

Expression of miRNA in 5-FU resistant esophageal cancer

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Abstract. Fluoropyrimidine plus platinum (FP) are chemotherapeutic drugs that are most frequently used to treat esophageal squamous cell carcinoma (ESCC). However, drug resistance often occurs, and the mechanisms of resistance to 5-FU is yet to be determined. The role of micro (mi)RNAs has been well established in a variety of human cancers. The aim of the present study was to investigate the expression profile of ESCC, revealing the differential expression between ESCC and 5-FU resistant ESCC. The establishment of a 5-FU resistant (5-FUR) cell lines model provides a way of analyzing the expression of miRNAs in drug resistance. The miRNA expression indicated 50 miRNAs that were upregulated in TE10-5-FUR compared with TE10, while 119 miRNAs were downregulated. The TE11-5-FUR demonstrated 140 miRNAs were upregulated compared with TE11, which exhibited 12 downregulated miRNAs. Both cell lines share the 2 candidate upregulated miRNAs (miR-146a and miR-483-5p) and 5 downregulated miRNAs (miR-34a, miR-141, miR-200b, miR-200c and miR-205). Further studies are required to analyze and evaluate the function of the miRNAs.

Introduction

Cancer is a leading cause of human death. Esophageal cancer is the nine most common cancer worldwide, with an estimated 572,034 new cases and 508,585 deaths in 2018. Men have a substantially higher incidence than women (1). The cancers arise from the esophageal mucosa. There are two main histological types: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the predominant histological type in southeast Asian countries, including Thailand (2). Major risk factors for ESCC are smoking and excessive alcohol consumption (3,4). These risk factors may

lead to esophageal cancer through multiple genetic alterations, such as activated oncogenes and inhibited tumor suppressor genes (5).

Despite recent advances in surgical techniques and perioperative management, the prognosis of patients who undergo surgery alone for esophageal cancer remains poor. Neoadjuvant chemotherapy and chemoradiotherapy followed by surgery have emerged as a promising strategy for advanced esophageal cancer, and, in fact, good responders to such preoperative therapy show improved survival (6-9). Cisplatin/5-fluorouracil (5-FU) has been accepted as a standard treatment in for ESCC (10). However, following Cisplatin/5-FU based chemotherapy, non-responders are likely to receive no survival benefit (11,12). The ability to predict the response to chemotherapy before treatment should limit the application of chemotherapy to selected ESCC patients who are likely to show benefits. However, the prognosis of patients who are resistant to 5-FU treatment is poor. Resistance to treatment with anticancer drugs results from a variety of factors, including individual variations in patients.

miRNAs are noncoding RNAs that are approximately 22 nucleotides in length. They act through repressing the translation of target mRNAs by binding to the 30-nucleotide untranslated region of those mRNAs (13). miRNAs exist stably in various tissues and play pivotal roles in differentiation and development (14,15). The role of miRNAs has been well established in various human cancers. The evidence has shown that miRNA mutations or misexpression correlates with various human cancers, indicating that miRNAs can function as tumor suppressors or oncogenes. In addition, aberrant expression of miRNAs has been reported in various types of cancers (16,17). Recent studies of ESCC reported the oncogenic microRNAs: miR-21, miR-10b, miR-31, and miR-373; the oncosuppressor microRNAs: let-7, miR-34a, miR-133a, miR-150, miR-375, miR-205, miR-145, miR-29c, and miR-210 (18). The mi-R-25, miR-99a, miR-133a and miR-133b showed good potential as diagnostic markers and interestingly the mi-R-21, miR-27b, miR-126, miR-143 and miR-145 appeared to be useful both as diagnostic and prognostic/predictive markers (19). A recent publication showed the involvement of several miRNAs in resistance to 5-FU treatment as follows: The miRNA profiles of neoadjuvant radiochemotherapy non-responders showed upregulation of has-miR-1323, has-miR-3678-3p, hsv2-miR-H7-3p, has-miR-194, has-miR-3152, kshv-miR-K12-4-3p, has-miR-665 and has-miR-3659, and downregulation of has-miR-126, has-miR-484, has-miR-330-3p and has-miR-3653 (20). The aim of this study was to investigate

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the expression profile of ESCC, revealing differential expression between ESCC and 5-FU resistant ESCC.

