AEG-1 Promotes Metastasis Through Downstream AKR1C2 and NF1 in Liver Cancer

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Liver cancer is one of the most lethal cancers, but our knowledge of the molecular mechanism underlying this process remains insufficient. Through deep sequencing and expression regulation analysis in liver cancer cells, we identified two novel factors, AKR1C2 (positive factor) and NF1 (negative factor), as the AEG-1 downstream players in the process of metastasis in liver cancer. They were experimentally validated to have the capacities of regulating cell migration, cell invasion, cell proliferation, and EMT. Further clinic expression and animal model evidence confirmed their functions. Together, our findings provide a new insight into the pharmaceutical and therapeutic use of AEG-1 and downstream AKR1C2 and NF1.

Key words: Metastasis; AEG-1; AKR1C2; NF1

INTRODUCTION

Liver cancer or hepatocellular carcinoma (HCC) is one of the most lethal cancers, and there is still no effective treatment. Patients with more than a solitary tumor might not live for more than 6 months, while those with no metastasis might be cured after surgery. Former research found a few components involved in liver cancer metastasis, such as DLC1, CCR1, and SATB1, but our understanding of the metastasis molecular mechanism remains unclear (1–3).

Metastasis is a complex and multifaceted process and consists of several stages such as tumor invasion, tumor cell dissemination, colonization to distant organs, and metastatic outgrowth (4). Those processes are interactive with several pathways such as cell proliferation and angiogenesis. It is suggestive that there is a systematic gene network underlying this process (5). A few factors have been identified, such as KISS1, VEGF, and MMP, as positive or negative regulators (6–8). But our understanding of the molecular mechanism behind it is far from

enough. We still lack effective clinical methods to efficiently control metastasis in late stage cancer patients.

AEG-1/MTDH orthologs have been found in most vertebrate species, and their sequence remains conservative. Human MTDH/AEG-1 is a protein with 582 amino acids and a molecular mass of 64 kDa (9). It is the downstream of Ha-Ras and c-Myc, and its overexpression leads to activation of downstream PI3k/Akt, NF-κB, and Wnt pathway. AEG-1 was identified to be involved in the metastasis process, and the expression level of AEG-1 is high in most tumor tissue, including colorectal cancer, ovarian cancer, and breast cancer (10–12). Inhibition of AEG-1 leads to suppression of metastasis in lymph node cancer (13). AEG-1 is also involved in Huaier polysaccharide's inhibition of metastasis in HCC cells, possibly as a potential target (14,15). But there still remains much to know about the detailed downstream players in liver cancer.

Here, we identified two novel AEG-1 downstream factors, AKR1C2 and NF1, through high-throughput

sequencing. Both of those two genes were strongly involved in the process of metastasis in vitro and in vivo. AKR1C2 was a positive regulator, and NF1 was a negative regulator. Further clinical evidence and animal model results provided solid support to our results. This work provides us with new visions into the metastasis process and potential efficient clinical targets in the future.

MATERIALS AND METHODS

siRNA and Plasmid

siRNA duplexes were obtained from View-Solid Biotech (Beijing). The silence efficiency of these siRNAs for their targeted mRNAs was tested by quantitative real-time PCR at 24–48 h after transfection. All reactions were run in triplicates. During RNAi experiments, we made use of two specific siRNAs and a scrambled siRNA as a negative control (siNC). Two specific siRNAs showed similar effect (data not shown), and we just showed the results of one siRNA.

The human AEG-1, AKR1C2, and NF1 cDNA clones were constructed into the pcDNA3.1 vector (Addgene). During overexpressing experiments, we made use of the empty vector as a negative control (Vector NC).

Cell Culture and Transfection

The two types of HCC lines (MHCC-97H and Huh7 cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines including MHCC-97H and Huh7 were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin under humidified conditions in 95% air and 5% CO_2 at 37°C. Transfection experiments followed Lipofectamine™ 3000 (Invitrogen) transfection reagent protocol.

Transwell Migration and Invasion Assay

For migration assays, MHCC-97H or Huh7 cells were seeded into the upper chamber of a Transwell insert (pore size, 8 μm; Costar) in 100 μl serum-free medium per well. Then 600 μl medium containing 10% serum was placed in the lower chamber to act as a chemoattractant. Nonmigratory cells were removed from the upper chamber by scraping with a cotton bud. The cells remaining on the lower surface of the insert were fixed with 4% formaldehyde (Sigma-Aldrich) and stained by DAPI (Roche). For invasion assays, cells were seeded in a Matrigel (Bio-Rad)-coated chamber and were incubated at 37°C. The incubation time was 12 h.

