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Study on relationship between genetic abnormalities and clinicopathological features in K hospital's patients with colorectal cancer

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ABSTRACT

The MAPK-ERK, as well as PI3K-AKT signaling transduction pathway, represents a pivotal function in tumorigenesis. Genetic alterations of potential tumor-driven genes, for instance, *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* can result in uncontrolled cell proliferation and progression. The main aims of the study were not only to identify the prevalence of *KRAS*, *BRAF*, *NRAS*, *PIK3CA* molecular modifications but also to evaluate the relationship between gene changes and clinical and/or pathological characteristics of 251 Vietnamese colorectal cancer. Genetic abnormalities on *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* were detected through the utility of Realtime PCR, Pyrosequencing, and Direct sequencing methods, respectively. The frequency of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations were 34.3%, 6.4%, 7.2%, and 17.5%, in turn. *KRAS* mutation was mutually exclusive against that of *NRAS* and *BRAF* mutations in CRC. *BRAF*, as well as *RAS*/*RAF* mutations, were more usual in older age. A significant association between *PIK3CA* mutations and age together with differentiation of CRC was determined. In addition, *PIK3CA* mutation tended to coexist with *KRAS* but not with *NRAS* and *BRAF* mutation. Our results indicate the information of molecular markers that contribute to self-sufficient oncogenic mechanisms in the carcinogenesis of CRC.

INTRODUCTION

Worldwide, colorectal cancer (CRC) is one of the majority types of cancer, which is the third most widely examined and the fourth malignant neoplastic disease-related mortality. The percentage of Caucasians CRC has been given a picture of being more superior to the Asian Ethnic. For the time being, the occurrence of cancer from parts of the large intestine was considerably accelerated in Asian countries including China, the Republic of Korea, and Vietnam, and there is a speedily rising tendency in the future, which may potentially be related to risk elements such as nutritional factors, diet modification, physical inactivity, the habit of smoking and extravagant alcohol dependence and environmental contamination [1, 2].

Activating mutations in the RAS-RAF-MAPK pathway including *KRAS*, *BRAF*, and *NRAS* abnormalities have been demonstrated to be major prognostic factors about resistance in the expectation of anti- Epidermal Growth Factor Receptor (anti-EGFR) medications. Patients with wild-type *KRAS*, *NRAS*, and *BRAF* display clinical sensitivity to this targeted therapy [3]. Since it's important to determine *RAS*/*RAF* mutation before using cetuximab and panitumumab. This allows us to precisely predict the efficacy of anti-EGFR monoclonal antibodies (mAb) as well as understand the molecular characteristics of CRC [3].

In addition, Phosphoinositide-3-kinase (PI3K) is the family of lipid kinases in the PI3K/AKT/mTOR transduction route, that assumes a variety of cellular functions and is often dysregulated in solid tumors. Abundant studies have been evidence of activated tumor-derived *PIK3CA* mutations were observed in many malignancies including CRC [4, 5]. *PIK3CA* mutation is present in 10-20% of colorectal cancer, in which approximately 80% of variant regions on the subject of the helical along with kinase domains of exon 9 and 20, correspondingly [5]. The *PIK3CA* mutation is closely associated with *KRAS* mutations and epigenetic modifications, in particular coincidental hypermethylation of numerous CpG-rich promoters of several genes (the CpG island methylator phenotype, or CIMP) [6]. Monoclonal antibody drugs targeting EGFR such as cetuximab and panitumumab are major target therapy in malignant colorectal cancer, however, *PIK3CA* pathogenic variant carriers could potentially belong less susceptible toward these target drugs [7]. This suggests that genetic abnormalities of RAS/RAF and PI3K pathway should be evaluated to guide the anti-EGFR treatment. Furthermore, identifying interactions between genetic changes in *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* oncogenes may help to understand the detailed carcinogenesis mechanism of colorectal tumors, in addition to explaining differences in healing response among individual patients. *RAS*, *RAF*, and *PIK3CA* abnormalities induce to activate of the MAPK and PI3K signaling transduction paths, resulting in the interior of consolidative or conglomerative impact on the edge of being alive of CRC sufferers [8, 9]. Although new insights into the mechanisms have emerged from recent studies, information about molecular changes in Vietnamese CRC patients remains unclear. Hence, this research was designed to meet the needs of frequency in tandem with the dispensation of genetic variations in *KRAS*, *NRAS*, *BRAF* along *PIK3CA*, on top of that correlation of each with the clinicopathological parameters of the Vietnamese CRC population.

