Original Paper

Impact of Preferentially Expressed Antigen of Melanoma on the Prognosis of Hepatocellular Carcinoma

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Key Words

Corepressor · Hepatocellular carcinoma · Preferentially expressed antigen of melanoma · Prognosis · Retinoic acid

Abstract

Background: Retinoids, vitamin A and its derivatives, have an antitumor effect on hepatocellular carcinoma (HCC). The function of retinoids is exerted by the complex of retinoic acid (RA) with the heterodimer of retinoid X receptor and the RA receptor. The preferentially expressed antigen of melanoma (PRAME) acts as a dominant repressor of RA signaling by binding to the complex. The significance of PRAME on the prognosis of HCC remains to be clarified. **Methods:** PRAME mRNA expression was examined by quantitative real-time polymerase chain reaction in both tumor and non-tumor tissues of 100 HCC patients who received surgical resection. The effect of PRAME knockdown on DR5-mediated RA transcriptional activity was examined. **Results:** In tumor tissues, there were significant associations among PRAME expression, clinical stage, tumor markers, and tumor numbers. In non-tumor tissues, there were significant associations among PRAME expression, overall survival, and disease-free survival. The knockdown of PRAME caused no reduction in DR5-mediated transcriptional activity of RA, suggesting that PRAME acts via other mechanisms than the DR5 RA-responsive elements. **Conclusion:** Our findings indicate that PRAME expression is a novel prognostic marker in HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death and is reportedly increasing worldwide [1]. The prognosis of HCC patients has been improved due to the progress of locoregional treatments such as tumor ablation and transcatheter arterial chemoembolization [2]. Since the biological features of HCC result in high rates of secondary occurrence of HCC, some interventions which decrease its malignant potential seem to be required.

Retinoids, vitamin A and its derivatives, play an important role in many cell types. Retinoic acids (RAs), metabolites of retinoids, exert a variety of actions through their binding to the heterodimer of RA receptor (RAR) and the retinoid X receptor (RXR). Since retinoids have tumor-suppressive activity, defects in RA signaling are implicated in cancers [3, 4]. Indeed, many epidemiological studies have reported that vitamin A acts as an inhibitor of carcinogenesis in several cancers [5–8]. The transgenic mice expressing RAR α dominant negative form in hepatocytes developed steatohepatitis and liver tumors, and RAs regulate iron metabolism, disorders of which cause reactive oxygen species [9, 10]. Twenty-six RA-responsive genes were identified by means of in silico analysis using RA-responsive element (RARE) [11]. Of these 26 genes, downregulation of the *OTU domain-containing 7B* (*OTUD7B*) gene, which was upregulated by all-trans RA (ATRA), in tumor tissue was associated with a low cancer-specific survival of HCC patients. Indeed, *OTUD7B* negatively regulates NF- κ B signaling and decreases the survival of HCC cells.

The preferentially expressed antigen of melanoma (PRAME) was first described as an antigen in human melanoma [12]. PRAME is overexpressed in a variety of other human cancers [13–16]. PRAME binds to RAR in the presence of RA, preventing ligand-induced receptor activation and target gene transcription [17]. PRAME is a dominant repressor of RAR signaling. Expression of PRAME serves as a poor prognostic marker in many cancers [18–21]. Consequently, the knockdown of PRAME is expected to be an interesting option for the development of new therapeutic strategies for cancer [22]. Since the clinical significance of PRAME expression in HCC is totally unknown, the aim of the current study was to clarify the significance of PRAME in HCC patients.

Patients and Methods

Patients and Characteristics

One hundred HCC patients who underwent surgical resection from 1998 to 2008 at Kyoto University Hospital were enrolled in this study. This study was approved by the ethics committees of Kyoto University Hospital and Tottori University Hospital. All patients gave written informed consent for the sample collection and analyses described in the present study in agreement with the Declaration of Helsinki. HCC tissues and their adjacent liver tissues were taken from the patients. The clinicopathological data of the patients are summarized in table 1. The clinical stage of HCC was assessed by the Japanese Tumor-Node-Metastasis (TNM) staging system for primary liver cancer [23].

