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ORIGINAL ARTICLE

An improved strategy to detect the epithelial-mesenchymal transition process in circulating tumor cells in hepatocellular carcinoma patients

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Abstract
Purpose We adopted a new strategy to explore the relationship between the EMT process of CTCs and hepatocellular carcinoma (HCC). Furthermore, we intend to illustrate the potential diagnostic value of CTCs of distinct phenotypes in HCC.
Methods The clinical data of 33 HCC patients and 10 healthy volunteers were collected retrospectively. By using the optimized CanPatrol CTC enrichment technique, patient blood samples of about 5 ml were collected, and CTCs were identified and characterized. The first step of this detection process was to isolate CTCs via a filter-based method; then, an RNA in situ hybridization (RNA-ISH) technique based on the branched DNA signal amplification technology was used to classify the CTCs according to EMT markers. The relationships between HCC CTCs and clinical characteristics were analyzed.
Results The number of epithelial CTCs was related to tumor size ($r = 0.456$, $p = 0.008$), epithelial-mesenchymal-mixed CTCs were related to tumor number ($r = 0.421$, $p = 0.015$), and mesenchymal CTC was associated with metastasis ($r = 0.375$, $p = 0.032$). There was no significant correlation between CTC number and other clinicopathological factors, such as age, serum AFP level or cirrhosis.
Conclusions Epithelial-mesenchymal-mixed CTCs seem to play an important role in EMT transition in HCC, mixed CTCs might be a vital factor for intrahepatic metastasis, and mesenchymal CTCs had the potential to be a predictor of extrahepatic metastasis.

Keywords Hepatocellular carcinoma · Circulating tumor cells · Epithelial-mesenchymal transition · Metastasis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common lethal malignancies and causes of cancer mortality worldwide [1, 2]; each year >600,000 people die from HCC [3]. Surgical resection is the preferred treatment modality for most patients with HCC. HCC is characterized by early metastasis, and most HCC patients have intrahepatic metastasis at the time of surgery that results in high rates of postoperative recurrence and metastasis. The development of overt metastasis is preceded by the dissemination of tumor cells from the primary tumor to distant sites such as the blood circulation or lymphatic system [4]. As hematogenous spread is the major route of HCC metastasis [5], detection of circulating tumor cells (CTCs) might be complementary to the current diagnostic techniques used for tumor staging and monitoring the response to treatment [6]. It has been reported that aberrant activation of epithelial-mesenchymal transition (EMT) has been implicated in this process

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of dissemination of tumor cells, based on studies with human cancer cell lines and mouse models [7, 8]. During the EMT process, the expression of epithelial genes, such as the epithelial cell adhesion molecule (EpCAM) and cytokeratins (CK), will be downregulated, while expression of mesenchymal genes, such as vimentin and twist, will be upregulated [9]. Tumor cells experiencing EMT will have several characteristics of stem cells, resulting in variation of adhesion and enhanced metastatic and invasive potential. EpCAM is a transmembrane glycoprotein that mediates cell-cell adhesion in epithelial tissues, and this protein has oncogenic potential via its capacity to upregulate c-myc, cyclin A and cyclin E [10]. CKs are the proteins of the keratin-containing intermediate filaments found in the cytoskeleton of epithelial cells. Both EpCAM and CK are commonly used biomarkers for CTCs from epithelial-derived neoplasms [11]. Vimentin, a member of the intermediate filament family of proteins, is ubiquitously expressed in mesenchymal cells [12], and expressing vimentin in cancer cells increases tumor growth and invasiveness [13]. Twist is a helix-loop-helix protein that is transcriptionally active during cell differentiation [14], and increased expression of twist has been observed in many types of tumor cells, such as prostate, gastric and breast cancer [15]. CTCs are a very heterogeneous population of cells, and one of the most common approaches for isolating CTCs is the EpCAM-based enrichment technique [16, 17]. However, recent studies have demonstrated that this technique has failed to detect CTC subpopulations that have undergone EMT [18, 19]. In Yu's study [18], they established a quantifiable, dual-colorimetric RNA-in situ hybridization (ISH) assay to analyze EMT in CTCs from breast cancer patients. Inspired by their research, we adopted the technique (optimized CanPatrolTM CTC enrichment, Surexam Biotech, Guangzhou, China) [20] by using an 8-μm-diameter-pore calibrated membrane and designing a different mRNA ISH array in order to explore the relationship between the EMT process of CTCs and hepatocellular carcinoma (HCC). Furthermore, we intend to illustrate the potential diagnostic value of CTCs of distinct phenotypes in HCC.

