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The role of c-Src in the invasion and metastasis of hepatocellular carcinoma cells induced by association of cell surface GRP78 with activated α_2M

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Abstract

Background: Emerging data have suggested that cell surface GRP78 is a multifunctional receptor and has been linked to proliferative and antiapoptotic signaling cascades. Activated α_2 -macroglobin (α_2M^*) is a natural circulating ligand of cell surface GRP78. Association of cell surface GRP78 with α_2M^* is involved in the regulation of cell proliferation, survival and apoptosis in human cancers.

Methods: The invasion and metastasis of HCC cells were examined using transwell and wound healing assay; Cell surface expression of GRP78 was detected by in cell western assay. Translocation of GRP78 from cytosol to cell surface was observed by transfection of GRP78-EGFP plus TRIRC-WGA staining. The levels of Src, phosphor-Src, FAK, phospho-FAK, EGFR, phospho-EGFR, phospho-Cortactin, phospho-Paxillin were determined by western blot. Cell surface expression of GRP78 in HCC tissue samples was observed by immunofluorescence. The distribution of Paxillin and Cortactin in HCC cells was also observed by immunofluorescence. The interaction between GRP78 and Src were detected by far-western blot, co-immunoprecipitation and GST pulldown. GRP78 mRNA was detected by RT-PCR.

Results: In the current study, we showed that association of cell surface GRP78 with α_2M^* stimulated the invasion and metastasis of HCC. Cell surface GRP78 could interact directly with c-Src, promoted the phosphorylation of c-Src at Y416. Inhibition of the tyrosine kinase activity of c-Src with PP2 reverted the stimulatory effect caused by association of cell surface GRP78 with α_2M^* . Moreover, association of cell surface GRP78 with α_2M^* facilitates the interaction between EGFR and c-Src and consequently phosphorylated EGFR at Y1101 and Y845, promoting the invasion and metastasis of HCCs. However, inhibition of the tyrosine kinase of c-Src do not affect the interaction between EGFR and Src.

Conclusion: c-Src plays a critical role in the invasion and metastasis of HCC induced by association of cell surface GRP78 with α_2M^* . Cell surface GRP78 directly binds and phosphorylates c-Src. As a consequence, c-Src phosphorylated EGFR, promoting the invasion and metastasis of HCCs.

Keywords: Cell surface GRP78, Hepatocellular carcinoma, c-Src, EGFR, Invasion, Metastasis

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Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Invasion and metastasis contributed largely to the high mortality of HCC [2]. Therefore, exploring the mechanisms regulating the invasion and metastasis is critical for searching new strategies to improve the outcome of HCC.

Human α_2 -macroglobulin (α_2 M) is a typical member of the pan-proteinase inhibitors of the α_2 M family, which is mainly synthesized by the liver [3]. Many data have reported that α_2 M is overexpressed in HCC with the background of hepatitis B infection and the increased serological α_2 M is associated with HCC in humans, identifying α_2 M as a cytochemical marker for the diagnosis of HCC [4]. α_2 M is activated by intracellular proteinases. When activated, α_2 M binds directly with corresponding cell surface receptors and functions as a regulator of many signaling pathways and plays a growth factor-like role in many human cancers. So far, two cell surface receptors that specifically bind with activated α_2 M (α_2 M*) have been identified, namely cell surface glucose-regulated protein 78 (GRP78) and LDL receptor related protein (LRP) [5]. Upon most occasions, GRP78 is regarded as an endoplasmic reticulum chaperone, whose major function is to fold and process the unfolded or misfolded proteins [6]. However, it is also presented on the cell surface under stress condition [7]. Cell surface GRP78 acts as a multi-functional receptor which plays critical role in the proliferation, viability and apoptosis [8, 9]. For example, association of cell-surface GRP78 with α_2 M* triggers MAPK and Akt signaling cascades, promoting cellular proliferation of 1-LN prostate cancer cells [3, 10, 11]. Ligation of cell surface GRP78 with α_2 M* activates the NF- κ B signaling pathway, decreases p53 level and plays a stimulatory role in the proliferation and viability of prostate cancer cells [11, 12].

Although a large body of evidence has linked cell surface GRP78 to proliferative and antiapoptotic signaling cascades, little is known about the role of cell surface GRP78 in the invasion and metastasis of human cancer cells. Cellular Src (c-Src), a non-receptor protein tyrosine kinase, is overexpressed and hyperactivated in many human cancers [13, 14]. Increasing evidence has demonstrated that c-Src is implicated in the regulation of a variety of cellular functions, such as tumor invasion and metastasis, by interacting with and phosphorylating a wide range of intracellular proteins including epithelial growth factor receptor (EGFR) [15]. EGFR is a member of the ErbB family of receptor tyrosine kinases and is overexpressed in many types of human cancers including HCC [16]. Accumulating evidence has suggested that Src could form a complex and mediated EGFR phosphorylation, regulating the tumor progression [17].

In this article, we found that GRP78 is overexpressed on the cell surface in HCC tissue samples. Association of cell surface GRP78 with α_2 M* promotes the invasion and metastasis of HCC in hepatocellular carcinoma cell lines QGY-7703 and PLC by stimulating the translocation of GRP78 from the cytosol to plasma. Downstream, cell surface GRP78 interacts directly with c-Src and promotes the phosphorylation of c-Src at Y416. Moreover, association of cell surface GRP78 with α_2 M* facilitates the interaction between EGFR and c-Src. As a consequence, c-Src phosphorylated EGFR at Y1101 and Y845, promoting the invasion and metastasis of HCCs.

Methods

Antibodies and other reagents

The following antibodies were used: anti-Src, anti-pSrcY416, anti-FAKpY397, anti-pPaxillin Y118, anti-Paxillin, anti-pCortactin Y486, anti-pCortactin Y466, anti-Cortactin were all from life technologies; anti-GRP78 N20, antiGRP78 C20 (for antibody blocking), anti- β -actin were from Santa Cruz. Anti-GRP78 (for immunoprecipitation and in cell western analysis), anti-pEGFR Y1101, anti-pEGFR Y1068, anti-pEGFR Y845, anti-EGFR, anti-p-Tyr and rabbit isotype IgG were obtained from Abcam. Anti-EGFP was obtained from Origen. All the secondary antibodies except for IRDYE680RD-conjugated antibody (LI-COR) were all from abcam. α_2 M was purchased from Sigma-Aldrich and activated as previously reported [18]. PP2, lipofectamine 2000, TRITC-WGA and fibronectin were from life technologies. Protein A/G agarose beads and G-sepharose beads were purchased from GE healthcare. RT-PCR kit was from Takara. Plasma protein isolation kit was purchased from Pierce. GRP78-EGFP recombinant and corresponding pEGFP-N1 were kindly given by the Cell Biology Department of China Medical University.

Cell culture and treatment

Human hepatocellular carcinoma cell line QGY-7703 and PLC were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM supplemented with 10 % fetal bovine serum, 1 mM L-glutamine and 1 % penicillin/streptomycin antibiotics. For experimental purpose, cells were serum starved for 4 h. PP2 (10 μ M for 30 min) or blocking antibodies (4 μ g/ml for 1 h) were added for 1 h, following α_2 M* stimulation (50pM for 30 min), cells were harvested and subjected to subsequent analysis.

Human tissue specimens and ethics statement

All 10 cases of paired HCC tissue samples were obtained from the Department of Gastroenterology of the General Hospital of Chinese Liberation Army. The Ethics Committee of Liaoning Medical College approved and supervised

specimen collection procedures (No. 20130023). All the experimental performances related to the tissue samples were in compliance with Helsinki Declaration. We have got the permissions of all the patients before specimen collection. The differentiation extents were re-evaluated by two pathologists according to Edmondson-Steiner grading system. None of the patients has received chemotherapy or irradiation before surgery.

Transwell, wound healing assay

In vitro cell invasion and migration were analyzed using transwell assay and wound healing assay as previously described [19]. The experiments were repeated for 3 times and the data were represented as $\bar{X} \pm SD$.

