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ORIGINAL RESEARCH

Clinicopathological Associations of *K-RAS* and *N-RAS* Mutations in Indonesian Colorectal Cancer Cohort

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Abstract
Background *K-RAS* and recently *N-RAS* gene mutation testing are mandatory requirements prior to anti-epidermal growth factor receptor (EGFR) monoclonal antibody treatment of metastatic CRC. Mutation prevalence and distribution in Indonesian colorectal cancer (CRC) are not known.
Methods Combined methods of PCR high-resolution melt (HRM), restriction fragment length polymorphism (RFLP), and direct DNA sequencing were used to genotype exons 2, 3, and 4 of both *K-RAS* and *N-RAS* genes for routine clinical testing of CRC patients. Descriptive analytical review of 595 consecutive CRC patients (years 2013 to 2016) was performed to find associations between gene mutations and clinicopathologic features.
Results This retrospective study revealed overall *K-RAS* gene mutation in exon 2 (codon 12 and 13) rates being 34.9%. Women (42.5%), stages I and II (43.4%), and well and moderate differentiations (37.7%) had higher frequency of *K-RAS* exon 2 mutations than men (29%, $p = 0.006$), stages (III and IV 31.9%, $p = 0.05$), and poor differentiation (11.8%, $p = 0.002$), respectively. At later period (2015–2016), 121 of 595 patients were genotyped for the remaining exons 3 and 4 of *K-RAS* as well as exons 2, 3, and 4 of *N-RAS* mutations resulting in overall *RAS* mutation prevalence of 41%. Mucinous histology had highest frequency of *N-RAS* mutation.
Conclusions Combination of PCR HRM with either RFLP or direct DNA sequencing was useful to detect *K-RAS* exon 2 and extended RAS mutations, respectively. Frequency of all RAS mutations in stage IV Indonesian (41%) was similar among Asians (41–49%), which tend to be lower than western (55%) CRC.

Keywords *K-RAS* · *N-RAS* · Colorectal cancer mutation · Indonesia · High-resolution melting · Asian

Introduction

Colorectal cancer (CRC) is one of the most common and one of the leading causes of cancer-related death, especially in developing countries. Mean incidence rate in southeast Asian countries has been estimated to be 6.5 in 100,000 that is lower than developed nation such as UK [1]. The rising CRC incidence rates in Asia, especially among the young age group, have also raised concern [2].

CRC is among the most frequent malignancy in Indonesia with estimated age standardized rates per 100,000 of 19 in men and 15 in women [3]. Hospital-based data in Indonesia suggests that 35.2% of CRC patients were under 40 years old [4]. The underlying molecular mechanisms in these young patients are not entirely clear. Expression patterns of mismatch repair proteins (MLH1 and MSH2) were similar in native young and old Indonesian CRC patients [4].

Introduction of anti epidermal growth factor receptor (EGFR)-targeted therapy (e.g., cetuximab and panitumumab) has improved survival of metastatic CRC in patients with normal or wild-type alleles of both *K-RAS* and *N-RAS* genes

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[5]. *K-RAS* and *N-RAS* mutation testing has been endorsed by Asian clinical guidelines [6]. Beginning in 2011, Indonesian authority has mandated national health insurance agency to reimburse the cost of anti-EGFR therapy in metastatic CRC patients who have wild-type *K-RAS* gene.

Polymerase chain reaction-high resolution melt (PCR-HRM) method has been used to genotype *K-RAS* and *N-RAS* genes [7–9]. In this study, we used similar approaches and demonstrated utility HRM melt curve analyses in combination with restriction fragment length of polymorphism (RFLP) to genotype common mutations of *K-RAS* exon 2 (codons 12 and 13). In addition, PCR HRM melt curve and direct DNA sequencing had been used to genotype exons 3 and 4 of *K-RAS* and exons 2, 3, and 4 of *N-RAS* genes as routine methods of molecular testing serving patients living in major Indonesian cities. Our study also demonstrated association of *K-RAS* and *N-RAS* gene mutations with several clinicopathologic features of patients. These associations may fill in gaps of baseline information and understanding of underlying molecular pathways of Indonesian CRC.

