et al., 2007; Zhao et al., 2009). Accordingly, Hedgehog signaling inhibitors have been clinically tested and might be beneficial for patients with advanced medulloblastoma or basal cell carcinoma, although Smo mutations in cancer cells confer resistance against such inhibitors (Rudin et al., 2009; Von Hoff et al., 2009; Yauch et al., 2009).

2.1.3 Notch signaling

Notch signaling has a pivotal role in regulating cell-to-cell communication during embryogenesis (Artavanis-Tsakonas et al., 1999), and is known to regulate stem cell fate in various organs (Androutsellis-Theotokis et al., 2006; Fre et al., 2005). Mammalian Notch ligands consist of the two structurally distinct families Delta-like ligands (DLLs) and Jagged ligands (JAGs), and these ligands are bound to the cell membrane (Fig. 4). The activation of Notch signaling is initiated by the binding of these membrane-bound ligands to Notch receptors, which results in the release of the Notch intracellular domain into the cytoplasm and nucleus by the γ-secretase complex to activate the Notch-specific transcriptional program.

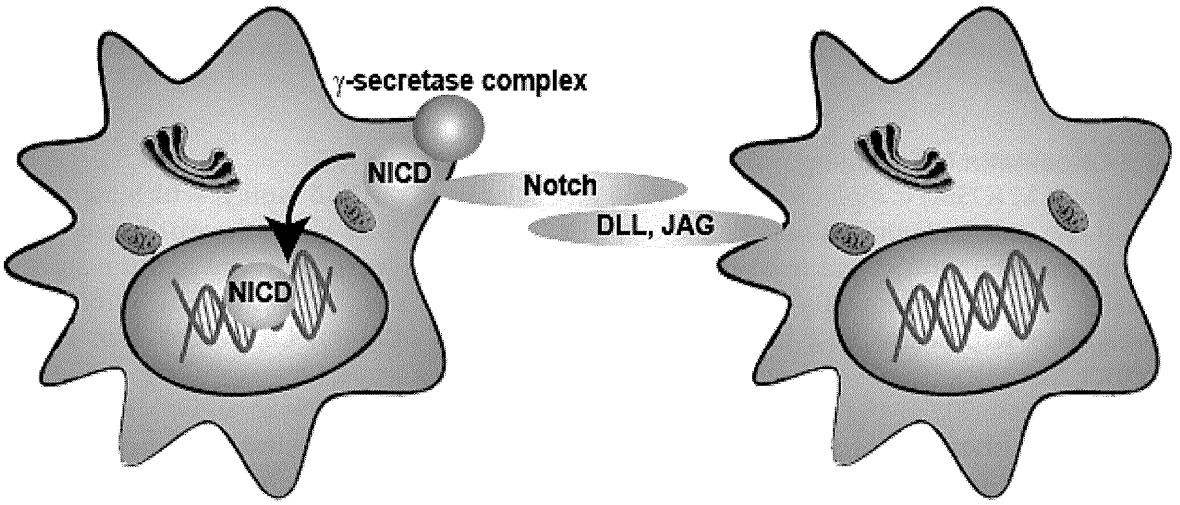

Fig. 4. Notch signaling. DLL, Delta-like ligand; JAG, Jagged; NICD, Notch intracellular domain

Notch signaling has been implicated in various types of cancers, including solid tumors and leukemia (Pannuti et al., 2010). A growing number of recent studies has demonstrated that the activation of the Notch signaling pathway can drive tumor growth via the expansion of the cancer stem cell population (Korkaya and Wicha, 2009; Peacock and Watkins, 2008; Wilson and Radtke, 2006). Indeed, the Notch signaling pathway has been demonstrated to be active in cancer stem cells and to play a critical role in the self-renewal of cancer stem cells (Fan and Eberhart, 2008; Fan et al., 2010; Wang et al., 2009). Thus, Notch signaling is considered to be a good target for pharmacological inhibition to eradicate cancer stem cells, and the effect of Notch inhibitors against Notch, including γ-secretase inhibitors or monoclonal antibodies, have been extensively evaluated (Pannuti et al., 2010).

2.2 Signaling pathways responsible for cancer stem cell differentiation

Although self-renewal pathways are considered to be critical targets for the eradication of cancer stem cells, it is still debatable if differentiation pathways are equally effective for their

eradication. Several recent studies have provided evidence of the utility and limitation of the cancer stem cell differentiation strategy by modulating the signaling pathways responsible for the differentiation of normal stem/progenitor cells.

2.2.1 Bone morphogenic protein signaling

Bone morphogenic protein (BMP) signaling is known to be activated during embryogenesis and to play a pivotal role in the differentiation of neural and intestinal stem cells (Varga and Wrana, 2005). BMPs belong to a subgroup of the transforming growth factor-β superfamily and activate signaling through the BMP-receptor (BMPR)-mediated phosphorylation of Smad proteins. Interestingly, recent studies have suggested the utility of BMPs to induce the differentiation of brain cancer stem cells and facilitate brain tumor eradication (Lee et al., 2008; Piccirillo et al., 2006). More recently, colorectal cancer stem cells have been shown to lack the expression of BMP4, and the administration of BMP4 enhanced the terminal differentiation, apoptosis, and chemosensitization of colorectal cancer stem cells (Lombardo et al., 2011). Interestingly, the effects of BMP4 on the differentiation of colorectal cancer stem cells appeared to be independent of the phosphorylation status of Smad, suggesting the importance of non-canonical signaling pathways activated by BMP4 for the differentiation of these cells.

2.2.2 Oncostatin M signaling

Oncostatin M (OSM) is a pleiotropic cytokine that belongs to the IL-6 family, which includes IL-6, IL-11, and leukemia inhibitory factor (LIF). These cytokines share the gp130 receptor subunit as a common signal transducer, and activate Janus tyrosine kinases and the signal transducer and activator of transcription 3 (STAT3) pathways. However, gp130 forms a heterodimer with a unique partner, for example, the IL6 receptor, LIF receptor, or OSM receptor (OSMR); thus, each cytokine uniquely induces a certain signaling pathway (Heinrich et al., 2003), and OSM is known to exploit distinct signaling in an OSMR-specific manner (Kinoshita and Miyajima, 2002). Of note, OSM is known to activate the hepatocytic differentiation program in hepatoblasts in an OSMR-specific manner (Kamiya et al., 1999; Kinoshita and Miyajima, 2002).

We recently identified that OSMR is expressed in a subset of liver cancer stem cells (Yamashita et al., 2010). Interestingly, OSMR-positive hepatocellular carcinoma (HCC) was characterized by the abundant expression of stem cell markers and poorly differentiated morphology, suggesting that OSMR is more likely to be expressed in HCC with stem/progenitor cell features (Yamashita et al., 2008a). Of note, the OSM-OSMR signaling pathway was maintained in these HCCs, and OSM induced hepatocytic differentiation in liver cancer stem cells (Fig. 5).

