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Original Article

Screening candidate metastasis-associated genes in three-dimensional HCC spheroids with different metastasis potential

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Abstract: Purpose: Previously, we have established a tissue-like HCC spheroid which better mirrors the biological features of tumorigenesis and metastasis. This study was to find out metastasis-associated genes between two 3D HCC spheroids with different metastasis potential using comparative PCR arrays. Materials and Methods: Two HCC spheroids derived from high-metastatic MHCC97H cells and low-metastatic Hep3B cells were formed respectively in a rotating wall vessel bioreactor after 3D culture for 15 days. The candidate metastasis-associated genes related to cell adhesion, matrix secretion and invasion in HCC spheroids were screened by RT^2 profiler PCR arrays. The expression patterns of several differentially-expressed genes were further confirmed by real-time RT-PCR. Results: Total of 123 differential expression genes (fold-change >2) were found between two HCC spheroids, including 70 up-regulated genes (VCAM-1, IL-1β, CD44, tenascin C, SPP1, fibronectin, MMP-2, MMP-7, etc) and 53 down-regulated genes (E-cadherin, CTNND2, etc) in the high-metastatic spheroid. Function classification showed that the number of up-regulated genes related to adhesion molecules mediating cell-matrix interactions and matrix secretion was significantly higher in high-metastatic spheroid than that in low-metastatic spheroid. In contrast, the expressions of adhesion molecules maintaining homotypic tumor cell adhesion were decreased in metastatic spheroid as compared with that in low-metastatic spheroid. In addition, the expression pattern of seven selected genes associated with tumor metastasis measured by real-time RT-PCR were consistent with results of PCR arrays. Conclusions: Obvious differences between two HCC spheroids in gene expression patterns of adhesion molecules, matrix secretion, invasion and other molecules may determine the different metastatic characteristics and malignant phenotype of HCC spheroid.

Keywords: Hepatocellular carcinoma, three-dimensional culture, spheroid, metastasis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide [1]. Most of HCC patients die from cancer metastasis even after receiving radical treatment. More than 75% of HCC patients experience tumor recurrence and metastasis within 5 years after surgical resection [2]. Hereby, it is critical to clarify the molecular mechanisms controlling HCC metastasis for effective therapeutic intervention.

Metastasis involves a complex cascade of signal events between tumor cells and stroma microenvironment. Adhesion molecules mediating cell-cell interaction and cell-matrix interaction play significant roles at the different stages of metastasis such as tumor cell-cell detachment, cell migration and matrix degradation, etc. Accumulating evidences also support that alterations in cell-cell or cell-matrix interactions contribute to the occurrence of cancer metastasis [3]. However, the conventional monolayer two-dimensional (2D) culture, in which only cell-cell flat contact and little ECM are involved, fails to produce a well-defined geometry with cell-cell and cell-matrix spatial interactions like a solid tumor in vivo. Accordingly, there seems a challengeable task to identify adhesion molecules and matrix protein related to invasion/metastasis only based on the data from 2D cell cultures. Three-

Table 1. Primers used for real-time RT-PCR analysis

Gene Name	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
CD44	GGTGAACAAGGAGTCGTC	TTCCAAGATAATGGTGTAGGTG
VCAM-1	AGTTGAAGGATGCGGGAGT	GCAAAATAGAGCACGAGA
SPP1	CAGTGATTTGCTTTTGCC	AGATGGGTCAGGGTTTAG
E-cadherin	ATTGAATGATGATGGTGGAC	GCTGTGGAGGTGGTGAGA
MMP-2	GTTCATTTGGCGGACTGT	AGGGTGCTGGCTGAGTAG
MMP-7	GGGACTCCTACCCATTTG	CCAGCGTTCATCCTCATC
MMP-9	CTTTGGACACGCACGAC	CCACCTGGTTCAACTCACT
GAPDH	TCCTCCACCTTTGACGC	CCACCACCCTGTTGCTGT

dimensional (3D) cultures *in vitro* resemble a solid tissue with cell-cell spatial contact and cell-matrix interactions, exhibiting high concordance with *in vivo* conditions [4]. As an established cancer cell model under 3D culture, the tumor spheroid can mimic tumor microenvironment close to that of tumors *in vivo* [5]. In the previous study [6], we also established a tissue-like HCC spheroid, apparently differed from HCC cultured in monolayers, which can better mirror the biological features of HCC tissue in cell morphology, specific gene expression, protein secretion, tumorigenesis and metastasis, suggesting that within 3D tissues, cell-cell and cell-matrix contact might influence the expressions of specific genes and further determine the pathological characteristics of tumor. This study was to investigate candidate metastasis-associated genes related to cell adhesion, matrix secretion and invasion between two 3D HCC spheroids with different metastasis potential using comparative PCR arrays.

