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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

full-length *RPGRIP1L* cDNA as a template. [^{35}S] Methionine-labelled products were analysed by SDS–PAGE followed by autoradiographic detection.

2.6. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

We isolated the total RNA from each sample using RNeasy kits (Qiagen, Valencia, CA, USA). An additional DNase I digestion procedure was included in the isolation of RNA to remove contaminating DNA following the manufacturer's protocol. One microgram of total RNA from each sample was subjected to cDNA synthesis using Superscript III reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR analysis was performed on an ABI 7500 Real-Time System (Applied Biosystems, Forster City, CA, USA). *GAPDH* was used as an internal control. PCR reaction was conducted using an SYBR PCR master mix reagents kit. Relative gene expression was determined based on the threshold cycles (Ct) of the gene of interest and of the internal reference gene. The mRNA levels of the genes of interest were expressed as the ratio of the gene of interest to *GAPDH* mRNA for each sample. The level of mRNA for each gene of interest in each transfectant was compared with the level in the empty vector transfectant. QRT-PCR primer sequences are summarised in Supplementary Table 1.

2.7. Formation of stable colonies of transfected cells

Cells were seeded in six-well plates and were allowed to reach 80% confluence. The cells were transfected with pcDNA-*RPGRIP1L*, pcDNA-*RPGRIP1L***M** (C-terminus truncated form) or empty vector controls using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. After 6 h of transfection, the medium was replaced with fresh serum-containing medium. For stable transfectants, the medium was replaced after 48 h with G418-containing medium, and transfectants were selected.

2.8. RNA Interference

Short hairpin RNA (shRNA) sequences were cloned downstream of the human H1 promoter in the vector pSuper as described[27]. The target sites for *RPGRIP1L*-shRNA were 5′-GGGACAATGTAGAAATGAT-3′ (shRNA1) and 5′-GGAAATTTCACCAGAGGTA-3′ (shRNA2). Cell lines (SK-Hep1) that expressed *RPGRIP1L* were transfected with different shRNA constructs to evaluate any effects on colony formation. The target site for *MAD2*-shRNA was 5′-CGCAATGGATATTTGTACTGTT-3′.

2.9. Soft agar assay

A soft agar assay with HCC cells was performed as previously described. HCC cells (from 2×10^4 to 10×10^4) were suspended in 1.5 ml of 0.3% agarose and poured onto a 1.5 ml of 0.7% agarose bed in 3.5 mm tissue culture dishes. Colonies were counted after 3–4 weeks.

2.10. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 3000 cells/well. At different time points, cell viability was determined by using an MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] assay (Promega Corporation, Madison, WI, USA).[27] Briefly, MTS reagent (20 μl/ well) was added to 100 μl of medium containing cells in each well of 96-well plates and left for 1 h at 37 °C under humidified 5% CO_2 in air. For colorimetric analysis, the absorbance at 490 nm was recorded using a microplate reader. Each condition was repeated at least three times. Total cells were harvested at the designated times after treatment.

2.11. Western blot

A standard protocol was used. Anti-V5 polyclonal antibody was purchased from Invitrogen (Carlsbad, CA, USA) and anti-Mad2 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin, anti-Cyclin B1 and anti-Securin monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-Survivin was purchased from Novus Biologicals (Littleton, CO, USA). We generated rabbit polyclonal antibody against the human RPGRIP1L sequence (peptides 983–998 and 1029–1043). Antibody specificity has been confirmed by recombinant RPGRIP1L. Protein extracts were prepared and examined as described.[1,27,28]

2.12. Statistical analysis

SPSS (version 13, SPSS, Chicago, IL, USA) was used for statistical analyses. For *RPGRIP1L* overexpression and shRNA knockdown analysis, data are shown as means ± SD. Student's *t* test was used for comparing activities of different constructs. Fisher's exact test was used for association analysis between Hepatitis B virus (HBV)-associated HCCs and *RPGRIP1L* downregulation. The significance level was defined as $P < 0.05$.

3. Results

3.1. Expression evaluation of genes from the minimal region of deletion

In this study, the Entrez gene database at the NCBI (http://www.ncbi.nlm.nih.gov) was reviewed. Four protein-coding genes (*CHD9*, *RBL2*, *AKTIP* and *RPGRIP1L*), one pseudogene *LOC100132875* and one hypothetical gene *LOC643802* were located within the 0.75-Mb minimal deletion regions (Fig. 1). To determine which gene is the best candidate for the 16q12.2-tumour suppressor gene; we first examined their expression in normal livers using the quantitative reverse-transcription PCR (RT-PCR) assay as a positive control. The expression of the hypothetical genes *LOC643802* and *CHD9* was not detectable in normal livers (data not shown). Next, we prescreened *RBL2*, *AKTIP* and *RPGRIP1L* in 11 HCC cell lines, using quantitative RT-PCR. Only *RPGRIP1L* was frequently downregulated in HCC cell lines (91%, 10/11) (Fig. 1D and Supplementary

