Detection of gene mutations in gastric cancer tissues using a commercial sequencing panel

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Received March 20, 2019; Accepted August 26, 2019

DOI: 10.3892/mco.2019.1926

Abstract. Predicting malignancy is important for adequate adjuvant therapy in patients with cancer. Due to cancer being a genetic disease, the detection of gene mutations could be helpful in predicting the prognosis and efficacy of drugs. Gastric cancer is the fifth most common cancer and is the third leading cause of cancer associated mortality worldwide. Mutations in genes may correlate with clinical information in patients with gastric cancer after surgery and, therefore, may be useful for predicting the prognosis of this disease. In the present study, to assess the usefulness of a commercial sequencing panel, TruSeq® Amplicon-Cancer Panel (Illumina), using a next-generation sequencer (Illumina MiSeq), mutation analysis of fresh as well as formalin-fixed paraffin-embedded (FFPE) gastric cancer tissues was performed retrospectively. The study group comprised of 4 patients who underwent gastrectomy for gastric cancer. Cancer and normal stomach tissues were collected immediately following surgical removal. Thereafter, the specimens were fixed in 10% neutral formalin for 24-72 h. Normal and FFPE cancer tissues were histologically examined and confirmed. A total of 3 mutations were identified in the driver genes (*KRAS*, *TP53* and *APC*) in cancer tissues from 2 of the 4 patients, using fresh samples. In addition, FFPE samples were analysed for the same tissues and the same results were obtained by setting the threshold for the percentage of the mutation rate to avoid detection of pseudo-positive mutations. In conclusion, the sequencing analysis using FFPE-derived DNA samples was successfully performed.

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Abbreviations: NGS, next-generation sequencing; ACRG, The Asian Cancer Research Group; TCGA, The Cancer Genome Atlas; FFPE, formalin-fixed paraffin-embedded; bp, base pair; SVC, somatic variant caller; GQ, genotype quality; VF, variant frequency; SB, variant strand bias; MSI, microsatellite instability; MSS, microsatellite stable, EMT, epithelial-to-mesenchymal transition; TP53+, tumor protein 53 functional activation, TP53-, tumor protein 53 function loss; APC, adenomatous polyposis coli; KRAS, V-KI-RAS2 Kirsten rat sarcoma viral oncogene homolog

Key words: gastric cancer, sequencing panel, cancer panel, next-generation sequencing, mutation, formalin-fixed paraffin-embedded, adenomatous polyposis coli, V-KI-RAS2 Kirsten rat sarcoma viral oncogene homolog, tumor protein 53 function loss

Introduction

Gastric cancer is the fifth most common cancer and is the third leading cause of cancer-related deaths worldwide (1). In East Asia, more than one million new cases are diagnosed each year. The methods for diagnosis of gastric cancer are improving and early-stage gastric cancer can be detected by upper gastrointestinal endoscopy. A part of early-stage gastric cancer tissue can be removed by endoscopic resection. However, surgeries for gastric cancer in Stage II and III are common. Sasako *et al* reported that postoperative adjuvant therapy with S-1, an oral fluoropyrimidine, improved the overall and relapse-free survival in patients with Stage II and III gastric cancer, who had undergone D2 gastrectomy (2). However, the overall prognosis of patients with gastric cancer is still poor. According to The Asian Cancer Research Group (ACRG) data (3), the 5 year prognosis of patients with Stage II, III and IV gastric cancer is 76, 59 and 24%, respectively. Combinations of chemotherapeutic drugs have a limitation in effective curing of patients with advanced gastric cancer. Therefore, molecular therapy is needed for good prognosis of this cancer.

Companion diagnostics provide information on the effective use of a drug or biological product that helps physicians decide the appropriate treatment for patients. Especially, in the field of cancer, new technologies, such as next-generation sequencing (NGS), are used to identify mutations in the genome (3-5). Currently, the most prevalent implementation of NGS in oncology is in the detection of mutations using targeted panels. NGS can be multiplexed to assay many genes simultaneously. It is, therefore, important to know the characteristics of cancer at the molecular level. The Cancer Genome Atlas (TCGA) analysis revealed that gastric cancer has many

DNA alterations, such as mutations, copy number variations, insertions, deletions, and translocations (4).

