High frequency of microsatellite instability and its substantial co-existence with *KRAS* and *BRAF* mutations in Vietnamese patients with colorectal cancer

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Abstract. Tumor heterogeneity and resistance to chemotherapy have been recognized as two major obstacles in the diagnosis and treatment of colorectal cancer (CRC). Microsatellite instability (MSI) and *KRAS* and *BRAF* mutations are common diagnostic factors that have been widely used to classify CRC for therapeutics. In the present study, 151 patients with CRC were analyzed from the two most populous ethnic groups of Vietnam, Kinh and Muong, for their MSI status, frequency of *KRAS* and *BRAF* mutations, and their clinical implications. MSI-high (MSI-H) was detected in 45.0% (68/151), while mutated *KRAS* and *BRAF* were identified in 37.1% (56/151) and 2.6% (4/151) of the cases, respectively. There was a substantial co-existence of MSI-H with *KRAS* (27/56; 48.2%) and *BRAF* (3/4; 75.0%) mutations. Statistical analysis showed that MSI-H tumors were significantly associated with colon location (P=0.011) and more advanced T stages (P=0.016). *KRAS* exon 2 mutations were significantly more likely to be detected in patients who belonged to the Muong ethnic group (P=0.013) or those with no/fewer lymph node metastasis (P=0.048) as compared with their counterparts. In summary, the data revealed typical molecular features of Vietnamese patients with CRC, including a strikingly high rate of MSI-H and its high co-existence with *KRAS* and *BRAF* mutations, which should be carefully considered in the future therapeutics for this type of cancer.

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Introduction

Colorectal cancer (CRC) is currently the second-most lethal cancer globally, with an estimated 880,792 deaths occurring in 2018, accounting for 9.2% of total cancer deaths worldwide (1). In Vietnam, CRC is the fifth most common cancer and the fourth leading cause of cancer death in both sexes combined (2). Generally, colorectal tumorigenesis undergoes a stepwise process of mutations and clonal expansion, subsequently leading to invasive and metastatic tumors (3-5). There are two major molecular pathways contributing to CRC tumorigenesis, namely chromosomal instability (CIN) and microsatellite instability (MSI) (6).

CIN appears to be the most common type of genetic change in CRC, accounting for 85% of sporadic cases (5). CIN is often associated with copy number variations (CNVs) and/or the mutations in cancer-associated genes, including *KRAS* and *BRAF* (7,8). MSI, on the other hand, accounts for 15% of all CRC (9), as a consequence of a post-replicative DNA mismatch repair (MMR) deficiency mostly caused by germline mutations (10) or epigenetic silencing of MMR genes (11). MSI high (MSI-H) CRC tends to be associated with a high frequency of replication errors due to the slippage of DNA polymerase (12). MSI-low (MSI-L) tumors, however, seem to occur through the CIN pathway, similar to microsatellite stable (MSS) tumors (13). MSI-H CRC has largely been recognized as a favorable prognostic factor (14-17).

One of the key early events during tumor progression is the acquisition of mutations in *KRAS* oncogene, leading to the activation of multiple epidermal growth factor receptor (EGFR) signaling pathways, including the RAS-RAF-ERK-MAPK and the PI3K-PTEN-AKT pathways (18). Activation mutations of the *KRAS* gene have been detected in 30-50% of CRC cases, mostly affecting codons 12 and 13 (19), resulting in a substitution of glycine with cysteine (p.G12C), valine (p.G12V), or aspartic acid (p.G12D, p.G13D) (19-21). These mutations diminish the intrinsic GTPase activity of KRAS and confer unresponsiveness to GTPase activating proteins (GAPs), causing the accumulation of KRAS-GTP and constitutively activating its downstream pathways, including cell prolifera-

tion and survival (5,22). BRAF is a serine/threonine protein kinase, which is associated with the MEK/ERK signaling pathway (23). Oncogenic mutations of *BRAF* have been detected in ~5-15% of CRCs (23-25), mostly involving a substitution of glutamic acid for valine at codon 600 (p.V600E) (26). Molecularly, *BRAF* mutations trigger its kinase activity that activates ERK through its effectors MEK1/2, which eventually induces cell proliferation (27). Mutations in *KRAS* and *BRAF* have been largely regarded as negative predictors for anti-EGFR therapy among patients with CRC (28-33). Detection of *KRAS* and *BRAF* mutations, therefore, has been performed in a routine CRC diagnosis as an aid for prognosis and therapeutic options (34-36).

Despite its significance, the MSI status and the prevalence of *KRAS* and *BRAF* mutations and their associations with the other tumor characteristics remain poorly known for Vietnamese patients with CRC. The present study aimed to determine the MSI status, mutation frequencies of *KRAS* exon 2 and *BRAF* exon 15, as well as the associations between MSI status and other clinicopathological features in 151 Vietnamese patients with CRC from two populous ethnic groups, Kinh and Muong.

