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Original Article

Comprehensive functional genomic analyses link APC somatic mutation and mRNA-miRNA networks to the clinical outcome of stage-III colorectal cancer patients

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ABSTRACT

Background: Colorectal cancer (CRC) is a major health concern globally, but exhibits regional and/or environmental distinctions in terms of outcome especially for patients with stage III CRC.

Methods: From 2014 to 2016, matched pairs of tumor and adjacent normal tissue samples from 60 patients with stage I–IV CRC from Chang Gung Memorial Hospital in Taiwan were analyzed using next-generation sequencing. The DNA, mRNA, and miRNA sequences of paired tumor tissues were profiled. An observational study with survival analysis was done. Online datasets of The Cancer Genome Atlas (TCGA) and The International Cancer Genome Consortium (ICGC) were also integrated and compared.

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Transcriptome
Exome

Results: The gene that exhibited the highest mutation rate was adenomatous polyposis coli (APC) (75.0%), followed by TP53 (70.0%), KRAS (56.6%), and TTN (48.3%). APC was also the most frequently mutated gene in TCGA and ICGC datasets. Surprisingly, for non-metastatic cases (stages I–III), CRC patients with mutated APC had better outcome in terms of overall survival ($p = 0.041$) and recurrence free survival ($p = 0.0048$). Particularly for stage III CRC, the overall survival rate was 94.4% and 67.7%, respectively ($p = 0.018$), and the recurrence free survival rate was 94.4% and 16.7%, respectively ($p = 0.00044$). Further clinical and gene expression analyses revealed that the APC wt specimens to a greater extent exhibit poor differentiation state as well as EGFR upregulation, providing molecular basis for the poor prognosis of these patients. Finally, based on integrated transcriptome analysis, we constructed the mRNA–miRNA networks underlying disease recurrence of the stage III CRC and uncovered potential therapeutic targets for this clinical condition.

Conclusion: For stage III CRC, patients with mutated APC had better overall and recurrence free survival.

At a glance commentary

Scientific background on the subject

Colorectal cancer (CRC) is a major health concern globally, but exhibits regional and/or environmental distinctions in terms of outcome especially for patients with stage III CRC. Although patients with this advanced stage of disease receive curative treatments, the outcome remains poor due to high recurrence rate.

What this study adds to the field

In the Taiwanese CRC patients, wild-type APC genotype was found to be a poor prognostic factor for stage III patients, implying a regional and/or environmental basis of this unique outcome. Further transcriptome-wide construction of mRNA–miRNA networks underlying CRC recurrence nominated candidate marker genes and pathways.

Colorectal cancer (CRC) is a major health concern worldwide. In Taiwan, CRC ranks second in incidence and third in mortality with >5000 deaths reported annually [1]. While colonoscopy remains the gold standard for CRC diagnosis, this procedure is associated with a 0.1%–1% complication rate, due to life-threatening colon perforation and post-polypectomy bleeding [2–4]. Alternative screening tests are also available in the clinical setting. For example, the immunochemical-based fecal occult blood test is regularly used for the detection of early-stage CRC. However, the immunochemical-based fecal occult blood test is characterized by barely satisfactory sensitivity [5,6].

Stage III disease constitutes 30%–40% of all CRC cases [7,8]. Although patients with this advanced stage of disease undergo curative surgical resection followed by the administration of adjuvant chemotherapy, the 5-year survival rate remains low (approximately 65%–70%) [9]. In general,

approximately one-third of patients with stage III CRC will develop tumor recurrence [10]. Currently, the sensitivity of the CEA marker for detecting the recurrence of CRC is approximately 65%–80% [11]. Therefore, there is an urgent need to discover effective biomarkers for accurate monitoring and patient stratification [12].

Given its nucleotide resolution and high throughput, next-generation sequencing has been used in deciphering the genomic alterations underlying many malignancies [13]. For the genomic landscape of CRC, numerous novel genetic mutations have been reported as potential diagnostic or prognostic markers. Notable examples include the KRAS/BRAF/PIK3CA cascade, (with implications in response to chemotherapy) [14]. Most of these genetic alterations represent potential indicators of poor prognosis or resistance to chemotherapy. Based on transcriptome analyses, several RNA markers have also been identified as detectors of CRC [15], indicators of tumor recurrence [16], or predictive markers of liver metastasis [17,18]. Despite this extensive body of evidence, the therapeutic value of most of these genetic markers remains under preclinical verification.

Previous studies have pointed to APC as the most frequently mutated gene in tumor tissue, an alteration that is compatible with the adenoma–carcinoma sequence [19]. However, inter-cohort or interracial distinctions in the mutational landscape of CRC have been reported by different research groups [20–22]. In this study, we performed whole-exome sequencing (WES), as well as mRNA and miRNA sequencing experiments on matched pairs of tumor and adjacent normal specimens collected from 60 patients with CRC at the Chang Gung Memorial Hospital. Notably, we demonstrated the importance of the APC gene mutation status of the non-metastatic primary tumor in the prognosis of survival outcome. In particular, our data on the Taiwanese CRC patients revealed that wild-type APC genotype was actually a poor prognostic factor for stage I–III CRC, especially in stage III patients. These observations also are in line with those reported for the early-onset cases of African American CRC but distinct from the Caucasian patients [23], implying a regional and/or environmental basis of this unique outcome. In addition, based on transcriptome profiling, we were able to construct mRNA–miRNA networks underlying CRC recurrence for the identification of candidate marker genes and pathways.

Materials and methods

Collection of clinical specimens from patients with CRC

Sixty patients with CRC were recruited at the Division of Colon and Rectal Surgery, Chang Gung Memorial Hospital, from December 2014 to May 2016. Blood and matched tissues samples were obtained prior to surgical intervention. All patients received surgery with curative intent. Standard management, including adjuvant or palliative chemotherapy, was provided according to the treatment protocol. All cases were reviewed in Tumor Combined Conference for adherence to guidelines. After surgical management and/or chemotherapy, patients were continually monitored at 3-month intervals in outpatient clinics for 2–3 years, 6-month intervals for another 3–5 years, and subsequently 1-year intervals until 10 years after treatment.

Ethics approval and consent to participate

This study was approved by the Chang Gung Memorial Hospital Institutional Review Board as a retrospective analysis (IRB 103-2529B) and was conducted within the guidelines of the Declaration of Helsinki. Patients/families were counseled with regard to the present study design, and all participants provided written informed consent to participate in the study.

Consent for publication

All individuals involved in this study provided consent for publication. We also obtained consent to publish the clinical information of all individuals presented in this study.

Next-generation sequencing and data analysis

For whole-exome sequencing, high-quality genomic DNA from tumor tissue and matched PBMCs (1 µg per sample) were subjected to capture using a TruSeq Exome Kit according to the manufacturer's protocol (Illumina). RNA libraries were prepared using the TruSeq® Stranded mRNA Library Prep Guide (Illumina) according to the instructions provided by the manufacturer. For the small RNA-sequencing, we prepared libraries using Illumina TruSeq small RNA library preparation kits according to manufacturer's instructions. Libraries were assessed using the Agilent 2100 Bioanalyzer instrument with the Agilent High SensitivityDNA Kit (Agilent Technologies). Equal amounts of libraries were pooled in molecular ratio and consequently sequenced by the NextSeq 500 sequencer. Furthermore, we performed RNA-seq using normal/tumor paired samples from 60 of the 104 patients with CRC [24]. The data analyses (including VCF/variant calling and differential gene expression) were performed according to our previous report [24,25].

