Original Article Human lung cancer-derived microparticles enhanced angiogenesis and growth of hepatoma cells in rodent lung parenchyma

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Abstract: This study tested the hypothesis that human lung cancer-derived microparticles (LcD-MPs) played an important role in tumor angiogenesis and growth. Fischer 344 rats (F344, n=18) were equally categorized into group 1 [Sham Control (3.0 mL normal saline intravenous injection (IV))], group 2 [hepatoma cell line (2.0 x 10⁶ cells, IV)], and group 3 [hepatoma cell line + LcD-MPs (3.0 x 10⁶, IV)]. Animals were euthanized by day 28 after hepatoma cells transfusion. Our result showed that the gross pathology confirmed growth of hepatoma cell line in lung parenchyma. The size and weight of the lungs were significantly increased in group 2 and further elevated in group 3 than in group 1 (all p<0.001). Histopathological analysis demonstrated that the lung crowded score and number of small vessel exhibited an identical pattern, whereas the number of alveolar sacs showed an opposite pattern compared to that of total lung weight among the three groups (all p<0.0001). The cellular expressions of CD34⁺, CXCR4⁺, c-Kit⁺, CK19⁺, VEGF⁺ and vimentin+ cells in lung parenchyma exhibited an identical pattern, whereas that of an identical pattern compared to those of total lung weight among all groups (all p<0.001). The protein expressions of apoptotic (Bax, cleaved caspase-3 and c-PARP), fibrotic (Smad3, TGF- β), and tumor suppression (PTEN) biomarkers were displayed an opposite pattern compared to that of total lung weight among all groups (all p<0.001). The MPs could enhance angiogenesis and accelerated hepatoma cell growth in rodent lung parenchyma.

Keywords: Lung cancer-derived microparticles, angiogenesis, lung parenchyma, tumor growth hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide [1-3]. Its global incidence is still on the rise because of the dissemination of hepatitis B and C viral infection [4-8]. Epidemiological studies have shown that the incidence of HCC is highest in Africa and Asia [7, 9], especially in Mainland China [10] and Taiwan [11]. Additionally, the mortality rate of HCC in these two

countries [10, 11] has not decreased in recent years principally because of uncontrolled tumor invasion and metastasis [12]. Advanced HCC is characterized by frequent metastasis and resistant to conventional chemotherapeutic agents and radiation. Thus, to clarify the biological factors that accelerate tumor growth is of utmost importance for the development of the new therapeutic strategies for HCC.

On the other hand, studies have previously demonstrated that an increase in the circulating number of microparticles (MPs) is strongly associated with a wide range of diseases [13], including hematologic malignancy [14, 15], breast cancer [16], and lung cancer [17]. Actually, it has been reported that MPs, which are plasma membrane fragments, are released by mammalian cells in response to chemical and physical stimulation (e.g., thrombin, endotoxin, and shear stress), or pro-apoptotic stress (e.g., growth factor deprivation or other apoptosis inducers) [13]. In fact, MPs, which are tiny particles ranging in size from 0.1 µm to 1.0 µm, have been previously shown to circulate in the blood and is able to participate in physiological and pathological processes, such as inflammation, thrombosis, and neovasculogenesis [13-18]. Studies have further demonstrated different effects of MPs on angiogenesis depending on their origins [13, 19-22]. Furthermore, the MPs that display pro-angiogenic properties by promoting the growth of capillary-like structures and production of pro-angiogenic factors are mainly derived from platelets, endothelial cells, and lymphocytes [13, 15-17, 22-24]. Our studies have previously revealed that circulating MP levels are significantly increased in lung cancer (Lc) patients as compared with those in normal subjects [25] and the circulating level of platelet-derived activated MPs is predictive of one-year morality in patients with end-stage non-small cell lung cancer [26]. Moreover, our experimental study [27] has recently shown that administration of Lc-MPs significantly augmented angiogenesis and restored blood flow in a rodent model of critical limb ischemia. Based on the results of previous studies from others [13-23] and those from our group [25-27], it is rational to hypothesize that MPs may play an important role in the promotion of tumor angiogenesis that helps in tumor growth and distal metastasis.

