Molecular Mechanism of Chemoresistance by Astrocyte Elevated Gene-1

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Abstract

Our recent findings show that astrocyte elevated gene-1 (AEG-1) is overexpressed in >90% of human hepatocellular carcinoma (HCC) samples, and AEG-1 plays a central role in regulating development and progression of HCC. In the present study, we elucidate a molecular mechanism of AEG-1–induced chemoresistance, an important characteristic of aggressive cancers. AEG-1 increases the expression of multidrug resistance gene 1 (MDR1) protein, resulting in increased efflux and decreased accumulation of doxorubicin, promoting doxorubicin resistance. Suppression of MDR1 by small interfering RNA or chemical reagents, or inhibition of AEG-1 or a combination of both genes, significantly increases in vitro sensitivity to doxorubicin. In nude mice xenograft studies, a lentivirus expressing AEG-1 short hairpin RNA, in combination with doxorubicin, profoundly inhibited growth of aggressive human HCC cells compared with either agent alone. We document that although AEG-1 does not affect MDR1 gene transcription, it facilitates association of MDR1 mRNA to polysomes, resulting in increased translation, and AEG-1 also inhibits ubiquitination and subsequent proteasome-mediated degradation of MDR1 protein. This study is the first documentation of a unique aspect of AEG-1 function (i.e., translational and posttranslational regulation of proteins). Inhibition of AEG-1 might provide a means of more effectively using chemotherapy to treat HCC, which displays inherent chemoresistance with aggressive pathology. Cancer Res; 70(8); 3249–58. ©2010 AACR.

Introduction

The observation that the expected incidence and mortality for hepatocellular carcinoma (HCC) runs closely parallel highlights the paucity of effective treatment modalities for this fatal disease (1). In the United States, the estimated new cases of HCC for 2008 were 21,370, of which 18,410 were expected to die (2). HCC is a tumor with rapid growth and early vascular invasion (3). Most HCC patients present with advanced symptomatic tumors with underlying cirrhotic changes that are not amenable to surgical resection or liver transplantation (4). Even after surgical resection, the recurrence rate is very high. Transarterial chemoembolization (TACE) and systemic therapy with doxorubicin alone or a combination of cisplatin, IFN, doxorubicin, and 5-fluorouracil (5-FU) are being used for advanced disease, with moderate improvement in overall survival duration varying between 6.8 and 8.6 months (5–7). These dismal figures point to chemoresistance as an inherent trait of HCC. Identification of key molecules contributing to this chemoresistance and creating strategies to counteract this process will help augment the efficiency of current modalities of treatment and increase patient survival.

Anthracyclines are the most effective anticancer chemotherapeutics, and doxorubicin is the first anthracycline isolated from Streptomyces peucetius early in the 1960s (8). Doxorubicin is the most common chemotherapeutic used for HCC (9). It is a component of TACE and systemic chemotherapy (6, 7). Although doxorubicin is widely used in diverse cancer indications, development of resistance to doxorubicin is a very common event. The most common mechanism of doxorubicin resistance is the enhanced efflux of drug by cancer cells, including HCC cells, mediated by increases in drug transporter family known as ATP-binding cassette (ABC) transporters (10).

ABC transporters are ATP-dependent efflux pumps with broad drug specificity. As yet, 48 human ABC genes have been identified and divided into seven distinct subfamilies (ABCA to ABCG) on the basis of their sequence homology and domain organization (11). The most common member of this family is ABCC1 [also known as multidrug resistance gene1 (MDR1)] that codes for the protein P-glycoprotein (12, 13). MDR1 is a broad-spectrum multidrug efflux pump...
that has 12 transmembrane regions and 2 ATP-binding sites (14). Overexpression of MDRI accompanied by a decrease in doxorubicin accumulation has been observed in HCC cell lines (15, 16). MDRI expression level is an adverse prognostic factor correlated with reduced survival (16, 17). The molecular mechanism of MDRI overexpression has not been fully elucidated. Epigenetic regulation by promoter demethylation leading to increased expression of MDRI mRNA is one mechanism of MDRI overexpression (18). In HCC cells, the riboregulator H19 has been implicated in regulating this process (19).

