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Alpha fetoprotein mediates HBx induced carcinogenesis in the hepatocyte cytoplasm

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Although tumor-associated fetal protein AFP has demonstrated utility as a clinical tumor marker, the significance of intracellular AFP is still unclear. The aim of this study was to explore the role of cytoplasmic AFP during HBx induced carcinogenesis, which had not previously been recognized; 614 HCC patients were analyzed for correlation of HBV infection with AFP level, and much higher AFP levels were found in HBsAg positive patients. Tumor tissue specimens from 20 HCC patients were used for analysis of AFP and GADD45α. Analysis of HCC specimens showed that upregulation of cytoplasmic AFP is associated with down-regulation of *GADD45α* in neoplastic tissue. Transfected HBx promotes transcription of *AFP* by acting on the elements in the *AFP* gene regulatory region. HBx itself did not directly impact transcription of *GADD45α*. However, the obstruction of RAR signaling by HBx induced elevation of AFP, which led to down-regulation of *GADD45α*. Cytoplasmic AFP was able to interact with RAR, disrupting its entrance into the nucleus and binding to the elements in the regulatory region of the *GADD45α* gene. Knockdown of AFP in siRNA-transfected AFP positive cell lines was synchronously associated with an incremental increase of RAR binding to DNA, as well as upregulation of *GADD45α* and it was contrary in *AFP* gene-transfected AFP negative cell lines. These results indicate cytoplasmic AFP is not only a histochemical tumor biomarker for human hepatoma but is also an intracellular signal molecule and potential participant in HBx induced hepatocarcinogenesis.

Key words: alpha fetoprotein, HBV, carcinogenesis, growth arrest and DNA damage 45α, all *trans* retinoic acid, nuclear receptor

Abbreviations: AFP: alpha fetoprotein; ATRA: all trans retinoic acid; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; RAR: retinoic acid receptor

Additional Supporting Information may be found in the online version of this article.

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Over the last decade, a multitude of studies have established circulating alpha fetoprotein (AFP) as a growth regulator during ontogenic growth and tumor progression.[1–6] While this is a still developing field replete with unanswered questions, compelling progress which has been made in determining the biological role of cytoplasmic AFP as a signal molecule has attracted considerable interest, as aberrantly elevated AFP disturbs the normal signaling network and results in hepatocyte proliferation. As shown in previous studies, regulation of cytoplasmic AFP in development and growth of malignant hepatic tumor cells is achieved through binding to signal molecules involved in growth or apoptosis. These findings have shown cytoplasmic AFP may interfere with the proliferative and apoptotic signaling of the caspase-3 cascade and PI3K signaling, and this leads to aberrant growth of hepatocellular carcinoma (HCC) cells.[7,8]

Findings in this study revealed a hitherto undiscovered role for cytoplasmic AFP in carcinogenesis and drug resistance. The regulatory attributes and clinical significance of AFP have been recently summarized and discussed.[9] However, to date findings have been insufficient for complete clarification of the intrinsic mechanism underlying the impact of cytoplasmic AFP, and in particular have been insufficient to clarify its clinical relationship to virus induced carcinogenesis.

HCC is the fifth most frequent neoplasm worldwide, and accounts for 5.6% of all human cancers.[10–12] Approximately

What's new?

Although tumor-associated fetal protein AFP has demonstrated utility as a clinical tumor marker, the significance of intracellular AFP remains unclear. This study set to explore the role of cytoplasmic AFP during hepatitis B virus (HBV)-induced carcinogenesis. The results show that HBx promotes transcription of AFP by acting on two elements in the regulatory region of the *AFP* gene. As a consequence, transcription of the tumor suppressor gene *GADD45α* is suppressed, through the disruption of RAR signaling. Cytoplasmic AFP is thus not only a histochemical biomarker for human hepatoma but also an intracellular signal molecule and potential participant in HBx-induced hepatocarcinogenesis.

75–80% of primary liver cancers are ascribed to persistent viral infections with hepatitis B virus (HBV).[13] Clinical research has shown the potential relation of AFP level to progression of HCC and its role in evaluation of treatment response in HCC patients.[14–16] HCC patients with higher AFP levels in fact show a higher mortality rate, which appears to be attributable to the growth promoting properties of AFP.[3] Evidence that AFP acts as a signal regulator involved in carcinogenesis has now become convincing, yet how AFP induces or initiates carcinogenesis under HBV infection is unclear. Shedding light on these questions may help advance understanding of AFP as a participating factor in the occurrence of HCC.

Given that AFP may interact with RAR and interfere with RAR signaling, AFP might therefore interfer with function of genes regulated by ATRA/RAR.[17] Included among the apoptosis/growth related genes regulated by ATRA/RAR which stimulated our interest is Growth arrest and DNA damage 45α (*GADD45α*), which is upregulated up to 9.5-fold after ATRA administration.[18] Moreover, as tumor suppressor gene *GADD45α* has a potential RAR recognition site in the upstream region, and was therefore chosen as a target gene for further evaluation of the effect of AFP on the RAR signal pathway.

GADD45α proteins have been implicated in cancer development and progression, and these proteins can give rise to cell cycle arrest, DNA repair, cell survival, senescence and apoptosis.[19–22] In view of these diverse functions, it is anticipated GADD45α influences the initiation and progression of malignancy and tumor response to therapy. Sequence analysis of the proximal upstream region of the *GADD45α* gene has shown that a canonical RAR recognition site is present, raising the possibility that this gene is regulated by RAR signaling.

In view of these findings, it is of considerable interest to determine whether AFP is involved in ATRA induced transcription of the *GADD45α* gene through its binding to RAR, which results in reduction of RAR nuclear translocation and binding to DNA. This also raises a question as to whether the regulatory effect of AFP in RAR mediated expression of the *GADD45α* gene is of clinical significance, as the expression of the *GADD45α* like *AFP* gene is altered in HBV induced HCC.[19] Therefore it is of particular importance to determine whether cytoplasmic AFP obstructs ATRA-induced apoptosis specifically by interfering with expression of the *GADD45α* gene under HBV infection, and as a part of its overall effect brings about uncontrolled proliferation and drug resistance. This study was therefore undertaken to explore the involvement of cytoplasmic AFP in RAR mediated transcription of *GADD45α*, as well as the relation of HBV to cytoplasmic AFP in the RAR signal network, and to further provide experimental support for clarification of the mechanism of hepatocarcinogenesis.

Material and Methods

Specimens

Tumor tissue specimens obtained from 20 patients undergoing partial hepatectomy for HCC at Henan Cancer Hospital were divided into high (>1000 ng AFP/mL, 10 cases) and low (<20 ng AFP/mL, 10 cases) AFP groups based on AFP serum concentration of. Tissue specimens from cancerous livers were collected together with adjacent noncancerous liver tissues in each case and stored in liquid nitrogen pending experimental use. All selected patients were biopsied to confirm the diagnosis of HCC by histopathologic evaluation. This study protocol was approved by the Ethical Committee of Henan Cancer Hospital. Informed consent was obtained from all patients before surgery.

Correlation of HBV infection with AFP level

A total of 614 patients diagnosed with HCC who had serum collected at Henan Cancer Hospital from 2008–2013 were analyzed for correlation of HBV infection with AFP level.

