



Original Article

Detection of *KRAS* Mutations among Filipino Patients with Colorectal Adenocarcinoma



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Abstract

Background and objectives: The activation of the Kirsten RAS (*KRAS*) oncogene is one of the factors responsible for the transition from intermediate adenoma to carcinoma in the colon. Approximately 30% to 60% of mutations in colorectal cancer (CRC) occur in the hotspot codons 12 and 13 of exon 1 and codon 61 of exon 2. This study aimed to characterize mutations of the *KRAS* gene among Filipinos with CRC.

Methods: Paired frozen normal and tumor tissues from 35 CRC patients who underwent surgical resection were included. Genomic DNA was extracted, and all five coding exons were amplified by polymerase chain reaction, followed by mutation screening using denaturing high-performance liquid chromatography and DNA sequencing.

Results: From sequencing, 18/35 (51%) samples showed mutations in exon 1 (A11R, G13C, L19W, and silent mutation L23), exon 2 (D54H), and codon 4B (silent mutation D173). Nine mutations could be considered pathogenic as they occurred within the conserved region, potentially contributing to the oncogenic potential of *KRAS*. Eight of these mutations were also found outside the hotspot region of the *KRAS* gene. Mutations were significantly associated with tumor stage III ($p = 0.007$) but not with other clinical parameters or survival.

Conclusions: This study characterizes *KRAS* mutations in Filipino patients with CRC, suggesting a possible difference in their cancer genetic profiles. Additionally, the use of easily accessible mutation screening techniques, such as denaturing high-performance liquid chromatography, may help increase reports of mutational profiles in Southeast Asian populations.

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide, ranking third in incidence and second in mortality among cancers. Over 1.9 million new colorectal cancer cases and 935,000 deaths were estimated to occur in 2020, accounting

for about one-tenth of cancer cases and deaths.¹ Despite progress in methodology for cancer screening and diagnosis, around half of CRC cases are diagnosed at late stages, for which few treatment options are available.^{2,3} Thus, the search for targeted therapy for managing the disease, both in early and advanced CRC, is needed.

CRC is a heterogeneous disease that develops through several molecular pathways.⁴ The chromosomal instability (CIN) pathway involves multi-step mutational activation of oncogenes or inactivation of tumor suppressor genes that drive the transition from normal mucosa to adenoma to carcinoma.^{5,6} CIN tumors are characterized by several mutations in tumor suppressor genes (*APC*, *TP53*) or oncogenes (*KRAS*, *PIK3CA*) that activate molecular pathways leading to the initiation and progression of CRC.⁷

The most frequently altered *RAS* proto-oncogene in human tumors is the *KRAS*, originally identified as retroviral oncogenes in rat sarcoma viruses.^{8,9} *KRAS* is located on chromosome 12p12.1,

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Table 1. Primer sequences and annealing temperatures used to amplify the entire coding region of the *KRAS* gene and the size of PCR products

Exon	Sequence (5'-3') F	Sequence (5'-3') R	bp	Optimum DHPLC (Tm)
1	ATGACTGAATATAAACTTGT	TCCACAAAATGATTCTGAAT	90	
2	GACTGTGTTTCTCCCTTCT	GGCAAATACACAAAGAAAG	161	60
3	GGTGTAGTGGAACTAGGAATTAC	GACATAACAGTTATGATTTGCAG	344	56
4a	CTCAAGCTCATAATCTCAAACCTCT	GTAGTTCTAAAGTGGTTGCCACC	305	58
4b	GACAAAACACCTATGCGGATGA	GCTAACAGTCTGCATGGAGCA	429	54

bp, base pair; DHPLC, denaturing high-performance liquid chromatography; PCR, polymerase chain reaction; Tm, melting temperature.

and comprises six exons, including one non-coding exon (exon 0) and five coding exons (exon 1 to 4A/4B), of which exon 4 has two different forms, directing the synthesis of two mRNAs via alternative splicing. *KRAS* encodes a small 21 kDa protein (p21 ras) involved in G protein-mediated signal transduction.¹⁰ RAS proteins control cellular signaling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation. RAS proteins belong to the large family of small GTPases, which are master regulators of a myriad of signaling cascades involved in highly diverse cellular processes.¹¹