Materials and methods

Patients and clinicopathological characteristics. Fifty pairs of primary CRC and adjacent normal tissues (at least 3 cm from the tumor) were collected from an unselected consecutive series of patients of the Kinh ethnic group undergoing surgical resection of CRC at Hue Central Hospital (Hue, Vietnam) between February 2017 and May 2018. These samples were stored at -80˚C after snap freezing in liquid nitrogen. The primary formalin-fixed, paraffin-embedded (FFPE) colorectal samples (n=101) of Kinh (n=37) and Muong (n=64) ethnic groups, who were surgically treated and histologically diagnosed with CRC between May 2011 and December 2016, were obtained from the pathology archives of the Hoa Binh Hospital (Hoa Binh, Vietnam). None of the patients underwent any other treatments before the surgery. Routine histopathologic staging of the resected specimens was done by an experienced pathologist, and all the cancer specimens contained at least 70% carcinoma cells.

The clinicopathological characteristics of the cohort, including the patient's sex, ethnicity, age at diagnosis, Tumor-Node-Metastasis (TNM) stage, tumor site and tumor grade (level of differentiation), are shown in Table I. The study was approved by the Ethics Committee of Duy Tan University. Written informed consent for the use of resected tissue and clinical data in research was obtained from all patients.

DNA extraction. For the FFPE CRC specimens (n=101), five to eight sections with a thickness of ~10 μm per sample were cut using a rotary microtome (HM 325 Rotary Microtome; Thermo Fisher Scientific, Inc.) and immediately collected into a sterile 2 ml microcentrifuge tube. After xylene/ethanol deparaffinization, samples were lysed with ~500 μl of lysis buffer (10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS and 200 μg/ml proteinase K) at 56˚C for ~3 h. DNA was then isolated using a mixture of phenol-chloroform, followed by ethanol precipitation, as previously described (37).

For the fresh-frozen paired samples (n=50), a pea-size tissue sample was cut and collected into a sterile 2 ml microcentrifuge tube containing 500 μl of ice-cold lysis buffer and a 2.5-mm diameter iron ball. The sample was homogenized by a TissueLyser LT (Qiagen Inc.) according to the manufacturer's instructions, and DNA was extracted as aforementioned. DNA samples were then dissolved in Tris-EDTA buffer or ddH_2O and kept at -20˚C for later use. DNA purity and concentration were assessed by a NanoDrop 2000 Spectrophotometer (ND-2000; Thermo Fisher Scientific, Inc.).

MSI analysis

Multiplex PCR amplification of short tandem repeat (STR) loci. Typing of MSI was performed using the standard Bethesda marker panel (38) and CAT25 (39), as previously described (40) (Table SI). Multiplex PCR reaction was done in a total volume of 25 μl using 12.5 μl Phusion U Multiplex PCR Master mix (Thermo Fisher Scientific, Inc.), 0.4 mM of each primer pairs (Integrated DNA Technologies, Inc.), and 50 ng of genomic DNA (gDNA). For set 1 (BAT25, BAT26 and CAT25), the cycling parameters were as follows: 30 sec at 98˚C; 30 cycles for fresh-frozen and 32 cycles for FFPE samples of 10 sec at 98˚C, 30 sec at 57˚C, 30 sec at 72˚C, and a final extension step at 72˚C for 7 min. For set 2 (D2S123, D5S346 and D17S250), the annealing temperature was adjusted to 52˚C.

Fragment analysis. The PCR products were subjected to the ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Inc.) for fragment analysis. The sizes and patterns of the PCR products were then resolved using GeneMapper 5.0 software (Thermo Fisher Scientific, Inc.). The occurrence of MSI in a CRC sample was specified by the presence of novel alleles as compared with the corresponding normal sample and/or the appearance of allelic imbalance at heterozygous loci. Tumors were considered as MSI-H if instability was detected in at least two out of six tested markers; as MSI-L if instability was observed in only one of the markers; and as MSS if no instabilities were detected (38,39).

Determination of allelic imbalance. For dinucleotide makers, allelic ratios (R) were calculated based on the fluorescent intensity peak height, as previously reported (41,42). In normal tissues, the peak height ratio of <70% could be considered as evidence of allelic imbalance (AI) or partial loss of heterozygosity (pLOH) (43). In practice, the AI thresholds were generally set from 70% to as low as 59% (42,44). In the present study, the AI thresholds were set at <59% or >125%. For simplicity, the smaller allele (in size) was always made the numerator, and the site with the allelic ratio of >1.7 or <0.8 was recognized as a site of AI or pLOH and will be reckoned as MSI (Fig. S1).

KRAS and BRAF mutation detection

PCR amplification. The amplification mixture consisted of 12.5 μl 2X DreamTaq PCR Master mix (Thermo Fisher Scientific, Inc.), 0.4 mM of each primer pairs (Table SII), and 50-100 ng gDNA. For *KRAS*, the PCR conditions were 3 min at 95˚C, followed by 32 (for fresh-frozen samples) to 35 cycles (for FFPE samples) of 30 sec at 95, 50 and 72˚C, sequentially. For *BRAF*, the annealing temperature was adjusted to 48˚C.

Table I. Clinicopathological characteristics of patients with colorectal cancer according to microsatellite instability status.

