Cancer panel analysis of circulating tumor cells (CTCs) in breast cancer patients

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Running title: Cancer panel analysis of CTCs from breast cancer patients.

Abstract

Purpose

Liquid biopsy using circulating tumor cells (CTCs) is noninvasive and repeatable; therefore, it can be useful for molecular assays. However, the rarity of CTCs has been a challenge. To overcome this issue, we have developed a new technology of isolating CTCs on the basis of cell size difference. In this study, we isolated CTCs from patients with breast cancer and used these cells for cancer gene panel analysis.

Methods

Blood samples from eight patients with breast cancer were collected, and CTCs were enriched using size-based filtration. Enriched CTCs were analyzed for counting using immunofluorescent staining with EpCAM and CD45 antibodies. Genomic DNA of CTCs was extracted, amplified, and screened for mutations in 400 genes using the Ion AmpliSeq Comprehensive Cancer Panel. WBCs from the same patient served as a negative control, and mutations in CTCs and WBCs were compared.

Results

EpCAM-positive cells were detected in seven out of eight patients, and the average number of EpCAM-positive cells was 8.6. The average amount of amplified DNA was 32.7 μg, and the percentage of reads mapped to any targeted region relative to all reads mapped to the reference was 98.6%. The detection rate of CTC-specific mutations was 62.5%. The CTC-specific mutations were EZH2, NOTCH1, ARID1A, STK11, FLT3, MYCN, APC, and PTEN.

Conclusion

We demonstrated that our technique is efficient for isolating CTCs, with purity enough for genomic analysis, and provide comprehensive cancer panel analysis as a possible application for precision medicine.

Key words: Circulating tumor cells, breast cancer, cancer gene panel analysis, liquid biopsy

Introduction

Breast cancer is the most common cancer in women, with mortality ranking of fifth among all types of cancers and first among female cancers worldwide (1). Moreover, the mortality rate of breast cancer at the age of 25–45 years in Korea is highest in the world (2).

Despite the fact that hormone receptor-positive breast cancer in the early stage may be treated with a wide variety of effective regimens and show relatively better survival rates, a significant portion of patients may have tumor recurrence and metastasis (3).

The genomic characteristics of a metastatic tumor are different from those of a primary tumor owing to the time interval between recurrence and metastasis and occurrence of the primary tumor. Further, this genomic difference can be intensified with preceding treatments, such as chemotherapy (4).

Trastuzumab, a targeted therapeutic agent, markedly improves the rates of progression-free and overall survival in patients with metastatic breast cancer having a poor prognosis in the short term. However, long-term observation over 30 months demonstrated similar recurrence and mortality rates in both general chemotherapy and targeted therapy (5).

Recent reports provided several hypotheses to explain the resistance caused by genomic changes in tumor cells during trastuzumab treatment (6), and other therapies for metastatic cancer that are resistant to trastuzumab have been continuously reported (7, 8).

There is an increasing necessity to monitor the genomic profiles of tumor cells during cancer onset, recurrence, and metastasis; however, repeated tumor tissue biopsy is not always practical.

Circulating tumor cells (CTCs) that have shed from a primary tumor are present in the blood

circulation and can cause tumor metastases (9, 10). Liquid biopsy using CTCs is noninvasive and repeatable; therefore, it can be useful for counting tumor cells, pathological characterization, and molecular assays. Furthermore, liquid biopsy using CTCs can replace metastatic tissue biopsy for the prediction of drug sensitivity and resistance, monitoring of drug responsiveness, and detection of metastasis (11, 12).

Previously, we have developed a new technology of enrichment and isolation of CTCs on the basis of cell size difference (13). In this study, we isolated CTCs from patients with breast cancer using this method and performed cancer panel analysis of isolated CTCs. Furthermore, we compared the genetic mutations of CTCs with those of WBCs from the same patient to evaluate cancer-specific mutations.

Materials and Methods

Clinical background of patients with breast cancer

Eight patients with breast cancer, with a median age of 45 years (range, 28–48 years), from the ASAN Medical Center, Seoul, Korea, were included in this study. Cancer stage was evaluated on the basis of the 7th American Joint Committee on Cancer (AJCC) Tumor, Node, and Metastasis (TNM) Classification (Table 1). All blood samples and medical data used in this study were irreversibly anonymized. This study was approved by the institutional review board (2013-1048).

