Monitoring of Circulating Tumor DNA in Serially Collected Plasma in Patients with Gastric Cancer

A Thesis Submitted to
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ABSTRACT

Monitoring of Circulating Tumor DNA in Serially Collected Plasma in Gastric Cancer Patients

Circulating tumor DNA (ctDNA) has emerged as a candidate biomarker for cancer screening. However, the studies on the usefulness of ctDNA for post-operative recurrence monitoring are limited. The present study monitored ctDNA in post-operative blood, employing cancer-specific rearrangements. Personalized cancer-specific rearrangements in 25 gastric cancers were analyzed by whole-genome sequencing (WGS) and were employed for ctDNA monitoring with blood until 12 month after surgery. Personalized cancer-specific rearrangements were identified in 19 cases. The median lead time, which is the median duration between ctDNA-positive detection and recurrence, was 4.05 months. Post-operative ctDNA prior to clinical recurrence was significantly correlated with cancer recurrence within 12 months of surgery ($P = 0.029$), in contrast to the finding of no correlation for pre-operative ctDNA, suggesting the clinical usefulness of post-operative ctDNA monitoring for cancer recurrence in gastric cancer patients However, the clinical application of ctDNA can be limited by the presence of ctDNA non-shedders (42.1%, 8/19) and by inconsistent post-operative ctDNA positivity.

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1. Introduction

1.1 Gastric cancer recurrence

Gastric cancer is one of the most commonly diagnosed and deadly cancers in the world (Rawla and Barsouk 2019). In 2018, the incidence of gastric cancer ranked fifth and mortality ranked third for all cancers, and all ages in worldwide (Figure 1). Curative surgical resection is the most effective treatment for gastric cancer (Shin et al. 2016). However, patients who have undergone surgical resection could experience recurrence, especially within 2 years. Recurrence of gastric cancer has a great effect on mortality. The recurrence rates of gastric cancer after curative surgical resection are various according to tumor size, stage of disease, and lymphatic invasion (Liu et al. 2016, Jung et al. 2014, Norio Shiraishi 2000). Early or timely detection of gastric cancer recurrence can help reduce incidences of cancer-related deaths (Hamashima et al. 2013)

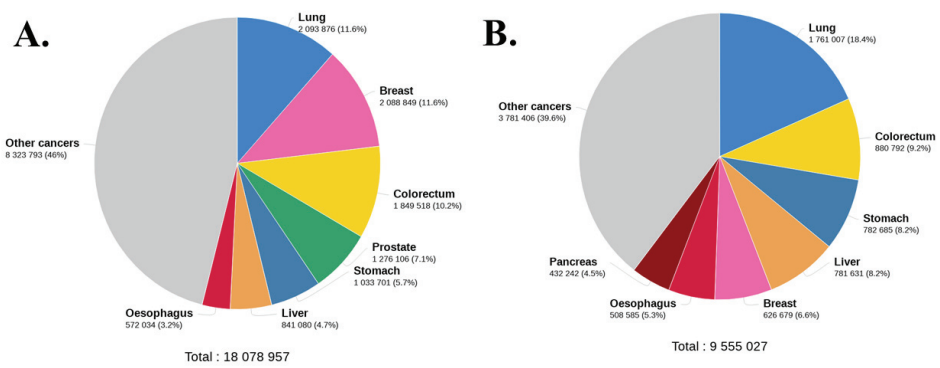

Figure 1. Cancer incidence and mortality in worldwide, Globocan 2018.

A. Cancer incidence for all cancers, and all ages in worldwide. B. Cancer mortality for all cancers, and all ages in worldwide.

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For detection of gastric cancer recurrence, computed tomography (CT), and positron emission tomography (PET) usually are used (Hallinan and Venkatesh 2013). However, those methods depend on morphological changes, and thus, those accuracy is poor. The most frequent recurrence patterns in early gastric cancer after surgery are peritoneal seeding and remnant tumors from surgical resection. But they are difficult to diagnosis with CT (Hamakawa et al. 2015, Choi et al. 2016).

Recently, liquid biopsy has been widely used in clinical monitoring for cancer diagnosis owing to various advantages, such as non-invasiveness, potential rapidity and precision. Liquid biopsy utilizes circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), samples derived from blood, urine, saliva, cerebrospinal fluid (CSF) and the like (Bai and Zhao 2018, Siravegna et al. 2017). The present dissertation was designed for detection of ctDNA in gastric cancer patients after curative surgical resection.

1.2 Emerging role of circulating tumor DNA

Circulating tumor DNA (ctDNA) is a fragment of genetic material shed from necrotic or apoptotic tumor cells, introduced thereby into systemic circulation, and found in the cell-free component of blood (Figure 2) (Bettegowda et al. 2014, Dawson, Rosenfeld, and Caldas 2013, Diehl et al. 2008). ctDNA contains tumor genetic alteration sequences such as point mutation or rearrangements.

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Rearrangements include deletions, insertions, translocations, and others (Lars Feuk 2006).

ctDNA has emerged as a candidate biomarker for screening of cancer patients, for monitoring of cancer recurrence, and for determining somatic mutations in cancer patients (Sung et al. 2017, Park, Cho, et al. 2018, Cohen et al. 2018). There are many techniques to detect alteration from ctDNA, among which are polymerase chain reaction (PCR)-based assays, and next generation sequencing (NGS)-based assays (Bettegowda et al. 2014).

ctDNA's high fragmentation state and low amounts makes it difficult to detect or analysis accurately (Vendrell et al. 2017). In order to remedy this problem, this dissertation employed cancer-specific rearrangements to enhance the sensitivity and specificity of ctDNA monitoring for cancer recurrence.

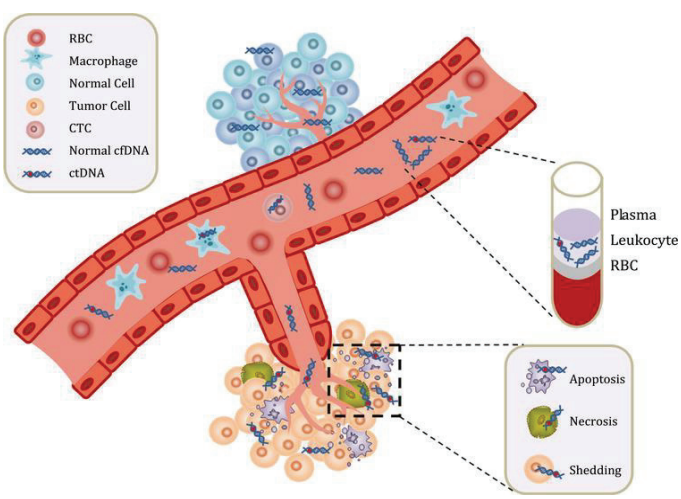

Figure 2. Circulating cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) are found in serum and plasma fractions from blood. The

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mechanism of ctDNA release is unknown, though apoptosis, necrosis, and active shedding from tumor cells have been hypothesized. Once ctDNA is isolated, it can be quantitated and analyzed for genomic alterations (Hahn et al. 2019)

1.3 Next generation sequencing

Next generation sequencing (NGS), also known as high-throughput sequencing, can determine the sequences of large numbers of DNA variation, in the thousands or millions at once (Figure 3) (Kwon 2012). AS such it offers much greater sensitivity and accuracy than can the Sanger sequencing techniques (Simona Serratì 2016). The clinical application of NGS has rapidly evolved, and widened, from diagnostics to prognostics (Kamps et al. 2017). Especially, NGS is actively employed in the field of cancer research for discovery of biomarkers that can be utilized as targets in personalized therapies (Basho 2015).

Whole genome sequencing (WGS), using NGS techniques, can be used to obtain information on the entire genome, including the intron and exome regions. WGS offers high resolution genetic alterations, and comprehensive evaluation of cancer genomics (Horak, Frohling, and Glimm 2016). With these features, WGS is predominantly applied for detection of genetic alterations in cancer (Nakagawa and Fujita 2018). The present dissertation employed WGS to analyze samples' cancer-specific rearrangements, especially translocations, in order to employ them as markers for detection of ctDNA in blood.

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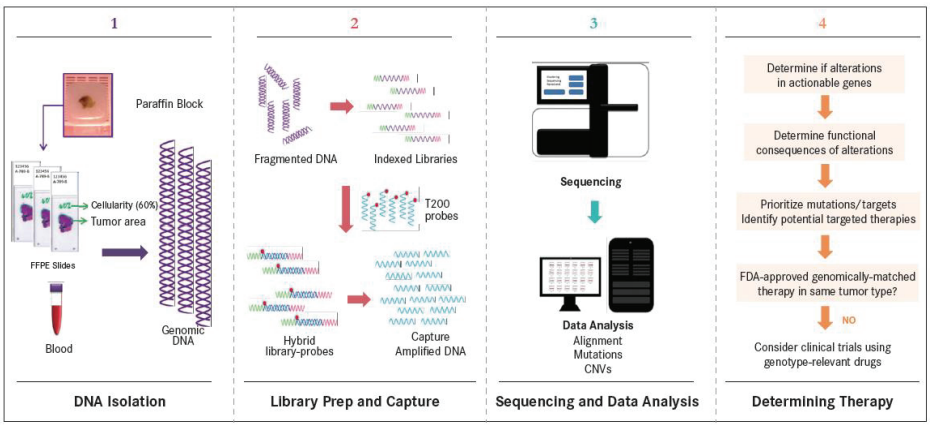

Figure 3. Overview of a Potential Next-Generation Sequencing Work Flow (Basho 2015).

1.4 Laser capture microdissection

Laser capture microdissection (LCM) is used to accurately separate specific cells of interest from tumor, stromal and normal tissue within a single biopsy specimen. Thereby, it is possible to obtain specific tumor enrichment cells (S Curran 2000, Virginia Espina 2006) (Figure 4). Such enrichment cells are well suited for genomic analysis (De Marchi et al. 2016).

In the analysis of cancer tissues containing low percentage of cancer cells, obtaining rearrangement information is may not be easy. Due to the fact that non-rearranged sequences outnumber rearranged ones. By enrichment of cancer cells and utilization of the resultantly enriched rearranged sequences, the chance of detection of rearrangements is enhanced. This dissertation employed LCM to increase the accuracy of WGS and to obtain specific tumor enrichment cells thereby.

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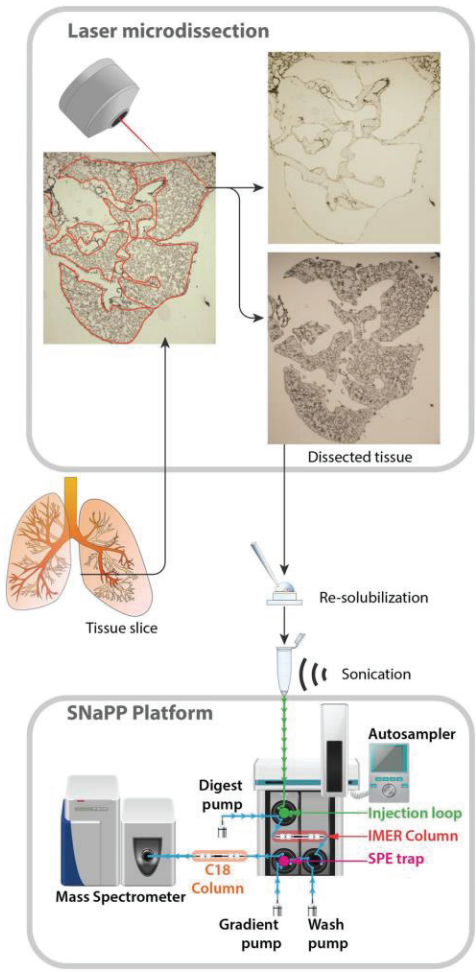

Figure 4. High-throughput LCM-proteomics platform for ultrasensitive analysis. Schematic of the LCM proteomics workflow (Clair et al. 2016).

1.5 Quantitative PCR

Quantitative polymerase chain reaction (qPCR), also known real-time PCR, is a method that can quantify target DNA by amplification (Dhanasekaran et al. 2010). qPCR is used to measure the emitted fluorescence of targeted DNA during

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a PCR. In general, traditional PCR detects only the presence or absence of target products at the end point. qPCR, by contrast, detects the amounts of PCR products in the exponential growth phase. Thus using qPCR, with its high technical sensitivity (<5 copies) and a high precision (<2% standard deviation) (Klein 2002). Both absolute quantification and relative quantification of target genes are possible. As absolute quantification can determine the absolute copy number of targets (Jie FU 2009). qPCR is applied for diagnosis of infectious disease, cancers and others (Espy et al. 2006). In the present dissertation, qPCR was employed for quantitation of the total amount of ctDNA in plasma.

2. Purpose of This Study

The aims of this study were 1) to conduct a feasibility test for detection of low-level post-operative ctDNA in serially collected blood samples in early phases of clinical recurrence in gastric cancer patients who had undergone surgical resection of primary tumor, and 2) to evaluate the usefulness of post-operative ctDNA for monitoring of cancer recurrence.

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3. Materials and Methods

3.1 Study design

This study retrospectively and preferentially selected 25 recurrent cases whose frozen primary tumor samples as well as serial plasma samples obtained up to 12 months after curative surgical resection were both available. 2 cases already had peritoneal metastasis and found after surgery. 19 cases had recurrence and 4 cases had not a recurrence after surgical resection within 12 month. DNAs were prepared after laser-capture microdissection (LCM). Rearranged sequences were analyzed from WGS, and were confirmed 19 cases by PCR sequencing. The presence of ctDNA was monitored by PCR amplification of personalized cancer-specific rearranged sequences in serially collected plasma samples (Figure 5). .

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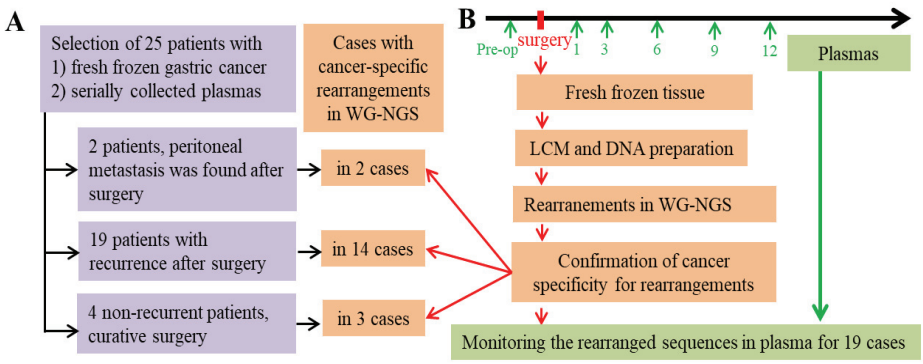

Figure 5. Study Scheme

A. Cases utilized in present study. B. Methodological procedure of present study.

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3.2 Patients sample collection

Plasma samples were prepared from whole blood on pre-operative day and at post-operative 1, 3, 6, 9, and 12 months after surgical resection of primary cancer. Fresh-frozen paired tumor and normal tissues were obtained from the Tissue Bank of the National Cancer Center, Korea. All of the patients had been diagnosed as gastric cancer stage II, III or IV according to the seventh edition of the AJCC TNM-staging system, and their clinical information is summarized in Tables 1 and 2. The use of plasma and tissue samples for the present study was approved by the Institutional Review Board of the National Cancer Center, Korea (NCC2014-0025), and all methods were performed in accordance with the relevant guidelines and regulations. The informed consents for all participants in the present study were obtained from our previous study (NCCTS-04-105) for plasma and from the Tissue Bank for frozen tissues, and waived for the present study.

3.3 Laser-capture microdissection (LCM) from fresh-frozen samples

A pathologist confirmed the gastric cancer cells for each sample and demarcated the tumor areas on Hematoxylin and Eosin (H&E)-stained slides. To obtain samples consisting of 70% or more tumor cells, tumor areas were dissected using a laser-capture microdissection (LCM) instrument (Ion LMD, Jungwoo F&B, Korea). The dissected tumor fragments were incubated in 1 M

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sodium thiocyanate overnight. Subsequently, DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The fresh-frozen tissues were used also for paired normal gastric tissue DNA preparation after confirmation on H&E stained slides by a pathologist.

3.4 Library preparation and WGS

Preparation of sequencing libraries using the TruSeq Nano DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and 150-bp paired-end sequencing by Illumina HiSeqX Ten with 30X average read depth were performed at Macrogen (Korea).