**Astrocyte elevated gene-1 (AEG-1)** is an important gene regulating development and progression of multiple cancers, including HCC (20, 21). Our recent results using immunohistochemical analysis of tissue microarray revealed that AEG-1 expression gradually increases with the stages and grades of HCC, whereas its expression is virtually undetected in normal liver (20). Among 109 HCC patients, >90% showed positive staining for AEG-1. In a subset of patients, the overexpression of AEG-1 mRNA and protein is caused by genomic amplification of the AEG-1 gene (20). Overexpression of AEG-1 in poorly aggressive HCC cells, such as HepG3, generated aggressive, highly vascular, and metastatic tumors in nude mice, whereas inhibition of AEG-1 by small interfering RNA (siRNA) in highly aggressive HCC cells, such as QGY-7703, markedly inhibited growth of the tumors in nude mice (20). In HCC cells, AEG-1 activates multiple prosurvival signaling pathways, including mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK, Akt, NF-κB, and Wnt signaling (20). Affymetrix microarray confirmed that AEG-1 modulated specific genes regulating invasion, angiogenesis, chemoresistance, and senescence, thus playing a major role in HCC progression (20).

Chemoresistance is a major effect attributed to AEG-1 expression (22–24). We have shown that AEG-1 confers resistance to 5-FU by increasing the transcription factor LSF that induces thymidylate synthase, the substrate for 5-FU, and by increasing the 5-FU–catabolizing enzyme dihydropyrimidine dehydrogenase (DPYD; ref. 22). In the present study, we document that AEG-1 also confers resistance to doxorubicin in HCC cells. We elucidate a molecular mechanism by which AEG-1 confers doxorubicin resistance and show that inhibition of AEG-1 effectively reverses doxorubicin resistance. Thus, AEG-1 inhibition might provide an effective strategy to improve doxorubicin efficacy in HCC patients.

**Materials and Methods**

**Cell lines, culture condition, viability assays, and chemical reagents.** HepG3 and QGY-7703 human HCC cells...
were cultured as described (20). Generation of Hep-AEG-1-14 and Hep-AEG-1-8 clones, HepG3 cells stably expressing AEG-1, and Hep-pc-4, HepG3 cells stably transduced with empty vector, has been described previously (20). Cell viability was determined by standard MTT assay as described (20). Doxorubicin was obtained from Sigma. MDR1 inhibitors verapamil, C-4, and cyclosporin A were obtained from Calbiochem and used at a working concentration of 10, 50, and 2.5 μmol/L, respectively. MEK/ERK inhibitor PD98059, NF-κB inhibitor quinazoline, and phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (all obtained from Calbiochem) were used at a concentration of 10, 1, and 10 μmol/L, respectively. The proteasome inhibitor MG132 (Calbiochem) was used at a concentration of 5 μmol/L.

**Patient samples.** Patient samples were obtained from the Liver Tissue Cell Distribution System, a NIH service contract to provide human liver and isolated hepatocytes from regional centers for distribution to scientific investigators throughout the United States (NIH contract N01-DK-7-0004/HHSN267200700004C).

**siRNAs and lentiviruses.** Scrambled siRNA and siRNA for AEG-1 and MDR1 were obtained from Santa Cruz Biotechnology and used at a concentration of 20 nmol/L. The 19-bp AEG-1 sequence used to generate AEG-1 short hairpin RNA (shRNA) is 5′-CAGAAGAAGAAGAACCAGGA-3′. Detailed description of lentivirus vector production is described previously (22, 25).

