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Original Article

Molecular characteristics of KRAS, BRAF, and PIK3CA mutation frequency in sessile serrated adenomas and polyps in patients from central Taiwan

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Background: Colorectal cancer (CRC) is one of the most common cancers, and patients with CRC usually present with several polyps that can be detected by colonoscopy, which is a commonly used screening tool. Sessile serrated adenomas (SSAs) were a polyp type considered to have a high risk of becoming interval colorectal cancer following negative colonoscopy.
Aim: The purpose of this study was to evaluate the molecular features of SSAs in central Taiwan.
Methods and Results: We used the FemtoPath primer kit to detect BRAF, KRAS, and PIK3CA mutations for 32 specimens: BRAF (V600E), KRAS (G12D, G12C, and G13D), and PIK3CA (H1047Y) mutations were detected.
Conclusion: Almost all SSA samples contained BRAF mutations, and KRAS gene mutations were detected to a statistical significance for patients with high dysplasia and for those with more than three polyps.

Keywords: Interval cancer, Sessile serrated adenomas (SSAs), KRAS, BRAF, PIK3CA

Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide (1, 2). In Taiwan, the Health Promotion Administration indicates that colorectal cancer is still one of the most commonly diagnosed cancers and in 2016 was the third most common cause of cancer-related deaths in men and women (3). Colonoscopy is utilized either as a primary screening tool or as a follow-up exam when other screening tests are positive and significantly contributes to reducing colon cancer development (4).

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However, colonoscopy is not a perfect screening tool for the detection of neoplasia or interval colorectal cancer (5).

Sessile serrated adenomas (SSAs) or sessile serrated polyps (SSPs) are early precursor lesions in the serrated neoplasia pathway and considered to be major contributors to interval cancers, which can rapidly become dysplastic or invasive carcinomas. Interval colorectal cancer is a CRC for which malignant colon cancer developed within 60 months after a negative colonoscopy (4). The development of interval colorectal cancer is related to lifestyle, sex, age, family history of bowel cancer, comorbidities, digestive tract diverticulum, and the skills of endoscopists (6). Hence, the incidence of interval colorectal cancer has been found to vary greatly, and no accurate and simple methods are available to detect or classify polyps for clinical operation (7). Moreover, the carcinogenesis process of interval colorectal cancer that might be regulated by genes and tumor microenvironment remains unclear (8). As a result, there is an increasingly urgent need for insight regarding the tumorigenesis of interval colorectal cancer and for interval colorectal cancer prevention strategies that reduce the risk of incidence within 5 years after a negative colonoscopy (9).

With the rapid development of personalized genetic testing, biomarker gene information is obtained to help doctors decide how to undertake surveillance and treatment and how to provide information on future drug replacement needs, and such information even serves as a reference for determining recurrence risk (10–12). The common genetic changes caused by colorectal cancer involve the mutation of APC/β-catenin genes, KRAS genes, BRAF genes, and PIK3CA genes, which mediate cellular responses to growth signals (13). Therefore, the aim of this study was to detect the mutation rates and mutation types for KRAS, BRAF, and PIK3CA genes in SSAs and SSPs with high-sensitivity amplification gene sequencing and to identify specific molecular characteristics and genotypes in patients in central Taiwan.

Materials and methods

Patients and samples

The study's 29 participants included 7 women and 22 men who had received polypectomies, biopsies, endoscopic mucosal resection, and endoscopic submucosal dissection and who had been diagnosed with SSAs in a national cancer screening program at Chung Shan Medical University Hospital in Taiwan, for which they had enrolled between 2013 and 2017. A total of resected polyps were 32 polyps that were classified according to World Health Organization criteria and general consensus by experienced pathologists. Two criteria were used for these polyps: One was that they be diagnosed as SSA type, and the other was that the sum of the length and width of all these samples must be more than 0.7 cm each. The study was a retrospective study conducted in Chung Shan Medical University and Hospital in central Taiwan and was approved by the Institutional Review Board of Chung Shan Medical University Hospital (CSMUH No. CS2-18028).

DNA extraction

All samples are formalin-fixed paraffin-embedded tissue and were dissected in two 10-μm-thick sections. The DNA extraction of formalin-fixed paraffin-embedded tissues was performed using the AccuPure FFPE Tissue DNA Manual Kit (AccuBioMed, New Taipei City, Taiwan). The extracted DNA quality and quantity was assessed using BioPhotometer Plus (Eppendorf AG, Hamburg, Germany).

