Functional Impairment of Melanoma-associated p16INK4a Mutants in Melanoma Cells despite Retention of Cyclin-dependent Kinase 4 Binding

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ABSTRACT

Purpose: Melanoma-associated germ-line mutations affecting the tumor suppressor and cyclin-dependent kinase (CDK) inhibitor, CDKN2A/p16INK4a, have been identified in >100 melanoma-prone families. To predict the melanoma risk for carriers of specific mutations, it is useful to test the function of the mutant proteins in biochemical assays; however, it is unclear how well these results correlate with their cellular effects. We examined the relationship between loss of CDK binding by mutant proteins and various measures of cellular growth in melanoma cells.

Experimental Design: The cellular activities of four melanoma-associated p16INK4a mutations (Arg24Pro, Ala36Pro, Met53Ile, and Val126Asp) were compared by use of inducible expression in stably transfected melanoma cells, deficient in expression of the endogenous protein, and compared with their ability to bind CDK4.

Results: The cell cycle-inhibitory activity of all of the mutants was profoundly reduced, and partially retained capacity for CDK4 binding in functional assays did not correlate with significant preservation of cell cycle-regulatory function.

Conclusion: Testing of p16INK4a interactions with CDKs in protein-binding assays is an unreliable predictor of mutant p16INK4a function in cells. In addition to exhibiting reduced stability, these mutant proteins may also be defective in interaction with cellular targets other than CDKs.

INTRODUCTION

The CDKN2A gene on chromosome band 9p21 encodes the CDK4 inhibitor p16INK4a. Somatic alterations affecting this gene occur in >50% of human tumors (1, 2). Inherited CDKN2A mutations are associated with susceptibility to melanoma and, in some cases, pancreatic carcinoma (3, 4). To date, at least 48 germ-line p16INK4a mutations have been identified in >100 melanoma-prone families worldwide. Although these mutations may segregate closely with the disease in melanoma-prone families, demonstration of functional defects in the p16INK4a protein remains clinically important in distinguishing disease-associated mutations from population polymorphisms. p16INK4a was initially identified as a binding partner of CDK4 (5). Although several functions of p16INK4a have been described in recent years, including the repression of the transcription factor NFκB (6), it is widely assumed that the main role of p16INK4a is to inhibit CDKs. In particular, p16INK4a binds to and inhibits the activity of CDK4 and CDK6. These kinases regulate the progression of eukaryotic cells through the G1 phase of the cell cycle. CDK4 and CDK6 initiate the phosphorylation of pRb, which permits cells to enter DNA replication in S phase. CDK4 may also be involved in cell cycle progression through the G2 phase of the cell cycle and into mitosis (M phase; Ref. 7).

To investigate various p16INK4a mutations for their ability to bind and inhibit CDK4 and CDK6, a number of CDK-binding and kinase assays have been applied. These assays used p16INK4a and CDKs fused to GST (8), polyhistidines (9), or proteins translated in vitro (9–11). Alternatively, in the yeast two-hybrid system, the proteins were fused to regulatory yeast peptides (12–14). These tests confirmed that most cancer-associated p16INK4a mutations are functionally impaired. However, different assays revealed variable degrees of functional loss for identical p16INK4a mutations. For example, the Gly101Trp mutation is strongly associated with melanoma in >20 families worldwide, but its cyclin D1/CDK4 inhibitory activity in different functional assays ranged from 5 to 73% of the wild-type protein (8, 9, 12, 14). Functional deficiency of p16INK4a mutant proteins has also been shown after the gene or protein was introduced into a number of nonmelanoma and melanoma cell lines (15, 16). However, these studies were also limited because the cell cycle-inhibitory effect of functional p16INK4a selects against cells expressing the transgene and thereby affects long-term studies. This growth-inhibitory effect can be overcome by use of an inducible expression system, and Stone et al. (17)

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3 The abbreviations used are: CDK, cyclin-dependent kinase; NFκB, nuclear factor κB; pRb, retinoblastoma protein; GST, glutathione S-transferase; IFTG, isopropyl-1-thio-β-D-galactopyranoside; SA, senescence-associated; CTD, COOH-terminal domain.
reported a detailed study of the effects of induced wild-type p16\(^{INK4a}\) expression in melanoma cells. Consequently, we chose to thoroughly assess the function of melanoma-associated mutant p16\(^{INK4a}\) proteins by stably introducing inductible constructs of the wild-type or mutant CDKN2A genes into melanoma cells lacking endogenous p16\(^{INK4a}\). The functional loss of all tested melanoma-associated p16\(^{INK4a}\) mutations was much greater than CDK4 binding studies suggested. We postulate that these mutants are defective in interaction with cellular targets additional to CDKs.

