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Detection of KRAS mutation at codon-12 and codon-13 associate with poor prognosis in colorectal cancer patients

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KEYWORDS

Codon-12 mutation;
Codon-13 mutation;
Colorectal cancer;
KRAS mutations;
Prognosis outcomes.

ABSTRACT

Colorectal cancer (CRC) associated with KRAS mutations relevance to clinical outcome of progression especially for codon mutation remain unclear, clinically. Thus this study aimed to detect KRAS mutations on specific codon-12 and codon-13. DNA extraction was performed from 40 formalin-fixed paraffin-embedded (FFPE) tissues. The KRAS codon-12 and codon-13 mutations were detected by quantitative multiplex real-time PCR consisting of seven primers of G12A, G12D, G12R, G12C, G12S, G12V, and G13D, specifically. Our results showed mutation of codon-12 at 37.5% (15/40 cases), especially for 86.7% (13/15 cases) were found G12D, G12V and G12R mutation for 40%, 40% and 6.67%, respectively. On the other hand, the codon-13 mutation found only G13D at 20% (3/15 cases). The present findings showed a statistically significant difference between KRAS mutations with lymph node metastases (p-value = 0.004), and mucosal lymphoma with other tumor types (p-value = 0.016). We noticed that KRAS mutations are associated with codon-12 and codon-13 and relevanced to prognostic outcomes of CRC patients on lymph node metastases as well as histological types. However, increased samples, novel prognostic biomarkers and more specific clinical information could be further explored to the clinical molecular biomarkers for monitoring and treatments.

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Introduction

Nearly 2 million cases of colorectal cancer (CRC) were diagnosed in 2020, making it the third most prevalent cancer form globally. Approximately 1 million people die from it each year, making it the second most frequent cause of cancer death including Thailand[1]. Over 10,000 new CRC cases occur annually, and about 40% are rectal cancer. The International Agency for Research on Cancer (IARC) estimates that the global burden of colorectal cancer will increase by 56% between 2020 and 2040, to more than 3 million new cases per year. For several reasons, CRC continues to be a significant healthcare burden in Thailand. It is the only malignancy with an increased incidence in both sexes in Thailand. Due to the lack of CRC screening and public awareness, non-metastatic cancer accounts only for 60%–70% of overall cases. The demand for general or colorectal surgeons outmatches the supply at a ratio of 1 general surgeon to 35,000 individuals. There are about 70 board-certified colorectal surgeons serving Thailand's population of nearly 70 million. As a result, more than 25% of cancer patients wait more than a month before surgery[2]. Although surgery is the main treatment modality for early CRC, adjuvant treatment is usually given to patients with advanced disease. Therapies with epidermal growth factor receptor (EGFR) inhibitors have been shown to be effective treatments in a subset of patients with metastatic colorectal cancer. Two anti-EGFR biologics, cetuximab and panitumumab, have been approved by the Food and Drug Administration for the treatment of refractory metastatic colorectal cancer.

The oncogene family RAS consists of three members (KRAS, HRAS and NRAS) that play important roles in human cancers. All RAS genes encode 21-kDa monomeric GTPases that relay extracellular signals to intracellular signal transduction cascades. The on/off state of rat sarcoma proteins (RAS) is determined by nucleotide binding[3], with the GTP-bound form exhibiting active signaling conformation. Missense mutations in RAS proteins alter the homeostatic balance of GDP and GTP binding toward the active state by either reducing GTP hydrolysis or increasing GTP loading rate[3]. Approximately, 40% of metastatic colorectal cancers (mCRC) have KRAS mutations, typically in exon 2 and codons 12 (nearly 80% of all KRAS mutations) and 13, and less frequently in exon 3 (codons 59 and 61) and 4 (codons 117 and 146). For decades, the different KRAS mutations were considered equivalent in terms of lack of anti-EGFR response and a negative prognostic factor[4]. It is known that most mutations in RAS are missense mutations in three hotspot residues, G12, G13, and Q61. The order of frequency of G12 is G12D, G12V, G12C, G12A, G12S, and G12R, with the G12C mutation most common in lung cancer and the G12D most common in PDAC[5]. In fact, KRAS G12D (KRAS G12D) is one of the most important therapeutic targets in tumors. Up to 40% of patients with KRAS wild-type CRC show at least a partial response to anti-EGFR therapy, an improvement over the 10% prior to KRAS mutation stratification. Whereas patients with KRAS mutated tumors do not benefit from anti-EGFR therapy. Thus, the advantage of determining KRAS mutation status is to identify those who will not respond to EGFR monoclonal antibody therapy[6]. Therefore, detection of KRAS mutation on the specific codon is an essential investigation for the further molecular biomarker's association, clinically.

Materials and methods

Patients and tumor samples

A total of 40 CRC patients from the lower part of the north-eastern Thai population were collected from January 2019 to June 2020. They were assembled with the approval from the hospital ethics committee (EC003/2021). The clinical information, pathological diagnosis, and formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from the CRC patient's registration.

