



Genome variation in colorectal cancer patient with liver metastasis measured by whole-exome sequencing

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Background: Liver metastasis of colorectal cancer (CRC) is an important cause of death from CRC, but its molecular mechanism is still unclear. In recent years, whole-exome sequencing has played an increasingly important role in the study of the occurrence and development of diseases, especially malignant tumors. Its high throughput and low cost advantages enable researchers to explore the pathogenic genes of diseases, and screen potential molecular markers and therapeutic targets from the level of genomics.

Methods: This study collected the primary tumor tissues, matched paracancerous, normal tissues, and liver metastases of 4 CRC patients admitted to the Department of General Surgery of the First Affiliated Hospital of Soochow University, and performed high-depth whole-exome sequencing, with the sequencing depth of each sample reaching 123× on average, then filtered the sequencing data, compared them, and analyzed the bioinformatics data.

Results: we found 8,565 single nucleotide variants (SNV) and 429 insertions/deletions (InDel) in the primary and hepatic lesion tissues, and the genes with the highest mutation frequency were titin (TTN), obscurin (OBSCN), and homeodomain-interacting protein kinase 2 (HIPK2). The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the mutant genes was conducted, and it was found that the mutant genes were mainly concentrated in the cells, cell parts, and cellular process of GO. The results of KEGG pathway analysis showed that mutations were mainly distributed in circadian entrainment, insulin secretion, and glutamatergic synapse. Further, we identified 723 SNV and Indel genes with high frequency mutations including TTN, OBSCN, and hydrocephalus-inducing protein homolog (HYDIN) across all tissues of liver metastases. The GO analysis showed that the mutated genes in liver metastatic tissues were mainly concentrated in cell, cell part, and cellular process. The KEGG pathway analysis showed that high frequency mutation genes were focused on gastric acid secretion, bile secretion, and melanogenesis.

Conclusions: This study found some candidate genes related to the occurrence of CRC and liver metastasis through whole-exome sequencing of relevant tissues in CRC patients with liver metastasis, which is expected to provide new markers and therapeutic targets for such patients.

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Keywords: Whole-exome sequencing; colorectal cancer (CRC); liver metastasis

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Introduction

Colorectal cancer (CRC) is one of the most common digestive system malignancies in the world. In China, nearly one fifth of CRC patients have been found to have liver metastasis (LM) at their first diagnosis, and half of CRC patients have LM during the disease progression. The involvement of LM is a very important factor in the prognosis of CRC (1,2), it is caused by the interaction of multiple factors, and its clinical phenotype, characteristics, and molecular mechanism of metastasis are also currently hot topics in both clinical and basic research. The variation of tumor cell genome is an important factor for the occurrence and metastasis of tumors (3-5). In recent years, whole-exome sequencing has been widely used in the study of various diseases, especially in oncology research. Several functional genes associated with lung cancer targeting or immunotherapy have been identified in non-small cell lung carcinoma (NSCLC) (6,7). It has also been repeatedly confirmed by whole-exome sequencing that protein 53 (P53), Kirsten rat sarcoma viral oncogene homolog (KRAS), adenomatous polyposis coli (APC), and other genes play an important role in the occurrence and progression of CRC (8,9). However, the genomic characteristics of LM from CRC are still unclear. Though there are many studies on the NGS of colorectal cancer, but relatively few studies on the gene detection of colorectal cancer with liver metastasis, and the data of exome sequencing of colorectal cancer with liver metastasis are even less in China.

In this study, primary tumor tissues, matched normal tissues, and liver metastasis specimens of 4 CRC patients with LM from the General Surgery Department of the First Affiliated Hospital of Soochow University were collected, and high-depth complete exome sequencing was performed to analyze the mutated genes, tumor mutation burden (TMB), molecular functions, and signal pathways related to CRC with LM. It is hoped that this study can deepen the understanding of CRC with LM and provide potential marker molecules and therapeutic targets for the diagnosis and treatment of CRC with LM. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/jgo-21-9>).

