Association between circ_0004365 and cisplatin resistance in esophageal squamous cell carcinoma

MOYURU YAMADA[1], KOJI TANAKA[1], KENICHI YAMAMOTO[2], HISATAKE MATSUMOTO[3], MAKOTO YAMASAKI[1], KOTARO YAMASHITA[1], TOMOKI MAKINO[1], TAKURO SAITO[1], KAZUYOSHI YAMAMOTO[1], TSUYOSHI TAKAHASHI[1], YUKINORI KUROKAWA[1], KIYOKAZU NAKAJIMA[1], YUKINORI OKADA[2], HIDETOSHI EGUCHI[1] and YUICHIRO DOKI[1]

Departments of [1]Gastroenterological Surgery, [2]Statistical Genetics and [3]Traumatology and Acute Critical Medicine, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

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Abstract. Cisplatin is one of the most predominant drugs for the chemotherapy of esophageal squamous cell carcinoma (ESCC); however, the underlying resistance mechanisms are still almost unknown. The present study performed RNA sequencing of human circular RNA (circRNA) in TE11 cells and cisplatin-resistant TE11 cells (TE11R). The expression profiles determined using CIRCexplorer2 revealed that the expression of circ_0004365, mapped on the Semaphorin 3C gene, was significantly greater in TE11R compared with in TE11. In reverse transcription-quantitative PCR, circ_0004365 expression was observed in human ESCC and non-tumor tissues and was significantly upregulated in ESCC tumor tissues after chemotherapy. Circ_0004365 expression was significantly upregulated in patients with poor pathological response (P=0.02). Furthermore, patients with advanced pT stage showed an upregulation in circ_0004365 expression after chemotherapy (P=0.02). The MTT assay revealed that knockdown of circ_0003465 in TE11 significantly decreased resistance to cisplatin. In conclusion, the present study suggested that circ_0004365 was associated with cisplatin resistance in ESCC and can be used as both a novel biomarker and a therapeutic target.

Introduction

Esophageal cancer ranks sixth in terms of cancer deaths with an estimated 572,000 new cases and 509,000 deaths yearly worldwide (1). Evidence has shown that preoperative chemotherapy with cisplatin and 5-fluorouracil (CF) followed by surgical resection improves survival of patients with esophageal squamous cell carcinoma (ESCC) (2-4). However, some patients show resistance to chemotherapy, the mechanism of which chemoresistance unclear, thereby hindering successful outcomes.

CircRNAs are a class of endogenous noncoding RNAs composed of single-stranded, covalently closed RNAs lacking 5' and 3' tails. It has been over 40 years since the existence of circRNAs in viroid had been initially reported (5). CircRNAs are generated through backsplicing events from linear primary transcripts, are resistant to exonucleases, are typically nonpolyadenylated, and are highly specific to cell type and developmental stage (6). Studies have revealed that circRNAs are abundant, conserved, and stable with a tissue-specific expression pattern (7-11). CircRNAs have been used as potentially ideal biomarkers of disease owing to various characteristics including universality, stability, conservatism, and specificity. With the development of high-throughput nonpolyadenylated and RNaseR-treated RNA sequencing (RNA-seq) (12) and the mapping method using bioinformatic tools to identify arrangements in an unbiased assessment (13-16), it has become possible to establish the profile of circRNAs expression, including novel transcript isoforms.

Memczak demonstrated that circRNAs form a large class of post-transcriptional regulators functioning as endogenous miRNA sponges (6). Several researches have revealed that the interaction between circRNAs and miRNAs are associated with proliferation in cancer (17-19). Furthermore, studies have shown that the interaction between circRNA and miRNA plays an important role in the resistance of gastric cancer to cisplatin (20).

In this study, we focused on the relationship between circRNA and the resistance of ESCC to cisplatin in order to provide new insights into circRNAs as potential biomarkers and therapeutic targets for ESCC. Firstly, we analyzed the expression profile of circRNAs in ESCC cells and cisplatin-resistant ESCC cells using RNA-seq. Next, we examined the characteristic expression of hsa_circ_0004365 in ESCC tissues and investigated its function *in vitro*.

