Advances in Brief

Aberrant Transcripts of the Cyclin-dependent Kinase-associated Protein Phosphatase in Hepatocellular Carcinoma

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Abstract

The cyclin-dependent kinase (Cdk)-associated protein phosphatase (KAP) is a human dual specificity protein phosphatase that dephosphorylates Cdk2 on threonine 160 in a cyclin-dependent manner. To investigate whether mutations of this enzyme occur in hepatocellular carcinoma (HCC), KAP mRNA was analyzed by reverse transcription-PCR (RT-PCR), followed by cloning and sequencing. Eight of 14 biopsy tissues obtained from advanced HCC, 6 of 13 surgically removed HCC tissues, and 2 of the adjacent noncancerous tissues contained aberrant KAP transcripts. Using the yeast two-hybrid system, five of seven representative KAP mutants were shown to be defective in interacting with Cdk2. These data suggest a possible role of KAP mutations in multiple-step hepatocarcinogenesis.

Introduction

Activation of Cdk2 requires binding of the Cdk to a corresponding cyclin and phosphorylation of Cdk2 at a conserved threonine residue (T160 in Cdk2; Refs. 1 and 2). The activity of an activated, T160 phosphorylated Cdk2 can be inhibited by phosphorylation of the other two conserved residues in the catalytic cleft (tyrosine 15 and threonine 14; Refs. 3 and 4). Such regulatory phosphorylation pathways are conserved among species, from yeast to humans (5). Several kinases have been shown to phosphorylate T160, such as Cdk7-cyclin H, which is present in the transcription factor TFIIH in humans and is conserved among species, from yeast to humans (5). Several kinases (6, 7). Therefore, it is believed that KAP binds to Cdk2 and dephosphorylates T160 to inactive Cdk2 when the associated cyclin subunit is degraded. Although the function of KAP was well characterized in vitro, its contribution in cell cycle regulation is still uncertain in vivo (8). Mutations of several tumor suppresser genes have been identified in HCC. According to the in vitro experiments mentioned above, KAP might function similarly to a tumor suppressor. In this study, we investigated whether mutations occurred in KAP genes in HCC by analyzing the nucleotide sequence of its mRNA. Strikingly, several aberrant transcripts of this gene were identified. The possible role of these mutants in multiple-step hepatocarcinogenesis was discussed.

Patients and Methods

Patients. Three groups of patients were included:
Group 1, 12 randomly selected patients who received liver biopsy because of chronic hepatitis B or C, and the pathological examination showed minimal hepatitis using the Knodelling scoring system (12).
Group 2, 14 randomly selected patients (accrued December 1997 to December 1998) who received liver biopsy under the clinical impression of advanced HCC, and the diagnosis was subsequently confirmed by pathological examination. None of these patients received surgical treatment because of intrahepatic spread of the tumors (in 11 cases), huge size of tumor (in 1 case), or both (in 2 cases).
Group 3, 13 patients (accrued June 1997 to December 1998) who received partial hepatectomy for HCC. These tumors were 2.8–5.2 cm in size, except for tumor T3, which was 14 cm in size. Cancerous and noncancerous parts of HCCs were separated by the surgeons and examined by a pathologist. All collected tissues were frozen in liquid nitrogen until analysis. The sex, age, and serological markers for hepatitis B or C are listed in Table 1.
Serology. Hepatitis B surface antigen was assayed using a commercially available RIA (Austria II; Abbott Laboratories, Chicago, IL). Antibody to hepatitis C virus (anti-hepatitis C virus) was assayed using an enzyme immunoassay (Abbott HCV EIA III; Abbott Laboratories).

Extraction of Total Cellular RNA. Total RNA was isolated from both cancerous and noncancerous liver tissues using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer’s instructions. To eliminate contaminated DNA, the extracted RNA was dissolved in diethyl pyrocarbonate-treated water containing 10 mmol/l of MgCl2 and incubated with 100 μg/ml of RNase-free DNase I for 30 min at 37°C. EDTA was added to a final concentration of 30 mmol/l, and the mixture was heated at 95°C for 5 min to stop the reaction.