MATERIALS AND METHODS

Obtaining tissue specimens

During the time between Nov 2019 and Oct 2021, we gathered 251 formalin-fixed paraffin-embedded (FFPE) clinical blocks according to the criteria each sample was pathological diagnosed based on the American Joint Committee on Cancer (AJCC) and operated surgical intervention on the edge of National Cancer Hospital K in Vietnam. The patient's tumor samples used in the study were not only obtained informed consent but also licensed all through the ordinances of the Vietnamese morality commission (Circular No.04/2008/TT-BYT). Sections (5µm thick) were cut from paraffin-embedded tumor tissue blocks and stained with Hematoxylin & Eosin using the Thermo Fisher Scientific system for histopathological examination, following the manufacturer's protocol.

DNA isolation from CRC tissue

QIAamp DNA FFPE Tissue Kit (Qiagen) was utilized for genomic DNA extraction from formalin-fixed paraffin-embedded tissues. The quality of DNA specimens was evaluated utilizing polymerase chain reaction (PCR) which amplified a single-copy gene, *β-globin*. Besides, the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) allows resolving the total DNA amount for this study.

Investigation of *KRAS, BRAF, NRAS,* and *PIK3CA* genetic changes from CRC tissue

Cobas® KRAS Mutation Test, Cobas® 4800 BRAF V600 Mutation Test (Roche) together with therascreen *NRAS* Pyro Kit (Qiagen) were used to identify mutations of *KRAS* exon 2-3; *BRAF* V600 on exon 15 and *NRAS* exon 2-3, respectively. *PIK3CA* transformations in the interior of the exon 9 in tandem with 20 were discovered through the utility of 3130 Genetic Analyzer (Applied Biosystems). All procedures were exactly performed as mentioned by the manufacturers' instructions. Primer sequences were detailed inward of Table 1.

Table 1. Primer sequences used for the study

Genes	Primers	Sequences
β-globin NC_000011.10	Globin F	CAACTTCATCCACGTTCACC
	Globin R	GAAGAGCCAAGGACAGGTAC
PIK3CA NC_000003.12	PIK3CA 9F	GGGAAAAATATGACAAAGAAAGC
	PIK3CA 9R	GAGATCAGCCAAATTCAGTT
	PIK3CA 20.1 F	CATTTGCTCCAAACTGACCA
	PIK3CA 20.1 R	TGTGCATCATTCATTTGTTTCA
	PIK3CA 20.2 F	TTGATGACATTGCATACATTCG
	PIK3CA 20.2 R	GGTCTTTGCCTGCTGAGAGT

Statistical analysis

The frequency of *KRAS*, *BRAF*, *NRAS,* and *PIK3CA* alterations accompanying the correlation between genetic abnormalities, and clinicopathological characteristics of colorectal cancer was evaluated by SPSS software version 20.0. In the present study, the association of variables is measured through the utility of the Fisher's exact test or else χ^2 test. The probability meaning in the expectation of the entirety of experiments was established at $p < 0.05$.

RESULTS

Clinicopathological parameters of patients with colorectal cancer

Clinicopathological features of 251 CRC patients in this study were showed in Table 2. Among 251 patients, the intermediate-age getting on for diagnosis was 59.3 years (ranging from 26 to 90 years). On the other hand, the proportion in respect to male to female patients was 1.28. Two hundred fifty-one patients with colorectal cancer including 136 (54.2%) and 115 (45.8%) were collected from the colon and rectum, respectively. Based on histological category, there were 187 (74.5%) adenocarcinoma (A), 54 (21.5%) mucinous adenocarcinoma (MA), 7 (2.8%) squamous cell carcinoma (SCC), and 3 (1.2%) signet ring cell carcinoma (SRCC) (Figure 1). As for tumor differentiation, 12 (4.8%) were well-differentiated, 160 (63.7%) moderately differentiated, and 15 (6.0%) poorly differentiated (excluding 54 mucinous adenocarcinomas, 7 squamous cell carcinomas, and 3 signet ring cell carcinoma). In our study, a predominant part of tumors (71.7%) was smaller than 5 cm in measurement, with a balanced lymph node metastasis status ratio. Pathologic stages showed 4 (1.6%) cases within stage I, 112 (44.6%) cases enclosed by stage II, 115 (45.8%) sufferers in stage III, in tandem with 20 (8.0%) patients in stage IV (Table 2).

