in vivo *33*: 447-452 (2019)
doi:10.21873/invivo.11493

Comprehensive Analysis of Somatic Mutations in Colorectal Cancer With Peritoneal Metastasis

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Abstract. *Background: To analyze for genetic mutations which may presage peritoneal metastasis by using targeted next-generation sequencing (NGS). Materials and Methods: Formalin-fixed, paraffin-embedded primary tumor specimens were obtained from 10 patients with small obstructing colorectal cancer and peritoneal metastasis (group A) and five with large non-obstructing colorectal cancer and no recurrence (group B). DNA was extracted for the sequencing of 409 cancer genes. The distribution of genetic mutations was compared between the two groups to find genetic mutations related to peritoneal metastasis. Results: When the samples were sorted based on similarity of gene expression by hierarchical clustering analysis, the samples were well divided between the two study groups. Mutations in AT-rich interactive domain-containing protein 1A (ARID1A), polycystic kidney and hepatic disease 1 (PKHD1), ubiquitin-protein ligase E3 component n-recognin 5 (UBR5), paired box 5 (PAX5), tumor protein p53 (TP53), additional sex combs like 1 (ASXL1) and androgen receptor (AR) genes were detected more frequently in group A. Conclusion: A number of somatic mutations presumed to be relevant to colorectal cancer with peritoneal metastasis were identified in our study by NGS.*

Peritoneal metastasis is encountered in approximately 7% of colorectal cancer patients at primary surgery, in approximately 4-19% of patients during follow-up after curative surgery, and in 40-80% of patients who die of colorectal cancer (1). Peritoneal metastasis, once established, is associated with poor prognosis in gastrointestinal cancer, with a median survival of 5.2-12.6 months in colorectal cancer and 1-9.4 months in gastric cancer, because there is still no effective treatment for peritoneal metastasis (1-3). Therefore, the prevention of peritoneal metastasis may likely be key to increasing long-term survival.

Clinically, the risk factors for developing metachronous peritoneal carcinomatosis are advanced stage at diagnosis, right-side cancer location, infiltrative or ulcero-infiltrative carcinomas, and history of perforation and obstruction in colorectal cancer (4-7), but these factors are not reliable predictors for peritoneal metastasis in individuals. Although many researchers have investigated the mechanism of peritoneal dissemination in terms of genetic variations (8-17), common changes of gene expression in tumor progression to peritoneal metastasis have not yet been clarified.

We previously reported that patients with small obstructing colorectal cancer (SOC) tend to have peritoneal metastasis at the time of surgery and have higher recurrence rates than patients with other types of tumor (small non-obstructing cancer and large obstructing or non-obstructing cancer, 45.5% *vs.* 18%, 14.3%, and 14.6%); peritoneal metastasis is the most common pattern of recurrence in patients with these cancer types (18). The conclusion is that SOC more easily invades the intestinal wall and has more aggressive biological traits than other types of tumors. Therefore, we surmised the discovery of high-risk gene mutations for peritoneal metastasis in patients with SOC might allow clinicians to predict prognosis and improve survival. Identification of specific gene mutations would also likely contribute to understanding the molecular mechanisms involved in peritoneal metastasis and provide new targets for therapeutic intervention to prevent metastatic disease progression in gastrointestinal cancer.

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Key Words: Colorectal cancer, peritoneal metastasis, somatic mutations.

Materials and Methods

Patients and tissue samples. Patients were selected from the colorectal cancer surgery databases of the Hanyang University Hospital, Seoul, Republic of Korea. Colonic obstruction was determined by clinical signs (abdominal distension, constipation, vomiting, and abdominal pain), radiological evidence (abnormal gaseous distension of the bowel), colonoscopic findings (inability to push the colonoscope past the lesion), and surgical findings (proximal bowel distension and edema). Our criteria for the group with SOC group were as follows: i) Primary colorectal adenocarcinoma, ii) tumor size ≤3 cm, iii) surgical resection from September 2004 to December 2008, and iv) synchronous or metachronous peritoneal metastasis. Patients with large non-obstructing colorectal cancer (LNOC, ≥9 cm) who underwent surgical resection in the same period were included as a contrast group to compare for differences in genetic mutations. Patient follow-up was reviewed from clinical records. All research was performed in consultation and agreement with the Institutional Review Board (No. 2017-05-026-004).

