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Research paper

A genome-wide assessment of variations of primary colorectal cancer maintained in metastases

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ABSTRACT

Colorectal cancer (CRC) is a highly heterogeneous disease that is the third leading cause of cancer-related deaths worldwide. This study presents a genome-wide assessment of variations in primary colorectal cancer maintained in metastases, even in distant metastases. The purpose of this study was to determine whether intratumor heterogeneity is related to disease progression and metastasis in CRC. The results showed that 882 single nucleotide polymorphism (SNP) associated genes and 473 copy number variant (CNV) associated genes specific to metastasis were found. In addition, 57 SNPs mapped to miRNAs showed significant differences between primary tumors and metastases. Functional annotation of metastasis-specific genes suggested that adhesion and immune regulation may be essential in the development of tumors. Moreover, the locus rs12881063 in the fourteenth chromosome was found to have a high rate of the G/C type in metastases. The rate of the G/C type in nearby lymph node metastases was 66.7%, while the rate of the G/C type in distance lymph node metastases was 83.3%. These results indicate that rs12881063 may be the basis for clinical diagnosis of CRC metastasis.

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1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide (Siegel et al., 2015). In recent years, the incidence of CRC has dramatically increased in Asia, especially in China (Pourhoseingholi, 2012). It is well known that metastases, rather than primary tumors, are the main cause of death in CRC patients (Chu, 2010). In early diagnosed CRC patients, the five-year survival rate is approximately 90%. However, in patients with distant metastases, the five-year survival rate decreases to <5% (Van Cutsem et al., 2014).

The incidence and development of CRC is a complex process affected by many factors including genetic and non-genetic risk factors (Sugarbaker, 2014). Genetic factors account for approximately 35% of all cases of CRC (Lichtenstein et al., 2000). Genome-wide association studies (GWAS) have been conducted for many years, and have helped identify single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) associated with the incidence of CRC (Zhang et al., 2014; Tenesa et al., 2008; Jia et al., 2013). Moreover, CRC is a highly heterogeneous disease, and exhibits both intertumor and intratumor genetic heterogeneity. Generally, intertumour heterogeneity refers to genetic and phenotypic variations between tumors of different tissue and cell types, even between individuals of the same tumor type. Intratumour heterogeneity refers to distinct subclonal diversity, expression and variation within a tumor (Burrell et al., 2013; Gerlinger et al., 2012). Intratumor genetic heterogeneity exists in almost all tumor types of each cancer (Gerlinger et al., 2012; Andor et al., 2016). Studies on genetic heterogeneity may give insight into the progression of disease, the prognosis and the risk of recurrence. However, the number of studies

Abbreviations: CRC, colorectal cancer; SNP, single nucleotide polymorphism; CNV, copy number variant; GWAS, genome-wide association studies; GO, gene ontology; BP, biological process; CAM, cell adhesion molecule; CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylator phenotype; NGS, next generation sequencing; CA2, carbonic anhydrase II; CEACAM7, carcinoembryonic antigen-related cell adhesion molecule 7; TEP1, TGF-regulated and epithelial cell-enriched phosphatase; PRKCD, protein kinase C; BRAF, B-Raf proto-oncogene serine/threonine kinase; IL11, interleukin-11; CTGF, connective tissue growth factor; CXCR4, chemokine receptor 4; CCL2, C–C chemokine ligand 2; CDH4, cadherin 4; ERBB2, V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2; FLT1, fms-related tyrosine kinase 1; IRAK1, interleukin-1 receptor-associated kinase 1; IRAK3, interleukin-1 receptor-associated kinase 3; CARD9, caspase recruitment domain-containing protein 9; TLR2, Toll-like receptor 2; CRNDE, colorectal neoplasia differentially expressed; TrxR1, thioredoxin reductase; HEPCAM, human epithelial cell adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule-1; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand; FDA, Food and Drug Administration; TLR, Toll-like receptor; RNASE6, ribonuclease A6; RNASE1, ribonuclease A1..

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on genetic heterogeneity between the primary tumor and metastases in CRCs has been relatively low, and differential SNPs and CNVs are not mentioned.

This study provides strong evidence of intratumor genetic heterogeneity in CRC. Heterogeneity was observed between spatially distinct metastases and primary tumors and was found for localized single nucleotide mutations, and for copy number alterations. The experimental findings also suggest that adhesion and immune regulation may be critical to the development of tumors. Moreover, a specific metastasis biomarker may have been found.

2. Materials and methods

2.1. Study subjects

CRC patients were recruited from the Zhujiang Hospital affiliated with Southern Medical University between 2012 and 2014. Pathologic diagnoses were reviewed by pathologists via biopsy reports. All the samples were taken from 18 patients, including 6 TxN1M0 (N1 subtype) patients and 12 TxN2M0 (N2 subtype) patients. The N (Node) term represents the involvement of regional lymph nodes. When lymph nodes are not involved, the type is represented by N0. With the increase in the degree and scope of lymph node involvement, the type is represented by N1–N3. All participants provided written, informed consent for this study. The ethics committee of Southern Medical University approved the protocol.

2.2. Colorectal tumors and metastases

The tumor and metastatic material was frozen in liquid nitrogen within 1 h after surgery. One slide was prepared for hematoxylin staining to select for samples with 60% or more tumor cells.

