Transcriptional Silencing of the p73 Gene in Acute Lymphoblastic Leukemia and Burkitt’s Lymphoma Is Associated with 5′ CpG Island Methylation

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Abstract

The p73 gene is located on 1p36.2–3, a region that is frequently deleted in human cancer. Because p73 encodes for a protein that is both structurally and functionally homologous to the p53 protein, p73 has been postulated to be a candidate tumor suppressor gene. To date, however, mutations of p73 have not been found. To study methylation of the p73 5′CpG island, a human bacterial artificial chromosome clone containing exon 1 and the 5′ region of p73 was isolated. There was no evidence for p73 exon 1 methylation in normal tissues. In contrast, p73 was aberrantly methylated in approximately 30% of primary acute lymphoblastic leukemias (ALLs) and Burkitt’s lymphomas. There was no evidence for methylation in any other types of hematological malignancies or solid tumors examined. In both leukemia cell lines and primary ALLs, methylation was associated with transcriptional loss of p73 by reverse transcription-PCR. We used single-strand conformational polymorphisms to screen for point mutations in a series of primary ALLs and found no mutations leading to a change in protein structure. Our results show that methylation of p73 is a frequent event in specific types of hematological malignancies and suggest that epigenetic silencing of p73 could have important consequences for cell-cycle regulation.

Introduction

p73, a gene with significant homology to p53, was recently cloned and localized to chromosome 1p36.2–3 (1). The p73 protein is structurally similar to the p53 protein within its DNA-binding, transactivating, and oligomerization domains. The p73 protein also shares some functional characteristics with p53, including the ability to promote apoptosis when overexpressed in vitro and up-regulate p53 responsive genes involved in cell-cycle control such as p21 (2). Loss of heterozygosity of 1p is common in human neoplasias including neuroblastoma, melanoma, hepatocellular carcinoma, colorectal carcinoma, and breast cancer (3), and it has been postulated that p73 is a candidate tumor suppressor gene (1). To date, however, somatic mutations of p73 have not been found in neural, colorectal, lung, or prostate tumors (4–8).

Genetic analysis of neuroblastomas with deletions of 1p36 show loss predominantly of the maternal allele, suggesting that the tumor suppressor gene(s) in this region is imprinted. In support of this finding, p73 seems to be monoallelically expressed in neuroblastoma cell lines, normal lymphocytes, and normal kidney (1, 9), which raises the possibility that inactivation of p73 would require only a single event leading to preferential loss of the expressed allele. In normal tissues, 5′ cytosine methylation of CpG dinucleotides located within promoter CpG islands has been associated with transcriptional silencing of imprinted genes and genes located on the transcriptionally silent X chromosome of the female (10). In the development of cancer, this epigenetic process occurs aberrantly and has been reported to inactivate a number of tumor suppressor genes and genes that preserve normal cellular function, including O6 methylguanine-DNA methyltransferase and tissue inhibitor of metalloproteinase-3 (11, 12). DNA methylation alters the binding of transcription factors to gene-regulatory regions and is associated with a repressive chromatin structure (13). Because of the interesting possibility that p73 is both an imprinted gene and a tumor suppressor, we have studied the methylation status of the p73 CpG island in both normal tissues and cancers.

In this study, we report evidence for aberrant hypermethylation of the p73 promoter in ALLs and Burkitt’s lymphomas. This finding led us to screen for p73 mutations in a series of ALLs.

Materials and Methods

Samples, Cell Lines, and Culture Conditions. The majority of primary leukemia and lymphoma samples used in this study have been described previously (14). In addition, 15 leukemia samples were obtained from the Cell Procurement and Banking Facility at The Johns Hopkins Oncology Center (Baltimore, MD) and 15 primary leukemia samples from patients presenting to the Pediatric Oncology service at Case Western Reserve University. The leukemia cell lines KG1A (AML), U937 (ALL), HEL (AML), ML-1 (AML), K562 (AML), HL-60 (AML), THP1 (AML), and RAJI (Burkitt’s lymphoma) were analyzed in this study. Cell lines were maintained in appropriate media and treated with 5 AzadC (Sigma) at a concentration of 1 μM for 3–5 days to achieve demethylation.

