

Cancer panel analysis of circulating tumor cells in patients with breast cancer

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Abstract. Liquid biopsy using circulating tumor cells (CTCs) is a noninvasive and repeatable procedure, and is therefore useful for molecular assays. However, the rarity of CTCs remains a challenge. To overcome this issue, our group developed a novel technology for the isolation of CTCs on the basis of cell size difference. The present study isolated CTCs from patients with breast cancer using this method, and then used these cells for cancer gene panel analysis. Blood samples from eight patients with breast cancer were collected, and CTCs were enriched using size-based filtration. Enriched CTCs were counted using immunofluorescent staining with an epithelial cell adhesion molecule (EpCAM) and CD45 antibodies. CTC genomic DNA was extracted, amplified, and screened for mutations in 400 genes using the Ion AmpliSeq Comprehensive Cancer Panel. White blood cells (WBCs) from the same patient served as a negative control, and mutations in CTCs and WBCs were compared. EpCAM⁺ cells were detected in seven out of eight patients, and the average number of EpCAM⁺ cells was 8.6. The average amount of amplified DNA was 32.7 μ g, 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 enhancer of zeste polycomb repressive complex 2 subunit, notch 1, AT-rich interaction domain 1A, serine/threonine kinase 11, fms related tyrosine kinase 3, MYCN proto-oncogene, bHLH transcription factor, APC, WNT signaling pathway regulator, and phosphatase and tensin homolog. The technique used by the present study was

demonstrated to be effective at isolating CTCs at a sufficiently high purity for genomic analysis, and supported the use of comprehensive cancer panel analysis as a potential application for precision medicine.

Introduction

Breast cancer is the most common cancer in women, with the fifth highest mortality rate among all types of cancer and the highest occurrence rate among female cancers globally (1). Furthermore, the mortality rate of breast cancer in patients aged 25-45 years in Korea is the highest worldwide (2). Despite the fact that it is possible to treat hormone receptor-positive breast cancer with a wide variety of effective regimens in the early stages of disease and obtain relatively improved survival rates, a significant number of patients may experience 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 the occurrence of the primary tumor. Furthermore, this genomic difference is intensified after treatment, including chemotherapy (4).

Trastuzumab, a targeted therapeutic agent, markedly improves progression-free and overall survival rates in patients with metastatic breast cancer, who have a poor prognosis, in the short term. However, long-term observation over 30 months demonstrated similar recurrence and mortality rates in patients treated with general chemotherapy and targeted therapy (5). Previous reports provided several hypotheses to explain the resistance to trastuzumab caused by genomic changes in tumor cells during treatment (6), and other therapies for metastatic cancer that are resistant to trastuzumab have been 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 may cause tumor metastases (9,10). Liquid biopsy using CTCs is noninvasive and repeatable; therefore, it is useful for counting tumor cells, pathological characterization and

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Table I. Clinical information of patients with breast cancer, and Whole Genome Amplification results.

Patient ID	Age, years	AJCC TNM stage	EpCAM ⁺ cells, n ^a	Cell type	DNA amount, μ g	Purity of DNA (A_{260}/A_{280})	On target, % ^b
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

^aPer 5 ml of blood. ^bReads mapped to any targeted region relative to all reads mapped to the reference. AJCC, the seventh American Joint Committee on Cancer; TNM, Tumor Lymph Node Metastasis; EpCAM, epithelial cell adhesion molecule; CTC, circulating tumor cell; WBC, white blood cell.

molecular assays. Furthermore, it is possible to use a liquid biopsy with CTCs to replace metastatic tissue biopsy for the prediction of drug sensitivity and resistance, monitoring of drug responsiveness, and detection of metastasis (11,12).

Previously, our group developed a novel technology to enrich and isolate CTCs on the basis of differences in cell size (13). In the present study, CTCs were isolated from patients with breast cancer using this method and a cancer panel analysis of isolated CTCs was performed. Furthermore, the genetic mutations of CTCs were compared with those of white blood cells (WBCs) from the same patient in order to evaluate cancer-specific mutations.

