Using bioinformatics approaches to investigate driver genes and identify BCL7A as a prognostic gene in colorectal cancer

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**Abstract**

Colorectal cancer (CRC) results from the uncontrolled growth of cells in the colon, rectum, or appendix. The 5-year relative survival rate for patients with CRC is 65% and is correlated with the stage at diagnosis (being 91% for stage I at diagnosis versus 12% for stage IV). This study aimed to identify CRC driver genes to assist in the design of a cancer panel to detect gene mutations during clinical early-stage screening and identify genes for use in prognostic assessments and the evaluation of appropriate treatment options. First, we utilized bioinformatics approaches to analyze 354 paired sequencing profiles from The Cancer Genome Atlas (TCGA) to identify CRC driver genes and analyze the sequencing profiles of 38 patients with >5 years of follow-up data to search for prognostic genes. The results revealed eight driver genes and ten prognostic genes. Next, the presence of the identified gene mutations was verified using tissue and blood samples from Taiwanese CRC patients. The results showed that the set identified gene mutations provide high coverage for driver gene screening, and APC, TP53, PIK3CA, and FAT4 could be detected in blood as ctDNA test targets. We further found that BCL7A gene mutation was correlated with prognosis in CRC (log-rank p-value = 0.02), and that mutations of BCL7A could be identified in ctDNA samples. These findings may be of value in clinical early cancer detection, disease monitoring, drug development, and treatment efforts in the future.

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1. Introduction

The prevalence of colorectal cancer (CRC) is quite high, with 776,120 men and 768,650 women diagnosed with CRC in the United States as of January 1, 2019 [1]. New case numbers were estimated at 104,610 and 43,340 for colon cancer and rectal cancer, respectively, diagnosed in 2020 in the US, according to an American Cancer Society report [2]. CRC, which is also known as bowel cancer, results from uncontrolled cell growth in the colon, rectum, or appendix. Most CRCs arise from adenomatous polyps. These neoplasms are usually benign, but some will further develop into cancer. The 5-year relative survival rate for patients with CRC is 65% (67% for rectal cancer and 64% for colon cancer). For CRC patients diagnosed with stage I and stage II disease, the 5-year relative survival rates are 91% and 82%, respectively. However, for patients diagnosed with stage IV CRC, the 5-year survival rate declines to 12% [1]. In other words, most long-term CRC survivors are diagnosed in the early stages, indicating the importance of early diagnosis and continuous monitoring. Regular screening can prevent death caused by CRC.

The current CRC screening methods have various limitations. For example, stool-based tests have a high false-positive rate (50%), and guaiac-based fecal occult blood tests (gFOBT) have sensitivity levels of only 24–40% [3]. One more advanced method, fecal immunochemical tests (FITs), can detect sources of lower gastrointestinal bleeding [4] but cannot prove cancer. A variety of serum markers have been associated with CRC, particularly carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA 19–9). However, they have a low diagnostic ability to detect primary CRC due to significant overlap with benign disease, as well as low sensitivity for early-stage CRC [5]. Colonoscopy is considered the gold standard for CRC screening; however, studies have indicated that colonoscopy will miss 2% to 6% of CRCs [6], some of which may be challenging to detect due to a proximal location or depressed appearance. Furthermore, colonoscopy is an invasive procedure that requires patients to undergo rigorous bowel preparation and the risk of pore formation, underscoring the need for a non-invasive method for diagnosing CRC.

Next-generation sequencing (NGS) is currently widely used in cancer genomics research. Many large-scale cancer projects, such as The Cancer Genome Atlas (TCGA), the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project, the International Cancer Genome Consortium (ICGC), and the Pediatric Cancer Genome Project (PCGP), use NGS technologies to generate large amounts of cancer genome data that can be used to form the basis for the development of precision medicines [7]. In recent studies, scientists have coined the term “driver mutations” to describe mutations known to cause cancer development and clonal expansion. By contrast, mutations that accompany cancer progression but do not cause cancer development and do not affect the cancer cells are referred to as “passenger mutations” [8–10]. Identifying driver mutations in cancer-related genes associated with colorectal cancer progression has been a central aim for the development of clinical early-stage detection techniques.