Methods

Participants and samples

CRC patient sample acquisition

We collected primary CRC tumor tissues, matched normal tissues, and liver metastatic tumor tissues of 4 patients from the Department of General Surgery of the First Affiliated Hospital of Soochow University from September 2019 to April 2020. Informed written consent was provided by all patients before inclusion in this study. Respective tumor tissue samples from a histologically confirmed adenocarcinoma by two molecular pathologists were matched with the inclusion criteria. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (No. 2021-023).

DNA extraction

The DNA was extracted from primary tumor tissues, liver metastatic tissues, and matched normal tissues using E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) and QIAamp DNA FFPE Tissue kit (Qiagen Sciences, Venlo, Netherlands). The DNA extracted from normal tissues were used as germline DNA control. The samples with cancer cell populations were estimated by pathologists to ensure more than 75% of cells were cancer cells. A bioanalyzer (Agilent, Palo Alto, CA, USA) was used to assess DNA quantity.

Whole-exome sequencing and data processing

Sample genome DNA was fragmented using NEBNext dsDNA Fragmentase (NEB, Ipswich, MA, USA) followed by DNA ends repairing. End-repaired DNA fragments were detailed and ligated with the NEBNext adaptor (NEB, Ipswich, MA, USA). Biotinylated RNA library baits and magnetic beads were mixed with the barcoded library for selection of targeted regions using the SureSelect Human All Exon V6 Kit (Agilent Technologies, Palo Alto, CA, USA). The captured sequences were further amplified for 150 bp paired-end sequencing in Illumina X-ten system (Illumina, San Diego, CA, USA).

Table 1 Clinicopathological participant characteristics

Characteristics	No. of cases [%]
Total number	
Age, years, mean [range]	64.5 [46–77]
Gender	
Male	3 [75]
Female	1 [25]
Pathological diagnosis	
Adenocarcinoma	4 [100]
Tumor stage at diagnosis	
III	0
IV	4 [100]
Histological grade	
Grade 2	2 [50]
Grade 3	2 [50]
Vascular invasion	
Yes	2 [50]
No	2 [50]
Lymphatic invasion	
Yes	4 [100]
No	0
Perineural invasion	
Yes	2 [50]
No	2 [50]
CEA value at diagnosis	
Normal	0
Increased	4 [100]

Somatic single nucleotide variant and insertion/deletion identification

To identify somatic single nucleotide variants (SNVs) and insertion/deletions (InDels), the Burrows-Wheeler Aligner (BWA) (10) was used to align the clean reads from each sample against the human reference genome (GRCh38). The picard tool (<http://picard.sourceforge.net/index.shtml>) removed the read duplicates derived from library polymerase chain reaction (PCR). Somatic SNV and InDel calling was processed for multi-sample by MuTect (<https://software.broadinstitute.org/cancer/cga/mutect>) (11). Tumor mutation burden (TMB) is calculated as the number of somatic mutations per million bases (MB), including

somatic single nucleotide variants (SNVs) and insertion/deletions (InDels).

SNV

The gene sets screened were used for functional annotation analysis using in-house script, which consisted of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (12) and Gene Ontology (GO) (13) database. The significance of gene group enrichment was defined by a modified Fisher's exact test and a P value <0.05 was considered to indicate a statistically significant difference.

Statistical analyses

The software SPSS (IBM Corp., Armonk, NY, USA) (14) was used to analyze all the correlated biological and clinical variables.

Results

Participant characteristics

The mean age of the 4 CRC patients with LM was 64.5 years (range, 46–77 years), and included 3 males and 1 female. All 4 were diagnosed with adenocarcinoma, and did not receive adjuvant chemoradiotherapy before Surgery. The tumor stage of 4 participants were stage IV. Half (n=2) of them were histological grade 2 and another 2 were histological grade 3. All 4 participants had lymphatic invasion and perineural invasion. The carcinoembryonic antigen (CEA) was increased in all 4 participants (*Table 1*).

Whole-exome sequencing

We collected the primary tumor tissues, matched paracancerous normal tissues, and LMs of 4 patients admitted to the Department of General Surgery of the First Affiliated Hospital of Soochow University, and performed high-depth whole-exome sequencing, with the sequencing depth of each sample reaching 123× on average (*Table S1*).

