MEK inhibitor enhanced the antitumor effect of oxaliplatin and 5-fluorouracil in MEK1 Q56P-mutant colorectal cancer cells

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Abstract. Mitogen-activated protein kinase kinase (MEK) small molecule inhibitors have been investigated in preclinical or clinical trials for the treatment of cancer. In the present study the genetic test results of 120 patients with colorectal cancer (CRC) were screened and the mutation rate of MEK1 was identified to be 1.67%. MEK inhibition by U0126 significantly decreased the growth of SW48 cells that harbored the MEK1 Q56P mutation, although it did not evidently affect the growth of NCI-H508 cells with MEK1 wild-type. In addition, U0126 increased the sensitivity of SW48 cells to 5-fluorouracil (5-FU) and oxaliplatin by producing more γH2AX foci and decreasing the expression of excision repair cross-complementation group 1 and thymidylate synthase. The results suggested that MEK inhibitors in combination with oxaliplatin/5-FU may offer an improved therapeutic effect in patients with MEK-mutant CRC.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortality worldwide. Despite advances in treatment methods, patients with CRC have a poor 5-year survival rate (1). Currently, 5-fluorouracil (5-FU) and oxaliplatin serve pivotal roles in treatment regimens for CRC (2). 5-FU increases DNA damage via inhibition of thymidylate synthase (TYMS). Oxaliplatin is a third-generation platinum-containing compound that may induce DNA cross-links, leading to DNA double-strand breaks (DSBs) (3). DSBs are one of the most important factors that threaten the integrity of the genome.

Mitogen-activated protein kinase kinase 1 (MEK1), also known as MAP2K1, is a protein kinase that is a known downstream target of Raf-1 proto-oncogene serine/threonine kinase and is upstream of extracellular signal-regulated kinase (ERK). A variety of small molecule inhibitors of MEK are currently investigated in preclinical or clinical trials for the treatment of malignancies (4). The small-molecule compound U0126 has been identified as a MEK1/2 inhibitor that directly suppresses MEK1/2 activation with well characterized off-target effects (5). Although the pharmacological characteristics of U0126 indicate render it unsuitable for clinical use, it has been demonstrated to be an efficient inhibitor in vitro and in vivo to study the functions of MEK1/2 (6).

Combination therapy is a common approach in cancer chemotherapy. However, the effect of an MEK inhibitor combined with oxaliplatin or 5-FU in MEK-mutant colorectal cells and the underlying mechanism remain unclear. Therefore, the present study aimed to evaluate the combined effect of the MEK inhibitor U0126 with oxaliplatin and 5-FU in CRC cells, and to further explore the underlying mechanisms involved.

Materials and methods

Reagents and cell culture. The human SW48 (cat. no. CCL-231) and NCI-H508 (cat. no. CCL-253) cell lines were obtained from the ATCC (Manassas, VA, USA). SW48 cells were cultured in Leibovitz's L-15 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 50 µmol/l β-mercaptoethanol, and were maintained in an environment of 5% CO₂ at
37°C in Gibco RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS.

U0126 was purchased from Selleck Chemicals (cat. no. S1102; Houston, TX, USA). 5-FU and oxaliplatin were obtained from Tongtai Medicine Co., Ltd. (Shandong, China) and Chenxin Medicine Co., Ltd. (Shandong, China), respectively. Antibodies against Akt (cat. no. 4691S; dilution, 1:2,000), phospho-Akt (cat. no. 4060S; dilution, 1:2,000), p44/42 MAPK (cat. no. 4695; dilution, 1:2,000), phospho-p44/42 MAPK (cat. no. 4370; dilution, 1:2,000), H2AX (cat. no. 9718; dilution, 1:1,000) and phospho-H2AX (cat. no. 7631; dilution, 1:1,000) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). β-actin polyclonal antibody (cat. no. AP0606; dilution, 1:2,000) was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA), and goat anti-rabbit secondary antibody was obtained from Signalway Antibody LLC (cat. no. L3012; College Park, MD, USA). Excision repair cross-complementation group 1 (ERCC1) rabbit polyclonal antibody (cat. no. 14586-1-AP) and TYMS rabbit polyclonal antibody (cat. no. 15047-1-AP) were purchased from ProteinTech Group, Inc. (Wuhan, China).

