A Human Model of Epithelial to Mesenchymal Transition to Monitor Drug Efficacy in Hepatocellular Carcinoma Progression

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Abbreviation list:

aCGH, array comparative genomic hybridisation; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; GSEA, gene enrichment analysis; HCC, hepatocellular carcinoma; IC₅₀, half maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SPARC, secreted protein, acidic and
rich in cystein; TACE, transarterial chemoembolisation; VEGF, vascular endothelial growth factor.

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Abstract

The epithelial to mesenchymal transition (EMT) of malignant hepatocytes is a crucial event in hepatocellular carcinoma (HCC) progression and recurrence. We aimed to establish a human model of EMT to examine drug efficacy and specificity in HCC progression. Human HCC cell populations were characterized by immunofluorescence analysis, migration and invasion assays, array comparative genomic hybridization, whole-genome expression profiling and promoter methylation. Therapeutic agents clinically used against HCC were examined for efficacy by determination of IC$_{50}$ values. We show that liver cancer cell lines exhibited either an epithelial or mesenchymal phenotype of which latter showed strong migratory and invasive abilities in vitro. The common cellular origin of both cell types indicated that mesenchymal HCC cells have been derived from epithelial hepatocytes through EMT in the HCC patient. Drug exposure of mesenchymal HCC cells showed higher resistance to the targeted therapeutic agents sorafenib and erlotinib as compared to epithelial HCC cells, which were slightly more resistant to cytostatic drugs. Most remarkably, combined treatment with doxorubicin and sorafenib caused increased susceptibility of both HCC cell types resulting in enhanced drug efficacy. Taken together, this EMT model of human HCC allows the identification of molecular mechanisms and the assessment of therapeutic drug efficacy during liver cancer progression in pre-clinical studies.
Introduction

The epithelial to mesenchymal transition (EMT) enables carcinoma cells to invade into surrounding tissues and to form secondary tumors known as metastases. A particular characteristic of EMT is the downregulation of \textit{E-cadherin} expression, which causes disruption of cell-cell junctions and dissemination of cells from the primary tumor (1). Dysregulation of E-cadherin is provoked by its transcriptional repressors involving \textit{Snail/SNAI1}, \textit{Slug/SNAI2}, \textit{ZEB1/\AE 1}, \textit{ZEB2/SIP1} or \textit{Twist} (2). Receptor tyrosine kinase (RTK)/Ras and transforming growth factor (TGF)-\(\beta\) signaling as well as Wnt/\(\beta\)-catenin-, Notch-, Hedgehog- and NF-\(\kappa\)B-dependent pathways can induce and maintain EMT during cancer progression (1, 3).

The pivotal role of EMT in hepatocellular carcinoma (HCC) has been increasingly recognized and various molecular mechanisms of hepatocellular EMT have been identified (4, 5). In human HCC, EMT correlates with invasive tumors, intrahepatic metastasis and poor survival (6). This is of particular relevance since intrahepatic metastases were observed in more than 30\% of HCC cases after liver surgery and in 80\% of HCC autopsy cases (7).

Epidemiologically, HCC has a poor prognosis and represents the third most cause of death from cancer worldwide due to diagnosis at advanced stages and lack of effective therapy options (8). Thus, HCC therapy is hampered by the fact that the liver is central for xenobiotic metabolism resulting in rapid modifications and efflux of drugs. Curative therapies such as resection and liver transplantation are applicable in only 15\% of HCC patients (9) and show a high incidence of recurrence (10). Unresectable HCC are treated with locoregional therapies involving transarterial chemoembolization (TACE), percutaneous ethanol injection or radiofrequency ablation (11). TACE represents an intra-arterial administration of therapeutic drugs combined with embolizing agents which leads to a more selective distribution and a higher retention time of therapeutics within HCC. TACE has been established as the standard therapy for patients with intermediate stage cancer (12), although the efficacy of TACE is
limited and patients often suffer from recurrence (13). The most frequently used chemotherapeutics for TACE are doxorubicin (Adriamycin RDF®), cisplatin (Platinol®) and epirubicin (Ellence®), either alone or in combination (14), from which doxorubicin and cisplatin showed significant benefits (13). Moreover, targeted therapeutic agents such as the multikinase inhibitor sorafenib (Nexavar®) have been shown to have survival benefits especially in patients with advanced stage HCC (15-17). Those studies are the basis to develop treatment modalities to efficiently combat HCC progression that is considered as utmost treatment-resistant. However, suitable human HCC models are required to identify potential molecular targets, to test drug efficacy and to estimate the effective concentration of therapeutic agents.