Retrieval and analysis of data from public cancer databases

Data from The Cancer Genome Atlas (TCGA) were downloaded through the Genomic Data Commons Data Portal [26]. The data of CRC patients were further retrieved (colon adenocarcinoma, or COAD; rectal adenocarcinoma, or READ). All clinical and recurrence data were also downloaded and analyzed. The data

obtained from TCGA were processed and analyzed as follows. Overall survival indicates the time from the initial date to death or last follow-up. Disease-free survival indicates the time from the initial date to death, new tumor event or last follow-up. For the other Asian dataset, we retrieved CRC data from the International Cancer Genome Consortium (ICGC) [27], which archived Chinese patients with CRC. All clinical data from ICGC-China were downloaded and analyzed as described above.

Gene expression discrepancy and pathway analyses

For the gene differential expression analysis, we divided patients with stage III CRC into two groups according to their 2-year disease recurrence status. The expression levels of genes in each sample and the corresponding fold changes were estimated by Partek Folw with GENCODE V25 (for RNA seq) and mirbase v21 (for miRNA seq) annotation. Relative expression of each gene is represented by CPM (Counts Per kilobase Million). Partek Genomics Suite and statistical package were used for the statistical analysis, and differential expression analysis. To distinguish differential expression, the filter was set as fold change >1.5 or < -1.5, $p < 0.05$, which resulted in 769 genes and 14 miRNAs (additional file 1: Table S5 & Table S6). For the construction of mRNA–miRNA networks among these differentially expressed molecules, the microRNA target filter analysis tool installed in IPA was employed. The criteria were set to capture targeted mRNA genes with 1) expression patterns converse to those of miRNAs and 2) potential target sequences of the targeting miRNAs. Finally, the 14 miRNAs and 82 genes were co-aggregated into 99 miRNA–mRNA paired regulation networks (additional file 1: Table S7). The 82 genes uncovered from this network were further subjected to IPA canonical pathway analysis (additional file 1: Table S8).

Statistical analysis

The Fisher's exact test was used for categorical variables in the analysis of clinicopathological factors. The ANOVA was used for continuous variables in the analysis of clinicopathological factors. The Kaplan–Meier method was used to determine overall survival and recurrence free survival. Time-to-event probabilities were computed using univariate analysis. Differences were estimated using a log–rank test. Statistical significance was set at $p < 0.05$. Disease recurrence was defined as the first date of local recurrence detection and/or confirmation of distant metastases through histology, re-operation and/or radiological studies. All analyses were performed using the Statistical Package for the Social Sciences version 20.0 (IBM Corp., Armonk, NY, USA) statistical software.

Results

Clinical features of the enrolled patients with CRC

From 2014 to 2016, sixty patients with CRC were enrolled in this study, with the following clinical stage distribution: seven (11.6%) stage I, 15 (25.0%) stage II, 26 (43.3%) stage III, and 12 (20.0%) stage IV patients (Table 1). This cohort of patients is hereafter referred to as Taiwan CRC-60. During the 2-year

Table 1 Distribution of clinicopathological features according to the different stages of patients with colorectal cancer.

Clinical Stage	1	2	3	4	p-value ^a
Patients number	7	15	26	12	
Age					
Mean (\pm SD)	66.5 \pm 10.2	61.6 \pm 10.6	59.1 \pm 8.6	61.3 \pm 9.5	0.333
Gender					
Male	6 (85.7)	9 (60.0)	12 (46.2)	5 (41.7)	0.225
Female	1 (14.3)	6 (40.0)	14 (53.8)	7 (58.3)	
T stage					
T1-2	7 (100.0)	0 (0)	3 (11.5)	0 (0)	<0.001
T3-4	0 (0)	15 (100.0)	23 (88.5)	12 (100.0)	
Family history					
Yes	2 (28.6)	9 (60.0)	11 (42.3)	5 (41.7)	0.556
No	5 (71.4)	6 (40.0)	15 (57.7)	7 (58.3)	
Histological grade					
Well differentiation	1 (14.3)	0 (0)	1 (3.8)	0 (0)	0.428
Moderate differentiation	6 (85.7)	14 (93.3)	22 (84.6)	9 (75.0)	
Poor differentiation	0 (0)	1 (6.7)	3 (11.5)	3 (25.0)	
CEA					
< 5 ng/mL	5 (71.4)	11 (73.3)	18 (69.2)	3 (25.0)	0.038
\geq 5 ng/mL	2 (28.6)	4 (26.7)	8 (30.8)	9 (75.0)	
Overall survival					
\geq 2 years	5 (71.4)	14 (93.3)	21 (80.8)	5 (41.7)	0.041
< 2 years	1 (14.3)	0 (0)	3 (11.5)	5 (41.7)	
Not available	1 (14.3)	1 (6.7)	2 (7.7)	2 (16.6)	
Disease free survival					
\geq 2 years	5 (71.4)	14 (93.3)	18 (69.2)	0 (0)	<0.001
< 2 years	1 (14.3)	0 (0)	6 (23.1)	12 (100.0)	
Not available	1 (14.3)	1 (6.7)	2 (7.7)	0 (0)	

^a The p-value was calculated using ANOVA for the continuous variable (age). The p-values were calculated using Fisher's exact test for categorical variables (gender, T-stage, family history, histological grade, CEA, overall survival, disease free survival).

postoperative period, nine patients expired due to the tumor or other reasons, while 19 patients developed tumor recurrence (see survival information in Table 1). As shown in Table 1, there were no differences among the stages of disease in terms of age distribution, sex, family history, histological grade, and overall survival. In contrast, marked differences were found among stages for T stage ($p < 0.001$), CEA level ($p = 0.038$), 2-year overall survival ($p = 0.041$) and 2-year disease-free survival ($p < 0.001$). In addition, patients in stage III–IV exhibited higher rates of disease recurrence, as shown in Table 1.

Overall mutational landscape of Taiwan CRC-60

Among all the sequence alterations profiled by our approach, the predominant mutation types were single-nucleotide variations and deletions (Fig. 1). In terms of functional impact, the most notable types were missense, frame-shift, and nonsense alterations (additional file 1: Table S1). In line with previous findings, most nonsense mutation events were found in the APC gene (Fig. 1), which also showed the highest overall mutation rate in our cohort (75.0%). Most APC mutations were nonsense alterations that cause pre-mature stop codon. Other prevalently mutated gene loci included TP53 (70.0%), KRAS (56.6%), and TTN (48.3%). There were 3 hypermutators, CRC_032, CRC_034, and CRC_042 in the CRC-60 dataset (Fig. 1, the upper panel), which exhibit extensive genomic alterations. To resolve whether these cases of hypermutation were attributed to mutation and/or expression alterations in the

DNA repair genes, we focused our attention on five related genes POLE, POLE2, MSH2, MLH1 and PMS2. Our analyses revealed that all three CRC patients uniquely exhibit somatic mutations in POLE and MLH1, and that mutation in one of the other three genes was also detected (additional file 2: Figure S1). This observation is in line with the previous findings that alteration in DNA repair genes such as POLE contributes to hypermutation in CRC [28,29]. The expression profiles of these genes in the samples are also provided in Figure S1 but did not show variations in association with the mutations. Despite the high mutation rate, there is currently no report linking this hypermutator phenotype to the morbidity and outcome of the CRC [30]. In our cohort, the prevalence of this hypermutation status is low (3/60 = 5%), and there is no significant clinical outcome correlated with these hypermutators as they are still alive at present.