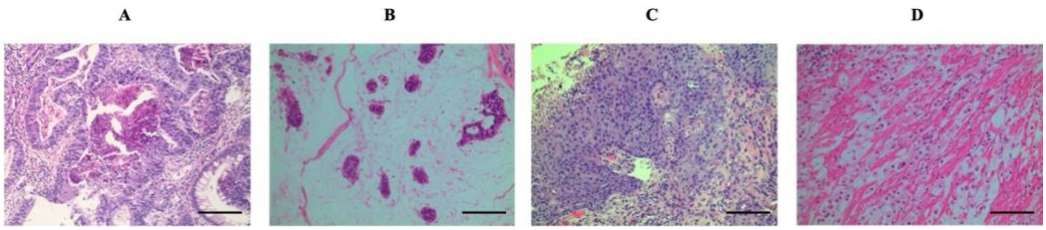

Figure 1. Representative images of H&E staining for histological subtypes. (A) Adenocarcinoma. (B) Mucinous adenocarcinoma. (C) Squamous cell carcinoma. (D) Signet ring cell carcinoma. Photographs were taken at ×200 magnification. Scale bar, 100 μm.

Table 2. Clinical and pathological parameters in the patients owing to CRC

Parameters		N	%
		251	
Age			
	> 59.3	135	53.8
	< 59.3	116	46.2
Gender			
	Male	141	56.2
	Female	110	43.8
Tumor Location			
	Colon	136	54.2
	Rectum	115	45.8
Histological subtypes			
	A	187	74.5
	MA	54	21.5
	SCC	7	2.8
	SRCC	3	1.2
Differentiation			
	Well	12	4.8
	Moderate	160	63.7
	Poor	15	6.0
	Unknown	64	25.5s
Lymph node metastasis			
	Yes	135	53.8
	No	116	46.2
Tumor size			
	≥ 5 cm	71	28.3
	< 5 cm	180	71.7
Stages			
	I	4	1.6
	II	112	44.6
	III	115	45.8
	IV	20	8.0

A: Adenocarcinoma, MA: Mucinous adenocarcinoma, SCC: Squamous cell carcinoma, SRCC: Signet ring cell carcinoma

The rate together with the distribution of *KRAS*, *NRAS*, *BRAF*, and also *PIK3CA* mutations in addition to their interrelations with clinicopathologic characteristics in patients with CRC

Table 3 detailed genetic abnormalities in 251 CRC tissue blocks. Our results showed that 86 cases had *KRAS* mutation, reaching 34.4%, which include 77 patients who harbored mutation situated at codon 12/13 belonging to the exon 2 combined with 9 sufferers found in codon 61 coming out of the exon 3. 17 out of 18 (6.8%) *NRAS* alterations were distributed in adenocarcinoma. Of 18 *NRAS* mutations, a greater part of changes was found at codon 12/13 from the exon 2 with regards to 14 patients,

achieving 77.8%. Only 4 patients carried an *NRAS* missense mutation at codon 61 from exon 3. There was no meaningful interaction amongst *KRAS* variations, *NRAS* mutation along with clinical and pathological features (agedness, sexual characteristics, tumor position, histological subtypes, differentiation, lymph node metastasis, tumor dimensions, and stage). *BRAF* mutation occurred in 6.4% (16/251) of cases in codon 600 of the exon 15. Compared with *RAF* wild-type tumors, *BRAF* mutant tumors were statistically associated with the younger group (p=0.023) (Table 3).

The mutation of either *RAS* (*KRAS* and *NRAS*) or *BRAF* was detected in 47.8% (120/251) of the cases examined. A critical correlation inward of *RAS*/*RAF* modifications with patients' age was observed within the bounds of our present study (p= 0.032). Regarding pathological parameters, *RAS*/*RAF* alterations tended also to be lightly correlated with histological subtypes (p=0.058), differentiation level (p=0.060), and lymph node malignancy status (p=0.059). Whereas *RAS*/*RAF* genetic changes in tandem with other clinicopathological features including patients' gender, tumor location, tumor size, and stages did not show any association in CRC tumors (p>0.05) (Table 3).

