

# EPA significantly improves anti-EGFR targeted therapy by regulating miR-378 expression in colorectal cancer

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**Abstract.** It is known that colorectal cancer (CRC) cells containing mutations of the genes KRAS and BRAF are predominate mechanisms causing resistance to epidermal growth factor receptor (EGFR) inhibitors, and commonly exhibit a lower expression of microRNA-378 (miR-378) when compared with the wild type. In the present study, the aim was to determine the possible mechanism which associates miR-378 with the mitogen-activated protein kinase pathway, and to determine the efficiency of eicosapentaenoic acid ethyl ester (EPA) in its ability to restore sensitivity towards cetuximab, an EGFR inhibitor. The results demonstrated that a combined treatment of 40  $\mu$ M EPA with 0.2  $\mu$ M cetuximab can significantly suppress the cell growth in KRAS-mutant and control wild-type cells. Furthermore, the higher phosphorylated protein level of extracellular-signal-regulated kinase 1/2 was notable in KRAS EPA-treated cells ( $P=0.006-0.047$ ) and resulted in significantly increased cell death; however, inconsistent results were indicated in EPA-treated BRAF-mutant cells, compared with the original cells (without treatment). KRAS-mutant and wild-type Caco-2 cells treated with EPA exhibited increased cetuximab response rates, but these response rates were reduced in the BRAF-mutant cells. In conclusion, upregulation of miR-378 induced by EPA may result in the significant restoration of sensitivity to cetuximab in the KRAS-mutant cells. The present data will contribute to a notable potential therapeutic solution for future clinical CRC treatments.

## Introduction

Lièvre *et al* (1) first disclosed colorectal cancer (CRC) with KRAS mutation as a predictor of poor response to anti-epidermal growth factor receptor (anti-EGFR) at 2006. The OPUS trial (2) in 2008 and the CRYSTAL trial (3) in 2009 reported KRAS mutations occurred at a frequency of 42 and 36.5% among metastatic CRCs. Those patients were insensitive to anti-EGFR therapy. Furthermore, BRAF mutations in CRCs were reported at a rate of 8.7% (4) and a 10% (5). The evidence identifies that ~50% of CRCs exhibit no response to anti-EGFR therapy, including cetuximab or panitumumab (1-11). KRAS mutations occur at an increased frequency, compared with BRAF mutations, and commonly occur at codon 12 (G12D) and 13 (G13D) of exon 2 in the KRAS gene (12-16); whereas, 90% of BRAF mutations occur in exon 15 (V600E) (17-19). According to previous studies, reduced expression of microRNA-378 (miR-378) may serve a crucial role in CRC, which is considered as an independent prognostic factor, and inhibits cell growth as well as invasion in tumor cells (20-23). It is known that miR-378 acts as an inhibitor in the mitogen-activated protein kinase (MAPK) pathway, which affect extracellular signal-regulated kinase (ERK) genes, such as ERK1/2; therefore, it is involved in cellular proliferation, differentiation, and transcription regulation and development (24). In our previous study, a reduced expression level of miR-378 was commonly observed in KRAS- or BRAF-mutant cells, compared with the wild-type CRC or normal control cells; however, following transfection of miR-378 into mutant CRC cells, to increase the expression level, it was observed that drug sensitivity to cetuximab was significantly restored and cell death was induced (25). The present data coincided with information from the databases TargetScanHuman ([www.targetscan.org](http://www.targetscan.org)) and miRbase ([www.mirbase.org](http://www.mirbase.org)), and further confirmed that miR-378 targets the 3'-untranslated region (UTR) of the ERK1/2 coding gene. Feng *et al* (26) also demonstrated that miR-378 suppressed the antitumor protein transducer of Erb-B2 receptor tyrosine kinase, which serves as a transcriptional repressor of cyclin D1, a downstream effector of the human epidermal growth factor receptor 2-Ras-ERK pathway. The precursors of miR-378/378\* are derived from the first intron of host gene peroxisome proliferator-activated

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receptor  $\gamma$  coactivator 1  $\beta$  (PGC-1 $\beta$ ) (27). Fatty acids can directly stimulate the gene PGC-1 $\beta$  expression, and as a result increase the co-expression of miR-378, which was demonstrated by our previous study (25).

