Cancer panel analysis of cultured circulating tumor cells and primary tumor tissue from breast cancer patients

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Abstract

Although many effective therapies have improved the survival rate of patients with breast cancer, a significant number of patients are unable to avoid recurrence and metastasis. Liquid biopsy using circulating tumor cells (CTC) is a non-invasive method to obtain tumor cells and can be a substitute for tumor tissue biopsy. This study was designed to determine whether CTC culture is an effective method to obtain ample amounts of CTCs for molecular analysis.

We developed a method to isolate and culture CTCs from patients with breast cancer and constructed a molecular profile of cultured cells using the Ion AmpliSeq Cancer Gene Panel V2. We also compared mutations observed in cultured CTCs with those observed in primary tumor tissues.

CTCs were successfully isolated and cultured from the blood of six patients with breast cancer. We detected Catalogue Of Somatic Mutation In Cancer (COSMIC) mutations in *PDGFRA, MET, PTEN, HRAS, SMARCB1, CDKN2A*, and *MLH1* in 5 of 6 samples. Comparison of mutations found in cultured CTCs with those found in primary tumor tissues demonstrated that a large portion of mutations found in CTCs is also detected in primary tumor tissues.

This report describes a new cell culture approach that can be used to obtain amounts of CTCs sufficient for molecular analysis. This new approach can be used as a tool for liquid biopsy during breast cancer treatment.

Introduction

Circulating tumor cells (CTCs) were first described in 1869 (1). CTCs, which are shed from primary tumor tissue (2), circulate in the bloodstream and cause metastasis (3, 4). CTCs have molecular characteristics similar to those of primary tumor tissue (5, 6); therefore, it is possible to monitor drug sensitivity and resistance and predict the prognosis of therapy through liquid biopsy using CTCs (7, 8).

Breast cancer mortality ranks fifth among all cancers and first among cancers that affect women (9). Although effective therapies based on hormone receptors and Her2 expression have shown high survival rates, recurrence and metastasis are unavoidable (10, 11). Because recurrent tumors and metastases have genetic characteristics different from those of the original tumor, therapies that are different than initial treatments are needed (12). CTCs from patients with breast cancer can be used as indicators for the detection of recurrence and metastasis (13), predictors of survival rate (14), and standards for use in making therapeutic decisions (15, 16).

A correlation between CTC counts and breast cancer recurrence or survival rate has been reported (17). Another report indicates that CTC count during therapy is a predictor for progression-free and overall survival (18). In addition, genomic profiles of CTCs can be used to predict therapeutic prognoses, make decisions regarding therapy, and analyze molecular variation during treatment (19). However, genomic analysis of CTCs has been difficult due to their rarity (20). In this study, we isolated live, intact CTCs on the basis of size difference and successfully cultured these CTCs to obtain sufficient quantities for genomic analysis.

Materials and Methods

Clinical information of patients with breast cancer

Six patients with breast cancer from the ASAN Medical Center, Seoul, Korea were included in this study. The median age was 44 years (range, 37–47 years). Cancer stages were evaluated using the Tumor, Node, and Metastasis (TMN) system based on the recommendations of the 7th American Joint Committee on Cancer (AJCC)/Tumor, Node, and Metastasis (TMN). All blood samples, tumor tissues, and medical data used in this study were irreversibly anonymized to ensure patient privacy. The protocol used in this study was approved by the institutional review board (IRB) of ASAN Medical Center, Seoul, Korea (IRB clearance No. 2013-1048).

Blood collection and CTC enrichment process

Blood (10 mL) from each patient was collected in a separate acid citrate dextrose (ACD) tube and processed within 4 h. The CTC culture kit (Cat# CIKC10; Cytogen, Inc.) was used to enrich CTCs from each blood sample. These CTCs were used in subsequent culture procedures. Briefly, blood samples were subjected to density gradient centrifugation, and the fraction containing peripheral blood mononuclear cells (PBMC) was diluted with a dilution buffer. Diluted cell suspensions were filtered through a high-density microporous (HDM) chip (21), and the cells retrieved from the HDM chip were used for cultivation.