Materials and methods

Cell culture. Human ESCC cell lines TE8 (RBRC-RCB2098) and TE11 (RBRC-2100) (RIKEN BioResource Center,

Correspondence to: Dr Koji Tanaka, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: ktanaka@gesurg.med.osaka-u.ac.jp

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Ibaraki, Japan) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere containing 5% CO_2 at 37˚C. To generate cisplatin-resistant cell lines, TE8 and TE11 cells were cultured with increasing concentrations of cisplatin for 24 weeks. The established cisplatin-resistant cell lines, TE8R and TE11R, were maintained under a constant cisplatin concentration of 6 μM. TE11 cells were also cultured with increasing concentrations of 5-fluorouracil and docetaxel for 24 weeks. TE11 5-FU was maintained at a 5-fluorouracil concentration of 4.0 μM and TE11 DTX was maintained at a docetaxel concentration of 3.0 ng/ml.

Patients and tissues. In total, 82 samples of ESCC tissues were collected from patients who were histologically diagnosed with primary thoracic ESCC at Osaka University Hospital (Osaka, Japan) between 2017 April and 2020 September. The 47 samples were obtained via biopsy before treatment, and the 35 samples were obtained by surgical resection after neoadjuvant chemotherapy (NAC). All samples were collected after obtaining written informed consent, and this study was approved by the ethics committee of Osaka University (approval no. 16305-4). Clinicopathologic findings were classified according to the UICC-TNM Classification, seventh edition (21). At our institution, patients with ESCC primarily receive a DCF regimen as NAC, which consisted of docetaxel 70 mg/m^2 and cisplatin 70 mg/m^2 on day 1 and 5-FU 700 mg/m^2/day on days 1-5, every 3 weeks (22).

RNA preparation and quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted from ESCC cells or tissues using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The quantity and quality of RNA samples were measured using NanoDrop ND-1000 (Thermo Scientific, DE, USA). The samples were reverse transcribed into cDNA using a reverse transcription system (Promega, Madison, WI) according to the manufacturer's protocol. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed on a ViiA™ 7 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green (TOYOBO, Osaka, Japan) via the following three-step protocol: 95˚C for 2 min, (95˚C for 15 sec, 65˚C for 15 sec, and 72˚C for 45 sec) x40 cycles, melting curve analysis from 60 to 95˚C with reads every 0.5˚C. PCR was performed in triplicate, with the expression values normalized to the mRNA expression of β-actin calculated using the $2^{-\Delta\Delta Ct}$ method (23). The genes and primers are specified in Table SI.

Next-generation RNA sequencing and analysis. Total RNAs were treated using the Epicenter Ribo-Minus rRNA Removal Kit (Illumina, CA, USA) and RNAse R (Epicenter, CA, USA) to remove ribosomal and linear RNA (6). Thereafter, RNA-seq libraries were constructed using TruSeq Stranded mRNA Library Prep (Illumina, CA, USA) following the manufacturer's protocol while skipping the process of purifying the polyA-containing mRNA molecules with oligo-dT attached magnetic beads. The BioAnalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) was used to confirm the quality and quantity of the libraries. The RNA-seq library was sequenced using an Illumina HiSeq 3000 instrument (Illumina, San Diego, CA), and 150 bp paired-end reads were generated. Sequencing reads were mapped independently using CIRCexplorer2 (13,14), which is one of the circular RNA detection output tools based on Python, a general-purpose programming language. Cirexplorer uses TopHat (15) and TopHat-Fusion (16) to detect backsplicing junction reads. To compare differences in circRNA expression profiles between TE11 and TE11R, we calculated the fold change between the groups for each circRNA. A t-test was used to estimate the statistical significance of the differences. A significant difference in the expression of circRNA was defined by a fold-change >3.0 or <0.5 with a P-value of <0.05.

