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Genetic Alterations in the Retinoblastoma Protein-Related p107 Gene in Human Hematologic Malignancies

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Genetic alterations in the p107 gene, a close relative of the retinoblastoma tumor suppressor gene, have never been identified in human malignancies. When we searched for such alterations in human hematologic malignancies by Southern blot analysis, 2 of 21 cell lines and 1 of 88 primary disorders had genomic alterations within the gene. Particularly, an altered p107 gene in a diffuse-large B-cell lymphoma cell line, KAL-1, harbored an intragenic deletion of about 15 kbp leading to the expression of an altered p107 mRNA devoid of 819 nucleotides of the coding sequences, which predicts to encode an approximately 87-kDa protein. This cell line was found to express solely a p107 derivative of 84 kDa by immunoblotting analysis. These results suggest that alterations in the p107 gene are probably related to a limited subset of human hematologic malignancies. © 1998 Academic Press

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The p107 protein was first identified by virtue of its ability to bind to the adenovirus E1A and to the SV40 and JC virus large T antigens (1–3). Sequence analysis of human p107 complementary DNA (cDNA) clones (4, 5) predicted a protein of 117 kDa and identified a region of 564 amino acids with a striking homology to the so-called "pocket domains A and B" of the human retinoblastoma gene product (pRB), a tumor suppressor that also binds to DNA tumor virus oncoproteins (6, 7). Identification and characterization of intracellular proteins that interact with p107 provided further insight into its function in cell cycle regulation. For example, p107 can complex with members of the E2F family of transcription factor (8), c-myc proto-oncogene protein (9), cyclinA-CDK2 and cyclinE-CDK2 complexes (10), and the cyclinD-CDK4 complex (11); many of which also interact with pRB.

The observation that over-expression of p107 inhibited proliferation in some cultured cell lines provided evidence for involvement of p107 in cell cycle regulation (5). Although these studies collectively indicate that p107 is likely to be a pivotal element in cell cycle regulation, *in vivo* tumor suppressor activity and genetic alteration of the p107 gene in human malignancies have never been demonstrated, in contrast to the cases of pRB.

We and others have found that the CAG repeat in a coding exon of E2F4 which can interact with p107 was altered selectively in human gastrointestinal cancers with microsatellite instability (12–14). We postulated that p107, the main interacting partner of E2F4 in growing cells (15, 16), might be altered in some human neoplasms.

We have extensively surveyed for alterations in the p107 gene in human hematologic malignancies. We report here the first evidence that the p107 gene is in fact affected by genomic rearrangements in some cell lines and a specimen derived from humans with lymphoid malignancies.

MATERIALS AND METHODS

Cells and specimens. Most of the established cell lines used in this study were obtained via Dr. J. Minowada from Fujisaki Cell Center, Hayashibara Bio-Science Inc. (Okayama, Japan), including six T-cell leukemias, one B-cell leukemia, two non-T, non-B-cell leukemias, five lymphomas, one chronic lymphocytic leukemia (CLL), and six myeloid leukemias. KAL-1, a diffuse large B-cell lymphoma cell line (17) was from Dr. S. Nakano of Kyushu University. Fresh specimens (lymph node biopsies, bone marrow mononuclear cells and peripheral blood mononuclear cells) from patients with hematologic disorders were collected at the Okayama University Hospital following acquisition of informed consent from each patient. Specimens (88 in total) were from 19 non-Hodgkin's lymphoma, 2 Hodgkin's disease, 19 acute myeloblastic leukemia (AML), 14 acute lymphoblastic leukemia (ALL), 10 myelodysplastic syndrome (MDS), 14 chronic myelogenous leukemia (CML), 3 CLL, 2

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multiple myeloma, 1 adult T cell leukemia, and 4 reactive lymphadenopathy.

DNA and RNA analyses. Southern and Northern blot analyses were done according to the standard procedure with 10 μg of DNA or 20 μg of RNA, respectively, and transferred to Hybond-N^+ filters (Amersham International plc, Buckinghamshire, England). Filters were then hybridized with the p107-specific cDNA probes described below.