Materials and methods

Cell lines and cell culture. Human ESCC cell lines (TE4, TE10, TE11 and TE15) were obtained from Tohoku University. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nacalai Tesque, Inc.) containing 10% fetal bovine serum (Life Technologies Inc.), 10% penicillin/streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) (Nacalai Tesque, Inc.) in a humidified atmosphere under 5% CO₂ at 37°C.

Establishment of 5-FU resistant cell lines. 5-FU resistant (5-FUR) cell lines were cultured through gradual increases in 5-FU concentration. The cultured cells were exposed to 5-FU at an initial concentration of 1 nM/ml. After 24 h, the cells were cultured in 5-FU free medium until confluence. Next, 5-FU concentrations were increased by 2- to 3-fold and the cycle was repeated.

Proliferation assay. The WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay was conducted as described by the manufacturer (Nacalai Tesque Inc.) and was used to determine the IC₅₀ (50% growth inhibition concentration) value of 5-FU. Cells were plated in 96-well microplates and cultured for 12 h before exposure to various concentrations of 5-FU (0, 0.5, 1, 5, 10, 50 µg/ml) for 48 h. The optical density (OD) value was detected by RAINBO SUNRISE (Wako Pure Chemical Industries Ltd.) at 450 nm test wavelength and 650 nm reference wavelength. The IC₅₀ value of 5-FU was calculated from the dose-response curve.

Isolation of miRNA and miRNA microarray. The miRNA was isolated from the cell lines using the *mirVana*TM miRNA Isolation kit according to the manufacturer's protocol (Ambion; Thermo Fisher Scientific, Inc.). The concentration of RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The miRNA expression profiling of human ESCC cell lines (TE10, TE11) and their corresponding 5-FU resistant (5-FUR) daughter lines (TE10 5-FUresistant cells: TE10-5-FUR; TE11 5-FU resistant cell: TE11-5-FUR) were examined by TaqMan[®] Human MicroRNA Array (Applied Biosystems; Thermo Fisher Scientific, Inc.). It contained 384 miRNA targets (and 7 control miRNAs) and was performed using MegaplexTM RT Primers. The miRNA microarray analysis was performed with Applied Biosystems 7900HT fast real-time PCR System and RT-PCR StatMinerTM software. The expression of each miRNA in 5-FU resistant cell lines was compared with that in the control parental cell line, and the ratio of miRNA expression in 5-FU resistant cells to control cells was calculated for all 384 miRNAs.

Statistical analysis. The significance validation data of miRNA expression are expressed as mean ± standard error of the mean. The cell viability is computed and differences between viability curves are compared. The parameters are compared using the χ^2 test for categorical data and continuous

variables are compared using Student's t-test. All data were analyzed with SPSS 22.0 data (IBM Corp.). A P-value <0.05 was considered to be statistically significant.

Results

The study first established 5-FU resistant ESCC cell lines (TE4, TE10, TE11 and TE15) by gradually increasing 5-FU concentration (starting from 0.1 µg/ml) and evaluating the cultures by WST-8 assay every 4 weeks. After 8 weeks, we determined the IC₅₀ values. The results showed a significant fold-increase in the concentration of 5-FU that inhibited TE10 and TE11 cell growth by 50%. TE10-5-FUR cells were relatively resistant to 5-FU, with an IC₅₀ of 42.66±2.38 µg compared to a value of 4.08±2.06 µg in the parent cells, a 10.5-fold increase in concentration (P<0.01). TE11-5-FUR cells were also relatively resistant to 5-FU with an IC₅₀ of 21.62±11.91 µg compared to 2.73±0.81 µg in TE11 parent cells, a 7.91-fold increase (P<0.01) (Fig. 1).