Materials and methods

Figure 1 shows the flow of the detection process.

Patients

We enrolled 33 HCC patients and 10 healthy volunteers in Guangdong General Hospital, Guangzhou, China.

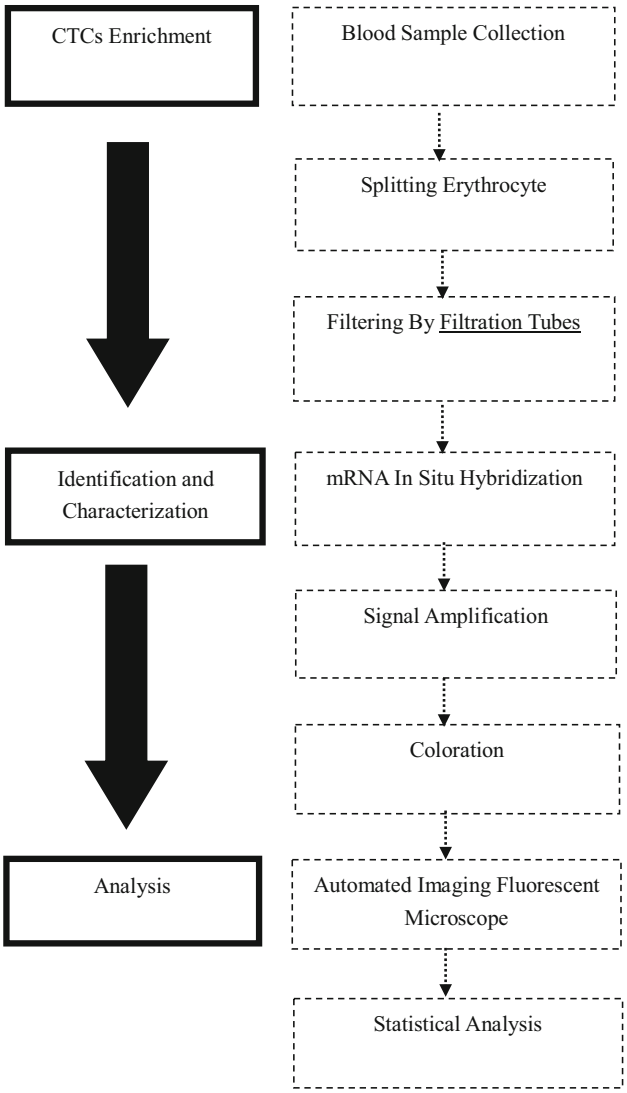

Fig. 1 The flowsheet of the detection process. *CTCs* circulating tumor cells

Blood sample collection

Patient blood samples of about 5 ml were collected in K2-EDTA tubes, transferred into sample preservative tubes (Surexam Biotech, Guangzhou, China) containing ammonium chloride-based lysing buffer by a tailored connection device (Surexam Biotech, Guangzhou, China) and incubated at room temperature for 30 min.

CTC enrichment by size-based membrane filters

The sample preservative tubes were centrifuged to collect the cell pellets. The supernatant was discarded, and the cell

pellets were suspended by adding 5 ml PBS into the tubes. The cell suspensions were filtrated by filtration tubes (Surexam Biotech, Guangzhou, China), which contained a membrane filter (Millipore, Billerica, MA, USA) [20] with 8-μm pore size. The cell suspensions flew through the filter under vacuum pressure, the circulating tumor cells were retained on the filter, and the blood cells went through the pores based on the fact that CTCs are larger than blood cells. The filter was taken out, and the cells were fixed by the 2 % formaldehyde retained on it.

