Published in final edited form as:

Mol Cancer Ther. 2016 April ; 15(4): 731–742. doi:10.1158/1535-7163.MCT-15-0706.

Targeting Androgen Receptor (AR)→IL12A Signal Enhances Efficacy of Sorafenib Plus NK Cells-Immunotherapy to Better Suppress HCC Progression

Liang Shi^{#1,2}, Hui Lin^{#1}, Gonghui Li^{#1}, Ren-An Jin¹, Junjie Xu^{1,2}, Yin Sun², Wen-Lung Ma³, Shuyuan Yeh², Xiujun Cai^{1,*}, and Chawnshang Chang^{2,3,*}

¹Chawnshang Chang Liver Cancer Center, Department of General Surgery, Sir Run-Run Shaw Hospital, Zhejiang University, Hangzhou 310016, China

²George Whipple Lab for Cancer Research, Departments of Pathology, Urology and Radiation Oncology, and The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY 14642, USA

³Sex Hormone Research Center, China Medical University/Hospital, Taichung 404, Taiwan

These authors contributed equally to this work.

Abstract

Gender disparity has long been considered as a key to fully understand hepatocellular carcinoma (HCC) development. At the same time, immunotherapy related to interleukin-12 still need more investigation before being applied in clinical settings. The aim of this study is to investigate the influence of androgen receptor (AR) on natural killer (NK) cells related innate immune surveillance in liver cancer, and provide a novel therapeutic approach to suppress HCC *via* altering IL-12A. By using in vitro cell cytotoxicity test and in vivo liver orthotopic xenograft mice model, we identified the role of AR in modulating NK cells cytotoxicity. Luciferase report assay and chromatin immunoprecipitation assay were applied for mechanism dissection. Immunohistochemistry is performed for sample staining. Our results showed AR could suppress IL-12A expression at the transcriptional level *via* direct binding to the IL-12A promoter region that resulted in repressing efficacy of NK cell cytotoxicity against HCC, and sorafenib treatment could enhance IL-12A signals *via* suppressing AR signals. These results not only help to explain the AR roles in the gender disparity of HCC but also provide a potential new therapy to better suppress HCC *via* combining sorafenib with NK cells related immunotherapy.

Keywords

Androgen receptor; IL12; Natural killer cells

*Co-corresponding authors: Xiujun Cai (; Email: cxjzu@hotmail.com) and Chawnshang Chang (; Email: chang@urmc.rochester.edu)
The authors disclose no potential conflicts of interest.

Introduction

Hepatocellular carcinoma (HCC), the major liver cancer, is the second leading cause for cancer death in men (1). The etiology of HCC could be attributed to many liver diseases including viral hepatitis, cirrhosis and other chronic liver damage (2, 3). Importantly, gender disparity involving the androgen receptor (AR) during HCC initiation and progression has also been well documented but detailed mechanisms remain to be further elucidated (4, 5).

While the androgen deprivation as therapy for HCC remains controversial (6, 7), using mice with knocked out AR in hepatocytes and AR degradation enhancer, ASC-J9[®], to degrade AR in selective cells, Wu et al. and Ma et al. found the dual roles of AR in HCC. They found that AR might enhance HCC initiation and early development, but suppress HCC metastasis at the later stages of the disease (8-10).

Recent studies also indicated that various immune cells in the tumor microenvironment might play important roles to influence the HCC progression (11-13) and the natural killer (NK) cells have been implicated to be effective for immunotherapy in HCC (14, 15). The detailed mechanisms how NK cells suppress HCC and their potential linkage to AR function, however, remain unclear. Furthermore, early studies indicated that interleukin 12 (IL-12), also called the NK cell stimulatory factor, might play important roles to activate and mediate the cytotoxicity of NK cells and CD8⁺ cytotoxic T cells (16, 17), and suggested that IL-12 gene therapy might represent an effective approach to suppress HCC progression (18-21). However, the development of lethal toxicity shown during Phase II clinical trials prevented further study (22, 23).

Here we revealed the relationship between cytotoxicity of NK cells in HCC and AR by demonstrating that AR could suppress IL-12A, one of the two subunits of IL-12. Importantly, we demonstrated that sorafenib, the only drug approved by FDA for advanced HCC, could enhance the cytotoxicity of NK cells by elevating the IL-12A levels through inhibition of AR.

Materials and Methods

Cell culture and transfection

The human HCC cells were maintained in DMEM (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS), 1% Glutamine; and 1% penicillin/streptomycin. NK-92MI cells were obtained from ATCC (Manassas, VA) on December 3, 2010 and ~25 ampules (2×10⁶ cell/ml) were frozen in liquid N₂. Resuscitated cells were used within 3 months. NK-92MI cells were maintained in α-MEM (Invitrogen) with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, horse serum to a final concentration of 12.5% and FBS to a final concentration of 12.5% based on ATCC guidelines. Human HCC SKAR3, SKAR7 and SK-pBABE cells were established and described previously (9). SK-Hep1 and HepG2 cells were obtained from ATCC and authenticated by a professional biotechnology company in 2015. SNU-423 cells were bought from ATCC on April 4, 2014 and all the experiments were performed within 3 months of resuscitation. HA22T cell (BCRC No.

60168) is a gift from Professor. Yuh-Shan Jou, Academia Sinica, Taiwan (24). All cell lines were cultured in a 5% (v/v) CO₂ humidified incubator at 37°C.

