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ORIGINAL ARTICLE

Intratumoral Heterogeneity of Somatic Mutations for *NRIP1, DOK1, ULK1, ULK2, DLGAP3, PARD3* and *PRKCI* in Colon Cancers

Eun Ji Choi[1] · Ju Hwa Lee[1] · Min Sung Kim[1] · Sang Yong Song[2] · Nam Jin Yoo[1] · Sug Hyung Lee[1]

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Abstract Both *NRIP1* and *DOK1* genes are considered candidate tumor suppressor genes (TSGs). Also, cell polarity-related genes *PARD3, PRKCI* and *DLGAP3*, and autophagy-related genes *ULK1* and *ULK2* genes are considered to play crucial roles in tumorigenesis. The aim of our study was to find whether these genes were mutated in colorectal cancer (CRC). In a genome database, we observed that each of these genes harbored mononucleotide repeats in the coding sequences, which could be mutated in cancers with high microsatellite instability (MSI-H). For this, we studied 124 CRCs for the frameshift mutations of these genes and their intratumoral heterogeneity (ITH). *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* harbored 18 (22.8%), 2 (2.5%), 2 (2.5%), 2 (2.5%), 5 (6.3%), 2 (2.5%) and 2 (2.5%) of 79 CRCs with MSI-H, respectively. However, we found no such mutations in microsatellite stable (MSS) cancers in the nucleotide repeats. We also studied ITH for the frameshift mutations in 16 cases of CRCs and detected that the frameshift mutations of *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* showed regional ITH in 5 (31.3%), 2 (12.5%), 0 (0%), 0 (0%), 1 (6.3%), 1 (6.3%) and 3 (18.8%) cases, respectively. Our data exhibit that candidate cancer-related genes *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* harbor mutational ITH as well as the frameshift mutations in CRC with MSI-H. Also, the results suggest that frameshift mutations of these genes might play a role in tumorigenesis through their inactivation in CRC.

Keywords Tumor suppressor gene · Frameshift mutation · Colon cancer · Microsatellite instability

Eun Ji Choi and Ju Hwa Lee contributed equally to this work.

✉ Sug Hyung Lee
suhulee@catholic.ac.kr

[1] Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, South Korea

[2] Department of Pathology and Translational Genomics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea

Introduction

Transcription cofactor NRIP1 (nuclear receptor–interacting protein 1) represses E2F1 activity and inhibits E2F1 target gene expression such as CCNE1 and CCNB2, subsequently inhibiting cell cycle progression [1]. Decreased expression of NRIP1 has been reported in many tumors including breast and colorectal cancers (CRC) [1, 2]. Docking proteins (DOK1, DOK2 and DOK3) belong to a tyrosine-phosphorylated adaptor protein family and inhibit tyrosine kinase activation [3]. DOK proteins are frequently downregulated in cancers [4]. Evidence exists that aberrant expression and localization of the polarity proteins are common in human tumors [5]. Atypical PKCs, PARD3 and PARD6 bind together and constitute the Pard complex that is central to the development of junctional structures and apical-basolateral polarization in epithelial cells [6]. *PARD3* and *PRKCI* encode PARD3 and protein kinase C iota (an atypical PKC), respectively [7, 8]. DLGAP3 is an interacting protein with DLG4, a Dlg family protein in the Scribble complex [9]. Autophagy is implicated in many human diseases, including neurodegenerative disorders, microbial infections and cancers [10]. ULK1 and ULK2 form Unc-51 like autophagy activating kinase 1 (ULK1) protein

kinase complex that is located at the most upstream position during autophagy process [10].

About one third of CRCs are classified as high microsatellite instability (MSI-H) cancers [11]. Many TSGs harbor frameshift mutations at monocleotide repeats in MSI-H cancers [11]. In the human genome database, we observed that *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* genes possess nucleotide repeats in coding sequences that might be mutated in MSI-H cancers. However, frameshift mutations of the repeats in these genes in CRC have not been reported. Intratumoral heterogeneity (ITH) is a common phenomenon in cancers, which may result in cancer evolution and influence on clinical outcomes [12–14]. Thus, identification of genetic ITH is important in understanding biological and clinicopathologic features of the cancers. The current study aimed to find whether *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* frameshift mutations are common and harbor ITH in MSI-H CRC.