		MSI sub-group				MSI-H vs. MSI-L/MSS	
Characteristics	Cases, n=151	MSI-H	MSI-L	MSS	P-value	MSI-L/MSS	P-value
Age, years					0.122[a]		0.066[a]
Mean ± SD (range)	59.94±12.36 (23-90)	58.35±12.15 (27-87)	62.29±13.79 (32-90)	60.51±11.51 (23-84)		61.24±12.44 (23-90)	
≤50	32 (21.2)	19 (27.9)	7 (20.6)	6 (12.2)		13 (15.7)	
>50	119 (78.8)	49 (72.1)	27 (79.4)	43 (87.8)		70 (84.3)	
Sex, n (%)					0.008[a]		0.170[a]
Male	84 (55.6)	42 (61.8)	11 (32.4)	31 (66.3)		42 (50.6)	
Female	67 (44.4)	26 (38.2)	23 (67.6)	18 (36.7)		41 (49.4)	
Ethnicity, n (%)					0.957[a]		0.786[a]
Kinh	87 (57.6)	40 (58.8)	19 (55.9)	28 (57.1)		47 (56.6)	
Muong	64 (42.4)	28 (41.2)	15 (44.1)	21 (42.9)		36 (43.4)	
Location, n (%)					0.040[a]		0.011[a]
Colon	104 (68.9)	54 (79.4)	20 (58.8)	30 (61.2)		50 (60.2)	
Rectum	47 (31.1)	14 (20.6)	14 (41.2)	19 (38.8)		33 (39.8)	
TNM stage, n (%)					0.536[c]		0.772[c]
I	7 (4.6)	1 (1.5)	1 (2.9)	5 (10.2)		6 (7.2)	
II	108 (71.5)	51 (75.0)	25 (73.5)	32 (65.3)		57 (68.7)	
III	28 (18.5)	12 (17.6)	7 (20.6)	9 (18.4)		16 (19.3)	
IV	1 (0.7)	-	1 (2.9)	-		1 (1.2)	
Missing	7 (4.6)	4 (5.9)	-	3 (6.1)		3 (3.6)	
T stage, n (%)					0.056[c]		0.016[c]
T2	10 (6.6)	2 (2.9)	2 (5.9)	6 (12.2)		8 (9.6)	
T3	84 (55.6)	34 (50.0)	23 (67.6)	27 (55.1)		50 (60.2)	
T4	57 (37.7)	32 (47.1)	9 (26.5)	16 (32.7)		25 (30.1)	
Lymph node invasion, n (%)					0.962[c]		0.797[c]
N0	115 (76.2)	52 (76.5)	26 (76.5)	37 (75.5)		63 (75.9)	
N1	28 (18.5)	13 (19.1)	7 (20.6)	8 (16.3)		15 (18.1)	
N2	2 (1.3)	-	-	2 (4.1)		2 (2.4)	
Missing	6 (4.0)	3 (4.4)	1 (2.9)	2 (4.1)		3 (3.6)	
Distant metastasis, n (%)					0.193[c]		0.364[c]
M0	145 (96.0)	66 (96.0)	33 (97.1)	46 (93.9)		79 (95.2)	
M1	1 (0.7)	-	1 (2.9)	-		1 (1.2)	
Missing	5 (3.3)	2 (2.9)	-	3 (6.1)		3 (3.6)	
Differentiation, n (%)					0.076[c]		0.103[c]
Well	25 (16.6)	6 (8.8)	5 (14.7)	14 (28.6)		19 (22.9)	
Moderately	17 (11.3)	9 (13.2)	4 (11.8)	4 (8.2)		8 (9.6)	
Poorly	5 (3.3)	2 (2.9)	2 (5.9)	1 (2.0)		3 (3.6)	
Missing	104 (68.9)	51 (75.0)	23 (67.6)	30 (61.2)		53 (63.9)	
BRAF, n (%)					0.350[b]		0.327[b]
Mutant	4 (2.6)	3 (4.4)	1 (2.9)	-		1 (1.2)	
Wild-type	147(97.4)	65 (95.6)	33 (97.1)	49 (100.0)		82 (98.8)	
KRAS, n (%)					0.516[a]		0.546[a]
Mutant	56 (37.1)	27 (39.7)	14 (41.2)	15 (30.6)		29 (34.9)	
Wild-type	95 (62.9)	41 (60.3)	20 (58.8)	34 (69.4)		54 (65.1)	

CRC, colorectal cancer; SD, standard deviation; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable. [a]χ^2 test; [b]Fisher's exact test; [c]Kruskal-Wallis test.