Cell Viability Assay

Cells were incubated with 10% CCK8 reagent (DoJinDo Laboratories, Japan) for 1 h at 37°C. Then plates were detected by the automatic spectrometer (Multimode Reader; Enspire) at 450 nm.

Luciferase Assay

For the luciferase assay, 4.0×10^4 MHCC-97H cells were cotransfected with 200 ng of the indicated pGL3 firefly luciferase construct and 20 ng of a pGL3 Renilla luciferase normalization control. The medium was changed 6 h posttransfection, and luciferase activity was measured after 48 h using the dual luciferase reporter assay system (Promega).

Immunoblotting

Lysates were resolved by electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore Corporation), and probed with antibodies against AEG-1 (ab45338; Abcam), AKR1C2 (ab131375; Abcam), and NF1 (ab186738; Abcam). The concentrations of the antibodies were 1:1,000 diluted.

Immunofluorescence

Cells were seeded onto sterile cover slides and allowed to attach overnight. Cells were then fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 2% bovine serum albumin for 1 h at room temperature. The expression of E-cadherin or vimentin was examined using their antibodies (anti-E-cadherin: ab76055; Abcam; anti-vimentin: ab8978; Abcam) and visualized using anti-rabbit IgG (H+L), F (ab)2 fragment (Alexa Fluor 488 conjugate; Cell Signaling Technology). Immunofluorescence was examined using a microscope (EcliPSETi-U; Nikon). The concentrations of the antibodies were 1:200 diluted.

Human Liver Sample and Immunohistochemistry

All human liver samples were obtained from the Beijing You An Hospital. Before surgery at the center, all patients were provided written informed consent to allow any excess tissue to be used for research studies.

Immunohistochemistry was accomplished at the Beijing You An Hospital. Tissue samples were fixed in 10% buffered formalin for 12 h, followed by a wash with PBS, and transferred to 70% ethanol and then embedded in paraffin. The immunohistochemistry detection with antibodies against AEG-1 (ab45338; Abcam), AKR1C2 (ab131375; Abcam), and NF1 (ab186738; Abcam) was performed on paraffin sections.

