Pathology International

Pathology International 2013; **63**: 233–235 doi:10.1111/pin.12051

Letter to the Editor

Intrahepatic cholangiocarcinoma with sarcomatous change producing granulocyte-colony stimulating factor

To the editor:

Granulocyte-colony stimulating factor (G-CSF) is a naturally occurring glycoprotein that stimulates the proliferation and maturation of precursor cells in bone marrow into fully differentiated neutrophils. Since G-CSF producing lung cancer was initially reported in 1977,[1] G-CFS producing malignant neoplasms, such as hepatocellular carcinoma (HCC), bladder cancer, uterine cervix cancer, pancreatic cancer, and so forth, has been reported. Intrahepatic cholangiocarcinoma (ICC) is a relatively infrequent tumor in most populations, but is the second most common primary hepatic malignancy following HCC. Only five ICCs have been reported to show G-CSF production with marked leucocytosis.[2–6] Herein, we report a case of ICC with sarcomatous change presenting marked leukocytosis and discuss the association between ICC and G-CSF with respect to hepatic stem/progenitor cells (HSPCs).

A 62-year-old woman was hospitalized for a large mass with a maximal diameter of 8 cm in the left lobe of the liver. She had a history of chronic hepatitis type C, and had been examined periodically in our hospital for 20 years. When she underwent examination by ultrasonography 5 months prior to the admission, the mass lesion was not detected in the liver. Physical examination on admission revealed high fever (38.4°C), epigastralgia, hepatomegaly and non-pitting edema in the lower extremities. Laboratory data on admission showed mild leukocytosis (11 900/μL), mild anemia, hypoalbuminemia and elevation of C-reactive protein. Serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 were markedly elevated to 104.9 ng/mL (normal: <2.5 ng/mL) and 5983.7 U/mL (normal: <37 U/mL), respectively. Abdominal computed tomography showed a large low density mass measuring 10 × 6 cm in the left lobe of the liver. Metastasis of lymph nodes or other organs was not detected.

Based on these findings, she was clinically diagnosed with ICC and underwent chemotherapy using gemcitabine (1400 mg/day). However, chemotherapy had no effect. With the tumor progression, white blood cell count and CEA were elevated up to 50 500/μL and 227.0 ng/mL, respectively. She died of liver failure approximately 3 months after admission.

Autopsy was performed. The liver weighed 2470 g. The cut surface showed a yellowish solid mass measuring 12.2 × 8.5 cm with massive necrosis and hemorrhage in the left lobe of the liver. Innumerable small nodules had diffusely invaded the bilateral lobes of the liver. Histopathologically, the tumor was composed of two different components. One was an adenocarcinoma component, the other was a sarcomatous component. In the former component, tumor cells were proliferative in glandular fashion with mucin production in a relatively limited part (Fig. 1a: left side). The latter component showed that tumor cells with loose cohesiveness and marked pleomorphism were diffusely proliferative (Fig. 1a: right side). Numerous atypical mitoses were encountered. Neutrophils infiltration was evident inside tubular structures of adenocarcinoma component and around sarcomatous component. The expression of G-CSF was observed both adenocarcinoma and sarcomatous components immunohistochemically (Fig. 1b). Both components were positive for vimentin (Fig. 1c). All of the tumor cells were positive for cytokeratin (CK) 7, CK19, CK CAM 5.2 and negative for hepatocyte paraffin-1 and epithelial cell adhesion molecule. These findings indicate this tumor is a moderately differentiated adenocarcinoma with sarcomatous change. The inner rims of small ducts in the adenocarcinoma component were positive for CD56 and CD 133, which are representative markers of HSPCs (Fig. 1d). The tumor metastasized to bilateral lung diffusely, bronchopulmonary and para-aortic lymph nodes. Background non-carcinomatous hepatic tissue showed the formation of regenerative nodules and infiltration of mononuclear cells in the portal area, which corresponded to cirrhosis associated with hepatitis C virus infection. There were no apparent infection foci causing severe leucocytosis in other organs.

The diagnostic criteria of G-CSF producing tumor are as follows: (i) extreme leukocytosis, (ii) elevated G-CSF activity, (iii) drop in white blood cell count after tumor resection, or (iv) proof of G-CSF production in the tumor.[1] Our present case revealed marked leucocytosis. Moreover, G-CSF production was confirmed by immunohistochemical staining although the serum G-CSF level was not examined. It is reasonable to regard this tumor as a G-CSF producing tumor because leukocytosis deteriorated with the tumor progression and there were no other factors causing leukocytosis. The prognosis of patients with a G-CSF-producing tumor is extremely poor. ICC rarely causes marked leucocytosis. To the best of our knowledge searched by PubMed, only five cases of G-CSF producing ICC with marked leucocytosis have ever been reported (Table 1). Out of these, squamous cell carcinoma, adenosquamous cell carcinoma and adenocarcinoma were pathologically diagnosed in one, one and three cases, respectively. The average prognosis of them was 49 days. G-CSF was considered to be a major autocrine growth factor in rapid tumor proliferation and metastasis. Autocrine

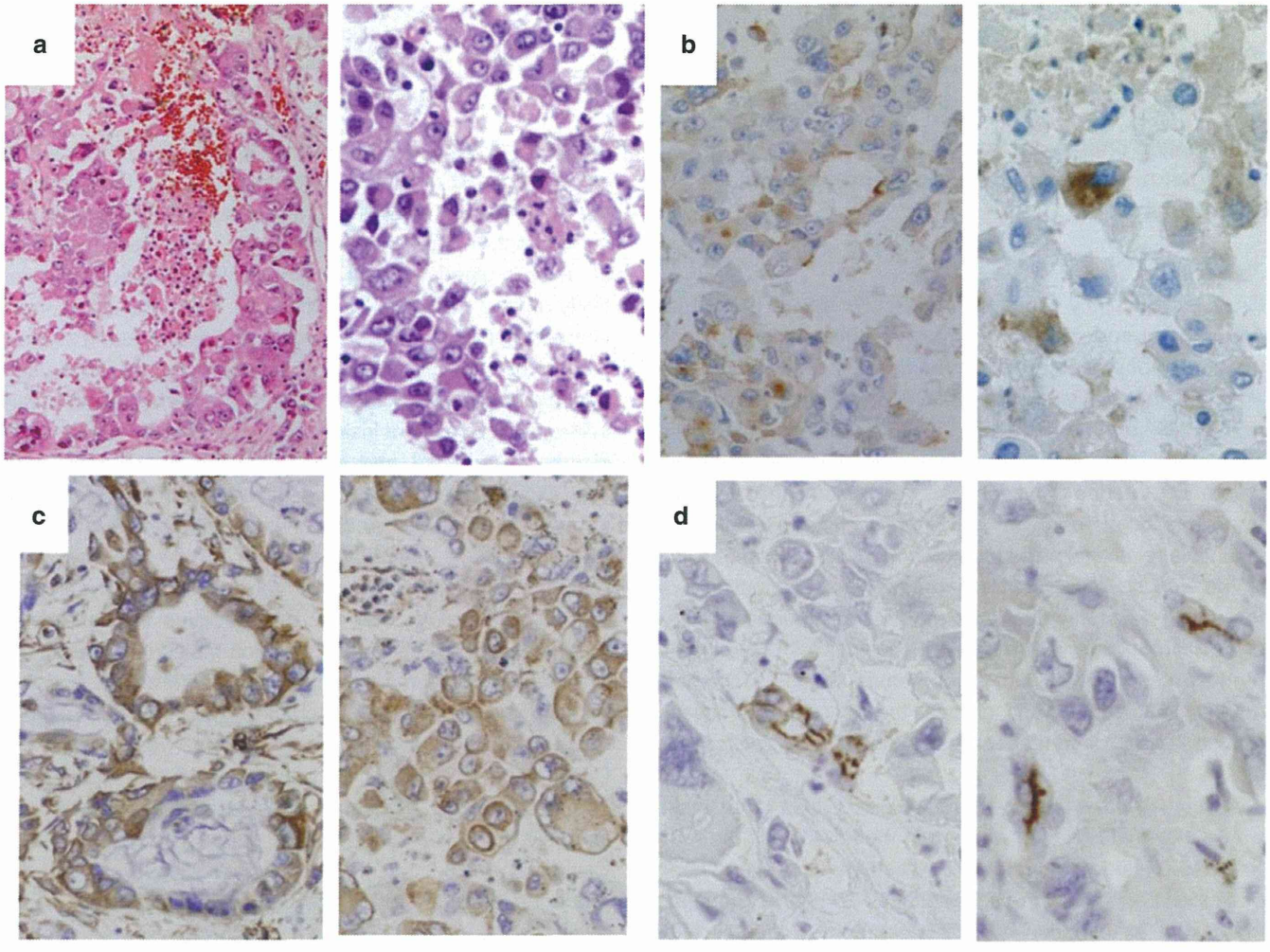

Figure 1 (**a**) Histopathological findings, the tumor cells form glandular arrangement with mucin production (left side: HE stain). In most parts, acidophilic tumor cells have a loose connection with marked pleomorphia and mitoses (right side: HE stain). (**b**) The expression of G-CSF was observed in cytoplasm of both adenocarcinomatous (left side) and sarcomatous component (right side). (**c**) The cytoplasm of both adenocarcinoma (left side) and sarcomatous component (right side) was positive for vimentin immunohistochemical staining. (**d**) CD56 (left side) and CD133 (right side) expression were observed in the inner rims of small ducts of ICC cells.