CircRNA-microRNA-mRNA network analysis. mRNAs involved in the pathways associated with conferring cisplatin resistance to tumor cells were selected for CircRNA-microRNA-mRNA network construction. The binding sites in the 3'-UTR between differentially expressed miRNAs and the selected mRNA were determined using Targetscan (http://www.targetscan.org/) to predict the mRNAs targeted by miRNAs filtered using a cumulative weighted context score of ≤0.8. We used Cytoscape software to visualize the data (24) and the bioinformatic database CircInteractome to predict the sequence of miRNA-binding sites of circ_0004365 (25).

Silencing of circ_0004365. Small interfering RNA (siRNA) of circ_0004365 was synthesized by Sigma-Aldrich (St. Louis, MO) targeting to sequence of the fusion region of circ_0004365. TE11 and TE11R cells were transfected with circ_0004365 siRNAs using the Lipofectamine® RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, cells were seeded at 2x10^5 cells/well in 6-well plates added to a final concentration of 50 nM using Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) and cultured for 24 h at 37˚C in 5% CO_2. The sequence of circ_0004365 siRNA was sense CUGUUUCUCGGAACAGGACdTdT and antisense GUCCUGUUCCGAGAAACAGdTdT.

In vitro drug sensitivity assay. A total of 4.0x10^3 cells per well were seeded in 96-well plates. Cell viability was then assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay 48 h after the addition of cisplatin. Absorbance of the converted dye was measured at a wavelength of 550 nm with background subtraction at 665 nm using an iMark™ Microplate Absorbance Reader (BIO-RAD, Tokyo, Japan). The concentration at which the drug produced 50% growth inhibition (IC50) was estimated using the relative survival curve.

Evaluation of histopathological responses to chemotherapy. Histopathological tumor response was evaluated according to the histological criteria of the Japanese Esophageal Society (26) and was classified into five categories according to the proportion of tumor degeneration and necrosis: Grade 3 (markedly effective; no viable cancer cells); grade 2

