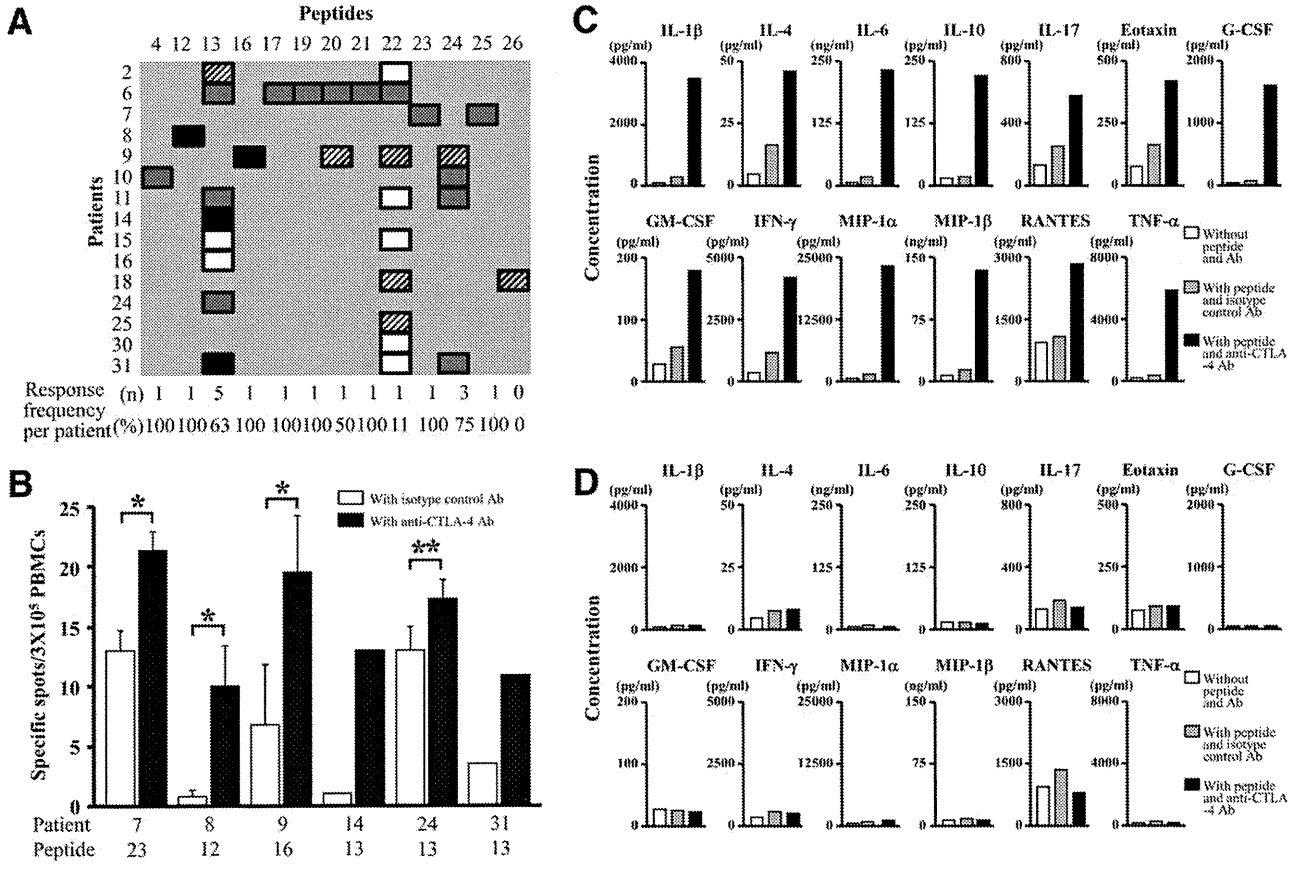

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^{-}/CCR7^{+}$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.

On the other hand, recent studies have shown the important role of $CD4^+$ helper T cells in optimal function and proliferation of $CD8^+$ T cells.[46] Therefore, the lack of $CD4^+$ helper T cells or anergic $CD4^+$ T cells may explain the limited TAA-specific $CD8^+$ T-cell responses in HCC. Further studies using $CD4^+$ T-cell-depleted PBMCs or $CD8^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

Acknowledgment: The authors thank Kazumi Fushimi, Maki Kawamura, Masayo Baba and Nami Nishiyama for technical assistance.

