Cancer Letters 412 (2018) 99–107

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Quantified postsurgical small cell size CTCs and EpCAM^{+} circulating tumor stem cells with cytogenetic abnormalities in hepatocellular carcinoma patients determine cancer relapse

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ARTICLE INFO

Article history:
Received 13 September 2017
Received in revised form
2 October 2017
Accepted 6 October 2017

Keywords:
iFISH
Aneuploid CTC and circulating tumor stem cell
Hepatobiliary malignancy
Hepatocellular and cholangiocarcinoma
Surgical resection

ABSTRACT

Detection of hepatocellular carcinoma circulating tumor cells performed with conventional strategies, is significantly limited due to inherently heterogeneous and dynamic expression of EpCAM, as well as degradation of cytokeratins during epithelial-to-mesenchymal transition, which inevitably lead to non-negligible false negative detection of such "uncapturable and invisible" CTCs. A novel SE-iFISH strategy, improved for detection of HCC CTCs in this study, was applied to comprehensively detect, *in situ* phenotypically and karyotypically characterize hepatocellular and cholangiocarcinoma CTCs (CD45^{-}/CD31^{-}) in patients subjected to surgical resection. Clinical significance of diverse subtypes of CTC was systematically investigated. Existence of small cell size CTCs ($\leq$5 μm of WBCs) with cytogenetic abnormality of aneuploid chromosome 8, which constituted majority of the detected CTCs in HCC patients, was demonstrated for the first time. The stemness marker EpCAM^{+} aneuploid circulating tumor stem cells (CTSCs), and EpCAM^{-} small CTCs with trisomy 8, promote tumor growth. Postsurgical quantity of small triploid CTCs ($\geq$5 cells/6 ml blood), multiploid ($\geq$pentasomy 8) CTSCs or CTM (either one $\geq$ 1) significantly correlated to HCC patients' poor prognosis, indicating that detection of those specific subtypes of CTCs and CTSCs in post-operative patients help predict neoplasm recurrence.

Introduction

Global morbidity of hepatobiliary malignancies, mainly including hepatocellular carcinoma (HCC), and cholangiocarcinoma (CC), etc. has recently increased. HCC shows the third highest mortality rate out of all major malignant carcinomas worldwide [1,2]. Cholangiocarcinoma is one of malignant cancers with the worst prognosis, its 5-year overall survival (OS) rate is as low as only 10–20% [3]. Though surgical resection is still the preferred therapeutic choice for hepatobiliary carcinoma treatment, its postsurgical recurrence has maintained as high as 50–70% for HCC [1,2], and 40–80% for CC patients [3], respectively. Currently, efficacy of conventional clinical approaches, such as imaging, pathology and serum tumor marker detection to monitor hepatobiliary

Abbreviations: HCC, hepatocellular carcinoma; CC, cholangiocarcinoma; CTC, circulating tumor cell; CTSC, circulating tumor stem cell; DTC, disseminated tumor cell; CTM, circulating tumor microemboli; EC, endothelial cell; CEC, circulating endothelial cell; nCAC, non-hematopoietic circulating aneuploid cell; EMT, epithelial-to-mesenchymal transition; CK, cytokeratin; SE, subtraction enrichment; iFISH, immunostaining-fluorescence *in situ* hybridization; DFS, disease-free survival.

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https://doi.org/10.1016/j.canlet.2017.10.004
0304-3835/

carcinoma recurrence and metastasis including minimal occult metastasis, is very limited. It is, therefore, imperative to develop an effective strategy with respect to early detecting malignant hepatobiliary carcinoma metastasis and relapse.

Circulating tumor cells (CTCs) play a fundamental role in tumor metastasis [4]. A series of recent studies indicated that, at the very initial stage of malignancy, early CTCs or disseminated tumor cells (DTCs) as seeds circulate in peripheral blood, then reach and stay in the target organ as dormant cells [5,6]. Those dormant tumor cells could be subsequently "awakened" and activated, then initiate metastasis [7]. It is anticipated that quantifying and molecular subtyping CTCs are beneficial for early monitoring or predicting cancer metastasis and recurrence. Moreover, cytogenetic and phenotypic subtyping of CTCs could help further understand mechanisms of cancer metastasis. Currently, CTC detection is mainly applied to either evaluate therapeutic efficacy or predict prognosis [8,9], rarely to predict and monitor cancer metastasis and relapse in patients following resection of primary malignant lesions.

Circulating tumor microemboli (CTM, a cluster of ≥2 CTCs), are derived from oligoclonal clumps of primary malignant carcinoma cells and constitute a very highly metastasis-competent subset of CTCs. The CTM is expected to have 50 times greater metastasis potential than that of single CTC [10,11].

Conventional CTC detection strategies are restricted and biased to only both cytokeratin (CK) and EpCAM double positive CTCs, showing overall positive detection of 10–30% for CTCs shed from limited types of solid tumor [12]. In view of dynamic [13] or absent [14] expression of EpCAM, as well as degradation of both CK and EpCAM in CTCs during epithelial-mesenchymal transition (EMT) [15,16], conventional CTC detection methods are not only unable to effectively detect multi-functional mesenchymal CTCs [17], which are usually deficient in epithelial marker expression as well as in small cell size (≤5 μm of WBCs) [18], but also lead to a significant false negative detection of CTCs, particularly with HCC CTCs expressing limited amount of stemness marker EpCAM [19–21].

Based on the well accepted concept that aneuploidy of chromosome, leading to genomic instability [22], is the most common characteristic of malignant cells [23,24], a novel CTC detection strategy, integrating subtraction enrichment and immunostaining-fluorescence *in situ* hybridization (SE-iFISH) has been recently established [25,26]. Such EpCAM-independent technology enriches non-hematopoietic cells from various types of patients' biofluid samples including peripheral blood [27], followed by *in situ* phenotypic and karyotypic characterization to effectively co-examine both chromosome aneuploidy as well as multiple tumor biomarker expression, despite their subcellular localization [28,29] in those non-hematopoietic circulating aneuploid cells (nCACs) mainly including CTCs ($CD45^-/CD31^-$) and CECs ($CD45^-/CD31^+$) [26], thus greatly increases CTC detection efficiency in terms of both sensitivity and specificity [30]. Moreover, SE-iFISH allows for classifying CTCs into diverse subtypes upon tumor biomarker expression and chromosome ploidy, and each subcategory of CTCs seems relevant to distinct clinical significance [25,30]. Our previous clinical studies performed with SE-iFISH on gastric cancer patients demonstrated that, in addition to increasing CTC detection sensitivity from 54.8% by *CellSearch* to 90.5% by SE-iFISH [25], distinct subpopulation of CTCs was demonstrated to correlate to patients' poor progression-free survival (PFS) and overall survival (OS) [31]. In addition, those diverse subtypes of CTCs revealed either intrinsic or acquired resistance to the chemotherapy agent cisplatin, in both gastric cancer patients [25] and gastric neuroendocrine cancer patient derived metastatic xenograft model (mPDX) [32].

