ARTICLE

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Imaging the Recruitment of Cancer-Associated Fibroblasts by Liver-Metastatic Colon Cancer

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ABSTRACT

The tumor microenvironment (TME) is critical for tumor growth and progression. However, the formation of the TME is largely unknown. This report demonstrates a color-coded imaging model in which the development of the TME can be visualized. In order to image the TME, a green fluorescent protein (GFP)-expressing mouse was used as the host which expresses GFP in all organs but not the parenchymal cells of the liver. Non-colored HCT-116 human colon cancer cells were injected in the spleen of GFP nude mice which led to the formation of experimental liver metastasis. TME formation resulting from the liver metastasis was observed using the Olympus OV100 small animal fluorescence imaging system. HCT-116 cells formed tumor colonies in the liver 28 days after cell transplantation to the spleen. GFP-expressing host cells were recruited by the metastatic tumors as visualized by fluorescence imaging. A desmin positive area increased around and within the liver metastasis over time, suggesting cancer-associated fibroblasts (CAFs) were recruited by the liver metastasis which have a role in tumor progression. The color-coded model of the TME enables its formation to be visualized at the cellular level in vivo, in real-time. This imaging model of the TME should lead to new visual targets in the TME. J. Cell. Biochem. 112: 949–953, 2011.

KEY WORDS: GFP AND RFP NUDE MICE; TUMOR MICROENVIRONMENT; STROMAL CELLS; CANCER-ASSOCIATED FIBROBLASTS; LIVER METASTASIS; COLOR-CODED IMAGING

The use of fluorescent proteins for imaging is revolutionizing in vivo biology [Hoffman, 2005, 2008]. Green fluorescent protein (GFP) and red fluorescent protein (RFP) have been shown to be able to be genetically linked with almost any protein providing a permanent and heritable label in live cells to study protein function and location. Many different colors of fluorescent proteins have now been produced in the laboratory or found in nature [Matz et al., 1999; Shaner et al., 2004]. With different color fluorescent proteins, many processes can be visualized simultaneously in cells. Thus, live cells can be multiply labeled for imaging processes that heretofore could be seen only on fixed and stained cells. The use of fluorescent proteins for imaging in vivo has been particularly useful to study tumor growth and progression [Hoffman, 2005].

With the use of multiple colored-proteins, we developed imaging of the tumor microenvironment (TME) by color-coding cancer and stromal cells. The TME is critical for tumor growth and progression. Indeed, cancer cells and stromal cells must replicate in parallel in order for the tumor to grow. Our original color-coded imaging technology of the TME used a GFP or RFP transgenic nude mouse as a host in which we transplanted cancer cells expressing a fluorescent protein not expressed by the host [Yang et al., 2003, 2004, 2009].

Recently, color-coded in vivo imaging has shown that stromal cells had higher motility in the microenvironment at the tumor periphery than within the tumor mass [Egeblad et al., 2008]. Solid tumors contain fibroblasts, lymphocytes, dendritic, macrophages, and other myeloid cells in their microenvironment [Egeblad et al., 2008].

Myofibroblasts and are a major population of carcinoma-associated fibroblasts (CAFs) [Kalluri and Zeisberg, 2006]. CAFs stimulate cancer cell growth, inflammation, angiogenesis, and invasion [Kalluri and Zeisberg, 2006; Gaggioli et al., 2007; Pietras

Abbreviations used: TME, tumor microenvironment; GFP, green fluorescent protein; RFP, red fluorescent protein; CAF, cancer-associated fibroblasts.

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et al., 2008; Erez et al., 2010]. As the tumor grows, it recruits CAFs [Orimo et al., 2005; Egeblad et al., 2010].

How the TME is formed in real time during cancer progression is not known. The present study utilizes color-coded fluorescent protein-based imaging to visualize the recruitment over time of stromal cells including CAFs by liver metastases of the colon cancer.

MATERIALS AND METHODS

CELL CULTURE

The HCT-116 human colon cancer cell line was originally obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 supplemented with 10% FCS. All media were supplemented with penicillin and streptomycin (Gibco BRL). The cell line was cultured at 37°C in a 5% incubator.

GFP TRANSGENIC MICE

Transgenic C57/B6-GFP mice [Okabe et al., 1997] were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). The C57/B6-GFP mice expressed the *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer. The GFP gene was crossed into nude mice on the C57/B6 background [Yang et al., 2004]. Transgenic C57/B6-RFP mice were obtained from Jackson Labs (Bar Harbor, ME). C57/B6-RFP mice expressed the RFP (DsREDT3) under the control of a chicken β-actin promoter and cytomegalovirus enhancer [Vintersten et al., 2004]. The RFP gene was crossed into nude mice (Harlan, Indianapolis, IN) on the BALB/c and NCR background [Yang et al., 2009]. All animal studies were conducted in accordance with the principles of and procedures outlined in the NIH guide for the care and use of laboratory animals under assurance number A3873-1.

NON-COLORED COLON CANCER CELL (HCT-116)-GFP HOST MODEL

Six-week-old GFP nude mice were used as the host for non-colored HCT-116 human colon cancer cells. Non-colored HCT-116 cells were first harvested by trypsinization and washed three times with cold serum-free medium and then resuspended with serum-free RPMI 1640 medium. GFP nude mice were anesthetized with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) injected into the peritoneal cavity. Non-colored human HCT-116 colon cancer cells (2.0×10^6) were injected in the spleen of GFP nude mice during open laparotomy in order for experimental liver metastases to form.

IN VIVO IMAGING

For in vivo imaging based on fluorescent proteins [Hoffman, 2005; Hoffman and Yang, 2006a,b,c], the Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) was used. The Olympus OV100, which contains an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for cellular imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macro-imaging as well as micro-imaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6× to 16× and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems) [Yamauchi et al., 2006].

HISTOLOGICAL ANALYSIS

The host livers were fixed with 10% buffered formalin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin. The fluorescent cell area was measured using the National Institutes of Health (NIH) Image analysis software program (available at http://rsb.info.nih.gov/nih-image/). Desmin was stained with anti-desmin antibody (Lab Vision, Fremont, CA) using avidin-biotin-peroxidase complex technique (Vector, Burlingame, CA). Measurement of positive area was performed using the NIH Image.

STATISTICAL ANALYSIS

All data represent the mean of at least three independent experiments ± SD. For the determination of statistical significance, unpaired Student's *t*-tests were performed. *P*-values of less than 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

FLUORESCENT ORGANS IN GFP AND RFP NUDE MICE

After sacrifice of the GFP and RFP transgenic nude mice, organs including lungs (Fig. 1A), kidney (Fig. 1B), esophagus (Fig. 1C), stomach (Fig. 1C), duodenum (Fig. 1C), small intestines (Fig. 1D), colon (Fig. 1D), the circulatory system, uterus and ovary, pancreas, brain, heart, and spleen (data not shown) were harvested, and imaged with the Olympus OV100 Small Animal Imaging System. All of the tissues from the RFP transgenic mouse, with the exception of erythrocytes, were red fluorescent under appropriate excitation light (Fig. 1). In contrast, although the other organs of the GFP-transgenic nude mice were brightly fluorescent, the liver of GFP nude mice fluorescence was weak except for the gallbladder (Fig. 1E).

IMAGING RECRUITMENT OF CANCER-ASSOCIATED FIBROBLASTS BY COLON CANCER LIVER METASTASIS

Human non-colored HCT-116-colon cancer cells were injected in the spleen of GFP nude mice. On day 28 after cancer-cell injection, GFP fluorescence was observed in the experimental liver metastatic colonies. High-magnification fluorescence microscopy showed extensive GFP fluorescence in the tumor. Liver sections were stained with hematoxylin-eosin, and GFP fluorescence of recruited GFP-expressing hosts cells in the liver metastasis was observed. There was a very large increase of GFP expression in the tumor compared to the non-tumor-bearing liver in nude mice. There was also significantly more GFP fluorescence in the liver metastasis

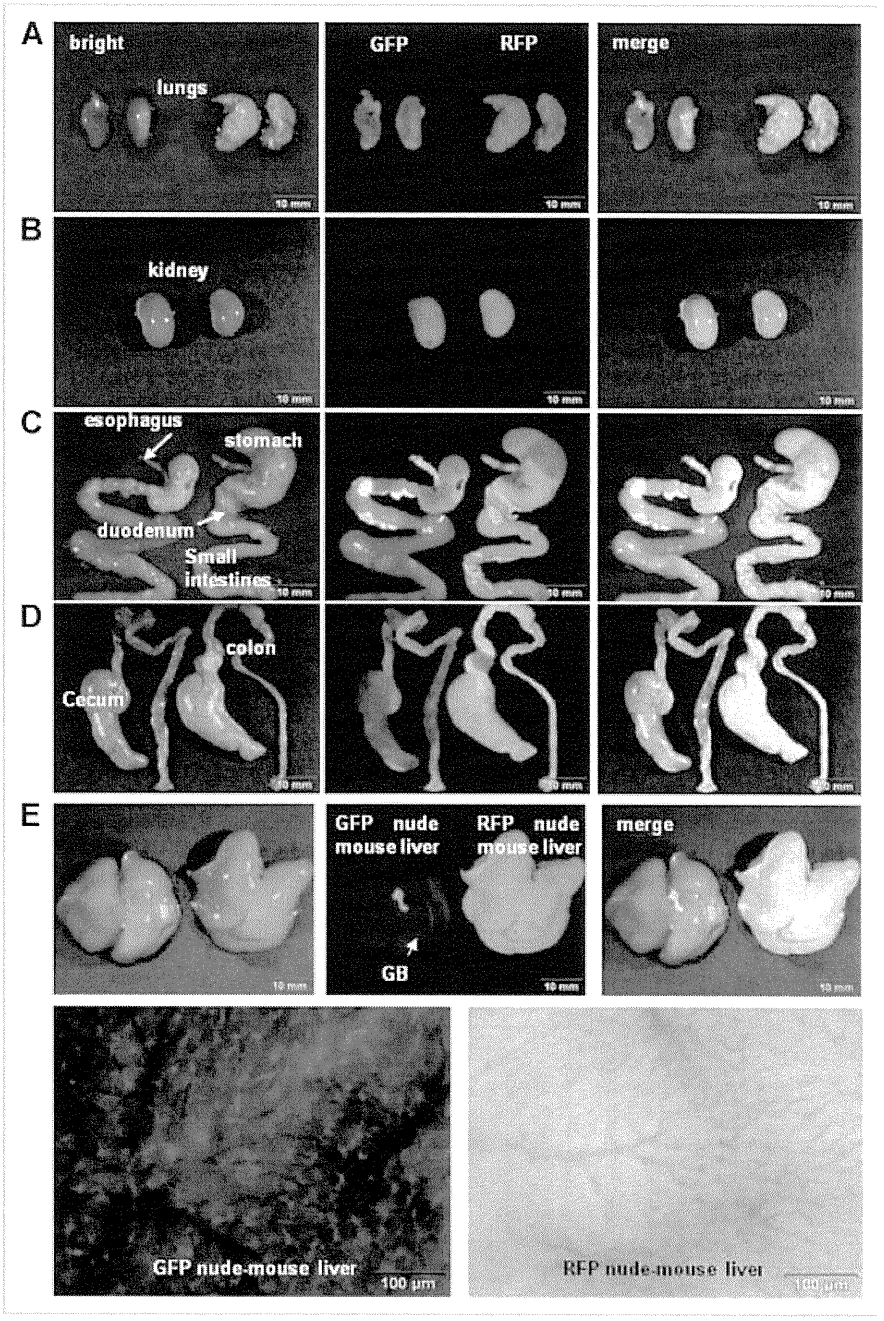

Fig. 1. Major organs of transgenic GFP and RFP nude mice. Fluorescence of organs after excitation with blue light. A: Lungs; (B) kidney; (C,D) digestive tract; (E) liver. Lower panel displayed high magnification (16×). The liver of GFP nude mice has very weak fluorescence.

