

Liver Regeneration Associated Protein (ALR) Exhibits Antimetastatic Potential in Hepatocellular Carcinoma

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Augmenter of liver regeneration (ALR), which is critically important in liver regeneration and hepatocyte proliferation, is highly expressed in cirrhotic livers and hepatocellular carcinomas (HCC). In the current study, the functional role of ALR in hepatocarcinogenesis was analyzed in more detail. HepG2 cells, in which the cytosolic 15 kDa ALR isoform was reexpressed stably, (HepG2-ALR) were used in migration and invasion assays using modified Boyden chambers. Epithelial-mesenchymal transition (EMT) markers were determined in HepG2-ALR cells *in vitro* and in HepG2-ALR tumors grown in nude mice. ALR protein was quantified in HCC and nontumorous tissues by immunohistochemistry. HepG2-ALR, compared with HepG2 cells, demonstrated reduced cell motility and increased expression of the epithelial cell markers E-cadherin and Zona occludens-1 (ZO-1), whereas SNAIL, a negative regulator of E-cadherin, was diminished. Matrix metalloproteinase MMP1 and MMP3 mRNA expression and activity were reduced. HepG2-ALR cell-derived subcutaneously grown tumors displayed fewer necrotic areas, more epithelial-like cell growth and fewer polymorphisms and atypical mitotic figures than tumors derived from HepG2 cells. Analysis of tumor tissues of 53 patients with HCC demonstrated an inverse correlation of ALR protein with histological angioinvasion and grading. The 15 kDa ALR isoform was found mainly in HCC tissues without histological angioinvasion 0. In summary the present data indicate that cytosolic ALR reduces hepatoma cell migration, augments epithelial growth and, therefore, may act as an antimetastatic and EMT reversing protein.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent form of the primary liver cancers, which in turn are the fifth most common cancers worldwide—contributing substantially to cancer mortality (1). HCC develops largely within an established background of chronic liver diseases, caused mainly by infections with hepatitis B and/or hepatitis C virus or alcoholic liver disease (2), but the molecular pathogenesis is not well understood. Although new therapies for chronic hepatitis have been established, the number of HCC patients

has not declined (1). Treatment success of HCC is dependent on the tumor stage at the time of diagnosis, with good curability at early stages and poor prognosis at advanced stages with tumor recurrence and metastasis after surgery or ablation therapy (2,3). Various factors are involved in the regulation of metastasis, and epithelial-mesenchymal transition (EMT) is characteristic for the most aggressive metastatic cancer cells (4,5). Therefore, biomarkers suitable for diagnostic purposes and new targets for developing therapeutic strategies are needed.

Human augmenter of liver regeneration (ALR) is a member of the newly discovered ALR/Erv1 protein family with FAD-linked sulfhydryl oxidase activity catalyzing disulfide bond formation (6,7). ALR is expressed ubiquitously with the highest levels found in testis and liver (8–10). Alternative splicing generates (11) a 21/23 kDa ALR isoform predominantly localized to the mitochondrial inner-membrane system (IMS). In concert with the redox-regulated receptor Mia40/TOM, ALR forms a disulfide relay system mediating the import of proteins of mitochondria into IMS (12). Furthermore, CXXC motif of ALR is essential for cell survival (13) and biogenesis of cytosolic Fe/S proteins (14). The short form of ALR (15 kDa) is found not only at extracellular sites but also at nuclear and cytosolic localizations participating in intracellular redox-dependent

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signaling pathways (15,16). ALR is a hepatotrophic factor stimulating proliferation of hepatocytes (17,18) and augmenting liver regeneration (15,16), thereby exerting beneficial effects in models of hepatic failure (19) and liver fibrosis (20).

Furthermore, enhanced ALR mRNA and protein expression is found in the liver of patients with cirrhosis, cholangiocellular and hepatocellular carcinoma. Here, ALR is localized predominantly in hepatocytes and bile duct cells (10). However, it is not clear yet whether the cytosolic isoform of ALR plays a role in the development of HCC. The aim of this study was to investigate the functional role and clinical importance of the short cytosolic ALR isoform in hepatocarcinogenesis.

MATERIALS AND METHODS

Liver Samples

Tissues of 64 patients undergoing liver surgery at the University Medical Center Regensburg were collected, including 53 patients with primary HCCs, three patients with liver cirrhosis and eight patients with colorectal liver metastases serving as healthy liver tissues. Tissue samples were fixed in formalin and paraffin-embedded for immunohistochemical analysis. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patients' consent (21) approved by the local ethical committee of the University of Regensburg.

Immunohistochemical Analysis of ALR Expression

Immunostaining was performed as described recently (10). Briefly, 2-mm sections of paraffin-embedded tissue were used, and unspecific binding was blocked by 30 min preincubation in phosphate buffered saline (PBS) with 3% human serum (Bio-Rad, Munich, Germany), sections were subsequently incubated with anti-ALR antibody (0.26 $\mu\text{g}/\text{mL}$) at 4°C overnight followed by incubation with secondary mouse antirabbit antibody (DAKO, Hamburg, Germany) for 1 h.

Immunostaining was developed using APAAP complex (DAKO) and Fast Red Chromogen staining (Roche, Penzberg, Germany). Counterstaining was performed with hematoxylin (Vektor, Burlingame, Canada). Rabbit IgG (Sigma, Munich, Germany) was used as isotype control. To evaluate ALR immunostaining, the percentage of positive cells and intensity of staining was analyzed independently by two pathologists. The following scores were defined: $\leq 10\%$ positive cells = 0; 10% to 25% positive cells = 1, 25% to 50% positive cells = 2; 50% to 75% positive cells = 3; and $\geq 75\%$ positive cells = 4. Signal intensity was defined as 0 when no staining was detected, as 1 when staining was weak, as 2 when staining was moderate and as 3 when staining was strong. The expression level of ALR was calculated by multiplying the scores: 0 = negative, 1–4 = positive, 6–12 = highly positive.