In cell western assay

In cell western were carried out as previously reported [10]. Briefly, confluent cells were harvested, diluted to 4×10^5 cells/mL with complete culture medium and dispensed to 96-well culture plate (100 μ l per well). After 24 h, cells were rinsed for three times with cold PBS and serum starved for 4 h. following $\alpha 2M^*$ stimulation (50pM for 30 min). Cells were fixed with 2 % formaldehyde in PBS, washed for three times with PBS with or without 0.05 % Tween 20, blocked with 3 % BSA for 2 h with gentle agitation. After blocking, cells were incubated with anti-GRP78 (1:50 dilution) in blocking solution overnight with gentle agitation, stained with IRDYE 800 D-conjugated secondary antibody in PBS for 60 min at RT and washed for three times with PBS and imaged by LICOOR Odyssey[®]. Cell surface GRP78 were quantified according to the manufacturer's instructions.

Western blot

For all western blot experiments, established protocols were performed [20], using antibodies that recognize phosphorylated or unphosphorylated EGFR, FAK, c-Src, Cortactin, Paxillin, β -actin.

Cell adhesion assay

The binding ability of tumor cells with fibronectin was detected using cell adhesion assay. Briefly, pretreated cells (10^4 each well) were trypsinized, re-plated on fibronectin-coated coverslips (10 μ g/ml) and incubated at 37 °C for 1 h. Following incubation, non-adherent cells were removed by washing for 3 times with PBS, adherent cells were fixed with 4 % paraformaldehyde solution, stained with crystal violet (1 %) and dissolved by 1 % SDS. Absorbances at 595 nm were determined using a microplate reader.

Transfection and plasma staining

Cells were cultured in a six-well culture plate and grown to 90 % confluent. Before transfection, Cells were cultured in complete medium without antibiotics overnight and transfected with 4 μ g GRP78-EGFP or pEGFP-N1 for 16 μ l lipofectamine 2000 in 500 μ l Opti-MEM (Gibco). After 48 h, cells were serum starved for 4 h, simulated with $\alpha 2M^*$ for 30 min stained with TRITC conjugated WGA (1:200 dilution) for 1 h under normal culture condition. Following WGA staining, cells were observed using inverted fluorescence microscope (DMI-4000B, Leica, Germany).

Immunofluorescence

The cellular distribution of Cortactin, Paxillin and GRP78 was observed using immunofluorescence [21, 22]. Immunofluorescence was performed as previously reported and observed using laser confocal microscope (SP5II, Leica, Germany).

Cell surface protein biotinylation

Confluence cells were rinsed with ice-cold PBS twice and serum starved for 4 h. PP2 was added for 30 min. following $\alpha 2M^*$ stimulation, EZ-link Sulfo-NHS-LC-Biotin (Pierce, USA) was added to cover the surface of the cell layer and the flasks were gently shaken at 4 °C for 30 min. The biotinylation reaction was stopped by adding Tris-HCl (pH 7.5) to a final concentration to 100 nM. Then, the cells were rinsed with ice-cold PBS twice and then lysed with RIPA buffer and Neutravidin-agarose beads (Pierce, USA) were added in whole cell lysate overnight and incubated at 4 °C with agitation. The beads were washed for 5 times with PBS buffer. The cell surface proteins were released by 1 \times SDS-PAGE sample loading buffer and heating at 100 °C for 5 min. The cell surface proteins were subjected to western blotting analysis.

RT-PCR

Conventional RT-PCR was performed as previously reported [23].

Far-western blot assay

Far-western blot was performed as previously reported [24]. Cell surface proteins (50 μ g each lane) were resolved by 10 % SDS-PAGE, transferred onto PVDF membranes (Millipore, USA). The membranes were incubated at room temperature for 2 h in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 % (v/v) Tween 20, 0.5 % (w/v) bovine serum albumin (TBST/BSA) and then overnight at 4 °C on a shaker with the biotin-conjugated recombinant human GRP78(rhGRP78) (5 μ g in 1 ml TBST/BSA). The membranes were washed for 3 times with TBST, incubated for 4 h with anti-biotin antibody (1:1000 dilution). Following incubation with primary antibody,

The membranes were washed and incubated for 1 h with appropriate AP-conjugated secondary antibody (1:5000 dilution). The membrane were stained with BCIP/NBT solution and photographed with a bioimaging system (I-BOX, UVP, USA).

Co-immunoprecipitation assay

One thousand micrograms of plasma membrane protein extract from each sample was pre-cleared with 50 μ l of protein A/G-Sepharose beads for 1 h at 4 °C and incubated with 5 μ g of anti-GRP78 or anti-EGFR (Abcam) overnight at 4 °C on a rotator. Following antibody incubation, 50 μ l of protein A/G-Sepharose beads (50 % slurry) were added and rotated at 4 °C for 3 h. The beads were then centrifuged at 12,000 g for 5 min and washed for 5 times with the lysis buffer. The precipitates were eluted by adding of 20 μ l of 1 \times SDS-PAGE sample loading buffer (50 mm Tris-HCl, pH 6.8, 100 mm DTT, 2 % SDS, 0.1 % bromphenol blue, 10 % glycerol), followed by heating at 100 °C for 5 min. The supernatant obtained after centrifugation was resolved by SDS-PAGE and subjected to Western blot analysis.

GST pulldown assay

Plasma membrane extract was isolated as described previously. The clarified plasma membrane extract was incubated with 50 μ l of a 50 % slurry of glutathione-Sepharose 4B (GE Healthcare, USA) and 25 μ g GST for 1 h at 4 °C. GST-Grp78-bound resin, GST-bound resin was incubated with cell lysates containing 1 mg protein extract overnight at 4 °C on a rotator. The resin was then washed 5 times with ice-cold lysis buffer. Proteins were eluted by adding 25 μ l of 2 \times Laemmli sample buffer at 100 °C for 5 min and centrifuged for 5 min at 12,000 g. The supernatant was resolved by SDS-PAGE and subjected to Western blot analysis using anti-c-Src (Abcam).

Statistical analysis

Comparison of the data was performed using one way ANOVA, student *t*-test and chi-square test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Association of cell surface GRP78 with α 2M* facilitated the invasion and metastasis of HCC

Increasing data have linked α 2M* to pro-proliferative and anti-apoptotic role. However, whether it plays a role in the invasiveness and metastasis of HCC remains unknown. To investigate the role of α 2M* in tumor invasion, QGY-7703 and PLC cells were serum starved for 4 h, followed by stimulation with α 2M* for 30 min (50 pM) [10]. Using transwell assay, we found that treatment of QGY-7703 and PLC cells with α 2M* caused significant increase in the invasiveness relative to that in cells

treated with vehicle, indicating that α 2M* stimulation facilitates the invasion of QGY-7703 (Fig. 1a) and PLC cells (Fig. 1b). We next assessed whether α 2M* modulate the metastasis of cancer cells using wound healing assay and found that stimulation with α 2M* significantly facilitated the metastasis of QGY-7703 (Fig. 1c) and PLC cells (Fig. 1d).

To investigate whether cell surface GRP78 is the surrogate of α 2M* in this process, serum starved QGY-7703 and PLC cells were pretreated with the antibody directed against the NH₂-terminal domain (NTD), COOH-terminal domain (CTD) of GRP78 or isotype IgG for 1 h followed by α 2M* stimulation [25, 26]. Transwell assay revealed that pretreatment with the GRP78 NTD antibody caused a marked decrease in the invasion potential as compared with vehicle upon α 2M* stimulation. However, pretreatment with CTD had no effect on the invasion potential as compared with pretreatment with isotype IgG or vehicle upon α 2M* stimulation (Fig. 1e-g). Using cell adhesion assay, we found that blockade of cell surface GRP78 with the NTD antibody decreased the binding ability of tumor cells to fibronectin in QGY-7703 cells (Fig. 1h). These data suggested that cell surface is the surrogate of α 2M* and cell surface GRP78 promotes the invasion and metastasis by its NH₂-terminal domain.

