

Overexpression of the androgen receptor in human hepatoma cells and its effect on fatty acid metabolism

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Received February 6, 2016; Accepted February 3, 2017

DOI: 10.3892/ol.2017.5973

Abstract. Hepatocellular carcinoma (HCC) is a predominantly male disease in which the androgen receptor (AR) serves an important pathogenic role in hepatocarcinogenesis. Fatty acid metabolism also contributes to hepatocarcinogenesis and is associated with the prognosis of cancer. The present study aimed to investigate the effects of the AR on fatty acid metabolism-associated gene expression in human hepatoma cell lines. AR-expression plasmids or control plasmids were transiently transfected into the human HCC cell lines Huh7 and HepG2. After 48 h, cellular protein and RNA were extracted and the expression of AR was confirmed by western blotting. Complementary DNA was synthesized and subjected to a quantitative polymerase chain reaction-based array to examine the expression of 84 fatty acid metabolism-associated genes. Overexpression of AR significantly downregulated the expression of 11 fatty acid metabolism-associated genes in Huh7 cells and 35 in HepG2 cells. The overexpression of AR also resulted in the upregulation of 6 fatty acid metabolism genes in HepG2 cells; however, it had no effect in Huh7 cells. Acyl-coenzyme A (CoA) thioesterase 7 and acyl-CoA oxidase 3 were downregulated in both cell lines. In conclusion, upregulation of AR via overexpression led to the disturbance of fatty acid metabolism-associated gene expression in human HCC cells. Therefore, the AR may serve a role in hepatocarcinogenesis via the regulation of hepatocellular fatty acid metabolism.

Introduction

Steroid sex hormones serve major roles as tumor promoters by promoting cell cycle progression in several types of cancer, including hepatocellular carcinoma (HCC) (1-3). The sex

hormone androgen and its associated androgen receptor (AR) are involved in the development of the lipogenic phenotype of cancer cells via the induction of proteins and enzymes, including fatty acid synthase (FASN), required for their autonomous nutrition (4).

A number of studies have implicated AR, a member of the nuclear steroid receptor superfamily, in hepatocarcinogenesis (1,3,5-7). AR is a transcription factor activated via ligand-dependent and ligand-independent mechanisms (8). Cytochrome P450, transforming growth factor β 1, vascular endothelial growth factor A and heat shock protein family A member 5 are target genes of ARs in the liver (3,7,9,10). Crosstalk between ARs and liver X receptor (nuclear receptor subfamily 1 group H member 2) has also been implicated in maintaining cholesterol homeostasis (11). It has also been demonstrated that the activity of ARs is coupled with the upregulation of FASN (4).

Abnormalities in cellular metabolism are closely associated with HCC occurrence and development (4,12) and it has been demonstrated that FASN is highly expressed in a number of cancer types, including HCC (12). In HCC patients, abnormalities in plasma phospholipid fatty acid profiles are occasionally observed, which may be attributed to the alteration of intrinsic fatty acid metabolism caused by the cancer itself (13). Dietary fatty acids may be implicated in the risk of developing non-viral hepatitis-associated HCC (14); however, there is data to refute this hypothesis (13).

Fatty acid metabolism also serves a role in human carcinogenesis and is associated with a poor prognosis in HCC patients. The present study aimed to investigate the effects of AR expression on fatty acid metabolism-associated gene regulation in human HCC cell lines.

Materials and methods

Cell culture. The human HCC cell lines HepG2 and Huh7, as described previously (3), were purchased from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and grown at 37°C with 5% CO₂.