Cell Culture and Stable Transfected Cells

Primary human hepatocytes (PHH) were isolated and cultured as described (22). The human hepatoma cell line *HepG2* (clone HB-8065) was obtained from American Type Culture Collection, (ATCC, Manassas, VA, USA) and grown at 37°C, 5% CO₂ in DMEM (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 units/mL), streptomycin (10 $\mu\text{g}/\text{mL}$) and 10% fetal calf serum. The human ALR cDNA encoding the short isoform was digested with *Bam*HI and *Eco*RI and subcloned into the expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany). *HepG2* cells were transfected using *siPORT XP-1* (Ambion, Austin, TX, USA) regarding to the manufacturer's instructions. *HepG2* cells stably expressing ALR were selected by 1 mg/mL G-418 (GIBCO BRL, Karlsruhe, Germany) over a period of 2 wks. Cells stably transfected with pcDNA3.1 served as controls (mock).

Immunocytochemical Analysis

Cells grown on BD BioCoat Collagen I culture slides (BD Biosciences, Heidelberg, Germany) were fixed and incubated with

anti-ALR (1:100) (10), anti-CDH1 (1:100) (Cell Signaling, Danvers, MA, USA), or anti-ZO-1 (1:100) antibody (Invitrogen). Cryosections (2 μm) from tumors derived from *HepG2*-ALR and *HepG2* cells were stained using anti-CDH1 (1:100) or anti-ZO1 (1:100) antibody. Fluorescent signals were visualized by an Alexa Fluor 488 dye-conjugated goat antirabbit (IgG) antibody (Invitrogen).

Migration and Invasion Assays

Briefly, 0.5×10^5 cells were resuspended in RPMI medium containing 1% FCS and seeded on inserts with 8-mm filter pores, which were either uncoated (migration assay) or matrigel-coated (invasion assay) (Becton Dickinson Bioscience, Heidelberg, Germany). 10% FCS served as chemoattractant. After 48 h, cells were fixed and stained (Diff-Quick reagent, Dade Behring, Newark, NJ, USA) and migrated and invaded cells were counted.

Scratch Assay 'Wound-Healing Assay'

Migration of cells was assayed by scratch assays. Cells were seeded in high density into six-well plates and scratched by a pipette tip in a definite array. Migration into this array was documented and measured after 48 h. Each analysis was performed in triplicate.

Nude Mouse Tumor Model

A model of inoculation of tumor cells into NMRI (nu/nu) mice to monitor tumor growth *in vivo* was performed as described previously (23). Ten-wk-old male athymic nude mice NMRI (nu/nu) from the universities breeding stock were used (mean body weight was 30 g). *HepG2*-ALR cells or mock-transfected *HepG2* cells (1×10^6 cells per mouse) were injected subcutaneously into nude mice, and six mice per clone were used. After 21 d, the formed tumors were removed, snap frozen and stored at -80°C for subsequent analysis.

RT-PCR Analysis

Total RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). One

μg of total RNA was reverse transcribed using the Reverse-Transcription System (Promega, Madison, WI, USA). Transcript levels of ALR, MMP1, MMP3 and 18S rRNA as internal control were quantified using real-time PCR technology (Light-Cycler, Roche, Penzberg, Germany). Primers used to amplify ALR were: forward 5'-CAC AAT GAA GTG AAC CGC AAG-3', reverse 5'-CAC CCA ACT GAG ACA CAA CAG-3', MMP1: forward 5'-TCA CCA AGG TCT CTG AGG GTC AAG C-3', reverse 5'-GGA TGC CAT CAA TGT CAT CCT GAG C-3', 18S rRNA: forward 5'-GTA ACC CGT TGA ACC CCA TT-3', reverse 5'-CCA TCC AAT CGG TAG TAG CG-3', MMP3 primers were obtained from TIB Molbiol (Berlin, Germany). The PCR reaction was evaluated by melting curve analysis. PCR was performed in duplicate and three independent experiments were performed.

Western Blot Analysis

Protein lysates (20 μg /lane) were separated by SDS-PAGE under reducing conditions using 100 mM DTT. Proteins were transferred to PVDF membranes and incubated with anti-CDH1 (1:1000), anti-ZO-1 (1:250), anti-SNAIL (1:250) (Santa Cruz Biotechnology, Heidelberg, Germany), anti-ALR (1:440) or anti- β -actin (1:1000) (Santa Cruz Biotechnology) antibodies. Positive signals were developed using enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Measurement of MMP1 and MMP3 Activity *In Vitro*

HepG2 and *HepG2*-ALR cells were cultured for 48 h and supernatants were collected. MMP1 and MMP3 activities were measured using the SensoLyte 520 MMP-1 Assay Kit and the SensoLyte 520 MMP-3 Assay Kit, respectively, following manufacturer's instructions (AnaSpec, Fremont, CA, USA). Briefly, MMP activity in culture supernatants was determined using the fluorogenic MMP specific substrate 5-FAM/QXL520 FRET peptide. Assays were performed in black 96-well plates with a clear flat bottom (Corning Inc, Corning, NY, USA) at 37°C.