Previous pan-cancer analyses of genomic alterations defined distinct mutational signatures as being genetic hallmarks of different cancer types [31–33]. By applying a similar algorithm to data for individual patient somatic SNVs discovered herein, we identified mutational signatures in CRC-60 in Taiwan (Fig. 1, the bottom panel, and additional file 2: Figure S2). Our CRC-60 patient cohort was enriched with the signatures 1, 3, 6 and 15. These mutation characteristics have the following implications: signature 1 has been found in all cancer types and correlates with age; signature 3 arises due to the DNA double-strand break-repair by homologous recombination; interestingly, signatures 6 and 15 are associated with defective DNA mismatch repair and frequently detected in

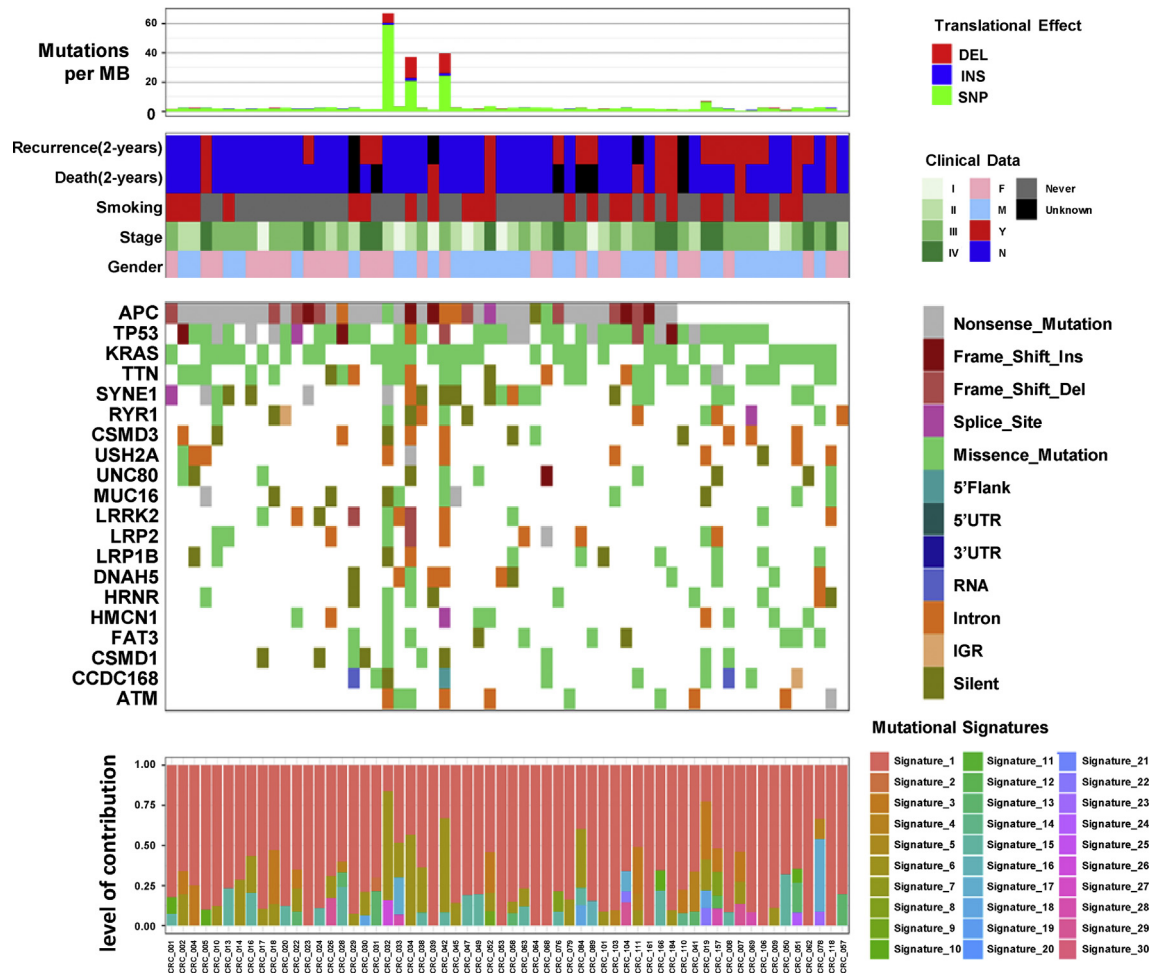


Fig. 1 Mutational profiles and signatures exhibited by the Taiwan CRC-60 data. For the 60 patients with CRC included in our cohort, attributes (e.g., mutation events per Mb, clinical data, mutation type, mutated genes, and mutational signatures) are depicted with different colors, as indicated. The graphical symbols are shown on the right side of each panel.

colorectal carcinoma. We further compared these signatures with those from the CRC cancer projects archived in the TCGA COADREAD [26] and ICGC COCA-CN [27] data portal (additional file 2: Figure S2), and discovered that the most prevalent signatures were similar across these three projects. However, data from the ICGC were enriched with additional mutation signatures, suggesting that there might be distinct environmental or other etiological factors.

Distinct impact of APC mutation on survival among the CRC-60, TCGA, and ICGC datasets

Next, we quantitatively compared gene mutation rates between the Taiwan CRC-60, TCGA COADREAD and ICGC COCA-CN. To this end, Fig. 2A illustrates the top 30 mutated genes in the Taiwan CRC-60 cohort and their relative occurrence in TCGA-COADREAD and ICGC COCA-CN. The most frequently discrepant alterations between TCGA dataset and our dataset included *FUT9* (19% more frequently in TCGA), as well as *SOX11*, *MUC16*, *FAT4*, *PIK3CA*, and *TTN*. On the other hand,

KRAS was 15% more frequently mutated in our dataset than in TCGA dataset (Fig. 2B).

We further discovered that, while *APC* was the most frequently mutated gene in all three datasets, marked differences were noted between these data in terms of top mutation events and corresponding frequencies. For the mutated genes uncovered in Colorectal Cancer-China (COCA-CN) from ICGC, which mainly comprised ethnically similar patient samples, the top mutated *APC* exhibited a lower mutation rate (<60%) compared with our data. Given that mutations in *APC* are reportedly less frequent in the right-sided CRCs [34–36], and that lack of *APC* mutation is associated with early onset of CRC [23], we then further compared patient distributions according to anatomical regions and diagnosis age (additional file 1, Table S2). With regard to tumor region, we did not find significant difference between the two cohorts. However, the proportion of diagnosis ages under 50 years in the China cohort was higher than that of the Taiwan CRC-60 group, providing a probable explanation for the lower rates of *APC* mutation in the ICGC COCA-CN data (additional file 1, Table S2).