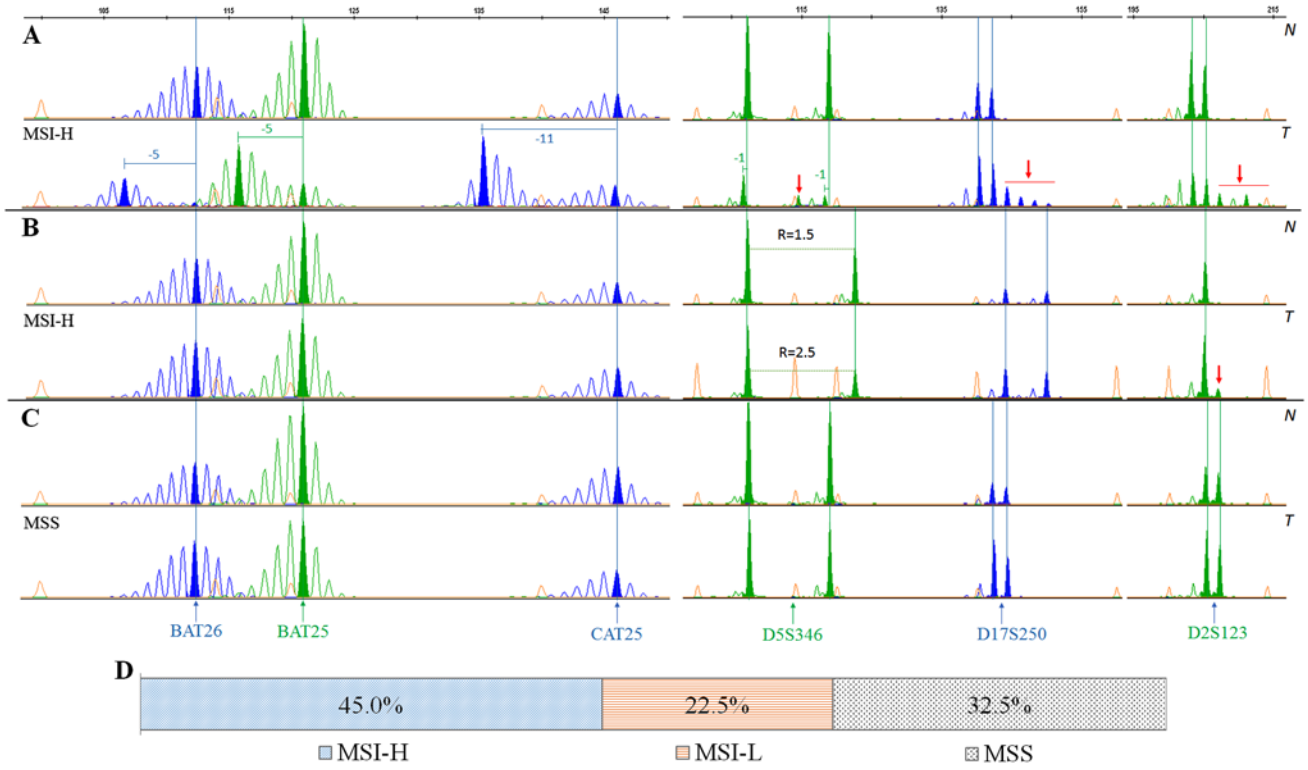

Figure 1. MSI detection by multiplex PCR using 6 primer pairs, comprising 3 mononucleotide markers (BAT26, BAT25 and CAT25) and three dinucleotide markers (D2S123, D5S346 and D17S250). (A) A colorectal tumor with MSI-H; all six tested markers showed instability. (B) A MSI-H colorectal tumor with a minimum number of unstable markers (two out of six). (C) A MSS colorectal tumor where no unstable markers were detected. (D) The frequency of MSI-H, MSI-L and MSS in Vietnamese patients with colorectal cancer. Orange peaks represent the internal length standard of 600 LIZ. Filled peaks span the largest area and are defined as the main products. Shift lengths are denoted above the corresponding product peaks. N, tumor-matched adjacent normal tissue samples; T, paired tumor samples; MSI-H, microsatellite instability-high; MSS, microsatellite stability; R, allelic ratio of a heterozygous locus, as calculated by dividing the peak height of the smaller allele by larger allele.

To avoid a cross-sample contamination, at least one negative control was included for each round of PCR amplification. PCR products were examined by 2% agarose gel electrophoresis to confirm the existence of amplified fragments before sequencing.

Sanger sequencing. All the successfully amplified fragments were subjected to unidirectional sequence analysis based on the Sanger method on an ABI 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) after being purified using the GeneJet PCR Purification kit (Thermo Fisher Scientific, Inc.).

Data analysis. Tumors were classified by MSI or *KRAS* mutation status. The clinicopathological features were assessed and compared between groups. Anatomic location was distinguished as the colon (from cecum to sigmoid colon) or rectum (rectum and anus). Tumor grade was categorized as: Well-differentiated (G1), moderately differentiated (G2), poorly differentiated (G3), and undifferentiated (G4) tumors. The node metastasis (N) in the TNM staging system was assigned by the number of metastatic lymph nodes as N1 (1-3 affected nodes) and N2 (≥4 affected nodes).

