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Frameshift mutations of *OGDH*, *PPAT* and *PCCA* genes in gastric and colorectal cancers

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Metabolic reprogramming is a hallmark of cancer. However, genetic alterations in metabolism-related genes are largely unknown. The aim of this study was to identify whether somatic mutations in *OGDH, PPAT* and *PCCA* genes known to be involved in amino acid or nucleotide metabolism are mutated in gastric cancer (GC) and colorectal cancer (CRC). By public database search, we identified that *OGDH, PPAT* and *PCCA* genes harbor mononucleotide repeats that may serve as mutation targets in cancers with microsatellite instability (MSI). We analyzed the repeats for the presence of the mutations in 90 GCs and 141 CRCs using single-strand conformation polymorphism (SSCP) and samples of 10 patients with shifted bands were sequenced. We found frameshift mutations of *OGDH* (3 cases), *PCCA* (5 cases) and *PPAT* (2 cases) in the cancers. These mutations were exclusively detected in MSI-high (MSI-H), and not in MSI-low or MSI-stable (MSI-L/MSS) cancers. We also analyzed 16 CRCs for the presence of intratumoral heterogeneity (ITH) and found that one CRC harbored regional ITH for *OGDH* frameshift mutation showing very rare frequency of *OGDH* mutation ITH in colorectal cancer tissues. Our data indicate that amino acid/nucleotide metabolism-related genes *OGDH, PPAT* and *PCCA* acquire somatic mutations in MSH-H GCs and CRCs and that mutational ITH may occur in at least some of these tumors. Collectively, our results may extend our insight into the involvement of amino acid/nucleotide metabolism in the pathogenesis of cancer for, in particular, MSI-H GCs and CRCs.

Key words: amino acid/nucleotide metabolism, OGDH, PCCA, PPAT, frameshift mutations, gastrointestinal cancers, microsatellite instability

Early observations of Warburg indicated that there were fundamental differences in the cellular metabolisms between normal and malignant tissue [1-3]. It is also clear that many key oncogenic pathways converge to adapt cancer cell metabolism for their growth and survival [1]. Several lines of evidence exist that metabolic reprogramming could be adopted for cancer therapies [3]. For example, cancer cells are addicted to glutamine [4], depletion of which has successfully been used for therapy in some cancers. Metabolism in cancer cells can be altered by several mechanisms such as somatic mutation of key enzyme genes in metabolic pathways [1]. For example, somatic mutations of *IDH1* and *IDH2* genes, encoding isocitrate dehydrogenases, commonly occur in glioblastomas and myeloid leukemias [5-7]. However, somatic mutations in amino acid and nucleotide metabolism-related genes have poorly been explored.

Through a search of a genome database (http://genome.cse.ucsc.edu/), we found that several genes involved in amino acid/nucleotide metabolism have mononucleotide repeats in their coding sequences that could serve as targets for frameshift mutations in cancers with microsatellite instability (MSI) [8]. Frameshift mutations in genes with mononucleotide repeats are well-known features of gastric cancers (GCs) and colorectal cancer (CRCs) exhibiting MSI [9]. Cancer becomes heterogeneous after subclonal expansions, which leads to intratumoral heterogeneity (ITH). The ITH plays a role in acquiring aggressiveness and impedes accurate diagnosis and proper selection of tumor therapies [10]. Here, we assessed whether somatic mutations in amino acid metabolism-related genes (2-oxoglutarate dehydrogenase (*OGDH*) and propionyl CoA carboxylase alpha (*PCCA*)) and a nucleotide biosynthesis –related gene phosphoribosyl pyrophosphate amidotransferase (*PPAT*) may contribute to development of GC and CRC, and ITH. OGDH catalyzes conversion of alpha-ketoglutarate to succinyl-CoA during the Krebs cycle. OGDH is known to induce neuronal cell

death [11, 12]. PPAT catalyzes the first step of de novo purine nucleotide biosythetic pathway. Loss of *PPAT* gene is related to early onset CRC [13]. PCCA is the alpha subunit of the heterodimeric mitochondrial enzyme propionyl-CoA carboxylase [14].