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Key words: androgen receptor, fatty acid, liver cancer

Transfection and RNA extraction. A total of 4×10^5 cells per well were placed in 6-well plates 24 h prior to transfection. The cells were transfected with 0.4 μg human AR protein-expressing plasmid (pSG5-AR) or 0.4 μg control vectors, which were kindly provided by Professor J.T. Isaacs of The Johns Hopkins University School of Medicine (Baltimore, MD, USA) (15), using Effectene Transfection Reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. At 48 h post-transfection, cellular RNA was collected using RNeasy (Qiagen GmbH), and RNA was stored at -80°C until use. RNA quality was measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction (qPCR) arrays. cDNA synthesis was performed by an RT² First Strand kit (Qiagen GmbH) using 1 μg of RNA per reaction. The cDNA synthesis reaction was incubated at 42°C for 15 min and then halted by heating at 95°C for 5 min. A human fatty acid metabolism PCR array (no. PAHS-007Z; Qiagen GmbH) was carried out according to the manufacturer's protocol. Briefly, amplification of cDNA was monitored with SYBR-Green by quantitative PCR analysis. PCR was performed in 25 μl of ROX PCR Master mix (Qiagen GmbH) containing primers and 1 μl of the reverse-transcription reaction mixture, using a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR reaction was performed as follows: 95°C for 1 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The data analysis was performed using the comparative threshold cycle method (16) and analyzed using PCR Array Data Analysis Software v2.1 (<http://www.sabiosciences.com/pcrarraydataanalysis.php>).

Western blot analysis. Cell lysates were prepared 48 h post transfection in 1X SDS sample buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Protein concentration was determined using the Bradford method. Cell lysate proteins (5 $\mu\text{g}/\text{well}$) were separated by 5-20% SDS-PAGE and transferred onto a nitrocellulose membrane (ATTO Corp., Tokyo, Japan). AR expression was analyzed by western blotting by incubating membranes with rabbit polyclonal antibodies against AR (no. sc-816; dilution, 1:1,000) or GAPDH (no. sc-25778; dilution, 1:2,000) (both from Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C for 16 h. Membranes were then incubated with anti-rabbit IgG HRP-linked secondary antibody (no. 7074; dilution, 1:3,500; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. Proteins were visualized using an enhanced chemiluminescence western blotting substrate (GE Healthcare Life Sciences, Chalfont, UK) and scanned with an image analyzer (LAS-4000) and Image Gauge software (version 3.1) (both from Fujifilm, Tokyo, Japan). The relative quantity of protein was determined by normalizing to GAPDH.

Statistical analysis. For statistical analysis, two-tailed Student's *t*-tests were performed with DA Stats software version PAF01644 (NIFTY Corp.; Fujitsu). $P < 0.05$ was considered to indicate a statistically significant difference.



Figure 1. Overexpression of AR in HepG2 cells. Western blot analysis of AR and GAPDH expression level in HepG2 cells transiently transfected with the AR-expressing plasmid, pSG5-AR, or a control vector. AR, androgen receptor.

Results

Overexpression of AR in human HCC cells. Overexpression of AR was induced in HepG2 and Huh7 cells to validate hypotheses of the effect of AR on the regulation of fatty acid metabolism-associated gene expression. In HepG2 cells transiently transfected with pSG5-AR, overexpression of AR was confirmed by western blot analysis (Fig. 1), and AR overexpression in Huh7 cells transiently transfected with pSG5-AR was demonstrated by western blotting in a previous study (3).

