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Advances in Brief

No *CDKN2* Mutations in Neuroblastomas[1]

Christian P. Beltinger, Peter S. White, Erik P. Sulman, John M. Maris, and Garrett M. Brodeur[2]

Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104-9786

Abstract

Mutations of *CDKN2* have been found recently in melanoma and many other tumor types. Neuroblastoma shares with melanoma a neuroectodermal origin and a high incidence of deletions of the short arm of chromosome 1. Therefore, we analyzed 18 primary neuroblastomas and 9 tumor-derived cell lines for mutations in *CDKN2*. We used PCR-single-strand conformation polymorphism to examine exons 1 and 2 of the *CDKN2* gene for mutations, but none were detected. Furthermore, no homozygous deletions were detected and there was no loss of heterozygosity at the closely linked *IFNA* locus. We conclude that disruption of the *CDKN2* gene is not required for malignant transformation of human neuroblastomas.

Introduction

The *CDKN2* (*MTS1*) gene encodes p16, a protein which inhibits the CDK4[3]/cyclin D complex. Inhibition by p16 decreases the phosphorylation state of the retinoblastoma gene product (pRb), and this cell cycle checkpoint limits the G_1-S transition and ultimately inhibits cell growth (1–3).

The *CDKN2* gene maps to 9p21, a site of frequent deletion in melanoma and other tumor types. Interestingly, mutations and homozygous deletions of *CDKN2* were found initially in cell lines from many different tumor types (4, 5). However, the overall incidence of mutations or homozygous deletions is generally less in primary tumors compared to cell lines (6). In primary tumors, homozygous deletions and intragenic mutations are frequently seen in pancreatic adenocarcinoma, glioblastoma, anaplastic astrocytoma (but not low-grade astrocytoma), esophageal squamous cell carcinoma, non-small cell lung cancer, bladder carcinoma, acute lymphocytic leukemia, and non-Hodgkin's lymphoma (4, 7–12). Intragenic alterations of *CDKN2* have been detected rarely, if at all, in primary breast cancer (12), primary renal cancer (11), and primary sporadic melanoma (13), although cell lines derived from these tumors often contain homozygous deletions (5). Although cell lines of osteosarcoma, ovarian tumor, and mesothelioma frequently show homozygous deletions of *CDKN2*, no data concerning primary tumors have been published yet for these cancers (4).

Homozygous deletions of *CDKN2* occur in 60% of melanoma cell lines (4, 5), and disease-specific germ line mutations are found in many 9p21-linked melanoma families (14, 15). Melanomas frequently contain deletions of the short arm of chromosome 1 (16), a region that also shows genetic linkage in several familial melanomas (15). Interestingly, deletion of the short arm of chromosome 1 is the most common genetic abnormality in human neuroblastomas (17, 18). Furthermore, melanocytes and neuroblasts are both derived from the neural crest. Because of these similarities, as well as the frequent involvement of *CDKN2* mutations in many other cancers, we examined the *CDKN2* gene in a series of neuroblastoma cell lines and primary tumors. However, no homozygous deletions or mutations were detected in the coding region, and no LOH was found at the adjacent *IFNA* locus, suggesting that CDKN2 is not involved in the pathogenesis of neuroblastomas.

Materials and Methods

Tumors and Cell Lines. Nine neuroblastoma cell lines were analyzed, six of which had amplification of the *MYCN* oncogene as well as 1p deletions or rearrangements (NGP, NLF, NAB, SMS-KAN, SMS-KCN, and LA-N-5). Three cell lines had neither *MYCN* amplification nor abnormalities of 1p (NBL-S, SK-NSH, and LHN). Eighteen primary tumor samples were selected to represent the distribution of stage and *MYCN* copy number typically found in neuroblastoma. One of six selected stage I–II tumors and 4 of 10 selected stage III–IV tumors had *MYCN* amplification. Two tumors were stage IV-S and not amplified. For LOH studies, constitutional DNA corresponding to the neuroblastoma samples was extracted and processed as described previously (17). DNA samples from unrelated probands of 31 Centre d'étude du polymorphisme humain pedigrees were provided by H. Donis-Keller. Constitutional DNA samples of eight patients with germ line *CDKN2* substitutions (one substitution in exon 1 and seven substitutions in exon 2) were a gift from N. Dracopoli (15).