Materials and methods

Animals, ethics, and immune suppression therapy prior to HCC cell implantation to the rats

Male Fischer 344 rats of age 4 to 6 weeks with body weight approximately 150-170 gm were purchased from Charles River Technology, BioLASCO Taiwan Co., Ltd., and housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAA-LAC)-approved animal facility in our hospital with controlled temperature (25°C), humidity (50-70%), and light cycle (12/12). All experimental procedures were approved by the Institute of Animal Care and Use Committee of Kaohsiung Chang Gung Memorial Hospital, Taiwan (IACUC No. 2013052701) and performed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

For the purpose of the present study, 24 male Fischer 344 rats were utilized to create a metastasis model (i.e., metastasis of HCC from liver to lung). The young animals (i.e., age 4-6 weeks) were utilized in the study because of their immature immunogenicity to facilitate the growth of implanted hepatoma cells. Additionally, after taking into account possible genetic influence on hepatoma growth and metastasis, the inbreed Fischer 344 strain was chosen in the present study to produce consistent and reproducible results. Furthermore, intravenous cyclosporine (20 mg/kg/day for total 5 days) was given with two dosages prior to and three dosages after the hepatoma cell line administration.

N1S1 cell culture and preparation for in vivo imaging study

The rat N1S1 cell line (CRL-1603; ATCC, Manassas, VA, USA) was first cultured in Iscove's modified Dulbecco medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 0.1% streptomycin (Gibco) and passaged three times per week. A transfection mixture composed of 5 μ g of pTag-RFP-C plasmid (Everogen) and 10 μ L of lipofetamin-2000 (Invitrogen) was diluted in 500 μ L of Opti-MEM (Invitrogen) and incubated at 25°C for 30 min according to the manufactory's instructions (Invitrogen). The transfection mixture (1 mL)



Figure 1. The final body weight pathological findings of lung by day 28 after hepatoma cell (HCC) implantation. A. Analytical results of final body weight (BW), * vs. †, p<0.01. B. Analytical results of lung weight (LW), * vs. other groups with different symbols (†, ‡), p<0.01. C. In Vivo Imaging System (IVIS) study showed a more disseminated red fluorescent intensity in the lung parenchyma of group 3 as compared with that of group 2. D. Gross anatomy exhibited a more diffuse and extensive tumor growth (black arrows) in group 3 than that in group 2. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.



Figure 2. Histopathological findings of lung parenchyma by day 28 after HCC cell implantation. A-C. Illustration of H&E staining of microscopic finding (100×) of lung parenchyma in three groups. D. Analytic result of number of alveolar sacs, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. E. Analytic result of crowded score of lung parenchyma, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. The scale bars in right lower corner represent 100 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, \uparrow , \ddagger) indicate significance (at 0.05 level). HPF = high-power field; SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

was then gently added to 10⁶ N1S1 cells (in 1 mL of IMDM with 1% FBS) in a 35-mm Petri dish at 37°C. Two days after transfection, the genetically engineered REF-N1S1 cells were selected using G418 (Geneticin, InvivoGen) in IMDM, and a single colony cell was picked up into each well of a 96-well plate in IMDM for culture. After clonal expansion for one week, the RFP-N1S1 cell lines were examined for red fluorescence by fluorescent microscopy (Olympus). The cells were then ready for utilization.

Microparticles (MPs) preparation from lung cancer patients

The procedure and protocol for the preparation of MPs have been described in details in our previous studies and the whole study protocol was approved by the Institutional Review Committee on Human Research at Kaohsiung Chang Gung Memorial Hospital (IRB number: 100-1024B and 100-0204C) [25-27]. In brief, peripheral blood was collected in acid citrate dextrose (ACD) vacutainer tubes. To prepare platelet-rich plasma, peripheral blood (1.5 mL) was centrifuged at 2500 ×g at 4°C for 15 min without acceleration or brake. Then 250 µL plasma samples were thawed and centrifuged for 10 min at 19,800 ×g at 4°C, and then collected for investigation of MPs smaller than 1.0 µm. Size calibration was conducted with 1.0 µm beads (Invitrogen, Carlsbad, CA). All buffers were filtered with a 0.2 µm filter. Additionally, since a large number of MPs were utilized for one animal, MPs were obtained from a pool of patients at advanced stages of non-small cell lung cancer (i.e., stage IIIb or staged IV). The dosage of MPs (3.0 x 10⁶ for one animal) was based on our previous report [27] with some modifications.

