Cloning and Characterization of a Novel Gene, \textit{DRH1}, Down-Regulated in Advanced Human Hepatocellular Carcinoma

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ABSTRACT

Few genes related to carcinogenesis and progression of hepatocellular carcinoma (HCC) have been identified to date. In the present study, we report the cloning and characterization of a novel gene, \textit{DRH1}, which is frequently down-regulated in HCC. The full-length \textit{DRH1} clone contains an open reading frame of 1257 nucleotides encoding 419 amino acids. The deduced \textit{DRH1} protein shows 41% identity to VDUP1, expression of which is rapidly induced by 1,25-dihydroxyvitamin D3. The \textit{DRH1} gene was localized to chromosome 15, and \textit{DRH1} protein was mainly observed in the cytoplasm of transiently transfected cells. Real-time quantitative reverse transcription-PCR analysis showed that the expression level of \textit{DRH1} was reduced in 29 of 35 (83%) HCCs compared with corresponding noncancerous liver tissue. The average (mean \pm SE) ratio of \textit{DRH1} expression level in tumor to corresponding noncancerous tissue was significantly different between well, moderately, and poorly differentiated HCCs (1.15 \pm 0.23, 0.69 \pm 0.10, and 0.19 \pm 0.04, respectively) and between HCCs without and with vascular invasion (0.94 \pm 0.16 and 0.46 \pm 0.07, respectively). These results indicate that the down-regulation of \textit{DRH1} occurs not at an early stage but rather at a late stage of HCC progression. Although the function of \textit{DRH1} protein is still unknown, our findings suggest that \textit{DRH1} is related to the progression of HCC and may provide a new prognostic factor.

INTRODUCTION

HCC is one of the most common cancers in Asia and Africa. Despite the remarkable advances in diagnostic and therapeutic techniques, the incidence of HCC remains on the increase. HCC occurs largely in chronically diseased livers (1), mainly resulting from hepatitis virus infection, and progression often leads to vascular invasion and intrahepatic metastasis. However, the mechanisms of carcinogenesis and progression of HCC remain unknown.

The accumulation of multiple genetic alterations, including activation of oncogenes and inactivation of tumor suppressor genes, causes carcinogenesis and further progression in various human cancers (2–4). Several methodologies, including mRNA DD-PCR (5, 6) and subtractive hybridization (7, 8), have been developed to analyze the genes differentially expressed between tumor and corresponding nontumorous tissue or among several cell lines. Using these techniques, several genes including CD24 (9), aldose reductase-like protein (10), MXR7 (11), and hLRH (12) have been reported to be up- or down-regulated in HCCs but have not been directly linked to the carcinogenesis and progression of HCC. Recently, a novel gene, \textit{gankyrin} (13), was identified as a candidate oncogene that is commonly activated in HCC. Because no previous reports show any oncogenes or tumor suppressor genes to be predominant responsibilities for hepatocarcinogenesis at present, the further study of \textit{gankyrin} is anticipated.

In an attempt to identify genes critical to HCC progression, we screened for genes differentially expressed between different HCC cell lines. We describe here the isolation and characterization of a novel gene, \textit{DRH1}, the expression of which was significantly down-regulated in advanced HCC and correlated with the extent of progression.

MATERIALS AND METHODS

Isolation of a \textit{DRH1} cDNA by mRNA DD-PCR and Cloning of Full-Length cDNA. mRNA DD-PCR analysis was performed on several HCC cell lines, using a Fluorescence Differential Display Kit Rhodamine version (TaKaRa Shuzo; Ref. 14) according to the manufacturer’s recommendations (details will be described elsewhere). We obtained a number of fragments that were differentially expressed among HCC cell lines and analyzed the nucleotide sequence. Among them, \textit{DRH1} was isolated from the human HCC KYN-2 cell line, which was primary cultured from moderately to poorly differ-

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3 The abbreviations used are: HCC, hepatocellular carcinoma; DD-PCR, differential display of PCR; RACE, rapid amplification of cDNA ends; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcription-PCR; AFP, \alpha-fetoprotein; LOH, loss of heterozygosity; HBV, hepatitis B virus; HCV, hepatitis C virus.
entilated HCCs (15). To isolate a full-length cDNA of DRH1, we proceeded to cDNA library screening, which was constructed for KYN-2 with oligo-dT primer and cloned into λZAPII vector (Stratagene, La Jolla, CA). The library was not amplified or normalized. More than $5 \times 10^5$ independent clones were screened with an $\alpha$-32PdCTP (Amersham, Arlington Heights, IL)-labeled cDNA fragment isolated by mRNA DD-PCR, using a Rediprime II (Amersham). Positive clones were subcloned into pBluescript II SK (--) and subsequently sequenced, using a Big Dye Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA). 5’ RACE was performed using the 5’ RACE System (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. The primer for first-strand synthesis was designed using the sequence acquired in the cDNA library screening, 5’-CAAT-GTCTTTAGGCCTGAATATGG-3’. The product amplified by PCR was cloned into pCRII (Invitrogen, San Diego, CA) and subsequently sequenced. The nucleotide sequence was analyzed using the BLAST programs of National Center of Biotechnology Institute, and protein localization was predicted by the PSORT algorithm.