PCR-SSCP and DNA Sequencing. PCR of *CDKN2* was performed as described previously (15). One primer pair covered exon 1. Three primer pairs spanning overlapping regions designated as A, B, and C covered exon 2. SSCP was performed as described (15), with the following modifications. Samples were electrophoresed at 25°C on a 4.5% polyacrylamide gel in 1X TBE (90 mM Tris-borate, 2 mM EDTA) at 25 W for 4 h. Duplicates were run at 25°C on a 0.5X MDE gel (AT Biochem, Malvern, PA) with 5% glycerol in 0.6X TBE at 6 W for 12 h. Variant bands were cut from the gel and used directly as a template for a second PCR reaction under the same conditions as described above. The PCR product was then sequenced in both directions with the same primers using methods described previously (19).

LOH Analysis. A microsatellite repeat within *IFNA*, which also maps to 9p21 and is adjacent to *CDKN2*, was PCR amplified from 17 paired normal and tumor DNA samples using primers derived from the Genome Data Base. One primer was end labeled with γ-^{32}P using T4 polynucleotide kinase. PCR was performed in 20-μl volumes containing 2 μl 10X PCR buffer II (Perkin Elmer/Cetus, Branchburg, NJ), 0.4 mM each primer, 0.1 mM each dNTP, 0.2 units AmpliTaq DNA polymerase (Perkin Elmer/Cetus), 1.5 mM $MgCl_2$, and 20 ng DNA. Reactions were amplified for one cycle at 95°C (3 min); for 16 cycles at 95°C (45 s) with the annealing/extension temperature starting at 70°C and decreasing by 0.7°C each cycle (1 min); 25 cycles at 95°C (45 s), 55°C (30 s), and 72°C (1 min); and for one cycle at 72°C (5 min). LOH analysis was performed as described (16).

Results

To first confirm the sensitivity of our PCR-SSCP method, we examined exons 1 and 2 of *CDKN2* in the constitutional DNA of eight melanoma patients with known germ line *CDKN2* substitutions. In each case, a variant band was clearly detectable by PCR-SSCP. Fig. 1 shows the three germ line samples with substitutions in exon 2 (region C) used as controls (*Lanes 6–9*). While not all mutations were seen on the polyacrylamide gels without glycerol, glycerol-supplemented MDE gels detected all substitutions.

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[2] To whom requests for reprints should be addressed, at Division of Oncology, Room 9000, Children's Hospital of Philadelphia, 324 South 34th Street, Philadelphia, PA 19104-9786.

[3] The abbreviations used are: CDK4, cyclin-dependent kinase 4; LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism.

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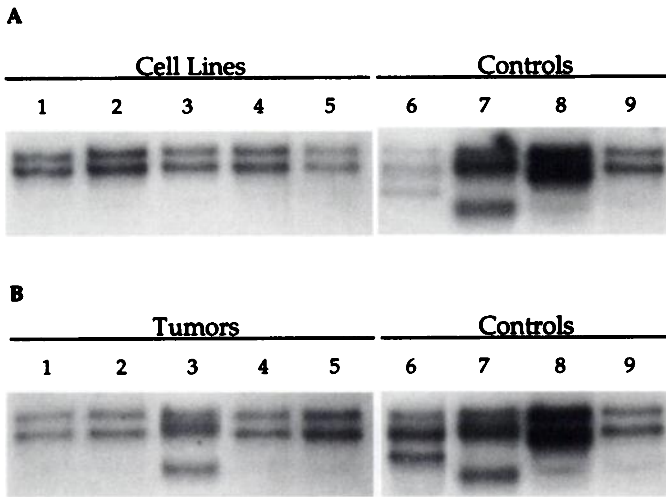