Evaluating the molecular status of patients, such as the relationship between mutant genes in the gastric cancer tissue and clinical characteristics, is important for understanding the mechanism of oncogenesis and for identifying biomarkers of gastric cancer, and thereby, for improving the clinical outcomes (6). Mutations of *CDH1* are useful for diagnosing diffuse-type gastric cancers (4). With regard to somatic mutations, it has been reported that *TP53*, *KRAS*, *ARID1A*, *PIK3CA*, *ERBB3*, *PTEN* and *HLA-B* are the commonly mutated genes in gastric cancer (4). In a previous study, *TP53* mutations were reported to be the most common in gastric cancer samples, followed by mutations of *EGFR*, *HNF1A*, *PIK3CA* and *ERBB2* (7). As of date, several drugs, with molecular targets, have been evaluated for their efficacy in treating cancers, which has been found to be associated with the genetic profile of patients. *ERBB3* mutation confers sensitivity to the anti-HER3 drug against gastric cancer (8). On the other hand, in the case of *ERBB2* amplification, Trastuzumab, in combination with chemotherapy, is effective for advanced gastric cancer (9). In advanced gastric cancer with low amplification of *ATM*, the poly (ADP ribose) polymerase inhibitor, Olaparib, in combination with chemotherapy in a Phase II trial was reported to be effective (10). *FGFR2* amplification is associated with resistance to LY2874455, a pan-FGFR inhibitor, in patients with advanced gastric cancer (11). In addition, esophagogastric cancer with *MET* amplification was reported to be sensitivite to Crizotinib (12). However, based on evidence, only Trastuzumab is effective for advanced gastric cancer.

Kuboki *et al* performed comprehensive analyses of advanced gastric cancer using NGS and immunohistochemistry (IHC), and found that the results of the amplification status obtained using NGS differed from those obtained using IHC (13). Therefore, it is important to perform a comprehensive analysis of the relationships of gene alteration status and patient's characteristics. Park *et al* performed an NGS analysis using a targeted gene panel to detect common as well as rare mutations, and showed that the accumulation of microsatellite instability status contributes to the genetic diversity and complexities in gastric cancer (7).

In the present study, to assess the usefulness of Illumina Cancer Panel, a commercial sequencing panel, using a next-generation sequencer (Illumina MiSeq), we performed a retrospective mutation analysis of fresh and formalin-fixed paraffin-embedded (FFPE) gastric cancer tissues from four patients. Using FFPE samples with next-generation sequencer is practically more useful than using fresh frozen samples. We hypothesized that mutations in some genes would be associated with clinical features in patients with gastric cancers, and evaluated such relationships.

Patients and methods

Patients. The study group comprised of four patients who underwent gastrectomy for gastric cancer at the Department of Surgery, Juntendo University Shizuoka Hospital, Japan between May 2012 and June 2012. We selected four patients who had diverse characteristics because we focused on an associated analysis of mutated genes and clinical characteristics using a commercial sequencing panel. Fresh cancer and normal stomach samples were collected immediately after surgical removal. Normal fresh tissues were collected from surgical margins that were distant from the cancer sites. Thereafter, the samples were fixed in 10% neutral formalin for 24-72 h. FFPE cancer tissues and normal tissues were examined histologically. Mutation analysis of both fresh and FFPE tissues was performed employing the cancer panel using a next-generation sequencer (Illumina MiSeq®). The medical records of the patients were reviewed retrospectively. Written informed consent was obtained from all the patients. This experiment was approved by the Ethics Committee of Juntendo University Shizuoka Hospital.

Amplicon library construction and deep sequencing. The TruSeq® Amplicon-Cancer Panel (Illumina, San Diego, CA, USA) provides pre-designed, optimized oligonucleotide probes for sequencing mutational hotspots in >35 kilobases (kb) of the target genome sequence. Forty-eight genes were targeted with 212 amplicons in a highly multiplexed, single-tube reaction (the gene list and the primer sequences are shown in Tables SI and SII).

DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen GmbH, Germany) for fresh tissues. QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Germany) was used for FFPE sections after deparaffinization with xylene and 100% ethanol. A pair of genomic DNA samples (250 ng each) consisting of genomes of tumor and matched normal (non-tumor) tissues, derived from the same patient was used for experiments, according to the manufacturer's instructions. Briefly, genomic DNA was initially hybridized with pairs of oligonucleotide-probes specific to the targeted regions and subsequently washed to remove the unbound probes. The pairs of oligonucleotide-probes were extended and ligated to form templates, which was followed by PCR amplification using primers that add adaptors and index tags for multiplex sequencing. The PCR products were then purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). The quality of the DNA libraries was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The quantity-normalized libraries were pooled and sequenced using the Illumina MiSeq system in 151-base-pair (bp) paired-end reads.