To assess miRNA expression levels, we used miRNA microarray to evaluate both 5-FU resistant and wild-type ESCC cell lines. The results of the miRNA expression study were subjected to a differential expression analysis and visualized using Expression Suite Software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The analysis showed 50 miRNAs upregulated in TE10-5-FUR compared to TE10, while 119 miRNAs were downregulated. The TE11-5-FUR demonstrated 140 miRNAs upregulated compared to TE11 with 12 miRNAs were downregulated. Among the most significantly upregulated miRNAs of TE10-5-FUR were has-miR-99a-4373008, has-miR-100-4373160, has-miR-125b-4373148, has-miR-140-5p-437374, has-miR-146a-4373132, has-miR-155-4375459, has-miR-196b-4395326, has-miR-302b-4378071, has-miR-499a-4373207, has-miR-483-5p-4395449 while the most downregulated were has-miR-34a-4395168, has-miR-130a-4373145, has-miR-141-4373137, has-miR-152-4395170, has-miR-183-4395380, has-miR-200a-4378069, has-miR-200b-4395362, has-miR-200c-4395411, has-miR-205-4373093, has-miR-429-4373203 (Table I). For TE11-5-FUR, the prominent upregulated miRNAs were has-miR-let7b-4395446, has-miR-let7c-4373167, has-miR-10b-4395329, has-miR-22-4373079, has-miR-137-4373301, has-miR-146a-4373132, has-miR-296-5p-4373066, has-miRs-449b-4381011, has-miR-483-5p-4395449, has-miR-522-4395524, while all those downregulated were has-miR-18a-4395533, has-miR-34a-4395168, has-miR-141-4373137, has-miR-200b-4395362, has-miR-200c-4395411, has-miR-203-4373095, has-miR-205-4373093, has-miR-331-5p-4395344, has-miR-429-4373203, has-miR-708-4395452 (Table II). The result in both cell lines observed at intersection of 2 miRNAs upregulated (miR-146a and miR-483-5p) and 5 miRNAs downregulated (miR-34a, miR-141, miR-200b, miR-200c and miR-205) (Fig. 2). Identification of potential target genes of miRNAs associated with 5-FU resistant ESCC cell lines was essential to investigate their biological functions. Candidate miRNAs of both cell lines were reviewed using the database of miRNA.org site (<http://www.microrna.org>) (21).

Table I. The list of differentially expressed microRNAs in TE10-5-FUR vs. TE10.

miRNAs	Fold-change	Regulation	microRNA family
has-miR-99a-4373008	5.25707	Up	miR-99a
has-miR-100-4373160	3.79643	Up	miR-100
has-miR-125b-4373148	4.09845	Up	miR-125b
has-miR-140-5p-437374	3.76036	Up	miR-140-5p
has-miR-146a-4373132	5.46298	Up	miR-146a
has-miR-155-4375459	4.67046	Up	miR-155
has-miR-196b-4395326	6.09578	Up	miR-196b
has-miR-302b-4378071	4.67347	Up	miR-302b
has-miR-499a-4373207	5.78943	Up	miR-499a
has-miR-483-5p-4395449	4.54962	Up	miR-483-5p
has-miR-34a-4395168	3.08635	Down	miR-34a
has-miR-130a-4373145	1.87463	Down	miR-130a
has-miR-141-4373137	2.98572	Down	miR-141
has-miR-152-4395170	0.56493	Down	miR-152
has-miR-183-4395380	1.59869	Down	miR-183
has-miR-200a-4378069	2.78942	Down	miR-200a
has-miR-200b-4395362	0.89423	Down	miR-200b
has-miR-200c-4395411	2.05483	Down	miR-200c
has-miR-205-4373093	1.68473	Down	miR-205
has-miR-429-4373203	1.87439	Down	miR-429

miR, microRNA.

