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Brief communication (Original)

Pyrosequencing analysis of *KRAS* codon 61 mutations in Thai patients with advanced colorectal cancer

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Background: *KRAS*, coding for a small G-protein downstream of epidermal growth factor receptor (EGFR) plays an important role in the EGFR signaling network. Mutation in *KRAS* is associated with resistance to anti-EGFR in patients with advanced colorectal cancer (CRC). According to the American Society of Clinical Oncology (ASCO) guidelines, screening for mutations in *KRAS* codons 12 and 13 in tumor samples is mandatory for all CRC patients who are candidates for anti-EGFR targeted therapy. However, some patients with undetectable mutations in codons 12/13 do not benefit from anti-EGFR treatment, and this might be because of mutations in codon 61, which is not currently recommended for screening.

Objectives: To develop an in-house pyrosequencing method to screen for *KRAS* codon 61 mutations, and examine the prevalence of mutations in Thai patients with advanced CRC with no detectable mutation in codons 12/13.

Materials and Methods: DNA extracted from FFPE specimens was screened for *KRAS* codon 61 mutations using pyrosequencing. Our method was suitable for routine clinical samples (formalin-fixed, paraffin-embedded tissue), and was able to detect 5 common mutations in codon 61 of the *KRAS* gene, including c.182AT (p.Q61L), c.182AG (p.Q61R), c.182AC (p.Q61P), c.183AC (p.Q61H), and c.183AT (p.Q61H).

Results: Of the 74 samples with undetectable codon 12/13 mutation examined, two (2.7%) were found to harbor mutation in codon 61.

Conclusion: Despite the low prevalence of *KRAS* codon 61 mutation in our population with advanced CRC, adding the mutation test into the routine molecular service deserves consideration because the cost of treatment is very expensive.

Keywords: Colorectal cancer, KRAS, mutation, pyrosequencing, targeted therapy

About 35% to 40% of patients with advanced colorectal cancer (CRC) have mutations in *KRAS*, which codes for a small G-protein downstream of the epidermal growth factor receptor (EGFR), particularly codons 12/13 [1-3]. The presence of *KRAS* mutation is associated with resistance to EGFR monoclonal antibody [4]. According to the American Society of Clinical Oncology (ASCO) guidelines, screening for codon 12 and 13 mutations of *KRAS* in the tumor sample is mandatory for all CRC patients who are candidates for anti-EGFR targeted therapy. In our hands, the prevalence of codon 12/13 mutations in Thai patients was 35.8% (139/388 cases).

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Additional mutation in *KRAS* codon 61 is also responsible for constitutive activation in the oncogenic rat sarcoma small GTPase (RAS)–serine/threonine-specific protein kinase (RAF)–mitogen-activated protein kinase (MAPK) cascade [5, 6], and the prevalence of mutations is low (2.1% to 5.1%) compared with codons 12/13 [1-3, 7, 8]. Despite this rarity, *KRAS* codon 61 mutation could also predict resistance to EGFR monoclonal antibody [7]. Because only the *KRAS* codon 12/13 mutations have been assessed in most of the laboratories in Thailand, the prevalence of mutation in codon 61 is unknown in our population. Here, we developed an in-house pyrosequencing protocol to screen for mutation in *KRAS* codon 61 in Thai patients with advanced CRC.

Materials and methods

Samples collection and DNA extraction

This study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB No.442/55). All anonymized tumor samples were from Thai patients with advanced CRC who underwent a *KRAS* codon 12/13 mutation test at Chulalongkorn GenePRO Center, Research Affairs, Chulalongkorn University, and were not found to carry a *KRAS* codon 12/13 mutation. Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue using a QIAmp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA). As detailed below, *KRAS* codon 61 mutation was screened using in-house pyrosequencing, and positive samples were subsequently confirmed using a commercial *KRAS* test kit.

PCR amplification

PCR amplification primers for pyrosequencing were as follows: KRAS61-F-Bio, a forward biotinylated primer, 5′-CTCTTGGATATTCTCGACACAGCAG-3′ and KRAS61-R, a reverse primer, 5′-AATGATTTAGTATTATTTATGGCAA-3′. Each PCR mixture had 60 µL of volume containing 1× Gold buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM primers, 1.5 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, Life technologies, Foster City, CA, USA) and 40 ng/µl of genomic DNA. The PCR products were amplified using initial denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 30 s, 56°C for 1 min, and 72°C for 35 s and then final extension at 72°C for 7 min. Five microliters of PCR product were visualized using 8% polyacrylamide gel electrophoresis stained with SYBR DNA gel stain (Invitrogen Life technologies, Carlsbad, CA, USA) to detect the 116 base-pair band PCR product.