Based on the results of fundamental research studies combined with new mutation detection technologies, the effectiveness of colon cancer screening panels has significantly increased, but their use is still currently limited to scientific research and personal testing, with only a few panels having received or still awaiting FDA approval. For scientific research purposes, Qiagen applies GeneRead DNASEq Targeted Panels V2 for CRC (<100 genes), while ThermoFisher Scientific applies the Ion AmpliSeq™ Colon and Lung Cancer Research Panel. For personal testing, Ambry Genetics™ applies ColoNext, which includes a 20-gene panel[11]. The Invitae company (https://www.invitae.com/en/), meanwhile, offers tests that can help people detect whether they have genetic mutations that are risk factors for several types of cancers, but not CRC.

Otherwise, HalioDx, a new French company, has developed a cancer assay, Immunoscore, that is an in vitro risk-assessment tool that provides independent and superior prognostic value and can predict the risk of relapse in early-stage colon cancer patients by measuring the host immune response at the tumor site [12]. However, as alluded to above, only two colon tests have obtained FDA approval thus far, namely, Cologuard [13], which was approved by the FDA in August 2014, and Epigenomics’ proColon, which is the first and only FDA-approved blood-based test for the detection of CRC [14]. Cologuard is a stool-based triple test method that includes a FIT test, a DNA mutation and methylation test, and a β-actin test, plus a hemoglobin immunoassay. Meanwhile, proColon is a blood-based CRC screening method that detects methylated SEPT9 in the blood. However, the coverage and the false-positive rates of these two tests remain unknown. We continue to require a more convenient and accurate screening method for the identification of early-stage CRC. Using gene mutations to detect cancer can improve the precision and specificity of cancer screening techniques. In addition to driver genes, the correlation between cancer prognostication and gene mutations has been studied [15–17]. The detection of the prognostic genes can aid drug selection and surgery evaluations in determining medical treatment strategies.

This study sought to identify driver mutations to develop an effective, early-stage cancer detection panel for CRC patients and identify prognostic genes for the evaluation of treatment options and prognostic assessments. First, we collected 354 CRC tumor-normal pairs sequencing profiles from TCGA and used the DRIVERDB [18] and Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA; http://www.ingenuity.com/) to find an eight-driver gene signature in CRC. To identify prognostic genes, we utilized the sequencing profiles of CRC patients in combination with prognostic data from TCGA, which resulted in ten genes that we classified as prognostic genes. Next, these eighteen genes, including eight driver genes and ten prognostic genes, were verified using tissue and blood samples from Taiwanese CRC patients. The results showed that the identified set of genes provides high coverage for driver gene screening, and one of the identified prognostic genes, BCL7A, was associated with a significant difference (log-rank p-value = 0.02) in survival. These findings could be used to improve CRC screening and provide guidance for drug development and treatment efforts in the future.

2. Material and methods

2.1. Data collection and mutation annotation

We collected data for 354 CRC patients, including 266 colon adenocarcinoma (COAD) and 88 rectum adenocarcinoma (READ) patients, with both tumor and normal sequencing data from our own database, DriverDB [18], in 2015. In brief, the mutation data, including single-nucleotide variants and insertions/deletions, of these patients were downloaded from the Cancer Genomics Hub [19] and mapped to published databases, including the dbSNP [20], 1000 genomes [21], NHGRI GWAS catalog [22], COSMIC [23], NHBLI GO ESP [24], ClinVar [25], and OMIM [26] databases, in order to annotate known variants. To predict the impacts of various mutations, such as synonymous mutations, frame-shift mutations, and stop/gain mutations, we used the SnvEff [27] and VEP [28] tools.
2.2. Identification of CRC driver genes

Based on mutation data from TCGA, we utilized 3 bioinformatics tools, as described in our previous study [18]: MutsigCV [29], Dendrix [30], and ActiveDriver [31], using default parameters. For ActiveDriver, the genes with a FDR adjusted P value < 0.05 are statistically unexpected mutated in protein phosphorylation sites or protein kinase domains. For MutsigDV, the genes with an FDR-adjusted P-value < 0.05 are significantly mutated. Dendrix identifies large numbers of genes in different K, where K represents a genaset within a module/pathway. The genes reported in at least 10% of modules in any K are identified as significant, based on our previous study [18]. The common genes identified by the three tools being selected for further analysis. For the COAD and READ datasets, there were 177 and 144 genes commonly identified by the 3 tools, respectively. Forty-seven genes were identified in both of the two datasets.