Material and Methods

Study Population and Cell Lines

Records of test request forms and genotyping results were retrieved and reviewed of all colorectal cancer patients (January 2013 to March 2016) referred to Kalbe Genomics Laboratory for *K-RAS* mutations in codons 12 and 13. This central laboratory is accredited for ISO15189 and registered in the European Society of Pathology *K-RAS* external quality control scheme (http://*K-RAS*.eqascheme.org). Patients who had failed test results were excluded. Average failure rate was 10% mostly due to insufficient tumor contents and poor DNA quality causing PCR failure. Average turn around time was seven workdays.

Successful *K-RAS* mutations in codons 12 and 13 test results of 395 patients (during the years 2013–2016) were included in this study as summarized in Table 1. In the later period of March 2015 to 2016, there were 121 of 395 colorectal cancer patients who had been tested for extended *RAS* encompassing both *K-RAS* and *N-RAS* gene mutations in all exons 2, 3, and 4 paying particular attentions to codons 12, 13, 61, 117, and 146. This study was determined to be exempt by the Institutional Review Board and performed in accordance to 1964 Helsinki Declaration and its later amendments. This retrospective study was approved by Institutional Review Board (IRB) of Stem-cell and Cancer Institute (SCI). Because of the retrospective nature of this study, informed consent was waived. Patient identities had been anonymized. Cell lines were obtained as gifts from Genomic Institute of Singapore (GIS) BT549 (*K-RAS* gene wild type), A549 (*K-RAS* gene G12S homozygous), and HCT116 (*K-RAS* gene G13D heterozygous).

Table 1 Patient demography (years 2013–2016; $N = 395$)

Indonesian cities/islands		Number	(%)
	Greater Jakarta (Jawa)	180	46%
	Surabaya, Bali, Malang (East Jawa)	99	25%
	Jogjakarta, Solo, Semarang (Central Jawa)	51	13%
	Bandung, Tasikmalaya (West Jawa)	32	8%
	Lampung, Medan, Padang, Palembang (Sumatera)	30	8%
	Others (Kalimatan)	3	1%
Age (years old)			
	Median	55	
	Mean	52.6	
	Range	17–78	
Gender			
	Male	221	56%
	Female	174	44%
Primary tumor sites			
	Colon	220	56%
	Rectum	129	33%
	Colorectal	46	12%
Histology			
	Adenocarcinoma	327	83%
	Mucinous	52	13%
	Signet	13	3%
	Not specified	3	1%
Grades/differentiation			
	Well and moderate	268	68%
	Poor	34	9%
	Not specified	93	24%
TNM stages			
	I and II	122	31%
	III	135	34%
	IV	127	32%
	Not specified	11	3%

Molecular Profiling

Tumor section from FFPE tissue samples were stained with hematoxylin-eosin (HE) and reviewed by two experienced pathologists. Clinicopathological information was obtained by reviewing the request forms to extract the following clinicopathology information such as age, gender, tumor sites, histological types, differentiation grades, and AJCC TNM stages. Right-sided tumors were defined as specimens originated in cecum, ascending colon to transverse colon, and left-sided tumors in splenic flexure, descending colon to rectosigmoid junction.

DNA Extraction

To obtain maximal tumor DNA, senior pathologists chose and marked areas of tumor components that were greater than 30%. DNA was then extracted from these tumor-enriched areas using the Qiagen QIAamp DNA Micro Kit (cat no. 56304, Qiagen, Germany) following the manufacturer's protocol.

High-Resolution Melting-Polymerase Chain Reaction

Polymerase chain reaction-high resolution melting (HRM) protocol was adopted from previous publication [10] with some modifications to screen for mutations in exon 2 (codons 12 and 13) of *K-RAS* gene. Commercial *RAS SplitSCAN* (KalgenDNA, Jakarta) HRM primers were used to screen hotspot mutation of exons 3 and 4 of *K-RAS* and exons 2, 3, and 4 of *N-RAS* genes. Samples were tested in duplicate using Rotor-Gene 6000™ (Corbett Life Science, Australia) or Rotor-gene Q (Qiagen, Germany). Melting curve was generated and scan for the presence of "split peaks" indicating mutations (Fig. 1). Controls containing DNA of *K-RAS* wild type (BT549) and *K-RAS* mutant (HCT116) were always included for melt curve comparisons. Samples showing split peak patterns similar to HCT116 were then genotyped using PCR-RFLP and/or direct DNA sequencing. Gel electrophoresis was performed on 2.5% (*w*/*v*) agarose to confirm the presence of single band of PCR amplicon.