Statistical analysis. SPSS 22.0 (SPSS, Inc.) was used for all statistical analyses. Continuous variables were presented as the mean±standard deviation. The associations between MSI, *KRAS* and *BRAF* mutational status and the clinicopathological factors were determined by the χ^2, Fisher's exact, or Kruskal-Wallis test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological features of patients. Of the 151 patients, 84 (55.6%) were male, and 67 (44.4%) were female; 87 (57.6%) were Kinh, and 64 (42.4%) were Muong ethnicity. There were 104 (68.9%) tumors at the colon and 47 (31.1%) tumors at the rectum. The pathological stage was defined according to the TNM stage classification; 7 patients (4.6%) had stage I, 108 patients (71.5%) had stage II, 28 patients (18.5%) had stage III, 1 patient (0.7%) had stage IV, and 7 patients (4.6%) had an undetermined stage tumor. Tumor grade was available for only 47 patients, of which 25 tumors (53.2%) were well-differentiated, 17 tumors (36.2%) were moderately differentiated, and 5 tumors (10.6%) were poorly differentiated. The patients' age ranged from 23 to 90 years (mean age, 59.94±12.36 years) (Table I).

Large proportion of Vietnamese patients with CRC are classed as MSI-H. All tumors were typed for MSI and classified as MSI-H, MSI-L or MSS based on their number of unstable markers (Fig. 1A-C). Of these 151 patients, there were 68 patients (45.0%) with MSI-H, 34 patients (22.5%) with MSI-L, and 49 patients (32.5%) with MSS (Fig. 1D).

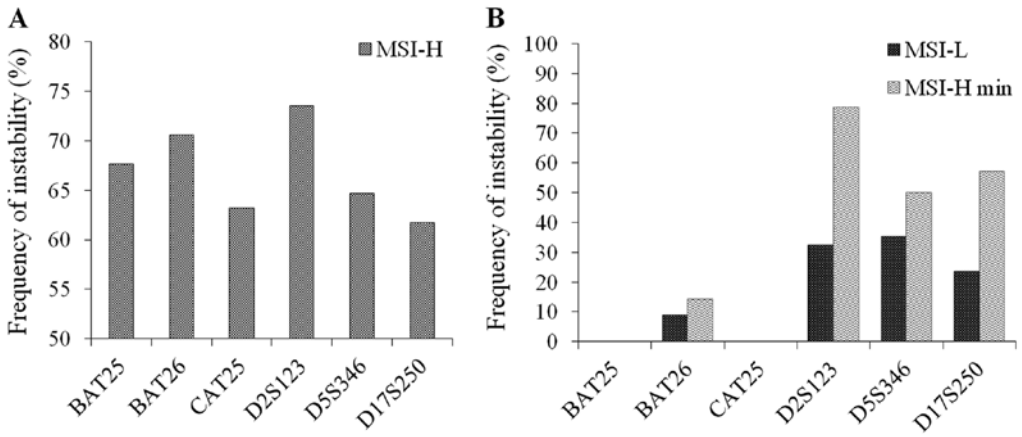

Figure 2. Frequency of instability (%) of six microsatellite markers used to assess MSI status of colorectal tumors. BAT25, BAT26 and CAT25 are mononucleotide and D2S123, D5S346 and D17S250 are dinucleotide markers. (A) The frequency of instability within MSI-H tumors. (B) The frequency of instability within MSI-L and MSI-H with a minimum number of two positive markers (MSI-H min). MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stable.

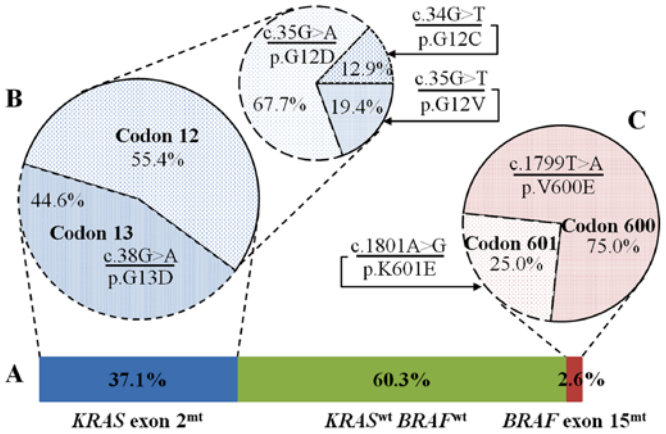

Figure 3. Status of *KRAS* and *BRAF* mutations in Vietnamese patients with CRC. (A) The frequency of *KRAS* exon 2 and *BRAF* exon 15 mutations in Vietnamese patients with CRC. The proportion of the specific substitution mutations detected in (B) codon 12 and 13 of *KRAS* exon 2 and (C) in codon 600 and 601 of *BRAF* exon 15. CRC, colorectal cancer patients; mt, mutant; wt, wild-type.

The mean age of patients at diagnosis in the MSI-H group (58.35±12.15 years) was lower compared with MSS (60.51±11.51) and MSI-L (62.29±13.79) groups. There was a significant association between MSI status and the patients' sex (P=0.008), and tumor location (P=0.040) (Table I).