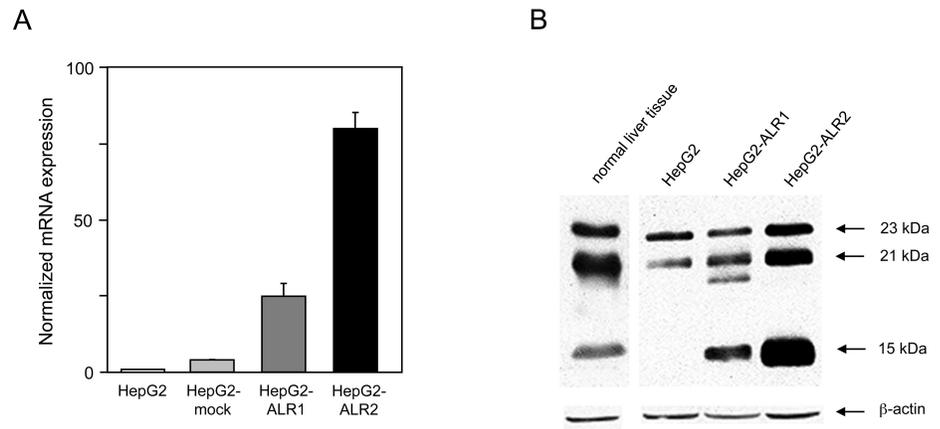


Figure 1. Stable reexpression of short isoform of ALR in HCC cells. *HepG2* cells stably expressing the 15 kDa ALR isoform (*HepG2*-ALR1 and *HepG2*-ALR2). (A) RT-PCR analysis revealed strong ALR mRNA expression in both ALR expressing *HepG2* cells compared with nontransfected *HepG2* or mock-transfected cells (three independent experiments, mean \pm SD). (B) Western blot analysis demonstrated reexpression of the 15 kDa isoform ALR in *HepG2*-ALR cell clones. Normal liver tissue served as a positive control. Expression of β -actin was used as loading control.

The increase in fluorescence resulting from enzyme mediated cleavage of the substrates was measured at 490/520 nm (excitation/emission)

Statistical Analysis

Significant differences in paired and nonpaired samples were evaluated by using Wilcoxon signed-rank *t* test and Mann-Whitney *U* test, respectively. Clinicopathological parameters were calculated by using Pearson chi-square test.

All supplementary materials are available online at www.molmed.org.

RESULTS

Reexpression of the Short Isoform of ALR in *HepG2* Cells

To gain more insight into the functional role of ALR in HCC, the short isoform of ALR (15 kDa) was reexpressed stably in the hepatoma cell line *HepG2* (*HepG2*-ALR). RT-PCR and Western blot analysis revealed strong induction of ALR mRNA and protein in *HepG2*-ALR cell clones compared with mock transfected and nontransfected *HepG2* cells, respectively (Figure 1A, B). All three iso-

forms (23 kDa, 21 kDa, 15 kDa) were detected in normal liver tissue whereas the 15 kDa ALR isoform was barely detected in *HepG2* cells (Figure 1B). Since *HepG2*-ALR2 cells showed a more intense 15 kDa ALR expression than *HepG2*-ALR1, this clone termed *HepG2*-ALR was used in further studies. Mock transfected *HepG2* cells were used as control. Immunocytochemical analysis identified a strongly enhanced expression of cytosolic ALR in *HepG2*-ALR compared with *HepG2* cells (Figure 2A i and v).

Reduced Migration and Invasiveness of HCC Cells after ALR Reexpression

Impact of ALR expression on cellular function was studied by analyzing cell motility. By performing Boyden chamber and matrigel invasion assays, a significant reduction in migration and invasion of *HepG2*-ALR cells compared with control cells was found (Figure 3A). In addition, scratch-assays (wound-healing assays) displayed a clearly diminished migration capacity of ALR reexpressing HCC cells compared with control cells (Figure 3B). Furthermore, cell adherence of *HepG2*-ALR after seeding was much faster compared with *HepG2* cells (data

