Clinical significance of circulating tumor cells via combined whole exome sequencing in early stage cancer screening: A case report

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Abstract. A newly-developed platform, integrating subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH), was applied to analyze the clinical significance of circulating tumor cells (CTCs) for early screening of cancer in healthy people. The present case report describes one healthy individual who accepted a CTC peripheral blood test, and 8 CTCs/7.5 ml blood were detected. However, various conventional cancer biomarkers were all negative, including cervical cytological inspection, alpha-fetoprotein, cancer antigen (CA)-125, CA19-9, carcinoembryonic antigen (CEA), CA15-3 and human papilloma virus. To explore the origin of the CTCs, whole exome sequencing was used to analyze the CTC variation spectrum. A total of 42 mutations were associated with cancer according to analysis in COSMIC (http://cancer.sanger.ac.uk/cosmic). The results revealed a high risk of tumor in the colorectum, stomach and breast (13, 12 and 6 variations matched, respectively). In this individual, an intestinal polyp was identified to be a hyperplastic polyp by pathological diagnosis. No lesions were discovered in the stomach and breast. No CTCs were detected in this patient’s blood at 1 and 6 months after removal of the lesions. This case indicates that CTC detection by SE-iFISH has potential in early stage cancer screening, and the mutation spectrum of CTC assists the tracking of its sources.

Introduction

Circulating tumor cells (CTCs) are cells that detach from primary or metastatic solid tumors into the vasculature, where they can be sampled from the circulating blood stream (1). CTCs are commonly identified in the peripheral blood supply of diverse solid tumors, including breast cancer (2,3), colorectal cancer (4), prostate cancer (5) etc. The migration of CTCs seems to be an early event in human carcinogenesis, even before the cancer is visible in clinical imaging. Experimental data in animal models has shown that tumors measuring less than 1 mm could be associated with the presence of CTCs in the bloodstream (6).

Zhangjiang Center for Translational Medicine published a series of studies of CTCs in breast cancer (7), pancreatic cancer (8) and colorectal cancer (9) using combined subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH), cooperated with other research teams. These studies demonstrated SE-iFISH CTC detection 91% positive rate for breast cancer patients (7), a sensitivity of 88% and specificity of 90% in pancreatic cancer and healthy individuals at the cutoff value of 2 cells/7.5 ml (8), and a sensitivity of 90.9% and specificity of 82.4% in colorectal cancer and healthy individuals at the cutoff value of one cell/7.5 ml (9). From these studies, CTC detection by SE-iFISH showed high sensitivity and specificity for distinguishing breast cancer, pancreatic cancer and colorectal cancer from healthy people. CTCs detection, up to date, provided potential biomarkers for screening of cancer or precancerosis.

SE-iFISH is a novel strategy to detect CTCs in blood (10), which enrich CTCs through the removal of WBCs using anti-CD45 antibody conjugated immunomagnetic particles, independent of EpCAM expression and tumor cell size. Centromere Probe 8 (CEP8), cytokeratin (CK), CD45, 4′,6-diamidino-2-phenylindole (DAPI) were combined to identify CTCs. Since aneuploidy is a typical common cytogenetic abnormality in tumor cells, this feature could be exploited for CTC detection. FISH was performed on CEP8 to identify aneuploidy cells. CTCs were confirmed to be negative for CD45, positive for DAPI and either positive for PanCK staining or aneuploidy chromosome 8. Cells with characteristics of CK-/CD45+/DAPI+/CEP8=2 were WBCs.
The present case report describes a healthy female who accepted a CTC test (by SE-iFISH platform) in Quanzhou No. 1 Hospital, and the result was 8 CTCs/7.5 ml, which indicated a high risk of cancer. WES of these CTCs was performed to analyze their mutation profiles to track the lesion.

Materials and methods

Subtraction enrichment of CTCs. 7.5 ml peripheral blood was collected by ACD anticoagulant tubes (BD Biosciences, Franklin Lakes, NJ, USA). Reagents for subtraction enrichment are Cytelligen CTC enrichment kit (Cytelligen, Inc., San Diego, CA, USA). In brief, peripheral blood (7.5 ml) was centrifuged at 800 x g for 8 min at room temperature, then supernatant was discarded. The left sample was transferred to a centrifuge tube containing 3 ml hCTC Separation Matrix. After centrifuging for 8 min at 450 g, the cell suspension was collected from the buffy-coat layer. 150 ul immunomagnetic particles conjugated anti-CD45 antibody was added into the cell suspension, which was incooled at room temperature for 10 min and then placed on a magnetic stand (Promega Corporation, Madison, WI, USA) till the liquid became clear. The supernatant was pipetted off the magnetic field (non-magnetic bead-containment cell suspension) to remove leukocytes by centrifuging at 500 rpm for 2 min. Sedimented cells were thoroughly mixed with cell fixative and applied onto the coated CTC slides for subsequent identification.