It has been recently reported that considerable amount of CTCs remain in circulation following resection of primary tumor in cancer patients [33,34]. Surgical resection may not be effective to significantly reduce quantity of CTCs in all the cases, suggesting some of those CTCs might not be derived from the resected primary malignant tumors, instead, they may pre-exist in circulation even at very early stage of carcinoma formation. It is speculated that those pre-existent malignant neoplastic cells may, at least partially, account for postsurgical cancer metastasis and relapse.

In the current study, we investigated clinical significance of the post-operative $EpCAM^+$ circulating tumor stem cells (CTSCs) [20,35] and $EpCAM^-$ CTC subtypes in cancer metastasis and recurrence in patients with hepatobiliary malignancies. It is for the first time in this study to demonstrate correlation of small cell size CTCs to clinical outcome. Obtained results indicated that post-surgical quantity of CTM (cut-off: ≥1 in 6 ml blood), $EpCAM^+$ CTSCs with pentasomy of chromosome 8 (≥1 cell), and $EpCAM^-$ small CTCs with trisomy 8 (≥5 cells), as well as increasing of small triploid CTCs compared to those quantified in pre-operative patients, significantly correlated to post-operative HCC patients' poor prognosis and cancer relapse.

Patients and methods

Patient enrolment and specimen collection

Total of 34, including 14 hepatocellular carcinoma, 16 cholangiocarcinoma and 4 gallbladder cancer patients were enrolled in this prospective study. Among those enrolled patients, 14 were stage I-II, and 20 were stage III-IV.

Consent forms signed by all enrolled patients were approved by the Ethics Review Committees (ERC) of Tsinghua University Changgung Hospital (TUCH), Beijing, China. The written informed consent forms were received from patients prior to enrollment in the study. The study was performed according to the Declaration of Helsinki Principles.

Blood collection (6 ml) was performed on patients prior to and a week after surgery according to previous description [25,27].

Immunofluorescence staining and SE-iFISH

Immunofluorescence staining of HepG2 HCC cell line cells and SW480 colon cancer cell line cells was performed according to the protocol previously described [26]. Briefly, cells plated on coverslips for 48–72 h were subjected to immunofluorescence staining performed with indicated antibodies conjugated to Alexa Fluor (AF) 488, at room temperature for 30 min. After washing, specimens were examined using a fluorescence microscope.

Detection of CTCs by SE-iFISH was performed essentially similar to that previously described with certain modifications [25,26], and according to the manufacture instruction (Cytelligen, San Diego, CA, USA). In principle, 6 ml of peripheral blood were centrifuged at 600×*g* for 5 min to separate plasma. All sedimented cells were subsequently loaded on the top of 3 ml of non-hematopoietic cell separation matrix [28,36], followed by centrifugation at 400×*g* for 5 min. Supernatants were collected and incubated with immunomagnetic beads conjugated to a cocktail of anti-leukocyte antibodies for 15 min at room temperature, and subsequently loaded entire reaction solution on the top of 3 ml separation matrix. Samples were centrifuged at 400×*g* for 5 min. Supernatants were collected and subjected to magnetic separation. Bead-free solution was spun at 500×*g* for 2 min. Cell pellet was mixed with 100 μl of cell fixative, and applied to the coated CTC slide, followed by air-drying.

For iFISH experiment, in general, air dried samples on the coated CTC slides were hybridized with centromere probe 8 (CEP8) (Abbott Laboratories, Abbott Park, IL, USA) for 3 h, followed by antibody staining performed by incubation with AF594 conjugated monoclonal anti-CD45, AF488-*anti*-indicated tumor biomarkers, and Cy5-*anti*-CD31 at room temperature for 30 min. After washing, samples were subjected to Metafer-iFISH® automated CTC 3D scanning and image analyses.

Automated CTC 3D scanning and image analysis performed with Metafer-iFISH®

iFISH CTCs fixed on the coated and formatted CTC slide (Cytelligen) were identified and analyzed using an automated Metafer-iFISH® CTC 3D scanning and analyzing system (Carl Zeiss, Oberkochen, Germany; MetaSystems, Altlussheim, Germany; and Cytelligen, San Diego, CA, USA) [26]. Briefly, CTC slides automatically loaded on a Zeiss fluorescence microscope (AXIO Imager. Z2) were subjected to automated full X-Y plane scanning with cross Z-sectioning of all cells performed at 1 μm step width depth, to acquire entire fluorescence signal of each multicolor channel. Automated image processing, classification and statistical analysis were performed comprehensively upon cell size, cell cluster, tumor biomarker(s) expression, and chromosome ploidy.

Identification criteria of positive CTC in this study: $DAPI^+/CD45^-/CD31^-/EpCAM^{+/-}$/aneuploid chromosome 8.

Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics (Version 21.0; IBM Corp., New York, USA). Correlation of CTC positivity with clinic pathological characteristics and clinical responses was examined with Fisher's exact test. The threshold of different CTC subtypes was established with nonparametric receiver operating characteristic (ROC) analysis, and optimal threshold values were determined to maximize the Youden index (sensitivity + specificity −1), which was determined by selecting a point that maximizes the number of subjects correctly classified, and giving equal weight to sensitivity and specificity [37]. DFS was defined as the duration from the time that surgery was performed to the time point of cancer relapse. OS was defined as the time that surgery was performed to the date that death occurred or the participants were censored. Kaplan-Meier survival plots for DFS were generated based on numbers of different subtypes of CTC before and after surgery, and survival curves were compared using log-rank tests. $^{*}p < 0.05$ and $^{**}p < 0.01$ were statistically significant and very significant, respectively. All p values were two-sided.

Results

Establishment of SE-iFISH suitable for HCC and cholangiocarcinoma CTC detection

In view of low detection efficiency for HCC CTCs performed with conventional strategies [19,21], SE-iFISH was developed and optimized to effectively detect HCC CTCs expressing various tumor biomarkers in this study. Depicted in Fig. 1A, immunofluorescence staining of HCC cell line HepG2 cells with anti-CK18, mesenchymal marker vimentin and PD-L1 showed distinct intracellular staining for CK18 and vimentin. PD-L1 had a plasma membrane staining. Due to very low expression of EpCAM on HepG2 cells, colon cancer cell line SW480 cells, as a proof of concept, were applied for anti-EpCAM staining. Plasma membrane distribution of EpCAM was revealed.

