MEG3 is a prognostic factor for CRC and promotes chemo sensitivity by enhancing oxaliplatin-induced cell apoptosis

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Abstract. A major reason for the failure of advanced colorectal cancer (CRC) treatment is the occurrence of chemoresistance to oxaliplatin-based chemotherapy. Recently, studies have shown that long non-coding RNAs (lncRNAs) play an important role in drug resistance. Using HiSeq sequencing methods, we identified that lncRNAs show differential expression levels in oxaliplatin-resistant (OxR) and non-resistant CRC patients. RT-qPCR was then performed in tissues and serum samples, and lncRNA MEG3 was verified to be downregulated in non-responding patients and to have considerable discriminating potential to identify responding patients from non-responding patients. Moreover, decreased serum MEG3 expression was associated with poor chemoresponse and low survival rate in CRC patients receiving oxaliplatin treatment. Subsequently, OxR cell lines were established, and MEG3 was significantly downregulated in HT29 OxR and SW480 OxR cells. In addition, overexpression of MEG3 with pMEG3 reversed oxaliplatin resistance in both CRC cell lines. Flow cytometric apoptosis analysis indicated that MEG3 promoted CRC cell apoptosis. More importantly, MEG3 enhanced oxaliplatin-induced cell cytotoxicity in CRC. In conclusion, our integrated approach demonstrated that decreased expression of lncRNA MEG3 in CRC confers potent poor therapeutic efficacy, and that MEG3 promotes chemosensitivity by enhancing oxaliplatin-induced cell apoptosis. Thus, overexpression of MEG3 may be a future direction by which to develop a novel therapeutic strategy to overcome oxaliplatin resistance of CRC patients.

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide (1). It is the second and third most commonly diagnosed cancer in females and males, respectively, and more than 1.2 million patients are diagnosed with CRC every year (2,3). Currently, oxaliplatin-based chemotherapy after surgical resection is one of the most widely used therapeutic strategies (4). However, a large proportion of patients receiving chemotherapy finally become metastatic and chemoresistant, and this has been a key barrier to the efficacy of CRC treatment (5). Hence, finding new therapeutic markers is warranted to evade drug resistance mechanisms, and possibly find a cure.

In recent years, a tremendous amount of effort has been devoted to understanding the mechanisms of chemoresistance and to elaborate the genes/pathways involved. Chemoresistance mechanisms are extraordinarily complex, including inefficient cellular drug uptake and accumulation (6), enhancement of DNA repair (7) and activation of the antioxidant glutathione system for detoxification (8,9). One major resistance mechanism utilized by tumor cells is to resist drug-induced cell death through disruptions in apoptotic pathways (10). Thus, it is also essential to better understand the pathways related to chemoresistance in CRC and discover novel strategies to further improve the effectiveness of oxaliplatin.

Long non-coding RNAs (lncRNAs) are defined as transcripts >200 nucleotides in length and are transcribed, but are non-translated non-coding RNAs in the human genome (11). Recent studies have demonstrated that lncRNAs play important roles in carcinogenesis and cancer metastasis, and some lncRNAs function as oncogenes, tumor-suppressor genes or both, depending on the circumstance (12). However, aberrant lncRNA expression has been detected and identified as a promising biomarker for diagnosis and prognosis in breast and gastric cancer, and CRC (13-15). The discovery and study of lncRNAs are thus of major relevance to human biology and disease, as they represent an extensive, largely unexplored and functional component of the genome (16,17). To date, lncRNA expression has not been extensively analyzed in CRC samples except in a few studies that have used microarrays from TCGA (18), and the genome-wide screening of relevant lncRNAs are essential for improving the prognosis of cancer.
informed consent was obtained from all patients according to local ethical regulations of the Ethics Committee of Zhongnan Hospital of Wuhan University.

