Research Article

RAS mutations that have a major impact on current cancer genomic medicine

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The status of rat sarcoma viral oncogene homolog (RAS) proteins is a negative predictive biomarker for anti-epidermal growth factor receptor (EGFR) therapy in metastatic colon cancer. In the phase 2 CHRONOS trial, patients with mutant gene(s) are ineligible for anti-EGFR therapy. However, our studies revealed that splicing caused by the RAS mutations, which were considered oncogenic, generates unfunctional RAS family. Especially, Kirsten Rat Sarcoma (KRAS) silent variants are of concern to be a serious problem in genomic cancer medicine.

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Short Title: Identification of cryptic splice donor sites caused by mutations for cancer genomic medicine

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Communication

The status of rat sarcoma viral oncogene homolog (RAS) proteins is a negative predictive biomarker for anti-epidermal growth factor receptor (EGFR) therapy in metastatic colon cancer. In the phase 2 CHRONOS trial, patients with mutant gene(s) are ineligible for anti-EGFR therapy.[1][2] However, our studies revealed that splicing caused by the RAS mutations, which were considered oncogenic, generates unfunctional RAS family. Especially, Kirsten Rat Sarcoma (KRAS) silent variants are of concern to be a serious problem in genomic cancer medicine.

The RAS superfamily (Ras protein, RAS subfamily) is a small guanosine triphosphate (GTP)-binding protein that regulates transcription, cell proliferation, and cell motility. Moreover, RAS is a molecule involved in many biological phenomena, such as the suppression of cell death. The RAS gene is a type of protooncogene because RAS mutants are significantly associated with the cancerization and carcinogenesis of cells. Mutations in the *KRAS* gene are the most frequent drivers of tumor development across the spectrum of human cancers.[3][4] Normally, RAS is inactivated by binding to guanosine diphosphate (GDP). However, when GDP is exchanged for GTP by a guanine nucleotide exchange factor (GEF), RAS is markedly activated in cancer cells. Moreover, when all tyrosine kinase receptors (TKRs), including platelet-derived growth factor (PDGF), nerve growth factor (NGF), EGF, *etc.*, are stimulated, RAS is constantly activated (Figure 1A).

The most common mechanisms of resistance identified upon rechallenge of panitumumab were mutations or amplifications in the *EGFR*, *KRAS*, and *NRAS* genes, observed in approximately 48% of patients with recurrent colon cancer. Suppose a missense mutation occurs in the RAS amino acid sequence in which G13 and Q61 in the depression to which GTP binds are replaced with another amino acid.[1][2] In that case, such mutant RAS can bind to GTP, but mutant RAS cannot hydrolyze GTP and are constantly activated. In the phase 2 CHRONOS trial, as expected, KRAS and NRAS mutations occurred frequently at position 61.[3][4] Therefore, mutations that are constitutively activated and result in oncogeneization are called oncogenic or pathogenic mutations.

The two antitumor agents covered by insurance as molecular-targeted therapies for colorectal cancer are bevacizumab, an anti-vascular endothelial growth factor (VEGF) agent, and cetuximab and panitumumab, which are anti-EGFR antibody agents (Figure 1A).[5] Approximately forty percent of patients with colon cancer have the pathogenic *KRAS* gene mutation(s), which leads to runaway cell proliferation and the rapid growth of cancer cells even without the EGF signal. In the case of colorectal cancer cells with the mutant *KRAS* gene, the response of molecular-targeted drugs to EGFR is not observed.[6] Therefore, the RAS status is a negative predictive biomarker for anti-EGFR therapy in metastatic colon cancer,[7] and identification of the *KRAS* gene mutations by genetic testing such as the oncoBEAM ™ RAS CRC kit is essential before anti-EGFR treatment.

Kobayashi *et al.* elucidated that RAS Q61K mutation caused a change in the generation (splicing) of *RAS* mRNA, such that the mature mRNA−− and thus the protein −− was truncated and

nonfunctional (Figure 1B).[8] However, in the case of a RAS Q61K mutation in which the codon of 60G has (GGA, GGC, GGG), splicing does not occur, so the RAS Q61K is oncogenic functional.[8]

The recent report states that although oligonucleotide drug technology is advancing rapidly, the therapeutic use of oligonucleotides for suppressing oncogenic KRAS signaling remains theoretical. [9] Recent report also shows that the development of small-molecule–specific inhibitors of splicing in exon 3 of RAS oncogenes is a candidate strategy for the growing portfolio of approaches that are being pursued to target cancers with oncogenic RAS; unfortunately the clinical applications of this work may be distant.[7]

