Peritumoral stromal neutrophils are essential for c-Met-elicited metastasis in human hepatocellular carcinoma

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Introduction

Tumor progression is now recognized as the product of evolving crosstalk between different cell types within tumors.\(^1\)\(^-\)\(^4\) Hepatocellular carcinoma (HCC) is usually present in inflamed fibrotic and/or cirrhotic liver with extensive leukocyte infiltration. Thus, the immune status at a tumor site can largely influence the biologic behavior of HCC.\(^5\)\(^-\)\(^8\) Recent studies have shown that activated monocytes/macrophages and IL-17-producing T cells in HCC can promote disease progression by stimulating cancer angiogenesis and B7-H1-associated immune privilege.\(^9\)\(^-\)\(^11\) These observations suggest that local immune environments are important determinants for disease progression and cancer metastasis in humans.

Although less characterized than tumor macrophages and IL-17-producing T cells,\(^12\)\(^-\)\(^13\) neutrophils are also emerging as important players in the pathophysiology of cancer.\(^14\)\(^-\)\(^16\) Neutrophils are the most abundant leukocytes and serve as essential effector cells in the first line of host defense against invading microorganisms.\(^17\)\(^,\)\(^18\) In addition to direct bactericidal activities, neutrophils can actively regulate angiogenesis and tissue remodeling by releasing multiple proteases.\(^19\)\(^,\)\(^20\) In a study of patients with HCC, it has been demonstrated that recruitment of neutrophils was regulated by IL-17.\(^17\) T cell-induced epithelium-derived CXC chemokines, and those neutrophils were associated with the progression of cancer angiogenesis.\(^21\) Notably, exposure of neutrophils to cancer environments also leads to autophagy-mediated survival of cells \textit{in vitro}.\(^22\) Thus, immune functional data and activated status of neutrophils in cancer environments are essential for understanding their roles and potential mechanisms in tumor immunopathogenesis.

Hepatocyte growth factor (HGF) is recognized as a potent mitogen for hepatocytes, and it plays important role in liver development and regeneration.\(^23\)\(^,\)\(^24\) HGF receptor, the tyrosine kinase c-Met, is also highly expressed by malignant cells, and...
targeting HGF/c-Met axis in mice could significantly reduce the growth and metastasis of malignant cells. However, such an approach is hampered in human cancers by the fact that the source and regulation of HGF are still unknown. The present study showed that high infiltration of neutrophils in HCC tissues determined malignant cell c-Met-associated clinical outcome of patients. Neutrophils were enriched predominantly in invading tumor edge of HCC tissues, and they were the major source of HGF in tissues. HCC environment-mediated activation was required for subsequent HGF production by neutrophils. Moreover, we also demonstrated that malignant cell-derived granulocyte-macrophage colony stimulating factor (GM-CSF) was an important determinant in neutrophil HGF production, which in turn enhanced the migration and invasion of malignant cells.

Results

**Neutrophil infiltration determines malignant cell c-Met-associated clinical outcome of HCC patients**

HGF/c-Met interaction activates a wide range of different cellular signaling pathways, including those involved in proliferation, motility, migration, and invasion of liver cells. In individuals with untreated HCC (n = 60; Table 1), we identified distinct expression patterns of c-Met in malignant cells by immunohistochemical staining in different paraffin-embedded samples (Fig. 1A). In 58.3% of the samples analyzed, the c-Met																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														\n
The presence of neutrophils was visualized by staining of CD15 in paraffin-embedded HCC tissues (n = 150; Table 1). CD15 cells were often predominant in tumor-invading edge rather than in the cancer nest (Fig. 1E). To further evaluate whether neutrophils contributed to c-Met-mediated tumor pathology, patients were stratified according to the median values of malignant c-Met expression and CD15 density in tumor-invading edge. As expected, in patients with high infiltration of CD15 cells, there was a striking inverse association between malignant c-Met expression and both OS and DFS (p < 0.001 for both; Fig. 1F; Table 2). By contrast, in patients with low infiltration of CD15 cells, malignant c-Met expression was unrelated to the prognosis of either OS or DFS (Fig. 1F; Table 2). Together, neutrophils in tumor-invading edge determine the malignant c-Met-associated clinical outcome of HCC patients.