Materials and methods

Cell culture

Human HCC cell line MHCC97H with highly metastatic potential was established in the Liver Cancer Institute, Fudan University [7]. MHCC97H cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (100 units/ml, each), and human HCC cells Hep3B with low metastasis potential, obtained from Cornell University of USA, were cultured in MEM containing 10% fetal bovine serum and 1% penicillin-streptomycin (100 units/ml, each). After growth reaching 80% confluence, the cells were harvested for further 3D cell culture. All culture media used here were supplied by GIBCO, USA.

Establishment of HCC spheroids under 3D rotating culture

Three dimensional rotating culture method and construction of HCC spheroid were carried out as the previously described with slight modification. Briefly, approximately 1×10^7 suspended HCC cells in 10 ml of DMEM medium (GIBCO, USA) and a sterilized PLGA scaffold of 4×4×1 mm were first seeded into the RWV bioreactor (Synthecon, Houston, TX, USA). The initial rotation speed of RWV bioreactor was set as 7-8 rpm for 18 h. Subsequently, it was gradually modulated to speed of 13-20 rpm to maintain cell aggregates in a freely suspended state within the vessel. After 15 days rotating culture, a tissue-like HCC spheroid was formed. The medium was replaced after 36 h with fresh medium.

PCR array analysis of gene expression in HCC spheroids

A HCC spheroid was pulverized in liquid nitrogen using a mortar and total RNA was extracted from it using Trizol according to the manufacturer's protocol (Invitrogen, USA). The RNA was further purified by RNeasy® MinElute™ Cleanup Kit (Qiagen). The purified RNA was reverse transcribed to cDNA using the Superscript III reverse transcriptase kit (Invitrogen, USA). The Human Extracellular Matrix & Adhesion Molecules RT^2 Profiler PCR Array and Tumor Metastasis RT^2 Profiler PCR Array (SABiosciences) were used to screen different gene related to cell adhesion, matrix secretion and invasion between two HCC spheroids with different metastasis potential. For data analysis, fold-changes in each gene expression were calculated using the $^{\Delta\Delta}$Ct method, and housekeeping gene controls were used for normalization of the results. Positive value indicates up-regulation of individual gene and negative value indicates down-regulation.

Validation of different expression genes by real-time RT-PCR

A HCC spheroid was pulverized in liquid nitrogen using a mortar and total RNA from it was extracted using Trizol reagent (Invitrogen, USA).