Animal grouping

Fischer 344 (F344) rats (n=24) were equally categorized into the sham control (SC) (3.0 mL normal saline injection; group 1), F344 rats + RFP-N1S1 (hepatoma cell line, 2.0 x 10^6 cells, by tail vein administration) at day 0 of study period (group 2), and F344 + RFP-N1S1+ Lc-



Figure 3. Numbers of small vessels in lung parenchyma by day by day 28 after HCC cell implantation. A-C. Microscopic finding (100×) of immunohistochemical staining [(i.e., α -smooth muscle actin stain (α -SMA)] for identifying the number of small pulmonary arterioles (PAs) in lung parenchyma (red arrows). D. Analytical result of number of small PAs, * vs. other groups with different symbols (†, ‡), p<0.0001. The scale bars in right lower corner represent 100 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

MPs (3.0 x 10⁶, by tail vein injection; group 3) from lung cancer patients. By intra-venous administration, the cancer cells were entrapped within the lung parenchyma to simulate the clinical setting of HCC metastasis from liver to the lung.

Lung specimen preparation

By the end of study period (i.e., at day 28 after RFP-N1S1 cell transfusion), the animals in each group were euthanized. The pathology features of the lungs were photographed and the total lung weight was measured for each animal.

The preparation of lung specimens for morphometric analyses was based on our recent reports [28-30]. Briefly, the left lung was inflated at a constant airway pressure (15-20 mmHg) and embedded within OCT (Tissue-Tek) for immunohistochemical staining and hematoxylin-eosin staining. The right lung was then cut into pieces and stored at -80° C for protein and mRNA analyses.

In vivo assessment: bioluminescence imaging (in vivo imaging system for small animals)

The procedure and protocol of In Vivo Imaging System (IVIS) was based on our recent report [31]. In brief, all animals in groups 2 and 3 underwent bioluminescence imaging on days 28 after RFP-N1S1 cell line implantation by using an animal imaging system (IVIS 200;



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Figure 4. IHC and IF staining for identification of endothelial progenitor cells in lung parenchyma by day 28 after HCC cell implantation. A-C. Immunofluorescent (IF) microscopic finding (200×) for identification of CXCR4⁺ cells (green color) in lung parenchyma (red color indicated implanted HCC cells). D. Analytical results of number of CXCR4⁺ cells, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. E-G. Immunohistochemical (IHC) microscopic finding (200×) for identification of CD34⁺ cells (gray color). H. Analytical results of number of CD34⁺ cells, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. The scale bars in right lower corner represent 50 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (\uparrow , \ddagger), pindicate significance (at 0.05 level). HPF = high-power field; SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

Xenogen) at multiple time intervals (1 minute, 5 minutes, and 15 minutes). The RFP-N1S1 cells appeared red on bioluminescence imaging. Most of the intraveneously infused RFP-N1S1 cells were found to be trapped within the lungs (**Figure 1C**). These findings suggested the successful creation of the animal model of HCC growth in lung parenchyma.

Western blotting for protein expression

An amounts (30 µg) of protein extracts from ischemic quadriceps of the animals (n=8 for each group) were loaded and separated by SDS-PAGE using 6% to 12% acrylamide gradients. The transferred PVDF membranes were incubated with indicated primary antibodies [CD31 (1:3000, Abcam), vascular endothelial growth factor (VEGF) (1:1000, Abcam), CXCR4 (1:1000, Abcam), stromal cell-derived factor (SDF)-1α (1:1000, Cell Signaling), Bax (1:1000, Abcam), cleaved caspase 3 (c-Casp3; 1:1000, Cell Signaling), cleaved poly(ADP-ribose) polymerase (c-PARP; 1:1000, Cell Signaling), Bcl-2 (1:200, Abcam), phosphorylated-Smad3 (1: 1000, Cell Signaling), transforming growth factor (TGF)-β (1:500, Cell Signaling), p-Smad1/5 (1:1000, Cell Signaling), bone morphogenic protein (BMP)-2 (1:5000, Abcam), PTEN (1:1000, Abcam)]. Signals were detected with HRP-conjugated goat anti-mouse or goat antirabbit IgG. Proteins were transferred to PVDF membranes which were then incubated in the primary antibody for two hours, followed by incubation with secondary antibody solution (1:3000) for one hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL: Thermo Scientific Pierce, Wa-Itham, MA, USA) which was then exposed to Super RX film (FUJIFILM Super RX, Minato-ku, Tokyo, Japan). For quantification, ECL signals were digitized using Labwork software (UVP, Waltham, MA, USA).