To demonstrate iFISH capability in terms of comprehensive *in situ* phenotypic and karyotypic characterization of tumor cells, HepG2 and SW480 cancer cells were spiked into blood, followed by subtraction enrichment and iFISH. Demonstrated in Fig. 1B, CK18 and PD-L1 on HepG2 cells with trisomy 8, and vimentin in multiploid HepG2 cells were observed. A triploid SW480 with positive EpCAM expression was depicted. WBCs (red arrows) did not express indicated tumor biomarkers.

EpCAM is the critical stemness marker, and of particular biological and clinical significance for HCC [20,35,38]. To investigate expression of EpCAM on CTCs/CTMs and its relevant clinical significance, we emphatically performed EpCAM-iFISH to examine EpCAM expression on CTCs and CTMs in this study. Depicted in Fig. 1C, an $EpCAM^{+}$ multiploid CTC enriched from a HCC patient was detected. WBC (red arrow) was negative for EpCAM staining. Diverse subtypes of CTCs or CTMs detected in patients were revealed in Fig. 1D–H, showing $EpCAM^{+}$ triploid CTC (D), $EpCAM^{+}$ CTM (a cluster of ≥2 CTCs) with multiploid chromosome 8 (E), $EpCAM^{-}$ multiploid CTC (F), $EpCAM^{-}$ triploid CTC (G), and $EpCAM^{-}$ CTM (H), respectively.

Analysis of quantified CTC subtypes in pre- and postoperative patients

Clinical and pathological characteristics of 34 patients enrolled in the current prospective study were summarized in Table 1. All

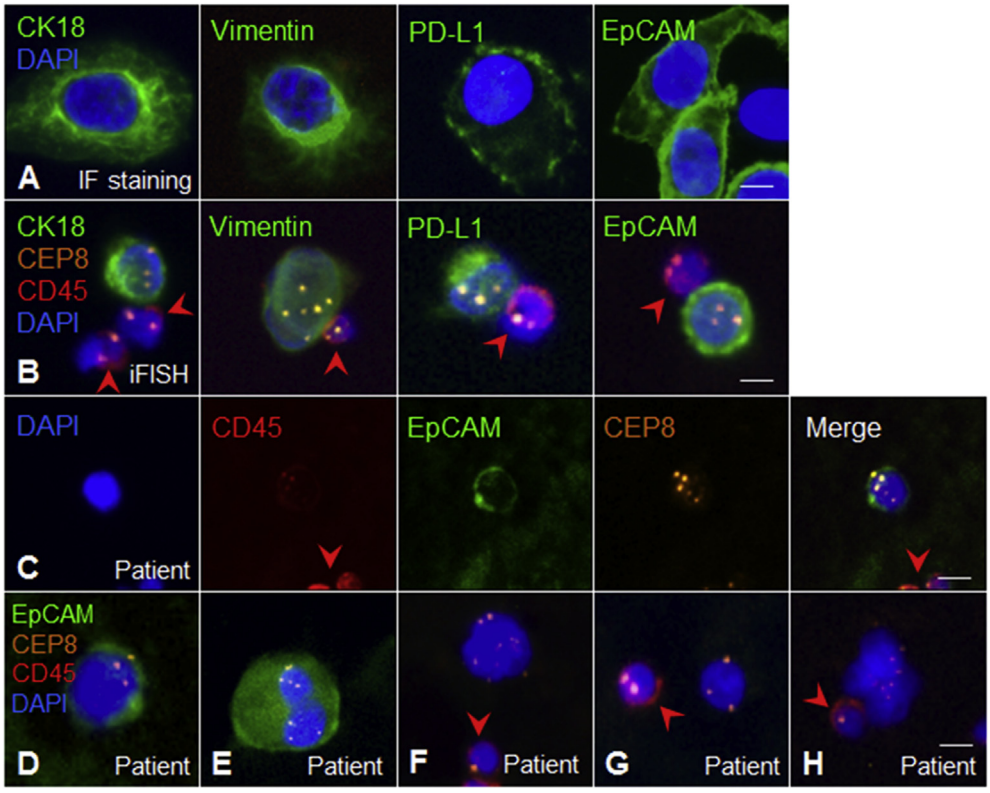

Fig. 1. Detection and *in situ* phenotypic and karyotypic characterization of hepatocellular carcinoma and cholangiocarcinoma cells, CTCs and CTMs by SE-iFISH. (A) Immunofluorescence staining of tumor biomarkers on cancer cells. Cancer cell line cells HepG2 and SW480 on coverslips were immunostained with the indicated monoclonal antibodies. CK18 and vimentin show intracellular staining in HepG2 cells, whereas PD-L1 (HepG2) and EpCAM (SW480) have a positive plasma membrane staining. (B) Tumor biomarkers-iFISH. Both protein expression of the indicated tumor biomarkers and aneuploid chromosome 8 in tumor cells enriched from blood are revealed. WBC ($CD45^{+}$, red arrow) does not show positive staining of each tumor biomarker. (C) A non-hematopoietic multiploid $EpCAM^{+}$ CTSC ($DAPI^{+}$, $CD45^{-}$, $EpCAM^{+}$, $CEP8^{+}$) enriched from a HCC patient blood was detected by EpCAM-iFISH. WBC ($CD45^{+}$, red arrow) is negative for EpCAM staining. (D) An $EpCAM^{+}$ triploid CTC from a cholangiocarcinoma patient. (E) An $EpCAM^{+}$ CTM from a HCC patient. (F) An $EpCAM^{-}$ multiploid CTC from a HCC patient. (G) An $EpCAM^{-}$ triploid CTC from a HCC patient. (H) An $EpCAM^{-}$ CTM from a cholangiocarcinoma patient. WBCs ($CD45^{+}$/$EpCAM^{-}$) in (H–G) are indicated by red arrows. Bars, 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Clinical and pathological characteristics of the enrolled patients.