Tumor samples were obtained from the archives of the Department of Pathology at the Hanyang University Hospital. Hematoxylin and eosin-stained primary tumor sections of formalin-fixed, paraffin-embedded surgical specimens were reviewed by a pathologist. Unstained tissue sections of 20 μm thickness were deparaffinized and manually microdissected using the hematoxylin and eosin-stained slide as a guide. DNA extraction was performed using PicoPure DNA extraction kit (Arcturus, Mountain View, CA, USA). Purity of the DNA was checked by NanoDrop instrument (NanoDrop Technologies, Wilmington, DE, USA). The ratio of absorbance at 260 nm to that at 280 nm was used as an indication of sample purity, and values of 1.8-2.0 were considered indicative of relatively pure DNA. Qubit DNA HS assay kit (Life Technologies, Carlsbad, CA, USA) was used to quantify purified DNA.

Mutation analysis using next-generation sequencing (NGS). Libraries were generated using Life Technologies Ion AmpliSeq™ Comprehensive Cancer Panel according to the manufacturer's recommendations. This panel consists of approximately 16,000 primer pairs covering 409 genes with known cancer associations. Genomic DNA (40 ng) from each sample was used to prepare barcoded libraries using IonXpress barcoded adapters (Life Technologies). Libraries were combined to a final concentration of 3 ng/ml using Ion Library Quantification Kit, and emulsion polymerase chain reaction was performed using the Ion Torrent One Touch TM 2 System (Life Technologies). Samples were sequenced on an Ion Torrent semi-conductor sequencer (Life Technologies) using Ion 316 or 318 chips. Sequencing reads were aligned to the 409 genes based on the Human Genome version 19 using Sequence Pilot v 4.2.0 (JSI medical systems GmbH, Ethenheim, Germany). In addition, the read depth and uniformity of coverage across individual amplicons was assessed. In data analysis, the cut-off was set at mutations found in ≥10% of the reads. Only non-synonymous and non-sense variations in coding regions were included.

Statistical analysis. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Descriptive data are presented as the mean±SD. The Chi-square test and Fisher's exact test were used for comparisons between the two groups. Data were considered statistically significant at $p \leq 0.05$. R 3.2.2 for Windows (Rstudio, Boston, MA, USA) was used to perform a hierarchical cluster analysis to confirm the genetic difference between the two study groups.

Table I. *Clinicopathological characteristics in study groups*.

	SOC	LNOC	*p*-Value
Age (years)			
Mean±SD	58.6±13.8	66.6±14.0	0.312
Gender			
Male	4 (40)	4 (80)	0.282
Female	6 (60)	1 (20)	
Tumor location			
Tumor location, n (%)			
Rt. colon	2 (20)	2 (40)	0.560
Lt. colon	8 (80)	3 (60)	
Tumor size (cm)			
Mean±SD	2.6±0.6	10.4±1.5	<0.001
T Classification#, n (%)			
T2	0 (0)	1 (20)	0.223
T3	8 (80)	4 (80)	
T4	2 (20)	0 (0)	
N Classification#, n (%)			
N0	1 (10)	1 (20)	0.526
N1	4 (40)	3 (60)	
N2	5 (50)	1 (20)	
M Classification#, n (%)			
M0	3 (30)	5 (100)	0.026
M1	7 (70)	0 (0)	
Degree of differentiation, n (%)			
Well	0 (0)	1 (20)	0.098
Moderately	9 (90)	2 (40)	
Poorly	1 (10)	2 (40)	
Lymphatic invasion, n (%)			
Absent	0 (0)	1 (20)	0.333
Present	10 (100)	4 (80)	
Vascular invasion, n (%)			
Absent	9 (90)	5 (100)	1.000
Present	1 (10)	0 (0)	
Perineural invasion, n (%)			
Absent	3 (30)	3 (60)	0.329
Present	7 (70)	2 (40)	

SOC: Small obstructing colorectal cancer; LNOC: large non-obstructing colorectal cancer; Rt.: right; Lt.: left. #According to the Seventh Edition of the American Joint Committee on Cancer Cancer Staging Manual (19).

Results

ClinicopathologicaI characteristics. Patient characteristics are listed in Table I. The mean size of tumor was 2.6±0.6 cm for the SOC group and 10.4±1.5 cm for the LNOC group. Among 10 SOC patients, seven had synchronous peritoneal metastasis and three had peritoneal recurrence after curative surgery. When other clinicopathological characteristics of the two groups were compared, there was no significant difference except for the M stage.