**MATERIALS AND METHODS**

**CDKN2A cDNA and Mutagenesis.** The originally cloned CDKN2A cDNA (5) was extended to full length (18) by PCR. Overlapping PCR, modified from Aiyar and Leis (19), was used to engineer four p16\(^{INK4a}\) mutations. The Arg24Pro (G071C) and Ala36Pro (G106C) variants were originally identified in our laboratory (20, 21), and the Arg24Pro mutation has now been reported in nine melanoma-prone families world-wide (11, 13, 20–25). The Met53Ile (G159C) variant was also originally detected in an Australian melanoma kindred (26), and melanoma association has now been reported in 12 families worldwide (11, 13, 21, 22, 24, 26–28). The Val126Asp (T377A) variant was one of the first CDKN2A mutations identified; its function has been examined with different CDK binding and kinase assays and after introduction into cell lines. It has been associated with melanoma in at least three families worldwide (24, 29, 30).

**Protein Expression in Bacteria.** CDKN2A cDNA was cloned into the pGEX-5X-I vector (Amersham Pharmacia), and the insert and adjacent vector sequences were completely sequenced. Apart from the inserted point mutations, all CDKN2A constructs had identical sequences. GST-fusion p16\(^{INK4a}\) (GST-p16\(^{INK4a}\)) was expressed in Escherichia coli (JM109), purified using glutathione-Sepharose beads (as described by the supplier) and stored in glycerol lysis buffer (25% glycerol, 100 mM NaCl, 1% Triton, 1 mM DTT, 1 mM EDTA, 50 mM Tris [pH 8]).

**CDK4 cDNA and Mutagenesis.** The originally cloned CDK4 cDNA insert was cloned into the pQE31 vector (Qiagen) and completely sequenced. Apart from the inserted point mutations, all CDKN2A sequences were completely sequenced. The insert and adjacent vector sequences were completely sequenced. GST-fusion p16\(^{INK4a}\) proteins bound comparable amounts of CDK4 glutathione-Sepharose. Both the wild-type and mutant GST-fusion p16\(^{INK4a}\) proteins copurified in this assay only when p16\(^{INK4a}\) was immobilized to glutathione-Sepharose. Both the wild-type and mutant GST-tagged p16\(^{INK4a}\) proteins bound comparable amounts of CDK4 when the proteins were allowed to interact at 20°C (Fig. 1). Because CDK4 binding to several p16\(^{INK4a}\) variants, including the Val126Asp mutation, has been reported to be temperature sensitive (9, 35), the binding assay was repeated at 37°C. Increasing the incubation temperature to 37°C led to decreased CDK4 binding by two p16\(^{INK4a}\) mutants [Met53Ile (35–45% of wild type) and Val126Asp (20–25% of wild type)], whereas CDK4 binding of the two remaining mutant proteins (Arg24Pro and Ala36Pro) was similar to that observed with wild-type p16\(^{INK4a}\) (Fig. 1).

**RESULTS**

**CDK4-binding Activity of Wild-Type and Melanoma-associated Mutant p16\(^{INK4a}\) Proteins.** Wild-type and melanoma-associated mutant p16\(^{INK4a}\) fused to GST were purified and tested for their ability to bind His-tagged CDK4. The amount of His-CDK4 bound to GST-p16\(^{INK4a}\) proteins was determined by immunostaining. As shown in Fig. 1, His-CDK4 copurified in this assay only when p16\(^{INK4a}\) was immobilized to glutathione-Sepharose. Both the wild-type and mutant GST-tagged p16\(^{INK4a}\) proteins bound comparable amounts of CDK4 when the proteins were allowed to interact at 20°C (Fig. 1). Because CDK4 binding to several p16\(^{INK4a}\) variants, including the Val126Asp mutation, has been reported to be temperature sensitive (9, 35), the binding assay was repeated at 37°C. Increasing the incubation temperature to 37°C led to decreased CDK4 binding by two p16\(^{INK4a}\) mutants [Met53Ile (35–45% of wild type) and Val126Asp (20–25% of wild type)], whereas CDK4 binding of the two remaining mutant proteins (Arg24Pro and Ala36Pro) was similar to that observed with wild-type p16\(^{INK4a}\) (Fig. 1).

