Expression and prognostic significance of insulin-like growth factor-2 receptor in human hepatocellular carcinoma and the influence of transarterial chemoembolization

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common human malignancies, the incidence of which is growing worldwide. The prognosis of HCC is very poor and it is often accompanied by a high rate of recurrence. Conventional chemotherapeutic approaches are largely inefficient. In order to develop novel effective methods for the early detection and prognosis of HCC, novel markers and therapeutic targets are urgently required. The present study focused on the effects of the expression of the tumor suppressor gene insulin-like growth factor-2 receptor (*IGF2R*) on patient survival and tumor recurrence in patients with HCC; this study paid specific attention to the influence of transarterial chemoembolization (TACE) prior to surgery. The mRNA expression levels of *IGF2R* were measured in primary human HCC and corresponding non-neoplastic tumor-surrounding tissue (TST) by reverse transcription-polymerase chain reaction (RT-PCR) (n=92). Subsequently, the associations between *IGF2R* expression and clinicopathological parameters, outcomes of HCC and TACE pretreatment prior to surgery were determined. Furthermore, the effects of the *IGF2R* gene polymorphisms rs629849 and rs642588 on susceptibility and on clinicopathological features of HCC were investigated. RT-PCR demonstrated that the mRNA expression levels of *IGF2R* were downregulated in HCC compared with in TST samples (P=0.004), which was associated with a worse recurrence-free survival of patients with HCC (P=0.002) and a lower occurrence of cirrhosis (P=0.05). TACE-pretreated patients with HCC (n=26) exhibited significantly higher *IGF2R* mRNA expression in tumor tissues (P=0.019). In addition, significantly more patients with HCC in the TACE-pretreated group exhibited upregulated *IGF2R* mRNA expression compared with in the non-treated patients (P=0.032). The *IGF2R* SNPs rs629849 and rs642588 were not significantly associated with HCC risk, whereas a homozygous *IGF2R* rs629849 GG genotype was associated with a significantly elevated risk of non-viral liver cirrhosis (P=0.05). In conclusion, these data suggested an important role for *IGF2R* expression in HCC, particularly with regards to TACE treatment prior to surgery.

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Abbreviations: HCC, hepatocellular carcinoma; IGF2R, insulin-like growth factor-2 receptor; TACE, transarterial chemoembolization; TST, tumor-surrounding tissue; IGF1, insulin-like growth factor-1; IGF2, insulin-like growth factor-2; IGF1R, insulin-like growth factor-1 receptor; TGF-β1, transforming growth factor-β1; PSQ, pyrosequencing; OR, odds ratio

Key words: IGF2R, HCC, TACE, single nucleotide polymorphism

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third most frequent cause of global cancer-associated mortality (1-3). Curative therapies for HCC include surgical resection and liver transplantation; however, <30% of patients are candidates for curative surgery (4,5). HCC is a highly aggressive tumor that is characterized by high-grade malignancy, early metastasis, infiltrating growth and a poor prognosis, even following liver transplantation (6-8). Furthermore, treatment options using systemic therapies are very limited. In general, HCC does not respond to classical chemotherapeutics. Due to the extensive molecular and genotypic heterogeneity of HCC, suitable biomarkers for surveillance, diagnosis and prediction of prognosis in patients with HCC remain to be identified, and are currently not ready for introduction into clinical practice. Therefore, novel markers

and therapeutic targets are urgently required to develop effective methods for early detection and prognosis of HCC, as well as for therapies to treat advanced HCC.

Transarterial chemoembolization (TACE) is a non-curative, but useful, palliative treatment option for nonsurgical patients with preserved liver function and large or multinodular non-invasive HCC (9). It is also used as a bridging strategy to limit tumor growth during the waiting times for liver transplantation (10,11). Intra-arterial infusion of a chemotherapeutic substance with a viscous emulsion, followed by the embolization of blood vessels with an embolic agent, results in a cytotoxic effect with ischemia (12). TACE has been reported to be effective in downstaging of cancer, leading to improved overall and recurrence-free survival following liver transplantation (13-16). Repeatedly performed TACE has been reported to be capable of selecting biologically favorable tumors and appears to reflect biological properties, such as tumor aggressiveness (17). At present, little is known regarding the molecular mechanisms that are induced during TACE treatment, and TACE-induced prognostic markers may influence the prognosis of HCC following liver transplantation. The identification of useful predictive markers for the prognosis of TACE therapy may be helpful in improving local tumor control, in order to better select patients suitable for liver transplantation, to reduce recurrence, and to prolong survival and quality of life for patients who remain unsuitable for resection (12).

The insulin-like growth factor (IGF) system is physiologically involved in the regulation of cellular proliferation and apoptosis, and is associated with tissue growth (18). The IGF axis is dysregulated in numerous types of cancer and is considered a key driver in hepatocarcinogenesis (19). Whereas the role of the ligands IGF1 and IGF2, as well as the IGF1 receptor (IGF1R) signaling pathway in hepatocarcinogenesis, has been the focus of numerous studies (20-26), few data exist regarding the specific role of the mannose 6-phosphate/IGF2 receptor (IGF2R, CD222). Many types of human cancer, including colorectal carcinoma (27), breast cancer (28) or HCC (29), are associated with a reduced IGF2R function. IGF2R is a transmembrane protein, which is predominantly located in the Golgi apparatus and pre-endosomal compartments (90%), and to a lesser extent at the cell surface (10%). It is ubiquitously expressed in tissues, but also present in the circulation (30,31). IGF2R binds IGF2, as well as proteins bearing mannose 6-phosphate residues (e.g. lysosomal proteins) at distinct sites on the receptor (32). The receptor participates in the internalization and lysosomal degradation of IGF2, a mitogen that normally acts through IGF1R to stimulate cell proliferation (33). As a cell surface protein, IGF2R lacks a tyrosine kinase domain, and was therefore originally assumed to solely act as a scavenger receptor lacking intrinsic signaling (34). Nonetheless, a few studies concerning cardiac pathophysiology (35-37) have indicated that IGF2R contains a putative G-protein binding site within its cytoplasmic domain. Therefore, IGF2R may not only function in degradation of IGF2, but may also trigger an intracellular signaling pathway through coupling with G-protein-coupled receptors. IGF2R also activates the latent precursor of transforming growth factor-β1 (TGF-β1), which is a potent growth inhibitor for several cell types (38,39).

The *IGF2R* gene is located on chromosome 6q26 in a large 140 kb locus, and is comprised of 48 exons and introns. There is considerable evidence supporting the importance of a genetic predisposition in patients with HCC (40). Numerous single nucleotide polymorphisms (SNPs) have been identified; however, their impact on HCC has yet to be fully elucidated (41). Notably, the SNP rs629849 (transition G → A at position +1,619) in exon 34, which is localized in the IGF2-binding domain, has been reported to be implicated in IGF2-dependent growth (42). Furthermore, the present study analyzed the role of the SNP rs642588 (transition C → T), which is located in the CTCF-binding site of the *IGF2R* promoter.

