IGF2 mRNA binding protein p62/IMP-2 in hepatocellular carcinoma: antiapoptotic action is independent of IGF2/PI3K signaling

Sonja M. Kessler,1 Juliane Pokorny,2 Vincent Zimmer,3 Stephan Laggai,1 Frank Lammert,3 Rainer M. Bohle,2 and Alexandra K. Kiemer1

1Saarland University, Department of Pharmacy, Pharmaceutical Biology, Saarland University, Saarbrücken, Germany; 2Department of Pathology, Saarland University, Homburg/Saar, Germany; and 3Department of Internal Medicine II, Saarland University, Homburg/Saar, Germany

Address for reprint requests and other correspondence: A. K. Kiemer, Saarland Univ., P.O. Box 15 11 50, 66041 Saarbrücken, Germany (e-mail: pharm.bio.kiemer@mx.uni-saarland.de).

The incidence of hepatocellular carcinoma (HCC) is rising in most industrialized countries not least because of metabolic risk factors such as obesity and diabetes mellitus and the increasing prevalence of nonalcoholic fatty liver disease (NAFLD) (12, 49). Reduced IGF2 expression was shown in patients with poor outcome in human hepatocellular carcinoma. p62 transgenic mice expressed the transgene exclusively in the liver and developed a fatty liver phenotype (52), suggesting a critical role for p62 in liver metabolism. Still, p62 transgenic livers do not show liver cell damage but exhibit a pronounced induction of expression and poor outcome in human hepatocellular carcinoma. p62 exerted antiapoptotic action in human hepatoma cells independent of PI3K signaling, but rather facilitated via extra-cellular regulated kinases (ERK) 1/2.

MATERIALS AND METHODS

Animals. All animal procedures and protocols were approved by an independent review committee (AZ: 391 2.2.2). Mice were kept under controlled conditions in terms of temperature, humidity, 12-h day-night rhythm, and food delivery. p62 transgenic mice were established as previously described (52). In short, mice carrying a liver-enriched activator protein promoter under the control of a tetracycline transactivator were crossed with p62 transgenic mice to create a doxycycline inducible mouse model. p62 expression was increased in doxycycline-induced caspase-3-like activity. Western blot analysis revealed increased levels of phosphorylated ERK1/2 in hepatoma cells overexpressing p62 and decreased levels in p62 knockdown experiments. When p62-overexpressing cells were treated with an ERK1/2 inhibitor, the apoptosis-protecting effect of p62 was completely abrogated. Our data demonstrate that p62 exerts IGF2-independent antiapoptotic action, which is facilitated via phosphorylation of ERK1/2. Furthermore, p62 might serve as a new prognostic marker in HCC.

IMP; hepatocellular carcinoma; apoptosis; ERK; chemoresistance

THE INCIDENCE OF HEPATOCELLULAR CARCINOMA (HCC) is rising in most industrialized countries not least because of metabolic risk factors such as obesity and diabetes mellitus and the increasing prevalence of nonalcoholic fatty liver disease (NAFLD) (12, 49). p62/IMP-2 is a member of the family of insulin-like growth factor II (IGF2) mRNA binding proteins and represents a splice variant of IMP-2, which exon 10 of the IMP2 gene is skipped (11). p62 was originally identified as an autoantigen in a patient with HCC (56) and was shown to be expressed in one-third to two-thirds of HCC (34, 57). Interestingly, p62 is also expressed in α-fetoprotein-negative HCC (34). The family member IMP3 has been shown to be implicated in growth promotion, carcinogenesis, angiogenesis, and tumor progression in different tumor types (6, 21, 23, 26). p62 transgenic mice expressing the transgene exclusively in the liver developed a fatty liver phenotype (52), suggesting a critical role for p62 in liver metabolism. Still, p62 transgenic livers do not show liver cell damage but exhibit a pronounced induction of expression and poor outcome in human hepatocellular carcinoma. p62 exerted antiapoptotic action in human hepatoma cells independent of PI3K signaling, but rather facilitated via extra-cellular regulated kinases (ERK) 1/2.

MTT assay. Cells were cultured on 96-well tissue culture plates and every 24 h a 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for up to 4 days. Cells were then incubated with MTT (0.5 mg/ml) solution for 2 h. MTT solution was
aspirated, and cells were lysed and measured at 550 nm and 690 nm as control wavelength.

