ORIGINAL ARTICLE

Characterization of Androgen Receptor Complex Associated Protein (ARCAP) in hepatocellular carcinoma and liver

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Abstract

Background: Hepatocellular carcinoma (HCC) ranks many tasks in clinical oncology due to possibly developing a general tumor in men and, usually lead to malignant to death within years. Researches had reported about major factors for being HCC was male sex and HCC associated with cirrhosis in childhood was found more common in males than females. In certain mouse strains as studied, breeding with testosterone significantly increases the development of HCC. Furthermore, castration of male mice diminished the frequency of the development of liver tumors. Meanwhile male hepatitis B virus transgenic mice have a greater occurrence of HCC than females.

Methods: We apply degenerate priming PCR to observe the expression of various steroid receptors in livers. Yeast-two hybrid screening to search a novel RNA fragment helps to find a new full-length gene by RACE experiment. RT-PCR is applied to detect various expressions in tissues and cell lines. In situ hybridization detects DNA in Chromosome mapping. GFP-constructs transfection proves the gene localization in cells. Immunoprecipitation pulldown assay verifies protein interaction. Gene transfection followed with luciferase assay demonstrates the interaction of genes within cellular signaling. Genomic alignment analysis for observing sequences data perform from NCBI database website (http:// www.ncbi.nim.nih.gov/genebank/).

Results: The androgen receptor (AR) expression level is found at the highest level among the steroid receptors families detected in liver tumors. By yeast-two hybrid screening, we cloned an Androgen Receptor Complex Associated Protein (ARCAP), of 95 Kd in molecular weight and its cDNA. ARCAP locates at Chromosome 1. Our findings indicate ARCAP is highly expressed in hepatoma cell lines and liver tumors and their adjacent tumors as observed. Yeast two-hybrid assay and in vitro immunoprecipitation assays demonstrated an interaction between AR and ARCAP.

Conclusion: We aim to search for different types and levels of steroid receptors expressed within human HCCs and in the adjacent liver tissues. To verify possible molecular mechanisms by which AR might affect hepatoma cells, we had characterized a novel protein ARCAP which functions as a coregulator to interact with AR within liver. The ligand-dependent AR with its cofactor, ARCAP, can induce a signal cascade by transactivation.

Keywords: Androgen receptor; Androgen Receptor Complex Associated Protein; Hepatocellular carcinoma; Protein interaction; Transactivation

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1.INTRODUCTION

Hepatocellular carcinoma (HCC) ranks many tasks in clinical oncology due to possibly developing a general tumor in men and, usually lead to malignant to death within years.[1] Research had reported major factors for being HCC was male sex and HCC associated with cirrhosis in childhood is found more common in males than females.[2,3] In certain mouse strains as studied, breeding with testosterone significantly increases the chemically induced hyperplastic nodules to the development of HCC.[4] Furthermore, castration of male mice diminished the frequent development of liver tumors.[5] Male hepatitis B virus transgenic mice have a greater occurrence of HCC than females.[6] The report describes sex hormone in animals regulated growth of HCC.[7] In addition, the

hormonal-additive environment at the initiation of tumor development may be based upon the linkage of the usage of anabolic steroids.[5] Other possible mechanisms, including sexual status with respect to body metabolism by environmental carcinogens, have been reported.[6] Such as the pathway leading to the active metabolite of aflatoxin is observed much more progressive in certain male animals compared to females.[3] In humans, however, the molecular developmental mechanisms between HCC and sexual status are not clear. Steroid[4] hormones initiate their functional cascade by binding to the receptors and turn on the transcriptional regulatory machinery within the cells.[8] A study finds that EGF-triggered growth and invasiveness of cells expressing estrogen and androgen receptors to be correlated to the cell malignancy and carcinogenesis.[9] Cofactors for steroid receptors have been confirmed to be involved in transcriptional signal which will define the difference of cell destiny.[10] Based on these observations, we will try to rule out the patterns of steroid receptors and possible cofactors expressed in liver, analysis of expression and characterization of their mechanisms in hepatoma would be important. Herein, we had observed high levels of androgen receptor in liver. After screen. liver cell library by AR, we found a novel ARCAP from hepatoma cells. Analysis of expression patterns of ARCAP and characterization of their mechanisms in hepatoma would be important. Our approach is to clarify the distribution of ARCAP and verify the expression in cells specifically. AR and ARCAP are expressed in liver. Analysis of their interaction patterns and characterization of their mechanisms during malignant process in liver would be important to understand genomic signaling in modulatory transactivation and thus leading to tumorigenesis.

2.METHODS

2.1. Liver samples

HCC were obtained from the Department of Surgery, Veterans General Hospital-Taipei Clinical information of each patient was recorded and informed consent was obtained. Samples obtained after surgery were stored in liquid nitrogen. This study was approved by review at Taipei General Veterans Hospital (IRB# 91-08-05).

2.2. Cell culture and cell proliferation assay

HepG2, HepA2, HA22T/VGH, Hep3B, SK-Hep-1, Huh7, 293T, PC3, and BNL were grown under standard conditions following ATCC protocols. Hepatoma G2, A2, HA22T/VGH, SK-Hep-1, Huh7 cells, BNL mouse normal liver cells, human embryonic kidney 293T cells, and human prostate cancer cell line PC3 were routinely subcultured in DMEM and NEAA medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). A Celltiter 96 (Promega, Madison, MA) was utilized to determine the number of viable cells in proliferation assays. Cells grew with different time courses and at different concentrations following the treatment with dihydrotestosterone (DHT; Sigma, St. Louis, MO) as various studies required. The proliferation assay was performed and analyzed with an ELISA reader Spectra Max 250 (Molecular Devices, Sunnyvale, CA) as the manual suggested.