Immunohistochemistry

Immunohistochemical staining was carried out following a standard protocol. Antibody against AFP or GADD45α and biotin-free HRP-labeled secondary antibody were purchased from Santa Cruze and Zhongshan Golden Bridge, Beijing, China, respectively.

Cell lines

HepG2 and PLC cells (AFP positive cells), the HLE hepatoma cell line (AFP negative cells) and L02 cells (normal human liver cell line, which produces no detectable AFP) were purchased from ShangHai MeiXuan biological technology Co., LTD. HeLa and 293T cells were used for evaluation of the impact of HBV/HBx transfection on transcription of

GADD45α. All cells were maintained in DMEM medium supplemented with 10% FCS.

Plasmid construction and transfection

A *GADD45α* fragment was amplified with PCR and inserted into pcDNA3.1(+) with HindIII/Xba I to create pcDNA3.1-GADD45α (Supporting Information Table S1). pZac2.1-HBV, which can transcribe and assemble HBV in host cells, was a gift from the Department of Microbiology, Peking University Health Science Center. pcDNA3.1-HBx was constructed by inserting an HBx fragment amplified from pZac2.1-HBV with *Hind*III/*Xba* I . Subsequently, serial 5′ deleted AFP-Luc fusion constructs (pLuc 1–4) were created using a deletional and PCR strategy (Supporting Information Table S1 and Fig. S1). *Kpn* I /*Mlu* I digested fragments (1871, 1004, 448 and 215 bp) were cloned into the pGL3-basic vector containing a firefly luciferase reporter gene, resulting in pLuc-1–4. Creation of serial *GADD45α*-Luc fusion constructs was carried out in the same manner as for *AFP*-constructs using *Kpn* I /*Mlu* I digestion and PCR strategy as described above (Supporting Information Table S1). Fragments (1626, 979, 646, 452 and 258 bp) were cloned into pGL3-basic vector resulting in pLuc-1–5. All constructs were verified by DNA sequencing. pcDNA3.1-*afp* was constructed for expression of *AFP*, as previously described.[7] Quantitative real-time reverse transcription PCR (qRT-PCR) and Western blotting were used to examine the expression of *AFP* and its impact on expression of the *GADD45α* gene in HLE or L02 cells. Laser Confocal Microscopy as described below was used to evaluate the transfection of pcDNA3.1-*afp*.

All the plasmids used in these transfection experiments were prepared with the Large-scale Purification Kit (Beijing ComWin Biotech, China) following the manufacturer's recommended protocol. For the luciferase reporter gene assay, transfection efficiency was monitored by cotransfection of the pRL-SV 40 promoter driven Renilla luciferase (Promega). Cells were transfected by Mega Tran 1.0 (Origene) following the Application Guide of the product. The corrected pLuc promoter driven luciferase activity was expressed as the ratio of pLuc promoter driven luciferase activity to renilla luciferase activity. The promoter-less luciferase reporter vector (pLuc-basic) served as a negative control.

qRT-PCR

qRT-PCR with primers listed in Supporting Information Table S1 was performed in a standard manner to evaluate the production of GADD45α, Bcl-2 and AFP mRNA as previously described (Supporting Information Table S1).[23] Relative content of GADD45α, Bcl-2 and AFP mRNA are presented as fold-change of samples as compared with the control.

Western blotting

The expression of *AFP, RAR* and *GADD45α* in tumor specimens and cell lines were examined by Western blotting by using anti-AFP, RAR and GADD45α antibodies purchased from Santa Cruz Biotechnology.[24] Tumor tissues were ground in powder in a mortar filled with liquid nitrogen, which were used for Western blotting as well as CoIP and ChIP assays (see experiments below). Antibodies against β-actin and Bcl-2 were purchased from EarthOx, and Bioworld, respectively. Appropriate secondary antibodies conjugated to horseradish peroxidase (Chongshan Biol Tech, Beijing) were incubated with the PVDF membrane. Immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer's instructions (Millipore) and exposed with ChemiDoc^{TM} XRS^{+} Imager.

Localization of RAR and AFP

Laser confocal microscopy was performed to evaluate intracellular localization of RAR and AFP in cell lines. Rabbit anti-RAR antibody and mouse anti-human AFP antibody and secondary goat anti-mouse or anti-rabbit IgG antibodies conjugated with rhodamine (TRITC) or fluorescence isothiocyanate were purchased from Santa Cruz Biotech and Zhongshan Boil Tech, Beijing, respectively. ATRA (80 μM) was added to observe the translocation of RAR into nuclei. Treated cells were viewed and captured with a Laser Confocal Microscope (Leica TCS-NT SP2, Germany).

Coimmunoprecipitation (CoIP)

The interaction of AFP and RAR in HCC specimens and cell lines was evaluated by CoIP assay with antibodies purchased from Santa Cruz Biotechnology as previously described.[25]

GST-pull down assay

This assay was used to further verify the interaction of AFP and RAR. A GST-RAR clone was purchased from Beijng FunGenome. GST pull down assay with the TNT T7 Quick Coupled Transcription/Translation System kit (Promega) followed by Western blotting were carried out to evaluate the interaction of the translated product of the TNT system and the GST-RAR fusion proteins.

RNA interference assay

We designed and synthesized three pairs of oligoduplexes targeting human AFP mRNA. Of these three anti-AFP-specific siRNA-expressing vectors, the anti-AFP siRNA construct (AFP–siRNA923) directed at the 923–944 region of the *AFP* gene was most effective, and its specificity was also confirmed by its knockdown effect on the expression of different proteins including RAR, Set7/9, CCR5, p53 and STAT3 (Supporting Information Figs. S2 and S3). The effects of AFP knockdown on AFP and GADD45α mRNA/protein and the binding capacity of RAR to DNA in HepG2 and PLC cells were monitored by qRT-PCR, ChIP-qPCR (see primers in Supporting Information Table S1), Western blotting and Laser Confocal Microscopy.

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR (ChIP-qPCR) assay

The ChIP-qPCR assay was performed in the standard manner to determine the influence of AFP on the capacity of RAR binding to DNA.[25] Briefly, HepG2, PLC, HLE and L02 cells were transfected with AFP-siRNA923 or pcDNA3.1-*afp*. Cells were fixed with formaldehyde and resuspended in cell lysis buffer after washing. The chromatin was sonicated (Ultrasonic Processor from Xinzhi Tech Ins, China) on ice. Sheared chromatin from cells was mixed with 50% protein A, which was blocked with salmon sperm DNA. (Sigma Chemical). The supernatant was collected and polyclonal antibody against RAR was added (Santa Cruz Biotechnology). The sample was then mixed again with 50% protein A and salmon sperm DNA. Pellets were consecutively washed. Antibody-protein-DNA complexes were eluted from protein A. DNA fragments were purified and subjected to qPCR analysis. Primers used for ChIP-qPCR are listed in Supporting Information Table S1. The ChIP assay with homogenized tumor specimens was carried out in a manner similar to that used for cultured cells (see primers in Supporting Information Table S1).

Flow cytometric analysis for apoptosis

Flow cytometry assay was performed in a standard manner to determine the effect of ATRA and GADD45α on apoptosis.[7] Briefly, after transfection of four cell lines for 36 hr with pcDNA3.1-GADD45α, apoptosis induced by GADD45α transfection was analyzed with flow cytometry. Cells were collected and resuspended in 70% ethanol after washing. Cells were filtered through 300 mesh and propidium iodide (PI) was added (final concentration 50 μg/mL). Relative fluorescent intensities of PI staining were measured using a FACScan-420 flow cytometer (Becton-Dickinson) with excitation at 488 nm and emission at 598 nm. The extent of cellular apoptosis was determined according to DNA analysis. This experiment was repeated at least three times.

