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Poly-(ADP-Ribose) Polymerase-1 Promotes Prothrombin Gene Transcription and Produces Des-Gamma-Carboxy Prothrombin in Hepatocellular Carcinoma

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Keywords

Des-gamma-carboxy prothrombin · Poly-(ADP-ribose) polymerase-1 · Hepatocellular carcinoma · Biomarkers · Prothrombin

Abstract

Background and Aim: Although des-gamma-carboxy prothrombin (DCP) is a well-known tumor marker for hepatocellular carcinoma (HCC), the mechanism of DCP production is unclear. This study aimed to investigate the mechanism how DCP is produced in HCC cells. ***Methods:*** Levels of mRNA and DCP were analyzed by real-time polymerase chain reaction and electro-chemiluminescence immunoassay respectively. Secreted alkaline phosphatase (SEAP) expression vectors including deletion mutants of the prothrombin gene promoter were constructed for reporter gene assay. The transcription factors bound to DNA fragments were analyzed by mass spectrometry. An electrophoretic mobility shift assay (EMSA) was performed using a biotin end-labeled DNA. ***Results:*** The prothrombin mRNA levels in all 5 DCP producing cell lines were appreciably high. However, those in 2 DCP non-producing cell lines were below detectable levels. A SEAP vector with −2985 to +27 showed a very high transcription activity in DCP-producing Huh-1 cells. However, transcription abruptly decreased when the vector with −2955 to +27 was transfected, and then remained at the similar levels with larger deletion mutants, indicating the existence of a cis-element at −2985 to −2955 (31-bp). Mass spectrometry analysis identified the protein that bound to the 31-bp DNA as poly-(ADP-ribose) polymerase-1 (PARP-1). Knockdown of the PARP-1 gene by small interfering RNA in Huh-1 cells induced marked inhibition of prothrombin gene transcription. The EMSA clearly showed that PARP-1 specifically binds to the 31-bp DNA fragment in the prothrombin gene promoter. ***Conclusions:*** Our data suggest that PARP-1 activates prothrombin gene transcription and that the excessive prothrombin gene transcription induces DCP production in DCP-producing HCC cells.

Introduction

Des-gamma-carboxy prothrombin (DCP) is well recognized as a tumor marker with high sensitivity and specificity in the surveillance and diagnosis of hepatocellular carci-

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noma (HCC) [1–4]. It has been reported that a high serum level of DCP is closely associated with a large tumor size, recurrence, vascular invasion, metastasis, and high mortality in patients with HCC [5–12], indicating that DCP is a prognostic indicator for HCC. Moreover, it has been reported that DCP directly binds to c-MET and stimulates cell proliferation as a potential autologous growth factor in HCC, and that DCP stimulates vascular endothelial cell proliferation and migration, promoting neovascularization in HCC tissue [13–15]. However, the precise molecular mechanism of DCP production is currently unclear.

DCP is an abnormal prothrombin that lacks the ability to interact with other coagulation factors, and it differs from normal prothrombin with respect to the composition of amino acid residues. The prothrombin molecule has a so-called Gla domain in its N-terminal that includes 10 gamma-carboxylated glutamic acid (Gla) residues. These Gla residues originate from glutamic acid (Glu) residues in prothrombin precursor protein, and they are completely synthesized by the vitamin K-dependent enzymatic reaction of gamma-glutamyl carboxylase (GGCX) via post-translational modification [16]. When this reaction is insufficient, in conditions, such as vitamin K deficiency, DCP with Glu residues remaining in the Gla domain without gamma-carboxylation are expressed and secreted extracellularly [17].

There have been 3 main mechanisms proposed for DCP production in HCC cells to date. The first is the theory of prothrombin precursor overproduction. Ono et al. [18] showed that the concentration of immune-reactive prothrombin (FII) in HCC tissues was significantly higher than that in the non-HCC tissues in patients with elevated plasma DCP levels, whereas there was no significant difference in FII concentration between HCC and non-HCC tissues in patients without DCP elevation, which is suggestive of a mechanism involving the overproduction of prothrombin. However, in that study, FII concentrations were measured by ELISA using an anti-prothrombin polyclonal antibody, which would therefore detect not only mature prothrombin but also DCP and prothrombin precursor proteins. Moreover, the study did not examine the tissue mRNA expression. Thus, it remains unclear whether or not mature prothrombin is overproduced. In addition, we are not aware of any published studies showing excessive prothrombin gene transcription in DCP-producing HCC cells.

The second possible explanation for DCP production in HCC cells is a decrease in GGCX activity. Huisse et al. [19] reported that GGCX activity is decreased in DCP-producing HCC tissues. Subsequently, Ueda et al. [20] reported that an alternative splicing variant, an exon 2 deletion of GGCX (Δ2GGCX) with no enzyme activity, is expressed in DCP-positive HCC cell lines. However, contradictory studies have reported that GGCX activity is not decreased in DCP-producing HCC tissues [21]. We have also found similar mRNA levels of the alternative splicing variant (Δ2GGCX) not only in DCP-producing cells but also in a DCP non-producing cell line (HLF), in DCP non-producing HCC tissues, and in non-cancerous liver tissues, suggesting that this variant exists to some extent in hepatocytes regardless of DCP production (unpublished data). Thus, this theory is currently debatable.

