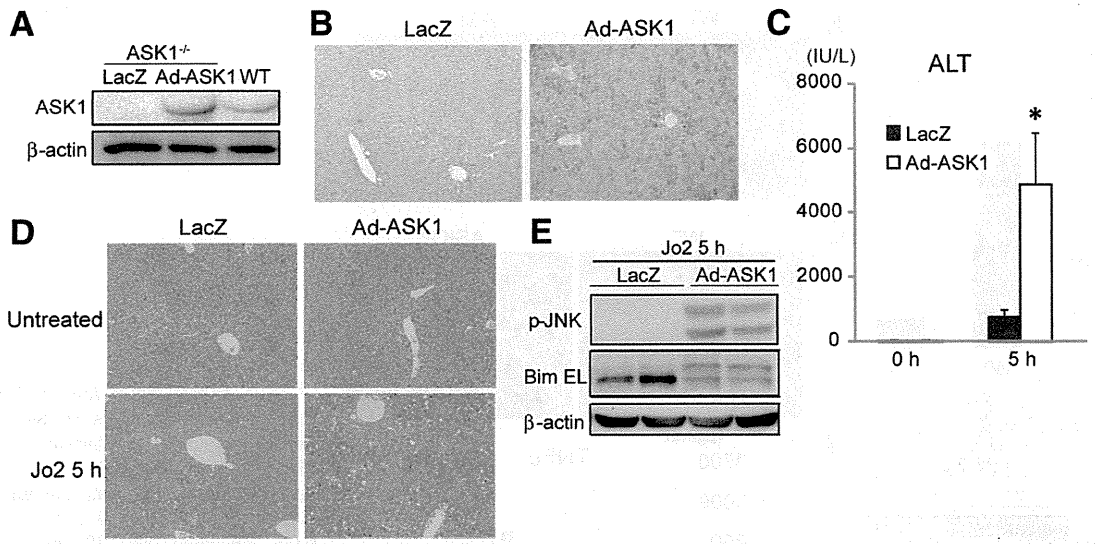

Fig. 6. Restoration of Fas sensitivity in ASK1-reintroduced ASK1$^{-/-}$ mouse liver. (A) Successful adenovirus-mediated expression of ASK1 in ASK1$^{-/-}$ mouse liver. ASK1$^{-/-}$ mice were tail-vein-injected with adenoviruses encoding β-galactosidase (LacZ) or ASK1 (Ad-ASK1). Anti-ASK1 immunoblots were performed to determine ASK1 expression in liver tissue. (B) Immunohistochemical analysis of ASK1 gene transduction in the liver using anti-HA antibody. (C) Serum ALT levels at basal level and 5 hours after Jo2 administration in ASK1$^{-/-}$ mice infected with LacZ or Ad-ASK1. Data are expressed as means ± SEM (n = 3 per group). *$P < 0.05$, compared with mice infected with LacZ. (D) Liver histology with H&E staining of pre- and posttreatment sections. (E) Western blot analysis of the phosphorylation of JNK and BimEL in the liver at 5 hours after Jo2 administration.

Next, to examine whether ASK1 may be involved in a Fas-induced mitochondria-independent apoptotic pathway, we used primary thymocytes, which are independent of mitochondria for Fas-induced apoptosis (so-called type I cells). Fas-induced activation of JNK and p38 was reduced in ASK1$^{-/-}$ thymocytes, whereas caspase-3 activation and cell viability were comparable between WT and ASK1$^{-/-}$ thymocytes (Supporting Fig. 1A,B), suggesting that ASK1 is not required for the mitochondria-independent apoptotic pathway.

Recently, Fas signaling was reported to play a role in not only cancer cell apoptosis, but also cancer cell proliferation.[26] JNK has also been shown to be one of the main mediators of Fas-mediated proliferative signals. To investigate whether ASK1 participated in Fas-mediated hepatocyte proliferation, we injected Jo2 to WT and ASK1$^{-/-}$ mice after partial hepatectomy, which is known to convert Fas signaling from apoptotic to proliferative.[27] As reported,[26,27] Jo2 injection after partial hepatectomy induced JNK phosphorylation and accelerated hepatocyte proliferation without liver injury (Supporting Fig. 2A,B). Although liver regeneration after partial hepatectomy and Jo2-induced JNK phosphorylation were slightly impaired in ASK1$^{-/-}$ mice (especially the upper band corresponding to JNK2), there was no significant difference in Jo2-mediated acceleration of hepatocyte proliferation (Supporting Fig. 2A,B). Thus, ASK1 seemed to regulate the apoptotic, but not proliferative, function of JNK in Fas signaling.

Restoration of Fas Sensitivity in ASK1-Reintroduced ASK1$^{-/-}$ Mouse Liver. To further confirm the involvement of ASK1 in Fas-induced hepatocyte apoptosis, we examined whether the reintroduction of ASK1 to ASK1$^{-/-}$ mouse liver restored sensitivity to Fas. We injected an adenoviral vector encoding either Ad-ASK1 or LacZ into the tail vein of ASK1$^{-/-}$ mice. ASK1 protein was successfully expressed in ASK1$^{-/-}$ mouse liver, as much as that in WT mouse liver, at 48 hours after Ad-ASK1 injection (Fig. 6A). Immunohistochemical analysis using anti-HA antibody revealed that ≈70%-80% of hepatocytes were transduced with the ASK1 gene (Fig. 6B). The reintroduction of ASK1 did not affect the serum ALT level or liver histology. These mice were injected intraperitoneally with Jo2 5 hours later; only mild serum ALT elevation and histological liver damage were found in LacZ-injected mice, whereas Ad-ASK1-injected mice revealed marked serum ALT elevation and severe histological damage (Fig. 6C,D). Furthermore, reintroduction of ASK1 restored Jo2-induced phosphorylation of JNK and BimEL in the liver (Fig. 6E).

Role of ASK1 in TNF-α-Induced Hepatocyte Apoptosis. To examine whether ASK1 is required for TNF-α-induced apoptosis of hepatocytes *in vivo*, we used an LPS/GalN liver injury model that depends on

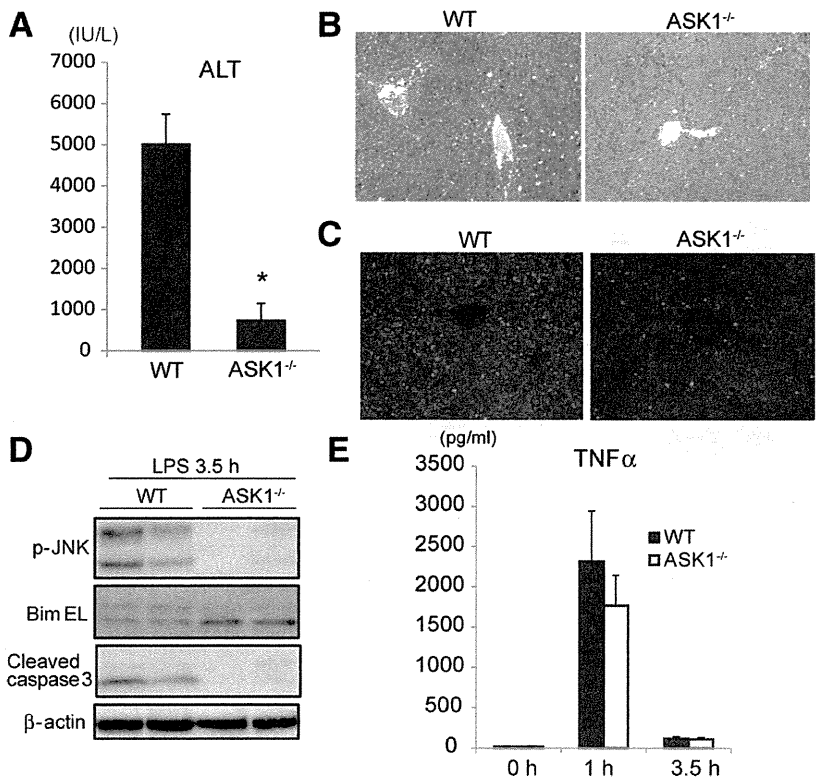

Fig. 7. Involvement of ASK1 in TNF-α-mediated hepatocyte apoptosis. (A) Serum ALT levels 6 hours after the injection of WT and ASK1$^{-/-}$ mice with LPS (20 μg/kg) and GalN (1,000 mg/kg). Data are expressed as means ± SEM (n = 3 per group). *$P < 0.05$, compared with WT mice. (B) H&E-stained sections of livers obtained 6 hours posttreatment. (C) TUNEL-stained sections of livers obtained 6 hours posttreatment. (D) Western blot analysis of caspase-3, JNK, and Bim activation in WT and ASK1$^{-/-}$ mouse liver obtained 3.5 hours after LPS/GalN administration. (E) Serum TNF-α levels after LPS/GalN administration. Data are expressed as means ± SEM (n = 3 per group).

TNF-α-induced apoptosis.[28] At 6 hours after LPS/GalN administration, WT mice exhibited marked ALT elevation, severe histological liver damage, and hepatocyte apoptosis, whereas these changes were significantly attenuated in ASK1$^{-/-}$ mice (Fig. 7A-C). As expected, LPS/GalN-induced phosphorylation of JNK and BimEL and cleavage of caspase-3 were significantly attenuated in ASK1$^{-/-}$ mice, as well as in Fas-induced liver injury (Fig. 7D). On the other hand, WT and ASK1$^{-/-}$ mice exhibited no significant difference in serum TNF-α levels (Fig. 7E). These findings provide further support for the hypothesis that ASK1 is required for death receptor-mediated hepatocyte apoptosis by way of the JNK–Bim-mediated mitochondrial apoptotic pathway. Furthermore, ASK1 silencing by siRNA attenuated TNF-α-induced sustained JNK and p38 activation, BimEL cleavage, and apoptosis in the HCC cell line HuH7 (Supporting Fig. 3A,B). Thus, resistance to death signaling may be a predominant cause of accelerated hepatocarcinogenesis in ASK1$^{-/-}$ mice.

Involvement of the ASK1-p38 Pathway in DNA Damage Response. Because DEN-induced acute phase reaction in the liver is known to be associated with future HCC development, we assessed the involvement of ASK1 in this phase.[29] Although the DEN-induced activation of JNK was slightly attenuated in ASK1$^{-/-}$ mouse livers, the increases in serum ALT levels were statistically similar in the WT and ASK1$^{-/-}$ mice (Fig. 8A, Supporting Fig. 4A). Bromodeoxyuridine labeling revealed that the numbers of compensatory proliferating hepatocytes in WT and ASK1$^{-/-}$ mice were similar after DEN administration (Supporting Fig. 4B). Furthermore, the level of DEN-induced p53 activation was similar in both groups (Fig. 8A). These findings suggest that DEN induces a similar extent of hepatocyte death, DNA damage, and compensatory proliferation in WT and ASK1$^{-/-}$ mice.

