C -reactive protein promotes tumor progression in Hepatocellular Carcinom by 1 interacting with Ephrin type B receptor 3

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Title: C-reactive protein promotes tumor progression in Hepatocellular Carcinom by interacting with Ephrin type-B receptor 3

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Abstract

C-reactive protein (CRP), an acute phase protein, has been increasingly implicated in various tumors, and the role of CRP is positively correlated with invasion and metastasis in hepatocellular carcinoma cells. However, the mechanism of CRP affecting HCC progression remains poorly investigated. The present study investigated the role of CRP in HCC and the underlying mechanisms. We first found that CRP was significantly upregulated in HCC tissues and HCC cells, the expression level correlated with the metastatic ability of HCC cells. Knockdown of CRP significantly suppresses migration and invasion capacity in HCC cells. Through a proteomic analysis of CRP co-immunoprecipitation complexes, the Eph receptor B3 was identified as a new CRP interactor. Then we found that the expression and functions of EphB3 were consistent with CRP in HCC. In addition, co-immunoprecipitation and immunofluorescence assays suggested that EphB3 was able to interact with MAPK/ERK to activate MAPK/ERK signaling pathways. Furthermore, we showed that CRP can induce the phosphorylation of MAPK/ERK by binding EphB3. Our findings showed that CRP increased HCC cells migration and invasion by binding EphB3 to activate MAPK/ERK signaling pathways. It suggested that CRP may become a prognostic factor and a potential therapeutic target for liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common human malignancy and the second leading cause of cancer-related death worldwide, with more than 50% of HCC cancer cases and deaths occurred in China [1-3]. Despite great advancements in HCC early diagnosis, intervention and prevention have been made, patients still have a high recurrence rate and poor prognosis. These poor outcomes are primarily due to metastases and recurrences [4,5]. Over the past decades, remarkable progress has been made to illuminate the pathogenesis of HCC [6,7]. However, the underlying mechanisms of recurrence and metastasis of HCC remain unclear. Therefore, it is necessary to develop a more reliable biomarker for predicting recurrence and understanding the mechanism of liver cancer metastasis as soon as possible.

C-reactive protein (CRP), the first acute-phase protein described and an ancient and highly conserved protein of the pentraxin family, is mainly synthesized by hepatocytes in response to various inflammatory stimuli [8]. Baseline circulating concentrations of CRP are significantly associated with cancer risk in the general population [9]. Recent clinical trials and basic research suggest that a close relationship between CRP and hepatocellular carcinoma. Elevated serum CRP level was an indicator of poor outcomes in HCC patients undergoing transplantation, transarterial chemoembolization, radiofrequency ablation, percutaneous ethanol injection and best supportive care [10-13]. Moreover, high levels of CRP in serum correlated well with liver cancer invasion and metastasis [14]. We have previously showed that CRP promoted migration and invasion of hepatocellular carcinoma cells in vitro [15]. However, an

underlying molecular mechanism between and HCC is still unclear.

With the development of Proteomics, more and more studies indicated that molecular function and biological process in cell were coordinated by protein complex or protein network, protein-protein interactions (PPIs) are crucial for all biological processes [16]. Therefore, compiling PPI networks provides many new insights into protein function and molecular mechanism of related diseases. Co-immunoprecipitation (IP) combined with mass spectrometry (MS) has become the method of choice for identifying protein-protein interactions [17,18].

In this study, we applied co-immunoprecipitation and iTRAQ-mass spectrometry techniques to identify the CRP-interacting protein network that may be related to the progression of liver cancer. 52 candidate proteins were identified to interact with CRP. Notably in our iTRAQ-mass spectrometry analysis, Ephrin type-B receptor 3(EphB3) may interact with CRP. EphB3 is a member of the transmembrane tyrosine kinase receptor subfamily, and affects the invasion and metastasis of tumor cells by activating the MAPK/ERK signaling pathway [19,20]. And EphB3 was overexpression in liver cancer tissue and HCC cells in our study. On these bases, our study undertaken in HCC cells to expound the molecular mechanism that CRP binds EphB3 to mediate invasion and migration in HCC, then providing a new direction for the mechanism of liver cancer development.

Results

CRP was overexpressed in hepatocellular carcinoma

To study the expression level of CRP in liver cancer, the mRNA level of CRP was

quantified by RT-PCR in 20 pairs of tumor and their matched normal liver tissues. Figure 1A shows that CRP mRNA levels were significantly increased in the liver tumor specimens. Results from Western blotting and immunohistochemistry further confirmed the upregulation of CRP in liver cancer specimens (Fig.1B, C). Furthermore, we also measured the CRP mRNA and protein levels in a panel of HCC cell lines and a normal liver cell line HL7702 by way of quantitative Real Time -PCR and Western blotting analysis. As shown in Fig. 1 D and E, CRP was overexpressed in HCC cell lines compared to the normal liver cell line HL7702.The results also indicated that HepG2 and Bel7402 (low metastatic ability) showed a relatively low expression of CRP, Huh7 and SMMC7721 (moderate metastatic ability) showed moderate levels of CRP whereas MHCC97H and MHCCLM3 (high metastatic ability) exhibited considerably higher expression levels of CRP. Therefore, it suggested that CRP was abnormally overexpressed in HCC and dysregulated expression of CRP might contribute to tumor progression.

CRP is involved in HCC cell invasion and migration

Most carcinomas, including hepatocellular carcinoma, progression toward malignancy is accompanied by tumor cell invasion and metastasis. The significantly increased expression of CRP in HCC tissues and cells prompted us to explore its biological role in HCC cells, Cell Migration and Invasion assay was used. We employed small interfering RNA to knockdown the expression of CRP, effective silencing with CRP-specific siRNAs was verified in SMMC7721 and Huh7 cells by Western blot analysis (Fig. 2A). Significantly silencing of intracellular CRP, the

migration and invasion ability of HCC cells was significantly reduced, the migration ability was reduced by 50% and 55%, compared with the control group, and the invasive ability was decreased by 40% and 50% (Fig. 2B, C, D). Similarly, the ability of CRP-silenced liver cancer cells to close scratch wounds was also reduced compared to control group (Fig. 2E, F). As CRP is a secreted protein, we used CRP-specific antibodies to treat liver cancer cells, and then decreased the ability of HCC cells to migrate and invasion as measured in a Cell Migration and Invasion assay (Supplementary Fig. S2). These results suggest that CRP plays an important role in HCC cell biological behaviour.