The third possible mechanism of DCP production in HCC cells involves vitamin K deficiency. Murata et al. [22, 23] reported that HepG2 cells, a DCP non-producing cell line, treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) produced DCP in a dose-dependent manner. They also found that TPA induced epithetial-to-fibroblastoid conversion in HepG2 cells, which accompanied cytoskeletal changes and impairment of endocytosis including vitamin K uptake, leading to vitamin K deficiency. However, their study did not measure cellular vitamin K uptake nor intracellular vitamin K concentrations. Moreover, since TPA is a strong cancer promoter, it may activate various signaling pathways that lead to DCP production regardless of vitamin K deficiency. Thus, the underlying mechanism of DCP production, particularly with respect to prothrombin transcription, remains unclear. Therefore, in this study, we first compared prothrombin mRNA levels between DCP producing and non-producing HCC cell lines. We then performed a secreted alkaline phosphatase (SEAP) reporter gene assay to analyze the region of the prothrombin gene promoter responsible for transcription in DCP-producing and non-producing cell lines as well as in normal hepatocytes. We also analyzed a novel transcription factor that binds to the 5′-flanking region of the prothrombin gene responsible for excessive DCP transcription in DCP-producing cells.

Materials and Methods

Cell Lines and Cell Culture

The human HCC cell lines, Huh-1, Huh-7, HepG2, PLC/PRF/5, HLF, and HLE, were obtained from Japanese Collection of Research Bioresources (Osaka, Japan). The Hep3B cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). All HCC cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) at 37°C. Normal human hepatocytes were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA) and cultured in Hepatocyte Maintenance Medium (HMM™, Lonza Walkersville, Inc.).

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DCP Quantitation by Electro-Chemiluminescence Immunoassay

After washing with PBS, each cell line (2×10^5/mL) was incubated with 3.0 mL of fresh FCS-free medium at 37°C for 48 h. The medium from each cell line was centrifuged, and DCP concentrations were determined by an electro-chemiluminescence immunoassay (ECLIA) (picolumi PIVKA™, Sanko Junyaku Co., Ltd., Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time-polymerase chain reaction (PCR) was performed as described previously [24]. The probe and primers from the TaqMan gene expression assay reagents (Applied Biosystems, Foster City, CA, USA) were used: prothrombin (Hs01011988_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) as an internal control.

Prothrombin Gene Promoter Constructs and SEAP Assay

This study was approved by the Committee of Gene Recombination in Tokushima University (22–60). Deletion fragments of the 5′-flanking region in the prothrombin gene promoter (–2985 to +27, –2955 to +27, –2485 to +27, –1259 to +27, –952 to +27, and –424 to +27) were amplified by PCR using each primer pair from genomic DNA of HepG2 cells. The sequences of each forward primer and reverse primer are shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000470837). Subsequently, each amplified fragment was ligated into the pSEAP2-basic vector (Clontech, Mountain View, CA, USA). Each construct was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and SEAP enzyme activity in the culture media was determined 48 h after transfection using Great EscAPe™ SEAP chemiluminescence assay kit (Clontech).

Extraction of Nuclear Protein

Nuclear extracts of the cells were prepared as described previously [25].

Mass Spectrometry

The 5′-end-biotinylated 31-base DNA fragment 5′-GCTTCAATTTCAGCAGAATTCATTTAGCTCT-3′ at –2985 to –2955 in the prothrombin gene and the 5′-end-biotinylated complementary reverse fragment 5′-AGAGCTAAATGAATTCTGCTGAAATTGAAGC-3′ were synthesized and annealed. They were incubated with each nuclear extract, and the DNA-protein binding complexes were collected using Dynabeads™ M-280 Streptavidin™ (Invitrogen). They were then separated on 2% SDS-PAGE, and silver-stained. Selected protein bands were cut into small pieces and digested with trypsin, as described previously [26]. Mass spectrometry analysis was carried out using a Q-Tof Ultima API spectrometer (Waters Corp., Milford, MA, USA). Data interpretation and protein identification were performed with the LC-MS/MS spectra data sets of the MASCOT server program version 2.0.05 (Matrix Science Ltd., London, UK). A protein "hit" was accepted as a valid identification when at least 2 LC-MS/MS spectra matched at a 95% level of confidence ($p < 0.05$).

Gene Knockdown by Small Interfering RNA

Double stranded poly-(ADP-ribose) polymerase-1 (PARP-1)-specific small interfering RNA (siRNA; SASI_Hs01_00159525, Sigma-Aldrich) was transfected into Huh-1 cells using Lipofectamine RNAiMAX (Invitrogen). After 24 h, SEAP vector P-2985 or P-2955 was transfected into the cells using Lipofectamine 2000 (Invitrogen), followed by medium exchange after another 8 h. SEAP assay was performed after incubation for an additional 40 h. Silencer Select GAPDH siRNA and Silencer Negative Control #1 siRNA (Applied Biosystems) were used as positive and negative controls respectively.

For the study of DCP production in PARP-1 knockdown cells, each cell line (2×10^5/mL) was transfected with PARP-1 specific siRNA followed by incubation with FCS-free media for 8 h and FCS-supplemented media for another 24 h. Conditioned medium was then prepared and DCP concentrations were determined by ECLIA after being incubated with FCS-free medium for 48 h.

Western Blot Analysis

Western blot analysis was performed using a rabbit anti-human PARP-1 monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) as described previously [27].

Electrophoretic Mobility Shift Assay

The 5′-end-biotinylated 31-bp double-stranded DNA fragment at –2985 to –2955 in the prothrombin gene was incubated with each nuclear extract, and electrophoretic mobility shift assay (EMSA) was performed using an EMSA Assay Kit™ (Signosis, Inc., Santa Clara, CA, USA). Anti-PARP-1 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used for the supershift assay.

Cell Viability Analysis

Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as described previously [27].

Results

DCP Production and Prothrombin mRNA Levels in Various HCC Cell Lines

We first investigated DCP production and prothrombin mRNA levels in various HCC cell lines. The DCP concentrations in the conditioned media of Huh-1, Huh-7, HepG2, Hep3B, and PLC/PRF/5 cells were 418 ± 74, 173 ± 82, 157 ± 112, 211 ± 72, and 386 ± 87 mAU/mL, respectively. However, the DCP concentrations in the conditioned media of HLF and HLE cells were below detectable levels (Fig. 1a). Thus, Huh-1, Huh-7, HepG2, Hep3B, and PLC/PRF/5 cells were DCP-producing cell lines, whereas HLF and HLE were DCP non-producing cell lines.