α 2M* induced GRP78 translocation from cytosol to cell surface in HCC

We next determined whether α 2M* affected the cell surface expression of GRP78 in QGY-7703 and PLC cells. Serum starved QGY-7703 and PLC cells were treated with α 2M* and vehicle. Using in cell western assay, cell surface and total GRP78 were determined with or without Tween 20 permeabilization. We found that α 2M* stimulation significantly elevated the cell surface fraction of GRP78, whereas did not affect total GRP78 in QGY-7703 and PLC cells (Fig. 2a). In QGY-7703 cells, α 2M* stimulation caused a ~2.7 fold increase in the cell surface expression of GRP78 as compared with vehicle. In PLC cells, a ~2.1 fold increase was observed upon α 2M* stimulation (Fig. 2b). Using RT-PCR, we found that GRP78 mRNA levels in cells treated with α 2M* were similar with that in QGY-7703 and PLC cells treated with vehicle (Fig. 2c). These data suggested that the elevated expression of cell surface GRP78 is not regulated at transcriptional and translational level and raised the possibility that α 2M* could induce the translocation of GRP78 from cytosol to plasma membrane. To answer this question, we transiently transfected GRP78-EGFP into QGY-7703 cells [27]. After 48 h of transfection, exogenous and endogenous GRP78 were examined using western blot (Fig. 2d). QGY-7703 cells transfected with GRP78-EGFP were serum starved for 4 h, followed by α 2M* stimulation, stained with TRITC-conjugated wheat germ

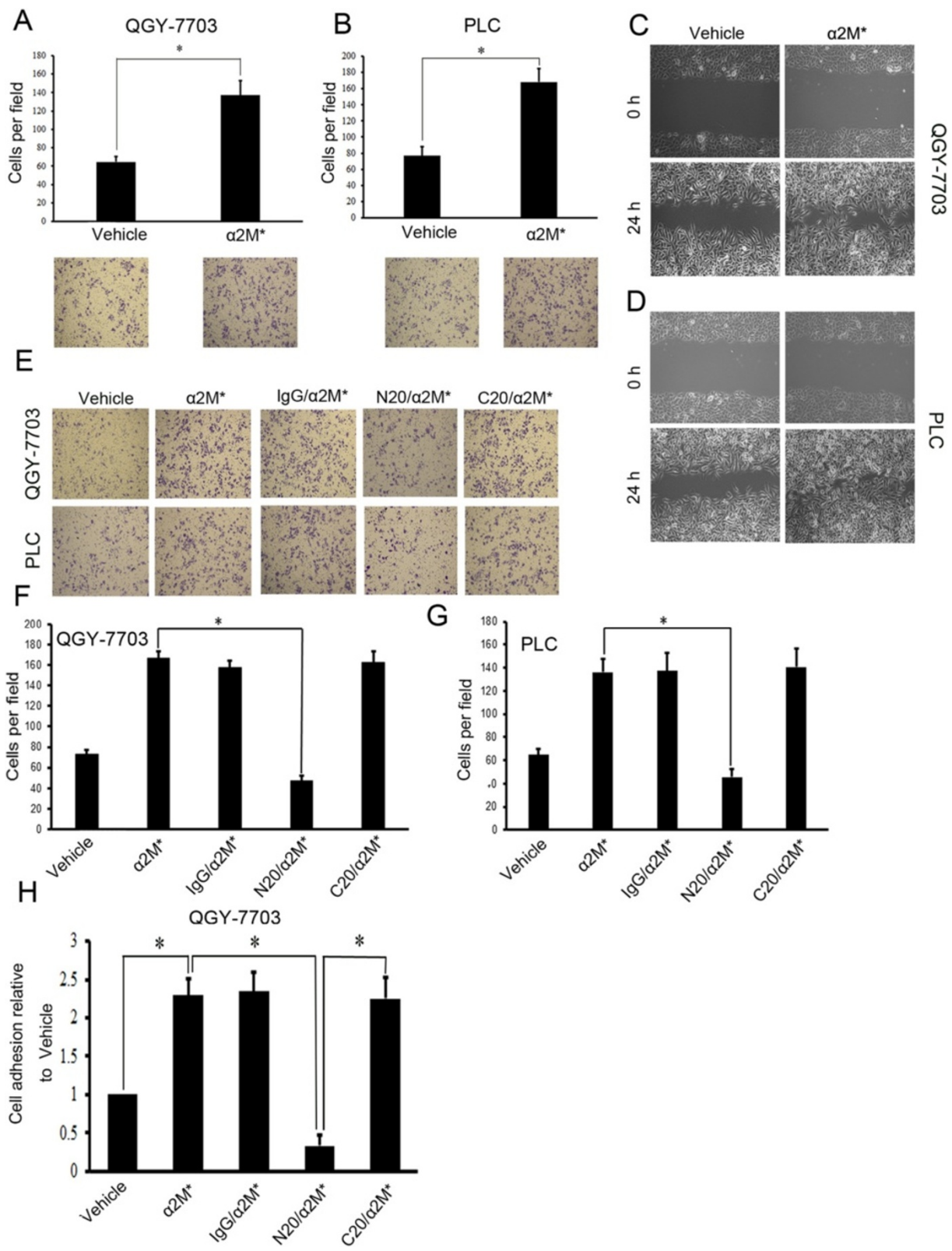


Fig. 1 (See legend on next page.)

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Fig. 1 Association of cell surface GRP78 with $\alpha 2M^*$ facilitated the invasion and metastasis of HCC. **(a-b)** Transwell analysis of the invasion of serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$. **(c-d)** Wound healing analysis of the migration of serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$. **(e)** Transwell analysis of the effect of antibody blocking on the invasion of serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$. **(f-g)** Quantitative analysis of the effect of antibody blocking on the invasion of serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$. **(h)** Cell adhesion analysis of the effect of antibody blocking on the binding ability to fibronectin in QGY-7703 cells. All these experiments were repeated for 3 times in triplicate. These data were presented as $\bar{X} \pm SD$ and analyzed using student's *t*-test and one-way ANOVA. The difference is regarded to be statistically significant when $p < 0.05$. *represented that the difference is statistically significant