Downregulated fatty acid metabolism-associated genes in AR-overexpressing HepG2 cells. Fatty acid metabolism-associated gene expression profiles were examined using a qPCR-based array. Expression levels between HepG2 cells transiently overexpressing AR and control vector-transfected HepG2 cells were compared. In HepG2 cells, AR overexpression significantly downregulated the expression of 35 fatty acid metabolism-associated genes, including 6 acyl-coenzyme A (CoA) dehydrogenases [acyl-CoA dehydrogenase family member (ACAD)10, acyl-CoA dehydrogenase, very long chain, acyl-CoA dehydrogenase, C-2 to C-3 short chain (ACADS), ACAD9, ACADS branched (B) and enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH)], 2 acyl-CoA oxidases [acyl-CoA oxidase (ACOX) 1 and ACOX3], 4 acyl-CoA synthetases [acyl-CoA synthetase long-chain family member (ACSL)1, acyl-CoA synthetase bubblegum family member (ACSBG)2, ACSBG1 and ACSL4], 4 acyl-CoA thioesterases [acyl-CoA thioesterase (ACOT)8, ACOT1, ACOT2 and ACOT7], 4 carnitine transferases [carnitine palmitoyltransferase (CPT)2, carnitine O-octanoyltransferase, carnitine O-acetyltransferase, CPT1B], 5 other fatty acid metabolism-associated genes [aldehyde dehydrogenase 2 family (mitochondrial) (ALDH2), 2,4-dienoyl CoA reductase 2, peroxisomal, enoyl CoA hydratase, short chain, 1, mitochondrial, enoyl-CoA δ isomerase 2 and methylmalonyl CoA mutase], 2 genes of fatty acid transport [CPT1A and solute carrier family 27 (fatty acid transporter), member 4], 4 genes of fatty acid biosynthesis regulation [protein kinase, cAMP-dependent, catalytic, α (PRKACA), protein kinase, AMP-activated, α 2 catalytic subunit, protein kinase, AMP-activated, γ 1 non-catalytic subunit and protein kinase, AMP-activated, α 1 catalytic

Table I. Fatty acid metabolism-associated genes are significantly downregulated in androgen receptor-overexpressing HepG2 cells examined by polymerase chain reaction arrays.

Symbol	Description	Fold change (vs. control)	P-value
GPD1	Glycerol-3-phosphatase dehydrogenase 1 (soluble)	0.40	0.0259
ACAD10	Acyl-CoA dehydrogenase family, member 10	0.44	0.0135
LIPE	Lipase, hormone-sensitive	0.50	0.0099
ACADVL	Acyl-CoA dehydrogenase, very long chain	0.54	0.0018
CPT2	Carnitine palmitoyltransferase 2	0.54	0.0181
CROT	Carnitine O-octanoyltransferase	0.54	0.0069
CPT1A	Carnitine palmitoyltransferase 1A (liver)	0.57	0.0039
ACSL1	Acyl-CoA synthetase long-chain family member 1	0.57	0.0375
ACSBG2	Acyl-CoA synthetase bubblegum family member 2	0.58	0.0096
ACADS	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	0.58	0.0448
ACAD9	Acyl-CoA dehydrogenase family, member 9	0.59	0.0177
ACADSB	Acyl-CoA dehydrogenase, short/branched chain	0.60	0.0475
ACOX1	Acyl-CoA oxidase 1, palmitoyl	0.60	0.0020
ACOX3	Acyl-CoA oxidase 3, pristanoyl	0.60	0.0199
PRKACA	Protein kinase, cAMP-dependent, catalytic, α	0.61	0.0012
PRKAA2	Protein kinase, AMP-activated, α 2 catalytic subunit	0.61	0.0347
SLC27A4	Solute carrier family 27 (fatty acid transporter), member 4	0.61	0.0428
BDH1	3-hydroxybutyrate dehydrogenase, type 1	0.61	0.0130
ACSBG1	Acyl-CoA synthetase bubblegum family member 1	0.61	0.0233
EHHADH	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	0.62	0.0212
ACSL4	Acyl-CoA synthetase long-chain family member 4	0.63	0.0341
ACOT8	Acyl-CoA thioesterase 8	0.63	0.0191
ACOT1	Acyl-CoA thioesterase 1	0.64	0.0017
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	0.64	0.0088
PRKAG1	Protein kinase, AMP-activated, γ 1 non-catalytic subunit	0.64	0.0321
CRAT	Carnitine O-acetyltransferase	0.66	0.0002
DECR2	2,4-dienoyl CoA reductase 2, peroxisomal	0.66	0.0412
ECHS1	Enoyl CoA hydratase, short chain, 1, mitochondrial	0.69	0.0368
ECI2	Enoyl-CoA δ isomerase 2	0.70	0.0347
GK	Glycerol kinase	0.72	0.0049
ACOT2	Acyl-CoA thioesterase 2	0.73	0.0252
CPT1B	Carnitine palmitoyltransferase 1B (muscle)	0.73	0.0038
PRKAA1	Protein kinase, AMP-activated, α 1 catalytic subunit	0.76	0.0409
MUT	Methylmalonyl CoA mutase	0.81	0.0453
ACOT7	Acyl-CoA thioesterase 7	0.81	0.0098