compared to the non-tumor area of the liver ($P < 0.01$). Thus, the host GFP cells were imaged extensively accumulating in the liver metastasis. Expression of desmin was determined by immunohistochemistry with anti-desmin antibody and found to be positive in the tumor area. Thus, the liver metastasis contained GFP- and desmin-expressing cells, suggesting that cancer-associated fibroblasts (CAFs) were recruited and grew in the tumor (Fig. 2).

It is well known that CAFs have an important role in tumor progression [Erez et al., 2010]. CAFs have an increased rate of proliferation and differential expression of extracellular matrix (ECM) components and growth factors compared to normal fibroblasts [Bhowmick et al., 2004; Kalluri and Zeisberg, 2006; Erez et al., 2010]. CAFs promote tumor growth by inducing angiogenesis, recruiting bone marrow-derived endothelial progenitor cells, and remodeling the ECM [Olumi et al., 1999; Allinen et al., 2004; Orimo et al., 2005; Pietras et al., 2008; Erez et al., 2010]. CAFs can confer resistance to antiangiogenic therapy [Crawford et al., 2009; Erez et al., 2010]. CAFs also mediate tumor-enhancing inflammation mediated by NF-kappaB signaling [Erez et al., 2010].

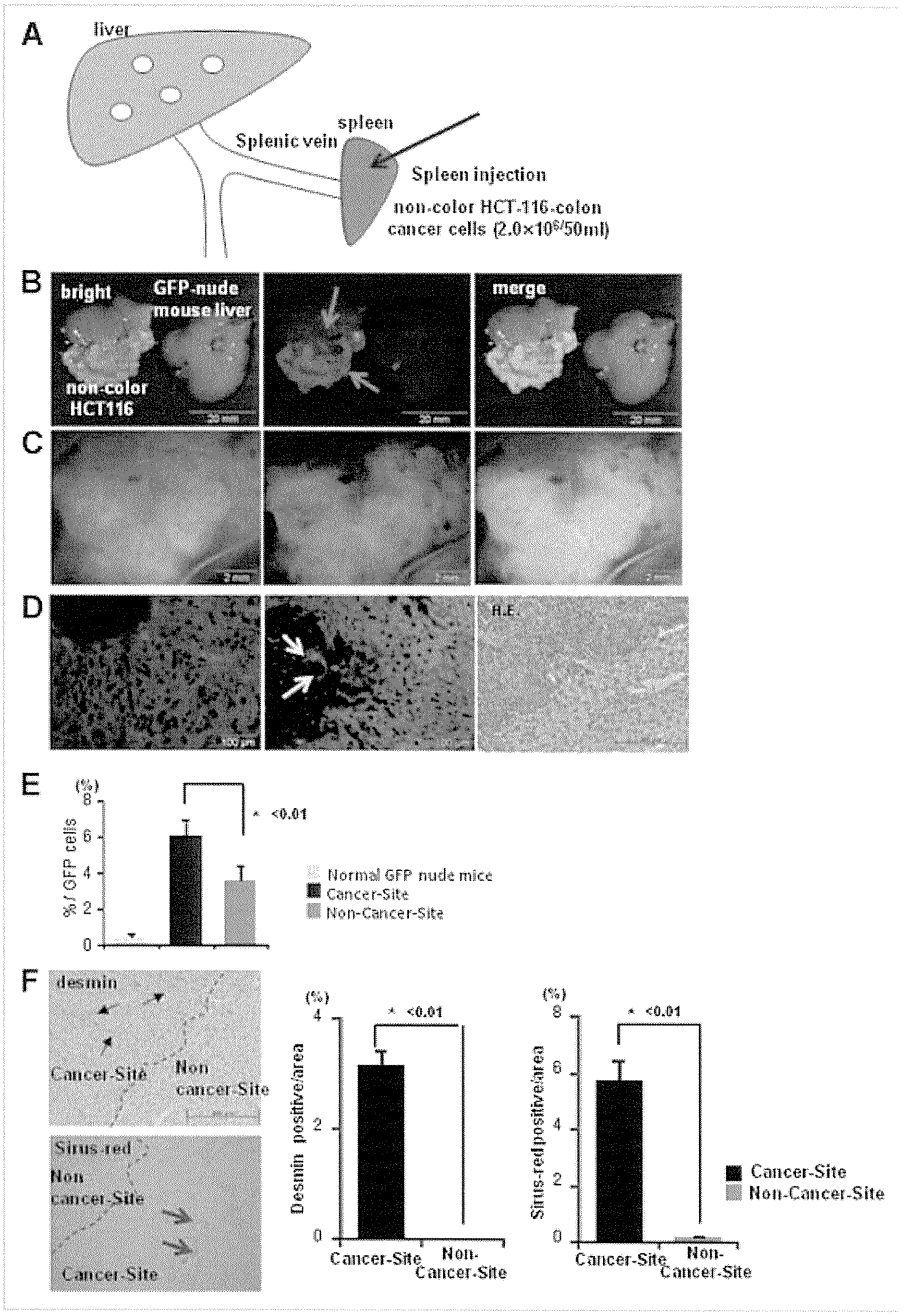

Fig. 2. Tumor–host interaction of non-colored human colon tumors in the liver of GFP nude mice. A: Schematic representation of experimental protocol. Human non-colored HCT-116-colon cancer cells (2.0×10^6/50 μl) were injected in the spleen of GFP nude mice during open laparotomy. B: On day 28 after cancer-cell injection, GFP fluorescence was observed in the liver metastasis (red arrows; left panel: bright field; central panel: fluorescence; right panel: merge; original magnification 0.14×). C: High magnification (left panel: bright-field; central panel: fluorescence; right panel: merge). D: Liver sections from GFP nude mice were stained with hematoxylin–eosin, and GFP fluorescence of non-parenchymal cells in the host GFP liver was observed as well (yellow arrows). E: There was significantly more GFP fluorescence in the metastasis than in the non-tumor part of the liver ($P < 0.01$). F: Expression of desmin was determined by immunohistochemistry with anti-desmin antibody (arrows; original magnification 100×). Measurement of the desmin-positive area was performed using the NIH Image analysis software program. Data are means ± SD from three independent experiments. Collagen deposition was assessed by Sirus red staining (blue arrows). The host GFP non-parenchymal cells were observed in the experimental liver metastasis. Non-parenchymal cells accumulated in tumors compared with normal liver. Liver metastasis contained GFP and desmin-expressing cells, suggesting that cancer-associated fibroblast cells have a role in metastasis.

The present report enables imaging of accumulation of CAFs of the tumor in real time.

The TME is critical for tumor growth and progression. However, the formation of the TME is largely unknown. This report demonstrates a color-coded imaging model in which the development of the TME can be visualized. In the GFP transgenic nude mice, only non-parenchymal cells of the liver have GFP fluorescence, which makes it a very useful model to image stromal development in the tumor, since GFP-expressing stromal cells were recruited by the metastatic tumors. A desmin-positive area increased around the

liver metastatic tumors over time, suggesting CAFs were recruited by the metastatic tumors and have an important role in tumor progression. Stromal cells essential for metastatic tumors to develop in the liver can be identified with the stromal imaging model described in this report.

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MOLECULAR CANCER

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Dual induction of caspase 3- and transglutaminase-dependent apoptosis by acyclic retinoid in hepatocellular carcinoma cells

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Abstract

Background: Hepatocellular carcinoma has a high mortality rate due to its rate of recurrence. Acyclic retinoid prevents recurrence of hepatocellular carcinoma in patients after surgical removal of their primary tumors by inducing apoptosis in hepatocellular carcinoma cells, although the molecular mechanisms of action are not understood.

Methods: Human hepatocellular carcinoma cells in culture, as well as nude mice transplanted with hepatocellular carcinoma cells and rats given with *N*-diethylnitrosamine were treated with acyclic retinoid. Changes in activated caspase 3 and transglutaminase 2 (TG2) levels, Sp1 cross-linking and its activities, expression of epidermal growth factor receptor, and apoptotic levels were measured.

Results: Acyclic retinoid simultaneously stimulated the activation of caspase 3, and the expression, nuclear localization and crosslinking activity of TG2, resulting in crosslinking and inactivation of the transcription factor, Sp1, thereby reducing expression of epidermal growth factor receptor and cell death in three hepatocellular carcinoma cell lines. These effects were partially restored by a caspase inhibitor, transfection of antisense TG2, restoration of functional Sp1, or an excess of epidermal growth factor. Nuclear expression of TG2 and crosslinked Sp1, as also activated caspase 3 were found in both hepatocellular carcinoma cells transplanted into nude mice and cancerous regions within the liver in *N*-diethylnitrosamine-induced hepatocarcinogenesis model in rats, following treatment of animals with acyclic retinoid.

Conclusions: Treatment with acyclic retinoid produces a dual activation of caspase 3 and TG2 induced apoptosis of hepatocellular carcinoma cells via modification and inactivation of Sp1, resulting in reduced expression of epidermal growth factor receptor.