Unexpectedly, we identified that the hepatocytic differentiation of liver cancer stem cells by OSM resulted in enhanced cell proliferation *in vitro* and modest anti-tumor activity *in vivo* when administered alone. However, we have further demonstrated that OSM-mediated hepatocytic differentiation of liver cancer stem cells effectively suppresses HCC growth when combined with conventional chemotherapy. It is possible that OSM may boost the anti-tumor activity of 5-FU by "exhausting dormant cancer stem cells" through hepatocytic differentiation and active cell division (Fig. 6). A similar chemosensitization effect was observed in colorectal cancer stem cells differentiated by BMP4 administration (Lombardo et al., 2011).

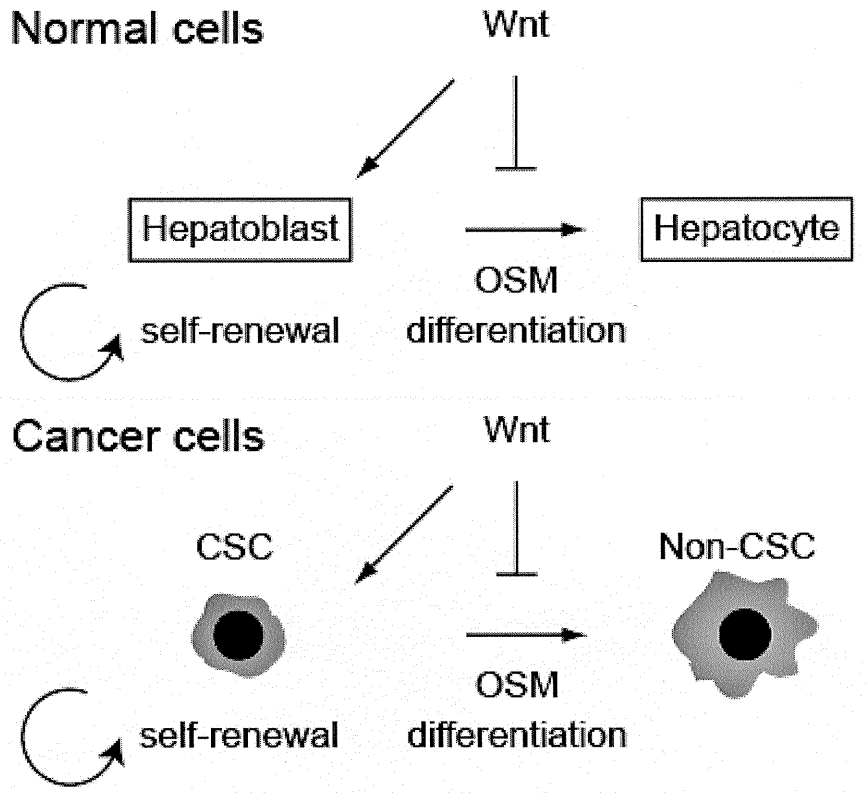

Fig. 5. Signaling pathways responsible for the self-renewal and differentiation of liver cancer stem cells. CSC, cancer stem cell; OSM, oncostatin M

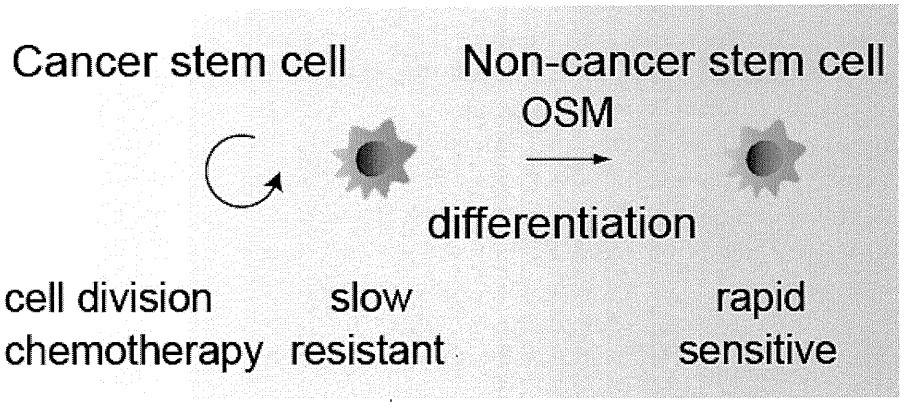

Fig. 6. Effect of oncostatin M (OSM) on exhausting dormant liver cancer stem cells

3. Limitation of cancer stem cell differentiation

As described above, some of the signaling pathways for the differentiation of normal stem cells may be maintained in cancer stem cells. To induce the differentiation of cancer stem cells by specific ligands, the expression of the corresponding receptors bound to ligands is clearly required, suggesting the importance of clarifying the mechanisms for receptor expression regulation. Interestingly, BMPRs and OSMR were detected in colorectal and liver

cancer stem cells, respectively, suggesting the possibility of ligand-induced differentiation therapy in the clinic. However, the expression of these receptors might be transcriptionally suppressed in a subset of cancers through methylation of their promoter regions (Deng et al., 2009; Kim et al., 2009; Lee et al., 2008). Indeed, a recent study suggested that BMP-mediated brain cancer stem cell differentiation failed in a subset of brain tumors in which BMP receptor promoters were methylated and silenced (Lee et al., 2008). Therefore, cancer stem cells may acquire resistance against differentiation therapy by additional epigenetic changes during the differentiation treatment.

It has been postulated that both normal stem cells and cancer stem cells are dormant and show slow cell cycles. Consistently, cancer stem cells are considered to be more resistant to conventional cytotoxic chemotherapeutic agents than non-cancer stem cells, possibly due to slow cell cycles as well as the increased expression of ATP-binding cassette (ABC) transporters, robust DNA damage responses, and activated anti-apoptotic signaling (Bao et al., 2006; Dean et al., 2005; Viale et al., 2009). Therefore, the induction of differentiation programs in cancer stem cells may result in cell proliferation of the tumor. Indeed, we recently demonstrated that differentiation of liver cancer stem cells by OSM increased cell proliferation, at least *in vitro* (Yamashita et al., 2010). Our data clearly suggested the necessity of conventional chemotherapy in addition to differentiation therapy to eradicate non-cancer stem cells originating from cancer stem cells. Furthermore, although the combination of OSM and conventional chemotherapy effectively inhibited tumor growth in our model, we did not observe tumor shrinkage (Yamashita et al., 2010). If both progenitors derived from a cancer stem cell lose their self-renewal capacity by the induction of differentiation, the tumor should subsequently shrink following the depletion of cancer stem cells. However, it is possible that ligand-based differentiation programs cannot completely inhibit the self-renewal programs of target cancer stem cells. Thus, the induction of differentiation in cancer stem cells with the eradication of non-cancer stem cells might not be sufficient for the eradication of the tumor, which may suggest the importance of inhibiting self-renewal as well as stimulating the differentiation of cancer stem cells.