DNA extraction

Genomic DNA was isolated from ti ssue samples using the PureLink® Genomic DNA kits following the manufacturers' instructions. All FFPE tissue sections were evaluated for tumor cell content more than 20% using hematoxylin and eosin (H&E) staining by pathologists. A total of 5-8

sections were taken from each sample followed by a thickness of each section was 10 µm. The FFPE tissue sections were placed into a 1.5 microcentrifuge tube and deparaffinized with xylene. Each sample was incubated with proteinase K at 56°C overnight until completed digestion. The lysate was subsequently incubated at 80°C for 4 hours to reverse formaldehyde crosslinks. DNA was precipitated with ethanol, fixed on the membrane by centrifugation, washed and eluted by using the elution buffer solution. The DNA concentration and purity were determined by spectrophotometer (Thermo Fisher Scientific) at 230, 260 and 280 nm using Nanodrop 2000, subsequently.

Detections of KRAS mutations

The KRAS gene mutations were detected using Therascreen RGQ PCR KRAS Kit (Qiagen, Manchester, UK). KRAS mutations in codons-12 and codon-13 were identified using a principle of multiplex real-time PCR that combines an amplification refractory mutation system. Scorpion fluorescent primers and probes system were used as directed by the manufacturer 12 GCT (Ala), 12 GAT (Asp), 12 CGT (Arg), 12 TGT (Cys), 12 AGT (Ser), 12 GTT (Val) and 13 GAC (Asp). The seven primer-specific reactions with one control reaction were produced in each sample. DNA template was used at concentration of 20-25 ng. The PCR profile consisted of an initial hold phase at 95 °C for 4 min, followed by 40 cycles of two-step amplification with denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min. The DNA samples with Ct-value between 23.5 and 29.5 were considered valid and suitable for subsequent KRAS analysis. The PCR reaction and data analysis were performed on the QMDx System (Qiagen, Hilden, Germany) and Rotor-Gene Q Software, version 2.3.1.49 (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Statistical analysis

All statistical analysis was calculated in SPSS v.25 (IBM Corp., Armonk, NY, USA). The relationships between clinicopathological variables and the presence of KRAS mutation were determined using Chi-square test. The significance difference was considered at *p*-value less than 0.05, statistically.

Results

Determine KRAS mutation detection by multiplex real-time PCR

The results showed 37.5% (15/40 cases) with KRAS mutations including of 86.7% (13/15 cases) with codon-12 mutations (p.G12D, p.G12V, and p.G12R was 40%, 40%, and 6.67%, respectively), and 20% (3/15) with codon 13 mutations (p.G13D was 5.08%). The most common mutation in codon 12 was glycine to aspartate (GGT to GAT) and glycine to valine (GGT to GTT), which was detected in 6 of 15 cases (40%), whereas the other mutation observed in codon 12 mutations resulting in a replacement of glycine by arginine (GGT to CGT; 1 case, 6.677%). On the other side, the most common mutation in codon 13 was glycine to aspartate (GGC to GAC) which occurred in 3 of 15 cases (20%), whereas the other mutation was glycine to cysteine (GGC to TGC).

Correlation between KRAS mutation and clinicopathological parameters

Our results showed a statistically significant difference between KRAS mutations and lymph node metastasis *p*-value = 0.004. Our results also found a statistically significant difference between KRAS mutations with mucinous adenocarcinoma and other tumor types *p*-value = 0.016. However, our findings did not show a significant difference between KRAS mutations and other pathological factors such as gender, age, clinical tumor markers, and degree of differentiation, as shown in table 1.

Table 1 Correlation between clinicopathological characteristics and KRAS mutation status (missense type) in 40 patients with CRC patients

Patient's information	n	KRAS genotypes (n)		*p*-value
		KRAS Mutation	KRAS Wild type	
Total CRC patients	40	15	25	
Sex				
Male	21	9	12	0.462
Female	19	6	13	
Age				
< 70 years	13	5	8	0.960
>70 years	27	10	17	
Primary tumor				
Right CRC	9	4	5	0.654
Left CRC	26	10	16	
Rectum	5	1	4	
Grade of differentiation				
Poor	1	0	1	0.575
Moderate	13	5	7	
Well	26	8	17	
Metastasis at diagnosis				
Lymph node	27	14	13	0.004
Peritoneum	1	1	0	
none	12	0	12	
Histology				
Mucinous	3	1	2	0.016
None	37	14	23	

Note: *p*-value by using chi-square test. KRAS, kirsten rat sarcoma; CRC, colorectal cancer.

Discussion

CRC is the third most common cancer and the leading cause of cancer-related deaths worldwide [7]. In Thailand, the incidence of CRC has increased in recent years. In CRC patients, KRAS mutations are present in 45% of metastatic tumors and in nearly 15-37% of early-stage tumors [8,9]. Recently, KRAS gene mutation in CRC has been intensively studied to clarify whether it is the reason for the tumors' expansive growth. Several researchers have reported that the KRAS gene mutation plays an important role in the protrusive growth of CRC [10].