Genomic Cloning. The CITB human bacterial artificial chromosome (BAC) library (Research Genetics, Huntsville, AL) was screened by PCR using p73 primers E2F: 5′-GCACCACTTGGAGCAGCTG-3′ and E3R: 5′-AGATGTAGTCAAGTCCATGAAGGC-3′, and clone 190018 was isolated. To isolate the 5′ region of the p73 locus, an EcoRI sublibrary was generated from BAC 190018 and was screened by colony hybridization using a 75-bp 5′ terminal Pst1 fragment of the p73 cDNA (GenBank Accession number Y11416) as a probe. This screen yielded a 7.3-kb fragment encoding p73 exon 1. A map of the p73 5′ region was generated by restriction digestion and by sequence analysis.

MSP. Analysis of methylation patterns within the CpG island of the p73 gene in exon 1 (sequence −110-bp to −42-bp relative to translation start, GenBank Accession number Y11416) was determined after the chemical
modification of genomic DNA with sodium bisulfite and MSP as described previously (15). Primer sequences for p73 for the methylated were 5’-GGACGTAGCAGATTGGGTT-3’ (sense) and 5’-ATCACACCTTCTCCCGGTT-3’ (antisense), and for the unmethylated reaction were 5’-AGGGGTATATGGTAACTGGGTTTT-3’ (sense) and 5’-ATCACACCTTCTCCCGGTT-3’ (antisense). Human placental DNA treated in vitro with Sssl methyltransferase served as a positive control for the methylated reaction. Control reactions without DNA were performed with each PCR.

**Southern Blot Analysis.** Genomic DNA (10 μg) was digested with methylation-dependent and methylation-independent restriction enzymes, separated in agarose gels, and transferred to nylon membranes as described previously (16). Membranes were hybridized with 32P-probes prepared by random hexamer primer extension, washed, and subjected to autoradiography.

**p73 Transcript Analysis.** Cytoplasmic RNA was isolated and reverse-transcribed as described previously (17). RT-PCR primers for p73 were 5’-CGGGACCGAGCGGCATG-3’ (sense, exon 1) and 5’-GAAGGTCGAGTAGTGCTGCTCGG-3’ (antisense, exon 3). GAPDH expression was analyzed as described previously (18).

**SSCP Analysis.** Screening of the entire 13 coding exons (2-14) and intronic splice donor and acceptor regions of p73 were performed using primers described previously (19). PCR reactions were carried out in a 10 μl-reaction volume containing 100 ng of genomic DNA and 1 μl of [32P]dCTP. Samples were diluted 1:10 in loading buffer (90% formamide, 10 mm NaOH, 0.05% bromphenol blue, and 0.05% xylene cyanol), heated at 90°C for 5 min, snap-frozen on dry ice, thawed on wet ice, and then loaded onto the gel (20). Electrophoresis was performed using 1x MDE agarose (FMC BioProducts, Rockland, ME) with 5% glycerol in 0.5x TBE. Gels were run at 5–10 W at room temperature for 12–16 h, transferred to 3-mm Whatman paper, dried, and autoradiographed with Kodak X-OMAT film.

**DNA Sequencing.** Ablombrantly migrating bands as confirmed on at least two separate PCR/SSCP reactions were excised from the gel, submerged in 100 μl of pH2O and heated to 80°C for 15 min. Ten μl of this elution containing single-stranded DNA was then reamplified using the appropriate primer set. Automated DNA sequence analysis was performed on PCR products directly or after cloning into pCR2.1-TOPO (Invitrogen).