Here, we established a human model of hepatocellular EMT which reflects important aspects of HCC progression and allows studying the underlying molecular mechanisms. The matched pair of epithelial and mesenchymal HCC cells enables us (i) to evaluate the efficacy of currently used therapeutic agents in single or combined treatments and (ii) to assess the effectiveness of novel anti-cancer agents during HCC progression.
Material and Methods

Cell culture

HCC-1.2 and HCC-1.1 cells, referred to as 3p and 3sp cells, respectively, were established from one male patient diagnosed with HCC (grade 2, T2N0M0, hepatitis C virus positive) at the age of 56 years. A fully documented patient’s history and informed consent was obtained. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the approval of the ‘Ethic Committee of the Vienna Medical University’. Briefly, the primary liver tumor tissue from surgical resection of the HCC patient was cut into pieces of approximately 0.5 mm³ and seeded on collagen I (Sigma, St Louis, USA) coated tissue culture plates by incubation in ACL4 medium plus 5% fetal calf serum (FCS) at 37°C and 5% CO₂. Outgrowing epithelial 3p and fibroblastoid 3sp cells were further separately cultivated. The propagation and immortalization of epithelial 3p and fibroblastoid 3sp cell populations were performed by passaging cells at a ratio of 1:2 once a week in ACL-4 medium supplemented with 5% FCS. Fibroblasts were eliminated from the epithelial 3p cell culture between 12 and 14 weeks by differential trypsinization over 2-3 cell passages. The established cell lines that have undergone more than 30 cell doublings were maintained in RPMI 1640 and 10% FCS. 3p hepatoma cells were further cultivated on collagen I coated tissue culture plates whereas 3sp cells were propagated on tissue culture plastic. 3p cells of passages 10 to 12 and 3sp cells of passages 7 to 13 are termed 3p early and 3sp early, respectively. 3p cells between passage 71 and 76 and 3sp from passage 72 to 87 were termed 3p late and 3sp late, respectively. Without particular designation of 3p and 3sp cells, passage numbers between 40 and 50 were employed. All cells were kept at 5% CO₂ and 37°C and routinely screened for the absence of mycoplasma.

Analysis of diploidy
Cells were trypsinized, washed with PBS and 1 x 10^6 cells were incubated with 1 ml hypotonic DNA staining buffer containing 5mg/ml propidium iodide (Calbiochem, San Diego, CA, USA), 0.1% sodium citrate, 0.3% Triton X-100 and 20 μg/ml ribonuclease A (all from Sigma, St. Louis, MO, USA) for 30 minutes on 4°C in the dark. Determination of the DNA content was performed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA)

**Short tandem repeat (STR) analysis**

PCR of seven short tandem repeats (Supplementary Table 1) was performed to verify the genetic origin of human 3p and 3sp hepatoma cells.

**Quantitative Real Time PCR**

PCR reactions were performed with Fast SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) in duplicates according to the recommendations of the manufacturer and quantified with the 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA). Arbitrary units were calculated by the dCT method. Forward and reverse primers are described in Supplementary Table 1.

**Confocal immunofluorescence microscopy**

Cells were seeded onto SuperFrostPlus glass slides (Menzel, Vienna, Austria) and fixed with either 4% paraformaldehyde or methanol/aceton. Collagen gels were fixed in 4% formaldehyde/PBS for 10 minutes at room temperature. After blocking for 30 minutes (1% PBS/0.5% Tween 20/0.2% fish gelatine with 0.2 mg/ml IgG1) at room temperature, the following primary antibodies were applied at a dilution of 1:100: anti-β-catenin (BD Transduction Laboratories, Lexington, UK), anti-E-cadherin (BD), anti-p 120catenin (p120catenin; BD), anti-keratin 8 (Progen, Heidelberg, Germany), Texas Red-X phalloidin (Invitrogen,
Carlsbad, USA) and anti-vimentin (Sigma, St. Louis, USA). The corresponding secondary antibody (Alexa 488; Invitrogen, Carlsbad, USA) was applied after one hour. Nuclei were visualized with To-Pro3 (Invitrogen, Carlsbad, USA) at a dilution of 1:10,000. Imaging was performed by confocal immunofluorescence microscopy (Zeiss, Jena, Germany).

**Proliferation analysis**

1.5 x 10⁴ cells were plated and cell numbers were determined after various time points with a multichannel cell analyzer (CASY; Schärfe Systems, Reutlingen, Germany). Three independent experiments were performed in triplicates.

**Cell migration and invasion assays**

Cell migration and cell invasion was determined by Platypus Technology according to the manufacturer’s protocol (Oris™ Cell Invasion & Detection Assay, Madison, USA). To analyze cell migration, 5 x 10⁴ cells were plated onto 8 not-coated wells. To examine cell invasion, same cell numbers were seeded into 8 basal membrane extract (BME; 3.5mg/ml)-coated wells and overlaid with undiluted BME. Migration and invasion of cells was microscopically analyzed and quantitatively evaluated by measuring the fluorescence after staining with CellTracker™ (Green CMFDA; Invitrogen, Carlsbad, USA).

**3-dimensional (3D) spheroid formation of HCC cells**

Spheroid formation and incubation was performed as described (18). Briefly, a cell suspension of 3 x 10² 3p cells per 100 µl or a 1:1 mixture of 3p and 3sp cells in RPMI containing 20% methyl cellulose (Sigma, St Louis, MO, USA) was incubated for 3 days at 37°C and 5% CO₂. After harvesting, 96 spheroids were mounted in collagen I (Sigma, St Louis, MO USA) and plated into flexiPERM conA wells (Greiner Bio-One GmbH, Kremsmuenster, Austria) to harden at 37°C for 15 minutes. Gels were then transferred into a
24-well plate and incubated with medium at cell culture conditions as outlined. CellTracker™
(Green CMFDA; Invitrogen, Carlsbad, USA) was applied at a concentration of 5 µM before
spheroid formation and incubated as outlined by the instructions of the manufacturer
(Invitrogen, Eugene, Oregon, USA). The fluorescence signal was visualized by microscopy
(Nikon, TE 300, Japan).