Materials and methods

Tissue samples and microdissection. Methacarn-fixed tissues of 90 sporadic gastric carcinomas (GCs) and 141 sporadic colorectal carcinomas (CRCs) were included in this study. All patients were of Korean descent. The male to female ratios of the GC and CRC patients were 48:42 and 80:61, respectively. The ages of GC and CRC patients ranged from 30-81 years (average: 55 years) and 32-85 years (average: 57 years), respectively. The cohorts consisted of 34 microsatellite instability-high (MSI-H) GCs, 56 MSI-low (MSI-L) or MSI-stable (MSS) GCs, and 79 MSI-H CRCs and 62 MSS/MSI-L CRCs. For the MSI evaluation five mononucleotide repeats (BAT25, BAT26, NR-21, NR-24 and MONO-27) were used, and the MSI status was determined as: MSI-H when two or more of the markers showed instability, MSI-L when one of the markers showed instability and MSS when none of the markers showed instability [15]. From 54/141 of the CRC samples, we collected four to seven different tumor areas and one normal mucosal area. The selected areas were 0.027-1 cm^3 in size and located at least 1.0 cm apart from each other. The remaining 87 CRC samples were collected separately and were not used for intra-tumor heterogeneity (ITH) analyses. The pathologic features of all cancers are summarized in Table 1. The histologic features of the MSI-H CRCs, including mucinous histology, tumor infiltrating lymphocytes, medullary pattern and Crohn's like inflammation, were for all cases evaluated by a pathologist. Malignant cells and normal cells were selectively procured from hematoxylin and eosin (H&E)-stained slides by microdissection using a 30G1/2 hypodermic needle as described previously [16, 17]. DNA extraction was performed by a modified single-step DNA extraction method using proteinase K. Approval of this study was obtained from the Catholic University of Korea, College of Medicine's institutional review board.

Table 1. Summary of the pathologic features of the carcinomas included in this study

Feature	MSI-H	MSS/MSI-L
Gastric carcinomas		
Total cases	34	56
TNM stage		
I	13	19
II	13	22
III	7	12
IV	1	3
Lauren's subtype		
Diffuse	4	25
Intestinal	20	25
Mixed	3	3
Indeterminate	7	3
EGC vs. AGC		
EGC	3	5
AGC	31	51
Colorectal carcinomas		
Total cases	79	62
TNM stage		
I	15	9
II	29	21
III	32	29
IV	3	3
Location		
Cecum	16	0
Ascending colon	46	3
Transverse colon	12	3
Descending & sigmoid colon	4	23
Rectum	1	33

EGC: early gastric cancer, AGC: advanced gastric cancer, TNM: tumor, lymph node, metastasis

Gene mutation and ITH analyses. We analyzed the repeats for the presence of the mutations in 90 GCs and 141 CRCs using single-strand conformation polymorphism (SSCP) and samples with shifted bands were sequenced. Three genes harboring mononucleotide repeats were selected for this study: *OGDH* exon 3 (T8), *PPAT* exon 6 (A7), *PPAT* exon 9 (A7) and *PCCA* exon 12 (T7). Genomic DNA from the microdissected cells was isolated and amplified by polymerase chain reaction (PCR) using specific primer pairs. A radioisotope ([^{32}P]dCTP) was incorporated into the PCR products for detection by autoradiogram. After SSCP, mobility shifts in SSCP gels (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) were detected by visual inspection. Direct DNA sequencing of both forward and reverse strands was performed in samples showing SSCP mobility shifts using a capillary automatic sequencer (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA). When a gene mutation was suspected by SSCP, DNA independently isolated from another tissue section of the same patient was selected for sequence analysis to exclude possible PCR artifacts.

Sixteen of the 54 CRC cases from which multi-region biopsies were obtained were identified as MSI-H. These multi-region biopsies (four to seven different tumor areas from the 16 cases with MSI-H) were analyzed for the presence of ITH based on the occurrence of frameshift mutations in the *OGDH, PPAT* and *PCCA* genes.

Results

Frameshift mutations of OGDH, PPAT and PCCA. Genomic DNAs isolated from normal and tumor tissues from the 90 GC and 141 CRC patients were assessed for the presence of mutations by SSCP analysis. We observed shifted SSCP bands in the *OGDH* (3 cases, 2.7% of MSI-H cancers), *PPAT* (2 cases,

1.8% of MSI-H cancers) and *PCCA* (5 cases, 4.4% of MSI-H cancers) genes. No band shifts were observed in DNAs from normal tissues from the same patients, indicating that the shifts had arisen somatically (Figure 1). Direct sequencing of DNAs confirmed that these band shifts resulted from somatic mutations, all of which were interpreted as being heterozygous (Figure 2). All the mutations represented deletion or insertion of a base in the repeats (frameshifts), resulting in premature translation termination (Table 2).