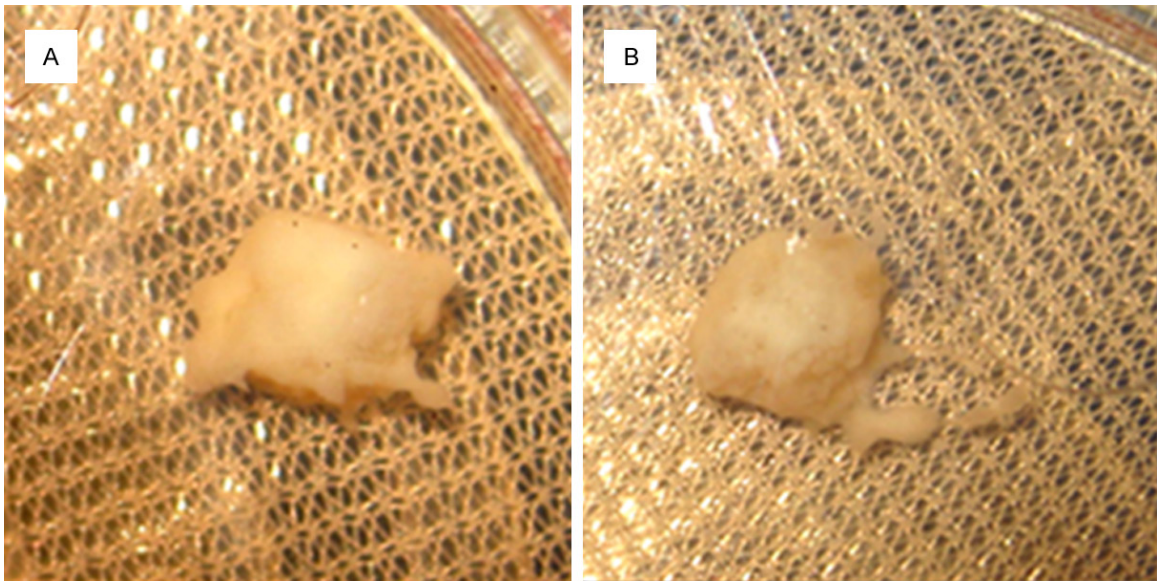

Figure 1. Macroscopic morphology of the HCC spheroids with a diameter of 1.0 cm. A: High-metastatic MHCC97H spheroid; B: Low-metastatic Hep3B spheroid.

Reverse transcription was performed with primer oligo(dT)18 (Fermentas, Thermo Fisher Scientific Inc). Selected gene transcripts were quantified by real-time RT-PCR using gene-specific primers and SYBR Green Mix (Invitrogen, USA). Samples were run in triplicate using a real-time RT-PCR thermo-cycler (Applied Biosystems, Foster, CA) and the results were analyzed by matched software. Relative expression of genes was determined by normalizing to internal control (GAPDH) expression and presented as the $2^{-\Delta Ct}$ formula ($^{\Delta}Ct=Ct_{(target\ gene)}-Ct_{(GAPDH)}$; Ct is the cycle threshold). All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services. Sequences of the gene-specific primers are listed in **Table 1**.

Statistical analysis

Data analysis was performed using SPSS 15.0 (SPSS, Chicago, IL). Data are given as mean ± SD. Comparisons of quantitative data between two groups were performed using the two-sample Student's t test. A two-sided *P* value ≤0.05 was considered statistically significant.

Results

The formation of HCC spheroids

HCC cells seeded in a RWV bioreactor first gathered at a low rotation speed of 8 rpm, and then turned into a loose aggregate within 48 h. On day 5, it shaped a spherical form by modulating the rotation speed of vessel. Subsequently, it gradually changed from a loose agglomeration to a compact spheroid at a high rotation speed of 20 rpm. On day 15, two tissue-like HCC spheroids with diameter of 1.0 cm from the metastatic HCC cells MHCC97H and the low-metastatic cells Hep3B were formed in the RWV bioreactors, respectively (**Figure 1**). Morphology and malignant characteristics of a MHCC97H spheroid on the day 15 were found close to that of solid tumor in our previous study [6].

Gene expression in the high-metastatic HCC spheroid was different from that in the low-metastatic spheroid

A total of 123 differential expression genes (more than 2-fold change), including 70 up-regulated genes (CD44, integrins, tenascin c, SPP1, fibronectin, collagen, laminin, etc) and 53 down-regulated genes (E-cadherin, CTNND2, etc), were identified in the high-metastatic MHCC97H spheroid compared with that in low-metastatic Hep3B spheroid (a list of partial differentially-expressed genes in **Table 2**). The number of up-regulated genes related to cell adhesion and matrix proteins was more increased in the high-metastatic MHCC97H spheroid than that in the low-metastatic Hep3B

Table 2. Partial list of candidate metastasis-associated genes with at least 2-fold change in the high-metastatic MHCC97H spheroids relative to the low-metastatic Hep3B spheroid