The HepG2 (ATCC, Manassas, VA) and HA22T AR stable transfectants were established based on a previous procedure (25). To generate AR knocked-down stable clones of SK-Hep1 (ATCC, Manassas, VA) and SNU423 cells (ATCC, Manassas, VA), HEK-293T cells were transfected with lentiviral vectors, pLKO1-AR-si/pLKO1-scr, with the psAX2 packaging plasmid, and pMD2G envelope plasmid, then transfected into 293T cells for 48 hours to get the lentivirus soup, which was frozen at -80°C for later use for infection of cells to produce stable clones.

For the luciferase reporter assay, cells were transfected using LipofectAMINE 3000 (Invitrogen) reverse transfection protocol, according to the manufacturer's instructions. See Supplemental Table for detailed reporter plasmid sequences.

MTT cell viability assay

HCC cells were placed in 24-well plates at a density from 0.4×10^5 to 1.0×10^5 cells/well based on the cell size. NK cells were co-incubated at an effector-to-target (E:T) ratio at 2:1 for 6 hours. Then, NK cells were washed away and 0.5 mL MTT (0.5 mg/mL) per well was added and incubated for another 2 hours. The absorbance at 570/630nm was detected. The percentage of survival was calculated using the following formula: survival (%) = $OD_E / OD_C \times 100\%$ (OD_E : OD value of the NK cells added cell group; OD_C : OD value of the HCC cells without NK cells added).

Cellular cytotoxicity assay

NK cells cytotoxicity against HCC cells was analyzed using a standard lactate dehydrogenase release assay. NK92-MI cells were starved in serum-free media for 24 hours and target cells were seeded in 96-well plates for 24 hours, then the serum starved NK92-MI cells were added to the target cells and incubated for 4 hours at 37°C. In rescue experiments, the starved NK cells were pre-activated with varying doses of recombinant human IL-12 (Sigma, St. Louis, MO) for 24 hours before adding to target HCC cells. In the interruption assay, human IL-12 neutralizing antibody (PeproTech, Rocky Hill, NJ) was used at a concentration of 0.1 µg/ml. An aliquot of 50 µL media was used for detection of lactate dehydrogenase (LDH) activity using the LDH cytotoxic assay kit (Thermo Scientific, Rochester, NY). Spontaneous release of target cells alone was <15% of the maximum release as determined with target cells lysed in lysis buffer. The experimental release was corrected by subtraction of the spontaneous release of effector cells at corresponding dilutions. %Cytotoxicity = (Experimental value - Effector Cells Spontaneous Control - Target Cells Spontaneous Control) / (Target Cell Maximum Control - Target Cells Spontaneous Control) × 100.

Fluorescent In Situ Detection of DNA Fragmentation (TUNEL)

We seeded 1×10^4 HCC cell per well in an 8-well chamber slide. After 24 hours, we added starved NK-92MI cells with an E:T ratio at 1: 1 and incubated for another 24 hours then performed TUNEL staining. Apoptotic cell death was determined using TUNEL staining

with an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, city, state), following the manufacturer's protocol. TUNEL-positive cells were calculated as the number of positive cells $\times$ 100% divided by the total number of cells per field in 10 random fields at $\times$ 100 magnification. The apoptosis rate was calculated based on numbers of positive cells and total cells.

ELISA

Serum starved NK-92MI cells were activated by conditioned media (CM) collected from HCC cells with different AR expression levels. After activation, the media from NK cells were collected for detection of IFN- γ using an ELISA kit (Thermo Scientific). The standard curve was made to determine the IFN- γ concentration. IL-12p35 was detected in culture media collected from HCC cells with an ELISA kit (Thermo Scientific). All the procedures were performed according to the manufacturer's instructions.

Quantitative Real-Time PCR Analysis

Total RNAs were isolated using Trizol reagent (Invitrogen). One μ g of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was conducted using a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA) with SYBR green to determine the mRNA expression level of a gene of interest. Expression levels were normalized to the expression of GAPDH RNA (see Supplemental Table for detailed sequence).

Western Blot Analysis

Cells were lysed in RIPA buffer and proteins (60 μ g) were separated on 8–10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking membranes, they were incubated with appropriate dilutions of specific primary antibodies, the blots were incubated with HRP-conjugated secondary antibodies and visualized using ECL system (Thermo Fisher Scientific, Rochester, NY). Anti-GAPDH (1:1000, 6c5) and anti-AR (1:1000, N20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IL-12A (Anti-IL-12-p35, 1:500, R12-2200) antibody was purchased from Assay Biotechnology Company (Sunnyvale, CA). Human IL-12 polyclone antibody (PeproTech, Rocky Hill, NJ) was used to test both IL-12A and IL-12B (1:500).

Plasmid Construction and Luciferase Assay

The full-length promoter of IL-12A was obtained from genomic DNA of 293T cell by Phusion® High-Fidelity DNA Polymerase (NEB, Beverly, NY) and ligated into pGL3-basic vector (Promega, Madison, WI) by the Gibson assembly method. For the 5th ARE mutation, we designed the primer as Forward: 5'-CAT GGT AAA AAT GTG GCC CCC TGG GTC AG-3', Reverse: 5'-CCA ACG AGG CGC GGC AGG ACT TTC-3', then used wild type IL-12A promoter pGL3 plasmid as the template to run PCR and performed self-ligation. For luciferase assay, cells were plated in 24-well plates and the cDNA transfected using Lipofectamine3000 (Invitrogen) according to the manufacturer's instruction. pRL-TK was used as internal control. Luciferase activity was measured by Dual-Luciferase Assay (Promega) according to the manufacturer's manual.

Chromatin Immunoprecipitation Assay (ChIP)

Cell lysates were pre-cleared sequentially with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and protein A-agarose. Anti-AR antibody from Santa Cruz (2.0 µg) was added to the cell lysates and incubated at 4°C overnight. For the negative control, IgG was used. Specific primer sets designed to amplify a target sequence within the human IL-12A promoter are listed in the Supplemental Table; PCR products were identified by agarose gel electrophoresis.