From December 2019 to April 2022, a total of 1689 cases (OncoGuide^{TM} NCC Oncopanel System* test: 299 cases, FoundationOne^{R} CDx** tissue test: 1245 cases, FoundationOne^{R} CDx liquid test: 145 cases) were investigated in cancer genomic medicine at a national university in Japan. Recently, our medical team examined total 318 patients with recurrent colon cancer in cancer genomic medicine, and then our medical team obtained the reports indicating detection result of KRAS Q61K, KRAS Q61L, and KRAS Q61H as oncogenic variants (also called druggable variant or pathogenic variant)*** in cancer genomic medicine by FoundationOne^{R} CDx for total 5 patients with recurrent colon cancer (Table 1). By retesting with ClinVar and OncoKB, KRAS Q61K, KRAS Q61L and KRAS Q61H were pathogenic variants. Therefore, our medical staff has abandoned the prescription of anti-EGFR inhibitors for recurrent colon cancer. However, after confirming the contents of the research by Kobayashi *et al.*[8], our medical team revealed by using whole DNA/RNA gene sequence analysis and Entrez Gene program that KRAS Q61K, KRAS Q61L and KRAS Q61H were KRAS GGT (G60), AAA (K61), CTA (L61) and CAT (H61), in short, these KRAS variants were nonfunctional KRAS caused by altered splicing from the cryptic splice donor site (Table 1). The response of panitumumab, an anti-EGFR inhibitor, has been confirmed for patients with colon cancer. Notably, the findings obtained from the research conducted by Kobayashi *et al.*[8] have already brought great benefits to the lives of cancer patients with recurrent colon cancer in clinical practice.

Currently, personalized medical care for patients with malignant tumors is being performed based on the results of cancer genome tests. As shown in the phase 2 CHRONOS trial, in the future treatment of recurrent colon cancer, liquid biopsies should prospectively be used to define treatment choice with an anti-EGFR antibody in patients with recurrent colon cancer. The results obtained from FoundationOne^{R} CDx tissue, FoundationOne^{R} CDx liquid, and other molecular tests to detect the

variants (i.e., mutations, high copy number of gene, loss of gene, *etc.*) are considered oncogenic, benign or variants of unknown significance (VUS) using updated databases including ClinVar, ConcoKB, cosmic, VarSome, and MGeND. However, the database used for cancer genomic testing, as in the case of KRAS this time, is not constantly updated. In the future, the contents of the genome-wide databases must be updated based on the results obtained from basic medical and clinical research.

Footnote

OncoGuide^{TM} NCC oncopanel System*; Gene mutation analysis set for cancer genome profiling test (Sysmex Corporation Kobe, Hyogo, Japan)

FoundationOne CDx**; cancer genome test (Foundation Medicine, Inc., Cambridge MA, USA)

Oncogenic variants (also called as druggable variants or pathogenic variant)***; These variants include oncogenic/pathogenic mutations, high-expression/high copy number, and gene loss.

Author Contributions

T.H. performed most of the clinical work and coordinated the project. T.H. conducted the diagnostic pathological studies. T.H. conceptualized the study and wrote the manuscript. T.H., N.Y. and I.K. carefully reviewed this manuscript and commented on the aspects of medical science. I.K. shared information on clinical medicine and oversaw the entirety of the study. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Institutional Review Board Statement

This study was reviewed and approved by the Central Ethics Review Board of the National Hospital Organization Headquarters in Japan (Tokyo, Japan) and Shinshu University (Nagano, Japan) on August 17, 2019, with approval codes NHO H31-02 and M192. The completion numbers for the authors are AP0000151756, AP0000151757, AP0000151769, and AP000351128. As this research was considered clinical research, consent to participate was required. After briefing regarding the clinical study and approval of the research contents, the participants signed an informed consent form.

Informed Consent Statement

Not applicable for studies not involving humans.

Data Availability Statement

The study did not report any data.

