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From Mosquito to Man: Identification of a Novel Protein Kinase, HsHPK, Which Is Highly Expressed in Human Hepatoma Tissues

Key Words
Protein kinase
Human liver cancer
Tousled gene

Abstract
Protein kinases play an important role in the signaling pathway of growth factors in most of the higher organisms. During the study of protein kinase profiles of mosquitoes using RT-PCR and degenerate primers for consensus catalytic domain motifs to amplify protein kinase genes, we have noticed that a novel mosquito kinase, AaPK-38, shares a stretch of amino acids identical to the corresponding domain in Tousled gene of *Arabidopsis thaliana* that is required for leaf and flower development. A 2.1-kb cDNA encoding human HsHPK gene, which is a homolog of AaPK-38, was isolated from human testis cDNA library. This cDNA contains an open reading frame of 563 amino acids, with a complete kinase domain in its carboxyl terminus. The expressed Flag-tagged HsHPK was shown to have kinase activity based on in vitro autophosphorylation. Northern blot analysis revealed that human HsHPK mRNA is most abundant in testes, much less in heart and skeletal muscle and almost undetectable in liver and lung. Finally, we found that the expression of HsHPK in 4 out of 6 human hepatoma tissues is much higher than that in the adjacent normal counterpart. This result suggests HsHPK may play a role in the development of human hepatoma.

Introduction

Liver cancer is the leading cause of cancer death among male Chinese in Taiwan. Infection with human hepatitis B virus has been associated with acute and chronic liver diseases and is considered as an etiologic factor for the development of human primary liver cancer [1, 26]. Integration of HBV DNA into human liver DNA has been demonstrated in many cases of liver cancer and in several cultured human hepatoma cell lines [2–4, 21, 22]. However, little is known about how the integrated HBV DNA could affect carcinogenesis of liver cancer. A better understanding of mechanisms for hepatocyte transformation and its growth control are urgently needed for developing new methods for early detection, diagnosis and therapy.

Protein phosphorylations are of central importance in the responses of cells to various internal and external sig-

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nals [reviewed in ref. 6, 11, 13]. Characterization of protein kinase commonly involves protein purification coupled with a suitable in vitro kinase activity assay. In some instances, protein kinase can be purified to a degree with quantity sufficient for amino acid analysis. However, in most cases, protein kinase activities have not been highly purified and the characterization is limited to biochemical studies.

The availability of amino acid sequence data for a number of protein kinases leads to an alternative approach to the identification and subsequent characterization of additional members of this family. Alignment of the sequences for maximum homology reveals that a few short stretches of amino acids within the catalytic domain are highly conserved throughout the protein kinase family [10, 11]. Therefore, a few approaches employing RT-PCR using degenerate primers for the conserved domain of protein kinases have been successfully developed [15, 16, 27].

The utilization of the same signaling components in multiple developmental pathways is emerging as a common theme in many organisms [5, 9, 23, 24]. Conservation of a structure motif of proteins across distant species often implies conservation of an important function of that protein [12, 14]. To identify a human homologue of evolutionarily conserved novel kinase [10, 11], we first identified a kinase in mosquitoes, AaPK-38, which exhibits a stretch of amino acids in its catalytic domain identical to a kinase in *Arabidopsis thaliana* that is required for leaf and flower development [17]. We then isolated the cDNA of a human homolog to AaPK-38, *Homo sapiens* hepatoma protein kinase (HsHPK), which also shows 100% conservation in that one region. We also found that HsHPK has a unique tissue expression pattern and is highly expressed in human hepatoma tissue.

Material and Methods

Rapid Amplification of cDNA Ends (RACE) and cDNA Library Screening

Total RNA was prepared from human hepatoma cell line Huh7 by the guanidinium isothiocyanate method [7]. RACE procedures were according to the user manual of the Marathon cDNA amplification kit (Clontech, Palo Alto, Calif., USA). First-strand cDNA was synthesized from 10 μg Huh7 total RNA by MMLV-reverse transcriptase with adapter-linked oligo-dT as a primer. The 3′-RACE was performed by PCR amplification of the cDNA with a primer specific for human HsHPK 5′ primer CCGGATCCATGGAGCTAACATCAC in conjunction with the adapter primer (AP1). The PCR condition was carried out for 25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 4 min. The PCR products were subcloned into pCRII (Invitrogen, Carlsbad, Calif., USA) and analyzed.