G-CSF/G-CSF receptor signaling in bladder cancer can significantly contribute to cancer cell adhesion and invasion in a β1-integrin-dependent manner.[7] These autocrine signals might be associated with rapid tumor progression in our case. The detection of G-CSF is very difficult by immunostaining because the G-CSF protein is generally retained in the cytoplasm for a short time and the antigenicity is vulnerable. However, in our present case G-CSF production was confirmed immunohisotochemically. In our case, microscopic findings revealed moderately differentiated adenocarcinoma with massive sarcomatous change. G-CSF is known to be associated with the transformation of the epithelial elements into a more immature and high grade phenotype. The expression of G-CSF was found in both components in our case. Moreover, the expression of vimentin, which is the representative marker of sarcomatous transformation or epithelial-mesenchymal transformation (EMT), was observed in both components. Thus, the adenocarcinoma component could already possess a sarcomatous transformation or EMT nature to keep adenocarcinomatous structures. G-CSF expression might enhance phenotypic changes to high grade tumor. Although the etiology of ICC remains unclear in most cases, hepatitis virus type C (HCV) related cirrhosis had a 1000-fold risk of ICC compared with the general population in Japan.[8] In addition, Sasaki *et al.* reported that the frequent expression of G-CSF and granulocyte macrophage colony-stimulating factor is characteristic of ICC with cirrhosis and the ICC element in combined hepatocellular cholangiocarcinoma.[9] They reported that these tumors might share several HSPCs or fetal parenchymal liver cells. Our present case was also accompanied by HCV related cirrhosis, indicating the tumor cells of our case might share characteristics with HSPCs. ICC with HSPC phenotypes has been proposed recently.[10] Indeed, this tumor also had a component showing

Table 1 Published cases of G-CSF producing ICC

First Author	Age (years)	Sex	HCV infection	Symptoms	WBC (/μL)	G-CSF (pg/ml) [normal value]	CRP (mg/dL)	Tumor size (cm) Location	Pathological diagnosis	IHC (G-CSF)	Therapies	Prognosis
[2]Aizawa	69	M	ND	Fever, weight loss	13700	82.5 (ND)	ND	Multiple small lesions	SCC	ND	Operation	1 month
[4]Masuda	48	M	(–)	Fever, abdominal pain	10500	213 [<9.8]	2.14	5 × 3 cm Left lobe	Por	(+)	Palliative care	2 months
[3]Kakinoki	66	F	(–)	Fever	14900	99.2 [<32.3]	13.0	11 × 13 cm Right lobe	Adenosq	(+)	Palliative care	2 months
[6]Sohda	56	M	ND	Fever, consciousness-disturbance	74300	264 [<27.5]	9.7	5 cm Left lobe	Por	(+)	Palliative care	5 days
[5]Shinojima	68	F	ND	Sweet Syndrome	11200	Normal	13.1	6 cm Right lobe	Adeno	(+)	Operation	ND
Present case	62	F	(+)	Fever, abdominal pain	11900	Not performed	4.46	10 × 6 cm Left lobe	Mode	(+)	Chemotherapy	3 months

Adeno, adenocarcinoma; Adenosq, adenosquamous cell carcinoma; CRP, C-reactive protein; F, female; G-CSF, granulocyte-colony stimulating factor; HCV, hepatitis C virus; ICC, intrahepatic cholangiocarcinoma; IHC, immunohistochemical staining; M, male; Mode, moderately differentiated adenocarcinoma; ND, not described; Por, poorly differentiated adenocarcinoma; SCC, squamous cell carcinoma; WBC, white blood cell count.

small sized acinar configuration as a minor component. This component, which was thought to be corresponding to bile ductular type of ICC, showed positive reaction for CD56 and CD133, which are representative markers of HSPCs.

We report a rare case of ICC with sarcomatous change producing G-CSF with severe leukocytosis. This case report provides novel information on the relationship between progression of ICC and G-CSF production.

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Translational Oncology
www.transonc.com

Volume 6 Number 5 October 2013 pp. 511–519 **511**

Metronomic Chemotherapy: Possible Clinical Application in Advanced Hepatocellular Carcinoma[1]

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Abstract

Hepatocellular carcinoma (HCC) is a hypervascular highly angiogenic tumor usually associated with liver cirrhosis. Vascular endothelial growth factor plays a critical role in vascular development in HCC. In contrast to the treatment of early-stage HCC, the treatment options for advanced HCC are limited and prognosis is often poor, which contributes to this tumor type being the third leading cause of cancer-related deaths worldwide. Metronomic chemotherapy, which was originally designed to inhibit angiogenesis, involves low-dose chemotherapeutic agents administered in a frequent regular schedule with no prolonged breaks and minimizes severe toxicities. We reviewed the potential effects and impact of metronomic chemotherapy in preclinical studies with HCC models and in patients with advanced HCC, especially when combined with a molecular targeted agent. Metronomic chemotherapy involves multiple mechanisms that include antiangiogenesis and antivasculogenesis, immune stimulation by reducing regulatory T cells and inducing dendritic cell maturation, and possibly some direct tumor cell targeting effects, including the cancer stem cell subpopulation. The total number of preclinical studies with HCC models shows impressive results using metronomic chemotherapy-based protocols, especially in conjunction with molecular targeted agents. Four clinical trials and two case reports evaluating metronomic chemotherapy for HCC indicate it to be a safe and potentially useful treatment for HCC. Several preclinical and clinical HCC studies suggest that metronomic chemotherapy may become an alternative type of chemotherapy for advanced unresectable HCC and postsurgical adjuvant treatment of HCC.

Translational Oncology (2013) 6, 511–519

Introduction

Systemic chemotherapy with cytotoxic agents remains the most common systemic therapy to treat patients with metastatic disease. Most anticancer agents are designed to inhibit growth or kill rapidly dividing tumor cells. These drugs are usually administered at the highest doses possible to induce the maximum therapeutic effect; this is referred to as maximum tolerated dose (MTD) therapy [1,2]. However, administration of anticancer agents at MTD requires prolonged breaks between cycles of the therapy to allow recovery from the induced adverse side effects in different tissues and organs. These gaps in chemotherapy can allow or facilitate tumor regrowth including growth of clones resistant to the therapy. The regrowth of tumor or drug resistance clones during such gaps can prevent or compromise improvement of overall survival of patients with advanced cancer even when the first cycle of MTD therapy is effective [1,3–6].

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[1]This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Supported Program for the Strategic Research Foundation at Private Universities. The authors have declared no conflicts of interest.
Received 18 June 2013; Revised 22 July 2013; Accepted 24 July 2013

DOI 10.1593/tlo.13481

A new concept of anticancer treatment that targets the tumor vasculature was first proposed by Folkman in 1971 [7]. This treatment concept is based on the indispensable role of the vasculature in tumor growth [8,9]. Antiangiogenic therapy has been investigated extensively in both preclinical and clinical studies [10,11]. In 1991, Kerbel [12] suggested that some conventional cytotoxic anticancer agents can suppress vascular development in tumors based on the immature and proliferative nature of endothelial cells present in the neovasculature. Klement et al. [13] and Browder et al. [14] reported that frequent repetitive low doses of chemotherapy drugs such as cyclophosphamide or vinblastine could markedly suppress tumor growth. Hanahan et al. coined the term *metronomic* therapy to describe this type of therapeutic schedule [15]. Metronomic therapy generally consists of the continuous administration of low-dose chemotherapeutic agents without extended intervals [2]. It was originally designed with the intention to inhibit tumor growth by antiangiogenic mechanisms, though other mechanisms can contribute to its antitumor efficacy as described below, and is usually associated with much less severe acute toxicities compared to conventional MTD chemotherapy [16]. So, recently, metronomic chemotherapy has been investigated in pediatric oncology [17]. Most new cancer cases and deaths now occur in low-income and middle-income countries [18]. As metronomic chemotherapy is a low-cost, well-tolerated, and easy-to-access treatment, it will be an attractive therapeutic option in resource-limited countries [19].