3.5 Analysis of rearranged sequences in WGS data

From the raw sequence data (FASTQ file), SAM files were prepared by the Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net) using the UCSC Human Reference Genome hg19. BAM files were generated with SAMtools (http://samtools.sourceforge.net/). Quality control with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was performed by trimming data with a sequence quality score less than 30. The trimmed BAM file was sorted with SAMtools according to the leftmost coordinates, and was indexed with SAMtools. The whole-genome data are summarized in Table 3.

Structural inter- and intra-chromosomal rearrangements were detected with Manta (Chen, Schulz-Trieglaff, et al. 2016) in the tumor-normal analysis mode.

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The analyzed structural rearrangements were then visualized with the Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/), after which the rearranged sequences were constructed based on the whole-genome information for rearrangements. Comparing the tumor and matched normal translocation results, the regions shown on both were excluded. The rearranged sites from the WGS are summarized in Table 4.

3.6 Confirmation of selected rearrangements in cancer DNA

For amplification of the rearranged sequences, PCR primers were designed with Primer3. PCR primers for longer rearranged sequences (200-1,000 bp) were designed for candidate rearranged sites from the WGS data (marked as long PCR in Table 5). After the amplification of the DNAs from the paired tumor and normal samples, the rearranged sequences were confirmed by Sanger sequencing of the amplified tumor-specific PCR products. After exclusion of non-specific amplifications, PCR primers for shorter rearranged sequences at confirmed rearranged sites were designed again (marked as short PCR in Table 5), and specific rearranged sequences were confirmed again by PCR with short primers and by Sanger sequencing by employing DNAs from the paired tumor and normal samples. PCR was performed for each sequence under the following conditions: initial incubation at 95°C for 10 min, followed by 45 cycles of 30 s at 95°C, 30 s at annealing temperature for each primer pairs, and 30s at 72°C in a mixture containing 1X PCR buffer II (Roche, Mannheim, Germany) with 1.5

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mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, and of 10 ng of genomic DNA in a final volume of 20 µl. For some PCR amplifications, modifications were made for specific amplification as indicated in Table 3. For the amplification controls, GAPDH primers (Table 5) were used. The amplified products were purified using the AxyPrep PCR Clean up kit (Axygen, Union City, CA) to remove leftover primers, and were then sequenced with forward or reverse primers used in the PCR reaction (Tables 5).

3.7 Detection of rearranged sequences in plasma cell-free DNA

Cell-free DNA (cfDNA) from plasma was prepared using the QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) according to the instruction manual, with an input plasma volume of 1 ml and an elution volume of 30 µl. PCR was performed under the same conditions as above, except that 2 µl of eluted cfDNA was used for each PCR reaction. The PCR product amplified from cfDNA for each sample was used for confirmation by Sanger sequencing.

To monitor the ctDNA levels in plasma, all available remnant plasma from the post-operative ctDNA-positive cases (GC4, GC8, GC9, GC14, GC15, GC17 and GC22) and plasmas from several selected post-operative ctDNA-negative cases (GC12, GC18, GC31, GC32, GC33 and GC34) was employed for the quantitative PCR. Quantitative PCR was performed at a one site for each sample, and the primer sequences were as indicated in Table 6. Quantitative PCR was carried out according to the manufacturer's protocol from FastStart Essential

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DNA Probes Master (Roche) by the LightCycler® 96 Real-Time PCR System (Roche) in a 25 µL reaction mixture constituted of 10 µL 2x FastStart Essential DNA Probes Master mix, 10µL template DNA (out of a total of 30 µl eluted cfDNA from 1 ml plasma), and primers (10 pmole each). To confirm the ctDNA negativity in pre-operative ctDNA negative cases (GC1, GC6, GC10, and GC12) by quantitative PCR, the 25 µL cfDNA (equivalent to 833 µL plasma) and 5 µL cfDNA (equivalent to 167 µL plasma) were employed for rearranged sequences and for the reference gene, *GAPDH*, respectively

3.8 Statistical analysis

In the analysis of pre-operative ctDNA positivity and clinical factors including T stage, N stage, clinical stage, and Lauren classification, Fisher's exact test was used. In the analysis of the correlation between post-operative ctDNA positivity and clinical recurrence, Fisher's exact test was also used with the consideration of post-operative ctDNA as positive 1) when any cancer-specific rearranged sequence was detected in any post-operative plasma sample within 12 months after surgery or 2) when ctDNA-positive cases detected only prior to clinical recurrence.

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Table 1. Analysis of personalized cancer-specific rearranged sequences in gastric cancer patients

ID	Sex	Age	Recur	Stage (TNM)	Rearranged sites in WG-NGS	Primers designed for PCR sites	Cancer-specific PCR	Confirmed by Sanger Sequencing	Finally validated sites	PreOp ctDNA	PostOp ctDNA	Lead time (months)
GC1	M	68	R	IIIA (T3N2M0)	48	12	7	4	4	-	-	
GC4	M	67	R	IIIC (T4aN3bM0)	44	7	3	3	3	+	+	9.4
GC6	M	64	R	IIIB (T3N3aM0)	3	3	3	3	3	-	-	
GC7	M	57	R	IIIB (T4aN2M0)	3	3	3	1	1	-	-	
GC8	M	53	R	IIIC (T4bN3aM0)	8	6	4	3	2	+	+	-1.4
GC9	M	44	R	GIST (T4N0M0)	8	8	3	3	3	+	+	5
GC10	M	73	R	IIIB (T4aN2M0)	31	5	3	3	3	-	-	
GC11	M	70	R	IIIB (T3N3aM0)	90	12	6	5	5	+	+	5.6
GC12	M	71	R	IIIC (T4aN3aM0)	3	3	3	2	2	-	-	
GC14	M	53	R	IIIB (T3N3bM0)	25	11	4	2	2	-	+	0.7
GC15	M	42	R	IIIC (T4bN3aM0)	7	7	5	3	2	+	+	3.1
GC17	M	71	R	IV (T4aN3bP1)	16	12	8	5	5	+	+	*
GC18	M	41	R	IIB (T3N1M0)	6	5	4	2	2	-	-	
GC21	M	77	R	IIIC (T4aN3aM0)	40	13	6	6	5	-	-	
GC22	M	49	R	IV (T4aN3bP1)	23	9	3	3	3	+	+	*

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GC31	**M**	**54**	**N**	**IIA (T3N0M0)**	**6**	**5**	**4**	**3**	**3**	+	-
GC32	**M**	**70**	**R**	**IIIC (T4bN2M0)**	**11**	**8**	**8**	**8**	**8**	+	-
GC33	**M**	**44**	**N**	**IIIC (T4bN3bM0)**	**6**	**6**	**6**	**6**	**6**	+	-
GC34	**M**	**63**	**N**	**IIIC (T4aN3aM0)**	**3**	**3**	**1**	**1**	**1**	+	-
GC2**	**F**	**62**	**R**	**IIB (T3N1M0)**	**0**	**0**	**0**	**0**	**0**		
GC3**	**M**	**64**	**R**	**IIIB (T4aN2M0)**	**0**	**0**	**0**	**0**	**0**		
GC5**	**F**	**72**	**R**	**IIIC (T4aN3bM0)**	**0**	**0**	**0**	**0**	**0**		
GC13**	**F**	**73**	**R**	**IIIA (T3N2M0)**	**0**	**0**	**0**	**0**	**0**		
GC23**	**M**	**60**	**R**	**IIIA (T3N2M0)**	**3**	**3**	**0**	**0**	**0**		
GC35**	**M**	**60**	**N**	**IIA (T3N0M0)**	**0**	**0**	**0**	**0**	**0**		
Total					**384**	**141**	**84**	**66**	**63**		

R, recurrence ; N, non-recurrence; PreOp, pre-operative; PostOp, post-operative ; -, negative ctDNA; +, positive ctDNA

*, peritoneal-seeding-positive cases ; ** no cancer-specific rearrangement was found in WGS, and no ctDNA monitoring was performed.

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Table 2. Clinical information for gastric cancer patients accrued in the present study

ID	Lauren	Histology*	Recurrent sites	Adjuvant Chemotherapy
GC1	Intestinal	Mod	Celiac axis LN	No
GC4	Intestinal	Poor + Mucin	Pancreas, Aorto-caval LN	Yes
GC6	Intestinal	Mod + Mucin	GJ Anastomosis , peritoneum, pleural	No
GC7	Intestinal	Mod	Duodenal stump, Porto-caval LN	No
GC8	Intestinal	Poor + Mucin	Peritoneum	Yes (palliative)
GC9		GIST	Liver, peritoneum	No
GC10	Intestinal	Mod + Neuro	Liver	No
GC11	Intestinal	Mod	Liver	No
GC12	Diffuse	Poor	Abdominal wall, mesentery	No
GC14	Intestinal	Poor	Aortico-caval and porto-caval LN	Yes
GC15	Diffuse	Poor	Para-aortic LN	Yes
GC17	Mixed	Poor + Mod	Bone, Peritoneum	Yes (palliative)
GC18	Diffuse	Signet	Colon	No
GC21	Intestinal	Mucin	Peritoneum	Yes
GC22	Mixed	Poor	Duodenal stump, para-arotic and thorax LN, peritoneum	Yes (palliative)
GC31	Intestinal	Mod	-	Yes
GC32	Diffuse	Poor	Gastrojejunostomy site	Yes
GC33	Intestinal	Poor	-	Yes
GC34	Diffuse	Poor + Mucin	-	Yes
GC2	Diffuse	Signet	Ovary	No
GC3	Intestinal	Mod	Peritoneum	No
GC5	Diffuse	Poor	LN (celiac, SMA) Peritoneum	Yes
GC13	Intestinal	Mod	Abdominal wall, Peritoneum	No
GC23	Intestinal	Poor	Liver	Yes
CG35	Diffuse	Poor	-	Yes

*Mod, moderate differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Mucin, mucinous adenocarcinoma; Signet, signet ring cell carcinoma; GIST, gastrointestinal stromal tumor.

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Table 3. The summary of whole genome shotgun data

Samples*	Sequencing reads**	Read length (bp)	Total yield (Mbp)	Throughput mean depth (X)	De-duplicated reads	De-duplicated reads % (out of total reads)	Mappable reads	Mappable reads % (out of De-duplicated reads)	Mappable yield (Mbp)	Mappable mean depth (X)	% ≥ 20X coverage	% ≥30X coverage
C1	709,303,590	150	106,395	37.2	621,425,520	87.6	596,493,644	96.0	89,474	31.3	87.9	59.3
C2	781,193,946	150	117,179	41.0	668,308,648	85.5	639,376,982	95.7	95,906	33.5	95.7	80.2
C3	712,376,406	150	106,856	37.4	626,052,682	87.9	598,921,057	95.7	89,838	31.4	91.7	69.3
C4	698,425,098	150	104,763	36.6	605,139,392	86.6	577,735,166	95.5	86,660	30.3	87.8	55.9
C5	791,615,928	150	118,742	41.5	680,288,418	85.9	650,897,946	95.7	97,634	34.2	95.4	77.7
C6	708,000,838	150	106,200	37.2	613,134,292	86.6	585,320,581	95.5	87,798	30.7	91.2	63.7
C7	812,888,522	150	121,933	42.7	693,087,484	85.3	660,310,511	95.3	99,046	34.6	93.9	80.3
C8	896,810,136	150	134,521	47.1	736,514,414	82.1	696,906,858	94.6	104,536	36.6	95.3	85.3
C9	844,658,256	150	126,698	44.3	705,211,798	83.5	670,322,267	95.1	100,548	35.2	91.3	76.3
C10	790,685,234	150	118,602	41.5	678,658,800	85.8	648,873,581	95.6	97,331	34.0	94.1	74.1
C11	793,476,142	150	119,021	41.6	728,996,018	91.9	703,929,460	96.6	105,589	36.9	92.5	68.7
C12	875,516,716	150	131,327	45.9	796,699,078	91.0	767,490,239	96.3	115,123	40.3	95.0	81.3
C13	833,771,924	150	125,065	43.7	753,325,656	90.4	720,937,639	95.7	108,140	37.8	97.2	83.9
C14	743,913,492	150	111,587	39.0	674,596,142	90.7	642,903,243	95.3	96,435	33.7	93.5	69.9
C15	774,265,852	150	116,139	40.6	706,541,954	91.3	671,262,631	95.0	100,689	35.2	94.7	73.3
C17	818,329,106	150	122,749	42.9	744,193,362	90.9	711,771,309	95.6	106,765	37.3	95.1	78.7
C18	832,615,954	150	124,892	43.7	757,690,626	91.0	721,345,196	95.2	108,201	37.9	96.2	86.2
C21	932,670,882	150	139,900	48.9	849,929,324	91.1	830,675,054	97.7	124,601	43.6	85.1	69.8
C22	792,898,934	150	118,934	41.6	729,640,526	92.0	689,123,721	94.4	103,368	36.2	89.0	63.7

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C23	896,658,266	150	134,498	47.0	820,943,624	91.6	786,588,769	95.8	117,988	41.3	79.5	59.9
C31	888,793,264	150	133,318	47	795,751,050	90	754,591,355	95	113,188	40	96	80
C32	850,050,766	150	127,507	45	757,429,632	89	720,588,360	95	108,088	38	89	73
C33	849,173,488	150	127,376	45	751,909,772	89	710,958,849	95	106,643	37	95	84
C34	761,028,374	150	114,154	40	654,636,996	86	611,376,192	93	91,706	32	94	76
C35	740,272,224	150	111,040	39	670,048,280	91	628,028,992	94	94,204	33	94	74
N1	755,041,068	150	113,256	39.6	629,405,906	83.4	598,474,285	95.1	89,771	31.4	93.3	74.7
N2	753,760,804	150	113,064	39.6	618,419,722	82.0	588,922,198	95.2	88,338	30.9	95.6	76.1
N3	890,317,982	150	133,547	46.7	719,723,830	80.8	687,210,434	95.5	103,081	36.1	95.9	87.3
N4	769,863,394	150	115,479	40.4	633,742,190	82.3	602,626,990	95.1	90,394	31.6	93.8	76.4
N5	756,558,900	150	113,483	39.7	626,999,518	82.9	596,709,074	95.2	89,506	31.3	95.0	75.7
N6	794,694,578	150	119,204	41.7	650,189,616	81.8	619,773,551	95.3	92,966	32.5	94.5	80.1
N7	780,488,730	150	117,073	41.0	634,288,872	81.3	600,064,513	94.6	90,009	31.5	94.0	78.1
N8	760,362,986	150	114,054	39.9	636,638,606	83.7	602,714,906	94.7	90,407	31.6	93.7	76.1
N9	725,814,732	150	108,872	38.1	638,267,818	87.9	608,036,087	95.3	91,205	31.9	92.3	71.0
N10	717,860,220	150	107,679	37.7	636,986,404	88.7	611,023,215	95.9	91,653	32.1	92.3	71.1
N11	832,545,506	150	124,881	43.7	763,407,340	91.7	712,859,717	93.4	106,928	37.4	95.3	83.9
N12	682,868,058	150	102,430	35.8	636,499,610	93.2	591,066,010	92.9	88,659	31.0	91.7	60.8
N13	755,065,092	150	113,259	39.6	699,560,412	92.6	656,750,764	93.9	98,512	34.5	96.5	76.7
N14	759,208,722	150	113,881	40	697512430	92	650696589	93	97604	34	94	75
N15	743,341,192	150	111,501	39	690719758	93	642778700	93	96416	34	94	73
N17	804,629,024	150	120,694	42	740433446	92	698284822	94	104742	37	95	83
N18	884,177,552	150	132,626	46	807540784	91	756408858	94	113461	40	96	88

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N21	797,243,836	150	119,586	42	738823662	93	696666129	94	104499	37	95	82
N22	737,750,022	150	110,662	39	682215916	93	641215766	94	96182	34	94	74
N23	757,958,182	150	113,693	40	698074374	92	656898693	94	98534	35	94	76
31N	923,352,638	150	138,502	49	855,038,666	93	807,365,803	94	121,104	42	97	91
32N	826,421,956	150	123,963	43	762,635,160	92	720,791,758	95	108,118	38	96	86
33N	810,046,520	150	121,506	43	739,885,782	91	699,652,296	95	104,947	37	96	84
34N	814,124,764	150	122,118	43	757,778,802	93	716,601,822	95	107,490	38	95	84
35N	819,133,546	150	122,870	43	757,192,374	92	708,490,893	94	106,273	37	96	84

*The tumor and matched normal genomes are discriminated with the use of 'C' and 'N', respectively.

**The mean and median coverage as well as the % of bases (>= 20 reads) were calculated onto the targeted regions.

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Table 4. Translocation sites identified by whole genome sequencing.