These previous findings have suggested that IGF2R possesses various growth inhibitory functions and is therefore considered a candidate tumor suppressor (43). The present study aimed to elucidate the effects of *IGF2R* expression on survival and tumor recurrence in patients with HCC. The expression levels of *IGF2R* were measured in HCC and corresponding non-neoplastic tumor-surrounding tissue (TST), and its association with clinicopathological parameters and outcomes was determined. To the best our knowledge, it is currently unknown as to whether the expression and regulation of *IGF2R* is altered following TACE. Therefore, the effects of TACE pretreatment on *IGF2R* mRNA expression were investigated. The possible effects of *IGF2R* gene polymorphisms (rs629849 and rs642588), and their combination, on susceptibility and on the clinicopathological features of HCC were also analyzed.

Materials and methods

*Patient tissue sample*s. HCC tumor samples and corresponding TST were obtained from 92 patients undergoing tumor resection (n=66) or liver transplantation (n=26) between March 2007 and December 2013 at the Department of Hepatobiliary and Transplantation Surgery and the Department of General and Abdominal Surgery (Johannes Gutenberg University Mainz, Mainz, Germany). A total of 26 patients underwent TACE prior to surgery. TACE was performed at 6-week intervals using mitomycin and lipiodol. Written informed consent was obtained from each patient, according to the agreement on transfer and scientific use of excess material of the University Medicine of the Johannes Gutenberg University Mainz. The present study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the State of Rhineland-Palatinate Medical Board [Number 847.243.17 (11077)]. Liver tissues were immediately flash frozen following resection and were stored in liquid nitrogen prior to analysis. All cases of HCC were diagnosed or confirmed by histology. Healthy normal and non-cirrhotic control liver tissues (n=31) were obtained from patients undergoing liver surgery for hepatic metastases following colon or breast carcinoma (male, n=21; female, n=10). The median age of the control patients was 63 years old (range, 47-85 years) at the time of surgery.

Tissue homogenization, RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Prior to RNA extraction, 20 mg tissue samples were homogenized using a Precellys 24 homogenizer (Bertin

Instruments, Montigny-le-Bretonneux, France) in CK14 tubes at 5,100 rpm for 2x20 sec. RNA was extracted from tissue samples using the PeqGOLD Total RNA kit (VWR International GmbH, Darmstadt, Germany). cDNA was prepared from 1 μg total RNA (20 μl total volume) using the qScript™ XLT cDNA SuperMix (Quantabio, Beverly, MA, USA). All aforementioned kits were used according to the manufacturers' protocols. Semi-quantitative analysis of *IGF2R* transcripts was performed by RT-PCR. The Absolute Blue QPCR SYBR-Green Mix kit (Thermo Fisher Scientific, Inc., Waltham MA, USA) and the following primers were used: *GAPDH*, forward 5'-TTTTGCGTCGCCAGCCGAG-3', reverse 5'-ACCAGGCGCCCAATACGACC-3'; and *IGF2R*, HS_IGF2R_1_SG Quantitect Primer Assay (Qiagen GmbH, Hilden, Germany). Cycling conditions were as follows: Initial denaturation at 15 min for 95˚C, followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 66˚C for *GAPDH* and 55˚C for *IGF2R* for 30 sec, and elongation at 72˚C for 30 sec. Samples were run on a LightCycler® 480 Real-Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany). PCR products were analyzed using the QIAxcel capillary gel electrophoresis system (Qiagen GmbH). The relative expression levels of *IGF2R* mRNA in HCC and TST samples were calculated by normalization to *GAPDH* gene expression using LightCycler® 480 Software Release 1.5.0 (Roche Diagnostics GmbH). For examination of *IGF2R* mRNA regulation, relative *IGF2R* mRNA expression in HCC tissues was compared with relative *IGF2R* mRNA expression in the corresponding TST.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections (size, 4 μm). For fixation, the tissue sections were incubated with 4% formalin at room temperature for 48 h. Following deparaffinization and rehydration, endogenous peroxidase activity was inhibited with 4% hydrogen peroxide in methanol for 30 min at room temperature. For antigen retrieval, tissue sections were incubated with 10 mM citrate buffer (pH 6.0) for 20 min in a steamer. Cells were permeabilized with 2% saponin (cat. no. 47036; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBS for 20 min at room temperature. For blocking of non-specific antibody binding, tissues were incubated for 30 min with protein blocking buffer [5% normal goat serum (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), 0.2% Triton X-100, 2% bovine serum albumin (BSA; SERVA Electrophoresis GmbH, Heidelberg, Germany)]. The following primary antibody was used for immunohistochemistry: Rabbit polyclonal anti-human IGF2R antibody (cat. no. NBP1-19465; Novus Biologicals, LLC, Littleton, CO, USA), which was used at a dilution of 1:100, was used to incubate the sections overnight at 4˚C in PBS (2% saponin, 5% BSA, 5% normal serum). For control sections, the specific primary antibody was omitted. The following day, sections were washed three times with Tris-buffered saline-0.5% Tween-20 and incubated for 20 min with Pierce Peroxidase Suppressor (Thermo Fisher Scientific, Inc.). After washing, sections were incubated for 1 h at room temperature with a secondary biotinylated goat anti-rabbit antibody (cat. no. E043201-8; Dako; Agilent Technologies, Inc.) at a dilution of 1:100, and were then treated with the avidin-biotin-peroxidase complex-based Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). Following incubation with horseradish peroxidase-conjugated streptavidin (cat. no. P039701-2; Dako; Agilent Technologies, Inc.) at a dilution of 1:300, tissues were stained at room temperature for 10 min with the liquid DAB+ Substrate Chromogen system (cat. no. K346711-2; Dako; Agilent Technologies, Inc.), according to the manufacturer's protocol. Counterstaining was performed with Gill's hematoxylin solution (Polysciences Inc., Warrington, PA, USA) at room temperature for 7 min and slides were mounted for examination under a light microscope.

Tissue homogenization and DNA extraction. Homogenization of 5-9 mg fresh frozen tissue blocks was conducted using a Precellys 24 homogenizer (Bertin Instruments) in CK14 tubes at 5,100 rpm for 2x20 sec following Proteinase K (Qiagen GmbH) digestion at 56˚C for 3 h. Genomic DNA was extracted using the QIAamp DNA Micro kit (Qiagen GmbH), according to the manufacturer's protocol, and was quantified by UV absorption via NanoVue (GE Healthcare Life Sciences, Little Chalfont, UK).