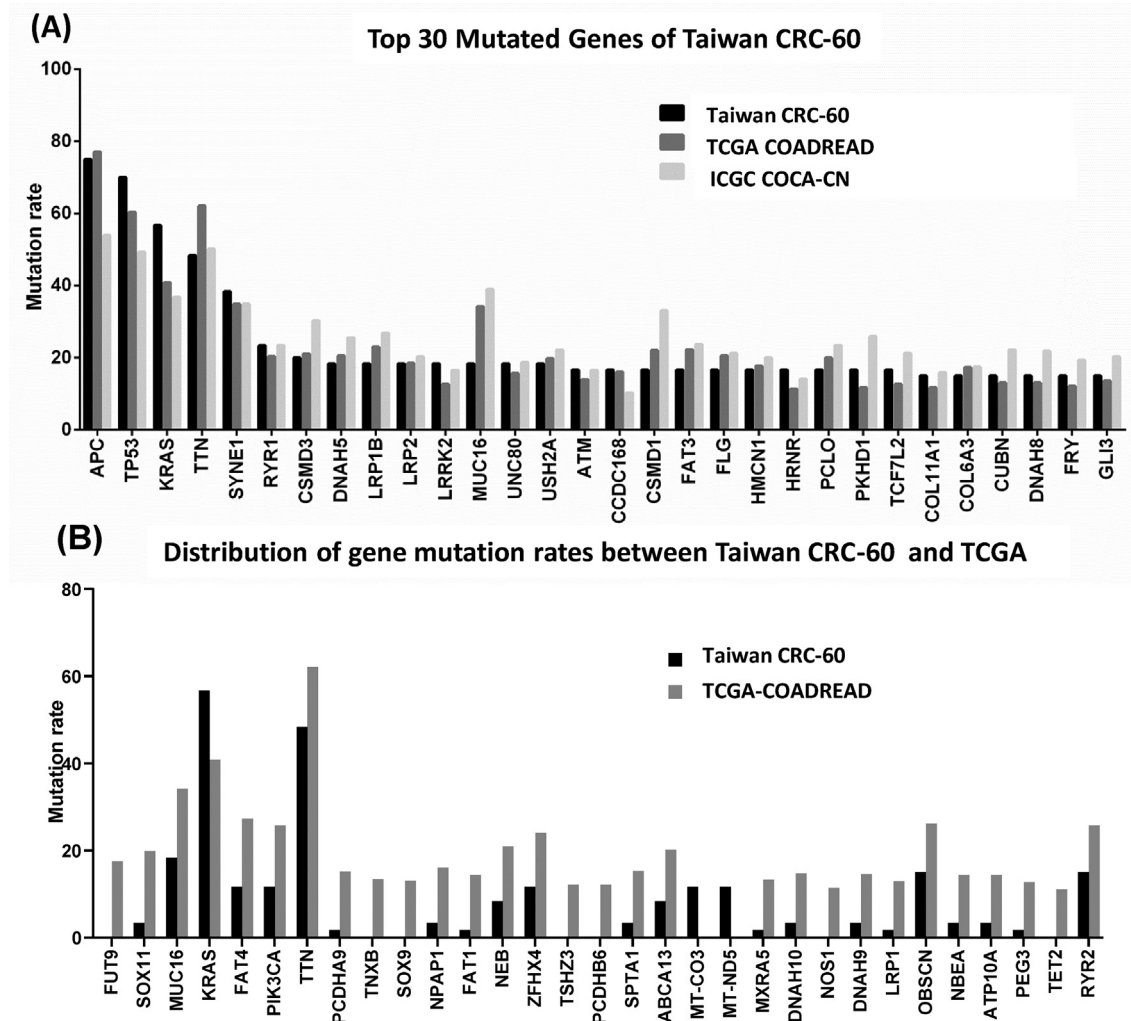


Fig. 2 Characteristics of mutation landscapes between different CRC datasets. (A) Top 30 most prevalently mutated genes in the Taiwan CRC-60 dataset, arranged in the order of their detected incidence. Corresponding mutation events detected in TCGA and ICGC datasets are also shown. (B) Distribution of the mutation rates for the most prevalently mutated genes shared between Taiwan and TCGA data.

Difference in survival among Taiwan CRC-60 patients with distinct APC mutation and CEA statuses

Next, we explored the possibility that these mutations could prognosticate the survival outcome in patients without distant tumor metastasis. Across I–III stages ($n = 48$), CRC patients with mutated APC ($n = 37$) had a better overall survival ($p = 0.041$) and recurrence free survival ($p = 0.0048$) (additional file 2: Figure S3). We further focused on the stage III patients, and discovered that the overall survival rate of these patients with mutated APC was significantly higher than that of patients with wild-type APC ($p = 0.018$) (Fig. 3A). A similar difference was observed for the recurrence free survival rates ($p = 0.00044$) (Fig. 3B). Based on these results, we then assessed the prognostic power of a conventional tumor marker CEA for patient outcome. The statistical power of using CEA to evaluate the overall survival of patients with stage III CRC was comparable to that of the APC genotype ($p = 0.017$ vs. $p = 0.018$, respectively) (Fig. 3A and C). However, the APC genotype was a

better indicator of recurrence free survival ($p = 0.00044$) than CEA ($p = 0.005$) (Fig. 3B and D)

To better understand the combined prognostic power of the APC genotype and CEA, we classified patients with stage I–III CRC into four subgroups based on the following molecular attributes: group 1, patients with mutated APC and abnormal CEA; group 2, patients with mutated APC and normal CEA; group 3, patients with wild-type APC and abnormal CEA; and group 4, patients with wild-type APC and normal CEA (Fig. 3E and F). For the overall survival, patients of groups 1, 2, and 4 exhibited comparable and better outcomes, whereas group 3 (wild-type APC and abnormal CEA) showed a markedly worse outcome ($p = 0.001$). In terms of recurrence free survival, group 2 showed the best outcome among the patient groups, whereas group 3 continued to have the poorest survival rate ($p < 0.001$).

We also compared our data with the ICGC COCA-CN, TCGA COADREAD datasets, with regard to the impact of the APC genotype on the survival of patients with stage I–III CRC