**Patient samples.** A total of 120 CRC patients, including 72 males and 48 females with a mean age of 62.3, were enrolled into the present study. Tumor tissues were obtained in Jiangsu Institute of Cancer Research (Nanjing, Jiangsu, China) between 2016 and 2017. The histological diagnosis of all samples was confirmed by pathologists. TNM classification of malignant tumors was used to determine tumor stage (7). Inclusion criteria were: i) Histologically confirmed diagnosis of CRC, ii) age ≥18 years, iii) availability of tumor tissue for next-generation analyses and iv) no prior therapy for CRC except surgery or radiotherapy, or any adjuvant chemotherapy had ceased for >12 months. Patients with incomplete records, no available tumor tissue or any other malignancy during the last 5 years were also excluded. All detailed information was recorded and summarized in Table I. All patients who participated in the study provided signed informed consent. The research using human tissue received approval from the Ethics Committee of Jiangsu Institute of Cancer Research.

**Mutation detection of cells and tissues by next-generation sequencing.** Gene mutations in formalin-fixed and paraffin-embedded tissues of 120 CRC patients were detected using Ion AmpliSeq™ Colon and Lung Cancer Panel (Ion AmpliSeq™ Community 4571815; Thermo Fisher Scientific, Inc.), including mutations in KRAS, EGFR, Braf, PIK3CA, ALK, NRAS, ERBB2, MET, MEK1, PTEN, SMAD4, STK11, FBXW7, ERBB4, DDR2, CTNNB1, AKT1, NOTCH1, FGFR1, FGFR2 and FGFR3. Next, DNA was extracted with E.Z.N.A.® Tissue DNA kit (Omega Bio -Tek Inc., Norcross, GA, USA), and DNA concentration was determined by Qubit® 2.0 fluorometer dsDNA HS assay kit (Thermo Fisher Scientific, Inc.).

A total of 15 ng DNA was then amplified, fragmented, ligated to adapters, barcoded and clonally amplified onto beads to create DNA libraries using Ion PGM™ ampliSeq kit 2.0 and IonXpress barcode adapters kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, library mixtures were enriched on an Ion OneTouch system with Ion PGM™ Hi-Q OT2 kit (Thermo Fisher Scientific, Inc.). Finally, the library pool was sequenced with Ion PGM™ Hi-Q sequencing kit (Thermo Fisher Scientific, Inc.) using Ion Torrent PGM system. The Ion Torrent variant caller plugin (version 4.0) was used to align reads to the reference genome hg19. The sequencing coverage of the tested genomic regions was >1,000 and the uniformity was >90%.

**Cell viability assay.** The cells were plated onto 96-well plates at a density of ~5,000 cells per well. After 24 h, different concentrations of oxaliplatin (0.5, 1, 5, 10, 20 and 50 µg/ml), 5-FU (0.5, 1, 5, 10, 20 and 50 µg/ml) or U0126 (0.1, 0.5, 1, 5, 10 and 20 µM) were added to the cells for 72 h. Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was then added to each well, and the optical density (OD) value was measured at a wavelength of 450 nm with an absorbance reader (BioTek ELx8080; BioTek Instruments, Inc., Winooski, VT, USA). The viability of untreated cells was set to 100%, and the data of treated cells are expressed as a percentage of the control.

**The calculation of combination index (CI).** The interactions between drugs were presented in terms of the combination index (CI), which was calculated by dividing the expected growth inhibition rate by the observed growth inhibition rate. A value of CI<1.0 was considered to indicate a synergistic interaction, while CI>1.0 indicated antagonistic drug effects. The CI analysis was performed using CalcuSyn software (version 1.0, Biosoft, Cambridge, UK).

**Flow cytometry analysis.** SW48 cells were seeded in 6-well plates at a concentration of 1x10⁶ cells per well. Next, the cells were incubated with oxaliplatin, 5-FU or U0126 alone, or with a combination of the drugs. Finally, the cells were stained with a FITC-Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA), and the apoptosis rate was detected with a BD Accuri C6 flow cytometer (BD Biosciences).