Statistical analysis

The results of multiple observations are presented as the mean ± s.d. of at least three separate experiments. Statistical significance was determined using the Student's *t* test (SPSS 17.0 software). Univariate analysis on the correlation of HBV infection and AF*P* values was carried out with the Wilcoxon rank sum test. p values <0.05 were considered statistically significant.

Results

AFP interacts with RAR and down regulates GADD45α in HCC clinical specimens

Immunohistochemistry analysis showed strong specific staining for AFP in cancerous tissues but not in adjacent non-neoplastic tissues in HCC patients with high serum AFP (Fig. 1a). By contrast, tissue sections from the same patient showed lower expression of *GADD45α* in cancerous tissue. As expected, neither cancerous nor adjacent tissues exhibited AFP staining in HCC patients with low serum AFP levels. The results of Western blot and qRT-PCR showed AFP was detectable in tumor tissues with high serum AFP but was undetectable or found only in trace amounts in adjacent non-cancerous liver tissues, and in HCC with lower serum AFP levels, which was consistent with immunohistochemistry results (Figs. 1b and 1c). Of note, the level of GADD45α as well as RAR proteins was apparently decreased in association with up-regulation of Bcl-2 protein in cancerous tissues in high serum AFP patients. At the same time, GADD45α in cancerous tissues in low serum AFP patients was higher than that in high AFP patients. AFP was able to interact with RAR in tumor tissues with high serum AFP, but not in adjacent noncancerous liver tissues or tumor tissues with lower serum AFP as determined by the CoIP assay (Fig. 1d). RAR bound to its elements in the promoter region of the *GADD45*α gene in tissues associated with high or low serum AFP (Fig. 1e). However, binding was apparently weakened in cancer tissues in view of the low nuclear RAR protein as shown with the ChIP assay, demonstrating interaction of elevated AFP with RAR in the cytoplasm leads to reduction of RAR translocation into nuclei.

HBV infection promotes AFP transcription

Six hundred and fourteen HCC patients were retrospectively assessed to evaluate the relationship of HBV infection and serum AFP levels. These data showed HBV(+) patients had an obviously high AFP level as compared with HBV(−) patients (Fig. 2a). Analysis with the Wilcoxon rank sum test demonstrated that the median AF*P* value (297.2) for HBV(+) patients was significantly higher than for HBV(−) patients (83.9; $p = 0.008$). In addition, transfection assays with hepatocyte cell lines with differing expression of AFP showed that both HBV and HBx constructs could elevate the level of AFP in HepG2 and PLC cells as compared with nontransfected cells (Fig. 2b). Although the fold change in *AFP* mRNA was elevated in all four cell lines as shown with qRT-PCR assays, the value of AFP in AFP negative cells was very low or undetectable by Western blot (Figs. 2b and 2c). It was of note that GADD45α was simultaneously reduced in HepG2 and PLC cells under HBx transfection (Figs. 2b and 2c).

Four AFP-Luc fusion constructs (pLuc 1 − 4) with different length fragments (1871, 1004, 448 and 215 bp) were created to analyze the activity of elements in the 5′ regulatory region of the *AFP* gene and the impact of HBx protein. Analysis with luciferase activity assay showed that deletion of 867 bp (Luc-2) from the 5′-terminal of Luc-1 resulted in an apparent decrease in firefly luciferase activity ($^{**}p < 0.01$ as compared with Luc-1), indicating there is a potential enhancer within this region (Fig 3a). Further deletion of 556 bp (Luc-3) from Luc-2 resulted in remarkable elevation of luciferase activity ($^{**}p < 0.01$ as compared with Luc-2), indicating there is a potential silencer within this region, which is consistent