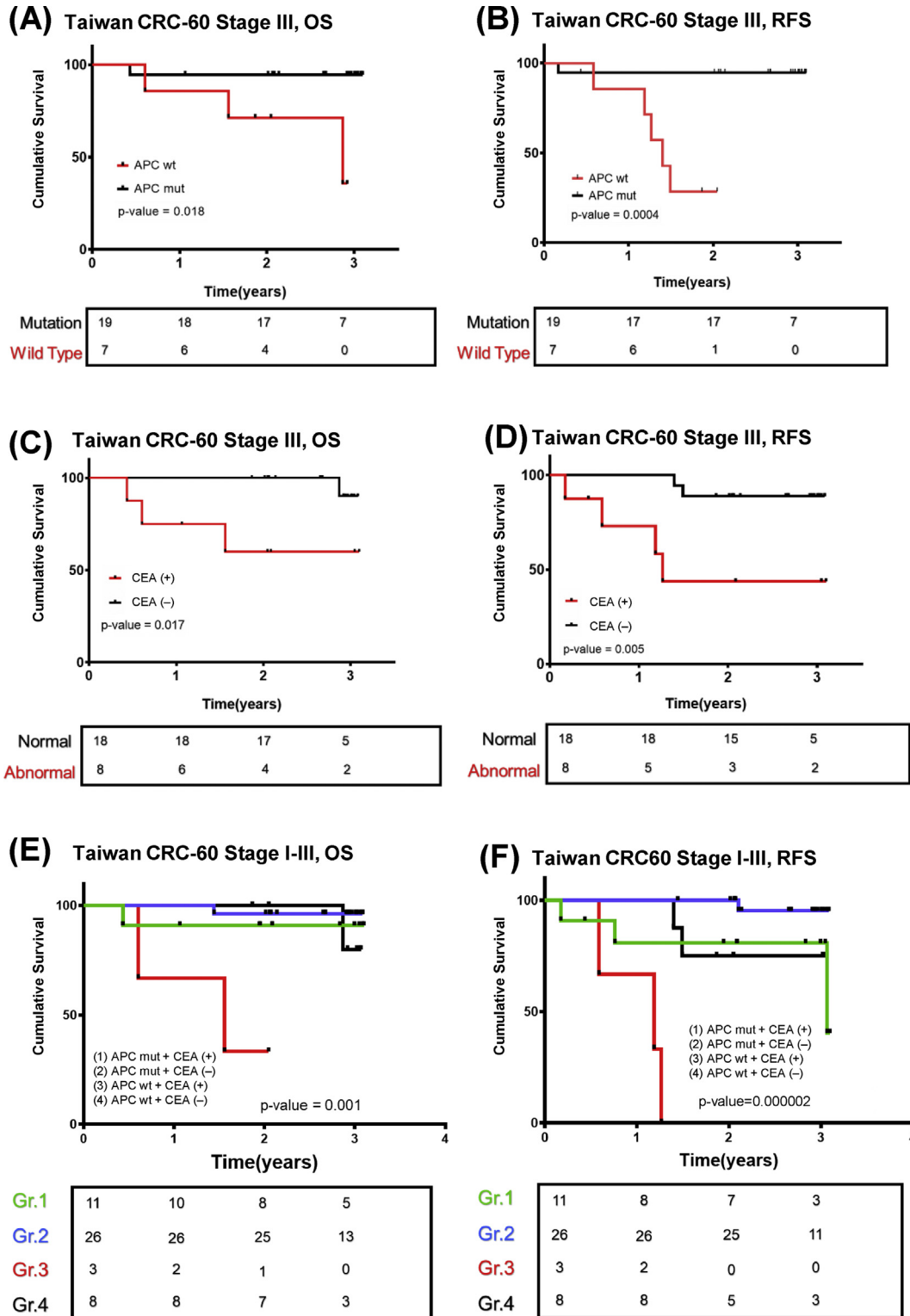


Fig. 3 The 2-year overall survival and disease-free survival of CRC-60 patients with stage III CRC were assessed on the basis of the somatic APC gene mutation or preoperative serum CEA statuses. The prognostic powers of the APC genotype and CEA were also compared individually (A to D) or in combination (E & F). For (E & F), patients with stage I–III CRC were divided into four groups using the APC genotype and CEA statuses, as indicated.

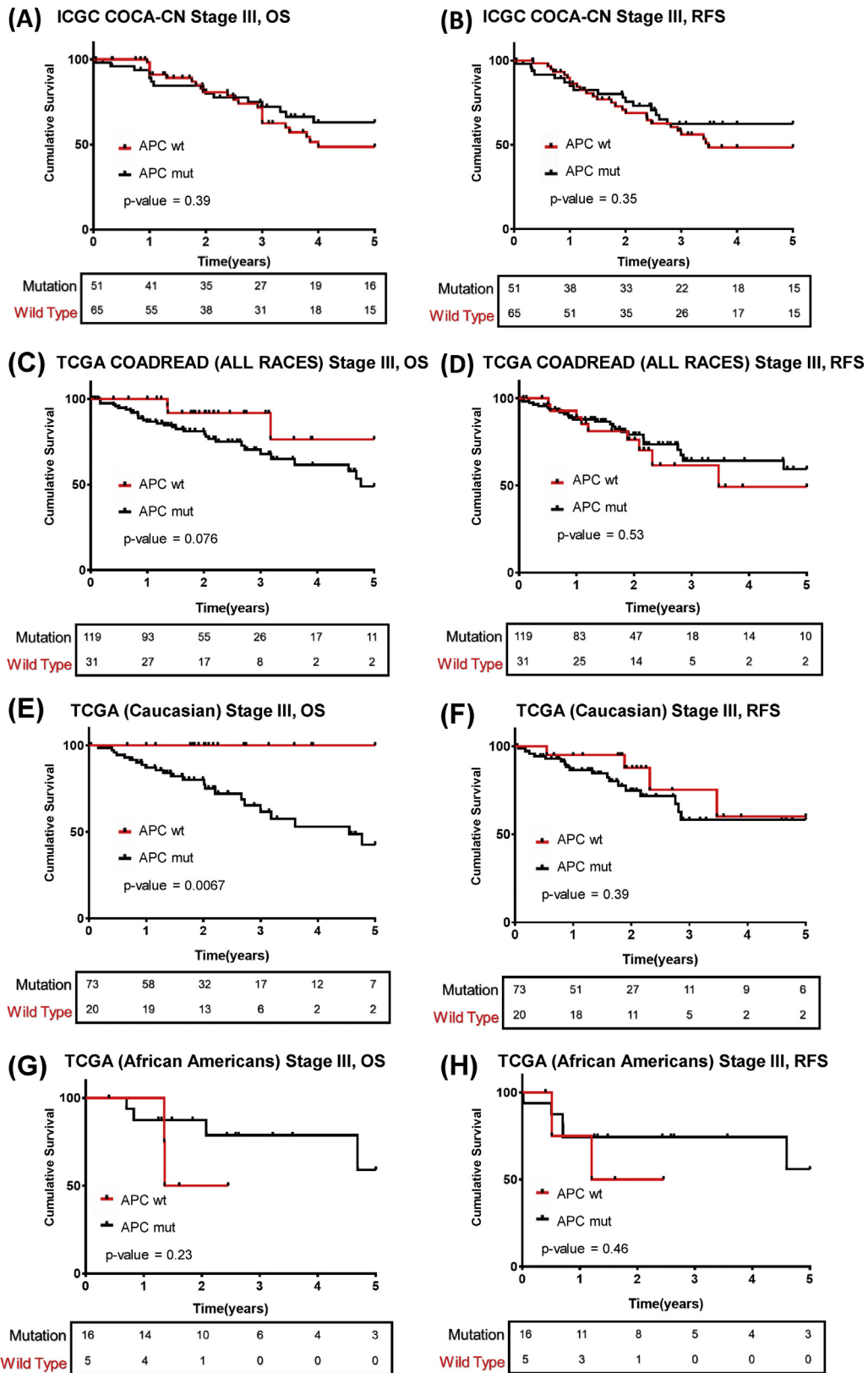


Fig. 4 Comparison of survival outcomes between the APC mutation (APC mut) versus APC wild-type (APC wt) status in the ICGC COCA-CN (A & B) and TCGA (C & D) databases. Patients with stage III CRC in these databases were analyzed. For the TCGA-COADREAD data, independent analyses were further performed on the Caucasian (E & F) or African Americans (G & H).

