Liver Cancer: EphrinA2 Promotes Tumorigenicity Through Rac1/Akt/NF-κB Signaling Pathway

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Eph/Ephrin family, one of the largest receptor tyrosine kinase families, has been extensively studied in morphogenesis and neural development. Recently, growing attention has been paid to its role in the initiation and progression of various cancers. However, the role of Eph/Ephrins in hepatocellular carcinoma (HCC) has been rarely investigated. In this study, we found that the expression of EphrinA2 was significantly up-regulated in both established cell lines and clinical tissue samples of HCC, and the most significant increase was observed in the tumors invading the portal veins. Forced expression of EphrinA2 in HCC cells significantly promoted in vivo tumorigenicity, whereas knockdown of this gene inhibited this oncogenic effect. We further found that suppression of apoptosis, rather than accelerating proliferation, was responsible for EphrinA2-enhanced tumorigenicity. In addition, EphrinA2 endowed cancer cells with resistance to tumor necrosis factor alpha (TNF-α)–induced apoptosis, thus facilitating their survival. Furthermore, we disclosed a novel EphrinA2/ras-related c3 botulinum toxin substrate 1 (Rac1)/V-akt murine thymoma viral oncogene homolog (Akt)/nuclear factor-kappa B (NF-κB) pathway contributing to the inhibitory effect on apoptosis in HCC cells. Conclusion: This study revealed that EphrinA2 played an important role in the development and progression of HCC by promoting the survival of cancer cells, indicating its role as a potential therapeutic target in HCC. (HEPATOLOGY 2010;51:535-544.)

Hepatocellular carcinoma (HCC) is a major health problem worldwide, ranking as the fifth most common cancer in the world and the third most common cause of cancer-related death.1 HCC shows great geographical variation, with a very high incidence in Asia and sub-Saharan Africa, but it is now becoming more common in the West. During the past 20 years in the United States, HCC has risen by 114%.2 This paralleled an increase in the incidence of chronic hepatitis, which serves as a main risk factor for HCC.3 At the initiation of hepatic oncogenesis, transformed hepatocytes must elude various cellular defense activities and acquire abnormal capabilities to survive and proliferate.4 Aberrant signaling through receptor tyrosine kinases plays a pivotal role in the development and progression of HCC.5 Eph receptors constitute one of the largest receptor tyrosine kinase families and have been reported to be involved in a variety of cancers. For example, up-regulation of EphA2 has been observed in many malignant tumors6-8 and is associated with accelerated cell prolifera-

Abbreviations: Akt, V-akt murine thymoma viral oncogene homolog; HCC, hepatocellular carcinoma; NF-κB, nuclear factor-kappa B; PARP, poly(adenosine diphosphate-ribose) polymerase; PCR, polymerase chain reaction; Rac1, ras-related c3 botulinum toxin substrate 1; siRNA, small interfering RNA; TNF, tumor necrosis factor.

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tion, enhanced neovascularization, and altered hormone
dependence. EphB4 is abnormally expressed in melano-
ma, bladder, colorectal, and breast cancers, although
the mechanism underlying the oncogenic effect remains
unclear. However, research of Eph/Ephrin mem-
bers in HCC is still rare.

EphrinA2, a cognate ligand to several Eph receptors,
including EphA3, EphA4, EphA5, and EphA7, is related
to neural development. In addition, EphrinA2 regulates
RhoA-dependent F-actin turnover, as well as the endo-
cytosis of growth cone through modulating Ras-related
C3 botulinum toxin substrate 1 (Rac1) activity. Although EphrinA2 has been found to be up-regulated in some breast cancer cell lines, its function in cancer re-
mains poorly investigated. In this study, we report that
the expression of EphrinA2 is dramatically increased in
HCC, especially in the tumors invading into the portal
veins. Furthermore, we demonstrate that EphrinA2 acti-
vates the Ras/C3 botulinum toxin substrate 1 (Rac1)/
Akt/murine thymoma viral oncogene homolog (Akt)/nu-
clear factor-kappa B (NF-kB) pathway in HCC cells, which suppresses apoptosis and thus facil-
itates cancer cell survival. Our study strongly highlights
the significance of EphrinA2 in the tumorigenesis of
HCC and therefore provides a potential drug target in
liver cancer therapy.