Background

Hepatocellular carcinoma (HCC) has high mortality rate because of it frequent rate of recurrence [1]. Acyclic retinoid (ACR), a synthetic retinoid, prevents the recurrence and development of HCC in patients after surgical removal of the primary tumors by inducing apoptosis in HCC cells [2,3]. Retinoid X receptor (RXR) α is highly phosphorylated and loses its activity as a transcriptional factor during carcinogenesis in HCC [4]. ACR prevents this aberrant hyper-phosphorylation of RXRα by suppressing the Ras-extracellular signal regulated kinase (Erk) pathway, thereby restoring RXRα's activity in response to physiological concentrations of 9-*cis* retinoic acid (9-*cis* RA) [5]. We therefore proposed that this restoration of RXRα transcriptional activity is a basis for ACR's activity to control aberrant cell growth and induce apoptosis. However, the possibility that genes under the control of RARα/RXRv are upregulated by

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ACR, thereby mediating ACR's effect in suppressing aberrant growth and/or inducing apoptosis, has not been fully elucidated. ACR downregulates epidermal growth factor receptor (EGFR) signals due to suppression of transforming growth factor (TGF) α in both HCC cells and human squamous cell carcinoma cells undergoing apoptosis [6,7]. ACR induces the expression of interferon receptor, and also the expression and activity of signal transducer and activator of transcription (STAT) 1 during suppression of cell growth and induction of HCC cell apoptosis [8]. However, it is unclear whether these phenomena are dependent on the restoration of RARα/RXRα.

Transglutaminase 2 (TG2) is a member of a family of crosslinking enzymes that catalyze a post-translational modification of proteins by a calcium-dependent crosslinking reaction that forms N-ε (γ-glutamyl) lysine bonds [9-12]. TG2 has been implicated in apoptosis, although the mechanisms are unknown. Recently, we demonstrated that TG2 induces caspase-independent apoptosis in ethanol-treated hepatocytes by crosslinking and inactivation of the general transcription factor, Sp1, thereby reducing Sp1-dependent expression of growth factor receptors [9,13]. However, whether TG2-induced apoptosis pathway is involved in apoptotic signaling in other cell types or is induced by stimulation with anticancer reagents remains unclear.

Piedrafita *et al.* [14] reported that retinoid-induced apoptosis of T cells accompanies degradation of Sp1 downstream of the caspase pathway. Shao *et al.* [15] found that ACR inhibits the growth of HCC cells by reducing the expression of an Sp1-transactiviable gene, fibroblast growth factor receptor 3 (FGFR3) [16].

These reports suggest that Sp1 and/or its regulating genes are important in ACR-induced apoptosis pathway in HCC cells. We have therefore tested the hypothesis that ACR can restore the expression of TG2 by preventing phospho-inactivation of RXRα, and downregulate the expression of growth factor receptors by inactivating Sp1 due to both caspase-dependent degradation and TG2-dependent crosslinking. We have used HCC cells in culture and *in vivo* models of both transplantation of HCC into nude mice and *N*-diethylnitrosamine (DEN)-induced rat hepatocarcinogenesis.

Methods

Materials

ACR (NIK-333) was supplied from Kowa Company, Ltd. (Tokyo, Japan). Anti-TG2 monoclonal antibody (TGase II, Ab-1) was purchased from NeoMarkers (Fremont, CA). Anti-TG2 polyclonal antibody was produced as previously described [13]. Mouse anti-Sp1 (IC6), rabbit anti-Sp1 (PEP2), anti-EGFR, anti-c-Met, anti-FGFR1 antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA).

Mouse anti-GAPDH antibody was from Millipore (Billerica, MA). Anti-Bcl-X_L and anti-cleaved caspase 3 antibodies were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Viable cells were measured using a cell counting kit-8 (Dojindo; Tokyo, Japan). 5-(biotinamido) pentylamine, a biotinylated primary amine substrate for TG2 was provided by Pierce Biotechnology (Rockford, IL). A caspase-3 specific inhibitor, zDEVD-fmk, and Hoechst 33258 came from Calbiochem-Novabiochem (La Jolla, CA). Anti-crosslinked Sp1 (CLSp1) antibody was made in rabbits, and purified as previously described [13].

Cells and plasmids

A HCC cell line, JHH-7 cells kindly supplied by Dr. Matsuura (Jikei University School of Medicine, Tokyo, Japan) [17] were maintained in ASF104 medium (Ajinomoto, Tokyo, Japan). HC cells, a normal human hepatocyte cell line purchased from Cell Systems (Kirkland, WA), were cultured in CS-C complete medium (Kirkland, WA) [4]. HuH-7, HepG2, and HelaS3 cells were maintained in RPMI 1640 medium containing 10% FBS. The expression vector for human Sp1 (*Sp1-pCIneo*) was constructed as previously described [18]. The TG2, Sp1, and EGFR siRNA-expressing lentiviral vectors were constructed in the pSIH-H1 shRNA vector (SBI System Biosciences, CA). A GC3-Luc vector, containing 3 sequential repeats of GC box motifs derived from the *EGFR* promoter [19] and its TATA box sequence upstream of the luciferase cDNA, was generated by inserting a synthesized oligodeoxynucleotide cassette into the pGL3 vector (Promega Corp., WI).

Transient transfection

Transfections and assays of luciferase activity were performed with a combination of UNIFECTOR lipofection reagent (B-Bridge International, Inc.; Mountain View, CA) and luciferase reporter genes (firefly- and *Renilla*-Luc) as previously described [20], with further details being provided in the Additional file 1.

TG2 knockdown

Knockdown of TG2 was performed by transfection of anti-sense (AS) or siRNA to TG2 in JHH-7 cells, suppressing the expression of TG2 protein ~50% and ~70%, respectively (Additional file 2 Figure S1)

Preparation of whole lysates and nuclear extracts

Whole lysates were prepared in Hepes buffer containing 10 mM CHAPS and protease inhibitors. Nuclear extracts were prepared as previously described [20].

Western blotting

Western blotting was carried out as previously described [20], using combinations of 1 μg/ml each of anti-Sp1, anti-CLSp1, or anti-TG2 antibody and HRP-conjugated goat anti-rabbit/mouse IgG (1:1,000 dilution). Reactants were detected with Enhanced Chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was done as before [18], using sets of specific primers summarized in Additional file 3 Table S1.

Staining of cells

Cells grown on cover slips were fixed with 10% formalin in culture medium. They were permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with the antibodies given in each figure legend. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method with the *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH; Mannheim, Germany). Digital images of cells were obtained by confocal microscope (Carl Zeiss, Inc. Germany), and digital images recorded.

Animal experiments

One week after JHH-7 cells (2×10^6 /50 μl) had been transplanted into the spleens of nude mice aged 6 weeks (Balb/c Slc-nu/nu, Japan SLC Inc., Shizuoka, Japan), ACR (100 mg/kg/day) or vehicle (soybean oil) was administrated by gavage at 10 μl/g body weight once a day on consecutive days for 3 weeks. The DEN-induced rat hepatocarcinogenesis model was used as previously described [21]. Briefly, 6-week old rats (F344/N SLC; Japan SLC Inc., Shizuoka, Japan) were given drinking water containing 40 ppm DEN (Tokyo Kasei Kogyo Co., Tokyo, Japan) for 15 weeks to produce liver neoplasms. ACR (40 and 80 mg/kg) or vehicle (soybean oil) was administered orally with a stomach tube at 5 μl/g body weight for 14 weeks. Experiments were performed in accordance with protocols approved by the RIKEN Institutional Animal Use and the Care Administrative Advisory Committees.

Immunohistochemistry

Immunohistochemistry were performed as before [13]. Livers were removed, fixed in 10% formalin, and embedded in paraffin wax. Sections were prepared and stained with anti-CLSp1, anti-TG2, anti-cleaved caspase 3, and anti-EGFR polyclonal antibodies. Staining signals were enhanced using an ABC kit (VECTASTAIN) and developed with DAB substrate.

Statistical analysis

Quantitative data are given as means ± SD. Student's *t* test was used to evaluate differences between 2 groups. In comparing data from the vehicle group with those from groups treated with ACR at doses of 25, 50, and 100 mg/kg body weight, the level of serum AFP and the number of AFP-positive mice were analyzed by Dunnett's multiple comparison and Fisher's exact probability test, respectively. A p-value of <0.05 was considered statistically significant.

Results

ACR induces both caspase- and TG2-dependent apoptosis pathways

ACR induced TUNEL-positive apoptosis of JHH-7 cells, but not normal hepatocyte HC cells (Figure 1A, *left panel*). Apoptotic JHH-7 cells were also positive for crosslinked Sp1 (CLSp1; Figure 1A, *right panel*). Strong immunofluorescent spots were obvious in cells undergoing severe apoptosis (Figure 1A, *right panel*, arrows). JHH-7 cells were the most sensitive to ACR of the 3 HCC cell lines (JHH-7, HuH-7, HepG2) and HelaS3 cells (Figure 1B). They showed similar TG2/TUNEL/CLSp1-positive apoptosis following ACR treatment (*data not shown*). Consistent with previous findings with another HCC cell line (HuH-7) [6], ACR treatment of JHH-7 cells, but not HC cells, suppressed phosphorylation of RXRα without affecting the expression of RXRα (Additional file 4 Figure S2A), prevented phospho-inactivation of RXRα, and enhanced the expression of TG2 (Additional file 4 Figure S2B).

Reciprocally in parallel with a dose- and time-dependent decrease in cell number (Figure 1C, *left panels*), both TUNEL (Figure 1C, *middle panels*) and TG2 positivity (Figure 1C, *right panels*) increased in ACR-treated JHH-7 cells undergoing apoptosis. ACR-induced apoptosis was partially blocked by either the inclusion of the caspase inhibitor, z-DEVD (Figure 1D and 1E, *sample 3*) or knocking down by 50% TG2 expression with antisense (AS) TG2 (Figure 1D and 1E, *sample 4*; Additional file 2 Figure S1A), whereas apoptosis was almost completely blocked by their combined inhibition (Figure 1D and 1E, *sample 5*). These results suggest that ACR-induced apoptosis is dependent on both caspase 3 and TG2 activation.