Mass spectrometry identification and western blot validation of candidate proteins

To clarify the mechanism of CRP in the progression of liver cancer, we used co-immunoprecipitation, iTRAQ labeling, and mass spectrometry to identify specifically interacting with CRP. CRP siRNA was used as the control group, and control siRNA was used as the experimental group, to treat SMMC7721, the knockdown efficiency was shown in Supplementary Fig. S3A. And the differential strip of the control and siCRP group sent for MS is shown in Supplementary Fig. S3B. Two or more peptides were used for quantification and protein identification. For Protein Pilot-based database searching and identification, the threshold [unused protscore (conf)] was set to achieve 95% confidence at 5% FDR. And, a ProtScore value of more than 1.3 was used to attain a confidence of 95%. When we classified the proteins as significantly regulated or not, an additional > 1.3 (1×1.3) -fold cutoff

was applied to all iTRAQ ratios to minimize false positives when determining proteins as up-expressed. This cutoff value was widely employed in the iTRAQ approach [21]. 52 unique proteins were successfully identified (Data not shown).

Gene Ontology analysis with DAVID suggested that the main molecular biological functions of these proteins were protein binding, cell adhesion, cytoskeletal organization, and small molecule activation. , modification; and the main biological processes involved are cell adhesion, regulation of translation, cytoskeletal organization, and angiogenesis (Supplementary Table S1).

To determine the reliability of the iTRAQ analysis data, we selected several interesting protein candidates and confirmed by co-immunoprecipitation (Co-IP) and western blot. As expected, UGDH, EphB3, ENO2, ANXA2, KRT5, MSH2, SHC1, and HSP90B1 were captured when using CRP as the bait protein (Supplementary Fig. S4).

EphB3 is overexpressed in hepatocellular carcinoma

Since literature data and bioinformatic analysis suggest the functional involvement of EphB3 in tumor metastasis [22], we hypothesized that CRP may bind to EphB3 leading to HCC progression. The relationship between EphB3 and CRP was explored. As detected in 20 pairs of tumor and their matched normal liver tissues, EphB3 showed overexpression in HCC tissues (Fig.3 A, B). Similarly, an obvious up-regulation of EphB3 was seen in HCC cells, the protein level of EphB3 were related to the metastatic potential of hepatoma cells (Fig.3 D, E). Furthermore, immunohistochemistry results showed that EphB3 was located on the cell membrane

of HCC tissues(Fig.3 C), and EphB3 was mainly located in the cell membrane and cytoplasm of HCC cells by Confocal assay(Fig.3 F). RT-PCR and Western blot analysis showed that the expression of EphB3 and CRP were consistent in HCC tissues and cells.

EphB3 is involved in HCC cell invasion and migration

To study the role of EphB3 in HCC cell motility, the expression of EphB3 was inhibited using siRNA transduction of SMMC7721 and Huh7 cells. Western blotting was performed to confirm the successful knockdown of EphB3 in these two cell lines (Fig.4A).The migration and invasion assay results indicated that knockdown of FOXC2 significantly reduced the mobility and invasiveness of HCC cells by 60-70% and 55-65%, 60-65% and 40-45% compared to controls ($P < 0.05$), respectively(Fig. 4B, C). Similarly, transfection of SMMC7721 and Huh7 cells with EphB3 siRNA resulted in a decrease in the ability to close scratch wounds, compared to control siRNA (Fig. 4D).

EphB3 binds to MAPK/ERK and activates MAPK/ERK signaling pathways

To examine whether EphB3 interacts with CRP in HCC cells, we performed co-immunoprecipitation assay and western blotting in SMMC7721 and Huh7 cells. EphB3 interacting with CRP and CRP binding EphB3 were observed in SMMC7721 and Huh7 cells (Fig.5A). Picco et al provide the evidence that EphB receptor forms a signaling complex with c-Src kinase to c activate MAPK/ERK and regulates tumor cell motility [23]. In our study, we used co-immunoprecipitation, immunofluorescence and western blot to verify if EphB3 could play its oncogenic role by activating an

MAPK/ERK signaling pathways. Co-IP assays indicated that EphB3 was able to interact with MAPK/ERK in HCC cells (Fig.5B). For subcellular location studies, the co-localization regions of CRP and MAPK/ERK were revealed in hepatoma cells by confocal microscopy (Fig.5C). Transfection of hepatoma cells with EphB3 siRNA revealed that the phosphorylation of MAPK/ERK was significantly attenuated when EphB3 was silenced in HCC cells (Supplementary Fig. S5). These results suggest that EphB3 may participate in the activation of MAPK/ERK.

CRP binds to EphB3 and activates MAPK/ERK signaling pathways

CRP binding EphB3 was confirmed in HCC cells. Then, we investigated the effect of CRP on the activation of MAPK/ERK signaling pathways. We examined the changes of MAPK/ERK phosphorylation in HCC cells after CRP treatment at different concentrations and timings. From the results we found that the expression of p-MAPK and p-ERK was decreased in a dose-dependent manner after CRP-specific antibodies treatment (Fig.6A). Inversely, it was observed that rCRP treatment can remarkably induce MAPK and ERK phosphorylation (Fig.6B). And MAPK/ERK phosphorylation was markedly changed at 30 min and peaked at 120 minutes after CRP treatment (Fig.6C, D).Then we found that co-incubation of cells with CRP and the pharmacological inhibitors of MAPK/ERK pathway (PD98059) could abrogate the effects of CRP-mediated phosphorylated kinases (Fig.6E). Transfection of HCC cells with EphB3 siRNA, the activation of MAPK/ERK pathway was also inhibited after rCRP treatment (Fig.6F). These results indicate that CRP activates MAPK/ERK signaling pathways via binding EphB3.

CRP upregulates MMP-9 expression by activating HIF-1α via the MAPK/ERK signaling pathways

Matrix metalloproteinases (MMPs) play pivotal roles in tumor cell migration and invasion via degrading most components of basement membranes and extracellular matrix [24]. Among the currently known 24 human MMPs, type IV collagen-degrading enzymes (MMP2 and MMP9) were most frequently overexpressed in cancer and were instrumental in cutting through basement membrane barriers [25]. And MMP2 and MMP9 are upregulated by hypoxia in breast and colon cancer cells via a HIF1-dependent mechanism [26]. Hence, we determined the levels of HIF1α and MMPs in HCC cells after CRP treatment. It was observed that CRP treatment can increase HIF-1α protein expression levels in HCC cells, further western blot analysis showed that CRP-induced regulation of HIF-1α expression can be suppressed by MAPK/ERK pathway inhibitor (Fig.7A, B). These results suggested that CRP promoted the expression of HIF-1α by activating MAPK/ERK signaling pathways. Furthermore, our study showed that CRP treatment can upregulate MMP-2 and MMP-9 protein levels in HCC cells (Fig.7C). However, knock-down the expression of HIF-1α in HCC cells significantly attenuated MMP-9 expression with or without CRP treatment (Fig.7D, E). Our study further showed that CRP stimulated MMP-9 expression through HIF-1α in HCC cells was linked to the activation of MAPK/ERK signaling pathways (Fig.8).