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2011.06723.x

MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

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Key words
hepatitis C virus, hepatocellular carcinoma, transcriptome.

Accepted for publication 10 March 2011.

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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-

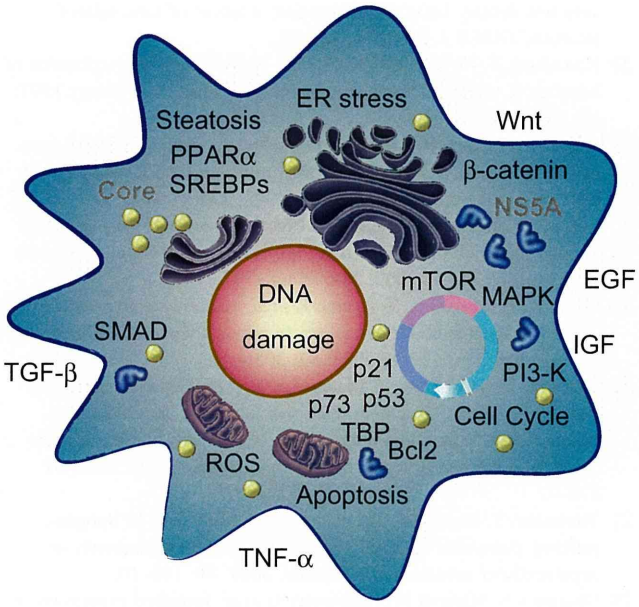

Figure 1 Signaling pathways potentially affected by hepatitis C virus (HCV) proteins. EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PPAR, peroxisome 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

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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

Key words: hepatocellular carcinoma, serial analysis of gene expression, *c19orf10*
Additional Supporting Information may be found in the online version of this article
DOI: 10.1002/ijc.25830
History: Received 14 Mar 2010; Accepted 15 Nov 2010; Online 2 Dec 2010
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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

