

Role of GLI-1 in epidermal growth factor-induced invasiveness of ARCaP_E prostate cancer cells

GUODONG ZHU^{1,2}, JIANCHENG ZHOU^{1,2}, WENBIN SONG^{1,2}, DAPENG WU^{1,2},
QIANG DANG^{1,2}, LINLIN ZHANG^{1,2}, LEI LI^{1,2}, XINYANG WANG^{1,2} and DALIN HE^{1,2}

¹Department of Urology, The First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, Xi'an 710061;

²Oncological Research Laboratory, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education of the People's Republic of China, Xi'an 710061, P.R. China

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Abstract. Epidermal growth factor (EGF) signaling and Hedgehog (HH) signaling are both involved in prostate cancer (PCa) progression, yet the mechanisms through which these two pathways are synergistically linked require elucidation. In the present study, we aimed to ascertain how EGF and the HH signaling transcription factor GLI-1 are linked in prostate cancer invasiveness. ARCaP human prostate cancer cells, which included ARCaP_E and ARCaP_M cells, were used as a model in the present study. The expression of EGF receptor (EGFR) and the HH signaling transcriptional factor GLI-1 were detected in ARCaP_E cells by immunofluorescence, and the ARCaP_E cells were treated with human recombinant EGF protein (hrEGF) for 4 consecutive days *in vitro*. Transwell invasion assays were performed in the ARCaP_E cells following treatment with DMSO (vehicle control), hrEGF, GANT61 (GLI-1-specific inhibitor), hrEGF plus GANT61 and in the ARCaP_M cells. The expression of phosphorylated extracellular signal regulated kinase (p-ERK), total ERK and GLI-1 was detected by western blotting in ARCaP_E cells at different time-points following treatment with hrEGF. The expression of EGFR and GLI-1 was detected in ARCaP_E cells, which exhibited a cobblestone-like morphology, while after treatment with hrEGF, the cell morphology was altered to a spindle-shaped mesenchymal cell morphology. Transwell invasion assays demonstrated that hrEGF dramatically enhanced the invasive capability of the ARCaP_E cells ($P < 0.05$). Additionally, western blot assay demonstrated that the expression levels of p-ERK and GLI-1 in ARCaP_E cells increased in a time-dependent manner after treatment with hrEGF ($P < 0.05$); however, the expression levels of total ERK in the cells remained relatively

unchanged. It also demonstrated that the GLI-1 inhibitor GANT61 could reverse the enhanced invasive effect induced by EGF in ARCaP_E cells ($P < 0.05$). Our preliminary *in vitro* study showed that EGF signaling may increase the invasive capability of ARCaP_E human prostate cancer cells via upregulation of p-ERK and the HH signaling transcriptional factor GLI-1. Additionally, this enhanced cell invasive effect was reversed by a GLI-1-specific inhibitor *in vitro*. Consequently, it indicates that both EGF and HH signaling are synergistically involved in the progression of human prostate cancer ARCaP cells, and GLI-1 may be one of the important effectors, which is activated by EGF downstream signaling, to promote the invasiveness of ARCaP_E prostate cancer cells.

Introduction

Prostate cancer is generally the second leading cause of cancer-related mortality in males of Western countries (1,2). Androgen ablation therapy is an effective treatment for hormone-dependent prostate cancer; however, a subset of patients ultimately develops hormone refractory disease (3-5). Therefore, it is necessary to identify and characterize important regulators of aggressive prostate cancer. Cellular heterogeneity is a common histopathological feature in prostate cancer, and cancer cells undertake progressive morphologic and behavioral changes during disease progression and metastasis. Many aggressive prostate cancers recapitulate normal developmental processes, such as epithelial-to-mesenchymal transition (EMT), to enhance cell migration and invasion. The conversion of an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, invasion and migration (6-9).

The prostate cancer ARCaP cell is a well recognized cell model for investigation of the molecular mechanisms of EMT in prostate cancer (10). ARCaP cells consist of two subtype cell lines, including ARCaP_E and ARCaP_M. The parental ARCaP_E cells were isolated from the ascites fluid of a patient with bone metastasis and display typical epithelial cell morphology and have only limited tumorigenic potential. However, ARCaP_E cells have a high propensity for rapid and predictable bone and soft tissue growth and metastases through orthotopic, intracardiac and intraosseous injections in athymic mice,

Correspondence to: Professor Dalin He, Department of Urology, The First Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an 710061, P.R. China
E-mail: hedlxjtu@yahoo.com.cn

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and can undergo EMT to become ARCAPE cells, which exhibit mesenchymal cell morphology and lose original epithelial cell markers but gain various mesenchymal cell markers (11,12).