These mutations were detected in the MSI-H samples, but not in the MSS/MSI-L samples (Table 2). A statistical difference in the frameshift mutation frequencies was found between the MSI-H (10/113) and MSS/MSI-L (0/118) samples (Fisher's exact test, $p = 0.001$). The *PCCA* frameshift mutation showed a difference between the MSI-H (5/113) and MSS/MSI-L (0/118) samples (Fisher's exact test, $p = 0.027$). In terms of tissue origin, no statistical difference in mutation frequencies was found between MSI-H GC (1/34) and MSI-H CRC (9/79) samples (Fisher's exact test, $p > 0.05$). No significant association was noted between the mutations encountered and the clinicopathologic characteristics of the patients tested (age, sex, histologic grade and stage) (χ^2 test, $p > 0.05$).

Intratumoral heterogeneity of OGDH frameshift mutations in MSI-H CRCs. Ninety-six regional fragments of 16 MSI-H CRC cases (4-7 fragments per case) were collected and analyzed with respect to their regional status of *OGDH, PPAT* and *PCCA* frameshift mutations. *OGDH* gene was found to be mutated in one CRC with ITH, whereas it was not mutated in any regional biopsies of the other 15 CRCs. The ITH data show very rare frequency of *OGDH* mutation ITH in colorectal cancer tissues. The *OGDH* mutation showed ITH in the case #51 (deletion of a base) in six out of seven regional biopsies (Figure 3). In contrast, neither the *PCCA* nor the *PPAT* mutations showed the ITH in the 16 CRCs.

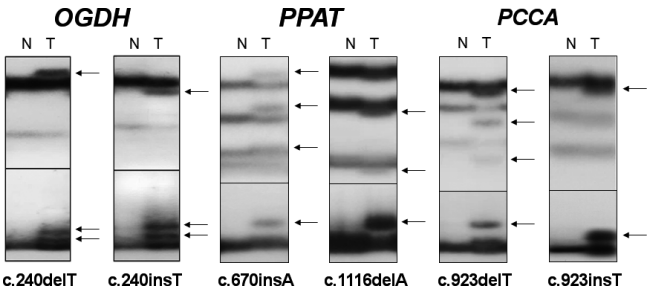

Figure 1. Representative SSCP of *OGDH, PPAT* and *PCCA*. SSCP of *OGDH* mutations (left), *PPAT* mutations (middle) and *PCCA* mutations (right) from tumor (Lane T) and normal tissues (Lane N). In the SSCP, the arrows (Lane T) indicate aberrant bands compared to the SSCP from normal tissues (N).

Discussion

In this study, we attempted to see whether somatic mutations in genes encoding proteins involved in amino acid metabolism (*OGDH* and *PCCA*) and nucleotide biosynthesis (*PPAT*) were present in GC and CRC. Next, we assessed whether these mutations could be related to the multiclonal development of tumors by finding ITH. These approaches are based on current knowledge that metabolic alterations are prominent features of cancer cells [1]. We found that all of the three genes harbored frameshift mutations in 1.8 – 4.4% of the MSI-H GCs and CRCs. Significant differences were observed in the mutation frequencies between the MSI-H and MSS/MSI-L cancers, indicating that association of the gene mutations with MSI-H phenotype was specific. We also found that one of the genes (*OGDH*) showed genetic ITH in an MSI-H CRC. These data indicate that *OGDH, PPAT* and *PCCA* genes encoding proteins in amino acid/nucleotide metabolism are altered in MSI-H GCs and CRCs by somatic frameshift mutations, and suggest that some mutations may exhibit ITH.

In our study, we observed both deletion and insertion within the nucleotide repeats of the genes analyzed (Table 2). All of them were found to result in premature stops and, hence, to represent typical loss-of-function mutations. OGDH is the rate-limiting component of the multi-enzyme OGDH complex (OGDHC) that includes the key regulatory enzyme of Krebs cycle. Due to the key position of OGDHC in mitochondrial metabolism, its cellular activity is controlled by many factors [18]. OGDH is involved in neuronal cell death, but its role in cancer development remains unknown. OGDHL, another molecule in OGDHC, suppressed cell proliferation and invasion, suggesting the role of OGDHC in tumor suppression [19]. It is possible that loss-of-function mutations of *OGDH* might similarly inactivate the tumor