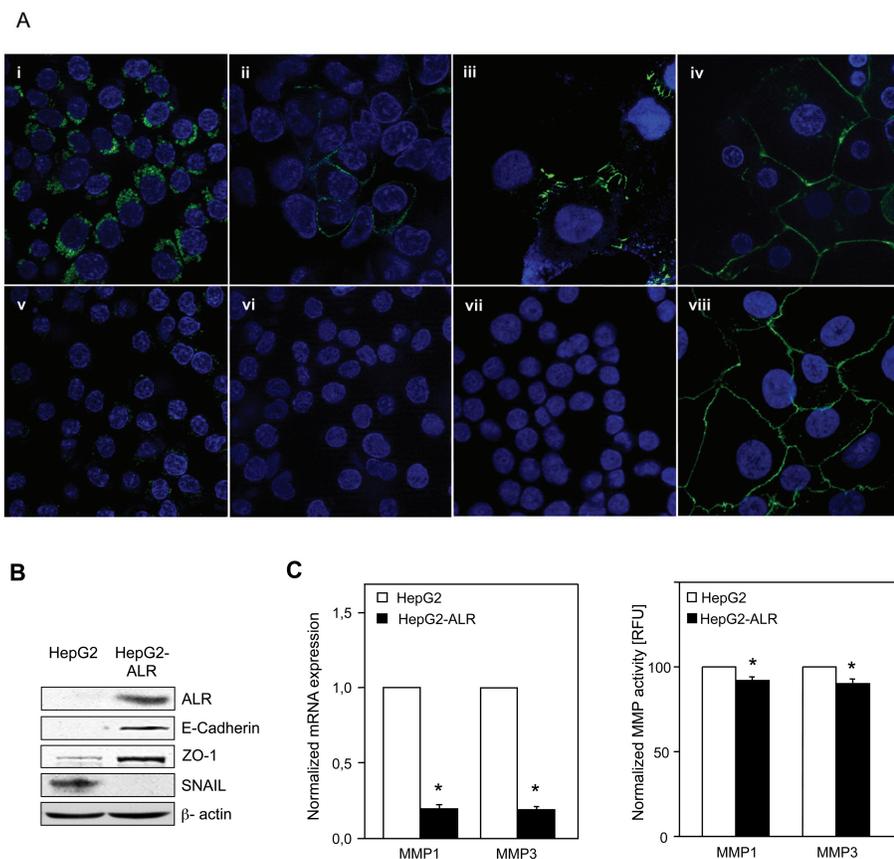


Figure 2. Expression of ALR in HCC cells affects markers of EMT. (A) Immunofluorescence analysis revealed strongly enhanced ALR expression in *HepG2*-ALR (i) compared with *HepG2* (v) cells. *HepG2*-ALR cells demonstrated reexpression of EMT markers E-cadherin (ii) and ZO-1 (iii), whereas *HepG2* cells showed no immunosignal (vi, vii). PHH were used a positive control for E-cadherin (iv) and ZO-1 (viii) immunostaining. (i, v-vii, magnification 400x; ii-iv, viii, magnification 650x). (B) Western blot analysis confirmed expression of E-cadherin and ZO-1 in *HepG2*-ALR compared with *HepG2* cells. Furthermore, expression of SNAIL was not detected in *HepG2*-ALR, but was found in *HepG2* cells. (C) RT-PCR and protein activity analysis of matrix metalloproteinases in *HepG2*-ALR compared with *HepG2* cells revealed reduction of MMP1 and MMP3 mRNA expression and activity, respectively (three independent experiments, mean \pm SD, * $P < 0.05$).

not shown). However, proliferation of *HepG2*-ALR and *HepG2* cells after 72 h of cultivation was similar (Supplementary Figure S1A).

Reexpression of ALR Affects EMT Markers

Alterations in cell motility often are accompanied by changes in the expression of specific markers of EMT. Therefore, we performed immunocytochemistry and demonstrated a reexpression of the epithelial cell marker proteins E-cadherin (Figure 2A ii and vi) and tight-junction

protein ZO-1 (Figure 2A iii and vii) in *HepG2*-ALR compared with control cells. Western blot analysis confirmed reexpression of E-cadherin and ZO-1 in *HepG2*-ALR, which were not detected in *HepG2* cells (Figure 2B). Furthermore SNAIL, a negative regulator of E-cadherin, was expressed in *HepG2* cells but was absent or in low abundance in ALR reexpressing *HepG2* cells (Figure 2B). In addition, matrix metalloproteinases MMP1 and MMP3 mRNA expression and protein activity were found to be reduced significantly in *HepG2*-ALR compared

with control-transfected *HepG2* cells (Figure 2C).

ALR Expression Attenuates Tumorigenicity of HepG2 Cells In Vivo

Growth behavior of *HepG2*-ALR and mock-transfected *HepG2* cells was analyzed in nude mice after subcutaneous injection of *HepG2*-ALR and control cells. One wk after injection, all mice developed tumors. Tumor size after 3 wks was similar for tumors derived from *HepG2*-ALR and mock-transfected HCC cells (Supplementary Figure S1B). Immunofluorescence and Western blot analysis of *in vivo* grown tumors confirmed reexpression of the epithelial cell marker proteins E-cadherin and ZO-1, as well as reduction of SNAIL expression in tumors originated from *HepG2*-ALR compared with mock-transfected *HepG2* cells (Figure 4A i, ii, v, vi; Figure 4B). Furthermore, immunohistochemistry demonstrated that tumors which originated from *HepG2*-ALR cells displayed a more epithelial-like cell growth and showed less necrotic area (Figure 4A iii) compared with *HepG2* derived tumors with signs of necrosis (Figure 4A vii). In addition fewer polymorphisms and atypical mitotic figures were observed in *HepG2*-ALR (Figure 4A iv) than in mock-transfected *HepG2* cell derived tumors (Figure 4A viii). Based on these findings and in-line with the *in vitro* results, we assume that diminished expression of 15 kDa isoform of ALR in HCC cells supports tumorigenicity and metastatic potential of HCC cells.

ALR Expression Correlates Inversely with Angioinvasive Activity of HCC

We have shown recently that ALR mRNA expression is enhanced in HCC tissues (10) and, therefore, we analyzed ALR protein expression by immunohistochemistry in a total series of 53 tissues of HCC patients including 41 HCC samples where the corresponding nontumorous tissue was available. ALR immunoreactivity (low and high) was found in all HCC tissues (n = 53) and surrounding cirrhotic (n = 25) tissues,

