Pathol. Oncol. Res. (2018) 24:477–481
DOI 10.1007/s12253-017-0272-9

ORIGINAL ARTICLE

Intratumoral Heterogeneity of Frameshift Mutations of *GLI1* Encoding a Hedgehog Signaling Protein in Colorectal Cancers

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Received: 18 December 2016 / Accepted: 22 June 2017 / Published online: 29 June 2017

Abstract GLI1 is a transcription factor for hedgehog signaling that plays a crucial role in signaling pathways for controlling cell proliferation, alterations of which are known to contribute to tumorigenesis. Aim of this study was to explore whether *GLI1* gene is mutated in gastric (GC) and colorectal cancers (CRC). In a public database, we found that *GLI1* had a G7 mononucleotide repeat in the coding sequences that could be a mutation target in the cancers with microsatellite instability (MSI). In this study, we analyzed frameshift mutation of *GLI1* in 79 GCs and 129 CRCs (high MSI (MSI-H) or microsatellite stable (MSS)) by single-strand conformation polymorphism analysis and DNA sequencing. We found 10 frameshift mutations in the repeat, nine for CRCs and one for GC. All of the mutations were detected in cancers with MSI-H and there was a statistical difference in the frameshift mutation frequencies between the cancers with MSI-H (10/113) and MSS (0/90). We also analyzed intratumoral heterogeneity (ITH) of the frameshift mutation in 16 CRCs and found that the mutations exhibited regional ITH in three of the CRCs (18.8%). Our data indicate *GLI1* harbored not only frameshift mutation but also its mutational ITH, which together could be a feature of GC and CRC with MSI-H.

Keywords GLI1 · Frameshift mutation · Colon cancer · Microsatellite instability · Intratumoral heterogeneity

Introduction

The GLI proteins (GLI1, GLI2 and GLI3) are the transcription factors for hedgehog signaling and are involved in cell fate determination, proliferation and patterning during embryo development [1–4]. Particularly, GLI1 plays an important role in human cancer development as an oncogene and are considered an important target of anti-cancer therapy [5–7]. Amplification and production of alternative splicing form that may promote GLI1 activity are reported in many cancers [8]. In colorectal cancer (CRC), GLI1 expression is increased in cancer tissues compared to the normal tissues [9].

About 10% of gastric cancer (GC) and CRC show microsatellite instability (MSI) phenotype that has defects in mismatch repair [10]. Genes are often observed to harbor frameshift mutations at monocleotide repeats in high-MSI (MSI-H) cancers [11]. There is a mononucleotide repeat (G7) in *GLI1* coding sequence that could be a mutation target in cancers with MSI-H. It is not known whether *GLI1* alterations are different between MSI-H and microsatellite stable (MSS) cancers. Intratumoral heterogeneity (ITH) is a common phenomenon in cancers, which may result in cancer evolution and influence on clinical outcomes [12]. Thus, identification of genetic ITH is important in understanding biological and clinicopathologic features of the cancers. The present study aimed to find whether *GLI1* gene harbored frameshift mutation within the repeat and ITH in GC and CRC.

Ju Hwa Lee and Sang Yong Song contributed equally to this work.

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Table 1 Summary of pathologic features of gastric and colorectal cancers

Feature	MSI-H	MSS
Gastric carcinomas		
Total cases	34	45
TNM stage		
I	13	15
II	13	18
III	7	11
IV	1	1
Lauren's subtype		
Diffuse	28	21
Intestinal	18	15
Mixed	3	6
Indeterminate	7	3
EGC vs. AGC		
EGC	3	4
AGC	31	41
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

EGC, early gastric cancer; *AGC*, advanced gastric cancer; *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 79 GCs and 124 CRCs. The GCs were 34 GCs with MSI-H, 45 GCs with microsatellite stable (MSS), 79 CRCs with MSI-H and 45 CRCs with MSS. These samples overrepresent MSI-H cases, because we collected MSI-H and MSS cases separately in different times. We adopted an MSI evaluation system using five mononucleotide repeats [13]. 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 *GLI1*. 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 [14–16]. 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 *GLI1* (G7 in exon 8). 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 [14–16].