In vivo orthotopic tumor model

A total of 32 Male 6-8 weeks old nude mice were used. SK-Hep1, SK-AR7 and SK-pBABE cells were engineered to express luciferase reporter gene (PCDNA3.0-luciferase) by stable transfection and the positive stable clones were selected with G418 and expanded in culture. Two groups of 6 mice each were injected with HCC cells (SK-AR7 or SK-pBABE) only (2×10^6 of luciferase expressing cells, as a mixture with Matrigel, 1:1), 2 groups of 6 mice each were co-injected with HCC cells (SK-AR7 or SK-pBABE) and NK-92MI cells (E:T ratio=1:5) and 8 mice were injected with SK-Hep1 cells, into the left lobe of liver. Tumor formation and metastasis were monitored by using a Fluorescent Imager (IVIS Spectrum, Caliper Life Sciences, Hopkinton, MA) starting 2 weeks after tumor injection and using mouse tail vein injection of 150 mg/kg Luciferin.

Mice injected with SK-AR or SK-pBABE cells w/wo NK-92MI cells were sacrificed after 8 weeks and liver tumors were isolated for further test. The mice injected with SK-Hep1 cells, after tumors formed as detected by IVIS, were divided into 2 groups randomly. Sorafenib tosylate or vehicle only were administered by oral gavage once daily for 16 days at dose levels of 30 mg/kg body weight. After treatment, mice were sacrificed and liver tumors were isolated for further testing. Sorafenib tosylate (BAY 43-9006) was dissolved in Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethyl alcohol) at 4-fold (4×) of the highest dose, foil wrapped, and stored at room temperature. This 4× stock solution was prepared fresh every 3 days. Final dosing solutions were prepared on the day of use by dilution of the stock solution to 1× with water. Lower doses were prepared by dilution of the 1× solution with Cremophor EL/ethanol/water (12.5:12.5:75).

All animal studies were performed under the supervision and guidelines of the University of Rochester Medical Center Animal Care and Use Committee.

Patient selection and Immunohistochemistry (IHC) staining

We randomly selected HCC samples from 81 male patients, which have been collected at the Sir Run-Run Shaw Hospital starting in February 2008 and this project was approved by the Institutional Review Board/Privacy Board of Sir Run-Run Shaw Hospital. We reviewed pathology records to identify samples with confirmed hepatocellular carcinoma. The IHC slides of all 81 patients used for AR and IL-12A scoring were reviewed by two pathologists in a double blind manner. The staining results were measured semiquantitatively on a scale of (–), (+), (++) and (+++). A stain was scored as follows: (–), there was less than 10% staining of nuclear AR in any of the tumor cells per field or no cytoplasm staining of IL-12A in tumor cells; (+), there was nuclear AR staining in 10% to 30% of the tumor cells with any

intensity, or faint, barely discernable cytoplasmic staining for IL-12A; (++) , there was staining in 30% to 50% of the tumor cells with moderate to strong intensity of nuclear AR, or moderate, smooth cytoplasmic staining of the tumor cells with moderate intensity for IL-12A; and (+++), there was staining in more than 50% of the tumor cells nuclei with strong intensity of AR, or apparent granularity, dark brown staining seen in cytoplasm for IL-12A. Representative examples of (-), (+), (++) and (+++) IHC staining for AR and IL-12A are demonstrated in Fig 4. For CD56 staining, we chose 33 patients' sample which could be clearly identified as non tumor, paritumor and intratumor region.

HE staining of tumor samples followed standard HE staining procedures. IHC stains for AR and IL-12A were performed using the standard streptavidin-biotin-peroxidase immunostaining procedure. The antibody used for anti-AR and anti-IL-12A was the same as used with Western blot with the concentration raised to 1:100. The CD56 antibody (bs-0736R) for NK cells staining was purchased from Bioss Antibodies (Woburn, MA). Appropriate positive and negative controls were used in each case.

Statistical Analysis

Data are expressed as mean $\pm$ SEM from at least 3 independent experiments. Statistical analyses involved unpaired t-test, one-way ANOVA and Spearman correlation with SPSS 17.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

AR decreases NK cells cytotoxicity in HCC

We first examined the endogenous AR expression in various HCC cell lines (these cell lines were all isolated from male HCC patients) and found AR was differentially expressed with higher expression in SNU423 and little expression in HepG2 cells (Supplemental Fig. S1). We then stably transfected full-length AR into SK-Hep1 cells (named SKAR3 and SKAR7) and applied MTT cell viability assay after treating HCC cells with NK-92MI cells for 6 hrs (E:T ratio at 2:1) (see Fig. 1A for detailed procedure). The results revealed that higher AR expression might lead HCC cells (both SKAR3 and SKAR7) to have better survival rates upon immunotherapy with NK cells (Fig. 1B). Similar results were also obtained when we replaced SKAR cells with the HCC HA22T cells with stable AR expression (Fig. 1B).

We also applied LDH cytotoxic assay (26) to these HCC cells after treating with NK-92MI cells for 4 hrs (E:T ratio at 15:1 based on multiple ratio results, see Supplemental Fig. S2), and results again confirmed that with higher AR expression these HCC cells were more resistant to NK cells cytotoxicity (Fig. 1C).

We then applied an opposite approach using AR-shRNA to knockdown AR in the two HCC AR-positive cell lines (SK-Hep1 and SNU423), and results confirmed previous studies showing targeting AR might result in HCC cells being more sensitive to NK cells treatment (Fig. 1D).