PCR and DNA sequencing of KRAS, BRAF, and PIK3CA gene mutation

The gene analysis was undertaken using the FemtoPath BRAF Exon 15 primer set, the FemtoPath KRAS exon 2 primer set, the FemtoPath PIK3CA exon 9 primer set, and the FemtoPath PIK3CA exon 20 primer set (HongJing Biotech. and PoloWang Biotech., New Taipei City, Taiwan). The PCR reaction mixtures were prepared in a solution of total volume of 20 μL containing 2X Master Mix, each primer mix (HongJing Biotech, New Taipei City, Taiwan), sterile water, and 100 ng of template DNA. The PCR conditions of BRAF exon 15, KRAS exon 2 and PIK3CA exon 9 was set up as following conditions: 95℃ for 10 minutes,

followed by 45 cycles of 94°C for 1 minute, 65°C for 3 minutes, 57°C for 1 minute, 72°C for 1 minute, and finally 72°C for 10 minutes, then preserved at 10°C. For PIK3CA exon 20, the PCR condition was 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute, and finally 72°C for 10 minutes, then preserved at 10°C. The PCR products were then detected using 2% agarose gel electrophoresis and stained with ethidium bromide for visualization. To analyze the presence of BRAF, KRAS, and PIK3CA gene mutations, all samples were directly assayed by DNA sequencing at Genomics Inc. (ABI 3730XL Genetic Analyzer, New Taipei City, Taiwan).

Statistical analysis

All statistical analyses were performed using SPSS software (version 13; IBM, Armonk, NY, USA). Group comparisons were carried out using the chi-square ($\chi 2$) test for each parameter. Two-tailed p values were calculated, and p values less than .05 were considered statistically significant.

Results

This study analyzed the mutation hotspots of BRAF exon 15, KRAS exon 2, PIK3CA exon 9,