Table 2. Summary of the mutations detected in the 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)
OGDH	Exon 3	T8	T7	MSI-H (2)	Colorectal: 2/79 (2.5)	c.240delT (p.Arg81Alafsx19)
		T8	T9	MSI-H (1)	Colorectal: 1/79 (1.3)	c.240insT (p.Arg81Serfsx68)
PCCA	Exon 12	T7	T6	MSI-H (4)	Colorectal: 4/79 (5.1)	c.923delT (p.Leu308Trpfsx14)
		T7	T8	MSI-H (1)	Gastric: 1/34 (2.9)	c.923insT (p.Leu308Phefsx35)
PPAT	Exon 6	A7	A8	MSI-H (1)	Colorectal: 1/79 (1.3)	c.670insA (p.Thr224Asnfsx15)
	Exon 9	A7	A6	MSI-H (1)	Colorectal: 1/79 (1.3)	c.1116delA (p.Lys372Asnfsx17)

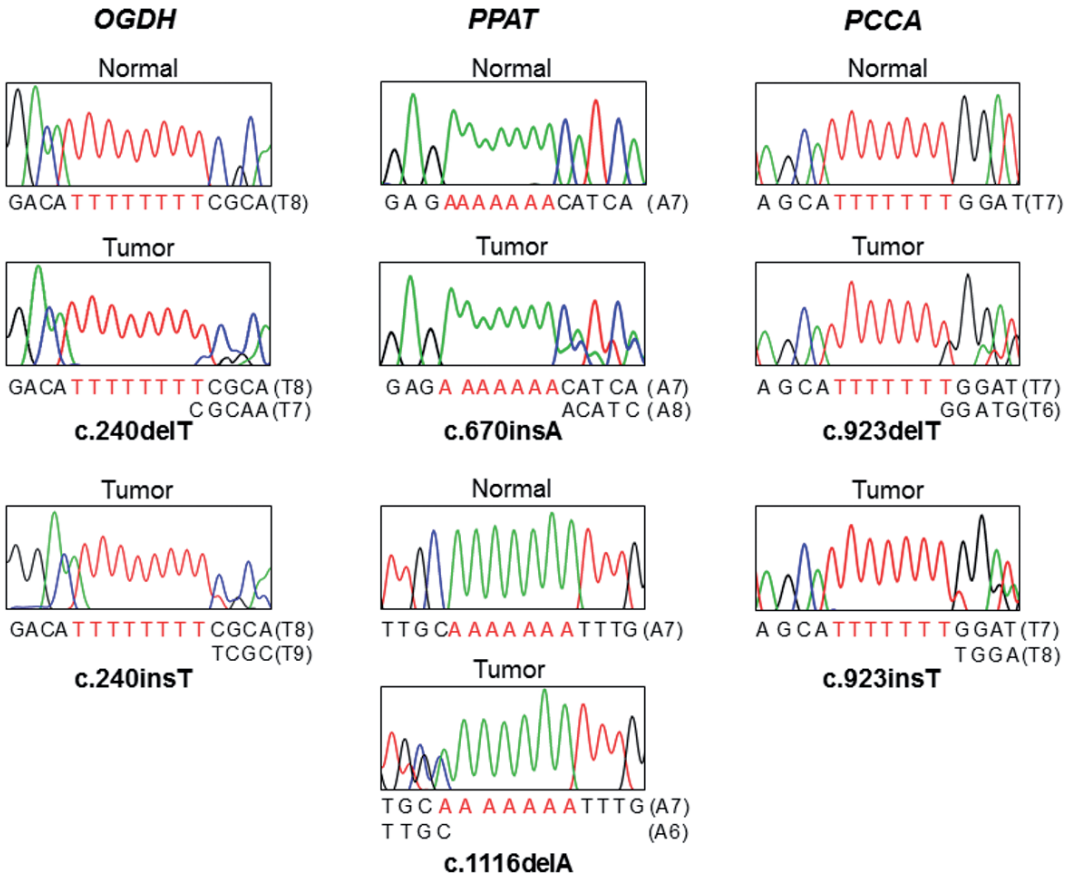

Figure 2. Representative DNA sequence data of the *OGDH* exon 3 (T8), *PPAT* exon 6 (A7), *PPAT* exon 9 (A7) and *PCCA* exon 12 (T7) repeats in colon carcinomas and matched controls. DNA sequences of *OGDH*, *PPAT* and *PCCA* from tumor and normal tissues. There are heterozygous mutations in tumor tissues compared to normal tissues.