Materials and methods

Cell culture. H358-GFP, MCF7, PC9 and KG-1 cell lines (American Type Culture Collection, Manassas, VA, USA), were maintained in RPMI (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% Antibiotic-Antimycotic (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured 37°C at 5% CO₂ in an incubator.

Clinical background of patients with breast cancer. From February 2015 to March 2015, 8 female patients with breast cancer, with a median age of 45 years (range, 28-48 years), and 4 female healthy volunteers with a median age of 34 years (range, 25-45 years) from the Asan Medical Center (Seoul, Korea) were included in the present study. A total of 4 patients (CG 237-240) received neoadjuvant systemic therapy and 4 patients (CG 242-245) did not receive treatment. Cancer

stage was evaluated on the basis of the seventh American Joint Committee on Cancer Tumor, Node, and Metastasis Classification (Table I) (14). All blood samples and medical data used in the present study were irreversibly anonymized. The present study was approved by the Institutional Review Board of Asan Medical Center (IRB no. 2013-1048).

Blood collection and CTC enrichment. Blood from each patient (10 ml) was collected in BD Vacutainer acid citrate dextrose-solution A tubes and processed within 4 h. The blood samples were divided into two groups as follows: One for immunofluorescent staining, and the other for the cancer panel analysis of CTCs. The samples were processed using the same procedure, with a CTC isolation kit (cat. no. CIKW10; Cytogen, Inc.) used according to the protocol. Briefly, blood samples were incubated for 20 min with an antibody cocktail against WBCs and red blood cells, and then mixed with preactivation buffer prior to density gradient centrifugation (400 x g for 30 min at 25°C). A cell suspension containing CTCs was collected and gradually diluted with dilution buffer. The diluted cell suspensions were then filtered through a high-density microporous (HDM) chip as previously described (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 μ l blood was layered onto a density gradient medium (Ficoll-Paque™ PLUS; GE Healthcare Life Sciences, Little Chalfont, UK) and centrifuged (400 x g for 30 min at 25°C). From the peripheral blood mononuclear cell

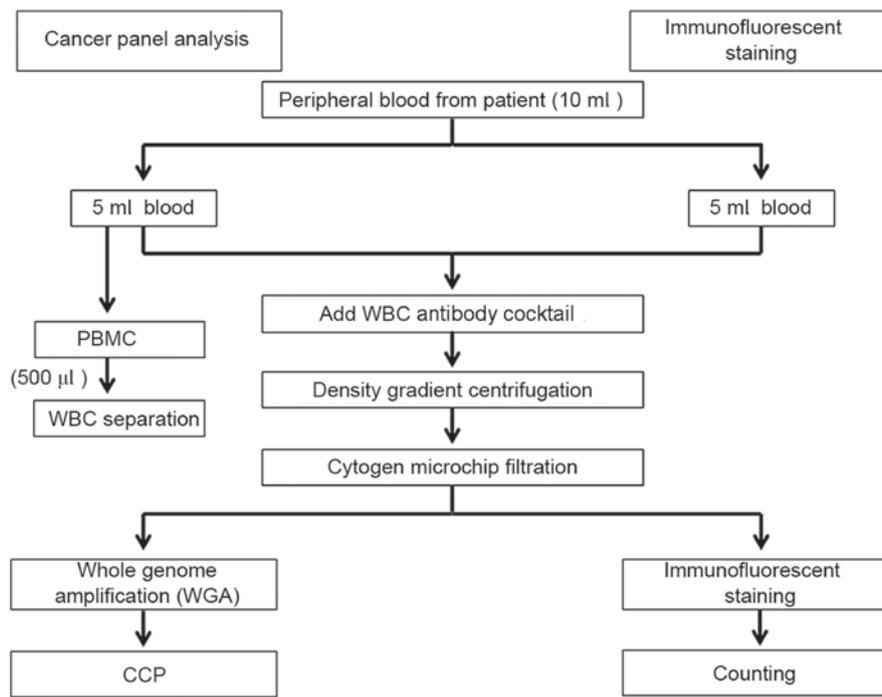


Figure 1. CTC isolation for CCP analysis flowchart. CTC, circulating tumor cell; CCP, comprehensive cancer panel; PBMC, peripheral blood mononuclear cell, WBC, white blood cell.