Pyrosequencing

The PCR product was bound to streptavidin-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, UK) then transferred to 70% ethanol, PyroMark denaturation solution (Qiagen), PyroMark wash buffer (Qiagen) and high-purity water respectively. The single-stranded PCR products were then annealed to 0.3 µM pyrosequencing primers and pyrosequencing was performed using PyroMark Q96 ID (Qiagen) following the manufacturer's instructions. The PCR products were sequenced in the reverse direction using two pyrosequencing primers as follows: KRAS61-Pyseq1, 5′-CTGGTCCCTCATTGCACT GTACTC-3′ and KRAS61-Pyseq2, 5′ GTCCCTC ATTGCACTGTACTCCTCT-3′. The nucleotide dispensation orders and sequence to analyze were 5′-GCTCGATCGA-3′ and 5′-CTC[T/G/A]TGACCTG ATGT-3′ for KRAS61-Pyseq1 and 5′-TACGTACT GC-3′ and 5′-[T/A/C/G]GACCTGCTGTG-3′ for KRAS61-Pyseq2 respectively (**Figure 1**). PyroMark Q96 software was used to generate histograms, and the sequence analysis was performed in the AQ (allele quantification) analysis mode. Samples with a mutant allele greater than 10% relative to the wild-type allele were considered as mutations detected in codon 61 of *KRAS*.

Figure 1. PCR amplification of exon 3 (codon 61) in *KRAS*. Codon 61 (CAA, nucleotides 181–183), which resides in exon 3 of *KRAS* (Transcript ID: ENST00000311936; Ensembl database) is shown in the square box. Mutations in nucleotides 182 and 183 (yellow highlighted nucleotide) reportedly result in amino acid changes including c.182A>T (p.Q61L), c.182A>G (p.Q61R), c.182A>C (p.Q61P), c.183A>C (p.Q61H), and c.183A>T (p.Q61H) in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database, version 63 release. Nucleotides 182 and 183 in codon 61 were sequenced in the reverse direction using KRAS61-PySeq1 and KRAS61-PySeq2 primers. **A:** KRAS61-Pyseq1 (5′-CTGGTCCCTCATTGCACTGTACTC-3′) and **B:** KRAS61-Pyseq2 (5′-GTCCCTCATTGCACTGTACTCCTCT-3′) primers complementary to red underlined nucleotides were used for pyrosequencing.

cobas KRAS Mutation Test

Samples with positive for a *KRAS* codon 61 mutation by the pyrosequencing were confirmed by a cobas *KRAS* Mutation Test according to the manufacturer's instructions (CE-IVD, Roche Diagnostics, Pleasanton, CA, USA).

Calculation of KRAS codon 61 mutation prevalence

The overall prevalence of codon 61 mutation (calculated from all 115 recent CRC cases tested in our laboratory) and percentage of codon 61 mutation in wild-type codon 12/13 (n = 74) were determined.

Results

Our developed in-house pyrosequencing could discriminate genotypes among wild-type and 5 mutation subtypes in *KRAS* codon 61, including c.182A>T (p.Q61L), c.182A>G (p.Q61R), c.182A>C (p.Q61P), c.183A>C (p.Q61H), and c.183A>T (p.Q61H) (**Figures 1 and 2**). Of the 74 most recent cases in our laboratory with wild-type codon 12/13,