Patients and Methods

This part is included in the Supporting materials.

Results

EphrinA2 Is Up-Regulated in Established Cell
Lines and Clinical Tissue Samples of Hepatocellular
Carcinoma. To explore the role of Eph/Ephrin members
in HCC, we first examined their expression levels in both
normal and cancerous hepatic cell lines by real-time poly-
merase chain reaction (PCR). Among all the tested mem-
bers, the expression level of EphrinA2 was significantly
up-regulated in the cancerous cell lines compared with the
normal ones (Fig. 1A). Then we checked its expression in
52 pairs of matched liver tissue samples and found that
the expression level of EphrinA2 was significantly higher
in HCC tissues compared with their normal counterparts
in most cases (Fig. 1B). The expression pattern of Eph-
rinA2 in both cell lines and clinical samples suggested its
involvement in the pathogenesis of HCC. The expres-
sions of receptors for EphrinA2 were also tested in both

![Graph of EphrinA2 expression levels in cell lines and tissue samples.](image)
cell lines and clinic samples. However, no significant change has been observed (Supporting Fig. 1).

HCC carries a high risk of invasion of the portal vein. Portal vein tumor thrombus markedly deteriorates hepatic function and serves as a poor prognostic factor, associated with frequent recurrences and intrahepatic metastasis.21 Thus, we assumed that the expression level of EphrinA2 may be further elevated in this context. As expected, we found that the protein level of EphrinA2 was lowest in normal liver tissues, relatively higher in the primary HCCs, and further increased in portal vein tumor thrombus, indicating its role not only at the onset but also in the progression of HCC (Fig. 1C, D).

**Expression of EphrinA2 in HCC Cells Positively Regulates In Vivo Tumor Growth.** To further investigate the function of EphrinA2 in HCC, we developed stable clones overexpressing EphrinA2 from 7404 cells, which exhibited relatively low expression level of EphrinA2 among HCC cell lines, and three 7404/EphrinA2 clones were selected for further studies (Fig. 2A). No significant difference was observed in in vitro proliferation between the control cells and 7404/EphrinA2 cells (Supporting Fig. 2A, 2B). However, 7404/EphrinA2 cells generated larger xenografts in nude mice than control cells (Fig. 2B, left panel), indicating that EphrinA2 stimulated proliferation of xenografts derived from either control or 7404/EphrinA2 cells indicated that it had no effect on proliferation (Supporting Fig. 2C), which was consistent with our previous observation. In contrast, cell apoptosis dramatically decreased in the xenografts derived from 7404/EphrinA2 cells, as suggested by the reduced level of cleaved poly(adenosine diphosphate-ribose) polymerase (PARP), a sensitive marker of apoptosis, whereas knockdown of the exogenous EphrinA2 effectively rescued the expression of cleaved PARP (Fig. 3A). The classic terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) assay also showed that the apoptosis DNA fragments were dramatically decreased in EphrinA2 overexpressing xenografts (Fig. 3B). These results suggested that the tumor-promoting effect of EphrinA2 was mainly attributed to its suppression of apoptosis in HCC cells.

HCC is usually associated with chronic inflammation induced by hepatitis virus infection, which often leads to elevated level of tumor necrosis factor alpha (TNF-α).22 TNF-α is closely involved in the induction of apoptosis and in triggering destruction of liver.23 To test whether EphrinA2 exerts a similar resistant effect in this cytokine-induced apoptosis, we performed TNF-α treatment on both control and EphrinA2-overexpressing cells. As shown in Fig. 4A, 7404/EphrinA2 cells exhibited stronger resistance to TNF-α-induced apoptosis compared with control cells, whereas this resistance was attenuated after EphrinA2 knockdown. With similar effects of overexpression of EphrinA2, exogenous purified EphrinA2-Fc protein also could increase the resistance to TNF-α in 7404 cells (Fig. 4B). Conversely, down-regulation of endogenous EphrinA2 in HepG2 cells, which showed relatively high levels of EphrinA2 (Fig. 1A), also resulted in hypersensitivity to TNF-α treatment (Fig. 4C), which was consistent with our observation in 7404/EphrinA2 cells. In the presence of TNF-α, the apoptotic marker cleaved PARP was down-regulated dramatically in the EphrinA2-overexpressing 7404 cell, as well as in the EphrinA2-Fc