Sample	Translocation number	Chromosome at site 1	Location at site 1	REF at site 1	Gene at site 1	Chromosome at site 2	Location at site 2	REF at site 2	Gene at site 2
1	1	2	145462032	G	TEX41	12	25128395	T	
1	2	2	133040364	A		17	43371479	C	MAP3K14
1	3	8	52307387	T	PXDNL	10	66095917	A	
1	4	8	53827530	A		10	59034193	C	
1	5	8	90474961	C		10	58859380	A	
1	6	8	92124417	C	LRRC69	10	66061499	T	
1	7	9	78556898	A	PCSK5	14	90634346	A	KCNK13
1	8	9	105124337	A		14	53007612	T	TXNDC16
1	9	15	25646343	T	UBE3A	17	37061676	T	LASP1
1	10	15	40391906	G	BMF	17	70845010	A	SLC39A11
1	11	15	41126725	G	RP11-532F12.5	17	55407764	G	MSI2
1	12	15	48245818	C	RP11-208K4.1	17	66029771	A	KPNA2
1	13	15	52314553	G	MAPK6	17	66049593	C	
1	14	15	53427433	G		17	48603566	C	MYCBPAP
1	15	15	63232698	A		17	43377923	G	MAP3K14
1	16	15	63244483	G		17	37031108	A	LASP1
1	17	15	63244902	C		17	55406950	G	MSI2
1	18	15	74119677	C		17	77338652	C	RBFOX3
1	19	15	74120551	G		17	74466269	A	RHBDF2
1	20	15	74274133	T	STOML1	17	48548049	G	ACSF2

1	21	15	74476625	A	STRA6	17	34026330	A	AP2B1
1	22	15	74478740	G	STRA6	17	70833969	G	SLC39A11
1	23	15	75054200	G		17	37056443	G	LASP1
1	24	15	75149084	G	SCAMP2	17	37059146	G	LASP1
1	25	15	75161399	G	SCAMP2	17	38288961	T	MSL1
1	26	15	79055858	C	ADAMTS7	17	55386803	T	MSI2
1	27	15	90748744	T	SEMA4B	17	48667643	C	CACNA1G
1	28	15	95556518	C		17	59788071	A	BRIP1
1	29	15	100573965	A	ADAMTS17	17	48603536	G	MYCBPAP
1	30	15	101636838	C		17	66032867	T	KPNA2
1	31	15	101684211	G		17	55780255	C	
1	32	15	40332699	C	SRP14	20	62407003	C	ZBTB46
1	33	15	63249297	C		20	57657700	T	
1	34	15	79070679	G	ADAMTS7	20	38851307	G	
1	35	15	85370560	C	ALPK3	20	34734321	T	EPB41L1
1	36	15	85377460	C	ALPK3	20	36981365	T	LBP
1	37	15	89737649	G	ABHD2	20	37083167	G	
1	38	17	30416248	T	RP11-640N20.6	20	51171125	T	
1	39	17	37031969	G	LASP1	20	57649834	T	
1	40	17	38283027	C	MSL1	20	62827275	T	MYT1
1	41	17	41445034	A		20	51170140	A	
1	42	17	48581302	C	RP11-94C24.6	20	37074268	T	SNHG11
1	43	17	55403234	T	MSI2	20	62503097	C	TPD52L2
1	44	17	55787511	A		20	21088452	A	
1	45	17	55841215	T		20	32794824	T	ASIP

1	46	17	56908130	T	PPM1E	20	40123512	A	CHD6
1	47	17	59491073	A	C17orf82	20	52195543	C	ZNF217
1	48	17	66066927	A		20	61040470	T	GATA5
4	1	1	972142	A	AGRN	11	27696494	A	BDNF
4	2	1	110197452	C	GSTM4	11	74521982	T	RNF169
4	3	1	22301578	C	CELA3B	12	27422600	G	STK38L
4	4	1	38241433	A		19	11422138	G	TSPAN16
4	5	1	42033303	G	HIVEP3	20	3020050	A	PTPRA
4	6	1	42078237	A	HIVEP3	20	3057090	T	
4	7	2	97217404	T	ARID5A	10	63863687	C	
4	8	2	97251183	T		10	63902185	T	
4	9	2	177913516	A	AC079305.11	12	112412651	A	TMEM116
4	10	2	177919086	A	AC079305.11	12	112118647	G	BRAP
4	11	2	178191754	A	NFE2L2	12	112118374	T	BRAP
4	12	3	103031286	A		6	13191135	T	PHACTR1
4	13	3	145450285	T		20	21440003	T	
4	14	3	189782119	T	LEPREL1	21	27253642	A	APP
4	15	5	153039080	A	GRIA1	6	13191167	A	PHACTR1
4	16	5	16964680	C		15	85597274	G	PDE8A
4	17	5	110190096	A		15	93158882	A	FAM174B
4	18	5	110190835	T		15	93143347	G	RP11-386M24.9
4	19	5	17297224	C		16	70094456	A	PDXDC2P
4	20	5	94351854	A	MCTP1	X	155234567	G	IL9R
4	21	6	160040535	A		7	29925262	A	WIPF3
4	22	6	130793704	C		15	45109941	T	Unknown

4	23	6	39222246	T		17	79874698	G	SIRT7
4	24	6	39227651	A		17	79951100	T	ASPSCR1
4	25	6	39229049	A		17	79938963	A	ASPSCR1
4	26	7	70911949	A	WBSCR17	8	73783868	T	KCNB2
4	27	7	71465814	A	CALN1	13	29472417	G	
4	28	7	116721614	G	ST7	22	34889664	A	
4	29	8	101525033	G	ANKRD46	10	27061543	A	ABI1
4	30	8	17494788	T	PDGFRL	11	74426544	A	CHRDL2
4	31	8	22273255	G	SLC39A14	12	69645633	A	CPSF6
4	32	8	22343195	C	PPP3CC	12	121757392	A	ANAPC5
4	33	8	73783971	A	KCNB2	16	60623988	T	
4	34	8	142263755	T	RP11-10J21.3	16	78455636	G	WWOX
4	35	10	32339793	G	KIF5B	12	113464446	G	
4	36	11	955876	G	AP2A2	15	45110387	C	
4	37	12	51591498	T	POU6F1	17	7297495	A	TMEM256-PLSCR3
4	38	12	51839109	T	SLC4A8	17	7296986	G	TMEM256-PLSCR3
4	39	12	11378799	C	RP11-711K1.8	Y	9872085	C	
4	40	12	19788516	C		Y	6633260	G	
4	41	14	34948250	C		16	8035894	C	
4	42	14	35316779	T	BAZ1A	16	7095851	G	RBFOX1
4	43	15	86131119	T	AKAP13	16	70426743	A	ST3GAL2
4	44	18	9029362	C		X	124625754	T	
6	1	1	187108178	C	LINC01036	21	20792897	C	
6	2	1	221173095	A		21	20793122	T	
6	3	8	31385804	T		14	59220711	A	

7	1	1	119344484	T		22	29066022	A	TTC28
7	2	2	105965462	T	C2orf49	7	104387039	C	LHFPL3
7	3	8	135411044	T		22	29065925	T	TTC28
8	1	6	143706913	C		13	30221916	T	
8	2	7	126098912	T	GRM8	14	59221115	T	
8	3	8	35416270	G	UNC5D	20	9699004	T	PAK7
8	4	8	36073941	T		20	16272773	A	KIF16B
8	5	8	38736522	C		20	11622313	G	
8	6	10	107808508	A		22	29065805	G	TTC28
8	7	11	189682	C	BET1L	17	30036	C	DOC2B
8	8	13	76433089	G	LMO7	17	64740469	T	PRKCA
9	1	8	29942495	A	TMEM66	9	131456462	A	SET
9	2	8	29980093	A	LEPROTL1	9	124210751	T	RP11-162D16.2
9	3	8	30259392	T	RBPMS	9	128966342	G	
9	4	8	30262787	G	RBPMS	9	135746039	T	AK8
9	5	8	30296784	A	RBPMS	9	135610016	G	AK8
9	6	8	41482627	A	AGPAT6	9	106011712	T	RP11-341A22.2
9	7	9	131288019	C	GLE1	16	30195284	T	CORO1A
9	8	9	138236266	G	C9orf62	16	29121678	T	CTB-134H23.3
10	1	1	201834999	C	IPO9	3	68794838	T	FAM19A4
10	2	1	58864562	C	DAB1	5	73145290	C	ARHGEF28
10	3	1	221173095	A		12	70184904	A	RAB3IP
10	4	1	22798838	G	ZBTB40	20	45979479	C	ZMYND8
10	5	1	23155464	A	EPHB2	20	45823894	G	
10	6	2	65309189	G	CEP68	11	129607610	G	

10	7	2	138364748	G	THSD7B	12	2993827	C	RHNO1
10	8	3	69493171	T		11	47240166	T	DDB2
10	9	3	116832711	T	LSAMP	15	56251788	A	NEDD4
10	10	4	1286816	A	MAEA	19	10917606	C	DNM2
10	11	5	31853395	A	PDZD2	19	29756889	A	
10	12	5	43011100	A		19	30062259	G	
10	13	5	45211237	A		19	29553655	A	
10	14	5	46016179	G		19	29011544	T	AC005307.3
10	15	6	13191211	C	PHACTR1	11	31663176	T	ELP4
10	16	6	44160440	T		20	3380426	C	C20orf194
10	17	7	7929063	G	RPA3-AS1	8	143828742	T	LYPD2
10	18	7	8003640	A	RPA3-AS1	8	143913477	A	GML
10	19	7	55022069	T		15	37316602	C	MEIS2
10	20	7	55033516	T		15	72836720	C	ARIH1
10	21	7	99192594	T	GS1-259H13.10	15	50910956	C	TRPM7
10	22	8	24890967	T		15	51282476	T	AP4E1
10	23	9	104201348	A		16	1351522	T	RP11-616M22.7
10	24	9	4946347	G		20	43116501	C	TTPAL
10	25	12	63569196	G		18	57071334	C	
10	26	14	92292467	A	TC2N	17	41541673	T	
10	27	16	1351669	C	RP11-616M22.7	19	29639170	T	
10	28	17	17622943	T	RAI1	20	43654122	C	STK4
10	29	18	9990974	G		19	44766293	T	ZNF233
10	30	18	9261632	A	ANKRD12	21	42913288	T	SNORA32
10	31	20	6681160	C		21	20793122	T	

11	1	1	84516969	T		7	37551672	T	
11	2	1	19249595	A	IFFO2	13	106685144	G	
11	3	1	43888449	C	SZT2	13	106683814	G	
11	4	1	44019669	C	PTPRF	13	106683809	A	
11	5	1	52658821	A	ZFYVE9	13	80174024	A	
11	6	1	204523693	A	MDM4	17	70560379	C	
11	7	1	205455606	A		17	70713020	A	SLC39A11
11	8	1	205458098	G		17	16346010	A	FAM211A
11	9	1	38167534	G	CDCA8	20	62355028	G	RP4-583P15.15
11	10	1	46105734	A	GPBP1L1	X	70730016	A	TAF1
11	11	1	71570880	A	ZRANB2-AS2	X	11732206	T	
11	12	1	84514368	C		X	34087817	T	
11	13	2	180678534	G	ZNF385B	4	75641755	C	
11	14	2	38820590	A	HNRNPLL	6	126348359	G	TRMT11
11	15	2	38821738	A	HNRNPLL	6	126347630	C	TRMT11
11	16	2	110899418	C	NPHP1	6	42891400	A	PTCRA
11	17	2	201679328	A	BZW1	7	73308857	G	
11	18	2	43165795	G		10	135150270	C	CALY
11	19	2	43166284	A		10	134833996	C	
11	20	2	43386215	G		10	134834006	G	
11	21	2	110927394	G	NPHP1	11	17766657	T	KCNC1
11	22	2	188515798	A	Unknown	19	29958128	A	CTC-525D6.1
11	23	2	216450615	T	AC012668.2	21	38824892	C	DYRK1A
11	24	2	216452331	T	AC012668.2	21	38792378	C	DYRK1A
11	25	2	216512759	A	LINC00607	21	38791413	C	DYRK1A

11	26	2	51426225	T	AC007682.1	22	29066121	A	TTC28
11	27	2	190789517	T	C2orf88	22	29066121	A	TTC28
11	28	3	167947978	T		7	84756115	T	SEMA3D
11	29	3	167948479	T		7	85033096	C	
11	30	3	186375138	A	HRG	12	119943377	T	CCDC60
11	31	4	43526907	T		6	24804903	A	FAM65B
11	32	4	21169457	A	KCNIP4	8	85608417	T	RALYL
11	33	4	75641494	T		12	15607116	C	PTPRO
11	34	4	75641987	G		12	66906252	C	GRIP1
11	35	5	604257	T	RP11-310P5.1	6	1925467	A	GMDS
11	36	5	6621387	A	NSUN2	6	1310844	C	FOXQ1
11	37	5	32313306	A	MTMR12	6	1310537	C	FOXQ1
11	38	5	12466	C		16	158794	G	NPRL3
11	39	5	161692794	C		20	17861598	A	
11	40	6	1720230	T	GMDS	7	77217122	T	PTPN12
11	41	6	1720631	A	GMDS	7	77231495	G	PTPN12
11	42	6	2150935	A	GMDS	8	141553819	A	AGO2
11	43	6	6828513	T		15	90041795	C	RHCG
11	44	6	42890019	A	PTCRA	15	89934031	T	LINC00925
11	45	6	43048371	G	PTK7	15	90051235	A	RP11-429B14.4
11	46	6	2004660	G	GMDS	17	48580141	G	RP11-94C24.6
11	47	6	86191584	T	NT5E	17	48713281	T	ABCC3
11	48	6	17205509	T		20	2651295	T	
11	49	6	17249851	T		20	2473331	G	ZNF343
11	50	6	92934476	C		X	11731565	C	

11	51	7	16213772	A	ISPD	8	87065565	T	PSKH2
11	52	7	18001843	T		8	57418796	C	RP11-17A4.2
11	53	7	21599734	C	DNAH11	8	104914209	A	RIMS2
11	54	7	31776852	C		8	142297346	T	SLC45A4
11	55	7	43841822	C	BLVRA	8	52060597	G	
11	56	7	17105549	C		11	65669340	A	FOSL1
11	57	7	17226219	G		11	65558240	C	OVOL1
11	58	7	68690430	C	RP11-3P22.2	11	48839015	A	
11	59	7	69026343	C		11	48839204	G	
11	60	7	74007211	A	GTF2IRD1	12	6050055	C	ANO2
11	61	8	11439657	A	LINC00208	9	139346288	C	SEC16A
11	62	8	31899424	G	NRG1	12	123444750	C	ABCB9
11	63	8	145273127	G	MROH1	16	958249	A	LMF1
11	64	8	145783242	C	ARHGAP39	16	958936	G	LMF1
11	65	8	15368088	A	TUSC3	17	73198934	G	NUP85
11	66	8	47916016	A		17	39757647	C	
11	67	8	128271195	A		X	95432614	T	
11	68	9	35998572	C		17	72743990	C	SLC9A3R1
11	69	9	35999257	A		17	72687738	A	CD300LF
11	70	9	100781031	G	ANP32B	17	28819295	T	GOSR1
11	71	9	116788352	A	ZNF618	17	512652	T	VPS53
11	72	9	136221936	A	SURF1	17	53075546	A	STXBP4
11	73	9	139331298	G	INPP5E	17	19357484	A	CTB-187M2.1
11	74	9	136231530	G	SURF4	18	9273113	A	ANKRD12
11	75	10	57004868	T	PCDH15	X	58375447	A	

11	76	10	58441082	A		X	11732262	T	
11	77	11	66168672	G		15	90670137	G	CTD-2315E11.1
11	78	11	15161188	T	INSC	20	62769851	G	
11	79	11	59398245	G	AP000442.1	22	42635927	G	
11	80	11	59511632	C	STX3	22	25126291	A	PIWIL3
11	81	11	59512091	G	STX3	22	42623287	A	
11	82	11	87934582	T		X	11731290	G	
11	83	12	95168027	A		13	30215705	T	
11	84	15	101463350	A	LRRK1	17	1272596	T	YWHAE
11	85	16	82141269	T	RP11-510J16.5	19	42541106	C	GRIK5
11	86	16	60774508	G		X	11732318	G	
11	87	17	56566123	A	HSF5	18	8833715	T	SOGA2
11	88	17	38820284	A	KRT222	20	60606771	T	TAF4
11	89	19	1728682	A		20	30892483	G	KIF3B
11	90	19	1745248	C	AC005256.1	20	30731363	T	TM9SF4
12	1	7	105813499	T		22	29065601	G	TTC28
12	2	8	146269287	A		15	89902939	G	
12	3	10	46964222	C	SYT15	22	20248979	G	RTN4R
14	1	1	41689552	T	SCMH1	3	14483301	G	SLC6A6
14	2	2	192274498	T	MYO1B	3	131875735	T	CPNE4
14	3	2	235734962	C		3	131876069	G	CPNE4
14	4	2	189847917	T	COL3A1	7	90330898	T	CDK14
14	5	2	133181372	G	GPR39	9	29634089	C	
14	6	3	101279866	T	TRMT10C	13	94807007	C	GPC6
14	7	3	107819921	C		14	68887971	G	RAD51B