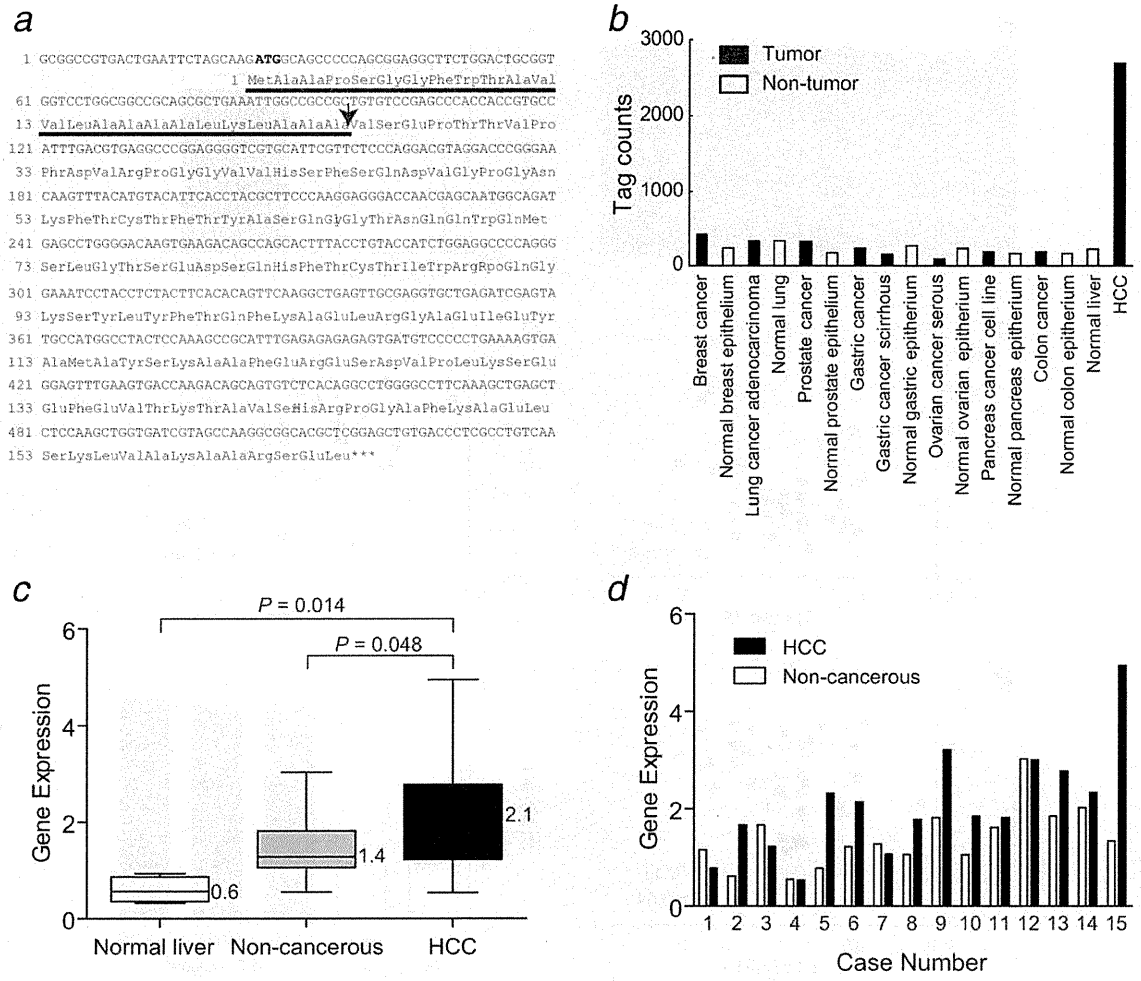

Figure 1. (*a*) Structure of a *c19orf10* gene and a c19orf10 protein. The DNA sequence of *c19orf10* and amino acid alignment of the encoded c19orf10 protein are shown. C19orf10 is predicted to have a molecular weight of 17 kDa and contain a signal peptide cleavage site (indicated as a black arrow). (*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