CoA, coenzyme A.

subunit], 1 gene of ketogenesis and ketone body metabolism (3-hydroxybutyrate dehydrogenase, type 1) and 3 genes of triacylglycerol metabolism [glycerol-3-phosphatase dehydrogenase 1 (soluble), lipase, hormone-sensitive and glycerol kinase; Table I].

Upregulated fatty acid metabolism-associated genes in AR-overexpressing HepG2 cells. In HepG2 cells, AR significantly upregulated the expression of 6 fatty acid metabolism-associated genes (Table II), including 3 fatty acid transports [Solute carrier family (SLC) 27A5, SLC27A2 and fatty acid binding protein 4, adipocyte], 1 fatty acid biosynthesis regulator [protein kinase, AMP-activated, γ 2

non-catalytic subunit (PRKAG2)], 1 acyl-CoA thioesterase (ACOT9) and 1 other fatty acid metabolism-associated gene [pyrophosphatase (inorganic) 1 (PPA1)].

Upregulated and downregulated fatty acid metabolism-associated genes in AR-overexpressing Huh7 cells. In Huh7 cells, AR overexpression significantly downregulated the expression of the following 11 fatty acid metabolism-associated genes: 4 acyl-CoA synthetases [acyl-CoA synthetase medium-chain family member (ACSM) 2A, ACSL6, ACSL3 and ACSM3], 3 acyl-CoA thioesterases (ACOT7, ACOT9 and ACOT6), 1 acyl-CoA oxidase (ACOX3), 1 fatty acid transporter (SLC27A2), 1 fatty acid biosynthesis regulator (PRKAG2)

Table II. Fatty acid metabolism-associated genes are significantly upregulated in androgen receptor-overexpressing HepG2 cells examined by polymerase chain reaction arrays.

Symbol	Description	Fold change (vs. control)	P-value
FABP4	Fatty acid binding protein 4, adipocyte	2.25	0.0015
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	1.51	0.0167
SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5	1.40	0.0156
ACOT9	Acyl-CoA thioesterase 9	1.25	0.0216
PPA1	Pyrophosphatase (inorganic) 1	1.23	0.0162
PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	1.20	0.0331

CoA, coenzyme A.

Table III. Fatty acid metabolism-associated genes are significantly downregulated in androgen receptor-overexpressing Huh7 cells.

Symbol	Description	Fold change (vs. control)	P-value
ACOX3	Acyl-CoA oxidase 3, pristanoyl	0.62	0.0180
PRKAG2	Protein kinase, AMP-activated, γ 2 non-catalytic subunit	0.65	0.0402
ACSM3	Acyl-CoA synthetase medium-chain family member 3	0.66	0.0249
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	0.69	0.0387
BDH2	3-hydroxybutyrate dehydrogenase, type 2	0.69	0.0015
ACSL3	Acyl-CoA synthetase long-chain family member 3	0.87	0.0127
ACOT6	Acyl-CoA thioesterase 6	0.88	0.0020
ACSL6	Acyl-CoA synthetase long-chain family member 6	0.89	0.0052
ACOT9	Acyl-CoA thioesterase 9	0.91	0.0434
ACOT7	Acyl-CoA thioesterase 7	0.92	0.0480
ACSM2A	Acyl-CoA synthetase medium-chain family member 2A	0.93	0.0018

CoA, coenzyme A.

and 1 regulator of ketogenesis and ketone body metabolism (3-hydroxybutyrate dehydrogenase, type 2; Table III). However, AR overexpression did not significantly upregulate the expression of any fatty acid metabolism-associated genes in Huh7 cells.