On the other hand, p38 activation was significantly attenuated in the ASK1$^{-/-}$ mouse livers (Fig. 8A), and p38 has been reported to play an important role in DNA damage responses, such as cellular senescence, by inducing cyclin-dependent kinase inhibitors through p53-dependent and -independent mechanisms.[30] Thus, we next compared induction of cyclin-dependent kinase inhibitors after DEN administration between WT and ASK1$^{-/-}$ mouse livers. As shown in Fig. 8B, p16 and p21 were slightly and remarkably induced after DEN administration, respectively, and p21 induction was significantly attenuated in ASK1$^{-/-}$ mouse livers. Because the p38 inhibitor, but not the JNK inhibitor, suppressed DEN-induced p21 up-regulation (Fig. 8C), we considered that the ASK1-p38 pathway may be involved in DNA damage-induced

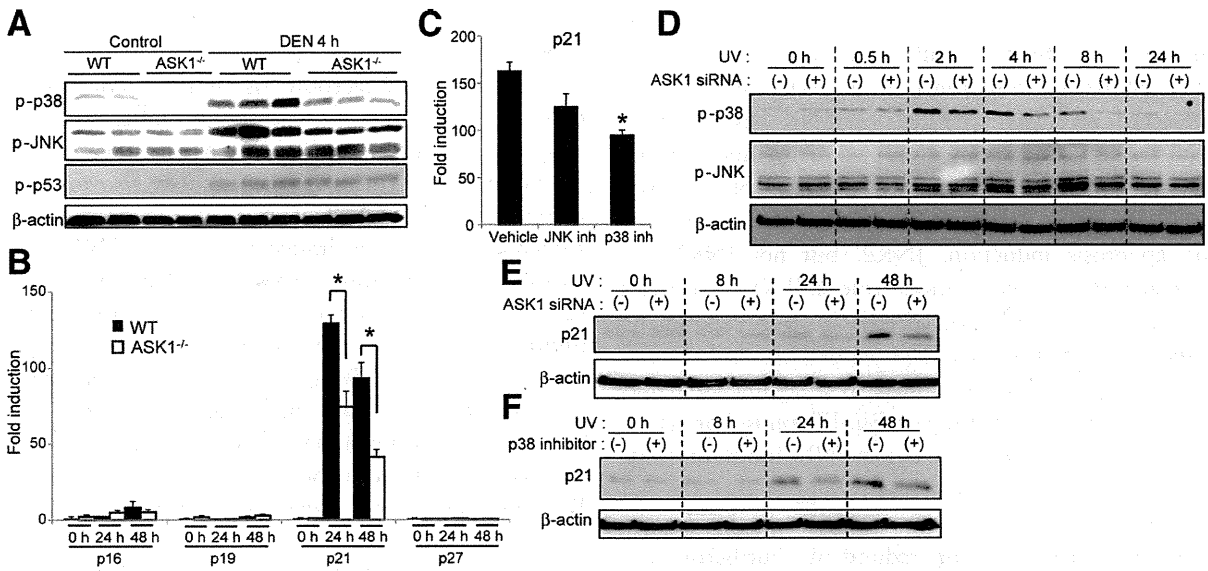

Fig. 8. Involvement of the ASK1-p38 pathway in DNA damage-induced p21 up-regulation. (A) Western blot analysis of phosphorylation of p38, JNK, and p53 in WT and ASK1$^{-/-}$ mouse livers obtained 4 hours after DEN administration (100 mg/kg). (B) mRNA levels of p16, p19, p21, and p27 were determined by real-time PCR in DEN-administered WT and ASK1$^{-/-}$ mouse livers. Data are expressed as means ± SEM (n = 4 per group). *$P < 0.05$ compared with WT mice. (C) Effect of JNK and p38 inhibitors on induction of p21 mRNA at 48 hours after DEN administration in WT mouse liver. Data are expressed as means ± SEM (n = 4 per group). *$P < 0.05$, compared with vehicle-treated mice. (D-F) The role of the ASK1-p38 pathway in p21 induction after DNA damage was analyzed by UVB irradiation (300 J/m^2 of 302-nm light) of an immortalized human normal hepatocyte line. (D) Western blot analysis of UV-induced phosphorylation of p38 and JNK in a human normal hepatocyte line after transfection with ASK1 or control siRNA. (E) Western blot analysis of the effect of ASK1 knockdown on p21 induction after UV irradiation. (F) Western blot analysis of the effect of a p38 inhibitor on p21 induction after UV irradiation. The human normal hepatocyte line was treated for 1 hour with the p38 inhibitor SB203580 (10 μM) or vehicle before UV irradiation.

p21 up-regulation. However, in addition to DNA damage, there are many other kinds of stimuli in the liver after DEN administration *in vivo*: inflammatory responses, liver regeneration signals, and toxic metabolites of DEN. Thus, we assessed the role of the ASK1-p38 pathway in p21 induction after DNA damage using UVB irradiation, which is a well-known direct DNA damage-inducer. UVB irradiation to the immortalized human normal hepatocyte line induced strong phosphorylation of p38 and very weak phosphorylation of JNK, and ASK1 silencing attenuated UVB-induced p38 phosphorylation, especially in the late phase (Fig. 8D, Supporting Fig. 5). Furthermore, UVB-induced p21 up-regulation was attenuated by ASK1 silencing and p38 inhibition (Fig. 8E F). These results suggest that ASK1 is involved in DNA damage-induced p21 up-regulation through p38 activation, and an impaired DNA damage response may be one reason for increased hepatocarcinogenesis in ASK1$^{-/-}$ mice.

Discussion

Dysregulation of the balance between cell proliferation and apoptosis plays a critical role in hepatocarcinogenesis.[31] Our results suggest that ASK1 plays only a minor role in cancer cell proliferation and a major role in death receptor-mediated apoptosis in the liver through the JNK pathway. Loss of ASK1 appears to cause an imbalance that accelerates chemical hepatocarcinogenesis in ASK1$^{-/-}$ mice. Furthermore, ASK1 is involved in the DNA damage response, through the p38 pathway. This study provides new insight into the regulation of stress-activated MAPK signaling in hepatocarcinogenesis.

JNK (primarily JNK1) has been reported to promote DEN-induced hepatocarcinogenesis by promoting cancer cell proliferation and neovascularization.[3,5] Although JNK activation was attenuated in ASK1$^{-/-}$ mice, the phenotype of ASK1$^{-/-}$ mice and JNK1$^{-/-}$ mice was opposite in hepatocarcinogenesis.[3,5] We suggest that the reasons may be as follows: (1) Although we observed attenuation of JNK activation in ASK1$^{-/-}$ HCC tissues, ASK1 appears to play only a minor role in HCC cell proliferation (Fig. 2). Additionally, vessel

density and VEGF expression in ASK1$^{-/-}$ HCC tissues were unaffected (Supporting Fig. 6A,B). Thus, the tumor-enhancing function of JNK1 seems to be preserved in ASK1$^{-/-}$ mice. (2) JNK has also been reported to act as a tumor suppressor by inducing cancer cell apoptosis.[32] JNK1 and JNK2 isoforms have distinct or redundant roles in some situations, including apoptosis induction. JNK2, but not JNK1, has been reported to play a major role in TNF-α-mediated hepatocyte apoptosis *in vivo*.[33] In our experiments using a Jo2-induced hepatitis model, the lack of neither JNK1 nor JNK2 resulted in a significant reduction in the ALT elevation or BimEL phosphorylation, unlike ASK1$^{-/-}$ mice or a pan-JNK inhibitor (Supporting Fig. 7A,B). These results suggest that the role of JNK1 and JNK2 in death receptor-mediated hepatocyte apoptosis may be redundant. Furthermore, a recent report demonstrated that JNK1 and JNK2 deficiency in hepatocytes increased DEN-induced HCC.[34] Thus, JNK1 and JNK2 have a wide range and redundant or distinct functions, and upstream molecules, such as MAP3Ks, must regulate the complex functions of JNK. We consider that ASK1 plays major roles in tumor-suppressing part of JNK in hepatocarcinogenesis. However, knockdown of ASK1 in HCC cell lines slightly decreased cell proliferation. This finding suggests that ASK1 may weakly promote the proliferation of some HCC cells, which could explain why the WT and ASK1$^{-/-}$ mice did not exhibit significant differences in tumor size.

On the other hand, mice with liver-specific p38 deficiency exhibit increased HCC development similar to ASK1$^{-/-}$ mice.[4,6] The accelerated hepatocarcinogenesis in p38-deficient mice is reportedly attributable to compensatory JNK activation and cancer cell proliferation. Although p38 activation was attenuated in ASK1$^{-/-}$ mice, JNK activation was also attenuated, unlike the liver-specific p38-deficient mice. Thus, the mechanisms of accelerated hepatocarcinogenesis in ASK1$^{-/-}$ mice and liver-specific p38-deficient mice appear to differ. p38 has also been reported to play an important role in DNA damage responses, such as cellular senescence, by inducing cyclin-dependent kinase inhibitors.[30] In this study, we showed that ASK1 is involved in DNA damage-induced p21 up-regulation through p38 activation. Furthermore, the ASK1-p38 pathway has been reported to have an inhibitory effect on malignant transformation of fibroblasts by triggering apoptosis in response to oncogene-induced reactive oxygen species (ROS).[35] Thus, the ASK1-p38 pathway may play a key role in the inhibition of tumor initiation in hepatocarcinogenesis.

Defective death-receptor signaling is considered a cause of tumor immune escape, so understanding its apoptotic mechanism is very important not only from the point of view of carcinogenesis, but also for cancer therapeutics.[21] Several *in vitro* studies have demonstrated that ASK1 is implicated in the TNF-α- and Fas-mediated apoptotic pathways,[11,36] but the *in vivo* role of ASK1 has not been determined. Our current findings provide the first evidence that ASK1 plays an important role in TNF-α- and Fas-mediated hepatocyte apoptosis *in vivo* and suggest that the JNK-Bim-mediated mitochondrial apoptotic pathway is an important downstream target of ASK1. JNK-mediated Bim phosphorylation triggers the proapoptotic activity of Bim by causing its release from sequestration to the microtubular dynein motor complex.[25] Bim initiates the mitochondrial apoptotic pathway by activating Bax and Bak directly and indirectly blocking prosurvival Bcl-2 family members.[37] Recent reports have shown that Bim plays an important role in Fas- and TNF-α-induced hepatocyte apoptosis.[19,20] Mitochondria are considered an important downstream target of ASK1 in apoptosis because overexpression of the active form of ASK1 in cell lines induces apoptosis through cytochrome *c* release from mitochondria and activation of caspase-9 and -3.[38] In our study, ASK1 was found to be involved in Fas-induced hepatocyte apoptosis but not in thymocyte apoptosis, suggesting that ASK1 is required for mitochondria-dependent apoptosis. Thus, we believe that the ASK1–JNK–Bim–mitochondrial pathway plays an important role in death receptor-mediated hepatocyte apoptosis. The observed attenuation of Bim phosphorylation and caspase-3 activation in ASK1$^{-/-}$ HCC tissues is consistent with the inhibition of death receptor-induced apoptosis.

Recently, death-receptor signaling, such as Fas signaling, has been reported to play a role in not only cancer cell apoptosis, but also cancer cell proliferation.[26] Our finding that Jo2-induced acceleration of hepatocyte proliferation after partial hepatectomy was comparable between WT and ASK1$^{-/-}$ mice suggests that ASK1 does not play a major role in Fas-mediated cell proliferation. Furthermore, the finding that WT and ASK1$^{-/-}$ HCCs exhibited no significant differences in cancer cell proliferation rates *in vivo* also supports this. Thus, ASK1 seemed to regulate the apoptotic, but not proliferative, function of JNK in Fas signaling, and ASK1$^{-/-}$ hepatocytes might alter death-receptor signaling to favor survival by escaping apoptosis. However, this is a relatively new concept, so

further study is needed to clarify the role of ASK1 in death receptor-mediated cancer cell proliferation.

In conclusion, ASK1 controls the tumor-suppressing function of stress-activated MAPK signaling, and thus acts as a tumor suppressor in hepatocarcinogenesis.