Variants calling. Somatic Variant Caller (SVC) ver 3.1 (Illumina) was used to align sequence reads to the human reference genome (GRCh37/hg19) and to perform somatic variant calling. Raw variant calls that failed to pass the following filters were eliminated: Genotype Quality (GQ) <30, Variant Frequency (VF) <0.05, Indel repeat length >8, Variant Strand Bias (SB) too high. We also removed indels that were detected by SVC at the boundaries of the amplicons with custom scripts.

Functional annotations of the Ensembl database GRCh37.72 (14) and the possible effects of variants were added using SnpEff version 3.3 h (15). Using these annotations, the variants were filtered; initially those that were predicted to alter amino acid sequences (missense, nonsense, and splice-site mutations, and indels in coding regions) were

Table I. Patient characteristics.

	Patient 1	Patient 2	Patient 3	Patient 4
Age (years)	77	80	71	71
Sex	Male	Female	Male	Male
Tumor location	M	U	M	M
Surgery	Distal gastrectomy	Total gastrectomy	Distal gastrectomy	Distal gastrectomy
Node dissection	D2	D2	D2	D2
Histology differentiation	Mod	Undiff	Mod	Undiff
Lauren's classification	Intestinal	Mixed	Mixed	Undetermined
Helicobacter pyroli	Negative	Positive	Positive	Negative
TNM stage (UICC 8th)	T3N0M0, IIA	T2N0M0, IB	T3N2M0, IIIA	T3N2M0, IIIA
Recurrence	None	None	None	Liver
Relapse free survival (months)	60	67	75	12
Outcome	Alive	Alive	Alive	Death
Overall survival (months)	60	67	75	17

M, middle third; U, upper third; Mod, moderately differentiated adenocarcinoma; Undiff, undifferentiated adenocarcinoma.

filtered, and then those that were rare [<1.0% Minor Allele Frequencies (MAF) in the HapMap-JPT (Japanese in Tokyo, Japan; http://hapmap.ncbi.nlm.nih.gov/) or the 1000 Genomes ASN (the East Asian population, composed mostly of Japanese and Chinese databases; http://www.1000genomes.org/)] were filtered.

Results

Clinical characteristics. The clinicopathological features of the four patients are listed in Table I. All the patients were over 70 years of age (the median age was 74) and had undergone R0 gastrectomy. Three patients were finally diagnosed with advanced gastric cancer at pStage II and III as per the TNM Classification of Malignant Tumors proposed by the UICC 8th edition (16). The fourth patient was finally diagnosed with pStage IB cancer. Two of the four cases were diagnosed as moderately differentiated adenocarcinoma, pathologically, and the others were diagnosed as undifferentiated adenocarcinoma (17). According to Lauren's classification (18), two of the four patients were diagnosed with 'mixed-type' and one patient was diagnosed with 'intestinal-type' adenocarcinoma. The other patient was classified as 'undetermined.' Only one patient had recurrence of liver metastasis, one year after surgery, and the survival time was 17 months. The other patients did not have any recurrence and were alive more than 5 years after the surgery.

Deep sequencing analysis. Forty-eight genes were analyzed for the four cases to detect the mutation and to compare the insertion/deletion between cancer and normal tissues for each sample using TruSeq Amplicon-Illumina Cancer Panel. The results of next-generation sequencing for fresh samples showed that the total read bases ranged from 134 to 174 M base, and the average sequence coverage was from 3498 to 4538 depth (Table II). There was no difference between the cancer and normal tissues. In contrast, the FFPE samples, which were originally from the same cancer tissues used in these analyses, had much lower total read bases (39 to 122 M base) and the sequence coverage was from 1020 to 3190 depth (Table II). The average percentage of the coverage of more than 100 depth in each sequence was not different between the fresh and FFPE samples. These results showed that despite the lower read depth in FFPE samples because of DNA damage, there were enough good quality reads for finding rare mutations (present only in very less percentage) in cancer tissues.

Mutation analysis for each sample. We found three hotspot mutations in cancer tissues from two patients by comparison with normal tissues (Tables III and SIII). Patient no. 1 had two mutations, R283H in *TP53* (COSM11483) and G12D in *KRAS* (COSM521), and the percentages of these mutations in the cancer tissue were 15.3 and 18.5%, respectively. Although these mutations could be damaging for SIFT prediction, the patient had no recurrence for 5 years after surgery. Patient no. 4 had one mutation, R876* in *APC* (COSM18852); the percentage of this mutation was 26.8%. This mutation would insert a stop codon, and was, therefore, damaging. This patient had liver metastases within one year of surgery. These three mutations were also detected in FFPE samples of the same tissues. Patients no. 2 and no. 3 had no mutation for the 48 genes in the cancer panel in cancer tissues in comparison to normal tissues.