**Patients, Cell Lines, and RNA Preparation.** We analyzed a total of 35 primary HCCs and their corresponding adjacent noncancerous liver tissues obtained from patients (mean age, 62.9 years; range, 20–76 years) who underwent surgical resection at the National Cancer Center Hospital (Tokyo, Japan) from July 1998 to June 1999. Surgical specimens were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored until use. The HCCs and noncancerous tissues were histologically confirmed by two independent pathologists. The main clinicopathological features are presented in Table 1. The histopathological grade of tumor differentiation was assessed by a modification of the Edmondson Grading System (16–18). Macroscopically, HCC was subclassified into three types: single nodule (type 1), single nodule with extranodular growth (type 2), and confluent multinodule (type 3; Refs. 17 and 18). The HCCs with vascular invasion included both tumor thrombus in the portal vein and intrahepatic metastasis, because intrahepatic metastasis is thought to be generated via the portal venous system (19). In cases of multicentric HCC, the largest nodule was representatively used in real-time quantitative RT-PCR analysis. Normal tissues were obtained from a male adult patient at autopsy who died with osteosarcoma and were used to examine gene expression in adult multiple organs. KYN-2 cells were cultured in RPMI 1640, and COS-7 cells were cultured in DMEM, both supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Total RNA from tissues and cell lines was isolated using an acid guanidinium thiocyanate-phenol-chloroform method (20).

**Northern Blot Analysis.** Total RNA (15 μg) was separated on a 1.0% agarose formaldehyde denaturing gel, followed by transfer to Hybond-N+ (Amersham) by capillary blotting. DNA probes for the full-length DRH1 and GAPDH were labeled with $\alpha$-32PdCTP as mentioned above. The hybridization and washing procedures were performed as described previously (21).

**GFP Assay.** The open reading frame of DRH1 was amplified by PCR using the forward primer, 5’-AGATCTCGAGGGATTCGGCAGAATTCG-3’, and the reverse primer, 5’-GGTGATCCATCTCCCATGCTAGAATTCGATATG-3’, and the reverse primer, 5’-GGTGATCCATCTCCCATGCTAGAATTCGATATG-3’. The PCR product was initially cloned into pCRII, and its sequence was confirmed and subcloned into the COOH-terminal enhanced fluorescent protein pEGFP-C1 vector (Clontech, Palo Alto, CA). KYN-2 and COS-7 cells were transfected with either vector alone as a control or vector with DRH1 insert, using FuGENE 6 Transfection Reagent (Boehringer Mannheim, Indianapolis, IN). Twenty-four h after transfection, the cells were fixed with 4% paraformaldehyde and examined with a LSM410 confocal microscope (Zeiss, Thornwood, NY).

**Real-Time Quantitative RT-PCR Analysis.** For RT-PCR analysis, all RNA samples were treated with DNase I (Promega Corp., Madison, WI) to remove genomic DNA. Real-time quantitative RT-PCR analysis was performed as reported previously (22, 23). In brief, cDNA of each tissue sample was synthesized from DNase-treated total RNA using oligo dT primer and AMV Reverse Transcriptase XL (TaKaRa). Each primer set for the PCR reaction was designed using the support of Primer Express software (Perkin-Elmer Applied Biosystems). For DRH1 cDNA, the following two primer sets were prepared. One primer set, 5’-ATGAGATATTTGGATGAGACATGCTAGTTG-3’ (forward) and 5’-GTTATCAAGCAGTACTCTGAGAT-3’ (re-
Fig. 1  Sequence analysis of DRH1. A, full-length DRH1 cDNA and partial clones acquired by each screening, which included mRNA DD-PCR, cDNA library screening, and 5' RACE, and the regions for RT-PCR analysis are shown. Closed rectangle, region of deduced open reading frame. B, full nucleotide sequence and corresponding amino acid sequence of DRH1. The deduced DRH1 protein sequence is shown below the nucleotide sequences. The potential polyadenylation site is underlined.
Down-Regulation of *DRH1* in Progressed HCC