Hepatocellular carcinoma (HCC) is the sixth most common solid tumor and the third leading cause of cancer-related death globally [20,21]. Although the major blood supply to HCC is the portal veins at the early stage of hepatocarcinogenesis, the main supply ultimately is provided by neoarteries that develop in parallel with tumor growth [22–24]. For advanced HCC, such as Barcelona Clinic Liver Cancer (BCLC) stage C, classical chemotherapy is sometimes selected [25]. However, HCC is usually associated with liver cirrhosis, and thus aggressive chemotherapy can cause severe side effects [26]. Unfortunately, the prognosis of patients with advanced HCC is usually poor even in those treated with sorafenib [27,28]. To improve the therapeutic efficacy and prognosis of patients with advanced HCC, new strategies are clearly needed.

In this review, we evaluate the potential effects and impact of metronomic chemotherapy in patients with advanced HCC, especially when combined with a molecular targeted agent such as sorafenib.

Treatment for Advanced HCC

The development of sophisticated diagnostic modalities, such as computed tomography, magnetic resonance imaging, and abdominal ultrasonography, has allowed early diagnosis of HCC [29–32]. Patients with small HCCs are usually treated by surgical resection, liver transplantation, percutaneous ethanol injection therapy, microwave coagulation therapy, or percutaneous radiofrequency ablation [33]. The prognosis of patients with small HCCs has improved following the application of these therapeutic modalities [33].

Treatment of advanced HCC includes transhepatic arterial chemoembolization, transhepatic arterial infusion chemotherapy, systemic chemotherapy, hormonal therapy, and immunotherapy [32,34–37]. However, only transhepatic arterial chemoembolization has been confirmed to improve long-term survival in BCLC stage B [38–41].

In large randomized trials, the median survival time (MST) of patients treated with doxorubicin were 6.8 and 7.4 months, respectively [42,43]. The MST of patients treated with PIAF regimen (cisplatin, interferon, doxorubicin, and fluorouracil) and FOLFOX4 regimen (oxaliplatin and fluorouracil) was 8.7 and 6.4 months, respectively. In three double-blinded, placebo-controlled trials, no survival benefit of tamoxifen was confirmed [44–46]. In several small studies, the MST of patients with HCC treated with capecitabine and gemcitabine was 10.1 and 6.9 months, respectively [47,48]. Other drugs such as cisplatin, 5-fluorouracil (5-FU), mitoxantrone, etoposide, paclitaxel, irinotecan, and fludarabine have also failed to demonstrate meaningful activity [49–55]. Despite maximum effort by many investigators, any definitive evidences that systemic chemotherapy is effective for advanced HCC have not been provided [56]. Sorafenib is an orally active multi-kinase inhibitor that targets vascular endothelial growth factor receptor 2 (VEGFR-2) and PDGF receptors, among others, and also blocks tumor cell proliferation by targeting the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinases (ERKs) signaling pathway by virtue of its targeting the intracellular threonine kinase Raf [57–59]. The efficacy of sorafenib for advanced HCC was confirmed for the first time in the phase III SHARP trial (MST; 10.7 months) and the Asian-Pacific phase III region trial (MST; 6.5 months) [27,28]. For advanced unresectable HCC with vascular invasion or extrahepatic metastasis (BCLC stage C), administration of sorafenib is now recommended worldwide [1,60,61]. Several trials with molecular target agents are underway. In the phase III trial, the MST of brivanib was 9.5 months and that of sorafenib was 9.9 months. In another phase III trial, the MST of linifanib and sorafenib was 9.1 and 9.8 months, respectively. In combination therapy, sorafenib and erlotinib (MST; 9.5 months) failed to prove the survival benefit comparing with sorafenib alone (MST; 8.5 months). Any other molecular target agents fail to surpass the efficacy of sorafenib so far. Due to the associated liver cirrhosis, patients with HCC sometimes develop severe side effects during conventional MTD chemotherapy, as noted above. Since metronomic chemotherapy is less toxic and, moreover, inhibits tumor growth through antiangiogenic mechanisms, this new therapeutic strategy using certain conventional chemotherapeutic drugs could be suitable for the treatment of advanced HCC.

Metronomic Chemotherapy

Preclinical Studies

The first preclinical studies of metronomic chemotherapy came from the laboratories of Folkman and Kerbel [14]. To date, there are more than 300 papers published on the preclinical effects of metronomic chemotherapy, as listed in PubMed. These reports describe the therapeutic efficacy of metronomic chemotherapy against at least 18 different types of cancers in the gastrointestinal tract, respiratory system, blood, brain, skin, and genitourinary systems. The most frequently selected anticancer drug for preclinical metronomic chemotherapy studies is cyclophosphamide. One interesting aspect of some of these studies is the potent antitumor efficacy of metronomic chemotherapy regimens in models of advanced metastatic cancer especially when combined with a targeted antiangiogenic drug which itself has minimal activity in this setting [62,63].

The main antitumor effects caused by metronomic chemotherapy are thought to be inhibition of tumor-associated vascular development and stimulation of immunity rather than direct cytotoxic effects on tumor cells (Figure 1) [12,64–66]. However, intriguingly, some recent reports have implicated direct targeting of cancer stem cells as a possible mechanism of metronomic cyclophosphamide [67], in contrast to MTD cyclophosphamide that does not target this subpopulation [68]. In the following section, we discuss recent information regarding

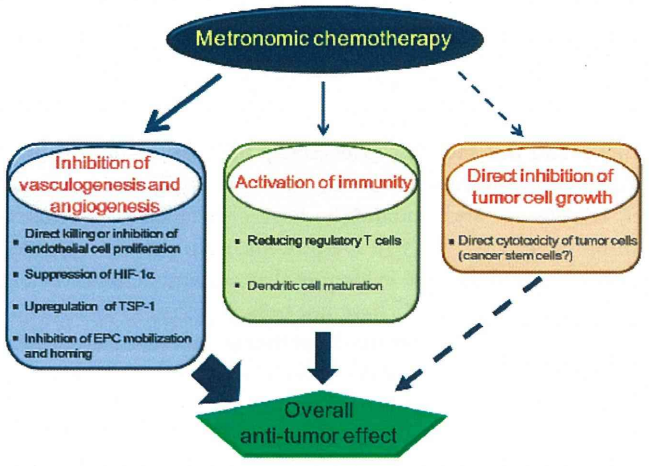

Figure 1. Mechanisms of action of metronomic chemotherapy. The beneficial effects of metronomic chemotherapy are mediated through inhibition of vasculogenesis and angiogenesis, activation of immunity, and probably direct inhibition of tumor cell proliferation. Inhibition of vasculogenesis and angiogenesis plays a critical role in metronomic chemotherapy. Inhibition of vasculogenesis and angiogenesis includes direct inhibition of endothelial cell proliferation, up-regulation of endogenous angiogenic inhibitor such as TSP-1, suppression of HIF-1α, and inhibition of EPC homing in tumor tissues. Metronomic chemotherapy also stimulates antitumor immunity by reducing the number of Treg cells and possibly inducing dendritic cell maturation.

mechanisms of metronomic chemotherapy, especially inhibition of vascular development and stimulation of immunity mediated by metronomic chemotherapy.

Inhibition of tumor angiogenesis/vascular development.

Direct cytotoxicity or inhibition of endothelial cell proliferation. Many conventional cytotoxic chemotherapeutic agents, such as cyclophosphamide, vinblastine, paclitaxel, docetaxel, tegafur/uracil (UFT), and tegafur/gimeracil/oteracil potassium (S-1), have antiangiogenic effects [69–75]. S-1 is composed of three compounds, namely, tegafur, gimeracil, and oteracil. UFT and S-1 decrease thymidine phosphorylase that is also called platelet-derived endothelial growth factor. The effect of UFT and S-1 seems to induce the antiangiogenic effect. The activated endothelial cells of newly formed blood capillaries are highly and selectively sensitive *in vitro* to very low concentrations of many conventional cytotoxic anticancer agents [76–81]. The antiangiogenic effects of conventional cytotoxic anticancer drugs seem to be optimized by administration of smaller doses without long breaks for prolonged periods [12].