Baseline characteristics	Patients No. (%)
Age (years)	
<60	17 (50.0)
≥60	17 (50.0)
Gender	
Male	24 (70.6)
Female	10 (29.4)
Types of cancer	
Hepatocellular carcinoma (HCC)	14 (41.2)
Cholangiocarcinoma (CC)	16 (47.0)
Gallbladder carcinoma	3 (8.8)
Gallbladder NEC	1 (2.9)
Differentiation degree[a]	
Well-to-moderately differentiated	3 (8.8)
Moderately differentiated	12 (35.3)
Moderate-to-poorly differentiated	13 (38.2)
Poorly differentiated	5 (14.7)
Microvascular tumor thrombus	
No	20 (58.8)
Yes	14 (41.2)
Ki67 (%)[b]	
≤20	4 (11.8)
>20	28 (82.3)
Edmondson stage	
I-II	14 (41.2)
III-IV	20 (58.8)

Enrolled patients (n = 34).
[a] Differentiation degree of 1 patient was unknown.
[b] Ki67 of 2 patients was unknown.

the patients were subjected to R0 resection, except 2 of CC patients for R1, and 1 of gallbladder cancer patients for R2 resection. Detection and characterization of CTC subtypes were performed on patients before and one week after surgery.

Analysis of quantitative composition of CTC subtypes (Fig. 2A) indicated that among entire detected CTCs, including both pre- and post-operative samples in all the patients, $EpCAM^+$ CTSCs were 8.03%, and remaining 86.35% were $EpCAM^-$ CTCs. Percentage of CTM among positive detection was 5.62%, including 0.97% $EpCAM^+$ and 4.65% $EpCAM^-$, respectively. Karyotyping demonstrated that among $EpCAM^+$ CTSCs, comparing to triploid CTCs (0.24% of total CTCs), most of $EpCAM^+$ CTSCs were multiploid (≥5 copies of chromosome 8) (7.55% of total). Among those $EpCAM^-$ CTCs, the ratio of tri-, tetra- and multiploid CTCs was 33.82%, 18.3%, and 34.3%, respectively. In addition to CTCs, existence of CTM (5.62% of total) was also detected, including 0.97% of $EpCAM^+$ and 4.65% of $EpCAM^-$.

Quantitative comparison of CTC subtypes composition (tri-, tetra-, and multiploid) in all the hepatobiliary patients (pre-surgery *vs* post-surgery) revealed, that no obvious variation was observed following resection (Fig. 2B). With respect to CTM and $EpCAM^+$ CTSC, though gallbladder carcinoma patients had all CTMs and CTSCs depleted after surgery (Table 2), both CTMs (Fig. 2C) and CTSCs (Fig. 2D) were still detected in post-operative HCC and CC patients (Table 2).

Correlation of quantified pre-operative CTCs with pathological and clinical staging of HCC patients

Correlation of pre-operative CTCs, including multiploid $EpCAM^+$ CTSCs, triploid CTCs and CTM to pathological staging was analyzed, and only triploid CTCs revealed positive correlation to staging. Shown in Fig. 3A, comparison of quantified triploid CTCs (cut-off value: ≥5) was performed among 14 HCC patients classified into the indicated subcategories. Number of triploid CTCs in patients with microvascular tumor thrombus, Edmondson stage III-IV, and BCLC stage B and C was significantly higher than those without microvascular tumor thrombus, Edmondson stage I-II, or BCLC stage A1-A4, respectively, and the difference is statistically significant ($^*p < 0.05$) or very significant ($^{**}p < 0.01$). Similar observation was obtained on CTM in Fig. 3B (cut off value: ≥1). Patients who had microvascular tumor thrombus or advanced staging (Edmondson or BCLC) showed significant higher number of CTM ($^*p < 0.05$). Obtained results suggest that the number of pre-operative triploid CTCs and CTMs correlates with primary tumor progression and tumor burden.

CC patients didn't show significant correlation between pathological staging and CTC numbers in this study.

Correlation of quantified postsurgical CTC subtypes to DFS

Significant amount of CTCs were observed in most of the post-surgical patients. Up to the time point of the latest CTC detection, 54.5% of postsurgical HCC patients had cancer recurred. Correlation of the postsurgical CTCs with cancer recurrence in HCC patients was investigated. Follow-up study with respect to investigating relapse on CC patients is in progress due to the limited number of recurrent patients to date.

Comparing to diverse subtypes of pre-operative CTCs, only postsurgical CTCs showed significant correlation with HCC patients' DFS. Demonstrated in Fig. 4A, DFS of patients with ≥5 postsurgical CTCs was significantly shorter than those with <5 CTCs. Cut-off value was established upon ROC and Youden index described in 2.4 Statistic analyses. Further karyotyping analysis shown in Fig. 4B revealed that patients who possessed ≥5 of post-operative $EpCAM^-$ triploid CTCs, had a shorter DFS than those who had <5 CTCs. Difference is statistically very significant ($^{**}p < 0.01$). Similar observation was obtained on those who had $EpCAM^-$ tetra- and multiploid CTCs (≥pentasomy 8) (data not shown). Analysis of $EpCAM^+$ CTSCs in HCC patients following resection was depicted in Fig. 4C, only those patients showing poor prognosis had post-surgical $EpCAM^+$ multiploid CTSCs detected (≥1 cell). In addition, positive detection of CTM (≥1 CTM/6 ml blood) in post-operative patients revealed a shorter DFS compared to patients with CTM negative (Fig. 4D).

Pre-operative CTCs were not found to significantly correlate to DFS in HCC patients (data not shown).

Analysis of small cell size CTCs and correlation of postsurgical triploid small CTCs with poor prognosis

Both small (≤5 μm WBC) and large (larger than the size of WBC) aneuploid HCC CTCs, with either positive or negative expression of EpCAM, were depicted in Fig. 5A. Quantitative comparison of small CTCs (pre-surgery *vs* post-surgery) in HCC and cholangiocarcinoma (CC) patients was revealed in Figs. 5B and C. Majority of the detected CTCs in HCC patients were in small size (red), with a proportion (small CTCs/total CTCs) of 67.3% (pre-operation) and 67.5% (post-operation) (Fig. 5B), and a median of 2 (pre-operation, Min 0/Max 7 cells), and 4 cells (post-operation, Min 0/Max 19 cells), respectively (Fig. 5C). No obvious variation in terms of ratio of small CTCs was observed in HCC patients following resection. However, with respect to small CTCs in CC patients after surgery, dramatic decrease of small CTCs (red) was observed, showing a proportion of 53.3% (pre-surgery) down to 24% (post-surgery) (Fig. 5B), with a median of 7 cells in pre-operative (Min 1/Max 228 cells), and 5 in post-operative patients (Min 1/Max 115 cells), respectively.