**Array comparative genomic hybridization (aCGH)**

Genomic DNA was isolated from early and late 3p and 3sp HCC cells using the QIAamp
dna Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol.
aCGH analysis was performed using human 4x44K whole genome oligonucleotide-based
arrays (Agilent, Santa Clara, USA). Male reference DNA (Promega, Madison, USA) and
sample DNA were digested with AluI and RsaI (Promega, Madison, USA) and labeled by
random priming with Cyanine 3- and Cyanine 5-dUTP (Perkin-Elmer, Massachusetts, USA),
respectively, by using the BioPrime Array CGH Genomic Labeling Kit (Invitrogen, Carlsbad,
USA). After purification with Microcon YM-30 filter units (Millipore, Billerica, USA), the
two labeled DNA species were hybridized together with human cot-DNA (Roche, Basel,
Switzerland) onto CGH arrays for 48 hours at 65°C. Slides were scanned with a G2505B
Micro Array Scanner (Agilent, Santa Clara, USA). Feature extraction and data analysis were
performed using the Feature Extraction and DNA Analytics software (Agilent, Santa Clara,
USA), respectively.

**Polymerase chain reaction (PCR) of genomic DNA**

Chromosomal DNA was isolated with GenElute Mammalian Genomic DNA Miniprep Kit
(Sigma; St Louis, MO, USA). PCR was performed using chromosomal DNA and illustra
PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) and 4 µM primer
mix of following primers: human TRPM3: exon 1, forward (for) 5'-
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CAGGCAAGGCTACTTGACTA-3', reverse (rev) 5'-CACCAGCCTCTTGTCTCTGA-3';
exon 3, for 5'-GGTGGACGGAGAGGAGAGA-3', rev 5'-
GGGGCAGGGGACTCACAGCA-3'; exon 6, for 5'-CTCCTAGCTCCCCGCCCCACCA-3',
rev 5'-TGCCAGTGAGAAACACCGGT-3'; exon 8, for 5'-
TCCACAATTAGACATGACGCTGCAA-3', rev 5'-GCTGGCCACCCCATGCGGAAT-3';
exon 9, for 5'-CCTGACTCATAGCAAGGGCCCA-3', rev 5'-
GCTGTCAGGATAGCCAAATCAATGTCC-3'; exon 10, for 5'-
ATGCCTCGGCCCCACACCA-3', rev 5'-AGATGGCGCCTGCAGAGGGA-3'; exon 12,
for 5'-TGAACCTCGCTCCACCCACAGGCA-3', rev 5'-GGTACAGTCAGCCAGGCCACCA-3';
exon 15, for 5'-AGGACAGCTGGGACCTGTCA-3', rev 5'-
CCTGTGTGTGCATCAGGGGT-3'; exon 18, for 5'-GCCTGGGCTTCCCAGGCTAC-3',
rev 5'-TGGACCTTGGCTTCTCTTGCC-3'; exon 21, for 5'-
TCCCTCTGCTTCCCCACCTC-3', rev 5'-TCAACAATGCGCCCTGGGAAT-3'; exon 24,
for 5'-TCTCTGACACCCCCACACCA-3', rev 5'-TCTGACAGGGGAGGTGTTCCA-3';
exon 25, for 5'-TCCTGCAACCCCGCTGGCAAC-3', rev 5'-
GGGAAGGGGACTGCTCAGGTCGCC-3'. Human AXIN1: exon 1, for 5'-
CCGGCTACCCGGATACCTCAGCA-3', rev 5'-CTCCAGGGCATCATGGCAGCAA-3';
exon 2, for 5'-CCCCACAGTGCTGGGTAGA-3', rev 5'-TGGGGCTCTCAGCGCCACTG-3';
exon 3, for 5'-TCCTGGCGGGAGCAGGACTG-3'; exon 5, for 5'-
GGCCGGACACAGAAAAAGGGG-3', rev 5'-CATCAGGACATCCACGGGCGC-3'; exon 6,
for 5'-GGGCGATCCTCGGAGAGA-3', rev 5'-GCAGCAGGATTGCGTGC-3';
exon 7, for 5'-AGGATGCGAGAGAAAAGCAG-3', rev 5'-
AGAAGTGCTACGCTCAGCA-3'; exon 8, for 5'-ACAGGGAAGGACACCGGCCCCCA-3',
rev 5'-TGCCCTCCAGGACCTCTGCCC-3'; exon 9, for 5'-AGACCGAGTTGCCACGACG-3';
Expression profiling using microarrays

Total RNA was isolated from duplicates of early and late 3p and 3sp HCC cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). The integrity and quantity of RNA was analyzed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). cDNA labeling and hybridization on Affymetrix GeneChip® Human gene 1.0 ST Array (Affymetrix, Santa Clara, USA) as well as scanning of signal intensities was performed according to the manufacturer’s protocol. The ratio of regulation was calculated and a minimum of 3-fold regulation was considered as significant. Pathway analysis was performed with Gene set enrichment analysis (GSEA) software by comparing the molecular profile data with existing as well as self-defined gene sets. Complete gene expression data have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible by GEO Series accession no. GSE26391.