2.3. Identification of CRC prognostic genes

We identified 38 CRC patients with at least 5 years of follow-up data or who died within 5 years of data collection by TCGA in 2015. We divided these 38 patients into 2 groups, according to >5-year and <5-year survival times, into “Good” and “Poor” survival groups, respectively. To identify prognostic genes in CRC, we used the 3 described bioinformatics tools to identify significantly mutated genes in the two groups. Ten significant genes identified in the good prognosis group but not the poor prognosis group were regarded as prognostic genes for CRC in this study.

2.4. Clinical samples collection

There were 56 tissue samples and nine blood samples collected from 56 Taiwanese CRC patients who served as the validation cohort in this study. The appropriate clinical samples for molecular analysis need to be considered in both qualitative and quantitative terms. This study was approved by the Institutional Review Board (IRB) of Taipei Veterans General Hospital (CF13041A-2), and all the participating patients signed and provided their informed consent before sample collection. During surgery, the tumor samples and adjacent normal parts were collected simultaneously by Dr. Jeng-Kae Jiang at Taipei Veterans General Hospital.

2.5. Genetic network analysis and canonical pathway analysis

The differential gene expression profiles were imported into the Ingenuity Pathways Analysis (IPA) software (Qiagen) to obtain functional regulatory networks and canonical pathways. The “core function” can interpret gene interaction networks based on its Ingenuity Knowledge Base (IKB), which is constructed by integrating findings from the past literature. It can in turn generate the gene-gene interaction/relationships and the pathways of focused genes by importing interesting genes to IPA. For canonical pathway analysis, IPA can help us discover genes incorporated into biological functions/pathways and generate a list of statistical scores as P values, indicating the connection strength between gene sets and pathways.

2.6. Target sequencing of the driver genes and prognostic genes

Utilizing Illumina TruSeq Custom Amplicon (TSCA), the amplicon design service provided by Illumina Inc., San Diego, CA, USA, we designed a target sequencing gene set consisting of the 8 driver genes and 10 prognostic genes (Fig. 1). Then, we performed target sequencing on the DNA from the tissue and blood samples of the aforementioned 56 Taiwanese CRC patients. According to the manufacturer’s protocol, libraries were prepared with TSCA. Preparation of these libraries was performed starting with up to 50 ng of genomic DNA, the adjacent normal tissue sample corresponding to the normal reference, or hg19 when germline DNA from the patient was not available. Briefly, the gDNA samples from different patients were amplified by numerous PCR primer sets designed by Illumina DesignStudio (http://www.illumina.com/informatics/research/experimental-design/designstudio.html), and the amplified DNA fragments were simultaneously added to adaptor sequences. The size distribution of the purified libraries was assessed using the 2100 Bioanalyzer with DNA assay kit (Agilent Technologies, Santa Clara, CA). The fluorescent method and Q-PCR (KAPA, USA, cat. KK4903, and KK4603) were used to quantify the libraries.