Quantitative variation of small CTCs with triploid chromosome 8 in both recurrent and non-recurrent HCC patients following resection was depicted in Fig. 5D. To those postsurgical non-recurrent patients, the median of Δsmall triploid CTCs (post-surgery cell number − presurgery cell number) was 0 (Min

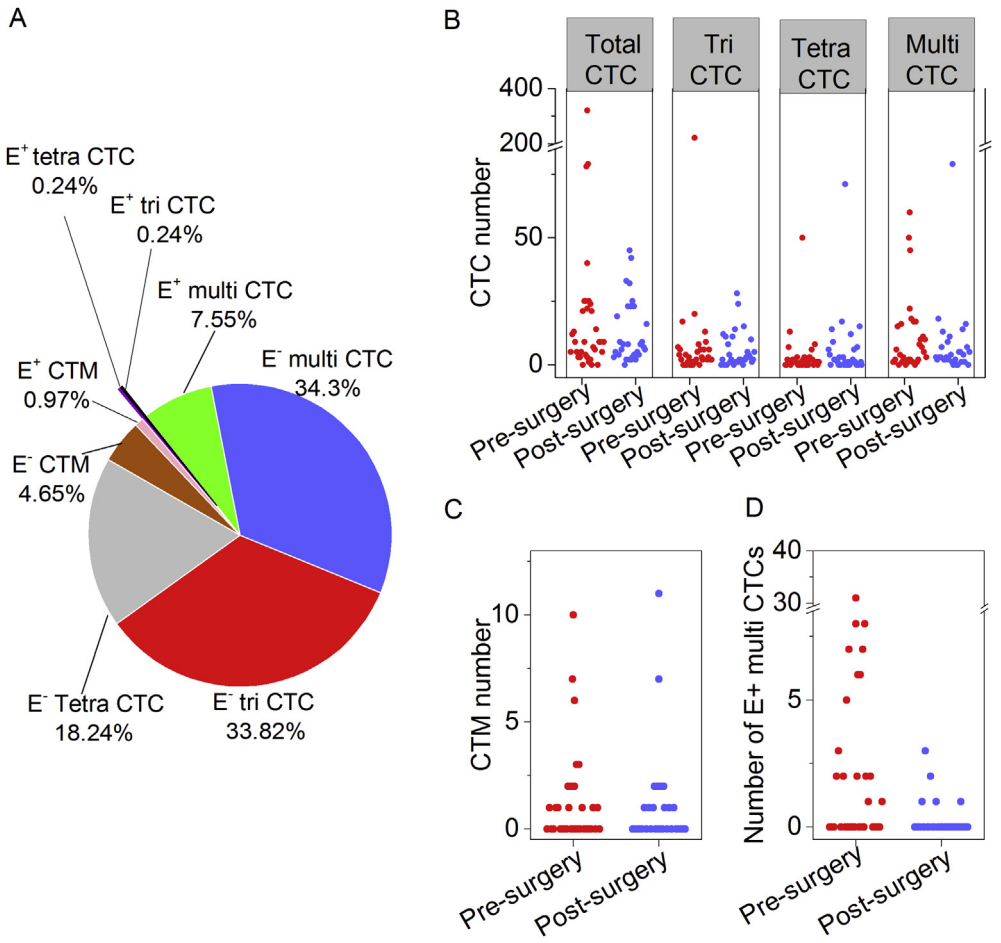

Fig. 2. Composition and quantitative comparison of CTCs and CTMs in all the enrolled carcinoma patients prior to and post-surgery. (A) Quantitative composition of diverse CTC subtypes in entire population of CTCs detected in all the pre- and post-operative patients. E^- tri CTCs, EpCAM$^-$ triploid CTCs; E^- tetra CTCs, EpCAM$^-$ tetraploid CTCs; E^- multi CTCs, EpCAM$^-$ multiploid (≥pentasomy 8) CTCs; E^+ tri CTCs, EpCAM$^+$ triploid CTCs; E^+ tetra CTCs, EpCAM$^+$ tetraploid CTCs; E^+ multi CTCs, EpCAM$^+$ multiploid CTCs. EpCAM$^+$ CTSCs are 8.03%, whereas 86.35% of CTCs are EpCAM$^-$. Remaining 5.62% are CTM, including 0.97% EpCAM$^+$ and 4.65% EpCAM$^-$, respectively. (B) Comparison of diverse CTC subtypes in overall patients pre- and post-surgery. Total CTC: total number of overall CTCs. No obvious quantitative variation of CTC subtypes is observed in overall patients (n = 34) before and after operation. (C) Comparison of pre- and postoperative CTM. No distinct variation is revealed in overall patients following resection. (D) Comparison of pre- and postoperative EpCAM$^+$ multiploid CTSCs. Number of EpCAM$^+$ multiploid CTSCs decreases in overall patients following surgery.

Table 2
Positivity of total and diverse CTC subtypes detected in hepatobiliary carcinoma patients.

Types of tumor	Overall subtypes (%)		Tri CTC (%)		Tetra CTC (%)		Multi CTC (%)		E^+ multi CTC (%)		CTM (%)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
HCC (n = 14)	78.6	78.6	64.2	71.4	42.9	42.9	78.6	64.2	42.9	14.3	35.0	35.7
CC (n = 16)	93.7	100	81.2	81.2	81.2	75.0	93.7	100	68.8	18.8	50.0	37.5
Gallbladder cancer (n = 4)	100	75.0	100	75.0	50.0	75.0	100	75.0	25.0	0.0	75.0	0.0
Total (n = 34)	88.2	88.2	76.5	79.4	85.3	85.3	88.2	82.3	53.0	14.7	53.3	32.3

Δvalue: −1/Max Δvalue: 2 cells), suggesting that postsurgical non-recurrent HCC patients did not have an obvious change in quantity of small triploid CTCs following resection. Δvalue = −1 indicates CTC number decreased in postsurgical patient compared to that enumerated in the same patient prior to surgery. Whereas recurrent patients showed a median of Δsmall triploid CTCs = 3 (Min Δvalue: 0/Max Δvalue: 8 cells), indicating that postsurgical recurrent HCC patients had a significant increased number of small triploid CTCs following surgery.

Depicted in Fig. 5E, postsurgical patients who had triploid small cell size CTCs (≥5 CTCs/6 ml blood) showed much shorter DFS, indicating that small triploid CTCs quantified in postsurgical patients significantly correlated with patients' poor prognosis. Cut-off value was established upon ROC and Youden index described in 2.4 Statistic analyses. Obtained results suggest that increasing of triploid small CTCs in postsurgical HCC patients compared to those CTCs detected in pre-operative patients, correlates to poor prognosis in terms of shorter DFS and rapid cancer recurrence.

