The analysis of the circRNAs in the progress of acquired resistance to Cetuximab

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Abstract
We established a Cetuximab-resistant cell lines by high-dose pulse method and searched for differentially expressed circular RNAs by RNA sequencing. Hundreds of circRNAs were altered between sensitive and resistant cells. Next, we chose six notably differential circRNAs and conducted quantitative real-time PCR by specific primers. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that differentially expressed circular RNAs are enriched in some tumor-related pathways, such as tumor transcription regulation, metabolism, PI3K-Akt, mTOR, and other signaling pathways. Our results explored differentially expressed circular RNAs associated with Cetuximab to find new targets for Cetuximab resistance therapy.

KEYWORDS
Cetuximab resistance, circRNA, colorectal cancer, RNA-seq

1 | INTRODUCTION

As one of the most common malignant tumors in the world, the global incidence rate of colorectal cancer (CRC) is estimated to be 10.0%, ranking the third and it is the second cause of cancer death (9.4%) in 2020.1 In China, with the continuous changes in people's lifestyles and dietary habits, the incidence and mortality of CRC have been increasing year by year. The five-year survival rate of CRC patients is only 31%, which is far lower than that of developed countries such as the United States and Japan.2 CRC has become an important problem that seriously endangers people health.

Noncoding RNAs (ncRNAs) have important roles in regulating the expression of genes that control fundamental biological functions. Compared with normal cells, altered levels of ncRNAs are frequently detected in cancer cells. Circular noncoding RNA (circRNA), covalently closed loops resulting from backsplicing of mRNA, is a new type of ncRNAs that is different from linear RNA.3,4 It is widely present in various cells. Most of it comes from the exon region of genes, and a small part is formed by intron splicing. Many circRNAs exert important biological functions by binding and acting as sponges of miRNAs or protein inhibitors to regulate protein function. They have been implicated in diseases such as diabetes mellitus, neurological disorders, cardiovascular diseases, and cancers.5,6 For instance, circRNA hsa_circ_0000523 was down-regulated in different CRC cell lines and suppressed apoptosis of CRC cells. In addition, it acted as a "sponge" of miR-31, indirectly regulating Wnt/β-catenin signaling pathway, which was involved in the progression of CRC.7 circ 0006528 affects Raf1 through "sponge adsorption" miR-7–5p, thereby regulating breast cancer resistance to Adriamycin.8

In this study, we cultured a Cetuximab-resistant cell lines by high-dose pulse method and searched for differentially expressed circular RNAs by RNA sequencing and RT-PCR. Our research may find new targets for clinical treatment of Cetuximab resistance.

2 | MATERIALS AND METHODS

2.1 | Cell culture

NCI-H508 cells and Cetuximab were purchased from the ATCC and Merck company (Germany), respectively. Cetuximab resistant...
NCI-H508 cells were generated as described previously\(^9\) and was named as H508/CR.

### 2.2 Cell counting kit-8 assays

Cell growth was determined by CCK8 assays as described previously.\(^9\) NCI-H508 and H508/CR cells were seeded into 96-well plates at a density of ~8000 cells per well and cultured for 24 h. Cells were treated for 72 h with different concentrations of Cetuximab including 1, 10, 20, 50, 100, and 200 μg/ml. Finally 10 μl CCK-8 reagent (Dojindo, Japan) was added to each well and absorbance was measured at 450 nm by BioTek ELx800 (BioTek Instruments, USA).

### 2.3 The circRNA sequencing

The circRNA sequencing was performed by Ribo Biotechnology Co., Ltd. (Guangzhou, China). TRIzol reagent (Cat. no. 15596026; Thermo Fisher Scientific Inc., USA) were used to extract cellular RNAs from two kinds of cells. The ribo-zero-magnetic-kit (Epicenter, France) was used to remove ribosomal RNA from the samples. To remove linear RNA, the samples were incubated for 1 h at 40°C with RNase R (Illumina, USA). Subsequently, RNA-seq libraries were prepared by NEBNext\textsuperscript{®} Ultra\textsuperscript{™} RNA Library Prep Kit (USA). RNA-Seq analysis was conducted with DESeq v1.18.0.

### 2.4 qRT-PCR assay

Specific primers were synthesized from RiboBio Co. Ltd. (Guangzhou, China). Total RNA were extracted and reverse transcribed into cDNA with PrimeScript\textsuperscript{™} RT Master Mix kit (Takara, Japan). PowerUp\textsuperscript{™} SYBR\textsuperscript{®} Green Master Mix (Thermo Fisher Scientific Inc., USA) and Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) were applied in qRT-PCR assay. Relative fold changes were determined using the ΔΔCT calculation method. Values were normalized to the internal control and ACTB (encoding β-actin) was set as a house-keeping gene.