**Doxorubicin efflux and accumulation assays.** Cells were treated with 3.44 μmol/L doxorubicin for 2 h, washed in PBS several times, and incubated with doxorubicin-free complete growth media for up to 120 min. Cell pellets were analyzed for intracellular doxorubicin accumulation, and the bathing media were used for efflux assay. The fluorescence images were obtained by an Olympus fluorescence microscope. Doxorubicin fluorescence intensity of intracellular retention and efflux was measured using λexcitation at 490 nm and λemission at 510 to 570 nm by using a fluorescence spectrophotometer (Turner Biosystems).

**Preparation of whole-cell lysates and Western blot analyses.** Preparation of whole-cell lysates and Western blot analyses were performed as described (20). The primary antibodies used were anti-AEG-1 (1:5,000; chicken polyclonal; in house; ref. 26), anti-CYP2B6 (1:2,000; mouse monoclonal; BD Biosciences), anti-AKR1C2 (1:1,000; rabbit polyclonal; Santa Cruz Biotechnology), anti-CYP2B6 (1:2,000; mouse monoclonal; in house; ref. 26), anti-CYP2B6 (1:2,000; mouse monoclonal; Abcam), anti-ABCC11 (1:1,000; mouse monoclonal; Abcam), anti-MDR1 (1:1,000; mouse monoclonal; Abcam), anti–phospho-eIF4G (1:1,000; rabbit polyclonal; Cell Signaling), anti–eIF4G (1:1,000; rabbit polyclonal; Cell Signaling), anti–phospho-eIF4E (1:1,000; rabbit polyclonal; Cell Signaling), and anti–phospho-4E-binding protein (4E-BP) 1 (1:1,000; rabbit polyclonal; Cell Signaling).

**Pulse-chase analysis and immunoprecipitation.** Hep-pc-4 and Hep-AEG-1-14 cells were cultured for 4 h in methionine-free medium, and then [35S]methionine was added at a concentration of 50 μCi/mL. The cells were incubated for 0 to 12 h, and cell lysates were prepared and subjected to immunoprecipitation using anti-MDR1 antibody as described (27). The immunoprecipitates were run on a SDS-PAGE gel, which was exposed to autoradiogram. Immunoprecipitation of ubiquitinated proteins was performed with anti-ubiquitin antibody (mouse monoclonal; Cell Signaling).

**Figure 2.** Inhibition of MDR1 or AEG-1 reverses resistance to doxorubicin. A, Hep-AEG-1-14 cells were transfected with siCon, siAEG-1, or siMDR1, and expression of AEG-1, MDR1, and β-tubulin was analyzed by Western blot. B, Hep-AEG-1-14 cells were transfected with siCon, siAEG-1, or siMDR1 or a combination or siAEG-1 and siMDR1 and treated with 3.4 nmol/L doxorubicin, #, P < 0.05 versus siCon; *, P < 0.01 versus siCon. C, Hep-AEG-1-14 cells were treated with verapamil (10 μmol/L), C-4 (50 μmol/L), or cyclosporin A (2.5 μmol/L) and treated with 3.4 nmol/L doxorubicin, #, P < 0.05 versus doxorubicin treatment only. D, Hep-AEG-1-14 cells were transfected with siCon or siAEG-1 and treated with verapamil (10 μmol/L) or cyclosporin A (2.5 μmol/L) and 3.4 nmol/L doxorubicin, #, P < 0.05 versus siCon + doxorubicin; *, P < 0.01 versus siCon + doxorubicin.
**Immunofluorescence and immunohistochemical analyses.** Immunofluorescence studies in cells and tumor sections and immunohistochemical studies in tumor sections were performed as described (20). For immunofluorescence, the primary antibodies used were anti-MDR1 and anti–Ki-67 (1:200; mouse monoclonal; BD Biosciences). The secondary antibody was Alexa Fluor 488–conjugated anti-mouse IgG or anti-rabbit IgG (Molecular Probes). The samples were mounted in Vectashield fluorescence mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were analyzed by a Zeiss confocal laser scanning microscope. For immunohistochemistry, anti–AEG-1 antibody was used at 1:200 dilution and anti-MDR1 antibody was used at 1:100 dilution. The sections were developed by avidin-biotin-peroxidase complexes with 3,3′-diaminobenzidine substrate solution (Vector Laboratories). The slides were costained with 10% hematoxylin solution. The images were taken by an Olympus microscope.