The mRNA levels of prothrombin in Huh-1, Huh-7, HepG2, Hep3B, and PLC/PRF/5 cell lines were 1.8 ± 0.3, 1.7 ± 0.4, 2.9 ± 0.5, 3.5 ± 0.4, and 4.0 ± 0.4, respectively. However, those of HLF and HLE cells were below detectable levels (Fig. 1b). These results suggested that prothrombin mRNA was over expressed only in DCP-producing cells. Thus, there was a close correlation between DCP production and prothrombin mRNA levels.

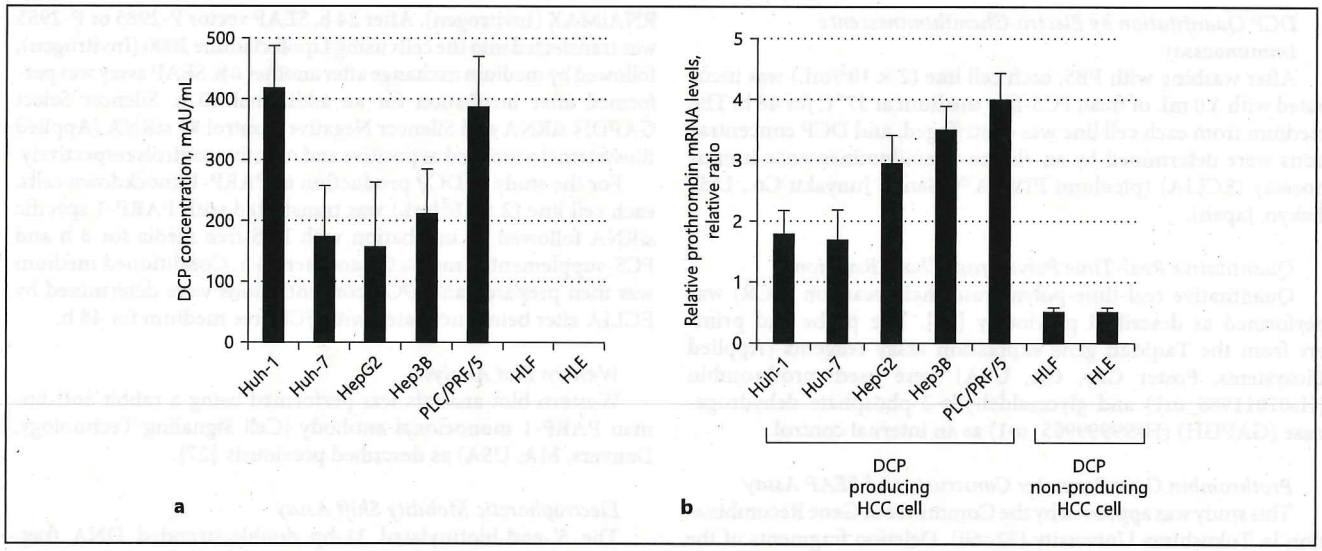

Fig. 1. Des-gamma-carboxy prothrombin (DCP) concentration and prothrombin mRNA levels in various hepatocellular carcinoma (HCC) cell lines. **a** After washing, each cell line was incubated in fresh medium for 48 h. The DCP concentration in conditioned medium was determined by electrochemiluminescence immunoassay. Data are represented as mean ± SD ($n = 5$). **b** Total RNA was extracted and reverse transcribed into complementary DNA with MuLV reverse transcriptase. Quantitative real-time polymerase chain reaction was performed with total RNA from each HCC cell line. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was used as a control. Data are expressed as the ratio to GAPDH mRNA and represented as mean ± SD ($n = 5$).

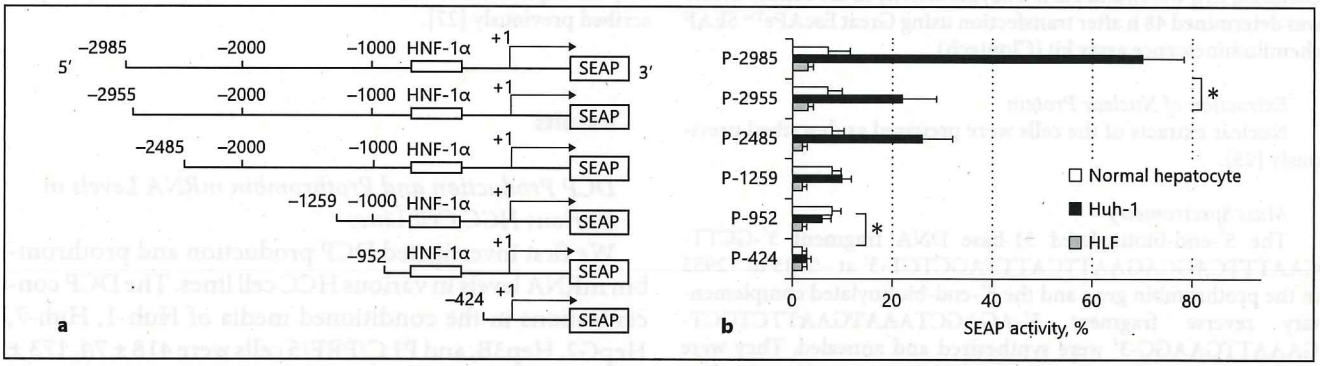

Fig. 2. a Schematic representation of the prothrombin promoter deletion mutants. Deletion mutants of the 5′-flanking region in the prothrombin gene promoter (−2985 to +27, −2955 to +27, −2485 to +27, −1259 to +27, −952 to +27, and −424 to +27) were amplified by the polymerase chain reaction using each primer pair. Each amplified fragment was ligated into the pSEAP2-basic vector. HNF-1α is reported to be located at −886 to −879. **b** A summary of SEAP assay activity. Each construct was transfected into Huh-1 cells, HLF cells, and normal hepatocytes, and SEAP activity in culture media was determined at 48 h after transfection. All results are expressed relative to the activity of pSEAP2-control. Data are represented as mean ± SD ($n = 5$). * $p < 0.05$.