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Receptor for Activated Protein Kinase C: Requirement for Efficient MicroRNA Function and Reduced Expression in Hepatocellular Carcinoma

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Abstract

MicroRNAs (miRNAs) are important regulators of gene expression that control physiological and pathological processes. A global reduction in miRNA abundance and function is a general trait of human cancers, playing a causal role in the transformed phenotype. Here, we sought to newly identify genes involved in the regulation of miRNA function by performing a genetic screen using reporter constructs that measure miRNA function and retrovirus-based random gene disruption. Of the six genes identified, RACK1, which encodes "receptor for activated protein kinase C" (RACK1), was confirmed to be necessary for full miRNA function. RACK1 binds to KH-type splicing regulatory protein (KSRP), a member of the Dicer complex, and is required for the recruitment of mature miRNAs to the RNA-induced silencing complex (RISC). In addition, RACK1 expression was frequently found to be reduced in hepatocellular carcinoma. These findings suggest the involvement of RACK1 in miRNA function and indicate that reduced miRNA function, due to decreased expression of RACK1, may have pathologically relevant roles in liver cancers.

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Introduction

MicroRNAs (miRNAs) are short (20-23-nt), endogenous, single-stranded RNA molecules, that regulate gene expression and control physiological and pathological processes, such as development and cancer. Mature miRNAs and Argonaute (Ago) proteins form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex that mediates post-transcriptional gene silencing [1] and then, complementary base-pairing of miRNAs guides the RISC to target messenger mRNAs, which are subsequently destabilized and sequestered from the translational machinery by Ago proteins [2–5]. Although insights into the regulatory function of miRNAs are beginning to emerge, their mechanisms of action and the genes involved in miRNA pathway have not yet been fully determined [6,7].

A large body of evidences suggests that the multigene regulatory capacity of miRNAs is dysregulated and exploited in cancer. Although several miRNAs are upregulated in specific tumors [8], a global reduction of miRNA abundance appears a general trait of human cancers, playing a causal role in the transformed phenotype [9,10]. In fact, the enzymes and cofactors involved in miRNA processing pathways may be targets of genetic disruption, further enhancing cellular transformation [9]. Moreover, the disruption of Dicer in mice promotes hepatocarcinogenesis [11] and the truncating mutations in TARBP2, which causes a defect in the processing of miRNAs, were identified in sporadic and hereditary colon carcinomas [12].

Retroviral insertion-mediated random gene disruption can be used to generate null alleles, resulting in diminished endogenous gene expression [13]. The use of such retroviral integration methods, combined with appropriate reporter constructs, has provided an efficient, comprehensive gene screening method [14,15]. In this study, we sought to identify new genes involved in miRNA function and determine its role in live cancers. To this end, we established reporter cell lines in which cellular miRNA function could be assessed by expression of a drug resistance gene. Using these cell lines and a random gene disruption method, we identified genes that have not previously been implicated in the regulation of miRNA function. We subsequently determined the role of one of these genes, RACK1, which encodes "receptor for activated protein kinase C" (RACK1), in miRNA function, as well as its expression in liver cancers. Collectively, our data suggest the potential involvement of RACK1 in pathological processes.

Results

Identification of the genes required in the miRNA pathway by random gene disruption

To identify genes involved in miRNA pathways especially in liver cells, we constructed a reporter carrying a hygromycin resistance gene with two miR122-responsive elements in its 3′-UTR (Fig. 1A). We chose miR122 because it is the most abundant and tissue-specific miRNA in the liver [16]. Binding of miR122 reduces the expression of this hygromycin resistance gene in this construct. However, if miR122 function is impaired by the disruption of genes that are important for miRNA signaling, hygromycin resistance gene expression increases. Cells carrying such disrupted genes will therefore survive hygromycin treatment. Additionally, to enhance the effects of miR122, we co-transfected a miR122 precursor-expressing plasmid (Fig. 1A) with the reporter construct and selected monoclonal cells containing both constructs to minimize the effects of their random integration. After infection with retroviruses carrying a blasticidin resistance gene to produce random gene disruption in the selected reporter cells, cells surviving hygromycin treatment were harvested. The disrupted genes in the surviving cells were identified by 3′ RACE. We infected $\sim 10^6$ Huh7-pBS-Hygro-miR122 cells established from Huh7 cells and obtained $\sim 10^4$ clones with random gene disruptions (confirmed by resistance to blasticidin) (Fig. 1B). After hygromycin selection, ten clones in which miRNA function was apparently impaired were obtained. In these ten clones, six disrupted genes were successfully identified (Table 1). One of them, RACK1 (also known as GNB2L1), appeared in duplicate and was found to be disrupted in two clones.

Requirement of RACK1 for microRNA function

Of the genes identified, RACK1 was found to be disrupted in two independent clones (Table 1). RACK1 was previously identified as the binding partner of eIF6, a ribosome inhibitory protein known to prevent proper assembly of the 80S ribosome, and contributes to miRNA silencing by associating with RISC [17–19]. However, the requirement for eIF6 in miRNA function is controversial because other models, in which miRNA silencing is mediated by Ago2 (eIF2C2) and an interaction with eIF4E [20], or by GW182 [21], have more recently been described.

To confirm the requirement for RACK1 in miRNA function, we measured miRNA activity using a reporter assay involving transient expression of an siRACK1 construct and an miRNA precursor-overexpression plasmid. Transient knockdown of RACK1 reduced the function of three miRNAs: miR122, miR140, and miR185 (Fig. 2A). To examine these effects using a natural 3′-UTR containing miR122 binding sites, we used a CatA-Luc reporter that carried the 3′-UTR of the CAT1 (cationic amino acid transporter 1) gene and a luciferase gene [22]. The CAT1 3′-UTR contains three predicted miR122 binding sites [22]. The requirement for RACK1 in miRNA function was confirmed using this reporter construct (Fig. 2B).

Next, we established stable RACK1-knockdown Huh7 cells (Fig. 2C) and compared the effects of miRNAs in control and RACK1-knockdown cells. Consistent with the results of the transient transfection assays, RACK1-knockdown cells showed weaker miRNA mediated-inhibition of target gene expression in a reporter system involving miR185 precursor-expressing plasmids and its reporter constructs (Fig. 2D). To measure changes in endogenous miRNA function in RACK1-knockdown cells, control and RACK1-knockdown cells were transfected with reporter constructs specific for several miRNAs and luciferase activity was then measured. We confirmed the inhibition of endogenous miRNA function in RACK1-knockdown cells (Fig. 2E). These results suggest that RACK1 is indeed required for miRNA function.

RACK1 may function after miRNA maturation but before the expression-inhibitory machinery

To identify the point at which RACK1 enhances miRNA function, mature miRNA levels were first measured in stable RACK1-knockdown cells. Levels of endogenous mature miR122, miR22, miR140-5p, -3p, and miR185, which are expressed at relatively high levels in liver cells [16], were comparable in control and RACK1-knockdown cells (Fig. 3A and Figure S1). This suggests that RACK1 may not be involved in miRNA maturation. We next showed that overexpression of artificial synthetic miRNA oligonucleotides replicated normal miRNA-mediated inhibition of gene expression in RACK1-knockdown cells (Fig. 3B). These results suggest that, in vivo, RACK1 may function after miRNA maturation, but before the expression-inhibiting machinery in the natural miRNA pathway, although the finding that the synthetic mature miRNAs were functional even in Ago2-knockdown cells (Figure S2A, B) indicates that their function might be Ago2-independent. Additionally, the expression of Drosha, DGCR8, Dicer, TRBP, Ago1, Ago2, Ago3, Ago4, and eIF4E, all of which are known to be involved in the miRNA pathway, were almost similar in control and RACK1-knockdown cells (Fig. 3C). Furthermore, the localization and number of the p-bodies, which may be involved in miRNA-mediated silencing [23,24], were not markedly affected by RACK1-knockdown (as determined by staining for the intracellular marker GW182) [23] (Fig. 3D). These results suggest that RACK1 functions after miRNA maturation, but before mature miRNAs exert their expression-inhibitory effects.

RACK1 interacts with KSRP and is required for the full recruitment of mature miRNAs to the RISC

To determine whether RACK1 interacts with miRNA pathway related-molecules, transiently-transfected myc-tagged RACK1 was first immunoprecipitated. While myc-tagged RACK1 was efficiently precipitated (Fig. 4A), RISC constituent proteins such as Dicer, DDX20 (Gemin3) and Gemin4 [25] were found not to interact with RACK1 (Fig. 4A). GW182 did not interact with RACK1 (Fig. 4A). eIF6, which was previously reported to interact with RACK1 [18] and which may be involved in miRNA silencing [19], did not show an interaction with RACK1 in the present study (Fig. 4A). However, Ago2 weakly interacted with RACK1 (Fig. 4A), as reported recently [26]. Moreover, KH-type splicing regulatory protein (KSRP; also known as KHSRP), a key mediator of mRNA degradation [27,28] and a component of the Dicer complex that promotes the maturation of a subset of miRNAs [29], interacted with RACK1, especially when Dicer was overexpressed (Fig. 4A and Figure S3). This interaction was also confirmed by immunoprecipitation of endogenous RACK1 (Fig. 4B). The interactions of RACK1 with Ago2 and KSRP were insensitive to RNase A treatment (Figure S4), suggesting that they were not mediated by RNAs. Next, because it seemed that RACK1 functioned after miRNA maturation and before mature miRNA recruitment into the RISC, we measured levels of miRNAs in complexes immunoprecipitated using an anti-Ago2 antibody. Levels of mature miRNAs examined in the Ago2-containing complexes were lower in RACK1-knockdown cells than in control cells (Fig. 4C and Figure S5). To determine the possible causes of impaired mature miRNA loading into Ago2-related complexes in RACK1-knockdown cells, we determined the intracellular localization of KSRP and Ago2 in RACK-1 knockdown cells (Fig. 4D). While KSRP is distributed both in the nucleus and cytoplasm and Ago2 localizes mainly in

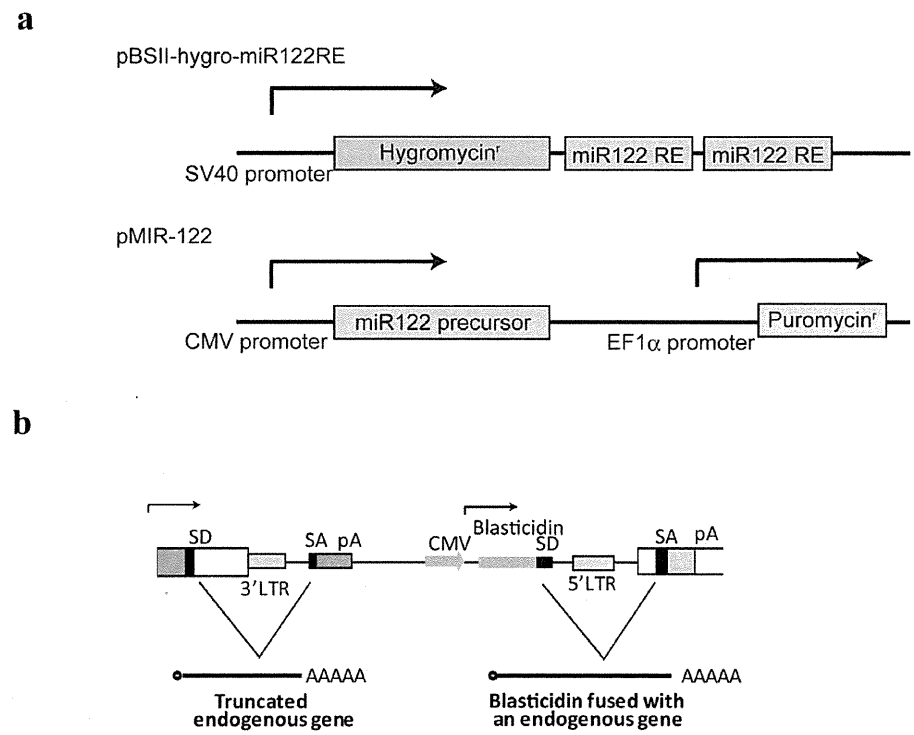

Figure 1. Constructs used in the study. A, Reporter and miR122 precursor-expressing constructs. A construct containing an SV40 promoter-driven hygromycin resistance gene with two tandem miR122-responsive elements (miR122 RE) in its 3′-UTR was used to assess miRNA function. To express miR122, a construct carrying a CMV promoter-driven miR122 precursor was used in conjunction with puromycin selection. **B,** pDisrupt vector structure and gene products resulting from viral integration. Splicing occurs between the SD (splicing donor) and SA (splicing acceptor) sites (i.e., between the 3′-end of the endogenous gene exon and the retroviral SA site, as well as between the retroviral SD at the 3′-end of the blasticidin gene and the endogenous SA site at the 5′-end of the downstream gene exon). pA, polyadenylation signal.
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cytoplasm in control cells, KSRP localizes more in the nucleus in RACK1-knockdown cells (Fig. 4D). The changes in the subcellular localization of KSRP in RACK1-knockdown cells were also confirmed by Western blotting using cell lysates prepared after subcellular fractionation (Fig. 4E). The function of synthetic mature miRNAs was not affected by KSRP knockdown (Figure S6A, B), suggesting that KSRP itself is not required for RISC activity. Thus, these localization changes with RACK-1 may be related to the impaired mature miRNA loading into Ago2 complexes from the KSRP-associated complexes, although, because the binding of KSRP to Ago2 could not be detected in our coimmunoprecipitation study (Figure S7), the precise mechanisms remain to be elucidated. Nonetheless, these results suggest that RACK1 interacts with KSRP and that the recruitment of mature miRNAs into the RISC is impaired in RACK1-knockdown cells.