**Preparation of polysomes and Northern blot analysis.** Polysomal fractions were purified essentially as described (28). Each fraction was taken at 500 μL, monitoring the absorbance at 260 nm, and polysome fractions were identified (typically fractions 10–20). RNA was extracted from each fraction with RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol, and Northern blotting was done as described using radiolabeled human MDR1 cDNA probe (28).

**Nude mice xenograft studies.** QGY-7703 cells were transduced with either a lentivirus expressing scrRNA or a lentivirus expressing AEG-1 shRNA at a concentration of 2 multiplicities of infection per cell for 48 h. One million cells were s.c. implanted in the flanks of athymic nude mice. Doxorubicin was injected five times per week for 2 wk at a dose of 4 mg/kg. Tumor diameter was measured with calipers at 2 wk later after injection, and the tumor volume in mm³ was calculated by the following formula:

\[
\text{volume} = \frac{\text{width} \times \text{length} \times \text{height}}{2}
\]

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay.** Apoptotic cell death was detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method using ApoAlert DNA fragmentation assay kit (Clontech) according to the manufacturer’s protocol. Images were analyzed by an Olympus immunofluorescence microscope.

**Statistical analysis.** Data were represented as the mean ± SE and analyzed for statistical significance using one-way ANOVA followed by Newman-Keuls test as a post hoc test. A P value of <0.05 was considered as significant.

**Results**

**AEG-1 increases MDR1 protein expression.** Human HCC cells HepG3 do not form tumors in nude mice and express low levels of AEG-1 (20). In contrast, QGY-7703 HCC cells form aggressive tumors in nude mice and express high levels of AEG-1 (20, 22). Stable overexpression of AEG-1 in HepG3 cells (Hep-AEG-1-14 and Hep-AEG-1-8) significantly increases invasion and anchorage-independent growth and generates highly aggressive vascular tumors in nude mice (20). To identify AEG-1 downstream genes conferring its oncogenic activity, we performed Affymetrix cDNA microarray analysis between Hep-AEG-1-14 and Hep-pc-4 cells, the latter is HepG3 cells stably transformed with empty pcDNA3.1-hygro vector. A list of the modulated genes has been discussed...
previously (20). Of relevance to the present study, we identified induction of several chemoresistance-associated genes by AEG-1 that include (a) DPYD, principal enzyme inactivating 5-FU; (b) cytochrome P4502B6 (CYP2B6), involved in metabolism of multiple drugs; (c) dihydropyridine dehydrogenase (AKR1C2), conferring resistance to doxorubicin and cisplatin; (d) the transcription factor LSF, which activates the transcription of thymidylate synthase, target of 5-FU; and (d) ABCC11/MRP8, a member of the ABC family transporter (20, 22). We extensively characterized the consequence of AEG-1–mediated induction of DPYD and LSF that promotes resistance to 5-FU (22). Real-time PCR and Western blot analysis confirmed the AEG-1–mediated induction of CYP2B6 (Fig. 1A; ref. 20). Because AKR1C2 and ABCC11 are known to mediate resistance to doxorubicin, we checked whether AEG-1 increases their expression at the protein level to confirm the microarray data. However, very modest increases were observed for AKR1C2 and ABCC11 in Hep-AEG-1 clones compared with the Hep-pc-4 clone. Although MDR1 mRNA upregulation was not detected by microarray or real-time PCR (data not shown), robust increase in MDR1 protein level was detected in Hep-AEG-1-14 and Hep-AEG-1-8 clones compared with Hep-pc-4 clone (Fig. 1A). This increase in protein level was confirmed by immunofluorescence analysis (Fig. 1B). Comparable levels of AEG-1 and MDR1 proteins were detected in Hep-AEG-1-14 and QGY-7703 cells (Supplementary Fig. S1A). We also checked AEG-1 and MDR1 protein expressions in sections of normal liver and matched HCC from five patients by immunohistochemistry. Significantly high level of AEG-1 and MDR1 was observed in HCC sections in comparison with normal liver in all five patients (representative result from one patient is shown in Supplementary Fig. S1B).