Discussion

Chronic inflammation often precedes or accompanies a substantial number of

cancers, and the pathogenic role for chronic inflammation has been verified in multiple tumor systems in tumor initiation, progression and metastatic potential [27,28].The infiltrating immune cells, cytokines and other soluble mediators were the key molecule to link between inflammation and carcinogenesis [29]. CRP, the first acute-phase protein, was found in 1930, laboratories including ours have shown that CRP is associated with hepatocellular carcinoma and HCC cells invasion and metastasis [13-15]. In this study, we investigated the role of CRP in HCC and HCC progression mechanism.

First, we found that CRP was over-expressed in human HCC tissues compared with that in adjacent non-tumor tissues. And the level of CRP in HCC may be correlated with high tumor Edmondson grade and TNM stage (data not shown).CRP was overexpressed in HCC cells, the level of CRP was correlated with the metastatic ability of HCC cells. Moreover, by manipulating CRP levels in SMMC7721 and Huh7 cell lines, we found that CRP can regulate HCC cells motility. Therefore, our results suggested that CRP acts as a tumor-promoting molecule during the progression of HCC. Then, using iTRAQ and mass spectrometry to screen CRP interacting proteins, we identified 52 proteins interacting with CRP, EphB3 was one of them and may be involved in regulating tumor cells motility.

EphB3 is a member of Eph receptors family, Eph receptors comprise the largest family of receptor tyrosine kinases [30,31]. Eph receptors and their interacting ligands (ephrin) together form an important cell communication system with widespread roles in normal physiology and disease pathogenesis [30]. Many studies have correlated Eph

and ephrin expression levels with cancer progression, metastatic spread and patient survival [30,32,33]. EphB3 was first investigated in the development of nervous system. It found that inhibition of EphB3 expression resulted in a disorganization of neural cell movement [34]. Another report showed that EphB3 promoted tumor cells migration and metastasis in Non–Small-Cell Lung Cancer [22]. Our study indicated that EphB3 was up-regulation in HCC, and silencing EphB3 expression could significantly inhibit cell migration and invasion. These results suggested that CRP may bind to EphB3 to effect HCC progression.

Eph receptors and ephrins signaling could activate or interact with the Src family kinase to regulate MAPK/ERK signaling pathways [35]. Our data found that EphB3 activated the phosphorylation of MAPK/ERK via indirect binding MAPK/ERK. And CRP regulated MAPK/ERK phosphorylation in a dose-dependent manner. Moreover, CRP- mediated p-MAPK and p-ERK expression can be attenuated by EphB3 siRNA. It indicated that CRP activated MAPK/ERK signaling pathways via binding EphB3. As we know, MMPs play key roles in tumor cell migration and invasion [24]. During the MMPs, MMP2 and MMP9 were most frequently overexpressed in cancer and positively correlated with a higher incidence of metastases in cancer [25]. Previous studies showed that Hypoxia is associated with an increase in the expression and the activity of MMP-2 and MMP-9. MMP9 is the downstream target gene of HIF-1α, which can induce the expression of MMP9 to increase degradation of extracellular composition and vascular basement membrane, thereby promoting tumor cells distant invasion and metastasis [36]. Hypoxia is a common condition in tumors, and which

formed hypoxic microenvironment in cancer. Hypoxia inducible factor-1 (HIF-1), as the most important transcriptional regulatory factor in hypoxic microenvironment, regulated the tumor cells to adapt to hypoxic microenvironment. HIF-1 is composed of an HIF-1α and an HIF-1β subunit, HIF-1α is the most commonly expressed and functions as a master oxygen balance regulator in many cell types [37,38]. HIF-1α widely involved in invasion and distant metastasis, angiogenesis, and metabolism by regulating the transcription of downstream target genes including VEGF and MMP-9 [39]. It was reported that MAPK/ERK signaling can stimulate HIF-1α synthesis and transactivation through the activation of phosphorylation [40]. In our study, CRP can increase HIF-1α protein expression in HCC cells by MAPK/ERK phosphorylation. Our results further showed that the addition of CRP significantly increased MMP-9 activity in HCC cells in a dose-dependent manner, and knock-down the expression of HIF-1α suppress CRP-stimulated MMP-9 protein expression. These results indicated that CRP can upregulate MMP-9 expression by activating HIF-1α via the MAPK/ERK signaling pathways.

In summary, we found that CRP was overexpressed in HCC tissues and cells, and up-regulation of CRP in HCC cells promoted cell migration and invasion. Mass spectrometry combination with co-immunoprecipitation identified EphB3 as a novel CRP interactor. CRP increased HCC cells migration and invasion by binding EphB3 to activate MAPK/ERK signaling pathways. CRP also significantly stimulated MMP-9 expression, mainly by activating HIF-1α via the MAPK/ERK pathways. Our findings give useful information into the mechanism of CRP in the development and

progression of HCC and pave the road for considering CRP as a novel therapeutic target.

Materials and Methods

Tissues and Cell Lines

We gathered liver tissue samples from nine HBV-related HCC patients undergoing hepatectomy at The Second Affiliated Hospital of Chongqing Medical University. HCC was confirmed by pathologic or radiographic examinations. Normal liver cell line HL7702 and HCC cell lines Bel7402, SMMC7721, MHCC97H, and MHCCLM3 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. HepG2 cell and Huh7 cell were purchased from ATCC (Rockville, MD). All cells were cultured in high-glucose DMEM (HyClone, Waltham, MA) supplemented with 10% fetal bovine serum (Gibco, San Diego, CA) and 100 IU/mL of penicillin, 100 μg/mL of streptomycin, maintained at 37 °C with 5.0% CO2.

Ethics approval and consent to participate. Patients with informed consent to participate. The study plan has been reviewed by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University, and it is considered to meet the ethical requirements of clinical research, and the study plan is approved. We confirming all the experiment protocol for involving humans was in accordance to guidelines of national in the manuscript.

Reagents and antibodies

Eight-plex iTRAQ reagent kits were acquired from Applied Biosystems (Foster

City, CA, USA). Monoclonal antibodies against human CRP were obtained from HyTest (Finland, Turku). Monoclonal antibodies against UGDH, EphB3, ENO2, ANXA2, KRT5, MSH2, SHC1, HSP90B1, MAPK, p-MAPK, ERK, p-ERK and HIF1α were purchased from Abcam (Cambridge, USA). CRP specific Stealth Select RNAi™ siRNA (NM_000567), Stealth RNAi™ Negative Control siRNA and Lipofectamine Max transfection reagent were obtained from Santa Cruz (California, USA). Small interfering RNA against human EphB3 (ID 146498, ID 146499) were purchased from Invitrogen (Grand Island, NY). IP lysis buffer was purchased from Beyotime (Shanghai, China). Protein A/G agarose beads from GE Healthcare (Little Chalfont, UK). CytoSelect™ 24-Well Cell Migration and Invasion Assay kits (8 µm, colorimetric format) were purchased from Cell Biolabs (San Diego, CA, USA).