Essential Region for Prothrombin Gene Transcription

To determine the region of the prothrombin gene promoter responsible for transcription, we constructed several deletion mutants with SEAP vector, and performed SEAP reporter gene assays using Huh-1 as a DCP-producing cell line, HLF as a DCP non-producing cell line, and normal hepatocytes (Fig. 2a). In normal hepatocytes, the constructs with −2985 to +27 (P-2985), −2955 to +27 (P-2955), −2485 to +27 (P-2485), −1259 to +27 (P-1259), and −952 to +27 (P-952) exhibited relatively low transcription activities ranging from 6 to 8%, but the construct with −424 to +27 (P-424) showed significantly lower activity (3.2 ±

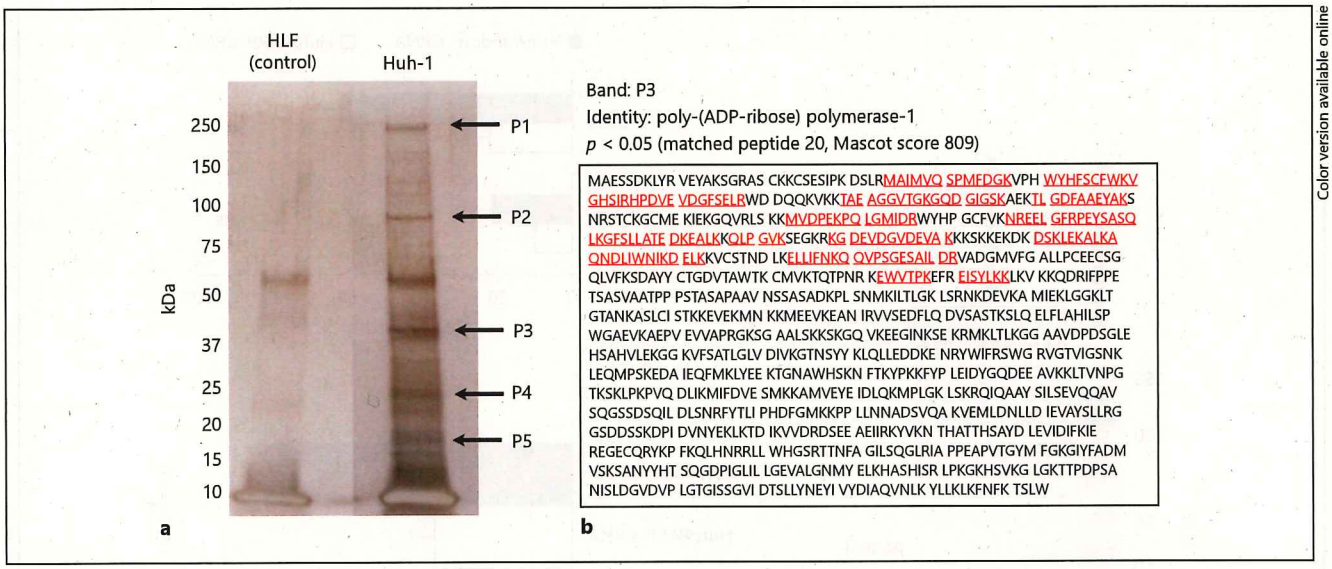

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Fig. 3. Mass spectrometry analysis of Huh-1 nuclear protein associated with the 31-bp DNA fragment. **a** Huh-1 was used as a des-gamma-carboxy prothrombin-producing cell, and HLF was used as a control. The protein bands marked P1–P5 were collected for LC-MS/MS analysis. **b** The detailed analysis of P3 is shown, and highlights in red show the matching sequences.

0.3%, $p < 0.05$). The decrease may be explained by the existence of the HNF-1α-binding sequence AAATATTA at −886 to −879 in the prothrombin gene; that is, HNF-1α binds to the sequence and promotes transcription as previously reported [28]. P-2985 exhibited a very high transcription activity (70.1 ± 8.8%) in Huh-1 cells. However, transcription activity was abruptly decreased when they were transfected with P-2955 as well as P-2485, P-1259, P-952, or P-424, indicating the existence of an important DNA sequence at −2985 to −2955 (31-bp) for transcription activity. Little transcription activity was detected when these constructs were transfected into HLF cells (Fig. 2b).

Identification of PARP-1 as a Candidate Transcription Factor

To identify the transcription factor that binds to the 31-bp DNA (−2985 to −2955) fragment, we performed mass spectrometry analysis of the nuclear proteins that bound to it. Nuclear proteins extracted from Huh-1 or HLF cells were incubated with the 31-bp DNA fragment and fractionated in SDS-PAGE. Five major protein bands, designated P1 to P5, were specifically identified in Huh-1 nuclear extract as compared with HLF nuclear extract (Fig. 3a). In the mass spectrometry analysis of P1, P2, P4, and P5, no candidate protein with ≥95% homology was found (online suppl. Table 2). However, analysis of the P3 (approximately 40 kDa) revealed 4 candidate proteins with homology greater than 95% (online suppl. Table 2). Of these, PARP-1 showed the highest number of matched peptides (amino acids), and exhibited the highest Mascot score (i.e., 809; Fig. 3b). It is known that there are several sizes of PARP-1 proteins including the 40 kDa fragment. Our results indicate that the 40 kDa PARP-1 is the most plausible transcription factor that binds to the 31-bp DNA fragment.