| Table 1. Summary of Clinicopathologic Variables |
|-----------------------------------------------|
| Characteristic | No. of Patients | Characteristic | No. of Patients |
| Sex            |                | Tumor size     |                |
| Male           | 44             | <=5            | 30             |
| Female         | 19             | >5             | 33             |
| Age (years)    |                | TNM stage      |                |
| <=51           | 41             | I/II           | 28             |
| >51            | 22             | III/IV         | 35             |
| Hepatitis status |             | Tumor number  |                |
| Yes            | 55             | Single         | 40             |
| No             | 8              | Multiple       | 23             |
| Liver cirrhosis |             | Tumor capsulation |        |
| Yes            | 53             | Complete       | 13             |
| No             | 10             | Incomplete     | 50             |
| Child-Pugh score |            | Vascular invasion |    |
| A              | 58             | Yes            | 23             |
| B              | 5              | No             | 40             |
| AFP (ng/mL)    |                | Lymph node metastasis |    |
| <=20           | 20             | Yes            | 7              |
| >20            | 43             | No             | 56             |

TNM, tumor-node-metastasis.
protein-treated 7404 cells. In contrast, its level was increased in the EphrinA2-deficient cells (Fig. 4D). 5-Fluorouracil is another drug commonly used in chemotherapy, and we also found that the expression level of EphrinA2 in HCC cells negatively correlated with the cell sensitivity to 5-fluorouracil-induced apoptosis (Supporting Fig. 3), suggesting that EphrinA2 may participate in the regulation of apoptosis induced by a variety of chemotherapeutic agents in HCC.

**Rac1/Akt/NF-Kappa B Pathway Is Regulated by EphrinA2 and Is Responsible for EphrinA2-Induced Alteration in Cell Survival.** The PI3K/Akt is a crucial pathway that can deliver anti-apoptotic signals and block induction of apoptosis. Up-regulation of this pathway through the phosphorylation of Akt has been documented as a frequent occurrence in several human cancers; therefore we examined whether this alteration also occurred in HCC. The level of phosphorylated Akt was significantly elevated in 7404/EphrinA2 cells (Fig. 5A), whereas it was reduced when EphrinA2 was knocked down in either 7404/EphrinA2 cells or HepG2 cells. The purified EphrinA2-Fc protein also could activate Akt in HCC cells. Furthermore, this positive correlation between EphrinA2 expression and Akt phosphorylation was also observed in paired clinical samples (Fig. 5B), suggesting their cooperatives in HCC development. Exposure of
7404/EphrinA2 cells to LY294002, a specific inhibitor of PI3K/Akt pathway, resulted in loss of resistance to TNF-α treatment (Fig. 5C), indicating that activated Akt was responsible for the in vitro apoptotic resistance endowed by EphrinA2. More importantly, blockage of PI3K cascade in vivo by rapamycin is able to dramatically impede the tumor growth of EphrinA2-overexpressing cells (Fig. 5D). The data show that biological effects mediated by EphrinA2 used the activated PI3K/Akt pathway.

Rho family members are well-known upstream regulators of PI3K/Akt pathway, and the activity of Rho family proteins are modulated by some Eph/Ephrins in several types of cells; therefore we hypothesized that overexpression of EphrinA2 in HCC cells may stimulate the activity of Rac1 (an important member of the Rho family). As expected, the level of active Rac1 was indeed increased in 7404/EphrinA2 cells compared with control cells, whereas knockdown of EphrinA2 led to a decreased level of the active form of Rac1 (Fig. 5E). Furthermore, blocking the activity of Rac1 with a specific inhibitor NSC23766 dramatically decreased the level of activated Akt in EphrinA2 expressing cells, whereas it only slightly affected the control cells, which was accorded with the level of active Rac1 in these cells.