Up-regulation of endogenous antiangiogenic factors and down-regulation of endogenous angiogenic factors. Angiogenesis is thought to be switched off or downregulated when levels of endogenous antiangiogenic factors such as thrombospondin-1 (TSP-1) and angiostatin exceed those of angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF) [82]. Bocci et al. [83,84] reported that protracted exposure of endothelial cells *in vitro* to low concentrations of various anticancer chemotherapeutic agents and ceramide analog caused marked induction of gene and protein expression of TSP-1. A number of other groups have reported up-regulation of circulating levels of TSP-1 in mice or patients exposed to metronomic chemotherapy [85]. TSP-1, a component of the extracellular matrix produced by endothelial cells, tumor cells, and infiltrating stromal cells, seems to act by binding to CD36 expressed on the cell membrane of endothelial cells [86–88]. TSP-1 also binds to VEGF and sequesters its angiogenic activity [89]. Hypoxia-inducible factor 1 (HIF-1) regulates the expression of angiogenic factors such as VEGF, bFGF, and stromal cell–derived factor 1 (SDF-1). Continuous administration of low-dose topotecan was reported to decrease the expression of HIF-1α [34], VEGF, and SDF-1 [90]. Administration of low-dose anthracycline chemotherapeutic agents also inhibited HIF-1 transcription and the expression of VEGF and SDF-1 [91]. VEGF is the major factor in angiogenesis/vascular development in many tumors [92,93]. A decrease in serum VEGF levels was observed in patients with advanced breast cancer treated with metronomic cyclophosphamide [94]. In addition, metronomic chemotherapy with weekly platinum and daily etoposide administration in patients with non–small cell lung cancer resulted in a decrease in VEGF level during treatment [95].

Inhibition of vasculogenesis by reducing the number and viability of circulating endothelial progenitor cells. Vascular development in tumor tissues consists of angiogenesis and vasculogenesis. Vasculogenesis is generally defined as the contribution to the formation of new blood vessels by circulating bone marrow–derived cells, possibly including endothelial progenitor cells (EPCs) [96,97]. Accumulating evidence suggests that circulating bone marrow–derived EPCs migrate into tumor tissues to support vascular formation and tumor growth [98,99]. In addition, local release of VEGF and SDF-1 induce the migration of EPCs to tumor tissues through VEGFRs and CXCR4 on the cell surfaces of EPCs [100].

Bertolini et al. [101] reported that the administration of MTD cyclophosphamide induced a robust EPC mobilization a few days after the end of treatment in tumor-bearing mice bearing human lymphoma cells. In marked contrast, metronomic chemotherapy of cyclophosphamide, using lower doses given daily, was associated with consistent decreases in the numbers and viability of EPCs, with a much more durable and marked inhibition of tumor growth [101].

Stimulation of Immunity

Metronomic chemotherapy with certain chemotherapeutic agents can stimulate the immune response by reducing regulatory T (Treg) cells and inducing dendritic cell maturation [65,66,102]. Treg cells are $CD4^+CD25^+$ lymphocytes known to accumulate in variety of cancers [103]. Increased frequency of Treg cells correlate with tumor progression and lack of treatment response [103]. Metronomic chemotherapy with cyclophosphamide and temozolomide was shown to increase the antitumor immune responses by suppressing the number and activity of Treg cells and also by increasing lymphocyte proliferation and memory T cells [65,66,104–106]. The reduction in Treg cell number was specific, and the treatment had no effects on other types of lymphocytes [106]. This effect was specific for metronomic chemotherapy. However, conventional MTD or high-dose chemotherapy can result in depletion of all types of lymphocytes. Reduction of Treg cells by metronomic chemotherapy restored the antitumor immune response by recovering the activity of both tumor-specific (cytotoxic T lymphocytes and helper T cells) and tumor-nonspecific effect cells (natural killer and natural killer T cells) [106]. Other immunostimulatory effects of metronomic chemotherapy have been proposed recently.

As an example, Tanaka et al. [102] reported that vinblastine, paclitaxel, and etoposide promoted dendritic cell maturation at nontoxic concentrations. They also found that local injection of low-dose vinblastine induced the maturation of tumor-infiltrating dendritic cells and stimulated antitumor immune responses *in vivo* [107]. However, the involvement of dendritic cell maturation by metronomic chemotherapy needs to be further investigated and confirmed. Preclinical studies using immunodeficient mice have shown that metronomic chemotherapy can result in marked tumor growth suppression. Such results indicate that the involvement of the immune system in metronomic chemotherapy is not necessarily critical [108,109]. Nevertheless, it is interesting to consider the potential benefits of combining metronomic chemotherapy with immunotherapeutic treatments, e.g., tumor vaccines [110].

Metronomic Chemotherapy: Studies Using HCC Models

The potential efficacy of various metronomic chemotherapy protocols using cyclophosphamide, UFT, cisplatin, and doxorubicin have been investigated in animal models of HCC [111,112], as summarized in Table 1. Park et al. [113] reported that metronomic chemotherapy with cyclophosphamide inhibited HCC growth and prolonged survival without inducing major toxicities using a rat HCC model with accompanying liver cirrhosis. Tang et al. [111] reported that single or doublet metronomic chemotherapy using cyclophosphamide, UFT, and/or doxorubicin without any added antiangiogenic agents did not have survival benefits. In contrast, they reported a significant improvement of overall survival in animals that received various combinations of metronomic chemotherapeutic regimens with DC101, an anti–VEGFR-2 targeting antibody that potently inhibits angiogenesis. They also reported that metronomic chemotherapy with metronomic UFT and sorafenib delayed the onset of tumor progression (i.e., delayed development of resistance to chemotherapy) [114]. Zhou et al. [115] also reported that metronomic doxorubicin in combination with bevacizumab had a profound effect on tumor growth inhibition and survival of HCC xenograft model. The appearance of resistance to molecular targeted agents, such as sorafenib, is an inevitable problem in the treatment of advanced unresectable HCC. Thus, this report may be hopeful with respect to the clinical application of metronomic chemotherapy with sorafenib for advanced HCC. Iwamoto et al. [116] demonstrated that metronomic chemotherapy with S-1 inhibited tumor growth and prolonged survival of hepatoma tumor–bearing mice and that these effects were enhanced by the addition of vandetanib, an oral inhibitor of both the epidermal growth factor receptor and VEGFR-2. The antitumor effects of metronomic chemotherapy with S-1 alone were shown to be mediated mainly through inhibition of angiogenesis by upregulation of TSP-1 expression and direct inhibition of endothelial cell proliferation in tumor tissues. With regard to the toxic effects of such therapies, the use of MTD S-1 caused body weight loss and myelosuppression, whereas S-1 metronomic chemotherapy or S-1 metronomic chemotherapy with vandetanib did not cause any severe toxicity. Metronomic chemotherapy with a single agent did not cause an antitumor effect in one study by Tang et al. [111]. However, not only S-1 metronomic chemotherapy with vandetanib but also metronomic S-1 monotherapy caused significant antitumor effects in the study by Iwamoto et al. Perhaps these differences might be due to greater antitumor effects caused by S-1 compared with UFT [116], although the different models could be another explanation.

Jang et al. [117] used a chemically induced model of HCC in rats and compared an MTD *versus* metronomic chemotherapy protocol using cyclophosphamide. The metronomic protocol was more effective in prolonging survival than the MTD method and also suppressed metastasis formation, not just intrahepatic tumor growth. Among the mechanisms implicated for the results included suppression of HIF-1α levels and matrix metalloproteinases (MMPs), including MMP-2 and MMP-9, and also of the MMP-2 activator, tissue inhibitor of metalloproteinase-2 (TIMP-2). In a previous study by the same group using the rat HCC model, suppression of VEGFR-2 caused by metronomic cyclophosphamide was also reported [113].

Metronomic Chemotherapy: Clinical Studies

To date, more than 50 clinical trials, mostly phase II trials, of metronomic chemotherapy have been reported in adult patients with breast cancer, lung cancer, prostate cancer, malignant brain tumor, colon cancer, multiple melanoma, malignant lymphoma, HCC, and other types of tumors [118–120]. Many of those clinical trials included both chemotherapeutic and antiangiogenic agents. About 80% of the trials have reported positive efficacy of metronomic chemotherapy. In addition to the improvement in therapeutic response rate (complete response + partial response) and/or clinical benefit (complete response + partial response + stable disease), Orlando et al. [121] showed that 27% of patients with advanced breast cancer who were already resistant to trastuzumab responded to treatment using doublet metronomic cyclophosphamide and methotrexate, in combination with trastuzumab. Furthermore, Kato et al. [122] and Watanabe et al. [123] reported that continuous daily administration of nontoxic doses of UFT was safe and effective as postoperative adjuvant treatment in randomized phase III adjuvant trials undertaken in patients with non–small cell lung cancer and breast cancer, respectively. UFT was administered daily with no breaks for 2 years and can be viewed as a

Table 1. Preclinical Studies Evaluating Metronomic Chemotherapy Regimens in Rodent Models of HCC.

