miR-297 modulates multidrug resistance in human colorectal carcinoma by down-regulating MRP-2

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Colorectal carcinoma is a frequent cause of cancer-related death in men and women. miRNAs (microRNAs) are endogenous small non-coding RNAs that regulate gene expression negatively at the post-transcriptional level. In the present study we investigated the possible role of miRNAs in the development of MDR (multidrug resistance) in colorectal carcinoma cells. We analysed miRNA expression levels between MDR colorectal carcinoma cell line HCT116/L-OHP cells and their parent cell line HCT116 using a miRNA microarray. miR-297 showed lower expression in HCT116/L-OHP cells compared with its parental cells. MRP-2 (MDR-associated protein 2) is an important MDR protein in platinum-drug-resistance cells and is a predicted target of miR-297. Additionally miR-297 was down-regulated in a panel of human colorectal carcinoma tissues and negatively correlated with expression levels of MRP-2. Furthermore, we found that ectopic expression of miR-297 in MDR colorectal carcinoma cells reduced MRP-2 protein level and sensitized these cells to anti-cancer drugs in vitro and in vivo. Taken together, our findings suggest that miR-297 could play a role in the development of MDR in colorectal carcinoma cells, at least in part by modulation of MRP-2.

Key words: colorectal carcinoma, microRNA (miRNA), microRNA array, multidrug resistance (MDR), multidrug resistance-associated protein 2 (MRP-2).

INTRODUCTION

CRC (colorectal carcinoma) is the fourth most common form of cancer in the United States and the third leading cause of cancer-related death in the Western world [1] and it is also one of the most common malignant tumours in China. The main method of treatment is chemotherapy; however, cancerous cells frequently develop MDR (multidrug resistance) to chemotherapy agents [1a]. MDR is the ability of tumour cells to resist several unrelated drugs after exposure to a single chemotherapy drug [2], and is the leading cause of cancer-related death. MDR has frequently been associated with elevated expression levels of one or more ABC (ATP-binding cassette) transporters. The four well-known transporters are: ABCB1 [MDR-1/p-gp (P-glycoprotein 1)] [3], ABCC1 [MRP (MDR-associated protein 1)] [1], ABCC2 (MRP-2) [1] and ABCG2 [BCRP (breast cancer-resistance protein)] [4]. The mechanisms of MDR in CRC have not been completely characterized so far and some studies suggest that mechanisms are likely to be multifaceted and extremely intricate [4a,4b]. These factors suggest that the most practical and long-term solution for overcoming the vast majority of CRC today is not likely to be achieved by creating new drugs. Instead, an increased focus on the dominant causes of CRC drug resistance is warranted, especially with regard to the exploration of novel MDR mechanisms.

miRNAs are approximately 21–23 bp of single-stranded non-coding RNA, and are post-transcriptional regulators that bind to complementary sequences in the 3′-UTRs (untranslated regions) of target messenger RNA transcripts (mRNAs), usually resulting in gene silencing [5,6]. Accounting for about 1% of all of the expressed human genes, miRNAs are predicted to regulate the expression of up to 1/3 of human protein-coding genes [7–10]; in fact, miRNAs have been shown to function as regulatory molecules by inhibiting protein translation and to play an important role in development, differentiation, cell proliferation and apoptosis [6]. More recently, miR-328 was shown to regulate negatively expression of BCRP [11]. miR-451 and miR-27 have been demonstrated to be involved in resistance of the MCF-7 breast cancer cells to the chemotherapeutic drug DOX (doxorubicin), a mechanism mediated by MDR-1 [12,13]. miR-326 was shown to negatively regulate the expression of MRP-1 [14].

In the present study, we reported that a limited set of miRNAs were differentially expressed in a MDR human CRC cancer cell line HCT116/L-OHP (oxaliplatin) and its parental cell line HCT116. For the first time miR-297, which is down-regulated in MRP-2. Taken together, our findings suggest that miR-297 could play a role in the development of MDR in colorectal carcinoma cells, at least in part by modulation of MRP-2.

MATERIALS AND METHODS

Cell lines and human samples

The human ileocaecal colorectal adenocarcinoma cell line HCT-8 and the human CRC cell line HCT-116 (obtained from the Cell Bank of the Chinese Academy of Sciences) and their MDR variants, HCT-8/VCR (vincristine) and HCT-116/L-OHP respectively (established and maintained in our laboratory), were maintained in RPMI 1640 medium (Gibco) with 10% FBS (fetal

Abbreviations used: ABC, ATP-binding cassette; BCRP, breast cancer-resistance protein; CRC, colorectal carcinoma; DOX, doxorubicin; EGFP, enhanced green fluorescent protein; 5-FU, 5-fluorouracil; L-OHP, oxaliplatin; MDR, multidrug resistance; miRNA, microRNA; MMC, mitomycin C; MRP, MDR-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NC, non-tumorous colon; P-gp, P-glycoprotein 1; PI, propidium iodide; siRNA, small interfering RNA; snRNA, small nuclear RNA; UTR, untranslated region, VCR, vincristine.

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bovine serum) (Gibco) at 37°C in a humidified atmosphere with 5% CO2. The HCT-8/VCR cells and HCT-116/L-OHP cells were seeded in the medium that additionally contained 0.5 μg/ml VCR and 5 μg/ml L-OHP respectively so as to maintain its drug resistance phenotype.