Gene symbol	Name of gene	Fold up- or down-regulation
VCAM-1	Vascular cell adhesion molecule 1	2750.00
IL-1β	Interleukin 1, beta	1846.15
MMP-7	Matrix metallopeptidase 7	1389.11
CD44	CD44	1232.95
COL6A1	Collagen, type VI, alpha 1	733.33
TNC	Tenascin C	637.50
COL6A2	Collagen, type VI, alpha 2	562.50
MMP-1	Matrix metallopeptidase 1	462.50
uPAR	Plasminogen activator, urokinase receptor	444.44
MET	hepatocyte growth factor receptor	318.18
MMP-2	Matrix metallopeptidase 2	256.82
SPP1	Secreted phosphoprotein 1 (osteopontin)	221.05
LAMB3	Laminin, beta 3	154.55
COL16A1	Collagen, type XVI, alpha 1	76.25
FN	Fibronectin	74.55
LAMA3	Laminin, alpha 3	68.46
COL4A2	Collagen, type IV, alpha 2	51.93
ITGA5	Integrin, alpha 5	30.00
COL5A1	Collagen, type V, alpha 1	27.50
ITGB3	Integrin, beta 3	23.18
TGF-β1	Transforming growth factor, beta 1	21.21
IL-18	Interleukin 18	12.64
ITGA6	Integrin, alpha 6	8.38
ICAM-1	Intercellular adhesion molecule 1	8.04
ITGA2	Integrin, alpha 2	6.76
LAMA2	Laminin, alpha 2	6.50
ITGB1	Integrin, beta 1	6.00
HGF	Hepatocyte growth factor	5.73
LAMB1	Laminin, beta 1	4.50
ITGB5	Integrin, beta 5	4.23
MDM2	Mdm2	3.64
CTSK	Cathepsin K	3.33
COL1A1	Collagen, type I, alpha 1	3.29
VEGFA	Vascular endothelial growth factor A	3.19
MYC	V-myc myelocytomatosis viral oncogene homolog	3.00
ITGAV	Integrin, alpha V	2.31
SRC	V-src sarcoma viral oncogene homolog	2.27
CTNND2	Catenin (cadherin-associated protein), delta 2	-1931.26
VTN	Vitronectin	-162.64
SSTR2	Somatostatin receptor 2	-67.49
HPSE	Heparanase	-30.08
COL15A1	Collagen, type XV, alpha 1	-22.68
COL8A1	Collagen, type VIII, alpha 1	-22.68
ITGA4	Integrin, alpha 4	-22.68
ITGA8	Integrin, alpha 8	-22.68
CTSL1	Cathepsin L1	-22.28
COL11A1	Collagen, type XI, alpha 1	-17.32

spheroid, suggesting that the highly metastatic spheroid secreted more matrix proteins and was much more inclined to interact with matrix proteins (**Table 3**). Specifically, some different genes encoding adhesion molecules and matrix proteins were up-regulated in the high-metastatic HCC spheroid. Among them, collagen I and IV, two important abundant collagen protein components in liver stroma, and integrin αv, β1, β3 mediating cell-matrix interactions, were all highly expressed in the metastatic MHCC97H spheroid. In contrast, expression of some homotypic tumor cell adhesion genes (eg. E-cadherin, CTNND2, etc) was decreased in the MHCC97H spheroid compared with that in the Hep3B spheroid, implying that loss of tight tumor cell-cell adhesion facilitates tumor metastasis. Of note, expression of adhesion molecules mediating heterotypic cell adhesion (eg. VCAM-1, ICAM-1) was increased in the high-metastatic MHCC97H spheroid. High expressions of VCAM-1 and ICAM-1 on the surface of tumor cells indicates that it has high potential

KISS1R	KISS1 receptor	-14.02
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	-13
HTATIP2	HIV-1 Tat interactive protein 2	-11.41
COL14A1	Collagen, type XIV, alpha 1	-9.38
MCAM	Melanoma cell adhesion molecule	-9.33
COL7A1	Collagen, type VII, alpha 1	-8.03
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	-5.4
NCAM1	Neural cell adhesion molecule 1	-5.14
CDKN2A	Cyclin-dependent kinase inhibitor 2A	-4.98
E-Cadherin	Cadherin 1, type 1, E-cadherin (epithelial)	-4.09
MMP-9	Matrix metallopeptidase 9	-3.33
NF2	Neurofibromin 2 (merlin)	-3.03
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-2.62
BRMS1	Breast cancer metastasis suppressor 1	-2.59
COL12A1	Collagen, type XII, alpha 1	-2.5
APC	Adenomatous polyposis coli	-2.21

Positive values indicate up-regulation of individual genes; negative values indicate down-regulation.

MHCC97H spheroid, whereas inhibiting cell proliferation (CDKN2A), disrupting angiogenesis (ADAMTS1, ADAMTS13, ADAMTS8) and tumor metastasis suppressor (BRMS1, KISS1R, HTATIP2) were up-regulated in the Hep3B spheroid. It hinted that the highly metastatic HCC spheroid has stronger capabilities of pro-inflammation, proliferation, pro-angiogenesis and metastasis compared with the low-metastatic spheroid. As for ECM degradation, types of ECM proteinases were expressed differently between two HCC spheroids. For example, MMP-1, MMP-2, MMP-7 and cathepsin K were over-expressed in the MHCC97H spheroid while MMP-9, heparanase and cathepsin L1 were up-regulated in the Hep3B spheroid.