**Caspase-3-like activity assay.** In primary murine hepatocytes apoptosis was induced by addition of 0.4 μg/ml actinomycin D (Act D) for 15 min and 100 ng/ml TNF-α for 20 h. HepG2 cells were treated with 12.5 μg/ml, PLC/PRF/5 with 50 μg/ml doxorubicin for 20 h. Cells were then washed twice, lysed, and centrifuged. The substrate solution containing benzoyloxyxycarbonyl-Asp-Glu-Val-Asp-aminofluoromethylcoumarin was added to the supernatant, and generation of free fluorescent 7-amino-4-trifluoromethyl coumarin was determined according to Kulhanek-Heinze et al. (29).

**Western blot analysis.** Western blots were performed as previously described (52). Antibodies used were specific to phospho-AKT (Ser473), phospho-ERK1/2, total ERK1/2, phospho-insulin receptor, total insulin receptor (New England Biolabs, Frankfurt, Germany), and α-tubulin (Sigma, Taufkirchen, Germany). IRDye 680-conjugated anti-rabbit IgG (LiCor Bioscience, Bad Homburg, Germany) and IRDye 800-conjugated anti-mouse IgG (LiCor) were used as secondary antibodies. Signal intensities were determined by using the Odyssey infrared image system (LiCor).

**Caspase-3-like activity assay.** In primary murine hepatocytes apoptosis was induced by addition of 0.4 μg/ml actinomycin D (Act D) for 15 min and 100 ng/ml TNF-α for 20 h. HepG2 cells were treated with 12.5 μg/ml, PLC/PRF/5 with 50 μg/ml doxorubicin for 20 h. Cells were then washed twice, lysed, and centrifuged. The substrate solution containing benzoyloxyxycarbonyl-Asp-Glu-Val-Asp-aminofluoromethylcoumarin was added to the supernatant, and generation of free fluorescent 7-amino-4-trifluoromethyl coumarin was determined according to Kulhanek-Heinze et al. (29).

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**Table 1. Real-time RT-PCR**

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**Table 2. Clinical data of HCC patients**

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Clinical data of hepatocellular carcinoma (HCC) patients including age, gender, Barcelona Clinic Liver Cancer (BCLC) and TNM staging, nodularity (uni- or multinodular), tumor size (< or >5 cm), grading, and follow-up. NASH, nonalcoholic steatohepatitis; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection.
Fig. 1. p62 and IGF2 expression in human hepatocellular carcinoma (HCC) samples. A: p62 and IGF2 mRNA expression in human HCC samples (n = 32) compared with matched normal tissue. B: correlation of p62 mRNA and IGF2 mRNA in human HCC samples. C: Kaplan-Meier survival analysis of the upper quartile (high p62) vs. the lower quartile (low p62) of p62 mRNA expression in HCC samples. D: p62 mRNA expression in HCC samples ordered by different clinicopathological parameters of poor prognosis of the corresponding patient: Barcelona Clinic Liver Cancer (BCLC), TNM classification and staging, grading, uni- or multinodularity, tumor size, and recurrence. E and F: correlation data of p62 expression to tumor stage for BCLC (E) and TNM (F) staging systems. G: comparison of p62 mRNA expression with prognostic clinical data from HCC patients, such as BCLC, TNM staging, multinodularity, and tumor size.
**Human HCC tissues.** Paraffin-embedded liver samples from randomly selected pseudonymized HCC patients who underwent liver resection at the Saarland University Medical Center between 2005 and 2010 were obtained. The study protocol was approved by the local Ethics Committee (Kenn-Nr. 47/07). Table 2 summarizes the clinical data. TNM classification of malignant tumors and staging was performed according to the NCCN Hepatobiliary Cancers Clinical Practice Guidelines in Oncology (NCCN Guidelines for Hepatobiliary Cancers V2.2012 © 2011 National Comprehensive Cancer Network cited with permission from the NCCN). T classification was ascertained on the resected specimen, whereas N and M classifications were deter-
cined by clinical radiological staging for most of the patients.

**Statistical analysis.** Data analysis and statistics were performed with Origin software (OriginPro 8.1G; OriginLabs). All data are displayed as mean values ± SE. Statistical differences were estimated by independent two-sample t-test. Human formalin-fixed paraffin-embedded HCC samples were tested by Wilcoxon rank-sum test.