HiSeq sequencing. Total tissue RNA was extracted by one-step extraction using a TRizol reagent kit (Life Technologies, Carlsbad, CA, USA), and the purity and quantity of RNA were determined using UV spectrophotometry. cDNA library construction and sequencing were performed according to previously described methods (19). Briefly, after extraction of total RNA, ribosomal RNA was separated to isolate as much ncRNA as possible. RNA containing poly(A) was then removed. RNA fragments were broken into short fragments randomly. The first chain of cDNA was generated using RNA fragments as templates and 6-bp random primers. Second chain of the cDNA was synthesized according to the kit’s instructions (Takara Bio Company, Dalian, China). After purification, end repair, base A and sequencing joint adding, the generated cDNA was fragmented using uracil-N-glycosylase (UNG). cDNA fragments were chosen according to size, then PCR amplification was performed to establish the complete sequencing cDNA library. lncRNAs were sequenced using the high-throughput, high-sensitivity HiSeq 2500 sequencing platform (Illumina Inc., San Diego, CA, USA). Sequencing results were analyzed and treated using Trim Galore software to dynamically remove joint sequence fragments and low-quality segments from the 3’ end. FastQC software was used for quality control of the pretreated data.

Cell culture. Human CRC cell lines HT29 and SW480 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) in 2014. All CRC cell lines were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Wilmington, DE, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 g/ml streptomycin (Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO2 and 95% air.

RNA extraction. Total RNA was isolated from primary CRC tissues and cells using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Serum RNA was isolated using acid phenol according to the manufacturer’s instructions. The extracted total RNA was eluted in 20 µl nuclease-free water and the RNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific). The samples with A260/A280 nm ratios between 1.8 and 2.0 were used for further experiments.

Quantitative real-time PCR (RT-qPCR). For primary CRC tissues and cell lines, the cDNA was synthesized from 200 ng extracted total RNA using the PrimeScript RT reagent kit (Takara Bio Company) and amplified by RT-qPCR using LightCycler 480 SYBR-Green I Master (Roche, Mannheim, Germany) and GAPDH was used as the control gene. For serum cell-free MEG3 detection, we used a previously established RT-qPCR-D method without RNA extraction (20). Briefly, the 2X preparation buffer was prepared, which contained 2.5% Tween-20 (EMD Chemicals, Gibbstown, NJ, USA), 50 mmol/l Tris, and 1 mmol/l EDTA (both from Sigma-Aldrich). First, 5 µl of serum was mixed with an equal volume of 2X prepartion buffer. Subsequently, the above mixture was reverse
transcribed (RT) in triplicates in a 20 μl reaction volume. Finally, the RT product was 10-fold diluted and centrifuged at 16,000 x g for 5 min, and 5 μl supernatant solution was used as a cDNA template for qPCR. The reagents and reaction conditions were the same as those for RT-qPCR. The 2⁻ΔΔCT method was used to determine the relative quantification of gene expression levels. The primer sequences were as follows: MEG3 forward, 5'-CTCTTCCCGCCTACCTACCTCAG-3' and reverse, 5'-CTCTTCCCGCCTACCTACCTCAG-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, ATGGTGTTGAAGACGCGAGT.

Development of oxaliplatin-resistant (OxR) cell lines. Oxaliplatin (Sanofi-Synthelabo, Hangzhou, China) was purchased from the pharmacy at Zhongnan Hospital of Wuhan University. HT29 and SW480 cells were exposed to an initial oxaliplatin concentration of 0.1 μmol/l in RPMI-1640 medium plus 10% FBS. The surviving population of cells was grown to 80% confluence and passed twice over 9 days to ensure viability. The concentration of oxaliplatin that the surviving population was exposed to was then sequentially increased in the same manner to 0.5 μmol/l (15 days), 1.0 μmol/l (30 days), and finally to the clinically relevant plasma concentration of 2 μmol/l. For all in vitro studies, OxR cells were used at no higher than 15 passages from creation and were maintained and exposed to 2 μmol/l oxaliplatin unless otherwise indicated.