**p16\(^{INK4a}\) Expression in WMM1175 Melanoma Cells.** To determine the cellular function of melanoma-associated p16\(^{INK4a}\) mutants, wild-type and mutant CDKN2A genes were stably introduced separately into the p16\(^{INK4a}\)-null WMM1175 melanoma cell line. The use of an inductible mammalian expression system for controlled p16\(^{INK4a}\) expression allowed us to avoid growth arrest of the transfectants during the selection process. Selected CDKN2A-transfected WMM1175 clones expressed the protein on induction with IPTG. Titration experi-
ments showed no detectable p16\(^{\text{INK4a}}\) expression below 10 \(\mu M\) and maximal expression at 4 \(\mu M\) IPTG (data not shown).

**Effects of Wild-Type p16\(^{\text{INK4a}}\) Expression in Melanoma Cells.** The expression of wild-type p16\(^{\text{INK4a}}\) produced major changes in the transfected WMM1175 cells. Cells were growth-arrested within 24 h of transgene induction, and this was accompanied by a strong decrease of cell numbers in S phase and a clear G1-phase block. The S-phase reduction and G1 block persisted through all subsequent time points (48, 72, and 96 h after p16\(^{\text{INK4a}}\) induction) as long as IPTG remained present. After 48 h, a decrease in the number of G2-phase cells was also apparent as cells had traversed through G2 and accumulated in G1 phase. This shows that p16 INK4a induces potent G1 arrest but does not affect the G2-M transition in WMM1175 under these conditions (Fig. 2).

Clonogenicity was abolished completely in WMM1175 cells with induced wild-type p16\(^{\text{INK4a}}\) expression when compared with the parent cell line or selected p16\(^{\text{INK4a}}\)-negative clones (Table 1). Withdrawal of IPTG from p16\(^{\text{INK4a}}\)-grown, growth-arrested cells led to recovery, and cells reentered the cell cycle within 24–48 h (data not shown). p16\(^{\text{INK4a}}\)-induced growth arrest was unchanged, and cells did not lose viability when IPTG induction was maintained in the medium for up to 3 weeks. The morphology of wild-type p16\(^{\text{INK4a}}\)-inducible cells started to change after 24 h of p16\(^{\text{INK4a}}\) expression: cells initially became more dendritic, gradually enlarged, and acquired a flattened granular phenotype, suggesting that these cells might have entered senescence. However, histochemical analysis for the presence of SA-\(\beta\)-galactosidase, a marker for cellular senescence, was negative. In contrast, senescent human fibroblasts stained strongly positive in this assay (Fig. 3).

**Effects of Mutant p16\(^{\text{INK4a}}\) Expression in Melanoma Cells.** Expression screening of WMM1175 clones transfected with mutant \(\text{CDKN2A}\) cDNA for p16\(^{\text{INK4a}}\) revealed fewer
Table 1  Effects of wild-type or mutant p16\(^{INK4a}\) in WMM1175 melanoma cells and in CDK4 binding assays.