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Tables and Figures

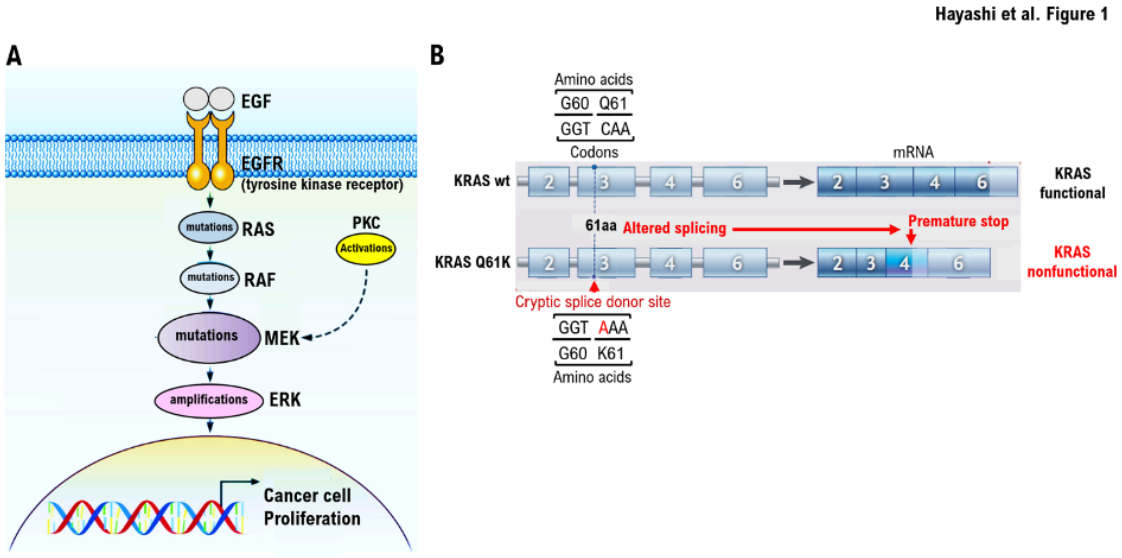

Figure 1. Identification of nonfunctional KRAS Q61K variant of patient with recurrent colon cancer by using whole gene sequence analysis. A. An overview of the EGFR pathway and its main downstream effectors, KRAS/BRAF/MEK/ERK. Notes: Activated MEK/ERK can induce cancer cell proliferation and invasion. Anti-EGFR mAbs, such as cetuximab or panitumumab, can bind EGFR and block its function. **B.** Recent research demonstrated that the oncogenic effect of an activating variant (the KRAS Q61K mutation) was dependent on a second, silent variant, G60, in KRAS. The mutation resulting in KRAS Q61K produces aberrant RNA splicing in exon 3, which results in a frameshift (in exon 4) and the introduction of an early stop codon. Abbreviations: BRAF; B-raf protooncogene, EGFR; epidermal growth factor receptor, ERK; extracellular signal-regulated kinase, KRAS; Kirsten murine sarcoma, MEK; mitogen-activated protein kinase

Results of FoundationOne CDx for KRAS mutations					
KRAS	Amino Acid		F1CDx	ClinVar	KRAS Activation
Wild Type	G60	Q61	-	-	normal
Patient #1	G60	K61	oncogenic	pathogenic	functional
Patient #2	G60	L61	oncogenic	pathogenic	functional
Patient #3	G60	H61	oncogenic	pathogenic	functional
Patient #4	G60	H61	oncogenic	pathogenic	functional
Patient #5	G61	L61	oncogenic	pathogenic	functional
Results of genome DNA/RNA sequence analysis and Entrez Gene program					
	codon of aa60	codon of aa61	RNA function		KRAS Activation
Wild Type	GGT (G60)	CAA (Q61)	normal function		Normal activation
Patient #1	GGT (G60)	AAA (K61)	altered splicing		nonfunctional
Patient #2	GGT (G60)	CTA (L61)	altered splicing		nonfunctional
Patient #3	GGT (G60)	CAT (H61)	altered splicing		nonfunctional
Patient #4	GGT (G60)	CAT (H61)	altered splicing		nonfunctional
Patient #5	GGT (G60)	CTA (L61)	altered splicing		nonfunctional

Table 1. Cases of patients with recurrent colon cancer

Table 1. *KRAS Q61K was determined to be an oncogenic variant in genomic cancer medicine by FoundationOne^{R} CDx for a patient with recurrent colon cancer. By retesting with ClinVar, KRAS Q61K was a pathogenic variant. The studies by using whole gene sequence analysis and Entrez Gene program revealed that KRAS Q61K, KRAS Q61L, and KRAS Q61H were KRAS GGT (G60) and AAA (K61), CTA (L61), and CAT (H61), and was nonfunctional KRAS caused by altered splicing from the cryptic splice donor site.*

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Declarations

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