Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Biological and clinical implications of retinoic acid-responsive genes in human hepatocellular carcinoma cells

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Background & Aims: Accumulating data from epidemiological and experimental studies have suggested that retinoids, which are vitamin A derivatives, exert antitumor activity in various organs. We performed a gene screening based on *in silico* analysis of retinoic acid response elements (RAREs) to identify the genes facilitating the antitumor activity of retinoic acid (RA) and investigated their clinical significance in hepatocellular carcinoma (HCC).

Methods: *In silico* analysis of RAREs was performed in the 5-kb upstream region of EST clusters. Chromatin immunoprecipitation analysis of the retinoic acid receptors and gene expression analysis were performed in HuH7, HepG2, and MCF7 cells treated with all-trans RA (ATRA). mRNA expression of RA-responsive genes was investigated using tumor and non-tumor tissues of clinical HCC samples from 171 patients. The association between gene expression and survival of patients was examined by Cox regression analysis.

Results: We identified 201 candidate genes with promoter regions containing consensus RARE and finally selected 26 RA-responsive genes. Of these, downregulation of OTU domain-containing 7B (*OTUD7B*) gene, which was upregulated by ATRA, in tumor tissue was associated with a low cancer-specific survival of HCC patients. Functional analyses revealed that OTUD7B negatively regulates nuclear factor κB (NF-κB) signaling and decreases the survival of HCC cells.

Conclusions: We identified RA-responsive genes which are regulated by retinoid signal and found that low-*OTUD7B* mRNA expression is associated with a poor prognosis for HCC patients. OTUD7B-mediated inhibition of NF-κB signaling may be an effective target for antitumor therapy for HCC.

Keywords: Hepatocellular carcinoma; Retinoid; Chemoprevention.
Received 9 November 2012; received in revised form 20 June 2013; accepted 24 June 2013; available online 2 July 2013

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Abbreviations: HCC, hepatocellular carcinoma; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; ChIP, chromatin immunoprecipitation; EST, expression sequence tag; ATRA, all-trans retinoic acid; RT-PCR, reverse transcription-polymerase chain reaction; DR5, direct repeat 5; TRRA, target RNA of retinoic acid; CYP26A1, cytochrome P450 family 26A1; OTUD7B, OTU domain-containing 7B; NF-κB, nuclear factor kappa B; siRNA, small-interfering RNA; RXR, retinoid X receptor.

Introduction

Hepatocellular carcinoma (HCC), a common and fatal malignancy, is increasing in incidence in developed countries. HCC generally arises in cirrhotic livers and has a poor prognosis because of the high incidence of disease recurrence after curative treatment [1]. To improve patient prognosis, identification of new targets for early detection, chemoprevention, and treatment is urgently required.

Accumulating data from epidemiological and experimental studies have suggested that retinoids, which are vitamin A derivatives, prevent cancer in various organs including the stomach, breast, lung, prostate, and liver [2–7]. HCC risk is 7-fold higher in hepatitis B surface antigen-positive persons with low serum retinol concentrations than in those with high concentrations [6]. In the pathogenesis of chronic liver disease, activation of hepatic stellate cells accompanied by decreasing vitamin A storage has been observed, leading to suggest that the loss of retinoid content is closely associated with the progression of chronic liver diseases including HCC [8–10].

Retinoic acid (RA), a physiologically active form of retinoid, and its retinoic acid receptor (RAR) exert their effects by regulating downstream gene expression in an RA response element (RARE)-dependent manner, namely via retinoid signaling [11]. In a previous study, we observed steatohepatitis and HCC development in transgenic mice that express a dominant-negative

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form of RAR in a liver-specific manner [12]. We found that impaired retinoid signaling in the liver resulted in decreased fatty acid β-oxidation and increased iron accumulation and oxidative stress [12,13]. These findings indicate that identifying genes regulated by retinoid signaling is important for the understanding of the molecular mechanisms of liver disease leading to hepatocarcinogenesis.

Although microarray analysis has identified a wide range of RA-responsive genes [14–16], whether this regulation is direct or indirect is uncertain [17]. Moreover, DNA chips display only a subset of genes transcribed from the genome, leaving open the possibility that unknown transcriptional regions may exist [17]. To overcome such limitations, it is important to establish whether functional RAREs are present in the promoters of transcriptional regions regulated by retinoid signaling. In this study, we referred to previous studies that investigated the response elements and target genes for transcription factors such as p53 [18], estrogen receptor [19,20], vitamin D receptor [21], and RAR [22], and developed a strategy of gene screening based on *in silico* analysis of RARE, chromatin immunoprecipitation (ChIP) analysis of RAR, and gene expression analysis. Moreover, we performed gene expression analysis using clinical HCC samples to investigate whether the RA-responsive genes identified in this study affect the prognosis of HCC patients.