<table>
<thead>
<tr>
<th>clone</th>
<th>cell cycle</th>
<th>p16(^{INK4a})</th>
<th>growth rate</th>
<th>colony forming ability</th>
<th>binding assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16(^{**})A(_4)</td>
<td>complete</td>
<td>complete</td>
<td>0%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>p16(^{**})A(_7)</td>
<td>complete</td>
<td>complete</td>
<td>96%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>p16(^{**})B(_7)</td>
<td>complete</td>
<td>complete</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Arg24Pro(^{**})A(_4)</td>
<td>some</td>
<td>some</td>
<td>90%</td>
<td>35-45%</td>
<td>35-45%</td>
</tr>
<tr>
<td>Arg24Pro(^{**})A(_7)</td>
<td>minor</td>
<td>minor</td>
<td>90%</td>
<td>35-45%</td>
<td>35-45%</td>
</tr>
<tr>
<td>Ala36Pro(^{**})A(_4)</td>
<td>some</td>
<td>some</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Met53Ile(^{**})A(_4)</td>
<td>minor</td>
<td>minor</td>
<td>90%</td>
<td>20-25%</td>
<td>20-25%</td>
</tr>
<tr>
<td>Met53Ile(^{**})A(_7)</td>
<td>minor</td>
<td>minor</td>
<td>90%</td>
<td>20-25%</td>
<td>20-25%</td>
</tr>
<tr>
<td>Val126Asp(^{**})A(_4)</td>
<td>no effect</td>
<td>no effect</td>
<td>100%</td>
<td>20-25%</td>
<td>20-25%</td>
</tr>
</tbody>
</table>

In three clones, wild-type p16\(^{INK4a}\) expression led to complete growth arrest, accumulation of cells in G\(_1\), and strong S-phase decrease. Some growth reduction was also observed for the high p16\(^{INK4a}\)-expressing Arg24Pro\(^{**}\)A\(_4\) and the medium-expressing Ala36Pro\(^{**}\)A\(_7\) clone. The medium-expressing Arg24Pro\(^{**}\)A\(_4\) clone and two medium-expressing Met53Ile clones showed minor but reproducible growth inhibition. Only the Val126Asp mutation showed no effect on cell growth. The growth rate changes correlate closely with the cell cycle distribution changes. Wild-type p16\(^{INK4a}\) induction completely abolished colony formation, whereas expression of the mutant proteins had no effect; only Met53Ile expression produced a reduction of colony formation by 10%. For the clonogenic assay, the data for the corresponding clones used in the study have been combined, and colonies formed on 4 mM IPTG induction are presented as a percentage of the colonies formed by the same clones without IPTG treatment. The parent cell line and cells that had been transfected and genetin selected but were not p16\(^{INK4a}\) inducible produced 102% and 104% colonies, respectively, when used as controls for this assay.

Clones expressing p16\(^{INK4a}\) protein; in addition, the levels of expression were usually lower than in clones transfected with wild-type p16\(^{INK4a}\). For additional experiments, clones expressing the highest mutant p16\(^{INK4a}\) levels were selected; only the clone Arg24Pro\(^{**}\)A\(_4\) reached expression levels close to the levels of wild-type p16\(^{INK4a}\) in p16\(^{**}\) clones (Fig. 4).

In contrast to the induced wild-type p16\(^{INK4a}\) expression, none of the mutant proteins abolished clonogenicity and only the Met53Ile mutation led to a minor reduction (10%) in colony-forming ability. Furthermore, none of the mutant proteins induced the changes in cell morphology that were characteristic for wild-type p16\(^{INK4a}\) expression. When more detailed measurements for cell proliferation were used, the four mutant p16\(^{INK4a}\) proteins differed in their ability to induce cell cycle inhibition in the WMM1175 melanoma cell line. Induced expression of three p16\(^{INK4a}\) mutations (Arg24Pro, Ala36Pro, and Met53Ile) had a reproducibly weak inhibitory effect on the growth rate over 7 days when compared with wild-type p16\(^{INK4a}\) (Fig. 2 and summary in Table 1). These effects correlate closely with the consistently weaker G\(_1\) cell cycle block induced by the mutants when compared with the G\(_1\) arrest observed when the wild-type p16\(^{INK4a}\) protein was induced (Fig. 2). The Val126Asp mutant showed more severe functional loss, failed to inhibit the growth of the WMM1175 cell line up to 7 days postinduction, and did not alter cell cycle distribution (Fig. 2 and Table 1).