Discussion

Conventional CTC detection technologies rely on EpCAM expressed on the plasma membrane of tumor cells. However, expression of EpCAM is very dynamic [13] or even absent [14] on

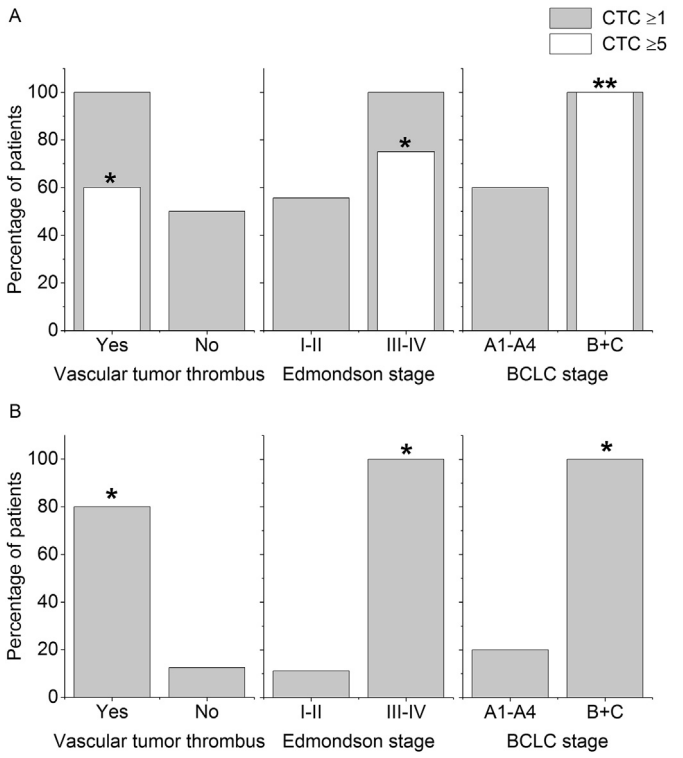

Fig. 3. Correlation of pre-operative triploid CTCs and CTMs with microvascular tumor thrombus, pathological and clinical staging in HCC patients. Detected triploid CTCs (A, cut-off: ≥5 CTCs) and CTMs (B, ≥1) correlate to existence of microvascular tumor thrombus and pathological staging. Number of triploid CTCs (≥5) (A) and CTMs (B) are higher in patients showing microvascular tumor thrombus, Edmondson stage III-IV, and BCLC stage B + C than those without microvascular tumor thrombus, Edmondson stage I-II, and BCLC stage A1-A4, respectively. The difference is statistically significant ($^{*}p < 0.05$) or very significant ($^{**}p < 0.01$).

different types or stages of carcinoma cells, especially during the process of EMT [15,16]. Such intrinsic biological properties of tumor cells inevitably results in significant false negative detection of CTCs with EpCAM heterogeneously expressed [30,39], particularly for HCC CTCs. SE-iFISH described in this study is able to enrich non-hematopoietic CTCs with high efficiency, followed by *in situ* karyotypic and phenotypic co-examination of chromosome ploidy, and a series of biomarkers, such as epithelial markers EpCAM/CK, mesenchymal marker vimentin, and tumor immunologic escape molecule PD-L1 on the same HCC cell (Fig. 1A). Comparing to low detection sensitivity for HCC CTCs due to inherent technical hurdle of conventional technologies fully relying on EpCAM expression [19,21], SE-iFISH showed higher detection sensitivity of 78.6, 93.7 and 100.0% for HCC, cholangiocarcinoma, and gallbladder carcinoma CTCs, respectively. Among the detected neoplastic cells in circulation, both EpCAM positive and negative populations of CTCs, CTSCs and CTM were identified. Further karyotypic characterization indicted that majority of $EpCAM^{+}$ CTSCs had multiploid chromosome 8 (≥pentasomy 8), whereas most of $EpCAM^{-}$ CTCs showed heterogeneous composition of tri-, tetra- and multiploid chromosome 8.

EpCAM, the critical stemness marker and an active signaling molecule for cancer cells including HCC, is of particular biological and clinical significance [20,35,38]. Positive expression of EpCAM on CTSCs is related to tumor metastasis and recurrence [19]. To further investigate clinical relevance of EpCAM expression on CTSCs, we took advantage of EpCAM-iFISH to emphatically investigate aneuploid hepatobiliary CTCs and CTMs with either positive or negative EpCAM expression in the present study. Multiple subtypes of CTCs/CTSCs/CTMs with heterogeneous EpCAM expression and diverse chromosome ploidy were detected in hepatobiliary carcinoma patients.

CTCs could be effectively detected by SE-iFISH in pre- and post-operative hepatobiliary patients in this study. Clinical significance

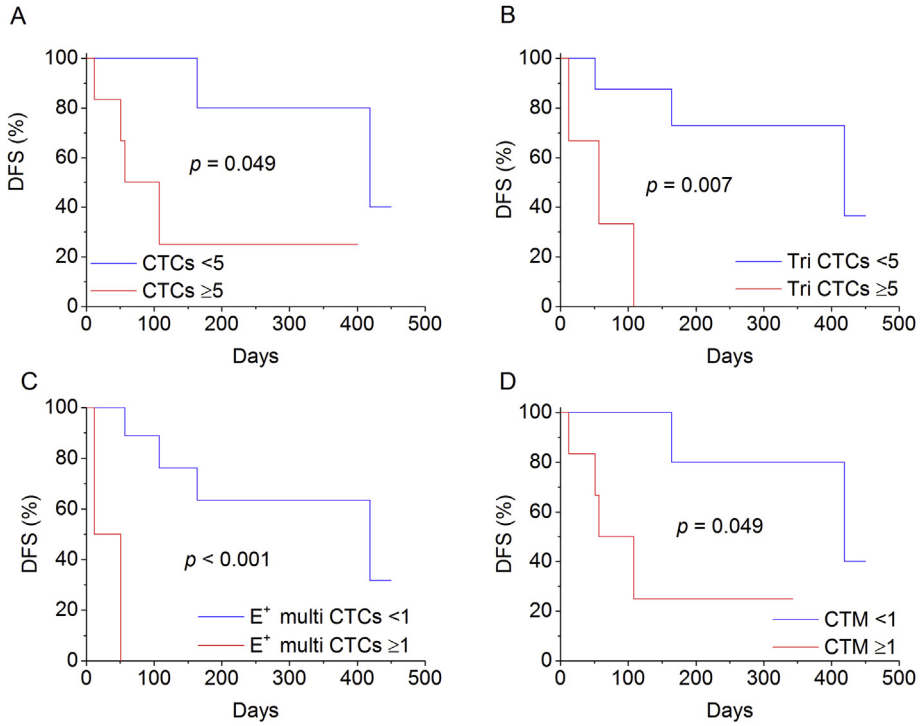

Fig. 4. Correlation of DFS to diverse CTC subtypes detected in postsurgical HCC patients. Post-operative patients with (A) total CTC number ≥5, (B) triploid CTC number ≥5, (C) $EpCAM^{+}$ multiploid CTSC number ≥1, and (D) CTM number ≥1 shows poor prognosis with a significant shorter DFS (red) than those with indicated different cut-off value of CTC or CTM numbers (blue), respectively. The difference is statistically significant ($^{*}p < 0.05$), or very significant ($^{**}p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