Results

Mutational Analysis

Genomic DNAs of the 79 GCs and 124 CRCs were studied to detect frameshift mutations in the *GLI1* (G7 in exon 8) mononucleotide repeat. In SSCP, we observed aberrant bands in 10 cases among the 79 GCs and 124 CRCs (Table 2 and Fig. 1). Normal tissues of the corresponding patients revealed no aberrant migration in SSCP, which indicated that the aberrant bands had risen somatically. Sanger sequencing of the cancer tissues revealed that they were frameshift mutations of *GLI1* (Fig. 1). All detected mutations of *GLI1* were considered heterozygous mutations by SSCP and direct sequencing patterns (Fig. 1). These mutations were a deletion mutation, which would lead to premature termination of GLI1 translation

Table 2 Summary of *GLI1* mutations in gastric and 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)
GLI1	Exon 8	G7	G6	MSI-H (10)	Colorectal: 9/79 (11.4) Gastric: 1/34 (2.9)	c.821delG (p.Gly274AlafsX6)

MSI-H, high microsatellite instability

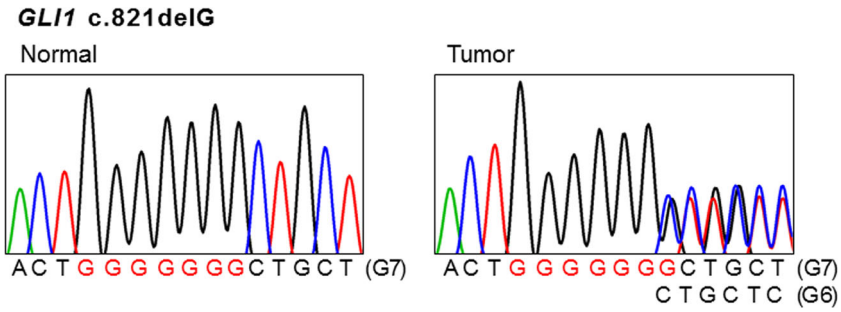

Fig. 1 Representative DNA sequencings of *GLI1* G7 repeat in a colon carcinoma. DNA sequencing analysis of *GLI1* from tumor (*Lane T*) and normal tissues (*Lane N*) show heterozygous G deletion within the G7 in tumor tissue as compare to matched normal tissue

(Table 2). Nine of 79 CRCs (11.4%) and 1 of 34 GCs (3.0%) with MSI-H exhibited the *GLI1* frameshift mutation. We found *GLI1* somatic frameshift mutations in 9 CRCs (9/79, 11.4%) and one GC (1/34, 3.0%) with MSI-H, but not in CRCs (0/45) and GCs (0/45) with MSS (Fisher's exact test, $p = 0.002$) (Table 2). These mutations were not detected in their normal tissues. The 10 mutations found were a same deletion mutation (c.821delG (p.Gly274Alafsx6)) in the coding region.

Intratumoral Heterogeneity of GLI1 Frameshift Mutation

Ninety-six areas from 16 CRCs with MSI-H were analyzed to find the ITH in the *GLI1* frameshift mutation. In 3 (#35, 45 and 49) of the 16 CRCs (18.8%), the G7 repeat in *GLI1* exhibited one base-pair deletion mutation (c.821delG) in one or more regional areas (Table 3 and Fig. 2), indicating ITH of the *GLI1* mutation existed in CRC. Clinical and histopathological parameters, however, could distinguish neither *GLI1* frameshift mutation (+) and (−) cancers, nor the ITH (+) and (−) cancers.

Discussion

The hedgehog signaling regulates cell fate determination and proliferation, alterations of which are features of cancers [17]. The hedgehog signaling is important not only in cancer development, but also in cancer therapy [1, 5]. The aim of our study was threefold. First, we attempted to find frameshift mutations in hedgehog signaling-related gene *GLI1* where mutational alterations had largely been unknown in cancers. We found frameshift mutation of this gene in CRCs (11.4%) and GCs (3.0%) with MSI-H. In addition, we found a significant difference of the mutation prevalence between the cancers with MSI-H and MSS, indicating that this mutation was MSI-H-specific. Third, we identified regional ITH of the frameshift mutation in 18.8% of CRC with MSI-H. Together, these