**Western blot analysis.** Subsequent to harvesting, cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China), and then lysates were centrifuged at 12,000 x g for 20 min at 4°C. Protein content was determined by DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and protein extracts (50 µg) were subjected to electrophoresis on a NuPAGE 10% Bis-Tris gel (Thermo Fisher Scientific, Inc.). Following protein transfer onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), membranes were incubated in 5% bovine serum albumin for 1 h and then incubated overnight at 4°C with the primary antibodies mentioned in the reagents section. Subsequently, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody and visualized with an enhanced chemiluminescence solution (EMD Millipore) and a BioRad ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.** Total RNA was extracted from cells with TRIzol® reagent (Thermo Fisher Scientific, Inc.) and RNA concentration was measured by OD-1000+ (Wuyi Technology Instruments, Inc., Kumamoto, Japan) was then added to each well, and the optical density (OD) value was measured at a wavelength of 450 nm with an absorbance reader (BioTek ELx8080; BioTek Instruments, Inc., Winooski, VT, USA). The viability of untreated cells was set to 100%, and the data of treated cells are expressed as a percentage of the control.

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Co., Ltd., Nanjing, China). Following RT with Takara PrimeScript™ RT Master Mix kit (cat. no. RR036Q; Takara Bio Inc., Otsu, Japan), the PowerUp™ SYBR® Green Master Mix (cat. no. A25742; Thermo Fisher Scientific, Inc.) and an Applied Biosystems 7300 Real-Time PCR system were applied for qPCR analysis. The cycling conditions comprised 2 min at 95°C, 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Experiments were conducted in triplicate, and β-actin was used as an internal control. The primer sequences used in this assay were as follows: ERCC1, 5'-GGCGACGTA ATTCGCCAGAT-3' (forward) and 5'-GGATGTCAGTCTG GGTGCAGGT-3' (reverse); TYMS, 5'-TTTGGAGGATT GCTGTTGGT-3' (forward) and 5'-GATCCATGCGATCC CAGAT-3' (reverse); and β-actin, 5'-TTCTCAATGAGCTG CGTGTG-3' (forward) and 5'-CAGCCTGGATAGCAGCT ACA-3' (reverse). The relative expression of RNA was calculated using the comparative Cq method (8). The experiments were replicated three times.

Immunofluorescence staining. Cells were seeded on coverslips, which were kept in a 24-well plate at a concentration of 1x10^4 cells for 24 h before treatment, and treated with different drugs for 72 h. Next, the cells were washed and fixed in 4% paraformaldehyde in PBS for 1 h at 37°C. The coverslips were then washed three times with PBS and blocked in immunofluorescence staining blocking buffer (cat. no. P0102; Beyotime Institute of Biotechnology) for 1 h. Subsequently, cells were incubated with a primary antibody against rabbit anti-phospho-H2AX (1:200) at 4°C overnight and washed three times with 0.3% Triton X-100 in PBS. FITC-labeled Goat Anti-Rabbit IgG (cat. no. A0562; JingAn Biological, Jiangsu, China) was used for visualization of phospho-H2AX staining, while the nuclei were stained with 4,6-diamidino-2-phenylindole. The samples were immediately examined using a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

Results

MEK1 gene mutations in CRC cell lines and patients. The colon and lung panel was used to screen gene mutations in SW48 and NCI-H508 cell lines. All gene alterations are listed in Table II. The MEK1 Q56P mutation was identified in SW48 cells. In addition, the presence of this mutation of MEK in patients with CRC was examined in the current study by retrospectively summarizing the genetic test results of 120 patients with CRC. Genomic profiling of these 120 samples revealed two MEK1 mutations in the included CRC patients, including p.D67N and p.Q56P. The total mutation rate of MEK1 was 1.67%.