Cell transfection. The MEG3 overexpression plasmid (pMEG3) and control vector (pVector) were purchased from Addgene (Cambridge, MA, USA). CRC cells were plated in 24-well plates at 1x10⁵/well. Forty-eight hours after plating, 100 nM of si-MEG3 or pMEG3 as well as the negative controls were transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell viability assay. Cell viability was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) assay. Briefly, 100 μl of cells from the different groups was seeded onto a 96-well plate at 1x10⁵/well. After 24 hours, 100 μl of 10% FBS were added. Cells were incubated at 37°C. At different time point, the optical density was measured at 450 nm using a microtiter plate reader, and the absorbance was assessed using flow cytometry (BD FACSCalibur; BD Biosciences). Statistical analysis. Statistical analyses were performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to determine the normality of the distribution of data in each group. Data are presented as median (interquartile range). Difference in lncRNA levels among multiple groups in HiSeq sequencing was determined using Bonferroni adjustment method. Mann-Whitney U test or Kruskal-Wallis test was employed to compare differences of lncRNAs among clinical cohort groups. A log-rank test was used to analyze the statistical differences in survival as deduced from Kaplan-Meier curves. Cox proportional-hazard regression analysis was performed to calculate hazard ratio (HR) and 95% confidence interval (CI) for each covariate. All differences were regarded as statistically significant when P<0.05.

Results

Identification of candidate lncRNAs by high-throughput HiSeq sequencing. The HiSeq sequencing with 8 tissue samples pooled from CRC patients showing response and 6 from patients showing no response to oxaliplatin treatment was conducted. In total, 735 lncRNAs were identified with significant differential expression (fold-change, ≥2.0). To identify the lncRNAs that are potential biomarkers, we concentrated on the top 60 most upregulated and 60 downregulated lncRNAs that were differentially expressed between CRC patients showing response or no response (Fig. 1). Starting from those lncRNAs with the greatest fold-change, we filtered appropriate candidate lncRNAs in descending order. Candidates should be plausible for primer designing, and only those having steady expression in tissue samples were selected. Finally, we chose 3 candidate lncRNAs from the upregulated group and 3 from the downregulated group as well (Table I). Another 4 lncRNAs were also tested by RT-qPCR since they were previously shown to be dysregulated in CRC chemoresistance (21-24). Thus, 10 lncRNAs were selected as candidates for further testing via RT-qPCR.

MEG3 is downregulated in CRC patients showing response to oxaliplatin treatment by RT-qPCR. The expression of all 10 candidate lncRNAs was evaluated by RT-qPCR, using 80 CRC tissues from patients showing response to oxaliplatin and 80 from patients showing no response. Among these, 4 lncRNAs (LOC286467, MEG3, LOC285194 and LincRNA-p21) were found to be significantly dysregulated in responding tissues compared with non-responding tissues (Table II). Subsequently, these 4 lncRNAs were further validated in an independent cohort of 140 serum samples from 70 CRC patients showing response and 70 showing no response. Among the 4 candidate lncRNAs, only MEG3 was significantly deregulated with a markedly suppressed expression in non-responding patients compared to responding patients (Fig. 2). Thus, we focused on the role of MEG3 in CRC chemoresistance.

Decreased serum MEG3 expression predicts poor response to oxaliplatin treatment in CRC patients. Predictive biomarkers are better when blood-based, as blood is easily available and provides the chance to monitor cancer progression. Thus, receiver operator characteristic (ROC) curve analysis was firstly performed to investigate the potential value of serum MEG3 in distinguishing the chemotherapeutic responding and non-responding CRC patients. Our data showed that the area under the curve (AUC) was 0.784, providing a diagnostic
sensitivity of 72.86% and a specificity of 61.43% (Fig. 3A). Under the stratification criteria (1.14) established by the ROC curve, patients were stratified into a high (n=62) and low (n=78) MEG3-expressing group. The proportion of patients that responded to oxaliplatin treatment was significantly higher in the high MEG3-expressing group than in the low MEG3-expressing group.