verse), was designed against the 3' untranslated region identified by mRNA DD-PCR, and another set, 5'-CACCTGTTACTCCATCATCCT-3' (forward) and 5'-GGTGCAAAACATTATATAGGA-3' (reverse), was designed against the deduced coding region of the full-length *DRH1*. For standardization of the amount of RNA, the expression of GAPDH in each sample was quantified using the primer sets, 5'-GAAGGTGGAAGGTGAGTGAG-3' (forward) and 5'-CCGGATCATTCCCTGGA-3' (reverse). All PCR reactions were performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems) under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 1 min. Real-time detection of the emission intensity of SYBR Green was performed with an ABI prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) as reported previously (24). Quantitative RT-PCR was performed at least three times, including a no-template control as a negative control. To analyze the correlation between the *DRH1* expression and the clinicopathological features, the ratio of *DRH1* mRNA expression level in tumor to corresponding noncancerous tissue (T:N ratio) was calculated in each case.

**Statistical Analysis.** To analyze differences in the distribution of T:N ratios of each clinicopathological factor, we performed the unpaired t-test between two groups, and the one-way ANOVA and Fisher’s PLSD test as Post hoc test between three groups, using Stat View (version 5.0) software (Abacus Concepts, Berkeley, CA). Differences with *P* < 0.05 were judged to be significant.

**RESULTS**

**Cloning and Sequence Analysis of *DRH1*.** To obtain a full-length cDNA clone for *DRH1*, we screened a cDNA library constructed from mRNA isolated from KYN-2 cells, using the fragment originally isolated by mRNA DD-PCR as a probe. After screening 5 \( \times 10^7 \) independent clones, we acquired two positive clones. These clones were shorter than the expected size of 4.2 kb, formerly analyzed by Northern blot of some HCC cell lines (data not shown), and had no clear open reading frame. Therefore, we performed 5' RACE using total RNA from KYN-2 cells and successfully obtained a full-length, 4084-bp *DRH1* clone (Fig. 1A), which contained a 419-amino acid open reading frame encoding a protein with an approximate molecular weight of 45,000 (Fig. 1B). Sequence analysis revealed 41% identity between the deduced proteins of *DRH1* and *VDU1* (Ref. 25; accession no. NM_006472; Fig. 2). By PSORT program, *DRH1* protein was predicted to be a cytoplasmic protein.

**Characterization of *DRH1*.** Distribution of *DRH1* expression was examined using total RNA from various human adult tissues. On Northern blot analysis, a 4.2-kb transcript could be observed in all human organs examined (Fig. 3A). The chromosomal localization of the *DRH1* gene was determined by Southern blot analysis using National Institute of General Medical Sciences human \( \times \) rodent somatic cell hybrid mapping panel 2 (26, 27). The *DRH1* gene was localized to chromosome 15 (data not shown). We could ascertain that the pieces (accession number AC024651), including the *DRH1* sequence, was localized to the q arm of chromosome 15, using the database of National Center for Biotechnology Information. To determine the subcellular localization of *DRH1*, an expression vector containing *DRH1* cDNA was transiently transfected in COS-7 and KYN-2 cells. Under confocal microscope, the *DRH1*-EGFP fusion protein was predominantly distributed outside the nucleus of COS-7 cells (Fig. 3B). On the other hand, EGFP protein alone showed a diffuse localization pattern in COS-7 cells. The same pattern of localization of *DRH1* fusion protein was seen in transfected KYN-2 cells, as in COS-7 cells (data not shown).

***DRH1* mRNA Expression in HCC.** Real-time quantitative RT-PCR analysis was used to examine *DRH1* mRNA expression (Fig. 4). In 29 of 35 cases (83%), the expression level in HCC was down-regulated when compared with corresponding noncancerous liver, and the average expression level in HCCs was significantly lower than that in noncancerous liver (4.98 ± 0.73 versus 9.02 ± 0.71; *P* = 0.0001). Almost identical results were obtained by using two different primer sets, as described in “Materials and Methods” (Fig. 1A). Noncancerous liver was histologically comprised of 3 normal, 16 chronic hepatitis, and 16 liver cirrhosis, and the average expression level of *DRH1* in each situation was 8.37 ± 1.79, 8.41 ± 0.49, and 9.75 ± 1.44, respectively (data not shown). Therefore, we could not find any significant difference between the expression level of *DRH1* and the histological situation of noncancerous liver. Subsequently, we statistically assessed the averages of the T:N ratio of *DRH1* mRNA in subgroups divided by clinicopathological parameters and analyzed the distribution of the T:N ratio (Table 1 and Fig. 5). The mean T:N ratio in 35 cases was 0.64 ± 0.13. There were prominent statistical differences in the average T:N ratio between histological grades (well versus moderate, moderate versus poor, and poor versus mixed), and among histological types (hemangioma versus HCC, metastasis versus HCC, and HCC versus normal liver). The statistical significance was maintained when the data were analyzed with the unpaired t-test between two groups, and the one-way ANOVA and Fisher’s PLSD test as Post hoc test between three groups. Statistical significance was also maintained when the data were analyzed with the unpaired t-test between two groups, and the one-way ANOVA and Fisher’s PLSD test as Post hoc test between three groups.
P = 0.02; moderate versus poor, P = 0.002), vascular invasion (absent versus present, P = 0.006). As shown in Fig. 5, the T:N ratio was markedly decreased in all cases of poorly differentiated HCCs. By contrast, no cases of well-differentiated HCCs showed a low T:N ratio, although the number of cases was small. Moreover, in the subgroup of HCCs with vascular invasion, many cases showed an extremely low T:N ratio. When a low T:N ratio was arbitrarily defined as being ≤0.33, these differences were more clearly observed. A total of 78% of poorly differentiated HCCs had a low T:N ratio, versus 39% of moderately and no well-differentiated HCCs. A total of 62% of HCCs with vascular invasion had a low T:N ratio, versus only 21% of those with no invasion; and in the high-AFP and low-AFP groups, 71 and 29% had low T:N ratios, respectively. There were also significant differences in the average T:N ratio in other parameters, such as virus infection and tumor size (Table 1).