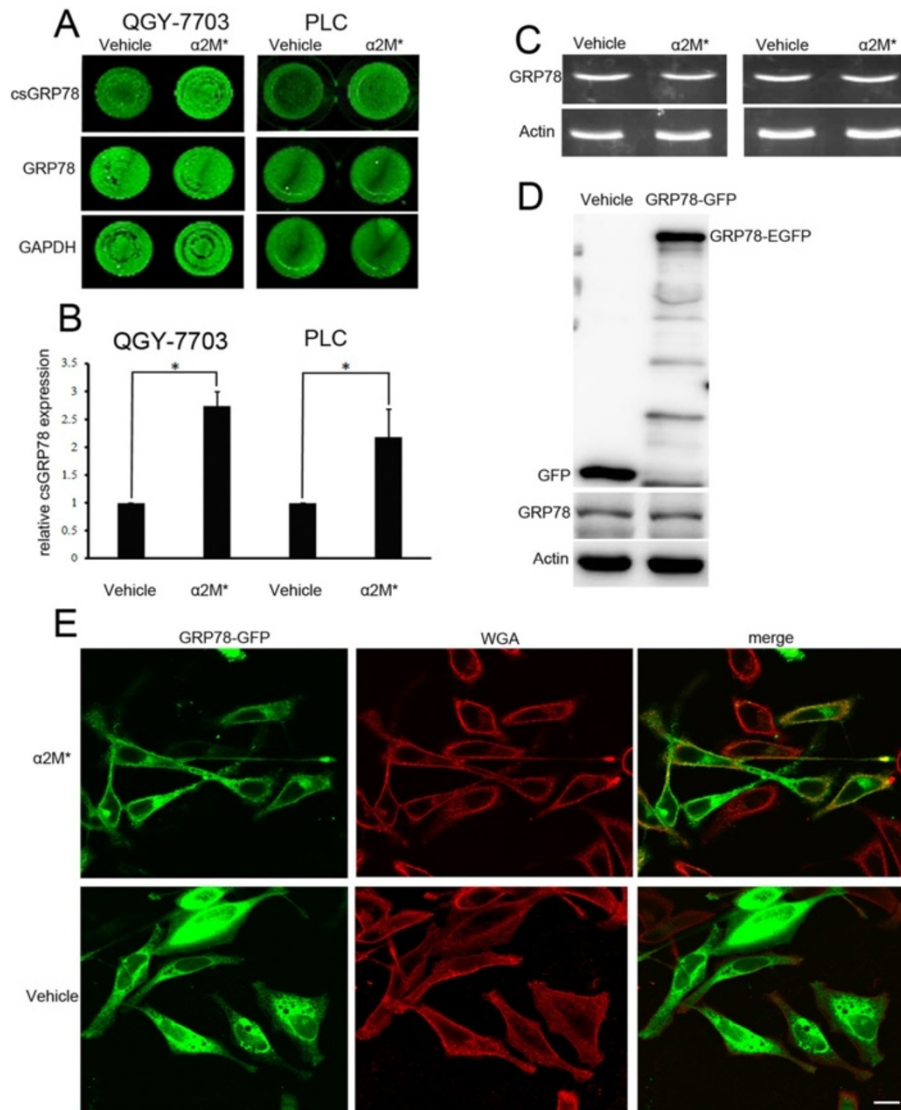


Fig. 2 $\alpha 2M^*$ induced GRP78 translocation from cytosol to cell surface in HCC. **(a)** In cell western analysis of cell surface GRP78 and total GRP78 in serum starved QGY-7703 and PLC cells treated with vehicle or $\alpha 2M^*$. **(b)** Schematic show of cell surface GRP78 expression in serum starved QGY-7703 and PLC cells treated with vehicle or $\alpha 2M^*$. The experiments were repeated for 3 times in triplicate. The data were presented as $\bar{X} \pm SD$ and analyzed using student's *t*-test. The difference is regarded to be statistically significant when $p < 0.05$. * represented that the difference is statistically significant. **(c)** RT-PCR analysis of GRP78 mRNA levels in serum starved QGY-7703 and PLC cells treated with vehicle or $\alpha 2M^*$. **(d)** Western blot analysis of exogenous and endogenous GRP78 in serum starved QGY-7703 cells transfected with GRP78-EGFP or pEGFP-N1. **(e)** Confocal microscopy observation of GRP78 translocation from cytosol to cell surface in serum starved QGY-7703 cells with or without $\alpha 2M^*$ stimulation. Scale Bar 25 μm

agglutinin (TRITC-WGA), a specific plasma membrane dye. Using confocal microscopy, we found that GRP78 colocalized with TRITC-WGA on the cell surface (yellow on the cell surface) in $\alpha 2M^*$ treated cells. By contrast, the colocalization of GRP78 and TRITC-WGA was not observed in cells treated with vehicle (Fig. 2e). These data demonstrated that $\alpha 2M^*$ induces the translocation of GRP78 from cytosol to cell surface.

c-Src interacts directly with GRP78 on the cell surface of HCCs

We have previously reported that cell surface GRP78 facilitates the invasion of HCC [28]. To further extend this finding, we sought to determine the downstream target of cell surface GRP78. For this purpose, we compared tyrosine-phosphorylated proteins in serum starved QGY-7703 cells treated with $\alpha 2M^*$ or vehicle. As determined by western blot using anti-p-Tyr antibody. A tyrosine phosphorylated band migrating at ~60 KDa was significantly induced in QGY-7703 cells treated with $\alpha 2M^*$, suggesting that cell surface GRP78 may regulate the phosphorylation of a unknown tyrosine kinase whose

molecular weight is ~60 KDa (Fig. 3a). Based on this result, we tried to determine whether cell surface GRP78 regulated the activity of c-Src in HCCs. We first identified GRP78 binding proteins in the whole cell lysates from QGY-7703 and PLC cells by far-western blotting using the biotin-conjugated rhGRP78 as the bait. For this purpose, we isolated plasma membrane proteins using cell surface protein biotinylation assay and performed co-immunoprecipitation experiment using the anti-GRP78 antibody (Abcam). As shown in Fig. 3b, a protein, migrating at ~60 kDa, could interact specifically with the biotin-conjugated rhGRP78. Moreover, we subjected the precipitated proteins to SDS-PAGE and found a ~60 kDa band in the precipitated proteins. Western blotting showed that c-Src is presented in the proteins co-precipitated with GRP78, suggesting that the cell surface GRP78 modulates the activity of Src by direct interaction (Fig. 3c). Finally, we performed GST-pulldown assay to verify the interaction between cell surface GRP78 and c-Src using GST-GRP78 as the bait. We found that a ~60 kDa band by SDS-PAGE and identified that this protein was c-Src by western blot (Fig. 3d).

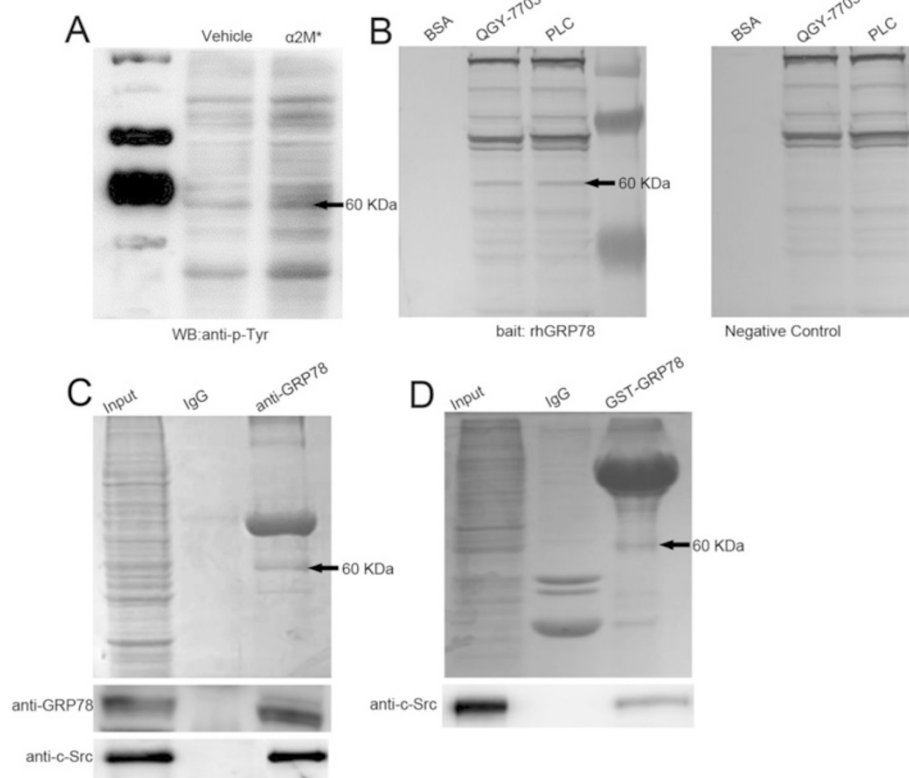


Fig. 3 c-Src interacts directly with GRP78 on the cell surface of HCCs. **(a)** Western blot analysis of the tyrosine-phosphorylated proteins in serum starved QGY-7703 cells treated with vehicle or $\alpha 2M^*$. **(b)** Far western blot analysis of GRP78 binding proteins in the whole cell lysates from serum starved QGY-7703 cells and PLC cells using biotin conjugated rhGRP78 as bait. **(c)** Co-immunoprecipitation analysis of the interaction between GRP78 and c-Src in the plasma membrane extracts from serum starved QGY-7703 cells using anti-GRP78 (Abcam) as precipitating antibody. Upper, SDS-PAGE; bottom, western blot. **(d)** GST pull-down analysis of the interaction between GRP78 and c-Src in the plasma membrane extracts from serum starved QGY-7703 cells using GST-GRP78 as the bait. Upper, SDS-PAGE; bottom, western blot

Association of cell surface GRP78 with $\alpha 2M^*$ activates c-Src and its downstream signaling pathways