Primary culture of CTCs

Enriched CTCs were collected, washed with PBS, and then cultured in 6-well ULA plates (Corning) containing mesenchymal stem cell growth medium (MSCGM, human Mesenchymal Stem Cell Growth BulletKit™ Medium; Lonza) at 37°C under an atmosphere containing 5% CO_2. After 16–18 days of culture, aliquots of cells were fixed in 4%

paraformaldehyde for use in immunofluorescent staining. The remainder of cell pellets was kept at −80°C until they were used for cancer gene panel analysis.

Immunofluorescence staining

Cells on a microscope slide were incubated with 0.2% Triton-X100 in PBS for 10 min and then quenched with 0.3% hydrogen peroxide for 30 min. After blocking with 1% BSA in PBS for 30 min, the cells were incubated with mouse anti-EpCAM antibody (Cell Signaling Technology). EpCAM signals were amplified with Tyramide Signal Amplification System (Life Technologies), which was used according to the manufacturer's protocol. The slides were mounted using Fluoroshield with DAPI (ImmunoBioScience). Stained cells were observed and photographed on a Nikon Eclipse Ti fluorescent microscope equipped with a 200× objective.

Whole genome amplification

Cellular DNA obtained from pellets maintained at −80°C was amplified using the REPLI-g Single Cell kit (QIAGEN) according to manufacturer's protocol. Briefly, cell pellets were mixed with a denaturation buffer and incubated at 65°C for 10 min. After adding a stop solution, resulting denatured DNA samples were mixed with REPLI-g sc DNA polymerase and a reaction buffer. This mixture was incubated at 30°C for 8 h and then at 65°C for 3 min.

Genomic DNA extraction from primary tumor tissues

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) primary tumor tissues. FFPE slides were initially examined by a pathologist to validate the presence of tumor cells. DNA present in the tumor cells was extracted using the Gentra Puregene DNA Isolation kit (Qiagen) according to the manufacturer's protocol.

Ion AmpliSeq Cancer Panel (ICP) analysis

Genomic mutations were analyzed using the Ion AmpliSeq Cancer Hotspot Panel v2 (CHPv2, Life Technologies). Briefly, genomic DNA was amplified using the primers contained within the Ion AmpliSeq Cancer Panel. The amplicons were purified using the Agencourt AM-Pure XP system (Beckman Coulter). Subsequent end-repair and ligation was performed with Ion Xpress barcode adapters (Life technologies). The median fragment size and the concentration of the final library were determined using a BioAnalyzer equipped with a High-Sensitivity Chip (Agilent). After the library was diluted to 10 pM using a low TE buffer, 5 µL of the library was used for emulsion PCR reactions using the Onetouch™ reagent kit (Invitrogen). The products of these emulsion PCR reactions were enriched using Dynabeads® MyOne™ Streptavidin C1 beads (Invitrogen). The final enriched Ion spheres were mixed with sequencing primers and polymerase and loaded onto a total of five 316 chips. Base calls were generated using Torrent Suite 3.0 software with tmap-f3 and maintained on the Ion Torrent server for further analysis. The base calling results were used to generate Bam and FASTQ (alignment) files that were used to identify gene variants, including single nucleotide polymorphisms and insertions/deletions.

Results

Expansion of CTCs through culturing

We cultured CTCs from six patients with breast cancer and obtained sufficient numbers of CTCs for characterization. During the early phase of culture (until day 9), cells were grown either attached or suspended as single cells (Fig.1A, B). Cells were expanded during the rest of the culture process until they reached a population of 4×10^5 to 8×10^5 cells (Fig. 1C, Table 1) and the attached cells showed cell membrane ruffling (Fig. 1D). These observations suggested the selective expansion of epithelial cells and improved cell motility.

CTC characterization

After 16–18 days of culture, we performed immunofluorescence staining for the epithelial cell marker EpCAM to evaluate the proportion of cultured cells that were CTCs (Fig. 2). The proportion of EpCAM-positive cells ranged from 35% to 86% (Table 1), demonstrating that CTCs were expanded with high purity.