EGF signaling is proposed to participate in the pathogenesis or maintenance of several types of human cancers of an epithelial origin (13). In prostate cancer cells, EGFR ligands are frequently elevated and EGFR itself is commonly overexpressed (14). Furthermore, EGFR expression increases during progression to a hormone-resistant stage (15-17). Previous studies have demonstrated that epidermal growth factor (EGF) signaling and Hedgehog (HH) signaling are both involved in prostate cancer tumorigenesis and progression (18-23); however, whether there is any 'crosstalk' between these two important pathways requires clarification. In the present study, we mainly explored the role of GLI-1, which is the transcription factor of HH signaling, in EGF-regulated enhancement of the invasiveness of ARCAPE prostate cancer cells *in vitro*. We found that GLI-1 may function as one of the important effectors, which is activated by EGF downstream signaling, to promote the invasiveness of prostate cancer ARCAPE cells. This finding indicates that EGF and HH signaling is synergistically integrated in prostate cancer progression.

Materials and methods

Cell culture. The human prostate cancer ARCAPE and ARCAPM cell lines were kindly provided as a gift by Dr Leland Chung from the Cedars Sinai Medical Center, USA. The ARCAPE and ARCAPM cells were maintained in T-medium (Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS) at 37°C supplemented with 5% CO₂ in a humidified incubator. To study the effect of human recombinant EGF protein (hrEGF) treatment, ARCAPE cells with 70% confluence were cultured in non-serum T-medium overnight, then 100 ng/ml hrEGF was added and treated for a consecutive 4 days. FBS and hrEGF were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Cell immunofluorescence staining. ARCAPE cells (5x10⁴) were added to poly-L-lysine-coated chamber slides and cultured for 24 h. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 in PBS and blocked with 10% donkey serum, then stained with goat polyclonal primary antibody against EGFR (sc-31156, Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit polyclonal primary antibody against GLI-1 (ab92611, Abcam, Cambridge, MA, USA). After rinsing, the primary antibodies were respectively detected with Alexa Fluor® 488 donkey anti-goat (A-11055, Invitrogen, Grand Island, NY, USA) or Alexa Fluor® 488 goat anti-rabbit secondary antibodies (Invitrogen), and the nuclei were labeled with DAPI (0.5 mg/ml) as previously described (24,25). Chamber slides were mounted and fluorescence images were visualized and captured with an Olympus IX50 inverted fluorescence microscope (Olympus, Tokyo, Japan) and processed using Adobe Photoshop 7.0 software.

Invasion assay. The invasive capability of prostate cancer cells was determined by the Transwell assay. Before seeding the cells, 1 ml of Matrigel (BD Biosciences, Shanghai, China)

was dissolved in 4 ml serum-free T-medium, and 60 µl diluted Matrigel was applied to the upper chamber of 8-mm pore size polycarbonate membrane filters (Corning Incorporation, Corning, NY, USA), and put into an incubator overnight. ARCAPE and ARCAPM cells were then harvested and seeded with serum-free T-medium into the upper chamber (1x10⁵ cells/well), and the bottom chamber of the apparatus contained T-medium with 10% FBS. The hrEGF or/and the GLI-1 inhibitor GANT61 (sc-202630, Santa Cruz Biotechnology, Dallas, TX, USA) were added to both the upper and bottom chambers for the ARCAPE cell treatment group at concentrations of 100 ng/ml and 10 µmol/l, respectively, and then incubated for 48 h at 37°C. Following incubation, the cells that had invaded and attached to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 1% toluidine blue. Cell numbers were counted in five randomly chosen microscopic fields (x100) per membrane using the IX50 inverted microscope (Olympus, Japan).