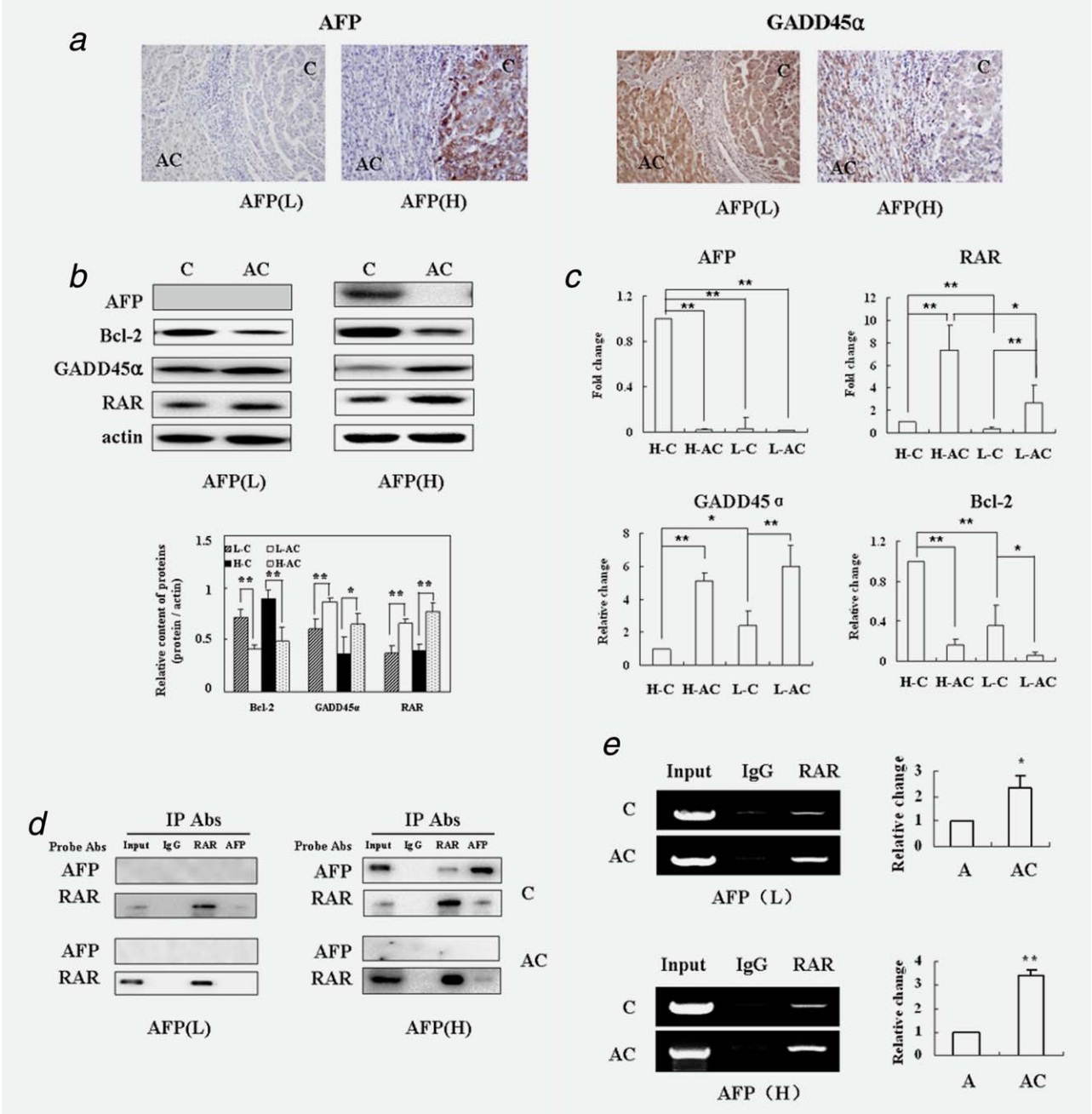

Figure 1. Regulation of *RAR* in *GADD45α* expression in clinical HCC specimens. (*a*) Immunohistochemistry analysis of expression of *AFP* and *GADD45α* in tumor tissues from HCC patients with high (H) and low (L) serum AFP. C: cancerous tissues; AC: adjacent noncancerous tissues. Tissue sections in the high AFP group were from the same patient. Western blotting (*b*) and qRT-PCR analysis (*c*) of expression of *AFP, RAR, Bcl-2* and *GADD45α* in cancerous and adjacent noncancerous tissues. The panel below the image of Western blotting is the densitometric and statistical analysis. (*d*) CoIP analysis of the interaction of AFP and RAR. E. ChIP analysis of RAR binding to its element in the regulatory region of the *GADD45α* gene (left panel). Right panel shows qRT-PCR analysis for the ChIP assay. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with control. Data are representative of an experiment that was repeated at least three times.

with what has previously been reported.[26] Deletion of 233 bp (Luc-4) from Luc-3 led to partial loss of the promoter sequence and consequent reduction of luciferase activity ($^{**}p < 0.01$ as compared with Luc-3; $\#p < 0.05$ or $\#\#p < 0.01$ as compared with Basic). In view of the fact that HBx transfection enhanced the luciferase activity of Luc-1 and Luc-3, it was presumed that HBx protein triggers the transcription of AFP by acting on two HBx sensitive regions including the promoter and enhancer in Luc-1 (Fig. 3b). HBx protein does not directly down-regulate GADD45α expression as shown by transfection assay with non-hepatocytic cells (HeLa and 293T cell lines) (Figs. 3c–3*e*). In view of the fact RAR

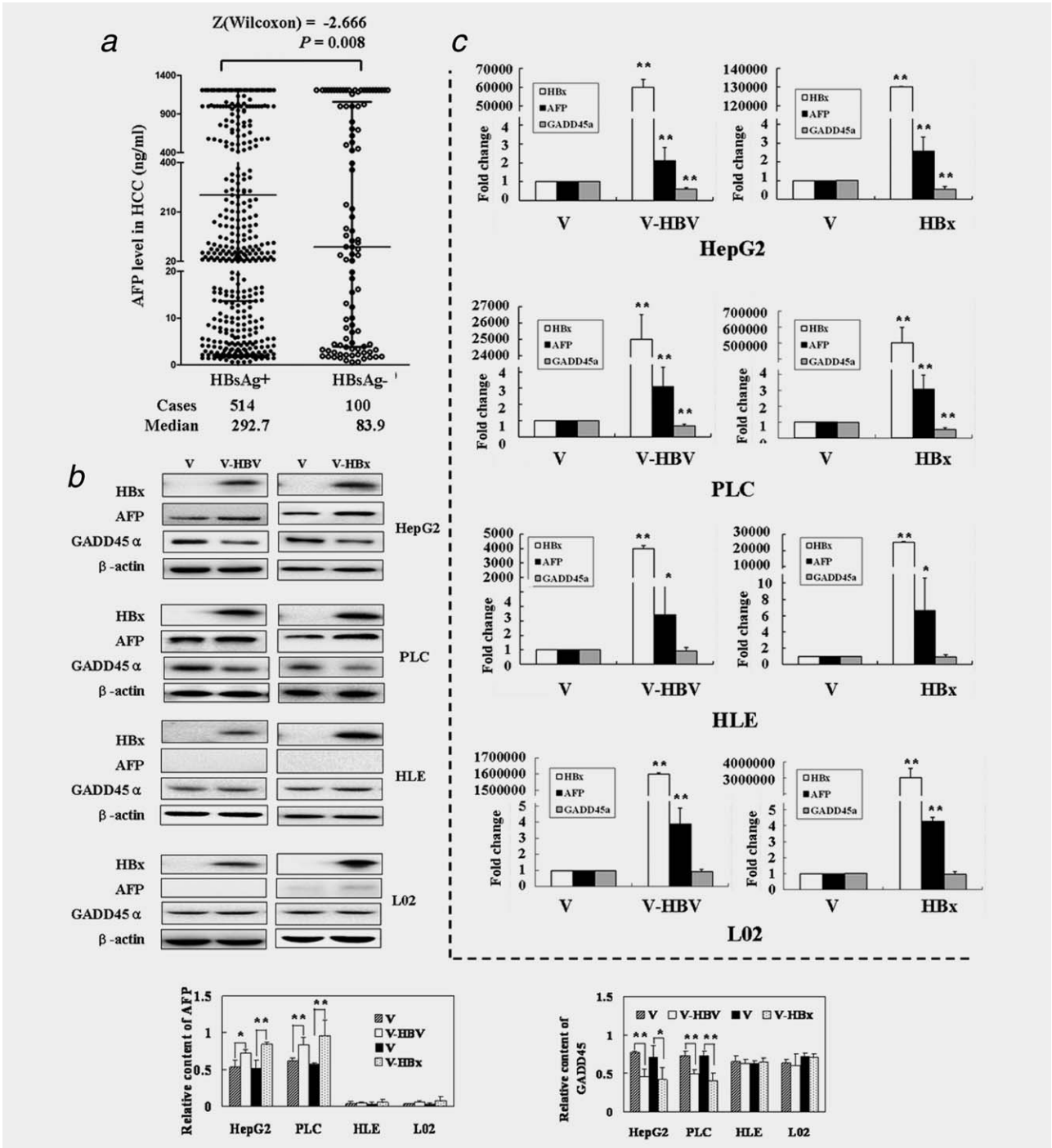

Figure 2. Relationship between HBV infection and AFP level in HCC patients and expression of *AFP* and *GADD45α* in HBV- and HBx-transfected cells. (*a*) Each scatter plot represents an individual HBsAg(+) or HBsAg(−) patient. Solid line among the points represents the median AFP value. The correlation between HBV infection and AFP values was analyzed by the Wilcoxon rank sum test. *p* values <0.05 were considered statistically significant. (*b*) Western blotting analysis of HBx and HBV transfected cell lines. The panel below the image of Western blotting is the densitometric and statistical analysis. (*c*) qRT-PCR analysis for AFP and GADD45α in HBx and HBV transfected cell lines. $*p < 0.05$ and $**p < 0.01$ as compared with empty vector control (V). Data are representative of an experiment that was repeated at least three times.

elements exist in the regulatory region of the *GADD45α* gene, which are potentially regulated by cytoplasmic AFP, there is a possibility elevated AFP is involved in the regulation of *GADD45α* expression.