PCR-RFLP Analysis

Polymerase chain reaction-restriction fragment length polymorphism (RFLP) to detect mutations in codons 12 and 13 of *K-RAS* gene was conducted as described [11].

Sanger DNA Sequencing

Sequencing primers were adopted from previous publication [12]. PC amplification products were purified using the HighPure PCR Product Purification Kit (Roche), according

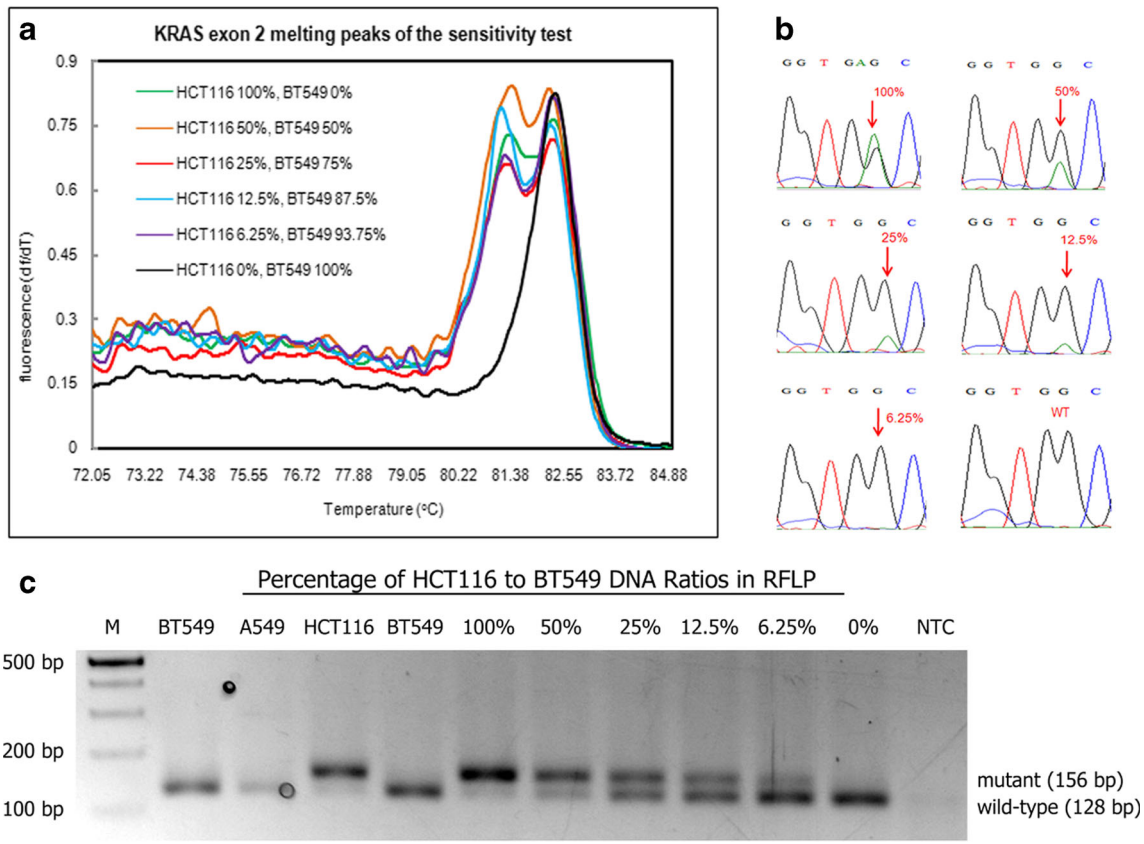

Fig. 1 Analytical sensitivity of high-resolution melt, direct DNA sequencing, and restriction fragment length polymorphism (RFLP) to detect mutations in *KRAS* exon 2 codon 12 and 13. HCT116 cell line harboring heterozygous *KRAS* mutant in codon 13 and BT549 cell line carrying normal *KRAS* gene was used as wild-type control. Serial dilutions were performed in order to obtain a mutant to wild-type allele ratio of 100, 50, 25, 12.5, 6.25, and 0%. **a** Melt curve graph. Single peak representing *KRAS* wild-type allele was shown in *black line*. Split peaks were shown in different colors representing different ratios of *KRAS* mutant to wild-type alleles. **b** Direct sequencing. *Red arrows* pointed to position of mutation *KRAS* allele due to single nucleotide substitution. **c** RFLP analyses. Digestion of PCR products by restriction enzyme led to either presence or absence of 156 and 128 base pairs (bp) representing mutated and wild-type *KRAS* alleles

to the manufacturer's protocol. Sequencing analysis was performed on an Applied Biosystem 3500 Genetic Analyzer.

Analytical Sensitivity Test

K-RAS gene mutant control DNA (extracted from both A549 and HCT116 cell lines) were mixed with wild-type DNA (extracted from BT549 cell line) and serially diluted as 50, 25, 12.5, and 6.25% mutant to wild-type DNA ratios. These mixtures were used for sensitivity detection of *K-RAS* gene mutant by PCR-HRM split peak, PCR-RFLP analysis, and Sanger sequencing.

Statistical Analysis

The data were processed using www.graphpad.com (GraphPad software, Inc., La Jolla, CA, USA). Individual information and baseline characteristics were summarized using descriptive statistics. The chi-square (χ^2) test or, where appropriate, the Fisher's exact test was used to compare the proportion of gene mutations among group with different clinicopathological parameters. Online calculator (http://vassarstats.net/kappa.html) was used to get kappa value and 95% CI as measure of diagnostic concordance between PCR HRM and RFLP and direct DNA sequencing methods.