(Additional file 2: Figure S3C–S3F). In the ICGC COCA-CN and TCGA COADREAD datasets, there were no difference noted in survival outcome between the mutated APC and wild-type APC groups. For the stage-III CRC in particular, the impact of APC mutation on the survival of patients in ICGC COCA-CN and TCGA COADREAD was similarly indistinguishable (Fig. 4A to D). However, an association of wild-type APC in stage III CRC with better outcome was noted, albeit without statistical significance (overall survival, $p = 0.076$).

Based on a recent report that a lack of APC mutation is associated with early-onset colorectal cancer in African American [23], we next sought to independently analyze the Caucasian and African American patients in TCGA database. We subsequently found marked discrepancies among different races in the impact of the APC genotype. For Caucasians, patients with stage III CRC carrying an APC mutation had a markedly worse prognosis – the overall survival rates were 100.0% and 40.0% for patients with stage III CRC with wild-type APC and mutated APC, respectively ($n = 93$; $p = 0.0067$) (Fig. 4E). Conversely, no difference was noted for

recurrence free survival (Fig. 4F). On the contrary, African American patients carrying an APC mutation showed better, but non-significant, survival outcomes for stage-III CRC (Fig. 4G and H). The observations in the African Americans were comparable to those in the Taiwan CRC-60 data, further implying a clinical association of the APC genotype status that is related to environmental and/or regional factor.

The poor outcome of the wild type APC genotype in our CRC-60 stage III patients raised the question of whether this APC status is associated with a distinct clinical attribute (Table 2). To this end, we discovered that there were no differences in the gender, age, tumor location and epidemiological features between the APC wt vs. APC mut tumors. On the other hand, histological grade was significantly different between the two groups: a greater proportion of the APC wt tumors exhibited poor differentiation state in either stage III ($p = 0.013$, Table 2) or stage I-III group ($p = 0.03$, additional file 1: Table S3), an attribute that is normally correlated with worse CRC prognosis. In addition, a greater proportion of the APC wt tumors exhibited positive lymph nodes in either stage III ($p = 0.038$,

Table 2 Comparison of features of stage III CRC with and without somatic APC mutation.

Features	All cases (n = 26)	APC mut (n = 19)	APC wt (n = 7)	p-value ^a
Clinico-pathological				
Gender, males/females	12/14	7/12	5/2	0.19
Age at diagnosis, mean (\pm SD)	59.1 \pm 8.6	60.3 \pm 8.3	55.8 \pm 9.1	0.25
Tumor location (R/L)	4/20	4/13 ^b	0/7	0.28
TNM stage (T1-2/T3-4)	3/23	3/16	0/7	0.54
Differentiation, (well + moderate)/poor	23/3	19/0	4/3	0.013
Positive lymph nodes, mean (\pm SD)	7.3 \pm 2.0	4.7 \pm 1.1	14.2 \pm 6.6	0.038
Tumor size, mean (\pm SD)	4.4 \pm 1.8	4.4 \pm 2.0	4.3 \pm 1.1	0.94
CEA, mean (\pm SD)	5.1 \pm 1.2	4.2 \pm 1.0	7.4 \pm 3.9	0.27
Epidemiological				
Body mass index, mean (\pm SD)	24.7 \pm 2.8	24.7 \pm 0.7	24.7 \pm 1.1	0.96
Smoking, ever/never	9/17	5/14	4/3	0.18
Alcohol consumption, ever/never	7/19	3/16	4/3	0.057
Molecular (ranking)				
(2) TP53 mutation (%)	21 (80.8)	16 (84.2)	5 (71.4)	0.58
(3) KRAS mutation (%)	13 (50.0)	10 (52.6)	3 (42.9)	1.00
(4) TTN mutation (%)	13 (50.0)	9 (47.4)	4 (57.1)	1.00
(5) SYNE1 mutation (%)	12 (46.2)	10 (52.6)	2 (28.6)	0.39
(6) RYR1 mutation (%)	8 (30.8)	6 (31.6)	2 (28.6)	1.00
(7) CSMD3 mutation (%)	7 (26.9)	3 (15.8)	4 (57.1)	0.057
(8) DNAH5 mutation (%)	2 (7.7)	1 (5.3)	1 (14.3)	0.47
(9) LRP1B mutation (%)	4 (15.4)	2 (10.5)	2 (28.6)	0.28
(10) LRP2 mutation (%)	6 (23.1)	6 (31.6)	0 (0)	0.14
(11) LRRK2 mutation (%)	4 (15.4)	3 (15.8)	1 (14.3)	1.00
(12) MUC16 mutation (%)	4 (15.4)	3 (15.8)	1 (14.3)	1.00
(13) UNC80 mutation (%)	3 (11.5)	3 (15.8)	0 (0)	0.54
(14) USH2A mutation (%)	4 (15.4)	2 (10.5)	2 (28.6)	0.28
(15) ATM mutation (%)	2 (7.7)	2 (10.5)	0 (0)	1.00
(16) CCDC168 mutation (%)	6 (23.1)	3 (15.8)	3 (42.9)	0.29
(17) CSMD1 mutation (%)	5 (19.2)	4 (21.1)	1 (14.3)	1.00
(18) FAT3 mutation (%)	3 (11.5)	1 (5.3)	2 (28.6)	0.16
(19) FLG mutation (%)	2 (7.7)	2 (10.5)	0 (0)	1.00
(20) HMCN1 mutation (%)	3 (11.5)	2 (10.5)	1 (14.3)	1.00

Numbers in boldface indicate statistical significance ($p < 0.05$).

^a We compared APC-mut and APC-wt in the table. The p-values to compare two groups were calculated using ANOVA for continuous variables (age at diagnosis, positive lymph nodes, tumor size, CEA, body mass index). The p-values to compare two groups were calculated using Fisher's exact test for categorical variables (gender, tumor location, TNM stage, differentiation, smoking, alcohol consumption, molecular).

^b There were 2 missing data of tumor location for patients with APC mutation.