Analysis of CpG methylation

Genomic DNA was isolated from 3p and 3sp HCC cells grown in quadruplicates and from primary peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The digestion of 600 ng genomic DNA with methylation-sensitive restriction enzymes (MSRE) was performed. In total, 327 cancer-specific promoter loci were analyzed by PCR. 600 ng genomic DNA of 3p and 3sp HCC cells and from primary peripheral blood cells were digested with methylation-sensitive restriction enzymes (MSRE) overnight at 37°C by employing a mixture of 6 units of each AciI (New England Biolabs, Frankfurt am Main, Germany), Hin6I (Fermentas, St. Leon-Rot, Germany) and HpaII (Fermentas). Quantitative
polymerase chain reaction (qPCR) was performed on MSRE-digested DNA covering the CpG rich area at chr5:151,066,289-151,066,501 (human genome annotation: hg 19), including part of the first gene coding exon. This sequence region contains 9 MSRE sites when using the mixture of 3 enzymes mentioned above. Completion of digestion was confirmed using a control PCR covering known differentially methylated regions (DMRs, H19, IGF2, ABL1, PITX2, XIST and FMR1) as published recently (19). 10 ng of MSRE-digested DNA was amplified in 10 µl PCR volume using 0.3 U HotStarTaq polymerase and buffer containing 1.5 mM MgCl₂ (Qiagen, Hilden, Germany) using a 384-well format and the LightCycler® LC480 (Roche, Vienna, Austria). Cycling was performed with 15 minutes of initial denaturation followed by 50 cycles with each 95°C for 40 sec, 65°C for 40 sec and 72°C for 80 sec including a final extension at 72°C for 7 minutes. A melting curve analysis was performed for confirming specificity of amplicons with a Tm of 88.4°C ±0.6°C. Cp values were extracted from qPCR-data using the LightCycler LC480 software. Boxplots were generated from quadruplicate “45-Cp” values using R statistical software.

Therapeutic agents

Doxorubicin hydrochloride (adriamycin hydrochloride), cis-diammineplatinum (II) dichloride (cisplatin) and epirubicin hydrochloride were purchased from Sigma, St Louis, USA and dissolved in 0,9% NaCl. Sorafenib (Nexavar®; Bayer HealthCare Pharmaceuticals, Wayne, USA), erlotinib (Tarceva®; LC Laboratories, Woburn, USA) and bevacizumab (Avastin®; Roche, Basel, Switzerland) were dissolved in dimethylsulfoxide (DMSO). Stock solutions were diluted in medium to concentrations indicated in the text.

Determination of the inhibitory concentration (IC)₅₀

Cell viability was determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in triplicates at a
density of $6 \times 10^3$ cells per well. After 24 hours, cells were incubated with drug-containing medium for three days. Cells were incubated with MTT solution (5mg/ml; Sigma, St. Louis, USA) and medium was replaced with DMSO after five hours. The absorbance was measured at 620 nm by employing a microplate reader (Asys HiTech, Salzburg, Austria). MTT assays were repeated 3 times for each drug application and untreated cells were used as reference. IC$_{50}$ values were obtained by log-linear interpolation of data points and are depicted by dose-response curves using the software GraphPad Prism$^®$ 5.01.

**Verification of IC$_{50}$ values by proliferation**

$6 \times 10^4$ cells /well were seeded in duplicates on collagen-coated 12-well plates and incubated with drugs at a concentration of determined IC$_{50}$ values for 3 days. Cells were trypsinized and counted with a cell counter (CASy, Roche, Basel, Switzerland). Untreated cells were used as reference to calculate the percentage of proliferation.

**Statistical analysis**

Data were expressed as means ± standard deviation (SD). The statistical significance of differences was evaluated using an unpaired, non-parametric Student’s t-test. Significant differences between experimental groups were * p<0,05, ** p<0,01 or *** p<0,005.
Results

HCC cells show an epithelial phenotype and a mesenchymal one correlating with enhanced cell motility

We analyzed various human hepatoma cell lines for their epithelial and mesenchymal characteristics in order to establish a model of HCC progression (Supplementary Table 2). Two distinct liver cell lines were of particular interest as these cell types have been isolated from the HCC of one patient (20). Phase contrast analysis suggested an epithelial cell type, termed 3p, and a mesenchymal cell population, designated 3sp (Fig. 1A). Both HCC cells showed diploid DNA content (Supplementary Fig. 1) and short tandem repeat analysis verified their common genomic identity (Supplementary Table 3). qRT-PCR analysis revealed that 3p cells express epithelial markers such as E-cadherin and keratin 8, whereas 3sp cells showed a mesenchymal expression signature by upregulation of the transcription factors LEF1, SNAI1, SNAI2 and ZEB1 (Fig. 1B). Immunolocalization demonstrated intact adherence junctions by expression of E-cadherin, β-catenin and p120catenin at cell borders of 3p cells, whereas 3sp cells failed to show this phenotype (Fig. 1C). In contrast to 3sp, 3p cells displayed cytoplasmic distribution of the epithelial marker keratin 8 by concomitant absence of the mesenchymal interfilament component vimentin. In addition, mesenchymal 3sp cells showed relocalization of actin from the cell membrane to stress fibers suggesting enhanced motility.