RNA Isolation and Gene Expression Analysis

RNA isolation and gene expression analysis were performed as previously described [9]. The mRNA expression level was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using the LightCycler 480 II detection system (Roche Diagnostics GmbH, Mannheim, Germany) and the LightCycler 480 SYBR Green I Master as the detection dye. Primers to detect PRAME were 5'-TAT CGC CCA GTT CAC CTC TC-3' and 5'-GGG ACT TAC ATC GGT CAG CA-3'. Primers to detect β -actin, a housekeeping gene, were 5'-CAC TCT TCC AGC CTT CCT TCC-3' and 5'-CGT ACA GGT CTT TGC GGA TGT C-3'. Each sample was standardized by β -actin and run in duplicate. Sample amplification for PRAME included a hot start (95°C, 5 min) followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 5 s, and extension at 72°C for 10 s. Sample

Table 1. Clinicopathological data of the HCC patients

Age, years (mean ± SD)	64.6 ± 10.6
Male:female ratio	70:30
Virus marker (HBsAg+/HCVAb+/both+/negative)	16/61/1/22
Liver cirrhosis (–/+/unknown)	49/34/17
Clinical stage (I/II/III/IVa/IVb)	5/52/24/16/3
Median tumor marker (range)	
AFP, ng/ml	26.8 (<3–639,256)
DCP, mAU/ml	159 (0.02–30,147)
Degree of differentiation (high/moderate/low/unknown)	17/57/24/2
Median tumor size, cm (range)	3.5 (1–17)
Tumor number (single/multiple)	69/31
Tumor factor (T1/T2/T3/T4)	5/52/26/17
PRAME gene expression	
Tumor tissue	27 (27%)
Non-tumor tissue	10 (10%)

amplification for β -actin included a hot start followed by 45 cycles of denaturation at 95°C for 1 s, annealing at 56°C for 5 s, and extension at 72°C for 4 s.

Small Interfering RNA Experiments

Specific small interfering RNAs (siRNAs) for PRAME and a scrambled negative control were purchased from Invitrogen (Tokyo, Japan). siRNA duplex and LipofectAMINE2000 reagent (Life Technologies, Carlsbad, Calif., USA) were mixed in OPTI-MEM Reduced Serum Medium (Life Technologies) to form a siRNA-LipofectAMINE2000 complex. The complex was then added to the culture medium and incubated for 24 h. PRAME mRNA levels were determined by qRT-PCR using β -actin as an internal control. Protein expression of PRAME was determined by Western blotting using polyclonal antibody against human PRAME (HPA045153) (ATLAS Antibodies, Stockholm, Sweden).

Luciferase Reporter Assay

Cells were transiently transfected with luciferase reporter vectors using LipofectAMINE2000. DR5-mediated transcriptional activity was measured using the firefly luciferase gene (Clontech, Takara Bio Inc., Kusatsu, Japan) downstream of the consensus RARE (pDR5-FLuc). The DR5 reporter vector was co-transfected with the pTK-RLuc (Promega Corp., Fitchburg, Wis., USA) to normalize transfection efficiency. Luciferase activity in the cell lysates was measured using the Dual Luciferase Reporter Assay Kit (Promega Corp.).

Statistical Analysis

All statistical analyses for clinical samples were performed using SPSS Ver.23 statistics software (IBM, Armonk, N.Y., USA). To examine associations between patient characteristics and PRAME expression, Mann-Whitney U tests were used for age and tumor marker, or Pearson's χ^2 tests for gender, clinical stage, tumor differentiation, tumor factor, tumor number and tumor recurrence. Kaplan-Meier survival curves and the log-rank test were used to compare the overall survival (OS) and disease-free survival (DFS) of patients. For in vitro experiments, Student's t test was used. A p value <0.05 was considered to be significant.