layer, 100 WBCs were isolated as a negative control for cancer panel analysis. In addition, different numbers (5, 10, 20 and 100) of MCF7 cells were spiked into 1 ml blood from healthy volunteers, isolated using the same CTC isolation procedure, and used as a positive control for evaluation of the Cytogen protocol.

Immunofluorescent 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% bovine serum albumin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in PBS for 30 min, and incubated with primary antibodies followed by secondary antibodies for 1 h each at room temperature. The primary antibodies were as follows: Mouse anti-epithelial cell adhesion molecule (EpCAM; dilution 1:200; cat. no. #2929; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti-CD45 (dilution 1:10; cat. no. SC-25590; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). EpCAM signals were amplified using the Tyramide Signal Amplification system (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The secondary antibody for CD45 was Alexa 594-conjugated goat anti-rabbit immunoglobulin G (H+L) (dilution 1:200; cat. no. A11012; Invitrogen; Thermo Fisher Scientific, Inc.). The slides were mounted with Fluoroshield with DAPI (Immunobioscience Corporation, Mukilteo, WA, USA). Stained cells were observed and photographed 3 fields using a fluorescence microscope (Eclipse Ti; Nikon Corporation, Tokyo, Japan) at a magnification of x400.

Whole genome amplification. The cell pellets that were kept at -80°C were amplified using the REPLI-g Single Cell kit (Qiagen GmbH, Hilden, Germany) according to the

manufacturer's protocol. Briefly, the cell pellets were mixed with a denaturation buffer (included in the kit) and incubated at 65°C for 10 min. Following the addition of a stop solution (included in the kit), the denatured DNA samples were mixed with REPLI-g sc DNA polymerase and a reaction buffer (included in the kit) and incubated at 30°C for 8 h and then at 65°C for 3 min.

Ion AmpliSeq comprehensive cancer panel (CCP) analysis. Genomic mutations were analyzed using the Ion AmpliSeq CCP (Thermo Fisher Scientific, Inc.), which is a next-generation sequencing assay that provides all-exon coverage of 409 oncogenes and tumor suppressor genes. The Ion AmpliSeq CCP 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 using the Ion AmpliSeq Cancer Panel and the amplicons were purified using Agencourt AM-Pure XP (Beckman Coulter, Inc., Brea, CA, USA). This was followed by end repairing and ligation with Ion Xpress barcode adapters (Thermo Fisher Scientific, Inc.). The median fragment size and concentration of the final library were detected using a BioAnalyzer instrument with a high sensitivity chip (Agilent Technologies, Inc., Santa Clara, CA, USA). The library was diluted to 10 pM by low TE buffer included in the kit; and the library (5 µl) was used for emulsion PCR reactions using the Ion PI™ Hi-Q™ OT2 200 kit (Invitrogen; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 80°C for 3 min, followed by 18 cycles of 99°C for 20 sec, 58°C for 30 sec, 72°C for 1 min, 99°C for 20 sec, 56°C for 30 sec and 70°C for 1 min, and 10 cycles of 99°C for 20 sec and 58°C for extended durations from 3-20 min. The emulsion PCR product was enriched using Dynabeads MyOne Streptavidin C1 beads (Invitrogen;