The scoring criteria were determined during a preliminary evaluation using a multiheaded microscope in order to reach a consensus. The staining results for each antibody were interpreted by two of the authors independently, without prior knowledge of the clinic pathological parameters. For each sample, at least five fields and >500 cells were analyzed. The number of immune-positive cells was semiquantitatively estimated. First, a scoring system according to the staining intensity was determined as follows: 0, colorless; 1, light yellow; 2, brown-yellow;
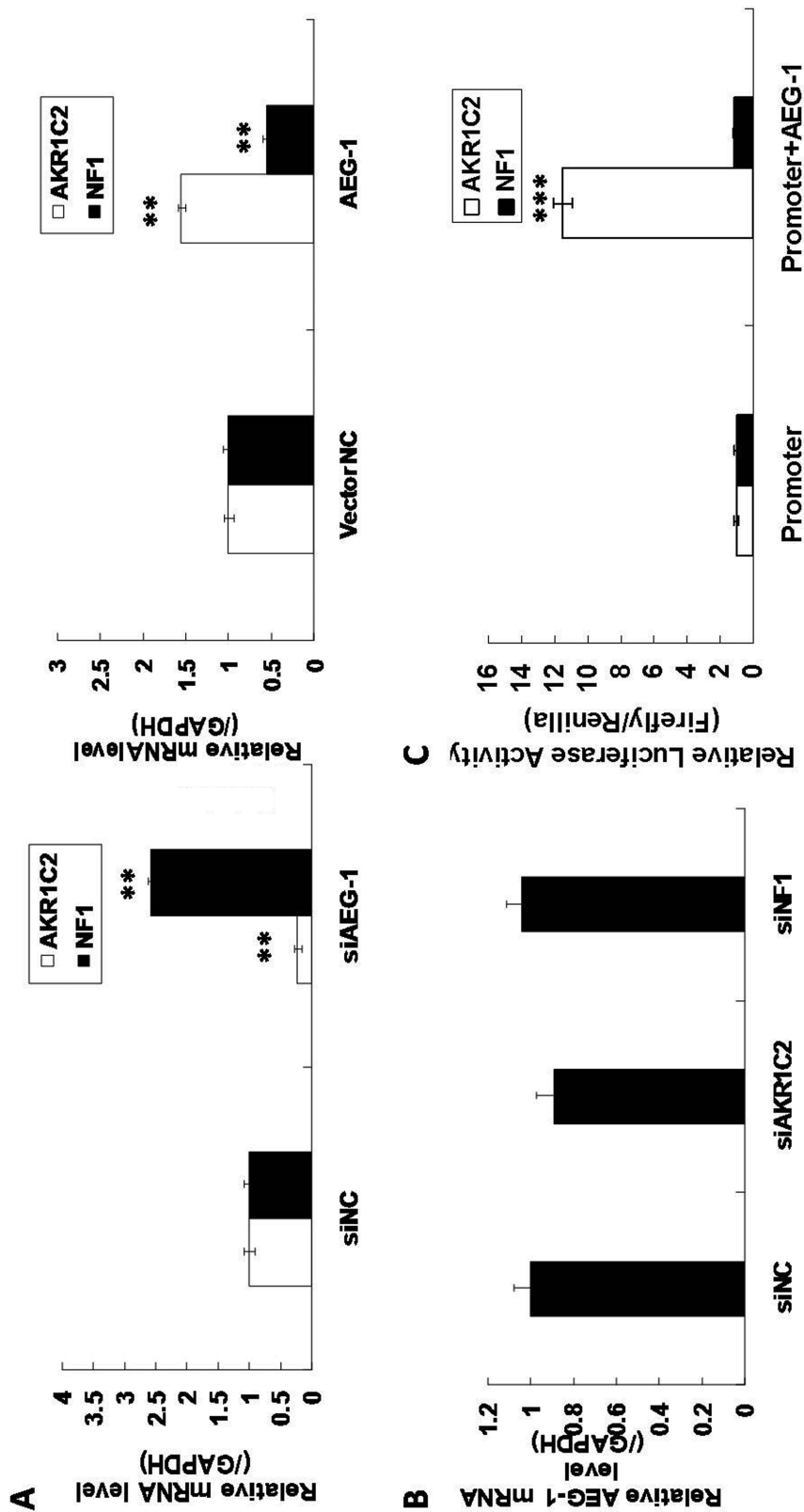
Figure 1. AEG-1 regulating downstream AKR1C2 and NF1. (A) Detection of AKR1C2 and NF1 mRNA level when AEG-1 was silenced or overexpressed by real-time PCR assay ($N=3$). Error bars represent SDs of independent experiments. (B) Detection of AEG-1 mRNA level when AKR1C2 or NF1 was silenced by real-time PCR assay ($N=3$). Error bars represent SDs of independent experiments. (C) Luciferase assay showing activity for cotransfection of AEG-1 and promoter vector in MHCC-97H ($N=3$). Error bars represent SDs of independent experiment. $**p < 0.01$, $***p < 0.001$.
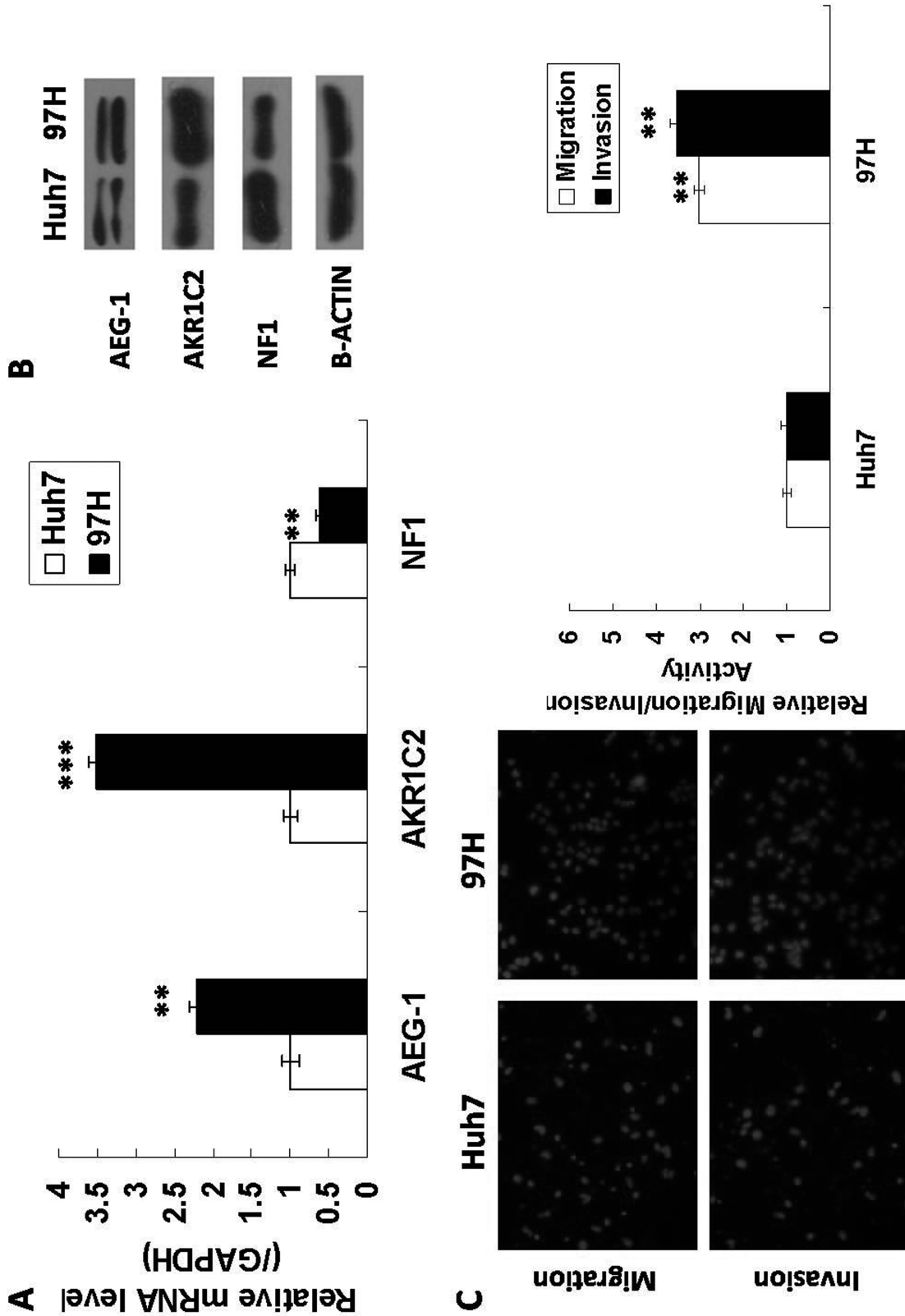
Figure 2. Expression of AEG-1, AKR1C2, and NF1 in high metastatic and low metastatic liver cancer cell lines. (A) Detection of mRNA level for AEG-1, AKR1C2, and NF1 in MHCC-97H and Huh7 cells by real-time PCR assay ($N=3$). Error bars represent SDs of independent experiments. (B) Detection of protein level for AEG-1, AKR1C2, and NF1 in MHCC-97H and Huh7 cells by immunoblotting. (C) Transwell migration and invasion assays showing that MHCC-97H is a high metastatic cell line, whereas Huh7 is a low metastatic cell line ($N=5$). Error bars represent SDs of independent experiments. $**p < 0.01$, $***p < 0.001$.