The hsa-miR-297 expression vector pEGFP-C1-miR-297, that contains pri-miR-297 and some of its flanking sequences, was cloned into a pEGFP-C1 vector. This vector can simulate the natural state of the stable expression of miRNAs. The primers used were: forward, 5′-CCCAAGCTTATGCCAAAGGCTTC-3′ and reverse, 5′-CGGGTTACACCCAGGCATAGTGATTGGG-3′. Stable cell lines were made by stable transfection of HCT116/L-OHP with the pEGFP-C1-Vector and pEGFP-C1-miR-297 vectors. Transfected cells were cultured in the presence of 800 μg/ml G418 for 4 weeks. The clones with G418 resistance and fluorescent label [EGFP (enhanced green fluorescent protein)] were selected and expanded and named as HCT116/L-OHP-EGFP-Vector and HCT116/L-OHP-EGFP-miR-297 respectively.

Human CRC and their corresponding NC (non-tumourous colon) samples were collected at the time of surgical resection at Putuo District Center Hospital, Shanghai University of Traditional Chinese Medicine, P.R. China. Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C. Use of human tissues was approved by the Institutional Review Board of the Putuo District Center Hospital.

miRNA microarray analysis

Prior to experimentation, HCT-116/L-OHP cells were cultured for 1 week without L-OHP. Total RNA from HCT-116 and HCT-116/L-OHP cell lines was isolated with TRIzol reagent (Invitrogen) and the miRNA fraction was further purified using a mirVana™ miRNA isolation kit (Ambion). The isolated miRNAs from the two cell lines were then labelled with Hy3 using the miRCURY™ Array Labelling kit (Exiqon) and hybridized respectively on a miRCURY™ LNA miRNA Array (v. 8.0, Exiqon) as described previously [15]. Microarray images were acquired using a Genepix 4000B scanner (Axon Instruments) and processed and analysed with Genepix Pro 6.0 software (Axon Instruments) and Excel.

Quantitative RT (reverse transcription)–PCR for miRNA

To prepare total RNA from tissues, the frozen tissues were ground into finely ground particles after 5-mm3 sections of each sample were cut, and then the tissue particles were subjected to extraction of RNA with TRIzol (Invitrogen) and the miRNA fraction was further purified using a mirVana™ miRNA isolation kit (Ambion). The isolated miRNAs from the two cell lines were then labelled with Hy3 using the miRCURY™ Array Labelling kit (Exiqon) and hybridized respectively on a miRCURY™ LNA miRNA Array (v. 8.0, Exiqon) as described previously [15]. Microarray images were acquired using a Genepix 4000B scanner (Axon Instruments) and processed and analysed with Genepix Pro 6.0 software (Axon Instruments) and Excel.

Western blot analysis

Proteins were resolved in an SDS/PAGE gel (7.5% gel) and subjected to immunoblot analysis using monoclonal antibodies against MRP-2, MRP-1, P-gp or β-actin (Cell Signaling Technology). All of the antibodies were used at 1 μg/ml

Luciferase activity assay

The 3′-UTR of human MRP-2 (GenBank® accession number NM_000392) cDNA containing the one putative target site for miR-297 was amplified by PCR using the primers sense, 5′−CCTCAGTCTGGTGAAGAAGGCT−3′ and anti-sense, 5′−CATGCCATCGTCCAGACCATGT−3′; and inserted into the Ncol and HindIII restriction sites, immediately downstream of the luciferase gene in the pGL3-promoter-vector (Promega). A mutant version with a deletion of 7 bp from the site of perfect complementarity was also generated by using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). Wild-type and mutant inserts were confirmed by sequencing. At 24 h after transfection, cells were seeded on to 96-well plates (8×103 viable cells/well) and allowed to attach overnight. A total of 200 ng of pGL3-p-MRP-2-3′UTR or pGL3-p-mutMRP-2-3′UTR plus 80 ng of pRL-SV40 (Promega) were transfected alone or in combination with control oligonucleotide (final concentration of 80 nM) or mimic (20, 30, 40, 60 and 80 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Luciferase activity was measured 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Three independent experiments were performed in triplicate.

RT–PCR and quantitative RT–PCR

Total RNA was extracted from cultured HCT-8, HCT-8/VCR, HCT116 and HCT116/L-OHP cells and tissues with TRIzol. The primer sequences for MRP-2, MRP-1, MDR-1 and β-actin were: MDR-1 (GenBank® accession number AF016535), 5′−GCTTCTCTGACTATGCCAAAGC−3′ and 5′−TCCTCACCTCCAGGCTCTAGT−3′; MRP-1 (GenBank® accession number NM_004996), 5′−AGGGTGACCTGTTTCTGTCGAC−3′ and 5′−ACCCTGTGATCCACCAAGG−3′; MRP-2 (GenBank® accession number NM_000392), 5′−TGAGCCTCTCCATAACCATGA−3′ and 5′−GGACTTCCAGATGCCTGACC−3′; and β-actin (GenBank® accession number NM_001101), 5′−CTCCATCTGGCCCTCGTGT−3′ and 5′−GCTGTCACCTTCACGTCTCC−3′. Quantitative and regular RT–PCR was performed as described previously [31]. For regular and quantitative RT–PCR, 500 ng of total RNA were transcribed into cDNA in a 20 μl reaction volume at 42°C for 45 min with a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems). The cycle conditions for cDNA PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and then 55°C for 45 s. For regular RT–PCR, reactions were performed with using a GeneAmp Gold RNA PCR Reagent kit. For SYBR Green quantitative PCR amplifications, reaction was performed in a 20 μl reaction volume containing 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems). The relative expression levels of each cell line of each group were measured using 2−ΔΔCt method as above and the relative expression levels of tissues were measured using the 2−ΔΔCt.