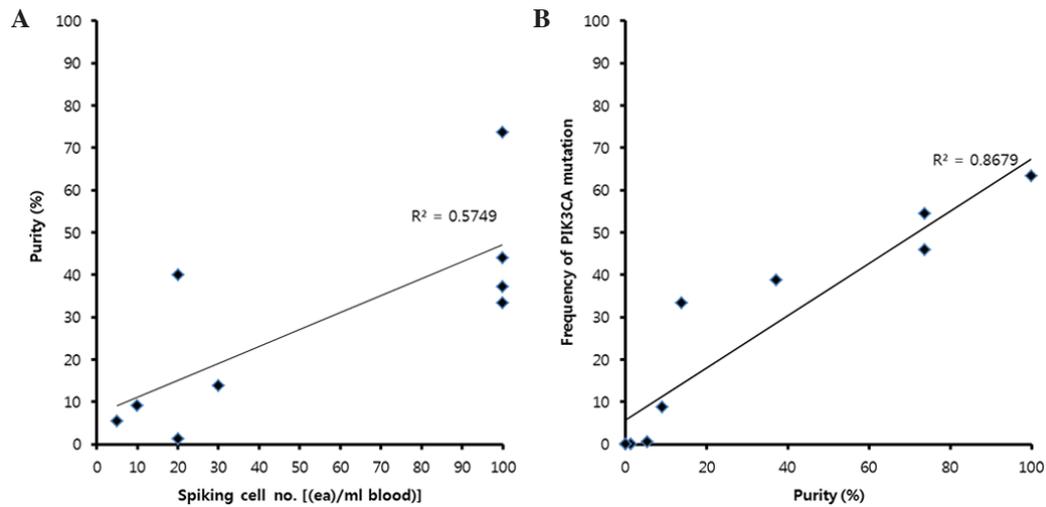


Figure 2. Spike-in experiment with the MCF7 cell line. (A) Association between purity and EpCAM⁺ cell number. (B) Correlation of PIK3CA mutation frequencies and purity of isolated cells. The purity was defined as the percentage of spiked-in cell number per total cell number. EpCAM, epithelial cell adhesion molecule; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α .

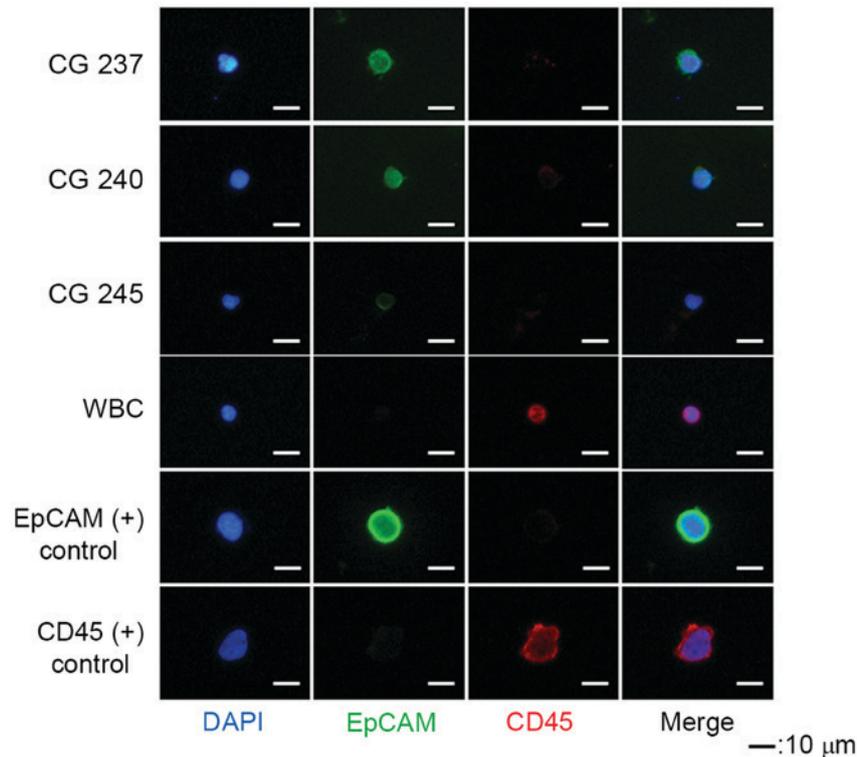


Figure 3. Representative images of circulating tumor cells from patients with breast cancer. CG, patient ID; WBC, white blood cell; EpCAM, epithelial cell adhesion molecule.