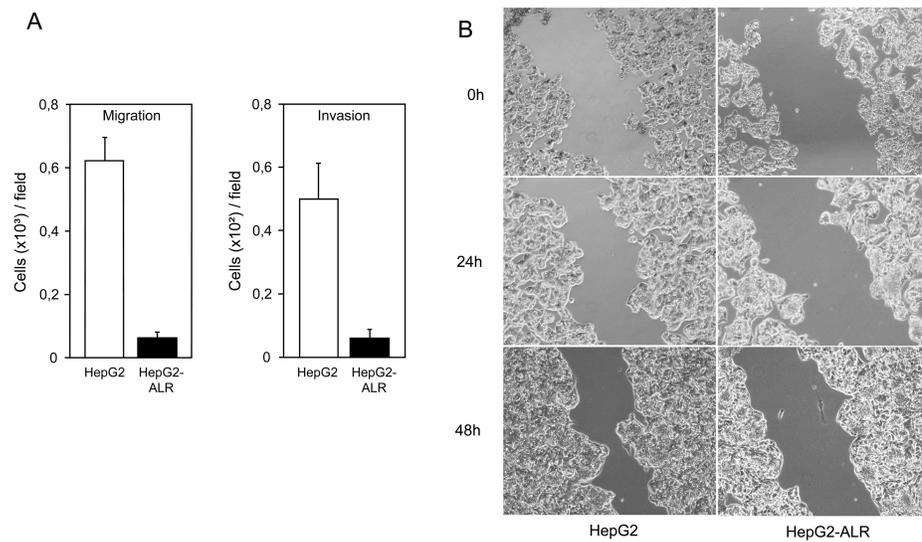


Figure 3. Expression of ALR attenuates HCC cell motility. (A) *HepG2*-ALR cells showed a strong reduction of their migratory and invasive potential compared with *HepG2* cells. Assays were performed in a modified Boyden chamber for 48 h (three independent experiments, mean \pm SD). (B) Scratch assays revealed a diminished “wound healing” of *HepG2*-ALR cells after 48 h of culture (one out of three experiments is shown, magnification 200x).

whereas in 37.5% (6/16) of the nondiseased tissue samples ALR expression was not detected. ALR was expressed significantly higher in HCC compared

with surrounding cirrhotic ($P < 0.001$) and nondiseased ($P < 0.001$) liver tissues. Clinico-pathophysiological characteristics and immunohistochemical results of

the HCC tumor cohort are summarized in Table 1. Positive ALR immunostaining was found to be associated significantly with higher tumor stage ($P = 0.024$). ALR expression correlated inversely with tumor grading ($P = 0.037$) and the rate of histological angioinvasion ($P < 0.001$). Western blot analysis revealed that the short isoform of ALR was present predominantly in samples without histological angioinvasion (Figure 5). ALR expression did not correlate with gender, age, tumor size, proliferation index or serum tumor markers (Table 1).

DISCUSSION

The three different isoforms of ALR with a molecular weight of about 15, 21 and 23 kDa were found to be expressed ubiquitously with the highest levels detected in liver, brain and testes (9,10,24). ALR and its homolog *Erv1* (essential for respiratory and vegetative growth) are essential for survival of hepatocytes (13) and *S. cerevisiae* (25), respectively. Knock-down of full length ALR expression in hepatocytes by transfection with antisense oligonucleotides led to mitochon-

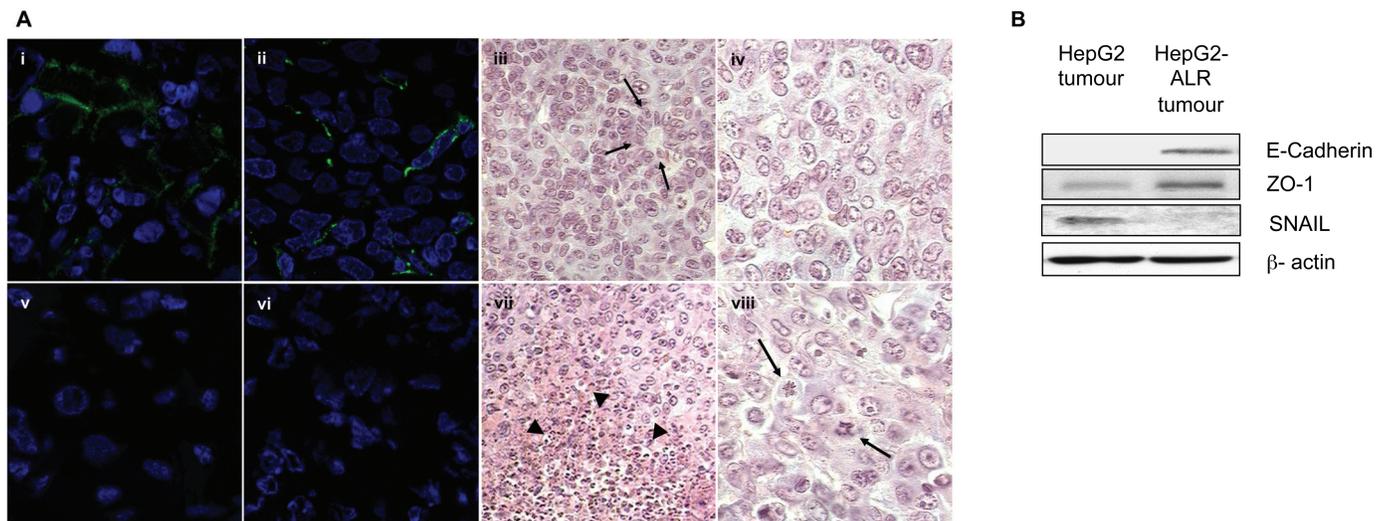


Figure 4. Tumorigenicity of ALR reexpressing HCC cells *in vivo*. (A) Subcutaneously grown tumors in NMRI (nu/nu) mice were analyzed 3 wks after injection of 1×10^6 *HepG2*-ALR (i–iv) or mock transfected *HepG2* cells (v–viii). HE staining of tumors derived from *HepG2*-ALR cells revealed more epithelial-like cell growth (iii arrow, iv) compared with tumors derived from mock transfected *HepG2* cells demonstrating signs of necrotic areas (vii arrow head), cell polymorphism and atypical mitotic figures (viii arrow). Performing immunofluorescence analysis we demonstrated reexpression of EMT markers E-cadherin (i, v) and ZO-1 (ii, vi) in tumors derived from *HepG2*-ALR cells. (iii, vii, magnification 400x; i, ii, iv–vi, viii, magnification 650x). (B) Western blot analysis confirmed reexpression of EMT markers E-cadherin and ZO-1 as well as reduction of SNAIL expression in tumors originated from *HepG2*-ALR cells (1 out of 3 experiments).