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of working concentration in PBS with 5% dried milk. The membrane was further probed with HRP (horseradish peroxidase)-conjugated rabbit anti-(mouse IgG) (1:2000 dilution; Santa Cruz Biotechnology) and the protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia). Quantification of protein bands was performed using the ImageJ (http://rsbweb.nih.gov/ij/) software.

**In vitro drug sensitivity assay**

The hsa-mir-297 miRIDIAN mimic, inhibitor and control oligonucleotides were purchased from Dharmaco. HCT-8, HCT-8/VCR, HCT116 and HCT116/L-OHP cells were plated on to six-well plates (4 × 10^5 cells/well) in antibiotic-free medium. HCT-8 and HCT116 cells were transfected with 80 nM control oligonucleotide or 80 nM inhibitor, and HCT-8/VCR and HCT116/L-OHP cells were transfected with 80 nM control oligonucleotide, 80 nM mimic or 80 nM MRP-2 siRNA (small interfering RNA) as a positive control (5'-GGCUGAUUAGUGCAAUCCCA-3'; Cell Signaling Technology) using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the cells were seeded on to 96-well plates (8 × 10^3 viable cells/well) and allowed to attach overnight. After cellular adhesion, freshly prepared anticancer drugs including L-OHP, VCR, DOX, 5-FU (5-fluorouracil) and MMC (mitomycin C) were added with the appropriate concentration gradient for each drug. After 48 h, cell viability was assessed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. The absorbance at 490 nm (A_490) of each well was read on a spectrophotometer. The concentration at which each drug produced 50% inhibition of growth (IC_{50}) was estimated by the relative survival curve. Three independent experiments were performed in quadruplicate.

**Flow cytometric analysis of DOX fluorescence**

HCT116/L-OHP cells were plated on to six-well plates (4 × 10^5 cells/well) in antibiotic-free medium and transfected with control oligonucleotide (80 nM), mimic (80 nM) or MRP-2 siRNA (80 nM) using Lipofectamine 2000. After 48 h DOX was added, with a final concentration of 20 μg/ml. After 2 h the cells were collected, washed with ice-cold PBS twice and then analysed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). Measurements were taken at 580 nm after excitation of cells by 488 nm with an argon ion laser.

**Flow cytometric analysis of apoptosis**

HCT116/L-OHP cells and HCT-8/VCR were plated on to six-well plates (4 × 10^5 cells/well) in antibiotic-free medium and transfected with control oligonucleotide (80 nM), mimic (80 nM) or MRP-2 siRNA (80 nM) using Lipofectamine 2000. After 24 h, L-OHP was added into HCT116/L-OHP cells with a final concentration of 80 μg/ml, and VCR was added into HCT-8/VCR cells with a final concentration of 20 μg/ml. After 48 h the cells were collected, washed with ice-cold PBS twice and gently resuspended in 400 μl of 1 × binding buffer. After the addition of 5 μl of Annexin V-FITC, the cells were gently vortexed and then incubated for 10 min at 4–8 °C in the dark. PI (propidium iodide; 10 μl) was then added followed by incubation for another 5 min at 4–8 °C in the dark. Flow cytometry was then conducted using a FACScan flow cytometer (Becton Dickinson). The fraction of the cell population in different quadrants was analysed using quadrant statistics. The cells in the lower-right quadrant represented early apoptosis and in the upper-right quadrant represented late apoptotic cells.

**Subcutaneous xenografts**

Male BALB/c nude mice (5-week-old and weighing 17–18 g) were purchased from Shanghai Laboratory Animal Resource Center, and were maintained in a pathogen-free environment. HCT116/L-OHP-EGFP-Vector cells (5 × 10^6) suspended in 100 μl of PBS were injected subcutaneously into the flank of BALB/c nude mice followed by 5 × 10^6 HCT116/L-OHP-EGFP-miR-297 cells suspended in 100 μl of PBS injected subcutaneously into the thighs of the same mice. L-OHP solution (10 mg/kg) was injected intraperitoneally every 4 days starting from the third week after subcutaneous implantation. Mice were detected by the Kodak In Vivo FX Pro (Kodak). The tumour volume of HCT116/L-OHP xenografts was detected weekly with fluorescence signalling. The mice were examined 5 weeks after the subcutaneous implantation. After the last in vivo optical imaging, all of the mice were killed. Tumour tissues were isolated for Western blot analysis. All of the experiments were carried out under the approval of the Administrative Panel on Laboratory Animal Care of the Putuo District Center Hospital.