2.3. Genotyping and CNV calling

Genomic DNA was extracted by GenPUR Multisource Genomic DNA (Fisher Scientific, China). A NanoDrop ND-2000 Spectrophotometer (NanoDrop Technology, USA) was used to evaluate DNA quality by measuring the purity and concentration. Genomic variations were detected by Illumina Human Zhonghua-8 Bead Chip (Illumina Inc., San Diego, CA, USA). Genotyping procedures were carried out according to the standard protocol of the manufacturer. The raw data were analyzed by Illumina BeadStudio v3.5.8 (Illumina Inc., San Diego, CA, USA) and Illumina GenomeStudio Software (Illumina Inc., San Diego, CA, USA).

2.4. Sample quality control

To ensure reliable results, several criteria were applied. First, samples with genotype rates of <95% were removed. Second, samples were excluded if an absolute value of the GC wave factor was larger than 0.05 or the standard deviation of the log R ratio was larger than 0.3.

2.5. Function annotation of metastasis-specific genes

Functional annotation was performed for metastasis-specific genes by using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009). Ref Seq annotations (UCSC, v. Feb. 2009, NCBI v37, hg19) were used to identify genes, and gene ontology (GO) functional annotation was performed on biological process (BP) with default settings.

2.6. SNP validation by sequencing

Sanger sequencing was carried out to detect differential SNPs. Samples validated by Sanger sequencing were from 18 CRC patients, among whom 16 CRC patients were new. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Primer sequences for rs12881063 were 5′-GGAGAATGGCGTGAACCC-3′ and 5′-TCCTAAATATCCACCAGAAGACAG-3′. The reaction system included 1 μL DNA (20 ng), 0.2 μL of 5 U/μL rTaq PCR Polymerase, 0.3 μL of 10 mM dNTP, 1.2 μL of 25 mM Mg^{2+}, 1 μL of both primers (5 μM) and 15.3 μL ddH_2O. Prior amplification conditions were as follows: an initial polymerase activation at 95 °C for 5 min, followed by 15 cycles at 95 °C for 30 s, with annealing at 65 °C (−1 °C/cycle) for 30 s and 72 °C for 45 s, and 20 cycles at 95 °C for 30 s, with annealing at 50 °C for 30 s and 72 °C for 45 s. PCR was performed on a PTC200 machine (BioRad). Sequencing was performed on an ABI 3730. Data were analyzed by GENtle (version 2.0, University of Cologne, Germany).

3. Results

3.1. Detection of CNVs and SNPs

To identify CNVs and SNPs present in metastasis, primary data were obtained from whole-genome chips, and analyzed by Illumina Bead Studio v3.5.8 and Illumina Genome Studio Software. The 1000 Genomes Project version of the hg19/GRCh37 human reference genome was compared with the data. CNV and SNP distributions are shown in Fig. 1. For the N1 subtype, we identified 183 CNVs in primary tumors and 23 CNVs in metastasis (Fig. 1A). For the N2 subtype, we identified 90 CNVs in primary tumors, 22 CNVs in nearby metastasis and 28 CNVs in distant metastasis (Fig. 1B). The 473 genes were associated with the CNVs between the primary tumor and metastasis. In both the N1 and N2 subtypes, SNPs of matched primary tumors and metastases were compared. In

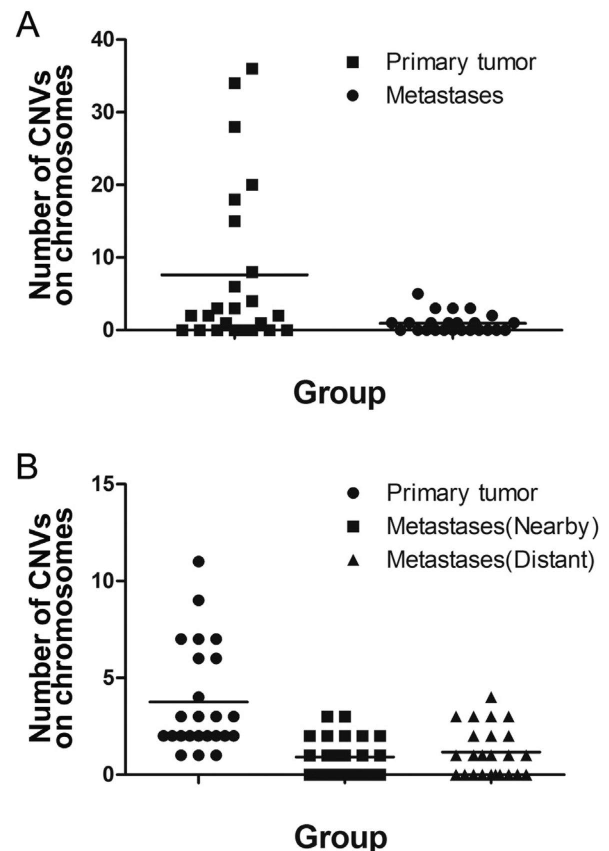

Fig. 1. CNV distributions. A: CNV distributions of N1subtype. Number of CNVs on each chromosome has been shown. In N1 subtype, there were 183 CNVs in primary tumor and 23 CNVs in metastasis. B: CNV distributions of N2 subtype. Number of CNVs on each chromosome has been shown. In N2 subtype, there were 90 CNVs in primary tumor, 22 CNVs in nearby metastasis and 28 CNVs in distant metastasis. There was no significant difference between metastasis nearby and metastasis in distance.

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