Results

Clinicopathological Data of the HCC Patients

The patients included 70 males and 30 females, and they were 64.6 ± 10.6 years old (table 1). The clinical stages of HCC were 5 patients in stage I, 52 in stage II, 24 in stage III, 16 in stage IVa and 3 in stage IVb. The degrees of differentiation of HCC were high in 17, moderate in 57, and low in 24 patients. The median tumor diameter was 3.5 cm. Sixty-nine patients had single tumor and 31 patients multiple tumors.

Table 2. Differences in clinicopathological findings between PRAME-positive and PRAME-negative patients

	PRAME-positive (n = 27)	PRAME-negative (n = 73)	p value
Age, years (mean ± SD)	62.5±9.7	65.4±10.9	0.122
Male:female ratio	14:13	56:17	0.016
Clinical stage (I/II/III/IVa/IVb)	3/7/7/9/1	2/45/17/7/2	0.005
Median tumor marker (range)			
AFP, ng/ml	1,753 (5.6–639256)	19.5 (3–107,579)	<0.001
DCP, mAU/ml	885 (13–30,147)	124.5 (0.02–12,100)	0.02
Degree of differentiation (high/moderate/low/unknown)	2/15/8/2	15/42/16/0	0.289
Tumor number (single/multiple)	13/14	56/17	0.006
Tumor factor (T1/T2/T3/T4)	3/7/7/10	2/45/19/7	0.001
Recurrence (–/+/unknown)	23/4/0	17/55/1	0.340

Differences in Clinicopathological Findings between PRAME-Positive and PRAME-Negative HCC Patients

The differences between PRAME-positive and PRAME-negative HCC patients are shown in table 2. The rate of males in PRAME-positive HCC patients was higher than that in PRAME-negative HCC patients ($p = 0.016$). PRAME-positive HCC patients tended to have advanced clinical stage ($p = 0.005$). The levels of tumor markers such as AFP and DCP in PRAME-positive HCC patients were significantly higher than those in PRAME-negative HCC patients ($p < 0.001$ and $p = 0.02$, respectively). The tumor number in PRAME-positive HCC patients was greater than that in PRAME-negative HCC patients ($p = 0.006$). The tumor factor in PRAME-positive HCC patients was higher than that in PRAME-negative HCC patients ($p = 0.001$). These data suggest that PRAME-positive HCC patients were in more advanced stages than PRAME-negative HCC patients.

PRAME Expression and Survival Period

The OS of PRAME-negative tumor-bearing patients did not differ from that of PRAME-positive tumor-bearing patients (fig. 1a). The DFS was also different between PRAME-negative tumor-bearing patients and PRAME-positive tumor-bearing patients (fig. 1b). However, the OS of patients with PRAME-negative non-tumor tissue was significantly longer than that of those with PRAME-positive non-tumor tissue; the median OS of patients with PRAME-negative non-tumor tissue was over 2,000 days while that of patients with PRAME-positive non-tumor tissue was 442 days ($p = 0.0001$, fig. 1c). The DFS of patients with PRAME-negative non-tumor tissue was also significantly longer than that of those with PRAME-positive non-tumor tissue; the median DFS of patients with PRAME-negative non-tumor tissue was 551 days while that of those with PRAME-positive non-tumor tissue was 145 days ($p = 0.0025$, fig. 1d). These data suggest that the PRAME-positive HCC patients were in more advanced stages than the PRAME-negative HCC patients. On the other hand, the patients with PRAME-positive non-tumor tissue had shorter OS and DFS than the patients with PRAME-negative non-tumor tissue.