In ACR-treated JHH-7 cells ACR had markedly increased levels of CLSp1 (Figure 1A, *right panel* and Additional file 5 Figure S3A, *lane 4*), whereas levels of the Sp1 monomer decreased (Additional file 5 Figure S3A, *lane 2*), thereby reducing its DNA binding activity (Additional file 5 Figure S3B) and transactivation activity (Additional file 5 Figure S3C), as previously seen in ethanol-induced hepatocyte apoptosis [13]. Impaired Sp1 activity was partially improved either by inhibition of caspase or TG2 knockdown by transfection of ASTG, and almost completely restored by their combination, as also by overexpression of Sp1. These results suggest that

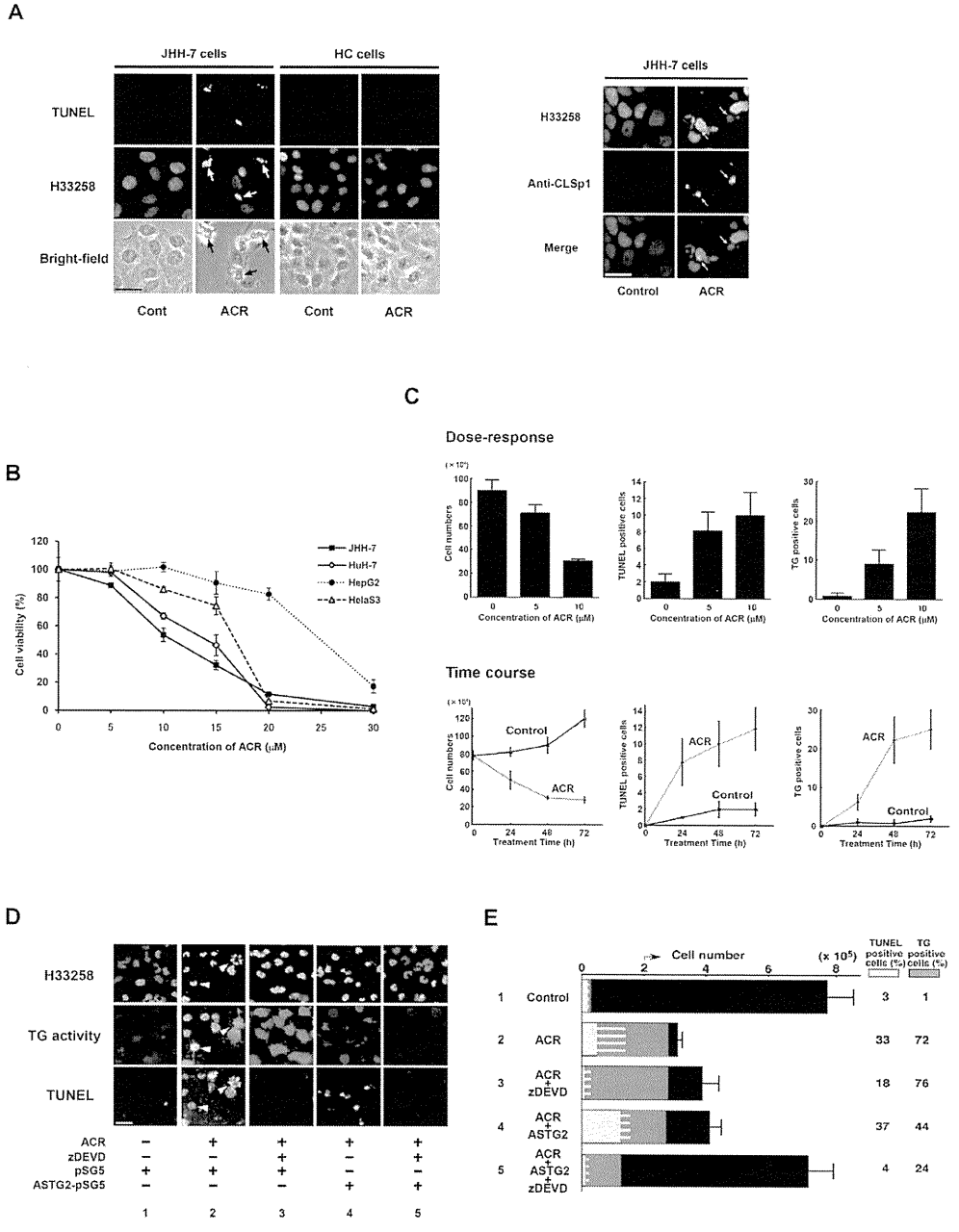

Figure 1 Induction of caspase 3 and TG2- dependent apoptosis by ACR in JHH-7 cell cultures. *A*, JHH-7 and HC cells were seeded in 35 mm dishes containing glass coverslips at 2 × 10^5/dish, and treated with 10 μM ACR or vehicle (0.1% ethanol) for 24 h. Cells were fixed and stained in combination with Hoechst 33258, and TUNEL (*left panels*) or anti-CLSp1 antibody (*right panel*). Scale bar, 50 μm. *B*, JHH-7, HuH-7, HepG2 and HelaS3 were seeded at 1 × 10^4 cells/96 well microplates and treated with the indicated concentrations of ACR or vehicle for 24 h. Viable cell counts are plotted as percentages of each control culture treated with vehicle. *C*, JHH-7 cells were seeded as before and treated either with the indicated concentrations of ACR for 48 h, or with 10 μM ACR for the indicated times. Cells were fixed and stained with Hoechst 33258, TUNEL, and anti-TG2 antibody. The numbers of total and apoptotic cells with TUNEL or TG2 positivity in each dish were counted and plotted. *D*, JHH-7 cells were seeded as before and transfected with either pSG5 or anti-sense (AS) TG2-pSG5. The next day cells were treated with either vehicle or 10 μM ACR for 24 h in the presence or absence of 100 μM zDEVD-fmk, with 1 mM 5-(biotinamido)-pentylamine being included during the last 2 h incubation. Cells were fixed and stained with Hoechst 33258 (*upper panels*), TRITC-conjugated streptavidin (*middle panels*), and TUNEL (*bottom panels*). Arrowheads indicate apoptotic cells with chromatin condensation. Scale bar, 50 μm. *E*, JHH-7 cells were treated as in (*C*). The numbers of total and apoptotic cells with TUNEL (green colors) or TG2 (orange colors) positivity in each dish were counted and plotted as bar graphs. Their percentages relative to total cell number are given on the right hand-side of each bar graph. Panels *A-E* show representative results from 3 different experiments with similar results.

both caspase- and TG2-dependent pathways lead to silencing of Sp1 activity, which correlates with cell viability (Additional file 5 Figure S3D).

Reduced expression of growth factor receptors as the major Sp1 transcriptional targets in ACR-treated JHH-7 cells undergoing apoptosis

ACR-treated JHH-7 cells expressed decreased levels of EGFR at both mRNA (Figure 2A; 2.5-fold reduction in quantitative PCR) and protein (Figure 2B) levels. Although protein levels of c-Met and FGFR1 remained largely unaltered, mRNA levels of c-Met and FGFR1 decreased slightly following ACR-treatment. mRNA of Bcl-X_L was unchanged, but moderately altered at the protein level. ACR induced activation of caspase 3, but not its expression (Additional file 6 Figure S4A and S4B, *respectively*). While a single treatment with either a caspase inhibitor, z-DEVD (Figure 2C, *lane 4*) or overloading EGF (Figure 2C, *lane 6*) partially prevented a reduction in cell number in ACR-treated JHH-7 cells, combined treatment completely prevented this reduction (Figure 2C, *lane 8*).

To determine whether reduced expression of EGFR was due to Sp1 inactivation, transactivation of a chimeric reporter gene-construct in which expression was driven by 3 tandem functional GC box motifs derived from the EGFR promoter was monitored. ACR-treatment decreased the transactivational activity of the EGFR gene promoter (compare Figure 2D, *lanes 1 and 2*), which was partially prevented by overexpressing Sp1 (compare Figure 2D, *lanes 2 and 4*) or downregulating TG2 expression by 70% (compare Figure 2D, *lanes 2 and 6*; Additional file 2 Figure S1B). It was partially reversed by overexpression of TG2 (compare Figure 2D, *lanes 2 and 8*) and Sp1 inactivation with siRNA (compare Figure 2D, *lanes 2 and 10*). Sp1 inactivation with siRNA also reduced expression of EGFR protein (Figure 3A). In hepatocytes, treatment with Sp1 siRNA had previously decreased cell viability ([13]; *data not shown here*). siRNA knockdown of EGFR led to apoptosis (Figure 3B-3D). These results suggest that transcriptional reduction of *EGFR* due to a reduction in Sp1 activity may partially explain ACR-induced apoptosis of HCC cells.

ACR suppresses both transplant of human HCC cells in nude mice and DEN-induced rat hepatocarcinogenesis by inducing apoptosis accompanying the emergence of nuclear TG2 and CLSp1

Finally, the *in vivo* effect of ACR was examined in the 2 animal models. Using the transplant model in mice, where ACR dose-dependent reduction of serum levels of a tumor marker for HCC, α-fetoprotein (AFP) and the incidence of HCC (Additional file 7 Table S2), nuclear

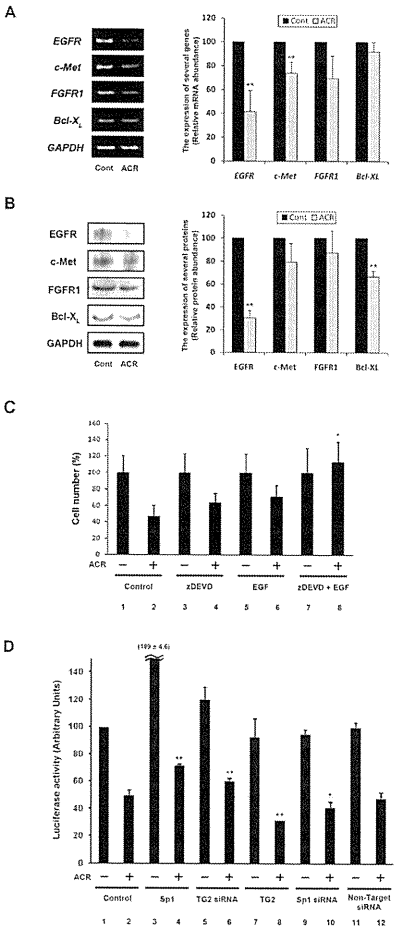

Figure 2 Induction of caspase 3- and TG2-dependent apoptosis by ACR via a reduction in the expression of EGFR due to silencing of Sp1. *A*, JHH-7 cells were treated with 10 μM ACR or vehicle for 12 h. Cells were harvested and mRNA expression of indicated genes was determined by RT-PCR. Bar graphs show densitometrically determined relative mRNA abundance normalized to *GAPDH* mRNA levels. **P < 0.01 compared to each control. *B*, JHH-7 cells were treated with 10 μM ACR or vehicle for 24 h. Cells were harvested and protein expression of indicated proteins was determined by Western blotting. The bar graph shows densitometrically determined relative protein abundance normalized to GAPDH protein levels. **P < 0.01 compared to each control. *C*, JHH-7 cells were treated with 10 μM ACR for 24 h in the presence or absence of 100 μM zDEVD, 50 ng/ml EGF or a combination of the two, and the numbers of viable cells were determined after trypsinization by Trypan Blue exclusion. Results shown are means ± SD (n = 3). *P < 0.05, compared to ACR-treated sample from control cells (*lane 2*). *D*, One day after transfection of JHH-7 cells with *EGFR* promoter GC3-Luc (1 μg/dish), cells were treated with 10 μM ACR for 24 h, co-transfected with Sp1 (*lanes 3 and 4*), TG2 shRNA (*lanes 5 and 6*), TG2 (*lanes 7 and 8*), Sp1 shRNA (*lanes 9 and 10*), and non-target siRNA (*lanes 11 and 12*) expression vector, and cell lysates were prepared. Luciferase activity of each cell lysate was determined. Results shown are means ± SD (n = 3). *P < 0.05, **P < 0.01 compared to ACR-treated control sample from control cells (*lane 2*). *Panels A-D* show representative results from three different experiments with similar results.