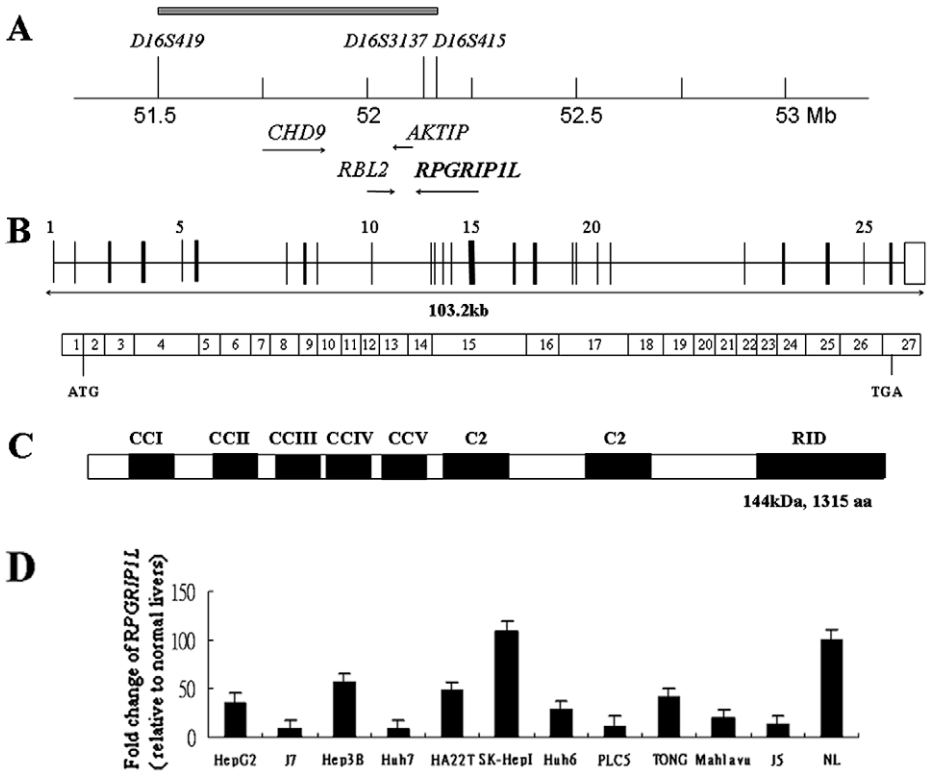

Fig. 1 – Gene structure and conserved domain of RPGRIP1L and expression of *RPGRIP1L* in HCC cell lines. (A) The minimal interval (gray bar) was defined between *D16S419* and *D16S415*, encompassed approximately by a 0.75 cM region of LOH. This interval contains four candidate genes (arrows indicate transcription direction). (B) Exon–intron structure of the human *RPGRIP1L* gene and transcript. RPGRIP1L is composed of 27 exons (vertical hatches) and extends over 103.2 kb. (C) Representation of the domain structure of the putative RPGRIP1L protein. CC, coil-coiled domain; C2, protein kinase C (PKC) conserved region 2 motif; RID, domain with homology to the RPGR-interacting domain of RPGRIP1. One RPGRIP1L mutation was located at the N-terminal C2 domain of RPGRIP1L. (D) *RPGRIP1L* expression in eleven HCC cell lines was analysed by quantitative RT-PCR. In comparison with the average of five normal livers, RPGRIP1L was frequently downregulated in HCC cell lines. *RPGRIP1L* expression was normalised with *GAPDH* as an internal control. Error bars indicate SD.

Fig. 1A) compared with RNA from five normal livers. Then, we analysed the expression of *RPGRIP1L* in 40 HCC case tissues and paired these with non-tumour components using quantitative RT-PCR (Table 1 and Supplementary Table 2). We found that *RPGRIP1L* was downregulated in 12 cases of 20 HBV-associated HCCs. Conversely, only two cases showed RPGRIP1L downregulation in twenty non-HBV-associated HCCs. There was a significant correlation between *RPGRIP1L* downregulation (<50%) and HBV-associated HCC (12/20 versus 2/20, $P < 0.05$, Supplementary Table 3).