Table 1 Summary of pathologic features of colorectal cancers

Feature	MSI-H	MSS
Colorectal carcinomas		
Total cases	79	45
TNM stage		
I	15	6
II	29	20
III	32	16
IV	3	3
Location		
Cecum	16	0
Ascending colon	46	3
Transverse colon	12	2
Descending & sigmoid colon	4	17
Rectum	1	23

TNM tumor, lymph node, metastasis, *MSI-H* high microsatellite instability, *MSS* stable microsatellite instability

Materials and Methods

Tissue Samples and Microdissection

In this study, we used 124 CRCs that consisted of 45 CRCs with microsatellite stable (MSS) and 79 CRCs with MSI-H. These samples overrepresent MSI-H cases due to our collection method (MSI-H and MSS cases were separately collected in different times). We adopted an MSI evaluation system using five mononucleotide repeats [15]. For 16 MSI-H CRCs, we picked 4–7 tumor areas and one normal area per CRC for the ITH analysis. Each ITH area was histologically confirmed under light microscope. These ITH areas were studied for detecting mutational ITH of *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2*. Pathologic features of the cancers were evaluated in all blocks of all cases by a pathologist and are summarized in Table 1. Tumor and normal cells were microdissected as described previously [16]. Approval of this study was obtained from the institutional review board of the Catholic University of Korea.

Single Strand Conformation Polymorphism (SSCP) Analysis

We analyzed DNA sequences in *NRIP1* (A8 and A7), *DOK1* (T), *DLGAP3* (G7), *PARD3* (A7), *PRKCI* (A8), *ULK1* (C7 and C5) and *ULK2* (A7). Genomic DNA was amplified using polymerase chain reaction (PCR). [^{32}P]dCTP was incorporated to the PCR products for visualization in autoradiogram. We determined aberrant gel motility in the SSCP (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) using visual inspection, which subsequently sequenced by Sanger DNA sequencing (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA). Other procedures in detail were described in our earlier studies [17, 18].

Results

Mutational Analysis

Genomic DNAs of the 124 CRCs (79 with MSI-H and 45 with MSS) were studied to detect frameshift mutations in the mononucleotide repeats by the PCR-SSCP analyses. On SSCP and Sanger sequencing, we observed that *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* harbored 18 (22.8%), 2 (2.5%), 2 (2.5%), 2 (2.5%), 5 (6.3%), 2 (2.5%) and 2 (2.5%) of 79 CRCs with MSI-H, respectively. Normal tissues of the corresponding patients from the slides were microdissected and analyzed, but there was no evidence of the mutations on SSCP and Sanger sequencing, which indicated that the mutations had risen somatically (Fig. 1 and Table 2). All detected mutations exhibited both wild-type and mutant signals in SSCP and Sanger sequencing, indicating that they were heterozygous mutations (Fig. 1).

The frameshift mutations detected in this study were found in MSI-H CRCs, but there was none in MSS CRCs (Table 2) (Fisher's exact test, $p < 0.001$). There was no significant association of these mutations with the grade, differentiation and tumor stage. In those with MSI-H, no correlation was observed between histological features (medullary pattern, mucinous histology and tumor-infiltrating lymphocytes) and the mutations.