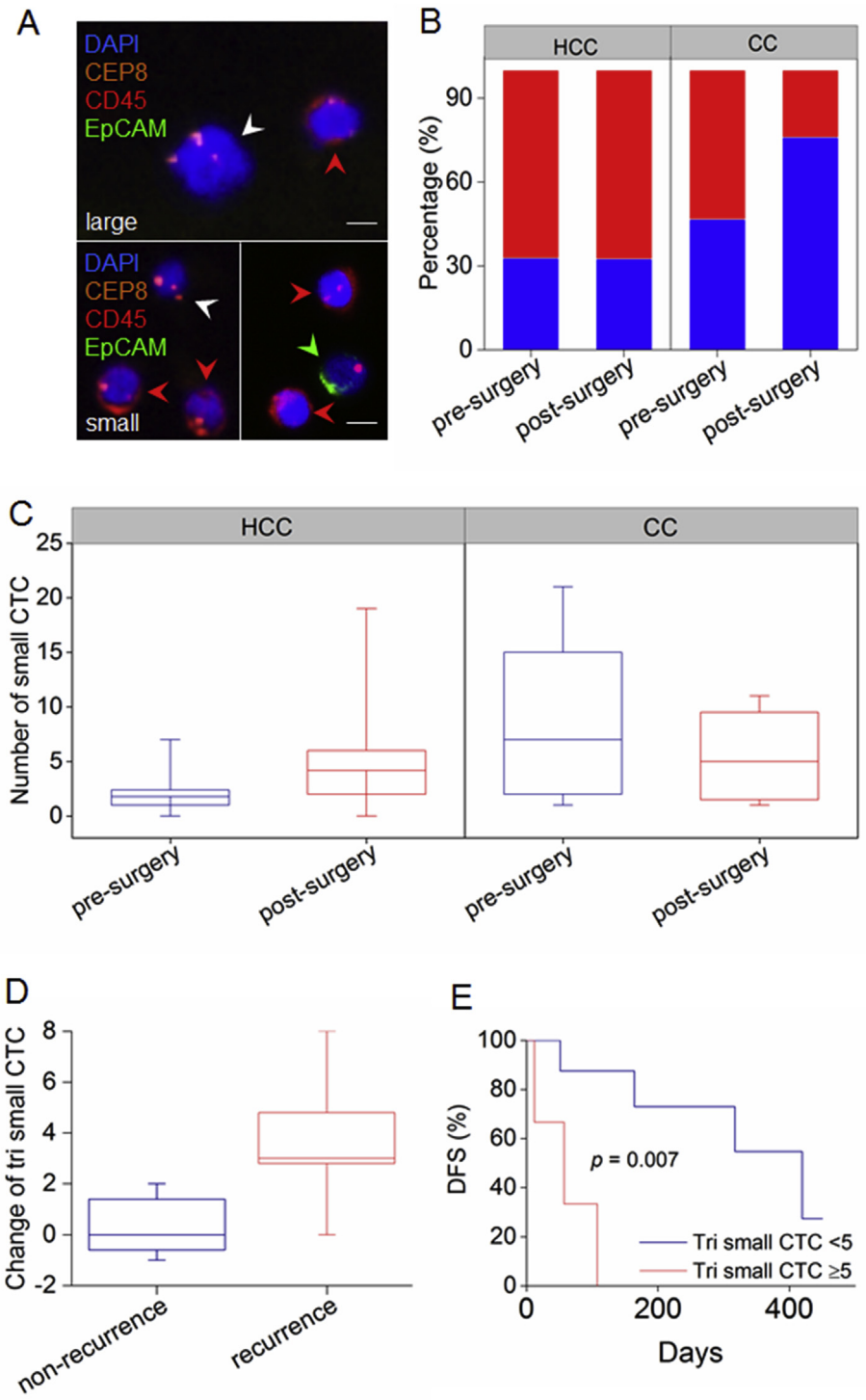

Fig. 5. Analysis of small cell size CTCs and correlation of small triploid CTCs to poor prognosis in postsurgical HCC patients. (A) Images of large and small HCC CTCs. Comparing to the size of WBCs (red arrows), an EpCAM$^-$ large (top) and small ($\leq$5 μm WBC) triploid CTC (bottom, left), as well as an EpCAM$^+$ small monoploid CTC (bottom, right), are indicated by white and green arrows, respectively. Bars, 5 μm. (B) Proportion of small CTCs (red) in HCC and CC patients. Majority of the detected HCC CTCs are small cells, showing 67.3% of the total pre-operative CTCs, and 67.5% of the total post-operative CTCs are in small size. The ratio of small CTCs in the total detected HCC CTCs is not changed following resection. CC patients have a dramatic decrease of small CTCs following surgical resection, showing 53.3% (pre-surgery) *vs* 24% (post-surgery). (C) Quantitative analysis of small CTCs. HCC patients have a median of cell number of 2 (pre-surgery, Min 0/Max 7 cells) and 4 (post-surgery, Min 0/Max 19 cells), respectively. CC patients show a median of 7 cells (pre-operation, Min 1/Max 228 cells), and 5 cells (post-operation, Min 1/Max 115 cells). (D) Postsurgical recurrent HCC patients have an increasing quantity of triploid small CTCs following resection. Comparing to postsurgical non-recurrent HCC patients, who show a median of Δsmall triploid CTCs (postsurgery cell number − presurgery cell number) = 0 (Min Δvalue: −1/Max Δvalue: 2 cells), postsurgical recurrent HCC patients have a median of Δsmall triploid CTCs = 3 (Min Δvalue: 0/Max Δvalue: 8 cells). (E) Quantity of triploid small CTCs in postsurgical HCC patients (cut-off value: $\geq$5 CTCs/6 ml blood) significantly correlates to patients' poor prognosis, showing shorter DFS (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of both pre- and postsurgical CTCs was investigated. Pre-surgical CTCs with trisomy 8 were demonstrated to only correlate to patients' microvascular tumor thrombus and pathological staging, but not to DFS in HCC patients in this study. Whereas post-surgical CTCs were highly relevant to HCC patients' poor prognosis and cancer relapse.