14	8	3	188717366	T	TPRG1	X	103516590	A	
14	9	4	19539	G		7	18094813	A	
14	10	5	166483237	C		12	71020366	T	PTPRB
14	11	5	16904768	G	MYO10	17	41390034	G	
14	12	5	149175572	A	PPARGC1B	20	32389923	C	RP4-553F4.6
14	13	6	51480953	A	PKHD1	7	18195957	G	HDAC9
14	14	6	107119204	G	QRSL1	8	11708277	G	CTSB
14	15	6	11474272	T	RP11-716O23.1	13	32093257	A	
14	16	6	30390038	A		14	104726488	T	
14	17	6	170811623	T		19	30379419	G	
14	18	6	13191016	A	PHACTR1	X	123827499	A	TENM1
14	19	6	13191453	A	PHACTR1	X	123827677	G	TENM1
14	20	7	69344263	G	AUTS2	10	106547242	G	SORCS3
14	21	11	16996591	A	PLEKHA7	17	17791698	T	TOM1L2
14	22	12	94548661	T	PLXNC1	X	130832463	G	
14	23	14	68907674	A	RAD51B	17	71217646	A	FAM104A
14	24	15	41270064	C	INO80	19	30480428	T	URI1
14	25	16	67388976	T	LRRC36	17	71217432	A	FAM104A
15	1	1	28242429	G	RPA2	17	40739970	T	FAM134C
15	2	7	5362803	C	TNRC18	17	40516485	T	STAT3
15	3	8	22693532	C	PEBP4	9	28470635	G	LINGO2
15	4	9	74301829	A	TMEM2	X	69832219	A	TEX11
15	5	12	39605710	A		X	69891944	G	TEX11
15	6	12	39606044	A		X	46764254	T	
15	7	16	59053066	C	RP11-410D17.2	17	49441698	T	

17	1	1	142698258	T	RP11-417J8.6	17	59691613	T	
17	2	1	143178580	A	RP11-782C8.2	17	47747622	T	SPOP
17	3	1	143516594	A	RP11-435B5.6	17	59051174	A	BCAS3
17	4	2	148845078	C	MBD5	6	85902185	T	
17	5	2	98602169	A	TMEM131	13	84098746	A	
17	6	3	112308675	C		20	3695210	T	
17	7	3	104825395	T		22	29066186	T	TTC28
17	8	3	120173018	A	FSTL1	22	29065922	C	TTC28
17	9	8	53555473	C	RB1CC1	14	86291396	T	
17	10	9	115556659	C	SNX30	13	26240677	C	ATP8A2
17	11	9	115556996	A	SNX30	13	26236992	A	ATP8A2
17	12	9	25510616	T		20	23413125	T	
17	13	11	10378409	C		22	29066057	A	TTC28
17	14	12	71021388	G	PTPRB	X	67833632	G	
17	15	17	59499143	A		21	9888133	G	
17	16	18	48862668	T		22	29065727	A	TTC28
18	1	1	18600517	A	IGSF21	5	166896298	T	TENM2
18	2	1	19293083	T		5	165549241	A	
18	3	1	168490369	A		X	147567266	T	
18	4	3	146611871	C		22	29065467	A	TTC28
18	5	8	82398260	C	FABP4	9	16039869	G	CCDC171
18	6	13	71081936	T		18	19350671	T	MIB1
21	1	1	94313296	C	BCAR3	6	24667349	C	ACOT13
21	2	1	94802777	T	RP11-148B18.3	6	25481542	G	LRRC16A
21	3	1	24375012	T	RP11-293P20.2	15	89912984	A	MIR9-3

21	4	1	24375698	C	RP11-293P20.2	15	90584634	G	ZNF710
21	5	1	46103503	T	GPBP1L1	18	10715	A	AP005530.1
21	6	1	145374188	T	RP11-458D21.1	18	26269737	T	
21	7	4	47613460	T	CORIN	7	30979173	T	
21	8	4	7844076	T	AFAP1	19	55365839	T	KIR3DL2
21	9	4	87336675	G	MAPK10	22	29066000	A	TTC28
21	10	9	11642124	T		22	29065627	A	TTC28
21	11	11	74902343	T	SLCO2B1	17	27039456	G	PROCA1
21	12	11	75257094	A		17	27039871	G	PROCA1
21	13	11	75257968	G		17	26621480	G	KRT18P55
21	14	11	66428317	A	RBM4	18	23508567	G	
21	15	11	55368019	T	OR4C11	20	49321807	A	
21	16	15	65214910	A	AC069368.3	16	8550599	G	
21	17	15	59724727	G		20	52616969	C	BCAS1
21	18	15	60110685	T		20	52616969	C	BCAS1
21	19	15	60111153	G		20	52618859	T	BCAS1
21	20	16	64500896	C	RP11-467L24.1	19	36727188	A	ZNF146
21	21	17	25685640	T		19	54901602	C	
21	22	17	25686017	T		19	40150157	A	LGALS16
21	23	17	26494043	C	NLK	19	38363850	G	
21	24	17	28190455	C	SSH2	19	29956520	A	CTC-525D6.1
21	25	17	36999319	A	C17orf98	19	56278844	G	RFPL4AL1
21	26	17	37246338	C	PLXDC1	19	53433416	A	ZNF321P
21	27	17	38173903	G	MED24	19	36638121	A	CAPNS1
21	28	17	38365946	G		19	36453076	T	AF038458.5

21	29	17	50769740	T		19	54060721	A	ZNF331
21	30	17	54775901	T		19	39044331	G	RYR1
21	31	17	58177916	A		19	39028706	G	RYR1
21	32	17	58218082	G	CTD-2319I12.5	19	39598597	A	PAPL
21	33	17	59541634	G	TBX4	19	37098153	T	ZNF382
21	34	17	60179678	A		19	39598051	T	PAPL
21	35	17	60194438	C		19	58647008	T	ZNF329
21	36	17	61344955	G	TANC2	19	55595888	A	EPS8L1
21	37	17	61565439	T	ACE	19	56616341	A	ZNF787
21	38	17	66791107	A	RP11-118B18.2	19	30053169	C	VSTM2B
21	39	17	68046147	G	LINC01028	19	56179847	G	U2AF2
21	40	17	72571389	G		19	38435979	A	SIPA1L3
22	1	1	25521097	T		5	173352955	A	CPEB4
22	2	1	33666411	C		5	173439896	C	
22	3	1	10872415	T		18	23498008	A	
22	4	1	143460457	T	RP11-435B5.4	21	9705589	T	
22	5	2	87898318	A		10	31731836	T	ZEB1
22	6	2	112109608	A		10	31731836	T	ZEB1
22	7	2	41116247	A		X	11732460	T	
22	8	3	191570163	A		19	58123488	A	ZNF134
22	9	3	95912354	T		X	11733230	C	
22	10	4	171234526	C		8	82944416	A	
22	11	4	190874364	A	FRG1	10	34511110	G	PARD3
22	12	4	156101631	T		14	37887028	A	MIPOL1
22	13	4	126702	A	ZNF718	15	68062181	T	MAP2K5

22	14	5	103963127	T	RP11-6N13.1	X	11732697	T	
22	15	5	132379607	A		X	53221663	C	KDM5C
22	16	8	5503321	A		11	12979234	T	
22	17	9	18635792	G	ADAMTSL1	10	37144716	T	
22	18	9	18912570	C	ADAMTSL1	10	36306928	A	
22	19	9	20410507	A	MLLT3	10	33550559	A	NRP1
22	20	9	37810397	T	DCAF10	10	33413787	A	
22	21	9	66262518	T		10	60970453	T	PHYHIPL
22	22	10	73442031	A	CDH23	19	52287943	G	
22	23	18	24598152	T	CHST9	22	29065756	A	TTC28
23	1	9	11124	G		12	80669072	A	OTOGL
23	2	11	134914930	G		21	45313853	C	AGPAT3
23	3	12	123299698	C	CCDC62	17	46018530	T	PNPO
31	1	4	177853100	C		14	37810739	T	MIPOL1
31	2	6	39175861	C	KCNK5	9	32999075	C	APTX
31	3	6	41712723	G	PGC	11	1021668	T	MUC6
31	4	6	127886305	A	C6orf58	11	69640202	G	
31	5	14	42437279	G		17	3811957	C	P2RX1
31	6	22	29065760	T	TTC28	X	65132456	G	
32	1	1	168764386	G	LINC00626	11	65757548	C	
32	2	1	35052583	A		12	120197007	C	CIT
32	3	2	133516150	T	NCKAP5	17	3362886	G	SPATA22
32	4	2	194974003	G		18	28658710	C	DSC2
32	5	3	180878044	T		12	109918025	A	UBE3B
32	6	7	66088367	A		21	14560336	T	

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32	7	7	74564154	A	GTF2IRD2B	21	10898037	C	
32	8	7	158620742	A	ESYT2	21	18359927	T	
32	9	8	11610320	A	GATA4	11	70768757	G	SHANK2
32	10	8	14033505	G	SGCZ	18	47803005	A	MBD1
32	11	9	76686985	A		18	781544	A	YES1
33	1	3	166123308	A		16	78629977	T	WWOX
33	2	8	121544042	A		10	4748952	A	
33	3	11	65244593	A		22	43563464	C	TTLL12
33	4	11	68622656	C		22	37970728	G	LGALS2
33	5	11	69050618	A		22	20918689	C	MED15
33	6	11	96602253	A		22	43564962	C	TTLL12
34	1	1	161086765	C	PFDN2	10	77719467	T	C10orf11
34	2	3	43129443	A	POMGNT2	22	29065650	A	TTC28
34	3	4	85859671	G	WDFY3	22	29065908	T	TTC28

Table 5. Rearranged sites for the confirmation by PCR amplifications.

Sample	Long or Short PCR	Primers	Fusion site 1		Fusion site 2		Primer sequences for fusion site 1	Primer sequences for fusion site 2	Modified PCR conditions	Second primer sequences for fusion site 1***	Second primer sequences for fusion site 2***
			chromosome	location	chromosome	location					
GC1	Long PCR	GC KN1-4	8	52307387	10	66095917	TGACACATCCTTTCCCTTCC	ACATGGCTTATGCCCTTCAG			
GC1	Long PCR	GC KN1-5	9	78556898	14	90634346	TCCCTGTCCTATCGGTTTTG	TTCCTGGCTTCAAGCAATCT			
GC1	Long PCR	GC KN1-2	9	105124337	14	53007612	TGAGATTCTGGGGGACTATCA	AAAAGGACAGGGGCTTAGTCA			
GC1	Long PCR	GC 1-4	15	52314553	15	79070679	AGCTCCAGGGCTCAAGCAATCT	GGGAGAGAACCCAGGGCAACCAT			
GC1	Long PCR	GC N1-6	15	25646343	17	37061676	TCACACCTGTAATCCCAGCA	AACATGGTTTTGGCCTTTGT			
GC1	Long PCR	GC KN1-3	15	74478740	17	70833969	AGCCTTAGCCTGTGAAAGCA	ATCAACGAAACTTGGAAGCA			
GC1	Long PCR	GC N1-7	15	75149084	17	37059746	CAGCATATCCCTCCAAGGAA	TCTGTAAACTGAGGGGGTCA			
GC1	Long PCR	GC 1-1	15	79055858	17	55386803	CTGAACCCCTCAGTTCCAAACA	GAAAATGCACTGTAGAAGAATCTTTGAAC			
GC1	Long PCR	GC N1-8	15	89131649	17	38288961	TGGTGGCTCATGCCTATAAA	TGAGACAGGGAGAATTGCTTG			
GC1	Long PCR	GC 1-3	15	40332699	20	62407003	GCCTAAGCAGGGCTGTATAATGAG	TGTGAGAAACATTCCAGCAAGCACT			
GC1	Long PCR	GC 1-2	15	85377460	20	36981365	GCAAGTTGCACAAAGGGATTGAGTC	TTTATGTTCCGTGAATTTCCTATCAA			
GC1	Long PCR	GC KN1-1	17	56098130	20	40123512	ATGGTGAAACCCTGCCTCTA	TTAGGCTGCAAGGCACTTTT			
GC4	Long PCR	GC 4-3	2	178191754	12	112118374	CTATAGGGTCTGAAGCTCAAGAGAAG	GGAAGGTGATGGGGAATTGAAAG			
GC4	Long PCR	GC 4-6	6	160040535	7	29925262	TGGGCGGAGTATAGGAGTTG	AGGAATATGTGTGTTGGGGG			
GC4	Long PCR	GC 4-7	6	160040535	17	79874698	ACACTCTGCGCTCTTGGAGT	CCATCATTCTTGGAGCGTTT			
GC4	Long PCR	GC 4-2	7	71465814	13	29472417	CCAAGCCTAAATAGTTACATTGGAAAATTC	GATAATGTCACAATCAGCAGGGATAC			
GC4	Long PCR	GC 4-5	10	32339793	12	113464446	ACCGTGAAGGGCCTATATCC	ATGCAGAACTGCCCTTGAGT			

GC4	Long PCR	GC 4-4	12	51839109	17	7296986	GGTTGGGATGGCTGTATTGAAGA	GGCTGAGGAATAAGGGGATGAG
GC4	Long PCR	GC 4-1	14	35316779	16	7095851	TATATACAGAAGGAGCAGGATTGAGAA	GTTTTCAAACTATCCTTTGTGACATACAG
GC6	Long PCR	GC 6-2	1	187108178	21	20792897	GCTCATTGCTACCAGGGTGATCAGAG	AATCTTCCTCCCCTCCCTTTCACTTAC
GC6	Long PCR	GC 6-1	1	221173095	21	20793122	TGGAATGCACACCTGAGGTAAG	AAAACAGAGCCTGAGGCAAAGAAC
GC6	Long PCR	GC 6-3	8	31385804	14	59220711	GACCCTCAAGTGGCTCTGGTAGTC	CCAAATTCCTATTAGAGGCAGTCCAAATG
GC7	Long PCR	GC 7-2	2	105965462	7	104387039	GCACCAGCACACCCAGCTTCTAC	GTTTGCCCAAACTCACAGAGCTAG
GC7	Long PCR	GC 7-5	8	135411044	22	29065925	GAAATGGTGCCATTGCTACATGA	TTGGCCCTAACTGGTCACACTTC
GC7	Long PCR	GC 7-3	1	119344462	22	29065689	CAAGCATATGGAAAACTTATTTAATCCAGTA	GAATCATAGGAGGGACCATAAATTC
GC8	Long PCR	GC KN8-1	6	143706913	13	30221916	TCAGCTCATCTGAAGGGAGA	CCGGTACCTCAGATGGAAAT
GC8	Long PCR	GC N8-3	7	126098912	14	59221115	CTGCATCCTAAGATTGCCATT	TAATGGGGGATATGCTGGAG
GC8	Long PCR	GC KN8-2	8	35416270	20	9699004	CGATGCTCTGATCTTGAAGC	TTTTTCCCCTCCTCACACTC
GC8	Long PCR	GC 8-4	8	36073941	20	16272773	TGTGTGTGTTTATGTGTTTTGACAAATG	CACAGCATATGGTGCAATCCTTTG
GC8	Long PCR	GC 8-2	11	189682	17	30036	GGCGTGCCCCGACTACACTG	CACATTTGATGCTTGGGAAACTC
GC8	Long PCR	GC 8-1	13	76433089	17	64740469	TATGTGTCTGACCTCAGGCCTTTTAG	GCTATTTGTCGGAACAGGAGAGAC
GC9	Long PCR	GC 9-5	8	29942495	9	131456462	GGCAAACAAGGCCATAACTGAAG	TTTGACCAGCTACACTGCCTATATTC
GC9	Long PCR	GC 9-4	8	29980093	9	124210751	GGTTTTGGGTGGTTTGCTATG	CAAATGTGGGAGCAGGAGGTATATG
GC9	Long PCR	GC 9-1	8	30259392	9	128966342	GCCACGGCAAGACTATGATTGTGATG	CGGAAGGAAGATTTACTATCAGCTCTAC
GC9	Long PCR	GC 9-2	8	30262787	9	135746039	AACCAAGGGCTAAGGGAAAAGAAGAC	GCTAAGCCCAATCCTCATGTGTC
GC9	Long PCR	GC KN9-3	8	30296784	9	135610016	TTGCTGAGGGGGAATATCTG	CCGTGCCCAACCTATTTATG
GC9	Long PCR	GC KN9-1	8	41482627	9	106011712	GTACGTGCCATCTCAGAACG	AACAAGTTTCACCCCTCTGG