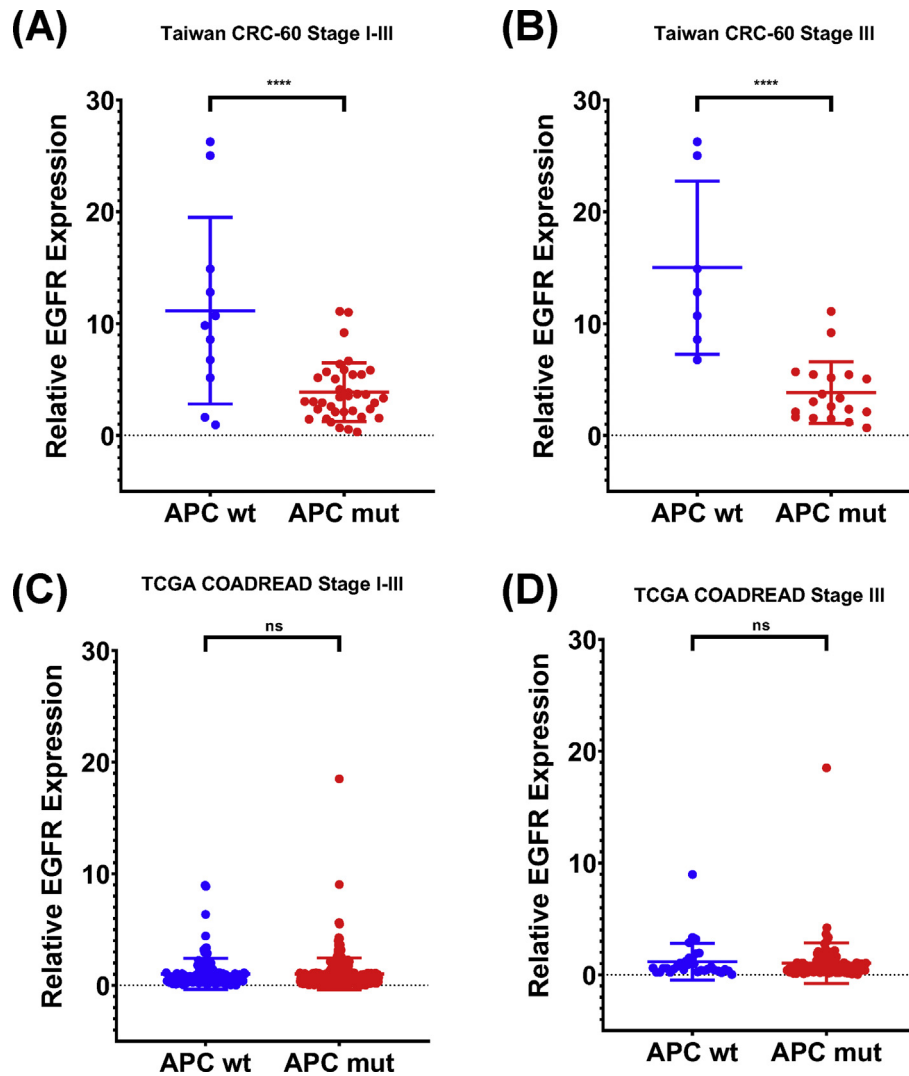


Fig. 5 Comparative expression patterns of the EGFR gene between the APC mutation (APC mut) versus APC wild-type (APC wt) status in the Taiwan CRC-60 (A & B) and TCGA COADREAD (C & D) data. Patients across from stages I to III (A & C) or with stage III CRC (B & D) were independently analyzed for the relative expression levels of EGFR (as normalized by the abundance of TUB3).

Table 2) or stage I-III group ($p = 0.02$, [additional file 1: Table S3](#)), another factor that is usually correlated with worse CRC prognosis. We further analyzed the differential gene expression profiles between APC mut vs. APC wt in the stage III patients, and observed that there was a distinct enrichment of genes associated with “Colorectal Cancer Metastasis Signaling” in the APC wt tumors ([additional file 1: Table S4](#)). Moreover, a key gene of this signaling pathway, EGFR, was found to be upregulated in the APC wt vs. APC mut comparison in either stage III or stage I-III group (Fig. 5A and B). In this regard, a previous report on Taiwanese CRC patients has found that an elevated EGFR expression corresponds to poor patient survival and a higher disease recurrence [37]. Interestingly, this clinical association is not evident in the TCGA data (Fig. 5C and D). While it remains unresolved as to how Taiwanese CRC patients without APC gene mutation display higher EGFR, the observed upregulation of EGFR may be an

underlying cause of the poor prognosis of Taiwan CRC patients with wild-type APC.

Construction of a microRNA-mRNA regulatory network associated with recurrence status in stage III CRC

To identify novel molecular biomarkers for monitoring patient outcome, we next set out to construct a stage-III recurrence-related mRNA-miRNA regulatory network. For that purpose, an integrative transcriptome analysis was performed using RNA-seq and small RNA-seq data to uncover stage-III recurrence-associated differentially-expressed genes (DEGs; $n = 769$, [additional file 1: Table S5](#)) that are also candidate targets of the differentially expressed miRNAs (DEMs; $n = 14$, [additional file 1: Table S6](#)).

To this end, we used the microRNA target filter analysis tool installed in IPA, with criteria set to capture DEGs with: (1)

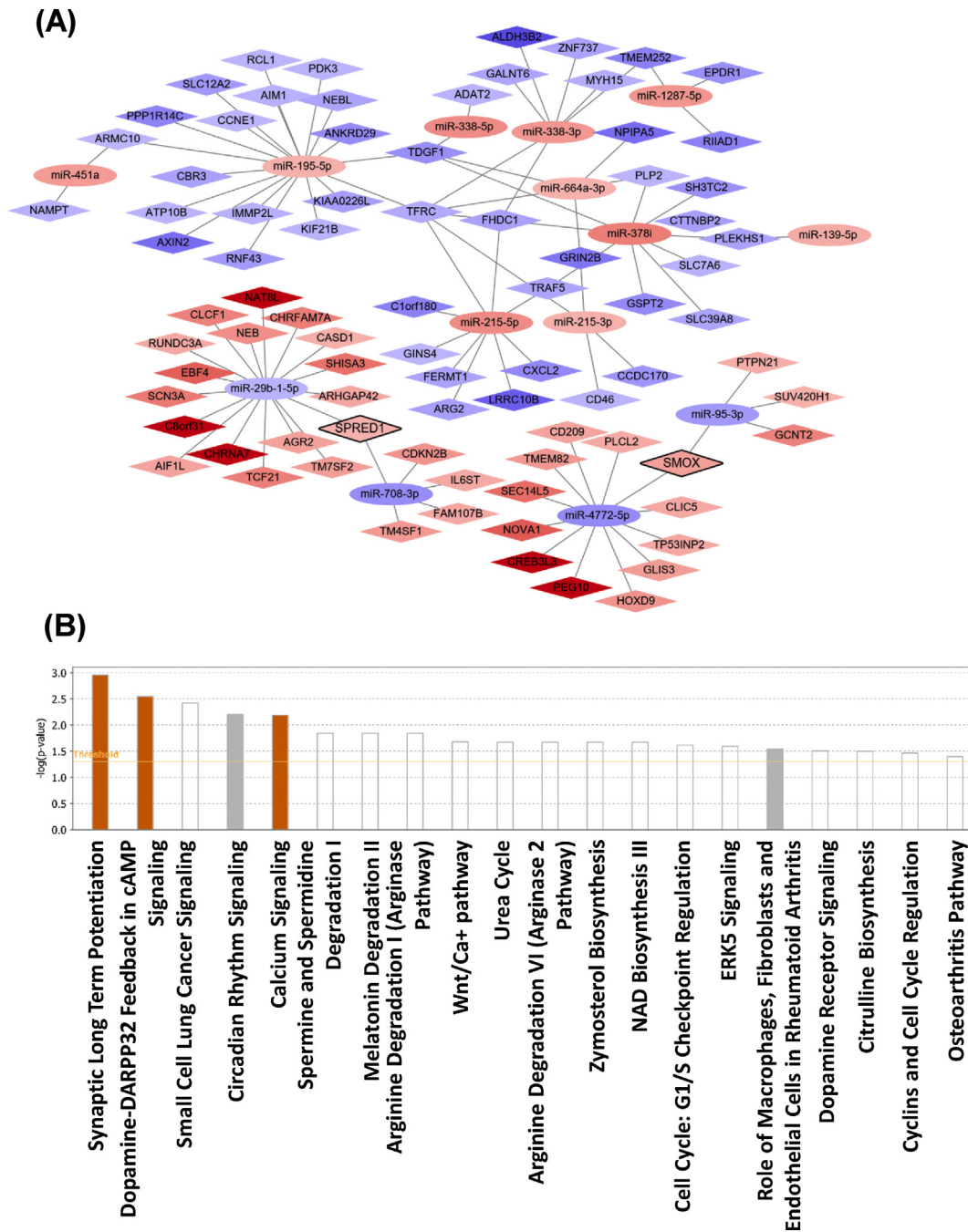


Fig. 6 An integrative analysis of transcriptome alterations associated with CRC survival outcome. Networks comprising 14 miRNA targets (oval) and 82 mRNA targets (diamond) found by this analysis are shown in (A), from which 99 miRNA-mRNA paired regulation networks were constructed. Red and blue nodes indicate the upregulated and downregulated targets, respectively. Pathway analysis of the 82 targets is shown in (B).