A 1-kb fragment derived from the 3′-RACE experiment was labeled with [α-^{32}P]dCTP by random priming (Amersham, UK) and used as probe to screen a human testis λDR2 cDNA library (Clontech). The cDNA clones were isolated by in vivo excision.

DNA Sequencing

The DNA sequence analysis was performed by dideoxy-chain termination method [20].

In vitro Phosphorylation of FLaG-Tagged HsHPK

The HsHPK cDNA was subcloned into pFlag-CMV2 vector (Eastman Kodak, New Haven, Conn., USA) by *Hind* III and *Xba*I and named as pFlag-CMV2-HsHPK. pFlag-CMV2 and pFlag-CMV2-HsHPK were transfected into human hepatoma cell line Huh7 using a calcium phosphate method [8]. The transfected cells were lysed after 48 h by lysis buffer containing 50 m*M* Tris-HCl, pH 7.8; 4 m*M* EDTA; 2 m*M* EGTA; 5 mg/ml leupeptin; 2 m*M* phenylmethyl-sulfonyl-fluoride; 5 mg/ml aprotinin; 1 m*M* sodium vanadate; 40 m*M* pyrophosphate; 50 m*M* sodium fluoride; 1% Triton X-100. To immunoprecipitate the FLaG-tagged HsHPK protein, 300 μg cell lysate were incubated with 15 μl anti-FLaG M2 affinity gel (Eastman Kodak) at 4 °C for 3 h. The immuno-precipitates were washed with lysis buffer four times, and rinsed with kinase buffer (20 m*M* Hepes, pH 7.2, 2 m*M* DTT, 0.1 m*M* EGTA, 0.1 μg/ml BSA, 0.1 m*M* sodium vanadate, 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$). For kinase reaction, the precipitates were resuspended in 20 μl kinase buffer containing 20 μ*M* ATP, 5 μCi [r-^{33}P] ATP. The reactions were incubated at 30 °C for 30 min and terminated by boiling in Laemmli-SDS-PAGE sample buffer followed by electrophoresis in a 10% SDS-polyacrylamide gel. Phosphorylation of HsHPK was visualized by autoradiography.

Northern Blot Analysis

The 2,145-bp HsHPK cDNA insert released by *Bam*HI and *Xba*I digestion was labeled with [α-^{32}P]dCTP by random priming (Amersham). The radio-labeled probe was hybridized to human multiple tissue RNA blots (Clontech) for Northern blot analysis as described [25].

Expression Level of HsHPK RNA in Different Tissues Isolated from Hepatoma Patients by RT-PCR

Total RNA was prepared from different hepatoma patients by the guanidinium isothiocyanate method [7]. For cDNA synthesis, 10 μg total RNA were reverse transcribed by incubation for 1 h at 42 °C in the presence of 25 units of AMV-reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) in 20 μl buffer containing 50 m*M* Tris-HCl (pH 8.5), 8 m*M* $MgCl_2$, 30 m*M* KCl, 1 m*M* dithiothreitol, 1 m*M* dNTP mix, and 0.5 μg of oligo-dT. PCR were carried out in 25 μl reaction mixture containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 1.5 m*M* dNTP, 100 pmol of each primer (5′ primer of HsHPK: ATGAGCAAGAAACTCCTTAT, 3′ primer of HsHPK: AACGAGTCAGTATCCAGAG, 5′ primer for transferrin: GGAACATTTTGGCAAAGACA and 3′ primer for transferrin: ATTCATGATCTTGGCGATGC), 1 μl of cDNA, and 2 units Taq DNA polymerase (Promega, Madison, Wisc., USA).

The PCR reactions were carried out for 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. The PCR products were analyzed with 1% agarose gel.