Immunohistochemical (IHC) and immunofluorescent (IF) staining

Both IHC and IF staining, rehydrated paraffin sections of in vivo rat lung specimens were first treated with 3% $\rm H_{_2}O_{_2}$ for 30 minutes and incubated with Immuno-Block reagent (BioSB, Santa Barbara, CA, USA) for 30 minutes at room temperature. Sections were then incubated with primary antibodies specifically against CD34 (1:100, BioSS), C-kit (1:300, Santa Cruz), cytokeratin (CK)-19 (1:100, Abcam), VEGF (1:400, Abcam), CD31 (1:500, AbD Serotec), vimentin (1:400, Abcam), and CXCR4 (1:100, Abcam) while sections incubated with the use of irrelevant antibodies served as controls. Three sections of lung specimen from each rat were analyzed. For quantification, three randomly selected HPFs (200× or 400× for IHC and IF studies) were analyzed in each section. The mean number of positive-stained cells per HPF for each animal was then determined by summation of all numbers divided by 9.

Histological assessment of lung injury

The lung specimens were sectioned at 5 µm for light microscopy. Hematoxylin and eosin (H&E) staining was performed to estimate the number of alveolar sacs in a blinded fashion as we previously reported [28-30]. Three lung sections from each rat were analyzed and three randomly selected high-power fields (HPFs; 100×) were examined in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9. The extent of crowded area, which was defined as region of thickened septa in lung parenchyma associated with partial or complete collapse of alveoli on H&E-stained sections, was also performed in a blinded fashion. The following scoring system [28-30] was adopted: 0 = no detectable crowded area: 1 =<15% of crowded area; 2 = 15-25% of crowded area; 3 = 25-50% of crowded area; 4 = 50-75%



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Figure 5. IHC and IF staining for identification of stem cells and endothelial cell marker in lung parenchyma by day 28 after HCC cell implantation. A-C. Immunohistochemical (IHC) microscopic finding (200×) for identification of c-Kit⁺ cells (gray color). D. Analytical results of number of c-Kit⁺ cells, * vs. other groups with different symbols (†, \ddagger), p<0.0001. E-G. Immunofluorescent microscopic finding (200×) for identification of vascular endothelial growth factor (VEGF)⁺ cells (green color) (red color indicated implanted HCC cells). H. Analytical results of VEGF⁺ cells, * vs. other groups with different symbols (†, \ddagger), p<0.0001. The scale bars in right lower corner represent 50 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

of crowded area; 5 = >75%-100% of crowded area/HPF.

Vessel density in lung parenchyma

The procedure and protocol for identification of small vessel in lung parenchyma were basic on our previous reports [27, 29, 31]. In details, IHC staining of small blood vessels was performed with α -SMA (1:400, Abcam) as primary antibody at room temperature for 1 hour, followed by washing with PBS thrice. Ten minutes after the addition of anti-mouse-HRP conjugated secondary antibody, the tissue sections were washed with PBS thrice. Then 3,3' diaminobenzidine (DAB) (0.7 gm/tablet) (Sigma) was added, followed by washing with PBS thrice after one minute. Finally, hematoxylin was added as a counter-stain for nuclei, followed by washing twice with PBS after one minute. Three lung sections were analyzed in each mouse. For quantification, three randomly selected HPFs (100×) were analyzed in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9.