Results

Analytical Sensitivity of PCR HRM

A simple melt curve of PCR amplicons of *K-RAS* gene showed different patterns for wild-type (single peak) and mutated allele (separated or split peak). As shown in Fig. 1a, black tracer line representing wild-type *K-RAS* from BT549 cell line was noticeably different from orange line (mutant codon 13 of *K-RAS* gene HCT116 cell line). Titration experiment (Fig. 1a) showed that split patterns of mutant alleles were detected as low as 6.25% HCT116. As expected, DNA sequencing ability to detect mutated *K-RAS* dropped off after 25% HCT116 (Fig. 1b) and completely failed to detect mutations at 6.25% mutant allele. Both PCR HRM and PCR RFLP (Fig. 1c) had shown better limit of detection than direct DNA sequencing.

To determine diagnostic sensitivity and specificity PCR HRM/RFLP, we used 147 archived specimens comprising of 92 *K-RAS* wild-type and 55 mutant cases that had been genotyped using direct DNA sequencing. PCR HRM/RFLP detected *K-RAS* mutations in 6 of 92 wild-type specimens and

Table 2 Association of KRAS mutation in exon 2 (codons 12 and 13) with clinicopathology characteristics

Clinicopathological features		Total *N* (%)	KRAS exon 2		
			Mutant	Wild type	*P* value
Overall		395 (100)	138 (34.9)	257 (65.1)	
Gender	Male	221(55.9)	64(29.0)	157(71.0)	0.006
	Female	174(44.1)	74(42.5)	100(57.5)	
Age (years old)	>median (56)	181(45.8)	69(38.1)	112(61.9)	0.288
	≤median	209(52.9)	68(32.5)	141(67.5)	
	Unspecified	5(1.3)	1(20.0)	4(80.0)	–
Tumor site	Colon right	91(23.0)	36(39.6)	55(60.4)	0.481
	Colon left	129(32.7)	42(32.6)	87(67.4)	
	Rectum	129(32.7)	42(32.6)	87(67.4)	
	Not specified	46(11.6)	18(39.1)	28(60.9)	–
Histological type	Adenocarcinoma	327(82.8)	116(35.5)	211(64.5)	0.328
	Mucinous carcinoma	52(13.2)	18(34.6)	34(65.4)	
	Signet cell	13(3.3)	2(15.4)	11(84.6)	
	Not specified	3(0.7)	29(66.7)	1(33.3)	–
Differentiation	Well and moderate	268 (67.8)	101(37.7)	167(62.3)	0.002
	Poor	34 (8.6)	4(11.8)	30(88.2)	
	Not specified	93 (23.5)	33(35.5)	60(64.5)	–
TNM stages	I and II	122 (30.9)	53(43.4)	69(56.6)	0.053
	III	135 (34.1)	43(31.9)	92(68.1)	
	IV	127 (32.1)	38(30.0)	89(70.0)	
	Not specified	11 (2.7)	4(36.4)	7(63.6)	–