3. Results

3.1. Identifying driver genes in CRC

We collected tumor-normal paired sequencing data for 266 colon adenocarcinoma (COAD) and 88 rectum adenocarcinoma (READ) patients from the TCGA portal. The tumor-normal paired sequencing data of these 354 patients was then used to find CRC mutated genes, including those predicting variant effects, and to find variants that might cause amino acid changes or lead to protein dysfunction (the “Driver Genes Identification” module in Fig. 1). These mutations were further mapped to the gene-level to predict driver genes. After identifying driver genes, 177 genes and 134 genes were annotated from the COAD and READ patients, respectively, and 47 genes that were annotated from both the COAD and READ patients were considered the common driver genes in CRC (Fig. 2A). These 47 genes are listed in Table S1. By taking advantage of IPA, we examined the relationships among these common driver genes in CRC. Eight genes related to COAD were recognized, namely, APC, BRAF, CDH8, FAT4, KRAS, NEFH, PIK3CA, and TP53 (P-value = 7.12E-07) (Fig. 2B). These 8 driver gene candidates were mapped back to the original TCGA data and showed a high coverage rate in COAD (93.98%) and READ (94.32%). The coverage rates of these eight genes in the 354 patients were as follows: APC (300/354), TP53 (249/354), PIK3CA (177/354), KRAS (172/354), NEFH (108/354), BRAF (96/354), FAT4 (67/354) and CDH8 (33/354) (Fig. 2D). These results indicated that these genes could cover most of the CRC patients. The gene network analysis of the aforementioned 47 genes showed that 5 of the 8 driver genes, including APC, BRAF, KRAS, TP53, and PIK3CA, had hub characteristics that were connected by more than three edges in our IPA results (Fig. 2C).
3.2. Validation of identified CRC driver genes by using Taiwanese samples

We collected clinical samples from 56 CRC patients at Taipei Veterans General Hospital in Taiwan. The baseline information of these 56 CRC patients is listed in Table 1. In terms of gender, 57.1% of the patients were male, and 42.9% were female, indicating no substantial gender imbalance in this data. The pathological examination results showed that most of these patients had T3N0M0 stage adenocarcinomas. After identifying the CRC driver genes, we designed a mutated gene sequencing set containing the eight genes related to CRC development and performed targeted sequencing with this focused gene set. The results revealed a high coverage rate of 96.4% in the 56 patient tissue samples and coverage of eight out of nine patient blood samples (Fig. 3). The coverage rates for the eight genes in the tissue samples were 33/56 (APC), 26/56 (FAT4), 23/56 (KRAS), 21/56 (TP53), 16/56 (CDH8), 12/56 (PIK3CA), 10/56 (NEFH), and 5/56 (BRAF). Thirty of the 56 patients had at least three mutations while 15 of the patients only had one gene mutation in this mutated gene sequencing set. Among these 15 patients, the KRAS gene was mutated in five patients, three patients had APC gene mutations, and two patients each had TP53 and FAT4 mutations. For the nine blood samples, the circulating cell-free tumor DNA (ctDNA) was...
tested, and the results showed that the driver gene mutations could be detected in the blood samples (Fig. 3B). The mutated eight-gene sequencing results showed that four gene mutations (APC, TP53, PIK3CA, and FAT4) could be detected in plasma. The APC and TP53 genes have been used as target genes in cancer screening using the ColoNext method of Ambry Genetics™, but the PIK3CA and FAT4 genes have not been used in that approach. Therefore, based on our findings, PIK3CA and FAT4 could be new targets for ctDNA analysis development in CRC.

### 3.3. Identifying prognostic genes in CRC

To find the genes correlated to prognostication, we collected characteristic clinical data from TCGA. According to their survival times, the sequencing profiles of 38 CRC patients were classified into two groups: a good prognosis group and a poor prognosis group. By taking advantage of DriverDB, we annotated the known genes and predicted the driver genes. We looked for genes in the poor prognosis group and filtered out the genes that intersected in the good and poor prognosis groups simultaneously. In the end, we identified ten prognostic genes, namely, AP3D1, APLP1, BCL7A, C8B, CTNNB1, FMNL2, KCNA1, PIK3R1, RET, and SYNE1 (the “Prognostic Genes Identification” module in Fig. 1). To evaluate the power of the ten prognostic genes, the 38 patients were divided into patients with the prognostic gene mutations and patients without the prognostic gene mutations. The Kaplan–Meier survival curve showed a significant difference (log-rank P = 0.0027) between these two groups (Fig. 4A). This result meant that the survival rate of the patients with the prognostic gene mutations was significantly decreased. The IPA showed that nine out of these ten genes were identified as abdominal adenocarcinoma-related genes (P = 1.26E-04) and digestive system cancer-related genes (P = 1.33E-03), while four of the genes (CTNNB1, PIK3R1, RET, and SYNE1) were correlated with rectal cancer (P = 1.47E-04) (Fig. 4B).