**Exposure to HCC environments leads to neutrophil activation and subsequent HGF production**

Having established the HGF production by neutrophils in HCC environments, we next set out to establish conditions under which this process can be reliably reproduced in vitro. Human neutrophils of ~98% purity were left untreated or incubated with culture supernatants from primary HCC cells, three established hepatoma cell lines, and a normal liver cell line. Consistent with our in vitro findings, exposure of neutrophils to 30% tumor culture supernatants (TSNs) from both primary and established hepatoma cells, including HepG2, QGY-7703, and SK-Hep-1, resulted in a marked HGF production in a time-dependent manner (Fig. 2A). By contrast, neutrophils cultured in medium alone or incubated with supernatant from normal liver cells (L02) marginally secreted those factors (Fig. 2A). Such increased HGF production in neutrophils exposed to culture supernatants from primary and established hepatoma cells was further confirmed by real-time PCR (Fig. 2B).

It has been demonstrated that phosphoinositide 3-kinase/AKT (PI3K/AKT), mitogen-activated protein kinase (MAPK), and NF-κB pathways are implicated in the regulation of neutrophil functions. To further probe the mechanisms involved in the induction of neutrophil HGF production by cancer environment, we examined the activation of PI3K/AKT, MAPK, and NF-κB pathways in neutrophils. The activation patterns of the PI3K/AKT, MAPKs, JNK, Erk, and p38, and the NF-κB pathways was selectively enhanced in neutrophils stimulated...
Figure 1. Infiltration of neutrophils in HCC determines c-Met associated clinical outcome of patients. (A) Paraffin-embedded HCC samples (n = 60) were stained with anti-c-Met antibody. The representative micrographs of 60 HCC samples for c-Met staining are shown in down panel. (B) Expression of c-Met was unrelated to OS or DFS of HCC patients. 150 patients were divided into two groups according to the median value of transcriptional level of c-Met in tumor tissues. (C) 24-h production of HGF by HCC tumor-derived CD3^+ T cells, CD14^+ monocytes/macrophages, CD56^+ NK cells, CD66b^+ neutrophils, and fibroblasts (n = 4). (D) 24-h production of HGF by healthy or HCC blood-derived CD66b^+ neutrophils (n = 4). (E) Paraffin-embedded HCC samples were stained with anti-CD15 antibody (n = 150). (F) Expression of c-Met was inversely correlated with OS or DFS in HCC patients with high peritumoral neutrophil infiltration. The patients were divided into four groups according to the median value of c-Met expression and peritumoral neutrophil infiltration in HCC tissues. Scale bar, 100 μm. Results represent mean ± SEM. *p < 0.01.

Table 2. Univariate and multivariate analyses of factors associated with survival and recurrence.

| Variables               | OS          | DFS          |
|------------------------|-------------|--------------|
|                        | Univariate  | Multivariate | Univariate  | Multivariate |
|                        | p value     | HR 95% CI    | p value     | HR 95% CI    |
| Age, years             | >50 vs. <50 | 0.735 NA     | NA          | 0.899 NA     |
| Gender                 | female vs. male | 0.612 NA | NA          | 0.711 NA     |
| HBeAg                  | positive vs. negative | 0.085 NA | NA          | 0.554 NA     |
| Liver cirrhosis        | present vs. absent | 0.819 NA | NA          | 0.838 NA     |
| ALT, U/L               | >45 vs. ≤45 | 0.488 NA     | NA          | 0.577 NA     |
| AFP, ng/mL             | >25 vs. ≤25 | 0.117 NA     | NA          | 0.159 NA     |
| Tumor size, cm         | >5 vs. ≤5   | <0.001 NS    | NS          | <0.001 NS    |
| Tumor multiplicity     | multiple vs. solitary | 0.061 NA | NA          | 0.082 NA     |
| Vascular invasion      | present vs. absent | 0.002 2.231 1.183–3.244 0.014 | 0.004 2.358 1.154–3.652 0.002 |
| Intrahepatic metastasis| yes vs. no  | <0.001 NS    | NS          | <0.001 NS    |
| TNM stage              | III–IV vs. I–II | <0.001 0.389 0.201–0.574 0.002 | <0.001 0.399 0.198–0.612 <0.001 |
| Tumor differentiation  | III–IV vs. I–II | 0.077 NA | NA          | 0.049 NS     |
| Fibrous capsule        | present vs. absent | 0.778 NA | NA          | 0.812 NA     |
| Combine neutrophils    | <0.001 NA |
| and c-Met^-            | <0.001 NA |

