Epidermal growth factor receptor transactivation is involved in the induction of human hepatoma SMMC7721 cell proliferation by insufficient radiofrequency ablation

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Abstract. Our previous study revealed that insufficient radiofrequency ablation (RFA) promotes the malignancy of human hepatocellular carcinoma (HCC) SMMC7721 cells via the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)/extracellular signal-regulated kinase (ERK)-induced overexpression of vascular endothelial growth factor (VEGF). The aims of the present study were to address the involvement of epidermal growth factor receptor (EGFR) transactivation in the enhanced SMMC7721 cell proliferation induced by insufficient RFA, in addition to its association with the CaMKII/ERK/VEGF signaling cascade. SMMC7721 cells were subjected to a 47°C treatment regimen to simulate insufficient RFA. Cell proliferation was determined using MTT and colony formation assays. The expression levels of VEGF, CaMKII, phosphorylated (phospho)-CaMKII, ERK, phospho-ERK, EGFR and phospho-EGFR were analyzed using western blotting. The results demonstrated that the enhancement of SMMC7721 cell proliferation by the 47°C treatment regimen was significantly inhibited by exposure of the cells to AG178 (a specific inhibitor of EGFR). Furthermore, AG1478 exposure prevented the overexpression of VEGF and phosphorylation of ERK, but had no significant effects on CaMKII phosphorylation. By contrast, 47°C treatment-induced EGFR phosphorylation was inhibited by treatment with KN93 (a specific inhibitor of CaMKII). Overall, the results of the present study have suggested a role for EGFR transactivation in the RFA-promoted growth of residual HCC. Thus, targeting EGFR may represent a useful preventive and therapeutic strategy for RFA-induced HCC progression and recurrence.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver tumor and the fifth most common type of cancer worldwide, representing the third leading cause of cancer-associated mortality (1-3). Although hepatic resection is currently regarded as the standard therapeutic modality for HCC, its application is limited due to severe liver dysfunction in the majority of hepatic cancer cases. Liver transplantation is an alternative treatment for small, unresectable HCC, although its application is restricted by the shortage of liver graft donors (4,5). Consequently, non-surgical methods, including image-guided radiofrequency ablation (RFA), have been introduced for HCC treatment. As the most widely used non-surgical treatment approach for HCC, RFA has numerous advantages, including its definitive therapeutic effect, minimal damage to functioning liver, simplicity, repeatability and shorter associated hospital stays (4,6). However, increasing laboratory and clinical studies have reported that residual tumor tissue following RFA exhibits more malignant growth and accelerated local recurrence compared with non-RFA treated tumors (5-8). Therefore, further investigations into the molecular mechanisms underlying the undesirable effects of RFA on HCC are warranted.

The epidermal growth factor receptor (EGFR) signaling pathway is important for cancer cell oncogenesis, proliferation, maintenance and survival. In addition to being directly activated by its cognate ligand, epidermal growth factor (EGF), increasing evidence has revealed that EGFR also serves as a central element in the signal transduction networks activated by other stimuli, including cytokines (9), ammonia (10), H_2O_2 (11) and G-protein-coupled receptors (12). Currently, this nonclassical EGFR stimulation process is generally termed 'EGFR transactivation' (12). Transactivated EGFR subsequently integrates signals from the various extracellular stimuli into a limited number of downstream signal transduction molecules, including mitogen-activated protein kinase (MAPK)/extracellular signal-regulated

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kinase (ERK), phosphatidylinositol 3-kinase/Akt, phospholipase C/protein kinase C, and Ca^{2+} (10,13). Our previous study demonstrated that insufficient RFA promotes hepatoma cell malignancy via the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)/ERK-induced overexpression of vascular endothelial growth factor (VEGF) (6). Thus, the current study aimed to investigate whether EGFR signal transactivation is involved in HCC malignancy induced by incomplete RFA, and determine its association with the CaMKII/ERK-VEGF signaling cascade.

Materials and methods

Cell culture. Human HCCSMMC7721 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cancer cells were maintained in RPMI-1640 medium (Corning Incorporated, Corning, NY, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed 2-3 times/week.