PCR and pyrosequencing (PSQ). Primer sets with one 5'-biotinylated primer were used for the amplification of SNP regions. All primers used for PCR and sequencing were generated using PyroMark Assay Design Software 2.0 (Qiagen GmbH). PCR was performed using the PyroMark PCR kit (Qiagen GmbH), according to the manufacturer's protocol. The following primers were used: rs629849, forward 5'-biotin-AAATCC GGCCTGAGCTATAAG-3', reverse 5'-AGCATGAGTCTT GAGCAATTACTG-3'; and rs642588, forward 5'-biotin-CAC ATGGGGATTATGGGAACT-3' and reverse 5'-AGCATG AGTCTTGAGCAATTACTG-3'. PCR reactions were run with 0.15 μM primer under the following thermal cycling conditions: 95˚C for 15 min, followed by 45 cycles at 94˚C for 30 sec, at the optimized primer-specific annealing temperature (60˚C) for 30 sec and at 72˚C for 30 sec, followed by a final extension step at 72˚C for 10 min. Amplification of the correct DNA product was confirmed by high-resolution capillary electrophoresis using a QIAxcel Advanced system (Qiagen GmbH). Subsequently, a standard PSQ sample preparation protocol was applied: Streptavidin beads (1.5 μl; GE Healthcare Life Sciences), 40 μl PyroMark binding buffer (Qiagen GmbH), 20 μl PCR product and 18.5 μl water were mixed and incubated for 10 min at room temperature with agitation (1,200 rpm). According to the manufacturer's recommendations, amplicons were denaturated using PyroMark denaturation solution (Qiagen GmbH), washed with PyroMark Wash buffer (Qiagen GmbH) and added to 20 μl annealing buffer containing 0.375 μM sequencing primer (rs629849: 5'-TGGGCCTATTGGTTG-3'; rs642588: 5'-AGCAATTAC TGATTAATATG-3') using the PyroMark Q24 Vacuum Workstation (Qiagen GmbH) at room temperature. Primer annealing was performed by incubating the samples at 80˚C for 5 min and then cooling to room temperature prior to PSQ. PyroMark Q24 Advanced Reagents (Qiagen GmbH) were used for the PSQ reaction, and the signal was analyzed using the Pyromark Q24 system (Qiagen GmbH). Genotype analysis of SNPs was conducted using PyroMark Q24 Advanced Software version 3.0.0 (Qiagen GmbH).

Statistical analysis. Data management and all statistical analyses were performed using SPSS program (version 23.0; IBM Corp., Armonk, NY, USA). For categorical variables, between-group differences were analyzed by χ^2 test or Fisher's exact test. Continuous data were expressed as the median and range. Two independent groups were compared using the Mann-Whitney U test with subsequent Bonferroni correction. For the comparison of multiple independent samples, the Kruskal-Wallis test and Bonferroni correction was performed. Overall survival rates were calculated using the Kaplan-Meier method and were compared using the log-rank test. The distributions of genotypic frequencies between cases and controls were analyzed by χ^2 test or Fisher's exact test. Odds ratio (ORs) and 95% confidence intervals (CIs) were calculated. Univariate analysis was performed to assess the association between genotype frequencies and the clinicopathological features of HCC. $P \leq 0.05$ (two-sided) was considered to indicate a statistically significant difference.

Results

IGF2R mRNA expression in HCC. To analyze the role of *IGF2R* in HCC, the present study detected the mRNA expression levels of *IGF2R* in HCC tissue and corresponding non-neoplastic TST (n=92). Semi-quantitative RT-PCR results demonstrated significant differences in *IGF2R* mRNA expression between HCC and TST (Fig. 1A). *IGF2R* mRNA was highly expressed in TST and was significantly downregulated in cancerous tissues (P=0.004). In HCC tissues, median *IGF2R* mRNA expression was reduced by 20.4% compared to that in the corresponding TST. Normal liver served as a control for the expression of *IGF2R* mRNA. TST exhibited comparable *IGF2R* mRNA expression to normal healthy liver tissue (n=31; P=1.000). In the present cohort, *IGF2R* mRNA was downregulated in 61% of HCC samples (n=56), whereas *IGF2R* mRNA expression was upregulated in 39% (n=36) (Fig. 1B). Details of clinical and pathological characteristics of patients and tumors are summarized in Table I, according to World Health Organization specifications (44).

Patient survival and tumor recurrence in patients. According to the RT-PCR results, the patients were divided into two groups: i) Patients with HCC that exhibited downregulated *IGF2R* mRNA expression (*IGF2R* mRNA expression in HCC/*IGF2R* mRNA expression in TST <1; n=56), and ii) patients with HCC that exhibited upregulated *IGF2R* mRNA expression in HCC (*IGF2R* mRNA expression in HCC/*IGF2R* mRNA expression in TST ≥1; n=36).

Overall 5-year survival of the patients with downregulated *IGF2R* mRNA tumor expression was 34%; conversely, it was 66% for patients with upregulated *IGF2R* mRNA tumor expression. Despite this pronounced difference, statistical significance could not be achieved (P=0.085; Fig. 2A). Significant downregulation of *IGF2R* mRNA expression markedly affected the risk of tumor recurrence within 5 years following surgery (Fig. 2B). Recurrence-free 5-year survival was 16% in patients with downregulated *IGF2R* mRNA expression, whereas it was 76% in patients with upregulated *IGF2R* mRNA expression (P=0.002).

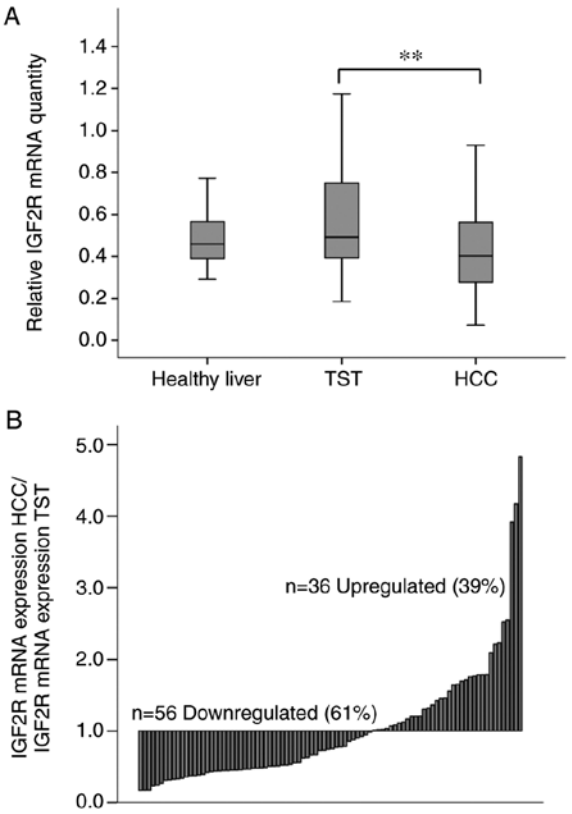

Figure 1. *IGF2R* mRNA expression in HCC tissue, corresponding TST and healthy liver tissues (from patients undergoing liver surgery for hepatic metastases). (A) *IGF2R* mRNA expression was downregulated in HCC tissues (n=92) compared with in the corresponding non-neoplastic TST (P=0.004) and in healthy liver tissues (n=31) (P=0.423). For statistical analysis, Kruskal-Wallis test followed by Bonferroni correction was used. **P<0.01. (B) Individual regulation pattern of *IGF2R* mRNA expression in HCC (n=92). A total of 56 HCC samples exhibited downregulated *IGF2R* mRNA expression compared with TST (61%), whereas 36 HCC samples exhibited upregulated *IGF2R* mRNA expression (39%). HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; TST, tumor-surrounding tissue.