2.3. RNA extraction and reverse transcription

Total RNA was extracted from tissues and cells using a Trizol Kit (Life Technologies, Inc.). cDNA was synthesized from 10 μg of total RNA. Reverse transcription was performed in a total volume of 30 μΛ containing RNA in 1x first strand buffer (Life Technologies, Inc.) with 10 μM DTT, 500 μM dNTPs, 50 ng/μΛ oligo-dT, and 100 units MMLV reverse transcriptase at 37°C for 1 hour. The samples were then denatured for 5 minutes at 95°C.

2.4. Degenerate priming

One pair of degenerate primers of TGYGARGGYTGYAARRG and CATICCIACIIIVADRCA encoding the zinc finger DNA binding domain from the p box to the second finger domain, sized around approximately a 170 bp PCR product, was utilized in the PCR reaction.

2.5. RT-PCR

cDNA (1 μΛ) was used for a first PCR amplification in a volume of 25 μl containing 0.8 μM of primer sets, 50 μM of each dNTP (GE Pharmacia, Piscataway, NJ), 1x PCR buffer, and 1.25 units Taq Polymerase (GE Pharmacia). The primers for human transferrin gene are shown as followed. F: TGAGAACTTGGCAAACAAG R: ATTCATGATCTTGGCGATGC. The PCR consisted of a denaturation beginning at 94°C for 3 minutes and 35 cycles of 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C, and with a final extension for 10 minutes at 72°C, using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA). The PCR products were resolved on 1% ethidium bromide-stained agarose gels (FMC Bioproducts, Rockland, ME).

2.6. Multiple Tissue Northern blot

Three Multiple Tissue Northern blots Cat. # 7760-1, 7759-1, and 7767-1 were ordered from BD-Clontech for the hybridization experiments with ARCAP probe. Hybridizations are performed as the user manual.

2.7. Northern hybridization

Samples of total RNA (10 μg) were size-fractionated in 1% agarose-0.67% formaldehyde gels and then transferred to nitrocellulose membranes. Hybridization to α-^{32}P dCTP (GE Amersham Corp., Piscataway, NJ)-labeled DNA probe was performed in 5X SSC, 50% formamide, 2X Denhart's solution containing 250 μg/μL salmon sperm DNA and 0.1% SDS overnight at 42°C. Membranes were washed in different stringent conditions and exposed to X-ray film (Fuji, Tokyo, Japan).

2.8. Fluorescence in situ hybridization

Cells were prepared as a logarithmic phrase and then by adding 10 μL/mL Colecemid to cell flasks. Cells were broken and hybridized with Cy5 label ARCAP probe. Wash after hybridization and visualized by fluorescence microscope.

2.9. Microscopic analysis of GFP-containing proteins

Full-length ARCAP and ARA70 were cloned into the EcoR1 site in a pEGFP-C1vector (Clontech) to generate GFP-ARCAP and GFP-ARA70. Cells were seeded on chamber glass culture plates (Corning) in a volume of 2 mL at densities of 1×10^5 cells/well. After24 hours, cells were washed with PBS and then transfected with 1 μg of the GFP-containing plasmid constructs using the FuGENE 6. After 6 hours, cells were then washed twice with incubating overnight in fresh media supplemented with 10% FBS. Following PBS wash once, cells were placed in serum-free RPMI 1640 medium for a further 18 hours. Cover slips were washed with PBS, fixed in methanol for 10 minutes at120°C, dried, and inverted on a microscope finally. Slides were analyzed using an Olympus AX80 microscope with 100× magnification.