RACK1 expression is frequently decreased in liver cancers

Recent results linking reduced global expression of miRNAs and reduced miRNA function with tumorigenesis [12,30] encouraged us to examine RACK1 expression in cancers. To this end, we

Table 1. List of genes identified in this screening.

Gene ID	Gene symbol	Gene title	Known function	Also known as
100288263	LOC100288263	hypothetical protein	Unknown	
10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	Translation	RACK1, PIG21
10055	SAE1	SUMO1 activating enzyme subunit 1	Sumoylation	AOS1, SUA1, UBLE1A
10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	Translation	RACK1, PIG21
6228	RPS23	ribosomal protein S23	Ribosome 40S	FLJ35016
6135	RPL11	ribosomal protein L11	Ribosome 60S	DBA7, GIG34
5358	PLS3	plastin 3	Actin binding	T-plastin

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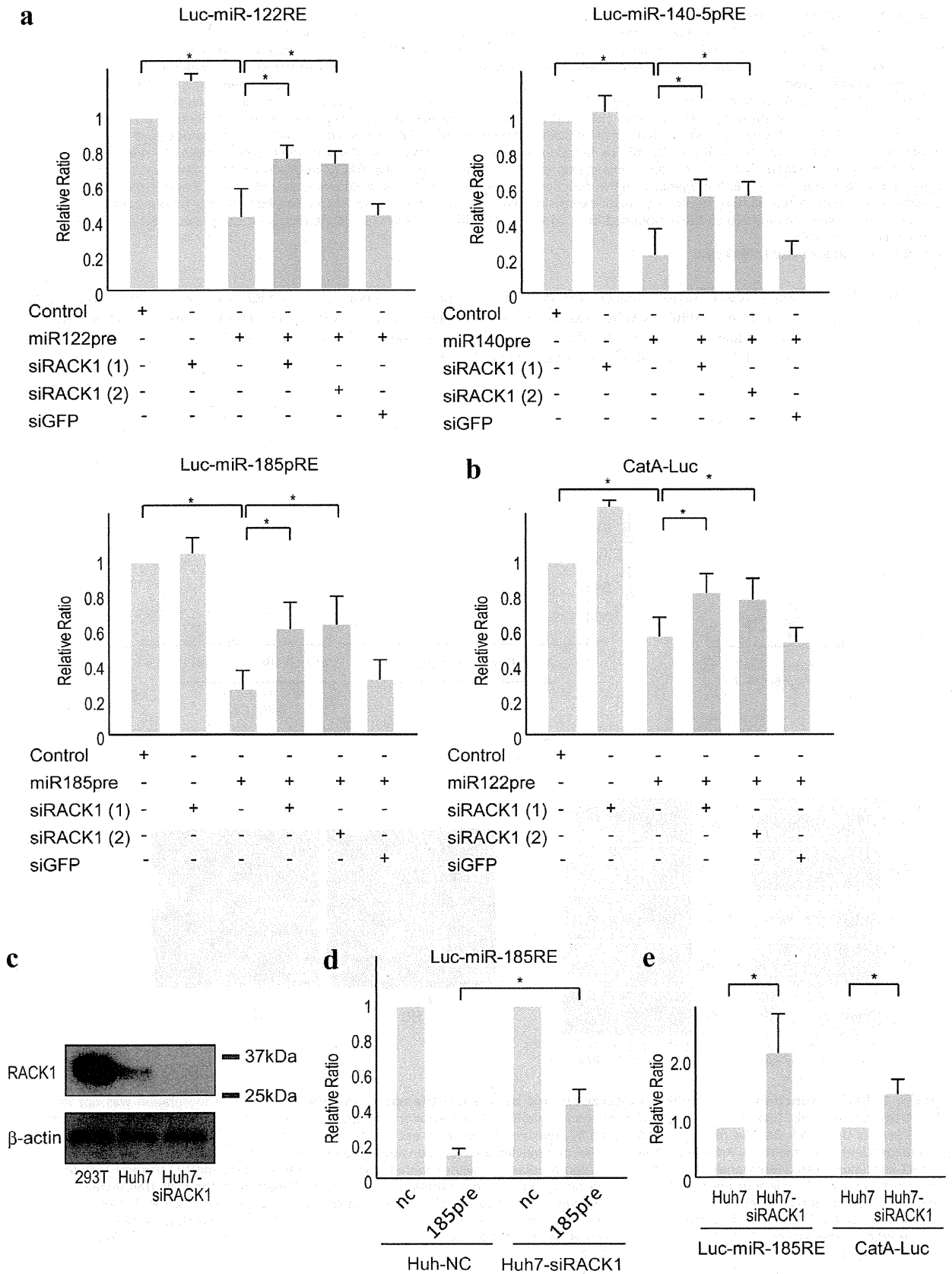
a
Luc-miR-122RE
Relative Ratio
Control
miR122pre
siRACK1 (1)
siRACK1 (2)
siGFP
Luc-miR-140-5pRE
miR140pre
Luc-miR-185pRE
miR185pre
b
CatA-Luc
c
RACK1
β-actin
37kDa
25kDa
293T Huh7 Huh7-siRACK1
d
Luc-miR-185RE
nc
185pre
Huh-NC
Huh7-siRACK1
e
Huh7 Huh7-siRACK1
Luc-miR-185RE
CatA-Luc

Figure 2. RACK1 is required for miRNA function. A, Overexpression of miR122, miR140, and miR185 precursors suppressed activity of the corresponding reporters. RACK1 knockdown partially blocked these effects. miRNA reporter plasmids were transfected, with or without the corresponding miRNA precursor- and two types of siRACK1-expressing plasmids (siRACK1 (1) and (2)), into Huh7 cells. Values were normalized to those obtained from cells transfected with a miRNA precursor-non-expressing control vector, which were set to 1. Data represent the mean ± SD of three independent experiments. siGFP was used as a control, and it had no effect. Similar results were obtained using PLC/PRF/5 cells. **B,** CatA-Luc plasmids, which contain endogenous miR122 target sites derived from the CAT1 gene in its 3′-UTR, were transfected, with or without miR122 precursor- and siRACK1-expressing plasmids, into Huh7 cells. Data were generated and are presented as described in (A). Similar results were obtained using PLC/PRF/5 cells. **C,** Confirmation of the efficient knockdown of RACK1 expression in stable RACK1-knockdown Huh7 cells. 293T cell lysates were used as a positive control. **D,** miRNA function is impaired in stable RACK1-knockdown cells. miRNA185 reporter plasmids were transfected, with or without miR185 precursor-expressing plasmids, into control and RACK1-knockdown cells. Data were generated and are presented as described in (A). **E,** Endogenous miRNA function is impaired in RACK1-knockdown cells. miRNA185 reporter plasmids and CatA-Luc plasmids were transfected without miRNA precursor-expressing plasmids into control and RACK1-knockdown cells to assess endogenous miRNA function. Values were normalized to those obtained from control cells, which were set to 1. Data represent the mean ± SD of three independent experiments. *, $p<0.05$.
doi:10.1371/journal.pone.0024359.g002

determined RACK1 expression in various cancers and in healthy tissues by immunohistochemistry. While RACK1 expression was essentially normal in most cancers, it was frequently reduced in hepatocellular carcinoma (HCC) (Fig. 5A, B, C), consistent with a previous report [31]. KSRP expression levels were relatively higher in liver tissues than in other organs, but no remarkable expression differences between HCC and adjacent tissues were observed (Figure S8A, B). These results suggest that decreased