3.2. *Cloning and characterisation of RPGRIP1L*

The BLAST and PSIBLAST algorithms were used to search for matches in GenBank databases. Interestingly, we identified four conserved domains within the putative RPGRIP1L protein (amino acids 47–580, 615–712, 791–896 and 1025–1315) (Fig. 1C). The first domain is a coil-coiled domain and the second domain is C2, which exhibits features of protein kinase C (PKC) conserved region 2. The third domain is another C2, which shows further homology to the C2 domain of PKC. The fourth domain is RID, a domain with homology to the RPGR-interacting domain of RPGRIP1. To elucidate the possible tumour suppressor function of RPGRIP1L, we constructed recombinant *RPGRIP1L* and used an *in vitro* transcription–translation system and western blotting to check whether recombinant *RPGRIP1L* could produce the predicted protein. The SDS–PAGE and Western blot analysis of the translated product revealed a single protein product of about 145 kDa (Supplementary data Fig. 1B). An interaction between the C-terminal C2 domain of RPGRIP1L and nephrocystin-4 has been demonstrated by coimmunoprecipitation.[23,24] Therefore, we constructed a C-terminal truncated *RPGRIP1L* mutant (*RPGRIP1L***M**), which contains 381 amino acid (Supplementary data Fig. 1B) and used it as a dominant negative mutant.

3.3. *Overexpression of RPGRIP1L in HCC cells suppresses colony formation*

Because *RPGRIP1L* is frequently downregulated in human HCCs, we next tested whether overexpression of *RPGRIP1L* affected the HCC cell growth and survival potential. Four different cell lines were employed to evaluate this possibility. Firstly, HepG2 cells stably expressing V5-tagged *RPGRIP1L* full length or C-terminus truncated *RPGRIP1L***M** were established in culture. Stable transfectants with increasing levels of

Table 1 – Relative quantitation using the comparative Ct method.

Patient No.	*RPGRIP1L* (T/N)	HBsAg	AntiHCV
1T	0.13	+	–
1N			
2T	0.08	+	–
2N			
3T	4	+	–
3N			
4T	0.4	+	–
4N			
5T	2.51	+	–
5N			
6T	1.06	+	–
6N			
7T	0.07	+	–
7N			
8T	0.01	+	–
8N			
9T	0.38	+	–
9N			
10T	1.21	+	–
10N			
11T	0.181	+	–
11N			
12T	0.48	+	–
12N			
13T	0.99	+	–
13N			
14T	1.9	+	–
14N			
15T	0.94	+	–
15N			
16T	0.2	+	–
16N			
17T	2.86	+	–
17N			
18T	0.3	+	–
18N			
19T	0.4	+	–
19N			
20T	0.3	+	–
20N			
21T	0.9	–	+
21N			
22T	0.87	–	+
22N			
23T	0.9	–	+
23N			
24T	1.06	–	+
24N			
25T	0.71	–	+
25N			
26T	0.96	–	+
26N			
27T	0.96	–	+
27N			
28T	0.9	–	+
28N			
29T	0.25	–	+
29N			
30T	0.71	–	+
30N			
31T	0.9	–	–
31N			
32T	0.47	–	–
32N			
33T	1.33	–	–
33N			
34T	4.6	–	–
34N			
35T	0.97	–	–
35N			
36T	0.99	–	–
36N			
37T	0.91	–	–
37N			
38T	0.9	–	–
38N			
39T	1.15	–	–
39N			
40T	0.95	–	–
40N			

N, non-tumour part; T, tumour part. The range given for RPGRIP1L tumour part relative to non-tumour part is determined by evaluating the expression: $2^{-\Delta\Delta Ct}$.

RPGRIP1L were selected (Fig. 2A and B) and these were examined to explore whether these stable cells affect HCC cell growth and survival. The growth of these transfected HepG2 cells was determined at various time points using a cell proliferation assay. The growth of *RPGRIP1L*-HepG2 and *RPGRIP1L***M**-HepG2 transfected cells was slightly different from the cells transfected with vector control (Supplementary Fig. 2), but not statistically significant. Similar results were found in Hep3B, Huh6 and Huh7 cells (Supplementary Fig. 2). The colony formation results showed that vector controls and *RPGRIP1L***M** HepG2-stable transfectants could attach and grow well in soft agar; however, the *RPGRIP1L* HepG2-stable transfectants formed very few colonies in soft agar (Fig. 2C). Vector controls grew approximately 10-fold better than *RPGRIP1L*-HepG2 transfectants; and *RPGRIP1L***M** HepG2-stable transfectants grew approximately 1.5-fold better than vector controls. Similar results, suggesting that *RPGRIP1L* overexpression suppresses cell survival in soft agar, were found for Hep3B, Huh6 and Huh7 cells (data not shown).