Identification of somatic single nucleotide polymorphisms and InDels

The normal tissue samples data of all participants matched the original site of cancer tissue samples data. To compare the LMs sample data analysis, we took the first participants' normal tissue and named it CRC01C (CRCO1 CONTROL), the original cancer organization

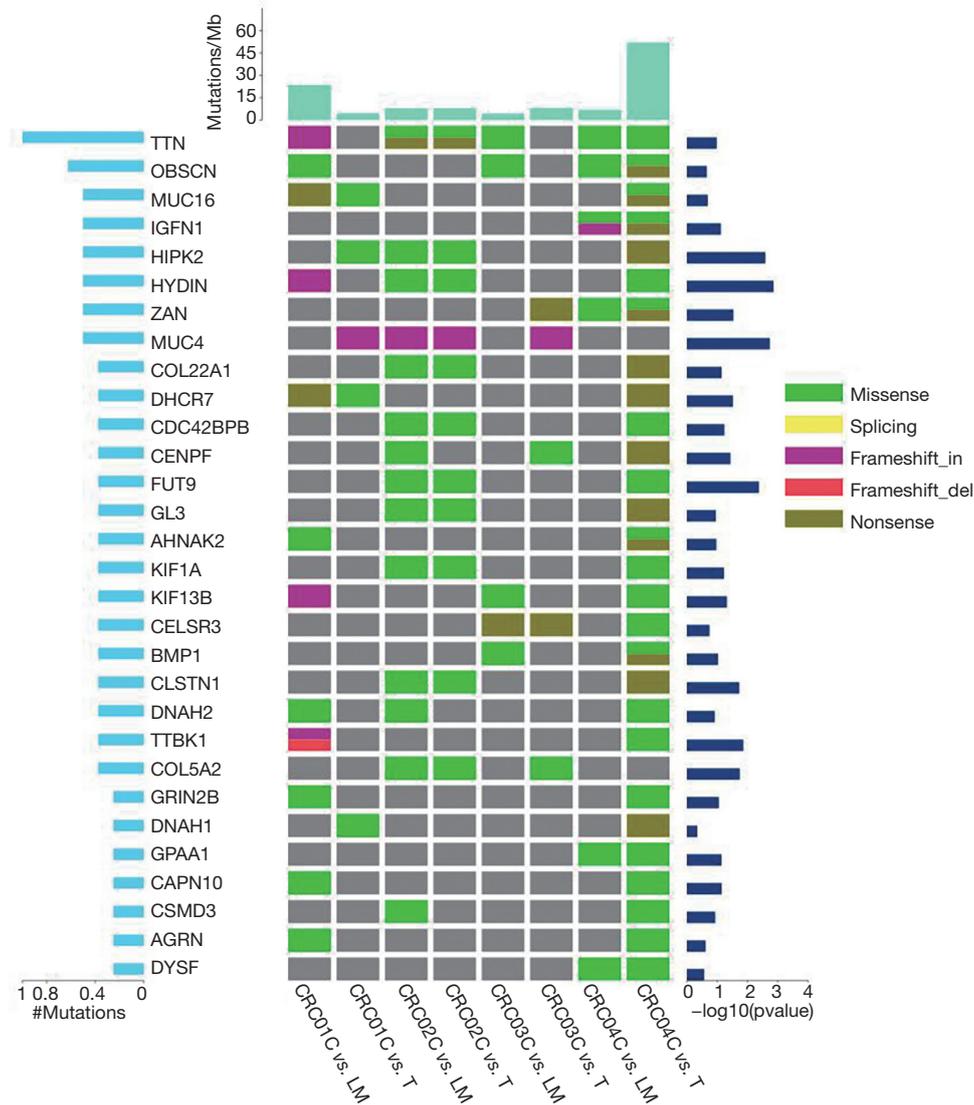


Figure 1 Landscape of somatic mutations in all samples. Figure left: proportion of the number of gene mutations. Figure right: significance of high-frequency mutations. Up: Sample mutation frequency. The types of mutations are shown, including (I) non-synonymous mutations (green), (II) shear site mutations (yellow), (III) causes code shift insertion (purple), and (IV) causes code shift deletion (red).