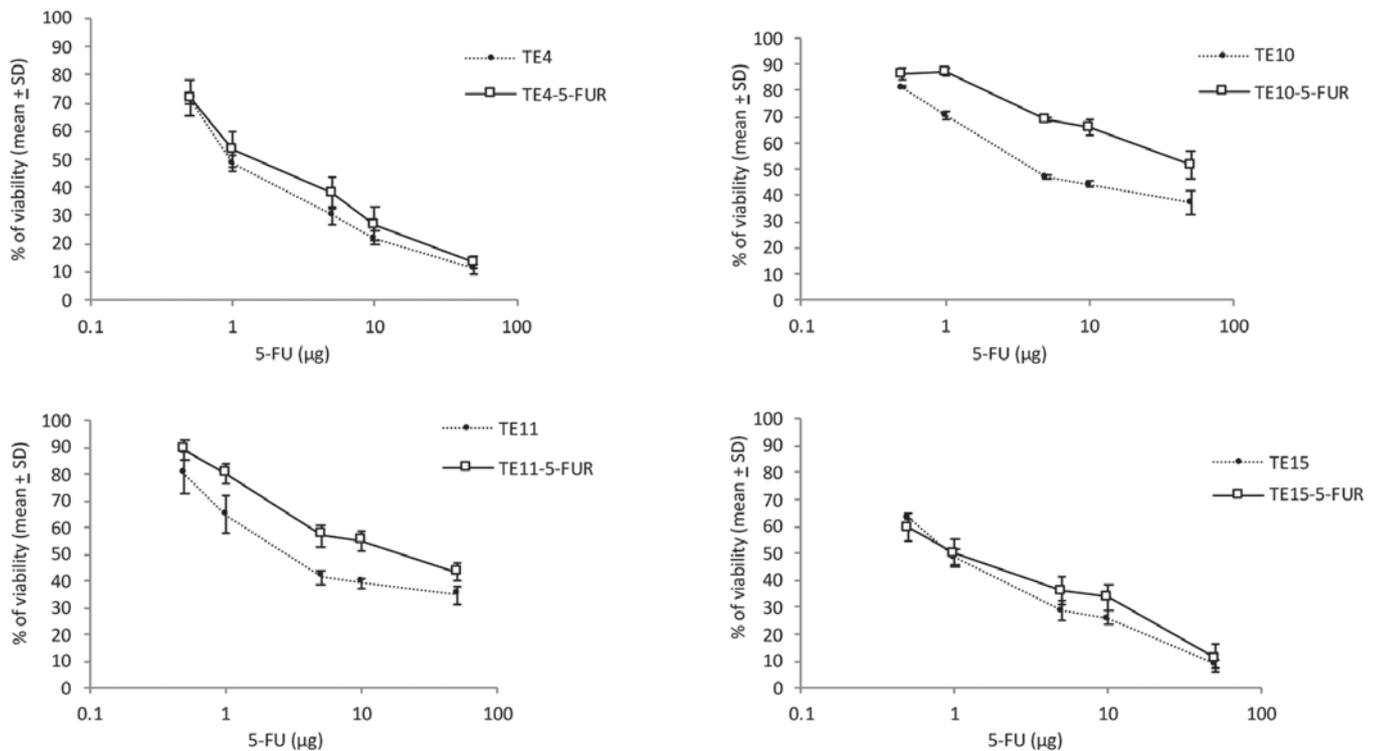


Figure 1. Quantitation of 5-FU resistance of esophageal squamous cell lines. TE4, TE4-5-FUR, TE10, TE10-5-FUR, TE11, TE11-5-FUR, TE15 and TE15-5-FUR cells were seeded into 96-well microplates (5×10^3 per well) 12 h before treatment and were then exposed to different concentrations (0, 0.5, 1, 5, 10 and 50 μg) of 5-FU for 48 h. The percentage of cellular proliferation was evaluated with WST-8. The 5-FU resistant TE10-5-FUR cells showed resistance to 5-FU with an IC_{50} value of $42.66 \pm 2.38 \mu\text{g}$ and TE11-5-FUR achieved an IC_{50} value of $21.62 \pm 11.91 \mu\text{g}$, both $P < 0.01$ compared to their parental cells. For 5-FU resistant TE4-5-FUR cells and TE15-5-FUR, the results were not difference to their parental cells. Data are presented as means \pm SD and evaluated using Student's t-test. WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; SD, standard deviation.

Table II. The list of differentially expressed microRNAs in TE11-5-FUR vs. TE11.

miRNAs	Fold-change	Regulation	microRNA family
has-miR-let7b-4395446	6.43275	Up	let7b
has-miR-let7c-4373167	7.59883	Up	let7c
has-miR-10b-4395329	5.58848	Up	miR-10b
has-miR-22-4373079	6.15343	Up	miR-22
has-miR-137-4373301	5.42084	Up	miR-137
has-miR-146a-4373132	5.58873	Up	miR-146a
has-miR-296-5p-4373066	5.02579	Up	miR-296-5p
has-miR-449b-4381011	4.96571	Up	miR-449b
has-miR-483-5p-4395449	6.19015	Up	miR-483-5p
has-miR-522-4395524	7.06593	Up	miR-522
has-miR-18a-4395533	0.47938	Down	miR-18a
has-miR-34a-4395168	0.30295	Down	miR-34a
has-miR-141-4373137	3.15697	Down	miR-141
has-miR-200b-4395362	3.18372	Down	miR-200b
has-miR-200c-4395411	4.10116	Down	miR-200c
has-miR-203-4373095	0.73426	Down	miR-203
has-miR-205-4373093	1.49047	Down	miR-205
has-miR-331-5p-4395344	0.80691	Down	miR-331-5p
has-miR-429-4373203	3.88948	Down	miR-429
has-miR-708-4395452	0.56551	Down	miR-708