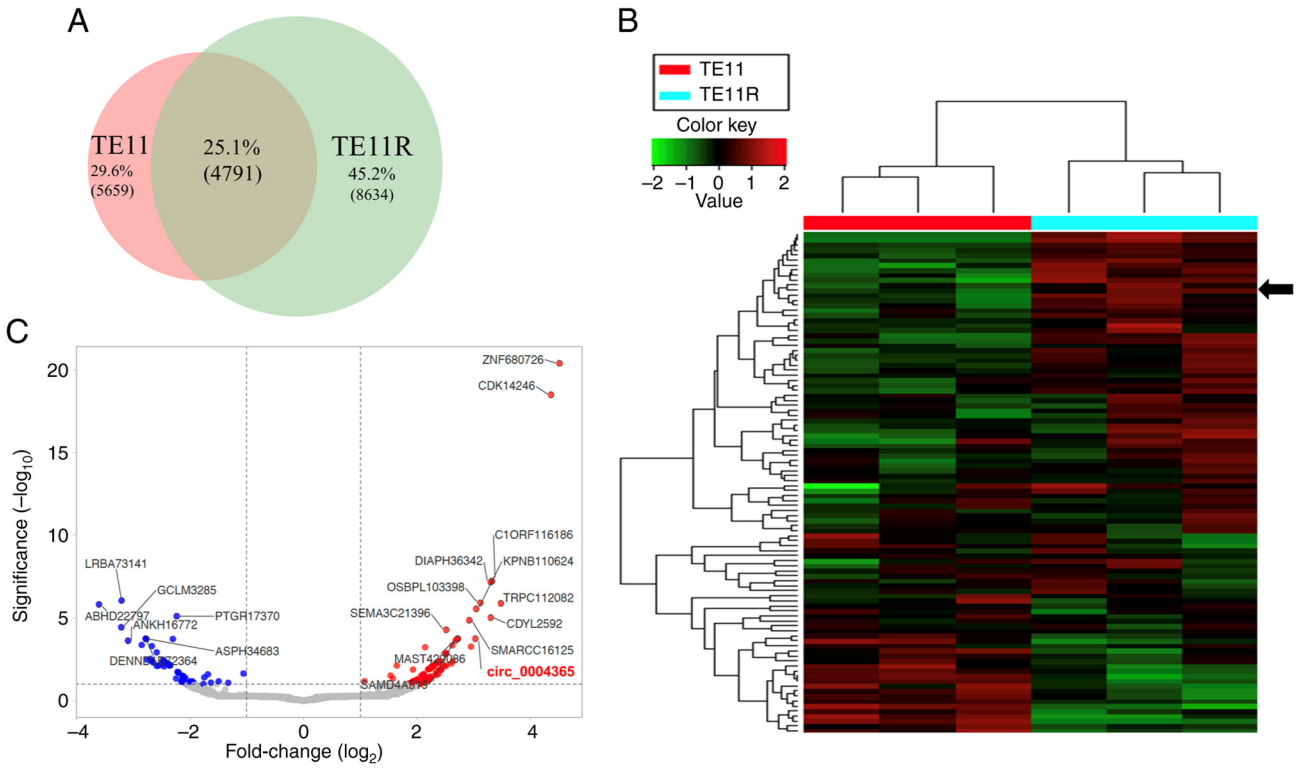

Figure 1. (A) Venn diagram analysis showing the common expression between TE11 and TE11R. (B) Clustered heatmap for upregulated and downregulated circRNAs, with rows representing circRNAs and columns representing cell lines. The arrow indicates circ_0004365. (C) Volcano plot comparing the expression fold changes of circRNAs between TE11 and TE11R. The red/blue dots represent significantly upregulated/downregulated circRNAs. Each circRNA is shown with its gene and length (bp). circRNA, circular RNA.

(moderately effective; viable cancer cells accounting for less than 1/3 of the tumor tissue while other cancer cells showed severe degeneration or necrosis); grade 1 (slightly effective; apparently viable cancer cells accounting for 1/3 or more of the tumor tissue, but some evidence of degenerating cancer tissue or cells was present), and grade 0 (ineffective; denoting no discernible therapeutic effect on cancer tissue or cells). Grade 1 lesions can also be subclassified into grade 1a (viable cancer cells accounting for 2/3 or more of the tumor tissue) and grade 1b (viable cancer cells accounting for 1/3 or more, but less than 2/3, of the tumor tissue).

Statistical analysis. To test for statistically significant differences between the two groups, continuous data were compared using the unpaired Student's t-test. Differences were considered significant at two-sided P-values of <0.05. All analyses were performed using JMP version 14.0 (SAS Institute, Cary, NC).

Results

RNA-seq analysis revealed circRNA expression profile in TE11 and cisplatin-resistant TE11. To identify circRNA expression patterns associated with cisplatin resistance in ESCC cells, we performed RNA-seq of three pairs of TE11 and cisplatin-resistant TE11 (TE11R). A total of 10451 circRNAs were consistent with Circexplorer2 (16). Thereafter, we compared circRNAs expression profiles between TE11 and TE11R to extract candidate circRNA. Accordingly, 4791 circRNAs were expressed both in TE11 and TE11R cells (Fig. 1A). Through expression intensity sorting within TE11 and TE11R cells, the mostly greater and lesser circRNAs in TE11R cells compared to those in TE11 cells are shown via hierarchical clustering (Fig. 1B). Variations in circRNA expression are demonstrated in the volcano plot (Fig. 1C). Among them, we selected 10 candidate circRNAs that were significantly upregulated and downregulated in TE11R relative to TE11 (Tables I and II).

Circ_0004365 is upregulated in cisplatin-resistant TE11 cells. To validate the expression profile established by RNA-seq and CIRCexplorer2 (16), we designed divergent primers of each circRNA candidate to specifically target the circular junction and performed RT-qPCR using ESCC cells and tissues. Consistent with the RNA-seq results, we found that the expression of circ_0004365, mapped on the SEMA3C gene (907 bp) (Fig. 2A, B), was significantly greater in TE11R than in TE11. At the same time, the upregulating expression pattern of circ_0004365 in resistant cells was observed in TE8 and TE8R (Fig. 3A). In the meanwhile, there was no difference in circ_0004365 expression between TE11, TE11 5-FU, and TE11 DTX (Fig. S1). Furthermore, the expression of circ_0004365 was broadly detected in human ESCC and nontumor tissues both before and after NAC (Fig. S2A, B). Interestingly, the relative expression of circ_0004365 was significantly greater in ESCC tumor tissues after NAC than in matched tumor tissues before NAC (Fig. S2C). Thus, the present study focused on the relationship between cisplatin resistance and the expression of circ_0004365 in ESCC.