DISCUSSION

We report the cloning and characterization of the full-length cDNA for a novel gene, DRH1. Clinicopathological analysis using real-time quantitative RT-PCR showed that DRH1 expression was significantly decreased in HCCs showing poor differentiation and with vascular invasion. In contrast, the reduction of DRH1 expression was not observed frequently in HCCs showing well differentiation and without vascular invasion. It is widely known that HCCs often show dedifferentiation (from well-differentiated HCCs to poorly differentiated HCCs) during multistep progression (18, 28, 29), and that some HCCs acquire metastatic potential during this progression, resulting in vascular invasion (30, 31). Our results suggest that the down-regulation of DRH1 is closely associated with the later events in hepatocarcinogenesis. Because vascular invasion is generally thought to be an important prognostic factor (32, 33), the DRH1 expression level may provide a useful prognostic marker. A marked reduction in DRH1 expression was also observed in HCCs showing elevated serum AFP levels. This result was compatible with previous findings that the elevation of serum AFP was more frequently observed in HCCs showing less differentiation (34, 35). We also found differential DRH1 expression between HCCs with HBV and HCV infection. The mechanisms of carcinogenesis induced by HBV and HCV are not well understood, and the further study of the DRH1 gene may help clarify these mechanisms.

From the deduced full-length DRH1 amino acid sequence,
we found a sequence similar to the consensus nuclear export signal, which was initially proposed in 1996 as a leucine-rich region (36, 37). Although this consensus sequence does not always function as a nuclear export signal, this is compatible with our observation of the DRH1-GFP fusion protein, mainly in cytoplasm, and with the prediction of the PSORT algorithm. DRH1 may possibly act as a cytoplasmic protein or as a transporter of other proteins from the nucleus to the cytoplasm. No other clues as to the function of the DRH1 protein could be inferred from the primary structure. DRH1 showed 41% identity with VDUP1 at the amino acid sequence level. VDUP1 was reported previously as a gene that was rapidly induced in HL-60 cells by 1,25-dihydroxyvitamin D3 treatment, which is often used to induce hematopoietic cell differentiation. Although we do not know the function of the VDUP1 protein in detail, we assume that up-regulation of VDUP1 under the presence of the inducer of cell differentiation does not contradict our finding that a marked down-regulation in DRH1 expression was observed frequently in less differentiated HCCs but not in well-differentiated HCCs. DRH1 expression was observed in all organs tested, similar to the housekeeping gene. Therefore, a study of DRH1 expression in other tumors must be informative for the role of DRH1 in tumor progression.

The DRH1 gene was localized to the q arm of chromosome 15. Although frequent genome loss and LOH of the q arm of chromosome 15 has not been observed in HCC (38–40), there are several reports in other tumors about LOH on chromosome 15 (41–43). Moreover, this LOH was observed as a late event of tumor progression in breast cancer. These observation raises the possibility that this region includes the important gene showing responsibility for the progression of cancer. Therefore, although we still do not know which of the mechanisms, such as LOH, hypermethylation of the promoter region, and so on, affected the down-regulation of DRH1, it is interesting that DRH1 was down-regulated only in advanced HCCs.

In the present study, we cloned a novel gene, DRH1, the expression of which is down-regulated in advanced HCC. The DRH1 expression level appropriately reflects the extent of HCC progression, suggesting that DRH1 plays an important role in HCC progression. Further studies of the biological function of DRH1 will be helpful in understanding the pathogenesis of HCC.

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