Cancer gene panel analysis

We detected COSMIC mutations in *PDGFRA, MET, PTEN*, *HRAS, SMARCB1*, *CDKN2A*, and *MLH1* from five of the six samples of cultured CTCs (Table 2). To evaluate whether the cultured CTCs maintained genomic profiles similar to those of primary tumor tissues, we compared mutations observed in cultured CTCs with those observed in primary tumor tissues. In samples taken from patient AMC-15-02, we confirmed the same mutation of *HRAS* in both cultured CTCs and primary tumor tissues (Table 3). Likewise, 60% of novel mutations were shared by cultured CTCs and primary tumor tissues (Table 4). Although the cultured CTCs obtained from patient AMC-15-06 did not have any COSMIC mutations in common with primary tumor tissue, 80% of novel mutations were found in both cultured CTCs and primary tumor tissues (data not shown).

Discussion

It has been reported that CTCs retain the genomic characteristics of the primary tumor. Consequently, they can be used as a substitute for tissue biopsy to monitor drug responsiveness and make therapy decisions (7, 8). We have already successfully conducted cancer gene panel analyses using uncultured CTCs (unpublished data, submitted), suggesting that CTCs, although rare, can be sufficient in number for molecular analysis without culturing. However, expansion of the CTC sample is essential for chemosensitivity assays or in the use of the PDX model.

To overcome issue of cell numbers, we have developed a new methodology of isolating and culturing CTCs. EpCAM-positive cells consisted of 35%–86% of total cells obtained using our culture method, and the final number of cultured cells was between 4×10^5 and 8×10^5 (Table 1). We successfully achieved cultivation of at least 1×10^5 CTCs, which can be used for cancer gene panel analysis. Furthermore, these CTCs can be useful for chemosensitivity assay and in the patient-derived xenograft (PDX) model of breast cancer. This number of CTCs was presumably underestimated because this method was not able to detect mesenchymal CTCs that should be present because a significant portion of CTCs may have undergone the epithelial to mesenchymal transition (22).

We detected mutations in *PDGFRA*, *MET*, *PTEN*, *HRAS*, *SMARCB1*, *CDKN2A*, and *MLH1* from genomic analysis of cultured CTCs. Mutations in these genes have been reported in breast tumor tissues (23-25), suggesting that cultured CTCs maintain genetic characteristics similar to those of primary tumor tissues. *PDGFRA* and *HRAS*, which were mutated in three of the six cultured CTC samples, are known to be related to breast cancer progression (24-26).

Furthermore, we analyzed the genomic profiles of primary tumor tissues along with those of the corresponding cultured CTCs and found that a large portion of mutations detected in

CTCs was also detected in primary tumor tissues. Although the cultured CTCs obtained from patient AMC-15-06 did not have any COSMIC mutations shared with the primary tumor tissue, mutation of *HRAS* that have been reported to affect breast cancer recurrence and metastasis (26, 27) was detected in CTCs of this patient.

To evaluate whether cultured CTCs maintain genomic characteristics similar to those of the primary tumor is the first step for the application of cultured CTCs to breast cancer treatment. Here we isolated and cultured CTCs with high purity and performed genomic analysis of these CTCs. We also confirmed that cultured CTCs maintain genomic profiles similar to those of primary tumor tissues, suggesting that the use of cultured CTCs is an appropriate technology for breast cancer treatment.

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Table 1. Clinical information from patients with breast cancer, including immunofluorescence staining analysis of EpCAM-positive cells

Patient ID	Age	AJCC/TMN stage[a]	No of cultured cells	
			No. of total cells	No. of EpCAM+ (%)
AMC-15-01	47	IIA	4.0×10^5	34.92
AMC-15-02	38	IIA	5.0×10^5	53.74
AMC-15-03	43	IIA	5.0×10^5	53.76
AMC-15-04	51	IIB	5.2×10^5	41.20
AMC-15-05	37	IIIC	8.3×10^5	86.54
AMC-15-06	46	IIB	4.5×10^5	86.14

[a]AJCC/TMN stage**:** the 7th American Joint Committee on Cancer/TMN: Tumor Metastasis lymph Nodes

Table 2.Ion AmpliSeq Cancer Panel V2 of cultured CTCs from patients with breast cancer.