Using western blot, we examined p-Src phosphorylation at Y416 in cell lysates from serum starved QGY-7703 and PLC cells treated with $\alpha 2M^*$ or vehicle. We found that $\alpha 2M^*$ stimulation significantly increased p-c-Src levels as compared with vehicle in both QGY-7703 and PLC cells, indicating that cell surface GRP78 facilitates c-Src phosphorylation in HCC (Fig. 4a). We next determined whether the tyrosine kinase activity of c-Src is involved in the regulation of adhesion and invasion of HCC cells in QGY-7703 cells. Serum starved QGY-7703 cells were pretreated with PP2 at a concentration of 10 μM for 30 min followed by $\alpha 2M^*$ stimulation. Cell adhesion assay revealed that pretreatment of serum starved QGY-7703 cells with PP2 caused a significant decrease in binding ability to fibronectin as compared with vehicle upon $\alpha 2M^*$ stimulation. No difference was observed between cells treated with PP2 alone and cells treated with PP2 followed by $\alpha 2M^*$ stimulation (Fig. 4b). The invasion potentials were analyzed using transwell assay. As shown in Fig. 4c, pretreatment of serum starved QGY-7703 cells with PP2

caused a marked decrease in the invasion potential as compared with vehicle upon $\alpha 2M^*$ stimulation. It is worth to note that QGY-7703 cells treated with PP2 alone share similar inhibitory extent on tumor invasion as compared with cells pretreated with PP2 followed by $\alpha 2M^*$ stimulation. Similar results were obtained in PLC cells (Fig. 4d-e). These data suggested that c-Src is the downstream signaling molecule of $\alpha 2M^*$. We also examined whether cell surface GRP78 could activate c-Src downstream signaling pathways [29]. As shown in Fig. 4f, treatment of serum starved QGY-7703 and PLC cells with $\alpha 2M^*$ caused significant increase in the phosphorylation levels of FAK (Y397), Cortactin (Y486 and 466) and Paxillin (Y118) as compared with vehicle treated cells.

Although many data have demonstrated that $\alpha 2M^*$ could bind with cell surface GRP78 and stimulate the signaling pathways downstream of cell surface expression of GRP78, we still need to preclude the possibility that $\alpha 2M^*$ binds with other cell surface protein and facilitates c-Src phosphorylation. To obtain this goal, serum starved QGY-7703 and PLC cells were incubated with the antibody directed against the NH2-terminal domain (NTD)

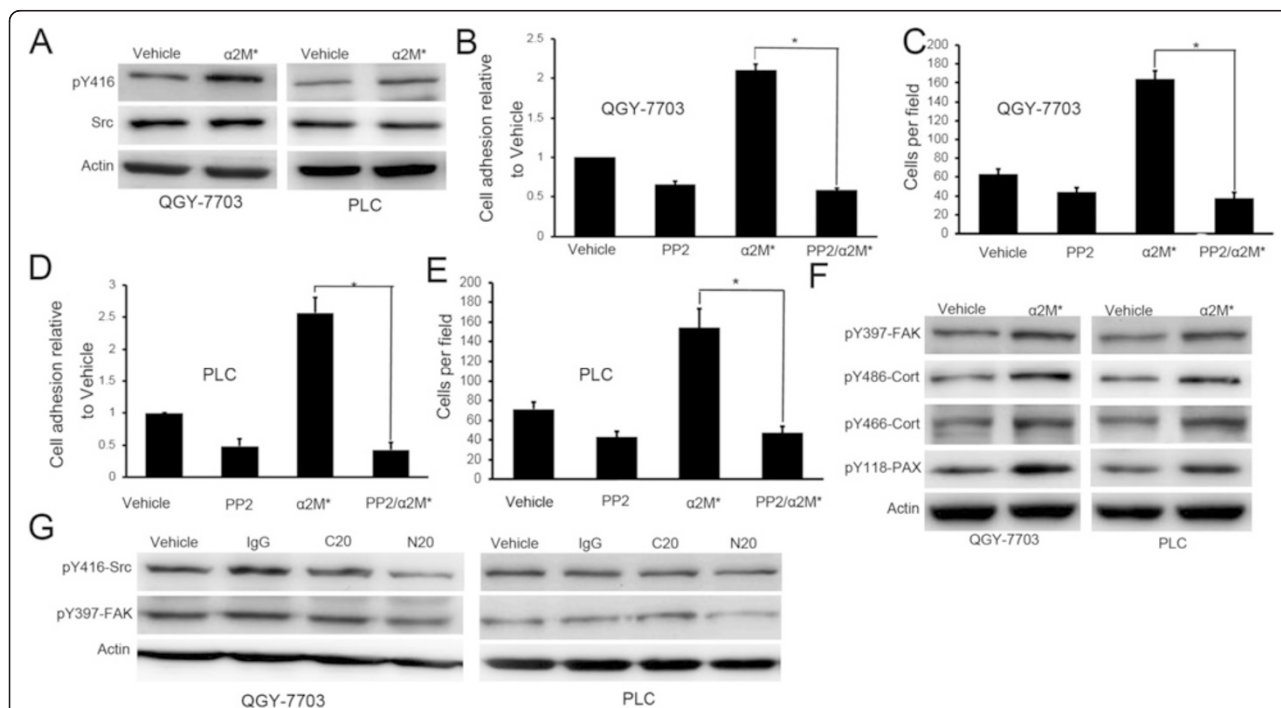


Fig. 4 Association of Cell surface GRP78 with $\alpha 2M^*$ activates c-Src and its downstream signaling pathways. **(a)** Western blot analysis of the expression levels of c-Src and pY416-Src in serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$. **(b)** Cell adhesion analysis of the effect of PP2 pretreatment on the binding ability of serum starved QGY-7703 cells to fibronectin upon $\alpha 2M^*$ stimulation. **(c)** Transwell analysis of the effect of PP2 pretreatment on the invasion of serum starved QGY-7703 cells upon $\alpha 2M^*$ stimulation. **(d)** Cell adhesion analysis of the effect of PP2 pretreatment on the binding ability of serum starved PLC cells to fibronectin upon $\alpha 2M^*$ stimulation. **(e)** Transwell analysis of the effect of PP2 pretreatment on the invasion of serum starved PLC cells upon $\alpha 2M^*$ stimulation. **(f)** Western blot analysis of the phosphorylation status of c-Src downstream signaling pathways in serum starved QGY-7703 and PLC cells treated with vehicle and $\alpha 2M^*$ **(g)** Western blot analysis of the effect of antibody blocking on the phosphorylation levels of c-Src at Y416 and FAK at Y397 in serum starved QGY-7703 and PLC cells. These experiments were repeated for 3 times in triplicate. The data were presented as $\bar{X} \pm SD$ and analyzed using student's *t*-test. The difference is regarded to be statistically significant when $p < 0.05$. *represented that the difference is statistically significant