MSI-H vs. MSI-L/MSS CRC showed significant differences in the tumor location and T stage. Accordingly, MSI-H tumors were often found to be located at the colon (54/68; 79.4%) compared with MSI-L/MSS groups (50/83; 60.2%) (P=0.011). Furthermore, MSI-H was positively associated with more advanced T stages (P=0.016), especially for T4 stage tumors, which has been detected in 32 out of 68 (47.1%) patients with MSI-H compared with 25 out of 83 (30.1%) patients with MSI-L/MSS (Table I). In parallel, pairwise comparisons between MSI groups also revealed the significant differences (P≤0.05) between MSI-H vs. MSI-L and MSI-H vs. MSS tumors in both tumor location and T stage (Table SIII). Additionally, the results revealed significant differences in sex between MSI-H vs. MSI-L (P=0.005) and MSI-L vs. MSS (P=0.006) tumors (Table SIII). Specifically, male were more often in the MSI-H (42/68, 61.8%) and MSS (31/49, 66.3%) groups compared with the MSI-L tumors (11/34; 32.4%) (Table I). Moreover, the statistical analyses have also uncovered associations between MSI status and the tumor grade and age at onset (Table SIII). Particularly, MSI-H (P=0.027) tumors appeared to be significantly less differentiated and younger onset (P=0.041) compared with MSS tumors (Table SIII).

Taken together, the data revealed that a substantial proportion of Vietnamese patients with CRC were MSI-H. MSI-H tumors exhibited with more advanced T stage tumors and colon-located tumors. Furthermore, MSI-H tumors occurred more in male compared with MSI-L tumors, and less differentiated and younger age at onset compared with MSS tumors. These distinct clinicopathological features of MSI-H tumors specified that the carcinogenic pathway underlying MSI-H tumors might be different from that of MSI-L/MSS tumors.

Diagnostic role of each individual marker. The diagnostic role of microsatellite markers was assessed by analyzing the frequency of instability of these markers on three groups: MSI-H, MSI-L and MSI-H cases with a minimum number (2 out of 6) of unstable markers (MSI-H min, Table SIV). Noticeably, the frequency of instability was similar between mononucleotide and dinucleotide markers within MSI-H group. Of which, D2S123 and BAT26 were the most unstable markers that showed evidence of instability in 73.5 and 70.6% of the MSI-H cases, respectively (Fig. 2A). However, for MSI-L and especially for MSI-H min group, the dinucleotide markers appeared to be much more sensitive in the detection of instability compared with mononucleotide markers. Particularly, while BAT25 and CAT25 did not show any evidence of instability in MSI-L and MSI-H min CRC, the frequency of instability detected in BAT26, D2S123, D5S346 and D17S250 in MSI-L and MSI-H min were 8.8, 32.4, 35.3, 23.5%, and 14.3, 78.6, 50, 57.1%, respectively (Fig. 2B).

KRAS and BRAF mutations and their co-existence with MSI-H. Electropherograms of *KRAS* and *BRAF* gene sequences carrying hotspot substitution mutations were illustrated in Fig. S2A and B.

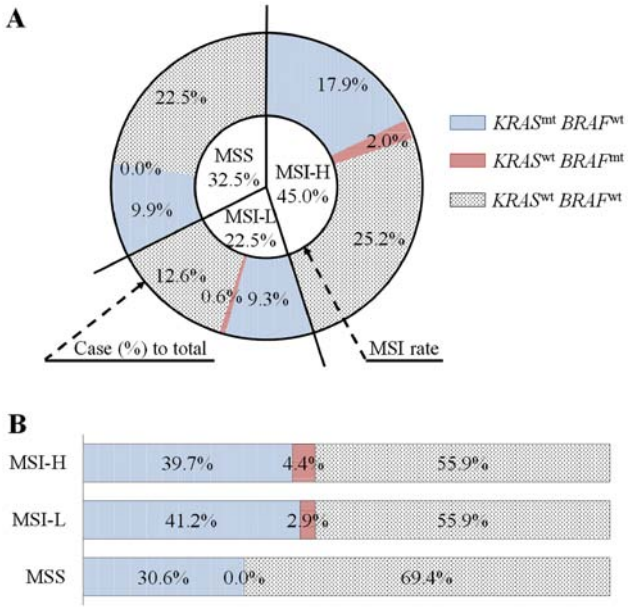

Figure 4. Association between MSI and *KRAS* exon 2 and *BRAF* exon 15 mutations. (A) Frequency of MSI-H, MSI-L and MSS in patients with CRC (the core circle) and the proportion of *KRAS* exon 2 and *BRAF* exon 15 mutations represented as the percentage (%) of total cases (the second circle). (B) The proportion of *KRAS* exon 2 and *BRAF* exon 15 mutations represented as the percentage (%) within each MSI group. MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stable; mt, mutant; wt, wild-type.

Sequence analysis of *KRAS* exon 2 showed single point mutations in 56 of 151 tested samples (37.1%) (Fig. 3A), of which 31 tumors (55.4%) had mutations in codon 12 and 25 tumors (44.6%) had mutations in codon 13 (Fig. 3B). The most frequent alteration in codon 12 was c.35G>A (21/31; 67.7%) transition, causing a replacement of glycine with aspartic acid (p.G12D). Two other mutations at codon 12 were c.35G>T (6/31; 19.4%) and c.34G>T (4/31; 12.9%) transversion, leading to the substitution of valine (p.G12V) or cysteine (p.G12C) for glycine, respectively (Fig. 3B). Mutations in codon 13 were exclusively c.38G>A (25/25) transition, resulting in an exchange aspartic acid for glycine (p.G12D) (Fig. 3B).