A recent paper suggested that leukemia-initiating cells are composed of genetically diverse, functionally distinct populations (Notta et al., 2011), suggesting the clonal evolution of leukemia-initiating cells. Accordingly, cancer stem cells in solid tumors may also have a distinct tumorigenic/metastatic capacity as well as chemoresistance with certain genetic/epigenetic changes in each subclone as a result of clonal evolution. Thus, the cancer stem cell model and the clonal evolution model are not considered to be mutually exclusive. Therefore, clonal selection of cancer stem cells resistant to differentiation therapy might occur with additional genetic/epigenetic changes during treatment as a result of clonal evolution. The effects of differentiation therapy on the clonal evolution or genetic diversity of cancer stem cells need to be clarified in the future.

4. Conclusion

The recent re-emergence of the cancer stem cell hypothesis has provided novel insights on the effect of differentiation programs on cancer stem cells for the potential eradication of tumors. Although the activation of several signaling pathways by certain cytokines may be effective for the differentiation of cancer stem cells, their utility and limitation for tumor eradication should be clarified in future to provide novel therapeutic opportunities for cancer patients.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

Taro Yamashita, Masao Honda and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

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Correspondence
Professor Shuichi Kaneko, Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan. Email: skaneko@m-kanazawa.jp

Abstract

Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and chronic liver disease worldwide. Recent developments and advances in HCV replication systems *in vitro* and *in vivo*, transgenic animal models, and gene expression profiling approaches have provided novel insights into the mechanisms of HCV replication. They have also helped elucidate host cellular responses, including activated/inactivated signaling pathways, and the relationship between innate immune responses by HCV infection and host genetic traits. However, the mechanisms of hepatocyte malignant transformation induced by HCV infection are still largely unclear, most likely due to the heterogeneity of molecular paths leading to HCC development in each individual. In this review, we summarize recent advances in knowledge about the mechanisms of hepatocarcinogenesis induced by HCV infection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide.[1] The majority of HCCs arise from a background of chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[2] Although both viruses are hepatotropic and regarded as causative agents of HCC, the underlying mechanisms of hepatocarcinogenesis are considered to be largely different, partly due to differences in the nature of DNA viruses (with an integration capacity for the host genome) and RNA viruses (with no genome integration capacity).

Hepatitis C virus is an RNA virus that is unable to integrate into the host genome but, instead, its proteins interact with various host proteins and induce host responses that potentially contribute to the malignant transformation of cells. In addition, HCC usually develops in the setting of liver cirrhosis after long-term continuous inflammation/regeneration processes; these accelerate the turnover of hepatocytes with increased risk of replication errors and DNA damage. Furthermore, recent genome-wide association studies have suggested that the natural course of HCV infection might be modified by the genetic background of the host.[3,4] Thus, both host and virus factors are considered to affect the process of hepatocarcinogenesis in a complex manner.

In this review, we summarize the current knowledge of the mechanisms of hepatocarcinogenesis induced by HCV infection. We also focus on recent findings of transcriptomic characteristics of HCV-related HCC and summarize the potential signaling pathways that are altered in this condition.

Epidemiology

Chronic HCV infection is a major risk factor for the development of HCC worldwide. According to the World Health Organization (WHO), approximately 170 million people are chronically infected with HCV. Although epidemiological evidence has suggested a clear, close relationship between HCV infection and HCC,[5,6] the prevalence of HCV infection in HCC patients differs noticeably between geographical regions. Thus, HCV infection is found in 70–80% of HCC patients in Japan, 70% in Egypt, 40–50% in Italy and Spain, about 20% in the United States (US), and less than 10% in China.[7–9] In industrialized countries including the US, a recent increase in HCC incidence and mortality has been observed, potentially due to the rising incidence of HCV infection transmitted through contaminated blood.[10]

Hepatitis C virus increases the risk of HCC by promoting inflammation and fibrosis of the infected liver that eventually results in liver cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of about 4–7%.[11] Other factors including alcohol intake, diabetes, and obesity have also been reported to increase the risk of HCC development by about two- to fourfold, indicating a strong life-style effect on the process of hepatocarcinogenesis.[12,13] Age and male gender are also contributing risk factors for HCV-related HCC, although the detailed mechanisms are still debatable.

Virus proteins and host responses

Hepatitis C virus belongs to the Flaviviridae family. It has a positive-stranded linear RNA genome of about 9.6-kb containing a

single large open reading frame encoding three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.[14] The structural proteins form the HCV virions, whereas non-structural proteins are involved in the processes of viral replication, assembly, and maturation. HCV proteins are known to be processed by host and viral proteases. Both structural and non-structural proteins can interact with various host cellular proteins to potentially promote the malignant transformation of hepatocytes (see recent reviews[7,15,16]). In this review, because of space limitations, we focus on the findings of core and NS5A proteins in terms of host responses potentially evoked during the process of HCV-related hepatocarcinogenesis.

Core protein

Hepatitis C virus core is a 21-kDa nucleocapsid protein with an RNA-binding capacity. In addition to its function in regulating HCV-RNA translation and HCV particle assembly, core protein is known to be involved in mediating the alteration of various host cell signaling pathways, transcriptional activation, modulation of immune responses, apoptosis, oxidative stress, and lipid metabolism.[7] Several recent studies have indicated the statistically significant high frequency of mutations in the *core* gene in HCV-infected patients who developed HCC.[17,18] However, the functional relevance of mutant core proteins on the malignant transformation of hepatocytes or the HCV life cycle has yet to be clarified.

Evidence of core protein as a causative agent of HCC was initially obtained from the transgenic mice model in which *core* gene overexpression, under the regulation of the HBV regulatory element used as a promoter, resulted in steatosis of mouse livers in early life, with subsequent development of adenoma and HCC.[19] However, another mouse model using a different promoter and of a different strain background resulted only in steatosis or different phenotypes without HCC development.[20,21] Similar controversial findings were reported in transgenic mice expressing HCV polyprotein or structural protein with regards to the development of HCC.[22,23] Thus, the role of core protein alone in the development of HCC remains unclear in transgenic mouse models.