All the p107 oligonucleotide primers used for reverse-transcription and polymerase chain reaction (RT-PCR) were prepared according to the published human p107 cDNA sequences (5). A full-length p107 RT-PCR fragment (3310 bp covering the entire coding sequence) was amplified using one-tenth aliquots of the sample after reverse-transcription of 5 μg of total RNA with Superscript II (Gibco-BRL, Rockville, MD), by long PCR technique with S1 primer (5′- TGGGAGGGAGAA-AGAAGTCGG) and AS1 primer (5′-TCTGCAGAACAATCTGAAAGTGC), under the following conditions; 35 PCR cycles of denaturing at 94°C for 30 s and extension at 64°C for 5 min, and final elongation at 72°C for 7 min with an extra-long-PCR kit containing rTth DNA polymerase (Perkin–Elmer, Foster City, CA). The 5′-half RT-PCR product (1764 bp) was raised by a combination of primer S1 and AS2 (5′-TGACTCCATGCTAAACTCTCCAA). The 3′-half product (1723 bp, 177 bp of which overlapped with the 3′-end of 5′-half fragment) was by primers S2 (5′-TGCCTATAGCTCACCTCGTACT) and AS1. The PCR products were cloned into a plasmid vector, pBluescript KS(−) by blunt-end insertion. Radioactive probes were prepared by labeling excised cDNA fragment with [α-^{32}P]dCTP and random primer (Amersham). To analyze aberrant transcripts in MOLT-4 cell line, p107 cDNA clones were isolated by screening a λgt10 cDNA library constructed from RNA of MOLT-4 (Clonetech, Palo Alto, CA) with the full-length p107 cDNA probe. Sequencing of PCR products and their clones were done with 50 ng of either gel purified PCR products or plasmid DNAs using either the same primers as PCR reaction or a sequencing primer set (M4 and RV) within the vector sequences, by the ABI-PRISM dye-terminator cycle sequencing system (Perkin–Elmer).

Protein analysis. Total cell-lysates were prepared from approximately 2×10^7 cells each by boiling in 500 μl of lysis buffer [62 mM Tris–HCl (pH 6.8)/2.3% sodium dodecyl sulfate (SDS)/5% 2-mercaptoethanol/10% glycerol], followed by centrifugation to remove debris. A hundred micrograms protein of total cell-lysate each was fractionated through 7.5% polyacrylamide-SDS gel, electrically transferred onto a nitrocellulose membrane, and reacted with an anti-p107 monoclonal antibody SD9 (5), a kind gift from Dr. Ed Harlow of Harvard University. Immunological detection was performed using the ABC Western blot kit (Vector Lab. Inc. Burlingame, CA).

RESULTS

Genomic alterations in the p107 gene. We searched for genomic alterations in the p107 gene in a collection of the cell lines and fresh specimens of human hematologic malignancies by Southern blot hybridization analyses using either the 5′-half or the 3′-half cDNA clone of the p107 as probes. Representative Southern hybridization profiles are shown in Fig. 1A. By using restriction endonuclease Hind III, 2 of 21 cell lines examined, a T-cell leukemia cell line, MOLT-4 (18) and a diffuse large B-cell lymphoma cell line, KAL-1(17) exhibited rearranged fragments (Fig. 1A), which were corresponding to the 5′- and the 3′- portion of the p107 gene, respectively. These abnormalities are neither from an incomplete digestion of DNA nor from an RFLP, since the altered fragments were reproducibly detected and the same alterations were never identified in more than 150 normal or malignant specimens. Moreover, Southern analyses of KAL-1 DNA with three other restriction endonucleases (Avr II, Xho I, Swa I) also showed rearrangements as shown in Fig. 1B (lanes 3, 6, and 9). All of these rearranged fragments appear to be derived by deletion of about 15 kbp segment from normal gene. In the case of MOLT-4, proofs for the rearrangement by Southern analysis with other enzymes have not been obtained. However, MOLT-3, a sib-line of MOLT-4 from the same patient (18), showed the same rearranged Hind III fragment as that of MOLT-4. This indicates that the abnormality had occurred in the original patient but not during *in vitro* culture. Then 88 specimens of hematologic disorders were surveyed for such alterations. One of 14 ALL specimens, #57L showed another type of rearrangement on the 3′-portion of the gene (Fig. 1A, lane 6).