Western blot analysis. The expression levels of p-ERK, ERK and GLI-1 were determined by western blot analysis according to previous studies (24-27). Briefly, the cells were washed with ice cold PBS (pH 7.4) and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA and 0.1% SDS) with the addition of mixed protease inhibitors. Supernatants were collected after centrifuging at 14,000 rpm for 10 min at 4°C. Protein concentration was determined by the Bradford method. Protein samples (20 µg) prepared in a final 1X sample buffer (50 mM Tris/pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue and 0.5% 2-β-mercaptoethanol) were denatured for 5 min at 100°C and resolved on 10% SDS-polyacrylamide minigels. Electrophoresis was initially carried out at 90 V through the stacking gel and then at 120 V through the separation gel. After electrophoresis, proteins were transferred to nitrocellulose filters (Bio-Rad Laboratories, Shanghai, China). Filters were subsequently blocked for 1.5 h at room temperature with blocking solution (50 mM Tris/pH 7.4, 150 mM NaCl, 0.05% Tween-20 and 5% skim milk), followed by incubation with rabbit anti-p-ERK, anti-ERK, and anti-GLI1 polyclonal antibodies (Santa Cruz Biotechnology) at 1:500 in blocking buffer and with mouse anti-GAPDH monoclonal antibody (KangChen Bio-Tech Inc., Shanghai, China) at 1:1,000 in blocking buffer for 1.5 h at room temperature. After a 4 x 5-min rinse with TBST buffer (50 mM Tris/pH 7.4, 150 mM NaCl and 0.05% Tween-20), blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (KangChen Bio-Tech) at 1:3,000 in blocking buffer for 1 h at room temperature and rinsed 4 x 5 min with TBST. The immunopositive bands were examined by an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Rockford, IL, USA), and the signals were transferred and analyzed using the Odyssey quantitative fluorescent imaging system (LI-COR Biosciences, Lincoln, NE, USA). Protein equal loading was confirmed by GAPDH expression.

Statistical analysis. All statistical analyses were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as means ± SEM, and the



Figure 1. Morphologic change in ARCaP_E cells following treatment with EGF. The parental ARCaP_E cells exhibited a cobble-stone, epithelial-like morphology and aggregated growth, while the lineage-derived ARCaP_M cells exhibited a spindle-shaped mesenchymal morphology and scattered growth. After treatment with human recombinant EGF protein (hrEGF, 100 ng/ml) for 4 days, the morphology of the parental ARCaP_E cells switched to a mesenchymal cell shape, which largely resembled the morphology of the ARCaP_M cells.

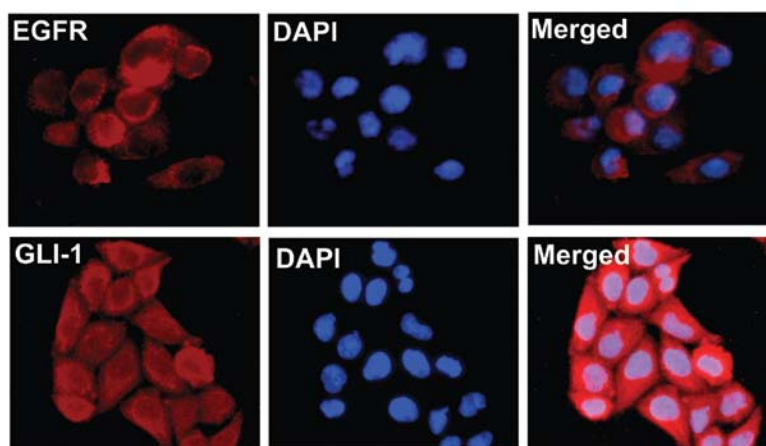


Figure 2. Expression of EGFR and GLI-1 in ARCaP_E cells. Using cell immunofluorescence staining, the expression of EGFR was detected in both the cell membrane and the cytoplasm of the ARCaP_E cells, while expression of GLI-1 was found in the cell cytoplasm and nucleus of the ARCaP_E cells.

differences between two groups were compared by the 2-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Morphology of the ARCaP_E cells is altered from an epithelial shape to a mesenchymal cell phenotype following treatment with hrEGF. When grown in a two-dimensional culture, ARCaP_E cells exhibited a cobble-stone, epithelial-like morphology and aggregated growth (Fig. 1), while the lineage-derived ARCaP_M cells exhibited a spindle-shaped mesenchymal morphology and scattered growth (Fig. 1). The morphologic changes observed in the ARCaP_M cells resembled that of cells undergoing EMT. However, after treatment with hrEGF (100 ng/ml) for 4 days, the morphology of the ARCaP_E cells displayed various characteristics of a mesenchymal cell-like phenotype with dispersed growth (Fig. 1), which largely resembled the morphology of the ARCaP_M cells (Fig. 1).