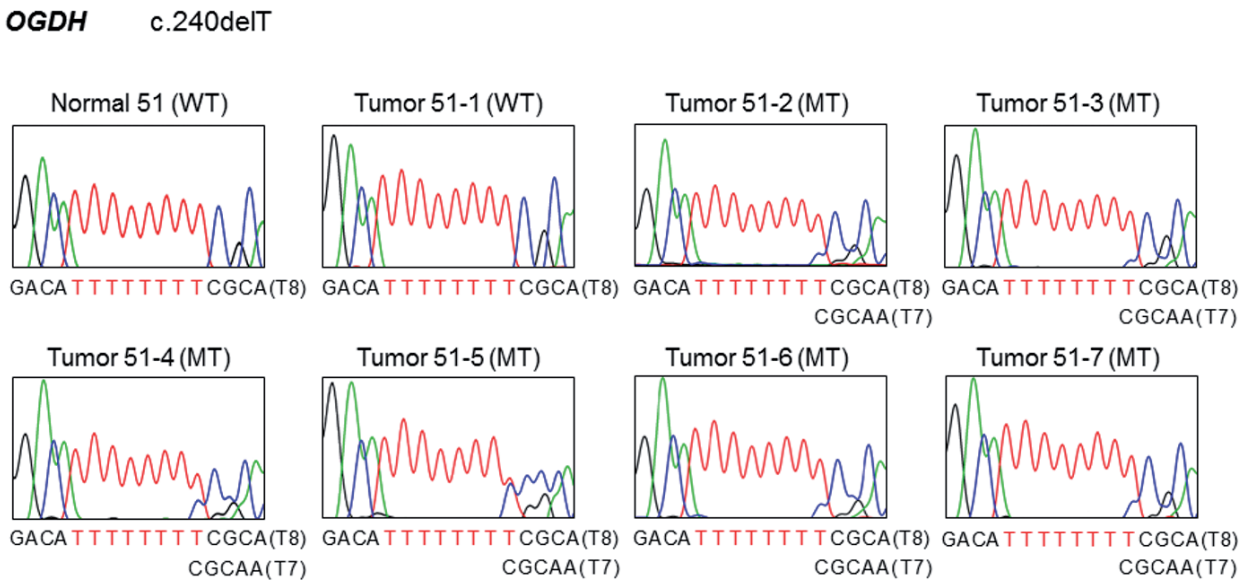

Figure 3. Intratumor heterogeneity of *OGDH* frameshift mutations in a colon carcinoma. A: Direct DNA sequencing analyses showing the *OGDH* c.240delT mutation (MT) in six regional biopsies (51-2, -3, -4, -5, -6, -7) and wild-type (WT) *OGDH* in another regional biopsy (51-1).

suppressor function of OGDHC and contribute to tumorigenesis. Loss of chromosome 4q12, where *PPAT* resides, and loss of PPAT mRNA expression are common in early onset CRC [13], suggesting that inactivation of *PPAT* gene might be involved in CRC development. The *OGDH, PPAT* and *PCCA* mutations detected in this study, however, were identified as heterozygous frameshift mutations. Heterozygous mutations do not necessary lead to disease pathology since the remaining wildtype allele may still allow sufficient amount of proteins to be translated. Heterozygous inactivating mutations of *PCCA* are known to cause propionic academia that results in acidosis and ketosis [14]. As for *OGDH* and *PPAT* there are no studies to support that their heterozygous mutations can cause diseases. Firm links between the mutations identified in our study and these or other cancer-related characteristics remain to be clarified.

In our analyses, we noted ITH for *OGDH* gene mutation in the CRC samples tested. These results are in accordance with previous studies showing that genetic ITH for microsatellite markers, as well as repeat sequences within coding genes, may be encountered [14, 20, 21]. The ITH data indicate that ITH of *OGDH* frameshift mutation may be a rare event in colorectal cancer tissues. After 3 years of surgery, no definite differences in clinical courses were observed between CRCs with or without *OGDH* gene mutation-related ITH. Probably due to the small number of CRCs with the *OGDH* mutational ITH (n=1), we were not able to define clinical feature of the ITH in this study. Also, it is not known that the ITH in this case is an incidental finding. Thus, we propose that its role should be clarified in conjunction with elucidation of the oncogenic role of *OGDH* gene and with a large number of cancer cases.

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