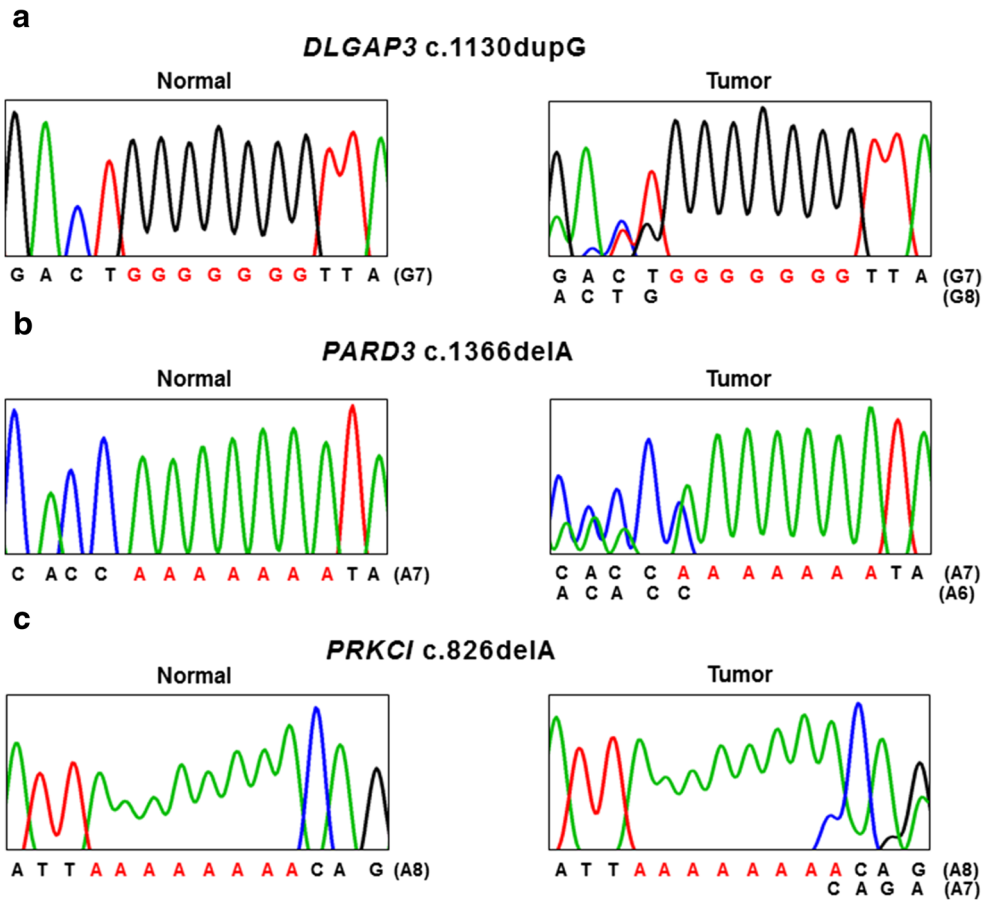

Fig. 1 Representative DNA sequencings of the repeats in *NRIP1* and *DOK1* in colon cancers. DNA sequencing analyses of A8 and A7 repeats in *NRIP1* and T7 repeat in *DOK1* from tumor (T) and normal tissues (N). **a-c** Direct DNA sequencing analyses show a heterozygous A deletion or a heterozygous A duplication for the *NRIP1* repeats in the tumor tissues as compared to normal tissues. D-E. Direct DNA sequencing analyses show a heterozygous T deletion or a heterozygous T duplication for the DOK1 repeats in the tumor tissues as compared to normal tissues

Intratumoral Heterogeneity of the Frameshift Mutations

Ninety-six areas from 16 CRCs with MSI-H were analyzed to find ITH in the frameshift mutations. We observed that the frameshift mutations of *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* showed regional ITH in 5 (31.3%), 2 (12.5%), 0 (0%), 0 (0%), 1 (6.3%), 1 (6.3%) and 3 (18.8%) cases, respectively (Table 3 and Fig. 2). There was no significant histological difference between mutation (+) and mutation (−) ITH areas.

Table 2 Summary of the frameshift mutations in colorectal cancers

Gene	Location	Wild type	Mutation	MSI status of the mutation cases (n)	Incidence in MSI-H cancers (%)	Nucleotide change (predicted amino acid change)
NRIP1	Exon 4	A8	A7	MSI-H (14)	Colorectal: 14/79 (17.7)	c.2184delA (p.Glu729Argfsx5)
		A7	A6	MSI-H (3)	Colorectal: 3/79 (3.8)	c.1547delA (p.Asn516Thrfsx11)
			A8	MSI-H (1)	Colorectal: 1/79 (1.3)	c.1547dupA (p.Asn516Lysfsx10)
DOK1	Exon 1	T7	T6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.35delT (p.Leu12CysfsX26)
			T8	MSI-H (1)	Colorectal: 1/79 (1.3)	c.35dupT (p.Leu12PhefsX33)
LK1	Exon 22	C7	C6	MSI-H (2)	Colorectal: 1/79 (1.3)	c.2299delC (p.Gln767Argfsx57)
		C5	C4	MSI-H (1)	Colorectal: 1/79 (1.3)	c.2279delC (p.Pro760Argfsx64)
ULK2	Exon 11	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.834delA (p.Lys278Asnfsx130)
			A8	MSI-H (2)	Colorectal: 2/79 (2.5)	c.834dupA (p.Lys279Ilefsx64)
DLGAP3	Exon 4	G7	G6	MSI-H (8)	Colorectal: 5/79 (6.3)	c.1130delG (p.Gly377Valfsx91)
PARD3	Exon 9	A7	A6	MSI-H (2)	Colorectal: 2/79 (2.5)	c.1366delA (p.Ile456fsx1)
PRKCI	Exon 9	A8	A7	MSI-H (1)	Colorectal: 1/79 (1.3)	c.826delA (p.Thr276Glnfsx7)
			A9	MSI-H (1)	Colorectal: 1/79 (1.3)	c.1130dupG (p.Thr276Asnfsx16)