Effect of formalin treatment. Cancer panel analysis was successful for the same tissues, which were fixed in 10% formalin for 24 to 72 h. The nucleic acid transition was more prevalent in samples fixed for 72 h, but the error was not much for samples fixed for 24 h (Tables IV and SIV). Also, when the threshold was set to more than 15% as positive mutation, almost same results were obtained in the comparison of fresh and FFPE samples. These results showed that precise

Table II. Read depth of the coverage of sequencing.

Sample	Total read (bases)	Depth average	Depth >10x (%)	Depth >50x (%)	Depth >100x (%)	Depth >200x (%)	Depth >500x (%)
Fresh							
#1 Cancer	169066751	4404.50	69.6	69.6	69.2	67.9	66.5
#1 Normal	149629388	3898.12	69.6	68.9	68.3	67.6	65.5
#2 Cancer	134301536	3498.8	69.6	69.2	68.9	67.6	65.9
#2 Normal	174209760	4538.49	69.6	69.6	68.9	67.9	66.2
#3 Cancer	169186734	4407.63	69.7	69.6	69.2	68.6	66.2
#3 Normal	155001180	4038.07	69.6	69.6	68.6	67.6	65.1
#4 Cancer	152395380	3970.18	69.7	69.6	69.2	68.3	66.2
#4 Normal	137063750	3570.76	68.9	68.0	66.1	62.8	58.0
FFPE							
#1 Cancer	122449437	3190.03	69.7	69.2	69.2	69.2	67.5
#2 Cancer	95050075	2476.23	69.6	69.2	69.2	67.2	65.1
#3 Cancer	117840924	3069.97	69.6	69.2	68.9	68.2	66.2
#4 Cancer	39178167	1020.66	68.3	67.9	66.9	65.5	55.7

FFPE, formalin-fixed paraffin-embedded.

Table III. Mutation reports.

Patient	Variant frequency (%)	Gene ID	Gene name	Codon number	COSMIC ID
1	18.5	ENSG00000133703	KRAS	12	COSM521
1	15.3	ENSG00000141510	TP53	283	COSM11483
4	26.8	ENSG00000134982	APC	876	COSM18852

KRAS, V-KI-RAS2 Kirsten rat sarcoma viral oncogene homolog; TP53, tumor protein 53; APC, adenomatous polyposis coli.

identification of mutations, without false positives, can be made by setting a high threshold for the mutation call in the FFPE samples. In contrast, there was no effect on the average coverage in sequence depth regardless of whether the tissues were fixed using formalin or not.

Discussion

The cancer panel analysis was performed for four gastric cancer patients. Three gene mutations in cancer tissues were found for two patients. Among these genes, *TP53* acts as a tumor suppressor gene and plays the most important role in maintenance of genome integrity (19,20). The p53 protein also performs many complex functions within the cell (21-23). According to the data available in TCGA database, in gastric cancer, the mutation rate for *TP53*, which is one of the most frequently mutated genes in human cancers, is about 50% (24). In the ACRG data set, the prognosis was compared on the basis of molecular features of gastric cancer (3). Four molecular subtypes have been classified, namely microsatellite instability (MSI), microsatellite stable (MSS) and epithelial-to-mesenchymal transition (14,23) (MSS/EMT), TP53 functional activation (MSS/TP53+), and TP53 function loss (MSS/TP53-). Among the four subtypes, MSS/TP53+ and MSS/TP53- exhibit intermediate prognosis. There was no difference in the prognosis between MSS/TP53+ and MSS/TP53-. The prognosis of MSI group was the best among all the groups whereas that of MSS/EMT group was the worst.

We also found *KRAS*, *TP53* and *APC* mutations in cancer tissues from two patients. Although *KRAS* mutations are common in pancreatic, lung, and colorectal cancers, they are rare in gastric cancer. In a previous study, the frequency of *KRAS* mutation in gastric cancer was reported to be approximately 8% (3). Inhibitors of the epidermal growth factor (EGFR) signaling pathway have a major role in the treatment of colorectal cancer patients who have wild-type *KRAS* (25,26). However, EGFR therapies could not improve the prognosis in patients with unresectable gastric cancer, in several clinical trials (27,28). In another study, *KRAS* mutations in colon cancer were reported to be more frequent in elderly patients, but there was no relationship with the prognosis (29).

