A Novel p16\textsuperscript{INK4A} Transcript\textsuperscript{1}

Li Mao, Adrian Merlo, Gauri Bedi, Geoffrey I. Shapiro, Christian D. Edwards, Barrett J. Rollins, and David Sidransky\textsuperscript{2}

Department of Otolaryngology—Head and Neck Surgery, Division of Head and Neck Cancer Research, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} were initially identified as potent inhibitors of activated cyclin/cyclin-dependent kinase complexes. These genes were colocalized to chromosome 9p21, and p16 was subsequently found to be mutated in familial melanoma and deleted in a wide variety of sporadic cancers. We recently found that de novo methylation of a 5' CpG island led to transcriptional block of full-length p16 in many neoplasms. However, the presence of a truncated p16 transcript in methylated cell lines led us to investigate the presence of an alternative promoter or initiation site. We have now identified an alternative truncated p16 transcript in both methylated and unmethylated cell lines generated from a novel sequence (exon 1\textbeta) potentially involved in the complex regulation of these critical cell cycle genes.

Introduction

The CDK\textsuperscript{2} inhibitors p16 (p16\textsuperscript{INK4A}/CDKN2/MTS-1; Ref. 1) and p15 (p15\textsuperscript{INK4B}/MTS-2; Ref. 2) are important components of cell cycle regulation. Transition through G\texttextsubscript{1} is promoted by activation of cyclin/CDK complexes that phosphorylate Rb, resulting in release of E2F and cell cycle progression (3). In addition to the more universal inhibitors (4), CDK4 and CDK6 are strongly inhibited by both p16 and p15. Isolation of the genes for these negative cell cycle regulators was quickly followed by their colocalization to chromosome 9p21 within a critical region commonly deleted in many types of human cancer (5, 6). Although suitable tumor suppressor gene candidates, somatic point mutations in these genes were found to be rare in many primary human tumors with hemizygous loss of 9p21 (7–11).

We recently identified de novo methylation of a CpG island that extends into exon 1 of p16 in cell lines and primary tumors. This methylation was precisely associated with transcriptional block of full-length p16 in cell lines (12), and reversal of methylation with 5-azacytidine resulted in reexpression of p16 message. However, methylated cell lines always expressed an abundant, shortened p16 transcript entirely devoid of exon 1 coding sequences. We sought to identify the transcription initiation site of this truncated product and found an alternative transcript derived from a novel upstream start site.

Materials and Methods

Cell Lines and Primary Tumors. Cell lines used in this study included those derived from primary head and neck squamous cell carcinomas at Johns Hopkins (003, 006, 011, 012, 020, 022, 029, and 030) or elsewhere (A439, A549, and UMSSC), HeLa, and a normal lymphoblastoid line L89. Primary tumors included 14 non-small cell lung cancers, 8 small cell lung cancers, 15 pancreatic adenocarcinomas, and 13 head and neck squamous cell carcinomas collected at the Johns Hopkins Hospital (diagnosis was confirmed by pathology). Tumors were microdissected to remove nonepithelial cells, and genomic DNA or total RNA was extracted as described previously (13, 14).

"Inverse" PCR. A specific antisense primer from the 3' untranslated region of p16 (5'-TCCCGAGGTTCCTCGAG-3') was used for reverse transcription of total RNA (5 \mu g each from tumor or normal lymphoblastoid cell lines) by using 200U Superscript II RNase H\textsuperscript{-}RT in the presence of dNTPs and double-stranded cDNA was then synthesized following the manufacturer’s protocol (GIBCO BRL, Gaithersburg, MD). Self-ligation of the blunt end cDNAs was performed in a 100-\mu l reaction volume containing 50U T4 DNA ligase (GIBCO BRL) as described previously (15). Five \mu l of each ligation product was used for PCR amplification as described (16) by using primers in exon 2 of p16: the sense 5'-CAUCACUACUAUGATGTCGCACGGA-AGATGCTC-3' and antisense 5'-CACCAAGAACCTGC-3'.

"Reversal" PCR. Two \mu g total RNA was subjected to reverse transcription with random hexamers, dNTPs, and 200 units Superscript II RNase H\textsuperscript{-}RT (GIBCO BRL) in a 20-\mu l reaction volume as above. PCR amplification was performed by using primers P1 (5'-AGTGCGGCTTGCCTAC-3') and P2 (5'-TCCCGAGGTTCCTCGAG-3') for the p16\textsuperscript{β} cDNA fragment. The product was run on a 1% agarose gel and visualized by ethidium bromide staining.

RNase Protection Assay. Forty hundred eighty-five bp of a p16 cDNA fragment (including exons 1, 2, and 3) was cloned into the pBSK vector (Strategene). An antisense p16 RNA probe containing [\textgamma\textsuperscript{32P}]UTP was then synthesized by using an in vitro transcription kit and isolated by gel electrophoresis (Ambion). One hundred \mu g of total RNA from each sample was coprecipitated with labeled probe and hybridized overnight at 45°C. After RNase treatment of hybridized products, samples were centrifuged at ethanol precipitation, separated on 5% acrylamide/8 M urea gel, and exposed to film.