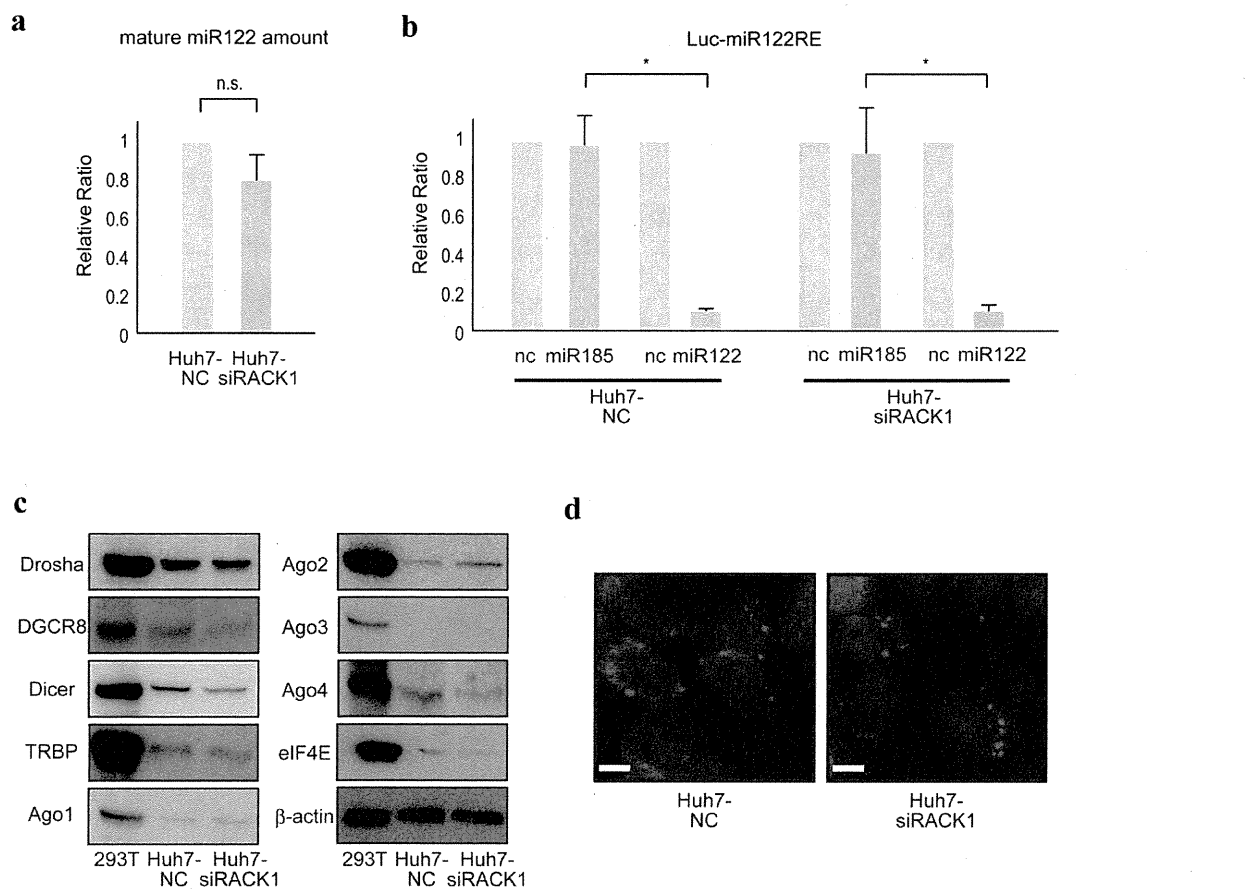

Figure 3. RACK1 functions after miRNA maturation and before the silencing machinery. A, miRNA maturation was not impaired in RACK1-knockdown cells. Total RNA was isolated from control and RACK1-knockdown cells. Levels of mature miR122 were measured and normalized to the level of U6 snRNA. Relative ratios were calculated by adjusting the value for each miRNA in control cells to 1. Data represent the mean ± SD of six independent experiments. **B,** Artificial synthetic miRNA oligonucleotides function appropriately in RACK1-knockdown cells. Control Huh7 cells and RACK1-knockdown cells were transfected with miR122 reporter plasmids with or without synthetic corresponding miR122 oligonucleotides and non-corresponding miR185 oligonucleotides (to verify specificity). Values were normalized to those obtained from the cells transfected with control synthetic oligonucleotides, which were set to 1. Data represent the mean ± SD of three independent experiments. *, $p<0.05$. Similar results were obtained using PLC/PRF/5 cells. **C,** Expression of proteins involved in the miRNA pathway was normal in RACK1-knockdown cells. 293T cell lysates were used as a positive control. **D,** Localization and expression of P-bodies were normal in RACK1-knockdown cells. Control and RACK1-knockdown cells were immunostained for the p-body marker GW182. Scale bar, 50 μm.
doi:10.1371/journal.pone.0024359.g003

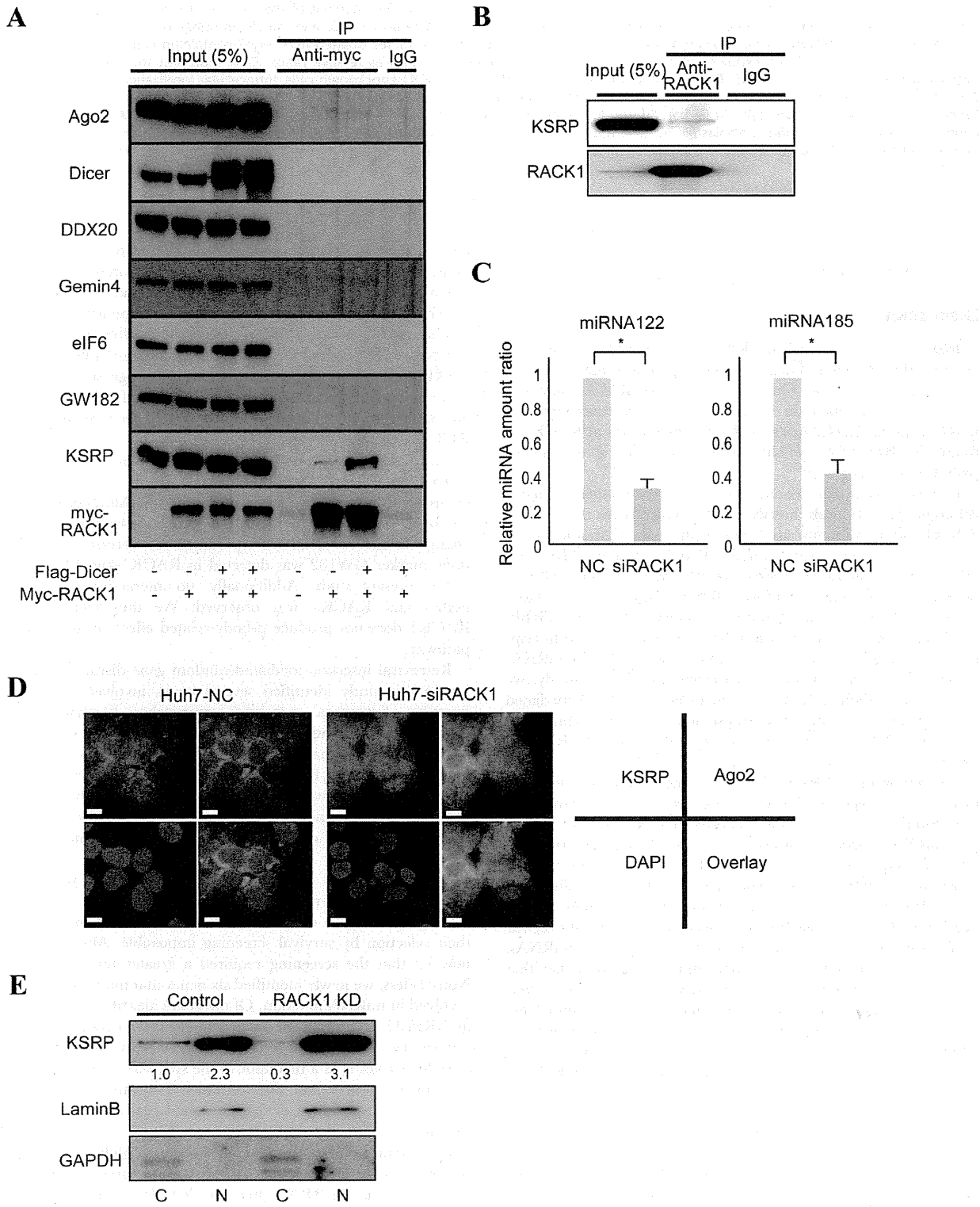

Figure 4. RACK1 binds to KSRP and may be involved in the recruitment of mature miRNAs to the RISC. A, Huh7 cells were transiently transfected with a control vector or a myc-tagged RACK1-expressing plasmid with or without a flag-tagged Dicer-expressing plasmid. Myc-tagged RACK1 was immunoprecipitated using anti-myc agarose. Normal mouse IgG was used as a control for immunoprecipitation. Co-precipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysates was loaded as "input." Representative results from two independent experiments are shown. Similar results were obtained using 293T cells. **B,** Endogenous RACK1 in Huh7 cells was immunoprecipitated using anti-RACK1 antibody and Protein A/G Sepharose. Normal mouse IgG was used as a control for immunoprecipitation.

Coprecipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysate was loaded as "input." Representative results from two independent experiments are shown. **C,** Levels of mature miR122 and miR185 in Ago2-containing complexes were reduced in RACK1-knockdown cells. Mature miRNA levels were measured in RNA samples isolated from Ago2-containing complexes from control Huh7 (NC) cells and RACK1-knockdown (siRACK1) cells. miRNA levels were calculated as relative ratios. Data represent the mean ± SD of six independent experiments. *, $p<0.05$. **D,** More KSRP localizes in the nucleus in RACK1-knockdown cells. Intracellular localization of KSRP (red) and Ago2 (green) were examined in control and RACK1-knockdown cells. KSRP was distributed both in the nucleus and cytoplasm, but more KSRP localized in RACK1-knockdown cells. Scale bar, 50 μm. **E,** Greater localization of KSRP to the nucleus in RACK1-knockdown (RACK1 KD) cells was confirmed by Western blotting. Cytoplasmic (C) and nuclear (N) fractions were blotted with anti-KSRP antibody. The numbers below the panel indicate relative KSRP protein levels. GAPDH (a cytoplasm marker) and Lamin B (a nucleus marker) were blotted to confirm the appropriate fractionation.
doi:10.1371/journal.pone.0024359.g004

RACK1 expression and, consequently, decreased miRNA function may play an important role in HCC.

Discussion

Here, we have shown that RACK1 is required for miRNA function through comprehensive gene screening using a random gene disruption method. Our results show that RACK1 interacts with KSRP and plays an important role in recruiting mature miRNAs to the RISC. Additionally, the expression of RACK1 is frequently decreased in HCC and may play a role in its pathogenesis.

RACK1 was initially identified as a major component of active ribosomes [18]. It binds directly to eIF6, which keeps the 40S and 60S ribosomal subunits apart, preventing the formation of a translationally competent complex. RACK1 bridges PKC and eIF6. Subsequent phosphorylation of eIF6 by PKC causes eIF6 to be released, an event that triggers 60S subunit activation. More recently, eIF6 was reported to be a component of a large TRBP-containing complex involved in miRNA-mediated post-transcriptional silencing [19]. However, the importance of eIF6 in miRNA-mediated silencing remains controversial [21]. In this study, we report that, although RACK1 is involved in miRNA-mediated silencing, it did not influence of translational machinery complexes containing eIF6, but instead appears to recruit mature miRNAs to the RISC.

The importance of RACK1 in regulating miRNA function was independently reported during the preparation of this manuscript [26]. Similar to our cases, they reported that RACK1 is required for miRNA function; however, the mechanism they reported differed from ours. They found that RACK1 contributes to the recruitment of RISC to the site of translation through binding with Ago2. While we could not detect the effects of RACK1 on miRNA-mediated translational repression as described above, our results were based on the use of synthesized mature miRNAs, which appear functionally Ago2-independent. Due to the fact that we also detected binding between RACK1 and Ago2, their proposed mechanism remains a possibility [26]. In addition, the importance of these diverse mechanisms might be dependent on different types of miRNAs. For example, the maturation of miRNAs used in our study, i.e., miR122, miR140, and miR185, is KSRP-independent [29]. Taking these points into consideration, in the future it may be necessary to determine if RACK1 is involved in different types of miRNA function under the same mechanisms, using various kinds of miRNAs.

We found that RACK1 interacts with KSRP particularly when Dicer is overexpressed. KSRP is a key mediator of AU-rich element-containing mRNA degradation [27,28] that has also been reported to bind to and promote the maturation of a subset of miRNA precursors [29]. In our study, however, maturation of miRNAs examined was preserved and the transfected synthetic miRNAs functioned appropriately in RACK1-knockdown cells. Moreover, levels of miRNAs in Ago2-containing complexes were decreased in RACK1-knockdown cells. Based on these observations, we speculate that RACK1 is only involved in the recruitment of mature miRNAs to RISCs from miRNA maturation to the point at which they exert their effects in vivo. Although the precise mechanisms by which RACK1 contributes to the recruitment of miRNAs to RISCs and the biological significance of its binding to KSRP remains to be determined, these results suggest that RACK1 may regulate miRNA function as a component of a KSRP complex and during the maturation of miRNAs and their recruitment to RISCs.

Translationally repressed mRNA accumulates in discrete cytoplasmic foci known as p-bodies [24] or in another class of cytoplasmic aggregates, stress granules [32]. Although RACK1 has been reported to mediate stress granule formation [33], no change in the localization of p-bodies or expression of the p-body marker GW182 was detected in RACK1-knockdown cells in the present study. Additionally, no interaction between p-bodies and RACK1 was observed. We thus conclude that RACK1 does not produce p-body-related effects in the miRNA pathway.

Retroviral insertion-mediated random gene disruption screening in this study identified several genes involved in miRNA function. Functional genomics approaches involving RNAi libraries have recently been used to identify genes involved in certain functional pathways [34–37]. However, RNAi libraries may not be suitable for identifying genes involved in miRNA- or siRNA-related pathways, because they affect the pathways being examined. Thus, random gene disruption, as used here, may be a useful alternative option for the functional screening of such genes.