Altered p107 transcripts in KAL-1 and MOLT-4. We next analyzed transcripts of the p107 gene in these affected samples. Northern blot analysis of total RNAs from most cell lines indicated that the major p107 mRNA appeared as a broad band between 4.6 and 5.0 kb (Fig. 1C, lanes 3–11). In addition, every cell line commonly produced two minor transcripts of 3.2 and 3.7 kb, probably of splicing variants because the full-length p107 mRNA is larger than 4 kb (5). On the contrary, the major p107 transcripts in KAL-1 and MOLT-4 were predominantly of smaller sizes ($\sim$3.7 kb) and the levels of normal sized mRNA were low (Fig. 1C, lanes 1 and 2).

We then did fine analysis of the messages by constructing cDNAs by RT-PCR (Fig. 1D). An RT-PCR product of the 5′-half of p107 gene from KAL-1 was indistinguishable from normal one, while RT-PCR products of the 3′-half contained two smaller fragments of 1.5 and 1.0 kbp, in addition to two faint fragments larger than the normal one of 1723 bp. The major 1.0 kbp fragment was likely to correspond to the major mRNA species observed in Northern analysis of KAL-1 RNA. Sequence analysis of this fragment clearly indicated that this was devoid of the 819 nucleotides (ntd) region of the 3′-half of the normal p107 cDNA. The deletion occurred at position 1958 ntd (counting from the 5′-end of the 3310 ntd RT-PCR product), however, did not destroy the open reading frame (ORF), predicts the production of MW 87 kDa protein truncated at the middle part of the intact p107 molecule, corresponding to the spacer and pocket domain B, as schematically illustrated in Fig. 2. Although the genomic rearrangement of the p107 gene in KAL-1 cell seemed to be heterozygous, we detected no normal-sized RT-PCR product from KAL-1. This indicates that the normal allele of the p107 gene in this cell line is