U0126 effectively inhibits the growth of SW48 cells. To verify the role of MEK1 mutation, SW48 and NCI-H508 cells were stimulated with a concentration gradient of U0126 (1, 5, 10 and 20 µM) for 72 h, and the cell viability was measured by a CCK-8 assay. Cell growth profiles demonstrated that inhibition of MEK by U0126 treatment significantly decreased the growth of SW48 cells, whereas U0126 exerted little effect on the growth of NCI-H508 cells (Fig. 1A). Approximately 82.8% of NCI-H508 cells survived with stimulation of 20 µM U0126. Therefore, the SW48 cell line was selected for use in subsequent investigations. Western blot analysis revealed that U0126 exposure decreased the phosphorylation of ERK in a dose-dependent manner, whereas Akt phosphorylation was not evidently affected (Fig. 1B). Furthermore, treatment with various concentrations of oxaliplatin or 5-FU, the most frequently used chemotherapeutic agents in CRC, was found to induce dose-dependent growth inhibition in SW48 cells (Fig. 1C and D).

Combined effect of MEK1 inhibitor with oxaliplatin and 5-FU. Compared with the control group (100%), the cell viability after stimulation with 2 and 5 µg/ml oxaliplatin decreased to 81.43±0.95 and 70.03±2.61%, respectively. However, the combination of U0126 (2 µM) with 2 or 5 µg/ml oxaliplatin

| Variable      | N  |
|---------------|----|
| Sex           |    |
| Male          | 72 |
| Female        | 48 |
| Age, years    |    |
| <60           | 45 |
| ≥60           | 75 |
| Histopathological grading |   |
| High/moderate | 39 |
| Low           | 81 |
| TNM staging   |    |
| I-II          | 46 |
| III-IV        | 74 |
| Distant metastasis |  |
| Yes           | 33 |
| No            | 87 |

Table I. Patient characteristics (n=120).

| Cell line | Gene | Mutation site | Protein position |
|-----------|------|---------------|------------------|
| SW48      | EGFR | c.2155G>A     | p.G719S          |
|           | CTNNB1 | c.98C>A   | p.S33Y           |
|           | MEK1 | c.167A>C     | p.Q56P           |
| NCI-H508  | BRAF | c.1786G>C    | p.G596R          |
|           | PIK3CA | c.1633G>A | p.E545K          |

Table II. Gene mutations detected by next-generation sequencing.

Statistical analysis. Statistical analysis was performed with IBM SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). Comparisons between pairs were performed using a Student's t-test, while multiple comparisons between the groups were analyzed using one-way analysis of variance followed by a Student-Newman-Keuls test. All the results are presented as the mean ± standard deviation of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.
significantly reduced cellular proliferation to 62.07±0.65 and 59.17±1.16%, respectively (Fig. 2A). Similarly, the cytotoxic effect in cells co‑treated with U0126 and 5‑FU (0.5 and 1 µg/ml) was increased compared with that in cells treated with either U0126 or 5‑FU alone (Fig. 2B). Furthermore, the CI values, shown in Table III, were both <1.0 for combined treatment with U0126 and oxaliplatin, and combined treatment with U0126 and 5‑FU, indicating synergism between the MEK inhibitor and two drugs.

The results of the apoptosis assay (Fig. 2C and D) were similar to those of the CCK-8 experiment. Compared with the control group (8.70±1.56%), single treatment with U0126 and oxaliplatin, and combined treatment with U0126 and 5‑FU, indicating synergism between the MEK inhibitor and two drugs.

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Figure 1. Effects of U0126, oxaliplatin and 5-FU on SW48 cells. Cell viability was measured using CCK-8 assay and is represented as the percentages of the untreated group value. (A) CCK-8 was performed following the treatment of SW48 and NCI-H508 cells with increasing concentrations of U0126 for 72 h. There was a statistical difference between the two groups (*P<0.05). (B) After 72 h of U0126 exposure, the cells were lysed and subjected to western blot analysis with relevant antibodies. Cell viability of cells treated with a concentration gradient of (C) oxaliplatin (0.5, 1, 5, 10, 20 and 50 µg/ml) and (D) 5-FU (0.5, 1, 5, 10, 20 and 50 µg/ml) for 3 days was assessed by CCK-8 assay. Values are expressed as the mean ± standard deviation of three individual measurements. *P<0.05 and **P<0.01, vs. the untreated control group. 5-FU, 5-fluorouracil; CCK-8, Cell Counting Kit-8; ERK, extracellular signal-regulated kinase; t-, total; p-, phosphorylated.