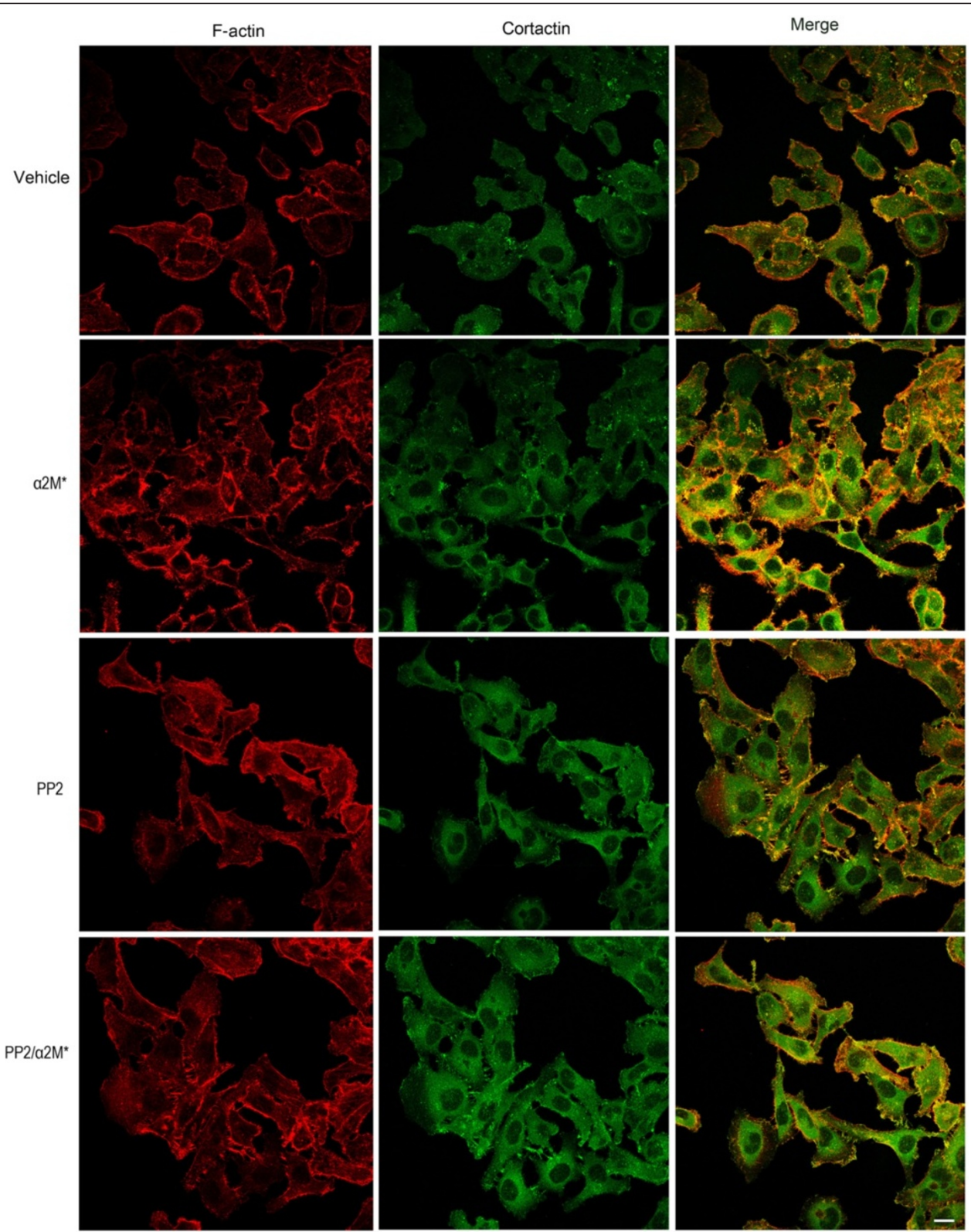


Fig. 5 Association of Cell surface GRP78 with α2M* induces invadopodia formation. QGY-7703 cells were treated with vehicle, α2M*, PP2 or PP2 in combination with α2M* and co-stained with TRITC-conjugated Phalloidin and anti-Cortactin antibody. The distribution of F-actin (red) and cortactin (green) was observed using a confocal microscope. The invadopodia was indicated as yellow patches. Scale Bar 25 μm

or COOH-terminal domain (CTD) of GRP78 for 1 h prior to $\alpha 2M^*$ stimulation. Many reports by other groups have demonstrated that the antibodies we used could block the binding of cell surface GRP78 with $\alpha 2M^*$. Western blot analysis showed significantly lower pY416-Src and pY397-FAK levels in cells pretreated with NTD antibody as compared with cells pretreated with isotype IgG upon $\alpha 2M^*$ stimulation. However, pretreatment with CTD antibody did not affect pY416-Src and pY397-FAK levels (Fig. 4g). These data suggested that cell surface GRP78 is the surrogate of $\alpha 2M^*$ induced c-Src phosphorylation and activates c-Src via its NH2-terminal domain.

Association of cell surface GRP78 with $\alpha 2M^*$ induces invadopodia formation and Paxillin redistribution

Invadopodia is a specialized invasive organelle for tumor cells undergoing invasion and metastasis [30]. To investigate whether cell surface GRP78 regulates the formation

of invadopodia, the distribution of Cortactin in serum starved QGY-7703 cells treated with $\alpha 2M^*$ or vehicle was observed using immunofluorescence [21]. By co-staining of Cortactin and F-actin, we observed that treatment with $\alpha 2M^*$ caused a marked increase in the number of speckles in cell cortex as compared with that treated with vehicle, while pretreatment with PP2 significantly decreased the number of speckles on cell cortex. Furthermore, $\alpha 2M^*$ stimulation caused a subtle increase the number of speckles in cell cortex in PP2 pretreated cells, indicating that c-Src is essential for the formation of invadopodia induced by association of cell surface GRP78 with $\alpha 2M^*$ (Fig. 5).

We also observed whether association of cell surface GRP78 with $\alpha 2M^*$ could cause the redistribution of Paxillin. Immunofluorescence microscopy revealed that Paxillin exhibited a dense punctate distribution on the cell periphery in serum starved QGY-7703 cells treated with $\alpha 2M^*$ as compared with that treated with vehicle,

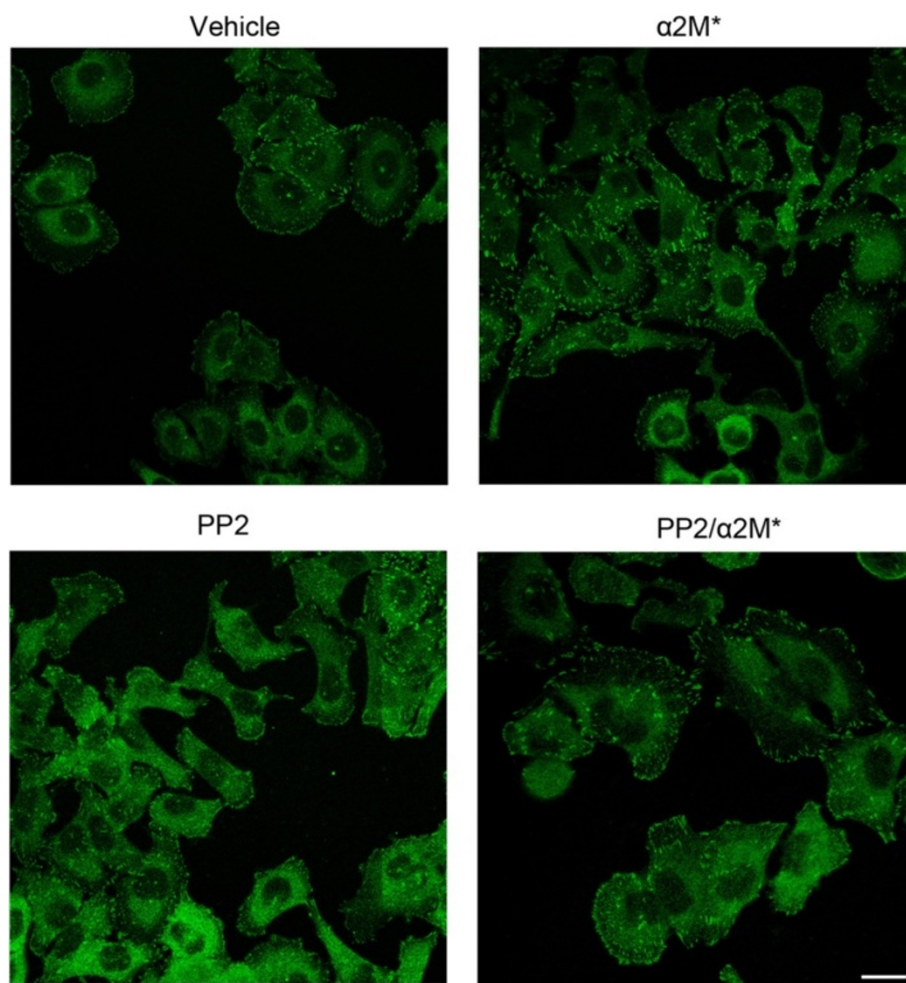


Fig. 6 Association of Cell surface GRP78 with $\alpha 2M^*$ induces paxillin formation. QGY-7703 cells were treated with vehicle, $\alpha 2M^*$, PP2 or PP2 in combination with $\alpha 2M^*$ and stained with anti-Paxillin antibody. The distribution Paxillin (green) was observed using a confocal microscope. Scale Bar 25 μm

indicating that cell surface GRP78 induced the redistribution of Paxillin. Pretreatment with PP2 decreased the cell periphery distribution. Moreover, $\alpha 2M^*$ stimulation caused a moderate increase in the cell periphery distribution of Paxillin in PP2 pretreated cells, indicating the critical role of c-Src in Paxillin redistribution caused by association of cell surface GRP78 with $\alpha 2M^*$ (Fig. 6).