Blood collection and CTC enrichment

Ten milliliters of blood from each patient were collected in ACDA tubes and processed within 4 h. The blood samples were divided into two parts: one for immunofluorescent staining and other for cancer panel analysis of CTCs. Both samples were processed through the same procedure with the CTC isolation kit (Cat# CIKW10, Cytogen, Inc.). Briefly, blood samples were incubated for 20 min with an antibody cocktail against WBCs and RBCs and then mixed with preactivation buffer before density gradient centrifugation. A cell suspension containing CTCs was collected and gradually diluted with dilution buffer. The diluted cell suspensions were filtered through a high-density microporous (HDM) chip (13). Cells on the HDM chip were retrieved and transferred to a microtube. For immunofluorescent staining, isolated cells were fixed in 4% paraformaldehyde for 5 min at room temperature. For cancer panel analysis, isolated cells were pelleted and kept at −80°C until further processing. From the same patient, 500 μL of blood was layered onto a density gradient medium and centrifuged. From the PBMC layer, 100 WBCs were isolated and saved as a negative control for cancer panel analysis. In addition, different amounts (5, 10, 20, and 100) of MCF7 cells were spiked into 1

ml of healthy volunteers' blood, isolated with the same procedure of CTC isolation, and used as a positive control for evaluation of Cytogen protocol.

Immunofluorescence staining

Cells on slides were permeabilized with 0.2% Triton-X 100 in PBS for 10 min and quenched with 0.3% hydrogen peroxide for 1 h. Cells were then blocked with 1% BSA in PBS for 30 min and incubated with primary antibodies followed by secondary antibodies. The primary antibodies were mouse anti-EpCAM (Cell Signaling Technology) and rabbit anti-CD45 (Santa Cruz). EpCAM signals were amplified with Tyramide Signal Amplification System (Life Technologies) according to the manufacturer's protocol. The secondary antibody for CD45 was goat anti-rabbit Alexa 594 (Invitrogen). The slides were mounted with Fluoroshield with DAPI (ImmunoBioScience). Stained cells were observed and photographed on a fluorescent microscope (Eclipse Ti, Nikon) at a magnification of ×400.

Whole genome amplification

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

Ion AmpliSeq comprehensive cancer panel (CCP) analysis

Genomic mutations were analyzed using the Ion AmpliSeq Comprehensive Cancer Panel (Life Technologies), a next-generation sequencing assay that identifying all-exon coverage of

409 oncogenes and tumor suppressor genes. Ion AmpliSeq Comprehensive Cancer Panel was designed to target all exons of key tumor suppressor genes and oncogenes most frequently cited and most frequently mutated. Briefly, genomic DNA was amplified by the Ion AmpliSeq Cancer Panel and the amplicons were purified by Agencourt AM-Pure XP (Beckman Coulter). This was followed by end repairing and ligation with Ion Xpress barcode adapters (Life technologies). The median fragment size and concentration of the final library were detected by BioAnalyzer using High Sensitivity Chip (Agilent). The library was diluted to 10 pM by low TE; 5 μL of the library was used for emulsion PCR reactions using Onetouch™ reagent kit (Invitrogen). Thereafter, the emulsion PCR product was enriched by Dynabeads® MyOne™ Streptavidin C1 beads (Invitrogen). The final enriched Ion spheres were mixed with a sequencing primer and polymerase and loaded onto a total of five 316 chips. Base calling was generated by Torrent Suite 3.0 using tmap-f3 on the Ion Torrent server for further analysis. Bam and FASTQ files (alignment) were generated on the basis of the base calling result and were used to report the variant calling, including single nucleotide polymorphisms and insertions/deletions.

Results

Spike-in experiment with MCF7 cell line

Immunofluorescent stained cells were counted for EpCAM-positive cells (Fig. 1). As expected, the purity of MCF7 cells increased with the number of cells (Fig. 2A). The recovery rate of our method using GFP cell lines was 84% (data not shown). PIK3CA mutation, a known mutation in MCF7, was detected in isolated MCF7 cells through the Cytogen protocol. The frequencies of mutations were increased when purity was high, but even in samples with low purity, gene mutation was detected (Fig. 2B).