Heat treatment. Incomplete RFA was simulated *in vitro* according to the authors' previously reported procedure (6). Briefly, SMMC7721 cells were seeded into 6-well plates at a density of $5x10^4$ cells/well. Following culture for 24 h in a CO_2 incubator with a humidified atmosphere containing 5% CO_2 at 37°C, the plates were sealed and submerged in a water bath at 47°C for 5 min. Thereafter, the cells were transferred into the CO_2 incubator for recovery. When the residual populations achieved 80% confluence, they were passaged into the 6-well plates and exposed to 47°C heat treatment for 10 min. This process was repeated with the stimulation duration increasing to 15, 20 and finally 25 min. Cells surviving the 47°C treatment regimen were designated as the 47°C treatment group. Cells in the control group underwent the same procedures; however, the temperature of the water bath was set at 37°C instead of 47°C. Subsequently, 100 nM AG1478, a specific inhibitor of EGFR, or 10 µM KN93, a specific inhibitor of CaMKII (both purchased from Sigma-Aldrich, St. Louis, MO, USA) was added to the 47°C treatment group, to investigate the roles of EGFR and CaMKII in incomplete RFA-induced SMMC7721 malignancy.

MTT assay. The trypsin-dispersed parent SMMC7721 or 47°C-treated SMMC7721 cells were seeded into a 96-well plate ($3x10^3$ cells/well in a volume of 100 µl RPMI-1640 medium supplemented with 10% fetal bovine serum) and cultured for 48 h at 37°C. Subsequently, MTT solution was added to each well to give a final concentration of 0.5 mg/ml, and incubated for 4 h at 37°C. Finally, the culture medium was removed and 150 µl DMSO/well was added. The absorbance was measured at a wavelength of 570 nm using an automated ELISA plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Colony formation assay. The SMMC7721 cells were seeded into 6-well dishes at a concentration of $1x10^3$/well and cultured in RPMI-1640 medium containing 10% fetal bovine serum for 2 weeks at 37°C. The obtained colonies were washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature, and then washed three times with PBS. Finally, colonies were stained with 0.1% crystal violet for 10 min at room temperature. Stained cells were washed with PBS and the colony number was counted.