and 3, dark brown. Scoring according to the percentage of positive cells was determined as follows: 0, no positive cells; 1, <10% positive-stained cells; 2, 11–50% positive-stained cells; 3, 51–75% positive-stained cells; and 4, >75% positive-stained cells. If the product of multiplication between staining intensity and the percentage of positive cells was ≥2, the sample was considered to be immune positive (+).

Animal Study

All research involving animals complied with the protocols approved by the Laboratory Animal Centre, Peking University. All BALB/C nude mice were purchased from the Animal Centre of Peking University. For tumor implantation, luciferase and GFP-labeled MHCC-97H cells were suspended in PBS and injected in the abdominal cavity or liver of mice. After 4 weeks, metastasis status was examined by the In-Vivo Imaging System (IVIS Spectrum CT; Caliper Life Sciences).

Statistical Analysis

All results were expressed as means, and n = derives from independent experiments. When comparing two groups, unpaired Student's t-test (two-tailed) was used. For all tests, a value of $p < 0.05$ was considered significant. Error bars represented SDs of at least three independent experiments.

RESULTS

Deep Sequencing Identifies AKR1C2 and NF1 as Downstream Players of AEG-1

In order to find potential downstream factors of AEG-1 in liver cancer cells, a stable MHCC-97H cell line with a low expression level of AEG-1 was constructed. As shown in supplementary Figure S1 (available at http://cas9.cbi.pku.edu.cn/20150707_OncologyResearch_SupplMaterial/20150707_OncologyResearch_SupplMaterial.html), lentivirus (Plko Vector) was used to infect MHCC-97H cells, and FACS was used to select cells. We constructed two stable MHCC-97H cell lines, including 97H-shAEG-1 (expressing AEG-1 shRNA) and 97H-NC (expressing shRNA with scramble sequence). The mRNA level was reduced 70%, and the protein level was reduced 60% in 97H-shAEG-1 cells compared with 97H-NC cells (Fig. S1).