RT-PCR analysis

RT-PCR analysis was performed as described previously [41]. Total RNA extraction was performed according to the manufacturer's instructions. The cDNA was produced by reverse-transcription of the total RNA by employing the Promega Reverse Transcription System A3500. An ABI 7900HT System (Applied Biosystems, Foster City, CA) was used for RT-PCR using gene-specific primers for CRP (Hs00437486_CE), EphB3 (Hs00244637_CE), GADPH (Hs02758991_g1). Fold enrichment of indicated gene expression was calculated using ΔΔCT method or $2^{-\Delta\Delta CT}$ [41]. The experiments were performed in triplicate.

Western blot analysis

Western blot analysis was performed as previously reported [42]. Tissues/cells were

lysed with IP lysis buffer, and a BCA Protein Assay Reagent Kit was used to determine the protein concentration. Protein samples were separated by SDS-PAGE and then electro-blotted onto PVDF membranes. Membranes were blocked with BSA in Tris-buffered saline solution with Tween-20 (TBS-T) for 2 h at room temperature. Subsequently, the membranes were incubated with the primary antibodies (1:1000-1:2,000 dilution), overnight at 4°C and then with HRP-conjugated secondary antibodies at a dilution of 1:5,000 after three washes with TBST buffer. Finally, membranes were visualized with an ECL detection instrument (Bio-Rad Laboratories, Hercules, CA, USA). Several blots were cut prior to antibody hybridization for the simultaneous detection of different target proteins in the same sample.

Immunohistochemistry

Immunohistochemistry analysis was performed as previously reported [43]. Briefly, HCC tissues were dehydrated in graded alcohol and embedded in paraffin wax. A thickness of 6 mm prepared on a glass slide was performed in xylene and rehydrated by ethanol and placed in PBS. Antigen retrieval was performed by heating in a microwave oven for 15 minutes with citric acid buffer (0.1 M, pH.6). After dewaxing the sections, endogenous peroxidase activity was blocked by 3% H2O2 in PBS. Non-specific adsorption was minimized by pre-incubating a portion of 10% normal donkey serum for 60 minutes, and CRP and EphB3 were detected using specific antibodies. Images were taken with a Nikon microscope (Nikon, Melville, NY, U.S.A.).

RNA Interference

Cells were trypsinized, then plated on a 6-well plate at an appropriate concentration per well with specific siRNAs or negative control siRNA using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Three days after the transfection, cells were harvested to examine the protein expression or used for experiments.

Migration, Invasion and wound healing assay

SMMC7721 cells and Huh7 cells were pretreated with 100 nm of CRP-siRNA as described above, then subjected to migration, invasion and wound healing assay. Cells' viability was determined by trypan blue dye exclusion to be over 95%.The migration and invasion assay were performed by using a Transwell Kit according to the manufacturer's instructions. For wound healing assays, cells were approximately 100% confluent in 6-well plate, then 200-μl pipet tip was used to scratch the cell monolayer. The resultant gap was monitored for up to 24 h via a microscope.

Co-Immunoprecipitation (Co-IP) and iTRAQ labeling

After SMMC7721 cells were pretreated with CRP-siRNA or a negative siRNA, cell Lysates were collected and the protein concentration was detected by BCA kit. Then the protein samples were mixed with goat antibodies against CRP, rotating vertically overnight at 4°C. Protein G beads were added to the immune complexes and incubated for 2 h under gentle rotation. The beads were pelleted and washed three times with lysis buffer. Bound protein-complexes were eluted using SDS sample buffer. The eluted protein (300 μg) was precipitated from each pooled group, dissolved, denatured, cysteine blocked, digested and labeled using iTRAQ reagents [Control siRNA, 118 and 121 tag; CRP siRNA 113 and 119 tag] (Supplementary Fig.

S1).

Mass spectrometry and Bioinformatic analysis

Labeled peptides were fractionated and purified by immobilized-pH-gradient isoelectric focusing (IPG-IEF), as previously described [44]. Purified peptide fractions were reconstituted in solvent A. The peptides were electrosprayed using a nanoelectrospray ionization source at an ion spray voltage of 2300 eV and analyzed by a NanoLC-ESI-Triple TOF 5600 system (AB Sciex). The mass spectrometer was set in the positive ion mode at a mass range of 300-1800 m/z. The two most intensely charged peptides above 20 counts were selected for MS/MS at a dynamic exclusion of 30 sec [44]. Data were processed by ProteinPilot v2.0 (AB Sciex) and the candidate proteins were identified. Protein identification was based on a threshold of protein score >1.3, a confidence limit of 95%, a false discovery rate of 5%, and an additional iTRAQ ratios >1.3. For quantitation, at least two unique peptides with 95% confidence and a P-value <0.05 were required. The bioinformatic analysis of gene ontology (GO) was performed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

Validation of CRP interacting proteins

Co-IP and western blot analyses were performed to validate the proteomic data on some randomly chosen CRP interacting proteins. SMMC7721 cells and Huh7 cells protein samples were collected in lysis buffer. Then CO-IP assay was performed as described above. The protein of UGDH, EphB3, ENO2, ANXA2, KRT5, MSH2, SHC1 and HSP90B1 which may interact with CRP was detected using

405 immunoblotting.

406 **Immunofluorescence assay**

407 Liver cancer cells with a concentration of 1×10^5 cells per dish were cultured in
408 confocal dishes with DMEM supplemented with 10% FBS for 24h. Then cells were
409 fixed with 10% (vol/vol) paraformaldehyde, perforated with 0.1% (vol/vol) Triton
410 X-100 and blocked with 10% (vol/vol) normal goat serum in PBS-T. Cells were
411 incubated for 18 hours in anti-EphB3, anti-MAPK and anti-ERK primary antibodies
412 and subsequently incubated in the appropriate species-specific Alexa fluorescent dye
413 conjugated secondary antibodies (Invitrogen) for 90 min at 37°C. The immunostained
414 cells were viewed by confocal microscopy (Nikon, Melville, NY, U.S.A.).

415 **Human recombinant protein, block antibody, Inhibitor and small interfering**
416 **RNA treatment**

417 After reaching 70-80 % confluence, HCC cells were seeded in six-well plates (2
418 $\times 10^5$cells/well). The cells were treated with the following reagents for different times
419 as needed for the experiment: (a) PBS solution as a control; (b) human recombinant
420 CRP protein (rCRP, ab111647) or CRP antibody or ERK inhibitor (PD098059, 10 μM)
421 alone; (c) rCRP plus ERK inhibitor or EphB3 siRNA or HIF1α siRNA. Doses of the
422 human recombinant protein, block antibody, inhibitor were determined according to
423 previous laboratory characterization and published data. Cell extractions were
424 collected in appropriate time after treatment.

425 All methods described above were in accordance to the institutional guidelines and
426 approved by Ethics Committee of The Second Affiliated Hospital of Chongqing

Medical University.