**Results**

**The p73 5’ Region.** To facilitate analysis of methylation at the p73 locus, we isolated a human BAC clone by screening the library for an exon 1 in a 0.5-kb Eagl-NotI fragment (Fig. 1A). Restriction mapping and further sequence analysis revealed the region surrounding exon 1 to be CpG- and GC-rich with abundant methylation. Primers were designed to detect methylation changes in exon 1, a region with high CpG density within the p73 region. We hypothesized that if p73 promoter methylation was associated with imprinting, normal tissues would be approximately 50% methylated, corresponding to one transcriptionally silent and one transcriptionally active parental allele. By MSP, however, there was no evidence for methylation of p73 in any samples obtained from normal lymphocytes, bone marrow, colon, kidney, brain, fetal lung, or adult lung (Fig. 2A). Southern blot analysis of restriction sites immediately flanking exon 1 confirmed the absence of methylation in normal tissues (data not shown). These data suggested that within the p73 CpG island, methylation in exon 1 and in the region immediately 5′ to exon 1 is not a mark for imprinting of this gene in the tissues examined.

**Fig. 1. A, a restriction map of the p73 5′ region. A 7.3-kb EcoRI fragment containing p73 exon 1 was isolated and mapped by restriction enzyme digestion and by sequence analysis. B, p73 exon 1; arrowhead, the orientation of transcription. III, an Alu element in intron 1; restriction endonuclease sites: E, EcoRI; P, PvuII; Ea, EagI, N, NotI, S, SacII; B, BamHI; above the map, the probe (pASE.5) used in methylation analyses; *, location of MSP primers. B, Southern blot analysis of p73 methylation in leukemia cells. Genomic DNA was digested with EcoRI and NotI, methylation-sensitive enzymes. Samples were separated on a 1% gel and hybridized with the probe pASE.5. A 7.3-kb band indicating methylation at the NorI site is clearly evident in lymphoid leukemia samples (Lanes 1–3 and 8) but not in myeloid leukemia samples. The very faint 7.3-kb band observed in three of the ALLs (Lanes 6, 7, and 9) represents minimal methylation; and these samples were not scored as positive.**

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**p73 Exon 1 Methylation in Cancer.** To determine whether aberrant methylation of p73 occurred in human cancers, we first studied a series of cancer cell lines. By MSP, three (60%) of five breast, one (20%) of five colon, and three (60%) leukemia cell lines showed evidence of aberrant methylation in exon 1 (Fig. 2B). Whereas the methylated solid tumor cell lines were partially methylated, two acute leukemia lines, KG1a and U937, were fully methylated and one leukemia cell line, HL60, was predominantly methylated. Consistent with these results, Southern blot analysis of leukemia cell lines at the NorI site immediately 5′ to exon 1 (Fig. 1A) revealed that two of four (KG1a and HL-60) were fully methylated and one of four (Raji) was partially methylated (data not shown). Because the NorI site is located upstream from exon 1, the complete methylation of HL-60 indicated by Southern blot analysis versus the near-complete methylation indicated by MSP analysis may reflect regional variations in methylation within the CpG island. In contrast, solid tumor cell lines derived from small cell lung (n = 4), non-small cell lung (n = 2), and neuroblastoma (n = 9) were unmethylated by MSP (data not shown).

The methylation patterns we observed correlated with transcriptional silencing of the gene. For the fully methylated cell lines, KG1a and U937, p73 transcript was undetectable by RT-PCR (Fig. 3A). Treatment with the demethylating agent 5 Aza-dC restored expression of p73 (Fig. 3A), confirming the functional importance of methylation in epigenetic silencing of this gene. After several days, the treated cell lines demonstrated a slower growth rate and evidence of cell death. We could not attribute these observations solely to the restoration of p73 function, however, because 5 Aza-dC has a general toxic effect on cells which was independent of their methylation status at the p73 promoter (data not shown).