was named CRC01CvsT (CRC01 CONTROL *vs.* tumor), LMs sample was named CRC01CvsLM (CRC01 CONTROL *vs.* liver metastasis), and then named the rest of the participant samples in the same way. By filtering the sequencing comparing and analyzing the data, 8,565 SNVs and 429 Indels were detected across all samples; CRC04CvsT had the largest number of SNVs (5,940), followed by CRC01CvsLM (1,094) (Table S2). Significantly mutated genes (SMG) are those genes whose mutation frequency is significantly higher than those in the background. Generally, differences of somatic SNV and InDel are analyzed comprehensively. MuSiC (The Genome

Institute, Washington University, USA) software (15) was used to search for genes with higher mutation frequency in tumor samples (compared with control samples). A significantly mutated gene (SMG) test was performed for each type of mutation, whose test method was convolution test (CT). We found that the genes with the highest frequency of mutation were titin (TTN), obscurin (OBSCN), and homeodomain-interacting protein kinase 2 (HIPK2). We identified the 30 genes with the highest frequency of mutations. We also calculated the TMB of each sample respectively on the average TMB over 16 mutations per MB (5–60 mutations/MB) (Figure 1).

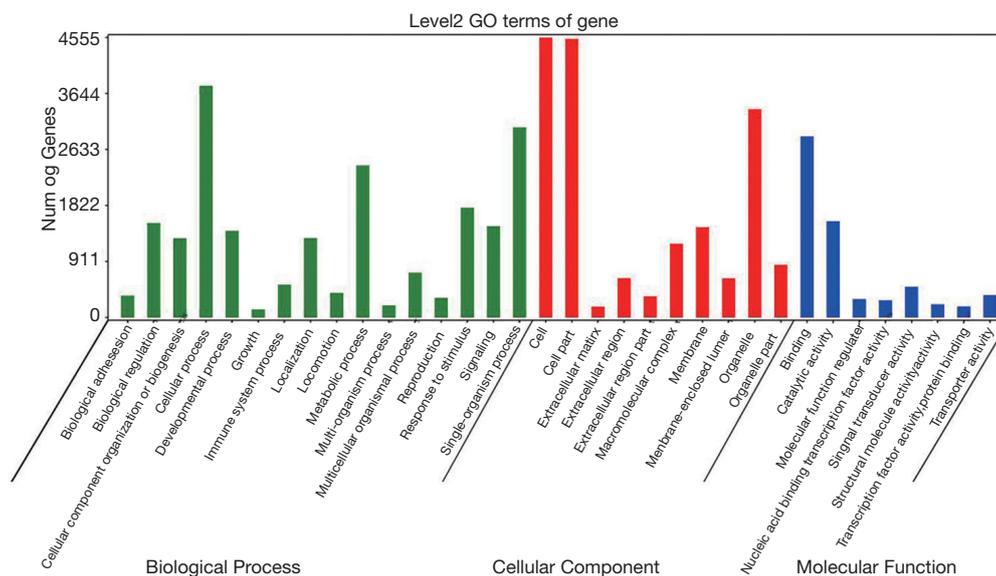


Figure 2 GO analysis histogram of the mutated genes of all samples. GO, Gene Ontology.

