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ORIGINAL ARTICLE

Frameshift Mutations in Repeat Sequences of *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* Genes in Colon Cancers

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Abstract Diminished ANK3 contributes to cell survival by inhibiting detachment-induced apoptosis. TP53BP1 that interacts with p53 and MFN1 that encodes a mitochondrial membrane protein are considered to have tumor suppressor gene (TSG) functions. HACD4 involving fatty acid synthesis and TCPL10 with transcription regulation functions are considered TSGs. Many genes involved in DNA methylations such as *LCMT2, RNMT, TRMT6, METTL8* and *METTL16* are often perturbed in cancer. 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). *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* harbored 11 (13.9%), 3 (3.8%), 0 (0%), 5 (6.3%), 1 (1.3%), 2 (2.5%), 4 (5.1%), 3 (3.8%), 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. There were ITH of the frameshift mutations of *ANK3, MFN1* and *TP53BP1* in 1 (6.3%), 1 (6.3%) and 1 (6.3%) cases, respectively. Our data exhibit that cancer-related genes *ANK3, HACD4, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* 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

Introduction

Ankyrins are a family of proteins that link the integral membrane proteins to the underlying spectrin-actin cytoskeleton and play key roles in cell motility, activation, proliferation, contact, and maintenance of membrane integrity [1, 2]. In cancers, somatic mutations across its entire coding region have been described as driver mutations in breast carcinoma, chronic lymphocytic leukemia, cutaneous melanoma and uterine carcinoma. 1.75% of colorectal cancers (CRCs) carry non-driver mutations of *ANK3* (https://www.intogen.org). Epithelial-mesenchymal transition (EMT) downregulates ANK3, a cytoskeletal protein, subsequently represses the tumor suppressor p14ARF and induces apoptosis resistance [3]. In addition, downregulation of *ANK3* correlates with poor prognosis in diverse human cancers [4].

TP53BP1 binds with damaged chromatin and carries out multiple roles such as recruitment of DNA double strand break (DSB)-responsive proteins [5]. Apart from the DNA repair, TP53BP1 interacts with p53 and contributes to tumor suppressor function of p53 [6]. In colorectal cancer (CRC), decreased TP53BP1 expression is associated with poor survival of the patients [7]. *Mitofusin 1* (*MFN1*) encodes a mitochondrial membrane protein that plays roles in mitochondria fusion and apoptosis [8]. ERK, which is constitutively upregulated in cancers, promotes phosphorylation of MFN1 protein and induces apoptosis [8].

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HACD4 belongs to a family of enzymes that catalyze the dehydration step of very long chain fatty acid synthesis [9]. Knockdown of *HACD4* increases STAT3 phosphorylation and cell proliferation [10]. TCPL10 encoding T-complex protein 10-like, a nuclear protein with transcription regulation functions, suppresses colony formation, cell cycle progression through G0/G1 phase and cell growth in vivo in liver [11]. Global and gene-specific DNA methylations are often perturbed in cancer. For example, a DNA methyltransferase-encoding gene *DNMT3A* is frequently mutated in acute myelogenous leukemia [12]. However, alterations of many other methyltransferase genes, such as carboxyl methyltransferase 2 (LCMT2), RNA guanine 7-methyltransferase (RNMT), methyltransferase like protein 16 (METTL16), methyltransferase like protein 8 (METTL8) and tRNA methyltransferase 6 (TRMT6) remain unclarified in cancer development. Together, these data suggest that genes listed above may possess tumor suppressor gene (TSG) activities.

About one third of CRCs are classified as high microsatellite instability (MSI-H) cancers [13]. Many TSGs harbor frameshift mutations at monocleotide repeats in MSI-H cancers [13]. In the human genome database, we observed that *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* 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 [14]. Thus, identification of genetic ITH is important in understanding biological and clinicopathologic features of the cancers. The current study aimed to find whether *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* frameshift mutations are common and harbor ITH in MSI-H CRC.