Table 1. ALR immunoreactivity (IR) in HCC tissues of 53 patients in relation to clinicopathological characteristics.^a

Variable	Categorization	n	%	ALR IR		P value
				Low	High	
Sex	Male	46	86.8	19	27	0.349
	Female	7	13.2	4	3	
Age (years)	≤60	21	39.6	10	11	0.412
	>60	32	60.4	13	19	
Tumor stage	T1	5	14.7	1	4	0.024^b
	T2	12	35.3	9	3	
	T3	16	47.1	4	12	
	T4	1	2.9	1	0	
Tumor size	≤5cm	19	48.7	11	8	0.213
	>5cm	20	51.3	8	12	
Histological grade	G1	11	24.4	2	9	0.037
	G2	26	57.8	14	12	
	G3	8	17.8	6	2	
Histological angioinvasion	No	13	40.6	2	11	0.001
	Yes	19	59.4	14	5	
Ki-67 staining intensity	≤5%	22	53.7	10	12	0.576
	>5%	19	46.3	9	10	
Serum α-fetoprotein (ng/mL)	<10	17	42.5	8	9	0.576
	10-500	16	40	10	6	
	>500	7	17.51	3	4	
Alkaline phosphatase (U/L)	≤124	22	53.7	12	10	0.442
	>124 L	19	46.3	9	10	

^aSignificance was evaluated by using Pearson chi-square test.

^bBoldfaces indicate significant differences.

drial depletion of ALR, reduced ATP levels and apoptotic cell death (13). Silencing of 23 kDa ALR had almost no effect on hepatocyte cell viability but significantly inhibited hepatoma cell growth and survival (26). The full length 23 kDa isoform of ALR has a putative N-terminal mitochondrial targeting sequence (25) and Erv1 also encodes a leader sequence for mitochondrial import (27). ALR (23 kDa) located in the IMS was shown to be involved in export of Fe/S clusters from the mitochondrial matrix, contributing to biogenesis of Fe/S proteins and cellular iron homeostasis (14,28). Furthermore, ALR/Erv1 play key

roles in generating disulfide bonds in the IMS, thereby facilitating import of proteins through the TOM complex and by Mia40, the latter, in its reduced form, is recycled by ALR (12,28). The 15 kDa ALR lacking N-terminal regions is neither involved in mitochondria associated function of full length ALR nor found in the IMS (28). The short isoform of ALR, which is located in the cytosol and released to the extracellular space was shown to act as hepatotrophic growth factor by stimulating hepatocyte proliferation thereby promoting liver regeneration (15). Recently, we reported that ALR induced c-myc expression and activated

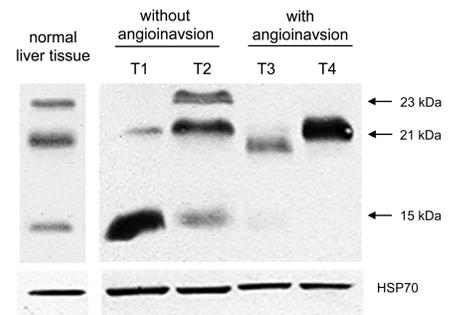


Figure 5. Expression of ALR in tissues of patients with hepatocellular carcinoma stratified for histological angioinvasion. Western blot analysis of hepatocellular carcinoma tissue samples (homogenates 25 μg of total protein extract) were performed. Short isoform of ALR (15 kDa) was expressed predominantly in HCC without histological angioinvasion (T1-T2) compared with samples with histological angioinvasion (T3-T4). Normal liver tissue served as control and expression of heatshock protein 70 (HSP70) was used as loading control.

polyamine metabolism with both being critically important in cellular growth (17). Furthermore this isoform modulated hepatic detoxification by cross-linking growth signals to the regulation of hepatic metabolism (18).

The proliferation augmenting effect of ALR was attributed to its ability to activate EGF receptor phosphorylation and the subsequent stimulation of the MAPK cascade (29). Furthermore, it has been elucidated that intracellular ALR may activate transcription factors via a MAPK independent pathway. ALR was shown to bind to JAB1 (c-Jun activating domain-binding protein 1), a coactivator of transcription factor AP-1, and thereby increased AP-1 activity (30,31). Additionally, ALR was found to interact with macrophage migration inhibitory factor (MIF) and inhibit its effect on AP-1. MIF reduced AP-1 activity by preventing JAB1 to bind to AP-1. On the other hand, the augmenting effect of ALR on AP-1 was inhibited by MIF, indicating a balanced regulation of JAB1-induced AP-1 activity by ALR and MIF (32). Interestingly, it was demonstrated that MIF is

expressed highly in HCC cells and stimulates tumor cell migration but not proliferation (33). Therefore, modulated cell migration of hepatoma cells upon ALR expression might be explained by an interaction of ALR and MIF as well as by altered JAB1 binding to AP-1. AP-1 has an indispensable role in activating MMP3 expression in hepatoma cells (34) and, further, it was shown that MMP3 activates SNAIL expression and subsequently downregulates E-cadherin (35). Therefore, we assume that ALR regulates AP-1 activity, which results in reduced MMP3 and SNAIL levels resulting in enhanced E-cadherin expression (35). On the other hand, it was reported that induction of EMT associated with loss of cell polarity, and promotion of invasive growth by AP-1 is attributed rather to c-fos than to c-jun expression (36), and therefore, further studies have to evaluate the role of ALR in AP-1 mediated EMT inhibition.