NOTE: Cox proportional hazards regression model. *Patients were classified into four groups according to their peritumoral neutrophil density and tumor c-Met expression.

Abbreviations: HR, hazard ratio; OS, overall survival; CI, confidence interval; DFS, disease-free survival; AFP, α-fetoprotein; NA, not adopted; NS, not significant.
with culture supernatants from both primary and established hepatoma cells (Fig. 2C). Accordingly, using inhibitors to block the signal transduction of Erk1/2, p38, and NF-κB effectively impaired such TSN-induced neutrophil HGF production, whereas abolishing the phosphorylation of AKT and JNK had only a marginal effect (Fig. 2D). These findings indicate that neutrophils are activated by HCC environments and subsequently acquire the ability to produce HGF.

**GM-CSF is required for tumor neutrophil activation and HGF production**

Our next endeavor was to determine the factor(s) involved in the induction of neutrophil HGF production by HCC environments. Recent studies have suggested that GM-CSF released by malignant cells contributes to the differentiation and protumorigenic functions of granulocytic MDSC in mice. Indeed, we also observed a marked increase of GM-CSF in plasma from HCC blood, and that the level of GM-CSF positively correlated with the patients’ TNM stage (Fig. 3A). Analyzing the GM-CSF produced by primary and established hepatoma cells revealed a marked accumulation of GM-CSF in the culture supernatants within 24 h (Fig. 3B). To investigate whether GM-CSF is also responsible for the generation of HGF-producing neutrophils in human HCC tumors, we initially tested the effect of recombinant human GM-CSF on HGF production by neutrophils. In support, GM-CSF, in a dose-dependent manner, did effectively induce HGF production (Fig. 3C). Correspondingly, exposure of neutrophils to GM-CSF triggered rapid activation of PI3K/AKT, MAPKs, JNK, Erk, and p38, and the NF-κB inhibitor IκBα as those displayed by neutrophils treated with culture supernatants from primary and established hepatoma cells (Fig. S1; Fig. 2C); as expected, inhibiting the activities of Erk1/2, p38, and NF-κB, but not the phosphorylation of AKT or JNK, also successfully attenuated HGF production by GM-CSF-incubated neutrophils (Fig. 3D). More importantly, using specific neutralizing antibody to abolish the effects of GM-CSF in culture supernatants from hepatoma cells and this treatment did efficiently inhibit HGF production by neutrophils (Fig. 3E).

We afterward established a mouse hepatoma model to investigate the roles of malignant cell-derived GM-CSF in the induction of neutrophil HGF production. The shRNA retroviral vectors were applied to stably suppress the expression of GM-CSF in mouse hepatoma Hepa1-6 cells. The expression of GM-CSF in Hepa1-6 tissue was extremely higher than in normal mouse liver; stable transfection of shGM-CSF retroviral vectors in Hepa1-6 cells could markedly attenuate the expression of GM-CSF in hepatoma tissue (Fig. 3F, left panel). Consistent with our hypothesis, the neutrophils derived from Hepa1-6 hepatoma that had been stably transfected with