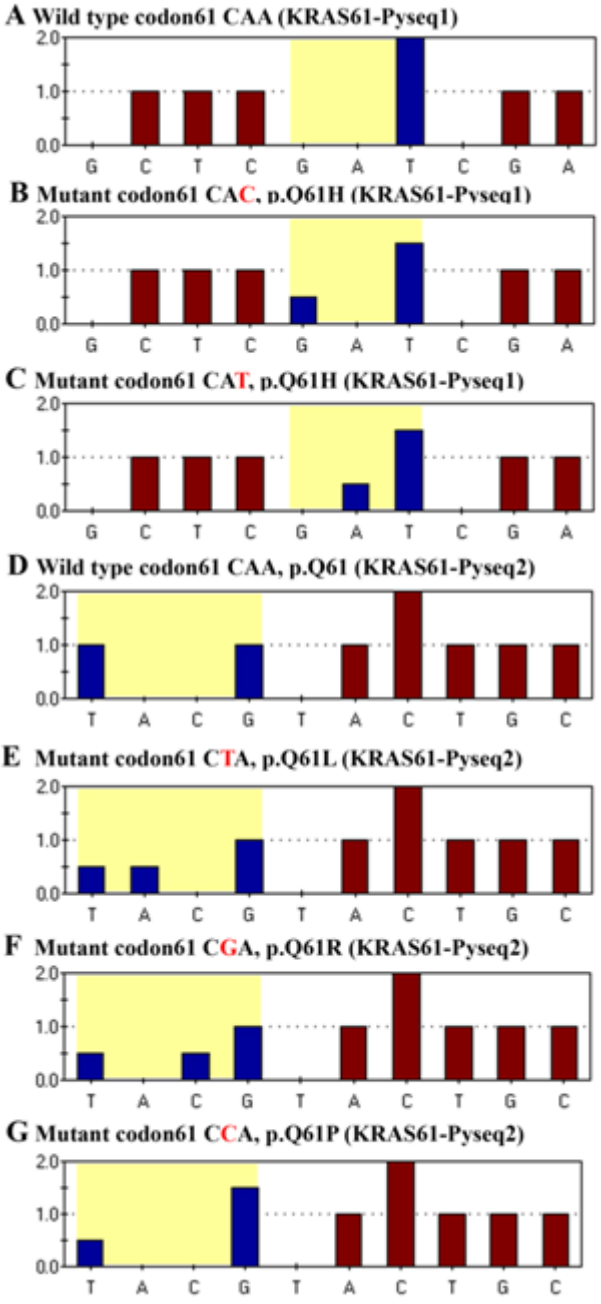

Figure 2. Histograms for *KRAS* codon61 wild-type and mutant discriminations. Histograms (**A–G**) were generated by PyroMark Q96 software using dispensation order and sequence to analyze as follows; (**A–C**) 5′-GCTCGATC GA-3′ and 5′-CTC[T/G/A]TGACCTGATGT-3′, (**D–G**) 5′-TACGTACTGC-3′ and 5′-[T/A/C/G]GACCTGCTG TG-3′, respectively.

two samples were found to harbor mutations in *KRAS* codon 61, one with heterozygous c.182A>T (p.Q61L) and the other with c.183A>T (p.Q61H) (**Figure 3**). The DNA samples with detectable mutation were independent reanalyzed using the pyrosequencing three times, and interassay validated with a cobas *KRAS* Mutation Test (CE-IVD, Roche). Mutations in the two samples were reproducibly detected by the additional pyrosequencing, and by the cobas *KRAS* Mutation Test, which also reported codon 61 mutation in both samples. The overall prevalence of codon 61 mutation in Thai patients with advanced CRC was 1.7% (2 from 115), and 2.7% (2 from 74) of samples with wild-type codon 12/13.