**Table I. Candidate lncRNAs selected on a basis of the HiSeq analysis.**

| Seqname   | Location     | Regulation (NR vs. R) | Fold-change   | P-value  |
|-----------|--------------|-----------------------|---------------|----------|
| LOC286467 | ChrXq26.2    | Up                    | 73.6341872    | 0.00006546 |
| CRNDE     | Chr16q12.2   | Up                    | 42.6729041    | 0.00109347 |
| SNHG7     | Chr9q34.3    | Up                    | 27.8730264    | 0.00960371 |
| LOC145837 | Chr15q23     | Down                  | 48.8710538    | 0.00054087 |
| MEG3      | Chr14q32.2   | Down                  | 46.4837692    | 0.00079283 |
| LOC285194 | Chr3q13.13   | Down                  | 22.7845924    | 0.01947853 |

NR, non-response; R, response.
the low MEG3-expressing group (P<0.01; Fig. 3B). More importantly, Kaplan-Meier survival analysis was performed to further investigate the effect of serum MEG3 on oxaliplatin treatment for CRC patients. The results indicated that low MEG3 expression was associated with poor OS (P=0.007; Fig. 3C) and RFS (P=0.012; Fig. 3D). Furthermore, we performed Cox regression univariate/multivariate analyses to identify whether MEG3 or another clinical parameter was an independent indicator for OS of CRC patients who received oxaliplatin chemotherapy. The results indicated that the serum MEG3 expression level and distant metastasis maintained their significance as independent prognostic factors for OS of CRC patients receiving oxaliplatin treatment (Table III).

MEG3 is downregulated in OxR CRC cell lines. As a follow-up to our patient data that revealed a lower expression

### Table II. Expression of 10 candidate lncRNAs in CRC patients showing response or non-response to oxaliplatin treatment [median (interquartile range)].

| lncRNA   | Response          | Non-response      | P-value |
|----------|-------------------|-------------------|---------|
| LOC286467| 1.39 (0.47-2.02)  | 1.86 (1.13-2.45)  | <0.05   |
| CRNDE    | 0.94 (0.43-1.95)  | 1.18 (0.49-2.03)  | 0.37    |
| SNHG7    | 1.14 (0.33-2.51)  | 1.57 (0.52-3.08)  | 0.24    |
| H19      | 0.87 (0.35-1.89)  | 1.27 (0.44-1.97)  | 0.06    |
| MALAT1   | 1.03 (0.42-2.07)  | 1.21 (0.66-2.49)  | 0.18    |
| LOC145837| 0.99 (0.38-2.15)  | 0.77 (0.23-1.85)  | 0.09    |
| MEG3     | 1.32 (0.40-2.31)  | 0.74 (0.35-1.62)  | <0.01   |
| LOC285194| 1.47 (0.46-2.28)  | 0.92 (0.37-1.89)  | <0.01   |
| LncRNA-p21| 1.22 (0.54-2.18) | 0.88 (0.33-1.76)  | <0.05   |
| SLC25A25-AS1 | 1.15 (0.60-2.22) | 1.02 (0.47-1.59)  | 0.44    |

*Intronic non-coding RNAs; CRC, colorectal cancer.*

Figure 2. (A-D) Relative expression of 4 identified serum lncRNAs in CRC patients showing response (n=70) and non-response (n=70) to oxaliplatin therapy using RT-qPCR assay in a validation cohort. Error bars represent median ± SD (standard deviation); *P<0.01.
of MEG3 in the OxR population, we further assessed the expression of MEG3 in the oxaliplatin-resistant CRC cell lines. HT29 and SW480 cell lines were constantly exposed to a high concentration of oxaliplatin (2 µM) to establish the HT29 OxR (OxR) cell line and SW480 OxR cell line. The established HT29-resistant cells were maintained and exposed to 2 µM oxaliplatin unless otherwise indicated. As shown in Fig. 4A, both HT29 OxR and SW480 OxR cells showed elevated cell viability compared with the HT29 and SW480 parental cells when incubated with culture medium.