Knockdown of PARP-1 Gene Inhibits Prothrombin Gene Transcription and DCP Production

To examine whether PARP-1 is involved in prothrombin gene transcription in DCP-producing cells, we knocked down the PARP-1 gene expression by using siRNA directed against the nucleotide sequence for 40 kDa PARP-1 in Huh-1 cells and investigated the transcription activity of P-2985 and P-2955 in those cells by SEAP assay. The 40 kDa PARP-1 as well as 113 kDa PARP-1 proteins in Huh-1 cells transfected with PARP-1 siRNA (Huh/PARP-siRNA) were almost negligible at 24, 48, and 72 h after transfection, as revealed by Western blotting (Fig. 4a). The transcription activity of P-2985 in Huh/PARP-siRNA cell was significantly lower than that in control cells with random siRNA (Huh/random-siRNA; Fig. 4b). There was no significant difference in transcription activity of P-2955 between Huh/PARP-siRNA and Huh/random-siRNA cells.

To further examine whether PARP-1 is involved in DCP production, we measured DCP concentrations in

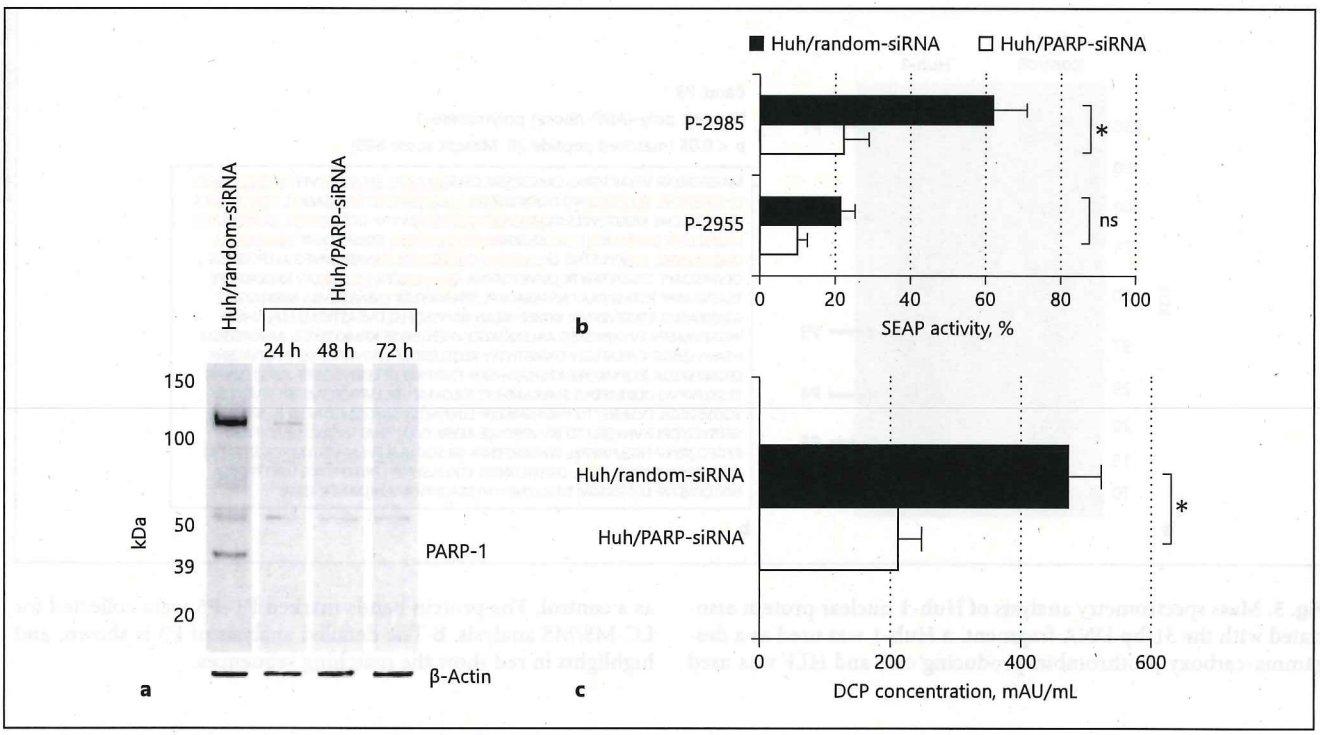

Fig. 4. Poly-(ADP-ribose) polymerase-1 (PARP-1) gene knockdown in Huh-1 cells, and SEAP activity of P-2985 and P-2955 in knockdown cells. **a** PARP-1 gene was knocked down by small interfering RNA (siRNA) (Huh/PARP-siRNA), and PARP-1 protein expression in the Huh/PARP-siRNA cells at 24, 48, and 72 h after transfection were examined by Western blotting. **b** Huh/PARP-siRNA cells and control cells with random siRNA (Huh/random-siRNA) were transfected with P-2985 or P-2955, and SEAP assay was performed. Data are represented as mean ± SD ($n = 3$). **c** Des-gamma-carboxy prothrombin concentrations in Huh/PARP-siRNA cells and Huh/random-siRNA were determined by electrochemiluminescence immunoassay. Data are represented as mean ± SD ($n = 5$). * $p < 0.05$. ns, not significant.

conditioned media from Huh/PARP-siRNA cells as well as Huh/random-siRNA cells. The DCP concentration in the conditioned media from Huh/PARP-siRNA cells (211 ± 45 mAU/mL) was significantly lower than that of Huh/random-siRNA cells (472 ± 51 mAU/mL, $p < 0.05$; Fig. 4c).

