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ORIGINAL ARTICLE

Basic Study

Mutation analysis of 13 driver genes of colorectal cancer-related pathways in Taiwanese patients

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Abstract

AIM: To investigate the driver gene mutations associated with colorectal cancer (CRC) in the Taiwanese population.

METHODS: In this study, 103 patients with CRC were evaluated. The samples consisted of 66 men and 37 women with a median age of 59 years and an age range of 26-86 years. We used high-resolution melting analysis (HRM) and direct DNA sequencing to characterize the mutations in 13 driver genes of CRC-related pathways. The HRM assays were conducted using the LightCycler® 480 Instrument provided with

the software LightCycler® 480 Gene Scanning Software Version 1.5. We also compared the clinicopathological data of CRC patients with the driver gene mutation status.

RESULTS: Of the 103 patients evaluated, 73.79% had mutations in one of the 13 driver genes. We discovered 18 novel mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53* that have not been previously reported. Additionally, we found 16 *de novo* mutations in *APC*, *BMPR1A*, *MLH1*, *MSH2*, *MSH6*, *MUTYH* and *PMS2* in cancerous tissues previously reported in the dbSNP database; however, these mutations could not be detected in peripheral blood cells. The APC mutation correlates with lymph node metastasis (34.69% *vs* 12.96%, P = 0.009) and cancer stage (34.78% *vs* 14.04%, P = 0.013). No association was observed between other driver gene mutations and clinicopathological features. Furthermore, having two or more driver gene mutations correlates with the degree of lymph node metastasis (42.86% *vs* 24.07%, P = 0.043).

CONCLUSION: Our findings confirm the importance of 13 CRC-related pathway driver genes in the development of CRC in Taiwanese patients.

Key words: Colorectal cancer; Driver gene; Colorectal cancer-related pathway; Mutation; High-resolution melting analysis

Core tip: In Taiwan, colorectal cancer (CRC) has had the highest incidences among cancers recently. In a study of 103 patients with CRC, we identified 18 novel mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53*. We assessed the frequency of non-pathological somatic mutations during oncogenesis, which has not been explored before. Our results indicated 16 *de novo* mutations that have been previously described in a public database and were detected in cancerous tissues only, but not in the patient's blood cells. We suggest these mutation sites may belong to a frequent mutational hotspot in both germline and cancerous tissues.

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INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of mortality and morbidity in Western and in Asian countries. In 2014, an estimated 136830 new cases were diagnosed, and 50310 deaths were due to CRC, making it the third most common cancer among men and women in the United States[1]. The lifetime risk of CRC is 6%, and the average age at diagnosis is 66 years old in the United States[2]. CRC has also become the third leading cause of cancer-related death in the Taiwanese population[3].

Inherited CRCs can be attributed to hereditary nonpolyposis CRC (HNPCC), familial adenomatous polyposis (FAP), and closely related variant syndromes[4]. Approximately 15%-30% of the patients may fall into this category, and their first- or second-degree relatives may have CRC[5]. New or *de novo* germ-line mutations of adenomatous polyposis coli (*APC*) occur in approximately 25% of FAP cases. The lifetime incidence of CRC in untreated FAP patients is approaching 100%[6]. The most common germline *APC* mutations are located at codons 1061 and 1309[7]. HNPCC is the result of germline mutations in DNA mismatch repair (*MMR*) genes, including mutL homolog 1 (*MLH1*), mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*) and PMS1 homolog 2 (*PMS2*), with mutations in *MLH1* and *MSH2* being more common than those in other *MMR* genes[8]. Juvenile polyposis syndrome is caused by mutations in the bone morphogenetic protein receptor, type 1A (*BMPR1A*) or SMAD family member 4 (*SMAD4*) tumor suppressor genes[9]. Cowden syndrome is associated with mutations in phosphatase and tensin homolog (*PTEN*)[10]. Homozygous mutations in the base excision repair (BER) pathway gene mutY DNA glycosylase (*MUTYH*) cause *MUTYH*-associated polyposis syndrome, and heterozygous *MUTYH* mutations are found in some cases of familial CRC[11].

Sporadic CRCs account for approximately 70%-85% of all cases, and these patients have no distinguishable genetic risk factors. The development of sporadic CRC is probably as a result of diet, lifestyle, and environmental factors as well as somatic mutations[12]. Sporadic CRCs have more biological variables compared with hereditary CRCs[13]. Chromosomal instability (CIN), microsatellite instability and CpG island methylator phenotype pathways are the major genetic mechanisms responsible for sporadic CRCs[14]. The CIN pathway implies the progression from adenoma to carcinoma. This pathway suggests a stepwise pattern of mutational inactivation of tumor suppressor genes, such as the *APC* and tumor protein p53 (*TP53*), and the activation of oncogenes, such as Kirsten rat sarcoma viral oncogene homolog (*KRAS*)[15]. Most sporadic CRCs (70%-80%) have *APC* somatic mutations, and the mutations appear to be enriched in the mutation cluster region (MCR, codons 1309 to 1450)[7]. Approximately 40% of CRCs have *KRAS* mutations, and almost all of these mutations are located at codons 12, 13 or 61[16,17]. The MSI pathway

is characterized by the inactivation of the MMR genes such as *MLH1*. Inactivation of MMR genes occurs either through *MLH1* promoter hypermethylation or point mutations in one of the MMR genes. *De novo* germline mutations or somatic mutations in MMR genes account for a small number of sporadic CRCs[8]. The Serrated Pathway is characterized by the presence of a mutation in the oncogene v-raf murine sarcoma viral oncogene homolog B (*BRAF*) and the hypermethylation of other genes[18]; 3%-13% of CRC patients have a mutation in the *BRAF* gene[19].

Multiple previous reports have revealed that several critical genes and pathways are important in the initiation and progression of CRC, these include WNT, RAS-MAPK, PI3K, TGF-β, P53, and DNA MMR pathways[20]. The Cancer Genome Atlas Network project has identified numerous recurrently mutated genes[21].

The aim of our study was to assess the genes known to be implicated in CRC and to compare the clinicopathological data with the molecular genetic profiles of the tumors. We used a high resolution melting (HRM) technique and direct DNA sequencing to analyze the *APC* exons 1-14 and part of exon 15, *BRAF* exon 15, *KRAS* exon 2, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) exon 9 and 20, the complete coding region of *BMPR1A*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PMS2*, *PTEN*, *SMAD4* and *TP53* in CRCs from 103 Taiwanese patients.

MATERIALS AND METHODS

DNA samples

One hundred and three colorectal adenocarcinomas were collected and analyzed. All samples were tested for sporadic and familial genetic changes in known CRC related genes (*APC*, *BMPR1A*, *BRAF*, *KRAS*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PIK3CA*, *PMS2*, *PTEN*, *SMAD4* and *TP53*). DNA was extracted using a commercially available kit (GE Healthcare, Little Chalfont, UK), following the manufacturer's recommendations. After extraction, DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington DE, United States). This study was approved by the Institutional Review Board of the China Medical University Hospital.