expression patterns converse to those of miRNAs and 2) potential target sequences of the targeting miRNAs. The results showed that 14 miRNAs and 82 mRNAs were co-aggregated into 99 miRNA-mRNA paired regulation networks (Fig. 6A and additional file 1: Table S7). We found that the pathway analysis of the 14 miRNA-targeted genes (82 mRNAs) revealed significant enrichment of several pathways, such as calcium signaling, Wnt/Ca + pathway, and G1/S checkpoint regulation pathway (Fig. 6B and additional file 1: Table S8). Within this

recurrence-related gene network, several potentially druggable targets were found to be upregulated (Fig. 6A). Of note, two of these candidate genes are simultaneously targeted by two miRNAs – SPRED1 by miR-29b-1-5p and miR-708-3p, while SMOX by miR-95-3p and miR-4772-5p (highlighted by solid outline in Fig. 6A). Our results in this part provided insights into the transcriptome networks underlying recurrence of stage-III CRC and illuminated on potential therapeutic targets.

Discussion

CRC is a major health concern worldwide and in Taiwan. A surge in incidence has been observed, and the frequent diagnosis in advanced stages of the disease is correlated with greater need for medical and surgical support. Thus, it is clinically pressing to discover effective biomarkers for the diagnosis and prognosis of CRC. In the present study, we analyzed the exomes and transcriptomes of 60 pairs of CRC tissue specimens. The mutational landscapes in Taiwanese patients with CRC were largely comparable with those previously reported. Nevertheless, a novel finding of our study was the correlation of the APC mutation status with the outcome in patients with CRC. Especially for stage III CRC, patients with mutated APC exhibited better 2-year overall survival and disease-free survival. The clinical implication of the APC genotype was not recapitulated by the analysis of TCGA COADREAD and ICGC COCA-CN dataset. However, we observed a similar (albeit nonstatistically significant) trend in TCGA African Americans, suggesting that the discrepancy may be attributed to regional and/or environmental differences. Xicola et al. (2018) reported a similar trend in African Americans [23]. They have identified a subtype of CRC that is associated with younger age of diagnosis, lack of APC mutation, lower mutation burden and distinctive methylation changes. In our Taiwan CRC-60 dataset, APC wt was found to be associated with greater proportion of poor differentiation state and higher EGFR expression, which reportedly corresponds to poor patient survival and a higher disease recurrence [37]. Importantly, this finding may serve as an important clinical recommendation for patients with CRC with wild-type APC during post-surgery follow-up.

Santos et al. have found a similar mutation pattern of APC gene with protein truncation, although they did not report its impact on prognosis [38]. Interestingly, using microarray data from 746 patients with stage I–IV CRC, Jorissen et al. recently reported that the wild-type APC status is a marker of poor prognosis in microsatellite stable proximal colon cancer [39]. For the stage III cases in their cohort, the 5-year recurrence free survival of patients with wild-type APC was 20.0%, markedly lower than the 54.0% observed in those carrying an APC mutation ($p = 0.0048$) [29]. While the clinical attributes (e.g., stages and anatomical sites) were generally similar between this study and ours, we lacked the sample size to further focus on subregional samples (i.e., proximal and distal colon cancer). Interestingly, despite both studies showing an inverse correlation between the APC genotype and patient survival, our study revealed a higher rate of KRAS mutation rate versus the previous study (56.6% vs. 35.1%, respectively). The negative contribution of a wild-type APC locus to the survival of patients with CRC is presently unresolved. Although mutation in the APC gene is frequently found in most CRC tissues, there are patients with CRC with an intact APC gene in their tumors [40,41]. These tumors may involve a different path of tumorigenesis and, when compounded by other genetic and/or environmental factors, result in an inferior disease outcome.

In our integrative transcriptome analysis, we uncovered two upregulated mRNA genes that were potential targeted by two different miRNAs (Fig. 6), suggesting that they are robustly connected in this recurrence-associated transcriptome network. One of the candidate genes is SMOX, a flavoprotein that is one of the key regulatory enzymes involved in polyamine homeostasis. It is involved in reactive oxygen species (ROS)/polyamine metabolism and could be used as a marker of ROS/polyamine metabolism. Interestingly, combination therapy using curcumin with the ODC inhibitor 2-difluoromethylornithine, a chemoprevention agent targeting polyamine metabolism, significantly inhibited the growth of gastrointestinal cancer cell lines [42], suggesting that SMOX might be a viable therapeutic target. A previous report has also shown that low SMOX RNA is related to better survival in colorectal cancer [43]. Moreover, the tumorigenic role of SMOX is demonstrated by the multiple intestinal neoplasia (Min) mouse model, in which treatment with MDL 72527 reduces *Bacteroides fragilis* ETBF-induced, SMOX-dependent colon tumorigenesis by 69% ($P < 0.001$) [44]. On the other hand, SPRED1 (or Sprouty related EVH1 domain containing 1) is a member of the Sprouty family of proteins and is phosphorylated by tyrosine kinase in response to several growth factors. The encoded protein can act as a homodimer or as a heterodimer with SPRED2 to regulate activation of the MAP kinase cascade. SPRED family genes are also key regulators of RhoA-mediated cell motility and signal transduction [45]. Defects in this gene are a known cause of neurofibromatosis type 1-like syndrome (NFLS). In addition, SPRED1 is negatively regulated by miR-196a in the progression of breast cancer [46], and was found to be up-regulated in metastatic prostate cancer [47], indicating that its expression is linked to malignancy. Further mechanistic understanding of the roles of these molecules in the clinical outcome of stage III CRC requires additional investigation.

Conclusions

Stage III disease constitutes a major part of CRC, which exhausts substantial medical resources due to tumor recurrence. Using NGS-based comparisons among different datasets, we set out in this study to decipher clinical and molecular attributes for markers discovery. APC mutation was prevalent among nearly all datasets, while its impact on prognosis was much different. Particularly for stage III CRC in the Taiwan cohort, the overall survival rate of patients with mutated APC was higher than those with wild type APC (94.4% and 67.7%, respectively; $p = 0.018$), and the recurrence-free survival rate was also (94.4% and 16.7%, respectively; $p = 0.00044$). Through clinical and gene expression analyses, we further discovered that the APC wt specimens to a greater extent exhibit poor differentiation state as well as EGFR upregulation, providing probably explanation for the poor prognosis of these patients. Finally, an integrated transcriptome analysis was used to construct the recurrence-associated mRNA-miRNA networks, from which potential druggable markers for recurrent stage III CRC was uncovered.

Conflicts of interest

The authors disclose no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bj.2021.03.001>.

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