Although the direct role of core protein in the malignant transformation of hepatocytes is still under investigation, it seems to be related to the development of hepatic steatosis.[19,24] Indeed, steatosis is one of the risk factors for the development of HCV-related HCC,[25,26] and activation of the lipogenic pathway has been reported in a subset of HCC cases.[27] Core protein is associated with the surface of lipid droplets in infected cells and might be directly related to steatosis through several factors responsible for lipid biogenesis and degradation, including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1.[21,28–30] Furthermore, core protein is reported to interact with endoplasmic reticulum (ER) or mitochondrial outer membranes and induce ER stress by perturbation of protein folding or by the accumulation of reactive oxygen species (ROS) through mitochondrial dysfunction.[31,32] ROS produced in this way might result in DNA damage to the host genome and accelerate the process of hepatocarcinogenesis. Increased hepatic iron deposition may also induce oxidative stress and lipid peroxidation, thus increasing the risk of HCC development in HCV polyprotein transgenic mice.[33]

Since the discovery of HCV, various studies have investigated the role of core on host cells. Its effects have been demonstrated on signaling pathways responsible for the cell cycle, and apoptosis through interaction with several tumor suppressors including p53, p73, and p21[34–39] as well as apoptosis regulators such as tumor necrosis factor-α (TNF-α) signaling or Bcl-2 members.[40–42] However, the data obtained from these studies are relatively inconsistent with each other and have varied across experimental models. Core protein may influence the growth and proliferation of host cells through activation of signaling pathways such as Raf/mitogen activated protein kinase (MAPK),[43] Wnt/beta catenin,[16] and transforming growth factor-β (TGF-β).[15,44] These pathways are known to be activated in HCC.[45] The findings therefore indicate a potential role for core in cell proliferation or suppression of apoptosis during malignant transformation of hepatocytes in the liver of chronic hepatitis C, where chronic inflammation and regeneration of hepatocytes continuously occurs.

NS5A protein

NS5A is a 56–58-kDa protein phosphorylated at serine residues by serine-threonine kinase[46] and is essential for replication of the HCV genome. NS5A protein forms part of the viral replicase complex and is localized mainly in the cytoplasm of infected cells in association with the ER. NS5A can become a lower molecular weight protein through post-translational modification, after which it can undergo translocation to the nucleus where it acts as a transcriptional activator. High frequencies of wild-type *NS5A* genes were reported to be dominant in liver cirrhosis patients who finally developed HCC compared with those who did not,[47] but the mechanistic significance of the *NS5A* wild/mutant genotypes in the process of HCV-related hepatocarcinogenesis remains uncertain.

NS5A protein has been suggested to interact with various signaling pathways including cell cycle/apoptosis[48] and lipid metabolism[28,49,50] in host cells and shares some signaling targets with core protein. NS5A is recognized as a transcriptional activator for many target genes[51] including p53 and its binding protein, TATA binding protein (TBP). Transcription factor IID activities were reported to be modified by NS5A in the suppression of p53-dependent transcriptional transactivation and apoptosis.[52,53] NS5A may also interact with pathways such as Bcl2,[54] phosphatidylinositol 3-kinase (PI3-K),[55] Wnt/beta catenin signaling,[56] and mTOR[57] to activate cell proliferation signaling and inhibit apoptosis.

Taken together, intriguing data concerning the function of core and NS5A proteins on host cell signaling pathways, transcriptional activation, apoptosis, oxidative stress, and lipid metabolism described above suggest a diverse role for HCV proteins in the pathophysiology of chronic hepatitis C that leads to malignant transformation in infected hepatocytes. Key findings and present concepts are summarized in Figure 1.

Transcriptomic characteristics of HCV-related HCC

As described above, HCV proteins can evoke various host responses in infected cells at transcriptional/translational/post-translational levels. Furthermore, enhanced cell death/regeneration processes are considered to induce DNA damage and accelerate replication errors that cause frequent mutations and genomic alter-

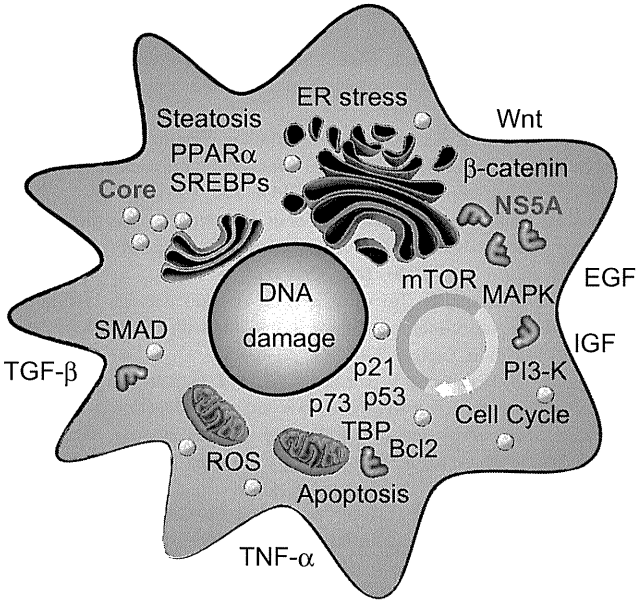

Figure 1 Signaling pathways potentially affected by hepatitis C virus (HCV) proteins. EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREBP, sterol-regulatory element binding protein; TBP, TATA binding protein.

ation in the host genome. The central dogma is defined as the flow of genetic information from DNA to mRNA and then to protein, so genetic/genomic alterations and transcriptional/translational modifications are ultimately considered to affect the cellular signaling pathway at the transcriptional level.

Over the past decade, several methods (including differential display, serial analysis of gene expression [SAGE], and microarray) have been developed to allow comparative studies of gene expression between normal and cancer cells on a genome-wide scale,[58] and the analysis of a set of all RNA molecules (mainly indicating mRNAs) is termed as whole transcriptome analysis. Extensive transcriptome analysis of HCC and corresponding noncancerous livers has been performed, and the results have greatly increased our knowledge about the transcriptome characteristics of HCV-related HCC.

Early microarray and SAGE studies investigating the gene expression patterns of chronic hepatitis B and C tissues indicated that these two chronic hepatitis tissues had distinct gene expression profiles; the genes activated in chronic hepatitis C were correlated with signaling pathways associated with apoptosis, oxidative stress responses, and Th1 cytokine signaling.[59,60] An early study comparing genes activated in HCV-related and HBV-related HCCs showed that the genes associated with xenobiotic metabolism were more abundantly expressed in HCV-related HCC,[61] suggesting a detoxification role, which is potentially induced by chronic inflammation and generation of ROS resulting from HCV infection. In contrast, HBV-related HCC might closely correlate with the activation of imprint genes, including insulin-like growth factor-II (IGF-II) as investigated by oligo-DNA microarray,[62] suggesting a role of de-differentiation or epigenetic alteration of the host genome in HBV-related HCC. Activation of genes associated with interferon, oxidative stress, apoptosis, and lipid metabolism signaling was detected in HCV-related HCC and chronic hepatitis C specimens,[27,60,63] consistent with numerous functional studies that have investigated the host response evoked by HCV structural and non-structural proteins.[48]

Transcriptome analysis has also recently shed new light on the transcriptional alteration events occurring in early stages of HCV-related hepatocarcinogenesis. *GPC3* (encoding Glypican 3) was identified as one of the most activated transcripts in the early stage of hepatocarcinogenesis,[60,64] while several recent studies showed that gene signatures including *GPC3* can successfully discriminate HCCs from pre-malignant dysplastic nodules and cirrhosis nodules.[65,66] Close examination of genes differentially expressed among cirrhotic nodules, dysplastic nodules, and early and advanced HCV-related HCC tissues has also suggested roles for Toll-like receptor signaling, Wnt signaling, bone morphogenetic protein (BMP)/TGF-β signaling, JAK-STAT signaling, and DNA repair/cell cycle responses in each step of the malignant transformation processes.[67] These processes might therefore provide candidate molecular targets for the chemoprevention of HCV-related HCC.