Furthermore, a previous study indicated that PGC-1 $\beta$  serves a function in lipid metabolism, in which the genes coding mitochondrial fatty acid oxidation and oxidative phosphorylation were diminished in liver specific-PGC-1 $\beta$  knock out mice (28). A number of miR-378/378\* target genes are associated with lipometabolism, including carnitine O-acetyltransferase, mediator complex subunit 13 and glucose transporter type 4 genes, and may also affect the development of lipogenesis in fatty cells (27,29). Additionally, a number of studies demonstrated that fatty acids could significantly upregulate the expression of the PGC-1 $\beta$  gene, in order to affect the metabolism of mitochondrial biogenesis (27,28,30).

EPA is one of omega-3 fatty acids commonly found in fish, including cod liver oil, salmon, herrings, sardines and various edible seaweeds. Based on a report by the European Food Safety Authority, a suggested dosage of intake for adults of EPA/docosahexaenoic acid (DHA) is 200 to 600 mg per day, and 40 to 250 mg/day for infants >6 months old, children and adolescents (31). Additionally, there are 0.2-1.2 mM free fatty acids in the human body (32,33) which provided an estimate of the EPA concentration selection in current study. A number of studies demonstrated that EPA and DHA can trigger the majority of the activities of the caspase family members, including caspase-8, which are associated with proteases and cell apoptosis, which has been indicated in CRC and pancreatic cancer cells (34,35). Notably, it has been observed that neoplastic oral keratinocyte cells are significantly suppressed by EPA through the inhibition of the expression of total protein ERK1/2, which increased the ERK1/2 phosphorylation (36). Based on our previous study, following restoring the expression level of miR-378, the CRC cells have a significant response to EGFR inhibitor cetuximab (25). In the present study, the aim was to firstly uncover the mechanism underlying the association between miR-378 and the MAPK pathway. Secondly, whether the gene expression of PGC-1 $\beta$  was also induced by EPA was investigated, with this gene indirectly increasing miR-378 co-expression and restoring cetuximab sensitivity. The results may directly benefit the treatment of patients with CRC containing KRAS or BRAF mutations.

## Materials and methods

**Cell lines and cell culture.** The CRC cell lines included in the present study were SW480, HCT116, HT29, and Caco-2. SW480 and HCT116 contain KRAS mutations (G5571T and G5574A, respectively), HT29 contains a BRAF mutation (T171429A) and Caco-2 is a wild-type (without gene KRAS or BRAF mutations) CRC cell line, which was used as the internal control in the experiments, when required. All cell lines were cultured according to our previous study (25). In brief, the cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glutamine, penicillin (12.5 U/ml), streptomycin (6  $\mu$ g/ml), 1 mM sodium pyruvate and 10% fetal bovine serum (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), then incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. SW480, HCT116,

HT29 and Caco-2 were selected in the present study due to them exhibiting aggressive growth, compared with other cell lines.