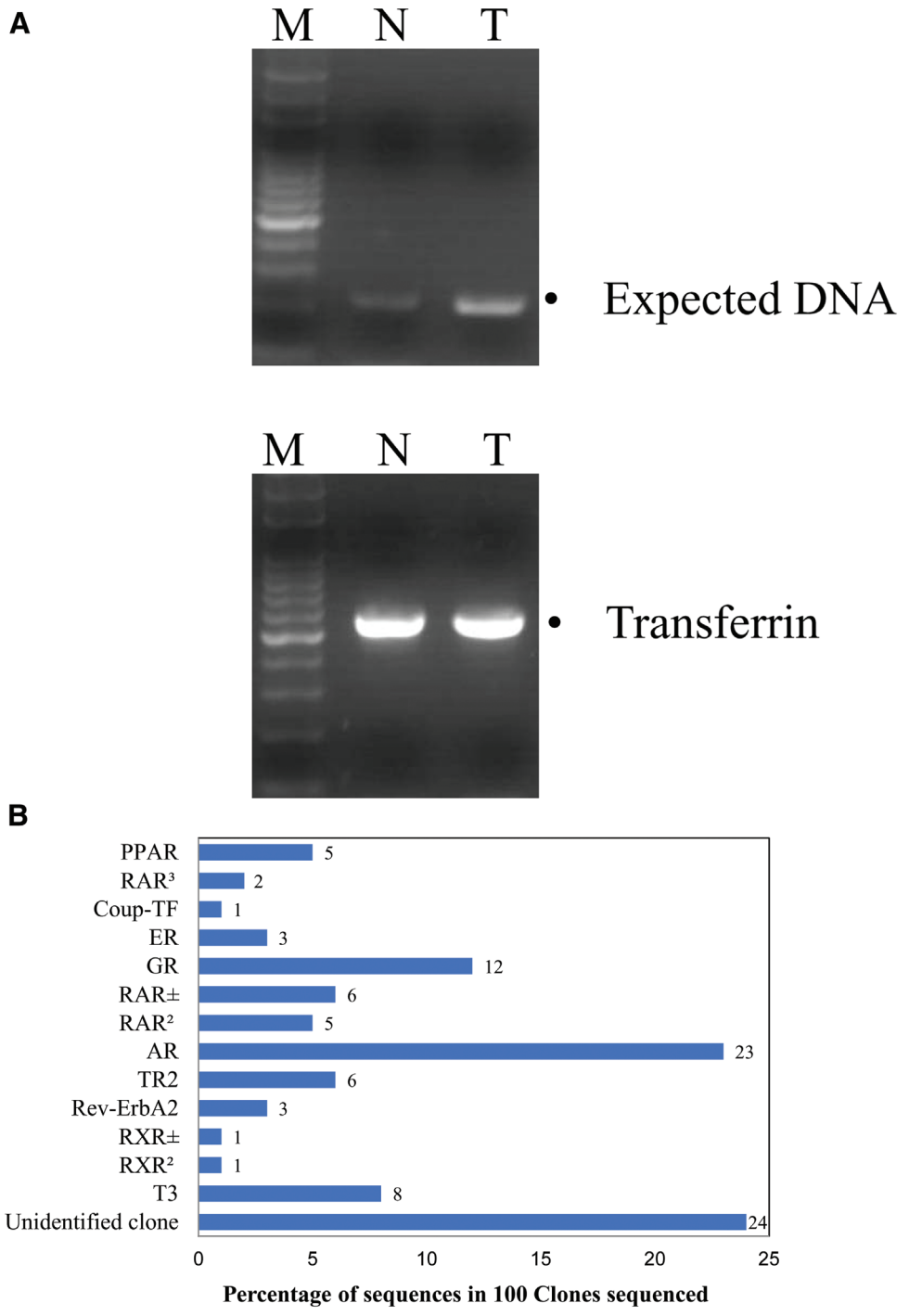


Fig. 1 A, Degenerate primer PCR was utilized to screen for steroid receptor genes expressed in HCC. Degenerate primers for many known steroid nuclear receptor genes were synthesized and used for RT-PCR. The data showed a difference in signal expression between that in liver tumors (T) and in its adjacent normal liver tissue (N). A 170 bp cDNA was amplified which was more highly expressed in hepatoma tissue than in the adjacent tissue. The expression level of human transferrin was included as controls. B, Comparison of the relative level of steroid receptors mRNAs in HCC. Nuclear receptor cDNA fragments isolated by degenerate primer RT-PCR in Italy, Korea, and Sweden. Fig. 1 from human hepatomas were cloned and sequenced. A compilation of identified cDNA clones encoding members of the steroid receptor superfamily is shown as the percentages found in 100 cDNA sequences examined.

2.10. Microscopic analysis of GFP-containing proteins

Immunoprecipitation pull-down assay was applied to study the interaction of the AR and the ARCAP. Recombinant constructs of AR-pCDNA3.1 in 293T cell lysate and ARCAP-containing GST-BL21 bacteria lysate respectively incubated with Sepharose-GST beads conjugates were recovered by centrifugation at 14,000 rpm for 3 minutes, and resuspended in 1 mL of buffer A (0.2% Triton X-100 and 350 mM NaCl in PBS), and re-spun. Samples were resuspended in 1 mL of buffer B (PBS containing 0.2% Triton X-100), re-spun, and resuspended in SDS sample

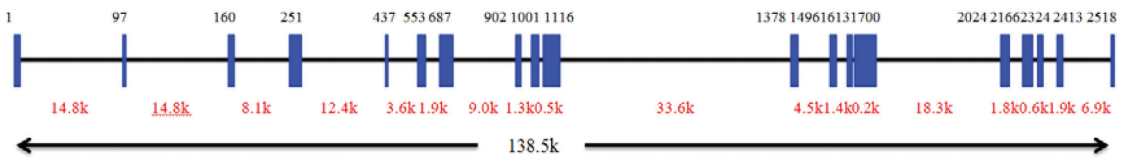


Fig. 2 The human ARCAP spans 19 exons within a 135 Kb genomic DNA structure (NCBI GenBank DQ768089.1) https://www.ncbi.nlm.nih.gov/nuccore/DQ768089.1, GenBank: ABG76793.1) https://www.ncbi.nlm.nih.gov/protein/110564287.