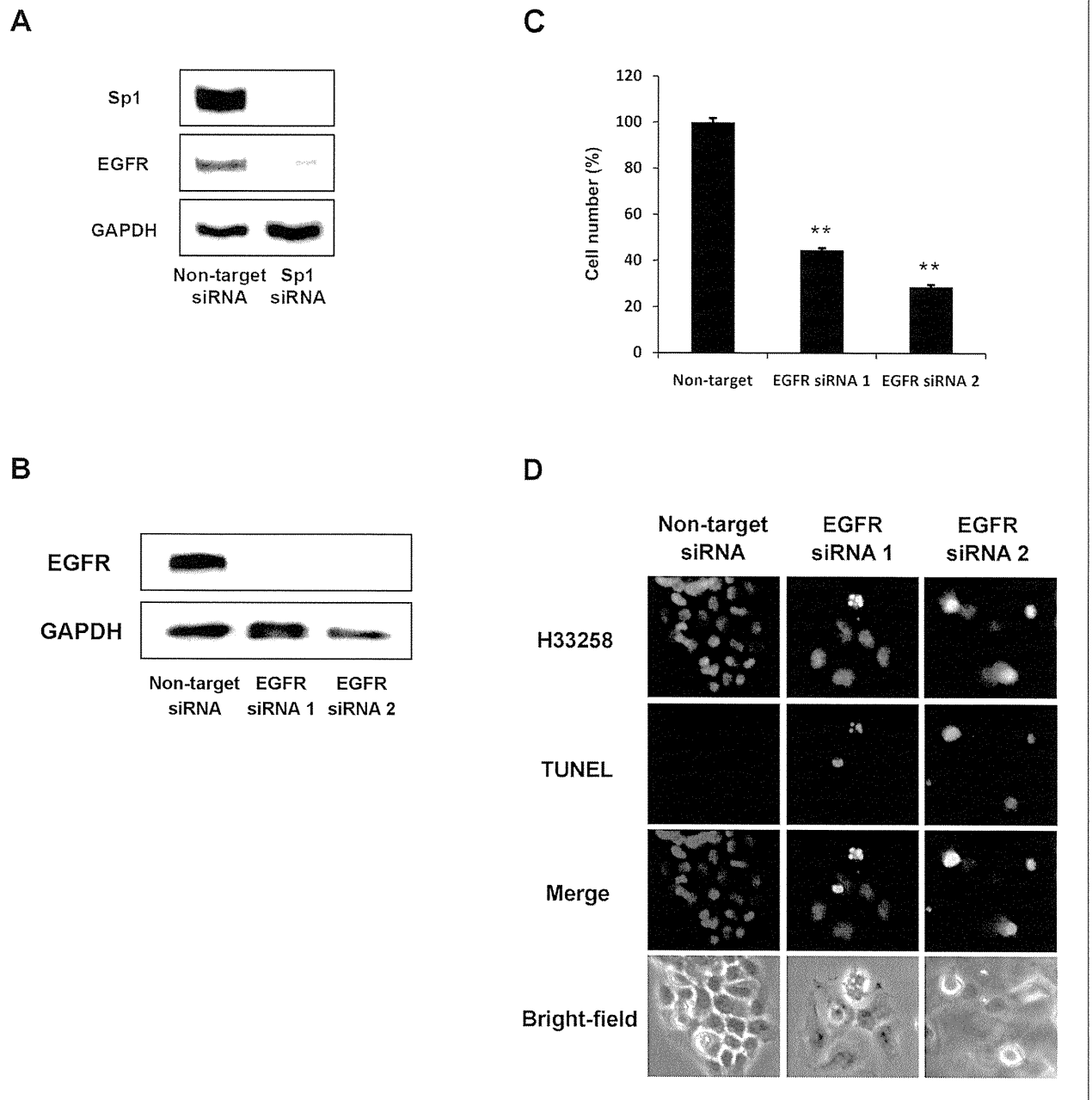

Figure 3 Induction of apoptosis in JHH-7 cells using Sp1 and EGFR siRNAs. *A*, JHH-7 cells overexpressing non-target or Sp1 siRNA were harvested and protein levels of Sp1, EGFR, and GAPDH determined by Western blotting. *B*, JHH-7 cells overexpressing non-target or 2 kinds of EGFR siRNAs were harvested and protein levels of EGFR and GAPDH determined by Western blotting. *C*, Numbers of viable cells were counted 2 days after seeding these cells on 35 mm dishes. Results shown are the number of viable cells relative to the controls, expressed as % ± S.D. **P < 0.01 compared to control cells. *D*, The seeded cells on cover slips in 35 mm dishes were fixed and stained with Hoechst (*upper panels*) and TUNEL (*second panels*). *Panels A-D* show representative results from 3 different experiments with similar results.

TG2 and CLSp1 increased in cancerous liver cells of ACR-treated nude mice transplanted with the JHH-7 cell line (Figure 4A, *panels A and B, respectively*) compared with adjacent normal liver (Figure 4A, *panels D and E*). Significant induction of TG2 and activation of caspase 3 occurred in metastatic areas in nude mice transplanted with JHH-7 cells after treatment with ACR (Figure 4A, *panels A and C, respectively*). Moreover, EGFR levels in the metastatic areas were lower than in normal areas of the same liver (compare Figure 4A, *panels G and J*). Similar results were obtained in the rat model of DEN-induced hepatocarcinogenesis, in which

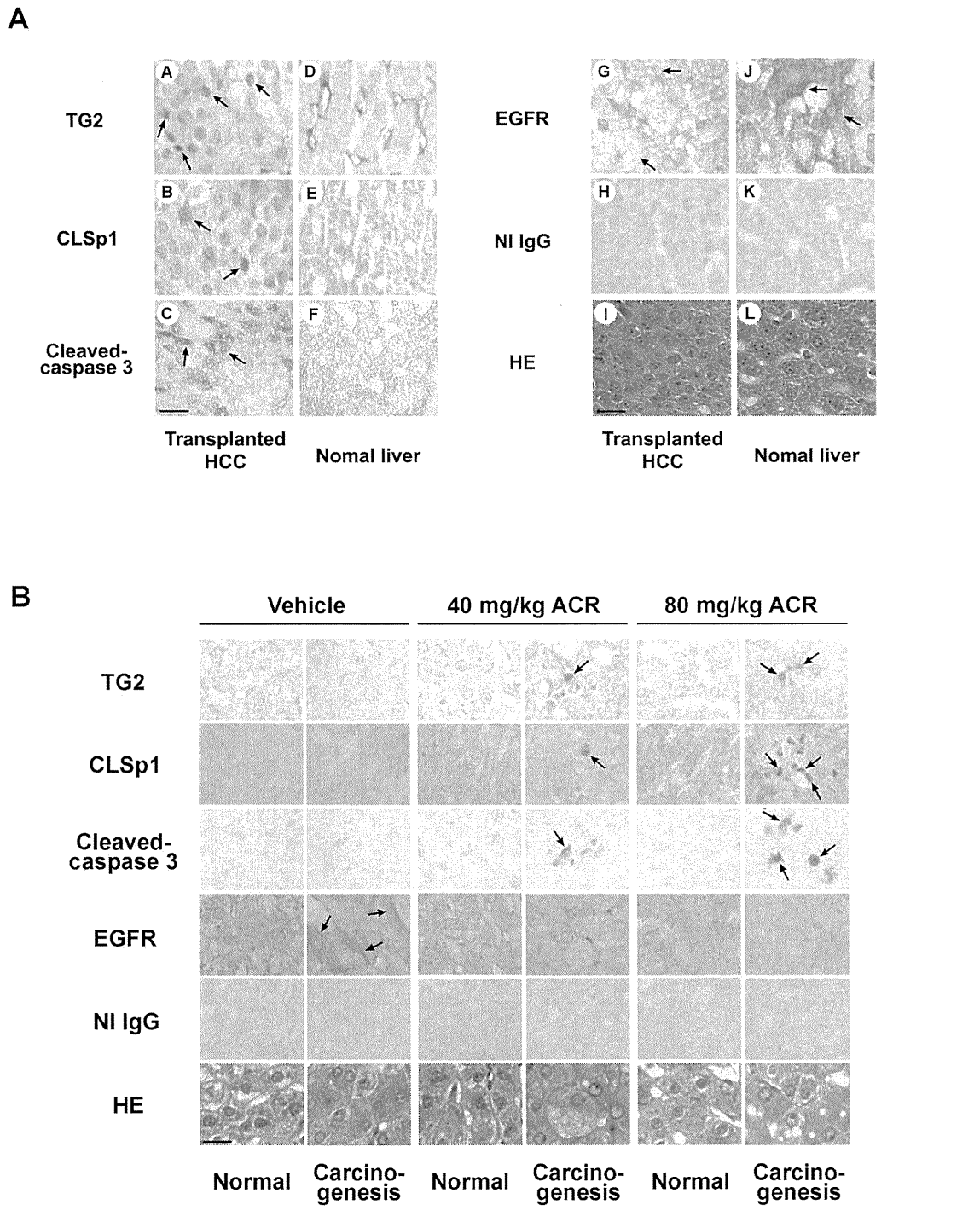

Figure 4 Nuclear accumulation of TG2 and CLSp1 observed in the liver of nude mice transplanted with JHH-7 cells, and in DEN-treated rats with liver cancer after ACR treatment. *A*, Liver sections including normal (*panels D-F and J-L*) and metastatic areas (*panels A-C and G-I*) from JHH-transplanted nude mice following treatment with ACR were stained with polyclonal anti-TG2 (30 μg/ml; *panels A, D*), anti-CLSp1 (30 μg/ml; *panels B, E*), anti-cleaved caspase 3 (10 μg/ml; *panels C, F*), anti-EGFR (10 μg/ml; *panels G, J*), and non-immune antibodies (NI IgG; 30 μg/ml; *panels H, K*). *B*, Liver sections from normal and neoplastic areas in DEN-treated rats following treatment with vehicle or ACR (at 40 and 80 mg/kg) were stained as in Figure 4A. The signals were enhanced with an ABC kit and developed with DAB substrate. Sections were counterstained with hematoxylin-eosin (HE; Figure 4A, *panels I, L*, and Figure 4B, *bottom panels*). Arrows indicate signals under the levels for each antigen. Scale bar, 50 μm.