Identification and characterization of CTCs by RNA-ISH

We established three groups of nucleic acid probes to identify and examine the expression levels of epithelial and mesenchymal genes in CTCs by multiplex mRNA in situ hybridization assay. Group 1 probes contained four pooled epithelial transcripts [cytokeratins (CK) 8, 18 and 19; epithelial cell adhesion molecule (EpCAM)]. Group 2 probes had two mesenchymal transcripts (vimentin and twist). The final group only contained a CD45 transcript, which was used to discriminate white blood cells and CTCs. The detailed capture probes synthesized by Invitrogen (Invitrogen, Shanghai, China) sequences are shown in Table 1. The details of the hybridization assay procedure were similar to those as in Yu's study [18]. Briefly, the cells retained on the filter were permeabilized and digested with protease. Then, the cells were subjected to a serial of hybridization reactions with a cocktail of probes specific to the intended examined genes described above. Finally, DAPI was used to stain the cell nucleuses. The samples were analyzed in an automated imaging fluorescent microscope using a 100× oil objective (Olympus BX53, Tokyo, Japan). The red and green dots of the fluorescent signal observed in the cells represented the epithelial and mesenchymal gene expression, respectively. The bright blue fluorescent dots showed that the CD45 gene expression was the marker of the white blood cells.

Statistical analyses

Spearman rank correlation analysis was used for nonparametric correlation analysis. $p < 0.05$ was considered statistically significant. All statistical tests were two-sided. All statistical processing was performed using the Statistical Package for Social Science, version 13.0 (SPSS v13.0).

Table 1 Capture probe sequences for the EpCAM, CK8/18/19, vimentin, twist and CD45 genes

Gene	Sequences (5′ → 3′)
EpCAM	TGGTGCTCGTTGATGAGTCA
	AGCCAGCTTTGAGCAAATGA
	AAAGCCCATCATTGTTCTGG
	CTCTCATCGCAGTCAGGATC
	TCCTTGTCTGTTCTTCTGAC
	CTCAGAGCAGGTTATTTCAG
CK8	CGTACCTTGTCTATGAAGGA
	ACTTGGTCTCCAGCATCTTG
	CCTAAGGTTGTTGATGTAGC
	CTGAGGAAGTTGATCTCGTC
	CAGATGTGTCCGAGATCTGG
	TGACCTCAGCAATGATGCTG
CK18	AGAAAGGACAGGACTCAGGC
	GAGTGGTGAAGCTCATGCTG
	TCAGGTCCTCGATGATCTTG
	CAATCTGCAGAACGATGCGG
	AAGTCATCAGCAGCAAGACG
	CTGCAGTCGTGTGATATTGG
CK19	CTGTAGGAAGTCATGGCGAG
	AAGTCATCTGCAGCCAGACG
	CTGTTCCGTCTCAAACTTGG
	TTCTTCTTCAGGTAGGCCAG
	CTCAGCGTACTGATTTCCTC
	GTGAACCAGGCTTCAGCATC
Vimentin	GAGCGAGAGTGGCAGAGGAC
	CTTTGTCGTTGGTTAGCTGG
	CATATTGCTGACGTACGTCA
	GAGCGCCCCTAAGTTTTTAA
	AAGATTGCAGGGTGTTTTCG
	GGCCAATAGTGTCTTGGTAG
Twist	ACAATGACATCTAGGTCTCC
	CTGGTAGAGGAAGTCGATGT
	CAACTGTTCAGACTTCTATC
	CCTCTTGAGAATGCATGCAT
	TTTCAGTGGCTGATTGGCAC
	TTACCATGGGTCCTCAATAA
CD45	TCGCAATTCTTATGCGACTC
	TGTCATGGAGACAGTCATGT
	GTATTTCCAGCTTCAACTTC
	CCATCAATATAGCTGGCATT
	TTGTGCAGCAATGTATTTCC
	TACTTGAACCATCAGGCATC