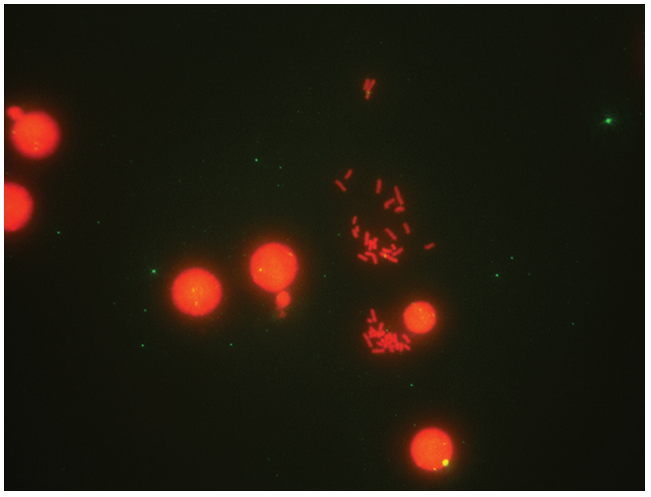


Fig. 3 The mapping of ARCAP locates within Chromosome 1q23.2-q24 as indicated as yellow spot.

buffer. Samples were resolved by electrophoresis in 10% polyacrylamide gels at 200 V for 45 minutes with equal loading Western transferred and coimmunoprecipitated with anti-AR antibody.

2.11. Transient transfection of 293T cells with various ARCAP constructs and luciferase reporter assay followed

Full-length ARCAP and N- and C-terminal truncates of ARCAP GST vector were constructed into pcDNA 3.1 vectors (Invitrogen, Carlsbad, CA). The N-terminal truncate (Y1) had deletions from the position in the amino acid residues 1 to 230 of ARCAP. C-terminus truncate (Y2) abolished the amino acid residues from 710 to 860 of the ARCAP. ARE-containing Luc, a luciferase reporter construct derived from the α-fetoprotein enhancer region, was used for the AR-based transactivation experiments in 293T cells. Beta-gal pC100 vector (Pharmacia, Piscataway, NJ) was included in the transfection for normalization of the luciferase activity. After transfection for a period of 24 hours, cells were washed by PBS, and medium containing vehicle or DHT was added as indicated. Cells were harvested at 48 hours after transfection and analyzed as protocols.

3.RESULTS

3.1. Degenerated priming method to detect the expression in HCC

Because the regulation of hormonal steroid receptors and cofactors and their actions are. not completely understood in the development of hepatomas, the expression patterns of steroid hormone receptors in hepatoma cells were investigated first. Degenerate primers encoding a conserved region of the zinc finger DNA binding domain of the steroid receptor superfamily were utilized to profile the expression of these receptors in hepatoma (Fig. 1A).

3.2. AR strongly expressed in HCC

A stronger DNA signal was observed in hepatoma tissue than in normal liver tissue after degenerate priming PCR. This PCR-amplified DNA fragment was isolated from the gel and ligated to a pGEM-T vector. Then, the DNAs were transformed into DH5α bacteria cells. To clarify the distribution of steroid receptor expression, we sequenced 100 clones from DNA-transformed DH5α bacteria. After data analysis, the AR ranked 24% of the steroid receptors expressed within hepatoma tissues (Fig. 1B).

3.3. A novel ARCAP cloned by yeast-two hybrid system

Since the AR was found to be expressed in hepatoma tissues and since no coregulators for the AR have been reported in liver, a yeast two-hybrid screening assay using the AR ligand-binding domain as the bait was performed. One candidate protein ARCAP (Androgen Receptor Complex Associated Protein) was found in the liver matchmaker library (BD Clontech). Originally, a short cDNA fragment was obtained. A longer cDNA fragment containing the original sequence was searched for by the screening of a HepG2 hepatoma cDNA library (StrateGene). Subsequently, the full-length ARCAP was obtained using 5' and 3' RACE (BD Clontech) with the RNA template from HepG2 cells. The human ARCAP has an open reading frame of 2583 bp that encodes 860 amino acids with a calculated molecular weight of 95 Kd (GenBank: DQ768089.1, GenBank: ABG76793.1).[11,12] Analysis of the coding sequence of the human ARCAP revealed that the genomic structure of ARCAP spanned 19 exons of 135 kb of genomic DNA at chromosome1q23.2-q24.3 region. (Fig. 2). Fluorescence in situ hybridization in hepatoma G2 cell indicates the mapping of ARCAP was located at Chromosome I which was shown as yellow spot within Chromosome 1 region (Fig. 3).

3.4. The expression of ARCAP

ARCAP is a novel gene. The tissue distribution of its transcriptional expression was determined by Northern and PCR techniques. ARCAP mRNA was not expressed in most normal human tissues, except it was weakly expressed in heart and skeletal muscle (Fig. 4A). There was a barely detectable level of ARCAP in normal liver, but it was highly expressed in all human hepatoma cell lines examined (Fig. 4B). ARCAP was more highly expressed in 13 HCC tissues with in the adjacent liver tissue from 15 pairs of the tumor and adjacent tissue samples studied (Fig. 4C). It's interesting that ARCAP is most expressed in liver tumor cells and hepatoma.

3.5. Localization of ARCAP

Ligand-bound AR is present in the nucleus.[8] The intracellular location of ARCAP was determined with an ARCAP-containing GFP fusion construct after it was transiently transfected into hepatoma G2 cells. Fluorescence microscopic observation, ARCAP is primarily localized in the nucleus of liver tumor cells (Fig. 5) with less located in the cytoplasm.