Western blotting. Whole-cell lysates were collected using freshly prepared lysis buffer [containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% sodium dodecyl sulfate, 1% Triton X-100, and 1% proteinase inhibitors (1:100, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)]. Following incubation on ice for 30 min, the cell lysates were centrifuged at 14,000 x g for 20 min at 4°C (Sorvall™ ST 16R; Thermo Fisher Scientific, Inc.). The protein content was subsequently determined using a Lowry protein assay with bovine serum albumin (Sigma-Aldrich; Merck KGaA) as the standard. For western blot analysis, solubilized proteins (30-50 µg/lane) were separated using SDS-PAGE (10% gel) and eletrophoretically transferred onto polyvinylidene difluoride membranes. Following transfer, the membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4°C with primary rabbit anti-human polyclonal anti-ERK (1:1,000; cat. no. sc-292838; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (phospho)-ERK (1:1,000; cat. no. sc-101760; Santa Cruz Biotechnology, Inc.), anti-CaMKII (1:500; cat. no. sc-13082; Santa Cruz Biotechnology, Inc.), anti-phospho-CaMKII (1:1,000; cat. no. V1111; Promega Corporation, Madison, WI, USA), anti-VEGF (1:200; cat. no. BA0407; Wuhan Boster Biological Technology, Ltd., Wuhan, China), anti-EGFR (1:500; cat. no. sc-03; Santa Cruz Biotechnology, Inc.), or anti-phospho-EGFR (1:500; cat. no. sc-101668; Santa Cruz Biotechnology, Inc.) antibodies. Subsequently, the membranes were incubated with polyclonal horseradish peroxide-conjugated secondary goat anti-rabbit antibodies (1:3,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) diluted in TBS with Tween-20 (20 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.1% Tween-20) for 2 h at room temperature. Finally, the membrane was rinsed with TBS and visualized using Pierce™ Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The optical density for each band was assessed using QuantityOne software (version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard error of the mean from ≥3 independent experiments. Statistical analysis for multiple comparisons was performed by one-way analysis of variance followed by Fisher's least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Incomplete RFA promotes SMMC7721 proliferation, which is significantly inhibited by AG1478. The MTT assay results presented in Fig. 1A demonstrated that the 47°C treatment regimen significantly promoted SMMC7721 cell proliferation compared with untreated cells. This effect was abrogated by treatment with AG1478, when compared with cells that had
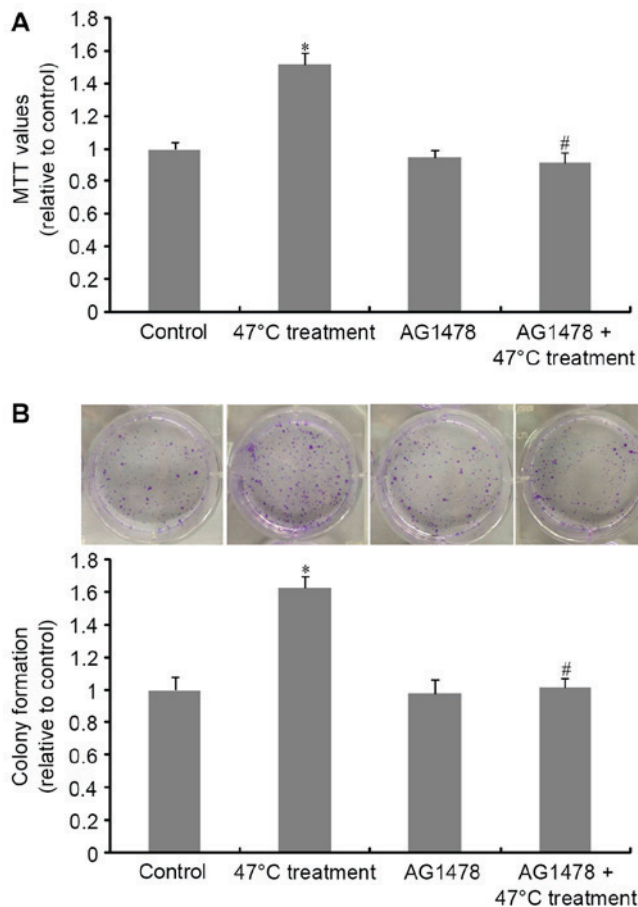
Figure 1. The induction of SMMC7721 cell proliferation by the 47°C treatment regimen is dependent on EGFR activation. The effect of AG1478 (a specific EGFR inhibitor) on cell proliferation and colony formation rates are shown, as detected using (A) MTT and (B) colony formation assays, respectively. Data are presented as the mean ± standard error of the mean from five independent experiments. *P<0.05 vs. control; #P<0.05 vs. 47°C treatment group. EGFR, epidermal growth factor receptor.
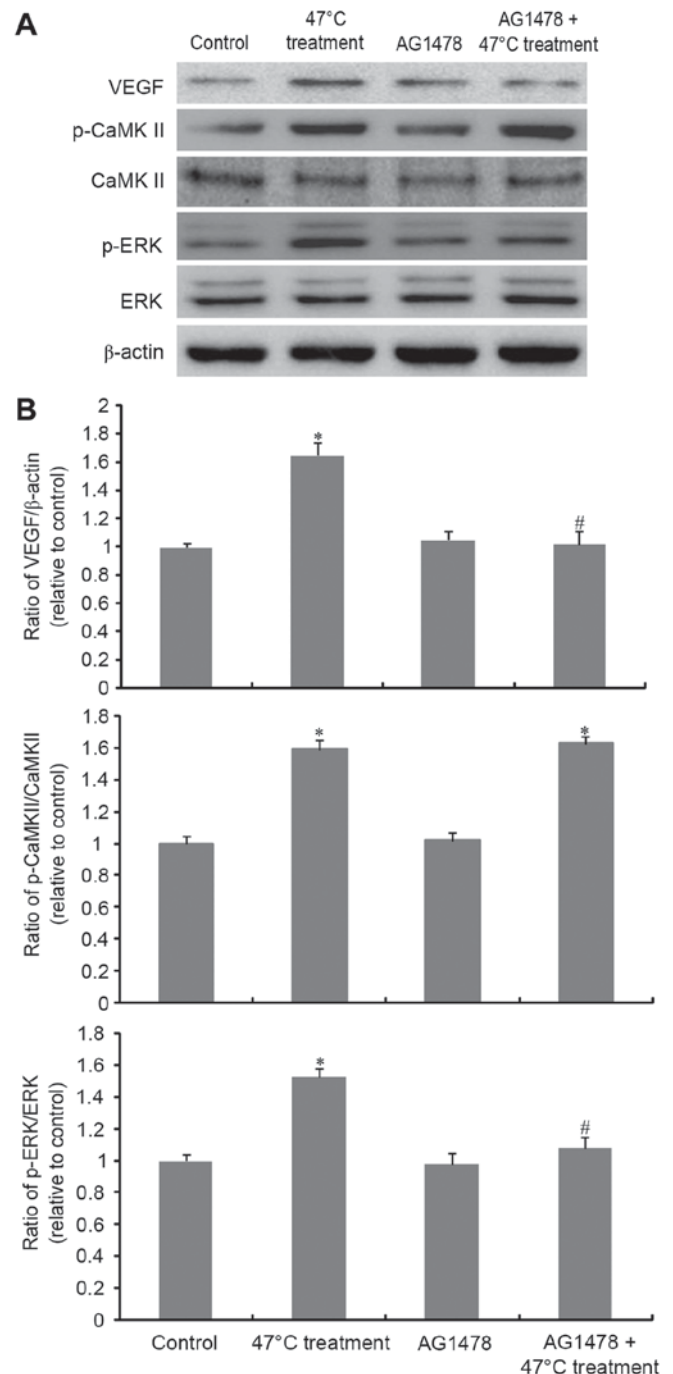