Protein expression of IGF2R, as determined by immunohistochemistry. To identify the localization of IGF2R expression in tumor tissue, protein expression was assessed by immunohistochemistry. As shown in Fig. 3A, IGF2R staining was detected at the tumor border in HCC tissues with downregulated *IGF2R* mRNA expression. In addition, predominant IGF2R staining in hepatocytes was detected in the non-neoplastic TST, whereas IGF2R staining was almost completely absent in tumor tissue.

Patient and tumor characteristics in association with IGF2R mRNA expression. Upregulation of *IGF2R* mRNA expression in HCC was associated with a better recurrence-free survival (P=0.017), a lower median age (P=0.013) and a higher occurrence of liver cirrhosis (P=0.05) (Table II). TACE pretreatment prior to resection or liver transplantation was carried out more often in the group with upregulated *IGF2R* mRNA tumor expression (P=0.032). *IGF2R* mRNA expression was not associated with any other tumor characteristics.

Patient and tumor characteristics in association with TACE pretreatment. As shown in Table I, 26 patients in the present

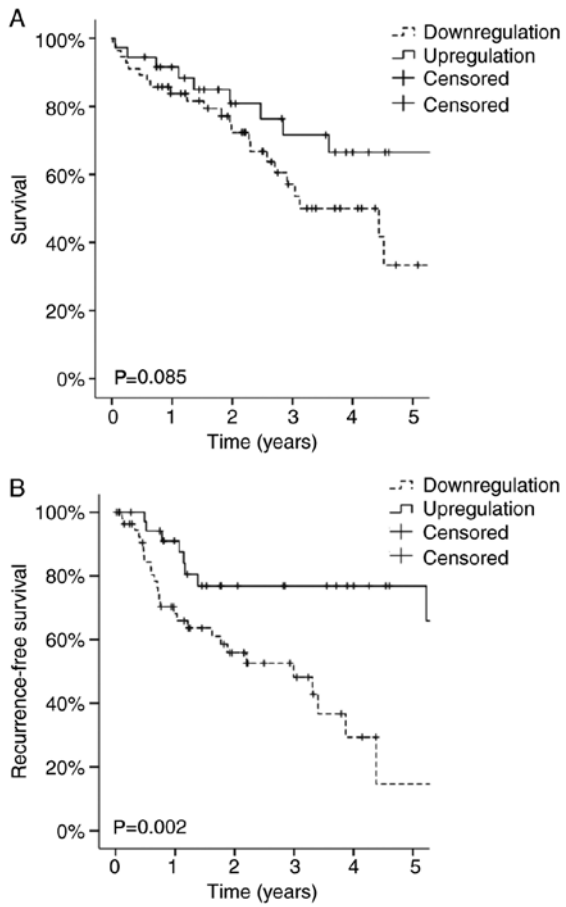

Figure 2. Survival and tumor recurrence of patients with HCC, according to intratumoral *IGF2R* mRNA up- or downregulation. The ratio of *IGF2R* mRNA expression in HCC and *IGF2R* mRNA expression in TST was used as a cut-off value. Patients with HCC were subdivided into an upregulated group (*IGF2R* mRNA expression HCC/TST ≥1; n=36) and a downregulated group (*IGF2R* mRNA expression HCC/TST <1; n=56). (A) Patient survival. The association between *IGF2R* mRNA expression and overall patient survival did not reach statistical significance (P=0.085). (B) Tumor recurrence-free survival. Downregulation of *IGF2R* mRNA expression in HCC was associated with significantly worse recurrence-free survival (P=0.002). HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; TST, tumor-surrounding tissue.

Table I. Patients and tumor characteristics.

Characteristics	Value
Total number, n	92
Median follow-up, days (range)	796 (4-2,615)
Median recurrence-free survival, days (range)	529 (4-2,615)
Male/female, n	79/13
Median age, years (range)	68 (35-86)
Nodules (1-3/multiple), n	75/17
Tumor diameter (<3 cm/≥3 cm), n	19/73
Median tumor diameter, cm (range)	5.0 (1.0-30)
T-classification (T1/T2/T3/T4), n	42/28/21/1
Grading (G1/G2/G3/Gx[a]), n	14/60/13/5
AFP (>100/<100), n[b]	15/71
Angioinvasion (yes/no), n[c]	24/67
Cirrhosis (yes/no), n	54/38
Child-Pugh grade (A/B/C), n	42/6/6
Cirrhosis (viral/non-viral), n	27/27
Pretreatment with chemoembolization, n	26
Liver transplantation/resection, n	26/66

[a]Gx, grade could not be assessed; [b]the status of six cases was unknown; [c]the status of one case was unknown. AFP, α-fetoprotein.

cohort were pretreated with TACE prior to liver transplantation or resection, whereas 66 patients did not receive any TACE pretreatment prior to surgery.

The present study analyzed the associations between patient and tumor characteristics, and preoperative TACE treatment; significant differences are presented in Table III. Notably, TACE-pretreated patients exhibited a significantly longer median follow-up time following surgery (P=0.023) and a prolonged median recurrence-free survival time (P=0.002). Furthermore, patients receiving TACE prior to surgery displayed less advanced HCC stages, since TACE-pretreated HCCs presented with smaller tumor diameters (P<0.001), with less advanced T-stage tumors (P=0.034) and with more well differentiated tumors (P<0.001). These findings coincided with a significantly higher expression of *IGF2R* mRNA expression in the tumor tissues of TACE-pretreated patients (P=0.028; Table III).

Notably, significant downregulation of *IGF2R* mRNA expression in HCC tissue compared with in TST could only

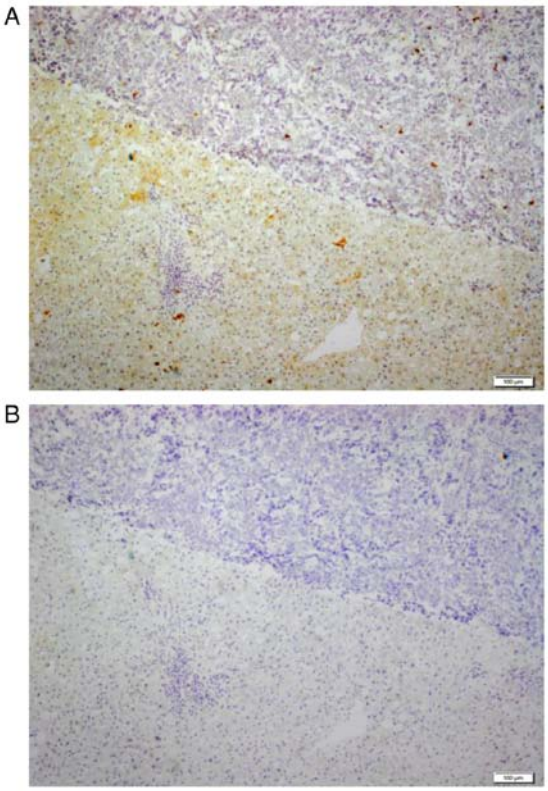

Figure 3. Immunohistochemistry of IGF2R protein expression in HCC and corresponding non-neoplastic TST. (A) IGF2R protein expression was detected at the tumor border in a patient with HCC and downregulated *IGF2R* mRNA expression. IGF2R exhibited pronounced staining of hepatocytes in non-neoplastic TST, whereas it was almost completely absent in tumor tissue. IGF2R expression was stained brown. (B) For the negative control, the specific antibody was omitted during the staining procedure. HCC, hepatocellular carcinoma; IGF2R, insulin-like growth factor-2 receptor; TST, tumor-surrounding tissue.