In addition, all data according to the rate in parallel with distribution concerning *PIK3CA* genetic changes were exhibited within the interior of this study. *PIK3CA* modification was identified in 44 samples (17.5%), of which 75.0% (33/44) and 25.0% (11/44) were occurred in exon 9 (including 7 E542 and 26 E545) and exon 20 (including 3 H1046, and 8 H1047), respectively (data not shown). The association between *PIK3CA* variant standing and clinical at the same time as pathological characteristics was not found in the Vietnamese patients with CRC. In contrast, a genetic abnormality in the *PIK3CA* gene had a higher incidence among males and moderately differentiated tumors (p<0.05).

Table 4 illustrated the interrelationship between somatic alterations of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* gene. Our results confirmed that *KRAS* mutation exhibited a mutually exclusive with *NRAS* and *BRAF* mutation pattern in CRC and was a strong association with *PIK3CA* mutation ($p< 0.05$). Meanwhile, no statistical correlation was found between *BRAF* and/or *NRAS* and *PIK3CA* mutations (p> 0.05).

Table 3. *KRAS, NRAS, BRAF* and *PIK3CA* somatic variations, as well as interrelationships together with clinical and pathological features

		KRAS mutation		*p-value*	*NRAS* mutation		*p-value*	*BRAF* mutation		*p-value*	*RAS/RAF* mutations		*p-value*	*PIK3CA* mutation		*p-value*
		Yes	%		Yes	%		Yes	%		Yes	%		Yes	%	
N	251	86	34.3		18	7.2		16	6.4		120	47.8		44	17.5	
Age				*0.464*			*0.518*			*0.023*			*0.032*			*0.825*
	> 59.3	135	49	36.3		11	8.1		13	9.6		73	54.1		23	17.0	
	< 59.3	116	37	31.2		7	6.0		3	2.6		47	40.5		21	18.1	
Gender				*0.248*			*0.956*			*0.598*			*0.385*			*0.002*
	Male	141	44	31.2		10	7.1		10	7.1		64	45.4		34	24.1	
	Female	110	42	38.2		8	7.3		6	5.5		56	50.9		10	9.1	
Tumor Location				*0.240*			*0.712*			*0.864*			*0.313*			*0.779*
	Colon	136	51	37.5		9	6.6		9	6.6		69	50.7		23	16.9	
	Rectum	115	35	30.4		9	7.8		7	6.1		51	44.3		21	18.3	
Histological subtypes				*0.334*			*0.113*			*0.838*			*0.058*			*0.114*
	A	187	65	34.8	*0.777*	17	9.1	*0.044*	13	*7.0*	*0.522*	95	50.8	*0.105*	39	20.9	*0.017*
	MA	54	17	31.5	*0.627*	0	0	*0.021*	3	*5.6*	*0.781*	20	37.0	*0.074*	4	7.4	*0.027*
	SCC	7	4	57.1	*0.196*	1	14.3	*0.459*	0	*0*	*0.483*	5	71.4	*0.204*	1	14.3	*0.819*
	SRCC	3	0	0	*0.208*	0	0	*0.628*	0	*0*	*0.649*	0	0	*0.095*	0	0	*0.422*
Differentiation				*0.314*			*0.239*			*0.486*			*0.060*			*0.043*
	Well	12	5	41.7	*0.580*	1	8.3	*0.873*	2	16.7	*0.135*	8	66.7	*0.180*	2	16.7	*0.936*
	Moderate	160	58	36.3	*0.380*	15	9.4	*0.073*	10	6.3	*0.915*	83	51.9	*0.087*	36	22.5	*0.006*
	Poor	15	2	13.3	*0.078*	1	6.7	*0.508*	1	6.7	*0.962*	4	26.7	*0.091*	1	6.7	*0.254*
	Unknown	64	21	32.8	*0.777*	1	1.6	*0.004*	3	4.7	*0.522*	25	39.1	*0.105*	5	7.8	*0.018*
Lymph node metastasis				*0.464*			*0.103*			*0.470*			*0.059*			*0.437*
	Yes	135	49	36.3		13	9.6		10	7.4		72	53.3		26	19.3	
	No	116	37	31.9		5	4.3		6	5.2		48	41.4		18	15.5	
Tumor size				*0.094*			*0.622*			*0.381*			*0.156*			*0.869*
	≥ 5 cm	71	30	42.3		6	8.5		3	4.2		39	54.9		12	16.9	
	< 5 cm	180	56	31.1		12	6.7		13	7.2		81	45.0		32	17.8	
Stages				*0.362*			*0.150*			*0.817*			*0.218*			*0.832*
	I	4	0	0	*0.146*	1	25.0	*0.164*	0	0	*0.599*	1	25.0	*0.357*	1	25.0	*0.692*
	II	112	37	33.0	*0.713*	4	3.6	*0.047*	6	5.4	*0.554*	47	42.0	*0.096*	17	15.2	*0.379*
	III	115	40	34.8	*0.873*	11	9.6	*0.176*	9	7.8	*0.387*	60	52.2	*0.203*	22	19.1	*0.540*
	IV	20	9	45.0	*0.292*	2	10.0	*0.609*	1	5.0	*0.793*	12	60.0	*0.255*	4	20.0	*0.762*