Four of 151 tumors (2.6%) comprised a point mutation in *BRAF* exon 15 (Fig. 3A). Three out of four mutations (75.0%) were c.1799T>A transversion replacing glutamic acid for valine in codon 600 (p.V600E). The remained mutation (25.0%) was c.1801A>G transition that switches a lysine to glutamic acid (p.K601E) (Fig. 3C). Noticeably, there was a substantial co-existence of mutant *KRAS* and MSI-H (27/151, 17.9%) compared with MSI-L (14/151; 9.3%) and MSS (15/151; 9.9%) (Fig. 4A). Within MSI-H group, mutant *KRAS* and mutant *BRAF* accounted for 39.7 and 4.4% (Fig. 4B); while within mutated *KRAS* and mutated *BRAF* groups, MSI-H accounted for 48.2% (27/56) and 75% (3/4), respectively (Table II).

The statistical tests disclosed significant differences in the ethnicity and lymph node metastasis rate between *KRAS* mutant and wild-type tumors. Accordingly, there were significantly more Muong (55.4%) than Kinh patients (44.6%) within the *KRAS* mutated tumors (P=0.013). Moreover, *KRAS* mutations were significantly associated with none (N0) or fewer number lymph node metastasis (N1) compared with *KRAS* wild-type (P=0.048). Lastly, the Kruskal-Wallis test has unveiled that *BRAF* mutant tumors were significantly less differentiated (P=0.046) compared with *BRAF* wild-type tumors. *KRAS* and *BRAF* mutations were mutually exclusive (Table II).

Discussion

Currently, it is well accepted that certain alterations at the molecular level favor CRC tumorigenesis and are used as prognostic markers. MSI phenotype, for example, was commonly proposed as a favorable prognostic biomarker for CRC (14,15,45-47) and mutations in *KRAS* and *BRAF* genes were decisively used as predictors of resistance to monoclonal antibodies targeting epidermal growth factor receptors (EGFRs) (48-50). Therefore, defining the status of MSI and the hotspot mutations in *KRAS* and *BRAF* is of utmost importance to support the prognosis and selection of treatment methods.

Among 151 patients with CRC, 45.0% of patients were classed as MSI-H, 22.5% were classed as MSI-L, and 32.5% as MSS. The proportion of MSI-H in Vietnamese patients with CRC in the present study is similar to that of African American (45%; 10/22) (51), substantially higher than Singaporean (30%; 32/109) patients with CRC (52), and strikingly higher than that of Japanese (4.5-6%) (53,54), Korean (5.5-9%) (55), and Western (10-20%) patients with CRC (56,57). These differences could be due to the genetic basis of the patient cohorts, microsatellite markers used for MSI detection, and/or the interpretation of the results (15,58). Several previous studies have suggested that BAT26 was a fairly good indicator of what would have been seen with the entire panel (16,59). However, in the present study, all six tested markers showed similar sensitivity in detecting MSI-H tumors, with ~70% of the cases, in which BAT26 and D2S123 were the most unstable markers (Fig. 2A). Particularly, in MSI-L and MSI-H min tumors, dinucleotides markers were found to be more sensitive than mononucleotide markers in detecting MSI (Fig. 2B). This finding was in concordance with one previous study in other Asian patients with CRC (52), indicating the significance of these dinucleotide markers in assessing MSI in patients with CRC.

Table II. Association of *KRAS* and *BRAF* mutational status with clinicopathological features in colorectal cancer.