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Author contributions

Conception and design: YX.Y. and H.R.; acquisition of data: S.S., M.Y., and SY.L.; analysis and interpretation of data: S.S., M.Y., and SY.L.; writing, review, and/or revision of the manuscript: S.S., M.Y., and HD.L.. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Statement of Data Availability

All data generated or analyzed during this study are included in this article.

References

1 Chen, W. Cancer statistics: updated cancer burden in China. *Chin J Cancer Res* **27**, 1, doi:10.3978/j.issn.1000-9604.2015.02.07 (2015).

2 Torre, L. A. *et al.* Global cancer statistics, 2012. *CA Cancer J Clin* **65**, 87-108, doi:10.3322/caac.21262 (2015).

3 Zhu, R. X., Seto, W. K., Lai, C. L. & Yuen, M. F. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. *Gut Liver* **10**, 332-339, doi:10.5009/gnl15257 (2016).

4 Forner, A., Llovet, J. M. & Bruix, J. Hepatocellular carcinoma. *Lancet* **379**, 1245-1255, doi:10.1016/S0140-6736(11)61347-0 (2012).

5 El-Serag, H. B. Hepatocellular carcinoma. *N Engl J Med* **365**, 1118-1127, doi:10.1056/NEJMra1001683 (2011).

6 Ye, Q. H. *et al.* GOLM1 Modulates EGFR/RTK Cell-Surface Recycling to Drive Hepatocellular Carcinoma Metastasis. *Cancer Cell* **30**, 444-458, doi:10.1016/j.ccell.2016.07.017 (2016).

7 Hou, G. *et al.* Aldehyde dehydrogenase-2 (ALDH2) opposes hepatocellular carcinoma progression by regulating AMP-activated protein kinase signaling in mice. *Hepatology* **65**, 1628-1644, doi:10.1002/hep.29006 (2017).

8 Volanakis, J. E. Human C-reactive protein: expression, structure, and function. *Mol Immunol*

38, 189-197 (2001).

9 Zhang, Y. & Jiang, L. CRP 1059 G/C and 1846G/A polymorphisms and cancer risk: a meta-analysis of 26,634 subjects. *Clin Res Hepatol Gastroenterol* **38**, 607-612, doi:10.1016/j.clinre.2014.04.002 (2014).

10 Kornberg, A. *et al.* Postoperative peak serum C-reactive protein is a predictor of outcome following liver transplantation for hepatocellular carcinoma. *Biomarkers* **21**, 152-159, doi:10.3109/1354750X.2015.1118548 (2016).

11 Rekik, S. *et al.* The CRP level and STATE score predict survival in cirrhotic patients with hepatocellular carcinoma treated by transarterial embolization. *Dig Liver Dis* **48**, 1088-1092, doi:10.1016/j.dld.2016.06.005 (2016).

12 Jiang, T., Zhang, X., Ding, J., Duan, B. & Lu, S. Inflammation and cancer: inhibiting the progression of residual hepatic VX2 carcinoma by anti-inflammatory drug after incomplete radiofrequency ablation. *Int J Clin Exp Pathol* **8**, 13945-13956 (2015).

13 Kinoshita, A., Onoda, H., Imai, N., Nishino, H. & Tajiri, H. C-Reactive Protein as a Prognostic Marker in Patients with Hepatocellular Carcinoma. *Hepatogastroenterology* **62**, 966-970 (2015).

14 Aino, H. *et al.* The systemic inflammatory response as a prognostic factor for advanced hepatocellular carcinoma with extrahepatic metastasis. *Mol Clin Oncol* **5**, 83-88, doi:10.3892/mco.2016.879 (2016).

15 Shen, S. *et al.* Molecular mechanism of C-reaction protein in promoting migration and invasion of hepatocellular carcinoma cells in vitro. *Int J Oncol*, doi:10.3892/ijo.2017.3911 (2017).

16 Stelzl, U. *et al.* A human protein-protein interaction network: a resource for annotating the proteome. *Cell* **122**, 957-968, doi:10.1016/j.cell.2005.08.029 (2005).

17 Free, R. B., Hazelwood, L. A. & Sibley, D. R. Identifying novel protein-protein interactions using co-immunoprecipitation and mass spectroscopy. *Curr Protoc Neurosci* **Chapter 5**, Unit 5 28, doi:10.1002/0471142301.ns0528s46 (2009).

18 Huang, B. X. & Kim, H. Y. Effective identification of Akt interacting proteins by two-step chemical crosslinking, co-immunoprecipitation and mass spectrometry. *PLoS One* **8**, e61430, doi:10.1371/journal.pone.0061430 (2013).

19 Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee. *Cell* **90**, 403-404 (1997).

20 Poliakov, A., Cotrina, M. L., Pasini, A. & Wilkinson, D. G. Regulation of EphB2 activation and cell repulsion by feedback control of the MAPK pathway. *J Cell Biol* **183**, 933-947, doi:10.1083/jcb.200807151 (2008).

21 She, S. *et al.* C-reactive protein is a biomarker of AFP-negative HBV-related hepatocellular carcinoma. *Int J Oncol* **47**, 543-554, doi:10.3892/ijo.2015.3042 (2015).

22 Ji, X. D. *et al.* EphB3 is overexpressed in non-small-cell lung cancer and promotes tumor metastasis by enhancing cell survival and migration. *Cancer Res* **71**, 1156-1166, doi:10.1158/0008-5472.CAN-10-0717 (2011).

23 Vindis, C., Cerretti, D. P., Daniel, T. O. & Huynh-Do, U. EphB1 recruits c-Src and p52Shc to activate MAPK/ERK and promote chemotaxis. *J Cell Biol* **162**, 661-671, doi:10.1083/jcb.200302073 (2003).

24 Curran, S. & Murray, G. I. Matrix metalloproteinases in tumour invasion and metastasis. *J*

Pathol **189**, 300-308, doi:10.1002/(SICI)1096-9896(199911)189:3<300::AID-PATH456>3.0.CO;2-C (1999).

25 Nelson, A. R., Fingleton, B., Rothenberg, M. L. & Matrisian, L. M. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* **18**, 1135-1149, doi:10.1200/JCO.2000.18.5.1135 (2000).

26 Krishnamachary, B. *et al.* Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* **63**, 1138-1143 (2003).

27 Kuper, H., Adami, H. O. & Trichopoulos, D. Infections as a major preventable cause of human cancer. *J Intern Med* **248**, 171-183 (2000).

28 Fidler, I. J. Modulation of the organ microenvironment for treatment of cancer metastasis. *J Natl Cancer Inst* **87**, 1588-1592 (1995).

29 Gonda, T. A., Tu, S. & Wang, T. C. Chronic inflammation, the tumor microenvironment and carcinogenesis. *Cell Cycle* **8**, 2005-2013, doi:10.4161/cc.8.13.8985 (2009).

30 Pasquale, E. B. Eph-ephrin bidirectional signaling in physiology and disease. *Cell* **133**, 38-52, doi:10.1016/j.cell.2008.03.011 (2008).

