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The basal body gene, *RPGRIP1L*, is a candidate tumour suppressor gene in human hepatocellular carcinoma

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ABSTRACT

Loss of heterozygosity (LOH) on chromosome 16q is one of the most frequent genetic alterations in hepatocellular carcinoma (HCC). Our previous data showed that the smallest common deleted region was between *D16S415* and *D16S419*, encompassed approximately by a 0.75 cM region on 16q12.2, suggesting that the putative tumour suppressor genes (TSGs) at this locus might be involved in the development of HCC. Of the four genes (*CHD9*, *RBL2*, *AKTIP* and *RPGRIP1L*) located in this region, only *RPGRIP1L* was downregulated in HCCs.

Downregulation of *RPGRIP1L* was found in 91% (10/11) HCC cell lines and in 35% (14/40) HCCs, respectively. To investigate the role of *RPGRIP1L* in HCCs, we used the overexpression of *RPGRIP1L* in four HCC cell lines (HepG2, Huh6, Huh7 and Hep3B). Overexpression of *RPGRIP1L* suppressed colony formation of tumour cells. Conversely, expression of *RPGRIP1L***M** (dominant negative form) in HCC cells enhanced colony formation. Furthermore, knockdown *RPGRIP1L* by RNA interference in SK-HepI cells promoted colony formation. Taken together, these data strongly suggest that *RPGRIP1L* might be the putative TSG in HCC. Moreover, we showed that Mad2, Survivin and Securin were elevated in *RPGRIP1L***M**-HepG2 transfectants and *RPGRIP1L*-shRNA-SK-HepI transfectants. After knockdown of *MAD2* in *RPGRIP1L*-shRNA-SK-HepI transfectants partly reverse cellular colony formation capability. These data suggest that *RPGRIP1L* suppresses anchorage-independent growth partly through the mitotic checkpoint protein Mad2.

1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide.[1–3] It is highly malignant, and death usually results within one year after the onset of symptoms. Despite intense study in the past decades, the detailed molecular mechanism of hepatocarcinogenesis is not fully understood.[4] It is now well recognised that tumourigenesis is a multistep process resulting from the accumulation of sequential genetic and epigenetic alterations.[4] In addition to oncogene activation, inactivation of tumour suppressor genes (TSGs) has been shown to play an important role in tumourigenesis.[4–8] Allelic deletion manifested as loss of heterozygosity (LOH) at polymorphic loci is recognised as a hallmark of tumour

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suppressor genes, whose other allele is inactivated by point mutations, methylation or by some other mechanism.[7,9,10] The delineation of such genetic alterations that occur in hepatocellular carcinoma may be important for both early detection and prognosis.[7,9–11]

In hepatocellular carcinoma, LOH has been described on one or both arms of multiple chromosomes, including 1p, 4q, 5q, 6q, 8p, 8q, 10q, 11p, 13q, 16q, 17p and 22q.[7–9,12–18] Among these alterations, LOH on chromosome 16q has been reported to occur more frequently in HCCs of poor differentiation or larger size, and with metastasis.[17,19] Recently, in the analysis of chromosomal abnormality in HCC[6] and in a comprehensive analysis of LOH,[8] we also observed that two of the most commonly affected areas spanned the region near the sequence tag site (STS) markers *D16S415* and *D16S419*.[8,20] The high incidence of LOH observed at an early stage of tumour development led us to speculate that candidate tumour suppressor genes located in this region may play an important role in early hepatocellular carcinoma. Other tumour types, such as prostate[21] and breast cancer,[22] also exhibit similar abnormalities. Due to these findings, considerable research effort has been made in an attempt to identify the putative tumour suppressor gene(s) that may reside in the long arm of chromosome 16.