	BRAF			*KRAS*		
Clinicopathological features	*BRAF*^{mt} n (%)	*BRAF*^{wt} n (%)	P-value	*KRAS*^{mt} n (%)	*KRAS*^{wt} n (%)	P-value
Age, years			1.000[b]			0.379[a]
≤50	1 (250.0)	31 (21.1)		14 (25.0)	18 (18.9)	
>50	3 (75.0)	116 (78.9)		42 (75.0)	77 (81.1)	
Sex			1.000[b]			0.465[a]
Male	2 (50.0)	82 (55.8)		29 (51.8)	55 (57.9)	
Female	2 (50.0)	65 (44.2)		27 (48.2)	40 (42.1)	
Ethnicity			0.638[b]			0.013[a]
Kinh	3 (75.0)	84 (57.1)		25 (44.6)	62 (65.3)	
Muong	1 (25.0)	63 (42.9)		31 (55.4)	33 (34.7)	
Location			1.000[b]			0.194[a]
Colon	3 (75.0)	101 (68.7)		35 (62.5)	69 (72.6)	
Rectum	1 (25.0)	46 (31.3)		21 (37.5)	26 (27.4)	
TNM stage			0.699[c]			0.086[c]
I	-	7 (4.8)		4 (7.1)	3 (3.2)	
II	3 (75.0)	105 (71.4)		41 (73.2)	67 (70.5)	
III	1 (25.0)	27 (18.4)		6 (10.7)	22 (23.2)	
IV	-	1 (0.7)		1 (1.8)	-	
Missing	-	7 (4.8)		4 (7.1)	3 (3.2)	
T stage			0.216			>0.999[c]
T2	-	10 (6.8)		5 (8.9)	5 (5.3)	
T3	4 (100.0)	80 (54.4)		29 (51.8)	55 (57.9)	
T4	-	57 (38.8)		22 (39.3)	35 (36.8)	
Lymph node metastasis			0.843[c]			0.048[c]
N0	3 (75.0)	112 (76.2)		45 (80.4)	70 (73.7)	
N1	1 (25.0)	27 (18.4)		6 (10.7)	22 (23.2)	
N2	-	2 (1.4)		-	2 (2.1)	
Missing	-	6 (4.1)		5 (8.9)	1 (1.1)	
Tumor grade (differentiation)			0.046[c]			0.114[c]
Well	-	25 (17.0)		44 (78.6)	21 (22.1)	
Moderately	2 (50.0)	15 (10.2)		4 (7.1)	11 (11.6)	
Poorly	1 (25.0)	4 (2.7)		6 (10.7)	3 (3.2)	
Missing	1 (25.0)	103 (70.1)		2 (3.6)	60 (63.2)	
BRAF						0.297[b]
Mutant				-	4 (4.2)	
Wild-type				56 (100)	91 (95.8)	
MSI			0.151[c]			0.356[c]
High	3 (75.0)	65 (44.2)		27 (48.2)	41 (43.2)	
Low	1 (25.0)	33 (22.4)		14 (25.0)	20 (21.1)	
Stable	-	49 (33.3)		15 (26.8)	34 (35.8)	

CRC, colorectal cancer; SD, standard deviation; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite; mt, mutant; wt, wild-type. [a]χ^2 test; [b]Fisher's exact test; [c]Kruskal-Wallis test.

On the other hand, CAT25, the additional marker suggested by Findeisen *et al* (39), did not seem to have added any value to the MSI diagnostics of this set of tumors (Fig. 2B).

Moreover, the current study showed significant associations between MSI-H and colon tumor location, males, younger onset, and more advanced T stages. The association between MSI-H and proximal tumor sites was noted by a number of previous studies (55,60), but contradictory findings that highlighted the association of MSI-H to female (51,61,62) and lower T stages (55,63) were also reported. These results

specified that MSI-H tumors possessed distinct clinicopathological features and possibly have been derived from a distinct carcinogenic pathway compared with MSI-L/MSS ones.

The sequencing data disclosed 37.1% patients with CRC carried a *KRAS* exon 2 mutation, which typically affects codon 12 (55.4%) and codon 13 (44.6%). This observation was in accordance with previous studies (64-66), and marginally lower than several other studies (50,53,67-69), where *KRAS* mutations were detected in ~35-37 and 40-42% of the tested patients with CRC, respectively. The present study also presented a significant difference in the frequency of this mutation in two ethnic groups by showing that 48.4% of Muong and 28.7% of Kinh patients with CRC had this mutation, suggesting a possible variation in the frequency and types of *KRAS* mutations among populations. Moreover, the present data also indicated that mutant *KRAS* was reversely associated with the rate of lymph node metastasis, which was in accordance with a study in the Chinese population (69). Mutations in *KRAS* exon 2 have been largely used as a predictive marker of unresponsiveness to EGFR therapies (48-50). However, more than 50% of CRC patients with a wild-type *KRAS* exon 2 are also resistant to this approach, possibly due to other 'rare mutations' in other exons of this gene or in other genes of the RAS/RAF family (25,67,69-72). These findings indicated the need for expanding the genetic test in CRC patients with a wild-type *KRAS* exon 2 before anti-EGFR therapy.

BRAF exon 15 mutations, on the other hand, were detected in only 2.6% Vietnamese patients with CRC. This ratio was similar to that of Greek patients with CRC (21), substantially lower than other Western countries (~9%) (25,50), and marginally lower than other Asian populations (~4.5-7%), including Japan (53,66) and China (69,73). In agreement with the previous studies, the present study also presented the mutual exclusivity of *BRAF* and *KRAS* mutations (23,66). Remarkably, the substantial rate of co-existence of MSI with *KRAS* and *BRAF* mutations found in the present study may indicate the poor prognosis of these cases (16,74). A future study on the associations between overall survival and/or recurrence-free survival and MSI with *KRAS* and *BRAF* mutations will gain some insight into this issue.

In summary, the present results revealed the typical molecular features and subgroups of Vietnamese patients with CRC. Particularly, the strikingly high proportion of MSI-H tumors and their substantial co-existence with *KRAS* and *BRAF* mutations should be taken into account for future diagnosis and clinical treatments for this type of cancer.

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Availability of data and materials

Data and materials available on request from the authors

Authors' contributions

HTN conceived the project, conducted most of the experiments and data analysis, and wrote the manuscript. DTL designed/selected primers and prepared figures. QHD and VBT helped with sample preparation and DNA extraction. BVN collected samples and patients' data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Duy Tan University (approval no. DTU-IRB/2019.15). Written informed consent for the use of resected tissue and clinical data in research was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The other authors report no conflicts of interest.

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