Mobility Shift of 31-bp DNA Probe by PARP-1

To confirm the direct interaction of the 31-bp DNA probe with PARP-1, EMSA was performed using a mixture of 31-bp DNA probe and nuclear extracts from Huh-1 or HLF cells. EMSA showed that the 31-bp DNA probe was shifted by nuclear extracts from Huh-1 cells (Fig. 5). When the mixture was incubated with anti-PARP-1 antibody, the band was supershifted indicating that the 31-bp DNA probe specifically binds to the PARP-1. In contrast, a much lower amount of the shifted band was observed in the mixture of the probe with nuclear extract from HLF cells.

Anti-Tumor Activity of PARP-1 Inhibitor against HCC Cell Lines

Since DCP has been reported to promote cell proliferation by an autocrine mechanism in HCC cells, it was expected that the PARP-1 inhibitor would have stronger anti-tumor activity against DCP-producing cells than against DCP non-producing cells due to the inhibition of DCP production. Therefore, we investigated the anti-tumor activity of iniparib, a representative PARP-1 inhibitor, on DCP-producing cells (Huh-1 and HepG2) and DCP non-producing cells (HLF and HLE). The IC50 values of iniparib for Huh-1 and HepG2 cells were 101.2 ± 10.6 and 96 ± 4.1 μM, respectively, while those for HLF and HLE cells were 161.3 ± 0.17 and 149.8 ± 0.17 μM, respectively (Fig. 6). There were significant differences in IC50 values between DCP-producing cells and non-producing cells ($p < 0.05$). Thus, our data indicate that PARP-1 inhibitor has approximately 1.5-fold stronger activity against DCP-producing cells as compared with DCP non-producing cells.

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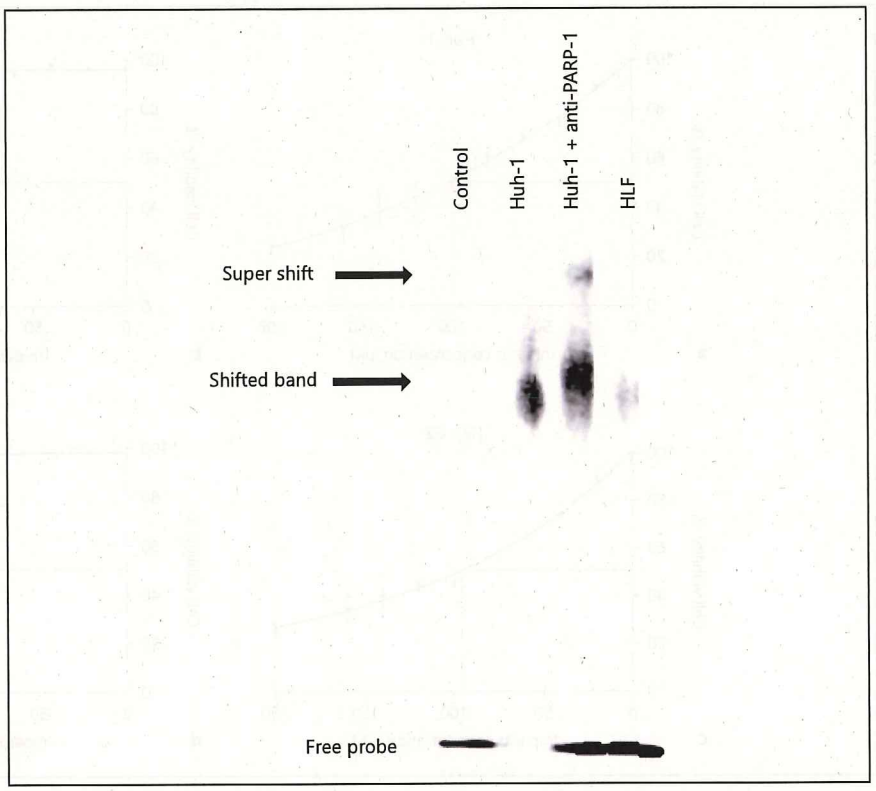

Fig. 5. Electrophoretic mobility shift assay of the 31-bp DNA fragment and nuclear extracts from Huh-1 or HLF cells. The 31-bp DNA fragment, whose 5′-end was biotinylated, was incubated with Huh-1 or HLF nuclear extract (± anti-poly-[ADP-ribose] polymerase-1 serum). The protein-DNA complex were electrophoresed in 6.5% nondenaturing polyacrylamide gels and transferred to a membrane. It was then incubated with horseradish peroxidase conjugate streptavidin and visualized using 3,3′-diaminobenzidine. Biotinylated DNA without nuclear extract was used as a control.

Discussion

In this study, we showed that PARP-1 plays a pivotal role in the excessive transcription of the prothrombin gene and induces DCP production in DCP-producing HCC cells. We also revealed that in normal hepatocytes, transcription factor HNF-1α promotes a basal level of prothrombin gene transcription, whereas PARP-1 drives transcription much more strongly by binding to the region at –2985 to –2955 of the prothrombin gene promoter in DCP-producing cells. This suggests that excessive production of prothrombin precursor exhausted intracellular vitamin K and/or GGCX, and caused impairment of carboxylation of Glu, leading to the accumulation of DCP. In DCP non-producing cells, however, only a low amount of prothrombin precursor is translated due to a low level of transcription, which does not exhaust them, and maintains normal carboxylation function with no DCP production (Fig. 7). Yamagata et al. [28] reported that prothrombin precursor and DCP levels in DCP-positive HCC tissues were significantly higher than in DCP-negative HCC tissues. This finding also supports our theory that the overproduction of prothrombin precursor is responsible for DCP production in HCC cells. We also found that DCP production significantly correlated with PARP-1 mRNA expression levels in HCC cell lines (online suppl. Fig. 1A) and in HCC tissues (online suppl. Fig. 1B).