Table II. Patient and tumor characteristics associated with intratumoral *IGF2R* mRNA expression.

Characteristics	*IGF2R* mRNA		P-value
	Downregulation (HCC/TST <1)	Upregulation (HCC/TST ≥1)	
Number of patients, n	56	36	
Median follow-up, (range)	796 (5-2,316)	826 (19-2,615)	0.247 (n.s.)
Median recurrence-free survival, days (range)	445 (5-2,316)	645 (19-2,615)	**0.017**
Male/female, n	48/8	31/5	1.000 (n.s.)
Median age, years (range)	69 (35-86)	66 (46-78)	**0.013**
1-3 nodules/multiple nodules, n	45/11	30/6	n.s. (0.789)
Tumor diameter (<3 cm/≥3 cm), n	9/47	10/26	n.s. (0.196)
Median tumor diameter, cm (range)	5.4 (1.0-20)	4.5 (2.0-30)	n.s. (0.203)
T-classification (T1/T2/T3/T4), n	22/18/16/0	20/10/5/1	n.s. (0.169)
Grading (G1/G2/G3/Gx[a]), n	9/39/7/1	5/21/6/4	n.s. (0.233)
AFP (>100/<100), n[b]	10/41	5/30	n.s. (0.577)
Angioinvasion (yes/no), n[c]	17/38	7/29	n.s. (0.331)
Cirrhosis (yes/no), n	28/28	26/10	**0.050**
Child-Pugh grade (A/B/C), n	23/2/3	19/4/3	n.s. (0.614)
Cirrhosis (viral/non-viral), n	10/18	13/13	n.s. (0.409)
Pretreatment with chemoembolization (yes/no), no	11/45	15/21	**0.032**
Liver transplantation/resection, n	11/45	15/21	**0.032**

[a]Gx, grade could not be assessed; [b]the status of six cases was unknown; [c]the status of one case was unknown. AFP, α-fetoprotein; HCC, hepatocellular carcinoma; n.s., non-significant; TST, tumor-surrounding tissue. Numbers in bold indicate statistical significance (P≤0.05).

Table III. Patient and tumor characteristics associated with preoperative TACE pretreatment.

Characteristic	No TACE	TACE	P-value
Number of patients, n	66	26	
Median follow-up, days (range)	724 (5-2,588)	1,198 (85-2,615)	**0.023**
Median recurrence-free survival, days (range)	418 (5-25,88)	1,127 (85-2,615)	**0.002**
Male/female, n	55/11	24/2	n.s. (0.337)
Median age, years (range)	71 (35-86)	60 (47-71)	**<0.001**
1-3 nodules/multiple nodules, n	56/10	19/7	n.s. (0.235)
Tumor diameter (<3 cm/≥3 cm), n	6/60	13/13	**<0.001**
Median tumor diameter, cm (range)	6.2 (2.0-30)	2.4 (1.0-18)	**<0.001**
T-classification (T1/T2/T3/T4), n	31/15/19/1	11/13/2/0	**0.034**
Grading (G1/G2/G3/Gx[a]), n	8/46/12/0	6/14/1/5	**<0.001**
AFP (>100/<100), n[b]	47/13	24/2	n.s. (0.137)
Angioinvasion (yes/no), n[c]	45/20	22/4	n.s. (0.189)
Cirrhosis (yes/no), n	29/37	25/1	**<0.001**
Child-Pugh grade (A/B/C), n	27/1/1	15/5/5	**<0.001**
Cirrhosis (viral/non-viral), n	8/21	15/10	n.s. (0.027)
Liver transplantation/resection, n	2/64	24/2	**<0.001**
IGF2R mRNA (down-/upregulated), n	45/21	11/15	**0.032**
Median IGF2R mRNA expression TST	0.53 (0.19-4.3)	0.48 (0.22-2.7)	n.s. (0.233)
Median IGF2R mRNA expression HCC	0.36 (0.71-3.5)	0.49 (0.14-1.1)	**0.028**
Median IGF2R mRNA expression HCC/TST	0.59 (0.17-4.8)	1.1 (0.27-2.2)	**0.019**

[a]Gx, grade could not be assessed; [b]the status of six cases was unknown; [c]the status of one case was unknown. AFP, α-fetoprotein; HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; n.s., non-significant; TACE, transarterial chemoembolization; TST, tumor-surrounding tissue. Numbers in bold indicate statistical significance (P≤0.05).

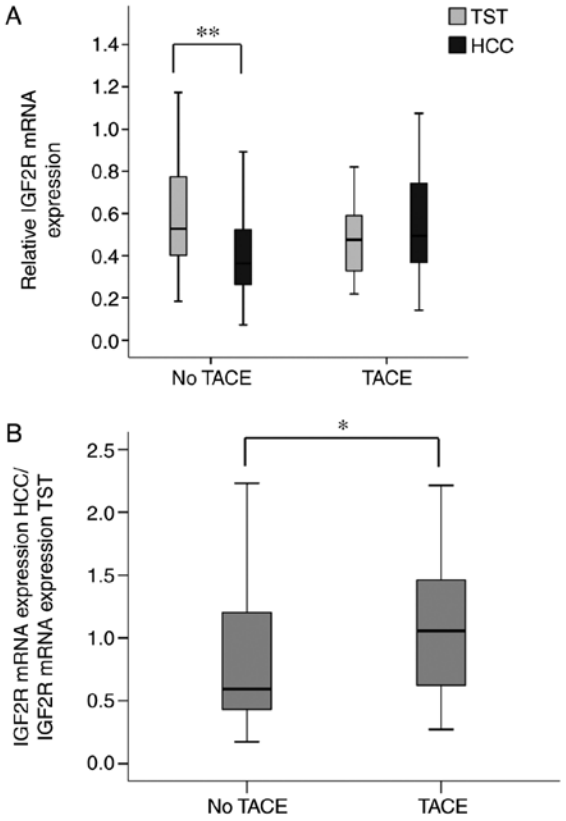

Figure 4. *IGF2R* mRNA expression in HCC and corresponding TST, and its association with TACE treatment prior to surgery. *IGF2R* mRNA expression was analyzed in HCC and TST in patients who did not receive TACE (n=66) compared with in TACE-pretreated patients (n=26). (A) *IGF2R* mRNA expression was significantly downregulated in HCC tumor tissues from the non-TACE-pretreated group (P=0.004). For statistical analysis, Mann-Whitney-U test with subsequent Bonferroni correction was used. (B) TACE-pretreated HCC samples exhibited significantly increased IGF2R mRNA expression compared with in non-pretreated HCC samples (P=0.019). For statistical analysis, Mann-Whitney-U test was used. *P<0.05, **P<0.01. HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; TACE, transarterial chemoembolization; TST, tumor-surrounding tissue.