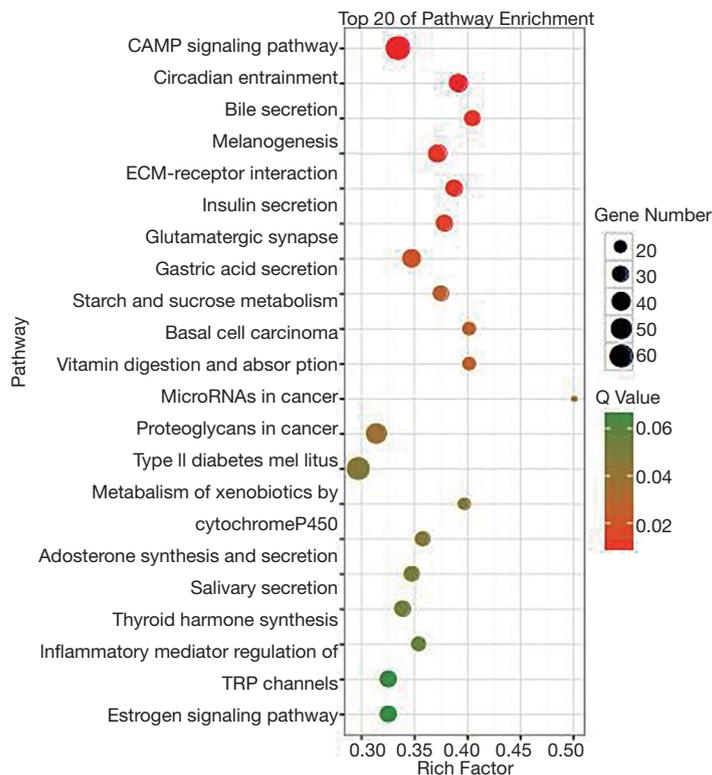


Figure 3 KEGG bubble of mutant genes of all samples. KEGG, Kyoto Encyclopedia of Genes and Genomes.

KEGG and GO analysis

We conducted GO and KEGG pathway analysis of the mutant genes, revealing that the mutant genes were mainly concentrated in the cells, cell parts, and cellular process

of GO (Figure 2). Results of KEGG pathway analysis showed that mutations were mainly distributed in circadian entrainment, insulin secretion, and glutamatergic synapse (Figure 3).