Table III. Univariate and multivariate Cox proportional hazards regression model analysis of factors for OS in patients with CRC in a validation cohort.

| Characteristics         | Univariate analysis | Multivariate analysis |
|-------------------------|---------------------|-----------------------|
|                         | HR                  | 95% CI                | P-value | HR                  | 95% CI                | P-value |
| Gender                  | 1.018               | 0.617-2.012           | 0.639   | 1.018               | 0.617-2.012           | 0.639   |
| Age (years)             | 1.533               | 0.741-2.882           | 0.202   | 1.533               | 0.741-2.882           | 0.202   |
| Tumor size              | 1.679               | 0.537-2.729           | 0.418   | 1.679               | 0.537-2.729           | 0.418   |
| Differentiation         | 1.885               | 1.029-3.352           | 0.087   | 1.885               | 1.029-3.352           | 0.087   |
| Local invasion          | 1.661               | 0.902-2.798           | 0.152   | 1.661               | 0.902-2.798           | 0.152   |
| Distant metastasis      | 2.771               | 1.580-3.998           | 0.008   | 2.771               | 1.580-3.998           | 0.008   |
| TNM stage               | 2.257               | 1.141-3.505           | 0.043   | 2.257               | 1.141-3.505           | 0.043   |
| Serum MEG3 level        | 1.353               | 0.321-2.107           | 0.007   | 1.353               | 0.321-2.107           | 0.007   |

OS, overall survival; CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.
containing 2 µM concentration of oxaliplatin. In contrast, the concentration-effect curve indicated that the IC₅₀ value of oxaliplatin for HT29 OxR cells was 11.6 µM, while the IC₅₀ value of oxaliplatin for the HT29 parental cells was 1.5 µM, which means that the HT29 OxR cells exhibited a 7.7 times higher ability of oxaliplatin resistance than the HT29 cells. Similarly, the SW480 OxR cells exhibited a 5.4 times higher ability of oxaliplatin resistance than the SW480 parental cells (10.3/1.9 µM; Fig. 4B). After the OxR CRC sub-lines were established, we determined the MEG3 expression level, and found that MEG3 was significantly downregulated in the HT29 OxR and SW480 OxR cells compared with the parental HT29 and SW480 cells, respectively (Fig. 4C).

Overexpression of MEG3 partially reverses the chemoresistance status of OxR cells. After having validating the disruption of MEG3 in OxR cells, we further investigated whether MEG3 plays a role during the formation of oxaliplatin resistance in CRC cells. MEG3 overexpression plasmid (pMEG3) was transfected into HT29 OxR and SW480 OxR cells, and RT-qPCR assay showed that the MEG3 expression level was significantly increased in cells transfected with pMEG3 (Fig. 5A). Moreover, the survival cell rate was significantly impaired when MEG3 was overexpressed in the HT29 OxR and SW480 OxR cells incubated with 2 µM concentration of oxaliplatin (Fig. 5B and C). This suggests that MEG3 overexpression partially reverses the oxaliplatin resistance in CRC.

MEG3 promotes oxaliplatin-induced apoptosis in CRC cells. Previous studies have demonstrated that MEG3 may have an effect on cell apoptosis during cancer angiogenesis (25). Thus, flow cytometric apoptosis analysis was performed to explore whether MEG3 reversed oxaliplatin resistance through promotion of cell apoptosis. Our results showed that pMEG3 significantly enhanced cell apoptosis in the HT29 and SW480 cells (Fig. 6A). To directly validate that MEG3 enhanced oxaliplatin-induced cell apoptosis in CRC, we treated HT29 and SW480 cells with a concentration gradient containing 2 µM concentration of oxaliplatin. In contrast, the concentration-effect curve indicated that the IC₅₀ value of oxaliplatin for HT29 OxR cells was 11.6 µM, while the IC₅₀ value of oxaliplatin for the HT29 parental cells was 1.5 µM, which means that the HT29 OxR cells exhibited a 7.7 times higher ability of oxaliplatin resistance than the HT29 cells. Similarly, the SW480 OxR cells exhibited a 5.4 times higher ability of oxaliplatin resistance than the SW480 parental cells (10.3/1.9 µM; Fig. 4B). After the OxR CRC sub-lines were established, we determined the MEG3 expression level, and found that MEG3 was significantly downregulated in the HT29 OxR and SW480 OxR cells compared with the parental HT29 and SW480 cells, respectively (Fig. 4C).