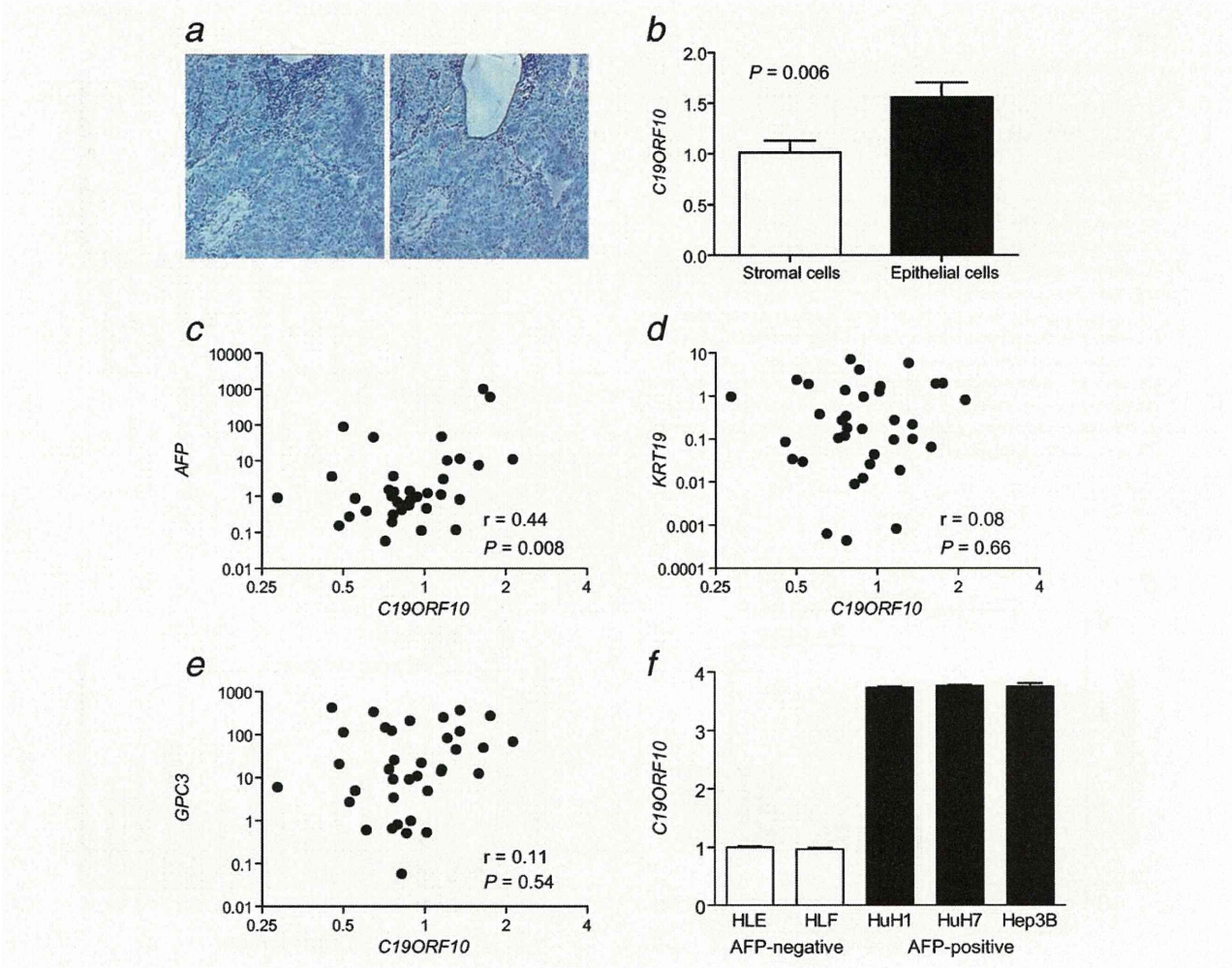

Figure 2. (*a*) Representative photomicrographs of an HCC tissue used for LCM (toluidine blue staining). Inflammatory mononuclear cells and stromal cells were separately captured (left: Pre-LCM, right: Post-LCM). (*b*) RTD-PCR analysis of *c19orf10* expression in inflammatory mononuclear cells and tumor 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