**In vivo optical imaging**

Prior to in vivo imaging, the mice were anaesthetized with phenobarbital sodium. Fluorescence imaging was obtained with an excitation wavelength of 465 nm and emission wavelength of 520 nm. Exposure times ranged from 1 min to 2 min. Fluorescence signals were simultaneously quantified using Kodak In Vivo FX Pro on days 7, 14, 21, 28 and 32 after subcutaneous implantation.

**Statistical analysis**

Each experimental value was expressed as a mean ± S.D. Statistical analysis was performed using Student’s t test to evaluate the significance of differences between the cell lines. All of the data points are the means of triplicate repeats. The statistical analysis of the tissues sample was performed using the Mann–Whitney test to evaluate the significance of differences between groups.

**RESULTS**

Expression of three MDR-related transporters in the HCT116/L-OHP and HCT116 cell lines

Prior to the analysis of miRNA expression, we analysed expression of mRNAs and proteins of three MDR-related transporters, MRP-1, MRP-2 and MDR-1 (P-gp), in HCT116 and L-OHP-resistant HCT116/L-OHP cells with RT–PCR and Western blot analysis (Figure 1A). The results showed that HCT116/L-OHP, which was selected by L-OHP, overexpressed MRP-1, MRP-2 and MDR-1 (P-gp) mRNA and protein in comparison with HCT116, and the highest expression of MRP-2 was observed.

**miRNAs have differential expression in MDR human CRC cell lines**

In order to determine whether miRNAs are involved in the development of MDR in human CRC cells, we performed a profile analysis of miRNA expression in HCT116/L-OHP cells compared with their parent cell line, HCT116, using a microarray containing 873 human mature miRNA probes. A total of 16 miRNAs showed an obvious differential expression between HCT116/L-OHP and their parent cells (fold change ≥ 3, Table 1). These miRNAs may play an important role in the development of MDR in colorectal carcinoma cells. We predicted the putative...
target of these miRNAs associated with MDR by TargetScan, (http://www.targetscan.org) and found that miR-297 and miR-630 may modulate MRP-2, which has the greater expression in HCT116/L-OHP cells. To verify the results obtained by microarray profiling, we performed quantitative RT–PCR analysis of these miRNAs expression in the four cell lines. Consistent with the microarray data, quantitative RT–PCR analysis showed that the expression of miR-297 was down-regulated compared with miR-630 in HCT116/L-OHP cells and HCT-8/VCR cells in comparison with their parental cell lines (Figure 1B).

miR-297 is significantly associated with the clinical stage in human CRC tissue samples

To determine the potential clinicopathological implications of altered miR-297 expression, we investigated the expression levels of miR-297 in 54 tissues samples by quantitative RT–PCR. The $2^{-\Delta \Delta C_t}$ was used to describe the expression level of miR-297. Table 2 shows the correlations between the miR-297 expression level and clinicopathological characteristics of CRC. A statistically significant association was observed in the present study only between the miR-297 expression level and clinical stage. The median expression of miR-297 was 0.0713 in the 11 cases with advanced stage (stage III) disease, whereas the median expression was 0.5479 ($P < 0.0001$, Mann–Whitney test) in the 16 cases with early-stage (stages I and II) disease.

### Table 1 miRNAs differentially expressed in the HCT116/L-OHP and HCT116 cell lines

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Up- or down-regulation</th>
<th>Mean fold change in HCT116/L-OHP cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-892b</td>
<td>Down</td>
<td>0.181</td>
</tr>
<tr>
<td>hsa-miR-1915</td>
<td>Down</td>
<td>0.215</td>
</tr>
<tr>
<td>hsa-miR-297</td>
<td>Down</td>
<td>0.238</td>
</tr>
<tr>
<td>hsa-miR-630</td>
<td>Down</td>
<td>0.248</td>
</tr>
<tr>
<td>hsa-miR-1274a</td>
<td>Down</td>
<td>0.283</td>
</tr>
<tr>
<td>hsa-miR-222</td>
<td>Down</td>
<td>0.290</td>
</tr>
<tr>
<td>hsa-miR-221</td>
<td>Down</td>
<td>0.297</td>
</tr>
<tr>
<td>hsa-miR-338-5p</td>
<td>Down</td>
<td>0.304</td>
</tr>
<tr>
<td>hsa-miR-513a-5p</td>
<td>Up</td>
<td>5.548</td>
</tr>
<tr>
<td>hsa-miR-1225-5p</td>
<td>Up</td>
<td>5.059</td>
</tr>
<tr>
<td>hsa-miR-1202</td>
<td>Up</td>
<td>5.025</td>
</tr>
<tr>
<td>hsa-miR-638</td>
<td>Up</td>
<td>4.348</td>
</tr>
<tr>
<td>hsa-miR-1275</td>
<td>Up</td>
<td>4.174</td>
</tr>
<tr>
<td>hsa-miR-1268</td>
<td>Up</td>
<td>3.501</td>
</tr>
<tr>
<td>hsa-miR-1207-5p</td>
<td>Up</td>
<td>3.027</td>
</tr>
</tbody>
</table>