Since NK cells could induce target cell apoptosis, we also examined the AR impacts on HCC cells apoptosis *via* tunnel assay (27). As shown in Fig. 1E, much higher apoptosis rates

were seen in HCC SK cells with lower AR expression as compared with those with higher AR expression. And similar results could be obtained when using SNU423 cells (Supplemental Fig. S3).

Together, results from Figs. 1, S1, S2 and S3 suggest that altering AR expression can influence NK cells cytotoxicity to kill HCC cells.

Targeting AR alters IL-12A expression at both mRNA and protein levels in HCC cells

To dissect the molecular mechanisms by which AR could influence NK cells activation to better kill HCC cells, we used qPCR focus array to screen NK cells related cytokines and ligands and found the mRNA of some selective cytokines and ligands were altered in HCC cells upon altering the AR expression. We narrowed down the targets by using different HCC cell lines with overexpressed or knocked down AR (Fig. 2A-E). We then focused on IL-12A since an early study indicated that IL-12 might play key roles in immunotherapy for HCC (20) and only changes of IL-12A were consistent in all the HCC cell lines we tested. We further confirmed these focus array results by western blot analysis, and results revealed IL-12A protein was suppressed after adding AR in HCC SK-AR3, SK-AR7, HA22T and HepG2 cells (Fig. 2F). In contrast, IL-12A protein was increased after knocking-down AR in SK-Hep1 and SNU423 cells (Fig. 2G). Such results were also confirmed when we used ELISA to detect IL-12A in culture media collected from HCC cells (Supplemental Fig. S4).

Interestingly, we found the IL-12B mRNA remained unchanged or changed in an opposite manner after altering the AR expression level (Fig. 2A, C and D). Western blot analysis using human IL-12 polyclonal antibody further confirm that only IL-12A, any not IL-12B, was suppressed after adding AR (Fig. 2H).

Because IL-12 was secreted into media during culture of HCC cells, we then examined if the conditioned media (CM) from higher AR expressed HCC cells could suppress the cytotoxicity of NK cells. The results revealed that the CM from cultured HA22T-AR, not HA22T-vector, made parental cells become more resistant to NK cells cytotoxicity (Fig. 2I, left panel). Similar results were also obtained when we replaced HA22T-AR cells with SKAR3 or SKAR7 cells (Fig. 2I, right panel).

Since activated NK cells could function through releasing more IFN- γ to exert their cytotoxicity, we then examined whether IFN- γ release was altered by stimulating NK-92MI cells with CM collected from HA22T-AR vs HA22T-vector control cells, and results revealed less IFN- γ release in HA22T-AR (as well as SKAR3 or SKAR7 cells) groups compared with vector-control groups (Fig. 2J).

Together, results from Fig. 2 suggest that targeting AR in HCC cells could suppress IL-12A expression at both mRNA and protein levels.

AR alters NK cells cytotoxicity *via* suppressing IL-12A expression in HCC cells

To prove targeting AR suppressed IL-12A expression in HCC cells is linked to altering the NK cells cytotoxicity, we then applied the interruption approach *via* adding recombinant human IL-12 to pre-activate serum starved NK-92MI cells and then performed the cytotoxic

assay (Fig. 3A). Results revealed adding exogenous IL-12 partially reversed the NK cells cytotoxicity in higher AR expressing HCC cells (Fig. 3B). We also applied the opposite approach using sh-IL-12A to knockdown IL-12A in HCC cells, and results revealed that after suppression of IL-12A, suppression of AR with sh-AR in HCC cells could no longer increase NK cells cytotoxicity in tumor cells (Fig. 3C).

Furthermore, we applied human IL-12 neutralizing antibody in culture media to treat HCC cells for 24 hrs, then added NK-92MI cells to perform cytotoxic assay. The results verified our conclusion that IL-12 was the major factor involved in AR induced cytotoxicity difference (Fig. 3D).

Together, results from Fig. 3A-D suggest that AR may function through modulating the IL-12A expression to alter the NK cells cytotoxicity in HCC cells.

IL-12A is negatively correlated with AR in human clinical HCC samples

To prove our above *in vitro* cell lines results in human clinical HCC, we applied the MAGNET V2.0 (<http://magnet.case.edu/>) (28), which is designed to analyze the possible linkage between genes based on published databases. We used the TCGA liver cancer database and performed Spearman test on this software and the results suggested there is a negative correlation (Correlation coefficient = -0.298 , $P=0.004564$) between AR and IL-12A among clinical HCC samples (Supplemental Fig. S5).

We then applied human HCC samples from Sir Run-Run Shaw Hospital and stained AR and IL-12A for correlation analysis. We classified the results into four grades based on the review from pathologists and representative images are shown in Fig. 4A and 4B. The results indicated the negatively correlated expression (Fig. 4C and D) and Spearman correlation analysis also identified a moderate negative correlation between AR and IL-12A in human HCC tumor tissues ($R=-0.3329$, $P=0.0024$).

We also tested for CD56+ NK cells in our patient samples, which showed a decreasing trend for NK cells through non-tumor to intra-tumor tissue (Supplemental Fig. S6). These results also confirmed a previous study about NK cells in HCC (29). Unfortunately, we failed to see that AR might have an effect on NK cells recruitment.

Together, results from Fig. 4A-D and Fig. S3 are consistent with our hypothesis showing IL-12A is negatively correlated with AR in human clinical HCC samples.