Statistical analyses

Quantitative data were expressed as mean \pm SD. Statistical analysis was performed by ANOVA followed by Bonferroni multiple-comparison post hoc test. All analyses were conducted using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

Pathological findings of the lung parenchyma by day 28 after HCC cell implantation (**Figure 1**)

The final body weight was significantly lower in groups 2 and 3 than in group 1 (SC), but it

showed no difference between groups 2 (i.e., RFP-N1S1 only) and 3 (RFP-N1S1+ patient's Lc-MPs). The total wet lung weight was significantly higher in group 3 than that in groups 2 and 1, and significantly higher in group 2 than that in group 1. Besides, IVIS examination demonstrated a more disseminated red fluorescent intensity in the lung parenchyma of group 3 as compared with that of group 2. Moreover, the gross anatomy showed a more diffuse and extensive tumor growth in group 3 than that in group 2. These finding suggested that MPs augmented the growth of the HCC cells (i.e., RFP-N1S1) with the lung parenchyma.

Histopathological findings of lung parenchyma and small blood vessels by day 28 after HCC cell implantation (**Figures 2** and **3**)

Light microscopic findings after H&E staining demonstrated that the number of alveolar sacs was significantly lower in group 3 than that in groups 1 and 2, and significantly lower on group 2 than that in group 1 (**Figure 2**). Conversely, the lung parenchyma was most crowded in group 3 and significantly more crowed in groups 2 than that in group 1 (**Figure 2**). Additionally, the number of small vessels in lung parenchyma was significantly higher in group 3 than in groups 1 and 2, and significantly higher in group 2 than in group 1 (**Figure 3**). These finding implicated that that MPs enhanced angiogenesis/ neovasculogenesis for tumor growth.

The IHC and IF staining for identification of cell markers in lung parenchyma by day 28 after HCC cell implantation (**Figures 4-6**)

IHC microscopy showed that the number of CD34 positively-stained cells in lung parenchyma, an indicator of endothelial progenitor cell (EPC), was significantly higher in group 3 than that in groups 1 and 2, and significantly higher in group 2 than that in group 1 (Figure 4A-D). Consistently, IF microscopy revealed that the



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Figure 6. IHC and IF staining for identification of stem cells and endothelial cell marker in lung parenchyma by day 28 after HCC cell implantation. A-C. Immunohistochemical (IHC) microscopic finding ($200\times$) for identification of cytokeratin (CK)-19⁺ cells (gray color). D. Analytical results of number of CK 19⁺ cells, * vs. other groups with different symbols (†, ‡), p<0.0001. E-G. Immunofluorescent (IF) microscopic finding ($200\times$) for identification of vimentin⁺ cells (green color) (red color indicated implanted HCC cells). H. Analytical results of vimentin⁺ cells, * vs. other groups with different symbols (†, ‡), p<0.0001. The scale bars in right lower corner represent 50 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). HPF = high-power field; SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.



Figure 7. Protein expressions of apoptotic and anti-apoptotic biomarkers in lung parenchyma by day 28 after HCC cell implantation. A. Protein expression of Bax, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.001. B. Protein expression of cleaved caspase 3 (c-Casp3), * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. C. Protein expression of poly (ADP-ribose) polymerase (PARP), * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. D. Protein expression of Bcl-2, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. D. Protein expression of Bcl-2, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. D. Protein expression of Bcl-2, * vs. other groups with different symbols (\uparrow , \ddagger), p<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, \uparrow , \ddagger) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancerderived microparticles.



Figure 8. Protein expression of fibrotic and anti-fibrotic biomarkers in lung parenchyma by day 28 after HCC cell implantation. A. Protein expression of phosphorylated (p)-Smad3, * vs. other groups with different symbols (†, ‡), p<0.0001. B. Protein expression of transforming growth factor (TGF)- β , * vs. other groups with different symbols (†, ‡), p<0.0001. C. Protein expression of p-Smad1/5, * vs. other groups with different symbols (†, ‡), p<0.0001. C. Protein expression of p-Smad1/5, * vs. other groups with different symbols (†, ‡), p<0.0001. D. Protein expression of bone morphogenetic protein (BMP)-2, * vs. other groups with different symbols (†, ‡), p<0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

number of cells positively-stained for CXCR4, another indicator of EPC, exhibited a pattern of expression identical to that of CD34⁺ cells among the three groups in lung parenchyma (**Figure 4E-H**).