3.4. *Knockdown of endogenous RPGRIP1L in HCC cells promotes colony formation*

To further explore whether *RPGRIP1L* might affect the cell growth and survival of HCC cells, we used an RNAi approach to inhibit expression of *RPGRIP1L* and assayed for growth rate of SK-HepI cells. To examine whether the *RPGRIP1L* shRNAs could inhibit endogenous *RPGRIP1L* expression in SK-HepI, we transfected two *RPGRIP1L* shRNA constructs (shRNA-1 and shRNA-2) and a negative control into SK-HepI for 72 h followed by western blotting and quantitative RT-PCR to detect the level of *RPGRIP1L* expression. The data showed a reduction in the *RPGRIP1L* protein and mRNA level with both shRNAs, whereas there was no significant reduction in *RPGRIP1L* when the vector control was used (Fig. 3A and B). The growth rate of these transfected SK-HepI cells was determined at various

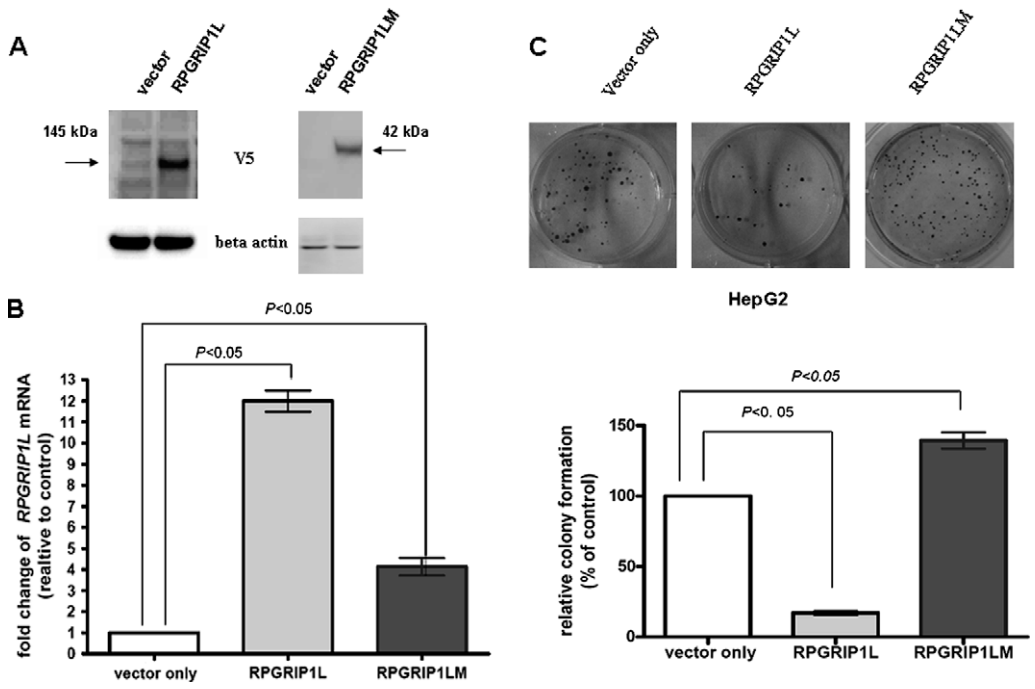

Fig. 2 – Overexpression of *RPGRIP1L* in HepG2 cells suppressed tumour cell survival in soft agar. (A) Western blot analysis was used to detect RPGRIP1L expression levels before and after overexpressing *RPGRIP1L* or *RPGRIP1LM* (upper panel). (B) Quantitative RT-PCR was used to confirm changes in mRNA expression of *RPGRIP1L* or *RPGRIP1LM* overexpression. (C) Number of colonies in the cells transfected with the *RPGRIP1L* decreased significantly ($P < 0.05$) compared with those in the vector controls. The bars show the means ± SDs.