### 3.4. CRC prognostic genes validation in Taiwanese samples

The ten genes identified as prognostic genes were then tested in the 56 patient samples we collected. The results showed a coverage rate of 71.4% in these 56 patient tissue samples (Fig. 4C) and coverage of six out of nine blood patient samples (Fig. 4D). The mutations of seven of the ten genes could be detected in ctDNA analysis, including KCNA1, RET, APLP1, C8B, SYNE1, BCL7A, and FMNL2.

Next, we inspected the Kaplan–Meier survival plot for the ten prognostic genes in 56 Taiwanese patients (Fig. S1). No significant difference in survival was observed between patients with and without these ten mutated genes. We then examined the survival rate for each prognostic gene. Among these ten genes, BCL7A was significantly correlated with the survival rate (P = 0.0204) (Fig. 4D). This is the first study in which BCL7A has been identified as a prognostic gene in CRC, and besides identifying its correlation with the survival rate, the mutation in BCL7A can be detected in ctDNA from blood samples (Fig. S1).

### 4. Discussion

This study performed driver gene identification for CRC by combining the DriverDB and IPA, analyzing 354 paired sequencing profiles from TCGA. A total of eight driver genes correlated to CRC were thus revealed, including APC, BRAF, CDH8, FAT4, KRAS, NEFH, PIK3CA, and TP53. In a recent study identifying CRC mutations, six genes were classified as CRC driver genes: APC, TP53, PIK3CA, KRAS, BRAF, and SMAD4 [35]. The most frequently mutated of those genes, namely, APC, TP53, PIK3CA, KRAS, were consistent with our findings.

A series of mutations accumulate in critical driver genes over time in CRC development [36,37]. APC plays a principal role in CRC development and contributes to tumor progression. A defect of the APC protein induces beta-catenin accumulation and the acti-
previous investigations, been reported to be correlated with other cancers besides CRC. In this study, the other three genes identified as prognostic and mutated with the late stage of CRC development, that is, the transition of carcinoma cells, while mutated genes have been found to results in the emergence of a malignant tumor with invasion potential. In this genetic development model of CRC, mutations of APC and BRAF are involved in transforming normal epithelial cells into adenoma cells, while mutated genes have been correlated with the late stage of CRC development, that is, the transition to carcinoma.

We found five genes with hub characteristics in our IPA results, namely, APC, BRAF, KRAS, TP53, and PIK3CA, a finding consistent with previous studies. The other three genes identified as prognostic genes in this study (FAT4, CDH8, and NEFH) have previously been reported to be correlated with other cancers besides CRC. In previous investigations, NEFH was found to serve as a tumor suppressor in hepatocellular carcinoma, esophageal squamous cell carcinoma, and breast cancer; FAT4 was found to be involved in the Wnt/PCP pathway and to play a role in HIF1-α regulation; and CDH8 expression was found to be correlated with renal cell carcinoma and lung squamous cell carcinoma. In this study, the FAT4 gene coverage rates were 18.9% and 46.4% for the 354 profiles from the TCGA dataset and the 56 profiles from the Taiwanese dataset, respectively, while the CDH8 coverage rates were 9.3% and 28.6%, respectively. The NEFH coverage rate for the 354 profiles from the TCGA dataset was 30.5% higher than the 17.9% coverage rate for the 56 profiles from the Taiwanese dataset. The differences between these two datasets could have been caused by racial differences and differences in the number of samples. In any case, the FAT4, CDH8, and NEFH genes could be potential targets for early-stage detection of CRC.