Patient ID	Gene ID	Mutations type	A.A. mutation[a]	Cosmic number
AMC-15-01	*PDGFRA*	SNP[b]	N659K	COSM22414
	MET	SNP	Unknown	COSM710
	PTEN	INS[c]	N323fs*2	COSM23626
	PTEN	INS	T321fs*3	COSM4994
	PTEN	INS	N323fs*2	COSM4990
AMC-15-02	*PDGFRA*	SNP	V824V	COSM22413
	HRAS	SNP	H27H	COSM249860
	SMARCB1	SNP	Unknown	COSM1090
AMC-15-03	*PDGFRA*	SNP	V824V	COSM22413
	HRAS	SNP	H27H	COSM249860
	SMARCB1	SNP	Unknown	COSM1090
AMC-15-04	N/A[d]			
AMC-15-05	*CDKN2A*	SNP	H66R	COSM14253
AMC-15-06	*MLH1*	SNP	V384D	COSM26085
	MET	SNP	Unknown	COSM710
	HRAS	SNP	H27H	COSM249860

[a]AA mutation: amino acid mutation

[b]SNP: single nucleotide polymorphism

[c]INS: insertion

[d]N/A: Not applicable

Table 3. Comparison of COSMIC mutations detected in primary tumor tissue with those detected in cultured CTCs from a patient with breast cancer (AMC-15002).

	Gene ID	**Mutation type**	**A.A mutation[a]**	**Cosmic number**
Primary tissue	*NOTCH1*	DEL[b]	V1578delV	COSM13047
	HRAS	SNP[c]	H27H	COSM249860
	TP53	SNP	H193Y	COSM10672
CTC	*PDGFRA*	SNP	V824V	COSM22413
	HRAS	SNP	H27H	COSM249860
	SMARCB1	SNP	Unknown	COSM1090

[a]AA mutation: amino acid mutation

[b]DEL: deletion

[c]SNP: single nucleotide polymorphism

Table 4. Complete comparison of Ion AmpliSeq Cancer Panel V2 between primary tumor tissue and cultured CTCs from a patient with breast cancer (AMC-15002).

Primary tissue				Cultured CTCs			
Gene ID	**Type**	**Allele Source**	**Cosmic number**	**Gene ID**	**Type**	**Allele Source**	**Cosmic number**
				ALK	SNP	Novel	---
ERBB4	SNP	Novel	---	ERBB4	SNP	Novel	---
VHL	SNP	Novel	---				
FGFR3	SNP	Novel	---	FGFR3	SNP	Novel	---
PDGFRA	SNP	Novel	---	PDGFRA	SNP	Novel	---
				PDGFRA	SNP	Hotspot	COSM22413
APC	SNP	Novel	---	APC	SNP	Novel	---
CSF1R	MNP	Novel	---	CSF1R	MNP	Novel	---
				EGFR	SNP	Novel	---
NOTCH1	DEL	Hotspot	COSM13047				
				NOTCH1	SNP	Novel	---
RET	SNP	Novel	---	RET	SNP	Novel	---
HRAS	SNP	Hotspot	COSM249860	HRAS	SNP	Hotspot	COSM249860
ATM	INS	Novel	---				
FLT3	SNP	Novel	---	FLT3	SNP	Novel	---
TP53	SNP	Novel	---				
TP53	SNP	Hotspot	COSM10672				
TP53	SNP	Novel	---	TP53	SNP	Novel	---
				STK11	SNP	Novel	---
				SMARCB1	SNP	Hotspot	COSM1090

Figure Legends

Figure 1. Representative microscopic images of CTC cultures at day 9 (A, B) and day 13 (C, D). (A, C) magnification, 100×; (B, D) magnification, 400×.

Figure 2. Immunofluorescence staining of cultured CTCs for EpCAM at day 9 (A, B) and day 13 (C, D). Magnification, 200×.