Besides, IHC staining also demonstrated that the number of cells positively stained for c-Kit, a stem cell surface marker, was significantly higher in group 3 than that in groups 1 and 2, and significantly higher in group 2 than that in group 1 (fiurev 5-A to 5-D). Similarly, IHC microscopy demonstrated that the number of cells positively-stained for VEGF, a marker index of endothelial cell, displayed an identical pattern of expression compared to that of c-Kit among the three groups (**Figure 5E-H**).

In addition, IHC microscopy revealed that the number of positively-stained cells positive for cytokeratin 19 (CK 19), a prognostic marker of HCC, was significantly higher in group 3 than



Figure 9. Protein expressions of angiogenesis biomarkers and PTEN in lung parenchyma by day 28 after HCC cell implantation (**Figure 9**). A. Protein expression of CXCR4, * vs. other groups with different symbols (†, ‡), p<0.001. B. Protein expression of stromal cell-derived factor (SDF)-1 α , * vs. other groups with different symbols (†, ‡), p<0.0001. C. Protein expression of vascular endothelial growth factor (VEGF), * vs. other groups with different symbols (†, ‡), p<0.0001. D. Protein expression of PTEN, * vs. other groups with different symbols (†, ‡), p<0.001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). SC = sham control; HC = hepatoma cell; HC-LcD-MPs = hepatoma cell + lung cancer-derived microparticles.

that of groups 1 and 2 and significantly higher in group 2 as compared with that in group 1 in lung parenchyma (**Figure 6A-D**). Moreover, IF microscopy showed that the number of cells positive for vimentin, a potential factor of metastasis in HCC³⁴, exhibited a pattern identical to that of CK+ cells among the three groups (**Figure 6E-H**). The protein expressions of apoptotic and antiapoptotic biomarkers in lung parenchyma by day 28 after HCC cell implantation (**Figure 7**)

The protein expressions of Bax, c-Casp 3, and c-PARP in lung parenchyma, three indices of apoptosis, were significantly higher in group 3 than those in groups 1 and 2, and significantly higher in group 2 than those in group 1. On the other hand, the protein expression of Bcl-2, an index of anti-apoptosis in lung parenchyma, displayed an opposite pattern of expression compared to that of apoptosis markers in all groups.

The protein expression of fibrosis and antifibrosis biomarkers in lung parenchyma by day 28 after HCC cell implantation (**Figure 8**)

The protein expressions of p-Smad3 and TGF- β in lung parenchyma, two indicators of fibrotic markers, were significantly higher in group 3 than those in groups 1 and 2, and significantly higher in group 2 than those in group 1. Conversely, the protein expressions of p-Smad1/5 and BMP-2 in lung parenchyma, two anti-fibrosis biomarkers, showed an opposite expression pattern compared to that of apoptosis markers among the three groups.

The protein expressions of angiogenesis biomarkers and PTEN in lung parenchyma by day 28 after HCC cell implantation (**Figure 9**)

The protein expression of CXCR4, SDF-1 α , and VEGF, three indicators of angiogenesis, were significantly higher in group 3 than those of groups 1 and 2, and significantly in group 2 than those in group 1 in lung parenchyma. Additionally, the protein expression of PTEN in lung parenchyma, a tumor suppression gene, exhibited an identical pattern of expression compared to that of angiogenesis markers among the three groups. This finding (i.e., an unregulation of PTEN in group 2 and especially in group 3) might imply an intrinsic response to HCC growth in the lung.

Discussion

This study, which investigated the impact of Lc-MPs from patients on enhancement of angiogenesis and tumor growth in an animal model to mimic the clinical setting of HCC metastasis to lung, yielded several striking implications. First, the animal model demonstrated that HCC cells successfully survived and grew in the lung parenchyma to serve the purpose of this study. Second, the results of the present study demonstrated that Lc-MPs from patients augmented growth of HCC in lung parenchyma. Third, Lc-MPs enhanced the expressions of EPC and angiogenesis biomarkers in the lung parenchyma.

One essential finding in the present study is that by day 28 after administration of HCC cell line, IVIS (i.e., live image) showed that the red fluorescence was found to be diffusely distributed in the lung parenchyma in the HCC cell line-treated group and more extensively spread in the lung parenchyma of the HCC cell line + MPs treatment group. The findings not only confirmed that the implanted tumor cells grew well in the lung parenchyma, but also supported that MPs treatment enhanced tumor growth and the spread of tumor cells within the lung parenchyma.