Figure 2. EGFR activation is involved in the induction of VEGF and ERK overexpression, but not in the activation of CaMKII, by 47°C treatment. (A) Representative blots are shown. (B) Quantified data from the western blot analysis are presented as the mean ± standard error of the mean from four independent experiments. *P<0.05 vs. control; #P<0.05 vs. 47°C treatment group. All data are normalized to β-actin. EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; p-, phosphorylated.

received incomplete RFA treatment alone. A similar result was obtained in the colony formation assay (Fig. 1B). These observations demonstrate the crucial role of EGFR transactivation in heat stimulation-induced tumor cell proliferation.

AG1478 significantly inhibits RFA-induced overexpression of VEGF and activation of ERK. Our previous study demonstrated that CaMKII/ERK-dependent VEGF overexpression was involved in the 47°C treatment-induced malignant proliferation of SMMC7721 cells (6). Thus, the present study further investigated the association between EGFR transactivation and the CaMKII/ERK/VEGF signaling cascade. As Fig. 2 demonstrates, VEGF expression was significantly increased upon 47°C treatment stimulation. AG1478 exposure significantly inhibited the overexpression of VEGF induced by incomplete RFA. Furthermore, AG1478 abrogated heat stress-induced ERK phosphorylation. However, CaMKII phosphorylation (activation) was not affected by EGFR inhibition.

KN93 significantly inhibits RFA-induced EGFR phosphorylation. Upon exposure of SMMC7721 cells to the 47°C regimen, EGFR phosphorylation levels were determined and the results are presented in Fig. 3. The heat stimulation regimen

triggered significant activation (phosphorylation) of EGFR, with no influence on total EGFR expression, confirming that 47°C treatment induces EGFR transactivation. Notably, this transactivation was significantly inhibited by KN93. This result, combined with the data in Fig. 2, suggests a CaMKII-dependent EGFR transactivation cascade upon 47°C treatment in SMMC7721 cells.
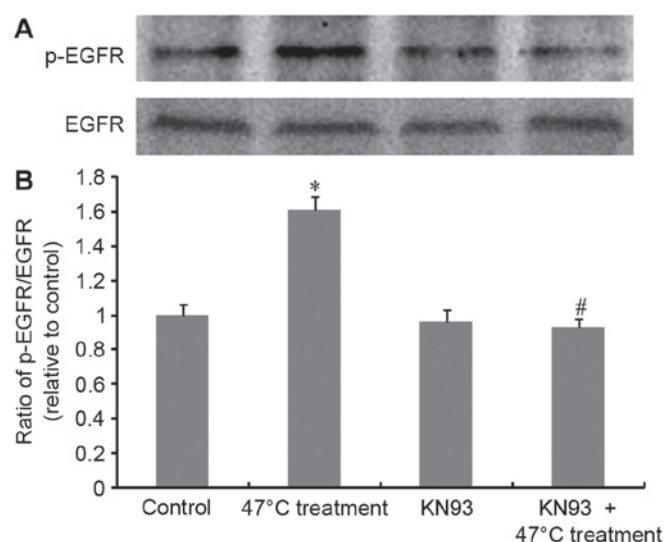
Figure 3. CaMKII activation occurs upstream of EGFR phosphorylation during 47°C treatment, as indicated by a significant decrease in p-EGFR expression following treatment with KN93, a specific inhibitor of CaMKII, and the 47°C treatment regimen. (A) Representative blots are shown. (B) Quantified data from the western blot analysis are presented as the mean ± standard error of the mean from four independent experiments. *$P<0.05$ vs. control; #$P<0.05$ vs. 47°C treatment group. All data are normalized to β-actin. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; EGFR, epidermal growth factor receptor; p-, phosphorylated.