ACR's anti-cancer effect has been reported [21]. Simultaneous induction of TG2, CLSp1, and activation of caspase 3 occurred in paralleled with a reduction in EGFR (Figure 4B).

Discussion

The data show that: (i) ACR suppresses the hyper-phosphorylation of RXRα, restored its transcriptional function, and enhanced the expression of TG2 and its nuclear accumulation, along with caspase 3 activation; (ii) Sp1 is crosslinked by TG2 and degraded by caspase 3, resulting in loss of its activity; and (iii) expression of Sp1-regulated target genes, such as EGFR (critical for cell survival), decrease, culminating in apoptosis of the cancer cells (Figure 5). The results of *in vitro* findings were confirmed by the *in vivo* models of nude mice transplanted with JHH-7 cells and DEN-induced hepatocarcinogenesis in rats (Figure 4). The recurrence of HCC in these animal models remains to be elucidated.

ACR treatment induced apoptosis in HCC cells (JHH-7 and HuH-7), but not in normal hepatocyte cells (HC cells) (Figure 1A and 1B). As a clue to a reason for the difference, we found that both expression and phosphorylation levels of RXRα were much higher in HCC cells than in HC cells, and that ACR suppressed its phosphorylation levels without altering its expression level (Additional file 4 Figure S2A), as previously shown [5]. In further previous work, we had demonstrated that 2 amino acids in RXRα, T82 and S260, were phosphorylated in HCC, but not in HC cells [4]. Therefore, phosphorylation of RXRα observed in JHH-7 cells was referred to as "hyperphosphorylation". However, RARα and RARβ were phosphorylated not only in JHH-7 cells, but also in HC cells, and ACR downregulated their phosphorylation in both cases (Additional file 4 Figure S2A). Phosphorylation was not detected in the other 3 subtypes of RXR and RAR (Additional file 4 Figure S2A). Therefore, phosphorylation of RXRα was only specific in cancer cells, which could be a reason for the selective apoptosis of cancer cells by ACR treatment.

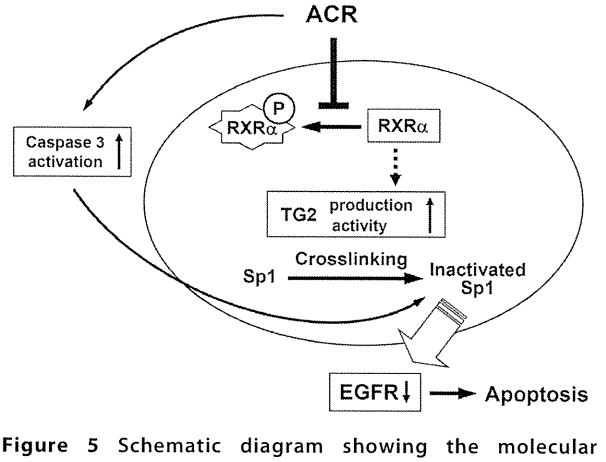

Figure 5 Schematic diagram showing the molecular mechanism by which ACR causes HCC apoptosis by restorating phospho-inactivated RXRα followed by enhanced TG2-mediated crosslinking/silencing of Sp1, thereby reducing EGFR-medicated EGF signaling.

It is noteworthy that treatment with either antisense of TG2 or inhibitors of caspase 3 only partially blocked ACR-induced apoptosis, whereas their simultaneous inhibition completely prevented apoptosis, suggesting that TG2 and caspase 3 contribute independently to the induction of apoptosis (Figure 1D and 1E). We measured the activity of caspase 3 and TG2 in the presence of an inhibitor of each other's enzyme, such as zDEVD and cystamine. When cystamine suppressed ~50% of ACR induction in TG2 activity (compare the differences between *lanes 1 and 4* with those between *lanes 2 and 5* in Additional file 6 Figure S4D), it suppressed 60% of ACR induction in caspase 3 activity (compare the differences between *lanes 1 and 4* with those between *lanes 2 and 5* in Additional file 6 Figure S4C). On the other hand, when zDEVD completely suppressed ACR-induced increase in caspase 3 activity (compare the differences between *lanes 1 and 4* with those between *lanes 3 and 6* in Additional file 6 Figure S4C), 50% of an increase in the TG2 activity remained (compare the differences with *lanes 1 and 4* with those between *lanes 3 and 6* in Additional file 6 Figure S4D). The data suggest that TG2 and caspase 3 influenced each other with a higher hierarchy of TG2 over caspase 3 in the contribution to the apoptosis of HCC induced by ACR. Synergism between inhibition in caspase and overloading of EGF in preventing apoptosis also suggests that both the caspase 3- and EGFR-dependent pathways exist (Figure 2C).

Expression of EGFR is regulated by Sp1 [19,22], and inhibition of EGFR signaling leads to growth inhibition, apoptosis, and cell cycle arrest of HCC cells [23,24]. We have linked these findings by showing that the downregulation of EGFR with siRNA induces apoptosis (Figure 3B-D), suggesting that inhibiting EGFR signaling via silencing Sp1 is a promising treatment strategy against HCC.

Induction of CLSp1 and the subsequent reduction in EGFR has been reproduced in ACR-treated HuH-7 cells (*data not shown*). In contrast, although Shao *et al.* [15] reported that ACR inhibits the cell growth through downregulation of FGFR3 expression and FGF-mediated signaling in HepG2 cells, this was not found to be the case in our ACR-treated JHH-7 cells (*data not shown*). These findings suggest that HCC cell lines differ in the way that growth factor receptors are involved in survival.

Whereas TG2 may be a substrate of caspase 3 during apoptosis of thymocytes, resulting in loss of transamidating function [25], TG2 in turn inhibits of apoptosis due to crosslinking and inactivation of caspase 3 in thapsigargin-mediated apoptosis of colon carcinoma cells [26]. In the latter article, thapsigargin treatment generated 2 additional biologically inactive species of caspase 3, viz. p40 and p64, via TG2-mediated crosslinking of caspase 3, thereby protecting cells from apoptosis. However, we failed to detect either p40 or p64 in our ACR-treated JHH-7 cells. We speculate that crosslinking of caspase 3 would be induced specifically by treatment with thapsigargin. Our data clearly shows that both caspase 3 and TG2 are functional in ACR-treated HCC cells, without apparent alteration of caspase 3 expression (Additional file 6 Figure S4A and 4B). These controversial results might be ascribed to differences in cell types and the nature of the apoptotic stimuli, although the precise mechanisms need to be elucidated.

Piedrafita and Pfahl [14] reported that caspase 3 directly cleaved and inactivated Sp1 in retinoid-treated T cells undergoing apoptosis. They showed that cleavages of PARP and Sp1 were simultaneously induced by caspase 3 and prevented with caspase inhibitors (zVAD-fmk and zDEVD-fmk). We anticipated that CLSp1 might also be partially cleaved by caspase 3; however, as molecular size differences would be too small to be recognized on the gel against a high molecular weight of CLSp1 detected at the top of the gel, we found no band shifts due to the cleavage. Hence, the possibility of simultaneous crosslinking and cleavage of Sp1 by TG2 and caspase 3, respectively, cannot be ruled out, even though we saw no truncated Sp1 with a Mw of 68 kD in ACR-treated HCC cells.

ACR-treated JHH show enhanced nuclear localization of TG2; nuclear localization of TG2 is also important for induction of TG2-dependent apoptosis. Peng *et al.* [27] reported that TG2 binds importin-α3, an important factor in nuclear translocation, and therefore we are investigating the detail mechanism of TG2 nuclear localization accompanying ACR-induced apoptosis.

Conclusions

Our new findings indicate that ACR induces both activation of caspase 3 as well as the expression and activation of TG2, which together initiate the apoptotic pathway via degrading/crosslinking and inactivation of the transcription factor, Sp1. Reduced expression of growth factor receptor genes (*e.g.* EGFR) also occurs. This dual activation of both caspase and TG-dependent apoptotic pathways could in part be central as mechanisms by which ACR inhibits tumor cell growth, resulting in the prevention of secondary tumors after treatment of primary HCCs (Figure 5). Future study should establish the possibility that regulation of TG2-dependent apoptotic pathway may help in the development of new therapies for the prevention of HCC.

Additional material

Additional file 1: Additional text. This text contains the additional "Methods" and "References"

Additional file 2: Figure S1: Efficiency of transfection with anti-sense and siRNA to TG2 in JHH-7 cells. *A*, JHH-7 cells were seeded in 60 mm dishes at 6 × 10^5/dish, and transfected with 4 μg of either empty vector (pSG5) or ASTG2-pSG5. Cells were harvested and the expression level of TG2 determined by Western blotting. Upper numbers in parentheses show the densitometrically determined relative protein abundance. *B*, JHH-7 cells were seeded in 60 mm dishes at 6 × 10^5/dish, and transfected with 4 μg of vectors expressing either non-target siRNA or TG2 siRNA. Cells were harvested and the expression level of TG2 determined by Western blotting. Upper numbers in parentheses show the densitometrically determined relative protein abundance. *Panels A and B* show representative results from 3 different experiments with similar results.

Additional file 3: Table S1: Primers for RT-PCR and quantitative-PCR experiments. The list of used specific primers for RT-PCR.

Additional file 4: Figure S2: ACR prevented phosphorylation and inactivation of RXRα, and stimulated the expression of TG2 in JHH-7 cells. *A*, JHH-7 cells (*lane 1 and 2*) and HC cells (*lane 3 and 4*) were treated with 10 μM ACR or vehicle for 12 h. Cells were harvested and nuclear extracts were prepared. Phosphoproteins affinity-purified from each nuclear extract using the Phosphoprotein Purification Kit (QIAGEN) (*left panel*) as well as whole nuclear extracts (*right panel*), were subjected to SDS-PAGE, followed by Western blotting using the indicated antibodies against 6 different RXR/RAR or GAPDH. *B*, JHH-7 cells were transfected with either an empty vector (*columns 1-4*) or vectors expressing wild-type RXRα (*columns 5-8*), its alanine mutant T82A (unphosphorylated form; *columns 9-12*), or its aspartate mutant T82 D (phosphomimic; *columns 13-16*). The next day cells were treated either with 9-*cis* RA (9cRA; 6 μM) or its vehicle, or with and/or ACR (10 μM) for 24 h. Subsequently, levels of *TG2* mRNA in cell lysates were quantified by RT-PCR (*upper panels*) and quantitative-PCR (*lower graphs*), where relative expression levels of TG2 were calculated in comparison with each control and then plotted. Treatment with 1 μM 9-cis-RA also gave basically similar results (data not shown), but the data obtained under treatment with 6 μM 9-cis-RA are shown here, giving the more significant differences. *Panels A and B* show representative results from 3 different experiments with similar results.