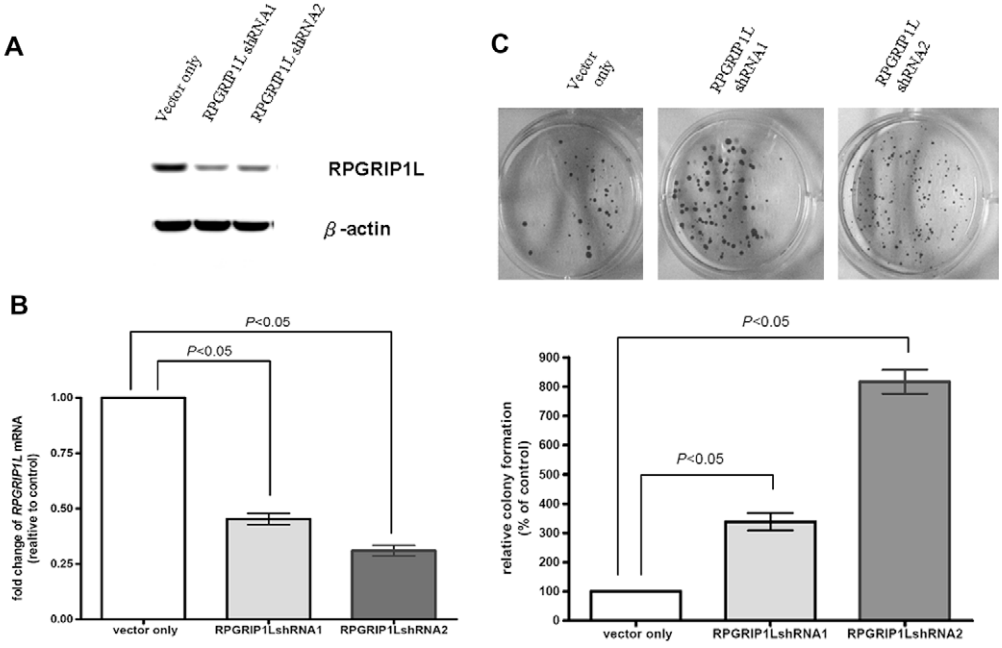

Fig. 3 – Knockdown of endogenous *RPGRIP1L* in SK-Hep1 cells promoted cell survival and transformation. (A) Western blot analysis was used to detect RPGRIP1L protein expression levels before and after *RPGRIP1L* shRNA treatment. (B) Quantitative RT-PCR was used to confirm shRNA-directed downregulation of *RPGRIP1L* expression. (C) Numbers of colonies in the cells transfected with the *RPGRIP1L* shRNA1 or *RPGRIP1L* shRNA 2 increased significantly ($P < 0.05$) compared with those in the vector controls. The bars show the means ± SDs. Experiments were performed in triplicate, and representative results are shown.

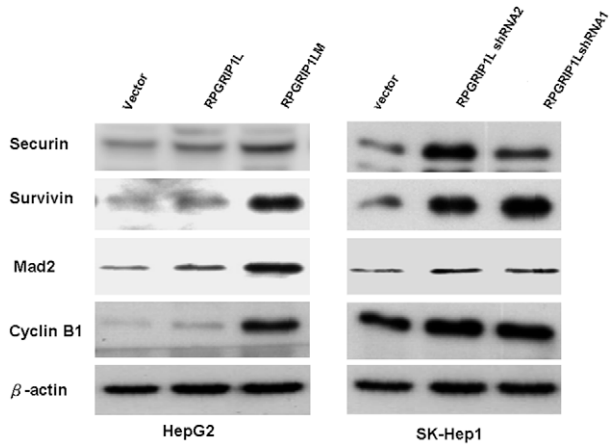

Fig. 4 – Western blot analysis of protein related with mitotic spindle checkpoint in *RPGRIP1L* M-HepG2 transfectants and *RPGRIP1L* -shRNA-SK-HepI transfectants. Western blotting was performed to demonstrate the expression levels of securin, survivin, Mad2 and cyclin B1 in these cells. Equal amounts of total protein extract from each cell clone were analysed by blotting with securin, survivin, Mad2 and cyclin B1 antibodies, and a β-actin antibody was used as an internal control.

time points using the MTS assay. The proliferation rate of *RPGRIP1L*-shRNAs transfected cells was slightly different from that in cells transfected with vector control, but the difference was not statistically significant (Supplementary Fig. 3). On the other hand, *RPGRIP1L*-shRNAs SK-HepI-stable transfectants could attach and grow well in soft agar; however, the vector control formed fewer colonies in soft agar (Fig. 3C). *RPGRIP1L*-shRNA transfectants grew approximately 3–8-fold better than vector controls. The data strongly support the idea that *RPGRIP1L* plays a role in cell survival.

3.5. *Possible mechanism by which RPGRIP1L suppresses cellular colony formation in HCC*

In our unpublished data, we found that the mRNA level of cell-cycle and mitotic checkpoint-related genes were upregulated in both *RPGRIP1L***M** HepG2-stable transfectants (dominant negative form) and *RPGRIP1L*-shRNA SK-HepI-stable transfectants. To further support this idea, we examined the protein levels of several mitotic checkpoint[25,29,30] related components in these transfectants. Overexpression of *RPGRIP1L***M** (dominant negative form) and knockdown of *RPGRIP1L* resulted in the elevated protein expression of Mad2, securin, cyclin B1 and survivin (Fig. 4). Next, we investigated whether *RPGRIP1L* suppresses tumour cell transformation through the mitotic checkpoint protein Mad2. To address this question, we first successfully reduced the expression of *MAD2* with *MAD2*-shRNA in *RPGRIP1L*-shRNA SK-HepI-stable transfectants (Fig. 5A and B). After *MAD2* knockdown, a significant reduction of about 50% in anchorage-independent