Discussion

As a novel therapeutic strategy, RFA exhibits numerous advantages and is now widely used for HCC treatment as an alternative to traditional surgical approaches. However, one of the major challenges associated with RFA is the rapid growth of residual HCC, thus leading to high recurrence rates associated with the cancer (6,8). As reported, post-RFA local recurrence rates can reach up to 60% (14). Previous studies have demonstrated that the rapid progression of residual HCC may be associated with a more malignant hepatoma cellular phenotype due to the activation of several signaling pathways. For example, a recent study revealed that Akt and ERK signaling activation served as an important mechanism underlying insufficient-RFA-induced HCC malignancy (8). Another study reported that suboptimal RFA accelerated residual tumor proliferation by inducing the overexpression of hypoxia inducible factor-1α and VEGFA (14). Similarly, our previous report showed that insufficient RFA could promote residual hepatoma cell proliferation via triggering the CaMKII/ERK-mediated overexpression of VEGF (6). In order to obtain a more detailed understanding of the signaling mechanisms, the involvement of EGFR transactivation in RFA-induced hepatoma cell malignancy and its association with CaMKII/ERK-VEGF signaling cascades were investigated in the present study.

In addition to being directly activated by its cognate ligand EGF, EGFR is involved in the signal transduction networks of other stimuli. A previous study has revealed that EGFR transactivation is required for the mediation of mitogenic effects of epoxyeicosatrienoic acid in various types of cancer cells (15). Previously, it was reported that heat stress induced a decrease in EGFR expression in intestinal epithelial-6 cells,

representing a protective mechanism in response to inflammation and/or injury in intestinal mucosa (16). However, the EGFR signaling response and its role in RFA-induced HCC malignant proliferation remain unclear. In the present study, it was demonstrated that 47°C heat stimulation significantly induced the proliferation of HCC cells, which was abrogated by the EGFR-specific inhibitor AG1478. Additionally, 47°C heat treatment resulted in significantly increased the levels of EGFR phosphorylation, as shown by western blot analysis. These data demonstrate the essential role of EGFR transactivation in 47°C treatment-enhanced malignant proliferation of liver cancer cells. This is further supported by preliminary data that revealed a significant inhibitory effect of genistein, a broad-spectrum tyrosine kinase inhibitor, on 47°C treatment-induced HCC over-proliferation (Dai et al, unpublished data).

In the present study, the association between CaMKII/ERK-VEGF signaling cascades and EGFR transactivation was investigated. The MAPK kinase (MEK)/ERK pathway is a common signaling pathway downstream of direct or indirect (trans-) activation of EGFR. In the majority of cases, EGFR-mediated MEK/ERK activation results in the malignant proliferation of tumors (17-19). Based on this, in addition to the significant inhibitory effect of AG1478 on HCC proliferation, it was hypothesized that 47°C treatment-induced ERK activation is mediated by EGFR transactivation. This hypothesis was confirmed by the significant inhibitory effect of AG1478 on heat stimulation-triggered ERK activation. Although it was initially hypothesized that CaMKII activation also occurred following EGFR activation, the data of the present study excluded this possibility. It is more likely that CaMKII serves as an upstream operator of EGFR activation, as its phosphorylation was eliminated by KN93, a specific inhibitor of CaMKII. This is in contrast with observations of transforming growth factor β1-stimulated CaMKII activation downstream of EGFR transactivation during the differentiation of fibroblasts to myofibroblasts (20). However, a CaMKII-dependent EGFR activation in the present study has been observed to underlie the following: i) The effect of hydrogen peroxide on lung alveolar epithelial A549 cells (21); ii) the effect of $α_{1A}$-adrenoceptorstimulation on Chinese hamster ovary cells (22); and iii) the effect of adenosine triphosphate on vascular smooth muscle cells (23).

In conclusion, the results of the current study suggest an important molecular mechanism involving EGFR transactivation in the incomplete RFA-induced proliferation of residual HCC cells. Thus, EGFR has been identified as a potential target for the prevention and treatment of RFA-induced HCC progression, and recurrence.

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