miR, microRNA.

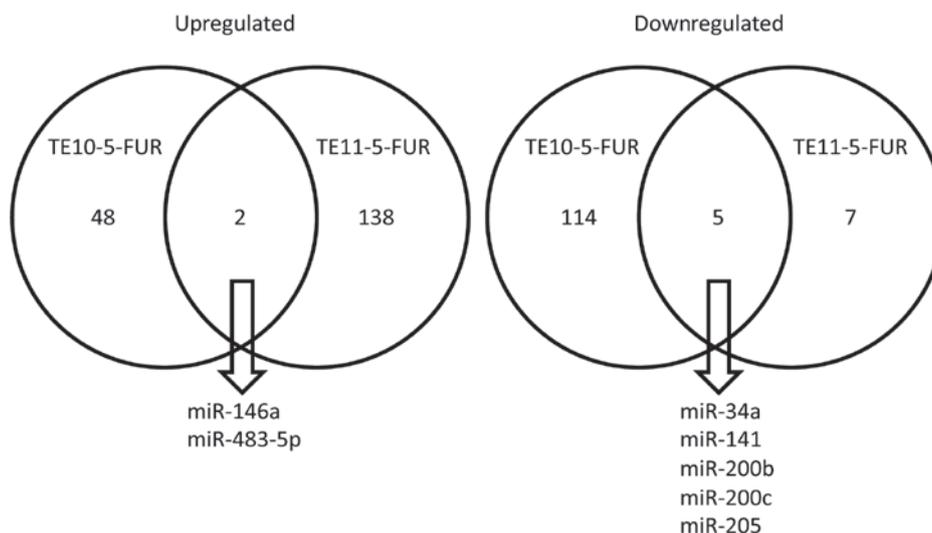


Figure 2. Diagrams of differentially expressed miRNAs shared between 5-FU resistance esophageal squamous cell carcinoma. The results demonstrated 50 miRNAs upregulated and 119 miRNAs downregulated in TE10-5-FUR, 140 miRNAs upregulated and 12 miRNAs downregulated in TE11-5-FUR, compared to their wild type. Both cell lines share the 2 candidate miRNAs upregulated (miR-146a and miR-483-5p) and 5 miRNAs were downregulated (miR-34a, miR-141, miR-200b, miR-200c and miR-205). miRNA/miR, microRNA.

Discussion

Esophageal cancer is a major global health problem. Squamous cell carcinoma is the main histological type. Current management of ESCC depends on the stage of the disease and includes surgery, chemotherapy and radiation therapy. In

multimodal treatment of esophageal cancer, chemotherapy has an important role in combination with radiation therapy and/or surgery. Fluoropyrimidine plus platinum (FP) are the chemotherapeutic drugs most frequently used to treat ESCC. This regimen has been reported to be effective, with improved overall survival (22,23). However, drug resistance

often occurs, and the mechanisms of resistance to 5-FU are still not clear.

The establishment of 5-FU resistant (5-FUR) cell lines model provides an approach to analyze the mechanism of drug resistance. The resistant cells were created from their parental lines by exposing them to gradually increasing 5-FU concentrations for 2 months. The TE10-5-FUR and TE11-5-FUR lines were partially resistant to 5-FU with IC₅₀ values of 42.66±2.38 μg and 21.62±11.91 μg.