**Figure 2.** Activation of Erk1/2, p38, and NF-κB is essential for the induction of HGF in tumor associated neutrophils. (A–C) Purified neutrophils were left untreated or stimulated with culture supernatant from primary HCC cells, three hepatoma tumor cell lines or a normal liver cell line (L02) for 48 h. (A) The release of HGF in the culture supernatants at the indicated times were determined by ELISA. (B) The expression of HGF in neutrophils after 2-h treatment was detected by real-time PCR. (C) The activation of Erk1/2, p38, JNK, AKT, and IκBα at 0.5 h was measured by immunoblotting. (D) Inhibition of Erk1/2, p38, and IκBα impaired HGF expression in neutrophils exposed for 24 h to culture supernatant from primary HCC cells. The illustrated results represent mean ± SEM of four separate experiments. *p < 0.05, **p < 0.01, compared with medium; #p < 0.05, compared with DMSO.
shGM-CSF retroviral vectors showed significant reduction of HGF production (Fig. 3F, right panel). Thus, malignant cell-derived GM-CSF is an important determinant in tumor-elicited neutrophil HGF production.

**Tumor neutrophils promote the metastasis of hepatoma cells via the HGF/c-Met axis**

The results described above suggested that HCC environments trigger neutrophil HGF production, which in turn elicits protumorigenic effects of these cells. To test this hypothesis, we assessed the influence of tumor neutrophils on hepatoma cell proliferation, migration, and invasion. We collected the conditioned media (CM) from tumor neutrophils (TCM) and paired blood neutrophils (BCM). The TCM, but not BCM, significantly enhanced the migration and invasion of hepatoma QGY-7703 cells, whereas neither TCM nor BCM could affect the proliferation of cells (Fig. 4A–B; Fig. S2). Supporting our hypothesis that tumor neutrophils promote hepatoma cell migration and invasion via the HGF/c-Met axis, using c-Met inhibitor SU11274 significantly impaired the increased hepatoma cell migration induced by TCM (Fig. 4C; left panel). Analogously, neutralizing HGF in TCM also efficiently attenuated its ability to induce hepatoma cell migration (Fig. 4C; right panel). In contrast, the control IgG did not affect that process (Fig. 4C; right panel). Similar results were obtained when we used CM from neutrophils pre-incubated with GM-CSF to stimulate hepatoma QGY-7703 cells: The CM significantly increased QGY-7703 cell migration and invasion; these processes could be abolished by c-Met inhibitor SU11274 (Fig. 4D). Notably, inhibiting the signals Erk1/2, p38, and NF-κB that regulated HGF production in GM-CSF-incubated neutrophils also effectively attenuated the ability of CM to induce hepatoma cell migration and invasion (Fig. 4E).

We finally used mouse hepatoma model to investigate the effect of neutrophil-derived HGF on intrahepatic metastasis. Depletion of tumor neutrophils by injecting anti-Gr1 Ab and suppression of HGF/c-Met axis by injecting anti-HGF Ab in mice both effectively impaired the incidence of intrahepatic metastasis, although anti-HGF Ab only weakly affected tumor sizes (Fig. 5A–B). Supporting the hypothesis that neutrophils are important cellular source of HGF in hepatoma environments, the combined usage of Abs against Gr1 and HGF in mice exhibited similar capability in reducing the incidence of intrahepatic metastasis as those displayed by injecting anti-Gr1 Ab alone (Fig. 5C). Consistently, peritoneal injection of supernatant derived from co-culture of neutrophils and Hepa1-6 cells partially restored the intrahepatic metastasis in mice treated with anti-Gr1 Ab, and this process could be abolished by adding anti-HGF Ab (Fig. 5C). In contrast, the supernatant from the culture of neutrophils alone did not influence the intrahepatic metastasis (Fig. 5C). These results together
indicate that release of HGF by tumor neutrophils contribute to c-Met-mediated HCC metastasis.

Discussion

Despite recent success in demonstrating the importance of HGF/c-Met axis in human cancers, little is known about the source and regulation of HGF in cancer environments. The current study demonstrated that neutrophils were the major source of HGF in HCC tissues and the density of neutrophils in tumors determined malignant cell c-Met-associated clinical outcome of patients. More importantly, hepatoma cell-derived GM-CSF was an important determinant in neutrophil HGF production, which in turn enhanced the migration and invasion of malignant cells.