Deep sequencing was conducted with 97H-shAEG-1 and 97H-NC cells. The AKR1C2 expression level was found to be reduced strongly, and the NF1 expression level was found to get lifted strongly (Fig. S2; available at http://cas9.cbi.pku.edu.cn/20150707_OncologyResearch_SupplMaterial/20150707_OncologyResearch_SupplMaterial.html). This result indicates AKR1C2 and NF1 probably function at the downstream of AEG-1 in liver cancer cells.

AKR1C2 and NF1 Are Regulated by AEG-1

To further understand the regulation mechanism of AKR1C2 and NF1 by AEG-1, we detect mRNA level on conditions of silencing or overexpressing of AEG-1. In MHCC-97H cells, AEG-1 silencing led to decreased AKR1C2 mRNA levels and increased NF1 mRNA levels, whereas overexpressing AEG-1 led to increased AKR1C2 mRNA level and decreased NF1 mRNA level (Fig. 1A). Silencing AKR1C2 or NF1 in MHCC-97H cells did not significantly affect AEG-1 mRNA levels (Fig. 1B). Protein levels accorded with mRNA levels (Fig. S3; available at http://cas9.cbi.pku.edu.cn/20150707_OncologyResearch_SupplMaterial/20150707_OncologyResearch_SupplMaterial.html). These results indicate both the genes are downstream of AEG-1.

Next, we cloned the 5-kb promoter region of AKR1C2 and NF1 and constructed it into a luciferase vector (pGL3-basic; Promega), then detected luciferase activity with coexpression of AEG-1 vector in MHCC-97H cells. The AKR1C2 promoter expression level was greatly lifted 12-fold, while the NF1 promoter expression level did not change significantly (Fig. 1C). This result indicates that AKR1C2, but not NF1, is directly regulated by AEG-1 on transcription level.

Expression Patterns of AEG-1 and Downstream Factors in Human Liver Cancer

In order to verify whether AKR1C2 and NF1 are related to AEG-1 in the metastasis process, expression levels of the three genes were detected in Huh7 and MHCC-97H cells. As known, MHCC-97H is more aggressive in cell migration and invasion (Fig. 2C). The mRNA level of AEG-1 and AKR1C2 was lifted twofold and 3.5-fold in MHCC-97H, while NF1 was reduced to 20%, compared with Huh7 cells (Fig. 2A). Protein levels accorded with mRNA levels (Fig. 2B).

Tissues from patient operations were used to identify the gene expression patterns in liver cancer. Those patients' liver tumors had transferred to other places, that is, metastasis had happened. As is shown in Figure S4 (available at http://cas9.cbi.pku.edu.cn/20150707_OncologyResearch_SupplMaterial/20150707_OncologyResearch_SupplMaterial.html), immunohistochemistry detection was conducted on tumor or normal adjunct tissues. In total, we tested 20 pairs of samples from 20 different liver cancer patients. In all 20 cases, AEG-1 and AKR1C2 were found to be expressed at high levels in tumor tissues, whereas NF1 was found to be expressed at high levels in normal adjunct tissues.

AEG-1 and Downstream Factors Affect Cancer Metastasis Behaviors In Vitro

In high metastatic MHCC-97H cells, AEG-1 or AKR1C2 was silenced, and NF1 was overexpressed in separate experiments. Both migration and invasion capabilities

were reduced to 30% compared with control groups (Fig. 3A), and cell viability was reduced to 70% compared with control groups (Fig. S5A; available at http://cas9. cbi.pku.edu.cn/20150707_OncologyResearch_Suppl Material/20150707_OncologyResearch_SupplMaterial. html). In low metastatic Huh7 cells, AEG-1 or AKR1C2 was overexpressed, and NF1 was silenced in separate experiments. Both migration and invasion capabilities were lifted by at least 50% compared with control groups (Fig. 3B), and cell viability was lifted by 30% compared with control groups (Fig. S5B; available at http://cas9. cbi.pku.edu.cn/20150707_OncologyResearch_Suppl Material/20150707_OncologyResearch_SupplMaterial. html). These results prove that the three genes are involved in the process of metastasis in liver cancer cells.

AEG-1 and Downstream Factors Are Involved in EMT

EMT is a key process of metastasis. In high metastatic MHCC-97H cells, silencing of AEG-1 or AKR1C2 and overexpressing of NF1 reversed the EMT process (Fig. S6; available at http://cas9.cbi.pku.edu.cn/20150707_Oncol ogyResearch_SupplMaterial/20150707_Oncology Research_SupplMaterial.html). Similarly, in low meta-static Huh7 cells, overexpressing of AEG-1 or AKR1C2, and silencing of NF1, promoted the EMT process (Fig. S7; available at http://cas9.cbi.pku.edu.cn/20150707_Oncol ogyResearch_SupplMaterial/20150707_Oncology Research_SupplMaterial.html). These results indicate that the three genes are involved in the EMT process in liver cancer cells.