AR modulates IL-12A expression at the transcriptional level

To dissect the molecular mechanism how AR modulates IL-12A protein and mRNA, we searched for the potential androgen receptor elements (ARE) in the IL-12A promoter (30) using PROMO analysis (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and found 5 AREs located in the IL-12A promoter region (Fig. 4E). We then applied chromatin immunoprecipitation (ChIP) assays to examine if AR could modulate the IL-12A expression *via* direct binding to its promoter region in SK-Hep1 cells, and results revealed that AR could bind to the 5th ARE (GGACAATTA), but not other AREs (Fig. 4F). We then constructed a luciferase reporter gene linked to the IL-12A promoter to

assay if AR can suppress this reporter, and results revealed that AR could suppress IL-12A expression at the transcriptional level (Fig. G, lane 2 vs 1). Importantly, when we mutated (9bp) this ARE and performed the promoter luciferase reporter assay, this mutation diminished the AR function on this promoter, suggesting AR may bind directly to this 5th ARE to modulate the IL-12A expression at the transcriptional level (Fig. 4G, lane 4 vs 3). Interestingly, while our results showed AR can bind directly to this 5th ARE, we detected there is only half of the canonical ARE (31). It will be interesting to determine in the future whether AR may interact with other factors to form a heterodimer or complex to bind to this half ARE to modulate the IL-12A transcription.

Combining Sorafenib with NK cells immunotherapy enhances efficacy to better suppress HCC progression

All above results concluded that targeting AR to increase IL-12A signals could lead to higher efficacy for NK cells to target HCC tumor cells. We were interested to see if any of the available drugs used in the current therapy for HCC can be applied here to target the AR expression. Interestingly, we noticed that sorafenib, a currently used drug to suppress HCC (32), was able to decrease AR in prostate cancer (33).

We first performed a relatively long term cell viability assay. We treated HCC cells with 4 μ M sorafenib and NK-92MI cells simultaneously with a E:T ratio at 1:2 for 48 hours, and results revealed that treating with sorafenib and NK cells together suppressed more HCC cells than either single therapy in both SNU423 and SK-Hep1 cells (Fig. 5A). Importantly, Western blots results revealed sorafenib could suppress AR expression in SNU423 and SK-Hep1 cells (Fig. 5B), which may then increase IL-12A expression (Fig. 5B). Detecting IL-12p35 in culture media also reached the same conclusion (Supplemental Fig. S7). Interestingly, the higher dose of sorafenib failed to further increase IL-12A expression, this may due to the obvious cytotoxicity to tumor cells when increasing the dosage. Using an interruption approach with AR-shRNA to knockdown AR blocked sorafenib-suppressed IL-12A expression, suggesting sorafenib may alter the IL-12A expression *via* decreasing AR (Fig. 5C). Also, supplying ectopic AR by lentivirus in SK-Hep1 and AR negative HepG2 cells could further prove that AR is necessary for sorafenib induced up-regulation of IL-12A (Supplemental Fig. S8). In AR negative HepG2 cells, sorafenib failed to increase IL-12A. And sorafenib could not increase IL-12A after providing ectopic AR in SK-Hep1. Next we treated two HCC cell lines (with/without knocked down AR) with 4 μ mol sorafenib and then tested their susceptibility to NK cells cytotoxicity. The results suggested without AR the sorafenib could only elevate the NK cytotoxicity against tumor cells in a limited manner (Fig. 5D). And we assumed this limited elevation may be explained by a previous finding about sorafenib function on NKG2D ligand shedding (34).

Together, results from Fig. 5 suggest that sorafenib could enhance cytotoxicity of NK cells against tumor through AR-IL12A signals.

AR expression level affected cytotoxicity of NK-92MI cells in orthotopic HCC mice model

To confirm all the above *in vitro* cell lines data in the *in vivo* mouse model, we developed an orthotopic xenograft nude mouse model by co-injecting HCC tumor cells (SK-Hep1, SK-

AR7 and SK-pBABE) w/wo NK-92MI cells at a ratio of 5:1 into the left lobes of nude mice livers. Using IVIS detection system, we found that groups only injected with tumor cells had tumor formation after 2 weeks while groups with NK-92MI cells injected could develop tumors only after 4 weeks.

Eight weeks after injection, the IVIS images showed SK-pBABE tumors were smaller than SKAR7 cells tumors when they were co-injected into liver with NK cells, and their control groups did not show obvious size differences on IVIS image (Fig. 6A). This may be explained by the tumor formation mechanism in our mouse model since previous we used DEN induced HCC in AR knock-out mice to determine the AR role in tumorigenesis (8). We then sacrificed mice and identified co-injected NK-92MI could effectively inhibit tumor formation and progression (Fig. 6B). Results from the tumor weight measurements and suppression rate calculation further verified our conclusion showing tumor cells with higher AR expression had less cytotoxicity from NK cells immunotherapy in our orthotopic xenograft mouse model (Fig. 6C). We also performed IHC staining in these tumor samples, and obtained positive signals in orthotopic tumors but not in adjacent normal liver cells (Fig. 6D), suggesting the repressed IL-12A expression in SKAR7 tumor, which supported our hypothesis (Fig. 6E and Supplemental Fig. S9 left panel).

Importantly, in the SK-Hep1 parental tumor model, we treated the mice with sorafenib or vehicle for 3 weeks followed by organ collection and examination of IL-12A expression with IHC staining. The results revealed that the IL-12A was up-regulated in the sorafenib treatment group (Fig. 6F and Supplemental Fig. S9 right panel).

Discussion

Since gender disparity of HCC was regarded as a key to fully understand initiation and progression of liver cancer, we investigated the potential linkage between AR and innate immune surveillance during HCC progression. By revealing IL-12A was transcriptionally suppressed as a down-stream gene of AR, we found AR in HCC not only promotes its initiation in an autonomous manner, but also regulates the tumor microenvironment through inhibiting the key component of the innate immune system, NK cells (Fig. 6G). These findings provide valuable information for future immune and hormone therapies for liver cancer.