The RAR signal cascade regulates the transcription of the GADD45α gene

To further analyze the potential impact of AFP on RAR mediated expression of *GADD45α*, cell culture based assays

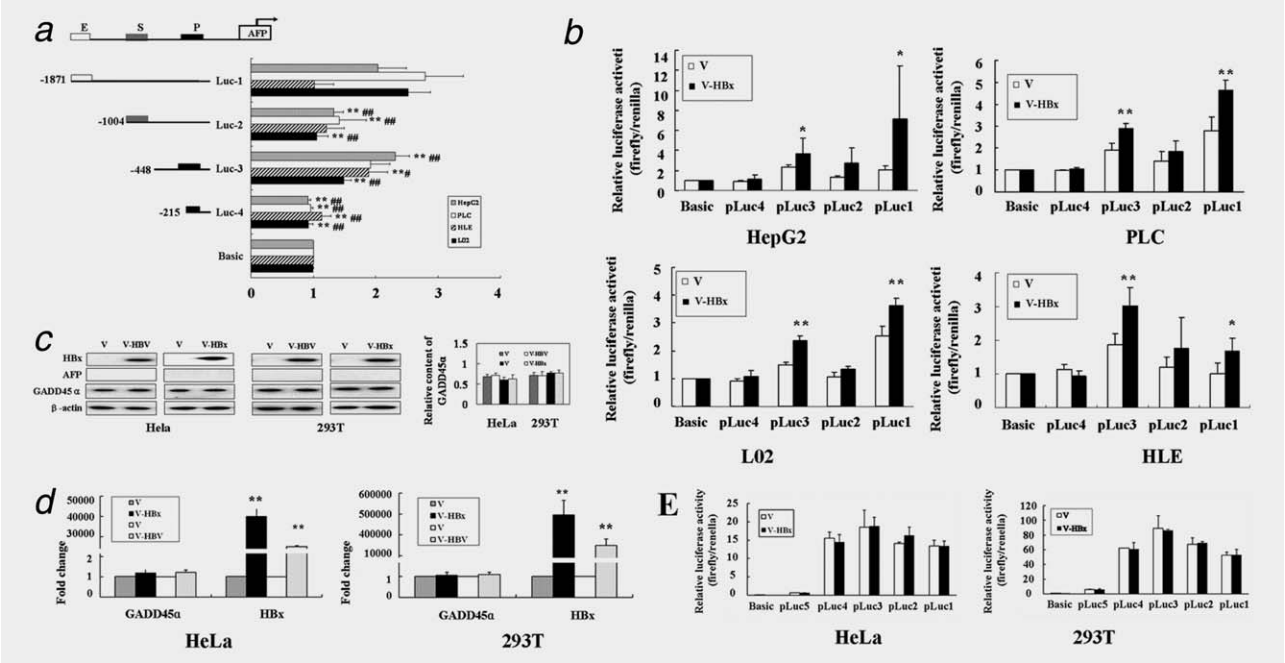

Figure 3. Effects of HBV and HBx transfection on the regulatory region of the AFP gene. A series of *AFP* gene 5′-regulatory region/luciferase constructs were prepared and introduced into four hepatocyte cell lines. (*a*) The activity of luciferase in different constructs. $^{**}p < 0.01$ was considered statistically significant among construct Luc-2 and Luc-1, or Luc-3 and Luc-2, or Luc-4 and Luc-3; $\#p < 0.05$ and $\#\#p < 0.01$ compared with Basic for each cell line; (*b*) Effects of HBx transfection on different constructs. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with empty vector control (pcDNA3.1 or pZac2.1). The bars show the mean ± sd of the results from 4 independent transfection experiments. Western blotting (*c*) and qRT-PCR (*d*) analysis for expression of *AFP* and *GADD45α* in HeLa and 293T cell lines under HBV or HBx transfection. Right panel of the image is the densitometric analysis. (*e*) Luciferase activity analysis for effect of HBx transfection on the regulatory region of the *GADD45α* gene in HeLa and 293T cell lines. Data are representative of an experiment that was repeated at least three times.

were carried out. As expected, *AFP* was expressed in HepG2 and PLC cells but not in L02 and HLE cells (Supporting Information Fig. S4). All four cell lines exhibited expression of GADD45α protein. Regulatory expression of *GADD45α* by ATRA/RAR was identified by assay with qRT-PCR and Western blotting. ATRA treated cells displayed an apparent increase in the expression of GADD45α mRNA and protein (Figs. 4a and 4b). As shown in Figures 4a and 4b, ATRA induced increase of GADD45α in HepG2 and PLC cells became evident in a dose-dependent manner, thus demonstrating sensitivity to treatment with ATRA. Although GADD45α is prone to incremental increase in HLE cells, sensitivity to ATRA was less than that in HepG2 and PLC cells. ATRA induced enhancement of GADD45α occurred at treatment time 24–36 hr (Figs. 4c and 4d). Ethanol, which served as the solvent for ATRA was used as a control at a corresponding concentration and did not itself influence the expression of the *GADD45α* gene (Fig. 4b).

Treatment with ATRA promotes nuclear influx of RAR as observed with confocal microscopy (Supporting Information Fig. S5). RAR-mediated regulation of GADD45α expression was further confirmed with the ChIP assay, which showed ATRA administration is able to promote greater binding of RAR to its element in the 5′-flanking region of the *GADD45α* gene in HepG2, PLC, HLE and L02 cells (Fig. 4e). Results of flow cytometric analysis (Supporting Information Fig. S6) and confocal microscopy (Supporting Information Fig. S7) showed that ATRA administration (80 μM, 36 hr) results in an increase of apoptotic cells. This was much more apparent in AFP negative cells owing to the absence of cytoplasmic AFP and associated morphologic changes including nuclear condensation and pyknosis. The impact of binding of AFP to RAR (see below) suggests strongly that ATRA induced RAR release from AFP-RAR complexes is a primary cause of increased nuclear influx of RAR into AFP-producing cells and subsequent apoptosis.

Cytoplasmic AFP interacts with RAR and interrupts its nuclear translocation

As shown by confocal laser microscopy, AFP co-localized with RAR in the cytoplasm of HepG2 or PLC cells (AFP positive) but not in HLE or L02 cells (AFP negative; Fig. 5a). However, RAR translocates into nuclei from the cytoplasm with the addition of ATRA (80 μM) (Supporting Information Fig. S5). This nuclear RAR translocation begins after 1 hr of incubation and is maintained for 2–4 hr (Supporting Information Fig. S8). AFP interacted with RAR in the cytoplasm of HepG2 and PLC cells, as demonstrated by CoIP analysis and GST pull down assay (Figs. 5b and 5c). This interaction was undetectable in HLE and L02 cells, which lack AFP. The interruption of RAR nuclear influx by AFP was confirmed through transfection of the *AFP* gene into non-AFP