Identification of CTCs. Reagents for CTC identification were provided by the Human Tumor Cell Identification kit (Cytelligen, Inc.). To identify aneuploidy CTCs, fluorescence in situ hybridization (FISH) and immunocytochemistry are used in combination. After a series of pre-treatment containing drying, washing and dehydration, 10 µl of probe solution containing fluorescence-labeled alpha-satellite probes for the centromeres of the chromosome (CEP8) (2 µg/ml) was added and then covered with a coverslip and sealed with neutral resin. The hybridization procedure was as follows: degeneration at 75°C for 5 min, followed by hybridization at 37°C overnight. Then the slide was rinsed with FR3 and added with monoclonal antibody anti-CD45 conjugated to Alexa Fluor 594 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) antibody anti-CD45 conjugated to Alexa Fluor 594 (Invitrogen; Thermo Fisher Scientific, Inc.) before inoculation at room temperature for 2 h. After rinsing with PBS, the slides were mounted with mounting medium containing DAPI and photographed with a fluorescence microscope (Nikon Corporation, Tokyo, Japan). CTCs were confirmed to be negative for CD45 and either positive for PanCK staining or aneuploid chromosome 8.

Laser capture microdissection and whole genome amplification of CTCs. The CTC fixed slide was put in ZEISS Palm MicroBeam Laser Micro Dissection System (Zeiss AG, Oberkochen, Germany). We found CTCs according to the coordinate recorded in process of CTC identification, and collected CTCs by laser micro dissection.

DNA amplification experiment of CTCs was according to the kit instruction of MalBac single cell genome amplification (YK001A/B; Yikon Genomics, Jiangsu, China). The product was quantified using DNA electrophoresis.

Whole exome sequencing. Whole exome sequencing was performed on DNA of blood, intestinal polyp and CTCs. For library construction, whole exome DNA capture was performed using Agilent SureSelect Human All ExonV5 kits following the manufacturer’s instructions (Agilent Technologies, Inc., Santa Clara, CA, USA). Subsequent to the quality test, the qualified library was sequenced as 125 bp paired-end reads on an Illumina Hiseq 2500 platform (Illumina, Inc., San Diego, CA, USA).

Data analysis of whole exome sequencing. For whole exome sequencing, clean data was obtained after filtering adapter, low quality reads and reads with proportion of N>10%. Reads were aligned to the reference human genome (UCSC hg19) through Burrows-Wheeler Aligner. Next, the Picard and Genome Analysis Tool kit (GATK) methods were adopted for duplicate removal, local realignment and base quality recalibration. Finally, the GATK Unified Genotyper was used for single nucleotide variation (SNV)/inDel annotation.

Somatic SNP/InDel detection was performed with Varscan2 software. Variants were annotated using the ANNOVAR software tool. Mutations of CTCs were aligned to COSMIC database (http://cancer.sanger.ac.uk/cosmic/). The data in COSMIC is curated from number of high-quality sources and combined into a single resource. The sources include: Peer-reviewed journal articles, CGP laboratories at the Sanger Institute, TCGA data portal, the ICGC data portal, IARC p53 database. We selected cancer-related mutations and annotated relevant primary organ.

Droplet digital PCR. Each PCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA) reaction system (in a total reaction volume of 20 µl) contained: 10 µl Bio-Rad 2xHdrPCR supermix, 1 µl primer and probe, 2 µl DNA template (100 ng) and 7 µl H2O. Droplets were generated and analyzed using the QX100 system (Bio-Rad Laboratories). Amplifications were performed using the following conditions: 1 cycle of 95°C for 10 min, 45 cycles of 94°C for 10 sec and 60°C for 45 sec, 1 cycle of 98°C for 10 min, and 1 cycle of 25°C for 10 sec. QuantaSoft analysis software (Bio-Rad Laboratories) enabled abundance to be calculated for each sample.