We found that AR can modulate IL-12A expression, suggesting that it is possible to increase IL-12 expression *via* suppressing AR, which may have clinical significance since IL-12 has been identified as a powerful activator for several cytotoxic immune cells for suppression of the tumor progression (35). To date, IL-12 alone still could not serve as a desirable clinical approach to treat malignances. Fortunately, researchers observed better tolerance of IL-12 in patients by an advanced approach using adenovirus encoded IL-12 (36), therefore shedding new light on IL-12 related therapy. The limitation for extensive application of IL-12 treatment for malignancy mainly comes from the severe toxicity (37). In this field, researchers mainly focused on genetically engineered approaches to overexpress IL-12 in tumors while the IL-12 naturally secreted by tumor cells themselves still remain largely unstudied due to the low abundance issue. Actually, cytokines secreted from tumor cells

have been identified long ago (38) and we believe our findings may provide novel understandings for future cancer immune therapy. Based on our results, we may have better criteria for patient selection for IL-12 treatment by considering the AR expression level in HCC tissues from each patient. And more importantly, considering AR performs as an important player in the tumor microenvironment, we could develop better IL-12 related immune therapies *via* effectively suppressing AR in HCC tumors and because of the low basal level of IL-12, patients may receive a relatively mild elevation of IL-12 with tolerable side effects.

An early study also indicated that cytotoxicity of NK cells might be modified by altering the NKG2D ligand status, and addition of sorafenib might suppress NKG2D ligand shedding on HCC cells then assisting NK cells to better target tumor cells (34). However, the cells they used, HepG2, are AR negative cells. It will be interesting to investigate more of the immune-regulation of chemotherapy drugs in liver cancer since this may provide valuable information to improve drug effects on advanced HCC by combining patients' immune system status (39).

Interestingly, there is only half of the canonical ARE identified by prediction and ChIP in the IL-12A promoter. Normally AR needs to function as a homo-dimer to bind to ARE. However, there is another report demonstrating that AR could bind to half-site-like sequences and still function transcriptionally (40), which may support our findings here.

In summary, these results demonstrated that AR could function through regulation of the innate immune system to influence HCC progression. Together with previous findings showing AR could also function through modulating HBV virus replication and HCC cells transformation, our results suggest AR may play multiple functions to influence HCC progression (5), and targeting AR with ASC-J9® or other small molecules may be developed to suppress HCC progression more effectively in near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Karen Wolf for help with manuscript preparation. We appreciate Dr. Zhinong Jiang and Dr. Tao Zhu from Sir Run-Run Shaw Hospital for kindly reviewing and scoring IHC staining of patient specimens.

Financial Support: This work was supported by NIH grants (CA155477 and CA156700) to C. Chang, George Whipple Professorship Endowment and Taiwan Department of Health Clinical Trial to C. Chang, Research Center of Excellence (DOH99-TD-B-111-004 to China Medical University, Taichung, Taiwan) to C. Chang, International scientific and technological cooperation projects (2012DFA30410, China) to X.J. Cai, Zhejiang Provincial Natural Science Foundation of China (LZ14H160002) to H. Lin, National Natural Science Foundation of China (81201942) to H. Lin, and the National Science-technology Support Plan Projects (2012BAI14B06, China) to X.J. Cai.

Abbreviations

AR	androgen receptor
NK cells	natural killer cells

IL-12	interleukin 12
HCC	hepatocellular carcinoma
E:T	effector cell:target cell
IFN-γ	interferon- γ
ARE	androgen receptor element

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA: a cancer journal for clinicians*. 2015; 65:87–108. [PubMed: 25651787]
2. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology*. 2004; 127:S35–50. [PubMed: 15508101]
3. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology*. 2004; 127:S5–S16. [PubMed: 15508102]
4. Shi L, Feng Y, Lin H, Ma R, Cai X. Role of estrogen in hepatocellular carcinoma: is inflammation the key? *Journal of translational medicine*. 2014; 12:93. [PubMed: 24708807]
5. Ma WL, Lai HC, Yeh S, Cai X, Chang C. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. *Endocrine-related cancer*. 2014; 21:R165–82. [PubMed: 24424503]
6. Yuan JM, Ross RK, Stanczyk FZ, Govindarajan S, Gao YT, Henderson BE, et al. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. *International journal of cancer Journal international du cancer*. 1995; 63:491–3. [PubMed: 7591255]
7. Yu L, Kubota H, Imai K, Yamaguchi M, Nagasue N. Heterogeneity in androgen receptor levels and growth response to dihydrotestosterone in sublines derived from human hepatocellular carcinoma line (KYN-1). *Liver*. 1997; 17:35–40. [PubMed: 9062878]
8. Wu MH, Ma WL, Hsu CL, Chen YL, Ou JH, Ryan CK, et al. Androgen receptor promotes hepatitis B virus-induced hepatocarcinogenesis through modulation of hepatitis B virus RNA transcription. *Science translational medicine*. 2010; 2:32ra5.
9. Ma WL, Hsu CL, Yeh CC, Wu MH, Huang CK, Jeng LB, et al. Hepatic androgen receptor suppresses hepatocellular carcinoma metastasis through modulation of cell migration and anoikis. *Hepatology*. 2012; 56:176–85. [PubMed: 22318717]
10. Ma WL, Jeng LB, Lai HC, Liao PY, Chang C. Androgen receptor enhances cell adhesion and decreases cell migration via modulating beta1-integrin-AKT signaling in hepatocellular carcinoma cells. *Cancer letters*. 2014; 351:64–71. [PubMed: 24944078]
11. Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, et al. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer cell*. 2006; 10:99–111. [PubMed: 16904609]
12. Yang JD, Nakamura I, Roberts LR. The tumor microenvironment in hepatocellular carcinoma: current status and therapeutic targets. *Seminars in cancer biology*. 2011; 21:35–43. [PubMed: 20946957]
13. Leonardi GC, Candido S, Cervello M, Nicolosi D, Raiti F, Travali S, et al. The tumor microenvironment in hepatocellular carcinoma (review). *International journal of oncology*. 2012; 40:1733–47. [PubMed: 22447316]
14. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. *Immunological reviews*. 2000; 174:5–20. [PubMed: 10807503]
15. Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology*. 2001; 120:250–60. [PubMed: 11208734]
16. Manetti R, Parronchi P, Giudizi MG, Piccinini MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *The Journal of experimental medicine*. 1993; 177:1199–204. [PubMed: 8096238]