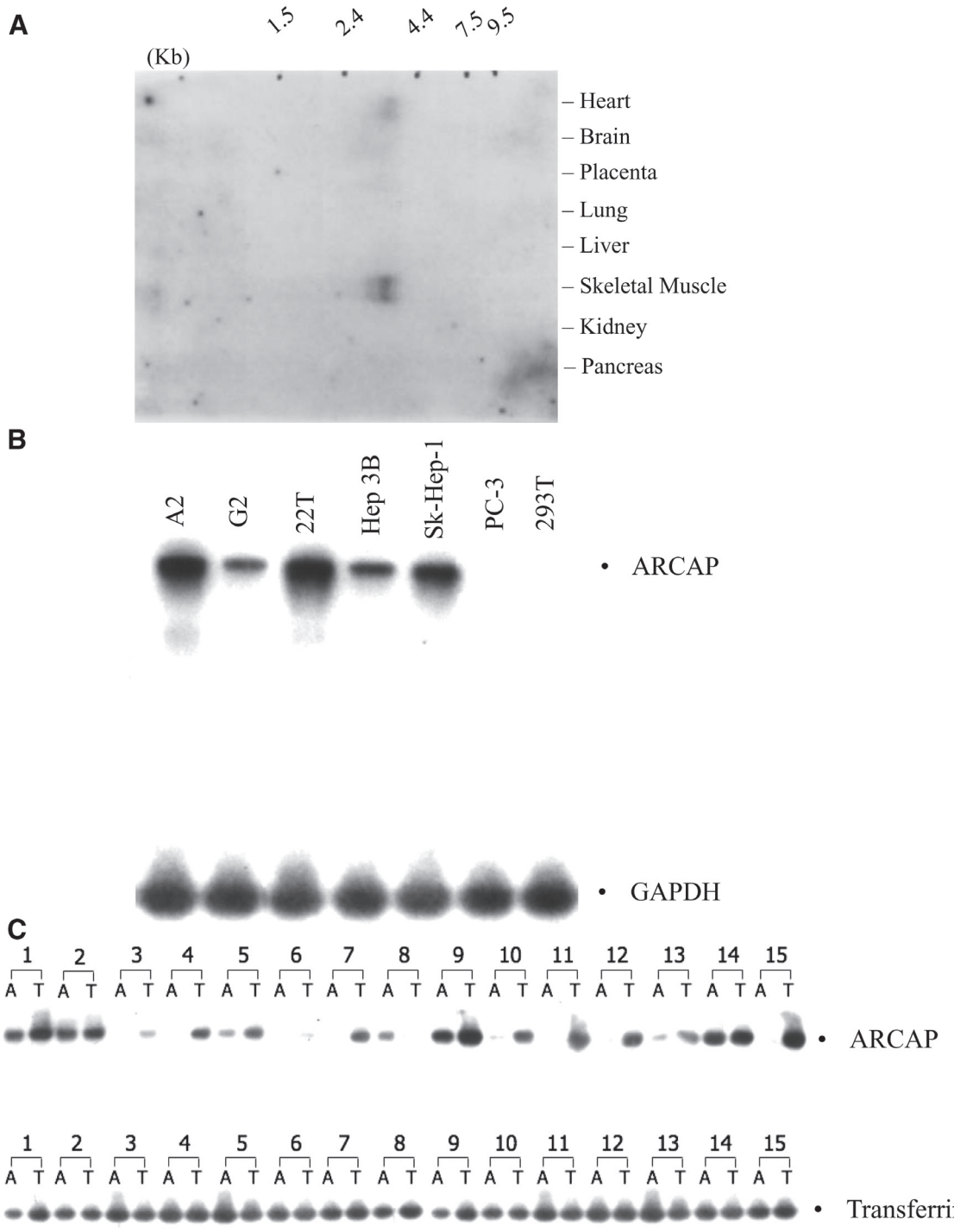


Fig. 4 The expression of ARCAP in normal tissues (A), cell lines (B), and HCC (C). A survey of ARCAP determined by Northern blotting in 23 normal tissue mRNAs as described in Materials and Methods is shown (A). ARCAP was weakly expressed in heart and skeletal muscle. The ARCAP expressed in all hepatoma cell lines was tested as sequences examined. determined by Northern hybridization as described in Materials and Methods, including A2, G2, 22T, Hep3B, and SK-Hep-1 cells, but not in non-hepatic cells: human prostate PC3 and 293T cells. GAPDH signal is included as an internal control (B). ARCAP mRNA was highly expressed in tumor tissue (T) of 15 different HCC samples tested, but at lower level expressed in their normal adjacent HCC samples tested as described in Methods. Transferrin signal is included as an internal control (C).

3.6. ARCAP interacts with AR to modulate AR transactivation in liver cells

To further examine the interaction between these two proteins, an ARCAP-containing GST expression BL21 bacteria lysate with an GST-express epitope and an AR subsequently fuse pcDNA3.1-containing 293T cell lysate are incubated with GST resin beads. Interaction complexes were purified and Western blotting to recognize the signal with the AR antibody against an immunoprecipitated. Our data demonstrate the in vitro pull-down can identify the interaction between ARCAP and AR, but not with the control protein (Fig. 6).