Chow et al. [28] investigated the prothrombin gene 5′-franking region using HepG2 cells and revealed that the transcription factor HNF-1α induces prothrombin gene transcription by binding to the consensus sequence of HNF-1α (AAATATTA) at –886 to –879. However, they examined only the limited region of the prothrombin gene from –1250 to –29. In this study, we examined a much wider upstream region from –3005 to +27 in the prothrombin gene, and found a pivotal sequence at –2985 to –2955 that strongly promotes transcription through binding with PARP-1. Similar results were obtained in the reporter gene assay using PLC/PRF/5, HepG2, and Huh-7 cells (data not shown). To our knowledge, there are no published studies that have examined prothrombin transcription in normal hepatocytes. In this study, we found that in normal hepatocytes there is an important region for basal transcription at –952 to –424, where an HNF-1α consensus sequence exists. This result is com-

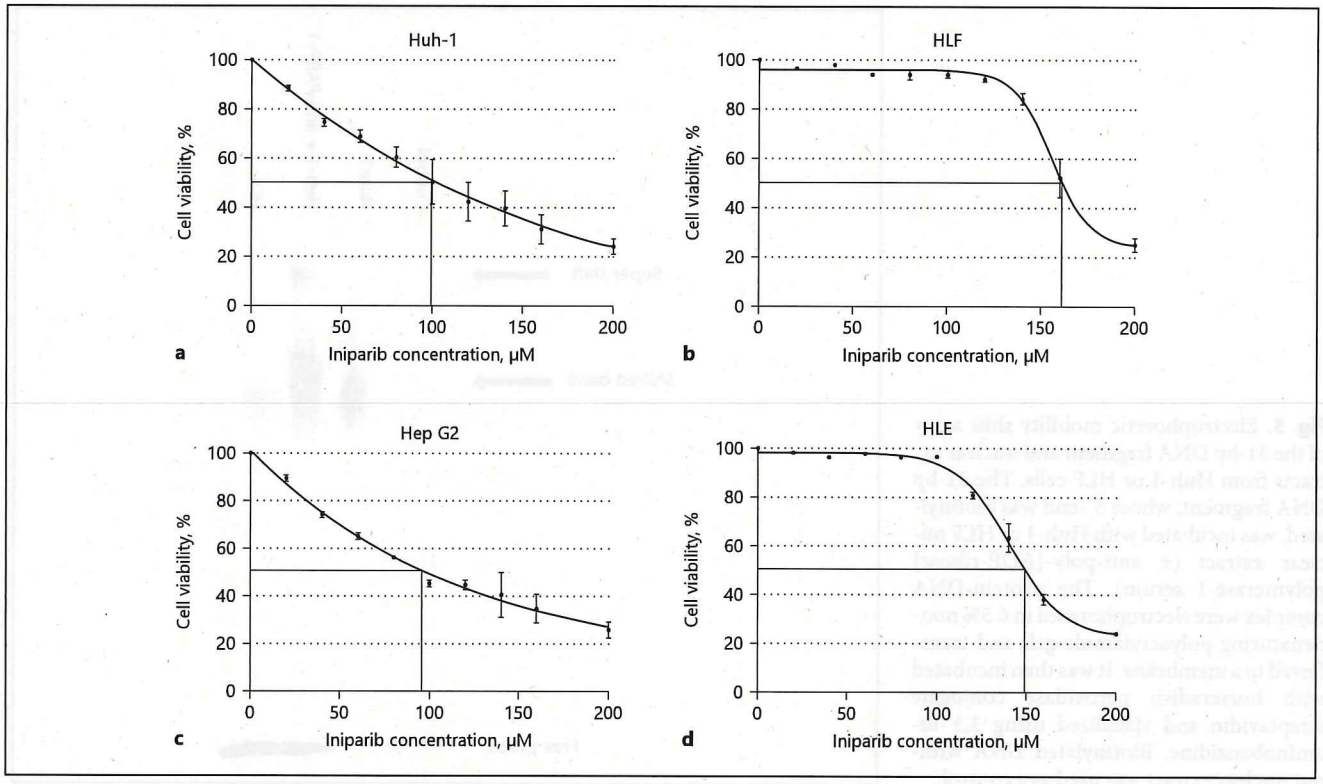

Fig. 6. a–d The anti-tumor activity of Huh-1, HepG2, HLF, and HLE cells to iniparib was assessed by MTT assay. The IC50 values were calculated by non-linear regression analysis.

patible with the previous report on HNF-1α by Chow et al. [28] However, in DCP-producing HCC cells, the 31-kb sequence found in this study plays an important role in the enhanced transcription. Interestingly, in normal hepatocytes, the upstream region at –2985 to –2955, including the 31-bp sequence did not drive prothrombin gene transcription (Fig. 2b). This indicates that the mechanism of prothrombin gene transcription in DCP-producing cells is different from that in normal hepatocytes.

The mass spectrometry analysis in this study identified PARP-1 as a fragment of approximately 40 kDa. It is well known that there are several fragments of PARP-1 including 113, 89, 64, 55, 40, and 24 kDa, which occur under physiological and pathological conditions. Of these, the 40 kDa fragment exists in the N-terminal region and includes the DNA-binding domain. It has been reported that the 40 kDa PARP-1 fragment serves as a transcription factor [29]. Moreover, the amino acid sequences of the 20 peptide fragments obtained were completely identical to the N-terminal amino acid sequence of PARP-1. Thus, it seems reasonable that the 40 kDa PARP-1 protein activates prothrombin gene transcription. In fact, we showed that the knockdown of the PARP-1 gene in Huh-1 cells decreased the transcription of P-2985 vector (Fig. 4b). We also found that the PARP-1 gene knockdown actually decreased the production of DCP in Huh-1 cells (Fig. 4c). There are 3 mechanisms by which PARP-1 has been proposed to activate transcription; (1) binding directly to the DNA sequence in the promoter/enhancer region; (2) binding to another transcription factor as a coactivator; and (3) modulating chromatin structure via a PARylation reaction [30]. We identified a TTCAA(T) sequence at –2983 to –2979 in the 31-bp sequence, which is similar to the PARP-1 consensus sequence TTCAAA [31]. Therefore, it is possible that PARP-1 directly binds to TTCAA(T) to activate prothrombin gene transcription, although the other 2 possible mechanisms cannot be ruled out and further examination is warranted.