NF-κB, a well-known mediator in the anti-apoptotic signaling downstream of Akt, has been implicated in liver carcinogenesis. We assumed that EphrinA2 could enhance cell survival by activating NF-κB. We examined the activation of NF-κB by using luciferase reporter assay. The cellular transcriptional activity of NF-κB was significantly increased once EphrinA2 was overexpressed or exogenous EphrinA2 protein was added (Fig. 6A). In contrast, when EphrinA2 expression was suppressed by siRNA in HepG2 cells, NF-κB activity decreased simultaneously (Fig. 6A). In most cell types, NF-κB is found in the cytoplasm as an inactive dimer bound to one of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) proteins that mask its nuclear localization signal. However, as assessed by immunofluorescence, marked nuclear localization of NF-κB was observed in 7404/EphrinA2 cells compared with control cells, whereas EphrinA2 knockdown reduced nuclear NF-κB (Fig. 6B,C), which further supported our assessment that EphrinA2 activated NF-κB. Activation of NF-κB can elicit the expression of various anti-apoptosis proteins. We found that the expressions of cIAP1, XIAP and Bcl2, all of which are well-known NF-κB targeted genes, were regulated by EphrinA2 in HCC cells (Supporting Fig. 4A-C). Blockage of NF-κB activation could significantly abolish the anti-apoptotic capability of 7404/EphrinA2 cells, demonstrating that NF-κB was crucial in the EphrinA2-mediated signaling (Supporting Fig. 4D). Furthermore, EphrinA2-induced activation of NF-κB was blocked by LY294002, NSC23766, dominant-negative Akt, and dominant-negative Rac1, respectively, despite varied inhibitory effects (Fig. 6D). These results indicated that the Rac1/Akt pathway participates in the modulation of NF-κB activity stimulated by EphrinA2, thus revealing an exquisite regulatory network between EphrinA2, Rac1, Akt, and NF-κB.
Discussion

We identified the relationship between EphrinA2 expression and the development of HCC. The level of EphrinA2 was lowest in normal hepatocytes and increased in primary HCC cells, and it reached the highest level in portal vein tumor thrombus cells. This gradually increasing expression pattern paralleled with deterioration of this disease, suggesting a potential role of EphrinA2 in the progression of HCC. In fact, an emerging body of evidence suggests an increasing role of Eph/Ephrins in cancer. For example, EphA2 can promote growth of breast, prostate, and pancreas cancer cells, perhaps by activating mitogen-activated protein kinases (MAPKs). EphA2 and EphB4 can stimulate cancer cell migration and invasion. The Eph/Ephrins also can enhance angiogenesis during cancer progression. In our study, we demonstrated that EphrinA2 could endow the HCC cells with resistance to both basal and cytokine-induced apoptosis, thus providing a growth advantage to cancer cells, which consequently enhanced the development and progression of HCC. Because the mechanism underlying the regulation of cell survival and apoptosis by Eph/Ephrins in cancer cells remains largely unknown, our study provides novel insights into this mechanism.

HCCs are often associated with chronic hepatitis, especially in Asia. TNF-α has been reported to be closely involved in the pathogenesis of chronic liver disease. Released by infiltrating lymphocytes, TNF-α can trigger both pro-survival and pro-apoptosis signaling.
through the NF-κB pathway and the caspase8 pathway. The cell fate determination mainly depends on the balance between these two pathways. We found that overexpression of EphrinA2 in HCC cells could activate NF-κB, leading to a shift from apoptosis to survival in the circumstance of TNF-α. This may explain how HCC cells tolerate the high level of such potential apoptotic factors.

A series of previous studies have suggested that TNF-α is a promising cytokine for cancer therapy34,35; however, the clinical outcome was disappointing, mainly because of the nonspecific toxicity of this factor at high dose. Efforts have been made to improve its application, such as modification of TNF-α to ameliorate its specificity aiming at tumor tissues.36 Another potential strategy is to increase