Thermo Fisher Scientific, Inc.). The final enriched Ion spheres were mixed with a sequencing primer (included in the kit) and polymerase (included in the kit) and loaded onto a total of five chips of Ion 316TM Chip kit. Base calling was generated by Torrent Suite 3.0 software (Thermo Fisher Scientific, Inc.), using tmap-f3 on the Ion Torrent server for further analysis. Bam and FASTQ alignment files 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.

Catalogue of somatic mutations in cancer (COSMIC) database. COSMIC is an online database of somatically acquired mutations in human cancer. It is the most comprehensive resource for exploring the impact of somatic mutations in human cancer (15).

Statistical analysis. Correlation analysis was performed by simple linear regression analysis. The equation used was $Y=0.6179x+5.6398$, and was calculated using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Results

Mutations are detectable in purified MCF7 cells. Fig. 1 depicts the methodologies employed by the present study. Immunofluorescent cells were counted to determine the number of EpCAM-positive cells. The purity of MCF7 cells increased with the number of cells (Fig. 2A). The recovery rate of our method using H358-GFP cell lines was 84% (data not shown). Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) mutation, which is a known mutation in MCF7, was detected in MCF7 cells isolated through the Cytogen protocol. The frequencies of mutation were increased when purity was high; however even in samples with low purity, mutations were detected (Fig. 2B). Therefore, R^2 (between the purity and frequency of PIK3CA mut. in cancer panel results) of Fig. 2B indicates that the panel analysis was reliable.

Successful isolation of CTCs. CTCs were defined as EpCAM⁺ and CD45⁻ cells (Fig. 3). EpCAM⁺ cells were detected in 7/8 patients, and the average number of EpCAM⁺ cells was 8.6 (1-23; Table I). PC9 (EpCAM⁺) and KG-1 (CD45⁺) cell lines were used as positive controls during immunostaining. CTCs isolated for cancer panel analysis were amplified using whole genome amplification, and the average DNA amount was 32.7 μ g with high purity (A_{260}/A_{280} 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 I).

Cancer gene panel analysis. COSMIC was used to confirm the CCP results. When mutations were in the WBCs analyzed as negative controls, they were considered germ line mutations. These mutations in CTCs were excluded from the analysis. CTC-specific mutations were validated by comparing mutations between CTCs and WBCs (Table II). CTC-specific mutations had a detection rate of 62.5%, and these were enhancer of zeste polycomb repressive complex 2 subunit (EZH2), notch 1 (NOTCH1), AT-rich interaction domain 1A, serine/threonine kinase 11, fms related tyrosine kinase 3, MYCN proto-oncogene, bHLH transcription factor, APC, WNT signaling pathway regulator, and phosphatase and tensin homolog (PTEN).

Discussion

Patients with early-stage hormone receptor-positive breast cancer may have several effective treatment options; however, multiple patients also develop recurrence and metastasis. Therefore, the early diagnosis of cancer, prognostication and monitoring of the genomic characteristics of tumor cells are essential (3). However, biopsies of tumor tissues are not always easy to repeat. CTCs may be able to overcome this limitation of tumor tissue biopsies as CTCs have similar characteristics to those of primary tumors, and may cause metastasis (16). Due to the presence of CTCs at low concentrations (1 in 1×10^9) (17), it is important to enrich or isolate them from the blood effectively. CellSearch (Menarini Silicon Biosystems, Bologna, Italy), a well-known commercial device, isolates CTCs through EpCAM⁺ selection (18). It is not possible to achieve this EpCAM⁺ selection technique

when tumor cells downregulate EpCAM expression (19). In addition, a previous study reported that a significant portion of CTCs are EpCAM⁻ (20).