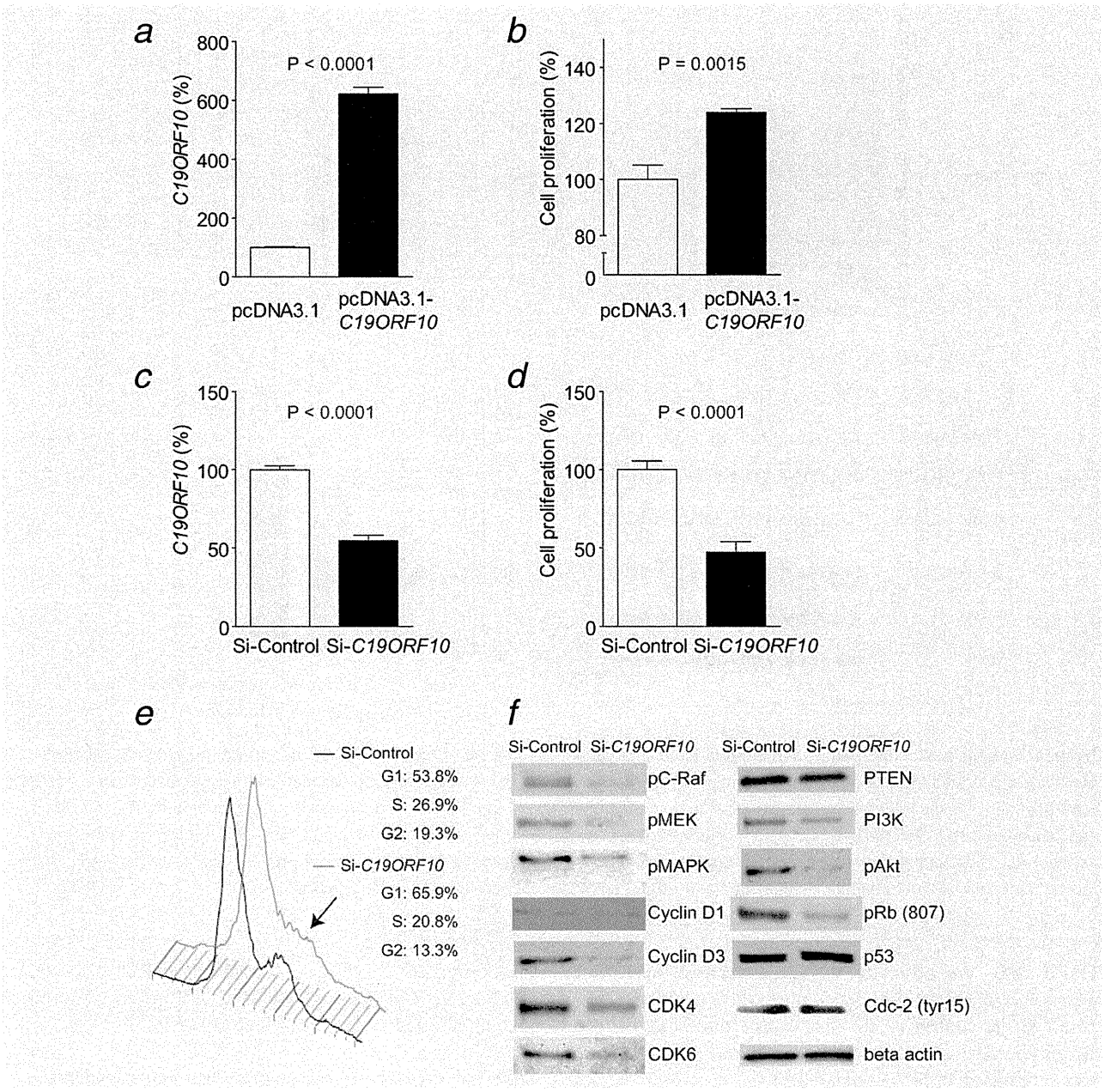

Figure 3. (*a*) RTD-PCR analysis of *c19orf10* expression in HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. (*b*) Cell proliferation assay of HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. Cell proliferation was evaluated 72 hr after each plasmid transfection. (*c*) RTD-PCR analysis of *c19orf10* expression in Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Gene expression was measured in triplicates 48 hr after transfection. (*d*) Cell proliferation assay of Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Cell proliferation was evaluated 72 hr after siRNA transfection. (*e*) Cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*. Cell cycle was evaluated 72 hr after siRNA transfection. A black arrow indicates the G2 phase peak. (*f*) Western blotting analysis of Huh7 cells transfected with Si-*Control* or Si-*c19orf10*. Cells were lysed by RIPA buffer 72 hr after siRNA transfection.

pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids, we identified an approximately sixfold overexpression of *c19orf10* when compared to the control 48 hr after transfection ($p < 0.0001$) (Fig. 3*a*). Interestingly, cell proliferation was modestly, but significantly, enhanced compared to the control 72 hr after transfection ($p = 0.0015$) (Fig. 3*b*).

We also transfected siRNAs targeting an irrelevant sequence (Si-*Control*) or *c19orf10* (Si-*c19orf10*) in Hep3B and