MSI-H high microsatellite instability

Table 3 Intratumoral heterogeneity of the frameshift mutations in colorectal cancers with MSI-H

Case	Regional biopsy sites							Mutation status	ITH status
	#1	#2	#3	#4	#5	#6	#7		
CRC3	DOK1 c.35dupT, ULK1 c.2279delC	WT ULK1 c.2279delC	WT ULK1 c.2279delC	WT WT	DOK1 c.35dupT, ULK1 c.2279delC	DOK1 c.35dupT, ULK1 c.2279delC	n.d. n.d.	DOK1 Mutation ULK1 Mutation	ITH ITH
CRC15	WT	WT	WT	WT	WT	WT	WT	WT	Non-ITH
CRC26	WT	WT	n.d.	WT	WT	WT	WT	WT	Non-ITH
CRC27	WT	WT	WT	WT	WT	WT	WT	WT	Non-ITH
CRC34	DOK1 c.35delT, DLGAP3 HET c.1130delG	WT, DLGAP3 HOMO c.1130delG	WT, DLGAP3 HOMO c.1130delG	WT, DLGAP3 HET c.1130delG	WT, DLGAP3 HET c.1130delG	WT, DLGAP3 HET c.1130delG	WT, DLGAP3 HET c.1130delG	DOK1 Mutation DLGAP3 Mutation	ITH ITH
CRC35	WT	WT	n.d.	n.d.	n.d.	WT	WT	WT	Non-ITH
CRC39	WT	WT	WT	WT	n.d.	WT	WT	WT	Non-ITH
CRC41	NRIP1 c.2184delA ULK2 c.834delA	n.d. n.d.	NRIP1 c.2184delA ULK2 c.834delA	WT ULK2 c.834delA	n.d. n.d.	WT WT	WT WT	NRIP1 Mutation ULK2 Mutation	ITH ITH
CRC43	WT WT	WT WT	WT WT	n.d. n.d.	n.d. n.d.	WT ULK2 c.834delA	n.d. n.d.	WT ULK2 Mutation	Non-ITH ITH
CRC45	NRIP1 c.2184delA	WT	NRIP1 c.2184delA	NRIP1 c.2184delA	NRIP1 c.2184delA	WT	WT	NRIP1 Mutation	ITH
CRC47	WT	WT	WT	WT	WT	WT	WT	WT	Non-ITH
CRC48	NRIP1 c.2184delA ULK2 c.834dupA	n.d. n.d.	n.d. n.d.	NRIP1 c.2184delA ULK2 c.834dupA	WT ULK2 c.834dupA	NRIP1 c.2184delA WT	NRIP1 c.2184delA ULK2 c.834dupA	NRIP1 Mutation ULK2 Mutation	ITH ITH
CRC49	n.d.	NRIP1 c.2184delA	NRIP1 c.2184delA	WT	NRIP1 c.2184delA	WT	NRIP1 c.2184delA	NRIP1 Mutation	ITH
CRC51	WT	WT	WT	WT	WT	WT	WT	WT	Non-ITH
CRC53	WT	WT	WT	WT	WT	WT	NRIP1 c.2184delA	NRIP1 Mutation	ITH
CRC55	WT	WT	n.d.	n.d.	WT	WT	WT	WT	Non-ITH

ITH intratumoral heterogeneity, *WT* wild type, *MSI-H* high microsatellite instability, *n.d.*: not done