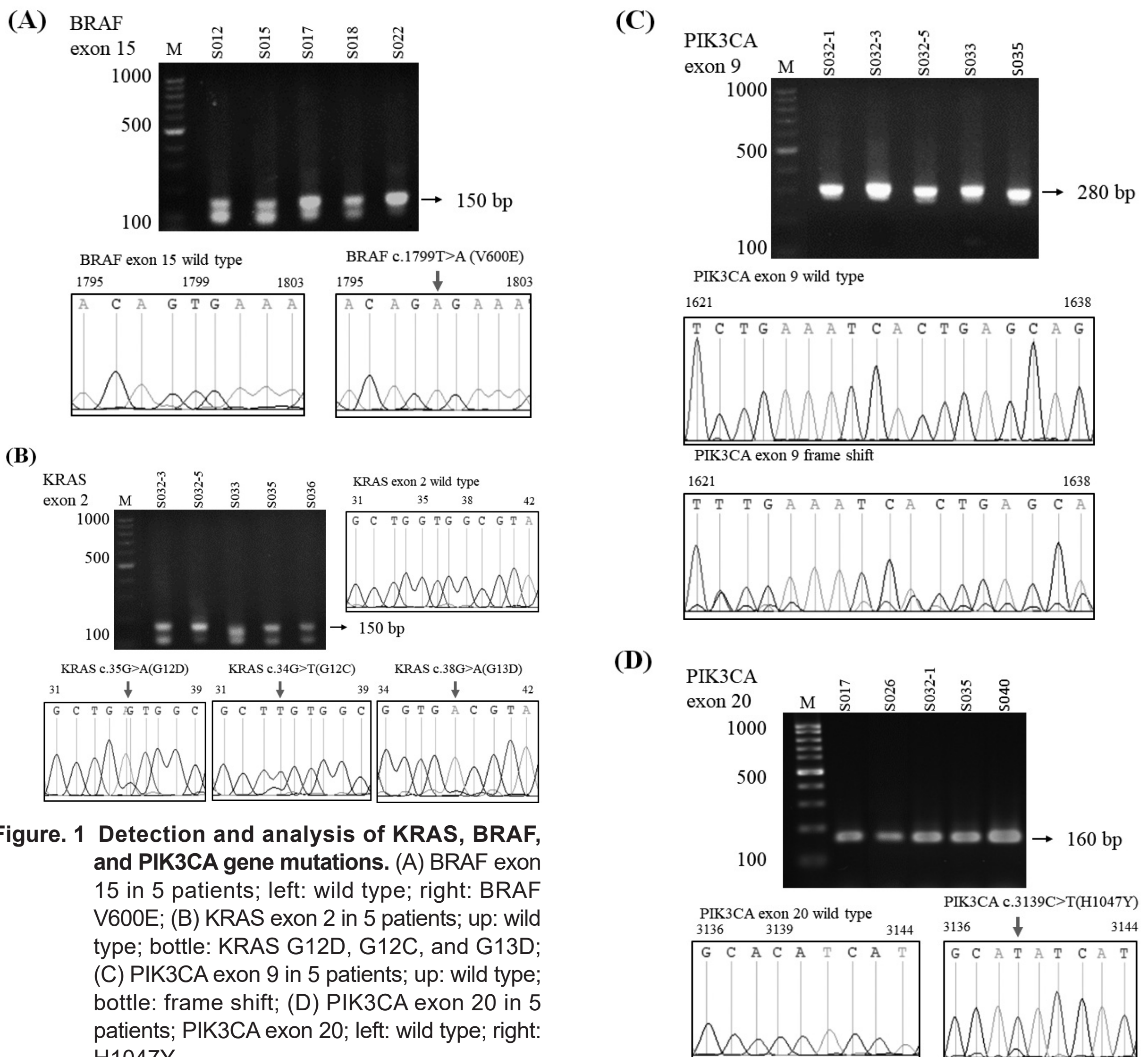

Figure. 1 Detection and analysis of KRAS, BRAF, and PIK3CA gene mutations. (A) BRAF exon 15 in 5 patients; left: wild type; right: BRAF V600E; (B) KRAS exon 2 in 5 patients; up: wild type; bottle: KRAS G12D, G12C, and G13D; (C) PIK3CA exon 9 in 5 patients; up: wild type; bottle: frame shift; (D) PIK3CA exon 20 in 5 patients; PIK3CA exon 20; left: wild type; right: H1047Y.

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Table I. BRAF mutation types in patients.

Sample	Codon	DNA change	Amino acid change	Type
S006	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S010	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S015	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S016	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S017	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S018	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S019	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S022	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S026	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S030	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S032-1	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S032-3	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S032-5	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S033	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S035	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S036	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S037	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S038	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S040	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S042-1	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S042-2	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S046	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S048	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S049	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S055	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S055	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
S057-2	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion
P013	600	GTG>GAG(T→A)	V600E (Val600Glu)	Transversion

and 20 in 32 SSA polyps from 29 patients. After PCR, we used 2% agarose gel to verify whether the PCR product had the targeted DNA fragment, then we sequenced the fragments. Figure 1 shows the results. The samples included one BRAF exon 15 (V600E) mutation type, three major KRAS exon 2 (G12D, G12C, G13D) mutation types, and one PIK3CA exon 20 (H1047Y) mutation type. The 32 SSA samples included no point mutations at PIK3CA exon 9 but did include one allele frame shift.

Tables I–III list all the mutation samples and types. Of the 32 samples, 28 (87.5%) contained BRAF V600E mutations. The high mutation rate

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Table II. KRAS mutation types in patients.

Sample	Codon	DNA change	Amino acid change	Type
S001	12	GGT>GAT(G→A)	G12D (Gly12Asp)	Transition
S012-2	13	GGC>GAC(G→A)	G13D (Gly13Asp)	Transition
S028	12	GGT>TGT(G→T)	G12C (Gly12Cys)	Transversion

Table III. PIK3CA exon 20 mutations in patients.

Sample	Codon	DNA change	Amino acid change	Type
S019	1047	CAT>TAT(C→T)	H1047Y (His1047Tyr)	Transition
S032-3	1047	CAT>TAT(C→T)	H1047Y (His1047Tyr)	Transition
S032-5	1047	CAT>TAT(C→T)	H1047Y (His1047Tyr)	Transition
S042-1	1047	CAT>TAT(C→T)	H1047Y (His1047Tyr)	Transition

of BRAF genes in SSA samples was similarly to other study results. However, the KRAS mutation rate (n = 3, 9%) was considerably lower than the BRAF mutation rate in SSA samples. The BRAF V600E mutation sample had no KRAS mutations; conversely, the KRAS mutation sample had no BRAF V600E mutations. Only one sample had neither BRAF V600E nor KRAS mutations. Moreover, the PIK3CA mutation rate (n = 4, 12.5%) was substantially lower than the BRAF V600E mutation rate. No samples contained PIK3CA exon 9 mutations, and only four samples contained PIK3CA exon 20 mutations.

We collected the clinical characteristics of every sample, as summarized in Table IV. In the results, patients over 50 years of age had significantly higher BRAF exon 15 mutation rates than younger patients did. Male patients also had higher BRAF exon 15 mutation rates than female patients did, but the difference was not statistically significant. Samples taken from the left colon had slightly higher BRAF exon 15 mutation rates than right colon samples did. The high-grade dysplasia sample had statistically significantly higher KRAS exon 2 mutation rates compared with the low-grade dysplasia sample rates. Patients with larger polyps (>6 mm) experienced higher BRAF exon 15 mutation rates.

In conclusion, we found that almost all SSA samples had BRAF mutations, especially codon 600 mutations. Patients who had more than three polyps or who had polyps larger than 6 mm had higher BRAF gene mutation rates. The KRAS exon 2 mutation rate was also significantly higher in the patients whose sample was diagnosed with high-grade dysplasia. However, the mutation rates for PIK3CA exon 9 and exon 20 were low, and were not statistically significant in all the parameters we analyzed.