**AEG-1 confers resistance to doxorubicin.** Because MDR1 overexpression is commonly associated with doxorubicin resistance, we analyzed cell viability of Hep-pc-4 and Hep-AEG-1-8 and Hep-AEG-1-14 clones as well as QGY-7703 cells (naturally AEG-1–overexpressing HCC cell line) on treatment with increasing concentrations of doxorubicin. In a 1-week assay, the IC_{50} of doxorubicin for Hep-pc-4 was 3.4 nmol/L, whereas that for Hep-AEG-1-8, Hep-AEG-1-14, and QGY-7703 cells was 60, 93, and 76 nmol/L, respectively, showing that AEG-1 overexpression results in profound resistance to doxorubicin (Fig. 1C). Detection of apoptosis by TUNEL assay showed a significant decrease in TUNEL-positive cells in the Hep-AEG-1-14 clone compared with the Hep-pc-4 clone on doxorubicin treatment (Fig. 1D). No TUNEL-positive cells were detected in either clone without doxorubicin treatment (Fig. 1D, left). It should be noted that we also observed resistance of these cells toward cisplatin, supporting MDR, but we used doxorubicin as a prototype drug to dissect the molecular mechanism of AEG-1–induced chemoresistance.

We explored the involvement of AEG-1 and MDR1 in mediating doxorubicin resistance through both loss-of-function
and gain-of-function experiments. Inhibition of AEG-1 by siRNA significantly decreased the levels of both AEG-1 and MDR1 protein, whereas MDR1 siRNA (siMDR1) only decreased MDR1 protein level, but not AEG-1 protein level, indicating that MDR1 is downstream of AEG-1 (Fig. 2A). Although siMDR1 alone did not affect viability of Hep-AEG-1-14 cells, it significantly (although modestly) increased the sensitivity to doxorubicin (Fig. 2B). AEG-1 siRNA (siAEG-1) alone resulted in a significant decrease in viability of Hep-AEG-1-14 cells, because AEG-1 regulates cell proliferation as shown previously (20), and increased sensitivity to doxorubicin significantly (Fig. 2B). However, a combination of siMDR1 and siAEG-1 markedly increased sensitivity to doxorubicin (Fig. 2B). Similar finding was also observed in QGY-7703 cells (Supplementary Fig. S2). The observation that siAEG-1 was more potent than siMDR1 in conferring sensitivity to doxorubicin might be explained by the regulation of other ABC transporters, such as ABCC11, and metabolic enzymes, such as AKR1C2, or other as yet identified genes by AEG-1 as shown in Fig. 1A. Chemical inhibitors of MDR1, such as verapamil, C-4, and cyclosporin A, also increased sensitivity of Hep-AEG-1-14 cells to doxorubicin (Fig. 2C). A combination of siAEG-1 with verapamil or cyclosporin A enhanced the sensitivity of Hep-AEG-1-14 cells to doxorubicin (Fig. 2D).

Because MDR1 functions by increasing drug efflux and decreasing drug accumulation, we next determined the efflux and accumulation of doxorubicin in Hep-pc-4 and Hep-AEG-1-14 cells. In a kinetic analysis, there was a significant increase in the efflux of doxorubicin and a significant decrease in accumulation of doxorubicin in Hep-AEG-1-14 cells when compared with Hep-pc-4 cells (Fig. 3A and B). In Hep-AEG-1-14 cells, doxorubicin accumulation was significantly increased and doxorubicin efflux was significantly decreased by treatment with siAEG-1 or siMDR1, and these effects were further augmented when treated with a combination of both siRNAs (Fig. 3C). Similarly, a combination of siAEG-1 and verapamil also significantly increased doxorubicin accumulation and decreased doxorubicin efflux in Hep-AEG-1-14 cells when compared with either agent used alone (Fig. 3D).