31 Taylor, H., Campbell, J. & Nobes, C. D. Ephs and ephrins. *Curr Biol* **27**, R90-R95, doi:10.1016/j.cub.2017.01.003 (2017).

32 Hirai, H., Maru, Y., Hagiwara, K., Nishida, J. & Takaku, F. A novel putative tyrosine kinase receptor encoded by the eph gene. *Science* **238**, 1717-1720 (1987).

33 Maru, Y., Hirai, H. & Takaku, F. Overexpression confers an oncogenic potential upon the eph gene. *Oncogene* **5**, 445-447 (1990).

34 Krull, C. E. *et al.* Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr Biol* **7**, 571-580 (1997).

35 Pasquale, E. B. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer* **10**, 165-180, doi:10.1038/nrc2806 (2010).

36 Kessenbrock, K., Wang, C. Y. & Werb, Z. Matrix metalloproteinases in stem cell regulation and cancer. *Matrix Biol* **44-46**, 184-190, doi:10.1016/j.matbio.2015.01.022 (2015).

37 Choi, J. Y., Jang, Y. S., Min, S. Y. & Song, J. Y. Overexpression of MMP-9 and HIF-1alpha in Breast Cancer Cells under Hypoxic Conditions. *J Breast Cancer* **14**, 88-95, doi:10.4048/jbc.2011.14.2.88 (2011).

38 Semenza, G. L. Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology (Bethesda)* **19**, 176-182, doi:10.1152/physiol.00001.2004 (2004).

39 Li, Y. Y. & Zheng, Y. L. Hypoxia promotes invasion of retinoblastoma cells in vitro by upregulating HIF-1alpha/MMP9 signaling pathway. *Eur Rev Med Pharmacol Sci* **21**, 5361-5369, doi:10.26355/eurrev_201712_13921 (2017).

40 Sang, N. *et al.* MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. *J Biol Chem* **278**, 14013-14019, doi:10.1074/jbc.M209702200 (2003).

41 Mounce, B. C. *et al.* Gammaherpesvirus gene expression and DNA synthesis are facilitated by viral protein kinase and histone variant H2AX. *Virology* **420**, 73-81, doi:10.1016/j.virol.2011.08.019 (2011).

42 Yang, Y. *et al.* Discovery of SLC3A2 cell membrane protein as a potential gastric cancer biomarker: implications in molecular imaging. *J Proteome Res* **11**, 5736-5747, doi:10.1021/pr300555y (2012).

43 Kim, E. S. *et al.* Inflammatory lipid sphingosine-1-phosphate upregulates C-reactive protein

via C/EBPbeta and potentiates breast cancer progression. *Oncogene* **33**, 3583-3593, doi:10.1038/onc.2013.319 (2014).

44 Yang, Y. *et al.* Cathepsin S mediates gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins. *J Proteome Res* **9**, 4767-4778, doi:10.1021/pr100492x (2010).

Figure Legends

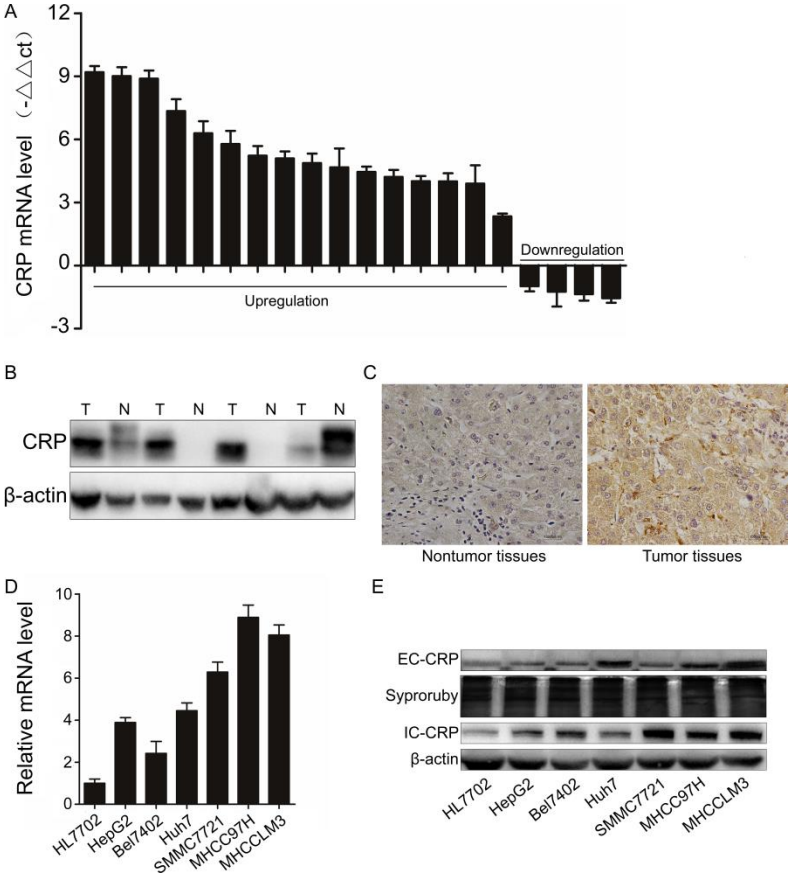
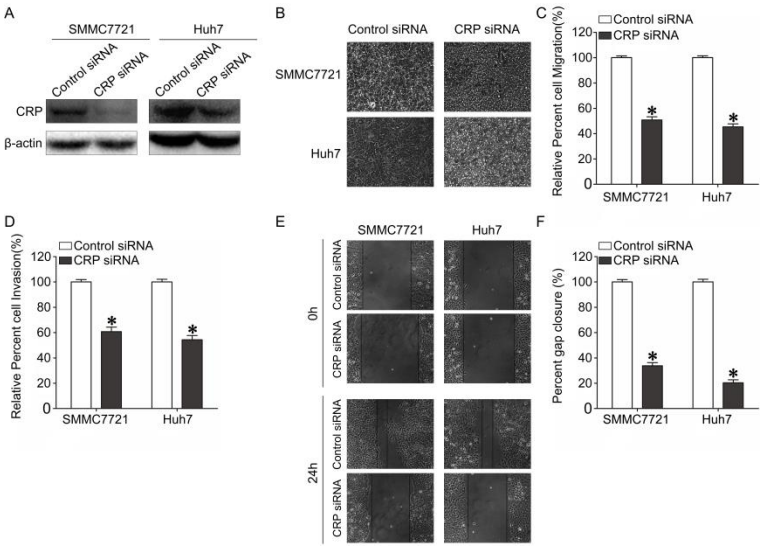


Figure1. CRP was overexpressed in matched hepatocellular carcinoma and normal liver samples, as well as HCC cell lines. A, RT-PCR detected the relative mRNA expression levels of CRP in 20 pairs of tumor and their matched normal liver tissues. GADPH was used as the normalization standard. B and C, expression of CRP in 4 randomly picked, paired HCC tissues were analyzed by Western blotting and immunohistochemistry. Proteins were separated on 10% acrylamide gels and immunoblots were probed with corresponding antibodies. D, RT-PCR detected CRP mRNA in 1 normal liver cell line and 6 HCC cell lines. E, protein level of EC-CRP and IC-CRP in normal liver cell line and HCC cell lines. Syproruby or β-Actin was used as a loading control.