Animal Model	Drug Used	Reference
Human HCC cell line orthotopic xenografts in SCID mice	Oral UFT + cyclophosphamide plus sorafenib or DC101	Tang et al. [111]
Human HCC cell line orthotopic xenografts in SCID mice	Oral UFT + sorafenib	Tang et al. [114]
Human HCC cell line orthotopic xenografts in nude mice	Intravenous doxorubicin plus bevacizumab	Zhou et al. [115]
Chemically induced HCC in rats	Oral cyclophosphamide	Park et al. [113]
Chemically induced HCC in rats	Cyclophosphamide	Jang et al. [117]
Human HCC cell line subcutaneous xenografts in nude mice	Oral S-1 + vandetanib	Iwamoto et al. [116]
Human HCC cell line xenografts and primary HCC cells from patients in Nonobese diabetic/SCID/interleukin-2 (IL-2) receptor γ null mice	Oral cyclophosphamide	Martin-Padura et al. [67]

SCID indicates severe combined immunodeficiency.
S-1 is an oral 5-FU prodrug; UFT is an oral 5-FU prodrug; vandetanib is an oral tyrosine kinase inhibitor that targets VEGFRs and epidermal growth factor receptors; DC101 is an anti-mouse VEGFR-2 neutralizing monoclonal antibody.

Table 2. Clinical Studies Evaluating the Therapeutic Efficacy of Metronomic Chemotherapy in Patients with HCC.

Drugs Used	Results/Comments	References
Octreotide, imatinib, oxaliplatin	Phase I/II study. Metronomic chemotherapy with oxaliplatin in combination with antiangiogenic drugs suppressed the increase of serum E-selectin, VEGF-A, PDGF-BB, and α-fetoprotein levels.	Treiber et al. [131]
UFT, sorafenib	Phase II study. Metronomic chemotherapy with UFT was safely combined with sorafenib and showed activity to improve the efficacy of sorafenib.	Hsu et al. [132]
5-FU, sorafenib, bevacizumab, thalidomide	Phase II study. An early α-fetoprotein response was a useful surrogate marker to predict treatment efficacy and prognosis of metronomic chemotherapy with 5-FU in combination with antiangiogenic agents.	Shao et al. [136]
Capecitabine	Case report. Metronomic chemotherapy with capecitabine induced complete remission with minimal toxicity.	Brandi et al. [140]
Capecitabine	Case report. Metronomic chemotherapy with capecitabine for HCC patient with Child-Pugh class B was effective and well tolerated.	Ballardini et al. [139]
UFT, sorafenib	Phase II study. Vascular response measured by dynamic contrast-enhanced MRI predicted tumor response and survival by metronomic UFT therapy with sorafenib.	Hsu et al. [133]
UFT, sorafenib	Phase II study. High baseline circulating EPC levels were associated with poor prognosis by sorafenib and metronomic chemotherapy with UFT.	Shao et al. [138]
Epirubicin, cisplatin, 5-FU	Prospective study. Metronomic chemotherapy might be a safe and useful palliative treatment for HCC patients with major portal vein tumor thrombosis.	Woo et al. [135]
UFT, thalidomide	Phase II study. High baseline IL-6 and IL-8 levels were associated with poor prognosis. Metronomic chemotherapy with UFT and thalidomide was safe and demonstrated modest activity.	Shao et al. [137]

MRI indicates magnetic resonance imaging.
UFT is an oral 5-FU prodrug.

metronomic chemotherapy-like trial. In contrast, a few other clinical trials of metronomic chemotherapy reported negative outcomes. In particular, malignant brain tumors seem to be resistant to metronomic chemotherapy [124–127]. With regard to adverse effects, metronomic chemotherapy was associated with minimal toxicity and severe adverse events are rare. The most common mild side effects were nausea, vomiting, fatigue, and bone marrow suppression [128,129]. In view of the encouraging preclinical and clinical findings evaluating metronomic chemotherapy or metronomic chemotherapy combined with targeted agents—especially antiangiogenic drugs—a number of randomized phase III trials have been initiated, four in breast cancer and two in colorectal cancer (www.clinicaltrials.gov) [130]. Two are adjuvant trials. The chemotherapy drugs involved include cyclophosphamide, methotrexate, and capecitabine, and the antiangiogenic drug, when used, is bevacizumab (Avastin), the monoclonal anti-VEGF antibody.

Metronomic Chemotherapy: Clinical Setting of HCC

To date, there are only four clinical trials evaluating metronomic chemotherapy for HCC (Table 2). One reported negative result, whereas others reported positive natures. Treiber et al. [131] randomly classified 38 patients with advanced HCC into the following four treatment groups: patients of group 1 received 30 mg of octreotide on day 1, group 2 received octreotide on day 1 and 400 mg of imatinib daily, group 3 received oxaliplatin (60-90 mg/m^2) on day 1, and group 4 received oxaliplatin (20-30 mg/m^2) on days 1, 8, and 15 combined with 30 mg of octreotide on day 1 and 400 mg of imatinib daily. The time to progression and overall survival were not different among the groups in this phase I/II trial. Hsu et al. [132,133] conducted another phase II study of the combination of sorafenib (400 mg twice daily) with metronomic UFT (125 mg/m^2 based on tegafur twice daily) for advanced HCC. They evaluated the efficacy and safety in 53 patients with Child-Pugh class A. The median progression-free survival was 3.7 months, and median survival was 7.4 months. Four patients showed partial response and 26 had stable disease. Treatment was associated with some severe toxicity including fatigue (15%), abnormal liver function (13%), elevated serum lipase (10%), hand-foot skin reaction (9%), and bleeding (8%). The authors concluded that metronomic chemotherapy with UFT could be safely combined with sorafenib and that such combination could improve the efficacy of sorafenib in patients with advanced HCC when compared to previous reports in similar patient cohorts treated with sorafenib alone [28,134]. The concurrent use of metronomic chemotherapy and sorafenib might augment antitumor efficacy but without a high incidence of severe side effects. Woo et al. [135] reported the results of a phase II trial involving infusion of metronomic epirubicin with cisplatin and 5-FU and found it to be a safe and potentially useful treatment for HCC patients with portal vein thrombosis (MST; 162 days). In addition, Shao et al. [136–138] undertook a metronomic UFT plus thalidomide, sorafenib, or bevacizumab trial in patients with advanced HCC and observed it to be safe, demonstrating modest activity (MST; 4.8 months). There are also two case reports reporting encouraging results in individual HCC patients treated with metronomic capecitabine [139,140]. In addition, Allegrini et al. [141] reported that metronomic UFT and cyclophosphamide plus celecoxib in heavily pretreated gastrointestinal patients including two patients with HCC were well tolerated and associated with interesting activity. To confirm the therapeutic efficacy and safety of metronomic chemotherapy in patients with advanced HCC, more (randomized) phase II trials with other anticancer agents and molecular targeted agents, including randomized controlled trials in larger populations, will be required.

Conclusions

In this review, we have attempted to outline the many reasons why we feel metronomic chemotherapy, especially when used in conjunction with an antiangiogenic drug such as sorafenib, is a potentially promising strategy to consider for the treatment of patients with advanced HCC. In summary, these reasons are given as follows:

1. HCC is a highly angiogenic tumor, driven by such proangiogenic growth factors such as VEGF and bFGF.
2. Sorafenib is already approved for treatment of patients with HCC.

3. Antiangiogenic drugs can augment the efficacy of metronomic chemotherapy and vice versa, as shown in a very large number of diverse preclinical studies—especially those involving treatment of mice with advanced metastatic disease—and also as suggested, or shown, in a number of phase II clinical trial results of other types of cancer.
4. Metronomic chemotherapy, which functions more as a biologic therapy, is now known to involve multiple mechanisms that include antiangiogenesis and antivasculogenesis, immune stimulation, and possibly some direct tumor cell targeting effects, including of the cancer stem cell subpopulation.
5. There is no effective standard chemotherapy for HCC when using conventional MTD treatment protocols, and in part, this is related to the toxicity of such treatments in patients with HCC who have the underlying comorbidity of liver cirrhosis; in contrast, the less toxic regimens associated with metronomic chemotherapy and the different cellular targets and mechanisms of action involved may make this an attractive and alternative type of chemotherapy to consider, especially for treatment of advanced HCC, but perhaps also for postsurgical adjuvant treatment of early-stage HCC, given the successes of metronomic-like protocol of UFT reported in adjuvant phase III breast and lung cancer trials.
6. The total number of preclinical studies showing impressive results using metronomic chemotherapy-based protocols, especially in conjunction with antiangiogenic drugs (even in models of advanced metastatic disease) along with the number of promising clinical study and trial results that have been published to date, argues strongly for giving more consideration to testing more extensively this type of treatment strategy for advanced HCC.
7. Some limited preliminary results of several preclinical HCC studies using metronomic chemotherapy in conjunction with antiangiogenic drugs indeed suggest that this treatment strategy can be highly active and, as such, should be given proactive clinical consideration.