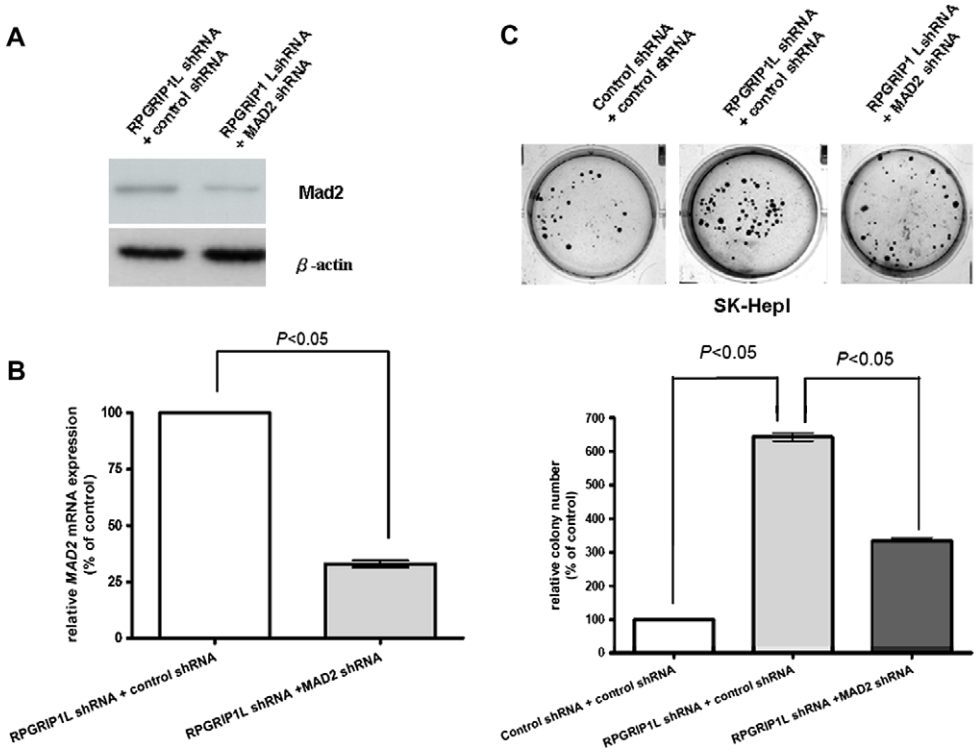

Fig. 5 – Knockdown *MAD2* expression in *RPGRIP1L*-shRNA-SK-HepI transfectants. (A) Western blot analysis was used to detect Mad2 protein expression levels before and after *MAD2*-shRNA treatment. (B) Quantitative RT-PCR was used to confirm shRNA-directed downregulation of *MAD2* expression. (C) Numbers of colonies in the *RPGRIP1L*-shRNA2 SK-HepI-stable transfectants treated with the *MAD2*-shRNA decreased significantly ($P < 0.05$) in comparison with control shRNA. The bars show the means ± SDs. Experiments were performed in triplicate, and representative results are shown.

growth in comparison with control shRNA was found (Fig. 5C, $P < 0.05$). These results may at least partly explain the increased tumour cells survival through Mad2 protein. Similar results were found in *RPGRIP1L***M** HepG2-stable transfectants (data not shown).

4. Discussion

In this study, we cloned and characterised a novel putative tumour suppressor gene, *RPGRIP1L*, located at chromosome 16q12.2 with high frequency of LOH in HCC. Downregulation of *RPGRIP1L* was found in 91% (10/11) of HCC cell lines and in 35% (14/40) of HCC clinical specimens. We have shown that this mutant form *RPGRIP1L***M** can enhance HCC cell transformation. A similar effect on HCC cells as *RPGRIP1L***M** transfectants was also found in *RPGRIP1L*-shRNA transfectants. Furthermore, we demonstrated that the possible mechanism by which *RPGRIP1L* suppresses tumour cell transformation may be partly through regulating Mad2.

From analysis of amino acid sequence of RPGRIP1L, its protein sequence shares similarity with RPGRIP1 (Retinitis Pigmentosa GTPase Regulator Interacting Protein 1). Recently, two papers have reported that *RPGRIP1L* is mutated in Joubert syndrome, an autosomal recessive disorder.[23,24] They identified missense and truncating mutations in RPGRIP1L in both CORS (cerebello-oculo-renal syndrome) and MKS (Meckel syndrome),[23,24] and showed that inactivation of the mouse ortholog Rpgrip1l (Ftm) recapitulates the cerebral, renal and hepatic defects of CORS and MKS.[24] Additionally, they found that RPGRIP1L colocalises at the basal body and centrosomes with the protein products of both NPHP6 and NPHP4,[23,24] which are the known genes associated with MKS and CORS. In addition, the RPGRIP1L missense mutations found in CORS individuals diminish the interaction between RPGRIP1L and nephrocystin-4.[23,24] It is worth exploring any biological relevance between this finding and the results presented in this report.