In CRC, multiple studies showed the frequency of *KRAS* gene mutation for more than 30% of all CRC cases, with the most common mutations occurring in hotspots codons 12 and 13 and less commonly in codon 61.¹² The most common *KRAS* gene point mutation is at glycine at position 12 (G12), followed by glycine at position 13 (G13), and glutamine at position 61 (Q61).¹² These mutations have been correlated with clinical outcomes, particularly poor prognosis and shorter overall survival.^{13–16} Furthermore, the *KRAS* mutational status directs treatment choices. *KRAS* mutations may lead to tumor resistance to cell surface receptor tyrosine kinase inhibitors as well as EGFR-targeted monoclonal antibodies.²

In contrast with the higher frequency of *KRAS* mutation reported in Western countries, a lower frequency has been noted in some Asian cases such as Taiwanese-Chinese (26.5%) and Japanese (23%) patients.^{17,18} However, very limited data have been presented on the mutational analysis of *KRAS* in Southeast Asian populations. This study aimed to characterize *KRAS* mutations in CRC tissues from Filipino patients. The results of this study would be significant, adding information to the limited data and understanding of the association of *KRAS* mutation with CRC clinical and tumor characteristics.

Materials and methods

Samples

Paired frozen normal and tumor tissues from the 35 previously identified CRC patients who underwent surgical resection at St. Luke's Medical Center (SLMC) in Manila, Philippines, were used in the study. Patients from whom these samples were obtained agreed to participate in the study by signing consent forms. Pertinent data such as gender, age, and family history of CRC were obtained. This project was approved by the Institutional Scientific Review Board and Institutional Ethics Review Board of SLMC, no. 99-006. Tumor and normal samples were stored in a -80°C biofreezer.

DNA extraction and quantification

DNA extraction was carried out according to the manufacturer's

instructions (QIAamp DNA Mini Kit, Qiagen). In summary, a lysis solution containing proteinase K was used to disrupt the cell membrane and release DNA. DNA was precipitated with ethanol and washed with wash buffers to remove residual contaminants from the isolated genomic DNA (gDNA). The purified gDNA was eluted from the column and consequently used for mutation analysis. DNA quantity and quality were assessed by spectrophotometry (Nanodrop® v.1000, Thermo Fisher Scientific). DNA yield and purity were determined based on the concentration of DNA in the eluate measured by absorbance at 260nm/280nm. Pure DNA typically has an absorbance at A260/A280 ratio of 1.7–1.9.

Polymerase chain reaction (PCR) of *KRAS* gene

KRAS gene was detected by PCR performed on a 96-well plate palm cycler (Corbett). A reaction mixture of 25 μL consisted of 0.05 U/ μL Taq polymerase and PCR reaction buffer (Taq PCR Core Kit, Qiagen), 2 mM MgCl_2 , 0.25 mM dNTP, and 0.10 μM of each primer. The PCR conditions were set as follows: one cycle of 94°C for 5 m; 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 45 s, and one cycle of 72°C for 7 m. The primer sequences used were based on previously published sequences in the National Center for Biotechnology Information GenBank with accession numbers: L0005, L0006, L0007, L0008, and L0009. The primer sequences of the *KRAS* gene are shown in Table 1. To confirm, the PCR product was electrophoresed on a 2% agarose gel and consequently stained with ethidium bromide. The stained gel was viewed under a UV (Ultraviolet) transilluminator (BioRad).

Denaturing high-performance liquid chromatography (DHPLC)

PCR products were screened for mutations in all exons of the *KRAS* gene using DHPLC (Helix™ System, Varian, Inc.). DHPLC identifies mutations and polymorphisms by detecting heteroduplexes that contain mismatched nucleotides, formed from two PCR products. Sequence variation creates a mixed population of homoduplexes and heteroduplexes when wild-type and mutant DNA are denatured and reannealed. A mix of approximately equal ratios of normal and tumor DNA amplicons required post-PCR processing to create partially denatured DNA strands. Amplified PCR fragments were denatured at 95°C for 5 m before being allowed to reanneal by gradually lowering the temperature from 95°C to 65°C in about 1 m. Initial column temperatures (Ti) for each of the five exons were calculated using the DHPLC Melt Program available on the Stanford DHPLC website (<http://insertion.stanford.edu/meltdoc.html>).