Perspective for Future Directions

In unresectable advanced HCC (BCLC stage C), sorafenib is recommended as the standard treatment. As HCC is usually accompanied with liver cirrhosis, a combination treatment with less adverse events will be required to improve the survival benefit of sorafenib. Metronomic chemotherapy will be a candidate treatment that meets these criteria. To confirm the synergy of metronomic chemotherapy, prospective trials of metronomic chemotherapy with sorafenib compared with sorafenib alone as the control arm will be necessary as soon as possible.

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ONCOLOGY REPORTS 30: 2049-2055, 2013

Effect of occult hepatitis B virus infection on the early-onset of hepatocellular carcinoma in patients with hepatitis C virus infection

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Received June 22, 2013; Accepted August 2, 2013

DOI: 10.3892/or.2013.2700

Abstract. Although overt hepatitis B virus (HBV) infection promotes the onset of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV)-infected patients, the effect of occult HBV infection remains unclear. The aim of this study was to investigate the effect of occult HBV infection on the early-onset of HCC in HCV-infected patients. A total of 173 HCC patients with HCV infection were enrolled and classified into 2 groups according to the median age of HCC onset: the early-onset group (n=91; 61.1±5.6 years) and the late-onset group (n=82; 73.8±3.7 years). Independent factors associated with the early-onset of HCC were assessed by multivariate analysis. In the overall analysis, independent risk factors for the early-onset of HCC were the white blood cell count and alanine aminotransferase level, but not the presence of HBV DNA. In a stratification analysis according to albumin levels of ≥3.5 g/dl, the presence of HBV DNA was a significant independent risk factor for the early-onset of HCC (OR 145.18, 95% CI 1.38-15296.61, P=0.036), whereas the presence of antibodies against hepatitis B core antigen was not found to be a risk factor. The presence of HBV DNA was not a risk factor for the early-onset of HCC in the overall analysis. However, its presence was an independent factor for the early-onset of HCC in HCV-infected patients with an albumin level of ≥3.5 g/dl. Thus, occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; BMI, body mass index; WBC, white blood cell; HbAlc, hemoglobin Alc; AST, aspartate aminotransferase; ALT; alanine aminotransferase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HOMA, homeostasis model assessment; APRI, AST to platelet ratio index; AUROC, area under the receiver operating characteristic curve analysis; MAPK, mitogen activated protein kinase

Key words: latent HBV infection, hepatoma, liver cancer, oncogenesis, white blood cell

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It ranks third in men and fifth in women as the cause of death from malignancies in Japan (1). Chronic hepatitis C virus (HCV) infection is the major cause of HCC and accounts for ~60-70% of HCC cases in Japan (2). In addition to hepatic inflammation and subsequent fibrosis, various other factors including aging, obesity and diabetes mellitus are involved in the hepatocarcinogenesis in HCV-infected patients (3-5).

Co-infection of HCV with hepatitis B virus (HBV) is thought to synergistically increase the development of HCC (6). The status of HBV infection is evaluated by the presence of hepatitis B surface antigen (HBsAg), antibodies against hepatitis B core antigen (HBcAb), and HBV DNA. In some cases, HBV DNA can be detected in the serum or liver tissue of patients who are negative for HBsAg, a condition referred to as 'occult HBV infection' (7,8). In Japan, the prevalence of occult HBV infection in HCV-infected patients is reported

Table I. Nucleotide positions and sequences of TaqMan PCR primers and probes.

Primer/Probe	Sequence	Position
S-sense	TGTACAAAACCTTCGGACGGAAA	442-464
S-antisense	TGCGAAAGCCCAGGATGATG	485-504
S-probe	CTGCACTTGTATTCCC	465-480
C-sense	ACTGTGGTTTCACATTTCCTGTCTT	2072-2096
C-antisense	GGCATTTGGTGGTCTGTAAGC	2163-2183
C-probe	CCACACTCCAAAAGAC	2132-2147
X-sense	CTACTGTTCAAGCCTCCAAGCT	1729-1750
X-antisense	GCTCCAAATTCTTTATACGGGTCAATG	1778-1804
X-probe	AAGCCACCCAAGGCAC	1751-1766

Nucleotide positions are based on the sequence of hepatitis B virus subtype adr4 (GenBank accession no. X01587) (29).

to be between 37.7% and 90% (9-11). Occult HBV infection is associated with a poor response to interferon therapy for chronic hepatitis C (12,13) and is also known to accelerate the progression of liver fibrosis, resulting in cirrhosis in patients with HCV infection (9,14,15). Several previous studies have examined the impact of occult HBV infection on the development of HCC in HCV-infected patients, but no clear conclusions have emerged (14,16,17). Moreover, the effects of occult HBV infection on the early-onset of HCC have not been investigated in HCV-infected patients.

Albumin is produced by hepatocytes, and the level of serum albumin is used to evaluate hepatic function (18). Albumin plays a significant role in maintaining colloid osmotic pressure and transports drugs and endogenous substances including bilirubin and unesterified free fatty acids (19). In addition, albumin exerts antioxidative properties (19), and hypoalbuminemia has been shown to be an independent risk factor for mortality among residents of a hyperendemic area of HCV infection in Japan (20). A serum albumin level of ≥3.5 g/dl is an independent predictor of survival in HCC patients (21,22) and in cirrhotic patients with a serum albumin levels of <3.5 g/dl, branched-chain amino acids increase serum albumin levels and subsequently suppress hepatocarcinogenesis (23,24). Thus, the serum albumin level is an important factor in hepatocarcinogenesis.

The aim of this study is to investigate the impact of occult HBV infection on the early-onset of HCC in HCV-infected patients. We also performed a stratification analysis according to the serum albumin level.

Subjects and methods

Subjects. We conducted a retrospective study to investigate the effect of the presence of HBV DNA on the early-onset of HCC in HCV-infected patients. Between 1995 and 2011, 325 patients underwent hepatic resection at the Kurume University Hospital. The inclusion criteria were histologically proven HCC, a positive result for serum anti-HCV, and a negative result for serum HBsAg. Exclusion criteria were the presence of autoimmune hepatitis, primary biliary cirrhosis, and hemochromatosis, no test results for serum HBV DNA, and a histological diagnosis of combined hepatocellular and cholangiocellular carcinoma. Although 214 patients met the inclusion criteria, 41 patients had to be excluded because of one or more of these reasons. The remaining 173 HCC patients with HCV infection were therefore enrolled in this study and classified into 2 groups according to the median age of HCC onset: the early-onset group (n=91; 61.1±5.6 years) and the late-onset group (n=82; 73.8±3.7 years).

The study protocol was approved by the institutional review board, and informed consent for participation in the study was obtained from each subject. None of the subjects were institutionalized.

Data collection. Demographic data were collected at the time of hepatic resection including age, gender, and alcohol intake. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2).

Venous blood samples were taken in the morning after a 12-h overnight fast. The presence of serum anti-HCV, HBsAg, and HBcAb was tested using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital). Blood platelet count, white blood cell (WBC) count, prothrombin time %, plasma glucose levels; hemoglobin A1c (HbA1c) levels, and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total bilirubin, insulin, α-fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) were also measured using standard clinical methods. Insulin resistance was evaluated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment for insulin resistance (HOMA-IR), as previously described (25).

The stage of hepatic fibrosis was assessed using the AST-to-platelet ratio index (APRI), which is calculated as the serum AST level (U/l)/upper limit of normal AST (U/l) x100/platelet count (x10^4/ml). Patients with APRI values of ≤1.5 were diagnosed as having chronic hepatitis, and patients with APRI values >1.5 were diagnosed as having liver cirrhosis, as previously described (26). The degree of liver cirrhosis was categorized according to the Child-Pugh classification (27). Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels >126 mg/dl or HbA1c levels >6.5%, in accordance with the Diagnostic Criteria for

Table II. Differences in the clinical characteristics between the early-onset and late-onset groups.