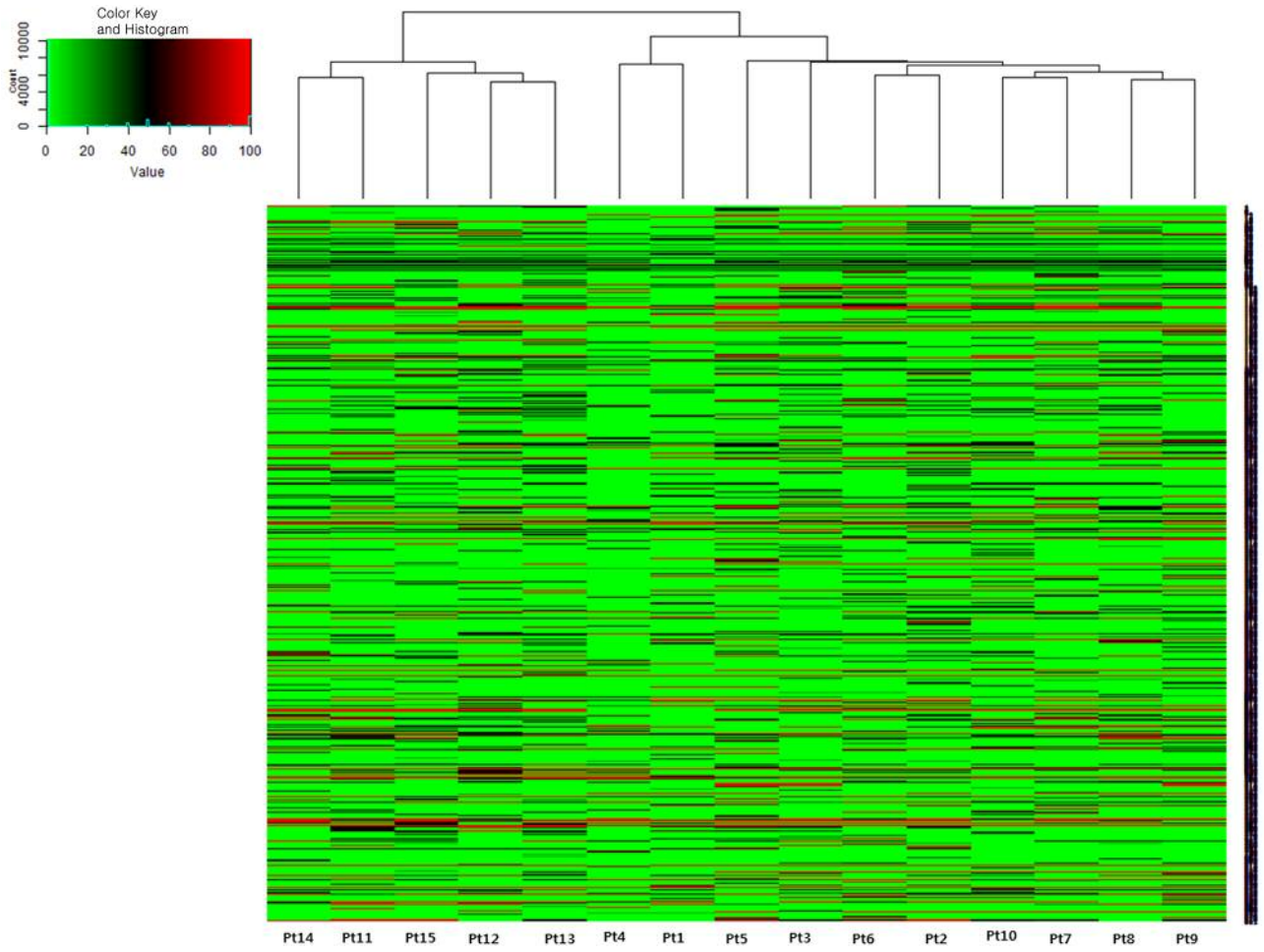

Figure 1. *Hierarchical cluster analysis with 883 somatic mutations in 10 patients with small obstructing colorectal cancer (pt1-10) and five patients with large non-obstructing colorectal cancer (pt11-p15).*