APC is a tumor suppressor gene in the Wnt signaling pathway, which is a regulator of several fundamental cellular processes, including cell division, cell attachment, and cell migration, in many cancers (30,31). Mutation of *APC* in cancer cells results in the accumulation of β-catenin, and

Table IV. Summary of mutation calls in each patient.

	Patient			
	1	2	3	4
Mutation				
Low quality (n)	14	49	6	33
Passed quality (n)	13	181	11	230
Passed quality (<15%) (n)	11	181	10	225
Passed quality (≥15%) (n)	2	0	1	5
Formalin treatment (h)	24	72	24	72

n, number of mutations.

transcriptional activation of an oncogene (32,33). The role of the Wnt signaling pathway has been observed in patients with germline mutations of *APC*, who present a 10-time higher risk of developing gastric cancer than the normal population (34). The *APC* mutation rate in gastric cancer was reported to be 12-32% (3,35,36). Moreover, a relationship was observed between *APC* mutations and the depth of invasion of gastric cancer (36). Although T1 tumor had more frequent *APC* mutation in gastric cancer tissue, there were no differences in any other clinical factor, including the stage. On the other hand, the *APC* mutation and decrease in APC protein expression in diffuse-type gastric cancer were associated with the advanced stage (37).

In the present study, one of the four cancer specimens had a *TP53* mutation. Therefore, the mutation of *TP53* was not related to the pStage and the prognosis. This result concurred with those of a previous study (3). *KRAS*, as well as TP53 mutations, were not affected in Patient #1. Patients #2 and #3 did not have any mutated gene in the cancer panel. This might be the reason for no recurrence of cancer and a good prognosis in these patients; however, it is possible that they have mutations in other genes. Patient #4 showed a recurrence and poor prognosis. Although there are no reports that the mutations of *APC* are associated with the prognosis in cancer patients, it might be possible that *APC* mutations had activated oncogenesis in this case. The fact that the histological type in this case was undifferentiated adenocarcinoma might relate with *APC* mutation and advanced stage.

We performed mutation analyses for both fresh and FFPE samples. In all cases, fresh samples had better quality DNA and less error in sequencing than FFPE. Nucleic acid fragmentation and cross-linking to proteins can reduce the quality of DNA and RNA extracted from FFPE specimens. In a previous study, it was reported that a better quality of DNA was obtained after fixing the samples in 10% formalin instead of fixing them in 20% formalin, as assessed by relative qPCR ratio (38). In addition, the fixation time is a very important factor for the quality of DNA. In the present study, the quality of DNA extracted from samples fixed in formalin for 24 h was much better than of those extracted from samples fixed for 72 h, suggesting that the best quality of DNA extracted from FFPE samples is obtained by fixation with 10% neutral formalin for one day. Using FFPE samples for next-generation sequencer is more useful than using fresh frozen samples in a clinical setting. It is not practical that frozen tissue is collected after or during surgery for a routine work (39). Recently, FFPE samples were used for the analyses of proteins by immunohistochemistry, as well as for DNA and RNA assays (38).

In conclusion, we successfully performed TruSeq® Amplicon-Cancer Panel with MiSeq® analysis and next-generation sequencing analysis using FFPE-derived DNA, even though a small sample size was used. The sequencing panel against most patients with advanced gastric cancer should be performed because understanding the molecular composition of cancer would be important in an era of molecular-guided targeted therapy. Further studies are needed to seek other gene mutations that are associated with the effectiveness of treatment of gastric cancer patients and to elucidate the relationship between the prognosis and such mutations.

Acknowledgements

Not applicable.

Funding

The present study was supported in part by a Grant-in-Aid (grant no. S1311011 and S1511008L) from the Foundation of Strategic Research Projects in Private Universities from the MEXT and a grant of the Institute for Environmental and Gender-Specific Medicine.

Availability of data and materials

The datasets used in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

TI, HO, and KS designed the experiments. RM performed the experiments. TI and RM analyzed the data and wrote the manuscript. RW, HM, TK, and MS contributed to the collection of clinical data and formalin-fixed paraffin-embedded samples. All the authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the declaration of Helsinki, and was approved by the Ethics Committee of Juntendo University Shizuoka Hospital (Rin-280). Written informed consent was obtained from the patients.

Patient consent for publication

Written consent for the publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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