Verification of differential expression genes in HCC spheroids

To assess the validity of the PCR array data, we confirmed seven differentially-expressed genes by real-time RT-PCR including adhesion molecules (VCAM-1, CD44, SPP1, E-cadherin), ECM degradation (MMP-2, MMP-7, MMP-9) (**Figure 2**). Increased expression of VCAM-1, CD44, SPP1, MMP-2, MMP-7, and decreased expression of E-cadherin and MMP-9 in the metastatic MHCC97H spheroid were consistent with results of PCR array.

Discussion

Disrupting cell-cell and cell-matrix interactions within tumor tissue is regarded as one of the most important events during the process of tumor metastasis, especially at the phase of tumor cell detachment, ECM degradation, and distant tumor cell colonization [3, 8]. However, data based on 2D cell culture cannot be convincing on explaining the roles of adhesion molecules in the tumor metastasis due to lacking of cell-cell and cell-matrix interactions. On the other hand, gene expression signature derived from the whole tumor tissues in many literatures cannot exactly define the specific contributions of each compartment in tumor metastasis because of impacts of stroma cell and matrix contents in tumor tissue. The 3D tumor spheroid better resembles *in vivo* tumor phenotypes and gradually becomes a promising model for studying tumor biology and cancer stem cells, screening anti-cancer drugs and developing new therapeutic approaches [5, 9-13]. Besides mimicking the important biological characteristics of tumor tissues, another unique advantage of tumor spheroid is to study the specific biological roles of cancer cell-cancer cell, or cancer cell-matrix directly with freeing from the effects of other stroma cells. In the previous study, we established a metastatic HCC spheroid model similar with real solid tumor in structure and biological function as specific gene expression, protein secretion, tumorigenesis and metastasis. Here we further produced two HCC spheroids from two HCC cells with different metastasis potential and investigate candidate metastasis-associated genes related to cell adhesion, matrix secretion and invasion. As far as we know, it is the first time to comparatively analyze candidate capability to combinewithreceptorsexpressed on stromal cells (eg. endothelial cell, macrophage) within tumor microenvironment.

In addition, as shown in **Table 3**, pro-inflammatory factors (IL-1β, IL-18), cytokines (TGF-β), growth factor and receptor (HGF/Met), angiogenesis-stimulating factors (VEGFA), oncogene (src, myc, Mdm2) were highly expressed in the

Table 3. Molecular function classification of 123 differential expression genes between two HCC spheroids

Category	Number and name of up-regulated genes in HCC spheroid	
	Metastatic MHCC97H spheroid (70 genes)	low-metastatic Hep3B spheroid (53 genes)
Cell-cell interactions		
homotypic cell adhesion molecules	2 (CTNNA1, CDH6)	4 (E-cadherin, CTNND2, CDH11, CNTN1)
heterotypic cell adhesion molecules	3 (VCAM1, ICAM1, PECAM1)	2 (NCAM, MCAM)
Cell-ECM interactions		
Transmembrane adhesion molecules or receptors	18 (CD44, Integrin α1, α2, α3, α5, α6, α7, αv, αm, β1, β3, β4, β5m, FXYD5, EPHB2, Selectin E, Selectin L, uPAR)	5 (Integrin α4, α7, α8, αL, SGCE)
Extracellular matrix protein (ECM)	17 (SPP1, Tenascin C, FN, COL1A1, COL4A2, COL5A1, COL6A1, COL6A2, COL16A1, LAMA2, LAMB1, LAMB3, LAMA3, ECM1, SPARC, VCAN, THBS3)	8 (Vitronectin, COL7A1, COL8A1, COL11A1, COL12A1, COL14A1, COL15A1, LAMA1)
ECM proteinases	7 (MMP1, MMP2, MMP7, MMP10, MMP14, MMP15, Cathepsin K)	9 (MMP3, MMP8, MMP9, MMP11, MMP12, MMP13, MMP16, Heparanase, Cathepsin L1)
ECM proteinases inhibitor	4 (TIMP1, TIMP2, TIMP4, CST7)	1 (TIMP3)
Pro-inflammatory factor	2 (IL1β, IL18)	1 (IL8RB)
Cytokines, chemokines and receptors	5 (TGF-β, TGFBI, HGF, Met, FLT4)	6 (IGF1, CCL7, CXCL12, CXCR4, SSTR2, FGFR4)
Cell cycle		1 (CDKN2A)
Angiogenesis-related molecules	1 (VEGFA)	3 (ADAMTS1, ADAMTS13, ADAMTS8)
Oncogene	3 (SRC, MYC, Mdm2)	1 (MYCL1)
Tumor suppressor gene	2 (TP53, RB1)	2 (APC, NF2)
Tumor metastasis suppressor	2 (MTSS1, CD82)	3 (BRMS1, KISS1R, HTATIP2)
Unknown function	4 (CTBP1, SPG7, EWSR1, TNFSF10)	7 (RORB, TRPM1, TSHR, NR4A3, KAL1, CLEC3B, PNN)