Mechanisms underlying the effect of U0126 on oxaliplatin/5-FU therapeutic efficacy in SW48 cells, the present study detected the effect of combination therapy on DNA repair. According to the results of CCK-8 and flow cytometry analysis, the doses of 2 µM U0126 combined with 5 µg/ml oxaliplatin or 1 µg/ml 5-FU were selected for subsequent investigations. Histone H2AX is a variant of the H2A histone family that is involved in chromosomal stability (9). H2AX is phosphorylated at serine 139 when cells are induced by irradiation or cytotoxic drugs, and is a key protein for DNA repair and genomic stability (10). In the current study, γH2AX foci were analyzed by immunofluorescence staining following single or combined treatment with U0126, oxaliplatin or 5-FU. There were significant differences between the cells treated with U0126/oxaliplatin/5-FU alone and control cells, suggesting that all three drugs were able to induce DSBs. Additionally, cells exposed to oxaliplatin or 5-FU combined with U0126 exhibited significantly more γH2AX foci compared with cells treated with monotherapy (Fig. 3A). The number of foci in 100 cells of each sample was calculated, and the mean number of foci per cell is shown in Fig. 3B. Treatment with U0126, oxaliplatin and 5-FU induced comparable amounts of γH2AX foci per cell (11.13±4.65 for U0126, 19.07±6.09 for oxaliplatin and 25.36±8.12 for 5-FU). Combination of U0126 and oxaliplatin or 5-FU triggers the formation of γH2AX foci. To further reveal potential
Furthermore, the induction of γH2AX was accelerated in SW48 cells treated with U0126 and oxaliplatin/5-FU together. The average number of foci following stimulation with U0126 and oxaliplatin was 33.96±6.53, whereas the number of foci was 38.58±10.53 following U0126 and 5-FU treatment (Fig. 3B).
U0126 decreases ERCC1 expression induced by oxaliplatin and enhances the inhibition of TYMS expression when combined with 5-FU. Removal of adducts from genomic DNA is mediated by the enzyme ERCC1, which serves an important role in the rate-limiting step or regulation of nucleotide excision repair (11). Increased expression of ERCC1 caused by platinum in several cancer types has been associated with improvement of DNA repair capacity and resistance to platinum-based drugs (12,13). In the current study, it was observed that oxaliplatin exposure increased ERCC1 expression by 1.62±0.02-fold. By contrast, the combination of U0126 and oxaliplatin significantly reduced ERCC1 mRNA levels to 0.86±0.06-fold (Fig. 4A). U0126 and 5-FU treatment alone inhibited TYMS expression to 0.36±0.07-fold and 0.45±0.02-fold, respectively. Furthermore, the combination of U0126 and 5-FU decreased TYMS expression by 0.21±0.03-fold (Fig. 4B). The results of protein expression were consistent with the mRNA expression (Fig. 4C and D).

Discussion

Individualized therapy facilitates the selection of the most suitable drug therapy for each patient according to differences in the gene composition or alterations in expression levels. Molecular targeted drugs selectively destroy tumor cells with specific mutated genes, leading to their death, without damaging the cells of the surrounding normal tissue (14). Selection of the appropriate molecular targeted drug subsequent to relevant gene testing can result in accurate and timely individualized treatment for patients.