Association of cell surface GRP78 with $\alpha 2M^*$ facilitated the maximal activation of EGFR in a c-Src dependent manner

For the critical role of EGFR in the invasion and metastasis of HCC, we wonder whether cell surface GRP78 could regulate EGFR phosphorylation. For this purpose, we examined EGFR phosphorylation levels at Y1068, Y845 and Y1101 in serum starved QGY-7703 cells treated with $\alpha 2M^*$ or vehicle. Using western blot, we found that $\alpha 2M^*$ stimulation significantly increased the phosphorylation level of EGFR at Y1068, Y845 and Y1101 as compared with vehicle, while did not affect EGFR expression. Pretreatment of serum starved QGY-7703 cells with PP2 caused a marked decrease in the phosphorylation of EGFR at Y845 and Y1101 as compared with cells pretreatment with vehicle upon $\alpha 2M^*$ stimulation. However, PP2 treatment did not affected EGFR phosphorylation at Y1068 (Fig. 7a, b).

Accumulating evidence has shown that EGFR is a downstream signaling molecule of c-Src and c-Src could directly bind with EGFR and activate EGFR [31, 32, 17]. We next determined whether cell surface GRP78 could regulate the interaction between EGFR and c-Src. For this purpose, whole cell lysates from serum starved QGY-7703 cells treated with $\alpha 2M^*$ or vehicle were immunoprecipitated with excessive anti-EGFR antibody (~5 μ g in a 1 ml volume containing 1 mg of total protein). The precipitations were subjected to western blot analysis and we found that c-Src level in the whole cell lysate from $\alpha 2M^*$ treated cells was higher than that from vehicle treated cells. It is worth to note that c-Src was not detected in isotope IgG precipitation. These data indicated that cell surface GRP78 facilitates the interaction between EGFR and c-Src (Fig. 7c). These data raised the question whether the tyrosine kinase activity of c-Src is essential for the interaction between EGFR and c-Src induced by cell surface GRP78. Using Co-immunoprecipitation in the lysates from serum starved QGY-7703 cells pretreated with PP2 or vehicle prior to $\alpha 2M^*$ stimulation, we found that the amount of c-Src co-immunoprecipitated with EGFR in the whole cell lysate from PP2 pretreated cells were paralleled with that from cells pretreated with vehicle, indicating that the tyrosine kinase activity of c-Src is not essential for the interaction between EGFR and c-Src (Fig. 7c, d).

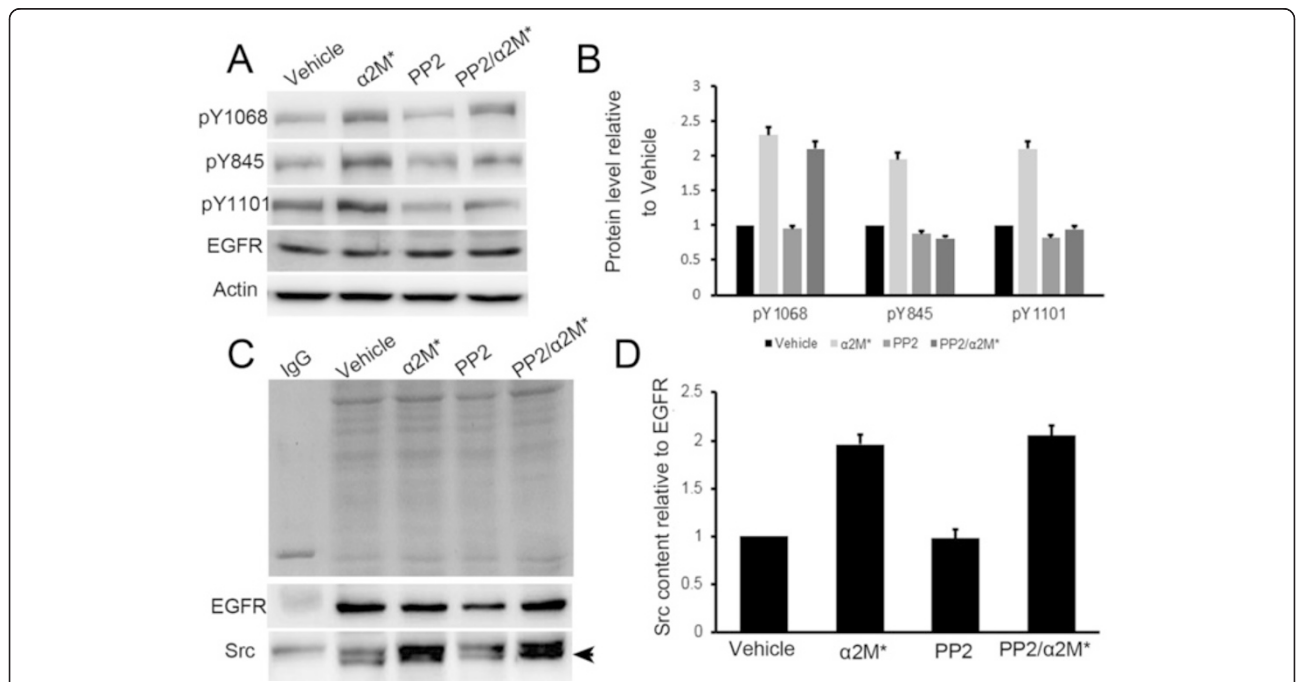


Fig. 7 Association of Cell surface GRP78 with $\alpha 2M^*$ facilitated the maximal activation of EGFR in a c-Src dependent manner. **(a)** Western blot analysis of the expression and phosphorylation of EGFR in serum starved QGY-7703 treated with vehicle, $\alpha 2M^*$, PP2 or PP2 in combination with $\alpha 2M^*$. **(b)** Quantitative analysis of the expression and phosphorylation of EGFR in serum starved QGY-7703 treated with vehicle, $\alpha 2M^*$, PP2 or PP2 in combination with $\alpha 2M^*$. **(c)** Co-immunoprecipitation analysis of the interaction between c-Src and EGFR in serum starved QGY-7703 cells treated with vehicle, $\alpha 2M^*$, PP2 or PP2 in combination with $\alpha 2M^*$. **(d)** Quantitative analysis of the interaction between c-Src and EGFR in serum starved QGY-7703 cells treated with vehicle, $\alpha 2M^*$, PP2 or PP2 in combination with $\alpha 2M^*$

GRP78 is highly expressed on the cell surface in tissue samples of HCC

Using immunofluorescence, we observed the cellular localization of GRP78 in ten cases of paired surgically resected HCC tissue samples. We found that GRP78 is expressed both in the cytosol and on the cell surface in 9 of 10 cases HCC tissue samples (Fig. 8c, d). By contrast, cell surface staining of GRP78 was not observed (7 of 10 cases) or very weak (3 of 10 cases) of paired peri-cancer tissue samples (Fig. 8a, b). Statistical chi-square test revealed that the difference is significant ($p < 0.01$). These data demonstrated that GRP78 is highly expressed on the cell surface of HCC.

Discussion

Cell surface GRP78 has been implicated in the progression of many human cancers including melanoma, breast, prostate cancer and hepatocellular carcinoma [9]. Lots of data have linked cell surface GRP78 to pro-proliferative and anti-apoptotic signaling pathway [33]. However, the role of cell surface GRP78 in tumor invasion and metastasis remains poor documented. In this article, we

stimulated hepatocellular carcinoma cell lines PLC and QGY-7703 with $\alpha 2M^*$ and demonstrated that c-Src is the surrogate in cell surface GRP78 induced tumor invasion and metastasis. Cell surface GRP78 facilitated the phosphorylation of c-Src at Y416 via direct interaction.