Small Interfering RNA Experiments and Luciferase Reporter Assay

To clarify the biological significance of PRAME positivity in tumor tissue, reporter gene analysis of DR5-RARE-dependent transcriptional activity was performed in PRAME-positive HLF cells (fig. 2). The capability of knockdown of PRAME mRNA was confirmed by three siRNAs targeting different sequences of PRAME mRNA (PRAME-1 to PRAME-3). Although only PRAME-1 and PRAME-3 siRNA suppressed PRAME mRNA levels, all three siRNAs

Fig. 1. Relations between survival periods and PRAME expression in tumor and non-tumor tissues. **a** OS of PRAME-positive tumor-bearing patients versus PRAME-negative tumor-bearing patients. Log-rank test: $p = 0.2870$. **b** DFS of PRAME-positive tumor-bearing patients versus PRAME-negative tumor-bearing patients. Log-rank test: $p = 0.1293$. **c** OS of PRAME-positive non-tumor-bearing patients versus PRAME-negative non-tumor-bearing patients. Log-rank test: $p = 0.0001$. **d** DFS of PRAME-positive non-tumor-bearing patients versus PRAME-negative non-tumor-bearing patients. Log-rank test: $p = 0.0025$.

suppressed PRAME protein expression at 24 h after transfection (fig. 2a). To clarify the down-regulation of PRAME expression on RA signal, HLF cells were co-transfected with siRNA, DR5-FLuc and pTK-RLuc with or without 5 μ M ATRA for 6 and 24 h, respectively (fig. 2b). However, the RA signal was not affected by downregulation by knockdown of PRAME. These data suggest that PRAME expression does not occur in a DR5-RARE-dependent manner.

Discussion

Although the clinical significance of PRAME expression in HCC has been unknown so far, this is the first report that PRAME expression in HCC and non-HCC tissues has clinical implications. These findings are in agreement with a previous report that PRAME acts as a tran-

Fig. 2. Reporter assay of RARE-dependent transcriptional activity in PRAME-knocked-down cells.

a In the upper side, validation of siRNA-mediated PRAME knock-down was shown in PRAME-positive HLF cells. Three siRNAs (PRAME-1 to PRAME-3) targeting different sequences of PRAME mRNA and scrambled control siRNA were transfected into HLF cells. In the lower side, PRAME protein expression was determined 24 h after transfection. * $p < 0.05$ versus control siRNA.

b Cells were co-transfected with siRNA (control or PRAME-3), DR5-FLuc and pTK-RLuc for 24 h in 1% FBS DMEM. Subsequently, cells were treated with 0.1% DMSO or 5 μ M ATRA for 6 and 24 h. Reporter activity was expressed as the FLuc/RLuc ratio and the values are shown in relative activity to those of DMSO as 1.0.

scriptional corepressor of RAR and contributes to cancer development by antagonizing RAR signaling [17]. The RARE-positive HCC patients were in more advanced clinical stages than the RARE-negative HCC patients. In addition, the OS and DFS in the patients with RARE-negative non-HCC tissues were significantly longer than in those with RARE-negative non-HCC tissues. These data suggest that RARE probably suppressed RA signals, leading to cancer development and progression. In the present study, the OS and DFS in the RARE-positive HCC patients did not differ from those in the RARE-negative HCC patients. Since it has been reported that the DFS after complete resection of HCC depends on the presence of liver cirrhosis and co-existing hepatitis [24, 25], PRAME expression in individual HCC per se may not affect the patients' OS and DFS. Since PRAME activation requires the recruitment of the polycomb protein enhancer of zeste homolog 2 (EXH2) [17, 26], complex formation of PRAME with EXH2 will have to be confirmed.

In the present study, downregulation of PRAME did not cause upregulation of the RA signal in PRAME-positive HLF HCC cells. Judging from the advanced clinical stages of the patients with PRAME-positive HCC tissue, PRAME presumably functioned as a repressor of RA signal even in our patients. The classical retinoid response element of a target gene is a direct repeat of the motif 5'-PuG(G/T)TCA-3' spaced by 1, 2 or 5 base pairs (DR1, DR2 and DR5, respectively) [27]. The DR2 and DR5 elements preferentially bind RXR/RAR heterodimer with RXR monomer binding the 5' motif, and other non-genomic mechanisms are involved in regulation of RA signal [28]. The other DR2 RARE or non-genomic mechanisms may be involved in RA signal in HCC.

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Disclosure Statement

The authors declare that no competing interests exist.

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