Variable	Reference value	Early-onset	Late-onset	P
Number of patients		91	82	
Age (years)		61.1±5.6	73.8±3.7	<0.001
AFP (ng/ml)	<8.7	1876±12163	769±3246	0.588
DCP (mAU/ml)	<40	1083±4120	1071±3845	0.378
Maximal HCC size (mm)	N/A	30.4±19.6	33.2±16.2	0.055
Gender (female/male)	N/A	23/68	20/62	0.893
BMI (kg/m^2)	18.5-22.0	23.6±3.6	22.4±3.2	0.045
Daily alcohol intake (none/0-60 g/>60 g)	N/A	21/42/14	23/36/10	0.676
Platelet count (x10^4/mm^3)	13-36	13.8±5.4	13.5±4.6	0.988
WBC count (/mm^3)	4000-9000	5009±1526	4420±1210	0.012
AST (U/l)	13-33	56.2±29.5	52.8±27.2	0.412
ALT (U/l)	6-30	62.2±40.9	51.7±31.7	0.104
Albumin (g/dl)	4.0-5.0	3.87±0.45	3.85±0.38	0.520
Prothrombin time (%)	70-130	90.0±11.2	91.7±12.2	0.272
Total bilirubin (mg/dl)	0.3-1.2	0.84±0.35	0.79±0.29	0.346
Chronic hepatitis/Child-Pugh class A/Child-Pugh class B	N/A	40/49/2	36/44/2	0.994
Complication of diabetes mellitus (yes/no)	N/A	30/61	20/62	0.214
Fasting blood glucose (mg/dl)	80-109	119±39	107±31	0.060
Insulin (μU/ml)	5-20	13.1±10.4	9.8±8.0	0.014
HOMA-IR	<2.5	3.05±2.47	2.11±1.03	0.622
HbA1c (%)	4.6-6.2	5.77±0.88	5.50±0.78	0.053
HBcAb positive/negative	N/A	49/42	50/32	0.344
HBV DNA positive/negative	N/A	6/85	3/79	0.385

Values are expressed as the mean ± SE. AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; BMI, body mass index; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; N/A, not applicable.

Diabetes Mellitus of the Japan Diabetes Society (28), or the use of antidiabetic agents.

Nucleic acid extraction from serum. Total nucleic acid was extracted from 300 μl of plasma using a commercially available kit (High Pure Viral Nucleic Acid kit; Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The extracted nucleic acid was eluted in 25 μl of elution buffer.

PCR for HBV DNA. Serum HBV DNA was analyzed for the presence of HBs, HBc, and HBx (S, C and X) regions using TaqMan real-time PCR according to the manufacturer's instructions (TaqMan Fast Universal PCR Master mix; Applied Biosystems, Tokyo, Japan). The oligonucleotide primers and probes that were optimized for the HBV subtype adr4 (29) were specific for the S, X and C region sequences are listed in Table I. The full-length HBV DNA (GenBank accession no. X01587) (29) was used as an internal standard in the quantitative real-time detection PCR. We used 8 μl of nucleic acid-containing serum in our study for better sensitivity. The limit of sensitivity of our TaqMan Real-time PCR methods was 4.5 copies/well, and the detection limit of our tests was 45 copies/ml (1.7 log copies/ml). A real-time PCR assay (COBAS TaqMan HBV Auto; Roche Diagnostics) was also performed to detect the core region of HBV DNA (limit of quantification, 1.8 log copies/ml). The presence of HBV DNA was defined as any positivity of S, X or C region.

Statistical analysis. Data are expressed as the absolute value or the mean ± SD. Differences between the early-onset and late-onset groups were analyzed using the Mann-Whitney U test. A logistic regression model with the Firth's correction 30 was used for multivariate stepwise analysis to identify independent variables associated with the early-onset of HCC, as previously described (31,32). All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. All statistical analyses were conducted using SAS version 9.2 (SAS Institute, Cary, NC, USA) or R packages version 2.15.2 (URL http://www.R-project.org/).

Results

Univariate analysis between the early-onset and late-onset groups. AFP levels, DCP levels, and maximal HCC size did not differ between the early-onset and late-onset groups (Table II). Furthermore, although BMI, WBC count, and serum insulin levels were significantly higher in the early-onset group than in the late-onset group, there were no significant differences

Table III. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma.

	Unit	Odds ratio	95% CI	P
HbA1c	1	1.37	0.91-2.07	0.136
BMI	1	1.08	0.98-1.19	0.133
ALT	10	1.10	1.00-1.21	0.045
DCP	20	0.99	0.98-1.00	0.091
WBC count	1000	1.35	1.06-1.73	0.014

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HbA1c, hemoglobin A1c; BMI, body mass index; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; WBC, white blood cell.

in the daily alcohol intake, platelet count, prothrombin time, Child-Pugh classification, presence of diabetes mellitus as a comorbidity, fasting blood glucose level, HOMA-IR value, HbA1c levels, and the serum levels of AST, ALT, albumin, and total bilirubin (Table II). The presence of HBcAb and HBV DNA did not differ either between the early-onset and late-onset groups (Table II).

Multivariate stepwise analysis for early-onset of HCC. Multivariate stepwise analysis showed that the serum ALT level and WBC count were independent risk factors for the early-onset of HCC (OR 1.10; 95% CI 1.00-1.21; P=0.045 and OR 1.35; 95% CI 1.06-1.73; P=0.014, respectively; Table III), but not the presence of HBcAb or HBV DNA.

Stratification analysis according to serum albumin level. Differences in the clinical characteristics between patients with the albumin level of ≥3.5 g/dl and <3.5 g/dl were summarized in Table IV. There were no significant differences in AFP levels, DCP levels, and maximal HCC size between the albumin level of ≥3.5 g/dl and <3.5 g/dl groups (Table IV). In the albumin level of ≥3.5 g/dl group, a significant elevation was seen in platelet count, prothrombin time and the number of patients with chronic hepatitis and a significant depletion was seen in AST level than in the albumin level of <3.5 g/dl group. However, other biochemical parameters and the

Table IV. Differences in the clinical characteristics between patients with the albumin level of ≥3.5 g/dl and <3.5 g/dl.

Variable	Reference value	Albumin level of ≥3.5 g/dl	Albumin level of <3.5 g/dl	P
Number of patients		138	35	
Age (years)		67.8±8.1	67.9±6.7	0.895
AFP (ng/ml)	<8.7	786±3219	3262±18195	0.248
DCP (mAU/ml)	<40	854±3269	1961±5977	0.306
Maximal HCC size (mm)	N/A	30.6±15.9	36.8±23.8	0.171
Gender (female/male)	N/A	35/103	8/27	0.759
BMI (kg/m^2)	18.5-22.0	23.0±3.5	23.0±3.4	0.918
Daily alcohol intake (none/0-60 g/>60 g)	N/A	21/58/38	3/20/6	0.172
Platelet count (x10^4/mm^3)	13-36	14.3±4.9	11.3±4.8	0.001
WBC count (/mm^3)	4000-9000	4798±1395	4291±1331	0.052
AST (U/l)	13-33	51.2±26.2	67.1±32.6	0.001
ALT (U/l)	6-30	54.9±37.4	63.4±32.7	0.057
Albumin (g/dl)	4.0-5.0	4.02±0.28	3.23±0.20	<0.001
Prothrombin time (%)	70-130	91.6±12.0	88.0±10.0	0.038
Total bilirubin (mg/dl)	0.3-1.2	0.82±0.34	0.80±0.28	0.822
Chronic hepatitis/Child-Pugh class A/Child-Pugh class B	N/A	69/69/0	7/24/4	<0.001
Complication of diabetes mellitus (yes/no)	N/A	38/100	12/23	0.431
Fasting blood glucose (mg/dl)	80-109	112±37	121±49	0.694
Insulin (μU/ml)	5-20	10.1±6.4	17.6±16.3	0.063
HOMA-IR	<2.5	3.12±3.87	4.23±2.23	0.315
HbA1c (%)	4.6-6.2	5.61±0.79	5.68±1.04	0.905
HBcAb positive/negative	N/A	75/63	24/11	0.129
HBV DNA positive/negative	N/A	6/132	3/32	0.315

Values are expressed as the mean ± SE. AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; BMI, body mass index; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; N/A, not applicable.

Table V. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of ≥3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HBcAb	Positive	0.59	0.27-1.26	0.169
HBV DNA	Positive	145.18	1.38-15296.61	0.036
Prothrombin time	10	0.76	0.54-1.08	0.109
ALT	10	1.08	0.97-1.21	0.145
Albumin	0.1	1.17	1.01-1.36	0.036
DCP	20	0.99	0.98-1.00	0.037
Platelet count	1	0.92	0.84-1.02	0.107
WBC count	1000	1.64	1.15-2.35	0.006

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; WBC, white blood cell.