### Table 2 The relationship between clinicopathological parameters and miR-297 expression in human CRC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of cases</th>
<th>Percentage</th>
<th>Median expression of miR-297/U6</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$\geq 60$</td>
<td>17</td>
<td>63%</td>
<td>0.3623</td>
<td>0.4313</td>
</tr>
<tr>
<td>$&lt;60$</td>
<td>10</td>
<td>37%</td>
<td>0.3915</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>48%</td>
<td>0.3912</td>
<td>0.4245</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>52%</td>
<td>0.3809</td>
<td></td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well and moderately differentiated</td>
<td>17</td>
<td>63%</td>
<td>0.3784</td>
<td>0.3674</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>10</td>
<td>37%</td>
<td>0.4342</td>
<td></td>
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<tr>
<td>Lymph node status</td>
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<td></td>
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</tr>
<tr>
<td>Metastasis</td>
<td>13</td>
<td>48%</td>
<td>0.3732</td>
<td>0.3866</td>
</tr>
<tr>
<td>No metastasis</td>
<td>14</td>
<td>52%</td>
<td>0.3901</td>
<td></td>
</tr>
<tr>
<td>Extent of invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasion</td>
<td>17</td>
<td>63%</td>
<td>0.3698</td>
<td>0.4987</td>
</tr>
<tr>
<td>No invasion</td>
<td>10</td>
<td>37%</td>
<td>0.3942</td>
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<tr>
<td>TNM stage</td>
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</tr>
<tr>
<td>Stage I/II</td>
<td>16</td>
<td>59%</td>
<td>0.5479</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>11</td>
<td>41%</td>
<td>0.0713</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

### Negative correlation of mi-297 and MRP-2 in a selected panel of human CRC tissues

We have observed the digressive expression of miR-297 in HCT116/L-OHP and HCT-8/VCR cells compared with HCT116 and HCT-8, and we predicted that MRP-2 was the target of miR-297 associated with by TargetScan, but what is the relationship between miR-297 and MRP-2? We found that miR-297 is significantly associated with the clinical stage in human CRC tissues samples (Table 2). To investigate further association of miR-297 to MRP-2, we measured expression levels of miR-297 and MRP-2 in 54 tissues samples with quantitative RT–PCR and Western blotting and analysed them by clinical stage. The $2^{-\Delta \Delta C_t}$ was used to describe the expression level of miR-297 and MRP-2 mRNA expression, and the detected bands intensity of MRP-2 protein/β-actin protein was used to describe the expression level of MRP-2 protein. Figure 2(A) shows the progressive decrease in miR-297 expression in tissues samples from NC ($n = 27$, median = 1.024) to stage I human CRC.
miR-297 modulates multidrug resistance in colorectal carcinoma

Figure 2  Expression levels of miR-297 and MRP-2 in tissue samples

(A) Progressive decrease in miR-297 expression in tissue samples from NC \((n = 27, \text{median} = 1.024)\) to stage I human CRC \((n = 7, \text{median} = 0.682)\), stage II CRC \((n = 9, \text{median} = 0.4767)\) and stage III CRC \((n = 11, \text{median} = 0.071)\). (B) Progressive increase in MRP-2 expression (mRNA level) in tissue samples from NC \((n = 27, \text{median} = 1.749)\) to stage I CRC \((n = 7, \text{median} = 3.489)\), stage II CRC \((n = 9, \text{median} = 6.531)\), and stage III CRC \((n = 11, \text{median} = 12.97)\). (C) The correlation between miR-297 and MRP-2 expression (mRNA levels) in CRC \((n = 27)\). Each point in this scatter graph represents an individual sample, in which relative miR-297 expression level indicate on y-axis and MRP-2 mRNA expression levels on x-axis. The correlation coefficient, \(r\), is \(-0.8171\) \((P < 0.01)\) indicating there is a strongly negative relationship miR-297 and MRP-2 expression (mRNA levels) in CRC. Data were analysed using the Spearman’s rank test. (D) MRP-2 protein expression level in CRC. \(\beta\)-Actin was used as an internal loading control. (E) Progressive increase in MRP-2 expression (protein level) in tissues samples from NC \((n = 27, \text{median} = 0.069)\) to stage I CRC \((n = 7, \text{median} = 0.0743)\), stage II CRC \((n = 9, \text{median} = 0.0952)\), and stage III CRC \((n = 11, \text{median} = 0.12)\). (F) The correlation between miR-297 and MRP-2 expression (protein levels) in CRC \((n = 27)\). Each point in this scatter graph represents an individual sample, in which relative miR-297 expression level indicate on y-axis and MRP-2 protein expression levels on x-axis. The correlation coefficient, \(r\), is \(-0.8942\) \((P < 0.01)\) indicating that there is a strongly negative relationship miR-297 and MRP-2 expression (protein levels) in CRC. Data were analysed using the Spearman’s rank test.