We next assessed the migratory and invasive potential of 3p and 3sp cells. First, we performed proliferation kinetics to exclude an influence of differential cell doubling rates on migration and invasion assays. No significant difference in proliferation of 3p and 3sp cells could be detected (Fig. 2A). Yet, a 4-fold increased migratory potential of mesenchymal 3sp cells was determined when compared to 3p cells (Fig. 2B and 2C). To analyze invasion, HCC cells were embedded into matrigel. While epithelial 3p cells showed no invasive potential, mesenchymal 3sp cells displayed a more than 5-fold stronger ability to invade into the matrix.
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(Fig. 2D and 2E). The invasive potential of mesenchymal 3sp cells was further analyzed by 3-dimensional (3D) spheroid formation in collagen gels (18). Epithelial 3p cells formed compact and round spheroids without cell invasion into the surrounding gel (Supplementary Fig. 2A), even though proliferation was not impaired (data not shown). On the contrary, mesenchymal 3sp cells failed to form spheroids on their own, however, these cells attached onto the surface of epithelial 3p spheroids after co-cultivation, and showed strong invasion into the surrounding matrix (Supplementary Fig. 2A and 2B). Immunofluorescence analysis of E-cadherin and β-catenin indicated epithelial characteristics of 3p-derived spheroids, whereas these markers could not be detected in invading 3sp cells due to disassembly of epithelial junctions (Supplementary Fig. 2C). Taken together, 3p and 3sp cells showed a distinct epithelial and mesenchymal phenotype correlating with poor and strong migratory and invasive abilities, respectively.

Mesenchymal HCC cells developed through undergoing EMT in the patient

We further investigated whether 3sp cells have been derived from epithelial 3p cells through EMT in the HCC patient. aCGH analysis showed that changes in the genomic DNA of these HCC cell lines were widely identical (Supplementary Fig. 3), which were exemplified by the loss of genomic DNA in the TRPM3 and AXIN1 loci of both cell types (Fig. 3A and 3B). PCR analysis of multiple exons could identify the exact chromosomal breaks which are located between exon 12 and 15 and between exon 2 and 3 in TRPM3 and AXIN1, respectively (Supplementary Fig. 4). This homozygous loss of chromosomal regions indicates an identical cellular origin of 3p and 3sp hepatoma cells, and thus demonstrates that 3sp cells have been developed from 3p cells via EMT in the patient.

To verify EMT, we analyzed the 3p/3sp HCC cell lines by whole-genome expression profiling (Fig. 4A and 4B). Remarkably, a large cluster of liver-specific genes were downregulated in 3sp cells compared to 3p cells (Supplementary Fig. 5), including CYP450...
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phase I enzymes, *alcoholdehydrogenase (ADH)* and *aldehyde dehydrogenase (ALDH)* and phase II enzymes such as *UDP-glucuronosyl-transferases (UGT)* and *glutathione-S-tranferase (GST).* Importantly, a cluster of epithelial markers involving components of tight junctions (*claudins*), desmosomes (*desmoplakin*) and adherence junctions (*E-cadherin*) were found to be strongly downregulated in mesenchymal 3sp cells (Fig. 4A). Moreover, EMT-specific genes such as *vimentin*, *LEF1* and *SNAI2* were highly upregulated in 3sp cells (Fig. 4B), as shown in Fig. 1B and 1C. In addition, expression profiling revealed (i) upregulation of growth factors and matrix *metalloproteinases (MMPs)* such as *connective tissue growth factor (CTGF), TGF-β2* and *MMP2,* (ii) upregulation of receptors associated with EMT such as *platelet-derived growth factor (PDGF)-Rβ* and *discoidin domain receptor tyrosine kinase (DDR)2,* and (iii) increase of EMT-associated proteins such as *fibroblast activation protein (FAP), lysyl oxidase, tenascin C* and *SPARC (secreted protein, acidic and rich in cysteine).* qPCR further confirmed the upregulation of *PDGF-Rβ, TGF-β2, DDR2* and *FAP* in 3sp cells as detected by microarrays analysis (Supplementary Fig. 6). From these data we conclude that mesenchymal 3sp HCC cells derived from epithelial 3p cells via EMT in the HCC patient.