HRM technique

To assess *APC*, *BMPR1A*, *BRAF*, *KRAS*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PIK3CA*, *PMS2*, *PTEN*, *SMAD4* and *TP53* mutations, we performed HRM of small amplicons using a LightCycler® 480 Instrument (Roche Diagnostics, Roche Instrument Center AG, Rotkreuz, Switzerland) in tumor samples from CRC patients. The primers used for HRM analysis are shown in Supplementary material. The amplifications were performed in 10 μL volumes containing 10 ng of genomic DNA, 0.25 μmol/L primers, 2.5 mmol/L $MgCl_2$ and 5 μL 2X LightCycler® 480 High Resolution Melting Master (reference 04909631001, Roche Diagnostics) buffer. Polymerase chain reaction cycling included an initial denaturation at 95 ℃ for 10 min followed by 45 cycles of 15 s at 95 ℃, 15 s at 60 ℃, and 15 s at 72 ℃. The melting program included three steps: denaturation at 95 ℃ for 1 min, renaturation at 40 ℃ for 1 min, and a subsequent melting cycle that consists of a continuous fluorescent reading from 60 to 90 ℃ at a rate of 25 acquisitions per ℃.

Gene scanning

Gene scanning analysis of the data using Gene Scanning Software consisted of three steps: (1) normalization of melting curves, which involved setting the initial fluorescence equal to 100% and the remaining fluorescence signal after DNA dissociation to 0%; (2) shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA was completely denatured; and (3) generation of difference plots, allowing capture of the melting profile difference between the reference sample curves and the test samples.

Direct sequencing

After HRM analysis, the samples were purified using the PCR-M™ clean up system (VIOGEN, Sunnyvale CA, United States). The sequence reaction was performed using 1 μL of the purified PCR product, 2.5 μmol/L of one of the PCR primers and 1 μL ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA) in a final reaction volume of 10 μL. The samples were sequencing using a 25-cycle PCR program (denaturation at 96 ℃ for 10 s, annealing at 50 ℃ for 5 s and elongation at 60 ℃ for 4 min). The sequencing detection was performed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Due to its size (2545 nucleotides) it was costly and time consuming to screen *MSH6* exon 4 using HRM; thus, direct DNA sequencing was performed.

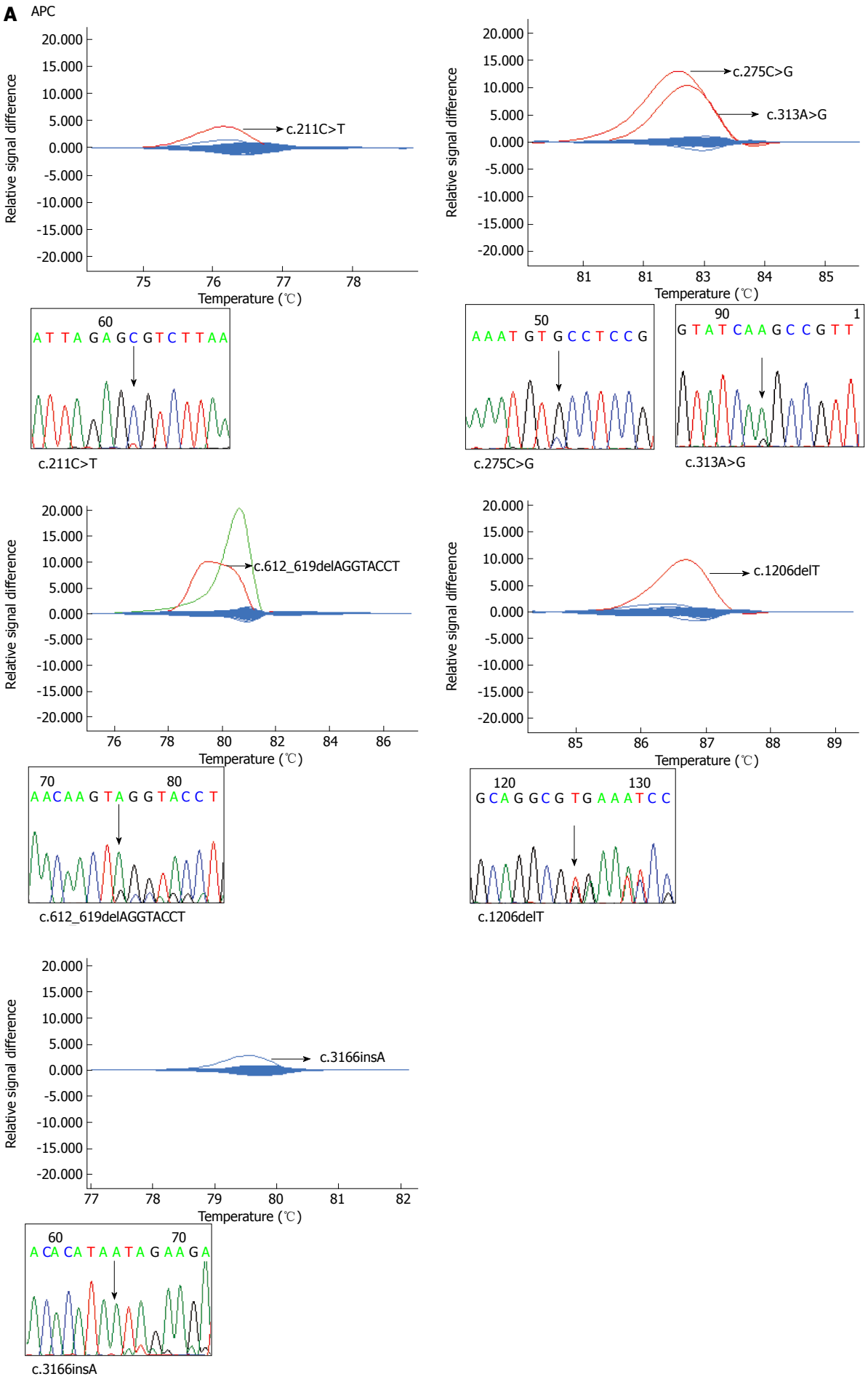
Statistical analysis

The results were analyzed using SPSS version 17.0 program. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Novel pathological mutations in the 13 driver genes

In total, we validated 18 mutations in six genes that have not been previously described in a public database (Figure 1). Six of these mutations occurred in the *APC* gene (c.211C>T, c.275C>G, c.313A>G, c.612_619delAGGTACCT, c.1206delT and c.3166insA), one in the *MLH1* gene (c.629C>T), three in the *MSH2* gene (c.166G>T, c.232G>A and c.718delG), one in the *PMS2* gene (c.1313A>C), three in the *SMAD4*