GC9	Long PCR	GC 9-3	9	131288019	16	30195284	TTCAGTATGGATGTGGTCCTTTAACTCG	GGAAAGGGGAACTTGGTCTTTTCTTG		
GC9	Long PCR	GC KN9-2	9	138236266	16	29121678	ATTCCTACCCAGCAAAGTGG	CAACACATACAAAAAGCAGCAA		
GC10	Long PCR	GC 10-3	1	23155464	20	45823894	CTAGCAGTAGGGGAAGGTGAC	CTGAATGTTCCCTGGAGAGATA	GACAACCCAAGATGACAGGCTCAC	AAAATCTCAGCCAGCAAAAAGATCC
GC10	Long PCR	GC 10-14	2	138364748	12	2993827	GTTAATGGAAGGAGAAGTGC	ATGTTTATCCTGAGTTCTTGCC		
GC10	Long PCR	GC 10-12	6	13191211	11	31663176	CTGGGATAACTGGGAGGGAA	GACCTGAGAAAGAGATTGTG		
GC10	Long PCR	GC 10-4	14	92292467	17	41541673	CGGAGTTTGCAATGAGCAGAGACCAC	ATATATCCACAGTCATCGTTGGAGTTTTC		
GC10	Long PCR	GC 10-11	18	9261632	21	42913288	GAGTTGTAGGTAGTTTGGTG	AATGGGTGGGTTTTTTTGCT		
GC11	Long PCR	GC N11-1	1	19249595	13	1066685144	TTGGGGACAGGAATCACAAT	AGCAATTATGTTGATGCCAAA		
GC11	Long PCR	GC11	1	204523693	17	70560379	TAGCAATGGCAAGCAGAATG	GTGTCAGCTTGCTGCTCTTG		
GC11	Long PCR	GC KN11-1	1	38167534	20	62355028	TGGAGATGGTTTTGGTTTAGG	CCCCAAAAGTGCTGGAGTTA		
GC11	Long PCR	GC KN11-2	1	46105734	X	70730016	AAAATAGCCGGGCATAATGG	TGGGGGCATCTATATCATCC		
GC11	Long PCR	GC KN11-3	2	38820590	6	126348359	TGGAAATGAATAAAGCAGGAA	TGGGTGCTCTTTTCATCTGTT		
GC11	Long PCR	GC11	2	110927394	11	17766657	AACACAATCTCATATTACTACTGCTTG	TGCTGAGTGAGGGTACATCG	tAACACAATCTCATATTACTACTGCTTGtaaattg	gccacTGCTGAGTGAGGGTACATCG
GC11	Long PCR	GC11	2	216512759	21	38791413	GACGGGATTTCACCATGTTC	GGCAAACTATAATGGTTGTTGGA		
GC11	Long PCR	GC11	4	21169457	8	85608417	CTTCCTGGCAATTGCATTC	CAAATACAGCATGTGAAAAGGTG	GATGGTTTAACAGCAGACCATCT	TTGTCATCTGGTTGATGTAATGC
GC11	Long PCR	GC11	8	11439657	9	139346288	GGTGGCAGGCACATGTAATC	CAGCACTCAGAATGCAAATGA		
GC11	Long PCR	GC11	8	145273127	16	958249	ATGACGGCCTGACTGGAATA	GGAAAGTGGGATGCTGTCTC		
GC11	Long PCR	GC11	10	58441082	X	11732262	TCATGAGTAAAGAAGATCACCAAAA	GGTCATTCAGGGTTTTGGTG		
GC11	Long PCR	GC11	15	101463350	17	1272596	CCTGGCTCTTCTAGCTCCAC	TTTTGTTCAAATTTCTGTGCTTT		

GC12	Long PCR	GC12	7	105813499	22	29065601	CAGCAAGTGTGAGCCAAAAG	TGGGTATATTTTGGGAAATAGTAGA		
GC12	Long PCR	GC12	8	146269287	15	89902939	GAATGGAGTTTTTCTCTTGTTGG	GGTTCCTGCCCCAAACAC		
GC12	Long PCR	GC N12-3	10	46964222	22	20248979	CCGTGGGTACTTTCCTGATG	GTTGGTGGCCTTCAAGACTG	CCTGATGCAAAGAGGAAGGA	GCACAAGGGTTGCTCTGTTT
GC14	Long PCR	GC N14-1	1	41689552	3	14483301	CAGATGGAAGGAGTCAGCAT	GGGGTTTTGTTGTGCTTTTG		
GC14	Long PCR	GC KN14-4	2	189847931	7	90330293	GGCCTACTTCATTTCCACTGA	CCACGAAGGAAAAAGGGTTA		
GC14	Long PCR	GC KN14-2	3	107819921	14	68887971	ATTTCTCCCCTCTGCACACA	GGCAGGAACATGAAAGCAGT		
GC14	Long PCR	GC14	4	19539	7	18094813	CAGTAGGAGAGCAGGGTGAT	AGTTAGCCAGGATGGTCTTG		
GC14	Long PCR	GC14	5	166483237	12	71020366	GCAAGCAAGAAAGGAGAAGA	TTCCCATCTATTCCAGTCAAG		
GC14	Long PCR	GC14	6	51481953	7	18195957	CACCACTGTAGGCCAACTCTAA	TTGTTTTGAGGCCAACTTGA	CTCCCATTGCTGATTTTCTGTAT	TTGTTTTGAGGCCAACTTGA
GC14	Long PCR	GC KN14-1	6	13191453	X	123827677	CACATGCTGCTGCGTAATTT	TAATAGCCAACCCCAAAGCA		
GC14	Long PCR	GC N14-2	7	69344263	10	106547242	AGGGATTGCTATTGCTGAGG	CATCATTGTCCCTTTTCATGG	TTGCTGAGGCTCTTTCTGTG	TCCCTTTTCATGGGAGAACA
GC14	Long PCR	GC14	11	16996591	17	17791698	CTTAGTTTGCCCACCATAGC	GACCCTGTCACTGGGAATAG		
GC14	Long PCR	GC14	14	68907674	17	71217646	CATGTTATTTCTGGTGCTGTGAA	ACTGAAAGTAAAGAACATTACTCCTTC		
GC14	Long PCR	GC KN14-3	16	67388976	17	71217432	CCAGCAATCATGTTCTTTGGT	CAATCCTGATCTTGGGCAAC		
GC15	Long PCR	GC15	1	28242429	17	40739970	CATCATTGTCCCTTTTCATGG	GTCAGGCTGGTCTCGAACTC	TGCTCTGAATGTCAAAGTGAAGA	GGGGTTAACAACATGTAACCAGA
GC15	Long PCR	GC N15-2	7	5362803	17	40516485	GAGCCTAGGCCTCGAGAGAG	CTGGCCAAAATGGTGAAACT	GGGTCTGGCAGGATAAACAA	CTGGCCAAAATGGTGAAACT
GC15	Long PCR	GC15	8	22693532	9	28470635	CTTTCAATGACCGGCTCTTC	TCATCCAGGCTCTCACACTG		
GC15	Long PCR	GC N15-3	9	74301829	X	69832219	AAATGGACCGTGAAGCCATA	TACATCATGCCCAGCTTTTAAG	AATGGACCGTGAAGCCATAG	GGTACATCATGCCCAGCTTT
GC15	Long PCR	GC N15-4	12	39605710	X	69891944	GATGCAGAAAAGGCCTTCAA	TTCTCCCCATTATTGCTGCT	AATTCAACAGCCCTTCAT	ATGGACATTTGGCTGT

									GC	TTCC
GC15	Long PCR	GC KN15-1	12	39606044	X	46764254	TGCCTCCAGCTTTGTTCTTT	TCTGAAGTTTCAGGCCTCCT		
GC15	Long PCR	GC KN15-2	16	59053066	17	49441698	AAGAAATGAGCACTGGAAATGA	AGCAAGTGGCAGAGTCAGAA		
GC17	Long PCR	GC17	1	142698258	17	59691613	ATTCCTCAGTGCCAAATGTGT	TTTCTGATTATTTTGGGAGAAGTACA		
GC17	Long PCR	GC KN17-1	1	143178580	17	47747622	CCATCGTAGGCACTTGTTGA	TCAGGGAAGCACACCTGTTT		
GC17	Long PCR	GC N17-3	2	148845078	6	85902185	AGTCACATGGACAAACTCAGGA	GTCTGCACATTTGCATGGTC	GGCAGCAGAATTCACTGTTG	GTCTGCACATTTGCATGGTC
GC17	Long PCR	GC KN17-2	2	98602446	13	84098990	ATGCCAACGGACTGAAGAAA	TTTGTCACCATGTGCTATCCA		
GC17	Long PCR	GC17	13	84098746	2	98602169	GTCTTTGCCCAAGAGCATTC	GGAGGGTCCACCACTTATTG		
GC17	Long PCR	GC17	3	120173018	22	29065922	CTGTCTGATTTTTGCCACTG	TTTCCACATACATGCTTAGTGA		
GC17	Long PCR	GC KN17-3	8	53555473	14	86291396	CGTTCTTGTTGCTGCATTGT	ATTGTCCTTCTCTCCCACCA		
GC17	Long PCR	GC17	9	115556659	13	26240677	AAGATGCCTTGAGAGGCTTTGTCA	TGATTCCAAGCTAAGTTTTGCTATTG	CCTTGAGAGGCTTTGTCAGG	CCAAGCTAAGTTTTGCTATTGGA
GC17	Long PCR	GC17	9	115556659	13	26240677	GGCCAACACATTCTTTAAGC	TGTCATAATGCCTTTTGGTG		
GC17	Long PCR	GC N17-5	11	10378409	22	29066057	CAAGTGGAACAAGCCACGTA	TCCAATTTAAATGTAAGTTCAAATACC	CATGCCAATTTTTGCAACTG	AGATGTTTTCCACATACATGCTT
GC17	Long PCR	GC KN17-4	12	71021388	X	67833632	GAATGGCTCCAAAGTTCAGG	CTTTAACCACAGTTTCTGGATAGAT		
GC17	Long PCR	GC17	17	59691613	17	142698258	TTTCTGATTATTTTGGGAGAAG	TATTCCTCAGTGCCAAATGT		
GC18	Long PCR	GC N18-1	1	18600517	5	166896298	AGGGAAAACATGAGCACAGC	CGCTACATGGAGGGCTTAAT		
GC18	Long PCR	GC KN18-1	1	168490369	X	147567266	TCTCACAATTCAACTCAAGCAA	CCCTGATAATATGATGTGGAACA		
GC18	Long PCR	GC N18-3	3	146611872	22	29066194	CCCTGCAAATGATGGTCAAT	GCCATAGATGGCCAGATCAA	TGTGCTCTCTGCTCATGCTT	TTTTTCCATATGCTTGAATTTTT
GC18	Long PCR	GC KN18-2	8	82398260	9	16039869	AACAGACTTTCCCGGATTGC	TGTTATCCGGTCCAATCTCC		
GC18	Long PCR	GC18	13	71081936	18	19350671	CAGGGTATGAAAGAGGCAGA	TCAATTGGCCAAATAGAATCTG	TTGAAGACCAGGGTATGA	ATCAATTGGCCAAATA

									AAGA	GAATCTG
GC21	Long PCR	GC N21-2	1	94313296	6	24667349	TCCCCTGCACTTTC CTTTAG	TGAGTCATGCTGGT CATCGT	GCTTCAGTG TCGCTTTTC CT	TTCGGAAG AGCTGGAA AGTC
GC21	Long PCR	GC21	1	24375012	15	89912984	TACCCAGAGAGAT GGTGGTT	GAATGCTAACACAC TCACAGG		
GC21	Long PCR	GC21	4	7844076	19	55365839	TAACCTTGGGGGA ATCATTG	GCGTGGGATTCTTA TGGAGA		
GC21	Long PCR	GC KN21-2	4	87336675	22	29066000	CCATTCCCGATCCA TATTGT	TTTTGGCCCTAACT GGTCAC		
GC21	Long PCR	GC21	9	11642124	22	29065627	GCAAAACTGAGAT GAGAAGACA	TCAAGCATATGGAA AAACTTATTTA		
GC21	Long PCR	GC21	11	74902343	17	27039456	CTGCACACCATGC ATTTAAC	ATGCGCAGAGTGT GTATGTT		
GC21	Long PCR	GC KN21-4	15	59794727	20	52616969	AACTTGTCCAAGGT CCCAAA	TGGAGTCCATGAAA GGAGAGA		
GC21	Long PCR	GC KN21-1	16	64500713	19	36727188	ATTTTGGGCTGAGA TGATGG	GCTTTCCCACATTC ACAAGG		
GC21	Long PCR	GC KN21-3	17	26494043	19	38363850	TAAAAACCCCCACA AAACCA	TCCCAAAGTGCCG AGATTAC		
GC21	Long PCR	GC N21-3	17	38173903	19	36638121	TTCACACACAGGC CTGACAT	ACAGGGTCTTGCTA TGTTGC		
GC21	Long PCR	GC21	17	50769740	19	54060721	ACAATGGAAATAAG CCCTCA	GTGAGGCCTTCTTC AAGGT		
GC21	Long PCR	GC21	17	61344955	19	55595888	GTACCCATGCCAA GTGTGAG	AGGCTGGAGTGTG ATCTCTTCT	TTTCATTTTA AATCTCAAAT CTACCA	TTCCAAGT CATGGTTC TACCAA
GC21	Long PCR	GC21	17	61565439	19	56616341	GTACCCATGCCAA GTGTGAG	AGGCTGGAGTGTG ATCTCTTCT		
GC22	Long PCR	GC22	1	25521097	5	173352955	GCGTTTCCCCCTGT GCAAGA	GTATCTGCAGCACA ATTAGATTTC	GTTTCCCCC TGTGCAAGA A	TGCAGCAC AATTAGATT TCACG
GC22	Long PCR	GC22	1	33666411	5	173439896	TCACATCATAGCCA ATCAAAA	TTTTTACATTTTGTT AGGTTTTGAA		
GC22	Long PCR	GC22	2	87898318	10	31731836	AGTTGCCATGGGTT TAATGT	GAAGTGGAGGAAA GAAACGA		
GC22	Long PCR	GC22	4	126702	15	68062181	GGACTTTGGAACTA TTGCTTTG	TTCGTGCGTCCGTT TAGTAG		
GC22	Long PCR	GC22	5	132379607	X	53221663	TTGTAAGTTGCAGG AATGAGATG	ACAACATGAACCCA GGAGGA		
GC22	Long PCR	GC22	8	5503321	11	89912984	GGTGTGAATAAGTA ACACATACTCG	GTGAGAACATGTGT TTCCATTT		
GC22	Long	GC22	9	20410507	10	33550559	AGATGAACATTTTA	TGAATACTTGGAAA		