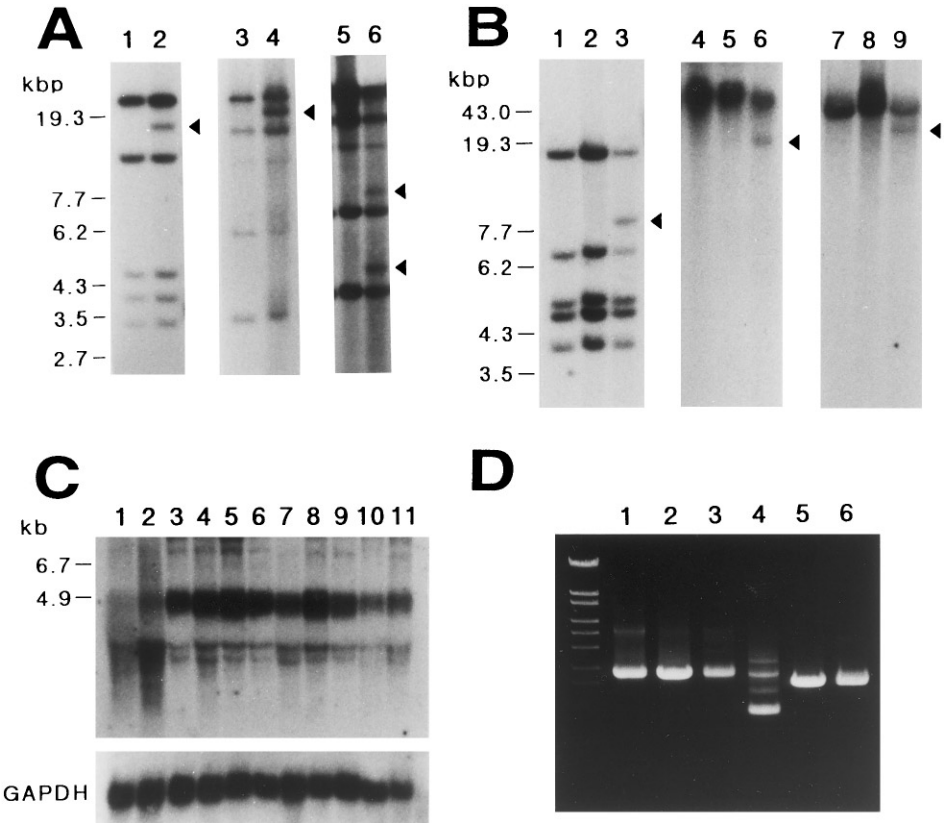

FIG. 1. Alterations of the p107 gene and its products. (A) Southern blot hybridization of the p107 gene. DNAs were digested with Hind III and hybridized with the p107 5′-half (lanes 1 and 2), or 3′-half (lanes 3–6) cDNA probes. Sources of DNA; normal peripheral blood mononuclear cells (PBMNC) (lanes 1, 3, and 5), MOLT-4 (lane 2), KAL-1 (lane 4) and PBMNC from an ALL patient (#57L) (lane 6). Rearranged fragments are denoted by arrowheads. (B) Southern blot profiles showing rearranged fragments of p107 with different restriction endonucleases. DNAs were digested with Avr II (lanes 1–3), Xho I (lanes 4–6), Swa I (lanes 7–9) and probed with the 3′-half p107 probe. Sources of DNA; normal PBMNC (lanes 1, 4,7) , MOLT-4 (lanes 2, 5, and 8), KAL-1 (lanes 3, 6, and 9). Rearranged fragments are denoted by arrowheads. (C) Northern blot hybridization of mRNAs. Total RNA (20 μg) were fractionated and probed with a full-length cDNA probe. Sources of RNA; KAL-1 (lane 1), MOLT-4 (lane 2), HSB-2 (lane 3), DHL-1 (lane 4), REH (lane 5), BV-173 (lane 6), RAJI (lane 7), NALM-6 (lane 8), DHL-4 (lane 9), PBMNC from a Burkitt lymphoma patient #27T (lane 10), and from an ALL patient #56L (lane 11). The same filter was reprobed with glyceraldehyde phosphate dehydrogenase (GAPDH) probe and shown below for reference. (D) RT-PCR products of p107 messages. RT-PCR reactions were done starting with 5 μg of total RNA from KAL-1 cells (lanes 1 and 4), from a specimen of non-Hodgkin's lymphoma (lanes 2 and 5) and from a specimen of reactive lymphadenopathy (lanes 3 and 6). One tenth amount of PCR product was electrophoresed through 0.7% agarose gel, visualized with ethidium bromide staining. Lanes 1–3, and 4–6 are 5′-half (1764 bp) and 3′-half (1723 bp) PCR products, respectively. The left-most lane includes a DNA size marker (Eco T14I-digested λ phage DNA).

probably down-regulated or inactivated by other mechanism(s).