be detected in the group of patients that did not receive TACE pretreatment (P=0.004; Fig. 4A). There was no difference between *IGF2R* mRNA expression in TST and HCC samples from TACE-pretreated patients (P=1.000). Furthermore, tumor tissues of TACE-pretreated patients exhibited a significantly higher median *IGF2R* mRNA compared with non-treated patients (P=0.019; Fig. 4B). A higher proportion of tumors in the TACE-pretreated patient group exhibited upregulation of *IGF2R* mRNA expression (P=0.032). Notably, 58% of TACE-pretreated patients exhibited upregulated *IGF2R* mRNA expression in HCC compared with only 32% of patients that did not receive TACE prior to surgery (P=0.032; Fisher's exact test; Table IV).

Association of TACE pretreatment with patient survival and tumor recurrence. Patients with HCC that received TACE therapy prior to surgery did not exhibit a significantly better overall 5-year survival compared with non-TACE-treated patients (P=0.097; Fig. 5A). Conversely, TACE pretreatment induced a significantly increased recurrence-free 5-year survival in patients with HCC (P=0.001; Fig. 5B). In the TACE group, 80% of TACE-pretreated patients exhibited

Table IV. Association of *IGF2R* mRNA expression in HCC with TACE pretreatment.

	IGF2R mRNA expression in HCC		
Group	Downregulated, n (%)	Upregulated, n (%)	P-value
No TACE	45 (68)	21 (32)	0.032
TACE	11 (42)	15 (58)	

HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; TACE, transarterial chemoembolization.

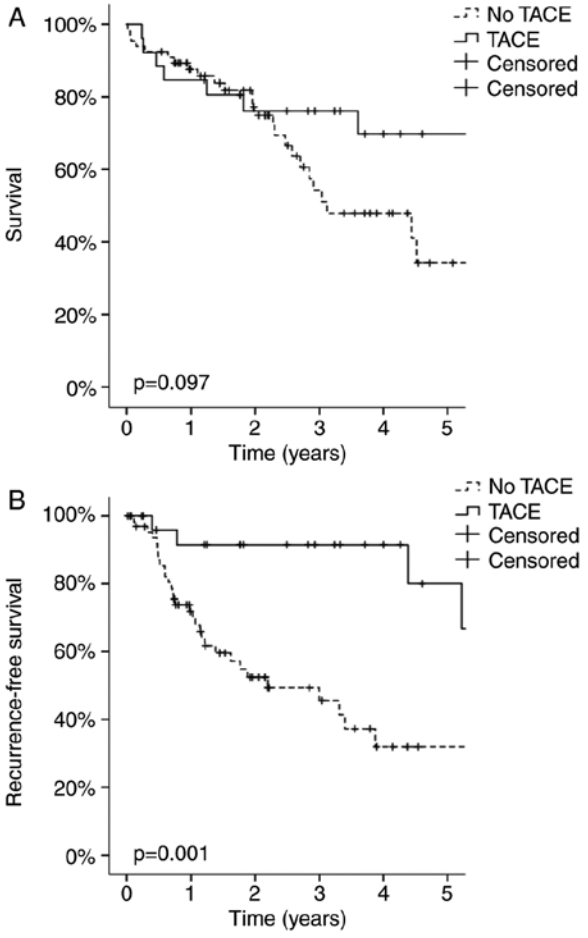

Figure 5. Survival and tumor recurrence of patients with HCC, and their association with TACE pretreatment. (A) TACE-pretreated patients with HCC (n=26) did not exhibit significantly improved survival compared with the non-treated patients (n=66). (B) TACE pretreatment of patients with HCC resulted in a significantly better recurrence-free survival (P=0.001). HCC, hepatocellular carcinoma; TACE, transarterial chemoembolization.

recurrence-free survival 5 years after surgery, whereas only 32% of patients who were not treated with TACE exhibited no recurrence after 5 years.

IGF2R SNPs as a mechanism for IGF2R mRNA expression. Two SNPs of the *IGF2R* gene, rs629849 and rs642588, were

Table V. Distribution frequency of *IGF2R* genotypes in 100 healthy controls and 83 patients with HCC.

Characteristic	Control, n=100 (%)	HCC, n=83 (%)	Total, n=183 (%)	P-value
IGF2R rs629849				
GG	79 (79.0)	61 (73.5)	140 (76.5)	0.145[a]
AG	21 (21.0)	19 (22.9)	40 (21.9)	
AA	0	3 (3.6)	3 (1.6)	
GG	79 (79.0)	61 (73.5)	140 (76.5)	0.388[b]
AG/AA	21 (21.0)	22 (26.5)	43 (23.5)	
IGF2R rs642588				
CC	60 (60.0)	58 (69.9)	118 (64.5)	0.068[c]
CT	38(38.0)	20 (24.1)	58 (31.7)	
TT	2 (2.0)	5 (6.0)	7 (3.8)	
CC	60 (60.0)	58 (69.9)	118 (64.5)	0.214[d]
CT/TT	40 (40.0)	25 (30.1)	65 (35.5)	

[a]P-value between the control and HCC groups with regards to GG, AG and AA genotypes, as determined by χ^2 test; [b]P-value between the control and HCC groups with regards to GG and AG/AA genotypes, as determined by Fisher's test; [c]P-value between the control and HCC groups with regards to CC, CT and TT genotypes, as determined by χ^2 test; [d]P-value between the control and HCC groups with regards to CC and CT/TT genotypes, as determined by Fisher's test. HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor.

investigated to analyze their association with HCC pathological characteristics. Frequency distributions were studied in 83 patients with HCC and were compared with those of 100 healthy controls; 12 patients with HCC could not be analyzed due to insufficient HCC tissue for DNA extraction. The alleles with the highest distribution frequency for rs629849 and rs642588 were GG (76.5%) and CC (64.5%), respectively. Differences in the frequencies of *IGF2R* genotypes were not statistically significant between patients with HCC and healthy controls (P=0.145 and P=0.068, respectively; Table V).

To estimate the ORs and 95% CIs of each *IGF2R* SNP in HCC, patients with HCC and healthy controls were classified into two subgroups: Those with at least one mutated allele and those with homozygous wild-type alleles. Results indicated no significant difference in any allele frequency distribution between patients with HCC and healthy controls (Table V). ORs with 95% CIs were estimated for each gene polymorphism for pathological characteristics, including T classification, grading and disease history of liver cirrhosis. The results revealed that at least one mutated A allele in rs629849 *IGF2R* gene polymorphism had a significantly lower risk for developing non-viral liver cirrhosis (OR=0.25, 95% CI=0.06-0.98) (Table VI). Nevertheless, no significant difference between the *IGF2R* genotype frequencies tested and any other clinicopathological variables was observed.