Fig. 1. PCR-SSCP analysis of *CDKN2* exon 2 region C in neuroblastomas. Radiolabeled primers were used to amplify region C of exon 2 by PCR. The amplified product was denatured and run on a 0.5X MDE gel containing 5% glycerol in 0.6X TBE at 25°C and detected by autoradiography. *A*, five representative neuroblastoma cell lines (*Lanes 1–5*): three different known germ line substitutions of melanoma patients serving as positive controls (*Lanes 6–8*) and genomic DNA of a healthy person serving as a negative control (*Lane 9*). No variant bands are seen in the neuroblastoma cell lines while all germ line substitutions of the controls are detected. *B*, five representative neuroblastoma tumors (*Lanes 1–5*), positive controls (*Lanes 6–8*), and negative control (*Lane 9*). *Lanes 3* and *6* have been substituted with longer exposures to normalize for unequal signal intensity. Only one tumor shows an abnormal SSCP pattern (*Lane 3*), subsequently shown to be a polymorphism, while all of the positive controls are detected.

Having confirmed the sensitivity of our PCR-SSCP method, we then examined the *CDKN2* coding region of nine neuroblastoma cell lines by PCR-SSCP. PCR amplification produced specific bands in all cell lines, indicating the absence of homozygous deletions. No aberrant bands were detected by SSCP, indicating the absence of mutations in the coding region. Fig. 1*A* depicts five representative samples (*Lanes 1–5*).

Subsequently, we investigated 18 primary tumor samples for the presence of *CDKN2* mutations. Specific bands were amplified in all tumors, again suggesting the absence of homozygous deletions. Only one tumor showed aberrant bands by SSCP, in region 2C of exon 2 (Fig. 1*B*, *Lane 3*). This variant appeared to be a polymorphism, because the SSCP pattern was identical to a genomic sample containing a known polymorphism in exon 2C (Fig. 1*B*, *Lane 7*). Furthermore, identical variant bands were also present in constitutional DNA from this patient as well as in 3 of 31 unrelated normal control DNAs (data not shown). This suspected polymorphism was confirmed by sequencing the variant bands of both tumor and constitutional DNA, both of which showed a G to A transversion at nucleotide position 436 in codon 140 as the only nucleotide change (data not shown). This transversion causes an alanine to threonine change and is a previously described, rare polymorphism (14, 15). The reported frequency of this polymorphism is similar to that which we observed in 64 chromosomes (heterozygote frequency of 6%).

To determine whether allelic loss occurs at 9p21 in neuroblastomas, we looked for loss of heterozygosity using a PCR-based polymorphism at the *IFNA* locus, which is closely linked to *CDKN2* (5). Nine of 16 examined tumors were informative for this microsatellite repeat at *IFNA*. However, no tumor showed evidence of LOH (data not shown).

Discussion

We did not detect homozygous deletion of *CDKN2* in any of the neuroblastoma cell lines, confirming preliminary data of Kamb *et al.* (5). Furthermore, we found no homozygous deletion of *CDKN2* in primary tumors, regardless of disease stage or the *MYCN* amplification status. The incidence of homozygous deletion in primary tumor samples might be underestimated by PCR because amplification of wild-type *CDKN2* from contaminating normal cells could occur within the tumors. However, amplification of a contaminating wild-type allele would not be expected in the clonal, homogeneous cell lines. The incidence of homozygous deletions for *CDKN2* is usually higher in cell lines compared to tumors, and the reverse has not been described. Thus, the absence of homozygous deletion in neuroblastoma cell lines makes it highly unlikely that the failure to detect deletions in primary tumors was an artifact of the PCR technique. This is supported by the absence of allelic loss at 9p21 in the vicinity of *CDKN2*, as determined by the absence of LOH for *IFNA*. Therefore, we conclude that *CDKN2* is not homozygously deleted in neuroblastoma.