This study also attempted to identify prognostic genes from the 38 profiles with associated survival data obtained from the TCGA dataset. After classifying these 38 sequencing profiles into a poor prognosis group and a good prognosis group, we used DriverDB to identify driver genes by searching for genes that were expressed in the poor prognosis group but not the good prognosis group. Ten genes (AP3D1, APLP1, BCL7A, CBB, CTNNB1, FMNL2, KCNA1, PIK3R1, RET, and SYNE1) were identified, and the Kaplan–Meier analysis showed that patients with mutations in these ten genes mutated had significantly poorer overall survival (log-rank P = 0.0027).

Kaplan–Meier analysis was applied to each of the ten identified prognostic genes in 56 Taiwanese patients. The KCNA1 gene, which encodes a voltage-gated delayed potassium channel, was mutated in 29 tissue samples from 56 Taiwanese patients and was detected in ctDNA derived from blood samples. A recent study revealed that the hypermethylation of the KCNA1 promoter might serve as a potential novel diagnostic biomarker for early CRC patients. Combined with our findings, this previous result suggests that KCNA1 might play an important role in CRC, although the mutation of KCNA1 was not associated with patient survival in the Taiwanese cohort. Patients with BCL7A mutations showed a worse survival rate (log-rank P = 0.0204). After Bonferroni correction, the conservative correction method which multiplies the raw P values by the number of tests, the posthoc adjusted p-value of BCL7A gene is 0.1836. Although the adjusted p-value is not small than 0.05, we believe that it’s due to the small sample size of the Taiwanese cohort. The BCL7A gene encodes a subunit of the SWI/SNF complex and has previously been reported to serve as a prognostic gene for early-stage cutaneous T-cell lymphoma and ovarian cancer. The result still provides insight of BCL7A as a prognostic gene. This study is the first to identify BCL7A as a prognostic gene in CRC and the first to report BCL7A ctDNA detection in plasma.

In recent years, the detection of circulating cell-free tumor DNA (ctDNA) has arisen as an attractive and minimally invasive method for detecting cancer-specific genetic differences in blood. This value of this approach has benefitted from the rapid development and application of NGS technology in cancer research and diagnostics. In our study, the highest coverage rate in the blood samples was for the TP53 gene (44%), followed by that for the APC gene (33%). The ColoNext (https://www.ambraygen.com/providers/genetic-testing/6/oncology/colonext) is a 20-gene guideline-based panel identifying patients with inherited risk for CRC. The only two genes included among both the 20 ColoNext genes and our
eight driver genes were APC and TP53, which showed that mutations of these two genes could be detected through blood screening. Moreover, the validated prognostic gene, BCL7A, could also be detected in the ctDNA samples. Owing to the rapid development of NGS technology, genetic tests will become cheaper and faster, and more and more patients will benefit from this technology. Relatively, developing precise gene panels with reasonable prices is an urgent need for the protection and improvement of human health. For the accurate early-stage detection of cancer, there is still a large amount of research that must be performed, and our findings could provide guidance for future research efforts.

5. Conclusion

The results of the current study showed that eight driver gene mutations were identified in CRC. Of those genes, APC, TP53, PIK3CA, and FAT4 could be detected in blood as ctDNA analysis targets. Moreover, this study discovered 10 prognostic gene mutations in CRC. Among these genes, BCL7A gene mutations are correlated with prognosis in CRC, and that these mutations could be found through the ctDNA sequencing. However, while our results and those of many previous studies have indicated several potential targets for ctDNA analysis, further large-scale research studies and further technological development are required. Furthermore, the plasma and ctDNA separation protocols need to be standardized for comparison between different studies in order to determine the clinical situations in which ctDNA analysis is superior to other methods and to further optimize these protocols for application in clinical early cancer detection or disease monitoring. Finally, the findings of the present study could provide valuable guidance for future studies involving disease screening, drug development, and treatment suggestions.

6. Author statement

W-C.C. and Y-F.L conceived and designed the experiments. JY-C.C, H-C.C., J-K.J., and C-Y.Y performed the experiments. W-C.C., F-H.C., Y-L.L, W-J.L, and C-Y.L analyzed the data. JY-C.C, M-H.Y and S-C.W. contributed reagents, materials, and/or analysis tools. W-C.C., Y-F.L. and JY-C.C wrote the paper. All authors reviewed the manuscript.

Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.06.044.

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