χ^2 test; Fisher's extract test; A: Adenocarcinoma; MA: Mucinous adenocarcinoma; SCC: Squamous cell carcinoma; SRCC: Signet ring cell carcinoma

Table 4. Correlation with regards to *KRAS*, *NRAS*, *BRAF* and *PIK3CA* abnormalities in CRC

		***NRAS* mutation**		***p*-value**	***BRAF* mutation**		***p*-value**	***PIK3CA* mutation**		***p*-value**	***RAS/RAF* mutations**		***p*-value**
		Yes	**No**		**Yes**	**No**		**Yes**	**No**		**Yes**	**No**	
N	251	18	233		16	235		44	207		120	131	
KRAS mutation				*0.001*			*0.003*			*0.002*			
Yes	86	0	86		0	86		24	62				
No	165	18	147		16	149		20	145				
NRAS mutation							*0.251*			*0.457*			
Yes	18				0	18		2	16				
No	233				16	217		42	191				
PIK3CA mutation							*0.894*						*0.008*
Yes	44				3	41					29	15	
No	207				13	194					91	116	

χ^2 test; Fisher's extract test

DISCUSSION

The EGFR signaling transduction path is involved in many important functions inside the range of cells, which dysregulate to lead to uncontrolled growth, appearing in solid cancers, including CRC [10]. Based on genetic alterations of this signaling pathway, cetuximab, panitumumab, nimotuzumab, and necitumumab is a group of targeting as concerns EGFR using a monoclonal antibody that has significantly improved the treatment, especially for patients with metastatic CRC [11]. However, De Roock W et al. (2010) confirmed that genetic changes belonging to *KRAS*, *BRAF*, *NRAS,* and *PIK3CA* genes were related further to a lower response rate after making utilization done by anti-EGFR monoclonal antibodies [12].

The frequency in regard to *KRAS*, *BRAF*, *NRAS,* and *PIK3CA* modifications was found in 86 of having 251 (34.3%), 16 of 251 (6.4%), 18 of 251 (7.2%), and 44 of 251 (17.5%) patients examined, respectively. Interestingly, there were 135 (53.8%) cases of patients who carried an oncogenic mutation in at the minimum one gene, including *KRAS*, *BRAF*, *NRAS,* and/or *PIK3CA.* Our results indicated that out of 165 *KRAS* wild-type CRC patients, 49 (29.7%) harbor *NRAS*, *BRAF*, or *PIK3CA* mutations. The reported *KRAS* mutation rate in patients with CRC varies widely between different populations worldwide, ranging from 13% to 66% [13–16]. In Vietnamese research, *KRAS* missense mutations at codons 12, 13, and 61 were detected in 34.3% of patients about CRC. Inside the range of our study, we discovered 34.3% of suffers harbored *KRAS* mutations, which was concordant according to reported data deriving out of Asian countries (i.e., China, Japan, and India) (20–66%), and lower than the one revealed surrounded by TCGA data (42%) [17].

Rat sarcoma virus (RAS) family members take the part of a key function in cell development. Any activating mutation at the hand of the *RAS* family, including *KRAS*, *NRAS,* and *HRAS* is an appropriate target for anticancer therapy [18]. Before the present time, there is a minority of studies on the subject of the prevalence of *NRAS* genetic modifications, ranging from 2.0 % to 10.0 % [14,16,19]. The frequency of *NRAS* mutations was 7.2% of the Vietnamese CRC patients. Similar to *KRAS* mutations, there was no meaningful relationship between *NRAS* mutations and clinical parameters were indicated in CRC tissue blocks. The extensive variability in frequency, as well as distribution of *KRAS* and also the *NRAS* mutation between studies, may perhaps be due to ethnicity, geographic factor, sample size, and mutation analysis techniques.