Isolation of CTCs from patients

We defined CTCs as EpCAM-positive and CD45-negative cells (Fig. 3). EpCAM-positive cells were detected in seven out of eight patients, and the average number of EpCAM-positive cells was 8.6 (1–23) (Table 1). For positive control of immunostaining, we included PC9 (EpCAM positive) and KG-1 (CD45 positive) cell lines. CTCs isolated for cancer panel analysis were amplified using whole genome amplification, and the average DNA amount was 32.7 μg with high purity (all above 1.80). On target, the percentage of reads mapped to any targeted region relative to all reads mapped to the reference was 98.6 % (range 97.8%–99.2%) (Table 1).

Cancer gene panel analysis

We used COSMIC to confirm the CCP results. Because mutations in WBCs were analyzed as negative controls, these were considered as germ line mutations. The same mutations in CTCs were excluded from the analysis. We validated CTC-specific mutations by comparing

mutations between CTCs and WBCs (Table 2). CTC-specific mutations had a detection rate of 62.5%, and these were EZH2, NOTCH1, ARID1A, STK11, FLT3, MYCN, APC, and PTEN.

Discussion

Patients with hormone receptor-positive breast cancer in the early stage may have several effective treatment options, but many patients also develop recurrence and metastasis. Therefore, early diagnosis of cancer, prognostication, and monitoring of genomic characteristics of tumor cells are essential. (3) However, biopsies of tumor tissues are not always easy to repeat. CTCs have been getting attention for overcoming this limitation of tumor tissue biopsy. CTCs have similar characteristics of those of the primary tumor and may cause metastasis (14). Because CTCs are present at very low concentrations (1 in 1×10^9) (15), it is very important to effectively enrich or isolate them from blood. CellSearch® (Veridex, USA), a very well-known commercial device, isolates CTCs through EpCAM-positive selection (16). This EpCAM-positive selection technique cannot be achieved when tumor cells downregulate EpCAM expression (17). In addition, a recent study reported that a significant portion of CTCs is EpCAM negative (18).

We have developed our own CTC enrichment technique based on cell size difference and double negative selection, which removes nontargeted cells with an antibody complex against WBCs. Using this technique, we effectively isolated CTCs with purity that is sufficient for genomic analysis (Fig. 2). In addition, we observed that COSMIC mutations were detected even in patients who did not have EpCAM-positive cells (Table 1), demonstrating that our technology can isolate EpCAM-negative CTCs. Among the CTC-specific COSMIC gene mutations that we identified, EZH2, Notch1, and PTEN have been reported to affect breast cancer status (19–22). Mutation of EZH2 causes abnormal DNA methylation and promotes mammary stem cell expansion and metastasis (19). Notch1 has been reported to regulate epithelial–mesenchymal transition and promote migration and invasion of breast cancer cells

(20); moreover, Notch1 expression in breast tumor tissue is higher than that in normal tissue (21). Mutated PTEN cannot inhibit the PI3K/Akt/mTOR pathway, thereby losing tumor suppressor activity (22).

There was a report on single-gene mutation analysis of CTCs and WBCs (23); another report showed cancer panel analysis of CTCs without WBCs as controls (24). To the best of our knowledge, our study is the first attempt on comprehensive analysis of cancer panel assay using CTCs along with WBCs. We identified CTC-specific COSMIC mutations and provided genomic information that may be useful for precision medicine.

Conclusion

This technique of CTC isolation was efficient in providing sufficient purity for genomic analysis and demonstrated that comprehensive cancer panel analysis is a possible application for precision medicine.

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Table 1. Patients' clinical information and WGA result.