Figure 4. MEG3 is downregulated in oxaliplatin-resistant CRC cell lines. (A) Both HT29 OxR and SW480 OxR cell lines showed elevated cell viability compared to the HT29 and SW480 parental cells when incubated with culture medium containing 2 µM concentration of oxaliplatin. (B) The concentration-effect curve indicated that the IC₅₀ values of oxaliplatin for HT29 OxR (11.6 µM) and SW480 OxR cells (10.3 µM) were significant higher than that for the HT29 (1.5 µM) and SW480 parental cells (1.9 µM). (C) The expression of MEG3 in HT29 OxR and SW480 OxR cells was significantly lower than that in the HT29 and SW480 parental cells, respectively. Error bars represent SD. *P<0.05; **P<0.01.
of 0.5 µM oxaliplatin after being transfected with pMEG3 or the negative control. The dose-effect curve showed that pMEG3 transfection was followed by significantly increased cell apoptosis compared with the pVector control (Fig. 6B). However, the IC_{50} values for oxaliplatin were 1.8 and 2 µM in the non-transfected HT29 and SW480 cells, and were markedly decreased to 1.2 and 1.4 after transfection with pMEG3, respectively. To conclude, MEG3 enhanced the chemosensitivity of oxaliplatin by promoting oxaliplatin-induced apoptosis in CRC cells.

**Discussion**

Despite recent chemotherapeutic regimens that have significantly increased the survival of patients with metastatic disease, invariably, nearly all CRC patients finally become chemoresistant accompanied by distant metastasis (5). Identification of new therapeutic markers and better understanding of the pathways related to chemoresistance are essential to improving the prognosis of CRC patients. In the present study, high-throughput HiSeq sequencing was firstly employed to provide basic information concerning the lncRNAs significantly dysregulated in CRC tissues. Candidate lncRNAs were selected and then evaluated by RT-qPCR in tissues and serum samples to validate their consistent pattern of dysregulation in these clinical materials. MEG3 was finally identified to show considerable discriminating potential to identify responding patients from non-responding patients with high AUC value. Serum MEG3 expression was associated with chemoresponse and predicted the prognosis of CRC.
MEG3 is also downregulated in nasopharyngeal and promoted cell apoptosis in CRC (13). Chak et al. MEG3 suppressed cell proliferation, caused cell cycle arrest partly consistent with previous studies. Yin et al. found that suppression of expression of MEG3 may be an important mechanism can reverse oxaliplatin resistance in CRC cells; thus, this study detected that MEG3 expression is downregulated in various types of cancers (27). Thus, MEG3 is a tumor-suppressor and overexpression of MEG3 could inhibit tumor cell proliferation and promote tumor cell apoptosis in different cancers (13,31-33). In our research, we confirmed the downregulation of MEG3 and found that serum MEG3 expression considerably distinguished responding patients from non-responding ones with high diagnostic efficiency. However, the proportion of patients that responded to oxaliplatin treatment was significantly higher in the high MEG3-expressing group than in the low MEG3-expressing group. Most importantly, low serum MEG3 expression was associated with poor OS and RFS in patients receiving oxaliplatin treatment. Collectively, we revealed that MEG3 is a lncRNA lowly expressed in non-responding CRC patients and closely correlated to the chemoresponse to oxaliplatin of CRC by enhancing oxaliplatin-induced cell cytotoxicity. We also revealed that MEG3 promoted the chemosensitivity to oxaliplatin of CRC by enhancing oxaliplatin-induced cell cytotoxicity. These findings indicate that downregulation of MEG3 confers a potent poor therapeutic efficacy. Thus, MEG3 may be a potential prognostic marker and therapeutic target in CRC patients.

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