We did not find mutations in the coding region of *CDKN2* in either neuroblastoma cell lines or primary tumors. Because the primers used span the intron-exon boundaries of exons 1 and 2, splice junction mutations should have been detected. In general, the sensitivity of SSCP analysis for intragenic mutations is less than 100%, and our SSCP assay was not designed to detect mutations in promoter regions and within introns, which might impair the expression of *CDKN2*. Furthermore, exon 3 of *CDKN2* was not examined, because no mutations have been described previously in the few base pairs of exon 3 that contribute to the coding region of *CDKN2*. Therefore, it is remotely possible that undetected mutations exist. However, the detection rate of 100% for eight known germ line *CDKN2* substitutions suggests a high sensitivity of the SSCP technique used. In addition, it also detected the most common reported *CDKN2* polymorphism in the tumor samples, with the expected frequency. Taken together, we conclude that the likelihood of undetected mutations is low and that the coding region of *CDKN2* is not commonly mutated in neuroblastoma.

Several studies have shown an inverse relationship between p16 and pRb expression (20–22), supporting the evidence of a negative feedback loop between pRb and p16 (20). Furthermore, these findings suggest that altering the activity of only one of the molecules involved in the control of the G_1-S transition, such as p16, CDK4, cyclin D, or pRb, might be sufficient to contribute to a malignant phenotype. This view is further supported by evidence that CDK4 amplification with overexpression is found in 20% of glioblastomas not containing homozygous *CDKN2* deletions, thus increasing the rate of glioblastomas with aberrations in either gene to 75% (8). Since *RB1* expression is readily detectable in most neuroblastomas,[4] homozygous deletion of *RB1* or point mutations leading to abrogation of *RB1* gene expression must be rare in neuroblastoma. Since we have not investigated the other components that regulate G_1-S transition, it is conceivable that alterations of CDK4, cyclin D, or p21 exist in neuroblastomas, which could lead to the same phenotype as mutations of *CDKN2*. Alternatively, alterations in the CDK4-mediated G_1-S checkpoint might not be important in neuroblastoma. In any case, disruption of the *CDKN2* gene apparently is not required for malignant transformation of neuroblastoma.

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[4] H. Shimada, personal communication.