**SPARC activation by CpG demethylation**

We next addressed the differences in the methylation status of gene promoters in 3p and 3sp cells. In order to exclude cell culture effects, genomic DNA of 3p/3sp cells from early as well as late passages was analyzed. Most notably, *SPARC* was identified to be epigenetically regulated, in line with a 128-fold upregulation of the transcript level (Fig. 4B). Methylation-specific PCR of the *SPARC* promoter revealed a lowered methylation status in both early and late passaged mesenchymal 3sp cells (Fig. 4C), which was verified by qRT-PCR analysis showing a >30-fold increase of *SPARC* mRNA in 3sp cells (Fig. 4D). Since the aCGH profile of the *SPARC* locus was unaffected, these data provide first evidence that the upregulation of *SPARC* is caused by demethylation during HCC progression.
The 3p/3sp EMT model is suitable for drug testing

We next used this human HCC model to assess the efficacy of clinically used anti-cancer agents. Dose response relationships and corresponding half maximal inhibitory concentrations (IC$_{50}$) of drugs were determined (Supplementary Table 4) and verified by viability assays (Supplementary Fig. 7A). Chemotherapeutics such as doxorubicin, cisplatin and epirubicin currently used for TACE were compared to targeted therapeutic drugs such as the multikinase inhibitor sorafenib, the EGFR-inhibitor erlotinib and the VEGF-inhibitor bevacizumab. 3sp cells showed a slightly increased susceptibility against chemotherapeutics when compared to 3p cells (Fig. 5A-C; Supplementary Table 4), which is explained by downregulation of multiple drug resistance genes in 3sp cells (Supplementary Fig. 8A). Interestingly, 3sp cells were more resistant to targeted therapies including sorafenib and erlotinib (Fig. 5D and 5E; Supplementary Table 4). Bevacizumab did not show an effect on neither of the cells (Fig. 5F; Supplementary Table 4). Of particular interest was the higher resistance of 3sp cells towards sorafenib, as expression analysis showed a 10-fold upregulation of PDGF-R$\beta$ (Fig. 4B). Additional targets of sorafenib including VEGFR-1, -2, -3 and RAF kinases were found unaltered between 3p and 3sp cells (data not shown). As PDGFR-\$\beta\$ is described to play an important role in migration (21), we analyzed the migratory potential of 3sp cells upon sorafenib treatment (Supplementary Fig. 7B and 7C). Interestingly, delivery of sorafenib at the IC$_{50}$ showed a strong reduction of migration, whereas control treatment of doxorubicin at the IC$_{50}$ did not impair migration. Together, these data indicate that sorafenib has a lower cytotoxic potential on 3sp cells, however, strongly represses their migratory abilities.

In recent clinical efforts, sorafenib and doxorubicin were frequently used in combination therapy. Thus, we investigated the effect of the combined treatment of these anti-cancer drugs in our model of HCC progression. We therefore treated cells with one drug in increasing concentrations, while the second drug was kept constant at its IC$_{50}$. First, variable amounts of
doxorubicin were applied in combination with the IC$_{50}$ of sorafenib on 3p and 3sp cells (Fig. 6A). Compared to doxorubicin monotherapy, cells showed a drastic susceptibility for the combined treatment, leading to a reduction of the IC$_{50}$ for doxorubicin of 60% and 39% in 3p and 3sp cells, respectively (Fig. 6B; Supplementary Table 4). The vice versa treatment, applying variable amounts of sorafenib in combination with the IC$_{50}$ of doxorubicin (Fig. 6C) showed a similar result, leading to a reduction of the IC$_{50}$ for sorafenib of 18% and 50% in 3p and 3sp cells, respectively (Fig. 6D; Supplementary Table 4). Most interestingly, we observed a normalization of the IC$_{50}$ values between 3p and 3sp cells after combined treatment in both approaches (Fig. 6B and 6D; Supplementary Table 4). Taken together, these results indicate that the monotherapy shows considerable differences in the susceptibility between HCC cells undergoing EMT. Our data provide evidence that the combined use of doxorubicin and sorafenib is highly efficient to target both epithelial and mesenchymal HCC cells.
Discussion

EMT has been increasingly recognized to play a crucial role in HCC progression by the acquisition of invasive properties. In line with the transdifferentiation of neoplastic hepatocytes to motile mesenchymal derivatives, HCC is described as a heterogenous tumor at advanced stages showing clonal expansion of genetically distinct malignant cell populations. Therefore, efficient anti-cancer therapy depends on targeting cancer cells at all stages of differentiation.

EMT has been suggested as the critical step in tumor cell dissemination and particularly associates with resistance towards chemotherapy and immunotherapy (1). Here we established and characterized the first human cellular model of EMT in HCC progression which correlates with the recently established molecular expression pattern of early and late TGF-β signatures (22). The late TGF-β signature of HCC was shown to associate with tissue invasion, EMT and metastasis, vascular transmigration and angiogenesis, earlier recurrence and shortened survival time as well as downregulation of glycolysis-regulating and acute-phase response genes. Aligning the gene expression sets of 3p and 3sp cells revealed a correlation with early and late TGF-β signature of more than 70 percent, respectively (data not shown). Moreover, we observed upregulation of TGF-β1 in mesenchymal 3sp cells and a resistance against TGF-β treatment suggesting an autocrine regulatory TGF-β loop. Thus, this model is a reliable tool to determine novel key players and to test drug efficacy of new and currently approved anti-cancer agents within HCC progression. By employing this model we show that epithelial cells are more sensitive to the targeted therapeutic agents such as sorafenib and erlotinib, whereas mesenchymal cells show a slightly more efficient susceptibility to chemotherapeutic drugs such as doxorubicin, cisplatin and epirubicin. This might be caused by the downregulation of various multiple drug resistance proteins in the 3sp cells (Supplementary Fig. 8A). In the same line, the high sensitivity towards erlotinib might
be explained by the higher phosphorylation status of *EGFR* found in 3p cells (Supplementary Fig. 8B).