	PCR						AAGAACCTTTTC	TCAATCAGAAC		
GC22	Long PCR	GC22	9	186355792	10	37144716	TCTGCTGGAGAGAAGTCCA	ACACTAATGAAAGAAATTAAAGATGC		
GC22	Long PCR	GC22	18	24598152	22	29065756	TGGTACCAGAATTACATGAACATAA	GGCCATTTTATGGAATGACAA	TGGTACCAGAATTACATGAACA	TGAGAAAATATAAAGGTTTATTACTGG
GC23	Long PCR	GC N23-1	9	11124	12	80669072	CAAGAGAGCCCTGCAGTTG	TCATTCTGACAATGTTCACAGC	TAAGCAAGAGAGCCCTGCAGTT	AAGAAGCAACTCCTCGTCCA
GC23	Long PCR	GC23	11	134914930	21	45313853	CCACCAACACTCACTGAAGG	AGCAGTCTCACTCACGAGCA	ATTCCCAGGACTCTTGTTCA	CTGACAAAAACAGGATGCAA
GC23	Long PCR	GC N23-3	12	123299698	17	46018530	TTTTGAGACAGCATCTTCCTCT	GCATGAGAGTGGGAGAGGTT	AGCTCACTGCAGCCTCAAAT	AAGGAAATTTGGGACCAAGG
GC31	Long PCR	GC31 L4	4	177853100	14	37810739	GCCCTTGTTTCCAACTAGTCA	CCAGGTATCCAAAACCAGCTT		
GC31	Long PCR	GC31 L1	6	39175861	9	32999075	TTGAGGGCTGAGGTTCTGAT	TGAACTCTGCCTCCATTTCC		
GC31	Long PCR	GC31 L2	6	41712723	11	1021668	AGGTTGCATATGAGGCAAGG	AAGCAGGGTATGAGAGACCAA		
GC31	Long PCR	GC31 L3	6	127886305	11	69640202	ACTCTCGAGGAAGTCCGAAA	TGGTAGATGCAAAGTAGCCACA		
GC31	Long PCR	GC31 L5	22	29065760	X	65132456	GCCAGTAATTGGGTATATTTTGG	TCAACCTAAGTGCCCATCAA		
GC32	Long PCR	GC32 L2	1	168764386	11	65757548	TGAAAGATAACAGGCCAGGTG	GTTTGGGCCTTGAATGAAGA		
GC32	Long PCR	GC32 L1	1	35052583	12	120197007	ATCTGGCCTGTGTCCATTTC	CGAGGGTTGTCATTTTCTGAG		
GC32	Long PCR	GC32 L3	2	133516150	17	3362886	ACCTCCCAGGTTCCAATGAT	ACCTTTGGAGCAGCTCTTTG		
GC32	Long PCR	GC32 L4	3	180878044	12	109918025	CATGATGAGTTAATGGGTGCAG	GGAGGTACTTGAGTGCACATTG		
GC32	Long PCR	GC32 L5	7	158620742	21	18359927	ACGCATTTCAAGGGCAGTAA	AAGGAGGTCCACTTTCACCA		
GC32	Long PCR	GC32 L6	8	11610320	11	70768757	CACCGACAGCAGTCTTTCAA	CCTTCCAAAGGTTTTCTTTCC		
GC32	Long PCR	GC32 L7	8	14033505	18	47803005	TGCAAATACCAAATGAAACTGT	AACCTCCAACCATGCCAGTA		
GC32	Long PCR	GC32 L8	9	76686985	18	781544	TTCTGCCTTGATGACCTGTCT	GCATCAGCTATGAGCCAACA		

GC33	Long PCR	GC33 L1	3	166123308	16	78629977	TCAACATTCAAAGGGGATAAGA	TCATCACCCAAGAGCTGTCA	
GC33	Long PCR	GC33 L2	8	121544042	10	4748952	AGCCACTTAGAGCCAACCAA	AAAGGAAATGAGGGTAAATCG	
GC33	Long PCR	GC33 L3	11	65244593	22	43563464	TCAGGAGGAATTGGAGCCTA	GACCCTCAGCAAACGAAAAG	
GC33	Long PCR	GC33 L4	11	68622656	22	37970728	CATTGTGGGAGTTCTGCTCA	GGCAAAGAGGCAATTTCACA	
GC33	Long PCR	GC33 L6	11	69050618	22	20918689	GCATCCTGATGGGTGAATGT	TGCTGCACTACTGCTTGGAA	
GC33	Long PCR	GC33 L5	11	96602253	22	43564962	TGCCTGACTTGTTTTGTCCA	TTGTCCCACTTCAGCATGAG	
GC34	Long PCR	GC34 L1	1	161086765	10	77719467	CCTGGCCAATATGGTGAAAC	AAGGAATCCCAAGACCCTGT	
GC34	Long PCR	GC34 L2	3	43129443	22	29065650	CACATCATCTCCCAAACCAG	TCACTAAGCATGTATGTGGAAA	
GC34	Long PCR	GC34 L3	4	85859671	22	29065908	AATTGGGGGACAACAACTAGA	TTGTGGAGTCAGCAGTTTCCT	
GC1	Short PCR	GC SKN1-1	17	56098130	20	40123512	TGAGGCAGGAGTATTGCTTG	GGCAAGCCTCTCAGATTCAA	*
GC1	Short PCR	GC SKN1-2	9	105124337	14	53007612	TTTCTACATCAAAAAGCATAGCTG	AGCTAGGCACTCAACAAAGG	
GC1	Short PCR	GC SKN1-3	15	74478740	17	70833969	TGCATGAGAATCACTGGGACT	GAAAGAAACCAGACACAAAAACA	*
GC1	Short PCR	GC SKN1-4	8	52307387	10	66095917	AGTGCCTTTGTTGGATCCTG	CAGATACCCGAGGGATATATGGT	
GC4	Short PCR	GC S4-5	10	32339793	12	113464446	CATTCAATTCTACAGGTGTGACTAA	GCCTTAGAAAGGGGTGGTAA	**
GC4	Short PCR	GC S4-6	6	160040535	7	29925262	GCGCATCTAGACACACTGACA	CCTGGGTAACACAGCGAAA	
GC4	Short PCR	GC S4-7	6	160040535	17	79874698	ATGAGGCACTCCAAGCAAAG	TGGGAGAGAAAGGAAGGTTTT	*
GC6	Short PCR	GC S6-1	1	221173095	21	20793122	TGGTAGCCCAAGTTGACTAAG	AGGCTGCTTGGAATTACTGC	
GC6	Short PCR	GC S6-2	1	187108178	21	20792897	GGCCTTCTTTGTCTCTTTTGA	TGTTAACATACGGGTTCTTTGC	
GC6	Short PCR	GC S6-3	8	31385804	14	59220711	GAAATTAGGCATCTGGCAAGT	AGCTTAAGTTGCATTCCACAC	
GC7	Short PCR	GC S7-5	8	135411044	22	29065925	CCAAAGAAGCTAAACCCAAAA	TTCCCCTTCCTGTGTCCAT	
GC8	Short PCR	GC S8-1	13	76433089	17	64740469	CACAGTCTGGATGTGTTTACTGA	GCCTGCTTAATACCTGTCATTT	
GC8	Short PCR	GC S8-4	8	36073941	20	16272773	AGCGTTCCATCACAGAATGA	CATTCCAGGCAACCAAAAAC	*

GC9	Short PCR	GC S9-5	8	29942495	9	131456462	TCCAGGTAGACGT GTCAAATAAA	TCCAGGTAGACGT GTCAAATAAA	*
GC9	Short PCR	GC S9-2	8	30262787	9	135746039	TGGGGTTAATGGT GATTGC	TGACTGGTGTAGA GAGAAAGAAGG	
GC9	Short PCR	GC SKN9-1	8	41482627	9	106011712	GCTGACGGAGATA AAGTTTGC	CCTCTGGGGAAAA TGTCAAA	
GC10	Short PCR	GC S10-11	18	9261632	21	42913288	CAACTCTAGGTTGG TGCAAAA	CCGGGAAAGTCTTT GAAGAAA	
GC10	Short PCR	GC S10-12	6	13191211	11	31663176	CCATGCTTGACCAA TATTCC	AAAATGCGCAGACT GAAAGG	
GC10	Short PCR	GC S10-14	2	138364748	12	2993827	CATTCCTGGTAAGA CTTCACTCG	TTGCCAGACTTTCA GCTCCT	
GC11	Short PCR	GC S11-1	1	19249595	13	1066685144	AATCATCTCTCCTG GCCACA	CCTCAGTTTAGATA AAGCTTGTGC	*
GC11	Short PCR	GC S11-2	1	204523693	17	70560379	CCCCTCATCTGCTA AACTTCA	TCATGAAGGAGGA GGAAGCA	*
GC11	Short PCR	GC S11-3	2	110927394	11	17766657	CGACCCTTAGGTTA GGTTTCA	TGTTAGACGTGAAG CCTTTCAG	*
GC11	Short PCR	GC SKN11-2	1	46105734	X	70730016	ATTGTGCCACTGCA TTCCA	TCTCCAGCTCTCCC AACAAT	
GC11	Short PCR	GC SKN11-3	2	38820590	6	126348359	ATACCTACCTGCCA AACACAGC	CAACTCTGGAGATC AGCCTTTT	
GC12	Short PCR	GC S12-1	7	105813499	22	29065601	CCTGGTCATCAAAT GGTCAA	CTTAACATTTTTAAT GCAAGCATATT	
GC12	Short PCR	GC S12-2	8	146269287	15	89902939	CCCAAGTAGCTGG GAAAACA	CCTCAGATGCACG TTCCA	
GC14	Short PCR	GC S14-3	11	16996591	17	17791698	AAAGTCCGGCGGG TTTTA	AAAGTCCGGCGGG TTTTA	
GC14	Short PCR	GC S14-4	14	68907674	17	71217646	TCTAAGTAGTTTTT ACCCATCCAAA	ATGGGTCATCAAAC AACTACAAAAG	*
GC15	Short PCR	GC S15-1	8	22693532	9	28470635	TCACAGTTACTGAG GGAGACAGA	GTTTTCCCAACACA CGAATG	*
GC15	Short PCR	GC SKN15-2	16	59053066	17	49441698	TGGCTAAGTGGAG AGAAATGG	TGGCTAAGTGGAG AGAAATGG	*
GC17	Short PCR	GC S17-1	1	142698258	17	59691613	GAAGTGGGTTCTTC TAATCAAGC	TGGTTCAGTTTCCG TATCTGT	
GC17	Short PCR	GC S17-2	13	84098746	2	98602169	CACTGCAGCTCAA CTTCTGTCT	TGTACTGGGATCTA TTTCTTGATTT	
GC17	Short PCR	GC SKN17-1	1	143178580	17	47747622	TGAGGAAATTACTG GGAACAGG	TGACACATTTGCCA ATAAGACC	
GC17	Short PCR	GC SKN17-2	2	98602446	13	84098990	GGGGTGTTAAGGT ATCAGAAAGG	CAGTTCTTTTTCCG GTTGTGA	*
GC17	Short PCR	GC SKN17-3	8	53555473	14	86291396	ATTGGTCTGGAACA AGAGAATAAT	TGAAAGAGTTTGTA GCATAATGAGG	

GC18	Short PCR	GC S18-1	1	18600517	5	166896298	TGTGTTGGGGGAA ATCACTC	CAAAATGCATATTT TCATGTCTATG	
GC18	Short PCR	GC SKN18-2	8	82398260	9	16039869	GGTCTCTTTGTATA TGACCTTCTCC	TCCTTCCTTCTGGC AATAGAA	
GC21	Short PCR	GC S21-1	4	7844076	19	55365839	GGGGAATCATTGC TCAAAAT	TGTCCTTCCATGCT GACTTT	
GC21	Short PCR	GC S21-3	17	38173903	19	36638121	CATGATTCCTCCTG TCTGCT	CATGTGGCCTTCTG AGTGTT	
GC21	Short PCR	GC SKN21-1	16	64500713	19	36727188	CGAACAAGCAATG AGGAAAG	TGATCTCCCAAATG CAGAGT	
GC21	Short PCR	GC SKN21-3	17	26494043	19	38363850	TGGGTTTCAAACTG ACTCCA	AGCTACCACACTCA GCCAAGAT	*
GC21	Short PCR	GC SKN21-4	15	59794727	20	52616969	TCCAAGGTCCCAAA GCTATTA	TACCATGTGCACAA GTCTGG	
GC22	Short PCR	GC S22-2	4	126702	15	68062181	TGATGCACATCACA TTTTGG	GGAAAAAGGCTCT GAAGTATGC	
GC22	Short PCR	GC S22-3	5	132379607	X	53221663	GGGTGGAGTTGGA ACGTTAG	AAAAACTGTGAGCA CGGCTA	
GC22	Short PCR	GC S22-4	9	20410507	10	33550559	CCTGTTTATATGGT AAAAGAGCAAA	GAATTCTGCTGTCT GGTGTCA	
GC31	Short PCR	GC31 S3	6	127886305	11	69640202	CCTCCCTTGGTTTC ATGACT	TTTGTTTTCATCAAT AAAATGTGG	*
GC31	Short PCR	GC31 S4	4	177853100	14	37810739	CCATATTCCTTTTG CCCTAGC	AGAACTTTGAACAA ACAGAGATACAG	
GC31	Short PCR	GC31 S5	22	29065760	X	65132456	GCCAGTAATTGGG TATATTTTGG	TTGGGAAACTAATG CAGGAAA	*
GC32	Short PCR	GC32 S1	1	35052583	12	120197007	TCCAAGCACTCCTG AGTTTC	GGTCTGCCACCTG TTATGAA	*
GC32	Short PCR	GC32 S2	1	168764386	11	65757548	TGGTGGCATACAC CTATTGC	GAAGACAAAACCC ACGGTTC	*
GC32	Short PCR	GC32 S3	2	133516150	17	3362886	TCCCAGGTTCCAAT GATTCT	CATATCAGGTGTCA GAGGAGGA	
GC32	Short PCR	GC32 S4	3	180878044	12	109918025	AACTCCCAGAAGAA AACATGG	GGAGGTACTTGAG TGCACATTG	
GC32	Short PCR	GC32 S5	7	158620742	21	18359927	TGAGCATTTTTATA TGGGATCG	CCACTTTCACCACC GTTATTC	
GC32	Short PCR	GC32 S6	8	11610320	11	70768757	CTGATCTAGAACCT GTACCTCAC	CATCCTTGTATACA TGCTTTGT	*
GC32	Short PCR	GC32 S7	8	14033505	18	47803005	TGCAAATACCAAAT GAAACTGT	ACCTGCTCCAGCT CCTATCT	
GC32	Short PCR	GC32 S8	9	76686985	18	781544	AAATTTGGGTGCTC CTGTGT	GCCAACACTAGGC TAAGCTACA	
GC33	Short PCR	GC33 S1	3	166123308	16	78629977	TGGGAAAAATACTA AATGGTAGGA	CTGCAGACCATAG ATCAAGGAG	

GC33	Short PCR	GC33 S2	8	121544042	10	4748952	AGACTTGGATGGA CAGCACA	CCTGTTCTATGTGG ATGTAGTTAGG	*
GC33	Short PCR	GC33 S3	11	65244593	22	43563464	TCAGGAGGAATTG GAGCCTA	AGCTGGAATGGGT GATAAGG	
GC33	Short PCR	GC33 S4	11	68622656	22	37970728	CATGATCTGAGATG CCCTGA	CAAAAGAGGAGCC GAGGAT	
GC33	Short PCR	GC33 S5	11	96602253	22	43564962	TGCCTGACTTGTTT TGTCCA	AAGGCCCGGAAGA TCTCA	*
GC33	Short PCR	Short PCR	11	69050618	22	20918689	TTTTTGAGATGGAG CCTCACT	TGGGCTGAGAAAA GACCAGA	*
GC34	Short PCR	GC34 S2	3	43129443	22	29065650	TCGACCTACTGCAT GTCCTTT	TGACAAAGGGCTA ATATCCAGA	*
Control1		GAPDH	12	6645634	12	6645743	TGCCTTCTTGCCTC TTGTCT	AATGAAGGGGTCA TTGATGG	

*, modified by using 1% Tween 20.

**, modified by using 1.5 mM 7-deaza-dGTP

***, Primer pairs for another PCR

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Table 6. Primer information for quantitative PCR.