**AEG-1 increases translation of MDR1 protein.** Based on our microarray data, it seems that AEG-1 increases MDR1 protein level without affecting MDR1 transcription. This increase in MDR1 protein by AEG-1 might be due to an increase in the translation of MDR1 protein or a decrease in ubiquitin-proteasome–mediated degradation of MDR1 protein. Pulse-chase experiment using 35S-labeled methionine clearly showed increased translation of MDR1 protein in Hep-AEG-1-14 cells compared with Hep-pc-4 cells (Fig. 4A). AEG-1 is known to activate MEK/ERK, NF-κB, and PI3K/Akt signaling pathways in HCC cells (20). Using chemical inhibitors of these three pathways, PD98059, quinazoline, and LY294002, respectively, it was observed that treatment with only LY294002 downregulated MDR1 protein level in the Hep-AEG-1-14 clone, indicating that activation of PI3K/Akt pathway by AEG-1 contributes to an increase in MDR1 level in these cells (Fig. 4B). Analyzing the association of MDR1 mRNA with polysomes further corroborated the importance of PI3K/Akt pathway in mediating MDR1 translation by AEG-1. Polysomal fractions were isolated from Hep-pc-4 and Hep-AEG-1-14 cells as well as Hep-AEG-1-14 cells treated with LY294002, and the association of MDR1 mRNA to the polysomes was analyzed by Northern blot analysis. Increased association of MDR1 mRNA to polysomes was clearly observed in Hep-AEG-1-14 cells compared with Hep-pc-4 cells, which was profoundly inhibited by LY294002 (Fig. 4C). Whereas treatment of Hep-AEG-1-14 cells with doxorubicin alone decreased cell viability by ~30%, in combination with a noncytotoxic concentration of LY294002 (5 μmol/L) the decrease in cell viability was increased to ~57%, providing further confirmation of the role of PI3K/Akt pathway in mediating doxorubicin resistance (Fig. 4D).
In addition to facilitating association of MDR1 mRNA to polysomes, we checked whether AEG-1 also affects the translational machinery. We checked the phosphorylation and activation status of key cap-dependent translational initiation factors [i.e., eukaryotic translation initiation factor 4E (eIF4E), eukaryotic translation initiation factor 4G (eIF4G), and 4E-BP] in Hep-pc-4 cells and Hep-AEG-1-14 cells untreated or treated with LY294002. The reason to check these particular proteins is that they are modulated by mammalian target of rapamycin (mTOR) pathway, a PI3K/Akt downstream signaling pathway, which plays a fundamental role in regulating translation initiation. Marked phosphorylation of eIF4G was observed in Hep-AEG-1-14 cells compared with Hep-pc-4 cells, which was not affected by LY294002 treatment that was confirmed by densitometric analysis (Fig. 5A). The phosphorylation of eIF4E and 4E-BP was not modulated by AEG-1.

To check if AEG-1 modulates ubiquitination and degradation of MDR1 protein, the total level of ubiquitinated MDR1 was determined in Hep-pc-4 and Hep-AEG-1-14 cells. The cells were treated with the proteasome inhibitor MG132, and the cell lysates were subjected to immunoprecipitation with anti-ubiquitin antibody followed by immunoblot with anti-MDR1 antibody. The level of ubiquitinated MDR1 was significantly lower in the Hep-AEG-1-14 clone compared with the Hep-pc-4 clone (Fig. 5B). As a corollary, treatment of Hep-pc-4 and Hep-AEG-1-14 clones with MG132 significantly increased the protein level of MDR1 in both clones (Fig. 5C). MG132 treatment increased MDR1 protein level of Hep-pc-4 cells to a level that is comparable with basal AEG-1 level in Hep-AEG-1-14 cells (Fig. 5C). These findings indicate that AEG-1 might interfere with ubiquitination and proteasomal degradation of MDR1 protein, thus increasing the total MDR1 protein level.