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References

1. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific-inhibition of cyclin-d/cdk4. Nature (Lond.), *366:* 704–707, 1993.
2. Hunt, T., and Sherr, C. J. Cell multiplication: editorial overview checks and balances. Curr. Opin. Cell Biol., *6:* 833–835, 1994.
3. Serrano, M., Gómez-Lahoz, E., DePinho, R. A., Beach, D., and Bar-Sagi, D. Inhibition of ras-induced proliferation and cellular transformation by $p16^{INK4}$. Nature (Lond.), *267:* 249–252, 1995.
4. Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature (Lond.), *368:* 753–756, 1994.
5. Kamb, A., Gruis, N. A., Weaverfeldhaus, J., Liu, Q. Y., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., Johnson, B. E., and Skolnick, M. H. A cell-cycle regulator potentially involved in genesis of many tumor types. Science (Washington DC), *264:* 436–440, 1994.
6. Spruck, C. H., III, Gonzalez-Zulueta, M., Shibata, A., Simoneau, A. R., Lin, M. F., Gonzales, F., Tsai, Y. C., and Jones, P. A. *p16* gene in uncultured tumours. Nature (Lond.), *370:* 183–184, 1994.
7. Caldas, C., Hahn, S., daCosta, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16* (*MTS1*) gene in pancreatic adenocarcinoma. Nat. Genet., *8:* 27–32, 1994.
8. Schmidt, E. E., Ichimura, K., Reifenberger, G., and Collins, V. P. *CDKN2* (*p16/MTS1*) gene deletion or *CDK4* amplification occurs in the majority of glioblastomas. Cancer Res., *54:* 6321–6324, 1994.
9. Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S., and Nakamura, Y. Frequent somatic mutation of the *MTS1/CDK4I* (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous-cell carcinoma. Cancer Res., *54:* 3396–3397, 1994.
10. Stranks, G., Height, S. E., Mitchell, P., Jadayel, D., Yuille, M. A. R., DeLord, C., Clutterbuck, R. D., Treleaven, J. G., Powles, R. L., Nacheva, E., Oscier, D. G., Karpas, A., Lenoir, G. M., Smith, S. D., Millar, J. L., Catovsky, D., and Dyer, M. J. S. Deletions and rearrangements of CDKN2 in lymphoid malignancy. Blood, *85:* 893–901, 1995.
11. Cairns, P., Tokino, K., Eby, Y., and Sidransky, D. Localization of tumor suppressor loci on chromosome 9 in primary human renal cell carcinomas. Cancer Res., *55:* 224–227, 1995.
12. Xu, L., Sgroi, D., Sterner, C. J., Beauchamp, R. L., Pinney, D. M., Keel, S., Ueki, K., Rutter, J. L., Buckler, A. J., and Louis, D. N. Mutational analysis of *CDKN2* (*MTS1*/$p16^{ink4}$) in human breast carcinomas. Cancer Res., *54:* 5262–5264, 1994.
13. Ohta, M., Nagai, H., Shimizu, M., Rasio, D., Berd, D., Mastrangelo, M., Singh, A. D., Shields, J. A., Shields, C. L., Croce, C. M., and Huebner, K. Rarity of somatic and germline mutations of the cyclin-dependent kinase 4 inhibitor gene, *CDK4I*, in melanoma. Cancer Res., *54:* 5269–5272, 1994.
14. Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., McClure, M., Aitken, J. F., Anderson, D. E., Bergman, W., Frants, R., Goldgar, D. E., Green, A., MacLennan, R., Martin, N. G., Meyer, L. J., Youl, P., Zone, J. J., Skolnik, M. H., and Cannon-Albright, L. A. Analysis of the *p16* gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat. Genet., *8:* 22–26, 1994.
15. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A., Ally, D. S., Sheahan, M. D., Clark, W. H., Tucker, M. A., and Dracopoli, N. C. Germline p16 mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.
16. Dracopoli, N. C., Harnett, P., Bale, S. J., Stanger, B. Z., Tucker, M. A., Housman, D. E., and Kefford, R. F. Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. Proc. Natl. Acad. Sci. USA, *86:* 4614–4618, 1989.
17. White, P. S., Maris, J. M., Beltinger, C. P., Sulman, E. P., Marshall, H. N., Fujimori, M., Kaufman, B. A., Biegel, J. A., Allen, C., Hillard, C., Valentine, M. B., Look, A. T., Enomoto, H., Sakiyama, S., and Brodeur, G. M. A region of consistent deletion in neuroblastoma maps within 1p36.2.-3. Proc. Natl. Acad. Sci. USA, in press, 1995.
18. Fong, C. T., Dracopoli, N. C., White, P. S., Merrill, P. T., Griffith, R. C., Housman, D. E., and Brodeur, G. M. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: Correlation with N-*myc* amplification. Proc. Natl. Acad. Sci. USA, *86:* 3753–3757, 1989.
19. Nakagawara, A., Liu, X. G., Ikegaki, N., White, P. S., Yamashiro, D. J., Nycum, L. M., Biegel, J. A., and Brodeur, G. M. Cloning and chromosomal localization of the human TRK-B tyrosine kinase receptor gene (NTRK2). Genomics, *25:* 538–546, 1995.
20. Li, Y., Nichols, M. A., Shay, J. W., and Xiong, Y. Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene-product pRb. Cancer Res., *54:* 6078–6082, 1994.
21. Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H., and Harris, C. C. Mutations and altered expression of p16(ink4) in human cancer. Proc. Natl. Acad. Sci. USA, *91:* 11045–11049, 1994.
22. Otterson, G. A., Kratzke, R. A., Coxon, A., Kim, Y. W., and Kaye, F. J. Absence of p16(ink4) protein is restricted to the subset of lung-cancer lines that retains wild type RB. Oncogene, *9:* 3375–3378, 1994.

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