Prior to running the samples, an evaluation of the system performance with pUC18 HaeIII (Sigma-Aldrich) digest was undertaken in a non-denatured condition at 50°C . The resolution of the 257 and 267 base pair (bp) peaks indicated adequate performance

Table 2. Frequency of samples/exons positive for mutation in DHPLC and sequencing

Exon	DHPLC		Sequencing	
	Mutant (%)	Wild type (%)	mutant (%)	Wild type (%)
2	5 (14)	30 (86)	3 (9)	32 (91)
3	1 (3)	34 (97)	0 (0)	35 (100)
4A	2 (6)	33 (94)	0 (0)	35 (100)
4B	9 (26)	26 (74)	6 (17)	29 (83)
total	17 (49)	18 (51)	9 (26)	26 (74)

DHPLC, denaturing high-performance liquid chromatography.

to run the samples. A triplicate injection of 2 μ L was performed for each sample. Results were presented as a chromatogram, a series of peaks corresponding to the DNA fragments. Separation of DNA fragments by size was achieved by differential absorption or partition between the liquid (mobile) phase and the matrix (stationary phase) of the column. DNA fragments were detected by UV absorbance at 260 nm, and sample analysis lasted for 10 m.

DNA sequencing

PCR amplicons found to have variations in DHPLC elution profiles attributable to sequence changes, along with their corresponding normal DNA samples, were purified using a DNA Gel Extraction Spin Column (BioRad). The purified DNA was sent to Microgen (Seoul, Korea) for DNA sequencing (ABI 3730xl, ABI Prism). Additionally, five tumor samples with single peaks (normal elution profiles) were also sequenced. The DNA sequences obtained were aligned and comparing with sequences from the databases of the National Center for Biotechnology Information. A consensus sequence per sample in each exon was obtained using the downloadable free software BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Chromatograms of the sequenced DNA were analyzed for mutations or sequence variations using DNAsis software (Hitachi Software Engineering Co., Ltd.).

Statistical analysis

Statistical analysis was calculated using GraphPrism version 5.01 (GraphPad Software, Inc.). Associations between clinicopathological characteristics (age, sex, tumor location, grade, and stage) and *KRAS* mutation status were determined using either Pearson's chi-square test or Fisher's exact test, depending on which was more appropriate. Survival relevance was analyzed using Kaplan-Meier curves and the log-rank test, with grouping based on *KRAS* mutation status. All tests were two-tailed, and significance was considered at a *p*-value of less than 0.05.

Results

CRC patient distribution

This study included 35 CRC patients of Filipino descent, comprising nineteen (54%) males and sixteen (46%) females with ages ranging from 34 to 77 years and a mean age of 60. Seventeen (49%) patients belonged to the age group younger than 60 years old, while eighteen (51%) were 60 years and above. Based on tumor node metastasis (TNM) staging, four (11%) were in stage I, twelve (34%) were in stage II, fourteen (40%) were in stage III, and five (14%) were in stage IV. Regarding tumor histological differentiation, three (9%) were poorly differentiated, twenty-six

(74%) were moderately differentiated, four (11%) were well differentiated, and two (6%) were of the mucinous type. Based on tumor location, seventeen (49%) were in the distal region (splenic flexure through the sigmoid, including the rectosigmoid), ten (29%) were in the proximal region (cecum through the transverse colon), and eight (23%) were in the rectum. No differences were noted between males and females for all parameters analyzed.

Detection of aberrant *KRAS* of CRC patients through DHPLC

Following PCR amplification of all five exons of the *KRAS* gene, paired normal and tumor DNA samples from all 35 CRC patients were screened using DHPLC. The data showed seventeen samples (49%) with aberrant chromatograms, comprising five samples (14%) for exon 2, one (3%) for exon 3, two (6%) for exon 4A, and nine (26%) for exon 4B. Mutation screening was not conducted for exon 1 due to its small product size (89 bp). DHPLC instrument can only detect PCR fragment sizes ranging from 150 to 500 bp. Multiple peaks in chromatogram outputs were observed in exon 2 and exon 4B from the DHPLC data. Consequently, all samples with aberrant chromatograms were analyzed by DNA sequencing.