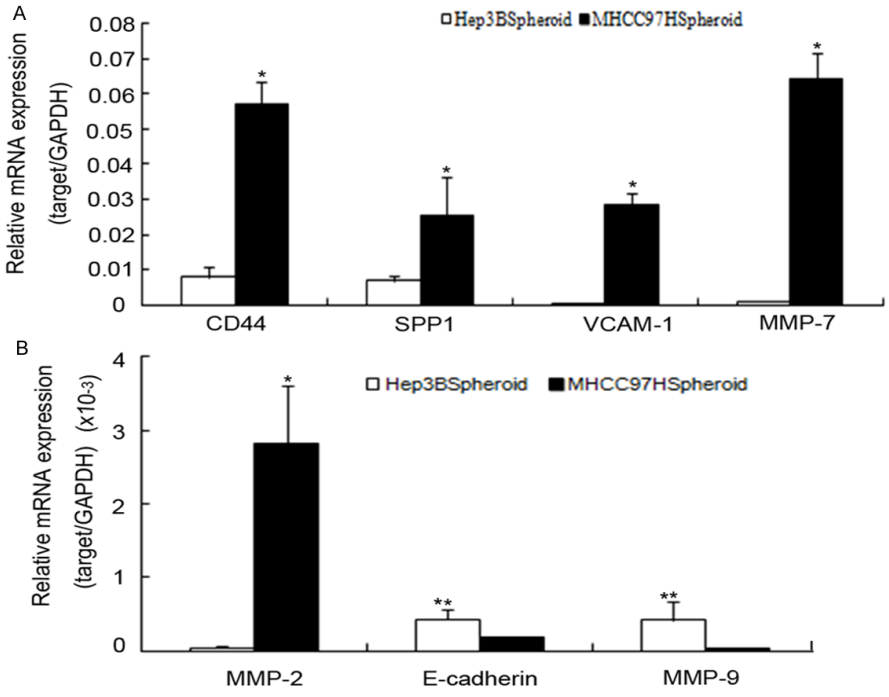

Figure 2. Expression of differentially regulated genes was confirmed in HCC spheroids by real-time RT-PCR. A: In comparison with that in Hep3B spheroid, expression of VCAM-1, CD44, SPP1 and MMP-7 was significantly increased in the high-metastatic MHCC97H spheroid. B: MMP-2 was highly increased while E-cadherin and MMP-9 were significantly decreased in the MHCC97H spheroid, compared with that in the Hep3B spheroid. Results were normalized to GAPDH. *, **P<0.05.

metastasis-associated genes related cell adhesion, matrix secretion in HCC spheroids.

The identified candidate metastasis-associated genes between the high-metastatic spheroid and the low-metastatic spheroid were categorized as (1) genes encoding adhesion molecules mediating cell-cell and cell-matrix interactions; (2) genes encoding ECM-degrading proteolytic enzymes; (3) genes encoding inflammatory factors such as IL-1β. (4) oncogene, tumor suppressor gene, tumor metastasis genes associated with angiogenesis, cell cycle and cytokines, etc.

Gene expression patterns of candidate metastasis-associated genes in the high-metastatic MHCC97H spheroid were obviously different from that of