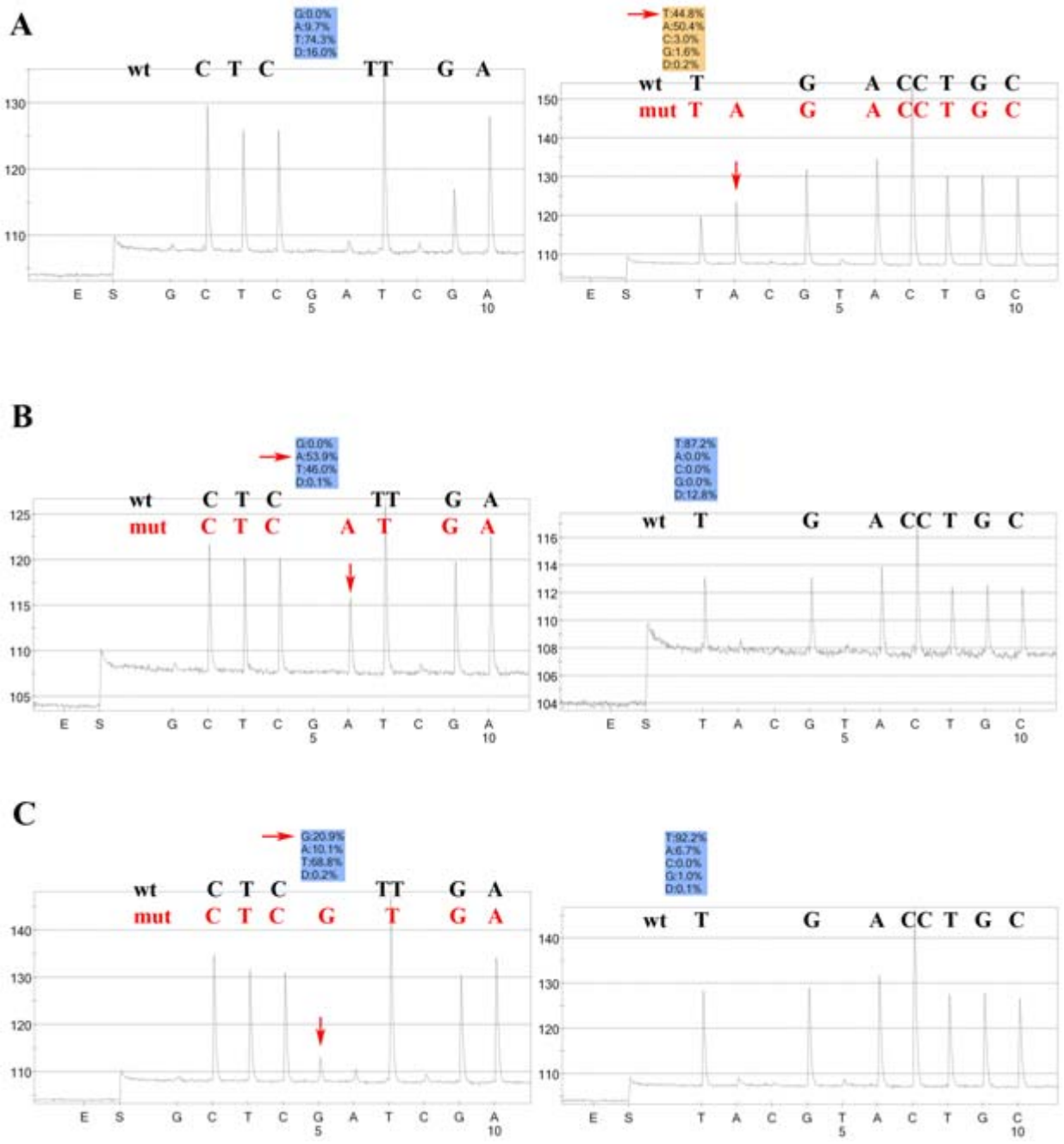

Figure 3. Pyrograms showing mutations in *KRAS* codon 61. (**A**) Heterozygous c.182A>T (p.Q61L); (**B**) heterozygous c.183A>T (p.Q61H) and (**C**) heterozygous c.183A>C (p.Q61H) in samples from Thai patients with advanced colorectal cancer. The red arrows indicate mutant peak and the percentage of mutant allele in the amplified PCR products. wt: wild type; mut: mutant

Discussion

EGFR monoclonal antibody has recently been used in combination with chemotherapy in the treatment of advanced colorectal cancer (CRC). The anti-EGFR can be used as a first-line treatment for patients whose tumors have wild-type *KRAS* [9, 10] or as a second-line for patients who are refractory to chemotherapy [11]. In addition, it can be used solely as a third-line treatment for patients who have failed or been intolerant to chemotherapy [12].