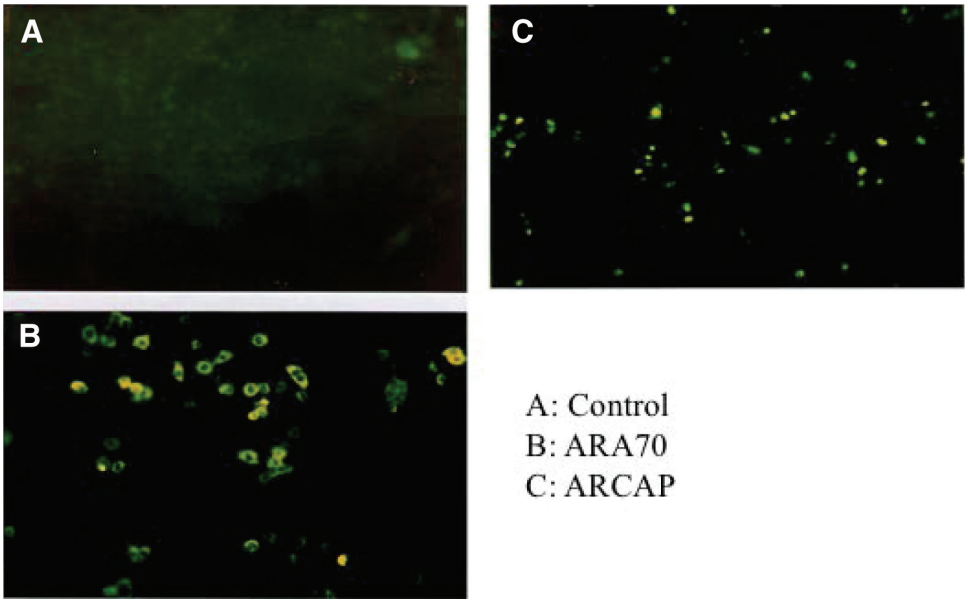


Fig. 5 Localization of the ARCAP in hepatoma A2 cells. ARCAP-GFP plasmid DNA was transiently transfected into hepatoma A2 cells. The ARCAP expression signal was visualized by fluorescence microscopy within the nucleus of the A2 cells. ARA70, a known AR coactivator (13), was included as a control for localizing expression within the cytoplasm.

3.7. Partial localization of the ARCAP binding regions with AR

Since there is no AR and ARCAP expressing in 293T cells, thus vector only, AR, and wild type of, truncates of the N-terminus (Y1) and the C-terminus (Y2) of ARCAP-containing DNA and an ARE-containing luciferase vector were transiently transfected into 293T cells. After 48 hours, cells were treated with different agents as 1% alcohol, or 10nM DHT and cells lysed after 24 hours, and then luciferase activity was determined. We demonstrate AR only can transactivate ARE in a DHT-dependent function. Meanwhile, data indicate a full-length of ARCAP DNA can co-transactivate with AR in a hormone-dependent manner as shown in Fig. 7.

3.8. Map ARCAP regions in regulating cell growth

There is no ARCAP expressing in mouse normal liver cells (BNL), thus vector only, and wild type of, truncates of the N-terminus (Y1) and the C-terminus (Y2) of ARCAP-containing DNA are transfected into BNL cells. After 48 hours, cells were treated with different agents as 1% alcohol in Fig. 8A; 100nM estrogen (E2) in Fig. 8B, or 100nM DHT in Fig. 8C. We observe overexpression of ARCAP stimulating BNL cell growth but truncates of ARCAP lose partial ability to increase BNL cell growth. Meanwhile, data indicate a full length of ARCAP can modulate liver cell proliferation independent of hormone treatment as shown in Fig. 8.

4.DISCUSSION

4.1. Novel AR CAP

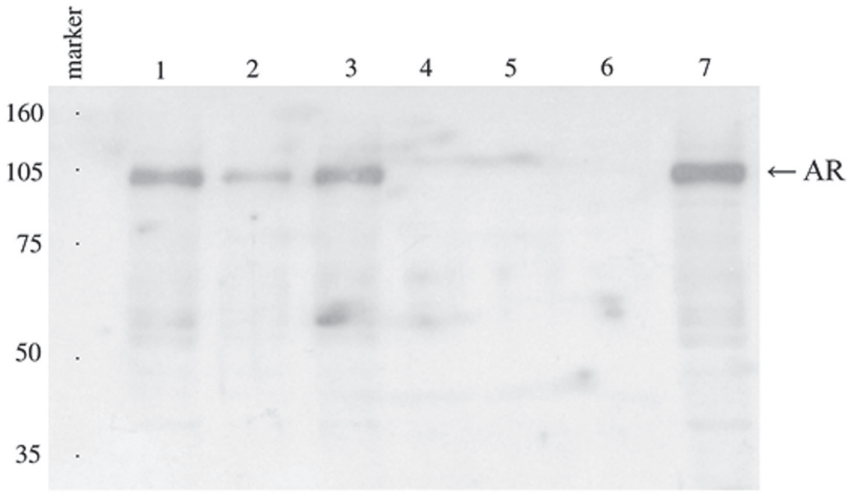
AR as known plays an important role in sexual development.[13] The distribution of AR in hepatoma has been reported by several laboratories.[14] A report described the presence of androgen receptors within liver tumor was significantly related to smaller tumor size.[14] As many reports about hepatomas, there is an elevation of both androgen receptors and estrogen receptors activities, and the progression from hyperplasia to cancer parallels suppresses of ER expression, but maintain of AR expression.[15] The signal pathways involving androgen signaling may affect the risk of HBV-related HCC among men.[16] The case of CAG polymorphism in exon 1 region within AR gene has demonstrated to be associated with a human male. HCC cases with shorter AR alleles found to confer a higher risk as reported.[17] Since the molecular mechanisms of AR seem to be important in liver tumor pathogenesis, but are still ambiguous, the understanding of these mechanisms should provide new avenues leading toward successful treatments for hepatoma. As reported, several studies have identified and characterized cofactors (coactivators or corepressors) for steroid receptors.[17,18] The interactions of the steroid receptor and the cofactors are essential for the regulation of target gene transcription.[18,19] AR complexes containing acetyltransferase and other coactivators have been reported.[20] Herein, we present ARCAP express a higher level in malignant liver cells and tumors. Our findings indicate a novel ARCAP cloned and mapped to Chromosome 1q23.2 may initiate the cascade pathway.