A
APC
Relative signal difference
Temperature (℃)
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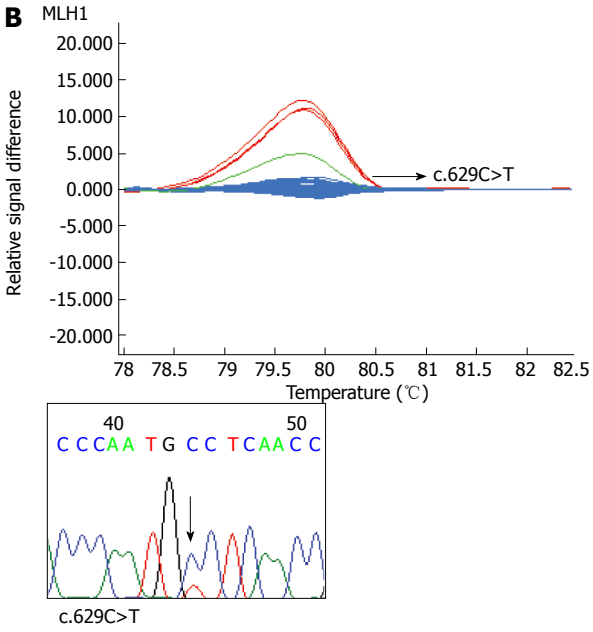
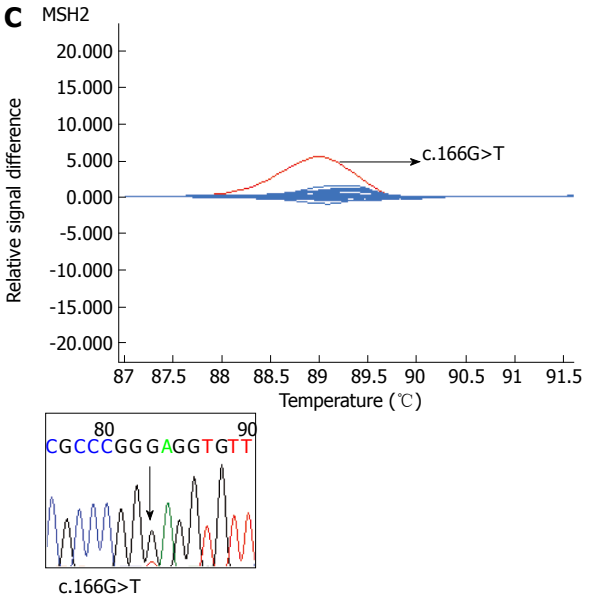
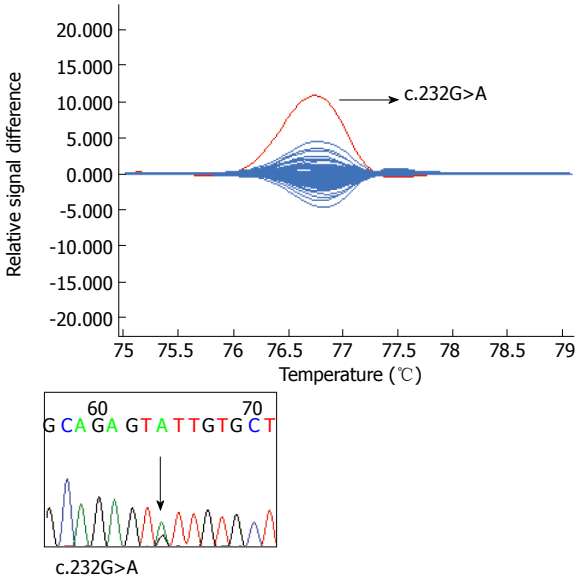
c.275C>G
c.313A>G
c.612_619delAGGTACCT
c.1206delT
c.3166insA


B
MLH1
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.629C>T
78 78.5 79 79.5 80 80.5 81 81.5 82 82.5
Temperature (℃)
40 50
C C C A A T G C C T C A A C C
c.629C>T


C
MSH2
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.166G>T
87 87.5 88 88.5 89 89.5 90 90.5 91 91.5
Temperature (℃)
80 90
C G C C C G G G A G G T G T T
c.166G>T


Relative signal difference
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15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.232G>A
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Temperature (℃)
60 70
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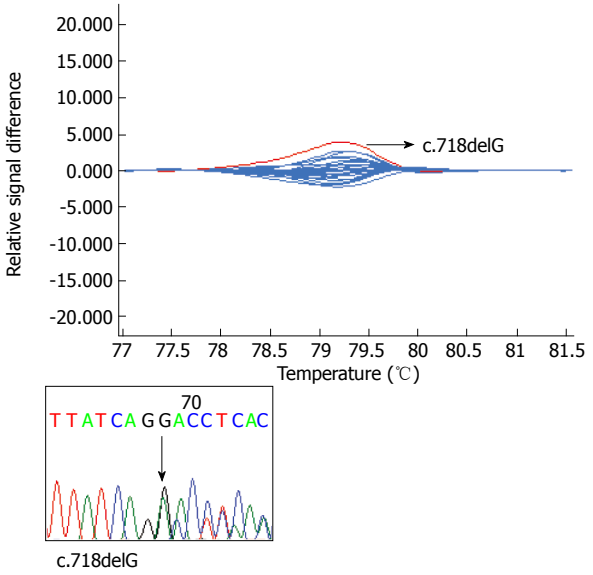
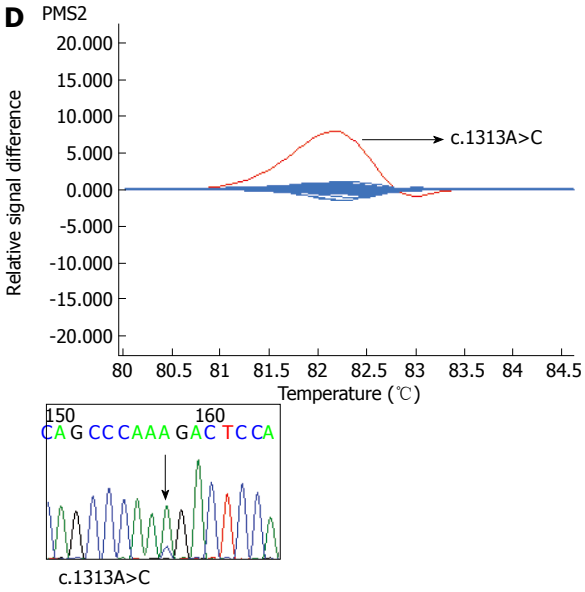
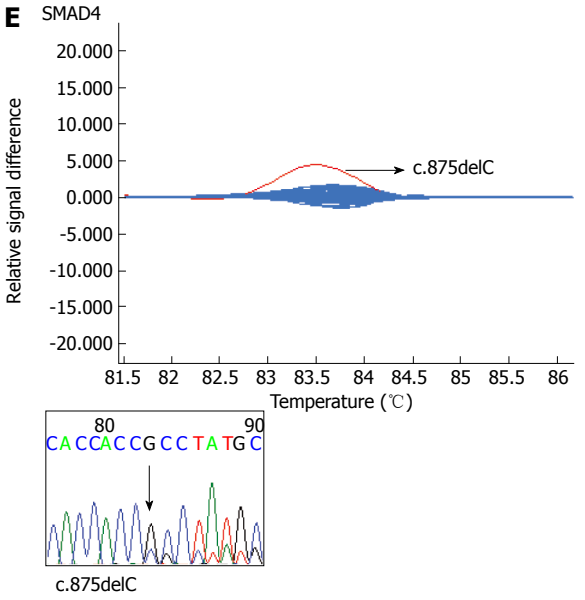
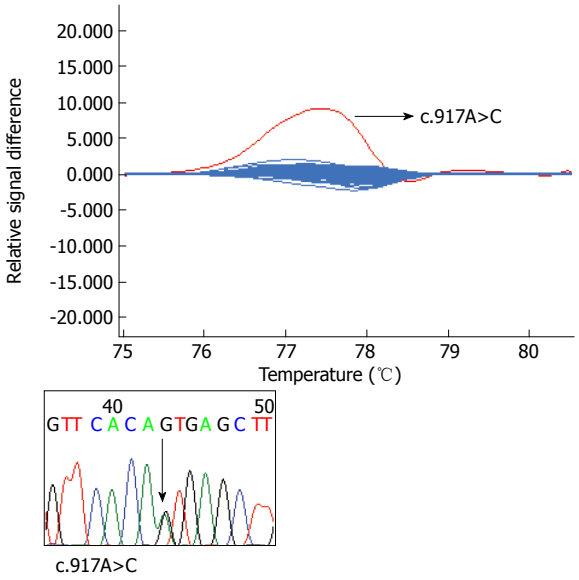
c.232G>A


Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.718delG
77 77.5 78 78.5 79 79.5 80 80.5 81 81.5
Temperature (℃)
70
T T A T C A G G A C C T C A C
c.718delG


D
PMS2
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.1313A>C
80 80.5 81 81.5 82 82.5 83 83.5 84 84.5
Temperature (℃)
150
160
CA G CC C AAA G AC TC CA
c.1313A>C


E
SMAD4
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.875delC
81.5 82 82.5 83 83.5 84 84.5 85 85.5 86
Temperature (℃)
80
90
CA CC AC C G C C TA TG C
c.875delC


Relative signal difference
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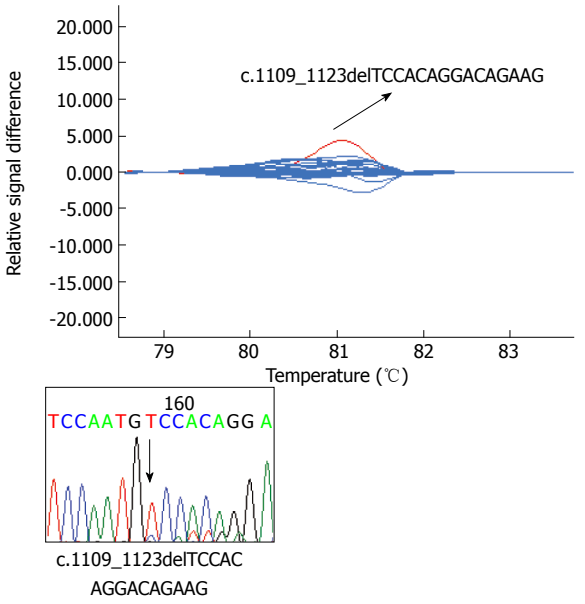
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.917A>C
75 76 77 78 79 80
Temperature (℃)
40
50
GTT C A C A GTGA G C TT
c.917A>C


Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.1109_1123delTCCACAGGACAGAAG
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Temperature (℃)
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c.1109_1123delTCCAC
AGGACAGAAG