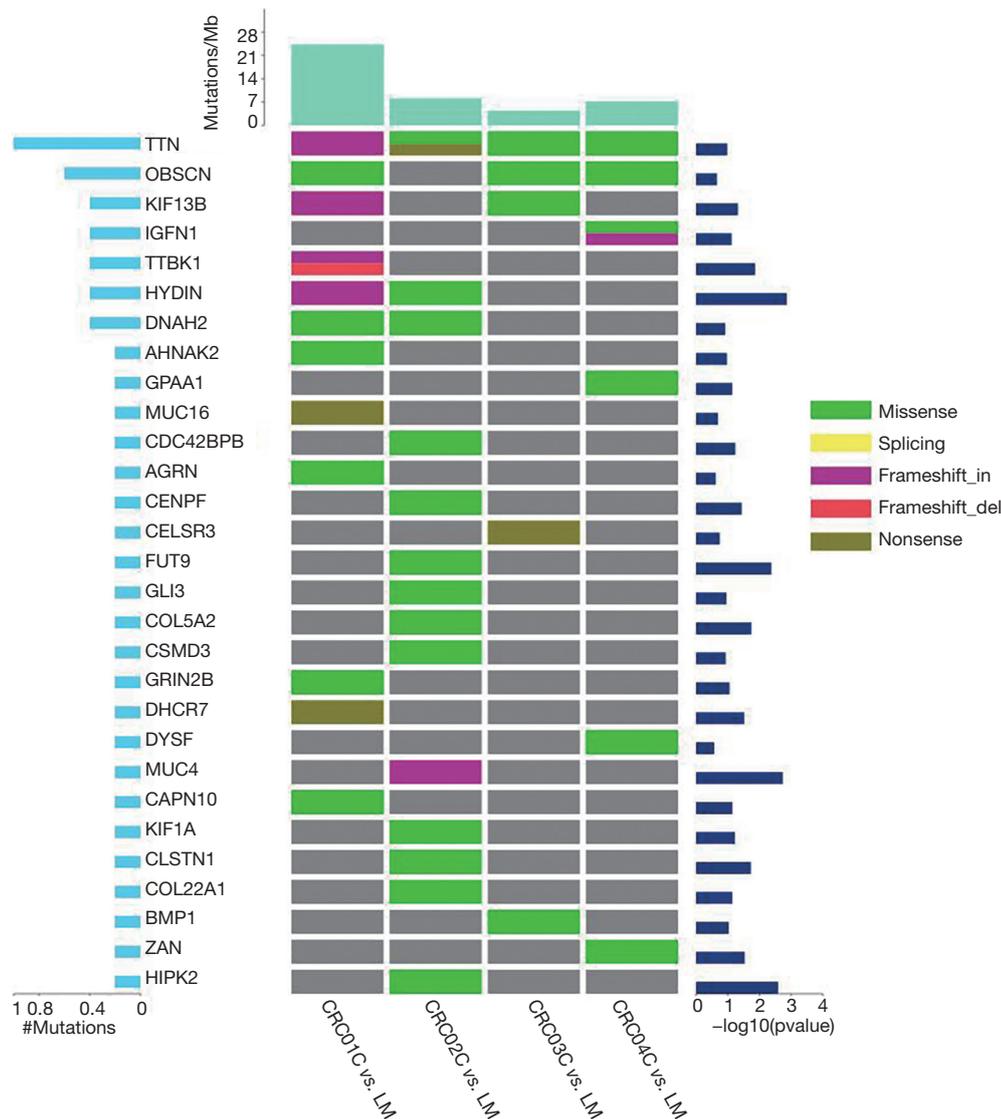


Figure 4 Landscape of somatic mutations in CRCLM samples. Figure left: proportion of gene mutations. Figure right: significance of high frequency mutations. Figure right: sample mutation frequency. The types of mutations are shown, including (I) non-synonymous mutations (green), (II) shear site mutations (yellow), (III) causes code shift insertion (purple), and (IV) causes code shift deletion (red). CVRCLM, colorectal cancer liver metastasis.

Gene mutation and KEGG and GO analysis in LM samples

We further analyzed the high frequency mutated genes of LMs, which were TTN, OBSCN and hydrocephalus-inducing protein homolog (HYDIN) (Figure 4). The GO analysis showed that the mutated genes in LM tissues were mainly concentrated in the cell, cell parts, and cellular process (Figure 5). The KEGG pathway analysis showed that high frequency mutation genes were focused on gastric acid secretion, bile secretion, and melanogenesis (Figure 6).

Discussion

Tumor metastasis is an important factor in tumor progression, and distant metastasis is often the main reason for tumor treatment failure. The process of LM of CRC is extremely complex, involving many tumor-related molecules, which are induced and regulated by their respective specific signaling pathways. With the continuous progress of all kinds of research techniques in recent years, a growing body of evidence has shown that tumor development

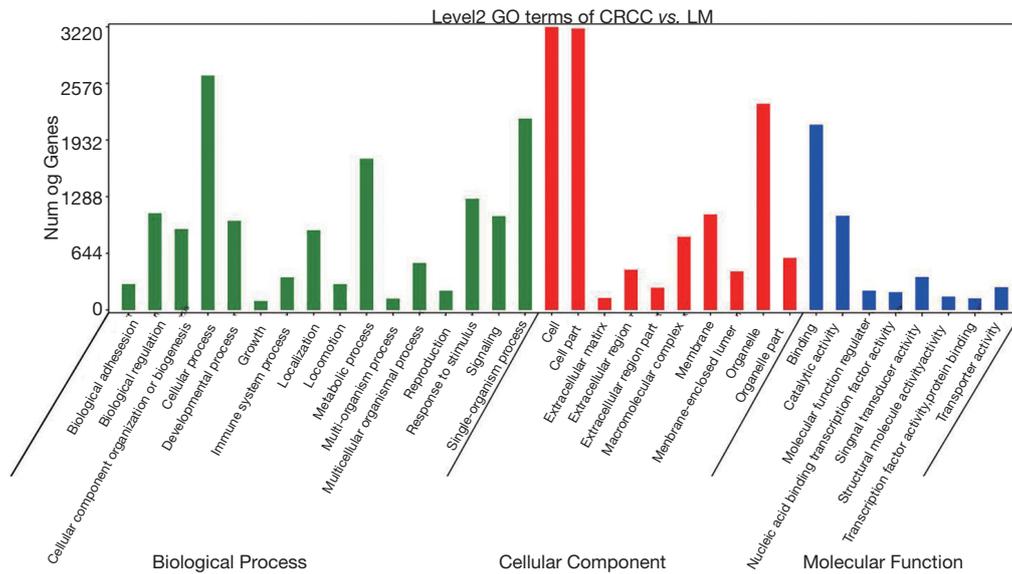


Figure 5 GO analysis histogram of mutant genes in colorectal cancer liver metastasis samples. GO, Gene Ontology.