Recent advances in transcriptome analysis have also provided detailed information on the status of small noncoding RNAs, microRNAs, that can regulate the expression of target genes and viral replication in normal and cancer tissues. Expression of microRNAs including miR-122 and −199a has been reported to modulate HCV replication,[68–70] and miR-122 expression can be regulated by host interferon signaling and responses.[71] HCV protein expression in turn could induce miRNAs and might affect the tumor suppressor DLC1 and the chemosensitivity of malignantly transformed cells.[72,73] Several microRNAs were also differentially expressed between HCV-related and HBV-related HCCs as well as their corresponding non-cancerous liver tissues. The candidate signaling pathways potentially altered by microRNAs in HCV-related tissues were those associated with antigen presentation, cell cycle, and lipid metabolism,[74] consistent with the mRNA microarray data described above. MicroRNAs have also recently been reported to successfully discriminate between HCC and cirrhotic liver tissues,[75] implicating their role in the early stages of malignant transformation. These data suggest that microRNAs may be good targets for the eradication of HCC as well as hepatocytes infected with HCV.

Conclusion

The heterogeneity of genetic/transcriptomic/proteomic events observed in hepatocytes or cell lines expressing HCV proteins and HCV-related HCCs reported thus far has suggested that complex mechanisms underlie malignant transformation induced by HCV infection. These potentially act through convoluted virus-host interactions including HCV replication with host cell cycles, apoptosis, proliferation, quality control of protein synthesis, lipid metabolism, and DNA damage responses. Indeed, HCC is a heterogeneous disease in terms of drug sensitivity, metastatic capacity, and clinical outcome. The heterogeneity of HCV-related HCC may closely correlate with the origin of malignantly transformed cells where multifaceted cellular reactions including apoptosis and

cell proliferation are induced by HCV infection. An in-depth understanding of these molecular complexities associated with HCV-related HCC may provide the opportunity for effective chemoprevention of HCC among those with HCV-cirrhosis, and to design tailor-made treatment options for HCV-related HCC patients in the future.

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Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma

Hajime Sunagozaka, Masao Honda, Taro Yamashita, Ryuhei Nishino, Hajime Takatori, Kuniaki Arai, Tatsuya Yamashita, Yoshio Sakai and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan

The identification of genes involved in tumor growth is crucial for the development of inventive anticancer treatments. Here, we have cloned a 17-kDa secretory protein encoded by *c19orf10* from hepatocellular carcinoma (HCC) serial analysis of gene expression libraries. Gene expression analysis indicated that *c19orf10* was overexpressed in approximately two-thirds of HCC tissues compared to the adjacent noncancerous liver tissues, and its expression was significantly positively correlated with that of alpha-fetoprotein (AFP). Overexpression of *c19orf10* enhanced cell proliferation of AFP-negative HLE cells, whereas knockdown of *c19orf10* inhibited cell proliferation of AFP-positive Hep3B and HuH7 cells along with G1 cell cycle arrest. Supplementation of recombinant c19orf10 protein in culture media enhanced cell proliferation in HLE cells, and this effect was abolished by the addition of antibodies developed against c19orf10. Intriguingly, c19orf10 could regulate cell proliferation through the activation of Akt/mitogen-activated protein kinase pathways. Taken together, these data suggest that c19orf10 might be one of the growth factors and potential molecular targets activated in HCC.

Hepatocellular carcinoma (HCC) is one of the most common cancers with an estimated worldwide incidence of 1,000,000 cases per year.[1] Most HCCs develop as a consequence of chronic liver disease such as chronic viral hepatitis due to hepatitis C virus (HCV) or hepatitis B virus (HBV) infection.[2–7] Liver cirrhosis patients with any etiology are considered to be at an extremely high risk for HCC.[8–10] Indeed, ~7% of liver cirrhosis patients with HCV infection develop HCC annually,[8,11] and the advancement of reliable HCC screening methods for high-risk patients is crucial for the improvement of their overall survival.[12]

Currently, imaging diagnostic techniques such as ultrasonography, computed tomography, magnetic resonance image and angiography are the gold standards for the early detection of HCC.[13,14] In addition, tumor markers such as alpha-fetoprotein (AFP) and des-gamma carboxyl prothrombin (DCP) have been used for the screening of HCC,[15–18] although their sensitivity and specificity are not sufficiently high. Recently, a gene expression profiling approach shed new light on Glypican 3, a heparin sulfate proteoglycan anchored to the plasma membrane, as a potential HCC marker, and its clinical usefulness as a molecular target as well as a tumor marker is presently under investigation.[19]

There are several options available for the treatment of HCC, including surgical resection, liver transplantation, radiofrequency ablation, transcatheter arterial chemoembolization and chemotherapy, while taking the HCC stage and liver function into consideration. Recently, molecular therapy targeting the Raf kinase/vascular endothelial growth factor receptor (VEGFR) kinase inhibitor sorafenib improved the survival of patients with advanced HCC,[20,21] emphasizing the importance of deciphering the molecular pathogenesis of HCC for the development of effective treatment options.

Here, we investigated the gene expression profiles of HCC by serial analysis of gene expression (SAGE) to discover a novel gene activated in HCC.[22–25] We identified a gene, *c19orf10*, overexpressed in HCC and determined that the encoded 17-kDa protein (c19orf10) is a secretory protein. Murine *c19orf10* was originally discovered to encode a cytokine interleukin (IL)-25/stroma-derived growth factor (SF20) in 2001.[26] The gene *c19orf10* was mapped in the H2 complex region of mouse chromosome 17 between *C3* and *Ir5*, and the hypothetical protein was predicted as globular protein.[26] However, the subsequent study failed to reproduce its proliferative effect on lymphoid cells, and the paper was retracted by the authors in 2003.[26,27] Nevertheless, independent studies revealed that c19orf10 was indeed produced by synoviocytes, macrophages and adipocytes, although the function of c19orf10 remained elusive.[28,29] In our study, we identified that *c19orf10* was overexpressed in AFP-positive HCC samples. Our data imply that c19orf10 could activate the mitogen-activated protein kinase (MAPK)/Akt pathway and

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Correspondence to: Shuichi Kaneko, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan,
Tel.: +81-76-265-2233, Fax: +81-76-234-4250,
E-mail: skaneko@m-kanazawa.jp

enhance cell proliferation in HCC cell lines, suggesting that c19orf10 may be a growth factor produced by tumor epithelial cells and/or stromal cells, and, therefore, would be a good target for the treatment of HCC.