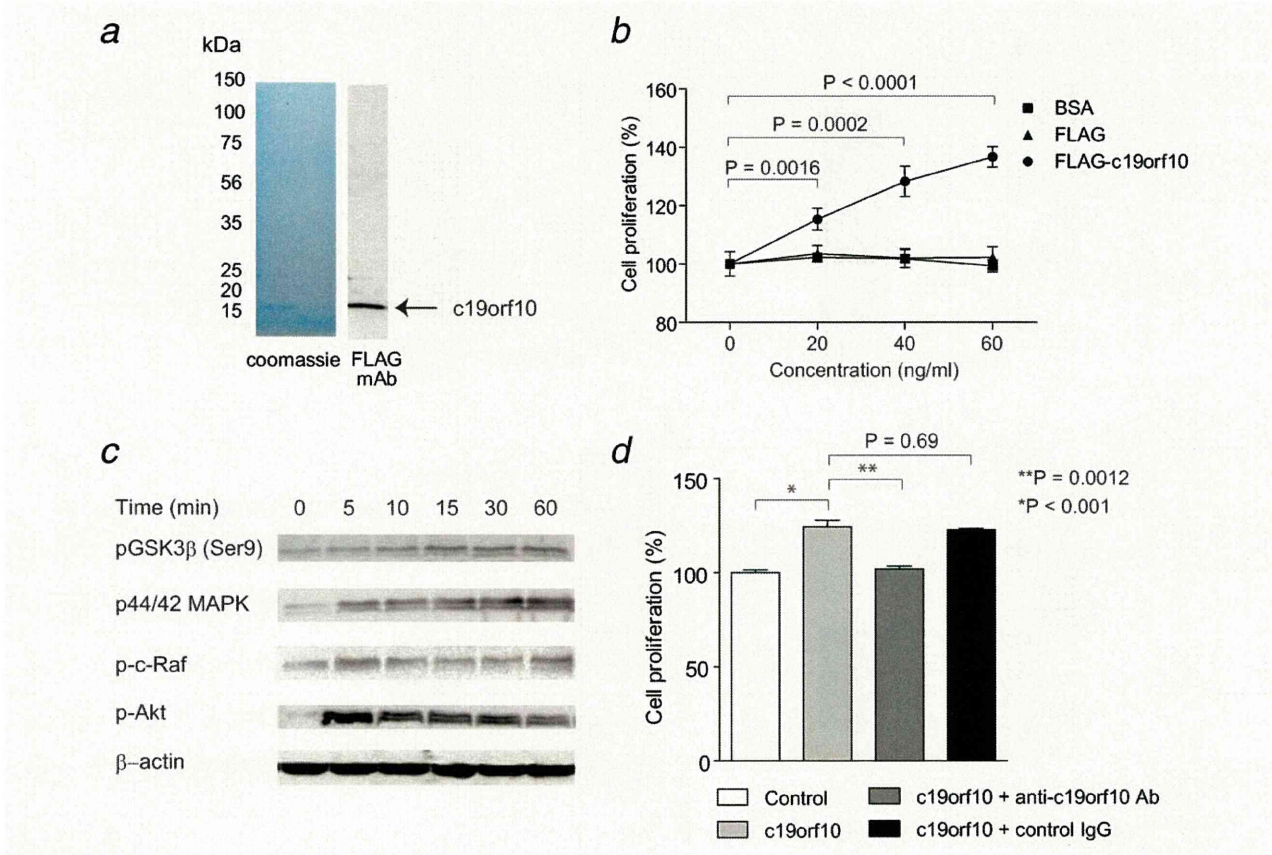

Figure 4. (*a*) Coomassie blue staining and Western blotting of culture supernatant of NIH3T3 cells transfected with pSI-*c19orf10-FLAG*. A black arrow indicates the 17-kDa c19orf10 protein. (*b*) Cell proliferation assay of HLE cells supplemented with recombinant c19orf10-FLAG, FLAG peptides or BSA. Cell proliferation was measured in quadruplicates 72 hr after supplementation. (*c*) Western blotting of HLE cells supplemented with c19orf10-FLAG (40 ng/ml). Cells were lysed at indicated time after c19orf10 supplementation. (*d*) Cell proliferation assay of HLE cells supplemented with control BSA (40 ng/ml) (white bar), c19orf10-FLAG (40 ng/ml) (light gray bar), c19orf10-FLAG (40 ng/ml) + anti-c19orf10 antibodies (gray bar) and c19orf10-FLAG (40 ng/ml) + control mouse IgG (black bar).

HuH7 cells. We observed an ~50% decrease in *c19orf10* expression in Hep3B cells transfected with Si- *c19orf10* compared to the control 48 hr after transfection with statistical significance ($p < 0.0001$). In this condition, cell proliferation was suppressed to 50% compared to the control 72 hr after transfection ($p < 0.0001$) (Figs. 3*c* and 3*d*). When we performed cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*, we identified an increase of G1-phase cells and a decrease of S- and G2-phase cells by *c19orf10* knockdown, suggesting that the G1 cycle arrest was caused by the knockdown of *c19orf10* (Fig. 3*e*).

We examined the representative MAPK/Akt pathway-associated proteins and cell cycle regulators using Western blotting 72 hr after siRNAs transfection (Fig. 3*f*). Interestingly, phosphorylation of c-Raf, MEK, MAPK, PI3K and pAkt was inhibited by knockdown of *c19orf10*, suggesting the involvement of *c19orf10* in the MAPK/Akt pathways. Furthermore, phosphorylation of Rb, CDK4 and CDK6 was also inhibited by knockdown of *c19orf10*, consistent with the observation of G1 cell cycle arrest by *C19ORF10* knockdown. PTEN, p53 and phosphorylated CDC2 protein expression was not affected by knockdown of *c19orf10*.