PARP-1 is a ubiquitous nuclear enzyme that is involved in cellular processes including DNA repair, apoptosis, gene expression, genomic stability, and cellular division [32]. PARP-1 plays an important role in repairing DNA single-strand breaks, and its inhibition causes an

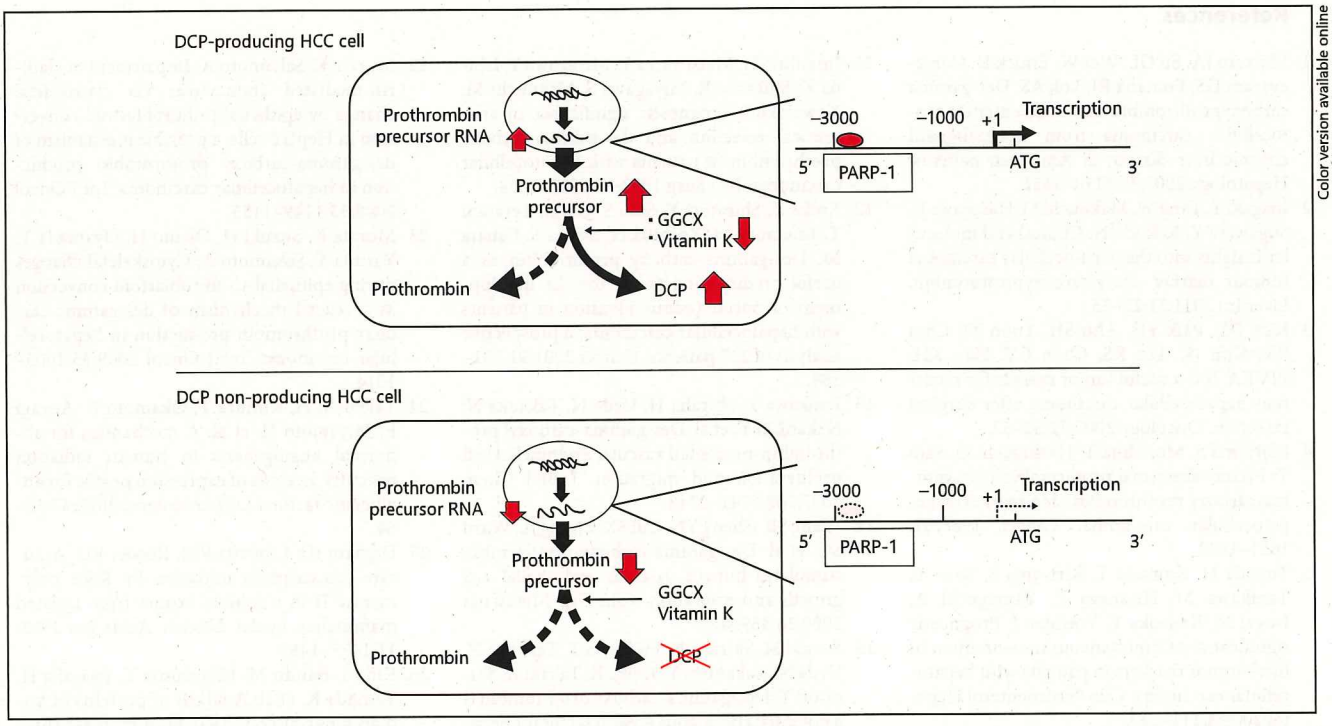

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Fig. 7. Schema of the mechanism of des-gamma-carboxy prothrombin (DCP) production in hepatocellular carcinoma. Poly-(ADP-ribose) polymerase-1 directly binds to the promoter region of the prothrombin gene and strongly promotes the prothrombin gene transcription. Excessive prothrombin precursor was translated and exhausted intercellular vitamin K and GGCX, thereby resulting in DCP production. On the other hand, a low amount of prothrombin precursor due to lower transcription does not exhaust them, thereby leading no DCP production in DCP non-producing cells.

accumulation of single-strand breaks, leading to cell cycle arrest and cell death. Thus, PARP-1 has received attention as a target for cancer therapy. It has been reported that PARP-1 is over expressed in some HCC cases [33]. We also analyzed PARP-1 mRNA expression levels in HCC tissues and clinicopathological factors, and found a significantly higher DCP concentration and a frequent tendency toward poor prognostic gross type in the PARP-1 high group than in the PARP-low group (online suppl. Table 3). Moreover, our data of this study indicate that PARP-1 inhibition directly suppresses prothrombin mRNA transcription and subsequently DCP production, possibly leading to the inhibition of cell proliferation, invasion and subsequent metastasis. This theory is consistent with the findings that a PARP-1 inhibitor was much more active in DCP-producing cells as compared with non-DCP producing cells. Thus, DCP may be a useful predictive biomarker for PARP-1 inhibitor in the treatment of HCC.

In conclusion, our study provided the first evidence that DCP is produced by excessive prothrombin gene transcription via PARP-1 in HCC cells. PARP-1 promotes the transcription of the prothrombin gene by binding to its upstream region in DCP-producing cells. PARP-1 inhibitors may be very effective for the treatment of DCP-producing HCCs, which are considered to be a poor prognostic subgroup of HCCs.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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