Knockdown of AEG-1 Strongly Inhibits Metastasis in Mouse Model

In order to further confirm AEG-1's role in liver metastasis, we constructed the 97H-shAEG-1 (stably expressing AEG-1 shRNA) cell line and 97H-NC (expressing shRNA with scramble sequence) cell line. When liver cancer cells were injected into the abdominal cavity of nude mice, the shAEG-1 group (nine mice) showed significantly reduced bioluminescence intensity, compared with the NC group (nine mice) (Fig. 4A). That is, tumor growth and local metastasis capabilities were inhibited by silencing AEG-1. When liver cancer cells were injected directly into the liver of mice, in the control group eight out of nine mouse liver tumors transferred to other organs, such as the lung, while in the shAEG-1 group none of the nine mouse liver tumors transferred (Fig. 4B). These

results further confirm that AEG-1 is a key player in liver cancer metastasis.

DISCUSSION

First of all, we reported AKR1C2 as the downstream factor of AEG-1 in the process of metastasis in liver cancer cells. AKR1C2 was formerly reported as aldo-keto reductase and may promote progesterone metabolism in ovarian endometriosis (16). It is the downstream of PGP2a and promotes apoptosis in human gastric cancer. It was reported to be regulated by AEG-1 in chemoresistance. However, this is the first time to identify AKR1C2 involved in the process of metastasis. Former research indicates the AKR1C2 expression level is reduced in breast cancer, which contradicts our results (17,18). This may result from tumor stage differences and tissue expression specificity. Therefore, these results provide a hint to find more about AKR1C2 functional mechanism.

Second, our results identified a novel downstream factor of AEG-1 in liver cancer cells. NF1 appears to function as a negative regulator of the Ras signal transduction pathway (19). Knockout of NF1 leads to diverse radiation-induced tumors modeling second malignant neoplasms. Our results proved NF1 is the downstream of AEG-1 in liver cancer cells for the first time. AEG-1 negatively regulates NF1. At the same time, we also proved NF1 functions as a negative regulator of metastasis. Probably, by inhibiting NF1, AEG-1 promotes the Ras signal transduction pathway, leading to rapid cancer cell proliferation. These findings indicate AEG-1 is involved in the process of metastasis in multifaceted ways.

Finally, our findings may provide novel potential clinical targets against metastasis in liver cancer patients. Since AEG-1 and AKR1C2 promote metastasis, inhibiting those two genes would effectively control metastasis. A small chemical inhibitor for both genes may be of potential clinical use and provide combination therapies with chemotherapy. Since AEG-1 locates in the cell membrane and functions in metastasis by physically interacting with other components, a neutralizing antibody may also act as an effective potential therapy. Our clinical immunohistochemistry detection and animal experiment results confirmed the antitumor function of AEG-1 in vivo. In sum, we believe that these findings provide new insight into the physiological and therapeutic importance of AEG-1 in liver cancer.