Expression of EGFR and GLI-1 in ARCaP_E cells. Using immunofluorescence detection assay, expression of EGFR and GLI-1 was detected in ARCaP_E prostate cancer cells. Expression of EGFR was predominate in the cell membrane and cytoplasm, while expression of GLI-1 was largely confined to the cytoplasm and cell nucleus (Fig. 2).

Treatment of EGF enhances the invasive capability of ARCaP_E cells. Using Transwell assay for 48 h, it was demonstrated that the number of invasive cells noted in the parental ARCaP_E cells, hrEGF-treated ARCaP_E cells, and ARCaP_M cells was 84 ± 3 , 148 ± 5 and 302 ± 18 , respectively. Compared to the number of invasive cells noted in the parental ARCaP_E cells, there was a significant difference in the invasive cell numbers noted in both the hrEGF-treated ARCaP_E and ARCaP_M cells (Fig. 3, $P < 0.05$). This result indicates that EGF treatment increased the invasive capability of ARCaP_E cells.

EGF activates ERK signaling in ARCaP_E cells. Following treatment with hrEGF, the expression of total ERK and phosphorylated ERK (p-ERK) in ARCaP_E cells was detected at a different time-point by western blot assay. The results showed that the expression level of p-ERK was initially upregulated after 15 min of treatment with hrEGF. The level continued to increase after 30 min and 1 h, but decreased to its baseline level after a 2-h treatment (Fig. 4, $P < 0.05$). However, the expression level of total ERK in ARCaP_E cells was relatively unchanged following treatment of hrEGF (Fig. 4).

Expression of GLI-1 is upregulated following treatment with EGF in ARCaP_E cells. The expression of GLI-1 in ARCaP_E cells was timely detected by western blot analysis and the cell lysates were harvested at 4, 12 and 24 h time-points after a

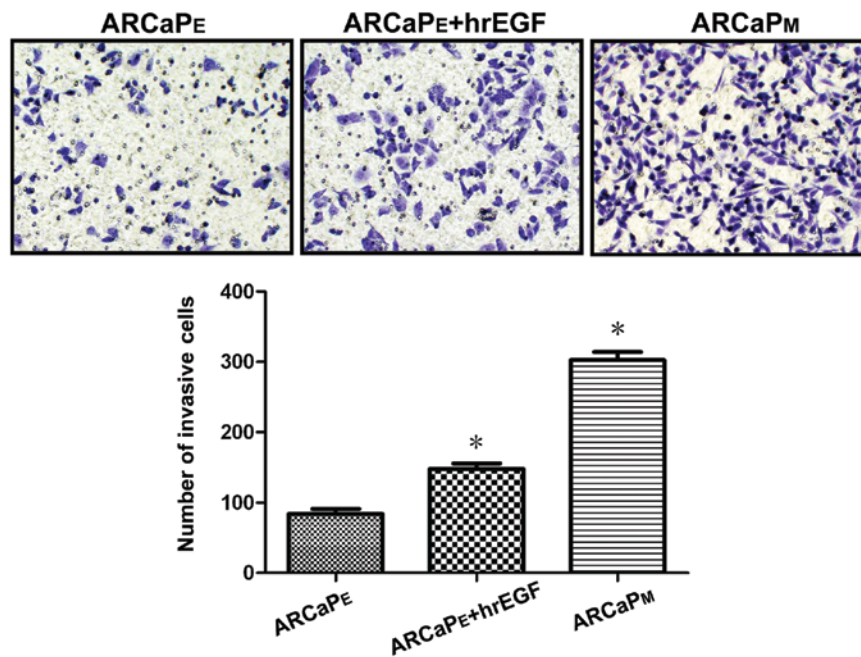


Figure 3. Effect of EGF on the enhanced invasiveness of ARCAPE cells. Using Transwell assay for 48 h, it was found that hrEGF dramatically elevated the invasive capability of ARCAPE cells. The number of invasive parental ARCAPE cells, hrEGF-treated ARCAPE cells, and ARCAPEM cells was 84 ± 3 , 148 ± 5 and 302 ± 18 , respectively. All experiments were conducted in triplicate (* $P<0.05$).