17. Scott P. IL-12: initiation cytokine for cell-mediated immunity. *Science*. 1993; 260:496–7. [PubMed: 8097337]
18. Harada N, Shimada M, Okano S, Suehiro T, Soejima Y, Tomita Y, et al. IL-12 gene therapy is an effective therapeutic strategy for hepatocellular carcinoma in immunosuppressed mice. *J Immunol*. 2004; 173:6635–44. [PubMed: 15557154]
19. Chang CJ, Chen YH, Huang KW, Cheng HW, Chan SF, Tai KF, et al. Combined GM-CSF and IL-12 gene therapy synergistically suppresses the growth of orthotopic liver tumors. *Hepatology*. 2007; 45:746–54. [PubMed: 17326190]
20. Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, et al. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology*. 2001; 33:52–61. [PubMed: 11124820]
21. Yamashita YI, Shimada M, Hasegawa H, Minagawa R, Rikimaru T, Hamatsu T, et al. Electroporation-mediated interleukin-12 gene therapy for hepatocellular carcinoma in the mice model. *Cancer research*. 2001; 61:1005–12. [PubMed: 11221826]
22. Sacco S, Heremans H, Echtenacher B, Buurman WA, Amraoui Z, Goldman M, et al. Protective effect of a single interleukin-12 (IL-12) predose against the toxicity of subsequent chronic IL-12 in mice: role of cytokines and glucocorticoids. *Blood*. 1997; 90:4473–9. [PubMed: 9373257]
23. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood*. 1997; 90:2541–8. [PubMed: 9326219]
24. Chen CF, Hsu EC, Lin KT, Tu PH, Chang HW, Lin CH, et al. Overlapping high-resolution copy number alterations in cancer genomes identified putative cancer genes in hepatocellular carcinoma. *Hepatology*. 2010; 52:1690–701. [PubMed: 20799341]
25. He D, Li L, Zhu G, Liang L, Guan Z, Chang L, et al. ASC-J9 suppresses renal cell carcinoma progression by targeting an androgen receptor-dependent HIF2alpha/VEGF signaling pathway. *Cancer research*. 2014; 74:4420–30. [PubMed: 24924778]
26. Wang XH, Qin Y, Hu MH, Xie Y. Dendritic cells pulsed with gp96-peptide complexes derived from human hepatocellular carcinoma (HCC) induce specific cytotoxic T lymphocytes. *Cancer immunology, immunotherapy : CII*. 2005; 54:971–80. [PubMed: 15965645]
27. Vujanovic NL, Nagashima S, Herberman RB, Whiteside TL. Nonsecretory apoptotic killing by human NK cells. *J Immunol*. 1996; 157:1117–26. [PubMed: 8757616]
28. Linderman GC, Chance MR, Bebek G. MAGNET: MicroArray Gene expression and Network Evaluation Toolkit. *Nucleic acids research*. 2012; 40:W152–6. [PubMed: 22669910]
29. Wu Y, Kuang DM, Pan WD, Wan YL, Lao XM, Wang D, et al. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology*. 2013; 57:1107–16. [PubMed: 23225218]
30. Liu J, Cao S, Herman LM, Ma X. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *The Journal of experimental medicine*. 2003; 198:1265–76. [PubMed: 14568984]
31. Dehm SM, Tindall DJ. Androgen receptor structural and functional elements: role and regulation in prostate cancer. *Molecular endocrinology*. 2007; 21:2855–63. [PubMed: 17636035]
32. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine*. 2008; 359:378–90. [PubMed: 18650514]
33. Oh SJ, Erb HH, Hobisch A, Santer FR, Culig Z. Sorafenib decreases proliferation and induces apoptosis of prostate cancer cells by inhibition of the androgen receptor and Akt signaling pathways. *Endocrine-related cancer*. 2012; 19:305–19. [PubMed: 22383427]
34. Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A, et al. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. *Hepatology*. 2010; 51:1264–73. [PubMed: 20099300]
35. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunological reviews*. 2004; 202:96–105. [PubMed: 15546388]

36. Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I, et al. Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2004; 22:1389–97. [PubMed: 15084613]
37. List T, Neri D. Immunocytokines: a review of molecules in clinical development for cancer therapy. *Clinical pharmacology : advances and applications*. 2013; 5:29–45. [PubMed: 23990735]
38. Stonans I, Stonane E, Russwurm S, Deigner HP, Bohm KJ, Wiederhold M, et al. HepG2 human hepatoma cells express multiple cytokine genes. *Cytokine*. 1999; 11:151–6. [PubMed: 10089137]
39. Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho HI, Antonia S, et al. Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *The Journal of clinical investigation*. 2010; 120:1111–24. [PubMed: 20234093]
40. Chen Z, Lan X, Thomas-Ahner JM, Wu D, Liu X, Ye Z, et al. Agonist and antagonist switch DNA motifs recognized by human androgen receptor in prostate cancer. *The EMBO journal*. 2015; 34:502–16. [PubMed: 25535248]