Presence of *KRAS* mutation in CRC tissues detected through DHPLC and DNA sequencing

Seventeen tumor DNA samples that exhibited aberrant chromatogram profiles in DHPLC were further analyzed by sequencing. Additionally, all purified PCR products for exon 1 were sequenced. As controls, five of the tumor samples with single peak (normal) chromatogram profiles were also sequenced to validate the sensitivity of the screening method used. The results of these single peak samples did not show any base changes in their DNA sequence. Table 2 shows the results of tumor samples analyzed by both DHPLC and sequencing. The frequency of *KRAS* mutations in exon 3 and exon 4A by DHPLC and sequencing were 3% (1/35) and 6% (2/35), respectively. For exon 2, they were 14% (5/35) by DHPLC and 9% (3/35) by sequencing. All tumor samples positive for mutation in exon 2 by sequencing were found to have mutations in DHPLC. The proportion of CRC samples positive for exon 4B by DHPLC was 26% (9/35), while only 17% (6/35) were positive for exon 4B by sequencing. Similar to exon 2, all exon 4B positive for sequencing were found to have mutations by DHPLC.

Table 3 shows a total of twenty (57%) *KRAS* mutations observed in eighteen (51%) of the 35 patients. Among all mutations, eleven mutations (55%) were noticed in exon 1 as follows: One in codon 13 (GGC→TGC; G13C), four in codon 19 (TTG→TGG; L19W), five in codon 23 (CTA→CTG; L23, silent mutation), and one in codon 11 (insertion C, A11R, frameshift mutation). In exon 2, three (15%) samples had mutations in codon 54 (GAT→CAT, D54H). In exon 4B, six (30%) samples had mutations in codon 173

Table 3. Sequencing analysis of *KRAS* gene

Codon	Nucleotide change	Amino acid change	Consequence	n (%)
11	Ins-C	A11R	Frameshift mutation; possible pathogenic mutation	1 (5%)
13	GGC→TGC	G13C	Missense mutation; possible pathogenic mutation	1 (5%)
19	TTG→TGG	L19W	Missense mutation; Possible pathogenic mutation	4 (20%)
23	CTA→CTG	L23	Silent mutation	5 (25%)
54	GAT→CAT	D54H	Missense mutation; possible pathogenic mutation	3 (15%)
173	GAT→GAC	D173	Silent mutation	6 (30%)

(GAT→GAC; D173, silent mutation).

Association of *KRAS* mutation and tumor characteristics

Table 4 presents the correlation between *KRAS* mutations (possible pathogenic mutations) and the clinical characteristics of the patients. Among nine cases with these pathogenic mutations, six (67%) were female, while only three (33%) were male. Regarding age, three (33%) were observed in CRC patients younger than 60 years old, while six (67%) were 60 years old and above. Mutations were predominantly found in moderately differentiated tumors (67%), with similar frequencies in poorly differentiated (11%), well-differentiated (11%), and mucinous type (11%) tumors. Based on location or site, more mutations were found in the proximal region (56%) than in the distal (33%) and rectum of

the large intestine (11%). *KRAS* mutations were significantly more prevalent in TNM stage III tumors (89%) than in stage II (11%), while there were no mutations observed in stage I and IV samples ($p = 0.007$, Chi-square test). Overall, except for TNM stage, there was no significant association between *KRAS* mutations and the clinical characteristics of the patients.

Association between *KRAS* mutation and CRC patient prognosis

Kaplan-Meier curve and log-rank test were performed to determine the prognostic values of *KRAS* mutations to the patient's overall survival. Twenty-six patients were followed for 60 months post-surgery, the timing defined as the cut-off for survival. The

Table 4. Correlation of possible pathogenic *KRAS* mutation with clinicopathological characteristic