While identified genes here did not include well-known components of the miRNA pathway such as Dicer and Ago2, these genes may be critical for cell survival, which would make their selection by survival screening impossible. Alternatively, it may be that the screening required a greater number of cells. Nonetheless, we newly identified six genes that may potentially be involved in miRNA function. Of the genes identified, two, RPS23 and RPL11, are ribosome-related molecules, encoding 40S and 60S subunit proteins, respectively. In view of the fact that one of RACK1's roles is as a mediator of the synthesis of 80S ribosomes (composed of 40S and 60S subunits) [18], these results may suggest the importance of the ribosomal machinery in miRNA function.

Consistent with recent evidence linking global microRNA depletion with oncogenesis [12,30], RACK1 expression was found to be reduced in HCC. Because RACK1 staining in non-cancerous liver tissues was stronger than that in other tissues, the involvement of RACK1 in physiological functions may be more relevant in the liver. These results suggest that the miRNA functional impairment plays an important role in oncogenesis in the liver and/or the maintenance of oncogenicity in HCC, similar to the deficiency of miRNA expression [11].

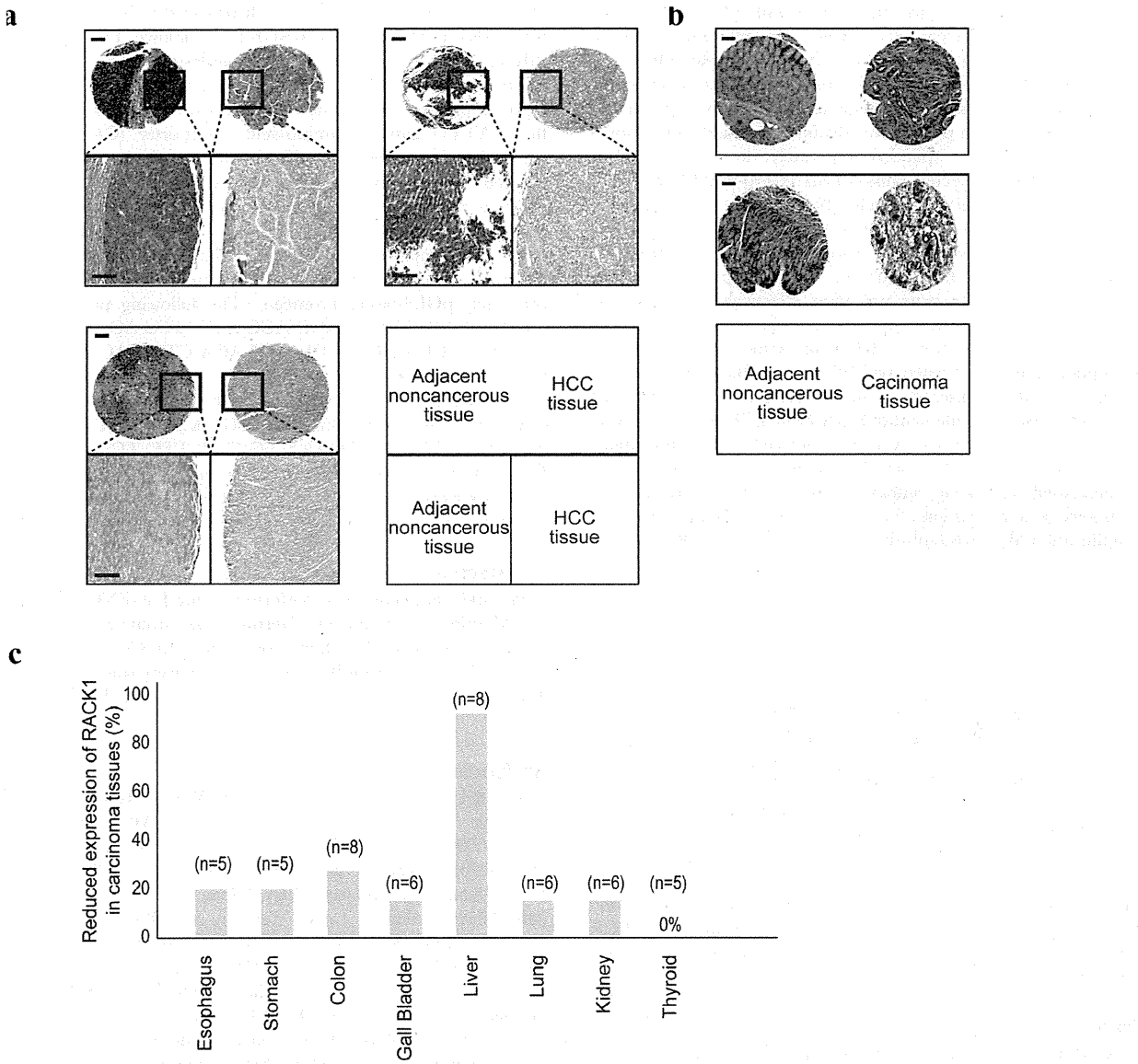

Figure 5. RACK1 expression is reduced in HCC. A, Immunohistochemical analysis of RACK1 protein expression in HCC and non-cancerous surrounding tissues. While strong staining was observed in the cytoplasm of hepatocytes in non-cancerous liver tissues (upper left (one panel)), HCC cells were stained more weakly (upper right). Lower panels: magnified images of the highlighted regions in the corresponding upper panels. Three representative cases are shown. Scale bar, 500 μm. **B,** Comparable expression of RACK1 in colon carcinoma tissues (right images) and non-cancerous surrounding tissues (left). Two representative cases are shown. Scale bar, 500 μm. **C,** Comparison of RACK1 staining in cancers and healthy surrounding tissues. Eight types of cancers were examined. Percentages of cases in which RACK1 expression was lower in cancerous tissues than in healthy tissues were calculated. The number of cases of each type of cancer studied is indicated.
doi:10.1371/journal.pone.0024359.g005

Materials and Methods

Cell culture

Huh7 and PLC/PRF/5 cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). 293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Stable cell lines derived from Huh7 and PLC/PRF/5 cells were established by retroviral or lentiviral infection. Clones were selected through the addition of 6 μg/mL puromycin, 400 μg/mL hygromycin, or 5 μg/mL blasticidin to the culture medium, unless otherwise specified.

Random gene disruption

The use of a pDisrupt retroviral vector carrying a blasticidin resistance gene for random gene disruption has been described previously [13]. Retroviruses were produced by transfection of the retroviral vector into virus-packaging Platinum A cells (Orbiden, San Diego, CA). Then, 48 h after transfection, the supernatants were harvested and viruses collected. To prepare reporter cell

lines, Huh7 cells were first transfected with pBSII-Hygro and selected on hygromycin. To avoid random integration in polyclonal cells, single clones were picked and expanded. The selected clones were then infected with MIR122-puro lentiviruses, which express a miR122 precursor and a puromycin resistance gene, and selected on puromycin. Again, several individual clones were isolated and expanded separately to avoid random integration. Using selected Huh7-pBSII-Hygro-miR122 cells and parental Huh7-pBSII-Hygro cells, titration of the hygromycin concentration required for total cell killing was performed to determine the clone showing the widest differences in hygromycin concentration (i.e., the clone in which expression of the hygromycin resistance gene was most effectively suppressed by miR122). Next, this clone was infected with retroviruses at low multiplicities of infection (~0.01) to achieve random gene disruption and then treated with blasticidin. Blasticidin-resistant cells carry viruses inserted in functional genes (otherwise, the blasticidin resistant gene cannot be expressed). Then, hygromycin was added to select hygromycin resistant cells, which potentially have impaired miR122 function as a result of gene disruption (which itself results in impaired suppression of hygromycin resistance gene expression). Such clones were picked and 3′-rapid amplification of cDNA ends (RACE) analysis of the mRNAs fused with the blasticidin gene used to identify the disrupted gene in each clone.

3′-RACE

We infected ~106 Huh7-pBS-Hygro-miR122 cells and obtained ~104 blasticidin-resistant clones. After selection, ten surviving clones were obtained. The endogenous gene sequence fused with the blasticidin gene was amplified by 3′-RACE. Total RNA was isolated and reverse transcribed using an RT primer (5-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC $(T)_{17}$ -3′). A nested PCR was performed using primers P0/Q0 (5′-GGT GTC GAC AGG TGC TTC TC-3′/5′-CCA GTG AGC AGA GTG ACG-3′) and P1/Q1 (5′-CTG GGA TCA AAG CGA TAG TG-3′/5′-GAG GAC TCG AGC TCA AGC-3′). P0 and P1 anneal to sequences in the blasticidin resistance gene and Q0 and Q1 to the anchor sequences of the RT primer. The PCR fragments were finally subcloned into a TA-cloning vector (Invitrogen, Carlsbad, CA) and sequenced.

Plasmids

A pBSII-Hygro plasmid containing miR122REs in its 3′-UTR was generated from pGL4-Hygro, purchased from Promega (Madison, WI). Synthetic oligonucleotides containing two tandem miR122-responsive elements were annealed and inserted in the 3′-UTR of this plasmid's hygromycin resistance gene (at the PmeI site). The primer used was 5′-AAA CAC *AAA CAC CAT TGT CAC ACT CCA* AAT TAC *AAA CAC CAT TGT CAC ACT CCA* CTC GAG-3′ (miR122-responsive sequences are shown in italics). Next, the gene cassette (containing an SV40 promoter, the hygromycin resistance gene, the miR122 responsive elements and polyA sequences) was excised using BamHI and SalI, and was inserted into pBlueScript II at the same restriction sites. pMIR-122-puro was constructed by replacing the eGFP gene of pMIRH122PA (System Biosciences, Mountain View, CA) at the FseI site by a puromycin resistance gene, which was PCR-amplified using pCDH-EF1α-puro (System Biosciences) as a template. The following primers were used: 5′-GGC CGG CCG CAT GAC GAG TAC AAG CCC AC-3′ and 5′-GGC CGG CCT CAG GCA CCG GGC TTG CGG GT-3′. pcDNA3-myc-RACK1 was used to achieve RACK1 overexpression. Two constructs targeting different RACK1 sequences, pSIH-H1-RACK1shRNA and pSuper.retro-RACK1 shRNA (both provided by Prof. Takekawa [33]), were used to achieve RACK1 knockdown. pSIH-H1-GFP shRNA was constructed as a control, as described previously [38]. To determine the effect of miR-122 on natural gene targets, a reporter plasmid (CatA-Luc) with natural 3′-UTR sequences of the CATT1 (cationic amino-acid transporter (CAT-1)) gene containing three predicted miR-122 binding sites was used. This plasmid was provided by Prof. W. Filipowicz [22]. Reporter plasmids used to analyze miRNA function were constructed by inserting annealed synthetic primers containing two tandem sequences complementary to each miRNA into the 3′-UTR of the firefly luciferase gene at the FseI site, driven by the CMV promoter (pGL3-basic; Promega). The following primers were used: miR-122, 5′-ACA AAC ACC ATT GTC ACA CTC CAA CTT CAC CCA ACC ATT GTC ACA CTC CAC TCG AGC CGG-3′; miR-140-5p, 5′-CTA CCA TAG GGT AAA ACC ACT GAA TTC TAC CAT AGG GTA AAA CCA CTG CTC GAG CCG G-3′; miR-185, 5′-TCA GGA ACT GCC TTT CTC TCC AAA TTT CAG GAA CTG CCT TTC TCT CCA CTC GAG CCG G-3′. The flag-tagged Dicer-expressing plasmid has been described previously [39]. MiR precursor overexpressing plasmids were purchased from System Biosciences.

Transfection

Plasmid transfection was performed using FuGENE6 (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol [40]. Synthetic mature miRNA oligonucleotides (miNatural, CosmoBio, Tokyo, Japan) were transfected using TransMessenger Transfection Reagent (Qiagen, Hilden, Germany).