Discussion

The present study demonstrated that overexpression of AR alters fatty acid metabolism-associated gene expression in human HCC cell lines. Previous studies (17,18) demonstrated that in patients with HCC, ACADSB and EHHADH expression are downregulated in tissue isolated from cirrhotic livers compared with control tissues from healthy patients. Acyl-CoA oxidase deficient-mice spontaneously develop HCC (19). Li *et al* (20) reported that cluster of differentiation (CD) 147 expression significantly contributed to the reprogramming of fatty acid metabolism in HCC cells, and that CD147 downregulated CPT1A and ACOX1 by activating the p38 mitogen-activated protein kinase signaling pathway to inhibit fatty acid β -oxidation. A previous study in phosphatase and tensin homolog null nonalcoholic steatohepatitis liver tissue isolated from mice revealed that ACSL1 expression

is decreased, and is almost non-existent in tumors isolated from these livers (21). A recent study (22) demonstrated that the presence of a specific mutation in ALDH2 (E487 K) led to increased protein turnover and promoted murine hepatocarcinogenesis. The presence of the DnaJ heat shock protein family (Hsp40) member B1-PRKACA chimeric transcript in fibrolamellar HCC suggests that this genetic alteration contributes to tumor pathogenesis (23). Therefore, the present study supports previous reports (17-23) (Table I).

Sakabe *et al* (24) reported that PRKAG2 enhances the effect of interferon (IFN)- α /fluorouracil (5-FU) and serves as a prognostic marker for IFN- α /5-FU therapy in HCC. The present study demonstrated that AR significantly upregulated the expression of PRKAG2 (Table II). ACOT7 has been implicated in the regulation of neuronal fatty acid metabolism to prevent neurotoxicity (25), and ACOX3 is expressed at particularly low levels in the liver (26). Notably, ACOT7 and ACOX3 were downregulated by the overexpression of AR in HepG2 and Huh7 cells (Tables I and III). These results support the results of a previous study by Bolton *et al* (27) that implicated AR in the regulation of cellular lipid metabolism.

In general, cancer cells synthesize a high amount of fatty acids via increased expression, and subsequent activity, of

lipogenic enzymes to fulfill their high energy requirements and to maintain cell membranes for growth (4,28,29). In patients with HCC, the alterations in lipid and lipoprotein metabolism can be clearly observed (30).

AR has been demonstrated to be associated with hepatocarcinogenesis and HCC development (1,3,5-7,9,10). The present study revealed that AR could serve a role in hepatocarcinogenesis via the regulation of hepatocellular fatty acid metabolism. Hepatitis C virus (HCV) augments AR-mediated signaling (7) and replicates in hepatocytes using lipid droplets, which are used for the storage of neutral lipids (31). In a previous study, eradication of HCV using an interferon-free antiviral regimen resulted in rapid changes in the metabolic signaling pathway in the liver, which suggested that there was a direct effect of HCV replication on lipid homeostasis (32); however, further studies are required to determine potential indirect effects of HCV on lipid homeostasis. Hepatitis B virus (HBV) has also been revealed to augment AR-mediated signaling (5,6) and to induce hepatic steatosis by influencing fatty acid metabolism (33). HBV serves a more minor role in hepatic steatosis compared with HCV (34).

In conclusion, improving the understanding of the association between AR and fatty acid metabolism in hepatocarcinogenesis and the development of HCC may aid in the creation of novel therapeutic and prevention strategies.

Acknowledgements

The authors would like to thank Professor J.T. Isaacs (The Johns Hopkins University School of Medicine, Baltimore, MD, USA) for providing the plasmid pSG5-AR. The present work was supported by grants from the Ministry of Health, Labour and Welfare of Japan. This study was partially supported by the Research Program on Hepatitis from the Japan Agency for Medical Research and Development (AMED).

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