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 *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16*. 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 *ANK3* (one A8 and two A7), *HACD4* (A8), *TCP10L* (G7), *TP53BP1* (A7), *MFN1* (A7), *LCMT2* (T7), *RNMT* (A7), *TRMT6* (A8), *METTL8* (A7) and *METTL16* (A7 and A6). 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

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

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)
ANK3	Exon 1	A7	A6	MSI-H (3)	Colorectal: 3/79 (3.8)	c.76delA (p.Arg26GlyfsX21)
	Exon 37	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.8251delA (p.Ile2751TyrfsX21)
		A8	A7	MSI-H (7)	Colorectal: 7/79 (8.9)	c.9349delA (p.Ile3117SerfsX2)
TCP10L	Exon 5	G7	G6	MSI-H (0)	Colorectal: 0/79 (0)	-
HACD4	Exon 7	A8	A7	MSI-H (3)	Colorectal: 3/79 (3.8)	c.689delA (p.Lys230Argfsx42)
TP53BP1	Exon 12	A7	A6	MSI-H (5)	Colorectal: 5/79 (6.3)	c.2145delA (p.Glu716AsnfsX12)
MFN1	Exon 4	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.401delA (p.Lys134ArgfsX3)
LCMT2	Exon 1	T7	T6	MSI-H (2)	Colorectal: 2/79 (2.5)	c.1356delT (p.Phe452LeufsX11)
RNMT	Exon 9	A7	A6	MSI-H (4)	Colorectal: 4/79 (5.1)	c.1180delA (p.Thr394HisfsX19)
TRMT6	Exon 4	A8	A7	MSI-H (3)	Colorectal: 3/79 (3.8)	c.458delA(p.Lys153AsnfsX9)
METTL16	Exon 6	A7	A6	MSI-H (2)	Colorectal: 2/79 (2.5)	c.721delA (p.Arg241AspfsX2)
METTL8	Exon 4	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.192delA (p.Val65fsX1)
	Exon 5	A6	A5	MSI-H (1)	Colorectal: 1/79 (1.3)	c.549delA (p.Gly184AspfsX18)

MSI-H high microsatellite instability

analyses. On SSCP and Sanger sequencing, we observed that of *ANK3, HACD4, TCP10L, TP53BP1, MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* harbored 11 (13.9%), 3 (3.8%), 0 (0%), 5 (6.3%), 1 (1.3%), 2 (2.5%), 4 (5.1%), 3 (3.8%), 2 (2.5%) and 2 (2.5%) of 79 CRCs with MSI-H, respectively (Table 2).
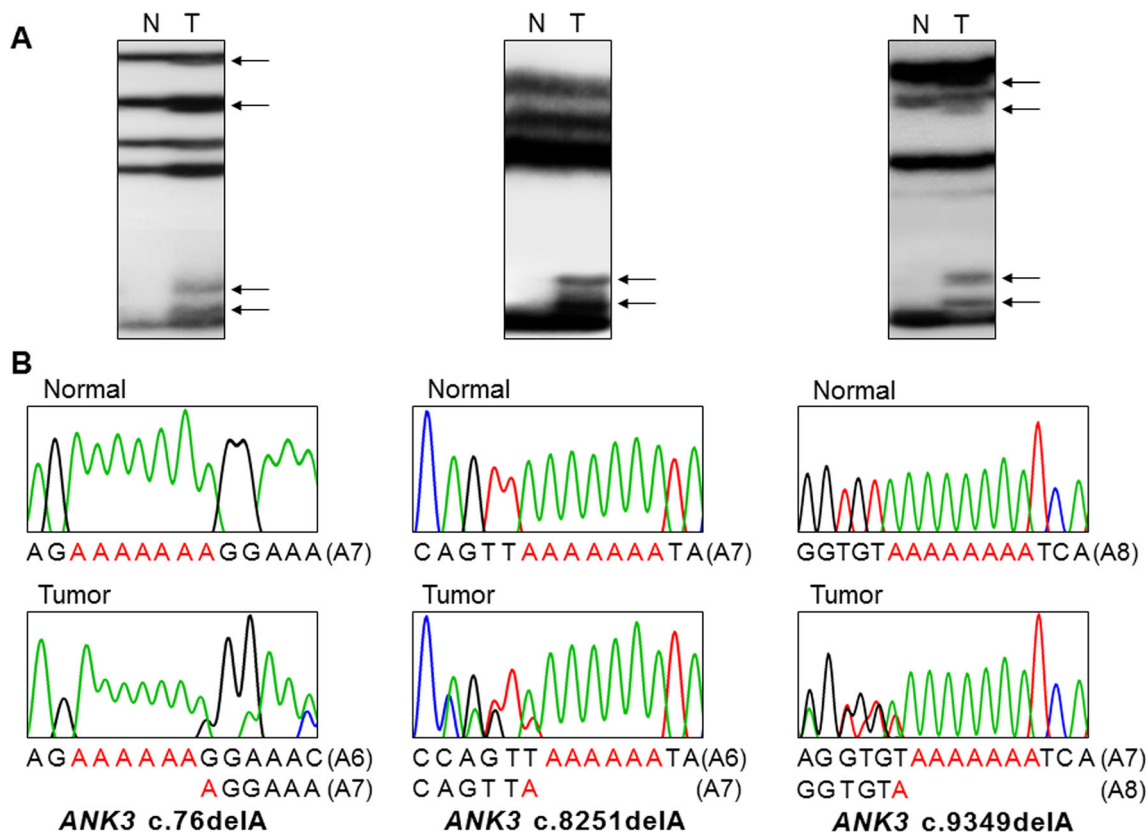

