types (35, 39, 53–55). The G1 arrest caused by ectopic p16 in melanoma cell lines with functional pRb and aberrant lack of p16 indicated their sensitivity to p16 and suggested how p16 loss could have provided a growth advantage to these cells. Apart from its indisputable biological significance, the occurrence of oncogenic mutations within the p16-cyclin D-Cdk-pRb pathway in the majority of, if not all cases, of a particular tumor type may raise a general question as to the potential utility of such information for the management of any particular patient suffering from the disease. Accumulating evidence now suggests that a precise identification of the aberrant component within the G1 pathway may have diagnostic (5, 56), prognostic (57, 58), and potentially therapeutic implications. The biological consequences of deregulating the individual gene products involved appear to be unequal, with the loss of pRb as the central downstream effector of the pathway having a more severe impact upon proliferation control than deregulation of any of the upstream regulators, such as cyclin D1 or p16 (25). It is also becoming clear from the functional studies such as this one that any attempts at gene therapy or other modes of therapy aimed at restoring the derailed G1 control must take into account that, e.g., reintroduction of p16 into cancers deficient in Rb or harboring the R24C mutation in Cdk4 is likely to fail in inducing any positive response, whereas such treatment could be beneficial in tumors with wild-type Rb and deficient for p16 and/or overexpressing cyclin D1 (20, 24, 25, 30, 31, 35, 39, 54, 55, and this study). There is little doubt that a more detailed understanding of the molecular principles governing the cell cycle can help elucidate their subversion in cancer cells and eventually lead to a significant practical impact on the diagnosis and management of a range of hyperproliferative and hypoproliferative diseases.

ACKNOWLEDGMENTS

We thank Dr. Klaus Hou-Jensen and Annette Birck (Department of Pathology, University Hospital, Copenhagen, Denmark) and the Danish Melanoma Group for discussions and for kindly making sections of the archival paraffin-embedded specimens available for our immunohistochemical analysis; Dr. Michael Strauss for helpful discussions and support; Anne-Arm Kjerulff for help with tissue culture; and Drs. Pierre van der Bruggen, Gosse J. Adema, Gordon Peters, Masao Seto, and Wen-Hwa Lee for donation of some of the cell lines and reagents.

REFERENCES

The p16-cyclin D/Cdk4-pRb Pathway as a Functional Unit Frequently Altered in Melanoma Pathogenesis

Jirina Bartkova, Jiri Lukas, Per Guldberg, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/23/5475

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.