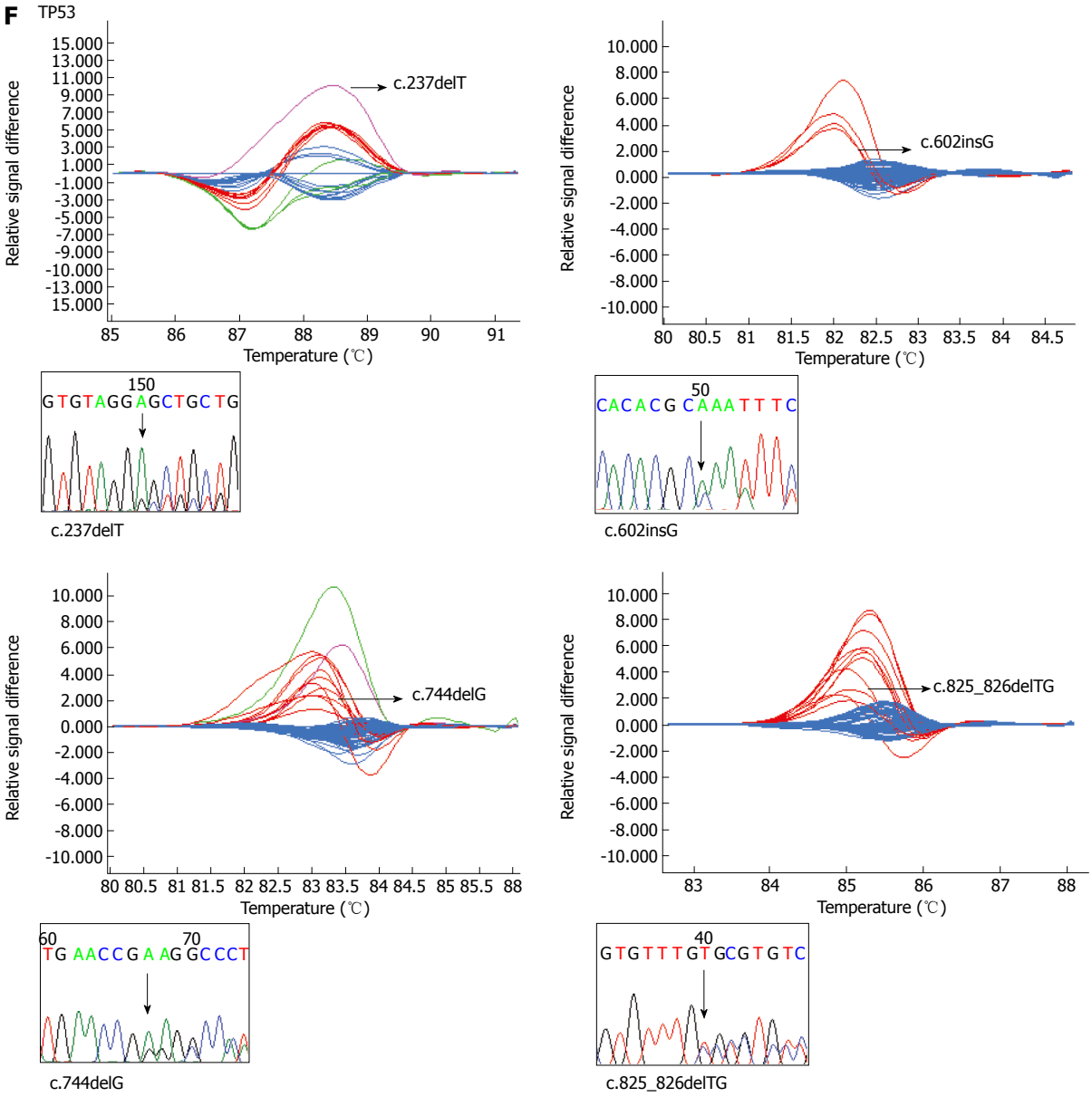

Figure 1 Direct sequencing was used to validate novel pathological mutations. A: *APC*; B: *MLH1*; C: *MSH2*; D: *PMS2*; E: *SMAD4*; F: *TP53*.

gene (c.875delC, c.917A>G and c.1109_1123delTCCACAGGACAGAAG) and four in the *TP53* gene (c.237delT, c.602insG, c.744delG and c.825_826delTG). To our knowledge, these mutations have not been detected in human cancers prior to this study.

De novo mutations in the 13 driver genes

In total, we found 16 *de novo* mutations in seven genes that have been previously described in a public database with the exception of *APC* c.465A>G, these mutations were detected in cancerous tissues only, and not in the patient's blood cells (Table 1). Three of these mutations occurred in the *APC* gene (c.465A>G, c.573T>C and c.1005A>G), one in the *BMPR1A* gene (c.1578A>G), five in the *MLH1* gene (c.462C>T, c.655A>G, c.1151T>A, c.1742C>T and c.2101C>A), two in the *MSH2* gene (c.23C>T and c.1886A>G), two in the *MSH6* gene (c.3488A>T and c.4065_4066insTTGA), two in the *MUTYH* gene (c.1422G>C and c.1440C>T) and one in the *PMS2* gene (c.1532C>T).

Polymorphisms in the 13 driver genes

In total, we validated 18 polymorphisms in six genes previously described in a public database (Table 2). One of them occurred in the *BMPR1A* gene (c.4C>A), three in the *MSH2* gene (c.471C>A, c.1168C>T and c.1690A>G), two in the *MSH6* gene (c.116G>A and c.3306T>A), one in the *MUTYH* gene (c.1014G>C), ten in the *PMS2* gene (c.59G>A, c.288C>T, c.780C>G, c.1408C>T, c.1454C>A, c.1621G>A, c.2253T>C, c.2324A>G, c.2340C>T and c.2570G>C) and one in the *TP53* gene (c.215C>G). Some of the frequencies are similar to those in a public database, but some are

Table 1 ***De novo*** **mutations of 13 driver genes detected in colorectal cancer**

Gene	Mutation	Protein change	rs number	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
APC	c.465A>G	p.Lys155=		1 (0.97)	0.49%		
	c.573T>C	p.Tyr191=	rs185154886	1 (0.97)	0.49%	NA	C = 0.06
	c.1005A>G	p.Leu335=	rs3797704	1 (0.97)	0.49%	G = 0%	G = 0.06
BMPR1A	c.1578A>G	p.Glu526=	rs202030576	1 (0.97)	0.49%	NA	G = 0.02
MLH1	c.462C>T	p.Asp154=	rs192938577	1 (0.97)	0.49%	NA	T = 0.02
	c.655A>G	p.Ile219Val	rs1799977	7 (6.8)	3.40%	G = 0%-37.5%	G = 12.96
	c.1151T>A	p.Val384Asp	rs63750447	4 (3.88)	1.94%	NA	A = 0.52
	c.1742C>T	p.Pro581Leu	rs63751684	1 (0.97)	0.49%	NA	T = 0.12
	c.2101C>A	p.Gln701Lys	rs63750114	1 (0.97)	0.49%	NA	A = 0.12
MSH2	c.23C>T	p.Thr8Met	rs17217716	3 (2.91)	1.46%	T = 0%-5%	T = 0.52
	c.1886A>G	p.Gln629Arg	rs61756468	3 (2.91)	1.46%	NA	G = 0.22
MSH6	c.3488A>T	p.Glu1163Val	rs63750252	2 (1.94)	0.97%	NA	T = 0.28
	c.4065_4066insTTGA	p.Lys1328Aspfs	rs55740729	1 (0.97)	0.49%	NA	TTGA = 0.8
MUTYH	c.1422G>C	p.Thr474=	rs74318065	1 (0.97)	0.49%	NA	G = 1.04
	c.1440C>T	p.Thr480=	rs150269172	3 (2.91)	1.46%	NA	A = 0.4
PMS2	c.1532C>T	p.Thr511Met	rs74902811	4 (3.88)	1.94%	NA	A = 3.69

Table 2 Polymorphisms of 13 driver genes detected in colorectal cancer

Gene	Mutation	Protein change	rs number	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
BMPR1A	c.4C>A	p.Pro2Thr	rs11528010	41(39.81)	19.9%	NA	A = 49.98
MSH2	c.471C>A	p.Gly157=	rs61756463	5 (4.85)	2.43%	NA	A = 0.24
	c.1168C>T	p.Leu390Phe	rs17224367	5 (4.85)	2.43%	T = 0%-4.7%	T = 0.28
	c.1690A>G	p.Thr564Ala	rs55778204	3 (2.91)	1.46%	NA	G = 0.06
MSH6	c.116G>A	p.Gly39Glu	rs1042821	1 (0.97)	0.49%	NA	A = 20.29
	c.3306T>A	p.Thr1102=	rs2020910	41 (39.81)	37.38%	A = 0%-22.9%	A = 4.93
MUTYH	c.1014G>C	p.Gln338His	rs3219489	51 (49.51)	27.67%	C = 45.2%-46.7%	C = 31.35
PMS2	c.59G>A	p.Arg20Gln	rs10254120	10 (9.71)	4.85%	NA	T = 7.57
	c.288C>T	p.Ala96=	rs12532895	58 (56.31)	33.5%	A = 28.2%-36%	A = 11.36
	c.780C>G	p.Ser260=	rs1805319	17 (16.5)	8.25%	G = 4.8%-8%	G = 16.87
	c.1408C>T	p.Pro470Ser	rs1805321	44 (42.72)	21.36%	T = 0%	T = 35.82
	c.1454C>A	p.Thr485Lys	rs1805323	44 (42.72)	21.36%	NA	T = 11.20
	c.1621G>A	p.Lys541Glu	rs2228006	15 (14.56)	7.28%	A = 4.4%-23%	A = 11.68
	c.2253T>C	p.Phe751=	rs1805325	2 (1.94)	0.97%	NA	NA
	c.2324A>G	p.Asn775Ser	rs17420802	8 (7.77)	3.88%	NA	NA
	c.2340C>T	p.Pro780=	rs142230276	8 (7.77)	3.88%	NA	A = 0.12
	c.2570G>C	p.Gly857ala	rs1802683	1 (0.97)	0.49%	NA	NA
TP53	c.215C>G	p.Pro72Arg	rs1042522	78 (75.73)	50%	G = 48.9%-61.4%	G = 45.71

not, which maybe due to ethnic differences.