C19orf10 encodes the secretory protein and stimulates cell proliferation

As the sequence of *c19orf10* suggested that it encodes a secretory protein, we transfected pSI-*c19orf10-FLAG* in NIH3T3 cells and examined the culture supernatant. Immunoprecipitation of the collected culture supernatant 48 hr after transfection using anti-FLAG antibodies indicated the existence of a 17-kDa protein (c19orf10), compatible with the molecular weight of the 142 amino acids protein encoded by *c19orf10* (Fig. 4*a*). We purified c19orf10-FLAG protein from the supernatant of HEK293 cells infected with Ad. *c19orf10-FLAG* using an anti-FLAG column. Supplementation of purified c19orf10-FLAG into the culture media for 72 hr enhanced the proliferation of HLE cells in a dose-dependent manner with statistical significance, whereas control FLAG peptides

and BSA had no effects on cell proliferation (Fig. 4*b*). Western blot analysis of HLE cells cultured with purified c19orf10-FLAG (40 ng/ml) or BSA control (40 ng/ml) indicated the immediate strong phospholyration of Akt peaked 5 min after supplementation (Fig. 4*c*). The modest phospholyration of GSK3β (Ser9) and p44/42 MAPK also followed and peaked 60 min after c19orf10 supplementation. These data suggest that Akt pathway might be directly involved in the c19orf10-mediated cell proliferation signaling with the subsequent activation of MAPK pathway. Furthermore, addition of antibodies against c19orf10 to the culture media abolished the cell proliferation induced by c19orf10, whereas control IgG had no effects (Fig. 4*d*). Taken together, these data suggest that c19orf10 may be a growth factor overexpressed in AFP-positive HCCs and activates the Akt/MAPK pathways, potentially through the activation of an unidentified c19orf10 receptor.

Discussion

SAGE facilitates the measurement of transcripts from normal and malignant tissues in a nonbiased and highly accurate, quantitative manner. Indeed, SAGE produces a comprehensive gene expression profile without *a priori* gene sequence information, leading to the identification of novel transcripts potentially involved in the pathogenesis of human cancer.[19] In our study, we identified seven SAGE tags potentially corresponding to novel genes activated in HCC. Among them, we identified the secretory protein c19orf10 activated in a subset of HCCs.

Several serum markers including AFP, DCP and Glypican 3 are currently used for the detection and/or the evaluation of the treatment for HCCs in the clinic.[15–18,35] These markers are known as oncofetal proteins, that is, expressed in the fetus, transcriptionally suppressed in the adult organ and reactivated in the tumor. We identified that the expression of *c19orf10* positively correlated with *AFP* expression but did not correlate with the expression of *GPC3* or the biliary marker *KRT19*. As *c19orf10* was rarely detected in the normal liver, it is possible that c19orf10 is also an oncofetal protein activated in HCC. We are currently developing a system to detect serum c19orf10 in HCC patients, and the significance of the serum c19orf10 value as an HCC marker should be clarified.

Recent advancement in molecular biology has revealed the considerable diversity of transcription initiation and/or termination of genes altered in the process of carcinogenesis. Indeed, using 5′ SAGE approach, we recently discovered the novel intronic transcripts activated in HCC.[36] Interestingly, when we investigated the transcription initiation of *c19orf10* using the 5′ SAGE database, we identified a potential 5′ splice variant initiated from the second exon of *c19orf10* (data not shown). Although we have not yet validated the presence of 5′ splice variants in *c19orf10* by PCR, examination of 5′ EST database also suggested the presence of the similar splice variants (GenBank Accession Number CR980295, BQ680744, BQ648461, *etc.*). Alteration of transcription initiation/termination in *c19orf10* might affect the abundance or function of c19orf10 protein, and the details of 5′ splice variants in *c19orf10* should be clarified in future studies.

Molecular targeting therapy has rapidly emerged for solid tumors as well as for leukemia.[37–39] Sorafenib is a multikinase inhibitor targeting Raf kinase in the MAPK pathway as well as VEGFR and the platelet-derived growth factor receptor.[40,41] In our study, we identified that c19orf10 activates the MAPK and Akt/PI3K pathways and contributes to the proliferation of HCC cell lines, although we still could not discover the potential receptor of c19orf10. Development of a neutralizing c19orf10 antibody may provide novel therapeutic options for HCC patients to inhibit these signaling pathways, and its efficacy should be evaluated in the future.

Recently, c19orf10 was found to be expressed in fibroblast-like synoviocytes in the synovium using a proteomics approach.[29] In addition, a recent article indicated that c19orf10 was expressed in preadipocyte cells and involved in adipogenesis using two-dimensional electrophoresis mass spectrometry analysis.[28] Thus, c19orf10 may have pleiotropic effects on various lineages of normal organs in various developmental stages, and the clarification of its distribution and biological properties in the whole body may provide more detailed information about the function of c19orf10.