**Supplements and reagents.** Ultra-pure free fatty acid of EPA (>99%) was purchased from Nu-Chek-Prep, Inc. (Elysian, MN, USA). The stock solution was diluted in 99% ethanol to a concentration of 1 mM and aliquoted in dark-colored glass vials stored at -20°C until used. EPA was added to DMEM to produce different testing concentrations from 0-40  $\mu$ M (0, 2, 5, 10, 20, 30 and 40  $\mu$ M), and was then used to treat the all cell lines for 24 h. at 37°C. The anti-EGFR cetuximab (Erbix<sup>®</sup>) was purchased from Merck KGaA (Darmstadt, Germany) and was added into the DMEM to produce final testing concentrations from 0-0.2  $\mu$ M (0, 0.01, 0.05, 0.1 and 0.2  $\mu$ M), and then incubated with all cell lines at 37°C for 48 h. The optimal EPA concentration was determined according to the IC<sub>50</sub> calculation, for which growth inhibition was observed in half of the tested cells, and it was determined as 40  $\mu$ M.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.** The primer sequences were as follows: MiR-378, 5'-CTCAACTGGTGTCTGGAGT-3' and 5'-GGGACTGGACTTGGAGTC-3' (37); RNU44, 5'-CCTGGATGATGATAGCAAATGC-3' and 5'-GAGCTAATTAAGACCTTCATGTT-3' (38). The miR-378 expression levels in cells were detected prior to treatment, and then after 24 h treatment with 40  $\mu$ M EPA/DMEM. RNA extraction with MirVana<sup>™</sup> miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.), An optical density of 260/280 nm absorbance ratio between 1.9-2.0 confirmed the quality of RNA (BioPhotometer plus; Eppendorf, Hamburg, Germany). TaqMan<sup>®</sup> MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to transcript RNA to cDNA-according to the manufacturer's protocols. In brief, the thermocycling conditions were: 5 min at 85°C; 5 min at 60°C; and lowered to 4°C which was immediately transferred to qPCR; or long term storage at -20°C. RT-qPCR of miR-378 was performed with the TaqMan Universal Master Mix II (no UNG) (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan<sup>®</sup> MicroRNA Assays kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for qPCR. The thermocycling conditions were: 10 min at 95°C for the first stage; 15 sec at 95°C for the second stage; and then 1 min at 60°C; and total reaction for 40 cycles at 25°C for 30 sec. The relative expression levels of targeted genes in cells were calculated by normalizing with RNU-44 expression levels through the comparative Cq method (39). Finally, collected data were analyzed using the BioRad iCycle iQ system software, version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Comparing the cell viability efficiency using an MTS assay.** All cell lines were grouped into four sets based on different treatments: Original cells treated with 1X PBS; cells treated with EPA only; cells treated with cetuximab only; and cells treated with EPA and cetuximab. CRC cells were firstly treated in gradient concentrations of EPA (0, 2, 5, 10, 20, 30, 40  $\mu$ M)/DMEM mixtures, and incubated separately using a 96-microplate (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 24 h, followed by treating with grading concentrations

of cetuximab (0, 0.01, 0.05, 0.1, 0.20  $\mu$ M) at 37°C for 48 h. The cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's protocols (Promega Corporation, Madison, WI, USA), and the absorbance was measured at 490 nm.

**ERK1/2 expression level and phosphorylation status detection by an ELISA assay.** To observe the MAPK pathway, the expression level of key protein ERK1/2 was determined. Following all cell lines being treated with 40  $\mu$ M EPA at 37°C for 24 h, ERK1/2 (Total/Phospho) InstantOne™ ELISA kit (cat. no. ABIN1981832; eBioscience; Thermo Fisher Scientific, Inc.), was used to detect total ERK1/2 protein expression and phosphorylation status (p-ERK1/2). In brief, the manufacturer's protocols of the ERK1/2 (Total/Phospho) InstantOne ELISA kit were followed with, and the absorbance was measured at 450 nm (Fig. 2).

**Statistical analysis.** SPSS 15.0 statistics software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis in the present study. Data are expressed as the mean  $\pm$  standard error of the mean and represent the fold differences among cells treated with EPA only, cells treated with cetuximab only and cells treated with EPA and cetuximab. Firstly, all three groups of cells were compared to their original cells, which were normalized at a base line of 100%. Secondly, to understand the efficiency of cetuximab with/without EPA, EPA-treated cells were further normalized and used as a base line for comparison. Comparisons within each cell group was assessed by one-way analysis of variance. Individual comparisons among subgroups were analyzed with Tukey's post-hoc test. The miR-378, ERK1/2 protein and p-ERK1/2 expression levels prior to and following treatment with EPA were analyzed with Student's t test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Efficiency of EPA on the viability of CRC cells viability.** Different concentrations of EPA (0, 2, 5, 10, 20, 30 and 40  $\mu$ M) were produced by mixing with DMEM and used to treat all cells lines. The results demonstrated that Caco-2 and SW480 cells remained more stable when the EPA concentration increased, compared with HCT116 and HT-29 cells. The cell viabilities indicated a range between 95.9-111.5% and 98.0-112.0%, respectively. The cell lines HCT116 and HT29 that were treated with 0-20  $\mu$ M EPA had cell viability ranges of 95.7-102.7%, and 93.4-102.0%, respectively; however, when the concentration increased to 30-40  $\mu$ M EPA, the cell viabilities decreased to 71.9-53.3%, and 81.3-79.0%, respectively. Notably, cells treated with 40  $\mu$ M EPA had the greatest dosage response to cetuximab.