the low-metastatic Hep3B spheroid. Of importance, the number of up-regulated differential genes related to cell adhesion and matrix protein in the high-metastatic HCC spheroid were apparently more than those in the low-metastatic spheroid, indicating that the highly metastatic spheroid secretes more matrix proteins and there are more interactions between cancer cell and matrix proteins, ultimately resulting in facilitating HCC metastasis. Specifically, these candidate genes play important roles in tumor metastasis. (1) Adhesion molecules CD44 and integrins expressed on the surface of cancer cells can bind to matrix such as hyaluronic acid, SPP1 or collagen mediating cell-matrix interactions to promote tumor metastasis. CD44 is also a surface mark for HCC stemness [14]. Integrin αv, β1, β3 are frequently involved in cell migration, tumor dissemination by mediating cell-matrix interactions. (2) Extracellular matrix protein secretion such as tenascin c, SPP1, fibronectin, collagen, laminin, was apparently up-regulated in the metastatic HCC spheroids. SPP1 over-expression is closely associated with HCC growth and metastasis [15]. SPP1 can bind to its receptors CD44 or integrins expressed on cancer cell, and activate many signal pathways and induce epithelial–mesenchymal transition (EMT) to facilitate tumor metastasis [16]. Under 3D culture, SPP1 is important for the formation of vasculogenic mimicry by HCC cells through activating MMP-2 and urokinase-type plasminogen activator (uPA) [17]. As similar way, tumor-derived tenascin C promotes cancer invasiveness via EMT regulation in colorectal cancer [18]. Collagen I and IV, two important collagen components in liver disease, can change the matrix stiff and induce cancer cell dissemination.

On the contrary, maintaining homotypic tumor cell adhesion (e.g. E-cadherin) was decreased in the high-metastatic spheroid, implying that loss of tight tumor cell-cell adhesion facilitates tumor metastasis. It has been shown that reduced E-cadherin expression is associated with HCC metastasis [19]. E-cadherin is an important switch in epithelial–mesenchymal transition (EMT) and EMT confers metastatic potential to tumor cells. However, adhesion molecules mediating heterotypic cell adhesion (e.g. VCAM-1, ICAM-1) was increased in the high-metastatic HCC spheroid. VCAM-1 and ICAM-1 expressed on the surface provide tumor cell the potential capability to bind with receptors on stromal cells (eg. endothelial cells, macrophage) within tumor microenvironment. Recent study has shown that VCAM-1 aberrantly expressed on breast cancer cells mediates lung metastasis by binding to its counter-receptor integrin α4β1 on metastasis-associated macrophages and providing the survival advantage to breast cancer cells [20, 21]. This suggests the importance of VCAM-1 in the tumor dissemination.

As for ECM degradation, specific types of ECM proteinases were expressed in the high-metastatic HCC spheroid. For example, MMP-1, MMP-2 and MMP-7 involved in tumor invasion and metastasis has been reported [22, 23]. Wu et al have also reported that in three-dimensional (3D) culture model, HCC cells secrete more MMPs to increase the invasion potentials [24]. Interestingly, another important matrix proteinase type, MMP-9 expressed lowly in the metastatic spheroid compared with that in the low-metastatic spheroid. However, MMP-9 level significantly up-regulated in a HCC invasion model established by 3D co-culture of HCC cells and a liver tissue fragment (data not shown), suggesting that MMP-9 might begin to increase once the detached HCC cells from primary tissue invade into matrix.

There were other genes of interest on the PCR array that warrant further investigation, for example, IL-1β and CTNND2. The pro-inflammatory cytokine IL-1β is a critical component of the persistent inflammatory milieu where HCC frequently derived. Recently, IL-1β has been described as a factor promoting cancer stemness and invasiveness of colon cancer cells [25]. Little is known regarding the specific roles of down-regulated CTNND2 in HCC metastasis. The CTNND2 gene encodes a protein called delta-catenin. This protein likely helps cells stick together to mediate cell adhesion and plays a role in cell movement. Identification and further characterization of these genes in human tumor tissues may allow a better understanding of HCC metastasis.

In conclusion, obvious differences in the gene expression patterns of adhesion molecules, matrix secretion, invasion and other molecules may determine the different metastatic potential of HCC spheroid. Screening candidate metastasis-associated genes in the metastatic HCC spheroid facilitate to investigate cell-cell and cell-matrix interactions during HCC metas-

tasis, ultimately aiming to find the target for inhibiting HCC metastasis.

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Disclosure of conflict of interest

None.

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References

[1] El-Serag HB and Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007; 132: 2557-2576.

[2] Tung-Ping Poon R, Fan ST and Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 2000; 232: 10-24.

[3] Spano D, Heck C, De Antonellis P, Christofori G and Zollo M. Molecular networks that regulate cancer metastasis. Semin Cancer Biol 2012; 22: 234-249.

[4] Baker BM and Chen CS. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci 2012; 125: 3015-3024.

[5] Kimlin LC, Casagrande G and Virador VM. In vitro three-dimensional (3D) models in cancer research: an update. Mol Carcinog 2013; 52: 167-182.