The present study also estimated the association of various combinations of these *IGF2R* SNPs with HCC susceptibility. As shown in Table VIII, no significant difference was observed in SNPs between healthy controls and HCC patients.

Discussion

It has been suggested that IGF2R may act as a tumor suppressor; therefore, the present study evaluated its role as a biomarker in the pathology of HCC. To the best of our knowledge, the present study is the first to analyze the expression profile of *IGF2R* in a large series of human HCC samples, and to investigate its association with clinical and tumor-specific data. In the human HCC samples analyzed in the present study, the mRNA expression levels of *IGF2R* were significantly downregulated in cancerous tissue compared with in the corresponding non-neoplastic TST. In addition, downregulation of IGF2R protein was detected in HCC tissues from one patient, which indicated that the results of RT-PCR were concordant with the protein expression experiment. The present study predominantly focused on the role of *IGF2R* mRNA expression as a potential marker in the pathogenesis of HCC; therefore, solely HCC tissue and TST samples were collected. One of the limitations of the present study is that tissue samples containing a tumor border, which would have been required for complete immunohistochemical analysis and subsequent statistical analysis, were not available. Further experiments are required to confirm the downregulation of IGF2R protein in HCC. The loss of IGF2R has already been described in some tumor types and its expression is associated with tumor suppression (28,45-47). Chen *et al* revealed that a decreased expression of *IGF2R* in human breast cancer cells, via infection with an adenovirus carrying a ribozyme targeted against *IGF2R* mRNA, enhances IGF2-induced proliferation and reduces susceptibility to tumor necrosis factor-induced apoptosis (28). In addition, radioimmunotherapy targeted to *IGF2R* in osteosarcoma cells suppresses tumor growth in a murine osteosarcoma xenograft model (45). Transfection experiments using breast cancer cells and choriocarcinoma cells demonstrated that overexpression of *IGF2R* decreases cellular growth rates *in vitro* and decreases tumor growth in nude mice (46,47). Whether the reduction of *IGF2R* levels in HCC may provide HCC cells with an important selective growth advantage should be investigated further and has not been reported in the present study. Furthermore, this study

Table VI. ORs and 95% CIs of clinical status and *IGF2R* genotype frequencies in patients with hepatocellular carcinoma (n=83).

	rs629849				rs642588			
Characteristics	GG n (%)	AG or AA n (%)	OR (95% CI)	P-value	CC n (%)	CT or TT n (%)	OR (95% CI)	P-value
Number of patients, n	61	22			58	25		0.21
T classification								
T1/T2	45 (73.8)	18 (81.8)	0.63 (0.18-2.13)	0.57	43 (74.1)	20 (80.0)	0.72 (0.23-2.25)	0.78
T3/T4	16 (26.2)	4 (18.2)			15 (25.9)	5 (20.0)		
Grading[a]								
G1/G2	51 (85.0)	16 (84.2)	1.06 (0.26-4.40)	1.00	44 (81.5)	23 (92.0)	0.38 (0.08-1.90)	0.32
G3	9 (15.0)	3 (15.8)			10 (18.5)	2 (8.0)		
Cirrhosis								
Negative	25 (41.0)	9 (40.9)	1.00 (0.37-2.70)	1.00	25 (43.1)	9 (36.0)	1.35 (0.51-3.55)	0.63
Positive	36 (59.0)	13 (59.1)			33 (56.9)	16 (64.0)		
Cirrhosis								
Viral	13 (36.1)	9 (69.2)	0.25 (0.06-0.98)	0.05	16 (48.5)	6 (37.5)	1.57 (0.46-5.32)	0.55
Non-viral	23 (63.9)	4 (30.8)			17 (51.5)	10 (62.5)		
Child-Pugh grade								
A	28 (77.8)	11 (84.6)	0.64 (0.12-3.48)	0.71	25 (75.8)	14 (87.5)	0.45 (0.08-2.40)	0.46
B or C	8 (22.2)	2 (15.4)			8 (24.2)	2 (12.5)		
Tumor diameter (cm)								
<3	10 (16.4)	6 (27.3)	0.52 (0.16-1.66)	0.35	11 (19.0)	5 (20.0)	0.94 (0.29-3.05)	1.00
≥3	51 (83.6)	16 (72.7)			47 (81.0)	20 (80.0)		
Nodules								
1-3	49 (80.3)	19 (86.4)	0.65 (0.16-2.54)	0.75	48 (82.8)	20 (80.0)	1.20 (0.36-3.96)	0.76
Multiple	12 (19.7)	3 (13.6)			10 (17.2)	5 (20.0)		
Angioinvasion[b]								
Negative	46 (75.4)	13 (61.9)	1.89 (0.66-5.43)	0.27	39 (68.4)	20 (80.0)	0.54 (0.18-1.67)	0.42
Positive	15 (24.6)	8 (38.1)			18 (31.6)	5 (20.0)		
AFP[c]								
<100	44 (81.5)	18 (81.8)	0.98 (0.27-3.53)	1.00	43 (81.1)	19 (82.6)	0.91 (0.25-3.25)	1.00
>100	10 (18.5)	4 (18.2)			10 (18.9)	4 (17.4)		
Surgery								
Liver transplantation	16 (26.2)	6 (27.3)	0.95 (0.32-2.84)	1.00	16 (27.6)	6 (24.0)	1.21 (0.41-3.57)	0.79
Resection	45 (73.8)	16 (72.7)			42 (72.4)	19 (76.0)		
Pretreatment								
No TACE	46 (75.4)	14 (63.6)	1.75 (0.62-4.99)	0.41	42 (72.4)	18 (72.0)	1.02 (0.36-2.91)	1.00
TACE	15 (24.6)	8 (36.4)			16 (27.6)	7 (28.0)		

[a]The status of four cases could not be assessed (Gx); [b]the status of one case was unknown; [c]the status of seven cases was unknown. AFP, α-fetoprotein; CI, confidence interval; *IGF2R*, insulin-like growth factor-2 receptor; OR, odds ratio. Numbers in bold indicate statistical significance (P≤0.05).

Table VII. Association of *IGF2R* gene polymorphism allele combinations with HCC.

IGF2R SNP rs629849	*IGF2R* SNP rs642588	Control, n=100 (%)	HCC, n=83 (%)	OR (95% CI)
GG	CC	45 (45.0)	41 (49.4)	Reference
GG	CT	32 (32.0)	15 (18.1)	1.94 (0.92-4.10)
GG	TT	2 (2.0)	5 (6.0)	0.36 (0.07-1.98)
GA	CC	15 (15.0)	14 (16.9)	0.98 (0.42-2.27)
GA	CT	6 (6.0)	5 (6.0)	1.09 (0.31-3.85)
AA	CC	0 (0.0)	3 (3.6)	-

None of these findings were significant. CI, confidence interval; HCC, hepatocellular carcinoma; *IGF2R*, insulin-like growth factor-2 receptor; OR, odds ratio; SNP, single nucleotide polymorphism.

demonstrated that patients with upregulated *IGF2R* mRNA expression were younger than those with downregulated expression; currently, we do not have any explanation for this. Therefore, it requires confirmation in further studies using a different and larger cohort. Nevertheless, the present study demonstrated a strong association between *IGF2R* mRNA expression and the risk of tumor recurrence within 5 years following surgery, indicating a functional consequence of *IGF2R* expression for patients with HCC. However, whether IGF2R serves a direct role in tumor suppression, or merely an indirect role as a transporter for ligands designated for degradation in the lysosomes, remains to be further elucidated.