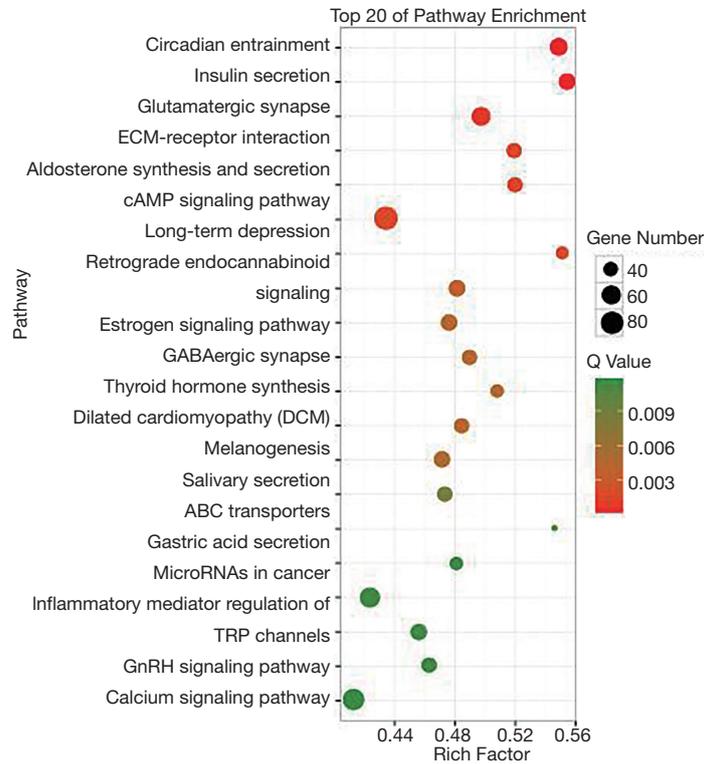


Figure 6 KEGG bubble diagram of liver metastases from colorectal cancer. KEGG, Kyoto Encyclopedia of Genes and Genomes.

and metastasis are related to the cancer genome (16), transcriptome (17), epigenetics (18), proteome (19), metabolome (20), and tumor microenvironment (21) interaction cross-regulation of a dynamic complex process.

This study collected the primary tumor tissues, matched paracancerous normal tissues, and metastatic tissues of 4 patients admitted to the General Surgery Department of the First Affiliated Hospital of Soochow University,

and conducted high-depth whole-exome sequencing, in an attempt to explore the molecular mechanism of CRC LM from the genomic level. We performed an average sequencing depth of 123× per sample. By filtering, comparing, and analyzing the data, we identified 8,565 SNVs and 429 Indels in primary and hepatic metastases, and we found that the genes with the highest frequency of mutation were TTN, OBSCN, HIPK2, and HYDIN. The TTN gene was the most frequently mutated gene and had mutations in all 4 participants with LM. The TTN gene is known to encode rhabdomyin, and mutations in this gene are also associated with familial hypertrophic cardiomyopathy (HCM) 9 (22). It has also been identified as a chromosomal structural protein, and TTN has been found to promote bone metastasis in breast cancer (23). We speculated that variation of TTN might lead to chromosome instability and thus promote the occurrence and metastasis of tumors. The OBSCN gene is over 150 kb long and contains over 80 exons encoding a protein of approximately 720 kDa that belongs to the sarcomeric signaling protein family (24,25). It is an important signaling protein that is involved in the modulation of multiple cellular signals. We hypothesized that OBSCN mutation might lead to abnormal intracellular signaling pathway transduction that catalyzed changes in cell function.

