RAPID COMMUNICATION

Deletion of Cyclin-Dependent Kinase 4 Inhibitor Genes P15 and P16 in Non-Hodgkin’s Lymphoma

By P.R.K. Koduru, M. Zariwala, M. Soni, J.Z. Gong, Y. Xiong, and J.D. Broome

B-cell non-Hodgkin’s lymphoma (NHL) is a heterogeneous lymphoid malignancy consisting of several histologic types. Alterations in proto-oncogenes caused by reciprocal chromosome translocations have been implicated in the etiology of specific histologic groups. In this study, we examined the contribution of the cell cycle inhibitor genes P15, P16, and P18 to pathogenesis in a large panel of 209 cytogenetically characterized B-cell NHL tumors representing various histologic groups. We identified the homozygous deletion of P15 and P16 genes in 13 tumors from 12 patients, all belonging to diffuse large-cell histology; 10 had this diagnosis made on presentation, 1 had transformed from small lymphocytic lymphoma, and 1 had transformed from Hodgkin’s disease. Treatment-specific point mutations were not identified in the coding regions of these genes. Cytogenetically, chromosome 9p was normal in all but one tumor. On the other hand, eight tumors hemizygous for 9p by cytogenetic analysis showed wild-type configuration of these genes. Our study, therefore, indicates that deletion of P15 and P16 occurs in about 15% of diffuse large-cell NHL and is not usually detected by cytogenetic analysis. P18 was wild-type in all tumors including the 13 tumors hemizygous for 9p.

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ALTERATIONS IN genes crucial for normal development and/or cellular differentiation have been implicated in the genesis of several types of tumors. In eukaryotes, orderly progression of cells through the cell cycle is controlled by a series of cyclin-dependent kinases (CDKs). The complex formed by CDK4 and D-type cyclins controls the passage of cells through G1 phase. Normal negative regulation of the cell cycle occurs at G1 and G2 checkpoints. The function of CDK4/CDK6 complexes is inhibited by a number of proteins, ie, p15, p16, p18, and p19, encoded by genes P15, P16, P18, and P19, respectively. That CDK4/CDK6 inhibitors can be tumor-suppressor genes is supported by two sets of data. First, recent studies have shown germline and somatic alterations of P16 in several types of cancer, such as melanoma, esophageal squamous cell carcinoma, non-small-cell lung cancer, pancreatic adenoma, bladder carcinoma, acute lymphoblastic leukemia, adult T-cell leukemia, and lymphoid transformation of chronic myelogenous leukemia. Second, transfection of wild P16 into human glioma cells that lack endogenous P16 suppressed growth. None-theless, homozygous deletion of P15 with P16 has been reported in a few cases of acute leukemia.

Characteristic molecular abnormalities of dominantly acting oncogenes, ie, BCL1, BCL2, BCL6, and CMYC, have been identified in specific histologic groups of B-cell non-Hodgkin’s lymphoma (NHL). However, the contribution of tumor-suppressor genes to lymphoma genesis and/or progression has been less well studied. Because abnormalities of 9p have been reported in about 10% of NHL, we examined the structure of the CDK4/CDK6 inhibitor genes P15, P16, and P18 in 209 tumors from various histologic groups of B-cell NHL by Southern blot analysis and polymerase chain reaction–single-strand conformation polymorphism analyses (PCR-SSCP) to determine whether somatic changes in these genes might contribute to lymphomagenesis and/or progression. Our study indicates that inactivation of P15 and P16 occurs through homozygous deletion.

MATERIALS AND METHODS

The 209 tumors examined in this study were collected between January 1989 and December 1994 for a comprehensive pathologic, immunologic, and genetic evaluation of NHL. Cytogenetic studies were performed on lymphocytes prepared by mincing the fresh tumor biopsies in RPMI 1640 and cultured overnight in RPMI 1640 supplemented with 15% fetal bovine serum, 1% L-glutamine, and 1% pen-strep. Standard air-dried chromosome spreads were trypsin-Giemsa banded and karyotypes were characterized following ISCN 1991.

Southern blot analysis. High molecular weight DNA was prepared from tissues frozen in liquid nitrogen. DNA was digested with HindIII and BglII restriction enzymes, electrophoresed in 0.8% agarose-TBE gels, and blotted to nylon. Probes used in hybridization were a 2.0-kb cDNA fragment covering the coding region of P15, an 800-bp cDNA fragment covering the coding region of P16 (both probes were a generous gift from Dr D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), a 525-bp cDNA probe covering the coding region of P18, and a 600-bp genomic DNA probe for BCL1. Probe DNA fragments were labeled with 32P-dCTP by random priming. Blots were hybridized for 16 hours to 20 hours at 65°C, washed in 0.1% sodium dodecyl sulfate (SDS) and 0.1× SSC at the same temperature, and autoradiographed as described previously.