Different expression of somatic mutations between SOC and LNOC. A total of 883 somatic mutations were detected after NGS. A hierarchical cluster analysis of patients' samples was performed using the detected gene mutations. When the samples were sorted on the basis of similarity of distribution of gene mutations, the samples were clearly separated into the two study groups, confirming their unique biology (Figure 1). Our interpretation is that the two study groups with different clinicopathological characteristics display distinctly different tendencies of somatic mutations. Seventeen somatic mutations with significantly different incidence between the two groups were identified. Mutations of in AT-rich interactive domain-containing protein 1A (*ARID1A*), polycystic kidney and hepatic disease 1 (*PKHD1*), ubiquitin-protein ligase E3 component n-recognin 5 (*UBR5*), paired box 5 (*PAX5*), tumor protein p53 (*TP53*), additional sex combs like 1 (*ASXL1*) and androgen receptor (*AR*) genes were detected more frequently in the SOC group with peritoneal metastasis, whereas mutations of tumor necrosis factor receptor superfamily member 14 (*TNFRSF14*), von Hippel-Lindau tumor suppressor (*VHL*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*), MLLT10 histone lysine methyltransferase DOT1L cofactor (*MLLT10*), baculoviral IAP repeat containing 2 (*BIRC2*), E1A binding protein p400 (EP400), insulin receptor substrate 2 (*IRS2*), period circadian clock 1 (*PER1*), transcription factor 3 (*TCF3*) and cytochrome P450 family 2 subfamily D member 6 (*CYP2D6*) genes were more common in the LNOC group without peritoneal metastasis (Table II). Among those gene mutations, ARID1A, *UBR5, TP53, ASXL1*, and *VHL* mutations are included in the COSMIC Cancer Database.

Table II. *Differentiation of gene expression between small obstructing (SOC) and large non-obstructing colorectal cancer (LNOC).*

	Gene ID*	Gene name	Encoded protein	Cosmic no.	Type of mutation	SOC: LNOC[#]	*p*-Value
More frequent in SOC	8289	*ARID1A*	AT-rich interactive domain-containing 1A	COSM51427	Frameshift deletion	9:0	0.002
	5314	*PKHD1*	Fibrocystin	---	Frameshift deletion	7:0	0.019
	51366	*UBR5*	E3 ubiquitin-protein ligase UBR5	COSM214501	Frameshift deletion	9:0	0.002
	5079	*PAX5*	Paired box protein pax-5	---	Nonsynonymous SNV	10:0	<0.001
	7157	*TP53*	Tumor protein p53	COSM4532, COSM44698	Frameshift deletion	8:0	0.007
	171023	*ASXL1*	Putative polycomb group protein ASXL1	COSM85923	Frameshift deletion	8:1	0.047
	367	*AR*	Androgen receptor	---	Frameshift deletion	7:0	0.019
More frequent in LNOC	8764	*TNFRSF14*	Tumor necrosis factor receptor superfamily member 14	---	Nonsynonymous SNV	3:5	0.019
	7428	*VHL*	Von Hipple-Lindau tumor suppressor	COSM18367	Frameshift deletion	0:4	0.004
	4552	*MTRR*	Methionine synthase reductase	---	Nonsynonymous SNV	2:4	0.047
	8028	*MLLT10*	Protein AF-10	---	Frameshift deletion	0:3	0.022
	329	*BIRC2*	Baculoviral IAP repeat-containing protein 2	---	Frameshift deletion	0:5	<0.001
	57634	*EP400*	E1A-binding protein p400	---	Frameshift deletion	0:3	0.022
	8660	*IRS2*	Insulin receptor substrate 2	---	Nonsynonymous SNV	0:5	<0.001
	5187	*PER1*	Period circadian protein 1	---	Frameshift deletion	0:3	0.022
	6929	*TCF3*	Transcription factor 3	---	Nonsynonymous SNV	3:5	0.026
	1565	*CYP2D6*	Cytochrome P450 2D6	---	Frameshift deletion	0:4	0.004

SNV: Single nucleotide variation. *NCBI gene ID. [#]The number of patients with each somatic mutation in SOC *vs*. LNOC groups.