BRAF gene composes of 18 exons, which performs the function of a downstream signal transduction component of triggering of the mitogen-activated protein kinase (MAPK) signal transduction. *BRAF* V600E (exon 15) is the most common activating mutation, interprets as 90% of the aggregate activating *BRAF* pathogenic variations [20]. All over the world, the described rate appertaining to *BRAF* alterations inward of dissimilar inhabitants fluctuates broadly, from 1.1% to 25% [13–16]. Within the confines of this study, the V600E *BRAF* variation was discovered in 16 patients, employing a percentage of 6.4% (16/251), which is more lightly outstanding than different Asian publications (1.1% to 5.8%). For the *BRAF* gene, of extraordinary consideration is the fact that the incidence of V600E mutation gave variety to in terms of age, as far as an outstandingly higher proportion in older convalescents (5.2%) compared to that in younger patients (1.2%) (p=0.023), it was similar to the previous report, showed that *BRAF* V600E mutation escalated from 10% in the interior of unselectable cases to 37% enclosed by females elder than the 70s [21].

Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) belongs to the PI3K family that is frequently mutated in solid tumors. In the present research, the rate of *PIK3CA* genetic changes was found in 44 patients, reaching 17.5% (44/251), consistent with the prevalence from 2% to 18% of metastatic CRCs [13,14,15]. Our study showed a significant association between *PIK3CA* mutation and patients' gender and differentiation ($p<0.05$). Ziv E et al. (2017) indicated that *PIK3CA* or *AKT* mutation carriers laid hold of poorer disease progression (55%) than wild-type groups (92%) after radiation, at 1-year post-embolization [22]. This finding suggests that activating mutations belonging to the PI3K signal transduction, especially *PIK3CA* genetic abnormalities, may potentially affect radiotherapy for CRC patients.

Our present study confirmed that *KRAS* mutation excludes *NRAS* and *BRAF* missense variations in CRC ($p< 0.05$), suggesting genetic alterations are involved in different oncogenic pathways for colorectal cancer tumorigenesis. This result could potentially be explained by the incompatibility between the mutations, just 1 mutation within the interior of the MAPK signaling pathway is enough to put a stop to the cell cycle [23]. Some genetic alterations may coexist, others are exclusive, such as the coexistence of *KRAS* mutations and *APC* inactivation leading to CRC progression [24]. Meanwhile, *BRAF* and *APC* pathogenic modifications are rarely found together in CRC. In the earliest precursor of CRC and adenomas, a considerable correlation out of *BRAF* alteration along with the serrated histological characteristic was detected [25]. In addition, we inaugurated a strong interrelationship between *PIK3CA* and *KRAS* mutations; *PIK3CA* to go with *RAS*/*RAF* mutations, similar to previous reports. For example, Li HT et al. (2011) indicated that *KRAS* and *PIK3CA* somatic co-variations are more popular surrounded by patients abreast of stage IV CRC than the early stages. This may be due to the complementary impact of mutations leading to activating the PI3K-AKT signaling pathway, resulting in metastasis [26]. Once *KRAS*/*PIK3CA* mutations are coexistence in the early stage, the patient has a poor prognosis such as developing distant metastasis and worse outcome [27]. Patients carrying mutations that activate the PI3K signaling pathway are commonly less susceptible to targeted therapy using anti-EGFR monoclonal antibodies. Thus, in addition to *RAS*/*RAF* mutations, the mutation status of components involving the PI3K signaling pathway is considered a biomarker for negative prognosis when it comes to anti-EGFR monoclonal antibodies therapy to approach progressive colorectal cancer.

In conclusion, our present study demonstrated the specific associations of alterations with *KRAS*, *NRAS*, *BRAF*, *PIK3CA* gene, and CRC patients' clinicopathologic parameters, suggesting to help individualized patient-oriented treatment for cancer

patients. Our results assist in better characterizing the Vietnamese CRC population to better announce to clinicians and researchers. Future molecular detailed studies should be carried out evaluating different outcomes by oncogenic abnormalities in CRC tumors.

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None.

AUTHOR CONTRIBUTIONS

L.D.V. and Q.N.N.: Conception and Design of the experiments. H.H.C.: Methodology and Data analysis, L.D.V.: Data curation and Writing – original draft, Q.N.N: Writing – review and editing, Supervision. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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