576

577 Figure2. CRP is involved in HCC cell invasion and migration. A, Western blotting
578 analysis showed that transfection of cells with CRP-specific siRNAs significantly
579 reduced CRP protein levels in SMMC7721 and Huh7 cells. Proteins were separated
580 on 10% acrylamide gels and immunoblots were probed with corresponding antibodies.
581 B and C, silencing of CRP significantly inhibited the migration properties of
582 SMMC7721 and Huh7 cells. D, matrigel invasion were significantly inhibited in HCC
583 cells transfected with CRP-siRNA after 72h. E and F, CRP knock-down led to a sharp
584 decreased in the ability of the SMMC7721 and Huh7cells to close the gap introduced
585 by a scratch wound, compared to control cells. *P＜0.05. Bars indicate SD.

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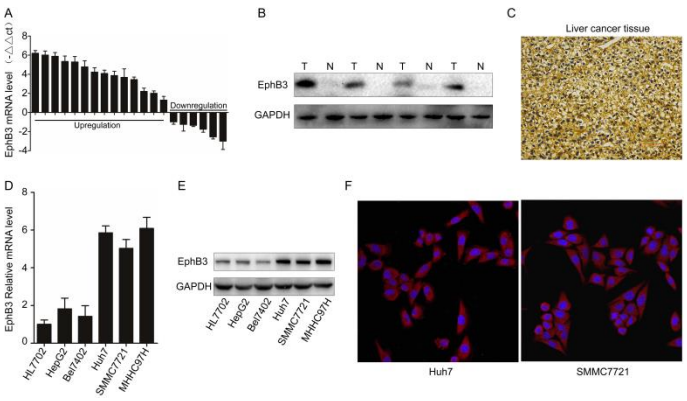
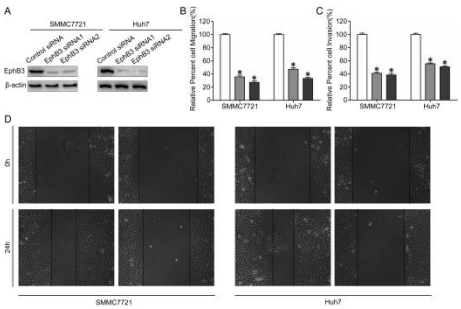


587

588 Figure3. EphB3 was upregulated in HCC. A, RT-PCR detected the relative mRNA
589 expression levels of EphB3 in 20 pairs of HCC tissues and their matched normal liver
590 tissues. GADPH was used as the normalization standard. B and C, protein expression
591 of EphB3 in HCC tissues were analyzed by Western blotting and

592 immunohistochemistry. Proteins were separated on 10% acrylamide gels and
593 immunoblots were probed with corresponding antibodies. D, EphB3 mRNA in normal
594 liver cell line and HCC cell lines was measured by RT-PCR. E, protein level of EphB3
595 in normal liver cell line and HCC cell lines. F, subcellular localization of EphB3 was
596 analyzed by immunofluorescence.
597



598

599 Figure4. Functional studies of EphB3 in liver cancer cell motility. A, the expression of
600 EphB3 was inhibited using EphB3-siRNA transduction of SMMC7721 and Huh7
601 cells. Proteins were separated on 10% acrylamide gels. B and C, silencing of EphB3
602 significantly suppressed the migration properties and matrigel invasion of
603 SMMC7721 and Huh7 cells. D, the ability of the SMMC7721 and Huh7cells to close
604 the gap was attenuated by wound healing assay after EphB3 siRNA treatment. *P＜
605 0.05. Bars indicate SD.
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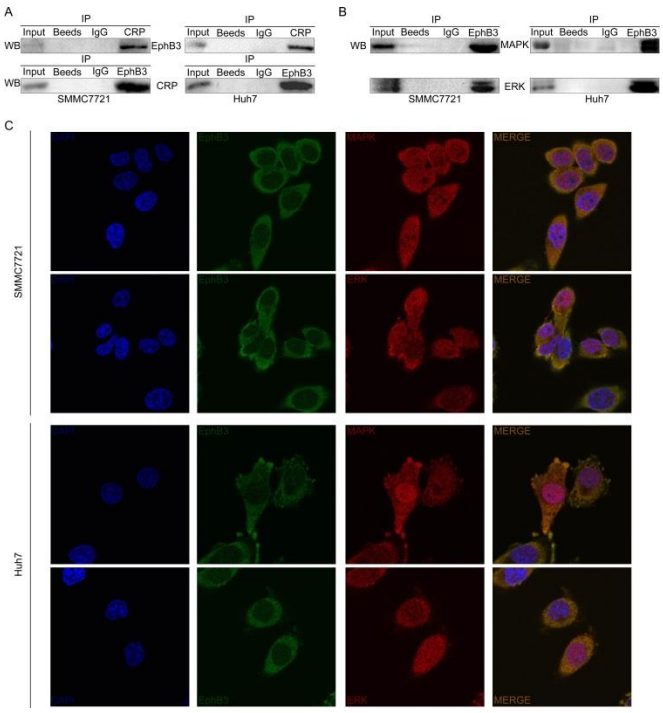

Figure5. EphB3 interacted with MAPK/ERK signaling pathways. A, EphB3 interacted with CRP. CRP-specific antibodies or EphB3 antibodies were used to capture the interacting protein, and co-immunoprecipitation and western blotting were detected the interaction between CRP and EphB3. Co-immunoprecipitation with normal rabbit IgG or not served as a negative control. B, EphB3 interacted with MAPK/ERK. EphB3 acted as a bait protein, interacting with MAPK/ERK was verified by co-immunoprecipitation and western blotting. Proteins were separated on 10% acrylamide gels and immunoblots were probed with corresponding antibodies. C, immunofluorescence was used to identified the co-localization of EphB3 with MAPK/ERK. SMMC7721 cells or Huh7 cells stained with anti-EphB3 antibody/Alexa Fluor 488 (green) and anti-MAP/ERK antibody/CY3 (red), and DAPI nuclear stain (blue).