Discussion

Research has shown that SSAs have a high risk of deteriorating to become malignant cancer (14). SSAs have a sessile or flat morphology, their borders are indistinct, and they occur predominantly in the right colon. The high risk associated with SSAs could be the difficulty of diagnosis with endoscopic detection and inadequate results of resection by polypectomy. Hence, incompletely resected SSAs or rapid SSA progression can lead to interval cancer.

Therefore, we attempted to utilize a high-sensitivity detection method to assist in clinical diagnoses. In the results, we observed that SSA samples had a high mutation rate for BRAF genes in Taiwan which is similar to the results of research in other countries (15). Nevertheless, no statistically significant differences were observed between BRAF exon 15 mutations for different ages, sexes, geographical locations, sample dysplasia grades, numbers of polyps, or polyp

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Table IV. Characteristics of KRAS, BRAF, and PIK3CA gene mutation rates in sessile serrated adenomas and polyps.

Variable	Total number	BRAF exon15 N=32 (%)		KRAS exon 2 N=31 (%)		PIK3CA exon 9 N=32 (%)		PIK3CA exon 20 N=30 (%)	
		wild	mutant	Wild	mutant	wild	mutant	wild	mutant
Age	32								
>50	23	4(12.5)	19(60)*	20(65)*	2(6)	23(72)	0	19(63)	2(7)
<50	9	0	9(28)	9(29)	0	9(28)	0	7(23)	2(7)
Gender									
Male	25	4(12.5)	21(66)	23(74)	2(6)	25(78)	0	20(67)	4(13)
Female	7	0	7(22)	6(19)	0	7(22)	0	6(20)	0
CEA maker									
>5	5	1(3)	4(12.5)	5(16)	0	5(16)	0	4(13)	0
<5	16	1(3)	15(47)	15(48)	0	16(50)	0	13(43)	2(7)
untested	11	2(6)	9(28)	9(29)	2(6)	11(34)	0	9(30)	2(7)
Site									
Right colon	13	2(6)	11(34)	11(35)	2(6)	13(41)	0	9(30)	2(7)
Left colon	17	2(6)	15(47)	16(52)	0	17(53)	0	15(50)	2(7)
Both	2	0	2(6)	2(6)	0	2(6)	0	2(7)	0
Dysplasia					***p*1=0.046**				
High	3	1(3)	2(6)	2(6)	2(6)	3(9)	0	3(10)	0
Low	29	3(9)	26(81)	27(87)	0	29(91)	0	23(77)	4(13)
Number of polyps					***p*2=0.030**				
>3	19	2(6)	17(53)	18(58)	1(3)	19(59)	0	15(50)	4(13)
<3	11	1(3)	10(31)	10(32)	0	11(34)	0	9(30)	0
unknown	2	1(3)	1(3)	1(3)	1(3)	2(6)	0	2(7)	0
Size of polyps									
>6 mm	19	2(6)	17(53)	17(55)	1(3)	19(59)	0	15(50)	2(7)
<6 mm	10	2(6)	8(25)	9(29)	1(3)	10(31)	0	8(27)	2(7)
unknown	3	0	3(9)	3(10)	0	3(9)	0	3(10)	0

Note: *: Results of groups with BRAF gene mutation and KRAS gene wild type are statistically significant ($p < 0.05$). *p*1: KRAS exon 2 gene mutations and dysplasia grade are statistically significant ($p = 0.046$). *p*2: KRAS exon 2 gene mutations and number of polyps are statistically significant ($p = 0.030$).

sizes. We speculated that two reasons for this were possible: (1) The sample size was not sufficient for statistically significant difference to emerge, or (2) the aforementioned parameters originally did not have a correction with BRAF mutation. The sample with BRAF V600E mutations did not have KARS exon 2 mutations. This result indicates that BRAF mutation is a key point in the serrated pathway. BRAF is an oncogene and encodes a protein called B-Raf. The B-Raf protein is involved in signaling transduction to direct cell growth. BRAF V600E mutation occurs in the activation site of the kinase domain and increases B-Raf protein kinase activity (16). Although only three SSA samples contained KRAS exon mutations, all three patients from whom these samples were obtained were diagnosed with high-grade dysplasia. This result was expected due to KRAS mutation and high-grade dysplasia being considered high-risk factors for malignancy. PIK3CA is also an important oncogene; 10%–20%

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of colorectal cancers involve PIK3CA exon 9 or 20 mutations (17). However, our study revealed no PIK3CA exon 9 mutations for SSA samples, and only four samples contained PIK3CA exon 20 mutations. Thus, we speculated that the pathway through which SSAs evolve to become malignant tumors was not via PIK3CA unlike the classical pathway of adenomas.

To conclude, almost SSA samples exhibited BRAF V600E mutation, but the KRAS and PIK3CA genes were wild types. Our study could provide diagnostic methods for clinical use in Taiwan to make accurate detection.

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