miRNAs are short noncoding RNAs that regulate gene expression and play an important role in human cancers. They can also modulate the sensitivity and resistance to anticancer drugs. This study demonstrated 50 miRNAs upregulated and 119 miRNAs downregulated in TE10-5-FUR, 140 miRNAs upregulated and 12 miRNAs downregulated in TE11-5-FUR, compared to their wild type. The result in both of cell lines found 2 candidate miRNAs upregulated (miR-146a and miR-483-5p) and 5 miRNAs were downregulation (miR-34a, miR-141, miR-200b, miR-200c and miR-205). Recent studies also showed the involvement of several miRNAs in resistance to anticancer treatment and roles in esophageal cancer. miR-146a has been reported as perhaps being associated with the cisplatin-base susceptibility to lung cancer by downregulating cyclin J (23,24) and as a potential therapeutic target for multidrug-resistant lung cancer by targeting DNA damage inducible transcript 3 (25). Polymorphism in miR-146a could be associated with the lymph node metastasis and prognosis of gastric cancer patients treated with oxaliplatin and fluoropyrimidines (26). miR-483-5p has been described upregulation with might be a tumor promoter of ESCC that correlated with TNM stage and survival (27). miR-483-5p could inhibit mitochondrial fission protein FIS1 with significant association with cisplatin sensitivity and with overall survival (28). miR-34a showed significantly expressed reduction in ESCC tissues and exerted its anticancer function by suppressing PLCE1 (29). miR-34a has shown upregulation in cisplatin sensitivity for lung cancer treatment via p53/miR-34a/MYC axis (30), mediates oxaliplatin resistance of colorectal cancer cells by inhibiting macroautophagy via the TGF-β/Smad4 pathway (31) and the patients with high levels of expression were found to benefit more from 5-FU based chemotherapy than patients with low levels of expression with the potential targets including CREB1, Bcl-2, Notch 1, Sirt1, and E2F3 (32). For miR-141, the overexpression could abolish the self-renewal ability and carcinogenicity of esophageal cancer stem-like cells and decrease cell invasion and migration by suppressing TM4SF1 (33). It enhanced the effect of 5-FU and suppressed the malignant biological behaviors of colorectal cancer by MAP4K4 signaling pathway (34). miR-141 was significantly decreased and correlated with advanced TNM stage and lymph node metastasis with predicted possible target MACC1 in gastric cardia adenocarcinoma (35). miR-200b has been reported down-regulated in the multi-drug resistance of small cell lung cancer via ZEB2 (36). For miR-200c, the serum levels in advanced ESCC patients were significantly increased and associated with poor outcome of platinum-based chemotherapy (37). miR-200c also related to 5-FU chemotherapy with the potential targets PTEN and E-cadherin in colorectal cancer (38-40). miR-205 has been published as a tumor suppressor in adenocarcinoma

and an oncogene in squamous cell carcinoma of esophagus through regulation of epithelial-mesenchymal transition (EMT) (41) and the Sp1-mediated transcriptional activation of miR-205 promotes radioresistance through PTEN via PI3K/AKT pathway in ESCC (42). For TE11-5-FUR, the let-7b and let-7c demonstrated upregulation with related to the previous publication that reported the let7 play the role of oncosuppressor microRNAs (18).

This study acknowledges its own limitations-the study, the sample size is too small to make any reasonable conclusion. The cell viability and miRNA microarray experiments are not performed on non-cancer cell lines as a control. This study is not verified the function analysis of miRNAs that have related to resistance to 5-FU. Their function should be testing by gene transfer or knockdown with in vitro studies. Alternatively, the association of these miRNAs with clinical efficacy of chemotherapy be examining in a cohort of patients with esophageal cancer. The current study revealed differentially regulated miRNAs that are involved in 5-FU resistant ESCC. The identification of miRNA expression profiles and candidates in 5-FU resistant ESCC could provide a better understanding of the mechanisms involved in chemo-sensitivity or resistance. By predicting the response to chemotherapy, one could offer another treatment option for patients who would otherwise be resistant. Further study is needed to select the potential targets and explore the pathways that are upregulated or downregulated after induction of 5-FU therapy. Moreover, these findings suggest that it may be helpful to develop novel strategies for targeted therapies in ESCC patients.

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Availability of data and materials

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

Authors' contributions

PM and PT contributed to the conception and design of the study. PM contributed to data collection, conduction of the study, performed the experiments, analysis and interpretation of the data. PM and PT reviewed the manuscript, designed the figures and tables. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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