FACING PAGE
Figure 3. AKR1C2 and NF1 modulating liver cancer metastasis in vitro. (A) Silencing of AEG-1 and AKR1C2 or overexpressing of NF1 leading to reduced migratory and invasive capability in MHCC-97H cells ($N=5$). Error bars represent SDs of independent experiments. (B) Overexpressing of AEG-1 and AKR1C2, or silencing of NF1 leading to enhanced migratory and invasive capability in Huh7 cells ($N=5$). Error bars represent SDs of independent experiments. **$p < 0.01$.
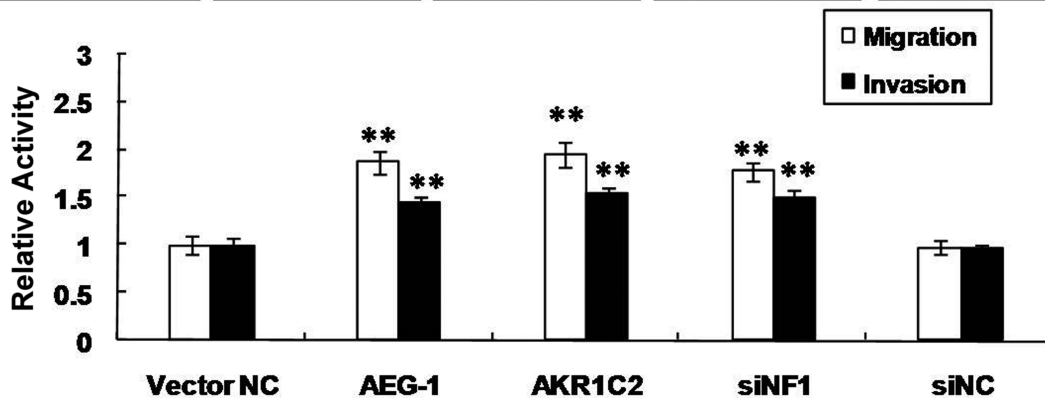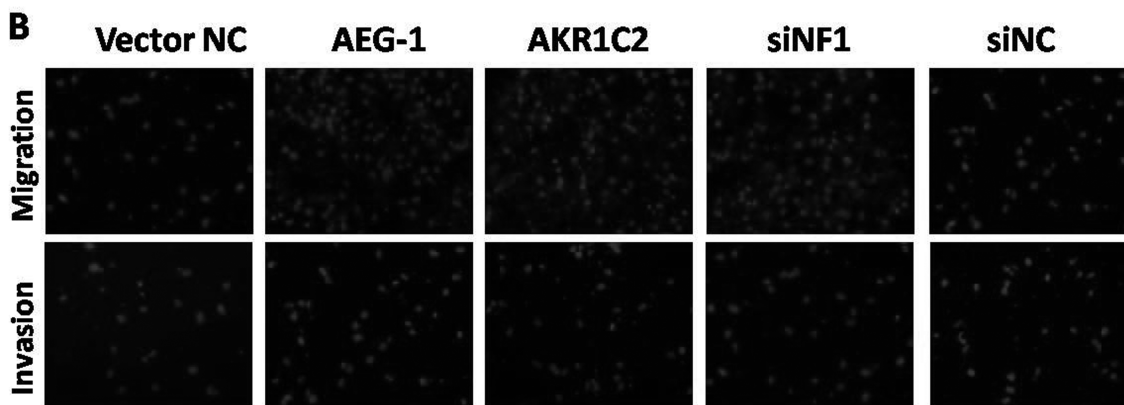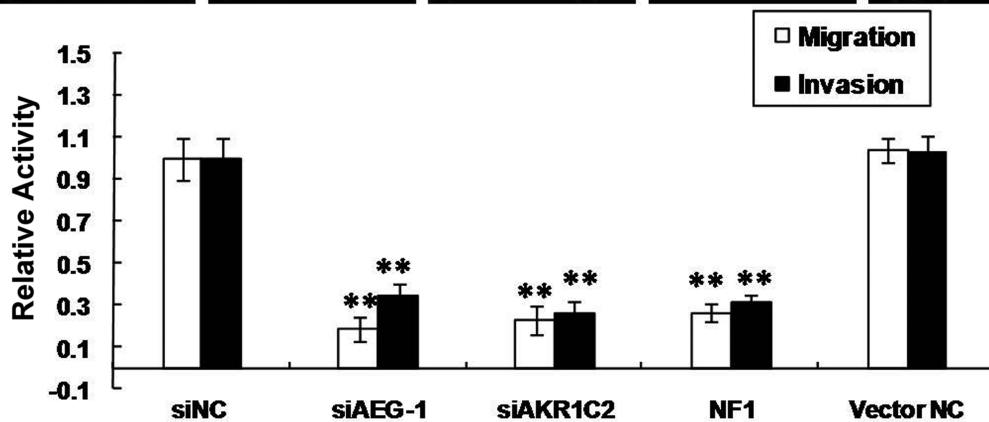