RPGRIP1L was frequently downregulated in HBV-associated HCCs with a significant correlation (Supplementary Table 3, $P < 0.05$). RPGRIP1L was also frequently downregulated in HCCs with LOH at 16q12.2 (80% (8/10) versus 5% (1/20), $P < 0.05$). This finding is consistent with our previous data showing that LOH at 16q12.2 is frequently found in HBV-associated HCC.[8,20] The putative promoter region of RPGRIP1L, which located at the upstream 1000 bp from transcription start site, was predicted by UCSC genome browser (http://genome.ucsc.edu/). We used methylation-specific PCR (MSP) (Supplementary Fig. 4) and bisulfite sequencing (data not shown) to analyse whether there is any promoter methylation in the putative promoter region of *RPGRIP1L*. Our data showed that promoter methylation of *RPGRIP1L* was not present in HCC samples. Therefore, we suggest that downregulation of *RPGRIP1L* is closely related to LOH at 16q12.2, not promoter methylation. However, *RPGRIP1L* was upregulated (>1.5-fold) in 5 of the 40 HCC cases (12.5%, 5/40) (Table 1). Further investigation is needed to explain this result.

Furthermore, we showed that Mad2, securin and survivin were elevated in *RPGRIP1L***M**-HepG2 transfectants (dominant negative form) and RPGRIP1L-shRNA- SKHepI transfectants, and promoted HCC cell survival and colony formation. After *MAD2* knockdown, *RPGRIP1L*-shRNA-SKHepI transfectants gave rise to a significant reduction of about 50% in colony formation ability in comparison with control shRNA (Fig. 5C, $P < 0.05$). Upregulation of *MAD2* is a frequent event seen in HCC cell lines (Supplementary Fig. 5). Mad2 expression must be tightly regulated because both reduced amounts and overproduction of this protein induce aneuploidy.[25,26,30] Mice overexpressing Mad2 undergo frequent chromosome missegregation and accumulate aneuploid cells, as do mice with low amounts of Mad2.[25,26] However, compared with mice with low Mad2 levels, mice with high levels of the protein have a much wider tumour spectrum than Mad2-haploinsufficient mice.[25,26] Taken together, our data suggest that *RPGRIP1L* might be involved in mitotic spindle checkpoint during chromosome segregation. Once inactivation of *RPGRIP1L* occurs, *MAD2* is activated and overexpressed in tumour cells. This could partly explain why inactivation of *RPGRIP1L* could enhance tumour cell survival and colony formation. Nevertheless, the mechanism underling these results may not be simply explained by the mitotic spindle checkpoint, and thus it needs further investigation to elucidate.

In summary, we have characterised a basal body gene, *RPGRIP1L*, located at chromosome 16q12.2, and provided evidence that *RPGRIP1L* is a reasonable candidate for the 16q12.2-tumour suppressor gene in HCC, especially in HBV-associated HCC.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.04.012.

REFERENCES

1. Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. *Clin Liver Dis* 2005;**9**(2):191–211.
2. Buendia MA. Genetic alterations in hepatoblastoma and hepatocellular carcinoma: common and distinctive aspects. *Med Pediatr Oncol* 2002;**39**(5):530–5.
3. El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004;**127**(5 Suppl. 1):S27–34.
4. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006;**6**(9):674–87.