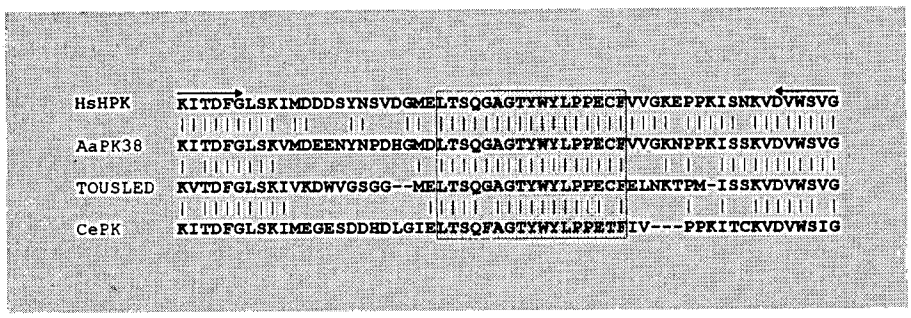

Fig. 1. Amino acid sequence alignment of partial HsHPK with homologous kinases from the databank. Deduced amino acid sequence of partial HsHPK is aligned with the corresponding region of homologous kinases from the mosquito *A. aegypti* protein kinase (AaPK-38), *A. thaliana* (Tousled), and *C. elegans* (CePK). Vertical lines indicate identical residues. Arrows denote primers used in PCR. The shaded area reveals extremely high homology among diverse species.

Results

Isolation and Characterization of Human HsHPK cDNA

In the course of studying protein kinase profiles of immunized mosquitoes, *Aedes aegypti*, using RT-PCR and degenerate primers for conserved catalytic domains to amplify protein kinase genes, we have noticed that one PCR fragment (AaPK-38) with 59 amino acids in length shares high homology with a stretch of amino acids (18 out of 19) in Tousled gene of *A. thaliana* (fig. 1). This stretch of sequence has not been found in any other known reported kinase gene. We then decided to search for the human homolog of AaPK-38. We first found that this conserved kinase fragment could be detected by RT-PCR in human hepatoma Huh7 cells. Then we used 3′-RACE to obtain a 1-kb cDNA fragment containing the unique conserved sequence and used that cDNA probe to screen human testis cDNA library. Seven overlapping cDNA clones were identified. Figure 2A shows the sequence of the longest cDNA clone isolated with 2.1 kb in length. It encodes 563 amino acid residues. The deduced protein sequence reveals the presence of an intact conserved kinase domain in the carboxyl terminus. Sequence alignment analysis shows that HsHPK shares 25% identity with Tousled in the NH_2-terminus and 54% in the kinase domain (fig. 2B). The data also indicates that the 2.1-kb cDNA clone may lack a portion of the N-terminus. Nevertheless, as shown below, this partial cDNA clone is biologically active.

Expression of HsHPK in Human Tissues

The expression pattern of the human homolog of Tousled kinase in various human tissues was examined by Northern blot analysis using a 2.1-kb human HsHPK cDNA clone as probe (fig. 3). One major 4.0-kb mRNA was detected, with the highest level found in the testis. Other tissues such as the heart, or skeletal muscles also express HsHPK but at a much lower level. Interestingly, in liver and lung the expression is barely undetectable. The expression pattern of HsHPK in human tissues supports the hypothesis that the normal function of HsHPK in humans is during development as is the case for Tousled kinase in plants.

Kinase Activity of HsHPK Protein in vitro

To ascertain that the isolated HsHPK cDNA is biologically active, we first tested the auto-phosphorylation activity in vitro. To express HsHPK protein in the cultured hepatoma cell line Huh7, a Flag-tagged HsHPK plasmid (pFlag-CMV2-HsHPK) was constructed. After transfection, the Flag-tagged HsHPK was immuno-precipitated with anti-Flag monoclonal antibody M2 and incubated in a kinase reaction containing [γ-^{33}P]ATP. As shown in figure 4, phosphorylation of a 60-kD protein band was detected only in the pFlag-CMV2-HsHPK-transfected cell lysate, but not in the mock-transfected cell lysate. The size of the phosphorylated protein is consistent with the predicted molecular weight of HsHPK cDNA. This result suggests that the kinase encoded by the 2.1-kb cDNA is active.