Variables	Freq	Mutations	No mutations	Total	χ^2	p
		n (%)	n (%)	n (%)		
Sex	Male	3 (33)	16 (61)	19 (54)		0.245
	Female	6 (67)	10 (39)	16 (46)		
	Total	9 (100)	26 (100)	35 (100)		
Age	< 60	3 (33)	16 (61)	19 (54)		0.245
	≥ 60	6 (67)	10 (39)	16 (46)		
	Total	9 (100)	26 (100)	35 (100)		
TNM stage	I	0 (0)	4 (15)	4 (12)	12.252	0.007
	II	1 (11)	11 (42)	12 (34)		
	III	8 (89)	6 (23)	14 (40)		
	IV	0 (0)	5 (19)	5 (14)		
	Total	9 (100)	26 (100)	35 (100)		
Tumor grade	Poor	1 (11)	1 (4)	2 (6)	1.677	0.795
	Moderate	6 (67)	20 (77)	26 (74)		
	Well	1 (11)	3 (11)	4 (11)		
	Mucinous	1 (11)	2 (8)	3 (9)		
	Total	9 (100)	26 (100)	35 (100)		
Location	Proximal	5 (56)	5 (19)	10 (29)	4.637	0.098
	Distal	3 (33)	12 (46)	15 (42)		
	Rectum	1 (11)	9 (35)	10 (29)		
	Total	9 (100)	26 (100)	35 (100)		

TNM, tumor node metastasis.

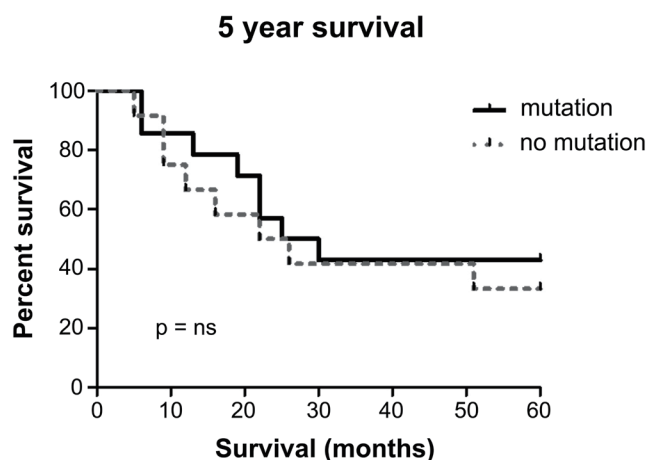


Fig. 1. *KRAS* mutational analysis for patients' prognosis. Kaplan-Meier analysis of colorectal cancer patients' overall survival 60 months after surgery. m, months; ns, not significant; *p*, *p*-value.

Kaplan-Meier curve analysis revealed no significant difference in patient survival, with the median values of survival of 27.5-month vs 24.0-month for *KRAS* mutation and no mutation group, respectively (Fig. 1).

Discussion

Mutations activating the *KRAS* gene are considered a key step in the progression from normal colorectal epithelium to carcinoma. These mutations may lead to increased proliferation of CRC cells, and in combination with other mutations (e.g., *APC*) can promote tumorigenesis.¹⁹ In fact, *RAS* is one of the most known proto-oncogenes, with its mutation occurring in around 30% of all human cancers.²⁰

In the present study, we screened and analyzed the entire five coding regions of the *KRAS* gene for mutations using DHPLC and direct DNA sequencing. DHPLC is an accurate, sensitive, and efficient screening technique used for distinguishing the variation in DNA sequence changes by heteroduplex analysis,²¹ making it suitable for detecting single nucleotide substitution or single nucleotide polymorphism. This technology has been used for mutation screening in genes causing diseases such as *BRCA1* and *BRCA2* genes in breast cancer,^{22,23} *CFTR* gene in cystic fibrosis,^{24,25} *RBI* gene in hereditary retinoblastoma,²⁶ and *BRAF* (V600E) in papillary thyroid carcinoma.²⁷ DHPLC presented better sensitivity than single-strand conformation polymorphism, with a range of about 92.5 to 100%, in the mutation screening of genetically heterogeneous diseases, such as Charcot-Marie-Tooth neuropathy.²⁸ Several studies have used DHPLC to verify *KRAS* mutations determined by high-resolution melt analysis or direct sequencing.^{29,30}

Nine mutations were identified (Table 3), dictating changes in amino acid and consequently altering the protein product, which may be considered pathogenic. All these mutations were located in the conserved region of the *KRAS* gene which carried the most important information responsible for the correct functioning of the protein.³¹ Point mutations in this conserved region block the GTPase activity, leading to constitutively active and oncogenic proteins.³²