Despite the recommendation for anti-EGFR targeted therapy for CRC patients with wild-type *KRAS*, approximately 40% to 60% of the patients with wild-type KRAS do not respond to the treatment [13]. This might be the result of somatic mutation in other genes in the RAS–RAF–MAPKs pathway such as the serine/threonine-protein kinase *B-Raf* gene (*BRAF*) (V600E) [7, 13-15] and phosphatidylinositol-4,5-bisphosphate-3-kinase, catalytic subunit α gene (*PIK3CA*) (exons 9 and 20) [16-18], and loss of phosphatase and tensin homolog gene (*PTEN*) expression [15, 17, 18]. However, before we proceed to examine other genes, we opted to screen for mutation in codon 61 of the *KRAS* gene in our patients with advanced CRC whose tumor did not carry mutation in *KRAS* codon 12/13. The overall prevalence of *KRAS* codon 61 mutation in our patients (1.7%) and 2.7% of cases with wild-type codon 12/13 are within the ranges of published studies [1-3, 7, 8].

Despite the low frequency of *KRAS* codon 61 mutation in our population, testing of the codon 61 is important not only because of the EGFR therapy benefits, but also the cost saving of the very expensive treatment in cases carrying mutation in codon 61. Currently, the cost of anti-EGFR is about 5,900 USD (190,000 Thai baht) per month for a single patient and it needs to be administered for at least 6 months or until disease progression, while the charge for complete *KRAS* mutation testing (codon 12,13, 61) is only 280 USD (9,000 baht) in our laboratory.

For a technical standpoint, somatic mutation screening in *KRAS* can be performed by various techniques, namely PCR-restriction fragment length polymorphism (RFLP) [19], direct sequencing [20], high-resolution melting (HRM) analysis [21], and allele specific real-time PCR [22, 23]. Although direct sequencing has long been considered a criterion standard for comparison with other diagnostic methods, it suffers lower sensitivity compared with the other methods including pyrosequencing [24, 25]. HRM analysis is highly sensitive [21], but requires certain mutant positive controls for the setup. A robust and rapid technique for fragmented DNA particularly that extracted from FFPE samples is allele specific real-time PCR, because the small PCR product was amplified and screened for the mutation [22, 23]. In addition, pyrosequencing is an alternative technique suitable for small PCR amplicon, with intermediate sensitivity between direct sequencing and allele specific PCR/HRM. While pyrosequencing is capable of quantifying wild-type or mutant alleles using reference peaks as an internal control for each sample, allele specific real-time PCR requires mutant controls to indicate type and quantity of the mutants. Also, pyrosequencing can detect subtypes of mutation by designed histograms (examples shown in **Figure 2**). In the present study, the histograms in pyrosequencing were designed to detect 5 mutation subtypes, including c.182A>T (p.Q61L), c.182A>G (p.Q61R), c.182A>C (p.Q61P), c.183A>C (p.Q61H), and c.183A>T (p.Q61H), which cover 91 percent (159 from 174) of the *KRAS* codon 61 mutation in the samples with CRC reported in Catalogue Of Somatic Mutations In Cancer (COSMIC) database, version 63 release. Other subtypes of *KRAS* codon 61 mutation in CRC including c.181C>A (p.Q61K), c.181C>G (p.Q61E), c.180_181TC>AA (p.Q61K), and other complex mutations should be screened with direct sequencing because of the cost of pyrosequencing setup for the additional rare mutations.

Compared with the commercial cobas *KRAS* Mutation Test (CE-IVD), our in-house pyrosequencing technique requires a comparable amount of DNA input. While the commercial test is automated, easier to perform, and provides a more rapid turn-around time; it provides only the mutation site whether it is detected in codon 12/13 or codon 61. By contrast, our pyrosequencing method can specify the specific mutation subtype. Some studies indicate that patients with tumors with a G13D mutation may benefit from anti-EGFR treatment and these patients appear to have a longer overall survival and progression free survival than those with other mutation patterns in codon12/13, but the relative treatment effects were similar for *KRAS* G13D mutant and wild-type tumors [2, 26]. It is our current practice to screen the KRAS gene mutation first with a cobas *KRAS* Mutation Test. The in-house pyrosequencing is performed when mutation is detected and the clinician makes a specific request for the mutation subtype (e.g. G13D) or when the cobas test shows an invalid result.

Conclusion

We reported the low prevalence of *KRAS* codon 61 mutation in the Thai patients with advanced CRC. Our pyrosequencing method is suitable for routine pathological samples. Because of the costly anti-EGFR treatment, extended *KRAS* screening for mutation in codon 61 deserves consideration. Future testing for other relevant genes e.g. *BRAF*, *PIK3CA*, and *PTEN* should also be considered.

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None of the authors has any conflict of interest to declare.

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