Case report

A female underwent circulating tumor cell detection using SE-iFISH platform in the surgical oncology department, Quanzhou No.1 hospital, Fujian, China, in Dec 22, 2014. She was 56-year-old, during menopause, and has no family history of cancer. The results were 8 CTCs/7.5 ml which indicated a high risk of cancer (Fig. 1). However, the levels of AFP, carcinoembryonic antigen (CEA) and CA15-3 were in normal range. This patient also accepted an HPV typing (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 6, 11, 42, 43, 44, 53, 66 and CP8304) test, and the results were negative. The thynprep cytology test showed no intraepithelial neoplasia. Written informed consent was obtained from this person for the present study.

To define the mutation spectrum of CTCs, we performed laser capture microdissection to isolate the CTCs, and whole genome amplification and whole exome capture DNA
sequencing (WES) on the 8 CTCs from this female. Sequencing achieved 81.5x mean coverage on targeted exons. 34215 SNVs and 6807 InDels were defined in the CTCs. Mapping these variations in COSMIC (http://cancer.sanger.ac.uk/cosmic/), 42 variations and InDels were correlated significantly with cancer (Table I). Notably, 13, 12 and 6 mutations were related to large intestinal cancer, gastric cancer and breast cancer, respectively (Fig. 2A). Fig. 2B shows 13 gene variations and their mutational frequency in large intestinal cancer, according to a TCGA study.

### Table I. Cancer-related variations annotation in COSMIC database.

| Gene       | Loci       | Ref     | Alt     | Histology         | Organ        |
|------------|------------|---------|---------|-------------------|--------------|
| HOXB3      | 46629737   | TG/TG   | TG/T    | Adenocarcinoma    | Colon        |
| CENPQ      | 49459978   | AA      | AG      | Adenocarcinoma    | Colon        |
| TSPAN10    | 79612161   | CC      | TT      | Adenocarcinoma    | Colon        |
| TCEAL8     | 102508779  | GG      | GT      | Adenocarcinoma    | Rectum       |
| MXRA5      | 3241256    | TT      | CC      | Adenocarcinoma    | Colon        |
| PRR25      | 855717     | CC      | GG      | Adenocarcinoma    | Rectum       |
| HCAR3      | 123200693  | AA      | GG      | Adenocarcinoma    | Colon        |
| PARL       | 183547404  | CT/CT   | CT/C    | Adenocarcinoma    | Colon        |
| KDM6B      | 7752901    | GC/CT   | G/G     | Adenocarcinoma    | Colon        |
| DCST1      | 155019710  | AA      | CC      | Adenocarcinoma    | Rectum       |
| RAI1       | 17696531   | GG      | CC      | Adenocarcinoma    | Colon        |
| ITGA4      | 182347350  | GG      | GA      | Adenocarcinoma    | Cecum        |
| ACKR2      | 42907112   | AA      | AC      | Adenocarcinoma    | Cecum        |
| FCRLA      | 161683136  | GG      | AA      | Adenocarcinoma    | Stomach      |
| TNRC18     | 5396715    | TT      | CC      | Adenocarcinoma    | Stomach      |
| ASAP1      | 131124559  | TT      | CC      | Adenocarcinoma    | Stomach      |
| C CCDC153  | 119063908  | CC      | TT      | Adenocarcinoma    | Stomach      |
| OR10G7     | 123909627  | TT      | CC      | Adenocarcinoma    | Stomach      |
| CLEC1B     | 10149406   | TT      | CC      | Adenocarcinoma    | Stomach      |
| BRCA1      | 41244000   | TT      | CC      | Adenocarcinoma    | Stomach      |
| LGALS14    | 40199914   | CC      | GG      | Adenocarcinoma    | Stomach      |
| CLC        | 40225646   | GG      | AA      | Adenocarcinoma    | Stomach      |
| CHGB       | 5904040    | GG      | AA      | Adenocarcinoma    | Stomach      |
| IGL1       | 23915652   | GG      | GA      | Adenocarcinoma    | Stomach      |
| GCNT2      | 10557242   | GA/GA   | GA/G    | Adenocarcinoma    | Stomach      |
| MINA       | 97664725   | CC      | TT      | Cancer            | Breast       |
| LINC01168, LOC100128127 | 134886618 | GG      | CC      | Cancer            | Breast       |
| WDR90      | 701656     | CC      | TT      | Cancer            | Breast       |
| CHTF18     | 840378     | AA      | GG      | Cancer            | Breast       |
| ZNF286A    | 15611495   | TT      | CC      | Cancer            | Breast       |
| BPIFB3     | 31656632   | CC      | CG      | Cancer            | Breast       |
| TNN        | 175067689  | GG      | AA      | Squamous cell carcinoma | Skin, face |
| MAN2B2     | 6602344    | GG      | GT      | Malignant melanoma | Skin, arm    |
| SMOC1      | 7042020    | GG      | GA      | Malignant melanoma | Skin         |
| SRSF4      | 29481412   | CC      | AA      | Hepatocellular carcinoma | Liver |
| COL15A1    | 101778265  | GG      | TT      | Astrocytoma       | Brain        |
| IDI2       | 1065491    | GG      | TT      | Renal clear cell carcinoma | Kidney |
| KRTAP4-7   | 39240627   | TT      | TC      | Transitional cell carcinoma | Bladder |
| LTBPI4     | 4118056    | AA      | GG      | Meningioma        | Meninges     |
| TGM3       | 2290333    | CC      | AA      | Adenocarcinoma    | Lung         |
| UMODL1     | 43346494   | GG      | GA      | Endometrioid carcinoma | Endometrium |
| TMEM37     | 120194651  | A/A     | AGTGTGC/AGTGTGC | Serous carcinoma | Ovary |