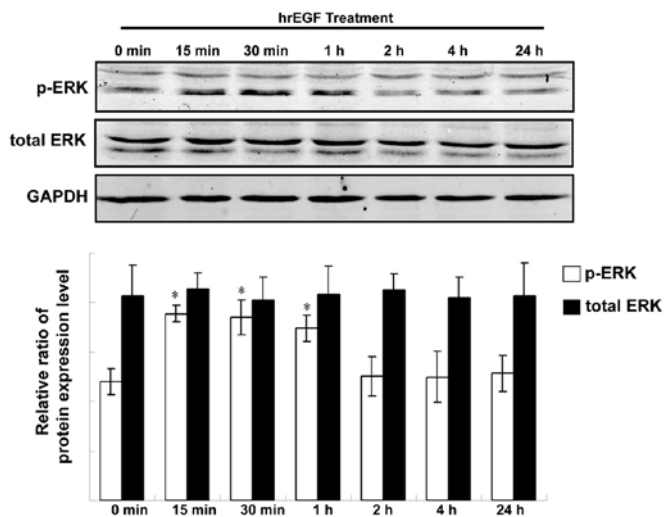


Figure 4. Increased expression of phosphorylated ERK (p-ERK) induced by EGF in ARCAPE cells. The expression level of p-ERK was initially upregulated after 15 min (* $P<0.05$) of treatment with hrEGF. The level continued to increase after 30 min (* $P<0.05$) and 1 h (* $P<0.05$), but decreased to its baseline level after a 2-h treatment. However, the expression level of total ERK in the ARCAPE cells was relatively unchanged following treatment of hrEGF. GAPDH was used as a loading control, and all experiments were conducted in triplicate.

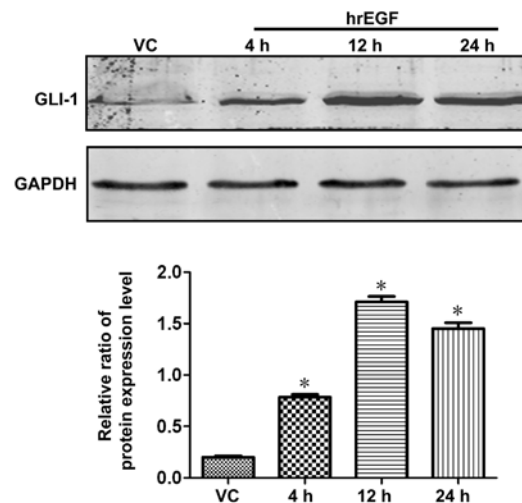


Figure 5. Increased expression of GLI-1 induced by EGF in ARCAPE cells. ARCAPE cell lysates were harvested at 4, 12 and 24 h time-points after a 30-min treatment with hrEGF (100 ng/ml), and were then transferred for western blot analysis. Compared with the vehicle control (VC) cells (parental ARCAPE cells treated with DMSO), the expression level of GLI-1 in ARCAPE cells treated with hrEGF was upregulated at a 4-h time-point following treatment, and the level gradually increased to a peak level at the time-point of 12 h and reached a relatively higher level at 24 h when compared to its baseline level (* $P<0.05$). GAPDH was used as a loading control, and all experiments were conducted in triplicate.

30-min treatment with hrEGF (100 ng/ml). Compared with the negative control cells (parental ARCAPE cells treated with DMSO), the expression level of GLI-1 in ARCAPE cells treated with hrEGF was upregulated at a 4-h time-point following treatment with EGF, and its expression was gradually increased to a peak level at the time-point of 12 h and reached a relatively higher level at 24 h when compared with its baseline level (Fig. 5; $P<0.05$).

GLI-1-specific inhibitor GANT61 reverses the enhanced invasive efficacy induced by EGF in ARCAPE cells. Using Transwell assay for 48 h, it was demonstrated that GLI-1 inhibitor GANT61 dramatically reversed the enhanced invasive effect induced by EGF in ARCAPE cells. The number of invasive ARCAPE cells treated with DMSO, GANT61, hrEGF and hrEGF+GANT61 was 85 ± 8 , 25 ± 2 , 177 ± 10 and 41 ± 4 ,