[6] Tang J, Cui J, Chen R, Guo K, Kang X, Li Y, Gao D, Sun L, Xu C, Chen J, Tang Z and Liu Y. A three-dimensional cell biology model of human hepatocellular carcinoma in vitro. Tumour Biol 2011; 32: 469-479.

[7] Tian J, Tang ZY, Ye SL, Liu YK, Lin ZY, Chen J and Xue Q. New human hepatocellular carcinoma (HCC) cell line with highly metastatic potential (MHCC97) and its expressions of the factors associated with metastasis. Br J Cancer 1999; 81: 814-821.

[8] Gupta GP and Massague J. Cancer metastasis: building a framework. Cell 2006; 127: 679-695.

[9] Lee J, Cuddihy MJ and Kotov NA. Three-dimensional cell culture matrices: state of the art. Tissue Eng Part B Rev 2008; 14: 61-86.

[10] Smalley KS, Lioni M and Herlyn M. Life isn't flat: taking cancer biology to the next dimension. In Vitro Cell Dev Biol Anim 2006; 42: 242-247.

[11] Achilli TM, Meyer J and Morgan JR. Advances in the formation, use and understanding of multicellular spheroids. Expert Opin Biol Ther 2012; 12: 1347-1360.

[12] Zhang L, Ye JS, Decot V, Stoltz JF and de Isla N. Research on stem cells as candidates to be differentiated into hepatocytes. Biomed Mater Eng 2012; 22: 105-111.

[13] LaBarbera DV, Reid BG and Yoo BH. The multicellular tumor spheroid model for high-throughput cancer drug discovery. Expert Opin Drug Discov 2012; 7: 819-830.

[14] Pang RW and Poon RT. Cancer stem cell as a potential therapeutic target in hepatocellular carcinoma. Curr Cancer Drug Targets 2012; 12: 1081-1094.

[15] Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC, Simon R, Li Y, Robles AI, Chen Y, Ma ZC, Wu ZQ, Ye SL, Liu YK, Tang ZY and Wang XW. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003; 9: 416-423.

[16] Bhattacharya SD, Mi Z, Kim VM, Guo H, Talbot LJ and Kuo PC. Osteopontin regulates epithelial mesenchymal transition-associated growth of hepatocellular cancer in a mouse xenograft model. Ann Surg 2012; 255: 319-325.

[17] Liu W, Xu G, Ma J, Jia W, Li J, Chen K, Wang W, Hao C, Wang Y and Wang X. Osteopontin as a key mediator for vasculogenic mimicry in hepatocellular carcinoma. Tohoku J Exp Med 2011; 224: 29-39.

[18] Takahashi Y, Sawada G, Kurashige J, Matsumura T, Uchi R, Ueo H, Ishibashi M, Takano Y, Akiyoshi S, Iwaya T, Eguchi H, Sudo T, Sugimachi K, Yamamoto H, Doki Y, Mori M and Mimori K. Tumor-derived tenascin-C promotes the epithelial-mesenchymal transition in colorectal cancer cells. Anticancer Res 2013; 33: 1927-1934.

[19] Zhai B, Yan HX, Liu SQ, Chen L, Wu MC and Wang HY. Reduced expression of E-cadherin/catenin complex in hepatocellular carcinomas. World J Gastroenterol 2008; 14: 5665-5673.

[20] Chen Q, Zhang XH and Massague J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell 2011; 20: 538-549.

[21] Chen Q and Massague J. Molecular pathways: VCAM-1 as a potential therapeutic target in metastasis. Clin Cancer Res 2012; 18: 5520-5525.

[22] Wang N, Zhu M, Tsao SW, Man K, Zhang Z and Feng Y. Up-regulation of TIMP-1 by genipin in-

hibits MMP-2 activities and suppresses the metastatic potential of human hepatocellular carcinoma. PLoS One 2012; 7: e46318.

[23] Ochoa-Callejero L, Toshkov I, Menne S and Martinez A. Expression of matrix metalloproteinases and their inhibitors in the woodchuck model of hepatocellular carcinoma. J Med Virol 2013; 85: 1127-1138.

[24] Wu YM, Tang J, Zhao P, Chen ZN and Jiang JL. Morphological changes and molecular expressions of hepatocellular carcinoma cells in three-dimensional culture model. Exp Mol Pathol 2009; 87: 133-140.

[25] Li Y, Wang L, Pappan L, Galliher-Beckley A and Shi J. IL-1beta promotes stemness and invasiveness of colon cancer cells through Zeb1 activation. Mol Cancer 2012; 11: 87.