Primer Extension Assay. An antisense primer of exon 1\beta (5'-GGGGTACCCAGAAGACTGCTC-3'; Fig. 1A) was end labeled with [\textgamma\textsuperscript{32P}]ATP and T4 DNA polynucleotide kinase (New England). One hundred \mu g total RNA was used for each reaction. After coprecipitation of the primer and RNA, samples were incubated in the hybridization buffer at 30°C overnight. Reverse transcription was performed at 42°C for 60 min with 200 units of Superscript II RNase H\textsuperscript{-}RT in the presence of dNTPs (GIBCO BRL). After treatment with RNase H, samples were concentrated by ethanol precipitation and separated on 6% acrylamide/8 M urea gel. A standard sequence of the p53 gene was used as a size marker after exposure to film.
Restriction Mapping. Exon 1 of p16 was labeled by random priming and used to probe the chromosome 9 cosmid library LL-9 constructed at the Biomedical Sciences Division, Lawrence Livermore National Laboratory, sponsored by the U.S. Department of Energy. The cosmids (217C4, 191G6, 190G8, 190D10, and 9C1) were cut with restriction enzymes EcoRI, PstI, and HindII, run on a 0.8% agarose gel, and transferred to nylon membrane. Exons 1 and 2 of both p16 and p15 and exon 1β were labeled by random priming and hybridized to the blots as described (12).

Sequence Analysis. Exon 1β of p16 from primary tumor or cell line DNA was amplified by PCR with primers 5'-TCCCATCTGAGATTAGG-3' and 5'-GCTTAAGTCTGTCGTTGTAACCCG-3' as described (16). Ten to 50 ng of amplified DNA was used for each sequence reaction. Sequencing primers 5'-AGTGCATCAGCACGAGGG-3' and 5'-AACATGGTGCGCAGGTTC-3' were labeled by [γ-32P]ATP at the 5' end and subjected to PCR amplification for 25 cycles using the AmpliCycle sequencing kit (Perkin-Elmer) according to manufacturer's protocol. Each amplified product (2.5 μl) was run on a 6% acrylamide/8 M urea gel and exposed to film.

TNT Assay. Two μg of total RNA was used for reverse transcription as described above in the presence of hexamers and dNTPs. PCR amplification was performed as described previously using primers TNT-P16 (9) and P2 for p16 cDNA and P4 (5'-GGATCCCTAATACGACTCACTATAGGAGACACCATGGGGCGTCTGCTACCTGTTGGT-3') and P2 for p16β cDNA in a 25 μl reaction volume. After phenol-chloroform extraction and ethanol precipitation, one-tenth of the product from each sample was subjected to a TNT in vitro translation and translation assay reaction in 10 μl volume by using a commercial TNT kit (Promega). Two μl of in vitro translated product was run on a 15% SDS-PAGE gel, enhanced with Amplify (Amersham), and exposed to film.

Immunoprecipitation. Two μl of in vitro-translated protein was incubated with either NH2-terminal or COOH-terminal polyclonal antibodies to p16 (Santa Cruz Biotechnology) in RIPA [10 mM Tris (pH 7.5), 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, and 0.1% SDS] at 4°C overnight and then shaken with Sepharose A beads for 60 min. After washing three times with RIPA buffer, the products were run on 15% SDS-PAGE gels and exposed to films.

Results and Discussion

To identify the transcriptional start site of p16, we used a specific antisense primer (from the 3' untranslated region) for reverse transcription of total RNA from a normal lymphoblastoid cell line. Following double-stranded cDNA synthesis, ligation was performed in large volume (15). The resulting circular product was amplified by PCR (so called "inverse" or "bubble" PCR) using primers in exon 2 of p16 oriented away from each other and then cloned into a plasmid vector. We then sequenced 10 individual clones completely; although we identified clones with the previously described exon 1 of p16, most clones (6 of 10) contained a novel 5' sequence spliced precisely onto (the first base of) exon 2 of p16. This novel 268-bp fragment exon 1β contained a theoretical open reading frame but was not in frame with the putative coding sequence of exons 2 and 3 of p16 (Fig. 1A). Thus, the originally described exon 1 of p16 was completely excluded from this alternative transcript. RNase protection with a full-length p16 probe demonstrated the presence of the originally described full-length p16 product (Fig. 2A). However, the smaller and more abundant product results from cleavage of the exon 1β sequence with protection of the shared p16 portion that contains exons 2 and 3. This predominant transcription initiation site was also confirmed by direct primer extension of total RNA (Fig. 2B). Reverse transcription-PCR of total RNA from eight tumor cell lines (without homozygous deletion of this region) confirmed the presence of the p16β transcript in all cases.