Materials and methods

In silico analysis

The human expression sequence tag (EST) cluster table (rnaCluster.txt.gz), which contains data regarding the genomic locus of clustered ESTs and mRNA, was downloaded from the genome database of the University of California Santa Cruz, California (UCSC; http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/). A total of 36,341 clusters were mapped to the human genome database (hg18, 2006) and the upstream 5-kb sequences from the 5′-terminal end (transcription start site) of clusters were collected as a promoter region of the genes. In the first step of the screening, consensus RARE DR5, a 5-bp-spaced direct repeat motif of RGKTCA (R = A/G, K = G/T) was searched in these promoter sequences. *In silico* analysis was performed by *In Silico* Biology, Inc. (Yokohama, Japan).

ChIP and quantitative polymerase chain reaction (PCR)

ChIP assays were performed as described in the Supplementary Data. The amount of RARE-containing DNA fragments immunoprecipitated by ChIP was measured by quantitative PCR analysis using specific primers (Supplementary Table 1) for the fragments.

Gene expression analysis

RNA isolation and quantitative RT-PCR analysis using gene-specific primers (Supplementary Table 2) were performed as described in the Supplementary Data.

Patient specimens

Gene expression analysis in human HCC specimens was performed as described in Supplementary Data. Clinicopathological data for the patients are summarized in Supplementary Table 3.

Statistical analysis

Statistical comparisons for *in vitro* experiments were performed using Student's *t*-test. $p < 0.05$ was considered statistically significant. All statistical analyses for clinical samples were performed using PASW statistics 18 software as described in Supplementary Data.

Additional Materials and methods are described in Supplementary Data.

Results

In silico analysis of RAREs identifies candidate RA-responsive genes

To identify RA-responsive genes directly regulated in a RARE-dependent manner, we established a screening strategy using a dataset of EST clusters and an *in silico* approach focusing on RAREs (Fig. 1). In the first step of the screening, we searched consensus RARE DR5 in upstream 5-kb sequences from the transcription start site of clusters. We identified 201 EST clusters containing the DR5 motif in their promoter regions as candidate genes controlled by RA, which we hereafter refer to as target RNA of retinoic acid (TRRA). Schematic illustration of the genomic structure of TRRA-99, which encodes cytochrome P450 26A1 (*CYP26A1*), a known RA-responsive gene, is shown as an example of TRRAs (Supplementary Fig. 1). Among the 201 TRRAs that we identified, 111 were RefSeq genes identified in the UCSC genome database (i.e., were known genes); the remaining 90 genes were unknown (Supplementary Table 4).

ChIP-quantitative PCR analysis identifies RAR-bound functional RAREs

In the second step of the screening, we examined the molecular binding of TRRAs to RAR to identify functional RAREs. To exclude possible artifacts resulting from cell death, we used 5 μM ATRA,

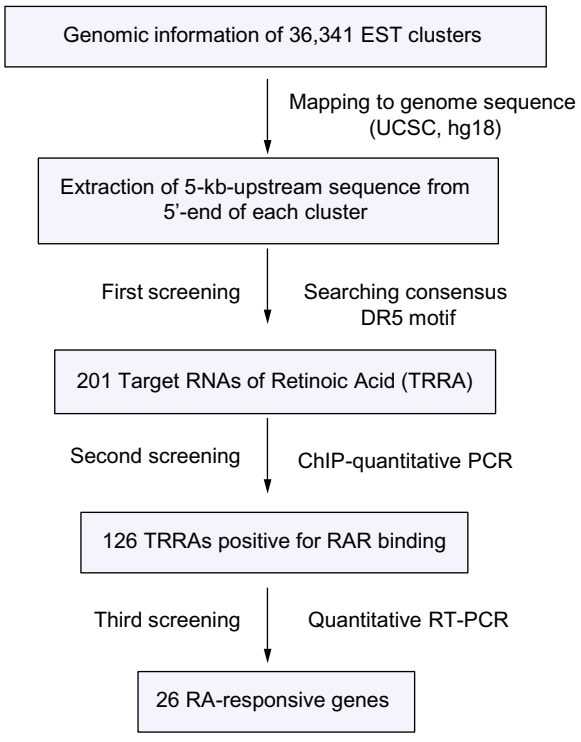

Fig. 1. Screening strategy to identify RA-responsive genes. Genomic information of EST clusters was mapped to the human genome sequence to extract the upstream 5-kb sequence from the 5′-end (transcription start site) of each cluster. In the first screening, consensus DR5 motif was searched in the 5-kb sequence to identify TRRAs. In the second screening, ChIP-quantitative PCR analysis was performed to examine the RAR binding to DR5. In the third screening, quantitative RT-PCR analysis after ATRA treatment was performed to identify RA-responsive genes.