In 2014, the United States Food and Drug Administration (https://www.fda.gov/) approved the combination of the MEK inhibitor trametinib with the BRAF inhibitor dabrafenib to treat patients with inoperable or metastatic melanoma with BRAF V600 E or V600 K mutation. MEK1 mutations are present in 1.5% of CRCs and the majority of mutations cause constitutive activation of this kinase (15). A comprehensive study of MEK1 somatic mutations in lung adenocarcinoma revealed that overexpression of MEK1 proteins with mutations in F53L, Q56P and K57N causes phosphorylation of ERK and increased colony formation, which may be inhibited by a MEK1/2 inhibitor (16). Transfection with MEK1 expression vectors illustrated that mutations in this gene, including Q56P and S72G, induced the phosphorylation of ERK1/2 and had a transforming potential, enhancing the tumorigenicity. It was also observed that use of a MEK inhibitor decreased the phosphorylation of ERK1/2 and induced apoptosis of cell lines with MEK1 mutations (17). In the present study, the mutation rate of MEK1 was observed to be 1.67%. MEK inhibition by U0126 significantly decreased the growth of SW48 cells that harbored a MEK1 Q56P mutation, although the effect on the growth of NCI-H508 cells with MEK1 wild-type was not marked. The results, to a certain extent, suggested that CRC patients with such oncogenic MEK1 mutations may be suitable for targeted therapy with MEK inhibitors.

Studies have reported that activation of MEK/ERK signaling is associated with increased...
resistance to fluoropyrimidines in breast and hepatocellular carcinoma (18,19). In recent years, chemotherapy regimens consisting of 5-FU in combination with oxaliplatin or irinotecan have served as the first-line options for treatment of metastatic CRC. However, 5-FU and oxaliplatin therapy exhibits problems with chemosensitivity (20). A MEK1/2 inhibitor, GSK1120212, exhibited an additive effect in combination with 5-FU or oxaliplatin, and a synergistic effect in combination with SN-38 in HT-29 cells (21). GSK1120212 and other retinoblastoma gene-reactivating agents have been reported to reduce thymidylate synthase expression and enhance the efficacy of 5-FU in \textit{BRAF}-mutated CRC (22).

Furthermore, MEK/ERK pathway inhibition enhanced the sensitivity to 5-FU in \textit{KRAS}-mutated and murine colorectal tumor xenograft models (23-25). Consistent with the findings of previous studies, the current study demonstrated that the MEK1 inhibitor U0126 increased sensitivity to 5-FU and oxaliplatin treatment in \textit{MEK1}-mutated CRC. The purpose of the present study was to provide experimental evidence for the combination of a MEK1 inhibitor and chemotherapeutic agents in patients with CRC harboring mutations in \textit{MEK1}.

\(\gamma\text{H2AX}\) foci were also detected by immunofluorescence staining to further investigate the mechanism of action of the investigated drugs. \(\gamma\text{H2AX}\) is a topic of primary research interest in the study of DNA damage stress responses. In 1998, Rogakou \textit{et al} (26) reported that irradiation and other treatments may cause rapid phosphorylation of Ser residues in H2XA, termed \(\gamma\text{H2AX}\). \(\gamma\text{H2AX}\) foci may be used to estimate the kinetics of DSBs rejoining and has become the gold standard for the detection of DSBs. It has further been revealed that there is no cell specificity for the formation of \(\gamma\text{H2AX}\) foci, and that induced DSBs are accompanied by H2AX phosphorylation and clustering (27). In the present study, SW48 cells treated with U0126, oxaliplatin or 5-FU alone exhibited an increased number of \(\gamma\text{H2AX}\) foci compared with the control cells. Combination treatment of U0126 with produced more \(\gamma\text{H2AX}\) foci as compared with the single drug treatments, which may be associated with the induction of DSBs. Phosphorylation of H2AX indicated the presence of a large number of DSBs and slow repair in SW48 cells exposed to combination therapy.

The present study demonstrated that combination treatment with the MEK inhibitor and oxaliplatin/5-FU increased DNA damage. Therefore, the expression levels of ERCC1 and TYMS, two critical enzymes involved in the nucleotide excision repair pathway, were detected. High expression of ERCC1 is associated with resistance to platinum-based chemotherapy (28). In addition, TYMS is considered an important predictor of 5-FU sensitivity (29) and it is important for maintaining the
All patients who participated in the study provided signed informed consent. The research using human tissue received approval from Jiangsu Institute of Cancer Research Ethics Committee.

Patient consent for publication
All patients participated in the study signed informed consent.

Competing interests
The authors declare that they have no competing interests.

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