Table VI. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of <3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HbA1c	1	1.83	0.75-4.47	0.183
HBV DNA	Positive	0.00	0.00-2.96	0.093
AFP	20	1.39	1.01-1.93	0.045

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HbA1c, hemoglobin A1c; HBV, hepatitis B virus; AFP, α-fetoprotein.

presence of HBcAb and HBV DNA did not differ between the albumin level of ≥3.5 g/dl and <3.5 g/dl groups (Table IV).

In patients with a serum albumin level of ≥3.5 g/dl, the WBC count and serum levels of albumin and DCP were identified as independent factors associated with the early-onset of HCC (OR 1.64; 95% CI 1.15-2.35; P=0.006, OR 1.17; 95% CI 1.01-1.36; P=0.036, and OR 0.99; 95% CI 0.98-1.00; P=0.037, respectively; Table V). Although the presence of HBcAb was not found to be a significant risk factor for the early-onset of HCC, the presence of HBV DNA was identified as a significant independent risk factor associated with the early-onset of HCC (OR 145.18; 95% CI 1.38-15296.61; P=0.036; Table VI).

In patients with a serum albumin level of <3.5 g/dl, the serum AFP level was the only significant risk factor found to be associated with the early-onset of HCC (Table V). The presence of HBcAb and HBV DNA was not found to be a significant risk factor for the early-onset of HCC.

Discussion

In the overall analysis, the presence of HBV DNA in serum was not identified as a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis according to a serum albumin level of ≥3.5 g/dl revealed that the presence of HBV DNA was an independent factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with a relatively low carcinogenic potential.

Although co-infection of HCV and HBV is thought to synergistically increase the risk of HCC (6), the overall analysis in this study showed that occult HBV infection was not significantly associated with the early-onset of HCC in HCV-infected patients. Similarly, several studies conducted in Asia have also failed to show any significant effect of occult HBV infection in these patients (33-35). Recently, Lok *et al* (36) performed a nested case-control study using a large number of patients enrolled in the HALT-C cohort and reported no significant difference in the prevalence of occult HBV infection between HCC and non-HCC patients with HCV infection. Taken together, these results suggest that occult HBV infection may not be an intensive promoter of HCC development in the presence of a potent carcinogenic factor such as HCV infection.

In contrast with these previous studies and with our own findings for all patients, a stratification analysis according to a serum albumin level of ≥3.5 g/dl showed that occult HBV infection was an independent risk factor for the early-onset of HCC. In patients with occult HBV infection, it is unclear whether a presence of HBV DNA is due to full-length HBV DNA replicated from covalently closed circular DNA in hepatocytes or fragmented HBV DNA integrated into the hepatocyte genome. However, the *HBx* gene is frequently integrated into cellular genes in HCC (37). The HBx protein upregulates the expression of proto-oncogenes including *c-jun*, *c-fos* and *c-myc*, all of which can promote hepatocarcinogenesis (38,39). In addition, albumin plays a crucial role in the development of various diseases, as it is a major antioxidant (19). In cirrhotic patients with a serum albumin level of <3.5 g/dl, branched-chain amino acids increase serum albumin levels, and this subsequently suppresses hepatocarcinogenesis (23,24). In this study, we found a significant association between occult HBV infection and the early-onset of HCC in patients with a serum albumin level of ≥3.5 g/dl, but not in patients with a serum albumin level of <3.5 g/dl. Taken together, these findings suggest that HBV DNA may promote hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

Although we designed this study to investigate the effect of HBV DNA on the early-onset of HCC in HCV-infected patients, we found instead that an elevated WBC count is an independent risk factor for the early-onset of HCC in HCV-infected patients. An elevated WBC count may reflect the consequences or underlying pathogenesis of the early-onset of HCC. One possible explanation is aging, because the WBC count declines in old age (40). Alternatively, an elevated WBC count still within the reference range is known to be associated with the development of various malignancies including gastric, colorectal, endometrial and lung cancers (41,42). The WBC count is a well-validated biomarker of inflammation. Chronic inflammation is a possible risk factor for hepatocarcinogenesis as it leads to the activation of receptors for chemokine and advanced glycation-end products (43,44). Another inflammation marker, C-reactive protein, is reported to be a diagnostic and prognostic marker of HCC (45,46). Taken together, these findings suggest that inflammation may promote the early-onset of HCC in HCV-infected patients.

A limitation of this study is that there were only a small number of HBV DNA-positive patients. Previous studies regarding occult HBV infection had a similar limitation (33,47,48). Since occult HBV infection is not frequently seen in HCV-infected patients with HCC, a multicenter study is needed to confirm our findings.

In conclusion, the presence of HBV DNA in serum was not a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis based on a serum albumin level of ≥3.5 g/dl revealed that presence of HBV DNA in serum was an independent risk factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

Acknowledgements

This study was supported, in part, by Health and Labour Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Discrete Nature of EpCAM$^+$ and CD90$^+$ Cancer Stem Cells in Human Hepatocellular Carcinoma

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Recent evidence suggests that hepatocellular carcinoma (HCC) is organized by a subset of cells with stem cell features (cancer stem cells; CSCs). CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been identified by the use of various stem cell markers. However, little information is known about the expression patterns and characteristics of marker-positive CSCs, hampering the development of personalized CSC-targeted therapy. Here, we show that CSC markers EpCAM and CD90 are independently expressed in liver cancer. In primary HCC, EpCAM$^+$ and CD90$^+$ cells resided distinctively, and gene-expression analysis of sorted cells suggested that EpCAM$^+$ cells had features of epithelial cells, whereas CD90$^+$ cells had those of vascular endothelial cells. Clinicopathological analysis indicated that the presence of EpCAM$^+$ cells was associated with poorly differentiated morphology and high serum alpha-fetoprotein (AFP), whereas the presence of CD90$^+$ cells was associated with a high incidence of distant organ metastasis. Serial xenotransplantation of EpCAM$^+$/CD90$^+$ cells from primary HCCs in immunedeficient mice revealed rapid growth of EpCAM$^+$ cells in the subcutaneous lesion and a highly metastatic capacity of CD90$^+$ cells in the lung. In cell lines, CD90$^+$ cells showed abundant expression of c-Kit and *in vitro* chemosensitivity to imatinib mesylate. Furthermore, CD90$^+$ cells enhanced the motility of EpCAM$^+$ cells when cocultured *in vitro* through the activation of transforming growth factor beta (TGF-β) signaling, whereas imatinib mesylate suppressed *TGFB1* expression in CD90$^+$ cells as well as CD90$^+$ cell-induced motility of EpCAM$^+$ cells. *Conclusion*: Our data suggest the discrete nature and potential interaction of EpCAM$^+$ and CD90$^+$ CSCs with specific gene-expression patterns and chemosensitivity to molecular targeted therapy. The presence of distinct CSCs may determine the clinical outcome of HCC. (HEPATOLOGY 2013;57:1484-1497)

The cancer stem cell (CSC) hypothesis, which suggests that a subset of cells bearing stem-cell–like features is indispensable for tumor development, has recently been put forward subsequent to advances in molecular and stem cell biology. Liver cancer, including hepatocellular carcinoma (HCC), is a leading cause of cancer death worldwide.[1] Recent studies have shown the existence of CSCs in liver cancer cell lines and primary HCC specimens using various stem cell markers.[2-7] Independently, we have identified novel HCC subtypes defined by the hepatic stem/progenitor cell markers,

Abbreviations: 5-FU, fluorouracil; Abs, antibodies; AFP, alpha-fetoprotein; CK-19, cytokeratin-19; CSC, cancer stem cell; DNs, dysplastic nodules; EMT, epithelial mesenchymal transition; EpCAM; epithelial cell adhesion molecule; FACS, fluorescent-activated cell sorting; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSCs, hepatic stem cells; IF, immunofluorescence; IHC, immunohistochemistry; IR, immunoreactivity; MDS, multidimensional scaling; NBNC, non-B, non-C hepatitis; NOD/SCID, nonobese diabetic, severe combined immunodeficient; NT, nontumor; OV-1, ovalbumin 1; qPCR, quantitative real-time polymerase chain reaction; SC, subcutaneous; Smad3, Mothers against decapentaplegic homolog 3; TECs, tumor epithelial cells; TGF-β, transforming growth factor beta; T/N, tumor/nontumor; VECs, vascular endothelial cells; VM, vasculogenic mimicry; VEGFR, vascular endothelial growth factor receptor.

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Received July 9, 2012; revised October 22, 2012; accepted November 6, 2012.

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23590967), a grant from the Japanese Society of Gastroenterology, a grant from the Ministry of Health, Labor, and Welfare, and a grant from the National Cancer Center Research and Development Fund (23-B-5) of Japan. X.W.W. is supported by the Intramural Research Program of the Center for Cancer Research, U.S. National Cancer Institute.