Fig. 1 Representative SSCP and DNA sequencings of repeats in *ANK3* in colon cancers. SSCP (**a**) and DNA sequencing analyses (**b**) of *ANK3* from tumor (Lane T) and normal tissues (Lane N). **a**. SSCPs of the PCR products from A7 repeat in exon 1 (left), A7 repeat in exon 37 (middle) and A8 repeat in exon 37 (right) show aberrant bands (arrows in lane T) as compared to SSCP from normal tissues (N). **b**. Direct DNA sequencing analyses (**b**) show heterozygous A deletions in exon 1 (left), A7 repeat in exon 37 (middle) and A8 repeat in exon 37 (right) in tumor tissues as compare to normal tissues

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). 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.

Intratumoral Heterogeneity of the Frameshift Mutations

Ninety-six areas from 16 CRCs with MSI-H were analyzed to find ITH in the frameshift mutations described above. We observed that the frameshift mutations of ANK3, MFN1 and TP53BP1 showed regional ITH in 1 (6.3%), 1 (6.3%) and 1 (6.3%) cases, respectively (Table 3 and Fig. 2). There was no significant histological difference between mutation (+) and mutation (−) ITH areas. HACD4, TCP10L, LCMT2, RNMT, TRMT6, METTL8 and METTL16 frameshift mutations did not harbor the ITH in these CRCs.

Discussion

In EMT, one of the most crucial phenotypes is alteration of cytoskeletal proteins, including loss of E-cadherin and cytokeratin expression [19]. Based on earlier reports that showed inactivation of ANK3 was related to EMT and to apoptosis resistance [3], we attempted to disclose whether somatic frameshift mutations of ANK3 gene was present in CRC. In the present study, we found ANK3 frameshift mutations in 13.9% of CRCs with MSI-H. TSG or anti-oncogene is a gene that protects a cell from one step on the path to cancer [20]. When a TSG is mutated, the cells can progress to cancer in combination with other genetic or epigenetic changes. Earlier studies identified that HACD4, TP53BP1 and MFN1 possessed TSG activities in cells [5–11]. Loss of cell polarity is a typical hallmark of cancer development and progression in epithelial cells [5]. The present study here identified that MSI-H CRC exhibited frameshift mutations within the repeats of HACD4, TP53BP1 and MFN1 in CRC. These results indicate that the functions of these genes are altered

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

| Case	Regional sites							Mutation status	ITH status
	#1	#2	#3	#4	#5	#6	#7		
CRC3	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	Non-ITH
CRC15	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC26	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC27	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC34	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC35	Wild type	Wild type	n.d.	n.d.	n.d.	Wild type	Wild type	Wild type	Non-ITH
CRC39	Wild type	Wild type	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	Non-ITH
CRC41	Wild type	n.d.	Wild type	Wild type	n.d.	Wild type	Wild type	Wild type	Non-ITH
CRC43	Wild type	Wild type	Wild type	n.d.	n.d.	Wild type	n.d.	Wild type	Non-ITH
CRC45	MFN1 c.del401A	MFN1 c.del401A	MFN1 c.del401A	MFN1 c.del401A	Wild type	Wild type	MFN1 c.del401A	MFN1 mutation	ITH
CRC47	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC48	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC49	n.d.	Wild type	Wild type	Wild type	Wild type	TP53BP1 c.2145delA	Wild type	TP53BP1 mutation	ITH
CRC51	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	Non-ITH
CRC53	ANK3 c.9349delA	Wild type	Wild type	Wild type	Wild type	ANK3 c.9349delA	Wild type	ANK3 mutation	ITH
CRC55	Wild type	Wild type	n.d.	n.d.	Wild type	Wild type	Wild type	Wild type	Non-ITH