In conclusion, we have identified the protein c19orf10 that regulates the Akt/MAPK pathways and cell cycle through an unidentified mechanism in HCC. Although further studies should be conducted to detect the potential c19orf10 receptor or signaling molecules binding to c19orf10, our study suggests that c19orf10 may be a novel growth factor, a potential tumor marker and also a potential target molecule for HCC treatment.

Acknowledgements

The authors thank Ms. Mikie Kakiuchi, Ms. Masayo Baba and Ms. Nami Nishiyama for their excellent technical assistance.

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Oncogene (2011) 30, 619–630

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Integration of cap analysis of gene expression and chromatin immunoprecipitation analysis on array reveals genome-wide androgen receptor signaling in prostate cancer cells

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The androgen receptor (AR) is a critical transcriptional factor that contributes to the development and the progression of prostate cancer (PCa) by regulating the transcription of various target genes. Genome-wide screening of androgen target genes provides useful information to understand a global view of AR-mediated gene network in PCa. In this study, we performed 5′-cap analysis of gene expression (CAGE) to determine androgen-regulated transcription start sites (TSSs) and chromatin immunoprecipitation (ChIP) on array (ChIP-chip) analysis to identify AR binding sites (ARBSs) and histone H3 acetylated (AcH3) sites in the human genome. CAGE determined 13 110 distinct, androgen-regulated TSSs ($P<0.01$), and ChIP-chip analysis identified 2872 androgen-dependent ARBSs ($P<1e-5$) and 25 945 AcH3 sites ($P<1e-4$). Both androgen-regulated coding genes and noncoding RNAs, including microRNAs (miRNAs) were determined as androgen target genes. Besides prototypic androgen-regulated TSSs in annotated gene promoter regions, there are many androgen-dependent TSSs that are widely distributed throughout the genome, including those in antisense (AS) direction of RefSeq genes. Several pairs of sense/antisense promoters were newly identified within single RefSeq gene regions. The integration of CAGE and ChIP-chip analyses successfully identified a cluster of androgen-inducible miRNAs, as exemplified by the miR-125b-2 cluster on chromosome 21. Notably, the number of androgen-upregulated genes was larger in LNCaP cells treated with R1881 for 24 h than for 6 h, and the percentage of androgen-upregulated genes accompanied with adjacent ARBSs was also much higher in cells treated with R1881 for 24 h than 6 h. On the basis of the Oncomine database, the majority of androgen-upregulated genes containing adjacent ARBSs and CAGE tag clusters in our study were previously confirmed as androgen target genes in PCa. The integrated high-throughput genome analyses of CAGE and ChIP-chip provide useful information for elucidating the AR-mediated transcriptional network that contributes to the development and progression of PCa.
Oncogene (2011) **30,** 619–630; doi:10.1038/onc.2010.436; published online 4 October 2010

Keywords: androgen receptor; prostate cancer; CAGE; Chip-chip

Introduction

Androgen action is essential for the development, proliferation and subsequent progression of prostate cancer (PCa). Androgen binds to its cognate receptor, androgen receptor (AR), a member of the nuclear receptor superfamilies that functions as a ligand-dependent transcriptional factor (Shang *et al.*, 2002; Wang *et al.*, 2005; Dehm and Tindall, 2006). It has been shown that AR and its downstream signals are deeply involved in the pathophysiology of hormone-dependent and hormone-independent PCas (Suzuki *et al.*, 2003; Chen *et al.*, 2004; Debes and Tindall, 2004). Therefore, elucidation of the entire AR signaling pathways will reveal the precise mechanisms underlying the development and the progression of PCa and will identify novel molecular targets for cancer therapy. Recent advances in high-throughput gene analysis technology have enabled to identify a number of target genes that are associated with diseases at various statistical thresholds. Our group and others have successfully determined *bonafide* AR binding sites (ARBSs) in the human genome by chromatin immunoprecipitation (ChIP) analysis combined with genome tiling arrays (ChIP-chip) (Massie *et al.*, 2007; Takayama *et al.*, 2007, 2009; Wang *et al.*, 2007, 2009). ChIP-chip analysis is also useful to determine epigenetic alterations in the genome. We

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Received 22 April 2010; revised 4 August 2010; accepted 12 August 2010; published online 4 October 2010