Expression of Human HsHPK in Human Hepatoma Tissues

Since the HsHPK transcript has been identified in the human hepatoma cell line Huh7 but was barely detectable in normal human liver tissue, we wished to determine whether HsHPK plays any role in the development of human hepatoma. We examined HsHPK mRNA in normal human liver, two cultured human hepatoma cell lines and 6 pairs of human hepatoma and their adjacent normal tissues by RT-PCR using specific primers for HsHPK. Similar reactions were performed using specific primers for transferrin as a control to normalize the amount of mRNA in each reaction (fig. 5). As we have observed before, the normal human liver sample expresses very little HsHPK mRNA, whereas the two human hepatoma cell lines, Hep3B and Huh7, express a significant level of HsHPK mRNA. In 4 out of 6 paired

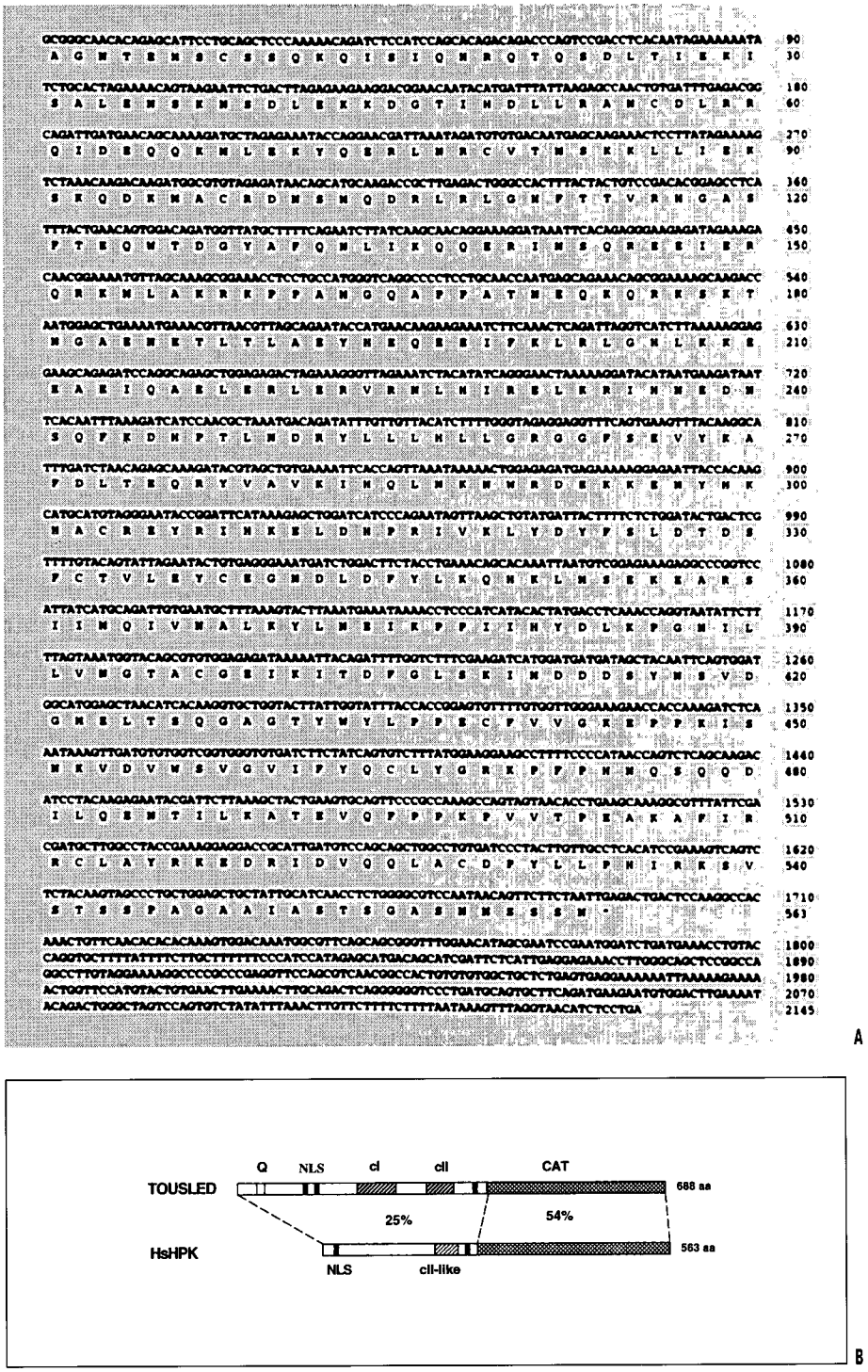