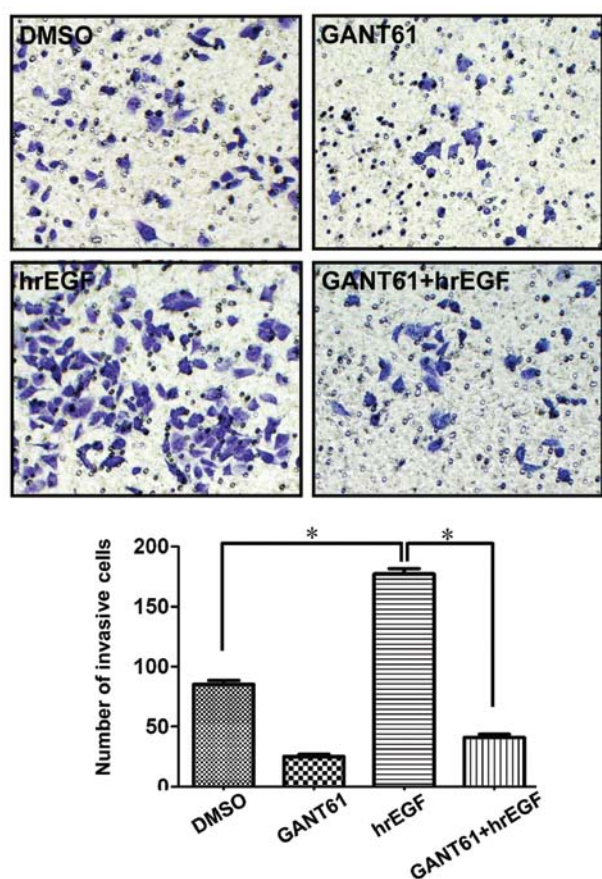


Figure 6. The GLI-1 inhibitor GANT61 reverses the enhanced invasion induced by EGF in ARCaP_E cells. Using Transwell assay for 48 h, it was found that the GLI-1 inhibitor GANT61 reversed the enhanced invasion induced by EGF in ARCaP_E cells. The number of invasive ARCaP_E cells treated with DMSO, GANT61, EGF, and EGF+GANT61 was 85±8, 25±2, 177±10 and 41±4, respectively. All experiments were conducted in triplicate (*P<0.05).

respectively. There was a statistically significant increase in the invasive cell number noted in the hrEGF-treated ARCaP_E cells when compared to the DMSO-treated control (Fig. 6, P<0.05), and a statistically significant decrease in the invasive cell number of hrEGF+GANT61-treated ARCaP_E cells (Fig. 6, P<0.05) was noted when compared to the invasive cell number of hrEGF-treated cells.

Discussion

GLI-1 is the key transcriptional regulator of HH signaling, which is very essential both for normal prostate development and prostate cancer progression (28-37). In mammals, the general schema of this signaling activation involves the binding of Hedgehog ligands to its receptor, Patched (PTC), which is released as an inhibitory function of the protein Smoothened (SMO), a co-receptor for HH signaling, and allows activation of downstream GLI-1 transcriptional factors to activate target genes (38-40). Previous studies have demonstrated that sonic Hedgehog (SHH) signaling pathway activity is common in localized PCa and that it may promote tumor cell proliferation by a combination of autocrine and paracrine signaling, and its activity is dramatically increased in advanced metastatic PCa (32-37). Specifically, Karhadkar *et al* (35) reported that

HH pathway activity is required for regeneration of prostate epithelium, propagation of prostate cancer in xenografts and expression of the stem cell renewal factors in cancer cells, and additionally it was also found that forced HH pathway activity could produce malignant transformation of primitive prostate epithelial progenitor cells, suggesting that prostate cancer might be initiated by trapping of a normal stem cell in a HH-dependent state of continuous renewal. Clinically, Shaw *et al* (36) reported that the HH pathway was activated in patients with androgen-independent prostate cancer (AIPC), and PTC-positive circulating tumor cells could be identified in patients with metastatic AIPC.

Based on the systematic and detailed studies concerning GLI-1 in cancer biology, a growing body of evidence suggests that activation of GLI-1 is not controlled exclusively by HH signaling itself but also by other pathways frequently activated in human malignancies (41-43). GLI-1 activity can be modulated by PI3K/AKT, MEK/ERK, protein kinase C, and transforming growth factor-β/SMAD, which affect stability, subcellular localization, or expression of GLI proteins in various types of cancers (41,44,45). However, the precise role of GLI-1 and its relationship with other signaling cascades in manipulating prostate cancer progression are largely unknown.