\((n = 7, \text{median} = 0.682)\), stage II CRC \((n = 9, \text{median} = 0.443)\) and stage III CRC \((n = 11, \text{median} = 0.071)\). Figure 2(B) shows the progressive increase in MRP-2 expression (mRNA level) in tissue samples from NC \((n = 27, \text{median} = 1.749)\) to stage I CRC \((n = 7, \text{median} = 3.489)\), stage II CRC \((n = 9 \text{ median} = 6.531)\), and stage III CRC \((n = 11, \text{median} = 12.97)\). We then analysed the correlation between miR-297 and MRP-2 expression (mRNA levels) in CRC \((n = 27)\). Each point in this scatter graph represents an individual sample; in which relative miR-297 expression level indicate on the y-axis and MRP-2 mRNA expression levels on the x-axis. The correlation coefficient, \(r\), is \(-0.8171\) \((P < 0.01)\), indicates there is a strongly negative relationship between...
Figure 3  MRP-2 is a target of miR-297

(A) MRP-2 protein level and mRNA level were assessed 48 h after transfection of control oligonucleotide or inhibitor (80 nM) in HCT116 cells, which were detected by Western blotting and real-time RT–PCR respectively. (B) MRP-2 protein level and mRNA level were assessed 48 h after transfection of control oligonucleotide or mimic (20 or 40 or 80 nM) or MRP-2 siRNA (80 nM) in HCT116/L-OHP cells. (C) MRP-2 protein level and mRNA level were assessed 48 h after transfection of control oligonucleotide or mimic (20 or 40 or 80 nM) or MRP-2 siRNA (80 nM) in HCT-8 cells and control oligonucleotide or mimic (20 or 40 or 80 nM) or MRP-2 siRNA (80 nM) in HCT-8/VCR cells. (D) MRP-2, P-gp and MRP-1 protein levels were assessed 48 h after transfection of control oligonucleotide, mimic (80 nM) or MRP-2 siRNA (80 nM) in HCT116/L-OHP cells, which were respectively detected by Western blotting. (E) The putative miR-297 targeted sequence in the MRP-2 gene. TargetScan predicts one binding sites in MRP-2 3′-UTR. (F) Luciferase reporter assays. The luciferase activity in HCT116/L-OHP transfected with the vector containing MRP-2 3′-UTR fragment with binding sequence of miR-297 was inhibited by transfection of miR-297 in a dose-dependent manner. Results are means ± S.D. from three independent experiments. *P < 0.05; **P < 0.01 compared with the control oligonucleotide group. *HCT-8 + control oligonucleotide; 2HCT-8/VCR + control oligonucleotide.

miR-297 and MRP-2 expression (mRNA levels) in CRC (Figure 2C). The data were analysed using the Spearman’s rank statistical test. To further confirm this result, we also investigated the MRP-2 protein expression (Figure 2D). Progressive increase in MRP-2 expression (protein level) in tissues samples from NC (n = 27, median = 0.069) to stage I CRC (n = 7, median = 0.0743), stage II CRC (n = 9, median = 0.0952) and stage III CRC (n = 11, median = 0.121) (Figure 2E). Figure 2(F) shows that expression levels of miR-297 also correspond negatively with MRP-2 protein in tissues samples [r = −0.8942 (P < 0.01)].
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Figure 4 Modulation of miR-297 expression altered the sensitivity of colorectal cancer cells to anti-cancer drugs

(A) HCT116 cells were transfected with control oligonucleotide or inhibitor (80 nM). (B) HCT116/L-OHP cells were transfected with control oligonucleotide or mimic (80 nM) or MRP-2 siRNA (80 nM). After incubation with five anti-cancer drugs for 48 h, cell viability was assessed using MTT assay and IC50 value to each drug was calculated. (E) Modulation of miR-297 expression altered the concentration of human CRC cells to DOX. The percentage of DOX fluorescence was increased in miR-297-mimic-transfected HCT116/L-OHP. Results are means ± S.D. from three independent experiments. *p < 0.05 and **p < 0.01 compared with control oligonucleotide group.

MRP-2 is a target of post-transcriptional repression by miR-297

It should be noted that the decreased expression of miR-297 in HCT116/L-OHP cells was concurrent with the overexpression of MRP-2 protein. Accordingly we transfected HCT116/L-OHP cells with miR-297 mimics or control oligonucleotide or MRP-2 siRNA which work as positive control to determine the change of MRP-2. The expression levels of MRP-2 protein determined by Western blotting were decreased in miR-297 miRIDIAN mimic-transfected HCT116/L-OHP and HCT-8/VCR cells relative with the control cells at a dose-dependent manner. The high concentration group (80 nM) almost played the same role as the positive control (MRP-2 siRNA) and the quantitative RT–PCR showed the same conclusion on the mRNA level (Figures 3A and 3C). On the other hand, the MRP-2 protein and mRNA level were increased in miR-297 miRIDIAN inhibitor-transfected HCT116 and HCT-8 cells (Figures 3B and 3C). To determine whether miR-297 also modulates other MDR-related transporters, MRP-1 and P-gp were tested by Western blotting in miR-297 miRIDIAN mimics or MRP-2 siRNA-transfected HCT116/L-OHP cells. Figure 3(D) shows that miR-297 mimics could not modulate other MDR proteins.

miR-297 have the identical seven nucleotides of the 5′ ‘seed’ region which are complementary to bases 64–86 of the MRP-2 3′-UTR (Figure 3E), thus it is a potentially target of MRP-2. To examine whether miR-297 directly targets MRP-2, a segment of the MRP-2 3′-UTR-containing miR-297-binding site was cloned into a luciferase reporter system. The resulting reporter vector was transfected into the HCT116/L-OHP cells together with increasing concentrations of miR-297. Figure 3(F) shows that miR-297 inhibited luciferase activity from the construct with the MRP-2 3′-UTR segment containing the miR-297-binding site in a dose-dependent manner. No luciferase activity change was observed when the cells were transfected with the plasmid with a MRP-2 3′-UTR fragment without the miR-297 binding sites (Figure 3F).