PCR-SSCP. For PCR-SSCP analysis of P15, the following primer pairs were used: P15-1, 5′-GAGGACTCCGCGACGTC- CG-3′/5′-TGGCTCAGCTCATTACCT-3′; P15-2, 5′-AGG-
GTAAAGACTGAGCCCA-3'/5'-GGCCGGGAGGCAACCCTAC-3' (the two pairs cover the first 350 nucleotides of the cDNA); and P15-4, 5'-AGGCTTCTGACGACGCT-3'/5'-CCGGTCTGGCACCTACG-3' (cover nucleotide 593 through the nucleotide 830 of cDNA). The part of P15 (nucleotide 370 through nucleotide 611) that has greater than 95% homology with P16 was not examined by PCR-SSCP analysis because of the potential pairing of these primers with the homologous sequences from P16.

For PCR-SSCP analysis of P16, the following pairs of primers were used: P16-1, 5'-AAATCCGACGCAGAGCA-3'/5'-TGATGCGCTGCTGCCAG-3'; P16-2A, 5'-CTTCTGCTTTGACTGCAAACG-3' (remaining 10 patients presented with diffuse large-cell lymphoma de novo. Repeat studies using probes for P16 and BCL1 genes hybridized simultaneously showed normal hybridization with BCL1 probe in tumors that showed no hybridization or very weak hybridization with P16 probe. These results indicate that the DNA was intact in these tumors and lack of hybridization with P16 probe was due to loss of this gene in tumor cells (Fig 1A). In three of these tumors (1399, 2590, and 3610), P16 and P15 probes showed very weak hybridization that was probably due to hybridization to DNA from nontumor cells present in them. In one case (2590/3499), deletion was identified in tumor obtained at diagnosis and after therapeutic relapse. PCR-SSCP analysis did not detect any tumor-related changes in the P16. An abnormal conformation in PCR-SSCP was identified in seven patients with exon 2 primers. Sequence analysis showed a G to A transition at codon 140 (GCC$^{\text{GGC}}$ at codon 140) (data not shown), which is a known polymorphism.

P15$^{\text{NSKB}}$ (MTS2). Hybridized P16 and BCL1 probes were stripped off and the blots were rehybridized with a P15 cDNA probe. No gross alterations were detected in this gene in any tumor. However, absence of hybridization or very weak hybridization indicating deletion of this gene was observed in the 13 tumors (Fig 1B), all of which showed deletion of P16. PCR-SSCP analysis did not identify any tumor-related changes in the coding region of P15. An abnormal conformation in PCR-SSCP was identified with primer set two in four patients (1.7%). Nucleotide sequence analysis showed a transition of G to A at nucleotide 240 located 88 bp upstream of the first codon (data not shown).

P18. Hybridized P15 probe was stripped off and the blots were rehybridized with a cDNA probe for P18. No genomic alterations were detected in this gene in any tumor. A polymorphic alteration in the PCR-SSCP pattern in exon 2 was identified in 19% of patients. Sequence analysis showed a C to T transition (GGT$^{\text{GGT}}$ at codon 114) (data not shown).

Cytogenetic changes. Among the 209 tumors included in this study, cytogenetic study was not performed on 20 tumors because of insufficient material. Metaphase spreads of suitable quality for cytogenetic analysis were not obtained from 35 tumors, and only normal metaphase spreads were observed in 9 tumors. Clonal cytogenetic abnormalities were identified in the remaining 145 tumors (Table 1). Specific recurring translocations affecting the site of cMYC (8q24), BCL1/PRAD1/CYCLIN D1 (11q13), BCL2 (18q21), or BCL6 (3q27) were identified in 52% of the cytogenetically abnormal tumors. Structural cytogenetic changes affecting 9p21 to which P15 and P16 have been mapped were identified in 9 tumors. These tumors were hemizygous for part of the short arm of chromosome 9 distal to band p13 due to the formation of a derivative chromosome 9p or, in one case, due to deletion. However, only one such tumor (3610) showed deletion of P15 and P16. In contrast to these findings,
Specific translocation breaks have been implicated in the genesis of different histologic types of NHL.\(^3\) Chromosomal regions 1p, 6q, 7q, 9p, 11q, 14q, and 17p have been identified as recurrent sites of deletions or structural changes.\(^3\)\(^3\) Alterations in the p53 gene, a gene involved in cell cycle regulation,\(^4\) have been reported in a few cases of transformed NHL,\(^4,\)\(^2\) whereas two distinct sites of molecular deletions, probably harboring tumor-suppressor genes, have been identified in 6q deletions.\(^4\) Therefore, we made a comprehensive study of CDK4 inhibitor genes P15 and P16, located at 9p21, and CDK6 inhibitor gene P18, located at 1p32, in cytogenetically characterized primary B-cell NHL.

Homozygous deletion of P15 and P16 was found in 15% of diffuse large-cell NHL. In three of these tumors (1399, 2590, and 3610), P15 and P16 probes gave very weak signal. Because the intensity of signal appeared to be less than 50% of that from the control probe (BCL1 probe), it is assumed that DNA breaks leading to deletion have occurred on 9p21 outside P15 and P16. On the other hand, cytogenetic hemizygosity for 9p was observed in 9 tumors (11\(^1\)4\(\times\)q13; q32) (Table 2).