**Efficiency of EPA on the expression of miR-378.** Our previous data (24) demonstrated that mutant cells, either KRAS or BRAF, had significantly reduced expression of miR-378, compared with wild-type CRC cells; however, the present data indicated that the cells with an increased expression of miR-378 were notably associated with the addition of EPA,

particularly at the concentration of 40  $\mu$ M, with the exception of Caco-2 wild-type cells. The results indicated a significant increase in expression of miR-378 in the cells of SW480, HCT116 and HT29, with an increase by 0.98- (P=0.005), 0.88- (P=0.016) and 1.05-fold (P=0.004), respectively, compared with their original control cells. Contrarily, miR-378 expression in wild-type Caco-2 cells did not demonstrate a significant difference to its original control cells (P=0.317) (Fig. 1).

**Cell ERK1/2 protein expression and p-ERK1/2 status following treatment with 40  $\mu$ M EPA.** ERK1/2 protein expression and the p-ERK1/2 status were detected following all CRC cell lines being treated with 40  $\mu$ M EPA mixture for 24 h. The results demonstrated that ERK1/2 protein expression was significantly decreased, compared with their original cells, except for BRAF-mutant HT29 cells. The KRAS-mutant CRC cells SW480 and HCT116 had a decreased ERK1/2 protein expression of 0.323- (P=0.035) and 0.226-fold (P=0.035), respectively. Furthermore, Caco-2 cells were also determined to have a decreased ERK1/2 protein expression of 0.484-fold (P=0.022). Contrarily, a significant increase in p-ERK1/2 was observed in all cell lines, except in HT29 cells (Fig. 2A). The expression of p-ERK1/2 in SW480, HCT116 and Caco-2 cells was increased by 1.61- (P=0.006), 1.32- (P=0.047) and 4.96-fold (P=0.033), respectively. Notably, a 0.42-fold decrease in p-ERK1/2 was determined in HT29 cells (P=0.029) (Fig. 2B).

**Cetuximab effect on EPA-treated cells.** Significant differences were observed in SW480 (P=0.0217), HCT116 (P<0.001) and Caco-2 (P=0.0064) cell groups. With the exception of HT29 cells (P=0.1185), the cell viability of CRC cells treated with EPA and cetuximab were significantly decreased, compared with their controls. Furthermore, the combined EPA with cetuximab-treated cells were compared with cells treated with EPA only, in which all cell lines except HT29 cells were observed to have significantly decreased cell viability (Fig. 3; P<0.05). With cetuximab in a combination with EPA, the efficiency of cell growth inhibition was greater than when treated with cetuximab alone for the wild-type Caco-2 cells. Additionally, the response rate to cetuximab following treatment with 0.1 or 0.2  $\mu$ M cetuximab in the SW480, HCT116 and Caco-2 cells treated with 40  $\mu$ M EPA were significantly improved, compared with their controls (P=0.0062, P=0.0176 and P=0.0054, respectively); however, the BRAF-mutant cell line HT29 (P=0.2116) remained insensitive to cetuximab following 40  $\mu$ M EPA treatment (Fig. 4).