EpCAM epithelial cell adhesion molecule, *CK* cytokeratins

Results

CTCs were detected in all 33 HCC patients. No CTCs were found in the blood of healthy volunteers. The mean age of the patients was 53.76 (range, 31 to 75) years, and the average tumor diameter was 7.63 (range, 1.7 to 17) cm. The average number of CTCs was 17.84 (range, 2 to 81), while the mean number of epithelial, epithelial-mesenchymal-mixed and mesenchymal CTCs was 0.81 (range,

Table 2 Clinical characteristics of the 33 HCC patients

Clinical variables	*N*	Minimum	Maximum	Mean
Age (years)	33	31	81	53.76
Epithelial CTCs	33	0	5	0.82
Mixed CTCs	33	1	75	13.82
Mesenchymal CTCs	33	0	21	3.21
Total CTCs	33	2	81	17.85
Tumor size (cm)	33	1.7	17	7.64
Total bilirubin (μmol/l)	33	6.9	67.7	22.88
Albumin (g/l)	33	23.9	43.31	34.30
AFP (ng/ml)	33	1.79	54,000	7686.19
Prothrombin time (s)	33	12.2	20.3	14.23

Clinical variables	Category	Number	Percentage
Gender	Female	4	12.1
	Male	29	87.9
Tumor number	Single	12	36.4
	Double	2	6.1
	Triple	1	3
	Multiple	18	54.5
Ascites	Absence	30	90.9
	Presence	3	9.1
HBV	Absence	3	9.1
	Presence	30	90.9
Cirrhosis	Absence	7	21.2
	Presence	26	78.8
Child-Pugh class	A	26	78.8
	B	7	21.2
BCLC staging for HCC	A	2	6.1
	B	14	42.4
	C	17	51.5
Metastasis	Absence	17	51.5
	Presence	16	48.5
Portal vein tumor thrombus	Absence	22	66.7
	Presence	11	33.3

AFP alpha-fetoprotein, *BCLC* Barcelona Clinic Liver Cancer, *CTCs* circulating tumor cells, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma

Table 3 Clinical characteristics of ten healthy volunteer

Clinical variables	*N*	Minimum	Maximum	Mean
Age (years)	10	28	70	50.6
Epithelial CTCs	10	–	–	–
Mixed CTCs	10	–	–	–
Mesenchymal CTCs	10	–	–	–
Total CTCs	10	–	–	–
Tumor size (cm)	10	–	–	–
Total bilirubin (μmol/l)	10	5.5	24.7	14.3
Albumin (g/l)	10	33.9	50.8	41.4
AFP (ng/ml)	10	1.83	18	5.83
Prothrombin time (s)	10	11.3	15.7	13.2

Clinical variables	Category	Number	Percentage
Gender	Female	2	20
	Male	8	80
Tumor number	–	–	–
Ascites	Absence	9	90
	Presence	1	10
HBV	Absence	8	80
	Presence	2	20
Cirrhosis	Absence	10	100
	Presence	0	0
Child-Pugh class	A	9	90
	B	1	10
BCLC staging for HCC	–	–	–
Metastasis	–	–	–
Portal vein tumor thrombus	–	–	–

AFP alpha-fetoprotein, *BCLC* Barcelona Clinic Liver Cancer, *CTCs* circulating tumor cells, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma

0 to 5, 4.54 %), 13.81 (range, 1 to 75, 77.41 %) and 3.21 (range, 0 to 21, 17.99 %), respectively (the details are shown in Tables 2 and 3). As shown in Fig. 2, I represented epithelial cells, II represented epithelial-mesenchymal-mixed cells, III represented mesenchymal cells, respectively, and IV represented leukocytes (negative control).

After Spearman rank correlation analysis, we found that in HCC patients, CTCs were related to tumor number ($r = 0.481$, $p = 0.005$). Besides, since total CTCs consisted of epithelial, epithelial-mesenchymal-mixed and mesenchymal CTCs, we also analyzed which factors were associated with these kinds of CTCs. Epithelial CTCs were related to tumor size ($r = 0.456$, $p = 0.008$), epithelial-mesenchymal-mixed CTCs were related to tumor number ($r = 0.421$, $p = 0.015$) as well, while mesenchymal CTCs were associated with metastasis ($r = 0.375$, $p = 0.032$). However, there was no significant correlation between the CTC number and other clinicopathological factors, such as age, serum AFP level or cirrhosis (Table 4).