No	Primer ID	Primer sequences*	Annealing temperature (°C)	PCR product
1	GC S4-7	F: 5'-ATGAGGCACTCCAAGCAAAG-3' R: 5'-TGGGAGAGAAAGGAAGGTTTT-3' Probe: 5'-CAGCAGCAAGAATGCAAAAA-3'	55	107bp
2	GC S8-4	F: 5'-AGCGTTCCATCACAGAATGA-3' R: 5'-CATTCCAGGCAACCAAAAAC-3' Probe: 5'-ACCGCCTTTGCAAAATTATG-3'	55	130bp
3	GC S9-5	F: 5'-TCCAGGTAGACGTGTCAAATAAA-3' R: 5'-TCCAGGTAGACGTGTCAAATAAA-3' Probe: 5'-TGAAGTTCAAAACTAAGGTAAATTTGG-3'	55	120bp
4	GC S12-2	F: 5'- CCCAAGTAGCTGGGAAAACA -3' R: 5'- CCTCAGATGCACGTTCCA -3' Probe: 5'-ACGACACCCGGCTAATTTTT-3'	55	126bp
5	GC S14-4	F: 5'- TCTAAGTAGTTTTTACCCATCCAAA -3' R: 5'- ATGGGTCATCAAACAACTACAAAAG -3' Probe: 5'-TTGCCCAAGATCAGGATTTG-3'	52	106bp
6	GC SKN15-2	F: 5'- TGGCTAAGTGGAGAGAAATGG -3' R: 5'- TGGCTAAGTGGAGAGAAATGG -3' Probe: 5'-TGAGGTTTTGATATTTCACGTGA-3'	55	121bp
7	GC S17-1	F: 5'- TGGTTCAGTTTCCGTATCTGT -3' R: 5'- GAAGTGGGTTCTTCTAATCAAGC -3' Probe: 5'-GATCTGAATTGTGTCATTCATTCA-3'	55	110bp
8	GC SKN18-2	F: 5'- GGTCTCTTTGTATATGACCTTCTCC -3' R: 5'- TCCTTCCTTCTGGCAATAGAA -3' Probe: 5'-TGGAGGTGGAGTTGTGTTCA-3'	55	130bp
9	GC S22-3	F: 5'- GGGTGGAGTTGGAACGTTAG -3' R: 5'- AAAAACTGTGAGCACGGCTA -3' Probe: 5'-GGCTAGGTGAGGAGTGTTGG-3'	55	115bp
10	GC31 S5	F: 5'- GCCAGTAATTGGGTATATTTTGG -3' R: 5'- TTGGGAAACTAATGCAGGAAA -3' Probe: 5'-TTCACTAAGCATGTATGTGGAAA-3'	55	127bp
11	GC32 S2	F: 5'- TGGTGGCATACACCTATTGC -3' R: 5'- GAAGACAAAACCCACGGTTC -3' Probe: 5'-GTGAGAGGATTGCTTGAGCC-3'	55	126bp
12	GC33 S3	F: 5'- TCAGGAGGAATTGGAGCCTA -3' R: 5'- AGCTGGAATGGGTGATAAGG -3' Probe: 5'-AGAGGATGAAGGGCGAGAAG-3'	55	124bp
13	GC34 S2	F: 5'- TCGACCTACTGCATGTCCTTT -3' R: 5'- TGACAAAGGGCTAATATCCAGA -3' Probe: 5'-TCATCAATGAAAATGGGGGT-3'	55	104bp
	GAPDH	F: 5'-TGCCTTCTTGCCTCTTGTCT-3'	55	110bp

*Primers for forward (F), reverse (R), and probe (Probe) sequences.

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4. Results

4.1 LCM and purification of DNA

Among 178 cases whose serial plasma samples up to 12 months after curative surgical resection were available (stage II, N=69; stage III, N=84; stage IV, N=24; GIST, N=1), all 21 recurrent cases (stage II, N=2; stage III, N=16; stage IV, N=2; GIST, N=1) and 4 non-recurrent cases (stage II, N=2; stage III, N=2) for which fresh-frozen paired tumor and normal samples were available from the Tissue Bank of the National Cancer Center were selected (Figure 6). Peritoneal seeding was diagnosed in two stage IV cases after surgical removal of the primary tumor. All of the patients' clinical information is described in Table 1. LCM was performed on the fresh-frozen primary tumors for enrichment of cancer cells (Figure 6), and the estimated cancer cell percentages after LCM were above 70%.

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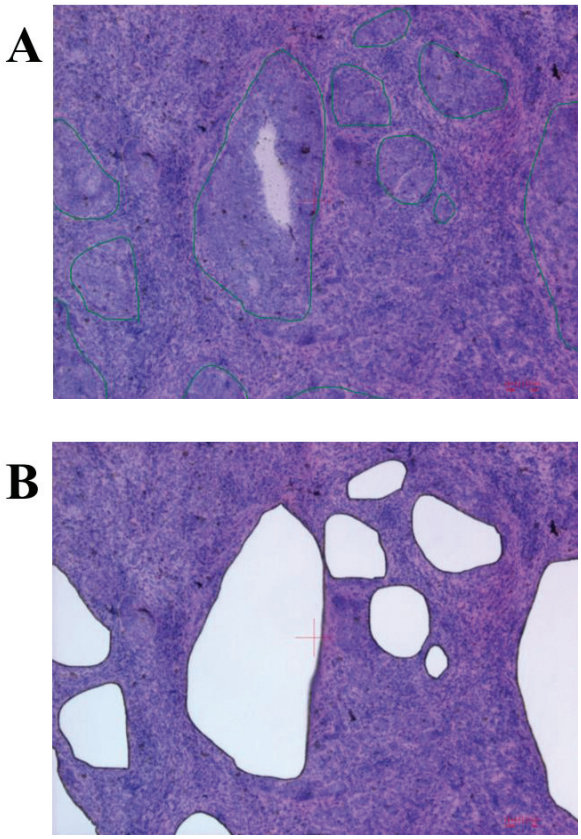

Figure 6. Fresh-frozen primary tumors, stained with haematoxylin and eosin. A. Cancer cells in primary tumor tissues before LCM. Cancer cell nests, marked in green lines. B. Remnant normal cells and inflammatory cells after LCM.

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4.2 Identification of tumor-specific rearrangement sequences

To identify personalized rearrangements that could serve as biomarkers, WGS was performed on DNAs isolated from 25 paired primary gastric cancer and normal gastric tissues. On average, 796 million DNA fragments were sequenced per tumor (range: 683- 933 million), yielding a mean genome sequence coverage of 41.7-fold (range: 35.8-48.9) (Table 3). After analysis of the WGS data, rearranged sequences specific to the tumor samples were identified (Figure7, Table 4). In 6 cases, no personalized cancer-specific rearrangement was identified in the WGS data, and no further analysis was performed.

4.3 Confirmation of selected cancer-specific rearrangement sequences

PCR primers were designed for 141 sites from 19 cases in which cancer-specific rearrangement was identified in the WGS data (Table 5). Out of 141 primer pairs, cancer-specific amplification was observed at 84 sites (Table 1). With Sanger sequencing, personalized cancer-specific rearranged sequences were confirmed at 66 sites (Figure7C, Table 1). With the Sanger sequencing data, specific primers were designed again for short-length PCR products. With the designed short primer pairs, rearranged sequences were confirmed finally by cancer-specific PCR and Sanger Sequencing at 63 rearranged sites (Table 5, Figure 8,9), and these personalized cancer-specific short primers were used for monitoring of ctDNA in plasma samples (Kang et al. 2015)

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4.4 Monitoring for presence of ctDNA in serial plasma samples

Circulating cell-free DNA was isolated from 83 plasma samples from 19 patients. Each personalized cancer-specific PCR was performed along with positive (tumor DNA) and negative (paired normal DNA) controls (Figure8). To confirm the rearranged sequences, the amplified products were sequenced by the Sanger sequencing method (Figure 9).

In pre-operative plasma, ctDNA was positive in 11 cases, and the positivity rate of pre-operative ctDNA in advanced gastric cancer patients was 58% (11/19) (P = 0.0587 by Fisher's exact test, Table 1). In the analysis of pre-operative ctDNA positivity and the clinical T stages (tumor size) of the gastric cancer patients, there was no significant correlation (P = 0.3189), though the case number was quite low. None of the other clinical factors, including N stage, clinical stage, and Lauren classification, was significantly correlated with pre-operative ctDNA positivity either. ctDNA was detected in post-operative plasma samples from 8 cases, and the median lead time from ctDNA positivity to clinical recurrence after ctDNA detection was 4.05 months (Table 1). Two clinical stage IV cases in each of which positive peritoneal seeding was found after surgical resection showed positive ctDNA in the post-operative plasma. In seven cases, no ctDNA was detected in pre- or post-operative plasma samples, even with 3-5 different markers (Figure 10).

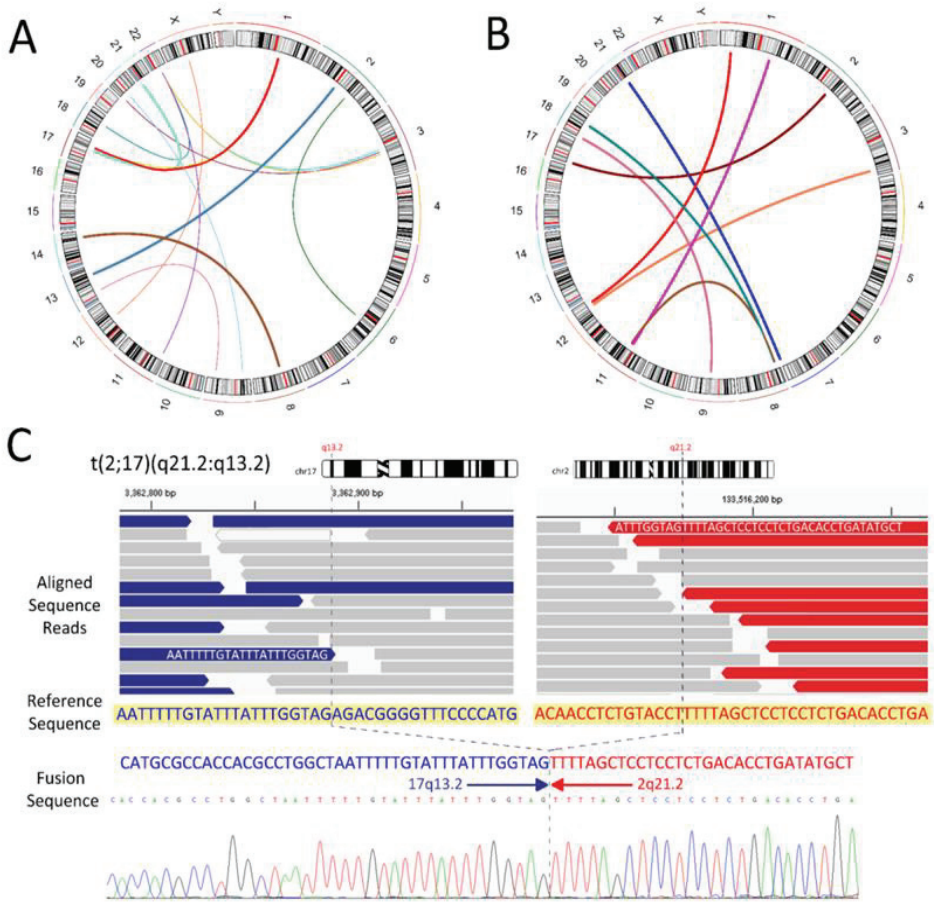

Figure 7. Identification of personalized cancer-specific rearrangements.

A. Circos diagram for rearrangements in GC17. The inter- and intra-chromosomal arcs in the center indicate chromosomal rearrangements. B. Circos diagram for GC32. C. Analysis of a rearranged sequence which is marked red in B for GC32 by Sanger sequencing.

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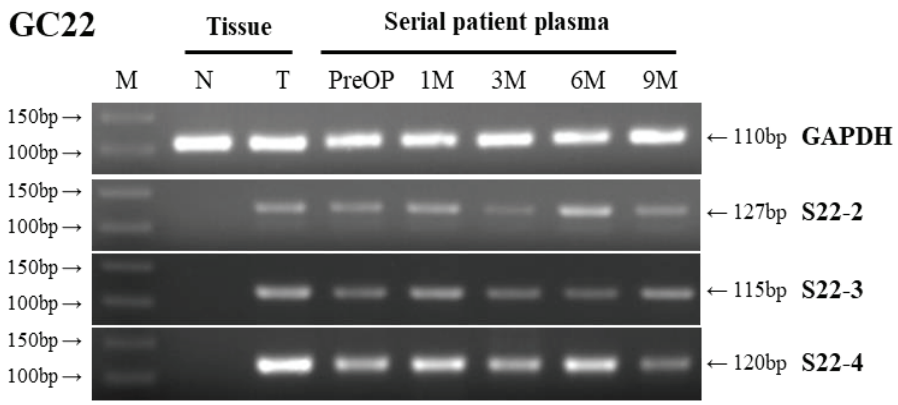

Figure 8. Monitoring of ctDNA in serially collected plasma samples. Confirmation of rearrangement sites by using PCR. Rearranged sequences (S22-2, S22-3, and S22-4) are amplified in pre-operative (PreOP) and serial post-operative plasma samples collected at 1-9 months (1M – 9M) after surgery, along with normal (N) and tumor (T) tissue samples. Mr, molecular size markers.

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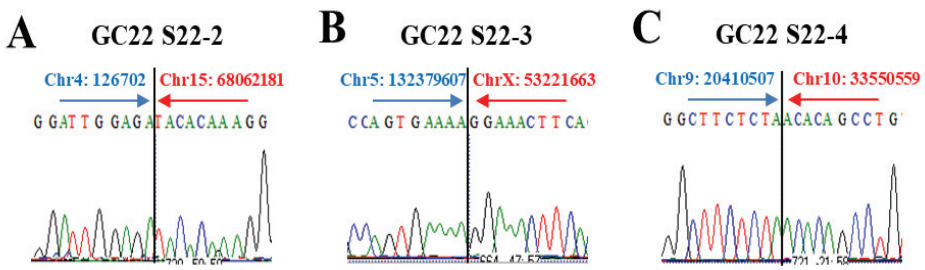

Figure 9. Confirmation of rearrangements by Sanger sequencing for 3 rearrangements.

Amplified products were sequenced by Sanger sequencing method. Rearranged sequences (S22-2 (A), S22-3 (B), and S22-4 (C)) of GC22 are confirmed.

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Sample ID	PCR		ctDNA						RFS (month)
	Tumor	Normal	PreOP	1M	3M	6M	9M	12M	
GC17	+	-	+	+		+	+	+	metastatic
	+	-	+	+		+	+	+	
	+	-	+	+	ND	+	+	+	
	+	-	+	+		+	+	+	
	+	-	+	+		+	+	+	
GC22	+	-	+	+	+	+	+		metastatic
	+	-	+	+	+	+	+	ND	
	+	-	+	+	+	+	+		
GC4	+	-	+	+	+	+	+	+	10.3
	+	-	+	+	+	+	-	+	
	+	-	+	+	+	-	+	+	
GC8	+	-	+	-	-	-	-	-	1.4
	+	-	+	-	+	-	-	-	
GC9	+	-	+	-	-	-	-	-	5.9
	+	-	+	-	-	-	-	-	
	+	-	+	+	-	-	-	-	
GC10	+	-	-	-	-	-	-	-	5.6
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
GC11	+	-	+	+	+	+			6.7
	+	-	+	-	-	+	ND	ND	
	+	-	+	+	+	+			
	+	-	-	-	+	+			
GC14	+	-	-	-	-	-	-	-	6
	+	-	-	-	-	+	+	-	
GC15	+	-	+	+	+	+	+	+	4
	+	-	+	-	+	+	-	-	
GC21	+	-	-	-	-	-	-	-	5.6
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
GC1	+	-	-	-	-	-	-	-	31.2
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
	+	-	-	-	-	-	-	-	
GC6	+	-	-	-	-				16.8
	+	-	-	-	-	ND	ND	ND	
	+	-	-	-	-				
GC7	+	-	-	-	-	-	-	-	13.1
GC12	+	-	-	-	-	-	-	-	15.2
	+	-	-	-	-	-	-	-	
GC18	+	-	-	-	-	-	-	-	30.4
	+	-	-	-	-	-	-	-	
GC32	+	-	+	-	-	-	-	-	18.2
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
GC31	+	-	+	-	-	-	-	-	non-recur
	+	-	+	-	-	-	-	-	
GC33	+	-	+	-	-	-	-	-	non-recur
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
	+	-	+	-	-	-	-	-	
GC34	+	-	+	-	-	-	-	-	non-recur

Figure 10. ctDNA positivity in 19 gastric cancer patients. Each line for a case indicates each personalized cancer-specific rearranged marker: +, positive ctDNA; -, negative ctDNA; RFS, relapse-free survival in months; ND, not determined.

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4.5 Correlation between post-operative ctDNA and clinical recurrence

In the analysis of the correlation between post-operative ctDNA positivity and clinical recurrence, the presence of post-operative ctDNA at any time within 12 months of surgical resection was significantly correlated with cancer recurrence within 12 months of surgical resection (P = 0.0023, Figure. 11B), in contrast to the finding of no significance for pre-operative ctDNA positivity (P = 0.6372, Figure 11A). For this analysis, ctDNA was considered as positive when any cancer-specific rearranged sequence was detected in any plasma sample. However, this correlation might not be properly indicative of the usefulness of ctDNA monitoring, because ctDNA-positive cases detected later than clinical recurrence also were included in the positive correlation. To remove this error, ctDNA-positive cases detected only prior to clinical recurrence were analyzed as post-operative ctDNA-positive cases, and the results once again indicated a significant correlation between ctDNA positivity prior to clinical recurrence and cancer recurrence within 12 months of curative surgical resection (P = 0.0294, Figure 11C), suggesting that ctDNA positivity can be an indicator of imminent clinical recurrence.