Structural cytogenetic abnormality affecting 1p32 to band p32 due to deletion (5 tumors), to a derivative chromosome lp (6 tumors), or to monosomy chromosome l distal to band p32 due to deletion (5 tumors), and abnormalities associated with aggressive disease.

DISCUSSION

Alterations in specific proto-oncogenes located at sites of specific translocation breaks have been implicated in the genesis of different histologic types of NHL.\(^3\)\(^2\)\(^3\) Chromosome regions 1p, 6q, 7q, 9p, 11q, 14q, and 17p have been identified as recurrent sites of deletions or structural changes.\(^3\)\(^5\)\(^4\)\(^6\) The pathologic significance of these secondary recurrent cytogenetic abnormalities has been little studied in NHL. Alterations in the p53 gene, a gene involved in cell cycle regulation,\(^4\) have been reported in a few cases of transformed NHL,\(^4\)\(^2\)\(^4\)\(^4\) whereas two distinct sites of molecular deletions, probably harboring tumor-suppressor genes, have been identified in 6q deletions.\(^4\) Therefore, we made a comprehensive study of CDK4 inhibitor genes P15 and P16, located at 9p21, and CDK6 inhibitor gene P18, located at 1p32, in cytogenetically characterized primary B-cell NHL.

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Specific cytogenetic changes

1p32 translocations

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<th>Cytogenetic Status</th>
<th>SmLy</th>
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Abbreviations: SmLy, small lymphocytic lymphoma; FSCC, follicular small cleaved cell lymphoma; Mixed FSC/CL, mixed follicular small-cell and large-cell lymphomas; FLC, follicular large-cell lymphoma; DSCC, diffuse small cleaved cell lymphoma; Mixed DSC/CL, mixed diffuse small-cell and large-cell lymphoma; DLC, diffuse large-cell lymphoma.

Table 1. Cytogenetic Status of 209 Lymphomas Examined for Changes in CDK4/CDK6-Inhibitor Genes

The frequency of P15 and P16 deletions now found was higher than that reported in three previous studies.\(^4\)\(^2\)\(^4\)\(^2\) These studies reported deletion of P16 in 2 of the 84 tumors examined. One of these was a case of transformed NHL, and the second was a case of NHL in a leukemic phase.\(^4\) However, the lack of histologic subclassification of tumors examined in earlier studies, particularly separation into low and high grades of malignancy, precludes comparison with our findings.

Recently, several investigators have examined the structure of P15 and/or P16 in several types of primary leukemias, including acute lymphoblastic leukemia (ALL) of B cells and T cells, T-cell prolymphocytic leukemia, adult T-cell leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, acute myelogenous leukemia (AML), myelodysplastic syndrome, and myeloproliferative disorders. Homozygous deletion, primarily of P15, has been identified in 64 of 142 T-cell ALL, 44 of 224 B-cell ALL, 10 of 37 adult T-cell leukemia, 5 of 10 chronic myelogenous leukemia in lymphoid blast crisis, and in 1 of 111 AML. Structural re-
Fig 1. Deletion of P15 and P16 in diffuse large-cell lymphoma. (A) Representative Southern blot of HindIII- and Bgl II-digested DNA from diffuse large-cell lymphomas simultaneously hybridized with probes for P16 and BCL-1. The probe for P16 showed no or very weak hybridization (indicated by arrowheads), whereas the probe for BCL-1 showed strong hybridization (indicated by arrows). These results suggest homozygous deletion of P16 in these tumors. Open arrows indicate a rearranged DNA band identified by BCL-1 probe in this tumor. The positions of size marker DNA fragments (λ DNA digested with HindIII) is shown on the left side. DNA from human placenta (P) and a diffuse lymphoma (2218) was used as negative controls. (B). The above-mentioned probes were stripped off and the blot was hybridized with a probe for P15. Note the absence or very weak hybridization of the probe, suggesting homozygous deletion of P15.

arrangements or point mutations have been identified very rarely. These data suggest that P15 and P16 deletions are associated with malignancies of immature lymphocytes.

Whether deletions of P15 and P16 occur early in disease or during progression is not known. A few studies examined sequential tumor samples. In one study of CML, deletion of P16 was found only in lymphoid blast crisis but not in the chronic phase. Fizzotti et al. found P16 deletions at diagnosis and at relapse in two patients with ALL. Hatta et al. noticed that disease progressed rapidly to acute phase in a patient who had deletion of P16 at chronic phase. In a second patient, P15 and P16 were normal in the chronic phase but deleted at acute phase. In two cases of NHL in which sequential samples were examined, P16 deletions occurred with transformation to immunoblastic lymphoma. In contrast to these findings, 10 of our 12 patients with deletion of P15 and P16 region at 9p21 might contribute to lymphomagenesis in this subset of NHL.
The above data indicate that loss of both alleles of P15 and P16 is the principal mechanism of inactivation of these genes when this occurs in primary leukemias and lymphomas. This is in contrast to the findings in P53 and Rb genes, whose inactivation is associated with deletion of one allele and mutation in the second allele. Because the occurrence of 9p21 abnormalities does not correlate with changes in P15/P16, it remains possible that this chromosome site may contain other genes of importance in oncogenesis.

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