misgenotyped 1 of 55 mutated cases. Therefore, diagnostic sensitivity and specificity was 98.9% (95%CI 93.8–99.8) and 90% (95% CI 79.9–95.3) and concordance rate estimated by Kappa value being 0.9 (95% CI = 0.8281 to 0.9721).

K-RAS Exon 2 Mutation Association with Clinicopathology Parameters

Combined PCR HRM and RFLP were used during routine genotyping of consecutive 395 colorectal cancer patients (year 2013–2015) for presence of mutations in *K-RAS* codons 12 and 13. As shown in Table 2, overall mutation rate of *K-RAS* mutations was 34.9%. High rate of *K-RAS* mutation in codons 12 and 13 was found in females (42.5% vs males 29%, $p = 0.006$), well and moderate differentiation (37.7% vs poor 11.8%, $p = 0.002$), and early stages I and II (43.4% vs stage III 31.9% vs stage IV 30%, $p = 0.05$). Frequency of *K-RAS* mutation was also higher in right colon (39.6%) than left colorectal (32.6%) although the difference rate did not reach statistical significance.

N-RAS Mutation Association with Clinicopathology Parameters

Beginning 2015, extended RAS genotyping comprised of *K-RAS* exons 3 and 4 and *N-RAS* exons 2, 3, and 4 were performed on 121 out of 395 consecutive colorectal cancer patients using PCR HRM and direct DNA sequencing. Examples of PCR HRM split peak tracers were represented in Fig. 2. As shown in Table 3, extended RAS mutations (in exons 3 and 4 of *K-RAS* mutations and exons 2, 3, and 4 of *N-RAS* mutations) contributed additional 10.7 to 30.6% of *K-RAS* mutation in exon 2 (codons 12 and 13), yielding an overall mutation rate of both *K-RAS* and *N-RAS* being 41.3% in 121 colorectal cancer patients. *N-RAS* mutation may be more frequent in patients with mucinous component (21.4%) than non-mucinous patients (3%, $p = 0.026$). There were no other clinicopathology parameters that held significant associations with *N-RAS* mutations.

Types of Mutations

One hundred twenty-one patients were genotyped using direct DNA sequencing for both K-RAS and N-RAS genes. Mutant-type *K-RAS* gene G12D (12.3%) was detected more frequently than G13D (4.9%) of *K-RAS* gene in exon 2. Mutation frequencies of K-RAS exons 3 (Q61H) and 4 (K117 N, A146T) were 0.8 and 5%, respectively. NRAS mutations in exons 2 (G12C, G12S), 3 (Q61R, Q61K), and 4 (A146T) each contributed 1.7%.

Discussions

We had validated PCR HRM coupled with RFLP and/or direct DNA sequencing to genotype *K-RAS* and *N-RAS* genes in consecutive Indonesian CRC patients. As previously observed, derivative melt graph of PCR HRM was the simpler method to scan and score mutation probability than were difference or normalized graphs [9]. We had observed and confirmed that specimens having mutant alleles showed split peaks when high-resolution melt data were analyzed using derivative melt graph. Split peaks were due to mixture of two populations (wild-type and mutant alleles) of PCR products having identical lengths (shown as single band in gel electrophoresis) but harboring single nucleotide variant due to substitutions or point mutations [9]. We also showed that PCR HRM analytical sensitivity to detect low-abundance mutant alleles (<10%) was better than direct DNA sequencing requiring 10–30% mutant alleles as summarized in recent meta analyses [13]. Moreover, HRM is also an attractive method for routine clinical testing because of high sensitivity and specificity, as well as rapid turn-around time by reducing burden of direct DNA sequencing [13]. In our experience, we saved up to 60% of incoming routine samples from genotyping *K-RAS* or *N-RAS*. Using these combination approaches (HRM, RFLP, and direct DNA sequencing), we were also able to gain several insights about associations of *K-RAS* and *N-RAS* with clinicopathology features of Indonesian colorectal cancer patients.