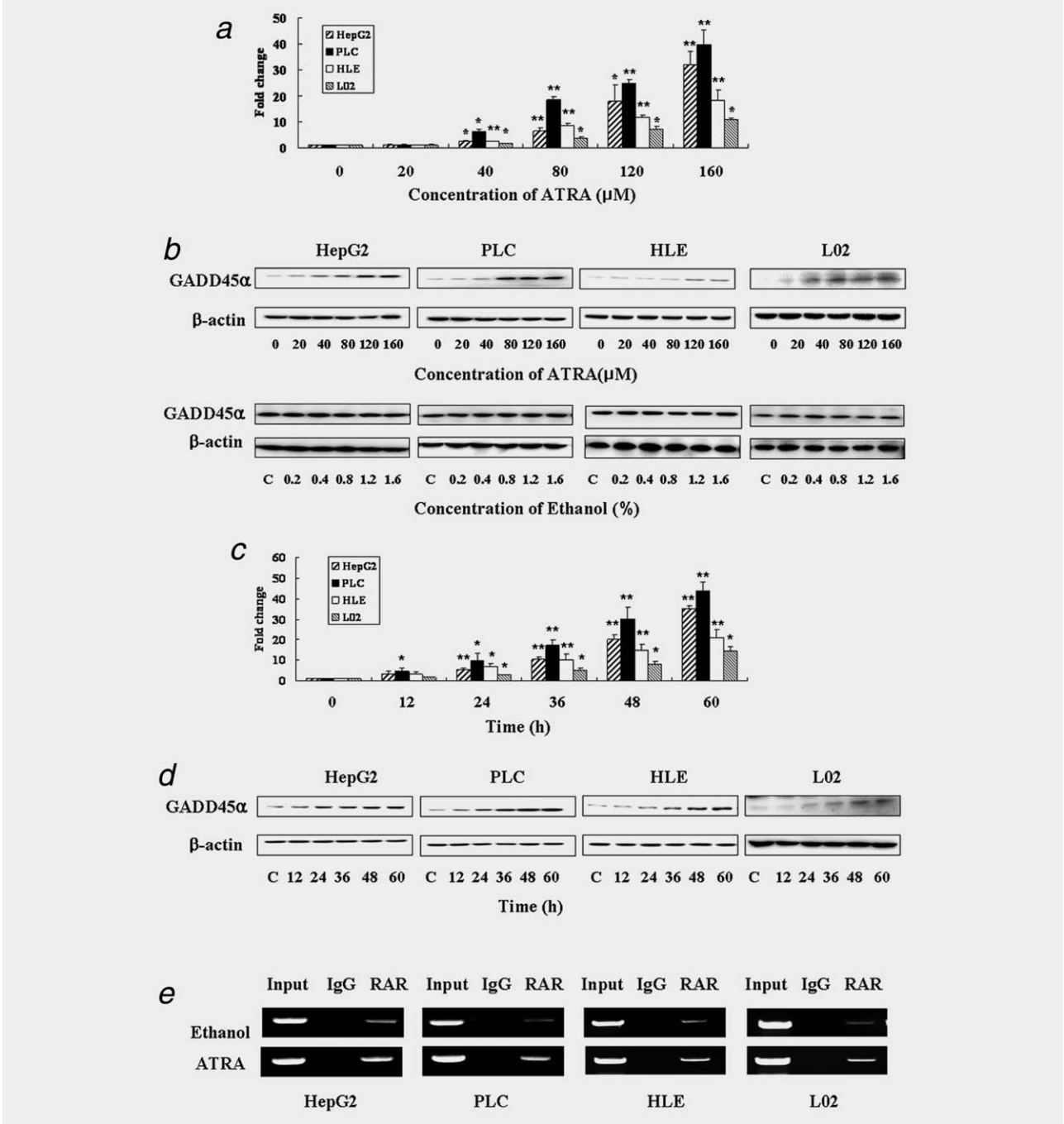

Figure 4. Effect of ATRA on expression of *GADD45* in HepG2, PLC, HLE and L02 cells. Effect of various concentration (0–160 μM) of ATRA and different times (12–60 hr) on expression of the *GADD45α* gene analyzed by qRT-PCR (*a* and *c*) and Western blotting (*b* and *d*). Ethanol was used as a solvent and as a control. (*e*) Effects of ATRA administration on RAR nuclear entrance analyzed by ChIP assay in HepG2, PLC, HLE and L02 cells. $*p < 0.05$ and $**p < 0.01$ as compared with the control. $*p < 0.05$ and $**p < 0.01$ as compared with the control. Data are representative of an experiment that was repeated at least three times.

producing cells. AFP levels were remarkably elevated in the cytoplasm of HLE and L02 cells after transfection of pcDNA3.1-*afp* (Fig. 5d). Upon appearance of AFP in pcDNA3.1-*afp* transfected HLE and L02 cells, interaction of AFP and RAR was detectable (Fig. 5e). These protein interactions argue that these molecules have high functional significance in a complex signaling network.

Expression of the gadd45 gene is inversely correlated with AFP

AFP interacted with RAR and as a consequence blocked ATRA induced nuclear translocation of RAR as described above. The effect of AFP on expression of the *GADD45α* gene was reversed by AFP knockdown. When intracellular AFP was decreased with small interference RNA in HepG2