MMPs and EMT related genes are regulated by several important signal-transduction pathways such as MAPK, PI3K, Rho-GTPase as well as Smad cascades (37) and we speculate that MAPK/Erk activation through ALR regulates cell migration of HepG2. Additionally, it was reported that tumor promoter TPA (12-tetradecanoylphorbol-13-acetate) can trigger EMT like cell scattering and migration of HepG2 cells by activation of Erk and reactive oxygen species (ROS) (38). Furthermore, recent findings implicate that the cellular redox state may be sensed by binding of ALR to thioredoxin (TRX) based on their ability to form disulfides, subsequently regulating AP-1 and NFκ-B activity (39). Most likely, ALR, by transferring oxidizing equivalents and forming disulfides bonds, either modulates TRX-mediated transcription factor activity or directly acts as an antioxidative agent. A protective and antiapoptotic effect of ALR was reported in neuroblastoma (40) and hepatoma cells (26,41) after hydrogen peroxide treatment (40,41) or irradiation-induced oxidative stress (26). This was mainly due to reduction of mitochondria-

mediated apoptosis characterized by increased mitochondrial membrane potential, reduced cytochrome c release and enhanced ATP levels (26,40,41). Therefore, we hypothesize that ALR may act as an antioxidant ameliorating oxidative stress, thereby regulating cellular ROS levels which consequently modulate redox-sensitive signaling pathways and hepatoma cell motility.

ALR expression was found to be increased in liver regeneration, in cirrhotic livers and HCCs. Although reports on ALR mRNA expression in HCCs vary (10,26), in the current study we confirmed our previously reported observation (10) that ALR protein expression is enhanced in HCC in a larger cohort of patients. Furthermore, an inverse correlation of ALR with tumor grading and angiogenesis was identified. The methods used were not suitable to discriminate between different ALR isoforms. Nevertheless, current *in vitro* data provide evidence that the short isoform of ALR regulates cell motility, a finding in accordance with increased expression of ALR most likely including higher abundance of the 15 kDa isoform in HCC tissues without angiogenesis. Recently it was reported that ALR synthesis and post-translational modification transport into and out of mitochondria as well as extracellular secretion (13) is a dynamic process. Therefore, it is assumed that the metastatic potential of HCC cells with enhanced growth activity and increased levels of ALR depends on the ratio of mitochondrial- and cytosolic-localized ALR. Cytosolic ALR might not protect from oxidative stress only, but also prevent EMT and maintain epithelial cell phenotype under conditions of cancerous growth as was shown in the current study.

In this report, we demonstrate that expression of 15 kDa ALR in HCC cells attenuates cell motility, supports and maintains epithelial cell growth both *in vitro* and *in vivo* and, therefore, abates the process of EMT. In addition, we found that ALR expression is inversely correlated with tumor grading and tumor angiogenesis. Therefore, we provide evidence

that 15 kDa ALR may be considered as an antimetastatic protein in HCC having the potential to become a marker in HCC diagnosis and a therapeutic target.

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DISCLOSURES

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. El Serag HB. (2004) Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology*. 127:S27-S34.
2. Farazi PA, DePinho RA. (2006) Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat. Rev. Cancer*. 6:674-87.
3. El Serag HB, Rudolph KL. (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*. 132:2557-76.
4. Thiery JP. (2002) Epithelial-mesenchymal transitions in tumor progression. *Nat. Rev. Cancer*. 2:442-54.
5. Huber MA, Kraut N, Beug H. (2005) Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* 17:548-58.
6. Senkevich TG, White CL, Koonin EV, Moss B. (2000) A viral member of the ERV1/ALR protein family participates in a cytoplasmic pathway of disulfide bond formation. *Proc. Natl. Acad. Sci. U. S. A.* 97:12068-73.
7. Senkevich TG, White CL, Koonin EV, Moss B. (2002) Complete pathway for protein disulfide bond formation encoded by poxviruses. *Proc. Natl. Acad. Sci. U. S. A.* 99:6667-72.
8. Giorda R, et al. (1996) Analysis of the structure and expression of the augmenter of liver regeneration (ALR) gene. *Mol. Med.* 2:97-108.
9. Klissenbauer M, Winters S, Heinlein UA, Lisowsky T. (2002) Accumulation of the mitochondrial form of the sulphhydryl oxidase