Tumor neutrophils exhibit distinct functional characteristics with impaired bactericidal activities, but increased potential in regulating angiogenesis and tissue remodeling. 

Figure 4. The effect of HGF/c-Met axis on the migration and invasiveness of tumor cells. (A and B) Effects of TCM or BCM on hepatoma QGY-7703 cell migration (A) and invasion (B); untreated QGY-7703 cells, Med. (C) Suppression of c-Met activity by inhibitor SU11274 (left panel) or neutralization of HGF in TCM (right panel) effectively impaired hepatoma QGY-7703 cell migration. (D) Suppression of c-Met activity by inhibitor SU11274 effectively impaired hepatoma QGY-7703 cell migration (left panel) and invasion (right panel) stimulated by CM from GM-CSF-treated neutrophils. (E) Suppression of Erk1/2, p38, and IkB activity in neutrophils treated with GM-CSF attenuated the effects of neutrophil CM on hepatoma QGY-7703 cell migration and invasion. All the data shown are representative of at least four separate experiments. Scale bar, 100 μm. * p < 0.01, compared with medium in D,E; # p < 0.05, compared with DMSO.

Figure 5. Effects of neutrophils and HGF on hepatoma growth and intrahepatic metastasis in tumor-bearing mice (n = 8 for each group). (A–C) The Hepa1-6 hepatoma were treated with PBS or injected with anti-Gr1 and/or anti-HGF in the presence or absence of culture supernatant (SN) from coculture of blood neutrophils and Hepa1-6 cells as described in the methods. The representative images hepatoma with intrahepatic metastasis was shown in (A). The numbers of intrahepatic metastasis in different group were determined by immunohistochemical staining of hematoxylin and eosin (C). n = 8 for each group; * p < 0.01.
study of human HCC, pro-inflammatory IL-17-producing cells have been found to recruit blood neutrophils into the peritumoral stroma of HCC by epithelium-derived CXC chemokines. In the present study, we observed that, under the influence of local HCC environments, neutrophils were activated and acquired the ability to produce significant levels of c-Met ligand HGF, and that these cells, but not hepatoma cells, CD14+ myeloid cells, CD3+ T cells, or CD56+ NK cells, represented the major source of HGF in tissues. In fact, these activated neutrophils were unable to kill tumor cells and instead they promoted the in vitro migration and invasion of malignant cells via HGF/c-Met interaction, which suggests that such neutrophils can actually benefit tumor progression. This notion is supported by our current finding that, in patients with high infiltration of CD15+ cells, the malignant c-Met expression was inversely associated both OS and DFS. Consistent with our results, other investigators reported that cancer environments stimulated neutrophils to produce oncostatin M and MMP9, which in turn increased the metastatic potential of malignant cells. P13K/AKT, MAPK, and NF-κB pathways are all tightly associated with activation-induced neutrophil survival, but the nature and regulation of these pathways in human tumor neutrophils remain largely unknown. The present study provided evidence that HCC environments could trigger rapid activation of P13K/AKT, MAPKs JNK, Erk, and p38, and the NF-κB inhibitor IκBα in neutrophils, which in turn promoted the HGF-mediated hepatoma metastasis. Agents that prevent Erk1/2, p38, and NF-κB activation were found to block HGF production. In addition, in vitro migration assays showed that suppression of these pathways also effectively reduced tumor neutrophil-mediated increased motility of malignant cells. Sequential neutrophil activation and enhanced malignant cell migration in tumors may reflect a novel immune-editing mechanism by which tumors render neutrophils able to perform a sustained protumorigenic function by stimulating neutrophil activation. This hypothesis is compatible with the studies showing that tumors educate macrophages to perform a suppressive role by inducing early activation of monocytes.