A statistical analysis on the correlation between adjuvant chemotherapy (Table 2) and post-operative ctDNA negativity was not significant, due to the limited case number. However, all three non-recurrent pre-operative ctDNA-positive cases with adjuvant chemotherapy were negative for

post-operative ctDNA, in contrast to all two pre-operative ctDNA-positive cases without adjuvant chemotherapy, which were positive for post-operative ctDNA.

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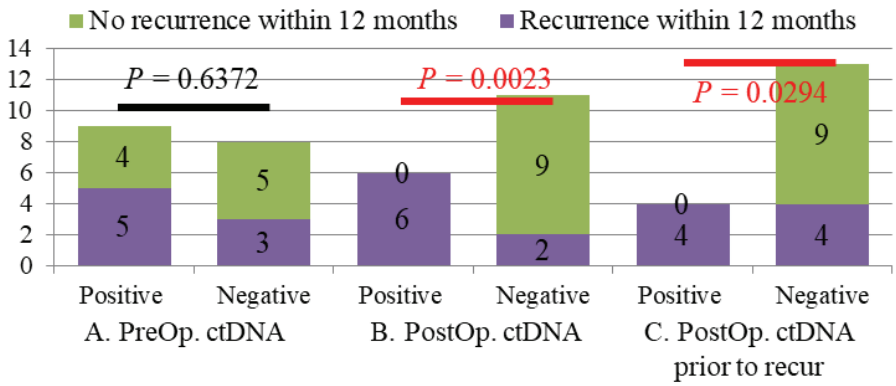

Figure 11. Correlation of ctDNA positivity with recurrent event within post-operative 12 months.

A. Correlation of pre-operative ctDNA positivity with recurrent event. B. Correlation of post-operative ctDNA positivity with recurrent event. C. Correlation of post-operative ctDNA positivity prior to clinical recurrence with recurrent cancer.

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4.6 Quantitative measurement of ctDNA in plasma

For quantitative measurement of ctDNA in plasma, quantitative PCR was performed for 13 cases. Amplification was confirmed in 96.1% (74/77) of plasma samples in which the presence of ctDNA was tested by PCR and Sanger sequencing (Figure 12, Table 7). Detection of ctDNA can help to predict clinical recurrence, as shown in Figure 12A; however, the cases shown in Figures 12B-12C would not be helpful, because the detection time is similar to or later than the date of clinical recurrence. In one case (Figure 12D), ctDNA was detected 1 month after surgery but not later than clinical recurrence. In order to check if there are more ctDNA-positive cases, quantitative PCR was performed in pre-operative bloods from 5 ctDNA-negative cases by the employment of sample amounts equivalent to 333 μL (for GC12 and GC18) or 833 μL (for GC1, GC6, GC10, and GC12) of plasma, but all were negative (Table 8).

In our quantitative results, the ctDNA level in most of the pre- and post-operative plasma samples was at the lower limit for quantitative PCR detection (mean Ct value: 37.8), which limits the quantitative value of the ctDNA. The difference in the ctDNA level between the pre-operative and post-operative plasma was not large (2-4 cycles) relative to the difference expected in light of the dramatic tumor size reduction after curative surgical treatment.

We performed droplet digital PCR (ddPCR) for two markers in the GC4 case, employing 10 μL cfDNA (equivalent to 333 μL plasma) to ensure the positive identification of ctDNA in post-operative plasma. In all of the post-operative

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plasma samples for the two markers by ddPCR, ctDNA was positive, which is quite correspondent to our results by quantitative PCR with employment of the same amount (10 µL) of cfDNA (Tables 7, and 9).

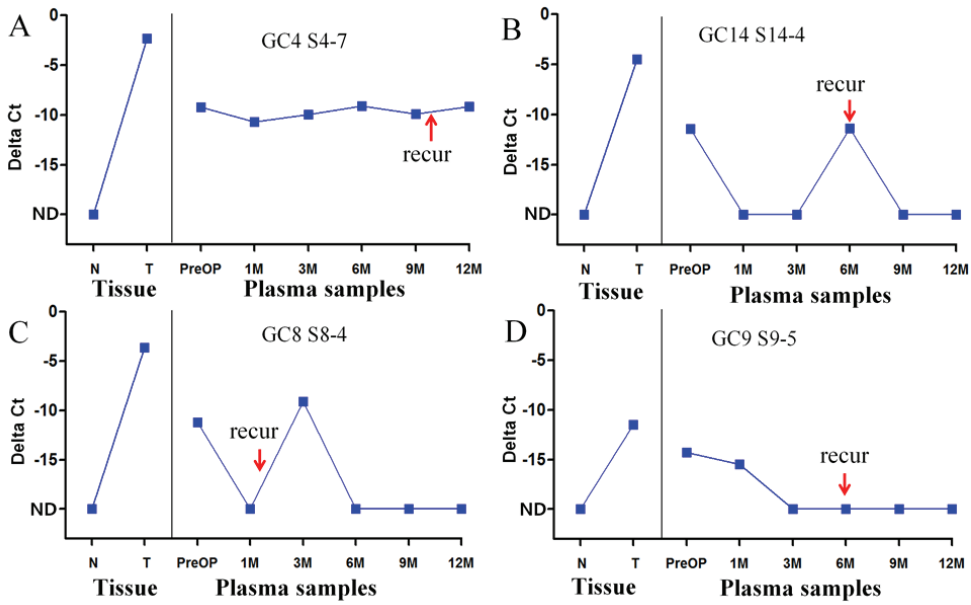

Figure 12. Quantitative measurement of ctDNA levels in serial plasma samples from gastric cancer patients.

ctDNA levels in bloods from cancer patients GC4 (A), GC14 (B), GC8 (C), and GC9 (D). *GAPDH*, amplification control. X-axis, DNAs from normal (N) and cancer (T) tissues, and from pre-operative (PreOP) and post-operative (PostOp) plasma samples at 1, 3, 6, 9, and 12 months after surgery. Y-axis, delta Ct (the difference of Ct values between the marker and *GAPDH*). The arrows indicate the time of clinical recurrence after surgery. ND,non-detectable.

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Table 7. Estimation of relative level of ctDNA by quantitative PCR.

Sample ID	Tissue (Ct)		ctDNA (Ct)					
	Normal	Tumor	PreOP	PostOP				
				1M	3M	6M	9M	12M
GC4	-	27.91	35.25	36.15	36.65	36.18	37.96	36.95
GC8	-	28.22	38.62	-	37.56	-	-	-
GC9	-	37.11	38.94	40.98	-	-	-	-
GC12	-	26.16	-	-	-	-	-	-
GC14	-	32.54	39.44	-	-	39.35	N.M	-
GC15	-	30.90	37.57	-	N.M	N.M	-	-
GC17	-	32.02	38.94	39.71	N.D	40.05	35.88	38.58
GC18	-	26.00	-	-	-	-	-	-
GC22	-	26.87	37.89	37.70	37.59	38.25	37.87	N.D
GC31	-	26.46	35.25	-	-	-	-	-
GC32	-	25.56	36.06	-	-	-	-	-
GC33	-	28.39	37.17	-	-	-	-	-
GC34	-	30.73	40.28	-	-	-	-	-

Ct, threshold cycles; -, not detected; N.M, not matched; N.D, not determined.

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Table 8. Estimation of the level of pre-operative ctDNA by quantitative PCR in 5 pre-operative ctDNA-negative cases.

Sample ID	*GAPDH* (Ct)			Target (Ct)		
	Normal sample	Cancer Tissue	Pre-operative plasma (sample amount µL*)	Normal sample	Cancer Tissue	Pre-operative plasma (sample amount µL*)
GC1	27.52	23.6	27.66 (167)	-	28.64	-833
GC6	25.65	24.12	27.42 (167)	-	26.7	-833
GC10	28.22	24.62	29.72 (167)	-	27.67	-833
GC12	26.86	25.18	30.45 (167)	-	26.85	-833
GC18	24.12	23.71	27.13 (333)	-	26	-333

* Sample amount of equivalent plasma employed for quantitative PCR

Ct, threshold cycles; -, not detected.

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Table 9. Quantitative measurement of ctDNA by digital droplet PCR

Marker	Tissue		ctDNA					
				PostOP				
	Normal	Tumor	PreOP	1M	3M	6M	9M	12M
GC4 S4-6	0	301	28	16	17	13	3	26
GC4 S4-7	0	354	19	15	10	5	9	11

The number of positive droplets is shown.

PreOP, pre-operative; PostOP, post-operative samples; 1M, 3M, 6M, 9M, and 12 M, plasma samples at 1, 3, 6, 9, and 12 months after surgery, respectively.

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5. Discussion

In the analysis of ctDNA levels in post-operative blood employing personalized cancer-specific rearrangements instead of mutations, we confirmed the presence of ctDNA at a median lead time of 4.05 months, and found that post-operative ctDNA positivity prior to clinical recurrence was significantly correlated with cancer recurrence within 12 months of radical surgery (P = 0.029). As such, our study can be considered to have confirmed the clinical usefulness of ctDNA monitoring for cancer recurrence in gastric cancer patients after curative surgical resection.

Although ctDNA has been detected in blood samples obtained from cancer patients, its usefulness for the detection of early recurrence after curative surgical resection has been in question, due to the possibility of low level of ctDNA shedding from microscopically remnant or recurrent cancer cells when the general correlation between the tumor burden and the ctDNA level is considered (Muhanna et al. 2017). Two studies on blood ctDNA for monitoring of recurrence in breast cancer (Garcia-Murillas et al. 2015) and colon cancer (Tie et al. 2016) suggested the possibility of the clinical application of mutation monitoring. The employment of mutations for serial ctDNA monitoring, however, can suffer from a high rate of inconsistency due to false positivity or negativity, or technical NGS problems in the detection of mutations, especially for low-allele-frequency mutants (Hudson et al. 2014). The proportion of ctDNA in blood is extremely low, and so NGS methods must be effective in detecting mutant allelic frequencies as

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low as 0.1% (Crowley et al. 2013), which fact might lead to inconsistency in ctDNA detection by NGS. A comparative study of mutations in primary tumors and ctDNA from the blood of advanced lung cancer patients also indicated that there would be inconsistency when mutation calls obtained from NGS are employed for monitoring of ctDNA: the concordance rate was only 50.4%, even in the blood from cancer patients who had not undergone surgical removal of primary tumors (Chen, Lou, et al. 2016). In order to alleviate the problem of inconsistency in NGS, personalized cancer-specific rearrangements have been employed for detection of ovarian cancer recurrence (Harris et al. 2016), the resultant data confirming the presence of ctDNA in post-operative blood; however, the clinical usefulness of ctDNA was not analyzed in that study. The present study, having employed cancer-specific rearrangements to increase specificity and sensitivity for detection of ctDNA in serially collected post-operative bloods, established the clinical usefulness of ctDNA monitoring for cancer recurrence: the presence of ctDNA was confirmed at a median lead time of about 4 months, which demonstrated the significant association between ctDNA presence in blood prior to clinical recurrence and cancer recurrence within 12 months of curative surgical resection. Therefore, our study can be considered to advocate for the utility of ctDNA monitoring for cancer recurrence after curative surgical resection.

Although previous studies have shown that ctDNA can be an excellent screening method for cancer recurrence, significant fractions of their recurrent cancer patients showed ctDNA negativity in their post-operative blood

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(Garcia-Murillas et al. 2015, Bettegowda et al. 2014). The main suggested factors behind those results were tumor heterogeneity and the relative paucity of remnant cancer cells after curative resection. Inconsistent post-operative ctDNA positivity for each rearranged sequence in some cases in the present study might indicate the possible heterogeneity in cancer cells, which would necessitate the employment of several rearranged markers for a case to increase the post-operative ctDNA positivity. In addition, ctDNA non-shedders, who do not have detectable ctDNA in pre-operative blood, may be one of the main reasons for the ctDNA-negativity in recurrent post-operative blood because a lot of cases in the present study showed no ctDNA in pre-operative blood though all recruited patients were at the T3 or T4 stage. Moreover, most of the pre-operative ctDNA-negative cases (7/8) and recurrent ctDNA non-shedders (4/5) remained ctDNA-negative in post-operative blood. Consistent with this, pre-operative ctDNA-negative cases and ctDNA non-shedders have already been reported (Cohen et al. 2018). Therefore, ctDNA-non-shedders might be an important reason for ctDNA negativity in recurrent cases. Inclusion of only pre-operative ctDNA-positive cases or ctDNA shedders for ctDNA monitoring might, accordingly, improve cost-effectiveness for early detection of cancer recurrence after curative surgical treatment.

In the present study on serial monitoring of ctDNA in post-operative blood, several issues arose. First, ctDNA in serial post-operative blood was not consistently positive during the follow-up periods. For example, in one case, ctDNA was positive at 1 month following surgical resection, but became negative

until clinical recurrence, which suggests that ctDNA levels during follow-up periods might continually change with ctDNA dynamics. Therefore, the meaning of ctDNA-positivity in the short term, as it relates to cancer recurrence risk, might be difficult to determine. At the very least, more frequent monitoring of ctDNA could increase the chances of correctly identifying recurrent cases, or could help to determine the risk for cancer recurrence. The second issue that arose in this study with respect to serial monitoring of ctDNA in post-operative blood was the fact that the level of post-operative ctDNA was not much different from its pre-operative level, although a large decrease in tumor burden after curative surgical removal of primary cancer was expected. Previous studies employing mutations for ctDNA monitoring also have reported cases showing small changes in ctDNA levels between pre- and post-operative blood (Garcia-Murillas et al. 2015, Hamakawa et al. 2015), suggesting that factors other than tumor size might also be important for determination of ctDNA levels. Although ctDNA levels have been reported to be correlated with tumor size (Crowley et al. 2013), there were no significant correlations in the present study between pre- or post- operative ctDNA positivity and T stage (tumor size), which supports the supposition that inherent biological or dynamic tumor factors determine ctDNA levels. Therefore, issues such as the presence of ctDNA in short-term follow-up and ctDNA dynamics independent of tumor size could be considered to interfere with accurate prediction of cancer recurrence by ctDNA monitoring.

In the present study, personalized cancer-specific rearrangements were

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employed for monitoring of ctDNA in post-operative blood samples obtained from cancer patients. We expected that monitoring of rearrangements in post-operative blood would be sensitive, simple, and rapid for more frequent monitoring of ctDNA. Although the sensitivity employing mutations have been dramatically increased (Lee et al. 2016, Park, Park, et al. 2018, Kinde et al. 2011), but the serial monitoring of mutations from post-operative blood by NGS or droplet digital PCR would take more time and cost than simple PCR. However, the burden of time and cost in obtaining information on cancer-specific rearrangements by WGS is high. Especially, high proportions of rearrangements detected in WGS analysis are negative in PCR confirmation or PCR sequencing. In the present study furthermore, WGS failed to find any cancer-specific rearrangements in 6 out of 25 cases, adding to the difficulty of employing rearrangements for ctDNA monitoring. Therefore, for employment of personalized cancer-specific rearrangements in monitoring of ctDNA, more time- and cost-effective screening methods are necessary.

The present study has several limitations. It was performed retrospectively, plasma samples having been collected until 12 months after curative surgical resection, and the available recurrence cases were enrolled preferentially, both of which conditions can incur bias. A prospective study with more extensive serial collection of plasma samples until cancer recurrence would yield more objective information on ctDNA monitoring for cancer recurrence. Additionally, the present study employed only limited amounts of plasma, about 1 ml in most cases, and only about 67 ul of plasma per PCR reaction for monitoring of ctDNA,

because several markers had to be checked at the same time. Employment of larger volumes of plasma for ctDNA monitoring would increase sensitivity.

In conclusion, we demonstrated the usefulness of ctDNA monitoring employing personalized cancer-specific rearranged sequences for detection of gastric cancer recurrence, having confirmed the presence of ctDNA, at a median lead time of 4.05 months, and its significant correlation with clinical recurrence. Our results also raise important issues that could limit the usefulness of ctDNA monitoring: 1) ctDNA non-shedders without any detectable pre-operative ctDNA, most of which remain as ctDNA non-shedders even after cancer recurrence; and 2) inconsistent post-operative ctDNA positivity in ctDNA shedders. In consideration of our overall results, ctDNA monitoring for cancer recurrence certainly warrants future prospective studies on its clinical utility, but the limitations due to ctDNA dynamics during pre- and post-operative periods should be considered for designing prospective studies.

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