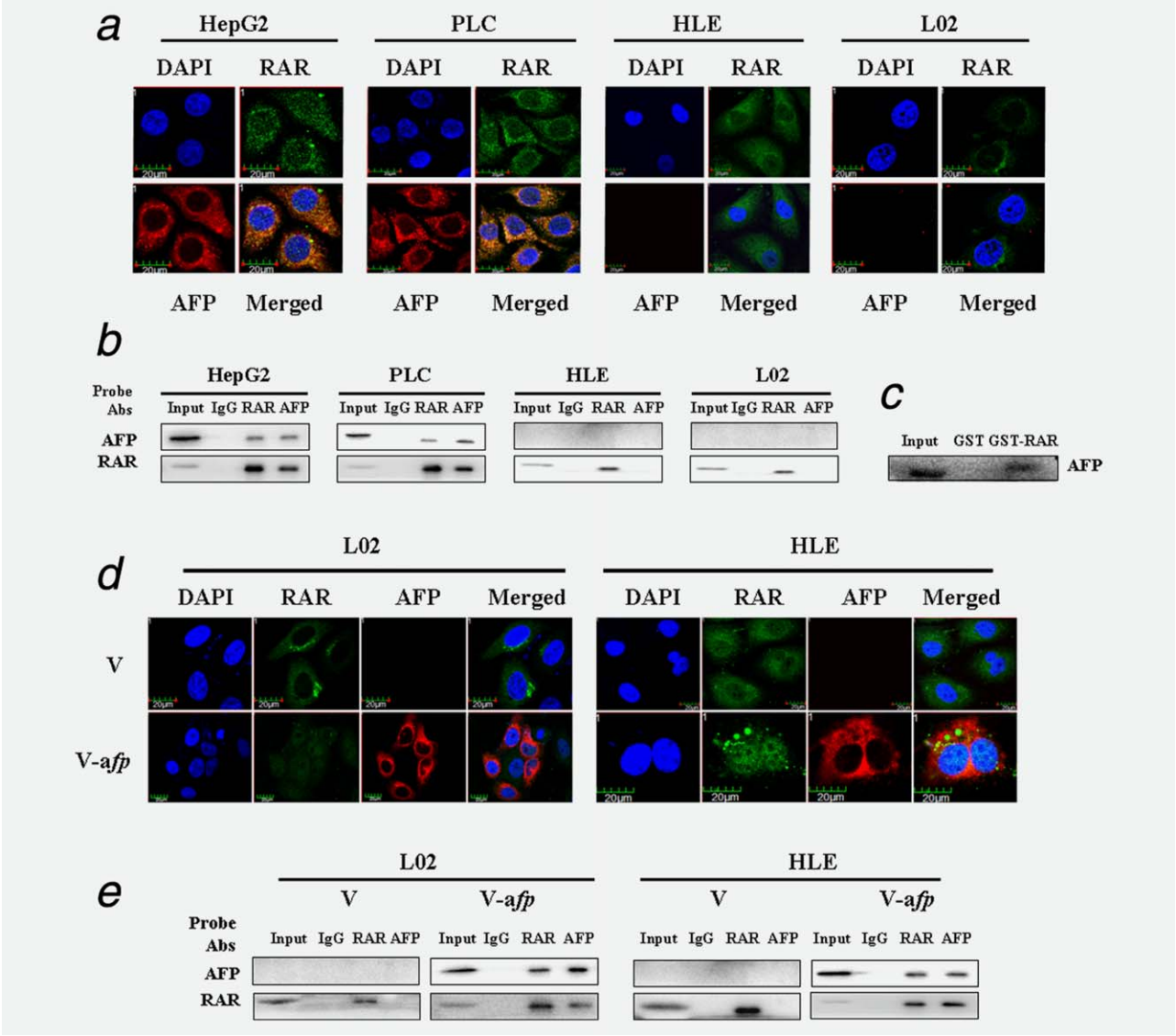

Figure 5. Expression of *AFP* and its interaction with RAR in HepG2, PLC, HLE and L02 cells. (*a*) Colocalization of AFP (red) and RAR (green) in HepG2, PLC, HLE and L02 cells captured under laser confocal microscopy. (*b*) CoIP analysis of the interaction of AFP and RAR in HepG2, PLC, HLE and L02 cells. (*c*) GST pull-down assay. (*d*) Effect of pcDNA3.1-*afp* transfection in HLE and L02 cells observed with confocal microscopy. Cells expressing AFP were labeled with TRITC (red). (*e*) CoIP analysis of the interaction of AFP and RAR in pcDNA3.1-*afp* transfected HLE and L02 cells. Data are representative of an experiment that was repeated at least three times.

or PLC cells, the change in *GADD45α* expression at the mRNA and protein levels increased by up to 2.72 and 2.01 fold (HepG2 and PLC cells, respectively) and by up to 2.11 and 1.82-fold (HepG2 and PLC cells, respectively) as compared with the control (scrambled RNA groups; $p < 0.01$; Figs. 6a and 6b). It was of interest that the product of the Bcl-2 oncogene was also reduced in the course of AFP knockdown. Reduction of AFP with siRNA resulted in greater binding of RAR to DNA in HepG2 and PLC cells (Figs. 6c and 6d). In addition, the effect of AFP was also confirmed through transfection of the *AFP* gene into non-AFP producing cells. Transfection of pcDNA3.1-*afp* into HLE or L02 cells resulted in apparent reduction of GADD45α mRNA and protein (Figs. 6a and 6b). qRT-PCR and Western blot analysis demonstrated 55% (33.1%) (HLE cells) and 63% (60.5%; L02 cells) decrease for cells transfected with the *AFP* gene as compared with the control (empty vector group; $p < 0.01$). Similarly, incremental increases in AFP were also accompanied by elevation of Bcl-2 protein. Elevated AFP in pcDNA3.1-*afp* transfected HLE or L02 cells led to an apparent reduction of binding of RAR to its elements as compared to input (Figs. 6c and 6d). For further confirmation of the effect of GADD45α on apoptosis, a GADD45α-expressing construct was generated and transfected into four cell lines. Results from flow cytometric analysis showed that over-expression of *GADD45α* may result in an elevation of the percentage of apoptotic cells in four cell lines (Fig. 6e). Compared with group transfected with empty vector, the