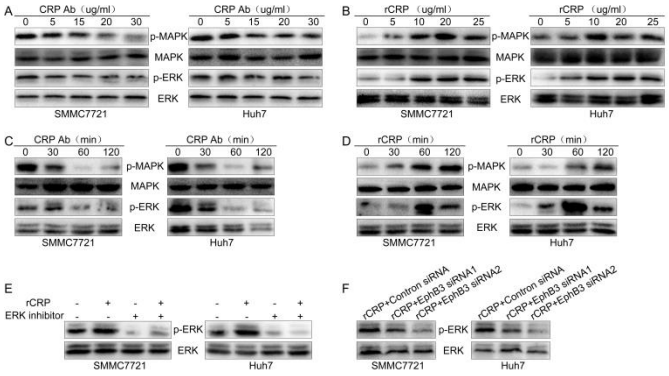

Figure6. CRP binds to EphB3 and activates MAPK/ERK signaling pathways. A and B, conditioned media of cells treated with CRP-specific antibodies or rCRP for 48h was subjected to immunoblot analysis by using antibodies against p-MAPK and p-ERK. C and D, SMMC7721 cells and Huh7 cells were treated with CRP Ab (30ug/ml) or rCRP (25ug/ml) for the indicated time. The phosphorylation of MAPK/ERK was detected by western blot. E, HCC cells were treated with 25μg/ml rCRP for 24h with or without 10μM PD98059. Phosphorylation of ERK was determined by immunoblot analysis. F, HCC cells were transfected with control siRNA, siRNAs targeting EphB3 (100nmol). The siRNA-transfected cells were treated with 25μg/ml rCRP for 24h. Phosphorylation of ERK was determined by western blot. Proteins were separated on 10% acrylamide gels and immunoblots were probed with corresponding antibodies.

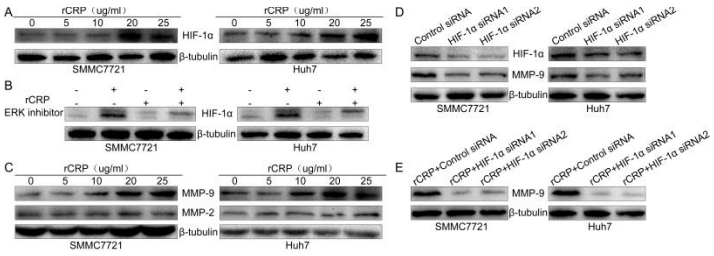

Figure7. CRP upregulated MMP-9 expression by activating HIF-1α via the MAPK/ERK signaling pathways. A, HCC cells treated with rCRP for the indicated concentration. The level of HIF-1α was detected by western blot. B, HCC cells treated with 25μg/ml rCRP for 24h with or without 10μM PD98059 were subjected to immunoblot analysis by using antibodies against HIF-1α. C, conditioned media of cells were treated 25μg/ml rCRP for 48h. The expression of MMP-2 and MMP-9 was analyzed by western blot. D, HCC cells transfected with control siRNA, siRNAs targeting HIF-1α (100nmol), and MMP-9 was detected by immunoblot analysis after

48h. E, HCC cells were transfected with control siRNA, siRNAs targeting HIF-1α (100nmol). The siRNA-transfected cells were treated with 25μg/ml rCRP for 24h. Expression of MMP-9 was determined by western blot. Proteins were separated on 10% acrylamide gels and immunoblots were probed with corresponding antibodies.

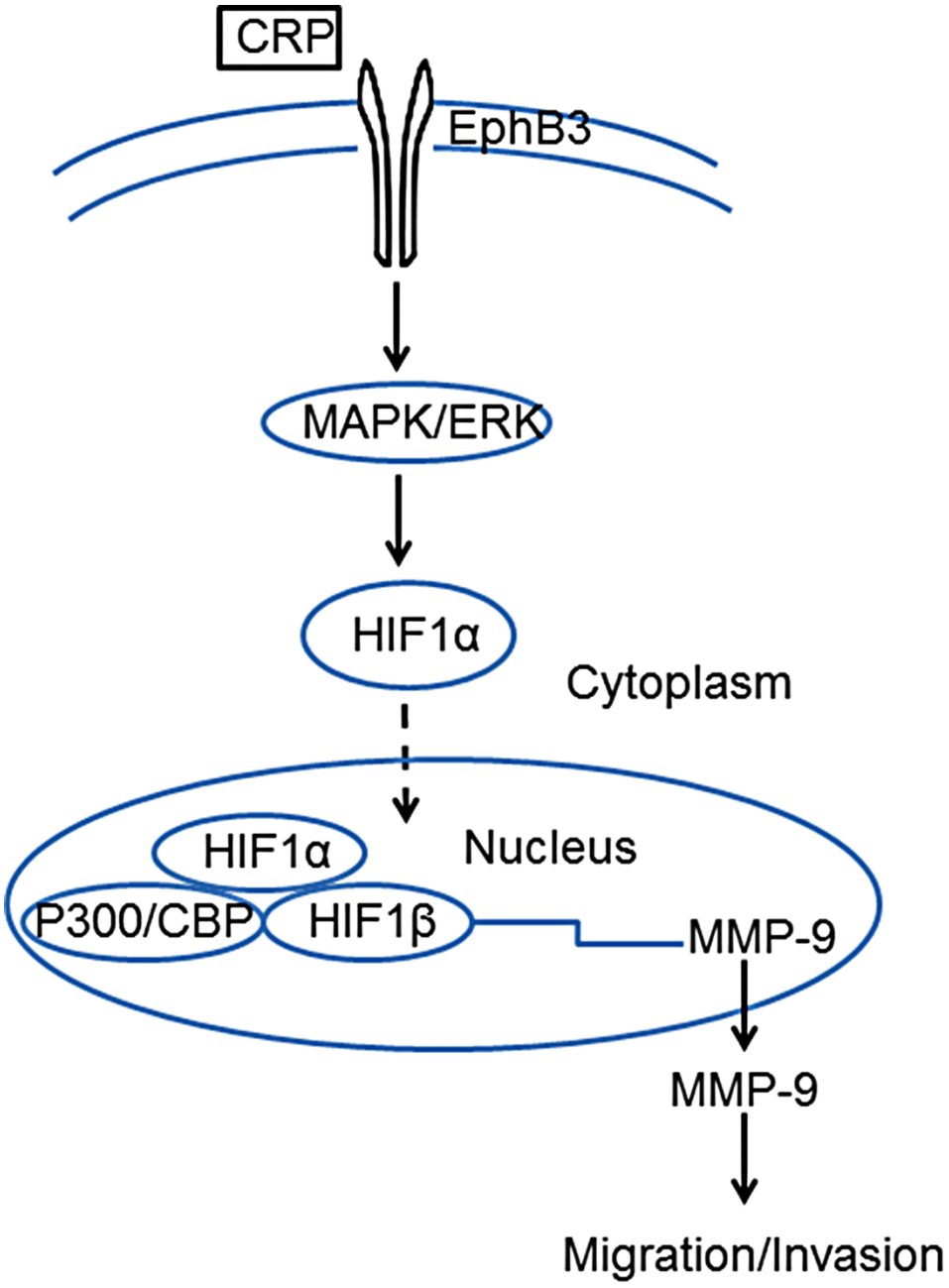

Figure8. Schematic representation of the molecular mechanism for CRP regulated HCC progression.

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