Antibodies

The following antibodies were used in Western blotting analyses: anti-Dicer (SAB4200087; Sigma, St. Louis, MO), anti-TRBP2 (SAB4200087; Sigma), anti-β-actin (A5316; Sigma), anti-Ago2 (#015-22031; Wako, Osaka, Japan), anti-Gemin3 (DDX20) (SC-57007; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Gemin4 (H00050628; Abnova, Taipei, Taiwan), anti-eIF4E (#9742; Cell Signaling Technology, Danvers, MA), anti-eIF6 (D16E9; Cell Signaling Technology), anti-KSRP (A302-021A; Bethyl, Montgomery, TX), anti-GW182 (MBL Nagoya, Japan), anti-Drosha (#3364; Cell Signaling Technology), anti-DGCR8 (SAB 4200088; Sigma), anti-Ago1 (clone1F2; Wako, Osaka, Japan), anti-Ago3 (SAB2104518; Sigma), anti-Ago4 (SAB2104338; Sigma), anti-Lamin B (SC-20682; Santa Cruz Biotechnology), anti-GAPDH (clone 3C2; Abnova), and anti-myc (Santa Cruz Biotechnology). An Atlas anti-RACK1 antibody was used in immunohistochemical analyses (HPA021676; Sigma).

Western blotting

Cell extract protein concentrations were measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Total protein (30 μg) was resolved by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Membranes were sequentially incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected using ImmunoStar reagents (Wako) [41].

Cell fractionation

To separate cytoplasmic and nuclear protein fractions, a ProteoExtract subcellular fractionation kit (Calbiochem-EMD Biosciences, San Diego, CA) was used according to the manufacturer's

protocol. To confirm the identities of the subcellular fractions, Lamin B was blotted for the nuclear fraction and GAPDH for the cytoplasmic fraction.

Immunocytochemistry

To determine the localization of p-bodies, cells growing on two-well chamber slides were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100. Fixed cells were then probed with an anti-GW182 antibody for 1 h after blocking with 5% normal goat serum for 30 min. They were then treated with an Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 30 min. Slides were mounted using VectaShield with DAPI (Vector Labs, Burlingame, CA). Numbers of p-bodies in the cells were determined in three independent views, and the average number or p-bodies per cell calculated. Similar procedures were applied to determine KSRP and Ago2 intracellular localization, except that anti-KSRP and anti-Ago2 were used as first antibodies, and Alexa Fluor 555-conjugated goat anti-rabbit antibody and Alexa Fuor 488-conjugated anti-mouse antibody were used as secondary antibodies.

Immunohistochemistry

Tissue arrays containing multiple organ carcinoma tissues and matched adjacent non-cancerous tissues (1.5 cm apart; #MC501 and #MC962) were purchased from US Biomax (Rockville, MD). Slides were incubated at 65°C for 1 h and deparaffinized. Endogenous peroxidase activity was blocked through incubation in 3% hydrogen peroxide buffer for 30 min. Antigen retrieval was achieved by incubating the slides at 89°C in 10 mM sodium citrate buffer (pH 6.0) for 30 min. To minimize non-specific background staining, slides were blocked in 5% normal goat serum (Dako, Glostrup, Denmark) for 10 min at room temperature. A primary anti-RACK1 antibody or anti-KSRP antibody, diluted 1:50 in Antibody Diluent (Dako), was applied for 1 h at room temperature. Slides were then incubated with an anti-mouse horseradish peroxidase-conjugated secondary antibody (Nichirei Bioscience, Tokyo, Japan) for 1 h. Bound antibody was visualized by incubation in 3,3′-diaminobenzidine (Nichirei Bioscience; diluted in buffered substrate) for 5 min. The slides were finally counterstained with hematoxylin, dehydrated with ethanol, and mounted using Clarion mounting medium (Biomeda, Foster City, CA).

Luciferase assay

Luciferase activity were measured using a Dual Luciferase Reporter Assay System (Promega) in conjunction with a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). pRL-TK, a control plasmid carrying the Renilla reniformis (sea pansy) luciferase gene, driven by the herpes simplex virus thymidine kinase promoter (Toyo Ink, Tokyo, Japan), was used as an internal control. Relative luciferase signals were calculated by dividing firefly luciferase activity by that of the internal control (sea pansy luciferase) (unless otherwise specified). All experiments were performed at least twice (each time in triplicate).

Lentiviruses and transduction

293T cells were transfected with pPACKH1 Packaging Plasmid Mix (System Biosciences) and pCDH (as a negative control), pSIH-H1-RACK1shRNA, or pMIR122-puro. After 2 days, supernatants were collected and the viruses concentrated using PEG-it Virus Precipitation Solution (System Biosciences) according to the manufacturer's protocol. Lentiviral particles expressing shRNAs specific for Ago2 (sc-44409) and KSRP (sc-44831) were purchased from Santa Cruz Biotechnology. Polybrene (Millipore, Billerica, MA) was used in lentiviral particle infection.

miRNA isolation, quantitation, and RIP assay

A Mir-X miRNA qRT-PCR SYBR Kit (Clontech, Mountain View, CA) was used in accordance with the manufacturer's protocol to measure levels of different miRNAs in cells. Total RNA was isolated from cells and tissues using the TRIzol reagent (Invitrogen). Levels of U6 snRNA were used in the normalization of cellular miRNA levels. Relative expression levels were calculated by the ΔΔCT method: ΔΔCT = ΔCTmiRNA – ΔCTU6. To purify miRNAs from Ago2-containing RISC-associated miRNP complexes, miRNA fractions were isolated using the Human Ago2 MicroRNA Isolation Kit (Wako), using antibodies raised against Ago2. The following primers were used in quantitative PCR analyses: miR122, 5′-TGG AGT GTG ACA ATG GTG TTT G-3′; and miR-185, 5′-TGG AGA GAA AGG CAG TTC CTG A-3′.

Immunoprecipitation

Huh7 cells were transfected with pcDNA3 (negative control) or myc-tagged RACK1-expressing plasmid with or without Dicer-expressing plasmid. When required, cell lysates were incubated at room temperature with RNase A (10 μg/mL; Promega) for 30 min. Cell extracts were prepared using immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin). Myc-tagged RACK1 was precipitated through incubation with anti-myc agarose (#SC-40; Santa Cruz Biotechnology) for 8 h. Anti-KSRP antibodies and Protein A/G Sepharose (#SC-2003; Santa Cruz Biotechnology) were used for endogenous KSRP precipitation. To immunoprecipitate endogenous RACK1 protein, anti-RACK1 antibodies and Protein A/G Sepharose were used. After being washed four times, the precipitated proteins were analyzed by Western blotting using the indicated antibodies. Five percent of each cell lysate was used as "input."

Statistical analysis

Statistically significant differences between groups were identified using Student's t-test (when variances were equal) or Welch's t-test (when variances were not equal).

Supporting Information

Figure S1 miRNA maturation was not impaired in RACK1-knockdown cells. Total RNA was isolated from control and RACK1-knockdown cells. Levels of mature miR22, miR140-5p, miR140-3p, and miR185, all of which are relatively abundant miRNAs in liver cells, were measured and normalized to the level of U6 snRNA. Relative ratios were calculated by adjusting the value for each miRNA in control cells to 1. Data represent the mean ± SD of six independent experiments.
(TIF)

Figure S2 Synthetic mature miRNAs were functional in Ago2-kcnockdown cells. A, Ago2-knockdown (Ago2 KD) Huh7 cells were established by shRNA-expressing lentiviral infection. **B,** Artificial synthetic miRNA oligonucleotides function normally in Ago2-knockdown Huh7 cells. Control and Ago2-knockdown (Ago2 KD) cells were transfected with miR122 or miR185 reporter plasmids with corresponding synthetic mature miRNA oligonucleotides. Values were normalized to those

obtained from cells transfected with control synthetic oligonucleotides, which were set to 1. Data represent the mean ± SD of three independent experiments. *, $p<0.05$.
(TIF)

Figure S3 RACK1 binds to KSRP. Huh7 cells were transiently transfected with a control vector or a myc-tagged RACK1-expressing plasmid, with or without a Flag-tagged Dicer-expressing plasmid. Endogenous KSRP was immunoprecipitated using anti-KSRP with Protein A/G Sepharose. Normal mouse IgG was used as a control for immunoprecipitation. Co-precipitated proteins were blotted using antibodies against myc tag. Five percent of the total cell lysates was loaded as "input." Representative results from two independent experiments are shown.
(TIF)

Figure S4 Coimmunoprecipitation of endogenous KSRP and Ago2 with myc-RACK1 is insensitive to RNase A treatment. Huh7 cells were transiently transfected with a control vector or a myc-tagged RACK1-expressing plasmid. Myc-tagged RACK1 was immunoprecipitated using anti-myc antibody conjugated to agarose. Normal mouse IgG was used as a control for immunoprecipitation. Coprecipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysate was loaded as "input." Representative results from two independent experiments are shown.
(TIF)

Figure S5 RACK1 may be involved in the recruitment of mature miRNAs to the RISC. Mature miRNA levels were measured in RNA samples isolated from Ago2-containing complexes from control Huh7 (NC) cells and RACK1-knockdown (siRACK1) cells. miRNA levels were calculated as relative ratios. Data represent the mean ± SD of six independent experiments. * $p<0.05$.
(TIF)

Figure S6 KSRP is not required for RISC activity. A, KSRP-knockdown Huh7 cells were established by shRNA-expressing lentiviral infection. **B,** Artificial synthetic miRNA oligonucleotides function normally in KSRP-knockdown Huh7 cells. Control and Ago2-knockdown cells were transfected with miR122 or miR185 reporter plasmids with corresponding synthetic mature miRNA oligonucleotides. Values were normalized to those obtained from cells transfected with control synthetic oligonucleotides, which were set to 1. Data represent the mean ± SD of three independent experiments. *, $p<0.05$.
(TIF)

Figure S7 KSRP does not bind with Ago2. Endogenous KSRP was immunoprecipitated using anti-KSRP with Protein A/G Sepharose. Normal rabbit IgG was used as a control for immunoprecipitation. Co-precipitated proteins were blotted using antibodies against Ago2.
(TIF)

Figure S8 The levels of KSRP expression are high in liver tissues. A, Immunohistochemical analysis of KSRP protein expression in hepatocellular carcinoma (HCC) and non-cancerous surrounding tissues. Lower panels: magnified images of the highlighted regions in the corresponding upper panels. Three representative cases are shown. For a comparison, the sections used here were almost the same parts used for determining RACK1 expression in Fig. 5. Scale bar, 500 μm. **B,** Relatively low expression levels of KSRP both in colon carcinoma tissues (right images) and non-cancerous surrounding tissues (left). Two representative cases are shown. Again, for a comparison, the sections used here were almost the same parts used for determining RACK1 expression in Fig. 5. Scale bar, 500 μm.
(TIF)

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Author Contributions

Conceived and designed the experiments: M. Otsuka M. Omata K. Koike. Performed the experiments: M. Otsuka AT TY K. Kojima TK CS MT HY. Analyzed the data: HY. Contributed reagents/materials/analysis tools: MT. Wrote the paper: M. Otsuka M. Omata K. Koike.

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A systematic review of hepatitis C virus epidemiology in Asia, Australia and Egypt

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Keywords
diagnosis – disease burden – epidemiology – HCV – hepatitis C – incidence – mortality – prevalence – systems modeling – treatment rate

Abbreviations
EDHS, egypt demographic and health survey; HCV, hepatitis C virus; HCVPWG, hepatitis C virus projections working group; I-C3, international conquer C coalition; IDU, injection drug use; IV, intravenous; MOH, ministry of health; NGHA, national guard health affairs in Saudi Arabia; NNDSS, national notifiable diseases surveillance system; RNA, ribonucleic acid.