4.2. Expression and localization of ARCAP

It has been speculated that differential levels of steroid receptor-coregulators among tissues contribute, at least partly, lead to the different responses elicited by these hormones.[20] The present findings indicate that the ARCAP is a cofactor with possible activation capability for AR. ARCAP is mostly expressed in liver tumor cell lines and liver tumor tissues. Although less is known about the functional domains of ARCAP yet, little input let us define the physiological roles in normal development and homeostasis, hormone regulation, and for the progression of cancer. Some of the characteristics of the ARCAP are studied further for understanding ARCAP might play a key point in possible liver tumorigenesis.

4.3. Interaction of ARCAP and AR

Our findings indicate that the ARCAP is a cofactor with activation capability for the AR. ARCAP not only stimulates the transactivating ability of AR, but stimulates proliferative growth of



Lane 1: Pass through
Lane 2: Elute
} Interaction of clone ARCAP-containing GST fusion protein with AR-overexpressed 293T lysate

Lane 3: Pass through
Lane 4: Elute
} Interaction of GST only protein with AR-overexpressed 293T lysate

Lane 5: Interaction of clone ARCAP -containing GST fusion protein with 293T lysate

Lane 6: Interaction of clone ARCAP-containing GST fusion protein with pcDNA3.1 vector only from 293T lysate

Lane 7: AR-overexpressed 293T lysate

Fig. 6 Immunoprecipitation of ARCAP and AR by pulldown assay. Immunoprecipitated-pull-down assay was applied to study the interaction of the AR and the ARCAP. Recombinant constructs of AR-pCDNA3.1 and ARCAP-containing GST respectively, were in vitro translated separately, and then coimmunoprecipitated with anti-AR antibody.

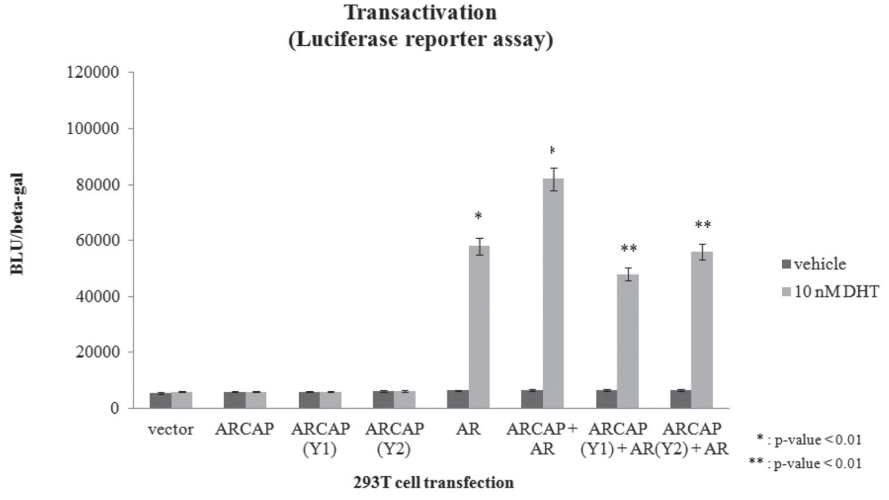


Fig. 7 Transactivation of the AR is coactivated by the ARCAP in a DHT-dependent manner. AR-containing and ARCAP-containing pCDNA3.1 DNA, ARE-containing luciferase vector, and a β-gal-containing internal control vector were transiently transfected into 293T cells. After 48 hours, the cells were lysed and luciferase and β-gal levels were determined separately as described in Methods. Transfected cells were grown in charcoal-treated serum or media with DHT (10 nM) as indicated. DHT = dihydrotestosterone.