Fig. 2. A HsHPK cDNA and deduced protein sequence. **B** Comparison of HsHPK and Tousled proteins.

human hepatoma tissues, HsHPK preferentially expressed in the hepatoma tissues, but at a much lower level in the adjacent liver tissues. In the other 2 paired tissues, both hepatoma and the adjacent liver tissues express equal amount of HsHPK transcripts.

Discussion

In this study, we describe the isolation of a novel human serine/threonin kinase clone, HsHPK, which shares sequence homology to that of the kinase AaPK-38

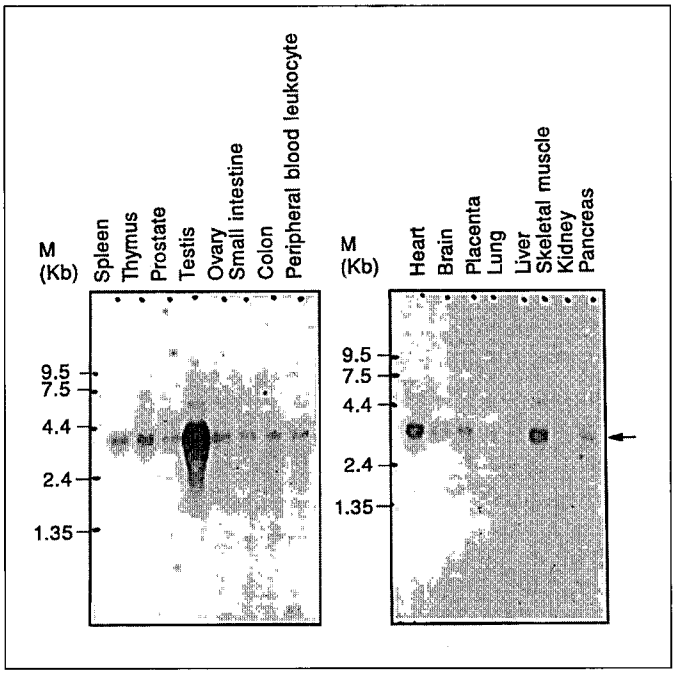

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Fig. 3. Tissue distribution of human HsHPK gene. Northern blot analysis was performed with human multiple tissue RNA blots (H, H1 from Clontech) by using 2.1-kb HsHPK cDNA fragment as probe. The location of HsHPK mRNA is indicated by arrow.