Material and Methods

SAGE and HCC samples

HCC and normal liver SAGE libraries that we had constructed were reanalyzed using SAGE 2000 software. The size of each SAGE library was normalized to 300,000 transcripts per library. Monte Carlo simulation was used to select genes whose expression levels were significantly different between the two libraries. Each SAGE tag was annotated using the gene-mapping website SAGE Genie database (http://cgap.nci.nih.gov/SAGE/) and the SOURCE database (http://smd.stanford.edu/cgi-bin/source/sourceSearch) as previously described.[30] An additional 15 SAGE libraries of normal and cancerous tissues from various organs were retrieved using the National Center for Biotechnology Information SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE/).

Fifteen HCC tissues (four HBV-related and 11 HCV-related) and the corresponding noncancerous liver tissues were obtained from HCC patients who received hepatectomy. Four normal liver tissues were obtained from patients undergoing surgical resection of the liver for the treatment of metastatic colon cancer. Additionally, 36 HCC tissues (17 HBV-related and 19 HCV-related) were obtained from HCC patients undergoing hepatectomy. These samples were snap frozen in liquid nitrogen immediately after resection and used for quantitative real-time detection PCR (RTD-PCR). Total RNA was extracted using a ToTALLY RNA™ kit (Ambion, Austin, TX).

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the institutional ethical review board committee. All patients provided written informed consent for the analysis of the specimens.

Laser capture microdissection and RNA isolation

Laser capture microdissection (LCM) was performed as previously described.[31] Briefly, 20 HCV-related surgically resected HCC tissues were frozen in OCT compound (Sakura Finetech, Torrance, CA).[32] Inflammatory cells and cancerous cells in HCC tissues were separately excised by LCM using a Laser Scissors CRI-337 (Cell Robotics, Albuquerque, NM) under a microscope. Total RNA was isolated from these cells using a microRNA isolation kit (Stratagene, La Jolla, CA) in accordance with the supplied protocol, with slight modifications.[31]

Construction of C19ORF10 expression plasmid and recombinant adenovirus vector

PCR was performed on a Marathon cDNA library from Huh7 cells using the following primers: sense primers: 5′-GACCCTAGTCCAACATGGCGGCGCCC-3′ (the first PCR), 5′-ATGGCGGCGCCCAGCGGAGGGTGGAACGGC-3′ (the nested second PCR) and antisense primers: 5′-CACCGGA GATGAGAAGGTGCCACCCGC-3′ (the first PCR), 5′-CAG GGCTGCTGGTCACAGCTCAGTGCGCG-3′ (the nested second PCR). The 5′ and 3′ends of the cDNA were isolated using a SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to the manufacturer's recommendations. The PCR products were cloned into a TA vector (Invitrogen, Carlsbad, CA) to generate the pcDNA3.1-*c19orf10* expression plasmid. Using this plasmid, a C-terminally FLAG-tagged construct of *c19orf10* was generated and inserted in a pSI mammalian expression vector (Promega, Madison, WI), which was driven by the SV40 promoter (pSI-*c19orf10*).

The replication-incompetent recombinant adenovirus vector expressing FLAG-tagged *c19orf10* (Ad. *c19orf10-FLAG*) was generated by homologous recombination using the AdMax system (Microbix, Toronto, Canada) as previously described.[33] The generated recombinant adenovirus was purified by limiting dilution, and the titer of viral aliquots was determined by the 50% tissue culture infectious dose method as previously described.[34]

RTD-PCR

RTD-PCR was performed as previously described.[31] Briefly, template cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RT (Invitrogen). RTD-PCR of *c19orf10* (Hs. 00384077_m1), *AFP* (Hs00173490_m1), *GPC3* (Hs01018938_m1), *KRT19* (Hs00761767_s1) and the *ACTB* internal control (Hs99999903_m1) was performed using a TaqMan® Gene Expression Assay kit (Applied Biosystems, Foster City, CA). The expression of selected genes was measured in triplicate by ΔΔCT method using the 7900 Sequence Detection System (Applied Biosystems).

Cell lines and transfection of plasmids

Human liver cancer cell lines HuH1, Huh7, Hep3B, HLE and HLF as well as HEK293 and NIH3T3 were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) in 5% CO_2 at 37°C. Transfection of plasmids was performed using FuGENE™ 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, 5×10^5 cells were seeded in a six-well plate 12 hr before transfection, and 3 μg of plasmid DNA was used for each transfection. All experiments were repeated at least twice.

Purification of c19orf10-FLAG fused protein and production of anti-c19orf10 antibody

Approximately 500 ml of culture supernatant obtained from HEK293 cells infected with Ad. *C19ORF10-FLAG* at a multiplicity of infection of 20 was applied to an anti-FLAG affinity gel column (Sigma-Aldrich, St. Louis, MO). The column was

Table 1. ESTs overexpressed in the HCC library

Tag sequence	*p* value	HCC	Normal liver	T/N ratio	Name	UniGene ID
TGGGCAGGTG	<0.00001	33	0	>33	Chromosome 5 open reading frame 13	Hs.483067
GCAAAATATC	<0.00001	31	2	15.5	Liver cancer-associated noncoding mRNA, partial sequence	Hs.214343
AGCCTGCAGA	0.0002	12	1	12	Chromosome 19 open reading frame 10	Hs.465645
TTGTGCACGT	0.000228	12	1	12	CDNA FLJ45284 fis, clone BRHIP3001964	Hs.514273
ACATTCTTGT	0.000042	12	0	>12	Transcribed locus, strongly similar to XP_496055.1	Hs.76704
ACAAGTACCC	0.001161	10	1	>10	Chromosome 5 open reading frame 13	Hs.483067
GAGGTGAAGG	0.000174	10	0	>10	KIAA1914	Hs.501106
GCTGGAGGAG	0.000114	10	0	>10	Transcribed locus	Hs.520115

subjected to elution by competition with FLAG peptide (5 μg/ml), and each 1 ml fraction of the eluted aliquot was collected to obtain the most concentrated c19orf10-FLAG protein in accordance with the manufacturer's protocol. The anti-c19orf10 antibodies were developed by immunizing rabbits with repeated intradermal injections of purified c19orf10-FLAG. Protein concentration was measured by the Bradford method.

Silencing gene expression by short interfering RNA

The selected short interfering RNA (siRNA) targeting *C19ORF10* (Si-*C19ORF10*; Silencer Select siRNAs s31855) and the irrelevant control sequence (Si-*Control*; Silencer Select siRNAs 4390843) was obtained from Applied Biosystems. Transfection of these siRNAs was performed using FuGENE™ 6 (Roche Diagnostics) as previously described.[30] Briefly, 2×10^5 cells were seeded in a six-well plate 12 hr before transfection. A total of 100 pmol/l of siRNA duplex was used for each transfection. The experiments were performed at least twice.