## Discussion

The crucial data of the present study indicated that 40  $\mu$ M EPA combined with 0.2  $\mu$ M cetuximab can significantly reduce the cell viabilities of KRAS-mutant CRC cell lines, with the exception of HT29 cells. Secondly, following treating cells with 40  $\mu$ M EPA, the cells exhibited significantly reduced the expression of protein ERK1/2 but increased the expression of phosphorylation ERK1/2 in KRAS-mutant CRC cells and also the wild-type CRC cells. Notably, contradicting data was observed in BRAF-mutant HT29 cells. Additionally, following treatment with EPA, the expression levels of miR-378 were



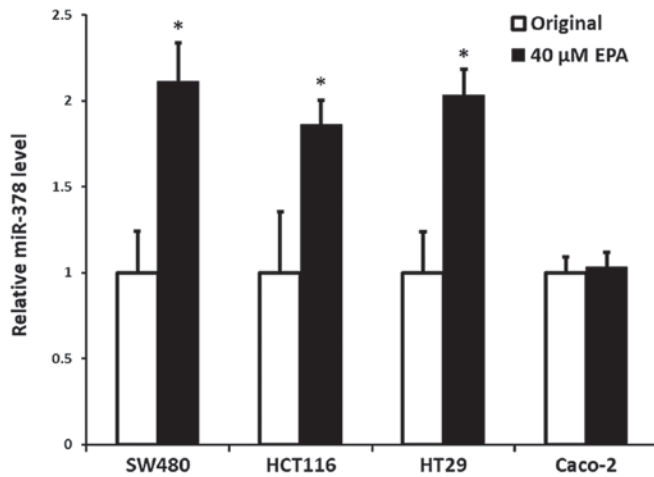


Figure 1. EPA induced the expression of miR-378 in CRC cells. All CRC cell lines were incubated in 40  $\mu$ M EPA culture medium for 24 h then compared with the untreated original cells. White bars represent the untreated original cells and the black bars represent EPA-treated cells. All the original cells were normalized to 1 then compared with the EPA-treated cells and displayed as relative miR-378 expression. All cell lines had a significantly increased expression of miR-378, with the exception of Caco-2 cells. Error bars represent standard errors, the 95% confidence interval. \* $P$ <0.05 vs. original cells. EPA, eicosapentaenoic acid.

determined to be significantly increased in all cell lines, with the exception of wild-type Caco-2 cells. These data were consistent with our previous study, in which the cells were treated with Lauric acid (25).

Based on the present results, the expression of miR-378 directly mediates the expression of ERK1/2, which is consistent to the TargetScanHuman database, where it is predicted that the 3'UTR of mRNA ERK1/2 is one of the miR-378 target binding sites; therefore, this results in the inhibition of protein ERK1/2 expression (24,26). However, the opposite results were determined for the ERK1/2 phosphorylation status, with the expression being increased in KRAS- and BRAF-mutant cells. Undetermined cellular mechanism may affect the MAPK signaling pathway, which may therefore trigger cell proliferation; nevertheless, the present results demonstrated a significant decrease in cell viabilities. Similar data has been reported by Nikolakopoulou *et al* (36), in which they produced a model regarding the reason why total protein ERK1/2 had the opposite expression level to the phosphorylation of ERK1/2. They concluded that EPA may be an important element to the EGFR ligand, and EPA may selectively inhibit the growth of pre-malignant and malignant keratinocytes by inducing a sustained activation of ERK1/2, but not have an effect on normal cells. Additionally, a number of studies also indicate that increased phosphorylation status of ERK1/2 is associated with cell death. For example, Yang *et al* (40) indicated that if the total protein expression level of ERK1/2 remained the same, the phosphorylation of ERK1/2 could still be increased, and consequently resulted in cell death. Similar data have been determined in CRC cells, lung cancer cells and cervical carcinoma studies, and this data agree that ERK1/2 phosphorylation could trigger the expression of caspase-3 and result in cell apoptosis (41-43). Although the potential mechanism is unknown between the ERK1/2 phosphorylation status and protein expression, which may be a result of unknown kinase effects. The MAPK/ERK