In previous study from Chinese population that represent KRAS mutation rate for codon 12 was 72.7%, of which G12D was the highest (47.5%) followed by G12V (30.6%), and the mutation rate for codon 13 was 22.0%, of which G13D was reported[11]. Like our study of KRAS mutations in codon-12 and codon-13 from the lower part of the north-eastern Thai population, we notice that a mutation in KRAS

codon-12 has much greater oncogenic potential than a mutation in codon-13. The CRC patients with KRAS codon-12 mutations are commonly present at G12V and G12D, while mutations in G12R are much less commonly found. Among the patients who carry on the KRAS codon-13 mutation, G13D is the most frequently observed mutation. From previous study of KRAS mutation detection on FFPE had been evaluated with a multiplex QPCR assay for the rapid detection of common KRAS mutations. Similar to our study that using multiplex real-time PCR has been shown to be more sensitive and cost effective than sanger sequencing in paraffin-embedded archival tissue [12]. It is a sensitive sequencing assay and can reliably detect mutant alleles at low frequencies (1% mutated) among wild-type alleles, which is often the case in solid tumors [12, 13].

To date, clinical information and biomarkers for drug responses are necessary for CRC managements. Treatments of mCRC with crucial prognostic biomarkers are one of the improvement and reduction risk of disease progression. Recently, Cetuximab and Panitumumab, as the highly effective antibodies targeting epidermal growth factor receptor (EGFR), have clinical activity in the mCRC patients' treatments[14-16]. So, we suggest that study of the molecular KRAS codon-12 and codon-13 mutations with chemotherapeutic drugs response for anti-EGFR could be further explored.

Conclusion

The association between KRAS codon-12 and codon-13 mutations and prognostic outcome of CRC patients should be used for the prognostic biomarkers, clinically. The clinical outcome of progression in the patients related to lymph node metastases as well as a mucinous colorectal adenocarcinoma histology type.

Take home messages

The mCRC patients including of both the sporadic and hereditary CRC patients with the specific clinical outcome of progression as well as the novel molecular biomarkers for diagnosis, prognosis, monitoring, and treatments could be further investigated, clinically.

Conflicts of interest

The authors declare no conflict of interest.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71(3): 209-49.
2. Lohsiriwat V, Chaisomboon N, Pattana-Arun J. Current colorectal cancer in Thailand. Ann Coloproctol 2020; 36(2): 78-82.
3. Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. Cell 2017; 170(1): 17-33.
4. Ciardiello D, Maiorano BA, Martinelli E. Targeting KRASG12C in colorectal cancer: the beginning of a new era. ESMO Open 2023; 8(1): 100745.
5. Plangger A, Rath B, Stickler S, Hochmair M, Lang C, Weigl L, et al. Cytotoxicity of combinations of the pan-KRAS SOS1 inhibitor BAY-293 against pancreatic cancer cell lines. Discover Oncology 2022; 13(1): 84.

6. Plesec TP, Hunt JL. KRAS mutation testing in colorectal cancer. Adv Anat Pathol 2009; 16(4): 196-203.
7. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin 2020; 70(3): 145-64.
8. Vaughn CP, ZoBell SD, Furtado LV, Baker CL, Samowitz WS. Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. Genes Chromosomes Cancer 2011; 50(5): 307-12.
9. Pang XL, Li QX, Ma ZP, Shi Y, Ma YQ, Li XX, Cui WL, Zhang W. Association between clinicopathological features and survival in patients with primary and paired metastatic colorectal cancer and KRAS mutation. Onco Targets Ther. 2017 May 19;10:2645-54.
10. Margonis GA, Kim Y, Spolverato G, Ejaz A, Gupta R, Cosgrove D, et al. Association between specific mutations in KRAS codon 12 and colorectal liver metastasis. JAMA Surgery 2015; 150(8): 722-9.
11. Li W, Liu Y, Cai S, Yang C, Lin Z, Zhou L, et al. Not all mutations of KRAS predict poor prognosis in patients with colorectal cancer. Int J Clin Exp Pathol 2019;12(3): 957-67.
12. Wolf WL, Ye F, Tran V, Yang Z, White R, Bloom K, et al. Sensitive multiplex detection of KRAS codons 12 and 13 mutations in paraffin-embedded tissue specimens. J Clin Pathol. 2011; 64(1): 30-6.
13. Tol J, Dijkstra JR, Vink-Börger ME, Nagtegaal ID, Punt CJ, Van Krieken JH, et al. High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. J Cell Mol Med 2010; 14(8): 2122-31.
14. Heinemann V, Stintzing S, Kirchner T, Boeck S, Jung A. Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. Cancer Treatment Reviews 2009; 35(3): 262-71.
15. Jones RP, Sutton PA, Evans JP, Clifford R, McAvoy A, Lewis J, et al. Specific mutations in KRAS codon 12 are associated with worse overall survival in patients with advanced and recurrent colorectal cancer. Br J Cancer 2017; 116(7): 923-9.
16. Passot G, Denbo JW, Yamashita S, Kopetz SE, Chun YS, Maru D, et al. Is hepatectomy justified for patients with mutant colorectal liver metastases? An analysis of 524 patients undergoing curative liver resection. Surgery 2017; 161(2): 332-40.