DISCUSSION

We initially tested the ability of wild-type and mutant p16\(^{INK4a}\)-GST fusion proteins to bind His-CDK4. The data obtained for CDK4 binding correlated in part with the results reported by Parry and Peters (9). Specifically, the CDK4-binding ability of the Val126Asp mutant decreased with increasing temperature. As expected from previous reports, the Met53Ile mutant, which is associated with melanoma in 12 families worldwide, had reduced capacity to bind CDK4 when compared with the wild-type protein (11, 13). In contrast, we found that the Arg24Pro mutant behaved normally in our CDK4 binding assay, despite cosegregation of this mutation with disease in nine melanoma-prone families. This contrasts with other studies that demonstrated reduced CDK-binding affinity for the Arg24Pro mutation (11, 13). It is possible that differences in the degree of functional loss are attributable to the type of fusion proteins used or the assay conditions applied, such as pH, temperature, or ionic strength. All of these parameters can influence protein conformation, stability, and surface charge and could thereby alter the degree of protein interactions. The Ala36Pro mutation, which has not been functionally tested previously, also showed wild-type CDK4 binding.

To compare the p16\(^{INK4a}\)-CDK4 binding results with the effects of expressing wild-type and mutant p16\(^{INK4a}\) variants in cells of melanocytic origin, we introduced the different CDKN2A constructs into p16-null melanoma cells. We applied an inducible mammalian expression system, which enabled us to obtain stable cell clones expressing either wild-type or mutant p16\(^{INK4a}\). In this model system, p16\(^{INK4a}\) can interact directly or indirectly with a range of cellular targets such as CDK4, CDK6, CDK7, and NFκB. Furthermore, any decreased stability or expression of the mutant RNA or the protein will affect its function and can be directly assayed. This is an important advantage and overcomes the limitation of CDK-binding assays in which protein concentrations are usually equalized, possibly masking the effect of low physiological concentrations of a mutant gene product.

Expression of wild-type p16\(^{INK4a}\) led to growth arrest within 24 h, which was shown to be attributable to G\(_1\)-phase
arrest. The growth arrest was maintained as long as IPTG was present and could be reversed by withdrawal of the inducer. These data confirm the findings by Stone et al. (17). Additionally we showed that wild-type p16 INK4a expression completely abolished colony-forming ability and that the senescence marker SA-β-galactosidase is not detectable despite morphological changes characteristic of senescence. Although several reports have shown that p16INK4a is involved in inducing or maintaining the state of cellular senescence (36), these data suggest that the up-regulation of SA-β-galactosidase requires other pathways. These pathways may be disrupted in the WMM1175 melanoma cell line, and this possibility is under investigation at present.

Screening of the WMM1175 cell clones transfected with mutant CDKN2A revealed fewer clones accumulating the p16 INK4a protein, and expression levels were generally lower. Because the constructs differed only by single point mutations, these results suggest that these melanoma-associated mutants show decreased RNA or protein stability. Ectopic expression of mutant p16INK4a protein in melanoma cells has previously been reported to be lower than wild type (37). It is possible that this lower expression of mutant p16INK4a is an important determinant of function. There are potentially important implications of this for understanding possible effects of the heterozygous state on melanocytes of mutation carriers. For example, the decreased concentration of mutant protein in cells expressing both wild-type and mutant p16INK4a may help minimize any potential dominant-negative effect from the mutant protein as long the wild-type allele remains intact. Such a negative effect could occur if the mutant protein binds to wild-type protein and prevents its normal function, as has been described for some p53 mutants (38). It would be important to determine whether such differences in protein concentrations are physiologically relevant in normal melanocytes.

In contrast to our CDK4 binding studies, the effects of mutant p16INK4a expression on melanoma cell growth were consistently different to those seen with the wild-type protein. None of the mutant p16INK4a proteins suppressed clonogenicity, apart from Met53Ile, which induced a small (10%) reduction. None of the striking changes in cell morphology observed with wild-type p16INK4a expression were seen in clones transfected with the mutant proteins (Table 1). In two previous studies, clonogenic assays were the main measurements for cell proliferation after p16INK4a expression. These studies had either introduced CDKN2A virally into fibroblasts with infection efficiencies of 50–80% (35) or had transfected plasmids
constitutively expressing p16\textsuperscript{INK4a} into melanoma cells, followed by a 14-day antibiotic selection period (despite the growth-inhibitory effect of p16\textsuperscript{INK4a}; Ref. 37). The fact that, under those conditions, not all cells would have expressed the transgene may explain why the data in these studies did not show the dramatic difference between wild-type and mutant p16\textsuperscript{INK4a} expression demonstrated here with stable p16\textsuperscript{INK4a}-inducible cell lines. In contrast to our results, the authors of these studies (35, 37) did not find complete inhibition of colony-forming ability by wild-type p16\textsuperscript{INK4a}, and expression of mutant protein led to more variable results in the clonogenic assays.