Eight of nine mutations reported in this paper were found outside the hotspot codons. Among these, five resulted in a non-conservative amino acid substitution (e.g., replacement of an amino

acid by another with different chemical properties) at residues most likely essential for the functioning of the protein.³³ Only one out of nine mutations reported in this paper occurred in the hotspot of *KRAS* gene (codon 13). A base substitution (G – T) in codon 13 of exon 1 brought about an amino acid change from Glycine to Cysteine (G13C). This mutation has been noticed in a small percentage of CRC patients in North Africa and the Chinese population.^{34,35}

Mutation L19W of exon 1 was found in four patients. A silent mutation codon 19 was previously reported in a CRC group of Dutch patients.³⁶ It was demonstrated that a single point mutation within the coding sequences has a transforming effect on the *KRAS* gene.³⁷ Meanwhile, a silent mutation in codon 173 of exon 4B (GAT→GAC) was noticed in six patients. This silent polymorphism was also seen in a group of Dutch individuals.³³ Interestingly, in this study, we found a novel point mutation in three CRC patients. This missense mutation in codon 54 in exon 2 resulted in an amino acid change from aspartic acid to histidine (D54H).

From statistical analysis, a significant association was noticed between the presence of *KRAS* mutation and tumor stage. *KRAS* mutations were more frequent in stage III tumors with regional lymph node metastases, with eight of the nine cases having potentially pathogenic mutations. However, this finding needs further validation. Our data are in agreement with the previous study indicating that *KRAS* mutations were significantly more common in advanced-stage tumors.³⁸ No significant associations were found between overall *KRAS* mutations and clinical characteristics of patients such as gender, age, tumor differentiation, and tumor site, in accordance with previous studies.^{35,39} There was also no association between mutations and overall patient survival. However, although not statistically significant, more male than female patients had *KRAS* mutation, and the most commonly affected site was the distal colon. However, in future works, the number of analyzed samples must be increased to indicate a better correlation between findings.

A recent study from the Philippines showed that using next-generation sequencing, mutations were reported in *KRAS* gene at codon 12 (G12S) and codon 59 (A59T) in Filipinos CRC patients, together with a novel mutation in codon 137 (Y137C). In the *in vitro* model, these mutations induced gross changes in F-actin cytoskeletal organization and cellular morphology of the cells.⁴⁰

Future perspectives

The overall survival is maximized when the highest standards of patient care are provided through a multidisciplinary team.⁴¹ Thus, information from gene mutation screening in CRC patients could be useful to guide a multidisciplinary team in delivering optimal results in the standard-of-care. It is important to note that this study was limited to screening for *KRAS* mutations. It could be useful to determine the association of the reported *RAS* gene mutations in this study with *RAF* (*BRAF*) mutation status to better identify possible *RAS/RAF* associations in CRC.⁴² Techniques like whole exome sequencing should also be considered to characterize the mutation frequency of the protein-coding region of interest. Despite being conducted with a limited number of patients, this report provides valuable information on the diverse genetic profiling of *KRAS* mutations in Filipino CRC patients, which could be useful for the treatment and knowledge of the disease.

Conclusions

This study was able to observe that Filipino CRC patients do

not show variation in the highly reported *KRAS* codon 12 and 13 hotspot mutations. The presence of *KRAS* mutation is associated with stage III tumors. This study also successfully demonstrated a novel *KRAS* mutation, D54H, resulting from DHPLC and DNA sequencing analysis. Such evidence, promote the use of denaturing high-performance liquid chromatography, for mutational screening which may help increase reports of gene mutational profiles in Southeast Asian populations. This study is one of the first few reports on the mutational status of *KRAS* in Filipino CRC patients, possibly defining a different *KRAS* mutation profile of Southeast Asian/Filipino populations.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

Study concept and design (MLDE), data collection (MLN, CAM, LKDC, ERC, FFN), statistical analysis (CAM), data interpretation (MLN, CHCS), manuscript preparation (MLN, LKDC, CHCS), literature search (MLN, LKDC, CHCS, MLDE), funds collection (ERC, FFN, MLDE). All authors have made significant contributions to this study and have approved the final manuscript.

Data sharing statement

No additional data are available.

Ethical statement

The procedures followed in this study were approved by the Institutional Scientific Review Board and Institutional Ethics Review Board of St. Luke's Medical Center, Philippines (no.99-006) in correspondence with the ethical guidelines of the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all participants before enrolled in this study.

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