Another essential finding was that the total body weight was significantly lower in groups 2 and 3 than in group 1. However, when compared with the SC group, the total lung weight was substantially increased in the HCC cell linetreated group and further substantially elevated in the HCC cell line + MPs treatment group. Additionally, gross pathological examination (Figure 1F-H) revealed that tumor grew more extensively in the HCC cell line + MPs treatment group than that in the HCC cell line-treated group. These findings once again implicate that tumor grew more rapidly and widespread in animals with MPs treatment compared to those without. Previous studies have clearly shown that increased tumor size is significantly related to worse overall survival [32, 33]. Intriguingly, we have previously shown that increased circulating level of MPs was predictive of poor prognostic outcome in patients with non-small cell lung cancer [26]. Moreover, our previous experimental study [27] has further demonstrated that Lc-MPs treatment enhanced angiogenesis and restored blood flow in a rat model of limb ischemia. These findings [26, 27, 32-34], therefore, suggest that MPs may be associated with a poor prognostic outcome in patients with HCC.

Angiogenesis has been identified to be an essential contributor to cancer cell differentiation, proliferation, invasion, and metastasis [35-37]. Additionally, hyper-vascularity has been demonstrated to be an important predictor of poor prognostic outcome in patients with HCC [38]. Furthermore, a body of evidence has demonstrated that cancer stem cell/EPCs were strongly associated with poor responses to chemotherapy in various types of cancers [39-41]. A principal finding in the present study is that the protein expressions of angiogenesis factors in the lung parenchyma were significantly enhanced in the HCC cell line-treated animals and further significantly enhanced in HCC cell line + MPs-treated animals than those in the normal controls. Besides, the expression pattern of EPCs was consistent with the protein expression of angiogenesis biomarkers among the three groups. Based on the results of our previous experimental study and those from others [34-41], we propose that MPs may participate in the proliferation, growth, and metastasis of HCC in human beings. Therefore, circulating level of MPs may be a prognostic factor for patients with HCC.

Previous studies have shown that over-expressions of CK19 [42, 43] and vimentin [44] were significantly associated with poor prognostic outcomes in patients with HCC regardless of the treatment strategies. Interestingly, the expressions of these two parameters were remarkably augmented in HCC cell line-treated group and further enhanced in the HCC cell line + MPs-treated group compared with those in normal controls. In addition, the protein expression of PTEN, an important tumor suppressor, was markedly enhanced in HCC cell line + MPs treatment group than in the other two groups. suggesting an intrinsic response against to tumor growth and spread in lung parenchyma. These findings, once again suggest that MPs are associated with a bad prognostic outcome in the setting of HCC.

Intriguingly, the protein expressions of apoptosis markers were found to be markedly increased in HCC cell line-treated group and further enhanced in the HCC cell line + MPstreated group compared to those in the normal controls. On the other hand, the protein expression of anti-apoptosis markers displayed an opposite pattern compared to that of apoptosis markers. These findings may reflect an imbalance between blood supply and demand from tumor overgrowth that caused tumor cell death and necrosis.

This study has limitations. First, this study did not provide evidence regarding whether the HCC had intra-pulmonary and or distal metastasis. Second, this study did not measure the arterial oxygen saturation in the animals so that the possible impact of hypoxia on animals with or without MPs treatment was not evaluated.

In conclusion, the results of the present study supported that MPs may play an important role in tumor growth, differentiation, and angiogenesis. The preclinical findings may encourage the use of circulating MPs level as a useful prognostic indicator for patients with HCC.

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Disclosure of conflict of interest

None.

Abbreviations

HCC, Hepatocellular carcinoma; LcD-MPs, lung cancer-derived microparticles; VEGF, vascular endothelial growth factor; SDF-1 α , stromal cell-derived factor -1 α ; c-PARP, cleaved poly (ADP-ribose) polymerase; TGF- β , transforming growth factor- β ; BMP-2, bone morphogenic protein-2; H&E, hematoxylin and eosin; HPFs, high-power fields.

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