We next examined primary cancers for evidence of p73 exon 1 methylation. There was no evidence of methylation in any of the solid tumors analyzed, including breast (n = 15), renal (n = 10), and colon cancers (n = 10; data not shown). Methylation of p73, however, was observed in specific types of hematological malignancies, including 31% (11 of 35) of ALLs, and 30% (3 of 10) of Burkitt’s lymphomas (Fig. 2C). In the ALLs, methylation was more frequent in T-cell than

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Fig. 2. Methylation of the p73 promoter region CpG island in cell lines and primary human samples. Visible PCR product in lanes marked U indicates the presence of unmethylated genes; visible PCR product in those lanes marked M indicates the presence of methylated genes. A, normal primary human tissues. All of the samples are unmethylated. B, MSP of p73 in human cancer cell lines. U937 (leukemia) is completely methylated, and HL60 (leukemia) is predominately methylated. HS578T (breast) and SW48 (colon) contain both methylated and unmethylated p73. T47D (breast) and MCF7 (breast) were both unmethylated. C, MSP of p73 in primary ALLs and Burkitt’s lymphomas. All of the methylated primary tumors have evidence of unmethylated p73, a result of the presence of normal contaminating tissue. +, in vitro methylated DNA, is a positive control for methylation.

Discussion

When p73 was originally cloned, its similarity to p53 suggested that it might be a new candidate tumor suppressor gene (1). Up to this point, however, numerous studies have not discovered any coding

B-cell ALL (62% versus 17%, respectively). In contrast, all of the acute myelogenous leukemias (n = 28), non-Hodgkins lymphomas (n = 27), and chronic lymphocytic leukemias (n = 9) that were examined were unmethylated (data not shown). Unlike with cell lines, methylated primary malignancies always displayed evidence for unmethylated p73, likely indicating the presence of normal lymphocytes present within the specimen.

Southern blot analysis of methylation at the NotI site immediately 5’ to exon 1 confirmed the above results and were concordant with the MSP analysis in 11 of 12 leukemia samples studied (Fig. 1B). In a group of nine ALLs, five were methylated by MSP, whereas six were methylated by Southern. The additional leukemia detected by Southern demonstrated a relatively faint 7.3-kb band indicative of methylation (Fig. 1B, Lane 8). Three AMLs were unmethylated by both techniques. In another group of leukemia samples analyzed only by Southern blot analysis, 22% (2 of 9) of ALLs were methylated, whereas all of the AMLs (n = 6) were unmethylated (data not shown). Thus, the incidence of p73 methylation that we observed in acute leukemias was comparable using the two different techniques.

The relatively high incidence of p73 methylation in ALLs and Burkitt’s lymphomas suggested that inactivation of p73 may be an important event in the etiology of these tumors. To further investigate the significance of this finding, we first studied whether methylation affected p73 expression in primary ALLs. p73 was fully expressed in normal lymphocytes and normal bone marrow (Fig. 3B). In contrast, as was observed in the leukemia cell lines, methylation of primary ALLs correlated directly with transcriptional repression of p73 (Fig. 3C). By RT-PCR, methylated ALLs demonstrated markedly lower levels of p73 transcript than an unmethylated ALL. Again, because some normal lymphocytes are invariably present in primary leukemia samples, this low-level expression in methylated tumors may reflect either transcription from unmethylated normal alleles or minimal expression from methylated tumor alleles.

While other tumor types have not been found to inactivate p73 by point mutation (4–8), epigenetic inactivation of p73 in ALL suggested that ALL is a malignancy in which p73 mutations might occur. SSCP analysis was performed on 31 primary ALLs and 4 leukemia cell lines. We observed silent polymorphisms in exons 7 (Val245Val, GTG→GTA), 9 (His349His, CAT→CAC), 14(ALA557ALA, GGC→GCA), and 14 (ALA610ALA, GGC→GCA) at frequencies of 6, 23, 11, and 17%, respectively (Fig. 4). In addition, we detected a single base deletion in intron 3 and one polymorphism in intron 9 at frequencies of 17 and 14%, respectively. Thus, we found no mutations that caused an amino-acid substitution or frameshift.
reasons, the role of p73 in lymphoid malignancies.