Discussion

In the past few years, the function of CTCs in the process of cancer metastasis has been under active investigation. Although many analytical approaches and techniques for CTC isolation, detection and characterization were developed in the past decade, no ideal method is currently available, especially for HCC [21]. Several studies recommended that the Cell Search System could be a standard

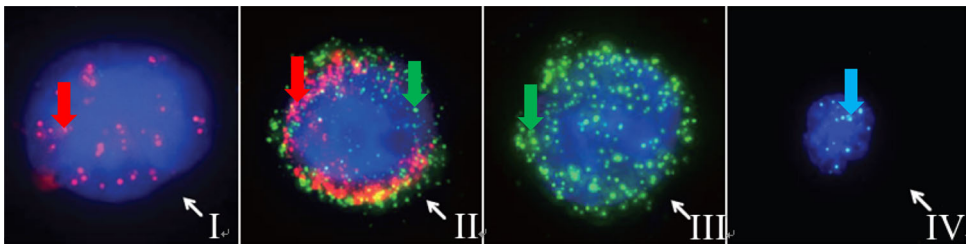

Fig. 2 Examples of different CTCs under the automated imaging fluorescent microscope. *I* epithelial circulating hepatoma cells stained for epithelial markers (*red dots, red arrowheads*); *II* epithelial-mesenchymal-mixed circulating hepatoma cells stained for epithelial markers (*red dots, red arrowheads*) and mesenchymal markers (*green dots, green arrowheads*); *III* mesenchymal circulating hepatoma cells stained for mesenchymal markers (*green dots, green arrowheads*); *IV* negative control, leukocytes stained for CD45 expression (*bright blue* fluorescence, *blue arrowheads*). ***CTCs*** circulating tumor cells

Table 4 Correlation between CTCs number and clinical variables in HCC patients

Clinical variables	Total CTCs (p value)	Epithelial CTCs (p value)	Mixed CTCs (p value)	Mesenchymal CTCs (p value)
Age	NS	NS	NS	NS
Sex	NS	NS	NS	NS
Tumor size	NS	0.008**	NS	NS
Tumor number	0.005**	NS	0.015*	0.032*
Metastasis	0.031*	NS	0.048*	0.017*
HBV	NS	NS	NS	NS
Cirrhosis	NS	NS	NS	NS
AFP	NS	NS	NS	NS
BCLC stage	NS	NS	0.024*	NS
Child-Pugh class	NS	NS	NS	NS
Portal vein tumor thrombus	NS	NS	NS	NS

** Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed)

AFP alpha-fetoprotein, *BCLC* Barcelona Clinic Liver Cancer, *CTCs* circulating tumor cells, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *NS* not significant

method for detecting CTCs in patients with cancers [22–24]. However, the Cell Search System has the vital limitation that it can only detect epithelial CTCs, which comprise just a small part (4.5 %) of all kinds of CTCs, according to our study. Studies [25] using this method tend to obtain poor CTC numbers, which might influence their conclusions. Compared to epithelial CTCs, epithelial-mesenchymal-mixed and mesenchymal CTCs seem to be more meaningful in explaining the process of dissemination of cancer cells. Based on the concerns above, we designed a new method to isolate and identify epithelial, epithelial-mesenchymal-mixed and mesenchymal CTCs by combining size-based membrane filters and RNA-ISH technology.

CK 8, 18 and 19, and EpCAM were demonstrated to be related to epithelial CTCs [16, 26–28], and twist and vimentin were mesenchymal-related transcripts [8, 29, 30]. By multiplex mRNA in situ hybridization assay, we designed three groups of nucleic acid probes to distinguish different kinds of CTCs: Group 1 probes contained four pooled epithelial transcripts (CK 8, 18 and 19; EpCAM). Group 2 probes had two mesenchymal transcripts (vimentin and twist). The final group only containing a CD45 transcript was used to discriminate white blood cells and CTCs. These probes were validated in cell lines (HepG2 cell line, ATCC, HB 8065, derived from a human hepatocellular carcinoma) to confirm the differential expression in epithelial versus mesenchymal cancer cells and the absence of expression in blood cells that contaminate CTC preparations; the details are shown in Fig. 3.