RPGRIP1-like protein (RPGRIP1L) is a homologue of RPGRIP1 (Retinitis Pigmentosa GTPase Regulator Interacting Protein 1). Recently, two groups have shown that RPGRIP1L colocalises at the basal body and centrosome with the protein product nephrocystin-4 (NPHP4) whose gene is known to be associated with cerebello-oculo-renal syndrome (CORS, also called Joubert syndrome type B).[23,24] CORS is defined by the features of Joubert syndrome with the addition of one or more of the following: renal involvement, retinal dystrophy, coloboma, polydactyly, tongue tumours, and liver fibrosis.[23,24] Our previous data demonstrated that the minimal common region of deletion was near locus *D16S415*, encompassed approximately by a 0.75 cM region on 16q12.2, suggesting that the putative tumour suppressor genes at this locus might be involved in the development of HCC.[8,20] Of the four known genes (*CHD9*, *RBL2*, *AKTIP* and *RPGRIP1L*) located in this minimal deletion region, only *RPGRIP1L* was considered an excellent candidate, as its product has been shown to be colocalised at the basal body and centrosome with NPHP4.[23]

Centrosomes have a crucial role in the formation of bipolar mitotic spindles, which are essential for accurate chromosome segregation.[25] The mitotic spindle checkpoint is a signal pathway which ensures that sister chromatids aligned at the metaphase plate do not separate prior to the bipolar attachment of all duplicated chromosomes to the mitotic spindle.[25,26] Mad2 is a central component of this pathway, because it is essential for inhibiting the E3 ubiquitin ligase cdc20-APC, which itself targets Securin, a negative regulator of separase, as well as Cyclin B for degradation.[25,26] Mad2 overexpression is a common event seen in many human cancers.[25,26] Mad2 overexpression in human fibroblasts and cell lines can stabilise Securin and Cyclin B, delay exit from mitosis and increase non-disjunction events and aneuploidy, thus contributing to tumourigenesis.[25,26] In this study, we performed genetic analysis and functional analyses, and found that the *RPGRIP1L* gene is a candidate tumour suppressor gene. We also found that *RPGRIP1L* suppresses tumour cell transformation partly through regulation of the mitotic checkpoint protein Mad2.

2. Materials and methods

2.1. HCC specimens

Forty HCC patients were selected for this study. A hematoxylin-and-eosin-stained section from each of the selected blocks was used to map the tumour and non-tumour areas. Both tumour and non-tumour parts were frozen immediately after surgery and were stored at −135 °C until use. The HCC tissues, DNA and RNA samples were provided by the Taiwan Liver Cancer Network (TLCN). The TLCN is funded by the National Science Council to provide with primary liver cancer tissue and their associated clinical information. The use of the 40 HCC tissues and paired non-tumour parts in this study was approved by our Institutional Review Board and the TLCN User Committee.

2.2. DNA and RNA extraction

Genomic DNA and total RNA were prepared from the tumour and non-tumour liver tissues using conventional procedures.[20] Forty pairs of qualifying RNA were further analysed by RT-PCR and quantitative RT-PCR. Total RNA from normal livers was purchased from three biotechnology companies including BD (BD Biosciences, Clontech, CA, USA), MDBIO (Taipei, Taiwan, ROC) and GenDiscovery (Taipei, Taiwan, ROC).

2.3. RPGRIP1L DNA construct and antibodies

We amplified *RPGRIP1L* (amino acid sequences 1–1315 of GenBank entry NM_015272) by RT-PCR; then cloned it into pcDNA3.1, a V5-tagged expression vector, and sequenced, aligned and compared the findings with a reference database. We also amplified *RPGRIP1L***M** (amino acid residues 1–381 of GenBank entry NM_015272) (C-terminus truncated form) and then cloned it into the same expression vector. A rabbit polyclonal antibody to RPGRIP1L was generated against the human sequence (peptides 983–998, HQSDETSPPPEDRKEI, and peptides 1029–1043 EVKENTEKMQQGKDD).

2.4. Predicted protein sequence analysis

The predicted *RPGRIP1L* amino acid sequence was analysed using the BLASTP and PSIBLAST algorithms in search for matches or homologies in GenBank protein databases. The prediction of protein domains was conducted using the Pfam domain models (PFAM: multiple alignments and profile HMMs of protein domains release 4.3. The Pfam Consortium, http://pfam.janelia.org/).

2.5. In vitro translation

In vitro translation was performed using an *in vitro* transcription–translation reticulocyte assay (TNT T7 Quick coupled Transcription/Translation System; Promega, CA, USA) with