Taken together, these results demonstrate that miR-297 directly inhibit MRP-2 expression at the post-transcriptional level through its 3′-UTR.

Modulation of miR-297 expression altered the sensitivity of human CRC cells to anti-cancer drugs

To investigate whether miR-297 has a direct function in MDR development or is simply differentially modulated in MDR human CRC cells, we transfected miR-297 inhibitor into HCT116 and HCT-8 cells, and miR-297 mimics or MRP-2 siRNA into HCT116/L-OHP and HCT-8/VCR cells, which express relatively low and high levels of miR-297 and MRP-2 respectively, and observed the effects on MDR phenotype thereafter. The MTT assay revealed that HCT116 cells transfected with inhibitor exhibited greatly enhanced sensitivity to VCR, DOX and L-OHP, but not to MMC compared with those transfected with a control oligonucleotide, as shown by significantly increased IC50 values (Figure 4A). On the other hand, transfection of the mimics or MRP-2 siRNA led to an increased sensitivity of HCT116/L-OHP cells to VCR, DOX, 5-FU and L-OHP (Figure 4B). The same results were found in HCT-8 and HCT-8/VCR cells (Figures 4C
and 4D). The above data indicate that modulation of miR-297 expression could alter the MDR phenotype of human CRC cells.

Modulation of miR-297 expression altered the concentration of human CRC cells to DOX

The results above mentioned showed that miR-297 could increase the sensitivity of human CRC cells to some anti-cancer drugs. To investigate whether miR-297 increase the sensitivity by down-regulating MRP-2 to inhibit drug efflux or the other ways, we transfected miR-297 mimics or MRP-2 siRNA into HCT116/L-OHP cells and added DOX, which shows red light excitation at 488 nm after 48 h. Figure 4(E) shows that miR-297 mimics could inhibit DOX efflux by down-regulating MRP-2.

Modulation of miR-297 expression altered the sensitivity of human CRC cells to apoptosis

To investigate whether miR-297 could increase apoptosis in MDR human CRC cells, we evaluated the effects of enforced miR-297 expression on L-OH–induced apoptosis in HCT116/L-OHP cells and VCR-induced apoptosis in HCT-8/VCR cells. A marked increase in apoptosis, as assessed by flow cytometry, was observed in miR-297 mimic-transfected HCT116/L-OHP cells after L-OHP treatment compared with the control oligonucleotide-transfected cells (Figure 5A), and HCT-8/VCR treated by VCR had the same increase in apoptosis (Figure 5B).

Modulation of miR-297 expression altered the MDR of human CRC in vivo

We observed that overexpression of miR-297 remarkably modulated the MDR in human colorectal carcinoma cells in vitro. To confirm this notion in vivo, HCT116/L-OHP cells were stably transfected with pEGFP-C1-cmiR-297 and its corresponding vector as described above, which closely mimicked the MDR mechanisms of human colorectal carcinoma. Re-expression of mature miR-297 in HCT116/L-OHP -EGFP-miR-297 and HCT116/L-OHP -EGFP-Vector cells was confirmed by quantitative PCR (Figure 6A). Then we implanted cells subcutaneously into nude mice. Successful transplantation of viable tumour pieces was confirmed by fluorescence image 1 week after the injection. L-OHP solution was injected in the third week and we found the size and fluorescence intensity of tumour in the miR-297 group was smaller than the Vector group; however, it was similar in these two groups before L-OHP added in the first two weeks (Figures 6B–6D). It indicated that miR-297 had no effect on human MDR CRC growth, but miR-297 could increase the tumour-inhibition rate by sensitizing L-OHP to the human MDR CRC in vivo. Figure 6(E) shows the expression of MRP-2 was inhibited in the tumour tissue which was implanted with HCT116/L-OHP -EGFP-miR-297 cells. This shows that MRP-2 was also the target of miR-297 in vivo. The above data indicate that modulation of miR-297 expression could alter the MDR of human CRC by MRP-2 in vivo.

DISCUSSION

Previous studies suggest that down-regulation of miRNAs may play a role in cancer progression [17–19]. In recent years much work has been intensely focused on the study of altered miRNA expression in human cancer. miRNA expression signatures seem to hold great promise in tumour characterization and could be potential diagnostic and prognostic markers for cancer diagnosis and treatment. It has been shown that some miRNAs in cancer cells could play a role as oncogenes to inhibit expression of tumour suppressors [20,21]. miR-21 has been shown to function as an oncogene and plays a critical role in tumorigenesis, invasion and metastasis of breast cancer cells, in part through the down-regulation of tumour-suppressor genes [22]. More recently, miR-126 and miR-335 have been demonstrated to be lost in a majority of primary breast tumours from recurrent patients and identified as suppressor miRNAs of breast cancer metastasis [23], whereas miR-373, miR-520c and miR-10b have been demonstrated to be metastasis-promoting miRNAs [24,25]. However, only a few of
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Figure 6 Modulation of miR-297 expression altered the MDR of human CRC in vivo