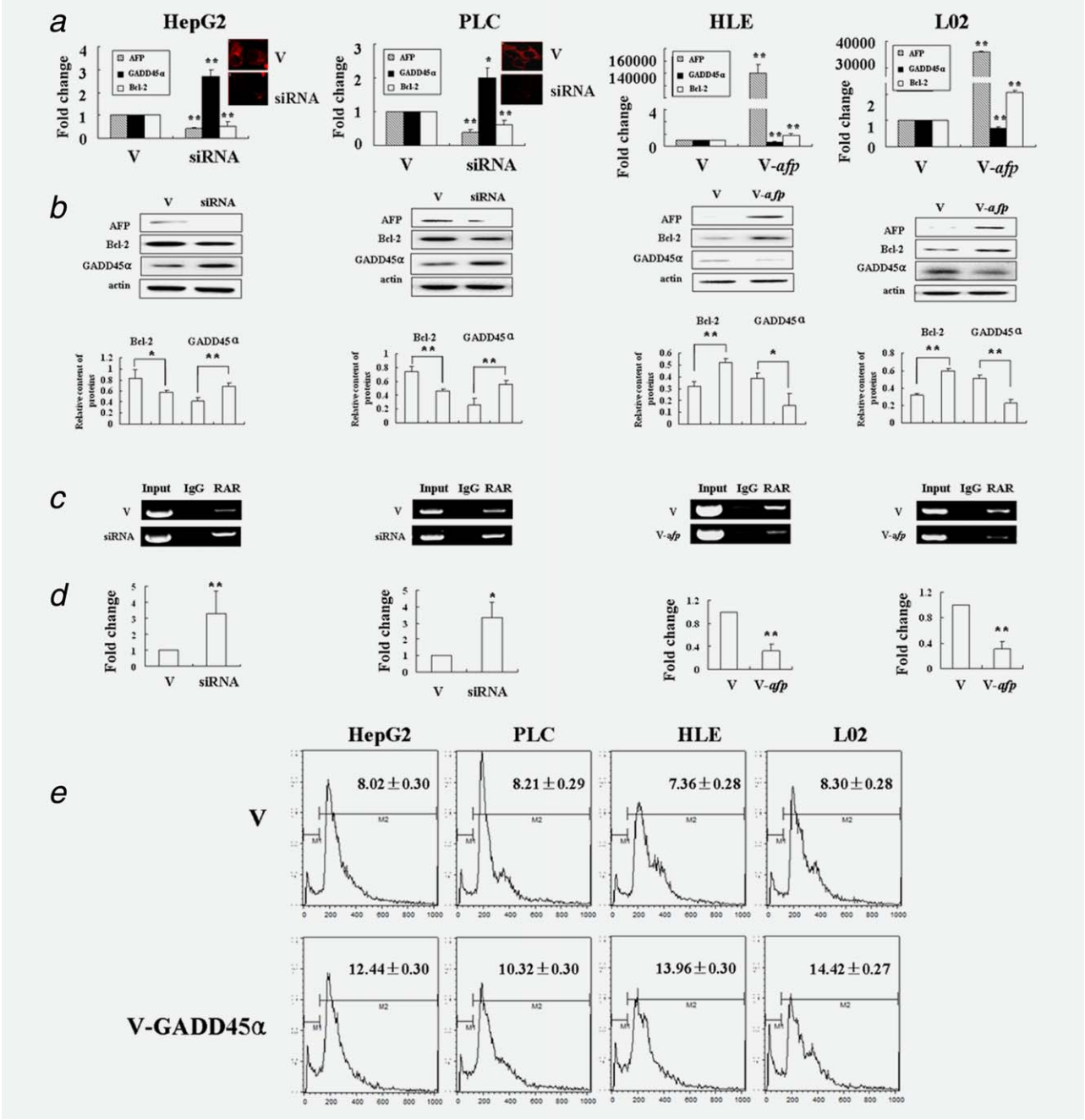

Figure 6. Expression of *GADD45α* after AFP knockdown or *AFP* gene transfection in HepG2, PLC, HLE and L02 cells. Analysis with qRT-PCR (*a*) and Western blotting (*b*) for effects of AFP knockdown in expression of the *GADD45α* gene in AFP–siRNA923-transfected HepG2 and PLC cells and pcDNA3.1-*afp*-transfected HLE or L02 cells. The panel below the image of Western blotting is the densitometric and statistical analysis. Images in panel A show expression of AFP (red). (*c* and *d*) Determination of capacity of RAR for binding to DNA in AFP silenced HepG2 and PCL cells, and *AFP* gene transfected HLE and L02 cells. ChIP assays (*c*) and qPCR for ChIP products (*d*) were performed to determine the capacity of RAR for binding to DNA in HepG2 and PCL cells with siRNA interference or *AFP* gene transfected HLE and L02 cells. 1: Input; lane 2: precipitation with nonspecific IgG; lane 3: precipitation with antibody against RAR. "V": scrambled siRNA (RNA interference assay) or empty vector; "siRNA": transfection with AFP–siRNA923; "V-*afp*": transfection with pcDNA3.1-*afp*. Data represent mean ± s.d. of three samples. $*p < 0.05$ and $**p < 0.01$ as compared with scrambled siRNA or empty vector control (*a*) or DNA complexes precipitated with non-specific IgG (D). E. GADD45α induced apoptosis in HepG2, PLC, L02 and HLE cells analyzed by flow cytometry after transfection with GADD45α. M1 and M2 peaks in each panel represent apoptotic and surviving populations of cells respectively. The number in each panel represents the percentage of apoptotic cells in each treatment group and the statistical analysis of data from flow cytometric assay. Data are representative of an experiment that was repeated at least three times.

percentages of survival cells were reduced to 87.6% (HepG2), 89.7% (PLC), 86.0% (HLE) and 85.6% (L02), respectively, after transfection with GADD45α-expressing construct, in which the percentages of apoptotic cells were obviously increased ($p < 0.01$ for all cell lines).

Discussion

Over the last two decades, there have been a number of studies, which have confirmed circulating AFP displays growth-regulatory properties in variety of cell types, governing processes such as cell differentiation, growth regulation and tumorigenesis, which has led to AFP being classified as a cytokine.[1,9,27,28] Cells that synthesize a growth factor and display its receptor have the potential for autocrine growth. Secreted AFP could act on neighboring cells as well as on the producing ones.[29] The higher local concentration created by secretion of AFP by neoplastic cells could allow its binding to the receptors present on adjoining cells. An AFP autocrine loop could thus contribute to the continued growth and proliferation of neoplastic cells by stimulating them.

Besides its growth regulator-like role in circulation, AFP may be recycled from blood into cells by receptor-mediated endocytosis. Previous studies showed AFP receptor was present in cell surface as an integral cell membrane protein. AFP has been detected in many fetal organs mostly as the result of its uptake from the blood.[9,30,31] Early uptake studies on the receptor-mediated endocytosis of AFP have shown the capacity for internalization of AFP by its receptor which is scarcely observed in differentiated cells and may reappear in neoplastic cells.[31–33] After uptake by its receptor, AFP then move to a tubular vesicular network situate close to the Golgi-centrosphere region.[34] Once released from the late endosomal vesicle, the receptor is separated and recycled back to the cell membrane or degraded. AFP can either be stored in the perinuclear spaces or be engaged in activating or inhibiting signal transduction pathways in the cytoplasm.[7,17,18,35,36]

To date, the majority of studies concerning the biological significance of AFP have focused mainly on the impact of circulating AFP in tumor development. Clinical observation has shown that HCC patients with high serum AFP levels consistently show high mortality rates and poor clinical outcomes.[3] However, studies now in progress are beginning to shed light on the role of cytoplasmic AFP, and there is accumulating evidence correlating the role of intracellular AFP with tumor growth.[7,17,18,37] The current research was conducted based on the prediction that cytoplasmic AFP has the capability for binding various ligands including retinoic acids, and it has confirmed that AFP is one of main binding partners of RAR.[38]

GADD45α participates in regulation of diverse cell functions under both physiologic and pathologic conditions, and it is a critical factor in the initiation and progression of malignancy.[21,22,39] Decrease or absence of *GADD45* expression may be a critical event allowing tumor cells or premalignant liver cells to bypass cellular senescence.[40] GADD45 has therefore been defined as an important molecule in tumorigenesis.[22] Nevertheless, the functional relation of intracellular AFP with altered *GADD45α* expression in HCC had been neglected, although serum AFP levels were occasionally cited in assessment of GADD45 in HCC patients.[41]

Although our previous study demonstrated that AFP colocalizes and interacts with RAR in the cytoplasm, the clinical significance of this finding, particularly under virus loading, was heretofore unknown.[17] This study showed that transfected HBx promotes the transcription of *AFP* and leads to elevation of AFP. The obstruction of RAR signaling by HBx induced elevation of AFP leads to down-regulation of the tumor suppressor gene *GADD45α*. This is consistent with our analysis of clinical data and tumor tissue specimens from HCC patients, in which upregulation of cytoplasmic AFP is associated with downregulation of *GADD45α* in neoplastic tissue.

Although previous work has shown that HBV positive HCC patients usually exhibit higher serum AFP than those who are HBV negative, few studies have sought to identify the connections among HBV, AFP and GADD45α in HCC. Recent work has shown that HBx protein is responsible for over-expression of AFP.[42] Current data also shows that HBx promotes transcription of AFP through a mechanism most likely involving promotion of the activity of the enhancer and promoter in the regulatory region of the AFP gene. Elevated AFP binds competitively to RAR as a partner and inhibits expression of *GADD45α*, and as a consequence brings about tumorgenesis. At the same time, decreased nuclear influx of RAR in the context of AFP over-expression further leads to elevation of Bcl-2, the antiapoptotic protein. The relationship of RAR and Bcl-2 level has been documented.[43] Early work revealed RAR binding sites located in negative regulatory elements ranging from −279 to −85 bp of the 5′-untranslated region of the *Bcl-2* gene, which inhibited the activity of the promoter.[44] The *Bcl-2* promoter could therefore be activated by retinoid acid antagonist.[45] These results together with the data presented in this study strongly argue that AFP mediated downregulation of *GADD45α*, as well as elevation of Bcl-2 act cooperatively to accelerate aberrant growth of HBV infected cells. Although evaluation of the regulatory effects of AFP on HBV induced HCC remains to be carried out, current data indicate the clinical significance of AFP is based on the HBV triggered AFP-RAR-GADD45 signal network.

Accordingly, we propose the concept that cytoplasmic AFP serves as a novel signal molecule and is a mediator in the RAR signal network, and is a factor, which leads to hepatocarcinogenesis and drug resistance. Clinical findings support the concept that HCC patients with higher serum AFP levels have higher resistance to ATRA therapy and higher mortality.[46,47] The development of a therapeutic strategy wherein the combination of *AFP* gene silencing or depletion of circulating AFP, together with chemotherapeutic treatment thus holds promise for effective control of tumor growth. As AFP is a competitor in the RAR pathway, it is likely that elimination of AFP will be beneficial for therapy of HCC patients. A more specifically detailed description of the involvement of AFP in HBV

induced hepatocarcinogenesis will contribute to a more elaborate and complex understanding of the intrinsic mechanism of tumor growth. Nonetheless, further in-depth study is needed to firmly establish this concept.

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