Patient ID.	Age	AJCC/TMN Stage[a]	No. of EpCAM(+) cells[b]	Cell type	DNA amount (μg)	Purity of DNA (A260/A280)	On target[c]
CG 237	41	IIA	20	CTC	35.97	1.8	97.83%
				WBC	33.22	1.84	98.89%
CG 238	45	IIIA	1	CTC	30.69	1.88	98.93%
				WBC	32.78	1.89	98.91%
CG 239	48	IIA	7	CTC	31.35	1.89	99.17%
				WBC	33.55	1.91	98.54%
CG 240	47	IIB	23	CTC	30.58	1.84	98.81%
				WBC	34.1	1.87	99.13%
CG 242	48	IIIA	2	CTC	33.44	1.88	98.67%
				WBC	30.69	1.92	98.74%
CG 243	30	IIA	6	CTC	31.02	1.91	98.21%
				WBC	31.9	1.86	98.64%
CG 244	45	IIA	0	CTC	31.02	1.88	98.87%
				WBC	34.43	1.85	98.42%
CG 245	28	IIA	1	CTC	35.09	1.86	98.19%
				WBC	33.55	1.88	98.19%

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

[b]No. of EpCAM(+) cells: Number of EpCAM positive cells per 5 ml of blood

[c]On target: the percentage of reads mapped to any targeted region relative to all reads mapped to the reference

Table 2. Comprehensive cancer panel analysis results.

Patient ID.	WBC		CTC		CTC specific	
	Gene	AA mutation	Gene	AA mutation	Gene	AA mutation
CG 237	MSH2	Unknown				
	ARID1A	p.D1850fs*4				
	HNF1A	p.G292fs*25				
			EZH2	p.D730fs*1	EZH2	p.D730fs*1
			NOTCH1	p.D1698D	NOTCH1	p.D1698D
CG 238	PDGFRA	p.V824V	PDGFRA	p.V824V		
	NOTCH1	p.D1698D	NOTCH1	p.D1698D		
	RET	p.T278N	RET	p.T278N		
	NF2	p.N371N	NF2	p.N371N		
	MSH2	Unknown				
	PTCH1	p.C727fs*11				
CG 239	NOTCH1	p.D1698D				
CG 240	MSH2	Unknown	MSH2	Unknown		
	PDGFRA	p.V824V	PDGFRA	p.V824V		
	FLT3	p.L561L	FLT3	p.L561L		
	STK11	p.T32T	STK11	p.T32T		
	SMARCB1	p.P383fs*4				
	SMARCB1	p.P383fs				
			NOTCH1	p.A2463fs*14	NOTCH1	p.A2463fs*14
			NOTCH1	p.D1698D	NOTCH1	p.D1698D

Table 2 continued.

CG 242	PDGFRA	p.V824V	PDGFRA	p.V824V		
	FLT3	p.L561L	FLT3	p.L561L		
	MSH2	Unknown				
	EZH2	p.D730fs*1				
	NOTCH1	p.D1698D				
			ARID1A	p.D1850fs*4	ARID1A	p.D1850fs*4
CG 243	ARID1A	p.D1850fs*4	ARID1A	p.D1850fs*4		
	FLT3	p.L561L	FLT3	p.L561L		
	SMARCB1	p.T372T	SMARCB1	p.T372T		
			NOTCH1	p.D1698D	NOTCH1	p.D1698D
			STK11	p.L282fs*3	STK11	p.L282fs*3
CG 244	MSH2	Unknown				
	PDGFRA	p.V824V				
	STK11	p.L282fs*3				
			ARID1A	p.K1072fs*21	ARID1A	p.K1072fs*21
			NOTCH1	p.D1698D	NOTCH1	p.D1698D
			FLT3	p.L561L	FLT3	p.L561L

Table 2 continued.

	MSH2	Unknown		
	STK11	p.L282fs*3		
CG 245			MYCN	p.P358L
			APC	p.R554*
			NOTCH1	p.D1698D
			PTEN	p.L57fs*6

Figure Legends

Figure 1. CTC isolation for cancer panel analysis

CTC, circulating tumor cells; PBMC, peripheral blood mononuclear cells; CCP, comprehensive cancer panel

Figure 2. Spike-in experiment with MCF7 cell line

Purity: Percentage of spiked-in cell number per total cell number

(A) Correlation of purity and EpCAM-positive cell number. (B) Correlation of PIK3CA mutation frequencies and purity of isolated cells.

Figure 3. Representative images of CTCs from patients with breast cancer