EGF signaling is well known for its multifaceted functions in development and tissue homeostasis. Binding of EGF to EGFR modulates cellular function by activating EGFR through autophosphorylation, which results in a downstream cascade that leads to increased cellular proliferation (46). EGF signaling results in activation of phosphoinositol-3 kinase. The latter activates the Akt family of kinases and signal transducer and activator of transcription (STAT), resulting in downstream events that regulate cellular proliferation, survival and migration (47). The EGF family has been extensively investigated for their roles in promoting tumorigenesis and metastasis in a variety of cancer types, including prostate cancer (18,48,49). Furthermore, its receptor, EGFR, is overexpressed in prostate cancers, and the EGF signaling pathway is involved in prostate cancer hormone resistance development (15-17).

Previous studies have demonstrated that EGFR is highly expressed in high grade prostatic intraepithelial neoplasia and in neoplastic cells. Both the ligand and its signaling receptor partner are frequently upregulated in advanced stages of prostate cancer, and are correlated with a high Gleason score and tumor progression from an androgen-dependent to an androgen-independent state (50,51). Targeting EGFR with monoclonal antibodies or with tyrosine kinase inhibitors suppresses the growth and invasion of androgen-dependent and independent prostate cancer cells *in vitro* (52). Although EGFR was demonstrated to play a key role in prostate cancer invasion and metastasis, the precise mechanism of its downstream signaling with other essential molecular pathways in prostate cancer progression is still unclear.

In the present study, we used the exogenous hrEGF to treat prostate cancer ARCaP_E cells *in vitro*, which dramatically increased the cell invasive capability. Additionally, we found that the expression of GLI-1 protein in ARCaP_E cells was upregulated after treatment with EGF when compared with the DMSO control, and p-ERK was also activated upon EGF treatment, although the total expression level of ERK was largely unchanged. Based on these findings, we hypothesized

that the signaling pathway, mediated via p-ERK 'crosstalk' with GLI-1, may play an important role in the elevated invasiveness of ARCAPE cells. Thus, we further blocked the function of GLI-1 using the specific inhibitor, GANT61. As expected, GANT61 dramatically reversed the enhanced invasive capacity of the EGF-treated ARCAPE cells. Thus, taken together, the role of EGF in ARCAPE cell invasiveness may partially depend on the induction of HH signaling transcriptional factor GLI-1 to achieve its function.

This novel finding is significant not only because it is consistent with published reports showing their specific roles in prostate cancer aggressiveness and metastasis, but it may also indicate the possible 'crosstalk' between these key molecules in prostate cancer progression. Actually, several similar studies have also implicated the EGFR pathway in the modulation of HH/GLI activity. For instance, EGF and Sonic HH cooperate to stimulate neural stem cell proliferation and invasive growth of keratinocytes (53-56), and recently Schnidar *et al* (57) reported that synergistic integration of GLI activator function and EGFR signaling is a critical step in oncogenic transformation and provides a molecular basis for therapeutic opportunities relying on combined inhibition of the HH/GLI and EGFR/MEK/ERK/JUN pathway in human basal cell carcinoma. However, few previous studies have shown the direct upregulation of HH transcriptional factor GLI-1 protein by EGF induction in prostate cancer cells as documented in our present study. Although the mechanisms by which EGF induces GLI-1 expression requires further investigation, our data clearly suggest that activation of GLI-1 by p-ERK leads to increased PCa cell invasion, and inhibition of GLI-1 may reverse this enhanced invasiveness induced by exogenous EGF in human ARCAPE prostate cancer cells.

In conclusion, our preliminary *in vitro* study showed that EGF signaling increases ARCAPE human prostate cell invasive capability via upregulation of p-ERK and the HH signaling transcriptional factor GLI-1. Additionally, this enhanced cell invasive capacity was reversed by a GLI-1-specific inhibitor *in vitro*. According to these results, we hypothesize that the EGF and HH pathway may have possible 'crosstalk' with their downstream effectors through unknown molecular mechanisms which synergistically contribute to the enhanced invasiveness of prostate cancer cells. Targeting this molecular 'crosstalk' may be a possible therapeutic strategy which warrants future exploration and drug design concerning anti-EGF signaling for the treatment of patients with advanced prostate cancer.

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