**RESULTS**

*p62 and IGF2 expression in human HCC samples.* The analysis of 32 human HCC tissues showed increased levels of p62 and IGF2 mRNA in tumor tissue compared with matched normal tissue (Fig. 1A). Furthermore, a significant correlation between p62 and IGF2 expression was observed (Fig. 1B). Kaplan-Meier survival analysis of patients with high p62 mRNA expression (upper quartile) vs. low p62 mRNA expression (lower quartile) was not significant but revealed a trend to shorter survival in patients with high p62 mRNA expression in liver tissue (Fig. 1C). Therefore, p62 mRNA expression was tested for different parameters of poor outcome of the corresponding patient. p62 mRNA expression was significantly higher in tumors with increased tumor size and in grade 2 and 3 tumors vs. grade 1 tumors (Fig. 1D). p62 mRNA expression correlated with tumor stage by both Barcelona Clinic Liver Cancer (BCLC) and TNM staging systems (Fig. 1, E and F).

Altogether, the subgroup with high p62 mRNA expression showed poorer outcome characterized by intermediate to advanced Barcelona staging (stages B or C), TNM staging III or IV, multinodularity, and increased tumor diameter (Fig. 1G).

**Relationship between p62 and IGF2 expression in human hepatoma cells.** To determine whether the presence of p62 induces IGF2 mRNA expression in the human system as...
previously observed in p62 transgenic mice (52), p62 was knocked down by siRNA in HepG2 cells. Knockdown of p62 decreased IGF2 expression (Fig. 2A). Vice versa, overexpression of p62 resulted in increased IGF2 mRNA levels (Fig. 2B).

Because of the proliferating and antiapoptotic features of IGF2 we investigated the effect of p62 on proliferation and apoptosis. After knockdown of p62, however, no effect on cell proliferation was observed (Fig. 2C).

Fig. 3. Phosphoinositide 3-kinase (PI3K) signaling. A: inhibition of doxorubicin-induced apoptosis by p62 determined by caspase-3-like activity. Inhibition was achieved by using either a neutralizing IGF2 antibody (Ab) or the PI3K inhibitors Ly294002 (Ly) and wortmannin (Wo). Data are expressed as percent inhibition of doxorubicin-induced apoptosis in p62 transfected cells (co). B: neutralizing effect of IGF2 antibody verified by Western blot analysis of phosphorylated (pIR) and total (tIR) insulin receptor levels in HepG2 after treatment with 75 ng/ml recombinant human IGF2 with and without treatment with IGF2 antibody (C and D). Phosphorylated AKT levels after knockdown (si co, random siRNA; si p62, p62 siRNA) (C) and overexpression of p62 (co-v, antisense construct; p62, sense construct) (D). E and F: densitometric analysis of Western blots from C and D.

Fig. 4. ERK signaling. A and E: inhibition of doxorubicin-induced apoptosis by p62 overexpression as determined by caspase-3-like activity in HepG2 (A) and PLC/PRF/5 (E) cells. Cells were treated with either a neutralizing IGF2 antibody (Ab) or the ERK inhibitors PD98059 (PD) and U126 (U). Data are expressed as percent inhibition of doxorubicin-induced apoptosis in p62 transfected cells (co). B–D and F–H: phosphorylated ERK levels after knockdown of p62 in HepG2 (B) and PLC/PRF/5 (F) cells (si co: random siRNA, si p62: p62 siRNA) and overexpression of p62 in HepG2 (C) and PLC/PRF/5 (G) cells (co-v, antisense construct, p62, sense construct). Densitometric analysis of Western blots from HepG2 (D) or PLC/PRF/5 (H) cells upon knockdown or overexpression of p62. Data are expressed as ratio of phosphorylated ERK to total ERK signal intensities with values for respective controls (si co or co-v) set as 100%. I: microphotography displaying a representative hepatocellular carcinoma case [a, hematoxylin and eosin (HE) staining; b, total ERK1/2 (tERK) immunostaining] with positive specific phospho-ERK1/2 immunostaining (pERK; c) and corresponding positive p62 immunostaining (d) in tumor tissue. Original magnification: ×100.
Basal levels of apoptosis were not altered in livers of transgenic animals (data not shown). TNF-α/Act D-induced caspase-3-like activity in primary murine hepatocytes from p62 overexpressing mice, however, was attenuated compared with control cells (Fig. 2D). In the human system overexpression of p62 in HepG2 cells decreased doxorubicin-induced apoptosis (Fig. 2E). Vice versa, knockdown of p62 in HepG2 cells resulted in increased doxorubicin-induced caspase-3-like activity (Fig. 2F).
**Antiapoptotic action of p62 is independent of PI3K signaling.**