We proceeded with genomic localization of the exon 1β sequence by using a specific oligomer derived from exon 1β to probe a chromosome 9 cosmid library. One of the hybridizing cosmid clones contained both p16 and p15. EcoRI restriction mapping of this cosmid and other hybridizing clones yielded a small contig (~80 kb) of the region. Successive Southern blot hybridization with all exons of p16 and p15 (and exon 1β) to the cosmid contig was performed. Exon 1β was found to be on the same 8.5-kb fragment with exon 2 of p15, and two cosmid contained exon 1β but not exon 1 from p15 (Fig. 1B). Another group has identified the same exon 1β sequence and localized it downstream of exon 2 of p15 (17). A more complete EcoRI restriction map of this region was described previously (5).

One of the cosmids was then used to derive the complete surrounding genomic sequence of exon 1β (Fig. 1A), which is notable in several respects: (a) the region is CpG rich, and there is a GT-rich region upstream of exon 1β suggestive of a GT box promoter element; (b) a consensus Kozak element is contained at an AUG site (underlined) are shown. The highly polymorphic (CA)n repeat is shown on the last line. B, genomic organization of exon 1β. Coding exons for p15 and p16 are designated by black boxes. E1, exon 1; E2, exon 2; E3, exon 3. Exon 1β is located downstream of exon 2 of p15 approximately 10–20 kb upstream of exon 1 of p16 (17). A more complete EcoRI restriction map of this region was described previously (5).
A 400bp or 500bp of exon Iß (HH)bp from the primer) in both a HeLa cell line and normal lymphoblastoid (see text). B. primer extension assay indicates the expected initiation site for transcription as the probe. 006 and 0/2. head and neck squamous cell carcinoma cell lines in which 

C To see if this product was translated in frame from the third Met of pl6 is recognized by both antibodies. 

To see if this product was translated in frame from the third Met of pl6 (just inside exon 2 and a consensus Kozak sequence), we proceeded with immunoprecipitation using polyclonal anti-pl6 antibodies that recognize either the COOH terminus or the NH2-terminal antibody. As expected, the TNT pl16b product was recognized by the COOH-terminal but not the NH2-terminal antibody, whereas pl16 is recognized by the COOH-terminal and not the NH2-terminal antibody, whereas p16 is recognized by both antibodies.

Fig. 2. A, an RNase protection assay was performed with a 485-bp p16 cDNA fragment as the probe. 006 and 0/2, head and neck squamous cell carcinoma cell lines in which methylation of the 5′ CpG island of p16 is associated with absence of full-length p16 mRNA (485bp Ref. 12). The HeLa cell line contains full-length p16 mRNA, and all cell lines contain a more abundant 355-bp smaller fragment from the protected portion of p16b (see text). B, primer extension assay indicates the expected initiation site for transcription of exon Iß (100 bp from the primer) in both a HeLa cell line and normal lymphoblastoid cell line (L89). C, immunoprecipitation of in vitro-translated pl6 and pl6b with a COOH-terminal antibody to pl6 (C) or an antibody to only the NH2-terminal portion (N). pl6b is recognized by the COOH-terminal but not the NH2-terminal antibody, whereas pl16 is recognized by both antibodies.

To see if this product was translated in frame from the third Met of pl16 (just inside exon 2 and a consensus Kozak sequence), we proceeded with immunoprecipitation using polyclonal anti-p16 antibodies that recognize either the COOH terminus or the NH2 terminus. As expected, the TNT p16b product was recognized by the COOH-terminal but not the NH2-terminal antibody, providing strong evidence that this product lacked the NH2-terminal exon 1 coding sequence. Preliminary studies have not identified the p16b protein product in cell lines (data not shown).

The presence of other AUG sites and a long untranslated region in exon 1ß probably favors diminished translation of the p16b transcript (18). In fact, our TNT studies reveal much weaker translation efficacy for p16b in comparison to p16 (Fig. 2C). Other cyclin/CDK inhibitors are known to undergo extensive transcriptional regulation and, in the case of p27Kip1, unusual posttranscriptional regulation (3). Thus, this exon 1ß sequence probably represents an untranslated open reading frame that plays a role in the complex regulation of the p16 cell cycle inhibitor. The presence of an abundant alternative transcript for p16 may provide an explanation for certain puzzling issues regarding their role as tumor suppressor genes. The absence of p15 and p16 point mutations in many primary tumors may reflect this complex genomic organization and regulation in the 9p21 region, perhaps resulting in strong selection for alternative mechanisms of inactivation in human cancers.

Acknowledgments

We thank Dr. Scott Kern for providing us with pancreatic carcinoma samples.

References

15. Zeiner, M., and Gehrig, U. Cloning of 5′ cDNA regions by inverse PCR. Biotec- 


2997
A Novel p16INK4A Transcript

Li Mao, Adrian Merlo, Gauri Bedi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/14/2995

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.