We propose that the molecular changes upon EMT are crucial for differential drug efficacy. In this regard, we could demonstrate for the first time *in vitro* that the combined application of sorafenib and doxorubicin shows two advantages. First, combined therapy is capable of targeting both, epithelial and mesenchymal cells, which might have the potential to reduce the risk of HCC recurrence. Second, the effective concentrations of drugs could be reduced and thus side effects can be minimized. Yet, both cell lines showed no response against bevacizumab, referring to the limitation of the HCC model to study angiogenic mechanisms *in vitro*.

The efficacy of combining sorafenib and doxorubicin was shown in a phase II trial, resulting in increased overall and progression free survival in patients with advanced HCC compared to those who received monotherapy with doxorubicin (23). Furthermore, a phase III randomized study which compares sorafenib together with or without doxorubicin treatment in advanced or metastatic liver cancer is currently recruiting HCC patients. Clinical trial information of the U.S. National Institutes of Health is available under the identifier NCT01015833.

Remarkably, our data obtained by using 3p/3sp HCC cells confirm these observations and thus underline the synergistic effects of sorafenib and doxorubicin in interfering with HCC progression. Other ongoing clinical trials investigate the combination of sorafenib and doxorubicin or other chemotherapeutics using TACE. Clinical trial information of the U.S. National Institutes of Health is available under the identifiers NCT01011010, NCT00478374 and NCT0085528. Interestingly, treatment of the 3p/3sp HCC model with both sorafenib and erlotinib (currently employed in a phase III study) also revealed to be effective at reduced concentrations while targeting both epithelial and mesenchymal HCC cells (data not shown).

Our data revealed a significant upregulation of *SPARC* in mesenchymal 3sp cells which has been reported in patient samples at advanced stages of disease (24). The role of this
protein in hepatocarcinogenesis, however, is rather ambiguous by acting either as a tumor promoter (25) or tumor suppressor (24). Notably, we could show for the first time that \textit{SPARC} is epigenetically upregulated in human HCC progression by demethylation. Further studies will reveal the consequences of \textit{SPARC} overexpression in hepatocarcinogenesis. In the same line, aCGH analysis revealed homozygous loss of the Wnt signaling component \textit{AXIN1} as well as of the cation-selective channel \textit{TRPM3}. While deletion of \textit{TRPM3} has not been reported in cancer progression, \textit{AXIN1} was found to be mutated in 5-25\% of HCC patients and associated with poor differentiation (26). These findings show that our EMT model is genetically well defined and thus particularly valid to study human HCC progression without unknown genetic variations in widely used human hepatoma cell lines.

Human models of hepatocellular EMT which reliably reflect HCC progression are invaluable tools in pre-clinical studies for (i) the identification of molecular mechanisms underlying HCC progression, (ii) the pharmacological determination of dose-effect relationships and thus the efficacy of single and combined treatments with novel and currently used anti-cancer drugs, and (iii) the (re)-evaluation of drug target specificity and pleiotropic effects after drug application.
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Disclosure of potential conflicts of interest

The authors declared no potential conflicts of interest.
References


Figure legends:

**Figure 1.** Epithelial and mesenchymal characteristics of 3p and 3sp HCC cells. A, Phase contrast images display an epithelial (3p) and a fibroblastoid phenotype (3sp; bar, 50 µm). B, qRT-PCR analysis of epithelial (*E-cadherin*, *keratin 8*) and mesenchymal markers (*LEF1*, *SNAI1*, *SNAI2* and *ZEB1*) in 3p and 3sp cells. C, Confocal immunofluorescence analysis of the epithelial markers *E-cadherin*, β-*catenin*, *p120catenin* and *keratin 8* as well as the mesenchymal marker *vimentin*. Localization of *actin* at cell membranes and actin stress fibers as indicated by phalloidin staining (red). Nuclei were counterstained with To-Pro3 (blue). Bar, 25 µm. dCT, delta CT value.

**Figure 2.** Mesenchymal 3sp HCC cells exhibit enhanced migration and invasion. (A) Proliferation kinetics of epithelial 3p and mesenchymal 3sp cells. (B) Migration assay using the Platypus® technology after 3 days and the quantification of cell migration by fluorometric analysis. (C) Invasion assays employing the Platypus® technology after 7 days. Upper left panel and lower left panel show the invasion area in the absence and presence of the mask, respectively. Quantification of cell invasion by fluorometric analysis is shown in the right panel. Cells were visualized with green CellTracker™. Bar, 250 µm; ***, p<0,005.