Combination of AEG-1 shRNA and doxorubicin significantly inhibits HCC xenograft growth in nude mice. The in vitro findings that inhibition of AEG-1 might overcome doxorubicin resistance were confirmed in in vivo nude mouse xenograft studies. QGY-7703 cells were ex vivo transduced with a lentivirus expressing control siRNA (Lenti.siCon) or siAEG-1 (Lenti.siAEG-1), and 2 days later, the cells were s.c. implanted on the flanks of athymic nude mice. After establishment of the tumor (~100 mm³ requiring ~7 days), the animals received i.p. injection of either PBS or doxorubicin (4 mg/kg) 5 days per week for 2 weeks. Inhibition of AEG-1 alone resulted in significant inhibition of tumor growth (Fig. 6A and B). Whereas doxorubicin treatment alone also resulted in inhibition of tumor growth, the combination of AEG-1 inhibition and doxorubicin treatment resulted in...
profound inhibition of tumor growth versus either agent alone (Fig. 6A and B). Analysis of tumor sections revealed that Lentivirus-AEG-1 treatment resulted in profound downregulation of AEG-1 and MDR1 proteins compared with Lentivirus siCon treatment in combination with PBS or doxorubicin (Fig. 6A). Kaplan-Meier survival curves showed that 100% siCon + PBS–treated animals died within 10 days of starting the treatment, whereas treatment with either siAEG-1 or doxorubicin alone resulted in 100% mortality within 14 days (Fig. 6C). Combinatorial treatment of siAEG-1 and doxorubicin resulted in a significant increase in the survival of the animals (Fig. 6C). Treatment with siAEG-1 + doxorubicin significantly downregulated Ki-67 staining, a marker for proliferation, in tumor samples (11.67 ± 3.5 Ki-67–positive cells per field) compared with siCon + PBS–treated tumors (41.33 ± 6.5 Ki-67–positive cells per field; Fig. 6D, left). Whereas siAEG-1 or doxorubicin alone increased TUNEL–positive cells in the tumor section, the combination of both agents markedly increased TUNEL–positive cells, indicating that the combination treatment decreases proliferation and increases apoptosis, therefore resulting in profound inhibition of tumor growth (Fig. 6D, right).

Discussion

The ability of AEG-1 to confer chemoresistance represents an important attribute of this cancer-promoting gene. We have documented that AEG-1 activates a specific pathway involving LSF transcription factor and the catalyzing enzyme DPYD to confer resistance to 5-FU (22). A study using neuroblastoma cells show that AEG-1 mediates resistance to doxorubicin and cisplatin, although the molecular mechanism of the resistance was not explored (24). Studies using breast cancer cells showed resistance to broad-spectrum chemotherapeutics conferred by AEG-1 that involves upregulation of aldehyde dehydrogenase 3 family, member A1 and hepatocyte growth factor receptor (23). These two candidates were identified by microarray analysis looking for AEG-1 downstream genes. In the microarray analysis, MDR1 mRNA upregulation was not observed because we now show that MDR1 upregulation by AEG-1 occurs at the protein level. Doxorubicin is the most common chemotherapeutic used for HCC. However, the clinical efficacy of doxorubicin is not profound, thus emphasizing an inherent resistance of HCC cells to doxorubicin that has been attributed to overexpression of MDR1 (16, 17). Our observation that AEG-1 increases MDR1 protein level and contributes to doxorubicin resistance has important potential clinical significance. Because AEG-1 confers a broad-spectrum resistance to chemotherapeutics and because AEG-1 is overexpressed in >90% of human HCC samples, inhibition of AEG-1, either by Lentivirus-AEG-1 or by small molecules, might be considered as a component of any combinatorial therapeutic strategy for HCC.