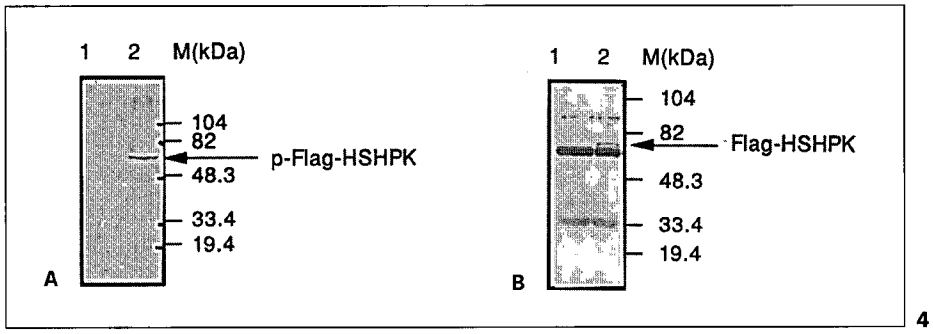

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Fig. 4. Autophosphorylation of Flag-tagged HsHPK protein in vitro. **A** Vector pFlag-CMV2 (lane 1) and pFlag-CMV2-HsHPK (lane 2) were transfected into human hepatoma cell line Huh7 and the Flag-tagged proteins were immuno-precipitated by anti-Flag antibody M2. The immuno-precipitants were subjected to auto-phosphorylation reaction, then applied to 10% SDS-PAGE and autoradiography. The phospho-Flag-tagged HsHPK is indicated by arrow. **B** The immuno-precipitants were also subjected to Western blot analysis by M2 antibody. Lane 1 shows pFlag-CMV2 immuno-precipitants, and lane 2 pFlag-CMV2-HsHPK immuno-precipitants. The Flag-tagged HsHPK is indicated by arrow.

isolated from mosquitoes and one from plant Tousled gene [17]. Even though the overall sequence homology between the human kinase and the plant kinase is only 41%, the presence of HsHPK in insect, plant and mammalian cells suggests that this type of kinase may serve an important role for the development of organisms and is well preserved during evolution. The most conserved domain of the human and plant kinase is localized in the catalytic domain VIII of the kinase. In this domain, the two kinases share almost 100% homology in a stretch of 19 amino acids. In the catalytic domain of the protein kinase, subdomain VIII has been suggested to be as a contact site for the substrate of the kinase [11]. The availability of a human clone paves the way to identify protein which may specifically interact with this conserved domain.

The highest expression of human HsHPK was found in the testis, a sex organ equivalent to the flower of plants,

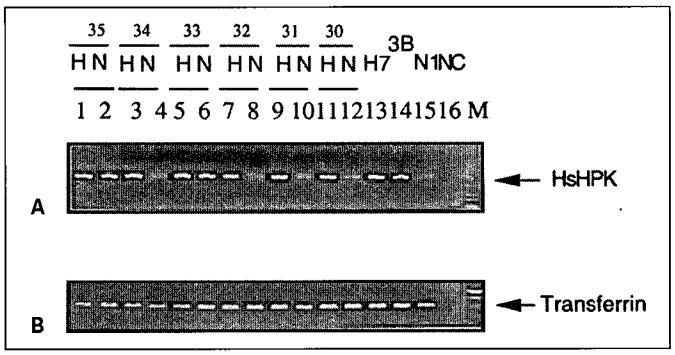

Fig. 5. HsHPK gene expression in hepatoma and tumor-adjacent tissues by RT-PCR. The RT-PCR reactions were set as described in the Methods. Six pairs of tissues were tested. The numbers represent the 6 patients. H = Hepatoma parts; N = tumor-adjacent tissues; NC = negative control. A normal liver tissue (N1) and two human hepatoma cell lines, Huh7 (H7) and Hep3B (3B), were also included. **A** RT-PCR result of HsHPK, **B** Transferrin.

suggesting that the normal function of HsHPK in human development may be similar to the Tousled kinase in plant. Mutation of the Tousled kinase in *A. thaliana* results in a complex phenotype, the most dramatic of which is the abnormal flowers produced in mutant plants. *tsl* flowers show a random loss of floral organs, and organ development is impaired [17–19]. The functional role of Tousled kinase in the leaf and flower development is still not clear. However, recent results suggest that the Tousled kinase is localized in the nucleus and may form oligomers by coiled-coil region [18]. It may function as a key regulator to modulate transcriptional factors and to direct a development program in the nucleus. Using specific antibody against an 18-amino acid peptide which corresponds to the most conserved amino acid sequence in kinase subdomain VIII of HsHPK, we also observed that the HsHPK is mainly localized in the nucleus of human hepatoma Huh7 and HeLa cells [unpubl. observation].

In contrast to normal human liver, where HsHPK was expressed at the lowest level, HsHPK is ubiquitously overexpressed in both cultured human hepatoma cell lines and human hepatoma tissues. Even though the number of human hepatoma tissues we examined is small, our observation raises the interesting possibility that deregulation of HsHPK may be involved in the development or the maintenance of the transformation phenotype of human hepatoma cells. Experiments are ongoing to study this possibility.

Acknowledgments

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