(A) Successful re-expression of mature miR-297 was confirmed by quantitative PCR. The values of miR-297 expression (miR-297/U6) were calculated as the fold change relative to the vector control as 1. (B) In vivo fluorescence imaging of HCT116/L-OHP-EGFP-Vector and HCT116/L-OHP-EGFP-miR-297 cells implanted subcutaneously in nude mice at day 7, 14, 21, 28 and 32 post-implantation. The intensity of fluorescence signals from subcutaneously xenografted tumour was quantified by Kodak In-Vivo FX Pro and calculated as the fold change relative to day 7 which was set as 1. (C) HCT116/L-OHP-EGFP-Vector. (D) HCT116/L-OHP-EGFP-miR-297. Each point in this graph represents an independent mouse. (E) Protein expression levels of MRP-2 in each mouse tumour tissue by Western blotting analysis. β-Actin was used as an internal loading control.

publications [12,13,20,26,27] are reported as to involvement of microRNAs in development of MDR in cancer. The role of microRNAs in development of cancer MDR remains largely unexplored. In the present study, our findings demonstrating the involvement of miRNAs in the development of MDR in colorectal carcinoma cells might support the suggestion that miRNAs could also serve as potential targets for chemosensitizing strategies.

The present study found that nine miRNAs shown an obvious differential expression between HCT116/L-OHP cells and their parent cells (fold change ≥ 3, Table 1), and eight of these miRNAs exhibited decreased expression levels, suggesting that the loss of these suppressor miRNAs may lead to overexpression of their targeted genes such as MDR-related genes in cancer cells.

At present, platinum drugs are the major clinical treatment drugs for CRC. Therefore platinum-drug resistance becomes an important clinical problem which needs to be solved. Previous studies have suggested that, with respect to anti-cancer drugs, overexpression of MRP2 in intact tumour cells is associated with platinum-drug resistance, whereas MDR1 and MRP1 are not [28]. Accordingly in the L-OHP-resistant CRC cell line HCT116/L-OHP, MRP-2 may play an important role as an MDR protein. In the present study, we also used the VCR-resistant CRC cell line HCT-8/VCR to allow us to make comparison. In HCT116/L-OHP cells, we observed that the down-regulation of miR-297 had the largest observed effect on MRP-2. Since miR-297 potentially regulates the target gene MRP-2, which has been widely accepted as drug resistance protein, we wondered if miR-297 might play a role in the development of MDR by targeting MRP-2.

Interestingly, we found that down-regulation of miR-297 was correlated significantly with the clinical stage of human CRC tissue samples, and there is a strongly negative relationship miR-297 and MRP-2 expression in CRC. The present study also showed that MRP-2 could also play a role of MDR, although HCT-8/VCR is not platinum-drug resistance cell line. Furthermore, the elevated levels of miR-297 in miR-297-mimic-transfected HCT116/L-OHP and HCT-8/VCR MDR cells not only down-regulated expression of the MRP-2 protein, but also increased sensitivity of these cells to VCR, DOX and L-OHP. However, it is worth noting that cytotoxicity was not modulated with MMC. A possible explanation for this phenomenon could be that MMC is not the substrate drug of MPR-2, so inhibition of miR-297 does not have any role in increasing the sensitivity of these cells.

The present study provides the first evidence that miRNAs may be involved in the development of MDR in CRC and is also the first report that miR-297 can act as a suppressor to modulate MRP-2-mediated MDR of tumour cells to chemotherapeutic agents in vitro and in vivo. Our results demonstrated that the ectopic expression of miR-297 in the HCT116/L-OHP and HCT-8/VCR MDR cell lines contributed to the suppression of MRP-2, sensitized HCT116/L-OHP and HCT-8/VCR MDR cells to
some anti-cancer drugs and indicated that modulation of miR-297 expression could alter the MDR of human CRC by MRK-2 in vivo.

Currently, a few investigations have successfully targeted oncogenes with artificial synthetic miRNAs [29–31]. Our results suggest that targeting MRP-2 by miR-297 may have significant implications for prevention and reversal of tumour cell MDR.

In conclusion, we have shown that expression of miR-297 is negatively correlated with MRP-2 expression in CRC cells. Furthermore, the elevated level of miR-297 by transfection of miR-297 mimics not only down-regulates expression of MRP-2 but also sensitizes HCT116/L-OHP and HCT-8/VCR MDR cells to some anti-cancer drugs and indicated that modulation of miR-297 expression could alter the MDR of human CRC by MRP-2 in vitro and in vivo. Our findings contribute further to the understanding of MDR regulation in cancer cells. Additionally, these findings may be beneficial for further research of predicting MDR in patients and designing personalized therapy for CRC patients.

AUTHOR CONTRIBUTION
Ke Xu performed experiments. Xin Liang, Ke Shen, Daling Cui and Yuanhong Zheng performed some on the in vitro experiments. Jianhua Xu, Zhongze Fan and Yanyan Qiu performed some on the in vivo experiments and the human tissues experiments. Qi Li and Lei Ni performed the animal experiments and human tissues experiment. Jianwen Liu supervised the project and wrote the paper.

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