### 2.5 Statistical analysis

GraphPad Prism 8 software (USA) were used to draw all the figures. The kobas 3.0 software was applied to analyze Gene Ontology (GO) and KEGG pathway. Statistical analysis was performed using SPSS 20.0. Two-way analysis of variance (ANOVA) and Student’s t test were performed for the comparison of cell viability between two groups under different drug stimulation and the differences between two groups, respectively. A p value of less than .05 is considered statistically significant.

### 3 RESULTS

#### 3.1 Validation of the Cetuximab-resistant cell line

The resistance index for H508/CR cells was determined by the CCK-8 assay. The IC50 values of H508/CR cells for Cetuximab was increased from 12.24 μg/ml for sensitive cells to 161.5 μg/ml. The resistance index was >10 (Figure 1).

#### 3.2 Profiles of the differentially expressed circRNAs

The volcano plots and heatmap showed distinct expression patterns of the circRNAs varied within NCI-H508 and H508/CR cells by RNA-seq analysis (Figure 2A,B). Two hundred and eight mRNAs were significantly increased, while 211 were declined in Cetuximab resistant group. KEGG pathways through bioinformatic tools revealed that significantly differentially expressed circRNAs are enriched in some tumor-related pathways, such as tumor transcription regulation, metabolism, PI3K-Akt, mTOR and other signaling pathways (Figure 2C).

#### 3.3 Real-time PCR validation of significantly differentially expressed circRNAs

Next, we selected three up-regulated and three down-regulated circRNAs to further determine circRNA expressions analyzed by RNA-seq. The expressions of hsa_circ_0000586, hsa_circ_00005236, hsa_circ_0019225 and hsa_circ_0000915 were measured by qRT-PCR (Figure 3). Except for one circular RNA, hsa_circ_00005236, the expressions of other circular...
RNAs detected by RNA sequencing were basically consistent with real-time quantitative PCR results. hsa_circ_0005236 expression was not increased in qRT-PCR.

4 | DISCUSSION

As an effective epidermal growth factor receptor (EGFR) antibody, Cetuximab competitively binds to the extracellular domain of EGFR with the epidermal growth factor to treat CRC and can effectively reduce the overall mortality rate in CRC patients. The acquired resistance to Cetuximab yet limits its application and causes tumor recurrence and metastasis. We do not fully understand the detailed mechanisms, expect for common genetic mutations.

High-throughput sequencing has been extensively used to identify differential RNAs including coding and noncoding RNAs that can be exploited for cancer treatment and drug development. In view of the fact that circRNA related research has just started, the
Furthermore, 50 CRC and 50 healthy Six chosen circRNAs were assessed by real-time PCR assay. At present, we chose six markedly expressed circRNAs and conducted qRT-PCR to confirm RNA-seq results. No reports before were concerned to some of these circRNAs. Many circRNAs participated in multiple signaling pathways such as tumor transcription regulation, metabolism, PI3K-Akt, mTOR. It has been reported that circular RNA is related to the growth, invasion and migration of tumor cells. Forced circ_0078607 expression was down-regulated in ovarian cancer cells and it inhibited ovarian cancer cell proliferation and induced apoptosis. Hsa_circ_0000586 was found to be downregulated in hepatocellular carcinoma tumor tissues and decreased cell viability, migration ability and promoted apoptosis, cell invasion in Huh7 and HepG2 cells by inhibiting HIF-1α expression. Hsa_circ_00005236 was associated with the different pathological grades of glioma. However, this study has some limitations. Because of lack of Cetuximab sensitive cells, only one resistant cell line was applied in vitro study for Cetuximab resistance and we did not reveal the detailed mechanisms. The role of the circRNAs need further investigations.

We further explore the new molecular target of Cetuximab resistance from the perspective of circRNAs, which is expected to reveal the new mechanism of drug resistance and provide new ideas for clinical treatment. It has positive theoretical significance and clinical application value.

AUTHOR CONTRIBUTIONS
Liping Yin wrote the manuscript and designed the study. Changwen Jing performed bioinformatic analysis. Yesong Guo contributed to the design of the study. All authors have approved the final manuscript.

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CONFLICT OF INTEREST
There is no conflict of interests for all the authors.

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