Known pathological mutations in the 13 driver genes

In total, we validated 58 mutations in 10 genes that have been previously described in a public database (Table 3). Fifteen of them occurred in the *APC* gene (c.95A>G, c.646C>T, c.694C>T, c.799G>T, c.832C>T, c.904C>T, c.3907C>T, c.3914C>A, c.3914delC, c.3934G>T, c.3935delG, c.3944C>A, c.3982C>T, c.4012C>T and c.4031C>A), two in the *BRAF* gene (c.1780G>A and c.1799T>C), six in the *KRAS* gene (c.34G>C, c.34G>T, c.35G>A, c.35G>C, c.35G>T and c.38G>C), one in the *MSH2* gene (c.1480T>C), on in the *MUTYH* gene (c.74G>A), four in the *PIK3CA* gene (c.1624G>A, c.1633G>A, c.1636C>G and c.3104A>G), one in the *PMS2* gene (c.2437C>T), one in the *PTEN* gene (c.19G>T), three in the *SMAD4* gene (c.1067C>G, c.1069T>C and c.1081C>T) and 24 in the *TP53* gene (c.318C>G, c.423C>G, c.440T>G, c.511G>T, c.514G>T, c.524G>A, c.536A>G, c.586C>T, c.638G>T, c.646G>A, c.700T>G, c.734G>A, c.742C>T, c.761T>G, c.772G>A, c.772G>T, c.817C>T, c.818G>A, c.841G>C, c.844C>T, c.853G>A, c.856G>A, c.857A>G and c.1015G>T).

Distribution of mutations in CRC-related pathways

To explore the patterns of mutations in the candidate pathways, we divided the 13 driver genes into six pathways: WNT, TGF-β, PI3K, RTK-RAS, P53, and DNA repair pathways. The *TP53* gene in the P53 pathway has a relatively high rate of mutation compared with genes in the WNT, TGF-β, PI3K, RTK-RAS, and DNA repair pathways.

In total, 76 patients (73.79%) had mutations in one

Table 3 Known pathological mutations of 13 driver genes detected in colorectal cancer

Gene	Mutation	Protein change	rs number in dbSNP/mutation id in COSMIC	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
APC	c.95A>G	p.Asn32Ser	rs539108537	1 (0.97)	0.49%	NA	G = 0.02
	c.646C>T	p.Arg216Stop	rs62619935	1 (0.97)	0.49%	NA	NA
	c.694C>T	p.Arg232Stop	rs397515734	1 (0.97)	0.49%	NA	NA
	c.799G>T	p.Gly267Stop	The UMD-APC mutations database	1 (0.97)	0.49%	NA	NA
	c.832C>T	p.Gln278Stop	The UMD-APC mutations database	1 (0.97)	0.49%	NA	NA
	c.904C>T	p.Arg302Stop	rs137854568	1 (0.97)	0.49%	NA	NA
	c.3907C>T	p.Gln1303Stop	COSM13728	1 (0.97)	0.49%	NA	NA
	c.3914C>A	p.Ala1305Glu	COSM1432302	1 (0.97)	0.49%	NA	NA
	c.3914delC	p.Ala1305Glufs	COSM19687	1 (0.97)	0.49%	NA	NA
	c.3934G>T	p.Gly1312Stop	COSM18817	1 (0.97)	0.49%	NA	NA
	c.3935delG	p.Gly1312Glufs	COSM18796	1 (0.97)	0.49%	NA	NA
	c.3944C>A	p.Ser1315Stop	COSM18777	1 (0.97)	0.49%	NA	NA
	c.3982C>T	p.Gln1328Stop	rs398123121	3 (2.91)	1.46%	NA	NA
	c.4012C>T	p.Gln1338Stop	rs121913327	3 (2.91)	1.46%	NA	NA
	c.4031C>A	p.Ser1344Stop	COSM19135	1 (0.97)	0.49%	NA	NA
BRAF	c.1780G>A	p.Asp594Asn	rs397516896	1 (0.97)	0.49%	NA	NA
	c.1799T>C	p.Val600Glu	rs113488022	3 (2.91)	1.46%	NA	NA
KRAS	c.34G>C	p.Gly12Cys	rs121913530	2 (1.94)	0.97%	NA	NA
	c.34G>T	p.Gly12Ser	rs121913530	2 (1.94)	0.97%	NA	NA
	c.35G>A	p.Gly12Ala	rs121913529	2 (1.94)	0.97%	NA	NA
	c.35G>C	p.Gly12Asp	rs121913529	11 (10.98)	5.34%	NA	NA
	c.35G>T	p.Gly12Val	rs121913529	12 (11.65)	5.83%	NA	NA
	c.38G>C	p.Gly13Asp	rs112445441	5 (4.85)	2.43%	NA	NA
MSH2	c.1480T>C	p.Ser494Pro	rs55653533	1 (0.97)	0.49%	NA	C = 0.02
MUTYH	c.74G>A	p.Gly25Asp	rs75321043	1 (0.97)	0.49%	NA	T = 0.18
PIK3CA	c.1624G>A	p.Glu542Lys	rs121913273	1 (0.97)	0.49%	NA	NA
	c.1633G>A	p.Glu545Lys	rs104886003	1 (0.97)	0.49%	NA	NA
	c.1636C>G	p.Gln546Lys	rs121913286	1 (0.97)	0.49%	NA	NA
	c.3140A>G	p.His1047Arg	rs121913279	2 (1.94)	0.97%	NA	NA
PMS2	c.2437C>T	p.Arg813Trp	rs375968016	1 (0.97)	0.49%	NA	A = 0.02
PTEN	c.19G>T	p.Glu7Stop	COSM5298	1 (0.97)	0.49%	NA	NA
SMAD4	c.1067C>G	p.Pro356Arg	COSM339351	1 (0.97)	0.49%	NA	NA
	c.1069T>C	p.Ser357Pro	COSM189735	1 (0.97)	0.49%	NA	NA
	c.1081C>T	p.Arg361Cys	rs80338963	1 (0.97)	0.49%	NA	NA
TP53	c.318C>G	p.Ser106Arg	COSM45944	1 (0.97)	0.49%	NA	NA
	c.423C>G	p.Cys141Trp	COSM44204	1 (0.97)	0.49%	NA	NA
	c.440T>G	p.Val147Gly	COSM44309	1 (0.97)	0.49%	NA	NA
	c.511G>T	p.Glu171Stop	COSM10996	1 (0.97)	0.49%	NA	NA
	c.514G>T	p.Val172Phe	COSM44240	1 (0.97)	0.49%	NA	NA
	c.524G>A	p.Arg175His	rs28934578	5 (4.85)	2.43%	NA	NA
	c.536A>G	p.His179Arg	COSM10889	1 (0.97)	0.49%	NA	NA
	c.586C>T	p.Arg196Stop	rs397516435	2 (1.94)	0.97%	NA	NA
	c.638G>T	p.Arg213Leu	COSM43650	1 (0.97)	0.49%	NA	NA
	c.646G>A	p.Val216Met	COSM10667	1 (0.97)	0.49%	NA	NA
	c.700T>G	p.Tyr234Asp	COSM43768	1 (0.97)	0.49%	NA	NA
	c.734G>A	p.Gly245Asp	rs121912656	2 (1.94)	0.97%	NA	NA
	c.742C>T	p.Arg248Trp	rs121912651	3 (2.91)	1.46%	NA	NA
	c.761T>G	p.Ile254Ser	COSM45035	1 (0.97)	0.49%	NA	NA
	c.772G>A	p.Glu258Lys	rs121912652	1 (0.97)	0.49%	NA	NA
	c.772G>T	p.Glu258Stop	COSM43568	1 (0.97)	0.49%	NA	NA
	c.817C>T	p.Arg273Cys	rs121913343	1 (0.97)	0.49%	NA	NA
	c.818G>A	p.Arg273His	rs28934576	2 (1.94)	0.97%	NA	T = 0.02
	c.841G>C	p.Asp281His	COSM10943	1 (0.97)	0.49%	NA	NA
	c.844C>T	p.Arg282Trp	rs28934574	5 (4.85)	2.43%	NA	NA
	c.853G>A	p.Glu285Lys	rs112431538	1 (0.97)	0.49%	NA	NA
	c.856G>A	p.Glu286Lys	COSM10726	1 (0.97)	0.49%	NA	NA
	c.857A>G	p.Glu286Gly	COSM43565	1 (0.97)	0.49%	NA	NA
	c.1015G>T	p.Glu339Stop	COSM11286	1 (0.97)	0.49%	NA	NA