High prevalence of *K-RAS* mutation in women CRC patients of our population was also seen in Brazil in certain age group (40–60 years old) [14] and in Japan [15]. Tumors with well and moderate differentiation also harbored more frequent *K-RAS* mutations than tumors with poor differentiation as observed in past studies [8, 16], but not others [17, 18].

Using the HRM method, several laboratories in Europe and Japan report overall *K-RAS* mutation (mostly exon 2 and codons 12 and 13) between 41 and 45% [7–9, 19]. However, an earlier Japanese study using direct DNA sequencing involving more than 5000 patients shows overall prevalence *K-RAS* exon 2 mutations being 37% [15]. While genotyping analytical sensitivities do contribute to variation in KRAS prevalence, recent study using mass spectrometry-based genotyping method demonstrates consistent trend of low frequency of KRAS mutation (Asian, 39%; Black, 53%; White, 43%) [20]. *K-RAS* mutation frequency (36%) in our cohort seemed to be similar within Asian populations in spite of using techniques (HRM and RFLP) that were more sensitive than DNA sequencing.

Alternatively, variation *K-RAS* mutation rate in Asian vs western cohort may be explained partly by incidence of proximal and distal tumors. *K-RAS* mutations tend to occur in the right or proximal colon instead of the left or distal colon [21]. As observed previously [22], the left or distal colorectal is more common in Asian than in western population.