RT-PCR, Cloning, and Sequencing. Total RNA derived from tumors or noncancerous liver tissues was subjected to RT-PCR. The primers were designed according to a published KAP sequence: 5’-ACTGGTCTCGAGT-GGGCGG-3’ (nucleotides 25–44, sense) and 5’-GGGGCG-3’ (nucleotides 46–65, reverse) and 5’-AGCTGGTTATGTCAGAGTC-3’ (nucleotides 406–387, reverse). The primer set was used for reverse transcription. The details of RT-PCR were described elsewhere (13, 14). After RT-PCR, the product was gel purified and cloned into a vector, pCR2.1-TOPO (Invitrogen,
ABERRANT KAP TRANSCRIPTS IN HEPATOCELLULAR CARCINOMA

Table 1. Basic data for patients included in this study

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Mean ± SD</th>
<th>HBsAg/anti-HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>M/F</td>
<td>(range)</td>
</tr>
<tr>
<td>Hepatitis control</td>
<td>12</td>
<td>9/3</td>
<td>48 ± 11</td>
</tr>
<tr>
<td>Advanced HCC</td>
<td>14</td>
<td>12/2</td>
<td>60 ± 13</td>
</tr>
<tr>
<td>Resectable HCC</td>
<td>13</td>
<td>11/2</td>
<td>51 ± 12</td>
</tr>
</tbody>
</table>

* P: positive; N: negative.
* P = 0.009.
* P = 0.04.

Carlsbad, CA). If multiple bands were found in the agarose gel, each band was gel purified and cloned into the vector separately. At least five clones were selected and sequenced for each PCR product. Nucleotide sequencing was performed using an automatic DNA sequencing (CEQ 2000; Beckman Instruments, Inc., Fullerton, CA).

Interaction Assay by the Yeast Two-Hybrid System. Protein–protein interaction between Cdk2 and KAP mutants was evaluated using the yeast two-hybrid system (Matchmaker LexA two-hybrid system; Clontech Laboratories, Inc., Palo Alto, CA). Primers containing an engineered EcoRI or XhoI site were first synthesized: P1, 5′-GGATTACCTGAGAAGCTTCAAGTCCTCAAAAGG-3′ (sense); P2, 5′-CCTCGAGTCTTGATACAGATCTTGATTG-3′ (antisense); P3, 5′-GGATTACCTGAGAAGCTTCAAGTCCTCAAAAGG-3′ (sense); and P4, 5′-CTCGAGTCTTGATACAGATCTTGATTG-3′ (antisense). The engineered sites were underlined. P1 and P2 were designed according to the 5′ and 3′ ends of the KAP coding region, whereas P3 and P4 were designed according to the 5′ and 3′ ends of the CAP coding region. The Cdk2 coding region was then amplified by RT-PCR using normal liver mRNA (purchased from Invitrogen), verified by nucleotide sequencing, digested with restriction enzymes, and inserted (in-frame) into EcoRI-XhoI sites of pLexA; a plasmid encoding the 202-residue LexA protein and HIS3 marker. The wild-type or mutant KAP coding region was amplified from the RT-PCR-derived clone used for mutation analysis (see the above section). The amplified coding region was verified by nucleotide sequencing and inserted (in-frame) into the EcoRI-XhoI sites of pBD42AD, a plasmid encoding the 88-residue B42 acidic activator and TRP1 marker. To demonstrate the presence of interaction between the two proteins, the two plasmids were cotransfected into EGY48[pop-lacZ] yeast cells, a yeast strain (EGY48) transformed with the autonomously replicating plasmid pop-lacZ (containing URA3 marker). This system allows double selection with both leucine prototrophy and lacZ reporter. Cotransformants were selected in SD/-His/Tsp1/-Ura medium. Protein–protein interaction was demonstrated by growth of the cotransformants as blue colonies on an SD/Gal/Raf/-His/-Trp/-Ura/-Leu plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Results

The basic data of the three groups of patients are summarized in Table 1. They were not significantly different in the number of patients, sex, and the distribution of hepatitis B and C markers. Group 2 patients were, however, significantly older than the other two groups. The tissues in group 2 were named BX-01 to BX-14. The samples in group 3 were named NT1 to NT13 (cancerous tissues). The short stretches of nucleotide deletions in nucleotides 6 to 52 were confirmed by RT-PCR using a second pair of primers (see “Materials and Methods”). The nucleotide sequence data were deposited with GenBank (accession nos. AF213033 to AF213035). The interpreted amino acid sequences of mutants are listed in Fig. 1.

All 12 patients in group 1 had wild-type KAP sequence, whereas 8 of 14 and 6 of 13 cancerous tissues of group 2 and 3, respectively, had mutant KAP sequences. Of group 2 tissues, 4 had nucleotide substitutions leading to either premature stop codons (Mps1; BX-03 and 07) or amino acid substitutions (Maas; BX-01, 05, 07 and 10). 1 had a single nucleotide insertion leading to a truncated reading frame (Mtrf-1; BX-11), and 2 had short stretches of deletions leading to either truncated reading frames (Mtrf-d; BX-02) or in-frame amino acid deletions (Mfid; BX-09). In group 2 tissues harboring KAP mutants, only one form of mutant was detected in each sample. A mixture of wild and mutant type sequences were detected in two samples: BX-11 and BX-03.