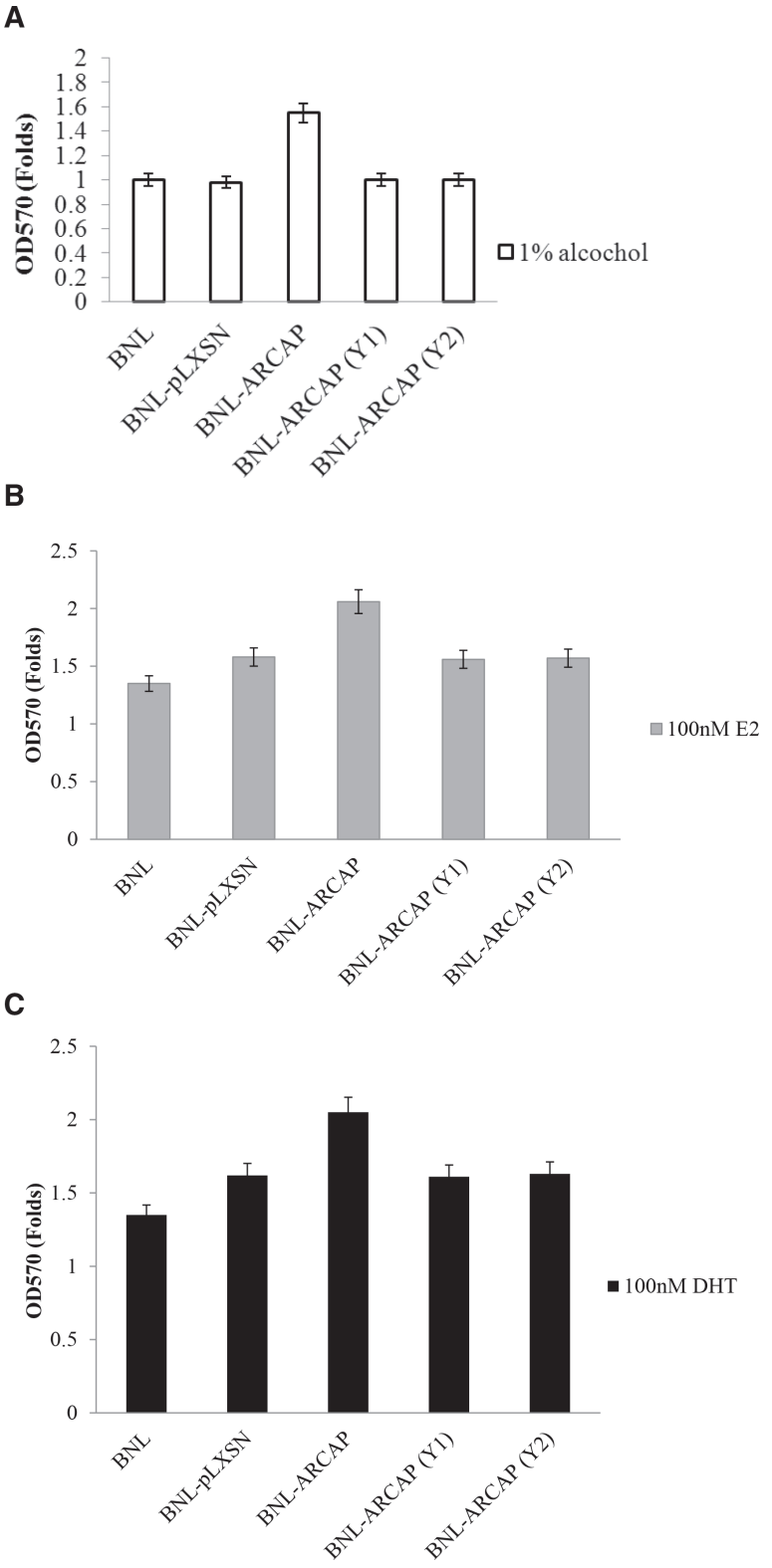


Fig. 8 Localization of ARCAP regions in regulating cell growth. Wild type, and truncates of the N-terminus and the C-terminus of ARCAP-containing DNA were transiently transfected into BNL cells. After 48 hours, cells were treated with different agents (1% alcohol in (A); 100nM E2 in (B), 100nM DHT in (C)) after 24 hours and then cell proliferation assay was determined. DHT = dihydrotestosterone.

liver cells. ARCAP is the first coactivator that specifically acts on AR in liver tumors. Whereas the ARCAP is highly expressed in liver tumors and hepatoma cell lines, in normal tissues, it is only expressed at very low heart and skeletal muscle,

The ability of ARCAP to affect AR transcriptional activation depends on the ARCAP and AR interacting regions, due to AR C-terminal ligand-containing interaction domain (C-terminal ligand-containing AR utilized as a "bait" dependent

transcription) is enhanced by ARCAP coexpression. Thus, the ARCAP represents a novel AR ligand domain-associated coactivator. Interestingly, ARCAP and AR are present in only a subset of hepatomas, suggesting that ARCAP may play a role in regulating AR-dependent liver function. Although we have searched and mapped the possible regions of ARCAP that might interact with the ligand-binding domain of AR, sequence analysis of the ARCAP has not revealed any classical interaction motifs found for other nuclear receptors. Several WD40 containing motifs[21,22] were found in both the N- and C-termini of the ARCAP which might contribute to the interaction of the ARCAP and the AR. These will be studied further. Deletion analysis of ARCAP failed to define a distinct interface within the ARCAP that mediates the association with AR.

One plausible explanation is that ARCAP may associate with AR ligand-binding domain through multiple weak interactions and that removal of any one of these contacts might make the ARCAP incapable of binding. ARCAP molecule may be involved in and the whole completeness of ARCAP may be necessary for the requirement of any region interacting with AR.

In conclusion, hepatocytes have a remarkable proliferative capacity that is little noted in normal liver. During tumorigenesis or following injury, the activation of the hepatocyte cell cycle can be directly stimulated by overexpression or combinations of genes. The critical steps required for the transition in liver from manipulating ARCAP-regulated to constitutive hepatocyte proliferation to malignancy are being studied. Verification of ARCAP performance may lead us to understand liver tumorigenesis.

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