Keeping in accordance with published studies, revealing that postsurgical CTCs shed from esophageal squamous cell carcinoma, breast cancer, and non-small cell lung cancer remained in circulation following resection [33,34], significant amount of CTCs and CTMs were also detected in postsurgical hepatobiliary patients in this study. Comparing to the number of CTCs and CTMs detected in pre-operative patients, quantity of post-operative CTCs and CTMs in the same population of subjects didn't significantly reduce following resection of primary lesions, indicating removal of primary tumor does not significantly impact on quantity of post-surgical CTCs in all the patients. Aside from potential surgical disturbing which might result in releasing some cancer cells from primary tumor mass into blood, it is reasonable to speculate that some of those stably existed postsurgical CTCs may not be derived from the resected primary lesions, instead, they are pre-existent in circulation. Similar to the reported dormant early disseminated cancer cells (eDCCs, or eDTCs) detected at very early stage of tumor evolution and formation, which is prior to manifestation of metastasis [5,6], some of the stably pre-existent hepatobiliary CTCs detected in this study, might belong to the similar category of dormant early CTCs (eCTCs), accounting for subsequent post-surgical cancer metastasis and recurrence once awakened [5,7].

Recently published studies indicated that dormant tumor cells could be awakened by a series of molecular and cellular mechanisms, and subsequently proliferate to metastatic lesions [7]. Among the “awakening stimulus”, critical role of chromosome 8 amplification (aneuploid chromosome 8) in malignant neoplastic cells has been reported [6]. Moreover, results obtained in the current study demonstrated that CTCs with trisomy 8 in preoperative HCC patients were apparently related to microvascular tumor thrombus, advanced staging and tumor burden in patients. Furthermore, diverse subtypes of CTCs correlating to patients' poor progression were confirmed in the current study. Particularly, $EpCAM^{+}$ multiploid CTSCs (cut-off: $\geq$1 cell in 6 ml of blood), $EpCAM^{-}$ small triploid CTCs ($\geq$5 cells), positive detection of CTM ($\geq$1), and increasing of triploid CTCs, all quantified a week after surgery, were highly relevant to poor prognosis. Therefore, in addition to CTM and $EpCAM^{+}$ CTSC, the specific subtype of small CTCs with trisomy 8, is another key player in cancer metastasis and relapse in HCC patients.

Our recently published study demonstrated that besides localizing in tumor mass, aneuploid $CD31^{+}$ tumor endothelial cells (ECs) existed in peripheral blood of cancer patients, i.e. existence of aneuploid circulating tumor endothelial cells (tumor CECs, $CD45^{-}$/$CD31^{+}$) [26,40]. However, it remains unclear how tumor ECs, and non-hematopoietic circulating aneuploid cells (nCACs) mainly including tumor CECs and CTCs, correlate to clinical endpoints. It is worthy of further investigating how aneuploid CTCs, CTSCs, tumor CECs and ECs in malignant lesions functionally interplay with tumor angiogenesis as well as cancer metastasis.

Though mesenchymal CTCs [18] or CTCs in patients following therapeutic treatment [41] were previously reported in small cell size, very few studies have been carried out to further carefully investigate how diverse CTC cell size correlate to clinical outcome, partially due to intrinsic drawbacks of the conventional CTC technology, such as cell size based filtration which loses significant amount of small CTCs [42]. Demonstrated in this study, unlike cholangiocarcinoma patients showing most of the detected CTCs were large cells ($\geq$5 μm of WBCs), surprisingly, majority (67%) of CTCs detected in either pre- or post-surgery HCC patients were in small size ($\leq$5 μm of WBCs). Additional karyotyping analysis indicated, postsurgical recurrent HCC patients showed increasing of triploid small CTCs following resection, and quantity of post-operative triploid small CTCs (cut-off: $\geq$5 cells) had a very significant correlation to HCC patient's poor prognosis.

Several clinical studies to further investigate how aneuploid $CD31^{+}$ tumor CECs [26] and different cell sizes of aneuploid CTCs expressing varieties of tumor biomarkers functionally cross-talk in cancer patients' poor prognosis, are currently performed by us on a large cohort of patients of different types of carcinoma.

Taken together, a novel integrated comprehensive SE-iFISH strategy, suitable for effective detection of hepatobiliary carcinoma CTCs expressing varieties of tumor biomarkers, was developed and optimized in this study. Diverse subtypes of nCACs, including $EpCAM^{+}$ CTSCs and $EpCAM^{-}$ CTCs, each with different ploidy of chromosome 8, were effectively detected in patients with hepatobiliary malignances. Correlation of CTC cell size to clinical outcome was demonstrated for the first time in this study. In post-operative patients, quantity of multiploid ($\geq$pentasomy 8) $EpCAM^{+}$ CTSCs (cut-off: $\geq$1 cell in 6 ml blood), small cell size $EpCAM^{-}$ CTCs with trisomy 8 ($\geq$5 cells), positive detection of CTMs ($\geq$1), and increasing of small triploid CTCs compared to those quantified in pre-operative patients, were demonstrated to significantly correlate to HCC patients' poor prognosis. Detection of those specific subtypes of CTSCs, CTCs and CTMs in postsurgical HCC patients, could help rapidly evaluate surgery efficacy, and predict cancer patients' poor prognosis as well as neoplasm recurrence.

Acknowledgements

This work was supported by Beijing Municipal Administration of Hospital's Clinical Medicine Development of Special Funding Support (No. ZYLX201712), and Beijing Municipal Administration of Hospital's Mission Plan (No. SML20152201) to JD; Tsinghua University Beijing Changgung Hospital Foundation (No. 12015C1033) to LW; Beijing Natural Science Foundation (No. 7161002) and the Capital Health Research and Development of Special (No. 2016-1-1021) to LS.

Authors thank staffs at Tsinghua University Affiliated Changgung Hospital (Beijing, China), Cytointelligen (China Medical City, Taizhou, Jiangsu, China), and Cytelligen (San Diego, CA, USA) for providing clinical and technical assistance. Please contact Dr. P. Lin for all technical issues in this study (plin@cytelligen.com).

Conflict of interests

i•FISH® is the registered trademark of Cytelligen. Dr. Peter P. Lin is president at Cytelligen. All the authors do not own Cytelligen's stock shares. No additional conflict of interest to be disclosed.

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