In order to increase the number of informative cases, both resected and transplanted livers were included for analysis, and approximately one-third of patients in the present cohort were pretreated with TACE prior to surgery. These patients most likely exhibited a good response to TACE, leading to subsequent liver transplantation in the majority of cases. The present study demonstrated that the TACE-pretreated patients possessed significantly higher mRNA expression levels of *IGF2R* compared with non-treated patients. To the best of our knowledge, this study is the first to describe this relationship; however, at present, it cannot answer whether *IGF2R* mRNA expression is directly influenced by the TACE procedure, because *IGF2R* mRNA expression was solely analyzed in liver tumor explants following TACE. It may be speculated that during TACE some factor could be induced, which may be responsible for disease stability. *IGF2R* may be considered a good candidate gene to further analyze in this context, as this study revealed that TACE-pretreated patients with HCC had a significantly higher expression of *IGF2R* mRNA compared with non-pretreated patients. It would be interesting to know whether tumors that respond to TACE therapy possess an overexpression of *IGF2R* mRNA already prior to TACE therapy or whether *IGF2R* mRNA expression is induced by TACE, e.g. by hypoxia- and/or chemotherapy-induced mechanisms. To analyze that, the expression of *IGF2R* mRNA in TACE-treated HCC samples has to be compared with pre-TACE biopsies taken at the time of diagnosis. This approach could determine whether *IGF2R* mRNA is induced by the TACE procedure or not.

Otto *et al* (48) proposed that TACE pretreatment may select patients with biologically less aggressive tumors. In this previous study, patients who experienced tumor response to TACE had a significantly prolonged disease-free survival compared to those with tumor progression during TACE. These findings are in accordance with the present study, which demonstrated that the TACE-pretreated patient cohort exhibited a significantly increased recurrence-free 5 year survival. Furthermore, Otto *et al* reported that the freedom of recurrence was not influenced by classification of the patient according to the Milan criteria or by downstaging, but rather by the stability of the disease during pretreatment with TACE (48). At present, it has not yet been analyzed as to whether *IGF2R* mRNA expression is associated with the prognosis of HCC following TACE pretreatment. Further investigations are required to address whether *IGF2R* mRNA expression before or after TACE is associated with the prognosis of HCC.

The molecular mechanisms that are induced through TACE therapy remain to be completely elucidated. It is well known that cells under stress, such as hypoxia, promote *IGF2R* expression (49). Whether *IGF2R* expression in HCC is affected by TACE-induced hypoxia remains to be analyzed. Nevertheless, the present data indicated that IGF2R may be a promising candidate marker, which may serve a role in TACE-induced hypoxia, as well as in tumor response. Most hypoxia-induced pathways not only promote tumor growth, but also induce apoptosis (50). Since IGF2R induces apoptosis, this is an interesting aspect to consider. If it could be shown that *IGF2R* is induced through hypoxia, and that *IGF2R* expression is associated with the prognosis of HCC following TACE pretreatment, this would be a further goal in improving TACE treatment by potentially increasing the proportion of patients able to undergo liver resection, reduce recurrence, and prolong survival and quality of life of patients who remain unsuitable for resection (12).

The genotypes of the SNPs rs629849 (located in the binding site for *IGF2*) and rs642588 (located in the CTCF-binding site of the *IGF2R* promoter) were not significantly associated with HCC risk in the present study. Weng *et al* (41) and Rashad *et al* (51) reported that the combination of *IGF2* rs10840452 (AA) and *IGF2R* rs629849 (GG) homozygosity exhibited a significant protective effect against HCC occurrence. The authors concluded that *IGF2* and *IGF2R* polymorphisms are significant IGF-system-associated factors in HCC development. The present findings concerning the SNP rs629849 are supported by Rezgui *et al* (42); this previous study analyzed the structure and function of the human SNP rs629849 and revealed that it fails to alter gene expression, protein half-life and cell membrane distribution of IGF2R, thus suggesting that the polymorphism has no direct effect on receptor function. Furthermore, the comparison of binding kinetics of 'wild-type' and 'mutated' (rs629849) *IGF2R* to *IGF2* revealed no differences in 'on' and 'off' rates, concluding that the rs629849 polymorphism is non-functional.

Notably, Rashad *et al* (51) demonstrated that the homozygous *IGF2R* rs629849 GG genotype is significantly associated with worse Child-Pugh grades. In the present study, no significant association was detected between the SNPs tested and the Child-Pugh classification grade. However, patients with a homozygous *IGF2R* rs629849 GG genotype were associated with a significantly elevated risk of non-viral liver cirrhosis (P=0.05). Furthermore, upregulation of *IGF2R* mRNA expression was associated with a higher occurrence of cirrhosis in patients with HCC (P=0.05) de Bleser *et al* (52) revealed that *IGF2R* is upregulated during liver fibrosis, which may be caused by the overexpression of *IGF2R* in hepatic stellate cells, which are the major cell type involved in liver fibrosis (53). Fibrogenesis is stimulated by TGF-β1, which is known to be activated by IGF2R (38). Activated stellate cells are also responsible for secreting collagen scar tissue, which can lead to cirrhosis (53). These findings support the role of IGF2R in fibrogenesis and the development of cirrhosis. However, data reporting the influence of rs642588 on the functionality of the CTFC-binding site in the *IGF2R* promoter region are currently not available.

The present study suggested an important role for *IGF2R* expression in HCC, particularly with regards to TACE pretreatment. These findings indicated that *IGF2R*

may be considered a good candidate for further investigation of TACE inducibility and as a marker for improved recurrence-free patient survival. Furthermore, this study suggested a pivotal role for *IGF2R* in the development of liver cirrhosis. Further studies are required to investigate the precise mechanisms underlying the effects of *IGF2R* on the progression of HCC, and to evaluate possible diagnostic and therapeutic consequences.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AL, FS and TZ made major contributions to the conception, analysis and interpretation of the data, and were major contributors in writing, drafting and revising the manuscript. HL, GO, JM, CD and PRG collected the clinical samples and the corresponding clinical data. AL, FS, JV and MHL performed the experiments and analyzed data. MHL conducted the clinical data collection and performed the statistical analysis. AS performed the histological evaluation. HL, TZ, GO, PRG and CD critically revised the manuscript. All authors read and approved the final version of the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the State of Rhineland-Palatinate Medical Board [Number 847.243.17 (11077)]. All patients provide written informed consent, according to the agreement on transfer and scientific use of excess material of the University Medicine of the Johannes Gutenberg University Mainz prior to data or specimen collection.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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