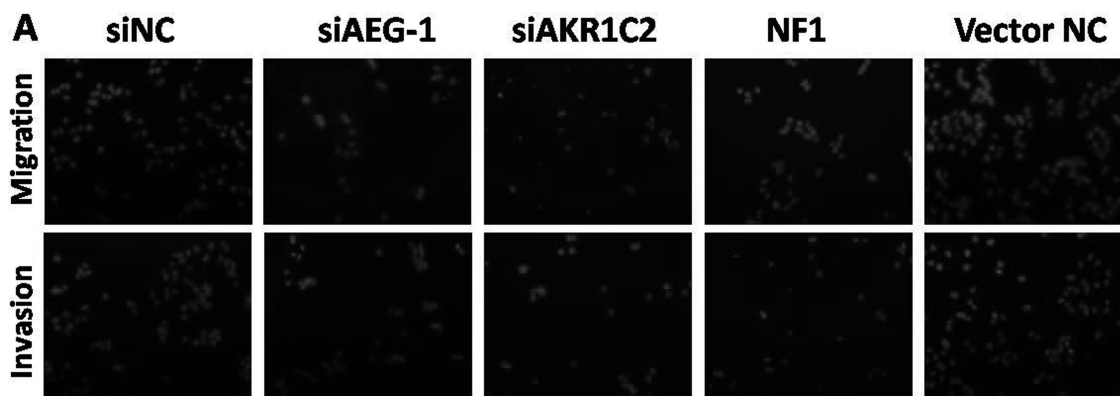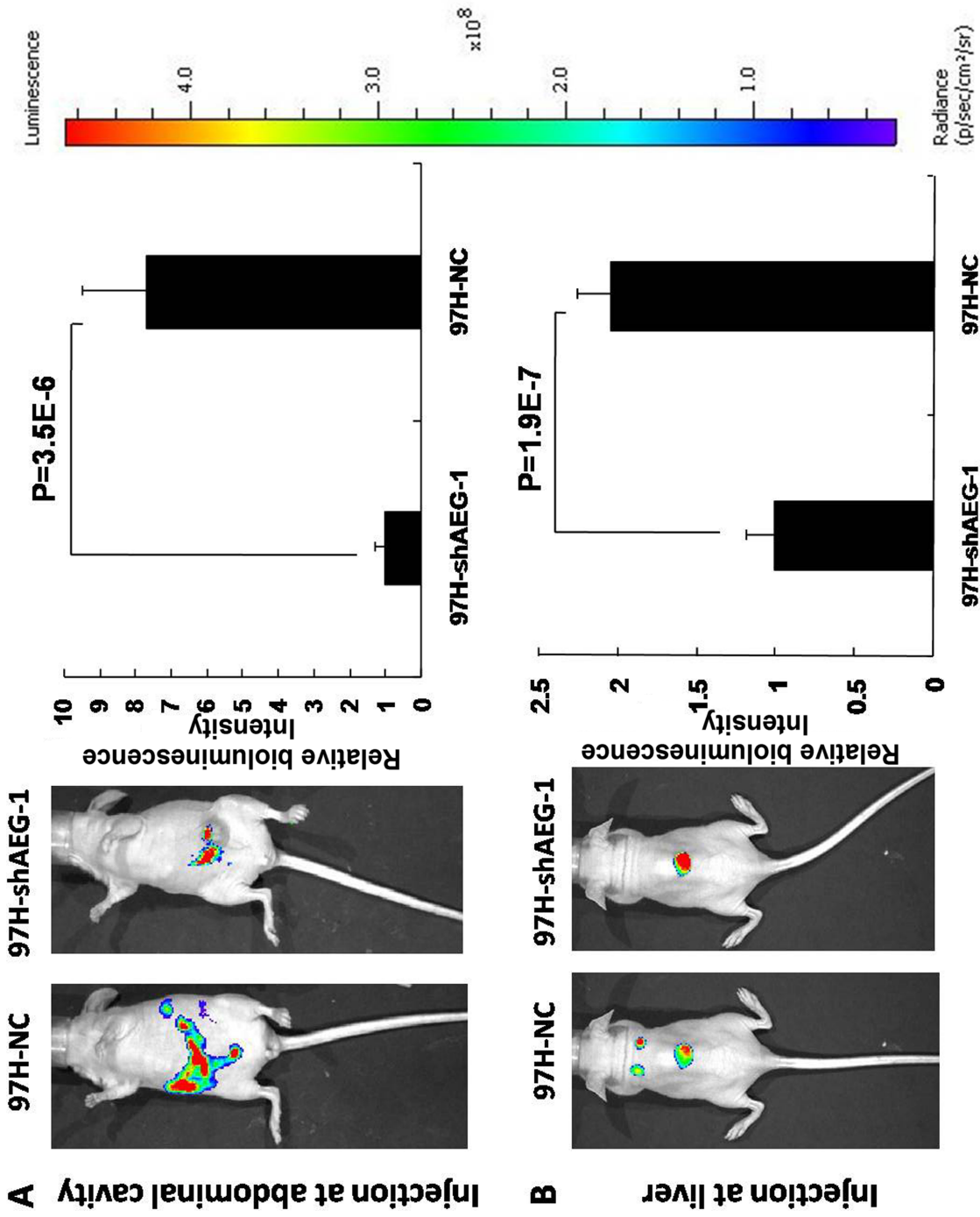
Figure 4. Knockdown of AEG-1 strongly inhibiting metastasis in vivo. (A) Primary tumor growth upon subcutaneous implantation of 3.0×10^5 luciferase-labeled MHCC-97H cells infected with indicated vectors. The experiment was terminated after 30 days ($N=9$). (B) Tumor metastasis upon liver implantation of 3.0×10^5 luciferase-labeled MHCC-97H cells infected with indicated vectors. The experiment was terminated after 30 days ($N=9$).

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