- Erv1p/Alrp during the early stages of spermatogenesis. *J. Exp. Biol.* 205:1979–86.
10. Thasler WE, et al. (2005) Expression of augments of liver regeneration (ALR) in human liver cirrhosis and carcinoma. *Histopathology.* 47:57–66.
 11. Polimeno L, Lisowsky T, Francavilla A. (1999) From yeast to man—from mitochondria to liver regeneration: a new essential gene family. *Ital. J. Gastroenterol. Hepatol.* 31:494–500.
 12. Bihlmaier K, Mesecke N, Kloeppe C, Herrmann JM. (2008) The disulfide relay of the intermembrane space of mitochondria: an oxygen-sensing system? *Ann. N. Y. Acad. Sci.* 1147:293–302.
 13. Thirunavukkarasu C, et al. (2008) Augmenter of liver regeneration: an important intracellular survival factor for hepatocytes. *J. Hepatol.* 48:578–88.
 14. Lange H, et al. (2001) An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. *EMBO Rep.* 2:715–20.
 15. Pawlowski R, Jura J. (2006) ALR and liver regeneration. *Mol. Cell. Biochem.* 288:159–69.
 16. Gatzidou E, Kouraklis G, Theocharis S. (2006) Insights on augmenter of liver regeneration cloning and function. *World J. Gastroenterol.* 12:4951–8.
 17. Dayoub R, et al. (2006) Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augmenter of liver regeneration. *Biochem. Biophys. Res. Commun.* 345:181–7.
 18. Thasler WE, et al. (2006) Repression of cytochrome p450 activity in human hepatocytes in vitro by a novel hepatotrophic factor, augmenter of liver regeneration. *J. Pharmacol. Exp. Ther.* 316:822–9.
 19. Margeli AP, et al. (2003) Hepatic stimulator substance administration ameliorates liver regeneration in an animal model of fulminant hepatic failure and encephalopathy. *Liver Int.* 23:171–8.
 20. Li Q, et al. (2005) Effects of augmentation of liver regeneration recombinant plasmid on rat hepatic fibrosis. *World J. Gastroenterol.* 11:2438–43.
 21. Thasler WE, et al. (2003) Charitable state-controlled foundation Human Tissue and Cell Research: ethic and legal aspects in the supply of surgically removed human tissue for research in the academic and commercial sector in Germany. *Cell Tissue Bank.* 4:49–56.
 22. Weiss TS, Pahernik S, Scheruebl I, Jauch KW, Thasler WE. (2003) Cellular damage to human hepatocytes through repeated application of 5-aminolevulinic acid. *J. Hepatol.* 38:476–82.
 23. Rothhammer T, Bataille F, Spruss T, Eissner G, Bosserhoff AK. (2007) Functional implication of BMP4 expression on angiogenesis in malignant melanoma. *Oncogene.* 26:4158–70.
 24. Tury A, Mairet-Coello G, Lisowsky T, Griffond B, Fellmann D. (2005) Expression of the sulfhydryl oxidase ALR (Augmenter of Liver Regeneration) in adult rat brain. *Brain Res.* 1048:87–97.
 25. Lisowsky T. (1996) Removal of an intron with unique 3' branch site creates an amino-terminal protein sequence directing the scERV1 gene product to mitochondria. *Yeast.* 12:1501–10.
 26. Cao Y, et al. (2009) Human augmenter of liver regeneration is important for hepatoma cell viability and resistance to radiation-induced oxidative stress. *Free Radic. Biol. Med.* 47:1057–66.
 27. Lee J, Hofhaus G, Lisowsky T. (2000) Erv1p from *Saccharomyces cerevisiae* is a FAD-linked sulfhydryl oxidase. *FEBS Lett.* 477:62–6.
 28. Daithankar VN, Farrell SR, Thorpe C. (2009) Augmenter of liver regeneration: substrate specificity of a flavin-dependent oxidoreductase from the mitochondrial intermembrane space. *Biochemistry.* 48:4828–37.
 29. Li Y, et al. (2000) Stimulation of the mitogen-activated protein kinase cascade and tyrosine phosphorylation of the epidermal growth factor receptor by hepatopoietin. *J. Biol. Chem.* 275:37443–7.
 30. Lu C, et al. (2002) Intracrine hepatopoietin potentiates AP-1 activity through JAB1 independent of MAPK pathway. *FASEB J.* 16:90–2.
 31. Wang Y, et al. (2004) Hepatopoietin interacts directly with COP9 signalosome and regulates AP-1 activity. *FEBS Lett.* 572:85–91.
 32. Li Y, Lu C, Xing G, Zhu Y, He F. (2004) Macrophage migration inhibitory factor directly interacts with hepatopoietin and regulates the proliferation of hepatoma cell. *Exp. Cell Res.* 300:379–87.
 33. Ren Y, et al. (2003) Macrophage migration inhibitory factor: roles in regulating tumor cell migration and expression of angiogenic factors in hepatocellular carcinoma. *Int. J. Cancer.* 107:22–9.
 34. Shih WL, et al. (2008) AMF/PGI transactivates the MMP-3 gene through the activation of Src-RhoA-phosphatidylinositol 3-kinase signaling to induce hepatoma cell migration. *Cancer Lett.* 270:202–17.
 35. Przybylo JA, Radisky DC. (2007) Matrix metalloproteinase-induced epithelial-mesenchymal transition: tumor progression at Snail's pace. *Int. J. Biochem. Cell Biol.* 39:1082–8.
 36. Li S, et al. (2009) MicroRNA-101 regulates expression of the v-fos/FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. *Hepatology.* 49:1194–202.
 37. Wu WS. (2006) The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev.* 25:695–705.
 38. Wu WS, et al. (2006) Reactive oxygen species mediated sustained activation of protein kinase C alpha and extracellular signal-regulated kinase for migration of human hepatoma cell Hepg2. *Mol. Cancer Res.* 4:747–58.
 39. Li Y, et al. (2005) Direct association of hepatopoietin with thioredoxin constitutes a redox signal transduction in activation of AP-1/NF-kappaB. *Cell Signal.* 17:985–96.
 40. Polimeno L, et al. (2009) Expression and localization of augmenter of liver regeneration in human muscle tissue. *Int. J. Exp. Pathol.* 90:423–30.
 41. Wu Y, Chen L, Yu H, Liu H, An W. (2007) Transfection of hepatic stimulator substance gene sensitizes hepatoma cells to H2O2-induced cell apoptosis via preservation of mitochondria. *Arch. Biochem. Biophys.* 464:48–56.