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Abstract
Background: The hepatitis C pandemic has been systematically studied and characterized in North America and Europe, but this important public health problem has not received equivalent attention in other regions. *Aim:* The objective of this systematic review was to characterize hepatitis C virus (HCV) epidemiology in selected countries of Asia, Australia and Egypt, i.e. in a geographical area inhabited by over 40% of the global population. *Methodology:* Data references were identified through indexed journals and non-indexed sources. In this work, 7770 articles were reviewed and 690 were selected based on their relevance. *Results:* We estimated that 49.3–64.0 million adults in Asia, Australia and Egypt are anti-HCV positive. China alone has more HCV infections than all of Europe or the Americas. While most countries had prevalence rates from 1 to 2% we documented several with relatively high prevalence rates, including Egypt (15%), Pakistan (4.7%) and Taiwan (4.4%). Nosocomial infection, blood transfusion (before screening) and injection drug use were identified as common risk factors in the region. Genotype 1 was common in Australia, China, Taiwan and other countries in North Asia, while genotype 6 was found in Vietnam and other Southeast Asian countries. In India and Pakistan genotype 3 was predominant, while genotype 4 was found in Middle Eastern countries such as Egypt, Saudi Arabia and Syria. *Conclusion:* We recommend implementation of surveillance systems to guide effective public health policy that may lead to the eventual curtailment of the spread of this pandemic infection.

The hepatitis C pandemic has been systematically studied and characterized in North America and Europe, but in other areas of the world this important public health problem has not received equivalent attention. The objective of this systematic review is to characterize hepatitis C virus (HCV) epidemiology in Egypt, Australia and selected countries in Asia, i.e. in a geographical area inhabited by over 40% of the global

population. Published studies from the medical literature as well as government or other institutional reports from Australia, China, Egypt, India, Japan, Korea, Pakistan, Saudi Arabia, Syria, Taiwan, Thailand and Vietnam were reviewed in order to provide a comprehensive overview of what is known regarding HCV epidemiology as well as to identify areas that require further investigation and study. By comparing best estimates of regional risk factors and HCV genotype distribution, our aim is to provide an understanding of the current transmission estimates and trends that could be used for projections regarding not only incidence and prevalence but also the overall disease burden, including the ominous complications of HCV infection such as cirrhosis, liver failure and hepatocellular carcinoma. Such an approach will inform health policy, resource allocation and healthcare delivery that may improve to the management of patients with HCV infection.

Methodology

A comprehensive review of the literature was used to gather country-specific data on risk factors, prevalence, number of diagnosed individuals and HCV genotype distribution. References were identified through two sources: indexed journals and non-indexed sources. Indexed articles were found by searching PubMed and regional databases using the following terms: 'hepatitis C AND country name AND (incidence OR prevalence OR mortality OR viraemia OR genotype OR diagnosis OR treatment OR sustained viral response).' Furthermore, references cited within the articles were used. Approximately 7770 abstracts and full articles were reviewed and 690 references were selected based on relevance. In addition, non-indexed sources were identified through searches of individual countries' Ministry Of Health (MOH) websites and international health agency reports. Finally, authors from each country provided government reports and proceedings of local conferences that were not published in the scientific literature. The search included publications in local languages, although reports in English accounted for over 90% of the data sources.

In every case, the prevalence values referred to the prevalence of anti-HCV antibodies that included spontaneously cured and treated/cured individuals. HCV genotype distribution values were based on studies in the viraemic, HCV ribonucleic acid (RNA)-positive, population. Community-based studies were reported, but the focus of this study was to identify/estimate prevalence in the general population. Because the first- and second-generation immunoassay tests provided false-positive results which overestimated the total infected population (1, 2), care was taken to use only studies that used the latest tests to estimate the country's prevalence. In some countries, blood donor data were the main source available for prevalence. The infected general population was composed of high-risk groups [e.g., persons with current or previous history of injection drug use (IDU), dialysis patients and immune compromised persons] as well as non-high-risk groups that contracted the disease through contact with infected blood (e.g. nosocomial infections, dental procedures, etc.). The blood donor population was a good proxy for the latter group. When multiple data sources were available, a systematic process using multi-objective decision analysis was used to rank and select the most appropriate sources (3). When insufficient data were available, data from other countries with similar risk factors and/or population composition were used. Unless indicated, the estimates were for 2004 because of lack of more recent data. When available, subtypes were assessed individually and summed to provide a value for the corresponding genotype. The adult population was defined as ≥ 20 years old.

Results

Australia

In 1998, the Australian Government formed the HCV Projections Working Group (HCVPWG), tasked with estimating incidence and prevalence of HCV (4). Models were developed by this group to estimate prevalence and long-term sequelae of the chronic disease using estimated high-risk populations and incidence of HCV infection. Newly diagnosed, anti-HCV-positive cases were reported to the National Notifiable Diseases Surveillance System (NNDSS) of the Australian Government.

Risk factors

Studies based on NNDSS data showed approximately 80% of infections occurred through IDU (5, 6). Blood transfusions before 1990 accounted for 5–10% of infections in the prevalent population. A study of 800 newly acquired hepatitis C cases reported to NNDSS between 1997 and 2000 found that 93% of all cases with documented risk factors were attributable to IDU, with the trend growing from 84% in 1997 to 95% in 2000 (7). Because transmission via blood supply was virtually eliminated, IDU was identified as the key risk factor along with immigration from endemic countries. Approximately 11% of the infected population were immigrants (8).

Prevalence

A number of publications reported prevalence estimates in the general population (4, 9–13). Studies considered the definitive source for prevalence and incidence were published by HCVPWG (4, 9, 10, 12). Their model considered IDU as well as estimates for infections attributable to immigration and other routes of transmission, such as needlestick injuries in healthcare workers and tattooing. The latest study estimated a prevalent population of 264 000 (all ages) in 2005 (1.3%) with 9700 new infections in the same year. This incidence represented a drop from a peak of 14 000 in 1999 as the result of a reduction in IDU. It was estimated that the total prevalence increased since 1960 and will continue to increase (9, 10).

In a separate analysis, blood samples submitted to all major public and private diagnostic laboratories throughout Australia from 1996 to 1998 were tested as part of a national serological survey for selected infectious diseases (11). Anti-HCV testing in 2800 samples showed an age-standardized prevalence of 2.3%, and a male-to-female ratio of 1.8:1. Peak prevalence occurred in individuals in their 20s to 40s, and the gender ratio was consistent with notifications identified from the NNDSS for the same period. The reported low prevalence in advanced age cohorts was consistent with surveys from England and the USA, all of which identify IDU as the most significant risk factor for HCV (14, 15). Extrapolating to the entire Australian population, 433 000 individuals were estimated to be anti-HCV positive. A number of sampling methodologies were used to minimize selection bias; however, this study most likely overestimated the prevalence as individuals with chronic hepatitis C were oversampled because of their higher utilization of

healthcare services (9). Studies in other subgroups (16–20) and blood donors (21–23) were reported elsewhere.

Diagnosed/incidence

The NNDSS reported the number of diagnosed cases of hepatitis C annually since 1995 (24). Cumulatively, 225 000 individuals were diagnosed and notified through 2005, implying a diagnosis rate of 85% when compared with an overall prevalence of 264 000 in the same year (9, 10). The incidence rates were described above.

Genotype distribution

The published studies were completed 1 year apart and all reported similar results (21, 25–27). A study of 425 patients from a single hospital population reported genotypes 1 (14%), 1a (15%), 1b (23%), 2 (9%), 3 (31%), 4 (5%), 6 (2%) and mixed (1%) (27). The results were consistent with other work conducted by a national reference laboratory (26), which also showed that genotype 3 was more prevalent in younger age cohorts (21–40 year olds), indicative of transmission via IDU, and that genotype 1b was identified more frequently among patients with transfusion-acquired HCV (26).

Summary

The epidemiology of acute and chronic HCV infection has been well characterized by a number of groups including the Australian government (primarily through the National Centre for HIV Epidemiology and Clinical Research) as well as reference laboratories and clinical groups at major tertiary hospitals. The research provided a more complete picture of the epidemic, which is notable for the relatively high proportion of genotype 3-infected individuals compared with other developed countries. IDU continued to be the main driver of HCV infection in Australia. The strength of the population estimates provided a strong basis for future public health planning.

China

HCV has been a notifiable infection in the Hong Kong region of China since 1996; cases are tracked through the Surveillance and Epidemiology Branch of the Centre for Health Protection (28). The use of paid blood donors has been banned in China, but anti-HCV screening reports were not regularly tracked among donation agencies (29).

Risk factors

There were a limited number of risk factor assessments (29–31). A 2009 study on 69 patients from around Anyang found the strongest risk factor to be intravenous (IV) injection, where 75.4% of HCV infections were associated with IV use of glass syringes or needles. A history of blood transfusions was also reported in 73.9% of the cases and was statistically significant after adjustment for other risk factors. An additional significant risk factor was oesophageal balloon use, found in 27.5% of infected individuals. All three risk factors point to under-regulated medical procedures conferring a large risk for HCV transmission (30).

A blood donor study also indicated continued iatrogenic transmission. Risk factor assessments suggested urban, educated individuals who were more likely to see a doctor were at higher risk for HCV, confirming continued transmission in the hospital/medical care-based setting (29).

Prevalence

The estimated HCV prevalence was 1–1.9%. Since 1992, a number of studies reported prevalence within a range of 0.29–9.6% (29, 30, 32–53); however, there were no systematic population-based estimates. Consistent with other countries, blood donor populations provided low prevalence rates because of selection bias. A study in 13 620 volunteer blood donors in one province reported a prevalence of 0.49% in 2003 (29). The prevalence was highest in the 40–49 year olds, at 0.86% (29).

Among non-blood donors, a 1998 study of 3902 individuals from Shenyang province reported a range of 0.42–1.66% (38). Others found a prevalence of 9.6% in 500 elderly individuals (> 55 years of age) in the rural Henan province (32), while Liu *et al.* (30) documented a prevalence of 0.90% in 8226 persons aged 25–65 participating in an endoscopic surveillance study for oesphageal cancer in the Anyang province. In another large study, a prevalence of 1.03% was reported in 12 280 patients admitted to the hospital for a transfusion or other surgical procedure (33).

Diagnosed/incidence

A single incidence study reported a rate of 24.2/100 000 in a sample of 89 647 blood donors in 2007 (54). However, data from other countries suggested that blood donor sampling underestimated the actual incidence rate. Thus, the number of new cases was likely to be higher.

Genotype distribution

Genotype distributions were reported by studies published in 1994–2006 (55–58). A study of 139 HCV patients sampled from nine regions in China (56) reported genotypes 1 (67.6%), 2 (14.4%), 3 (4.3%), 6 (13%) and other (0.7%). Genotype 1b was the most prevalent at 66.2%, and genotype 2a showed a prevalence of 13.7%. Statistically significant geographical differences were observed, and genotype 6 was only observed in the South (56).

A more recent study from Hong Kong sampled 1055 IDUs and non-IDUs in 1998–2004. The non-IDU population showed a genotype 1b prevalence of 63.6%. Genotypes 2a and 3 had prevalence rates of 3.1 and 3.9%, respectively, and genotype 6a was found in 23.6% of participants. The IDU population showed statistically different genotype distributions, where genotype 6a was seen in 58.5% and 1b in 33.0% (55).

Summary

HCV epidemiology in China is largely uncertain. No population-based prevalence or incidence rate estimate is available. Most investigations in HCV have been performed in subgroup studies or voluntary blood donor populations. There is evidence that genotype distribution and prevalence estimates are significantly different across the country, yet prevalence estimates appear relatively low by comparison to other countries in