Table 3 Intratumoral heterogeneity of *GLI1* frameshift mutation in colorectal cancers

Case	Regional biopsy sites							Mutation status	ITH status
	#1	#2	#3	#4	#5	#6	#7		
CRC3	WT	WT	WT	WT	WT	WT	n.d.	Wild type	Non-ITH
CRC15	WT	WT	WT	WT	WT	WT	WT	Wild type	Non-ITH
CRC26	WT	WT	n.d.	WT	WT	WT	WT	Wild type	Non-ITH
CRC27	WT	WT	WT	WT	WT	WT	WT	Wild type	Non-ITH
CRC34	WT	WT	WT	WT	WT	WT	WT	Wild type	Non-ITH
CRC35	WT	WT	n.d.	n.d.	n.d.	WT	WT	Wild type	Non-ITH
CRC39	WT	WT	c.821delG	WT	n.d.	WT	WT	Mutation	ITH
CRC41	WT	n.d.	WT	WT	n.d.	WT	WT	Wild type	Non-ITH
CRC43	WT	WT	WT	n.d.	n.d.	WT	n.d.	Wild type	Non-ITH
CRC45	c.821delG	c.821delG	c.821delG	c.821delG	WT	c.821delG	c.821delG	Mutation	ITH
CRC47	WT	WT	WT	WT	WT	WT	WT	Wild type	Non-ITH
CRC48	WT	n.d.	n.d.	WT	WT	WT	WT	Wild type	Non-ITH
CRC49	n.d.	c.821delG	c.821delG	WT	c.821delG	c.821delG	c.821delG	Mutation	ITH
CRC51	WT	WT	WT	WT	n.d.	WT	WT	Wild type	Non-ITH
CRC53	WT	WT	WT	WT	WT	WT	WT	Wild type	Non-ITH
CRC55	WT	WT	n.d.	n.d.	WT	WT	WT	Wild type	Non-ITH