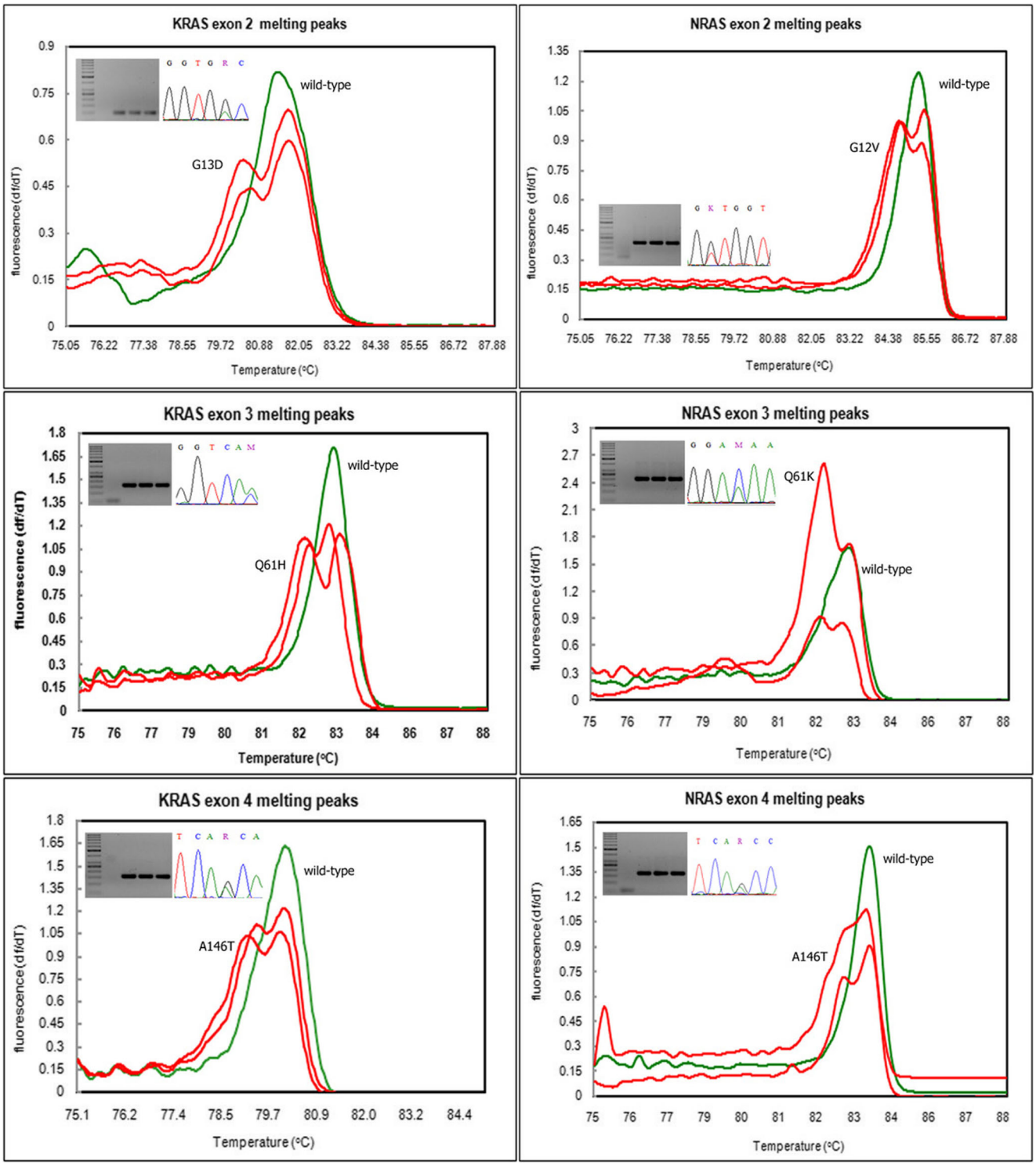

Fig. 2 Presence of split peaks in melt curve graphs to detect mutations in exons 2, 3, and 4 of *KRAS* and *NRAS* genes. Representative melt curve graphs of individual exons of RAS gene were shown together with sequencing traces (upper left corner) and gel electrophoresis of PCR products (lower right corner). *Arrows* pointed to positions of mutated nucleotides in sequencing traces. In the melt curve graphs, split peaks (*red lines*) and single peaks (*green lines*) indicated mutant alleles and normal/wild-type alleles, respectively. Gel electrophoresis images of PCR amplicons showed specific single band of PCR products

The low prevalence of *K-RAS* mutation in stage IV disease (30–33%) was even more apparent in this study and other Southeast Asian patients [18, 23] than in western patients (40–44%) [24]. It is not clear whether "westernized" dietary habits, environmental exposures, or inherent genetic susceptibility influence spectrum of gene mutation. A study in Japan

Table 3 RAS mutation prevalence ($N = 121$), years 2015–2016

Clinicopathological features		Total *N* (%)	All RAS			KRAS status exons 2, 3, 4			NRAS status exons 2, 3, 4		
			Mutation *N* (%)	Wild type *N* (%)	*P* value	Mutation *N* (%)	Wild type *N* (%)	*P* value	Mutation *N* (%)	Wild type *N* (%)	*P* value
Overall		121 (100%)	50 (41.3)	71(58.7)		44 (36)	79 (64)		6 (5)	115 (95)	
Gender	Male	64 (52.9)	24(37.5)	40 (62.5)	0.61	20 (31.3)	44 (68.7)	0.447	4 (6.3)	60 (93.7)	0.683
	Female	57 (47.1)	24 (42.1)	33 (57.9)		22 (38.6)	35 (61.4)		2 (3.5)	55 (96.5)	
Age (years)	>median (55)	54 (44.6)	19 (35.2)	35 (64.8)	0.36	17(31.5)	37(68.5)	0.567	2(3.7)	52(96.3)	0.691
	≤median	67(55.4)	29(43.2)	38 (56.8)		25(37.3)	42(62.7)		4(6.0)	63(94.0)	
Tumor sites	Colon right	31 (25.6)	14 (45.2)	17 (54.8)	0.9	12 (38.7)	19 (61.3)	0.714	2 (6.5)	29 (93.5)	0.974
	Colon left	32 (26.4)	13 (40.6)	19 (59.8)		11 (34.4)	21 (65.6)		2 (6.3)	30 (93.7)	
	Rectum	52 (43.0)	21 (40.3)	31 (59.7)		19 (36.5)	33 (63.5)		2 (3.8)	50 (96.2)	
	Not specified	6 (5.0)	0 (0)	6 (100)	–	0 (0.0)	6 (100.0)	–	0 (0.0)	6 (100.0)	–
Histological types	Non-mucinous	107 (88.4)	39 (36.4)	68 (63.6)	0.045	36 (35.0)	71 (65.0)	0.14	3 (3.0)	104 (97.0)	0.026
	Mucinous carcinoma	14 (11.6)	9 (64)	5 (36)		6 (42.9)	8 (57.1)		3 (21.4)	11 (78.6)	
Differentiation	Well and moderate	91 (75.3)	39 (42.8)	52 (57.2)	0.05	35 (38.5)	56 (61.5)	0.049	4 (4.4)	87 (95.6)	0.583
	Poor	17 (14.0)	3 (17.6)	14 (82.4)		2 (11.8)	15 (88.2)		1 (5.9)	16 (94.1)	
	Not specified	13 (10.7)	6 (46.1)	7 (34.9)		5 (38.5)	8 (61.5)	–	1 (7.7)	12 (92.3)	–
TNM	II	31 (25.6)	17 (54.8)	14 (45.2)	0.02	17 (54.8)	14 (45.2)	0.01	0 (0.0)	31 (100.0)	0.256
	III	21 (17.4)	6 (28.5)	15 (71.5)		4 (19.0)	17 (81.0)		2 (9.5)	19 (90.5)	
	IV	58 (48.0)	19 (32.7)	39 (67.3)		16 (27.6)	42 (72.4)		3 (5.2)	55 (94.8)	
	Not specified	11 (9.1)	6 (54.5)	5 (45.5)	–	5 (45.5)	6 (54.5)	–	1 (9.1)	10 (90.9)	–