NA: Not available.

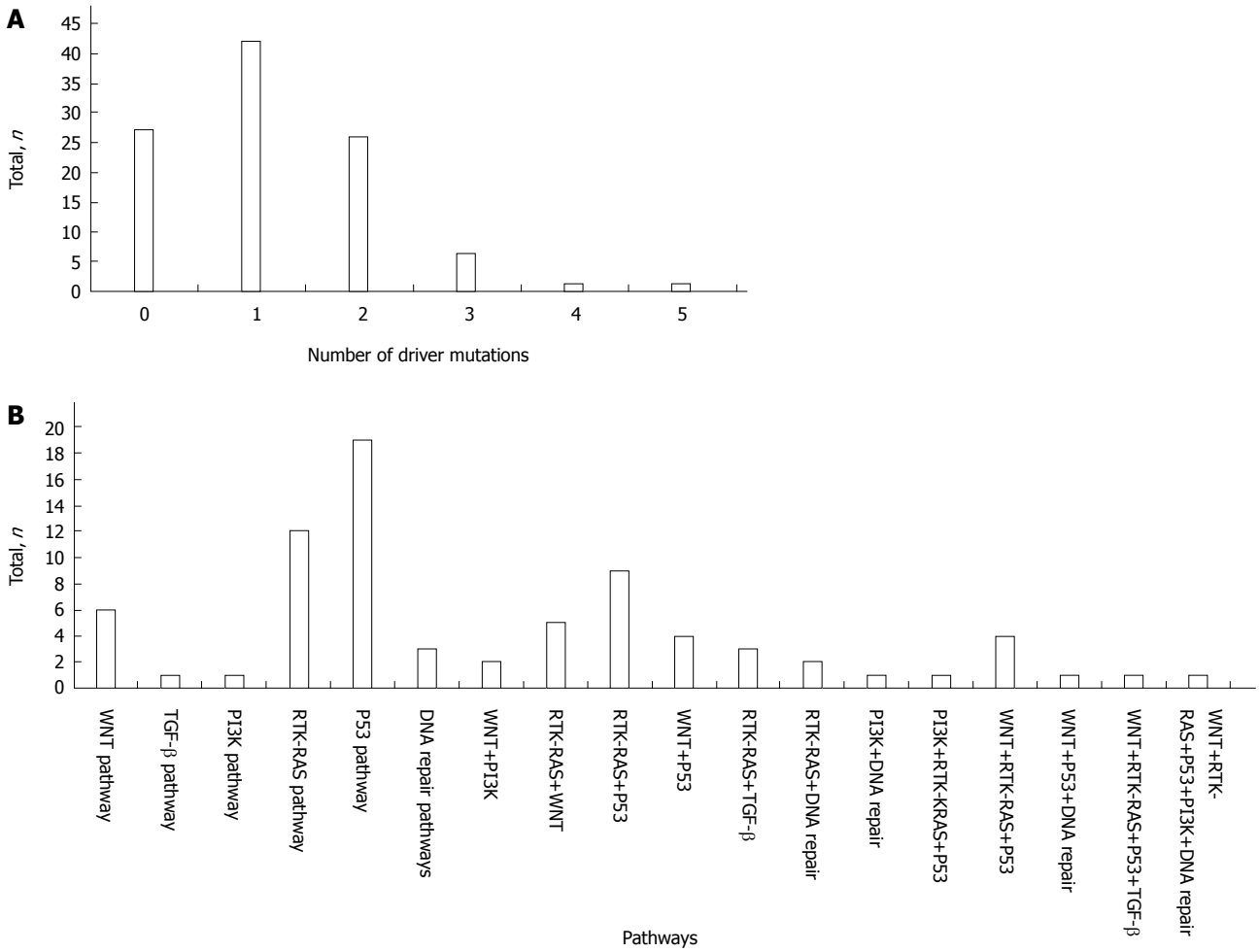

Figure 2 Mutation distribution in colorectal cancer-related pathways. A: Relationship between the number of driver mutations (horizontal axis) and number of patients (vertical axis); B: Relationship between the six major pathways (horizontal axis) and the number of patient (vertical axis).

of the 13 driver genes; of these, 42 patients (40.78%) had one driver gene mutation, and 34 patients (33.01%) had more than one driver gene mutation, including 26 patients (25.24%) with mutations in two driver genes, 6 patients (5.83%) with mutations in three driver genes, 1 patient (0.97%) with mutations in four driver genes, and 1 patient (0.97%) with mutations in five driver genes (Figure 2A).