For example, loss of expression of the DNA repair gene MLH1 is imprinted, cancer cells could lose the protein through selective loss of the normally expressed allele rather than through an inactivating mutation. Evaluating this hypothesis has been complicated by the fact that p73 seems to be monoallelically expressed in some, but not in all, tissue types (1, 9). Imprinting of p73, therefore, may be restricted to specific tissues. For these reasons, the role of p73 in human cancer has remained unclear. In the present study, we demonstrate that epigenetic modification of p73 via hypermethylation represents a critical alternative mechanism for inactivation of this gene in specific types of hematological malignancies.

We initially determined that methylation changes within, and immediately flanking, exon 1 in the 5’ Cpg island of p73 do not serve as a primary mark for the imprinting of p73. Although we did not directly examine the allelic origin of p73 transcripts in this study, we did study those tissues that have been reported to demonstrate monoallelic expression consistent with imprinting, including normal lymphocytes, normal kidney, and neuroblastoma cell lines (1, 9). Each of these tissues and all of the normal tissue types that we analyzed were completely unmethylated. Although imprinting is often associated with methylation of CpG islands within the promoter of the imprinted gene, in some cases the relevant methylation changes occur at a distance. This is certainly true for the imprinted gene IGF-2, in which selective methylation of a second gene, H19, controls the paternal expression of IGF-2. Of note, H19 is located 75 kb distal to IGF-2 on chromosome 7 and, when silenced via methylation, allows enhancer elements to initiate IGF-2 transcription (21). Thus, it remains possible that a methylated imprinting locus of p73 in normal tissues will be discovered outside the region we studied.

Importantly, we found that aberrant promoter methylation of p73 occurs frequently in ALL and Burkitt’s lymphoma. Promoter region methylation was tumor-specific since it was not observed in normal lymphocytes or bone marrow and resulted in markedly diminished expression of p73. Our results support previous observations that epigenetic silencing of tumor suppressor genes via methylation of promoter CpG islands is a common event in neoplasia. Indeed, in some cases, methylation changes are the predominant alterations that inactivate a tumor suppressor gene. For example, loss of expression of the DNA repair gene O6-methylguanine DNA methyltransferase in a variety of tumor types is caused primarily by promoter hypermethylation rather than deletion, mutation, or gene rearrangement (22). Similarly, in sporadic endometrial carcinomas with microsatellite instability, defects in the DNA mismatch-repair gene MLH1 are more often due to hypermethylation than mutation (23). Because we and others (4–8) have not detected any mutations of p73, aberrant methylation may be the only, or at least the most frequent, way that expression of this gene is altered in cancer.

Our results further suggest a tumor suppressor role for p73 in specific types of lymphoid hematological malignancies. Methylation changes were more common in, but not restricted to, T-cell tumors because B-cell ALLs and B-cell-derived Burkitt’s lymphoma were also involved. Loss of p73 could lead to defects in cell-cycle regulation and confer a selective growth advantage for clones in ALL and Burkitt’s lymphoma. Interestingly, although p53 abnormalities are the most common molecular lesions in human cancer (24), they are relatively less frequent in de novo ALL (2–19%; Refs. 25 and 26) and non-HIV-related Burkitt’s lymphoma (~30%; Ref. 27). Because p53 mutations are not common in these malignancies, one hypothesis is that loss of p73 represents an alternative event contributing to abnormal cell-cycle and cell-death regulation. The absence of p73 methylation in de novo AMLs, non-HIV NHLs, and CLLs and the fact that these tumor types also have a relatively low incidence of p53 mutations (28, 29) imply different mechanisms of pathogenesis in these hematological malignancies.

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References

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