and Singapore comparing *K-RAS* mutations among patients diagnosed in 1960s (0–25%) vs 1990s (28–36%) also revealed increasing trend of *K-RAS* mutations [25, 26] in Asia which mirrors rising incidence of CRC itself. Inherent difference of colorectal cancer genetic profile may also be reflected by relatively low frequency of BRAF mutation, a downstream effector of RAS signaling pathways in different ethnic groups [20].

In our cohort, stage III CRC had higher frequency of *K-RAS* mutations than advance stage IV. In our routine testing populations, stage III patients made up to 34%. Although *K-RAS* and *N-RAS* testing are primarily mandated for stage IV patients, there is consideration to test at earlier stage during routine clinical practice to anticipate progress to distant disease [27].

Five percent frequency of *N-RAS* mutation in our CRC cohort is also observed by many Asian studies with 2–5% range [8, 16, 28]. Combining *K-RAS* and *N-RAS* (extended or All RAS) mutation frequency in this study (41%) has similar rates with other Asian sites such as Korea (41%) [28], Japan (45%) [8], and China (49%) [16].

Sites of primary tumor have been shown as important prognostic factor of colorectal cancer [29]. Moreover, maximum benefits of targeted therapies (anti-EGFR or anti-angiogenesis) may also depend on primary tumor sites. *K-RAS* wild-type patients whose primary tumor sites were in the left sides had better survival than the ones in the right sides as shown in the Asian population [30]. While the frequency of *K-RAS* in our cohort seemed to be higher in the right side of the colon than in the left side of colorectal in our study, a large study in Japan suggests frequent *K-RAS* mutation in primary tumors located in the right side vs left side [15].

Conclusion

Melting graph analysis is a simple, rapid, cost-effective, and highly sensitive method for *K-RAS* and *N-RAS* mutation screening. To accompany HRM mutation screening, the RFLP method was useful to confirm mutation, but direct sequencing was still required for deeper and specific analysis. Overall rates of *K-RAS* and *N-RAS* mutations in Indonesian colorectal cancer patients were similar to other Asian populations, which tend to be lower than those in patients of European or western descents.

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Compliance with Ethical Standards This study was determined to be exempt by the Institutional Review Board and performed in accordance to 1964 Helsinki Declaration and its later amendments. This retrospective study was approved by Institutional Review Board (IRB) of Stem-cell and Cancer Institute (SCI).

Conflict of interest ML, GP, FS, and AU are employees of PT Kalbe Farma; GW and WG are senior pathologists receiving honoraria from PT Bifarma Adiluhung, Kalbe Farma; and all other authors have declared no conflicts of interests.

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