Another cell line, MOLT-4 also expressed predominantly smaller p107 mRNAs. In contrast to KAL-1, however, the smaller transcripts could not be converted to cDNAs by RT-PCR; only normal sized RT-PCR products were observed. The altered allele may be related to these small transcripts. In order to verify this possibility, we have screened for p107 cDNA clones from a cDNA library constructed from the cell line MOLT-4. We found that more than half of the cDNA clones (12 out of 21) contained only parts of the full length p107 cDNA, retaining incompletely spliced intronic sequences (not shown). This suggests that the normal splicing control of the p107 mRNA precursors is largely perturbed in MOLT-4 cells. The analysis of whole genomic structure of the p107 gene is currently underway, and we found there are at least 22 exons in the p107 gene encompassing about 90 kbp region of human genome (Ichimura *et al.*, unpublished). Since we also observed the normal sized mRNA and RT-PCR product in MOLT-4 cells, albeit to a much lesser amount compared to other cell lines (Fig. 1C), we examined the full sequences of this mRNA by isolating 10 nearly full-length cDNA clones with long PCR technique; 8 of 10 clones contained extra intronic sequences and/or deletion of exonic sequences distributing at several splicing junctions (not shown). Most of these splicing variants had premature termination codon downstream the alterations. Taken together, most, if not all, of the p107 mRNAs in MOLT-4 cells appeared to be non-functional for encoding the normal p107 protein.

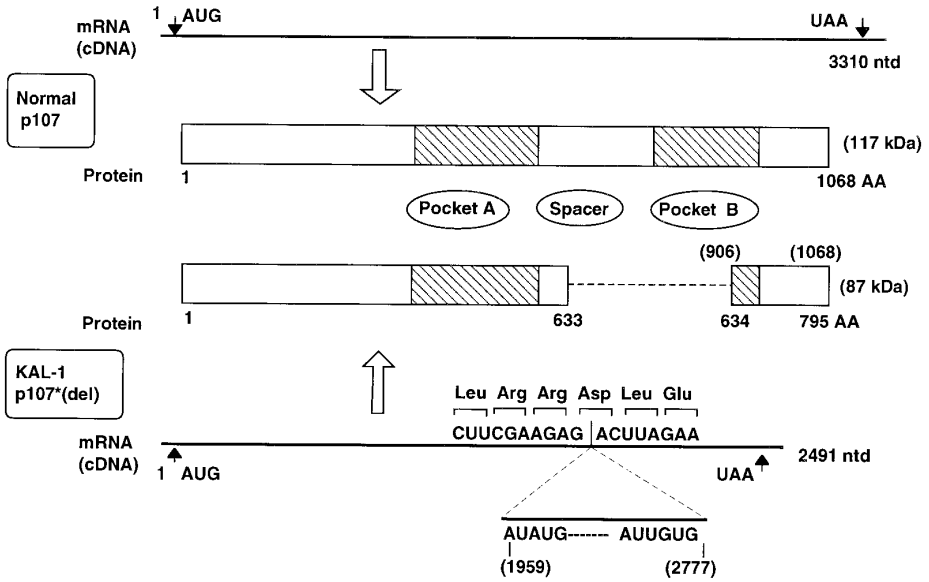

FIG. 2. Structure of the major deleted gene-products from KAL-1 cells. Structure of the truncated p107 protein, deduced from the sequence of the RT-PCR products (lower) is compared with normal (upper). Partial sequences around the deletion break point (1958 ntd) are depicted to show the restoration of ORF (lower).

Alteration in size or expression of p107 protein. Finally, to obtain support for all these findings, we conducted analyses at the protein level, using monoclonal antibody specific to p107. The total lysate of KAL-1 cells contained only the 84 kDa form of the p107 molecule (Fig. 3). This size is likely to accord, within an error range, with the estimated size of the predicted protein from the major p107 mRNA in KAL-1 (Fig. 2). The normal sized p107 was detected in three cell lines but not in KAL-1 and MOLT-4. Thus, lack of expression of normal p107 in both KAL-1 and MOLT-4 supports the results of RT-PCR analyses.

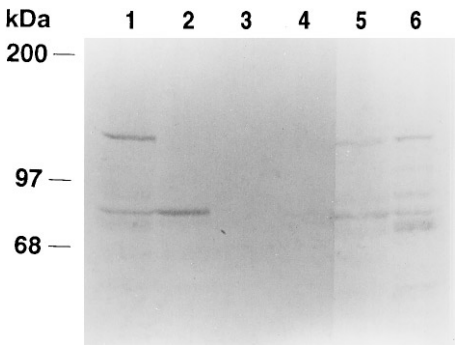

FIG. 3. Immuno-blot analysis of p107 proteins from several affected samples. A hundred microgram protein of total cell lysate each was fractionated, transferred onto a nitrocellulose membrane, and reacted with an anti-p107 monoclonal antibody SD9. Immunological detection was performed using the manufacture's instruction. Sources of protein; KB cells (oral carcinoma) (lane 1), KAL-1 (lane 2), MOLT-4 (lane 3), specimen #56L (ALL) (lane 4), NALM-6 (lane 5) and Lu-134 cells (lung carcinoma) (lane 6).