Fig. 1. Androgen receptor induces resistance of HCC cells to NK cytotoxicity. (A) Schematic diagram for procedure of cytotoxicity detection. (B) MTT cell viability assay was performed after co-incubating serum starved NK-92MI cells with HCC cells for survival rate calculations. (C and D) LDH cytotoxic assay was performed by adding serum starved NK-92MI cells into HCC cells with E:T ratio at 15:1 (upper panels). Overexpressed or knocked down AR efficiency were shown in lower panels using WB. %Cytotoxicity was calculated as described in method section. (E) For apoptosis signal detection, we added serum starved NK-92MI cells to HCC cells with an E:T ratio at 1:1 and incubated for 24 hours, then performed TUNEL staining. The left panel shows positive signals and the right panel is quantification data, mean $\pm$ SEM. Negative control groups were HCC cells without added NK cells. *** P< 0.001, ** P<0.01, * P<0.05.

Fig. 2.

Androgen receptor decreases IL-12 α at both mRNA and protein levels. (A-E) RT-qPCR screening results narrowed down the possible responsible factors related to NK cells activation. In all three AR-overexpressed HCC cell lines and two knocked-down cell lines, IL-12A was found negatively correlated with AR expression. (F and G) Western blots using IL12A-specific antibodies of selected factors also demonstrated. (H) Western blots performed with human IL-12 polyclonal antibody to show IL-12A changed while IL-12B did not. Recombinant IL12 was used as control. (I) We collected conditioned media (CM) from cells with higher or lower AR expressions and treated parental HCC cells, then performed MTT viability assay to test NK cells cytotoxicity (HA22T, left panel; SK-Hep1, right panel). (J) We also used AR CM and Vector CM to stimulate NK-92MI cells, then tested IFN- γ release by human IFN- γ ELISA kit. The control group directly tests IFN- γ concentration in CM before treating with NK cells. Data shown are mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$.

Fig. 3.

AR compromises NK cells cytotoxicity by modulating IL-12A in HCC. (A) Schematic diagram for procedure of recombinant IL-12 application rescue assay. (B) The result showed cytotoxicity of NK-92MI cells was partially rescued by supplying exogenous IL-12. (C) Knocking down IL-12A by shRNA could interrupt AR induced cytotoxicity changes in HCC cells. Right panel indicates knock down efficiency for AR and IL-12A. (D) Neutralizing IL-12A in HCC culture media could significantly lower the difference of cytotoxicity reaction between high and low AR cells. Data shown are mean±SEM. ** P< 0.01, * P<0.05, NS=Not Significant.

Fig. 4.

AR and IL-12A were negatively correlated in human HCC samples and AR could transcriptionally down-regulate IL-12A by binding to its promoter. (A) Representative images for scoring the AR IHC staining. (B) Representative images for scoring the IL-12A IHC staining. (C) Representative images to show the comparison of AR and IL-12A staining in the same patient. (D) Spearman correlation analysis for AR and IL-12A based in our stained clinical samples (P value= 0.0339). (E) Predicted localization of AREs in IL-12A promoter region. (F) Chromatin immunoprecipitation was performed with SK-Hep1 cells and only the 5th ARE showed a strong binding to AR. (G) Wild type or 5th ARE mutant IL-12A promoter reporter construct was co-transfected with TK with a ratio at 1000:1 into SKAR and Vector cells. Luciferase reporter assay was performed after 48 hours incubation. Data shown are mean±SEM. * P<0.05, NS=Not Significant.

Figure 5.

Sorafenib treatment could decrease androgen receptor expression, therefore up-regulate IL-12A in HCC cells. (A) We seeded HCC cells in 24-well plates, and treated cells with different combinations (4 μM Sorafenib and NK-92MI cell with E:T at 1:2) for 48 hours, then performed MTT cell viability assays. (B) We treated two HCC cell lines with multiple doses of sorafenib and extracted protein after 48 hours, then used Western Blot to test AR and IL-12A expression changes. (C) After knocking down AR in two cell lines and treating with 4 μM sorafenib, IL-12A could no longer be increased by sorafenib. (D) After 48 hours sorafenib treatment, HCC cells w/o shAR were seeded for another 24 hours, then we performed LDH cytotoxic assays with a E:T ratio at 15:1. Data shown are mean±SEM/ ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, NS=Not significant.

Figure 6.

AR expression level affected cytotoxicity of NK-92MI cells in orthotopic HCC mice model. (A) Orthotopic HCC tumor mice model was monitored by IVIS image system before sacrificed mice at 8 weeks after injection. Representative images were shown with four groups together (left panel) or only NK-92MI cells co-injected groups comparisons (right panel). (B) We sacrificed mice at 8th week after injection and checked tumor formation with naked eyes. Yellow arrow showed portal lymph node metastasis. (C) Isolated orthotopic tumors from mice livers (left panel). Tumors were kept in PBS before weighing. Suppression rate of NK cells was calculated based on tumor weight (right panel). $\text{Suppression rate} = (\text{weight of tumor co-injected with NK cells} / \text{average weight of same type tumor without NK cells}) \times 100\%$. (D) HE staining identified tumor formation (left panel) and IL-12A staining (right panel) in tumor edge area indicated the specificity of antibody. (E) Representative images of IHC staining in SK-AR7 and SK-pBABE tumor samples, which demonstrated IL-12A expression was negatively correlated with AR expression level. (F) Representative images of IHC staining in SK-Hep1 tumors treated with sorafenib or vehicle, which showed sorafenib could induce IL-12A expression. (G) Schematic diagram for major pathways we demonstrated.