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**Fig. 5.** The Rac1-Akt pathway is activated by EphrinA2 in HCC cells. (A) Vector control and EphrinA2 overexpressing 7404 cells, EphrinA2 overexpressing 7404 cells and their EphrinA2 knockdown counterparts, HepG2 cells and their EphrinA2 knockdown counterparts, and 7404 cells treated with or without EphrinA2-Fc protein were cultured and harvested for western blots for expression of Akt/pAkt. (B) Correlation of level of EphrinA2 expression and level of Akt phosphorylation in HCC samples. The upper panel shows representative western blot results of both EphrinA2 and pAkt in four pairs of samples. Expression levels of EphrinA2 and pAkt in all 11 pairs of samples were quantified, and the ratio of EphrinA2, as well as pAkt, between each T/N pair was analyzed by bivariate correlation using SPSS software, and the result is shown in the lower graph. (T, primary tumor; N, matched normal liver; P, portal vein tumor thrombus). (C) Inhibition of Akt phosphorylation reverses the resistance of TNF-α-induced apoptosis in EphrinA2 transfectants. Both vector control and EphrinA2 overexpressing 7404 cells were treated with or without 50 μg/mL LY294002 in combination with 20 ng/mL TNF-α and 667 nM actinomycin D for 4 days, and the cell growth was measured by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay. Results represent the means ± standard error of the mean from three independent experiments. (D) Blockage of PI3K/Akt cascade impairs the growth advantage of EphrinA2 overexpressing cells. The 7404 vector control and EphrinA2 overexpressing cells were injected into both flanks of eight nude mice in day 1. Ten days after injection, mice were randomly assigned into two groups and set for the rapamycin sensitivity assay. The tumor volume of each mouse was measured every 4 days, and the data at day 26 are shown. (E) Rac1 activation is involved in Akt phosphorylation. Vector control and EphrinA2 overexpressing 7404 cells (left panel), EphrinA2 overexpressing 7404 cells and their EphrinA2 knockdown counterparts (middle panel) were harvested and used for Rac1 activity measurement (pull-down assay), and the amount of cellular guanosine triphosphate binding Rac1 is shown by western blot. Right panel: Vector control and EphrinA2 overexpressing 7404 cells were treated with or without 100 μM NSC23766 for 48 hours, and then harvested for measurement of guanosine triphosphate binding Rac1 and phosphorylated Akt.
the sensitivity of cancer cells to TNF-α, which will decrease the effective dose of the cytokine and avoid unexpected systemic toxicity. According to the effects of EphrinA2 knockdown in our study, it is reasonable and hopeful to block EphrinA2 signaling with either its inhibitors or neutralizing antibodies to elevate the sensitivity of HCC cells to TNF-α in clinical use, suggesting that manipulation of EphrinA2 may facilitate the application of TNF-α in cancer therapy.

We further demonstrated that the EphrinA2/Rac1/Akt cascade was responsible for NF-κB activation and thus pro-survival of HCC. Activation of Akt is a risk factor associated with recurrence as well as poor prognosis of HCC.37,38 We noted a remarkable correlation between EphrinA2 expression and Akt phosphorylation in the clinical HCC samples, suggesting that Akt is regulated by EphrinA2 during HCC progression. On Akt activation, a spectrum of downstream factors can be triggered, which impact cell survival, including Bcl family proteins and FoxO members. Among them, we found that NF-κB was highly activated in HCC cells in an Akt-dependent manner and played an indispensable role in mediating the anti-apoptotic function of EphrinA2 in HCC cells. Thus, we demonstrated that activation of Akt/NF-κB cascade...
triggered by EphrinA2 conferred the anti-apoptosis capability on HCC cells.

Both EphrinA2 and TNF-α function as a ligand and exert their effects through ligation and activation of their cognate receptors. It has been reported that Rac1 is involved in the downstream signaling of either molecule. It is possible that EphrinA2 can interfere with the TNF-α-induced apoptotic pathway in an Rac1-dependent as well as receptor-involved manner, which eventually attenuated the cytotoxic effect of TNF-α. However, confirmation of this hypothesis and extensive clarification of the signal transduction induced and regulated by EphrinA2 are required for further investigation and will benefit the development of therapeutic strategies targeting EphrinA2 in HCC.

Except for HCC, the aberrant regulation of Rac1, Akt, and NF-κB occurs frequently in many other types of solid tumor, whereas its exact mechanism remains largely unknown. The finding that EphrinA2 is able to modulate them in HCC strongly suggests a possibility that EphrinA2 also may get involved in the progression of other malignancies through the Rac1/Akt/NF-κB pathway. Therefore, to explore the specific role of EphrinA2 in other cancers would undoubtedly expand our interpretation to the intricate process of oncogenesis.

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