Since p62 transgenic animals showed increased levels of phosphorylated Akt (52), we speculated that the IGF2/PI3K pathway is involved in the antiapoptotic effect of p62. To determine the involvement of IGF2 in the antiapoptotic effect, p62 wasoverexpressed in HepG2 cells followed by inhibition of the IGF2/PI3K pathway. Neither the PI3K inhibitors LY294002 and wortmannin nor a neutralizing IGF2 antibody abolished the antiapoptotic effect of p62 (Fig. 3A). The neutralizing capacity of the IGF2 antibody was confirmed by Western blot analysis of phosphorylated insulin receptor (Fig. 3B). The independence of p62 expression and PI3K/AKT signaling in the human system was confirmed by p62 knockdown or overexpression, and neither approach affected phosphorylation of AKT significantly (Fig. 3, C and D).

**p62 protects from apoptosis via phosphorylation of ERK1/2.**

Another survival pathway that is often altered in cancer is the mitogen-activated protein kinase (MAPK) pathway (30). When p62 overexpressing HepG2 cells were treated with ERK1/2 inhibitors, the apoptosis-protecting effect of p62 was completely abrogated (Fig. 4A). Knockdown of p62 in HepG2 cells resulted in significantly decreased phosphorylation of ERK1/2 (Fig. 4, B and G). Vice versa, overexpression of p62 activated ERK1/2 markedly (Fig. 4, C and G). To check the relevance of these findings in another hepatoma cell line, we investigated PLC/PRF/5 cells. p62 regulated IGF2 expression also in this cell line: p62 knockdown downregulated IGF2 mRNA by 40.88% (±6.66 P = 0.023) and p62 overexpression induced IGF2 mRNA 2.01-fold (±0.31 P = 0.016). Still, the IGF2 antibody did not affect chemosensitivity, whereas both ERK inhibitors significantly abrogated apoptosis induction (Fig. 4D). As seen in HepG2 cells, p62 induced ERK activation, as proven by knockdown and overexpression strategies also in PLC/PRF/5 (Fig. 4, E–H). Finally, in tissue samples from HCC patients activation of ERK was observed in hepatocytes, which showed high p62 expression (Fig. 4I).

**DISCUSSION**

The *Igf2* mRNA binding protein p62 was originally identified as a tumor-associated autoantigen with autoantibodies against p62 detected in 21% of HCC patients (56) and in several other types of cancer (41, 59). p62 protein was reported to be expressed in HCC tissue, but not in healthy surrounding tissue in 30% (34, 57) and up to 61.5% of HCCs (48). Concordantly, our data show significantly increased levels of p62 mRNA in HCC tissue.

The IMP family member IMP3 was suggested as a prognostic marker for tumor malignancy and prognosis in different cancers, such as pancreatic ductal adenocarcinoma, neuroblastoma, prostatic cancer, and colon cancer (7, 23, 25, 48). Similarly, IMP1 was observed to correlate with metastasis and shorter survival in colon cancer (15) and ovarian carcinoma (28). In line with these findings we observed a correlation between p62 expression and poor prognostic markers. Higher p62 expression was observed in tissues from intermediate or advanced tumors showing multinodularity, increased tumor size, poorer differentiation, and early recurrence of tumors, all of which indicate a more severe status of disease. Since p62 mRNA levels were increased in G2/G3 vs. G1 patients one might speculate that p62 expression might serve as a valuable tumor marker in the future. However, higher numbers of patients need to be analyzed.

Our HCC cases comprised different etiologies, hence the limited sample number did not allow correlating etiologies with the extent of p62 expression. Neither an association with viral infection (22) nor a potential correlation with steatohepatitis caused by NAFLD could be found for p62, although p62 overexpression in mouse livers induced a prominent steatotic phenotype (52).