Cell proliferation assay

Cell proliferation was evaluated in quadruplicate using a Cell Titer 96 MTS Assay kit (Promega). Briefly, 2×10^3 HLE or HuH7 cells were harvested in a 96-well plate 12 hr before the transfection or addition of the recombinant proteins. Transfection of siRNAs or plasmids was performed using FuGENE™ 6 (Roche Diagnostics). After incubation with MTS/PMS solution at 37°C for 2 hr, the absorbance at 450 nm was measured. The experiments were performed at least twice.

Cell cycle analysis

Cells were fixed using 80% ice-cold ethanol and incubated with propidium iodide for 10 min. DNA content was analyzed using a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA) counting 10,000 stained cells. The distribution of cells in each cell cycle phase was determined using FlowJo software (Tree Star, Ashland, OR).

Western blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and the extracts were subsequently electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gels and transferred onto protean nitrocellulose membranes. The blots were then incubated for 1 hr with an appropriate primary monoclonal antibody: phospho-PI3K (#4228), phospho-Akt (#4060), phospho-GSK-3β (#9323), phospho-c-Raf (#9427), phospho-MEK1/2 (#9154), phospho-p44/42 MAPK (Erk1/2) (#4370), Cdk4 (CDK4 (#2906)), Cdk6 (#3136), cyclinD1 (#2926), cyclinD3 (#2936), phospho-Rb (#9308), phospho-P53 (# 9286), phospho-cdc2 (#9111) and β-actin (#4970) (Cell Signaling Technology, Allschwil, Switzerland) and anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO). The blots were washed and exposed to peroxidase-conjugated secondary antibodies, such as anti-mouse or rabbit IgG antibodies, and visualized using the ECL™ kit (Amersham Biosciences, Piscataway, NJ). All experiments were performed at least twice.

Statistical analyses

Unpaired *t*-tests and Kruskal–Wallis tests were performed on the RTD-PCR and cell proliferation data using GraphPad Prism software (www.graphpad.com).

Results

Identification of *C19ORF10* overexpression in HCC by SAGE

To comprehensively explore the candidate novel genes activated in HCC, we reanalyzed two SAGE libraries derived from HCC tissues and normal liver tissues.[30] After normalization of each SAGE library size to 300,000 tags, we compared the HCC and normal liver libraries to obtain the list of genes overexpressed in HCC. We identified 79 genes significantly overexpressed in the HCC library by more than tenfold when compared to the normal liver library (Supporting Information Table 1). Among them, we explored expressed sequence tags (ESTs) as candidates for novel HCC-related genes to identify eight unique tags corresponding to seven ESTs (Table 1). We especially focused on the EST chromosome 19 open reading frame 10 (*c19orf10*) because the

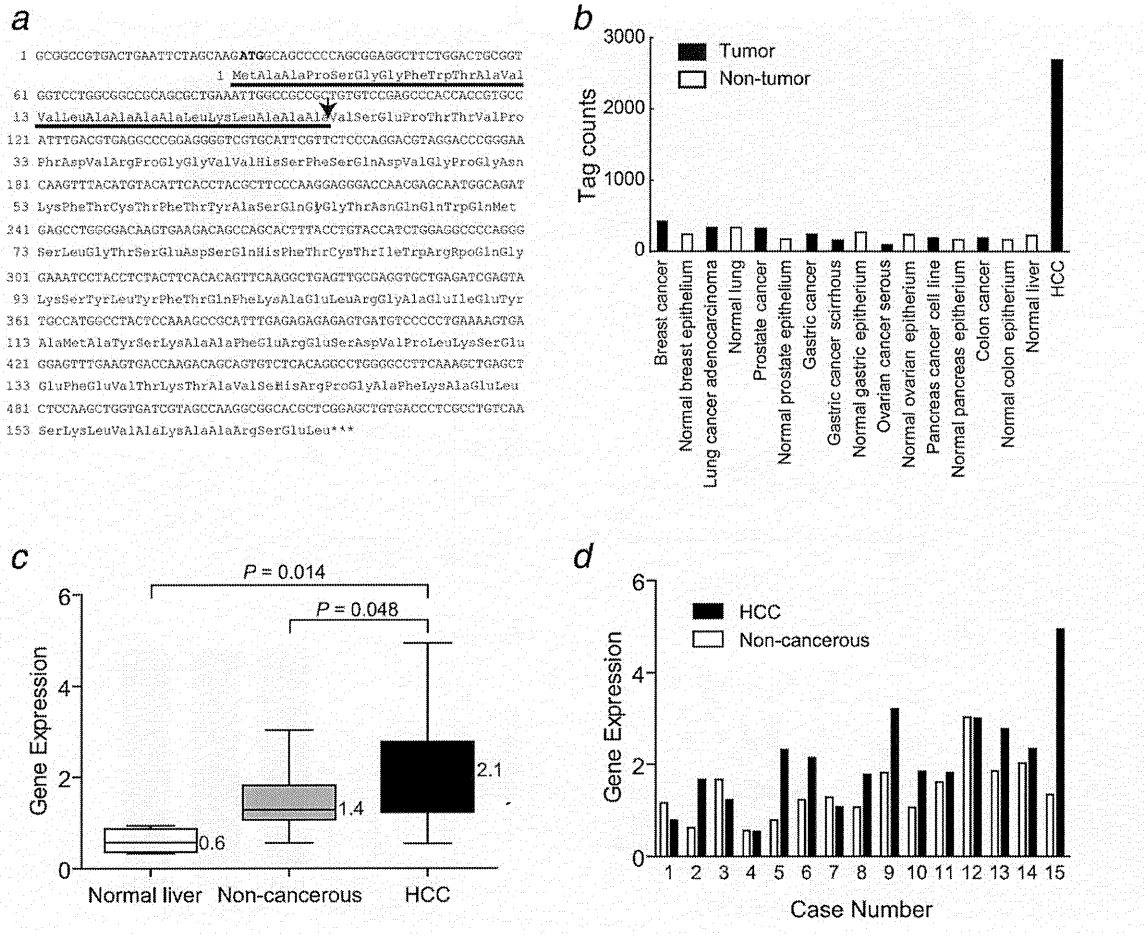

Figure 1. (*a*) Structure of a *c19orf10* gene and a c19orf10 protein. The DNA sequence of *c19orf10* and amino acid alignment of the encoded c19orf10 protein are shown. C19orf10 is predicted to have a molecular weight of 17 kDa and contain a signal peptide cleavage site (indicated as a black arrow). (*b*) *C19orf10* gene expression profiles in various tissues by SAGE. *Y*-axis indicates the number of tags corresponding to *c19orf10* in each tissue. (*c*, *d*) RTD-PCR analysis of *c19orf10*. RNA was isolated from 34 tissue samples: 15 HCC, 15 corresponding noncancerous liver samples and four normal liver samples. Differential expression of each gene among normal liver tissues, noncancerous liver tissues and HCC tissues was examined using the Kruskal–Wallis test and unpaired *t*-test. The mean value of gene expression data in each group is indicated (*c*). *C19orf10* was overexpressed in 10 of 15 examined HCC tissues compared to the noncancerous liver tissues (*d*).

sequence presumably encoded a secretory protein with a signal peptide sequence (Fig. 1*a*).