It is generally assumed that GM-CSF is efficient activator of M1 macrophage polarization, whereas M-CSF has been implicated for M2 macrophage polarization. However, emerging evidence reveals that GM-CSF participate in the differentiation of protumorigenic M2 macrophage. Indeed, studies in mice also showed that GM-CSF released by malignant cells contributed to the differentiation and protumorigenic functions of granulocytic MDSC. Supporting the protumorigenic roles of GM-CSF, our present study shows that induction of neutrophil HGF production by the hepatoma cell-derived GM-CSF plays a dominant role in maintaining prometastatic effect of neutrophils, as indicated by the results of four sets of experiments. First, significant GM-CSF was detected in plasma of HCC patients and in the culture supernatants of hepatoma cells, and that the levels of GM-CSF in plasma of patients were positively associated with their disease progression. Second, exposure of neutrophils to GM-CSF resulted in marked neutrophil activation and subsequent HGF production, and these neutrophils efficiently promoted migration and invasion of malignant cells via a HGF/c-Met-dependent manner. Third, Erk1/2, p38, and NF-κB were required for hepatoma cell-elicited neutrophil HGF production; these pathways were also responsible for GM-CSF-triggered neutrophil HGF production. Fourth, neutralizing GM-CSF effectively attenuated TSN-mediated neutrophil HGF production. These findings together suggest that increased GM-CSF in HCC environments may reroute neutrophils into a tumor-promoting direction by stimulating HGF production.

The biological effects of HGF are mediated by its interaction with its high-affinity tyrosine kinase receptor, c-Met, which is shown to be overexpressed and mutated in a variety of malignancies. For HCC, there is a close relationship between overexpression of the c-Met and HCC metastasis, experimental studies and clinical investigations have shown that c-Met is a potential therapeutic target in HCC. In the present study, elevated mRNA and protein expression of c-Met was observed in HCC tissues and hepatoma cell lines. In addition, our results showed that neutrophil-derived HGF induces significant migration of HepG2 and QGY hepatoma cells in vitro through interacting with c-Met receptor. However, the fact that anti-HGF antibodies did not completely abrogate such tumor cell migration suggests that other soluble motility factors are also involved. In accordance with our results, other investigators also reported that the neutrophil-derived oncostatin M and MMP9 promote motility and invasiveness of mammary cancer cells.

Our results give important new insights into the production of HGF by tumor neutrophils. GM-CSF derived from hepatoma cells can trigger a rapid activation of Erk1/2, p38, and NF-κB in neutrophils, and thereby induce the production of HGF, which in turn leads to the migration and invasion of cancer cells in human HCC, reflecting a positive regulatory loop between tumors and their stroma. In support of this, the number of CD15+ neutrophils in primary HCC was inversely associated with the OS of the patients. Therefore, it is possible that studies on the mechanisms that selectively modulate the activation of neutrophils will provide a novel strategy for anticancer therapy.

**Materials and methods**

**Patients and specimens**

HCC plasma and tissue samples were obtained from patients undergoing curative resection at the Cancer Center of Sun Yat-sen University (Table 1). Plasma samples (taken on day of surgery) were from 75 HCC patients who underwent surgical resections between December 2012 and June 2015 (Cohort 1; Table 1). An additional 150 HCC patients who had undergone curative resection between 2005 and 2010 and had complete follow-up data (Cohort 2; Table 1) were enrolled for analysis of OS and DFS. The inclusion criteria used in patient enrollment from the consecutive cohorts were absence of anticancer therapies or had distant metastasis prior to the operation, no concurrent autoimmune disease, HIV or syphilis, and availability of follow-up data. Of them, preoperative liver function was all classified as Child-Pugh class A. The clinical stage of tumors was determined according to the TNM classification system of International Union Against Cancer (edition 6). Tumor differentiation was graded by the Edmondson grading system. All
samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). Written informed consent was obtained from the patients, and the protocol was approved by the Review Board of Sun Yat-sen University.