The mutation combinations included 9 involved in the RTK-RAS/P53 pathways, 5 in the RTK-RAS/WNT pathways, 4 in the WNT/P53 pathways, 3 in the RTK-RAS/TGF-β pathways, 2 in the WNT/PI3K pathways, 2 in the RTK-RAS/DNA repair pathways, 1 in the PI3K/DNA repair pathway, 4 in the WNT/RTK-RAS/P53 pathways, 1 in the PI3K/RTK-RAS/P53 pathway, 1 in the WNT/P53/DNA repair pathway, 1 in the WNT/RTK-RAS/P53/TGF-β pathway, and 1 in the WNT/RTK-RAS/P53/PI3K/DNA repair pathway (Figure 2B).

Correlation of molecular findings with clinical data

APC mutations were significantly correlated with lymph node metastasis (P = 0.009) and cancer stage (P = 0.013) (Table 4). Other mutations did not show any significant correlation with sex, grade, lymph node involvement or stage. In addition, having mutations in two or more driver genes was correlated significantly with the degree of lymph node metastasis (P = 0.043).

DISCUSSION

The advent of next generation sequencing (NGS) has provided a powerful platform to investigate the genetic etiology of diseases. The HRM analysis is not practical for detecting mutations encompassing an entire exon or deletions of entire genes or exons; in contrast, NGS can identify the entire genetic coding sequence. NGS has been comprehensively applied in a variety of ways, including whole genome sequencing, targeted sequencing, chromatin immunoprecipitation sequencing, small RNA sequencing and transcriptome sequencing[22]. Although NGS has become the premier tool in genetic and genomic analyses, this approach can generate a large quantity of genetic data but often with high sequencing errors. Equipment, labor, reagent and supply costs for HRM analysis are significantly lower than those for NGS[23]. The HRM technique does not require complex protocols for experimental steps or data analysis, so it is faster and less expensive

Table 4 Correlation between clinicopathological features and *APC* mutation and two or more driver genes mutations

		Mutation of *APC*				Mutations of two or more driver genes			
		No	Yes	Total	*P* value	No	Yes	Total	*P* value
Gender	F	27	10	37	0.503	23	14	37	0.435
	M	52	14	66		46	20	66	
Grade	Well	4	3	7	0.443	4	3	7	0.579
	Moderate	67	19	86		57	29	86	
	Poor	8	2	10		8	2	10	
LN	-	32	17	49	0.009	28	21	49	0.043
	+	47	7	54		41	13	54	
Stage	Ⅰ, Ⅱ	30	16	46	0.013	27	19	46	0.108
	Ⅲ, Ⅳ	49	8	57		42	15	57	

P value by χ^2 test.

than NGS. We used the HRM technique to analyze the mutation profiles in CRCs of Taiwanese patients, and proved the concept.

No studies to date have assessed the frequency of non-pathological somatic mutations during oncogenesis. In our study, a *de novo* mutation was defined as a genomic alteration that was undetectable in peripheral blood and with nonpathogenic significance; however, the dbSNP database has shown the minor allele frequency for this group. Therefore, we suggest that these nucleotide changes may occur during/after cancer development, and that these mutation sites may belong to a mutational hotspot that occurs frequently in both germline and cancerous tissues.

We identified four point mutations and two deletions in the *SMAD4* gene in five CRC cases. SMAD4 plays a unique and pivotal role in the TGF-β pathway by mediating the transcriptional activation of target genes[24]. The mechanism by which mutation alters gene function is still unknown. Ling *et al*[25] proposed that a mutation in this gene may facilitate CRC progression. In our study, three samples were in the T3 stage and one was in the T4b stage, which may support this idea.

PTEN is a negative regulator of the PI3K pathway that induces cell survival and proliferation. Berg *et al*[26] found that *PTEN* mutations were more frequent in young CRC patients (< 50 years). However, in our study, we identified a *PTEN* nonsense somatic mutation in a male patient aged 66 years.

APC mutations play a critical role in CRC tumorigenesis. Some reports have indicated a potential interdependence of the two hits in *APC*, both in sporadic and FAP-associated CRCs[27,28]. In our study, one patient had two *APC* mutations outside the MCR, whereas most of patients had only one *APC* mutation. From these results, we suggest that one *APC* mutation is capable of initiating of CRC oncogenesis, similar to a *KRAS* mutation.

Tomasetti *et al*[29] showed that only three driver gene mutations are required for the development of advanced cancers in the lung and colon. In addition, they indicated that patients with MMR deficiencies that occurred through the sequential mutation of four or more driver genes significantly increased the incidence of CRC. In this study, we only analyzed 13 driver genes and were unable to confirm their findings, but we determined that 95 patients had fewer than three driver gene mutations. We suggest that further studies using NGS to sequence the exome may solve the discrepancies in these cases.

In conclusion, we identified mutations in genes such as *BRAF*, *KRAS*, *MUTYH*, *PIK3CA* and *PTEN*, as well as previously unreported point mutations or frameshift mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53* genes in a group of Taiwanese CRC patients.

COMMENTS

Background

Previous genetic studies on colorectal cancer (CRC) have revealed multiple critical mutations in candidate pathways; furthermore, statistical analysis has shown that the number of driver gene mutations plays an important role in the development of CRC. However, the genetic mutations associated with CRC in the Taiwanese population are unclear.

Research frontiers

Multiple previous reports and The Cancer Genome Atlas database have revealed that several critical genes and pathways are important in the initiation, progression and treatment of CRC, these include WNT, RAS-MAPK, PI3K, TGF-β, P53, and DNA MMR pathways, and some of these mutations may affect the results of treatment.

Innovations and breakthroughs

This is the first study using high-resolution melting analysis (HRM) technique to analyze the mutation profiles in CRCs of Taiwanese patients and evaluating the frequency of non-pathological somatic mutations during oncogenesis.

Applications

The studies show that HRM analysis can be used for high-throughput mutation screening for research, as well as for molecular diagnosis and clinical purposes.

Terminology

HRM analysis is a closed-tube method, indicating that PCR amplification and subsequent analysis are sequentially performed in 1 well.

Peer-review

The manuscript entitled "Mutational analysis of 13 driver genes of colorectal cancer-related pathways in Taiwanese patients" by Chang *et al* 2015 details the use of HRM and DNA sequencing techniques applied to CRC samples, and details the identification of novel genetic mutations, as well as characterization of the prevalence of other mutations in 13 driver genes of CRC-related pathways. This paper will be of interest to scientists working in the CRC field, recommend publication.

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