Due to the small number of patients with liver metastasis of colorectal cancer, it is difficult to obtain appropriate tissue samples of liver metastasis of colorectal cancer for sequencing. We will also increase the number of samples of liver metastasis of colorectal cancer for total exon sequencing in the future work to obtain more data. And due to financial constraints, this study failed to combine with other omics studies such as transcriptome and proteomics. We will try to address these issues in the future to further explore the molecular mechanism of CRC LM.

Conclusions

In this study, we found some candidate genes related to the occurrence of CRC and LM through whole-exome sequencing of relevant tissues in CRC patients with LM. These findings are expected to provide us with new markers and therapeutic targets for CRC patients with LM.

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Footnote

Reporting Checklist: The authors have completed the MDAR checklist. Available at <http://dx.doi.org/10.21037/jgo-21-9>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/jgo-21-9>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/jgo-21-9>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (No. 2021-023). Informed written consent was provided by all patients before inclusion in this study.

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Table S1 The sequencing depth statistics of all samples

Sample_ID	Total_ Target_Base	Mapped_ Target_Base	Average_ Target_Depth	Total_Near_ Target_Base	Mapped_Near_ Target_Base	Average_Near_ Target_Depth	Total_Near_ Target_Depth	Near_Target_ Coverage_ratio(%)	Target_ Coverage_ratio(%)	Target_ Coverage_5x (%)	Target_ Coverage_10x(%)	Target_ Coverage_20x (%)	Target_ Coverage_50x (%)	Target_ Coverage_100x (%)
CRC01LM	60456963	7.53E+09	124.61	76180188	1.18E+09	15.5	54.82	98.62	93.1	82.98	62.69	36.9	26.33	
CRC01control	60456963	1.32E+10	217.98	76180188	2.11E+09	27.76	65.91	99.04	96.21	91.73	83.56	70.36	58.34	
CRC01tumor	60456963	6.09E+09	100.72	76180188	1.06E+09	13.94	66.66	99.31	98.57	97.58	94.85	79.46	42.28	
CRC02tumor	60456963	5.95E+09	98.45	76180188	9.84E+08	12.91	62.93	99.32	98.52	97.4	94.24	77.21	40.3	
CRC02control	60456963	7.37E+09	121.84	76180188	1.21E+09	15.88	65.84	99.4	98.79	97.99	95.88	84.11	53.57	
CRC02LM	60456963	7.16E+09	118.47	76180188	1.21E+09	15.86	66.13	99.44	98.81	97.98	95.76	83.92	52.85	
CRC03control	60456963	6.45E+09	106.63	76180188	1.12E+09	14.69	66.54	99.32	98.58	97.62	95.07	81.41	46.62	
CRC03tumor	60456963	6.33E+09	104.7	76180188	1.07E+09	14.04	64.73	99.23	98.41	97.35	94.49	79.37	44.27	
CRC03LM	60456963	6.54E+09	108.2	76180188	1.11E+09	14.62	65.8	99.32	98.61	97.67	95.14	81.5	47.38	
CRC04LM	60456963	7.28E+09	120.41	76180188	1.23E+09	16.09	67.06	99.24	98.6	97.83	95.74	84.42	53.57	
CRC04control	60456963	7.47E+09	123.57	76180188	1.29E+09	16.92	68.16	99.18	98.53	97.79	95.88	85.92	57.01	
CRC04tumor	60456963	8.71E+09	144.14	76180188	1.41E+09	18.51	67.51	99.33	98.85	98.27	96.7	87.69	62	

Table S2 The single nucleotide variants (SNV) and insertions/deletions (InDel) numbers of all samples were counted

Sample name	SNV number	InDel number
CRC01CvsLM	1,094	326
CRC01CvsT	168	9
CRC02CvsT	303	19
CRC02CvsLM	300	17
CRC03CvsT	324	12
CRC03CvsLM	170	8
CRC04CvsLM	266	20
CRC04CvsT	5,940	18