Preparation of culture supernatants from hepatoma cells and liver cells

Human hepatoma cell lines (HepG2 and SK-Hep-1) and mouse hepatoma Hepa1-6 cells were obtained from the American Type Culture Collection (Manassas, VA). Human normal liver cell line LO2 and hepatoma cell line QGY-7703 were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. All cells were proved to be mycoplasma free as routinely tested by a single-step PCR method, and they were maintained in DMEM medium (Hyclone) supplemented with 10% FBS (Gibco). Culture supernatants from these cell lines were prepared as previously described.39

Culture supernatant from primary HCC cells was acquired by culture of completely digested HCC tumor biopsy specimens. All of the samples were from patients without concurrent autoimmune disease, HIV, or syphilis. Ten7 digested cells were resuspended in 10 mL of complete medium and cultured in 100-mm dishes. After 2 d, the supernatants were harvested, centrifuged, and stored at −80°C.

Immunohistochemistry

Paraffin-embedded formalin-fixed HCC samples were cut into 5-μm sections, which were processed for immunohistochemistry as previously described.18 The sections were incubated with Abs against human CD15 (Lab Vision Corporation), or c-Met (Santa Cruz Biotechnology), and then stained in an Envision System (DakoCytomation). Evaluation of immunohistochemical variables was performed by two independent observers who were blinded to the clinical outcome.

Immunoblotting

The proteins were extracted as previously described.46 Equal amounts of cellular proteins were separated by 10% SDS-PAGE, immunoblotted with Abs against AKT, p38, JNK, Erk1/2, phospho-AKT, phospho-p38, phospho-JNK, phospho-Erk1/2, phospho-1xB (Cell signaling Technology), and Actin (Boster), and then visualized with an ECL kit (Thermo Fisher Scientific).

Isolation and culture of neutrophils

Neutrophils of ∼98% purity in DMEM containing 10% FBS were plated at 5 × 10⁶ per well in the presence or absence of culture supernatants from hepatoma or liver cells. In some experiments, the cells were pretreated with a c-Met inhibitor SU11274 (Pfizer, 5μM), a GM-CSF neutralization antibody (R&D Systems, 10 μg/mL), an HGF neutralization antibody (R&D Systems, 100 ng/mL), an Erk1/2 inhibitor U0126 (25 μM), a p38 inhibitor SB202190 (50 μM), a JNK inhibitor SP600125 (100 μM), an IkB inhibitor Bay11-7082 (20 μM), or an AKT inhibitor Triciribin Hydrate (100 μM), and subsequently exposed to indicated stimuli. In other experiments, the neutrophils were directly treated with recombinant GM-CSF (R&D Systems) with or without different inhibitors.

Isolation of leukocytes and fibroblasts from tissues

For the isolation of tumor-infiltrating leukocytes and fibroblasts, fresh HCC biopsy specimens were cut into small pieces and digested in RPMI 1640 supplemented with 0.05% collagenase IV (Sigma-Aldrich), 0.002% DNase I (Roche), and 20% FBS at 37°C for 30 min. Dissociated cells were filtered through a 150-μm mesh and separated by density gradient centrifugation. Thereafter, the leukocytes were harvested and the tumor-infiltrating neutrophils, monocytes, T cells, NK cells, and fibroblasts were isolated by anti-CD66b, anti-CD14, anti-CD3, anti-CD56, and anti-fibroblast magnetic beads (Miltenyi Biotec or Stem Cell Technology), respectively.

In vitro assays of QGY-7703 cell migration and invasion

The migration and invasion assay was performed in a 24-well Boyden chamber with an 8-μm pore size polycarbonate membrane (Corning). For migration assay, QGY-7703 cells were left untreated or stimulated for 24 h with supernatants from neutrophils with indicated treatments. Thereafter, the cells were removed from culture dishes by trypsinization, then washed, pelleted by centrifugation, and resuspended in serum-free medium at a density of 3 × 10⁶/mL. 100 μL cell suspension was added to the upper chamber, whereas the lower compartment was filled with 600 μL of DMEM containing 10% FBS. After incubation at 37°C for 12 h, the cells remaining on the upper surface of the membrane were removed. The migrated tumor cells on the lower surface of the membrane were fixed and stained with crystal violet and subsequently counted under a light microscope. Invasion assay was done by the same procedure, except that the membrane was coated with 50 μg Matrigel to form a matrix barrier.