According to our results, epithelial-mesenchymal-mixed CTCs comprised 77.41 % of all CTCs, which indicated the importance of EMT transition in the formation and progression of CTCs. Besides, mixed CTCs might be a vital factor in intrahepatic metastasis, and mesenchymal CTCs had the potential to be a predictor of extrahepatic metastasis. These findings indicated that EMT transition in CTCs could help clinicians to develop better therapeutic regimens and improve the prognosis of HCC patients.

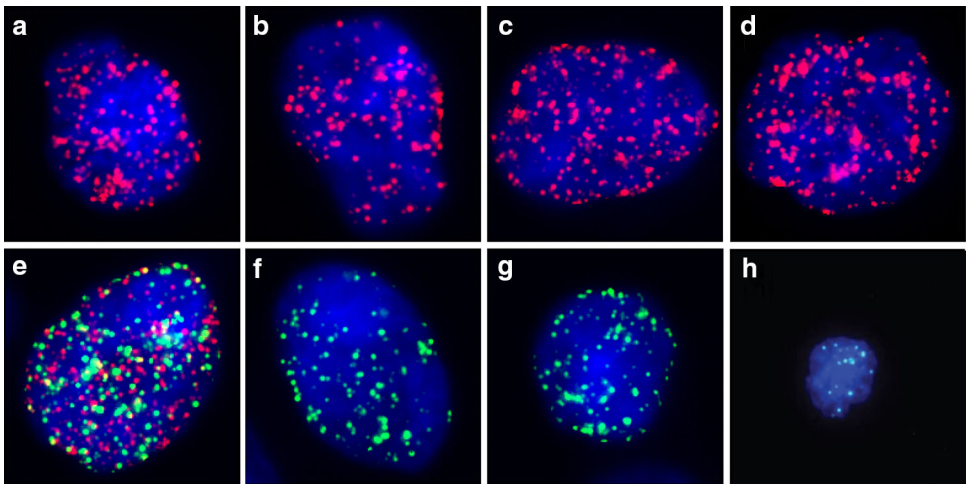

Fig. 3 CK8/18/19, EpCAM, twist and vimentin expression in HepG2 tumor cells and leukocytes. **a** HepG2 cells stained for CK8 expression (*red* fluorescence); **b** HepG2 cells stained for CK18 expression (*red* fluorescence); **c** HepG2 cells stained for CK18 expression (*red* fluorescence); **d** HepG2 cells stained for EpCAM expression (*red* fluorescence); **e** HepG2 cells stained for EpCAM, CK8/18/19, twist and vimentin expression (*red*/*green* fluorescence); **f** HepG2 cells stained for twist expression (*green* fluorescence); **g** HepG2 cells stained for vimentin expression (*green* fluorescence); **h** negative control, leukocytes stained for CD45 expression (*bright blue* fluorescence). The cells were analyzed by a 100× oil objective. *EpCAM* epithelial cell adhesion molecule, *CK* cytokeratin

To the best of our knowledge, this was the first time the EMT transition in HCC patients was detected by combining nanotechnology filters and mRNA ISH array. Besides, we also demonstrated that the EMT process was related to HCC evolution. However, we have to admit that the small sample number might have had an impact on our conclusions. According to our plan, this was the first step to prove the efficacy of this method in this study, and we will continue with further research to try to obtain more meaningful results.

Compliance with ethical requirements

Conflict of interest Written informed consent was obtained from each patient and volunteer, and the final protocol was approved by the independent ethics committee of Guangdong General Hospital & Guangdong Academy of Medical Sciences. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Li-gong Lu, Yong-kang Liu, Bao-shan Hu, Zhong-liang Li, Xu He and Yong Li declare that they have no conflict of interest.

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