Another interesting finding is that most cells produce, in addition to normal p107, altered forms of p107, prominently of 84 kDa species (Fig. 3). This may be the translation product from the alternatively spliced mRNAs widely expressed in most of the cell lines and in some informative fresh specimens as shown in Fig. 1C.

DISCUSSION

In this study, we report novel findings as to the structural aberration of the p107 gene in human hematologic malignancies. One of 21 cell lines examined, KAL-1, had a distinct rearranged p107 gene, giving rise to altered gene products at both mRNA and protein levels. In the case of another cell line, MOLT-4 (-3), in which p107 was not detected, the rearranged fragment was only detected with Hind III digestion but not with other enzymes, suggesting that the rearrangement might be a very subtle alteration leading to the aberrant splicing of p107 mRNA precursors and diminished p107 expression in this cell line. Furthermore, we also found another rearranged p107 gene in a fresh specimen of ALL, though the details of the aberration are yet to be analyzed. As a most notable finding of these three rearrangements, the nature of the rearranged p107 gene in KAL-1 cells appears to be deletion of about 15 kbp region of the gene. We have recently identified the deletion break points that reside within two *Alu*-family-like direct repeats encompassing a stretch of 15 kbp containing 5 coding exons (exons 15–19) and this missed part exactly accords with the deletion in the cDNA (Ichimura, K., *et al.*, unpublished). The resultant altered form of p107 in KAL-1 cells is devoid of the

spacer and pocket B domains which are thought to be essential for the normal function of p107 (19). These are, to our knowledge, the first indication for possible implication of the p107 gene, a candidate tumor suppressor, in cells derived from human neoplasms. One possibility that the acquired deletion of the p107 gene in KAL-1 cells had occurred during cultivation may not completely be excluded. However, even if this is the case, it is likely that the deletion might exert a positive effect on cell proliferation. Genetic alteration of another member of the pRB family, the p130 gene, has also been barely detectable in human cancers, but one case of such alteration was recently found in a cell line of small cell lung carcinoma (20). Since genetic aberrations of the p107 gene were found in only 3 cases of 109 samples derived from hematologic malignancies in our study, this might not be a very frequent event in human malignancies. This may be due to the functional redundancy of the pRB family proteins (16, 21). Studies with mice disrupted in the pRB family genes also suggested that the p130 and p107 are less involved in tumorigenesis (21, 22). Loss of p107, therefore, may not be a sole causative event for acquisition of the malignant phenotype. It would, however, still remain a possibility that loss of p130 or p107 could influence on cellular proliferation under some special circumstances such as pRB or other relevant genes were inactivated. Additionally, it may also be reasonable to speculate that the altered p107 derivative in KAL-1 cells exerts a dominant negative effect on function of the pRB family proteins, because the mutant protein retains more than 70% portion of intact p107.

Another interesting finding is the common appearance of altered p107 derivatives in many cell lines and fresh specimens including normal subjects, probably derived by alternative splicing. Similar but distinct splicing variants of mouse p107 have been reported (23). These observations suggest that, even in normal cells, the p107 gene tends to express altered forms of mRNA and protein species, though their roles remain to be determined. Some human hematologic disorders might have acquired more drastic alterations to enhance this tendency, as seen in cases of KAL-1 and MOLT-4 by genomic rearrangements. To gain insight into the mechanisms of these alterations and possible roles in tumorigenesis, functional studies on the altered forms of p107 are in progress.

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