When we examined the expression profiles of *c19orf10* using retrieved SAGE data from various cancers and their normal counterparts, we identified that *c19orf10* was abundantly expressed in human HCC (Fig. 1*b*). We further examined the publicly available EST profiles of *c19orf10* (http://www.ncbi.nlm.nih.gov/unigene) and confirmed its tendency to be overexpressed in HCC compared to the normal liver (data not shown). We validated the overexpression of *c19orf10* in 15 independent HCC tissues and adjacent noncancerous liver tissues by RTD-PCR. Gene expression of *c19orf10* was significantly higher in the HCC tissues than in the normal liver tissues and adjacent noncancerous liver tissues ($p = 0.014$ and 0.048, respectively; Fig. 1*c*). *C19orf10* expression was elevated in HCC tissues compared to the adjacent noncancerous liver tissues in 10 of 15 patients (66.7%; Fig. 1*d*).

Overexpression of *C19ORF10* in AFP-positive HCC

As HCC is a heterogeneous mixture of cancer epithelial cells and stromal cells, and a previous report indicated that *c19orf10* is expressed in fibroblast-like synoviocytes. We, therefore, evaluated the expression of *c19orf10* in tumor epithelial cells and stromal cells separately using LCM and RTD-PCR in 20 HCC tissues (Fig. 2*a*). Although tumor

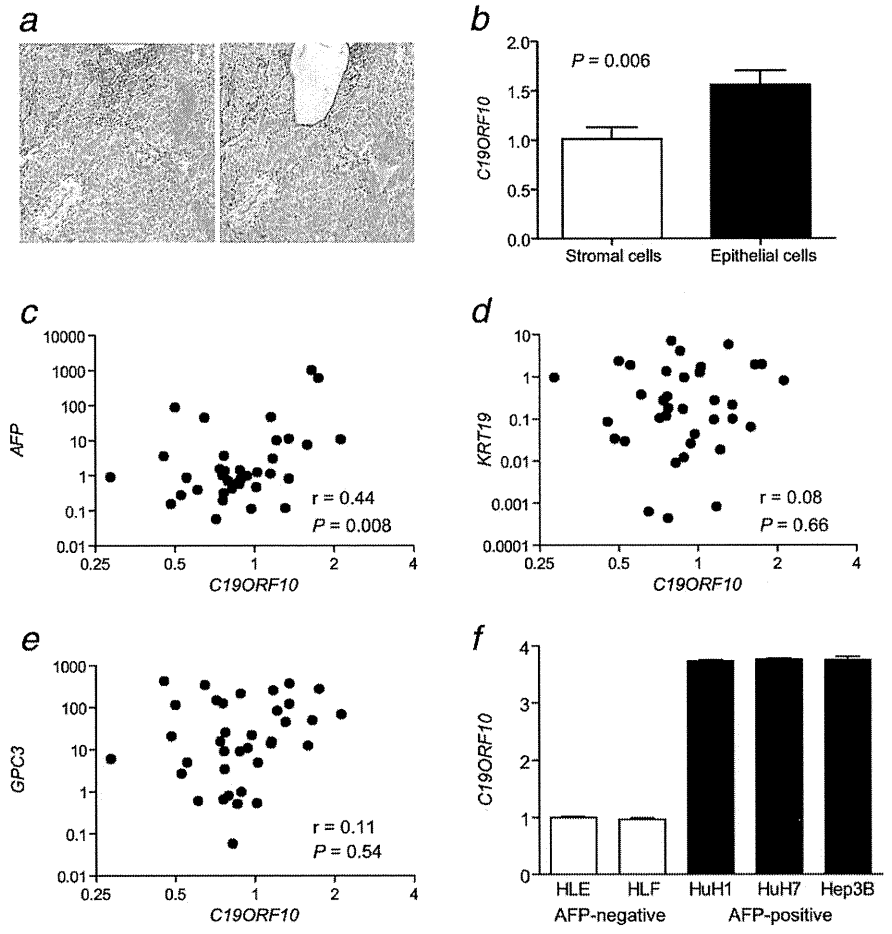

Figure 2. (*a*) Representative photomicrographs of an HCC tissue used for LCM (toluidine blue staining). Inflammatory mononuclear cells and stromal cells were separately captured (left: Pre-LCM, right: Post-LCM). (*b*) RTD-PCR analysis of *c19orf10* expression in inflammatory mononuclear cells and tumor epithelial cells in 20 HCV-related HCC tissues. Tumor-inflammatory mononuclear cells and stromal cells were isolated using LCM. RNAs were isolated from these cells as well as parenchymal tissues from the same liver, followed by RTD-PCR for *c19orf10* gene expression. Expression of the *c19orf10* gene was higher than that observed in HCC-infiltrating inflammatory mononuclear cells. $*p < 0.05$. (*c–e*) Scatter plot analysis of *c19orf10, AFP, KRT19* and *GPC3* expression in HCC. RNA was isolated from 17 HBV-related HCC and 19 HCV-related HCC. (*f*) RTD-PCR analysis of *c19orf10* in AFP-negative (HLE and HLF) and -positive (HuH1, HuH7 and Hep3B) liver cancer cell lines.

stromal cells expressed *c19orf10* at some level, the expression levels were significantly higher in tumor epithelial cells than in stromal cells ($p = 0.006$) (Fig. 2*b*).

To explore the relationship of *c19orf10* with other established HCC markers, we investigated the gene expression of *c19orf10*, *AFP* (alpha-fetoprotein), *KRT19* (cytokeratin 19) and *GPC3* (glypican 3). Because only 1 of 15 HCC tissues analyzed above (Fig. 1*d*) was AFP positive (data not shown), we further investigated the expression of *c19orf10* in an additional 36 HCC tissues using RTD-PCR. Interestingly, *c19orf10* expression was significantly positively correlated with *AFP* ($r = 0.44$, $p = 0.008$), but not with *KRT19* ($r = 0.08$, $p = 0.66$) nor *GPC3* ($r = 0.11$, $p = 0.54$) (Figs. 2*c*–2*e*). Furthermore, when we examined the expression of *c19orf10* in AFP-positive (HuH1, HuH7 and Hep3B) and -negative (HLE and HLF) HCC cell lines, we identified the overexpression of *c19orf10* in AFP-positive HCC cell lines (Fig. 2*f*). These data suggested that *c19orf10* is overexpressed and may play some role in AFP-positive HCCs.

C19orf10 regulates MAPK/Akt pathways and activates cell proliferation

To explore the functional role of c19orf10 in HCC, we performed *c19orf10* overexpression and knockdown studies using *c19orf10*-low HLE cells and *c19orf10*-high Hep3B and HuH7 cells, respectively. When we transfected HLE cells with