Discussion

The introduction of NGS technologies has revolutionized the speed and throughput of DNA sequencing (20, 21). While Sanger DNA sequencing is capable of limited target and limited read sequencing, NGS technologies can simultaneously sequence multiple samples for multiple genes using a limited amount of DNA, while also reducing costs of the analysis as well as the diagnostic response timing (22-24). In this study, we explored the mutational status of a series of 10 SOC compared to five LNOC as a contrast group using a commercially available NGS panel designed to screen all exons of 409 cancer-related genes using Ion Proton (Life Technologies). Recently, a high concordance of this 409-gene targeted panel with other NGS platforms and Sanger sequencing in a variety of solid tumors has been reported (25). Moreover, the method can detect potentially actionable cancer genes not evaluated in a traditional hot-spot cancer gene panel. As far as we are aware, our study is the first that screened high-risk gene mutations related to peritoneal metastasis in gastrointestinal cancer through this NGS technology. Paradoxically, it is one of the limitations of our study that the measurement was limited to only the genes included in this commercially available NGS panel even though it is possible for higher throughput NGS platforms to comprehensively screen larger numbers of genes, because an ever larger number of genetic mutations are being identified in virtually every tumor.

Peritoneal metastasis is an even more prevalent form of recurrence and metastasis in gastric cancer than in colorectal cancer (3, 26). Several molecules have been reported to be involved in gastric cancer with peritoneal metastasis (8, 12); however, the mechanisms underlying the more aggressive behavior of the tumor have yet to be elucidated. In this context, systematic profiling of gene expression was recently performed in gastric cancer by others (13, 14). In those studies, peritoneal metastasis was also found to be genetically complex; unfortunately, the genes related to peritoneal metastasis do not match between the reports. The results did not differ between reports on colorectal cancer (15-17). Consequently, the genes involved in peritoneal metastasis are not yet well known. In order to find predictive molecular markers for peritoneal metastasis, we investigated the differences in mutated genes occurring between SOC with peritoneal metastasis and LNOC without peritoneal metastasis by reference information identified in our previous study. The specific somatic variations of *ARID1A, PKHD1, UBR5, PAX5, TP53, ASXL1*, and *AR* were more frequently observed in SOC with peritoneal metastasis. On

the other hand, the somatic mutation of *TNFRSF14, VHL, MTRR, MLLT10, BIRC2, EP400, IRS2, PER1, TCF3*, and *CYP2D6* in NLOC were observed more commonly in LNOC without peritoneal metastasis. Among these significant somatic mutations, to our knowledge, there are no cancer-associated reports yet for *PKHD1, PAX5, AR, TNFRSF14, MTRR, MLLT10, BIRC2, EP400, IRS2, PER1, TCF3,* and *CYP2D6* mutations (Table II). Advances in NGS technology and economies seem to lead to the identification of novel therapeutic targets and biomarkers, as well as previously unknown oncogenes (27, 28). An example similar to our work is that of Zang *et al*., who found a number of potential cancer-driving genes for gastric cancer by whole-exome sequencing that included recurrent somatic mutations in the chromatin remodeling gene *ARID1A* and alterations in the FAT atypical cadherin 4 (*FAT4*) cell adhesion gene (28). These somatic mutations, which previously have not been described, may be molecular targets for understanding peritoneal metastasis in future studies.

Our study had some limitations. First of all, the number of patients with peritoneal recurrence was too small to be conclusive. A larger scale study should be conducted to confirm the relevance of these somatic mutational differences to peritoneal metastasis. Investigations of gastric or ovarian cancer in which peritoneal recurrence is relatively common may alternatively provide insight into the genetics and mechanisms of peritoneal seeding. Secondly, while NGS is an optimal method for identifying target genes, it cannot provide any information as to what the roles of the detected genes are or what function they perform at any time-point during disease progression. Gene-expression profiling, such as DNA microarray or RNA-seq, should be conducted in a follow-up research. As we mentioned previously, another limitation is that tested genes were limited to only those included in the commercial NGS panel, and as greater numbers of potential genes are identified and included in future panels, more candidate genes will likely be identified.

In conclusion, we found genetic mutations presumably associated with peritoneal metastasis in high-risk patients and other genes that may be related to suppression of metastasis in low-risk patients. The expression and functional roles of the detected somatic mutations should be investigated and further validation in cancer of other organs with peritoneal metastasis will likely prove fruitful in future studies.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

The manuscript has been seen and approved by all Authors and all Authors made contributions to conception and design, acquisition of data and analysis and Interpretation of data. The manuscript was revised several times by all Authors.

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Received December 30, 2018
Revised January 23, 2019
Accepted January 28, 2019