**Figure 3.** Mesenchymal 3sp HCC cells are derived from epithelial 3p HCC cells through EMT *in vivo*. aCGH analysis revealed a homozygous loss of genomic DNA in 3p and 3sp cells. (A) Homozygous loss of DNA in the *TRPM3* and (B) *AXIN1* gene locus. Red bars indicate exons and blue bars denote the positions of arrayed oligonucleotides on the chromosome. bps, basepairs.
Figure 4. Global transcriptome analysis shows regulation of EMT-specific genes in 3sp HCC cells. Gene Set Enrichment Analysis of a whole-genome Affymetrix GeneChip® was performed. (A) Downregulation (blue) of epithelial markers and (B) upregulation (red) of EMT regulators in 3sp cells were determined. The -fold upregulation of mRNA was calculated and depicted by the ratio of (A) 3p to 3sp cells (3p/3sp) and of (B) 3sp to 3p cells (3sp/3p). C, DNA methylation testing by qPCR upon methylation-sensitive digestion shows demethylation of the SPARC promoter in 3sp cells. 4 replicates of each cell type were analyzed. DNA from male and female blood (each n=4) were used as negative controls. D, Confirmation of SPARC upregulation in 3sp cells by qRT-PCR. dCT, delta CT value. ACTA2, smooth muscle actin; CDH1, E-cadherin; CLDN, claudin; CTGF, connective tissue growth factor; DDR2, discoid domain receptor; DSP, desmoplakin; EPCAM, epithelial cellular adhesion molecule; FAP, fibroblast activation protein; HNF4A, hepatocyte nuclear factor 4α; KRT, keratin; LEF1, lymphoid enhancing factor 1; LOX, lysyl oxidase; MMP, matrix metalloproteinase; PDGF-Rβ, platelet-derived growth factor receptor β; SNAI2, Slug; SPARC, secreted protein, acidic, cystein-rich; TGF-β, transforming growth factor-β; TNC, tenascin C; VIM, vimentin.

Figure 5. Increased resistance of mesenchymal HCC cells against targeted anti-cancer drugs. Cytotoxicity of (A) cytostatic agents and (B) targeted therapeutic drugs was analyzed by MTT assays and IC₅₀ values were calculated. Dose-response relationships of 3p and 3sp cells were compared. Dotted lines indicate IC₅₀.

Figure 6. Combined treatment with sorafenib and doxorubicin efficiently targets both, epithelial and mesenchymal HCC cells. Combined application was performed by delivering cells with one drug in increasing concentrations while the second drug was
kept constant at the IC\textsubscript{50} concentration. Dose response relationships for 3p and 3sp cells upon (A) treatment with doxorubicin alone or in combination with sorafenib (+ IC\textsubscript{50} sora) and (C) sorafenib alone or in combination with doxorubicin (+ IC\textsubscript{50} doxo) are shown. The resulting change of IC\textsubscript{50} values (arrow, %) of single to combined treatment are depicted for (B) doxorubicin and (D) sorafenib. Arrow bars indicate standard deviation of triplicates (A, C) or range of calculated IC\textsubscript{50} from at least three independent experiments (D, E).
Figure 1

A

B

C

E-cadherin

keratin 8

LEF1

SNAI1

SNAI2

ZEB1

E-cadherin

keratin 8

β-catenin

vimentin

pT20 catenin

phalloidin

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Figure 2

A

B

C

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A

TRPM3 (58556 bps)

3p early
3p late
3sp early
3sp late

B

AXIN1 (72025 bps)

3p early
3p late
3sp early
3sp late
Figure 4

A

Gene 3p/3p 3p/3p 3p/3p 3p/3p 3p/3p
KRT20 0.1 0.1 0.1 0.1 0.1
CCH1 0.2 0.2 0.2 0.2 0.2
EPCAM 0.0 0.0 0.0 0.0 0.0
SNP3A 0.0 0.0 0.0 0.0 0.0
CLCN4 0.0 0.0 0.0 0.0 0.0
CLDN1 0.0 0.0 0.0 0.0 0.0
D3P 0.0 0.0 0.0 0.0 0.0
KRT20 0.0 0.0 0.0 0.0 0.0
CLCN1 0.0 0.0 0.0 0.0 0.0

B

Gene 3p/3p 3p/3p 3p/3p 3p/3p 3p/3p
ACTA2 3.0 3.0 3.0 3.0 3.0
V8R 3.3 3.3 3.3 3.3 3.3
CTGF 5.7 5.7 5.7 5.7 5.7
PSMD18 7.0 7.0 7.0 7.0 7.0
TGFBR2 12.1 12.1 12.1 12.1 12.1
LRP1 24.8 24.8 24.8 24.8 24.8
DDR2 29.3 29.3 29.3 29.3 29.3
FAP 51.8 51.8 51.8 51.8 51.8
MMP2 101.3 101.3 101.3 101.3 101.3
LAM 205.3 205.3 205.3 205.3 205.3

C

D

BSPARC 40 30 20 10 0

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Figure 5

A

B

doxorubicin log[nM]  
sorafenib log[nM]  
cisplatin log[nM]  
erlotinib log[nM]  
epirubicin log[nM]  
bevacizumab log[nM]
Figure 6

A

B

C

D

% cell viability

IC₅₀ doxo [μM]

% cell viability

IC₅₀ sora [μM]

% cell viability

% cell viability

doxorubicin log[nM]
doxorubicin log[nM]
sorafenib log[nM]
sorafenib log[nM]

3p, 3p + IC₅₀ sora, 3p + IC₅₀ doxo

3p, 3p + IC₅₀ sora, 3p + IC₅₀ doxo

60%  39%

18%  50%
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Franziska van Zijl, Sabine Mall, Georg Machat, et al.

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