COSMIC, catalogue of somatic mutations in cancer.
SNV annotation in COSMIC showed a high risk of large intestinal cancer, followed by gastric cancer and breast cancer. To check for lesions, the patient underwent a series of imaging examinations, including colonoscopy, gastroscopy and color Doppler ultrasound (in the thyroid gland, cervical lymph node, mammary gland and draining lymph nodes, liver, spleen, gallbladder, pancreas, uterus and adnexa, bladder and adjacent tissue).

A hemispherical polyp (0.4 cm) was observed in the sigmoid 18 cm away from the anus (Fig. 3A), which was then resected via endoscopic therapy. Pathological diagnosis showed that it was a hyperplastic polyp (Fig. 3B). No other lesions were detected in the stomach or breast by both gastroscopy and color Doppler ultrasound.

WES was performed on DNA of resected intestinal polyp, and achieved 107.71x mean coverage on targeted exons. But none of 13 SNVs in CTCs was found in polyp, which were related with large intestine cancer in COSMIC database. For higher sequencing depth, we analyzed these 13 SNVs in polyp’s DNA using droplet digital PCR (ddPCR). ACKR2 c.A1118C, DCST1 c.A1534C, ITGA4 c.G1013A were positive in intestinal polyp (Fig. 4), other 10 SNVs were not detected.

CTCs in the peripheral blood of this patient were monitored after surgery, at 1 and 6 months during the follow-up. The number of CTCs reduced to 0 CTCs/7.5 ml, indicating a low risk of residual lesions (Fig. 5). And level of AFP, CA-125, CA19-9, CEA and CA15-3 in plasma were in normal range at 1 and 6 months during follow-up.

Discussion

CTCs in the blood have been suggested to be potential surrogate markers for minimal residual disease, and the precursor of metastatic disease (1). Their presence represents
an independent prognostic factor for reduced disease-free and overall survival (3,4). The migration of CTCs seems to be an early event in human carcinogenesis, even before the cancer is visible in clinical imaging. CTCs have been detected in the blood of model animals when the tumor size is <1 mm (6). The study of Ilie et al (11) showed that CTC+ patients with chronic obstructive pulmonary disease (COPD) were all diagnosed with lung cancer during a 1-4 years follow up period. The search for CTCs, at present, may facilitate an early diagnosis of cancer or precancers.

Many studies have proven that the SE-iFISH platform (identified by CEP8, CK and CD45 (12)) has a higher CTC detection rate than the Cellsearch system (7,8,10,13). The high detection rate of CTCs by the SE-iFISH platform was attributed to the following reasons, as previously reported (7).

Firstly, the subtraction enrichment of the SE-iFISH platform uses immunomagnetic particles conjugated with anti-CD45 antibody to wipe off the WBCs, which doesn't depend on the EpCAM expression of CTCs; the expression of EpCAM on CTCs may decrease during epithelial-mesenchymal transition (EMT). Secondly, the SE-iFISH system not only identifies CK+ CTCs by immunostaining, but also aneuploid CTCs by CEP8-fluorescence in situ hybridization; aneuploidy is a typical common cytogenetic abnormality malignant cells (14).