5. Nagai H, Pineau P, Tiollais P, Buendia MA, Dejean A. Comprehensive allelotyping of human hepatocellular carcinoma. *Oncogene* 1997;**14**(24):2927–33.
6. Lin YW, Sheu JC, Huang GT, et al. Chromosomal abnormality in hepatocellular carcinoma by comparative genomic hybridisation in Taiwan. *Eur J Cancer* 1999;**35**(4):652–8.
7. Lin YW, Lee IN, Chen CH, et al. Deletion mapping of chromosome 16q24 in hepatocellular carcinoma in Taiwan and mutational analysis of the 17-beta-HSD gene localized to the region. *Int J Cancer* 2001;**93**(1):74–9.
8. Sheu JC, Lin YW, Chou HC, et al. Loss of heterozygosity and microsatellite instability in hepatocellular carcinoma in Taiwan. *Br J Cancer* 1999;**80**(3–4):468–76.
9. Laurent-Puig P, Legoix P, Bluteau O, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001;**120**(7):1763–73.
10. Shih YL, Shyu RY, Hsieh CB, et al. Promoter methylation of the secreted frizzled-related protein 1 gene SFRP1 is frequent in hepatocellular carcinoma. *Cancer* 2006;**107**(3):579–90.
11. Jou YS, Lee CS, Chang YH, et al. Clustering of minimal deleted regions reveals distinct genetic pathways of human hepatocellular carcinoma. *Cancer Res* 2004;**64**(9):3030–6.
12. De Souza AT, Hankins GR, Washington MK, Fine RL, Orton TC, Jirtle RL. Frequent loss of heterozygosity on 6q at the mannose 6- phosphate/insulin-like growth factor II receptor locus in human hepatocellular tumors. *Oncogene* 1995;**10**(9):1725–9.
13. Fujimoto Y, Hampton LL, Wirth PJ, Wang NJ, Xie JP, Thorgeirsson SS. Alterations of tumor suppressor genes and allelic losses in human hepatocellular carcinomas in China [see comments]. *Cancer Res* 1994;**54**(1):281–5.
14. Elo JP, Harkonen P, Kyllonen AP, Lukkarinen O, Vihko P. Three independently deleted regions at chromosome arm 16q in human prostate cancer allelic loss at 16q241-q24.2 is associated with aggressive behaviour of the disease, recurrent growth, poor differentiation of the tumour and poor prognosis for the patient. *Br J Cancer* 1999;**79**(1):156–60.
15. Nose H, Imazeki F, Ohto M, Omata M. P53 gene mutations and 17p allelic deletions in hepatocellular carcinoma from Japan. *Cancer* 1993;**72**(2):355–60.
16. Yeh SH, Chen PJ, Chen HL, Lai MY, Wang CC, Chen DS. Frequent genetic alterations at the distal region of chromosome 1p in human hepatocellular carcinomas. *Cancer Res* 1994;**54**(15):4188–92.
17. Yeh SH, Chen PJ, Lai MY, Chen DS. Allelic loss on chromosomes 4q and 16q in hepatocellular carcinoma: association with elevated alpha-fetoprotein production. *Gastroenterology* 1996;**110**(1):184–92.
18. Yeh SH, Chen PJ, Shau WY, et al. Chromosomal allelic imbalance evolving from liver cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2001;**121**(3):699–709.
19. Cleton-Jansen AM, Moerland EW, Kuipers-Dijkshoorn NJ, et al. At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes Chromosomes Cancer* 1994;**9**(2):101–7.
20. Lin YW, Lee HS, Chen CH, Huang GT, Lee PH, Sheu JC. Clonality analysis of multiple hepatocellular carcinomas by loss of heterozygosity pattern determined by chromosomes 16q and 13q. *J Gastroenterol Hepatol* 2005;**20**(4):536–46.
21. Suzuki H, Komiya A, Emi M, et al. Three distinct commonly deleted regions of chromosome arm 16q in human primary and metastatic prostate cancers. *Genes Chromosomes Cancer* 1996;**17**(4):225–33.
22. Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA, Aldaz CM. WWOX, a novel WW domain-containing protein mapping to human chromosome 16q233-24.1, a region frequently affected in breast cancer. *Cancer Res* 2000;**60**(8):2140–5.
23. Arts HH, Doherty D, van Beersum SE, et al. Mutations in the gene encoding the basal body protein RPGRIP1L, a nephrocystin-4 interactor, cause Joubert syndrome. *Nat Genet* 2007;**39**(7):882–8.
24. Delous M, Baala L, Salomon R, et al. The ciliary gene RPGRIP1L is mutated in cerebello-oculo-renal syndrome (Joubert syndrome type B) and Meckel syndrome. *Nat Genet* 2007;**39**(7):875–81.
25. van Deursen JM. Rb loss causes cancer by driving mitosis mad. *Cancer Cell* 2007;**11**(1):1–3.
26. Sotillo R, Hernando E, Diaz-Rodriguez E, et al. Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. *Cancer Cell* 2007;**11**(1):9–23 [Epub 2006 Dec 28].
27. Shih YL, Hsieh CB, Lai HC, et al. SFRP1 suppressed hepatoma cells growth through Wnt canonical signaling pathway. 2007;**121**(5):1028–35.
28. Lee AY, He B, You L, et al. Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma. *Oncogene* 2004;**23**(39):6672–6.
29. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* 2005;**5**(10):773–85.
30. Weaver BA, Silk AD, Montagna C, Verdier-Pinard P, Cleveland DW. Aneuploidy acts both oncogenically and as a tumor suppressor. *Cancer Cell* 2007;**11**(1):25–36.