The p62 transgenic mouse model showed a pronounced expression of the metabolic and antiapoptotic growth factor *Igf2* (52). We now demonstrate that p62 expression correlates with *IGF2* expression also in human HCC. In fact, a causal effect of p62 on *IGF2* expression is proven by both knockdown and overexpression of p62 in two different hepatoma cell lines. *IGF2* is a well-described antiapoptotic factor overexpressed in HCC (5, 33, 42, 43, 51) and other tumor tissues (3, 9, 46). The balance between apoptosis and survival is often disrupted because of antiapoptotic signals occurring in cancer (17). Accordingly, hepatocarcinogenesis and promotion of HCC is characterized by defective apoptosis and increased cell proliferation (19, 39). Proliferation did not seem to be influenced by p62 expression, which has also been shown for IMP3 in a hepatoma cell line (26). Interestingly, however, both knockdown and overexpression of p62 in hepatoma cells verified an antiapoptotic effect of p62. The IMP family members IMP1 and IMP3 have recently been demonstrated to promote cell survival and cancer cell proliferation (27, 32), whereby *Igf2* seems to mediate apoptosis protection (32).

HCC is characterized by alterations in several important cellular signaling networks including the PI3K and the ERK pathways (53). Constitutive activation of the PI3K/AKT pathway has been firmly established as a major determinant of tumor cell growth and survival in several tumors (8). In HepG2 cells IGF1 has been demonstrated to have the ability to reverse apoptotic signaling by activation of the PI3K/AKT signaling (1). *Igf1* binds to the IGF1 receptor, whose autophosphorylation is followed by phosphorylation of intracellular targets and finally leads to activation of the PI3K and the ERK pathways (40). *Igf2* was also demonstrated to act via the PI3K/AKT pathway (31). Unexpectedly, the altered *IGF2* expression after knockdown or overexpression of p62 did not result in changes in AKT activation in HepG2 cells. Another recent study also showed independence of high *IGF2* mRNA expression and phosphorylation of AKT in human HCC tissue (51). Interestingly, p62 transgenic animals only exhibited increased Akt phosphorylation in 5-wk-old animals, although *Igf2* was strongly induced also in animals of other ages (52). Together with the lack of effect of a neutralizing anti-IGF2 antibody these findings strongly suggest no direct signaling axis p62-IGF2-PI3K/AKT. The different results between wortmannin vs. LY294002 regarding the extent of apoptosis protection in p62-overexpressing cells is probably due to the partial inhibition of proapoptotic p38 kinase by wortmannin (13).

Our data clearly indicate that the antiapoptotic effect of p62 is rather facilitated via ERK phosphorylation. In fact, in a rodent model tumor growth and apoptosis resistance of intra-peritoneally applied hepatoma cells were enhanced by overexpression of the ERK upstream kinase MEK1 (24), and over-activation of the MAPK pathway in liver tumor cells was reported to play a role in the initiation and development of HCC through resistance to apoptosis (4). Silencing ERK1/2 expression by using RNA interference also led to suppression
of cell proliferation in other tumors, such as ovarian cancer (47). In addition to these findings in experimental systems, two clinical reports displayed aberrant ERK phosphorylation, ranging from 23 up to 69% of HCCs (24, 44). Interestingly, Schnitz et al. (44) demonstrated ERK1/2 phosphorylation to correlate with poor prognosis. Among the human HCC samples we also observed phosphorylation of ERK in cases with high p62 expression and poor outcome. There were also samples that were negative for phospho-ERK in tumor vessels, serving as staining control. We suggest that the detection of phospho-ERK might have failed, because the stabilization of phosphorylated proteins in formalin-fixed paraffin-embedded tissues is not always successful because of slow tissue penetration (36), and phosphorylation of ERK seems to be less stable compared with other phospho-proteins (24). Therefore, an even higher frequency of HCC samples with ERK activation might be assumed.

ERK phosphorylation is not only altered in malignant tissues, but also in patients with severe chronic hepatitis B virus (HBV) infection (20), suggesting a transition step toward HCC. In fact, in the context of both HBV and hepatitis C virus (HCV) infection, activation of the ERK pathway was demonstrated to enhance cell cycle progression, cell proliferation, and survival (16, 60). Concordantly, ERK phosphorylation was strongly correlated to HCV infection (44). We demonstrate for the first time that ERK phosphorylation in hepatoma cells, which leads to resistance against apoptosis, is due to expression of the IMP p62. In line with these findings, ERK also seems to be involved in modulating drug resistance of HCC cells (54). Clinical relevance is underlined by the suggestion of a combined doxorubicin and ERK targeted therapy with enhanced anticancer effects in HCC (10).

Taking these findings together, p62 seems to play a dominant role in HCC progression and is accompanied by increased expression of the oncogene IGF2. Furthermore, p62-induced ERK activation seems to display a critical step in hepatocarcinogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