In this case, 8 CTCs/7.5 ml were detected in one healthy female by CK-/CD45-/DAPI+/CEP>2, indicating a high risk of cancer. Via mapping of the CTC mutation spectrum in the COSMIC database, we proposed that the cells may have derived from large intestinal cancer, gastric cancer or breast cancer, or their precancerous lesions. A 0.4 cm hemispherical
polyp was observed in the sigmoid, 18 cm away from the anus (Fig. 3A), which was diagnosed as a hyperplastic polyp by pathological determination (Fig. 3B). However, no lesions were found in the stomach or breast by gastroscopy and color Doppler ultrasound. There are small subsets of hyperplastic polyps with risk for development of colorectal cancer, although vast majority of hyperplastic polyps are innocent (15). There exists a degree of genetic and perhaps morphologic heterogeneity amongst hyperplastic polyps (16). It has been proposed that hyperplastic polyps may serve as the initial lesion in a serrated neoplasia pathway that results in the 15% sporadic colorectal adenocarcinomas that are microsatellite unstable (17-19).

A total of 13 SNVs above (Fig. 2B) weren't detected in resected intestinal polyp using WES, while three SNVs including ACKR2 c.A1118C, DCST1 c.A1534C, ITGA4 c.G1013A were detected by ddPCR (Fig. 4). That may be attributed to following reasons. Tumor evolve from benign to malignant lesions by acquiring a series of mutations over a long time. Genetic variations emerged in a minor fraction of a cell population before histology change during tumorigenesis (20). The cells with gene mutations may persist, but the cell numbers are very small compared with wild type cells. Sample for DNA extraction couldn't represent overall perspective of polyp because of heterogeneity. WES of hyperplastic polyp in this case achieved limited sequencing depth about 100x, which was hard to detect these rare mutations. While, ddPCR have a high degree of sensitivity, which is available for detecting 0.001% mutant fractions.

The CTC detection results were 0 CTC/7.5 ml in this patient's blood at 1 and 6 months after removal of the intestinal polyp. The study by Wu et al (9) reported that a decline of the CTC count after surgery indicated better prognosis, while an increase indicated fast recurrence for colorectal cancer patients. From the results above, we can deduce that the intestinal polyp could have been the main source of the 8 CTCs in this patient. And this intestinal polyp had risk for development of early colorectal cancer.

Sporadic CRC is a somatic genetic disease that may be influenced by the local colonic environment and the individual's background genetic makeup (21). Patients often present after 60 years of age, with most cancers originating from precursor initiating adenomas that, over 1-2 decades, transform into cancer. Because tumor cell dissemination appears to be an early event in tumor progression, CTCs may appear at very early stages of tumor development. Genomic alterations occur during the initiation and progression of a normal colonic cell into a neoplastic and malignant cell. Changes in the nucleic acids of the cancer cell might be detected from blood circulation, such as CTCs (22). Early screening of this population at age 50 years or older is effective and sustainable, reducing mortality from CRC and decreasing the incidence of CRC. Preventive surgery for adenoma, polyps or cancer is a vital approach towards a cure (23).

There is few study focused on CTCs' organ derivation for person who isn't previously diagnosed with cancer using WES, although some studies performed whole-exome sequencing of CTCs on patients with cancer (24,25). In summary, SE-iFISH CTC detection represents a potential tool for early stage cancer screening, and next generation sequencing of CTCs provides a window into the source of the CTCs and the properties of the solid tumor.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QXP and ZJS contributed to the study design; ZJS and JMZ were major contributors to writing the manuscript; ZJS interpreted the clinical data of patient; SYK, JZ and XYL collected samples and performed experiments; YW and JMZ analyzed the whole-exome sequencing data. QHS contributed to data analysis and manuscript revision.

Ethics approval and consent to participate

Ethical approval for the recruitment of human subjects was obtained from the Ethics Committee of First Hospital of Quanzhou Affiliated to Fujian Medical University and was consistent with ethical guidelines provided by the Declaration of Helsinki (1975). Written informed consent was obtained from each patient.

Patient consent for publication

All individuals whose data were used provided informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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