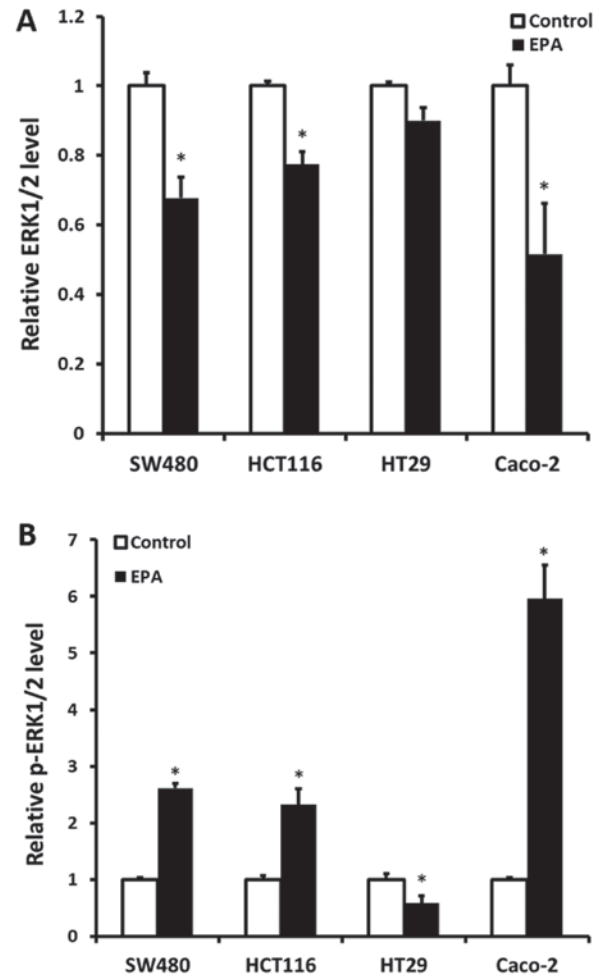


Figure 2. ERK1/2 protein and p-ERK between original cells and the cells cultured in 40  $\mu$ M EPA for 24 h were detected by an ELISA. (A) All cell lines exhibited significantly decreased ERK1/2 protein expression, with the exception of HT29 cells. (B) The p-ERK expression status was also determined simultaneously to ERK1/2. All cell lines exhibited significantly increased p-ERK expression, with the exception of HT29 cells, which exhibited the opposite data. White bars represent the untreated original cells; the black bars represent EPA-treated cells. Error bars represent standard errors, the 95% confidence interval. \* $P$ <0.05 vs. original cells. EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase.

pathway with a high phosphorylation ERK expression is correlated with an increased rate of cell apoptosis, as demonstrated by a number of studies (40-44). The present study further clarified that EPA was associated with miR-378. Regardless of suppressed expression of ERK1/2, the activation of phosphorylation ERK1/2 still significantly inhibited cell growth.

There are at least two major pathways, phosphoinositide 3-kinase (PI3K) and MAPK pathways, that are involved in anti-EGFR therapy (45,46). According to the present results, it was speculated the KRAS-mutant cells may be associated with the MAPK or PI3K/Akt pathways; therefore, miR-378 can trigger phosphorylation of ERK1/2 and result in cell apoptosis when the MAPK pathway is blocked or through an unknown mechanism of the PI3K/Akt pathway. This may explain the reason why the cells continued to survive following EPA treatment, but eventually succumbed to anti-EGFR cetuximab treatment; however, the BRAF-mutant cells exhibited a reduced expression level of phosphorylated ERK1/2, which is consistent to reduced