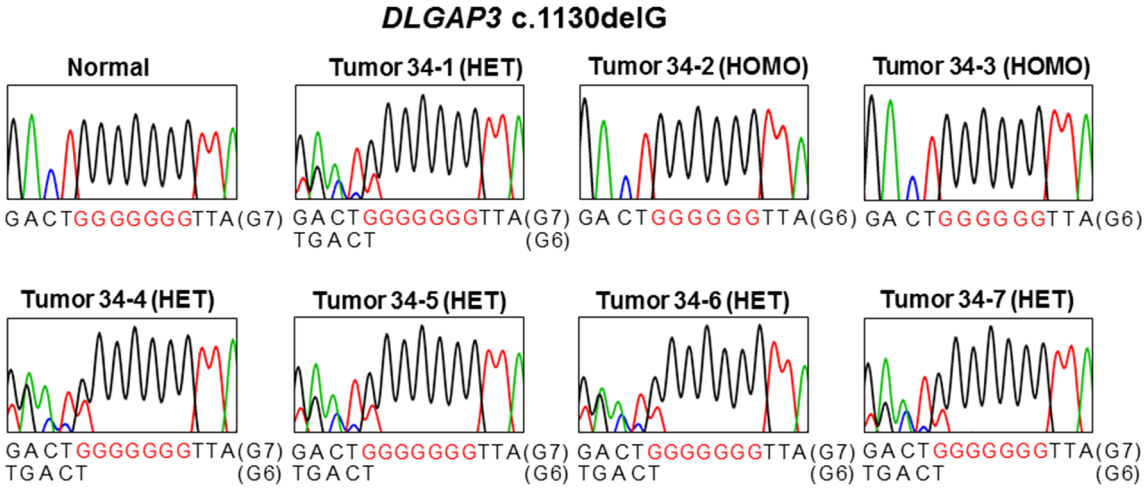

Fig. 2 Intratumoral heterogeneity of *NRIP1* frameshift mutation in a colon cancer. A: Sanger DNA sequencing analyses show *NRIP1* c.2184delA mutation (MT) in 4 regional areas (45–1, −3, −4 and −5) and wild-type (WT) in the other 3 areas (45–2, −6 and −7)

Discussion

Tumor suppressor genes (TSGs) or anti-oncogene is a gene that protects a cell from one step on the path to cancer [19]. When a TSG is mutated, the cells can progress to cancer in combination with other genetic or epigenetic changes. Earlier studies identified that both *NRIP1* and *DOK1* possessed TSG activities in cells [1–4]. Loss of cell polarity is a typical hallmark of cancer development and progression in epithelial cells [5]. *DLGAP3, PARD3* and *PRKCI* are polarity signaling-related genes that are related to TSG functions [5]. Loss of autophagy contributes to tumorigenesis [10]. Both ULK1 and ULK2 play crucial roles in autophagy formation [10]. The present study here identified that MSI-H CRC exhibited frameshift mutations within the repeats of *NRIP1, DOK1, PARD3, PRKCI, DLGAP3, ULK1* and *ULK2* in CRC. These results indicate that the functions of these genes are altered by frameshift mutations identified in this study and suggest that these inactivating mutations might inhibit their functions in MSI-H CRC as well. The *NRIP1, DOK1, PARD3, PRKCI, DLGAP3* frameshift mutations might play a role in the pathogenesis of MSI-H CRC by inhibiting their TSG activities. Also, the inactivating mutations of *ULK1* and *ULK2* might play a role in tumorigenesis of CRC by inhibiting autophagy activities of ULK1 and ULK2.

The frameshift mutations identified in the present study may truncate and disable their encoded proteins and hence may be considered loss-of-function mutations. It is also possible that both of these would be expected to lead to premature stop codons in the mRNAs but not necessarily the production of a truncated protein as these transcripts are more likely to be degraded by nonsense mediated decay [11]. Since cancers with MSI show extremely high mutator phenotype with frameshift mutation in microsatellite sequences, the frameshift mutations in this study could be passenger mutations. By contrast, many TSGs have been found to harbor mutations at nucleotide repeats in the coding sequences in the cancers with MSI (type II transforming growth factor β receptor, BAX, caspase-5, hMSH3, hMSH6 and TCF4) [20]. It remains undetermined whether the frameshift mutations detected in this study are driver or passenger mutations for the development of MSI-H cancers. We were not able to find any significant difference in clinocopathologic parameters between those with and without the mutations probably due to the small number of mutated cases. Analysis of a larger cohort with the mutations is required in future studies.

In this study, we identified mutational ITH of *NRIP1, DOK1, DLGAP3, ULK1* and *ULK2* in CRCs, which is consistent with previous studies that had reported frequent mutational ITH in CRCs with MSI-H [21]. Genomic instability in a cancer may result in an elevated level of somatic mutations and contribute to ITH development by providing a pool of mutations upon which selection can act in a given microenvironment [12–14]. The cancer ITH of driver mutations could result in poor clinical outcomes in patients as well. For instance, single or a group of mutations with a metastasis potential might redirect clinical outcomes since such rare clones may accomplish dominance during tumor progression [12–14]. Based on the cancer-related properties of the genes, our results suggest that loss of their properties may have regional heterogeneity in CRC that could be further selected and influence the clinical outcomes. However, presence of ITH of the frameshift mutations might suggest a possibility that there could be a mixed or ameliorated effect of the frameshift mutations in MSI-H cancers.

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Compliance with Ethical Standards

Conflicts of Interest and Financial Sponsorship and Support None.

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