Of the cancerous parts of the 13 paired samples in group 3, none had Mps1 and Mtrf-i mutants but 2 (T4 and T9) showed Maas, 2 (T6 and T7) showed Mtrf-d, and 4 (T2, T3, T4, and T6) showed Mfid mutants, respectively. Two different kinds of Mfid mutants were present in T4 (T4-1 and T4-2). Otherwise, only one kind of mutant was detected in other cancerous tissues of group 3. Wild-type KAP sequences coexisted with the mutants as a mixture in all of these cancerous tissues except for T3.

In the noncancerous parts of the paired tissues, mutations were detected in four samples. In one particular patient, a single amino acid substitution was found in the noncancerous tissue (NT1), but only wild-type sequence was present in the cancerous tissue (T1). In NT4 tissue, two amino acid substitutions were identified, which were also present in T4-1 and T4-2. In the remaining two cases, the noncancerous parts contained not only the mutants identical to the cancerous parts (NT2-1/T2 and NT3-1/T3) but also an additional distinct mutant for each sample (NT2-2 and NT3-2). In all these noncancerous tissues, wild-type KAP sequences coexisted with the mutants.

Using the yeast two-hybrid system, the interactions between Cdk2 and KAP proteins were studied. The wild-type KAP and seven KAP mutants (BX-02, BX-07, T2, T3, T4-2, T7 and NT2-2) were tested. Only wild-type KAP, T4-1, and T7 mutants were capable of interaction with Cdk2 protein (Table 2).

Discussion

KAP is capable of dephosphorylating T160 of Cdk2 and thus inhibiting its function in vitro (9). Although it is seemingly an important regulator in the cell cycle, solid evidence demonstrating its crucial role in vivo is lacking (8). In this report, we discovered that multiple forms of KAP mutants were present in HCC but not in hepatitis tissues.

The patterns of KAP mutants were somewhat different between the advanced HCC group (group 2) and the resectable HCC group (group 3): (a) there were two cases with Mps1 mutation in group 2 but none in group 3, whereas there was only one case with Mfid mutation in group 2 but four in group 3; and (b) except for 1 cancerous sample, all tissues harboring mutants in group 3 also possessed wild-type KAP sequences, whereas only two samples in group 2 contained a mixture of wild- and mutant-type KAP. Taken together, there seemed to be a trend that in the precancerous/early stage of HCC, multiple forms of KAP mutants, with a majority in the forms of Mfid, developed. These mutants coexisted with the wild-type KAP sequence. When the tumor grew to an advanced stage, the cell clones with truncated reading frames either caused by Mps1, Mtrf-d, or Mtrf-i mutation were gradually selected, whereas the clones with only wild-type sequence or Mfid were lost. The process of selection was reflected in NT2-1/T2 and NT3/T3, in which two forms of mutants were present in the precancerous parts, whereas only one form was found in the tumor part. The presence of single but not mixed forms of mutants at the advanced stage possibly attributed to deletion of one of the KAP alleles (loss of heterozygosity). Establishment of a valid assay is needed to verify this point. Although this was the likely scheme observed in our patients with KAP mutants, about half of our samples harbored only wild-type KAP sequence. It is still possible that one of the alleles was totally deleted, and thus only the remaining wild-type gene was detected, or the regions matched to our detecting primers were deleted so that the...
transcripts cannot be amplified. Alternatively, in such cases, other mechanisms unrelated to the disruption of the KAP gene were involved in hepatocarcinogenesis.

Because most of the mutants coexisted with the wild-type KAP, it is possible that such mutants interfere with the dephosphorylation ability of wild-type KAP through a competition mechanism. For instance, mutants such as T4-2 and T7 might still bind to Cdk2 but fail to dephosphorylate T160. On the other hand, all but one in-frame amino acid deletion occurred in amino acids 6–52, suggesting that a common mechanism was used. These mutants could be functionally defective, thereby facilitating the growth of tumor cells. Although it is possible to assay the phosphorylation status of T160 in HCC tissues, the results could be very difficult to decipher. Factors other than KAP were shown to regulate phosphorylation of T160 (see “Introduction”). It is therefore difficult to correlate the presence of KAP mutations and the status of T160 phosphorylation in HCC tissues. In contrast, using the yeast two-hybrid system, the interaction between Cdk2 and KAP mutants can be clearly demonstrated. Our results indicated that some KAP mutants failed to bind to Cdk2 and were thus functionally defective.

In summary, we have discovered that in HCC, multiple forms of aberrant KAP transcripts were present. In two cases, the aberrant...
transcripts in the cancerous tissues were also present in the nearby noncancerous tissues, suggesting that the mutations occurred in a precancerous stage and likely contributed to the process of multiple-step hepatocarcinogenesis.

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References

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