ITH intratumoral heterogeneity, n.d.: not done
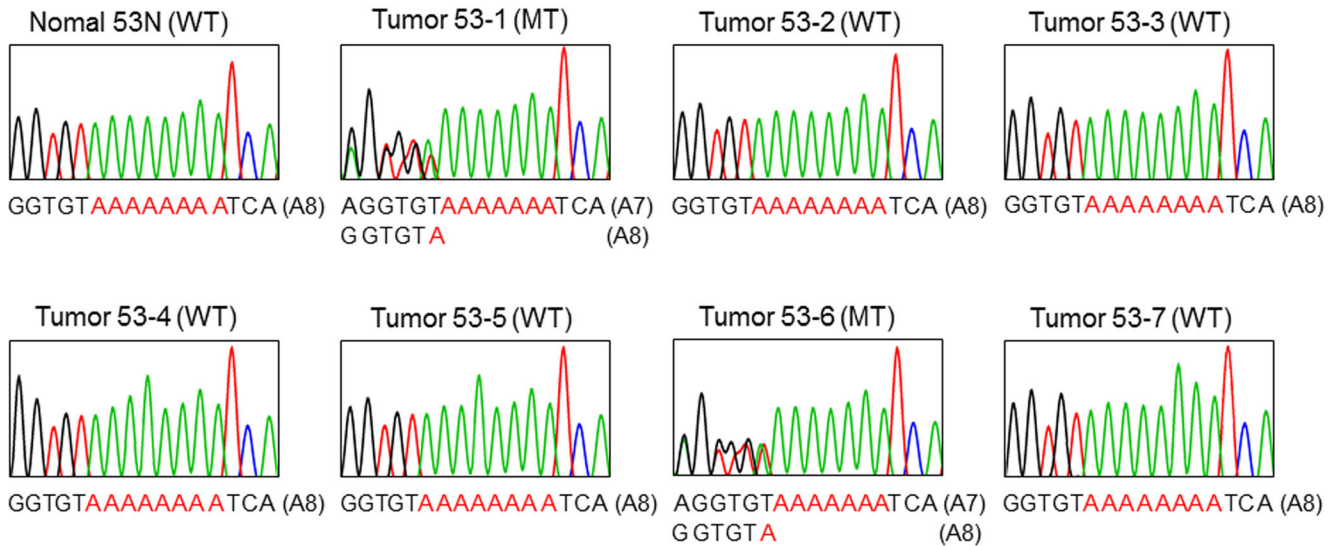
Fig. 2 Intratumoral heterogeneity of the *ANK3* frameshift mutation in a colon cancer. A: Direct DNA sequencings show *ANK3* c.9349delA mutation (MT) in two regional biopsies (53–1 and 53–6) and wild-type (WT) *ANK3* in the other five regional biopsies (53–2, 53–3, 53–4, 53–5 and 53–7)

by frameshift mutations identified in this study and suggest that these inactivating mutations might inhibit their TSG functions in MSI-H CRC as well. These frameshift mutations might play a role in the pathogenesis of MSI-H CRC by inhibiting their TSG activities. Also, the inactivating mutations of methyltransferase genes *MFN1, LCMT2, RNMT, TRMT6, METTL8* and *METTL16* might possibly play a role in tumorigenesis of CRC. However, consequences of the inactivating mutation of methyltransferase genes in MSI-H cancers remain unclear, because their normal biological functions are not known. Of importance, there was a significant difference in mutation frequencies between MSI-H and MSS cancers, indicating that these frameshift mutations with MSI-H were specific

All of the mutations detected in the present study would result in premature stops of amino acid synthesis in the proteins and hence resemble a typical loss-of-function mutation. 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 [13]. Since cancers with MSI show extremely severe mutation phenotype with frameshift mutation in microsatellite sequences, the frameshift mutations in this study could possibly 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 (BAX, type II transforming growth factor β receptor, caspase-5, hMSH3 and TCF4) [21]. 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. Analyses of a larger cohort with the mutations are required in future studies.

In this study, we identified mutational ITH of *ANK3, TP53BP1* and *MFN1* in CRCs, which is consistent with previous studies that had reported frequent mutational ITH in CRCs with MSI-H [22]. 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 [14, 23]. 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 [14, 23]. It is reasonable to conceive that other regions of these genes as well as other genes besides *ANK3, TP53BP1* and *MFN1* could harbor mutational ITH in these CRCs. 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.

Compliance with Ethical Standards

Conflicts of Interest None

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