n.d., not done; *ITH*, Intratumoral heterogeneity; *CRC*, colorectal cancer

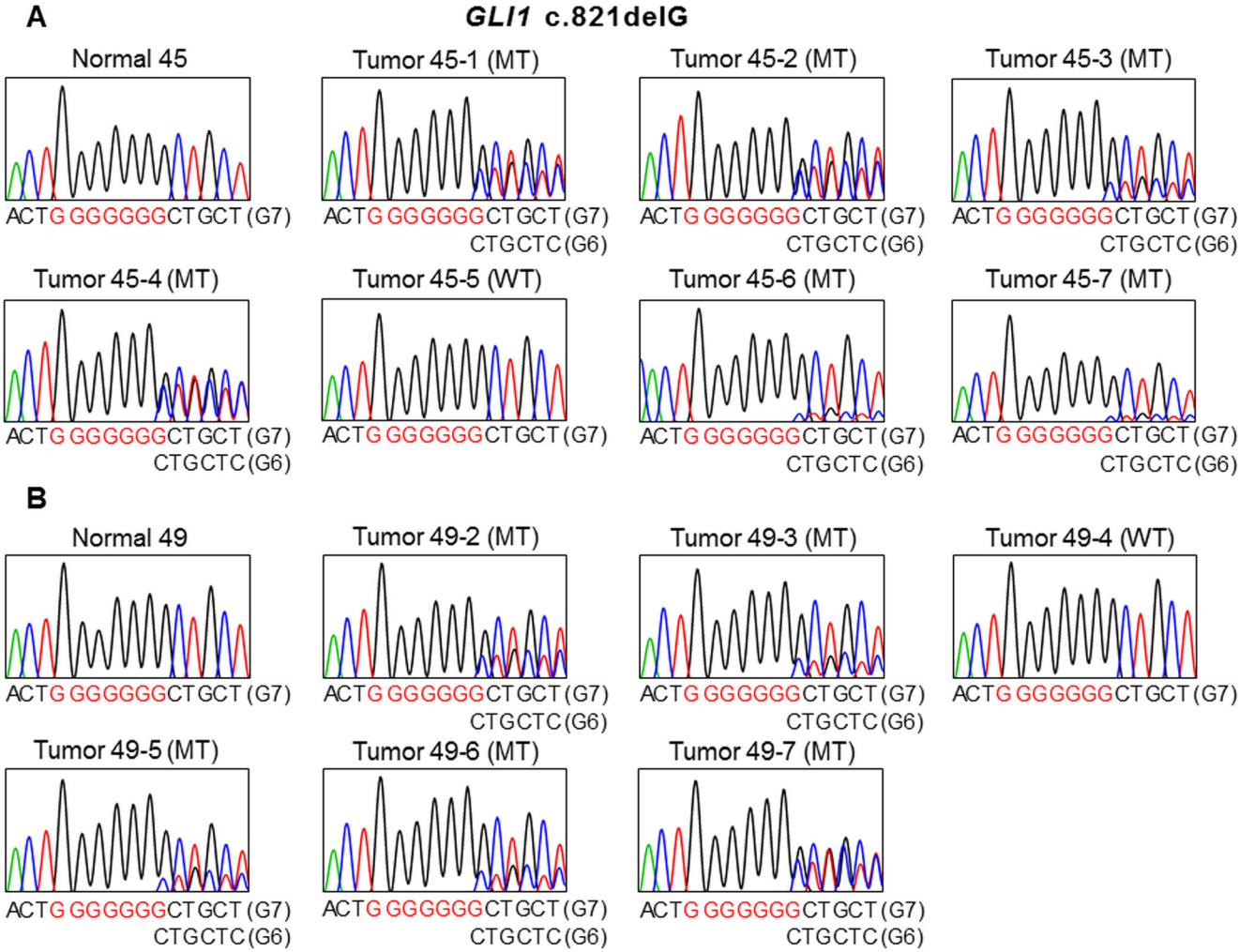

Fig. 2 Intratumoral heterogeneity of *GLI1* frameshift mutation in colon cancers. A: Direct DNA sequencings show *GLI1* c.821delG mutation (MT) in six regional biopsies (45–1, 45–2, 45–3, 45–4, 39–6 and 39–7) and wild-type (WT) *GLI1* in the other one regional biopsy (45–5). B: A: Direct DNA sequencings show *GLI1* c.821delG mutation (MT) in five regional biopsies (49–2, 49–3, 49–5, 49–6 and 49–7) and wild-type (WT) *GLI1* in the other one regional biopsy (49–4)

results exhibited that *GLI1* gene in hedgehog signaling could be a mutational target in MSI-H GC and CRC as well as mutational ITH, suggesting *GLI1* gene alteration might be a feature of MSI-H cancer.

The frameshift mutation identified in this study would result in truncation of GLI1 protein and hence would resemble a typical loss-of-function mutation (Table 2). At this stage, however, consequence of the inactivating mutation in MSI-H cancers remains unknown. Activation of hedgehog signaling is known to promote tumor development. For example, it leads to an increase in angiogenic factors, cyclins, anti-apoptotic genes and a decrease in apoptotic genes [18–20]. Provided that GLI1 behave as an oncogene, the frameshift mutation appears to reduce the oncogenic activity in cancer cells, possibly suggesting a rationale for explaining better prognosis of GC and CRC with MSI-H than those with MSS [10].

In this study, we identified mutational ITH of *GLI1* in 3 of 16 CRCs (18.8%), which is consistent with previous studies that had reported frequent mutational ITH in CRCs with MSI-H [21]. It is well known that cancer ITH of driver mutations could result in poor clinical outcomes in patients. 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, 22]. Based on the oncogenic natures of GLI1, our results might suggest a possibility that there could be a mixed or ameliorated effect of *GLI1* oncogenic effect in MSI-H cancers. However, we were not able to find any distinguished clinicopathologic features of *GLI1*-mutated or ITH-positive cancers. It was probably due to small number of *GLI1* mutated cases. Based on our preliminary data, further studies are needed to define

the clinical implication of *GLI1* mutation and its ITH in MSI-H cancers.

Acknowledgements This work was supported by a grant from National Research Foundation of Korea (2012R1A5A2047939).

Compliance with Ethical Standards

Conflicts of Interest None to declare.

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