Additional file 5: Figure S3: Crosslinking and silencing of Sp1 in ACR-treated JHH-7 cell cultures undergoing apoptosis and its reversion by overexpression of Sp1. *A*, JHH-7 cells were treated with 10 μM ACR for 24 h. The cells were harvested and nuclear extracts prepared. The levels of Sp1 and CLSp1 were assessed by Western blotting with an anti-Sp1 (*columns 1 and 2*) and CLSp1 (*columns 3 and 4*) antibodies, respectively. *B*, JHH-7 cells were transfected with 1.5 μg of either combination of *pCIneo*, *pSG5*, *Sp1-pCIneo*, or *anti-sense (AS) TG2-pSG5*. The next day they were treated with either 10 μM ACR or its vehicle in the presence or absence of 100 μM zDEVD-fmk for 24 h. Cells were harvested and nuclear extracts prepared. Sp1 DNA-binding activity of each nuclear extract (10 μg protein) was determined by gel-shift assay, using a consensus GC box as a probe (+cold; nuclear extracts + 50-fold excess of unlabeled probe, +anti-Sp1 IgG; nuclear extracts + 2 μg of anti-Sp1 antibody, +NI IgG; nuclear extracts + 2 μg of non-immune IgG). *C*, JHH-7 cells were transfected with 1.5 μg of a consensus GC3-Luc reporter and *Renilla*-Luc, plus a combination of *pCIneo*, *pSG5*, *Sp1-pCIneo* or *anti-sense (AS) TG2-pSG5*. The next day the cells were treated with 10 μM ACR for 24 h in the presence or absence of 100 μM zDEVD-fmk. Cell lysates were prepared and luciferase activity of each cell lysate determined. Results are means ± SD (n = 3). *D*, JHH-7 cells were

transfected with either a combination of *pCIneo, pSG5, anti-sense (AS) TG2-pSG5, Sp1-pCIneo, Sp1 C domain-pCIneo, ΔC Sp1-pCIneo*. The next day the cells were treated with 10 μM ACR for 24 h. The number of viable cells was determined. Results are means ± SD (n = 4). *Panels A-D* show representative results from 3 different experiments with similar results.

Additional file 6: Figure S4: ACR stimulated activation of caspase 3 and TG2 in JHH-7 cells and the crosstalk between these proteins. *A and B*, JHH-7 cells were treated with 10 μM ACR or the vehicle for 24 h. Cells were harvested and protein levels of activated caspase 3 and GAPDH determined by Western blots, using anti-cleaved-caspase 3 and anti-GAPDH antibodies (*A*); each of their mRNA expression was determined by RT-PCR (*B*). *C*, JHH-7 cells was seeded at 1×10^4 cells/96 well microplates and treated with 10 μM ACR or vehicle (0.1% ethanol) for 5 h in the presence or absence of either 100 μM zDEVD-fmk or 100 μM cystamine with 0.2 mM 5-(biotinamido)-pentylamine. Caspase 3 activity was measured using a Caspase-Glo 3/7 assay kit (Promega Corp., WI) as described in attached manual. Relative caspase 3 activity of each sample was calculated by normalization with the number of viable cells in the same sample measured with a cell counting kit-8 (Dojindo; Tokyo, Japan). *D*, JHH-7 cells seeded in 100 mm dishes at 1.6×10^6/dish were treated as in (C). TG2 activity was measured as described in Additional file 1. Relative TG2 activity of each sample was calculated by normalization with the number of viable cells in the same sample, measured with a cell counting kit-8 (Dojindo; Tokyo, Japan). *Panels A-D* show representative results from 3 different experiments with similar results.

Additional file 7: Table S2: Suppression by ACR of metastasis and growth of human HCC cell line, JHH-7 cells transplanted into nude mice. Nude mice that had been transplanted with JHH-7 were given orally with ACR with increasing concentrations (25, 50, and 100 mg/kg/day) as described detailed in the "Methods". Serum AFP was measured. Incidence was calculated based on level of the positive-AFP (more than 6 ng/ml). Cisplatin was used as a positive control. *$p < 0.05$ compared to control (Dunnett's multiple comparison test), #$p < 0.05$ compared to control (Fisher exact test)..

List of abbreviations

9-*cis* RA: 9-*cis* retinoic acid; ACR: acyclic retinoid; CLSp1: crosslinked Sp1; DEN: *N*-diethylnitrosamine; EGFR: epidermal growth factor receptor; FGFR3: fibroblast growth factor receptor 3; HCC: hepatocellular carcinoma; RXR: retinoid X receptor; TG2: transglutaminase 2.

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Authors' contributions

HT and TS performed the research, analyzed the data, and drafted the manuscript. YF helped with cell culture, transfection, immunostaining and Western blotting techniques. NI prepared the acyclic retinoid used in these studies. MW helped with immunostaining techniques. MO, HM and SK designed the research, interpreted the data, and revised the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Risk factors for recurrence after transarterial chemoembolization for early-stage hepatocellular carcinoma

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Abstract
Background Radiofrequency ablation (RFA) is a standard therapy for the treatment of hepatocellular carcinoma (HCC) with 3 or fewer tumors of up to 3 cm (early-stage HCC); when RFA is unsuccessful or unfeasible, transcatheter arterial chemoembolization (TACE) has often been performed. However, little information about the outcome of TACE for early-stage HCC has been reported and it is hard to decide whether to perform additional treatment following TACE in these difficult conditions. The aim of this study was to determine the risk factors for local or intrahepatic distant recurrence after TACE in early-stage HCC.
Methods Among 1,560 newly diagnosed HCC patients who were admitted to Okayama University Hospital, 43 patients with early-stage HCC who received only TACE in at least one nodule were enrolled in this study. We analyzed the risk factors for local and distant recurrence by the Cox proportional hazard model.
Results The local recurrence rates and intrahepatic distant recurrence rates at 3 months, 6 months, and 1 year were 18.6, 33.4, and 61.8%, and 2.8, 2.8, and 10.2%, respectively. Among 12 parameters examined as possible risk factors for recurrence, heterogeneous Lipiodol uptake (risk ratio 3.38; 95% confidence interval 1.14–10.60) and high serum des-gamma-carboxy prothrombin (DCP) (2.58; 1.03–7.14) were significantly correlated with local recurrence, and the presence of multiple tumors (10.64; 1.76–93.75) was significantly correlated with intrahepatic distant recurrence.
Conclusions Heterogeneous Lipiodol uptake, high serum DCP, and multiple tumors are risk factors for recurrence in patients with early-stage HCC who have undergone palliative TACE.

Keywords Hepatocellular carcinoma · Small HCC · TACE · Early-stage HCC

Introduction

Hepatocellular carcinoma (HCC) has become increasingly detected at an early stage with the growing use of surveillance systems. The guidelines established by the American Association for the Study of Liver Disease (AASLD) [1] and the European Association for the Study of the Liver (EASL) [2], and the Japanese "Evidence-Based Guidelines" recommend local treatment [3, 4], such as radiofrequency ablation (RFA) or operation, for HCCs with 3 or fewer tumors of up to 3 cm in patients with good liver functional reserve and performance status. Additionally, RFA combined with transcatheter arterial chemoembolization (TACE) has been reported to be an efficient and safe treatment that provides overall survival rates similar to those achieved with surgical resection [5–7]. If the HCC is a hypervascular tumor, HCCs with 3 tumors or fewer of up to 3 cm are often subjected to sequential TACE followed by RFA, percutaneous ethanol injection therapy (PEIT), or operation regardless of the size because TACE is expected to enhance the efficacy of local therapy by reducing arterial blood flow [8].

Occasionally, various factors such as poor liver functional reserve, difficult location for RFA treatment, and the presence

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of severe associated diseases in the elderly, or patients rejecting treatment, result in the selection of only TACE, even in candidates for local therapies. TACE is known to be effective for inoperable HCC [9, 10]; however, little information has been reported about TACE for early-stage HCC, and it is hard to decide whether to perform additional treatment following TACE in these difficult conditions.

The objective of this retrospective cohort study was to determine whether only TACE for HCC with 3 tumors or fewer of up to 3 cm could control HCC; to achieve this objective we examined the recurrence rate and the risk factors for local and intrahepatic distant recurrences in such cases.

Subjects and methods

Patients

Patients were enrolled from among 1,560 newly diagnosed HCC patients who were admitted to Okayama University Hospital between 2002 and 2010. Inclusion criteria were as follows: (1) no previous treatment of HCC; (2) 3 or fewer nodules of up to 3 cm (early-stage HCC); (3) at least one nodule treated only by TACE as an initial treatment; (4) no planned local treatment such as RFA, PEIT, or operation performed within 30 days of TACE; (5) no vascular invasion; and (6) no extrahepatic metastasis. Exclusion criteria were: (1) complete cure of all nodules by RFA, PEIT, or operation and (2) follow-up period less than 1 year. Finally, 43 patients were selected and enrolled in this study. Of these patients, 6 died in the follow-up period. Five of these patients died due to liver disease and 1 died of heart failure. Informed consent for the use of their clinical data was obtained from all patients in this study. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the ethics committee of the institute (approval # 458).

Diagnosis

HCC was diagnosed in accordance with AASLD guidelines. The diagnostic criteria for HCC via imaging were based on hyperattenuation in the the arterial phase and hypoattenuation in the portal phase on dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and tumor stain on angiography. Ultrasonography (US) with perfluorobutane (Sonazoid; Daiichi Sankyo, Tokyo, Japan) and/or gadolinium ethoxybenzyl MRI (Gd-EOB-MRI) was performed in 30 (69.8%) patients. When we could not diagnose HCC by imaging only, fine-needle biopsy using abdominal US was performed as histological proof (3 cases). Recurrence of HCC was diagnosed in the same way as at the initial diagnosis.

Treatment

TACE was performed using the Seldinger technique followed by arterial embolization. After introducing a 4-Fr catheter through the femoral artery, hepatic arteriography and superior mesenteric arterial portovenography were performed to evaluate portal flow and the location of the tumors. When portal flow was sufficient, a 1.8-Fr or a 2.0-Fr microcatheter was placed in the feeding arteries at the closest point to the HCC. An emulsion consisting of 30–60 mg of epirubicin (Kyowa-Hakko, Tokyo, Japan) and 2–6 mL of iodized oil (Lipiodol Ultrafluid; Terumo, Tokyo, Japan) was injected into the artery supplying blood to the tumor, followed by embolization with 1-mm gelatin sponge particles (Gelfoam; Nihonkayaku, Tokyo, Japan). After embolization, CT angiography was performed to determine the extent of vascular occlusion and to assess blood flow in other arterial vessels. Patients were observed carefully, and analgesia (pentazocine; Astellas, Tokyo, Japan) was administered if necessary.