Construction of viral vectors

The candidate sequence for mouse shGM-CSF and a scrambled sequence for shNC were cloned into pSiF-H1-CopGFP-shRNA lentiviral vectors (System Biosciences). Thereafter, the lentiviral vectors were transfected into HEK293T cells together with their helper virus vectors pFIV-34N and pVSV-G (System Biosciences) using calcium phosphate. After 2 d, the viral particles were harvested and enriched by ultracentrifugation.
**In vivo modulation of neutrophils and hepatoma cells**

Mouse hepatoma Hepa1-6 cells were left untreated or stably transfected with shGM-CSF retroviral vectors. Hepa1-6-derived hepatomas was inoculated under the liver envelope as described. After 25 d, tumor tissues were harvested and digested for isolation of tumor-infiltrating neutrophils. Thereafter, the production of HGF in tumor neutrophils was detected by ELISA. In another set of in vivo experiments, after 2-d inoculation of Hepa1-6-derived hepatoma, the animals were injected with Ab(s) against mouse Gr1 or HGF (10 mg/kg) or against Gr1 plus HGF in 100 μL buffered saline into the peritoneum every 3 d. In parallel, control animals were injected with 100 μL buffered saline. In some cases, the mice were first treated with an anti-Gr1 antibody (10 mg/kg) for 3 d. Thereafter, they were injected with anti-CD20 antibody plus 200 μL culture supernatant from co-culture of blood neutrophils and Hepa1-6 cells in the presence or absence of anti-HGF Ab every 3 d. After 21 d, tumors were harvested for subsequent immunohistochemical staining of hematoxylin and eosin. All mice were randomly grouped. Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Sun Yat-sen University.

**Analysis of gene expression**

Total RNA was extracted using the Trizol Reagent (Invitrogen) according to the manufacturer’s recommendations. The mRNA level of HGF and c-Met was detected using THUNDERBIRD SYBR qPCR Mix (TOYOBO). GAPDH was used as the endogenous control. All reactions were run in triplicate and repeated in three independent experiments. The Data were analyzed by using the comparative Ct study. The specific primers used in this assay were as follows: human HGF, 5′-CTC ACA CCC GCT GGG AGT AC-3′ forward and 5′-TCC TTG ACC TTG GAT GCA TTC-3′ reverse; human MET, 5′-CCC CAC CCT TTG TTC AG-3′ forward and 5′-TCA GGC TCT TCC CTG CT-3′ reverse; human GAPDH, 5′-CAC CAT CTT CCA GGA GCG AG-3′ forward and 5′-GGG GCC ATC CAC AGT CTT C-3′ reverse; mouse GM-CSF, 5′-TGCCTGTCAAGTGA ATG AAG A-3′ forward and 5′-TGG TGA AAT TGC CCC GTA GA-3′ reverse; mouse GAPDH, 5′-GTATGACTCCACT-CACGG-3′ forward and 5′-GGTCTGAGCTCTCTGGAAGA-3′ reverse.

**ELISA**

Concentrations of human HGF, mouse HGF, and human GM-CSF in the plasma or culture supernatants were determined using commercial ELISA kits (R&D system) according to the instructions provided by the manufacturer.

**Statistical analysis**

Statistical analyses were performed with the SPSS 17.0 (SPSS Inc., Chicago, IL) software. Results are expressed as means ± SEM. DFS was defined as the time from random assignment to recurrence, second primary cancer, or death without evidence of recurrence or second primary cancer. OS was defined as the time from random assignment to death as a result of all causes. Cumulative OS and DFS time were calculated using the Kaplan–Meier method and analyzed by the log-rank test. A multivariate Cox proportional hazards model was used to estimate adjusted hazard ratios and 95% confidence intervals and to identify independent prognostic factors. For categorical analysis, the median value was used as a cut point to dichotomize the series except serum AFP level and tumor size (for clinical applications). The χ² test was used to test for relationships between categorical variables. The statistical significance of differences between groups was determined by Student’s t-test. All data were analyzed using two-tailed tests unless otherwise specified, and p < 0.05 was considered statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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