Our group has developed a novel CTC enrichment technique based on cell size difference and double negative selection, which removes nontargeted cells with an antibody complex against WBCs (13). Using this technique, CTCs were effectively isolated at a purity that was sufficient for genomic analysis (Fig. 2). In addition, COSMIC mutations were detected even in patients lacking EpCAM⁺ cells (Table I), demonstrating that this technology was able to effectively isolate EpCAM⁻ CTCs. Among the CTC-specific COSMIC gene mutations that were identified, EZH2, NOTCH1 and PTEN have been reported to affect breast cancer status (21-24). EZH2 mutations cause abnormal DNA methylation and promote mammary stem cell expansion and metastasis (21). NOTCH1 has been reported to regulate the epithelial-mesenchymal transition and to promote the migration and invasion of breast cancer cells (22). Furthermore, NOTCH1 expression in breast tumor tissues is higher than in normal tissues (23). Mutated PTEN is not able to inhibit the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin pathway, thereby losing its tumor suppressor activity (24).

There was a previous report on single-gene mutation analysis of CTCs and WBCs (25), and another study performed cancer panel analysis of CTCs without WBCs as controls (26). However, to the best of our knowledge, the present study is the first attempt at a CCP using CTCs in conjunction with WBCs. CTC-specific COSMIC mutations were identified, and genomic information that may be useful for precision medicine was provided.

In conclusion, the CTC isolation technique used by the present study was effective, providing sufficient purity for genomic analysis, and demonstrated that CCP analysis is a potential application for precision medicine.

Acknowledgements

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SHC, MSK JL and BHJ directed and designed the study with contributions from CHL and SJL. DHL, DYH and PSP analyzed the circulating tumor cells from the patients. MSC and HKL maintained the cell lines and performed the spike tests. SHA, BHS, JWL and JHY provided patients' blood samples and clinical information. NJK, WCL and KSY performed the CCP (comprehensive cancer panel) analyses. CHL and MSK

Table II. 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
CG 242	PDGFRA	p.V824V	PDGFRA	p.V824V		
	FLT3	p.L561L	FLT3	p.L561L		
	MSH2	Unknown				
	EZH2	p.D730fs*1				
	NOTCH1	p.D1698D				
CG 243			ARID1A	p.D1850fs*4	ARID1A	p.D1850fs*4
	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
CG 245			FLT3	p.L561L	FLT3	p.L561L
	MSH2	Unknown				
	STK11	p.L282fs*3				
			MYCN	p.P358L		
			APC	p.R554*		
		NOTCH1	p.D1698D			
		PTEN	p.L57fs*6			

WBC, white blood cell; CTC, circulating tumor cell; AA, amino acid; CG, patient ID; MSH2, mutS homolog 2; PDGFRA, platelet derived growth factor receptor α ; FLT3, fms related tyrosine kinase 3; STK11, serine/threonine kinase 11; SMARCB1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1; NOTCH1, notch 1; EZH2, enhancer of zeste polycomb repressive complex 2 subunit; ARID1A, AT-rich interaction domain 1A; MYCN, MYCN proto-oncogene, bHLH transcription factor; APC, APC, WNT signaling pathway regulator; PTEN, phosphatase and tensin homolog.

wrote the manuscript with contributions from JL and BHJ. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Asan Medical Center (Institutional Review Board no. 2013-1048). All blood samples and medical data used in the present study were irreversibly anonymized.

Consent for publication

Not applicable.

Competing interests

CHL, SJL, SHC, DHL, DYH, MSC, PSP, HKL, MSK, JL and BHJ are employees of Cytogen, Inc. (Seoul, Korea), and the CTC isolation kit was supplied courtesy of Cytogen, Inc. The authors report no other competing interests.

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