Lipiodol uptake was categorized as either homogeneous or heterogeneous by plain CT after TACE. Homogeneous uptake was defined as complete uptake of Lipiodol in the tumor without any defect, and the CT value in these cases was more than 200 Hounsfield Units. Uptake that did not meet the definition of homogeneous was considered as heterogeneous. Two experienced investigators (K. N. and H. O.) reviewed the CT images and evaluated the Lipiodol uptake. If the two investigators had different diagnoses, they discussed the difference and reached agreement.

Follow up

Patients were assessed every 1–3 months by serum biochemistry, dynamic CT, dynamic MRI, or US. Local recurrence was defined as the appearance of a viable tumor in contact with or inside the treated area. Intrahepatic distant recurrence was defined as the occurrence of a new HCC in the liver that did not meet the definition of local recurrence. When recurrence was detected, TACE, RFA, PEIT, or surgical resection was performed depending on the condition of the recurrence and the background liver function. Patients were followed until loss to follow up, death, or 31 January 2011.

Statistical analysis

The following 12 parameters were used for analyzing the risk factors for recurrence: age, sex, viral markers (hepatitis B virus surface antigen and hepatitis C virus antibody), alcohol intake, liver function, size of tumors, number of tumors, location of tumors (within 10 mm of the surface of the liver or not), serum tumor markers [alpha-fetoprotein

(AFP) and des-gamma-carboxy prothrombin (DCP)], and the status of Lipiodol uptake.

Recurrence rates were estimated using the Kaplan–Meier method and differences between groups were compared using the log-rank test. The Cox proportional hazard model was used to analyze the predisposing factors for recurrence. All statistical analyses were performed using JMP version 9 (JMP Japan, Tokyo, Japan). All reported P values are 2-sided, with $P < 0.05$ considered statistically significant.

Results

Patient background

A total of 43 patients met the criteria of this study. The total number of HCC nodules was 54. There were 27 males (63%) and 16 females (37%) aged 50–85 years (mean: 71 years), and 34 patients (79%) were infected with hepatitis C virus and 6 (14%) with hepatitis B virus. Twenty-three patients (53%) were habitual drinkers. Thirty-four patients (79%) had a single tumor in the initial treatment. Thirty-six patients (84%) had recurrence (Table 1). Eleven (26%), 23 (53%), and 9 (21%) patients were treated with only TACE because of poor liver functional reserve, difficult location for RFA, and old age, respectively. Of the 11 patients who had poor liver function, 1 patient was Child C and 10 patients were Child B. Although the Japanese guidelines for treatment of HCC recommend RFA or operation for the treatment of small HCC in patients with Child A/B stage, these 10 Child B patients had poor conditions such as uncontrollable ascites or low albumin, so that it was quite difficult to perform RFA or operation. In the 23 patients who had a difficult location for RFA, the nodules were located beside the portal vein in 5 patients, beside the digestive tract in 4 patients, beside the gallbladder in 4 patients, beside the inferior vena cava in 3 patients, beside the collateral veins on the surface of liver in 3 patients, beside the bile duct in 3 patients, and beside the heart in 1 patient.

Table 1 Clinical background of 43 patients

Demographic variables	
Sex (male) (%)	27 (63)
Age (years)	71 (50–85)
Etiology (%)	
HCV	34 (79)
HBV	6 (14)
HCV + HBC	2 (4.7)
Alcohol	23 (53)
Unknown	1 (2)
Tumor size (mm)	18 (6–30)
Number of tumors (%)	
1	34 (79)
2	7 (16)
3	2 (5)
Location (distance from surface of the liver)	
≤10 mm	28 (65)
Recurrence (%)	
Local	22 (52)
Distant	7 (16)
Local + distant	7 (16)
No recurrence	7 (16)
Lipiodol uptake (%)	
Homogeneous	23 (53)
Heterogeneous	20 (47)
AFP (ng/mL)	124.1 (1.6–1,539)
DCP (mAU/mL)	157.7 (12–3,450)
Total bilirubin (mg/dL)	1.09 (0.37–3.13)
Albumin (g/dL)	3.47 (2.4–4.42)
ALT (IU/L)	43.5 (13–147)
AST (IU/L)	55.2 (23–119)
Child-Pugh score (A/B/C)	32/10/1

Numbers in the Tables are shown as medians (ranges) unless otherwise noted

HCV positive for hepatitis C virus antibody, *HBV* positive for hepatitis B virus antigen, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase

Recurrence rate

Local recurrence and intrahepatic distant recurrence were observed in 29 patients and 14 patients, respectively, and 7 of these patients showed both local and intrahepatic distant recurrences at the same time.

The total recurrence rates at 3 months, 6 months, and 1 year were 20.9, 35.3, and 68.5%, respectively. The local recurrence rates and intrahepatic distant recurrence rates at 3 months, 6 months, and 1 year were 18.6, 33.4, and 61.8%, and 2.8, 2.8, and 10.2%, respectively (Fig. 1). Thirteen patients had local recurrences within 180 days. Eleven patients received RFA and and 11 patients received TACE for the therapy of local recurrence. Eight patients received RFA, 5 patients received TACE, and 1 patient received PEIT for the therapy of intrahepatic distant recurrence. No difference in the therapies was observed between the local recurrence group and the distant recurrence group.

Factors related to local recurrence

Of the 12 factors analyzed, heterogeneous Lipiodol uptake (risk ratio 3.19; 95% confidence interval 1.41–7.90; $P = 0.004$) and high serum DCP (2.37; 1.06–5.83; 0.034)

were correlated with local recurrence by univariate analysis. The factor of location was not associated with local recurrence on univariate analysis. Factors exhibiting significance in the univariate analysis and reported to be correlated with recurrence; namely, DCP, age, number of HCCs, liver function, size of HCC, extent of Lipiodol uptake, HCV, and location of HCC, were further analyzed using the Cox multivariate proportional hazard model [11–20]. On multivariate analysis, only heterogeneous Lipiodol uptake (risk ratio 3.38; 95% confidence interval 1.14–10.60; $P = 0.027$) and high serum DCP (2.58; 1.03–7.14; 0.042) were significantly correlated with local recurrence (Table 2).

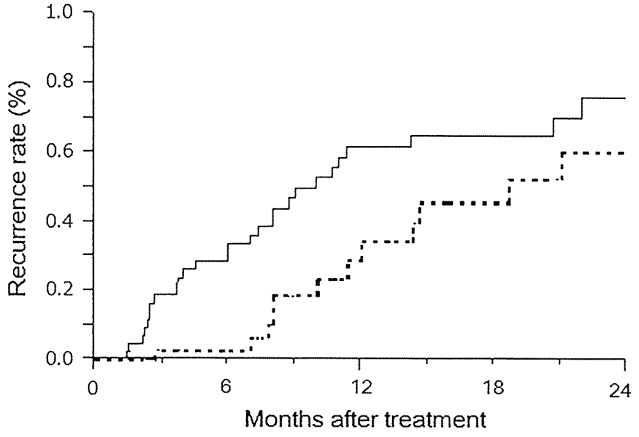

Fig. 1 Local and distant recurrences after transcatheter arterial chemoembolization. The local recurrence rates (*solid line*) and intrahepatic distant recurrence rates (*dotted line*) at 3 months, 6 months, and 1 year were 18.6, 33.4, and 61.8%, and 2.8, 2.8, and 10.2%, respectively

Factors related to intrahepatic distant recurrence

In the same way as for factors related to local recurrence, the 12 factors were analyzed, and the factor of multiple tumors (3.98; 1.02–13.50; 0.047) was correlated with intrahepatic distant recurrence by univariate analysis. On multivariate analysis with the factors serum DCP, age, tumor number, Child-Pugh score, tumor size, Lipiodol uptake, HCV, and tumor location, the presence of multiple tumors (10.64; 1.76–93.75; 0.010) was significantly correlated with recurrence (Table 3).

Discussion

While RFA is considered as the first choice for the treatment of early-stage HCC, TACE could be another option when RFA is unsuccessful or unfeasible. Livraghi [21] reported that RFA was not feasible in 6.0% of patients because of a high-risk tumor location or poor detection on ultrasonography (US). However, it is hard to decide whether to treat these patients with additional locoregional therapy because there have been few reported studies examining the outcomes of these patients in detail.

In the present study, we examined the clinical courses of 43 patients treated with palliative TACE for HCC with 3 tumors or fewer of up to 3 cm. More than 80% of the patients (36/43, 84%) had recurrence and most of them (29/36, 80%) exhibited local recurrence. The recurrence rate was higher than that for local ablation. The recurrence rates of the patients treated with RFA or operation at 3 months,

Table 2 Risk factors for local recurrence after TACE

Variables	Univariate		Multivariate	
	Risk ratio (95% CI)	*P* value	Risk ratio (95% CI)	*P* value
AFP (>10 ng/mL)	1.46 (0.69–3.23)	0.328		
DCP (>28 mAU/mL)	2.37 (1.06–5.83)	0.034*	2.58 (1.03–7.14)	0.042*
Age (≥75 years)	1.04 (0.50–2.24)	0.900	1.16 (0.42–3.11)	0.769
Tumor number (≥2)	1.72 (0.66–4.04)	0.249	1.18 (0.37–3.26)	0.759
Child-Pugh score (≥7)	1.25 (0.51–3.74)	0.650	1.65 (0.60–5.43)	0.347
Tumor size (≥20 mm)	1.58 (0.74–3.37)	0.230	1.66 (0.70–3.94)	0.245
Lipiodol uptake (heterogeneous)	3.19 (1.41–7.90)	0.004*	3.38 (1.14–10.60)	0.027*
Sex	1.01 (0.48–2.23)	0.976		
HCV	2.07 (0.74–4.99)	0.153	2.09 (0.55–7.65)	0.271
HBV	0.91 (0.32–3.85)	0.882		
Alcohol	0.99 (0.47–2.14)	0.975		
Location (within 10 mm from the liver surface)	0.66 (0.31–1.47)	0.302	0.80 (0.26–2.37)	0.687

TACE transcatheter arterial chemoembolization, *95% CI* 95% confidence interval. Other abbreviations are the same as those listed in Table 1 footnote

* Significant value