Although many data have identified $\alpha 2M^*$ as a natural ligand of cell surface GRP78, the mechanism by which $\alpha 2M^*$ activates cell surface GRP78 remains unclear. Here, we found that $\alpha 2M^*$ did not augment GRP78 expression at transcriptional and translational levels. Based on these results, we hypothesized that elevated presence of GRP78 on the cell surface may be caused by GRP78 redistribution. In consistent with our hypothesis, we demonstrated that $\alpha 2M^*$ could induce GRP78 translocation from cytosol to plasma. Furthermore, we demonstrated that cell surface GRP78 is the surrogate of $\alpha 2M^*$ induced invasion and metastasis in HCC. It is the NH₂-terminal domain of GRP78 but not the COOH-terminal domain played critical role in tumor invasion and metastasis. Other reports have demonstrated the critical roles of COOH-terminal domain of GRP78 in the regulation of proliferation and apoptosis in many types of human

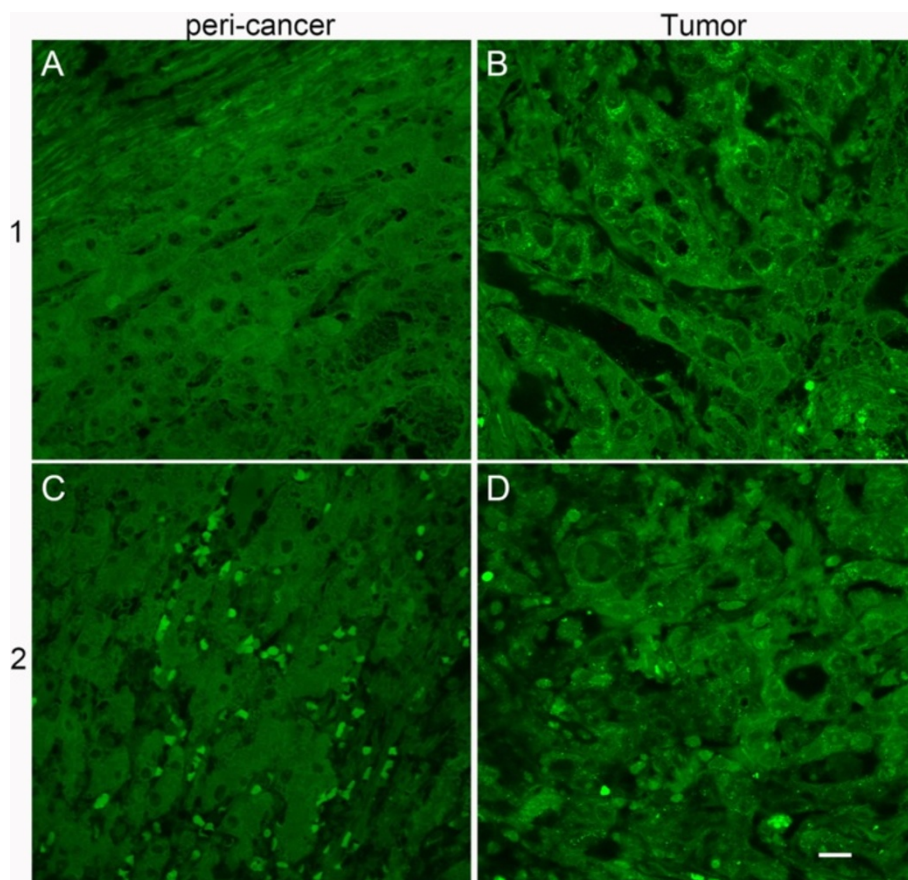


Fig. 8 Cell surface GRP78 expression in tissue samples of HCC. Immunofluorescence analysis of GRP78 expression in 10 cases paired HCC samples. GRP78 is localized on the cell surface in HCC tissue samples (b) and (d). On the contrary, it is not expressed (c) or expressed at very low level (a) in paired peri-cancer tissue samples. Scale Bar 25 μ m

cancers including melanoma, breast and prostate cancers [3, 10, 34]. For example, ligation of cell surface GRP78 with $\alpha 2M^*$ promotes the proliferation of cancer cells in prostate cancer cells, blockade of cell surface GRP78 using the antibody against the COOH-terminal domain of GRP78 reverted the pro-proliferative effect of $\alpha 2M^*$ [10, 11, 35, 36]. Taken together, these data suggested that cell surface GRP78 regulated the invasion, metastasis, proliferation and apoptosis with different mechanism. That is the NH₂-terminal domain of GRP78 is involved in the regulation of tumor invasion and metastasis. However, the COOH-terminal domain of GRP78 is involved in the regulation of proliferation and apoptosis of tumor cells.

Further investigation of potential downstream molecules of cell surface GRP78 revealed that cell surface GRP78 could interact directly with a ~60KDa protein and augment its phosphorylation in QGY-7703 and PLC cells. Furthermore, we identified the ~60KDa protein as c-Src. These raised the question of how cell surface GRP78 and c-Src interaction affected the invasion and metastasis of HCC. To answer this question, we analyzed whether pre-treatment with PP2 could inhibit $\alpha 2M^*$ induced adhesion and invasion in QGY-7703 cells. We found that inhibition of the tyrosine kinase activity of c-Src inhibited $\alpha 2M^*$ induced adhesion and invasion. The inhibitory extents were paralleled in cells treated with PP2 with or without $\alpha 2M^*$ stimulation. We further found that blockade of cell surface GRP78 using the antibody against the NH₂ terminal domain of GRP78 inhibited the phosphorylation of Src at Y416 and FAK at Y397. However, the antibody against the COOH terminal domain of GRP78 did not affect Src and FAK phosphorylation. These data further demonstrated that the NH₂-domain of cell surface GRP78 is involved in the regulation of tumor invasion and metastasis upon $\alpha 2M^*$ stimulation. Conflicting results have been described regarding the role of the NH₂-terminal domain of GRP78 in the regulation of cell proliferation and apoptosis. In NCCIT cells, blockade of Cripto binding to cell surface GRP78 using the antibody against the NH₂-terminal domain inhibited tumor cell proliferation, suggesting that the NH₂-terminal domain of cell surface GRP78 also plays a pro-proliferative role in NCCIT cells [26]. This could be explained by the difference in ligands that bind with GRP78 [37, 38]. This opinion was supported by the fact that binding of cell surface GRP78 with Par-4 played an pro-apoptotic role in prostate cancer [25, 39].

We also found that ligation of cell surface GRP78 with $\alpha 2M^*$ triggered EGFR signaling pathway in a Src-dependent manner. Inhibition of the tyrosine kinase activity inhibited EGFR phosphorylation at Y1101 and Y845 induced by $\alpha 2M^*$ stimulation. However, treatment of PP2 did not inhibit the phosphorylation at Y1068, indicating that Src plays critical role in the maximal activation of EGFR in HCC. This raised the question of how c-Src may regulate EGFR

activity induced by the association of cell surface GRP78 with $\alpha 2M^*$. Other reports have demonstrated that Src could regulate EGFR activity by direct interaction. We found that association of cell surface GRP78 with $\alpha 2M^*$ facilitated the interaction between c-Src and EGFR. Furthermore, inhibition of the tyrosine kinase did not affect the interaction between c-Src and EGFR. However, inhibition of Src tyrosine kinase activity inhibiting the maximal activation of EGFR by preventing the phosphorylation of EGFR at Y845 and Y1101. These data were consistent with the role of c-Src in EGFR activation and suggested that both tyrosine kinase activity and protein binding function of Src are essential for cell surface GRP78 induced EGFR activation.

Conclusion

Our research revealed that c-Src plays a critical role in the invasion and metastasis of HCC induced by association of cell surface GRP78 with $\alpha 2M^*$. Cell surface GRP78 directly binds and phosphorylates c-Src. As a consequence, c-Src phosphorylates EGFR, promoting the invasion and metastasis of HCCs.

Abbreviations

GRP78: Glucose regulated protein 78; EGFR: Epithelial growth factor receptor; FAK: Focal adhesion kinase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SR and LZ designed this research and drafted the manuscript. WQ, ZS, SC, LH, WG performed the experiments. All the authors have read and approved the final manuscript.

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