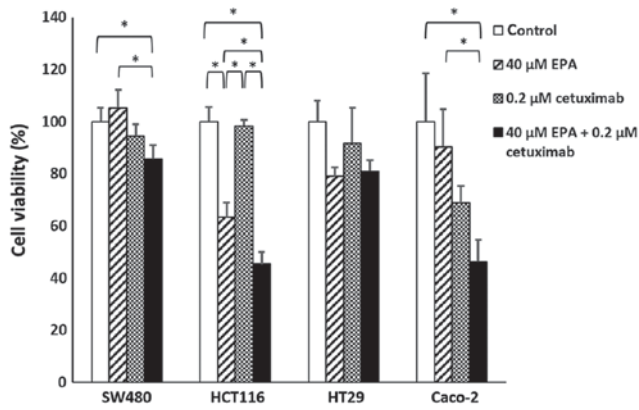


Figure 3. Comparison of the cell viability among the different group of cells. White bars represent original cell treated with 1X PBS. Oblique line bars represent cells treated with 40  $\mu$ M EPA. Meshed bar represent cells treated with 0.2  $\mu$ M cetuximab. Black bars represent cells treated with 40  $\mu$ M EPA 0.2  $\mu$ M cetuximab. Error bar represent standards error, the 95% confidence interval. \* $P < 0.05$ . EPA, eicosapentaenoic acid.

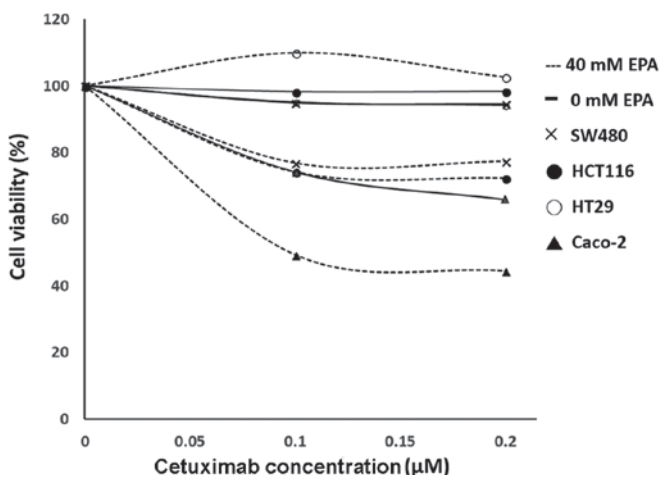


Figure 4. Efficiency of cetuximab test. Solid lines indicate cells without treatment with EPA. Dashed lines indicate cells treated with 40  $\mu$ M EPA. Cross marks represent SW480 cells. Dark circles represent HCT116 cells. Hollow circles represent HT29 cells. Dark triangles represent Caco-2 cells. All the cells have been normalized to their control as 100% cell viability. The dashed lines below 100% indicate that the improvement of cell response to anti-EGFR antibodies was statistical significant. EPA, eicosapentaenoic acid.

ERK1/2 protein expression following restoration of miR-378 expression. Additionally, the BRAF-mutant HT29 cells exhibited no significant response to anti-EGFR antibody following the EPA treatment. We hypothesized that BRAF-mutant cells may harbor an unknown mechanism underlying cell signaling pathways, which differ from the KRAS-mutant cells that will require further research in the future.

To conclude, the present results indicated that EPA could significantly induce the expression level of miR-378 in CRC mutant cells, but not in the wide-type cells. Restoration of the anti-EGFR antibody sensitivity of the KRAS-mutant cells was achieved following treating the cells with up to 40  $\mu$ M of EPA, which was not observed in the BRAF-mutant cells in the present study. Notably, the status of phosphorylation of ERK1/2 may serve an important role in mediating the cell response to cetuximab therapy; however, the bio-mechanism behind

this will require further study. This could also further reveal the discrepancy of clinical behaviors between KRAS- and BRAF-mutated colon cancer types.

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## Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

## Authors' contributions

WHW contributed to conception and design, acquisition of data, analysis and interpretation of data; and revising the article critically for important intellectual content; and giving final approval of the version to be published. WHL contributed to collecting samples, acquiring data and writing the article. YJP performed the experiments, and contributed to the acquisition of data and English editing. LWK performed the experiments and contributed to the acquisition of data. HHH contributed to the conception and design, and gave final approval of the version to be published.

## Ethics approval and consent to participate

Not applicable.

## Patient consent to participate

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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