Our use of inducible clones also permitted a more detailed examination of cell proliferation. Growth experiments over 7 days and examination of changes of cell cycle distribution at various time points after p16\textsuperscript{INK4a} induction showed that three mutations (Arg24Pro, Ala36Pro, and Met53lle) did retain small cell cycle-inhibitory function and that the Val126Asp mutation did not inhibit cell growth in any of the assays.

A comparison of our biochemical and cellular data revealed that the Ala36Pro and Arg24Pro mutations functioned as wild-type protein in the CDK4 binding assay but were functionally severely impaired in suppressing colony formation and induced only small growth reduction of WMM1175 cells. The Met53lle mutation had weak CDK4 binding affinity and showed insignificant effects on the growth rate and cell cycle of WMM1175 cells, although it was the only mutation inhibiting colony formation to a small degree. CDK4 binding of the Val126Asp mutation was reduced to only 20–25%, and the functional loss in melanoma cells was even more severe because it showed no impact on colony-forming ability, growth rate, or cell cycle distribution. The Val126Asp mutation has shown to have a disrupted secondary structure and the tendency to produce aggregates in vitro (39). Walker et al. (37) observed a “speckled appearance” of this mutation after immunostaining, suggesting that aggregation of p16\textsuperscript{INK4a} mutant proteins may be occurring intracellularly. This could help to explain the lack of activity of the Val126Asp protein in these melanoma cells.

Overall, these data indicate that testing of p16\textsuperscript{INK4a} mutations with CDK binding and kinase assays is an unreliable and inconsistent guide to the functional loss of these proteins. These assays are incapable of reflecting the effect of the mutations on cellular expression levels and on cellular half-life; in addition, being based on binding to a limited number of known target molecules, such as CDK4 or CDK6, these assays fail to measure the combined effects of altered gene products on a variety of potential protein targets. The ability of p16\textsuperscript{INK4a} mutations to differentially affect growth rate, cell cycle progression, and clonogenicity supports the concept that different mutations interfere with distinct functions of p16\textsuperscript{INK4a}.

A number of alternative target proteins for p16\textsuperscript{INK4a} mutations have been identified in recent years. p16\textsuperscript{INK4a} was shown to affect transcription by inhibition of phosphorylation of the CTD of TFIH by CDK7 (40). Recently this CDK7 inhibition has suggested to be linked to cell cycle regulation (41). p16\textsuperscript{INK4a} has also been shown to directly bind and inhibit the transcription factor NFκB (6). NFκB regulates the expression of a number of genes essential for cell cycle control, such as p53 and cyclin D1 (42, 43). The p16\textsuperscript{INK4a} interaction with specific target proteins may be influenced differently by various p16\textsuperscript{INK4a} mutations.

Indeed, it has been shown that NH\textsubscript{2}-terminal p16\textsuperscript{INK4a} mutations, such as Arg24Pro, are impaired in their inhibition of TFIH CTD phosphorylation by CDK7, whereas the Gly101Trp mutation located in the central region of p16\textsuperscript{INK4a} has no effect on this particular function (41). Interestingly, the Arg24Pro and Ala36Pro mutant proteins, which showed normal CDK4 binding in our assay but were functionally impaired in melanoma cells, carry mutations in the domain identified to be important for inhibition of TFIH CTD phosphorylation by CDK7. Therefore, the examined p16\textsuperscript{INK4a} mutations may be impaired for specific functions of p16\textsuperscript{INK4a}, such as inhibition of the pRB pathway via CDK4 and/or the inhibition of TFIH via CDK7. Furthermore, the mutation that reduced clonogenicity to a small degree, Met53lle, may still inhibit TFIH CTD phosphorylation by CDK7, which might specifically affect clonogenic survival. The presence of multiple targets for p16\textsuperscript{INK4a} may explain why cellular based assays give more reliable data about a possible cancer risk associated with specific p16\textsuperscript{INK4a} mutations than any protein binding test on its own.

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Functional Testing of p16 INK4a Mutations


Clinical Cancer Research

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