Our findings indicate that AEG-1 increases MDR1 protein at multiple levels. AEG-1 facilitates association of MDR1 mRNA to polysomes, thus increasing translation and inhibits polyubiquitination and subsequent degradation by proteasomes. Modulation of both processes by a single molecule is very intriguing. Indeed, AEG-1 is a distinctive molecule with pleiotropic properties. It resides on the cell surface facilitating adhesion to the endothelium, thus helping to facilitate metastasis (23, 29). It is also located in the nucleus where it functions as a transcriptional activator, increasing NF-kB activity, and inhibits transcriptional repression by PLZF, thereby augmenting c-Myc transcription (27, 30, 31). In addition, AEG-1 is also located in the endoplasmic reticulum as well as in the cytoplasm (32). Our present studies indicate potential functions of the cytoplasmic AEG-1 (i.e., regulation of translation and posttranslational modification of target proteins).

How does AEG-1 affect translation? We document that by activating the PI3K/Akt pathway, AEG-1 increases MDR1 translation by augmenting the association of MDR1 mRNA to polysomes. However, the mechanism by which AEG-1 activates the PI3K/Akt pathway remains poorly defined. Additionally, the mechanism by which the activated PI3K/Akt pathway increases association of MDR1 mRNA to polysomes also needs clarification. We document that AEG-1 causes phosphorylation of translation initiation factor eIF4G, and this phosphorylation was not affected by cotreatment with LY294002. On the other hand, we did not detect phosphorylation of eIF4E or eIF4E-BP by AEG-1. Hypophosphorylation or 4E-BP binds to the 5′ cap binding protein of eIF4E, thereby preventing its interaction with eIF4G and inhibiting translation (33). Phosphorylation of eIF4E by the mTOR pathway, which lies downstream of the PI3K/Akt pathway, releases 4E-BP from eIF4E, resulting in the recruitment of eIF4G to the 5′ cap and thereby allowing translation initiation to proceed (33). Because we did not detect eIF4E phosphorylation by AEG-1, the augmentation of translation by AEG-1 might be independent of the mTOR pathway. Several studies reported alterations in eIF4E-eIF4G association that were independent of changes in eIF4E-BP binding to eIF4E, and a potential mechanism for enhanced eIF4E to eIF4G assembly may involve phosphorylation of eIF4G (34–39). Increased phosphorylation of eIF4G correlates with conditions known to stimulate protein synthesis (38). Similarly, decreased eIF4G phosphorylation in the presence of mTOR activation is observed in skeletal muscle of septic rats, a condition that manifests resistance to stimulation of protein synthesis by insulin (40). Thus, eIF4G phosphorylation might occur by a mTOR-independent pathway. One possible mechanism by which AEG-1 facilitates translation initiation might be direct involvement of AEG-1 in the translational machinery. AEG-1 is a highly basic protein (26) that might function as a scaffold facilitating binding of nucleic acids, including mRNA. Our efforts aimed at defining AEG-1–interacting proteins have identified multiple proteins involved in translation initiation and elongation as well as ribosomal proteins as potential interacting partners of AEG-1.6 Our ongoing studies aim at establishing relevance of these potential interactions and deciphering the role of AEG-1 in regulating translation

6 B.K. Yoo and D. Sarkar, unpublished data.
initiation. The molecular mechanism by which AEG-1 interferes with ubiquitination and proteasomal degradation of MDR1 protein also requires further experimentation. Deciphering these molecular pathways will provide an in-depth understanding of the multitude of functions of AEG-1 and how it contributes to normal and abnormal cellular physiology.

In summary, the present study reveals novel aspects of AEG-1 function, identifies a novel regulator of MDR1, and ushers in new ways of potentially overcoming chemoresistance in HCC and other cancers. Because AEG-1 is being identified as a key player and prognostic indicator in diverse histologically distinct cancers (21), efforts are justified in developing strategies to effectively inhibit AEG-1 as a potentially important cancer therapeutic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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