Table I. Top 10 upregulated circRNAs in TE11R as compared with TE11.

Gene symbol	ID	Fold-change	P-value
ANKRD17	hsa_circ_0007883	3.12378	0.043183
CREBBP	hsa_circ_0007637	4.312371	0.045842
FAM185A	hsa_circ_0008271	3.093274	0.00134
GRHL2	hsa_circ_0085173	3.102087	0.000498
NFATC3	hsa_circ_0005615	4.819771	0.008801
NPEPPS	hsa_circ_0004622	3.09124	0.017307
RACGAP1	hsa_circ_0009035	3.424382	0.039387
SEMA3C	hsa_circ_0004365	5.401536	0.001506
TNFRSF21	hsa_circ_0001610	4.042647	0.004966
ZRANB1	hsa_circ_0000268	3.42829	0.038204

circRNA, circular RNA; miRNA, micro RNA; ANKRD17, ankyrin repeat domain 17; CREBBP, CREB-binding protein; FAM185A, family with sequence similarity 185 member A; GRHL2, grainyhead like transcription factor 2; NFATC3, nuclear factor of activated T cells 3; NPEPPS, aminopeptidase puromycin sensitive; RACGAP1, Rac GTPase-activating protein 1; SEMA3C, semaphorin 3C; TNFRSF21, TNF receptor superfamily member 21; ZRANB1, zinc finger RANBP2-type-containing 1.

Table II. Top 10 downregulated circRNAs in TE11R as compared with TE11.

Gene symbol	ID	Fold-change	P-value
ASPH	hsa_circ_0084615	0.442011529	0.049328
PUM12831	hsa_circ_0000043	0.492310713	0.012358
PTGR1	hsa_circ_0008043	0.364584794	0.039294
PTGR1	hsa_circ_0003731	0.192529045	0.000263
RAPGEF5	hsa_circ_0001681	0.321428355	0.010674
PTGR1	hsa_circ_0088030	0.266651031	0.040861
CELSR1	hsa_circ_0063809	0.383964597	0.030733
VWA8	hsa_circ_0000605	0.410925792	0.00274
IFAM53B	hsa_circ_0000267	0.175883872	0.001147
PSD3	hsa_circ_0004458	0.313919316	0.018126

circRNA, circular RNA; ASPH, aspartate beta-hydroxylase; PTGR1, prostaglandin reductase 1; RAPGEF5, rap guanine nucleotide exchange factor 5; CELSR1, cadherin EGF LAG seven-pass G-type receptor 1; VWA8, Von Willebrand factor A domain containing 8; FAM53B, family with sequence similarity 53 member B; PSD3, pleckstrin and Sec7 domain containing 3.

Circ_0004365 and cisplatin resistance in vitro. Firstly, we designed siRNA oligonucleotides targeting the specific junction of circ_0004365. RT-qPCR confirmed that they successfully knocked down circ_0004365 expression (Fig. 3B) without affecting the levels of endogenous linear SEMA3C transcript (Fig. 3C). Thereafter, the MTT assay revealed that TE11 with circ_0003465 knockdown had significantly lower resistance to cisplatin compared to control-transfected cells (Fig. 3D).

Similarly, TE11R cells transfected with circ_0003465 siRNA exhibited significantly lower resistance to cisplatin compared to control-transfected cells (Fig. 3E). Consequently, our findings suggested that circ_0004365 regulates the resistance of ESCC cells to cisplatin.

Circ_004365 expression in ESCC tissues. RT-qPCR revealed that circ_0004365 expression was observed in ESCC tissues and nontumor esophageal tissues. Firstly, no significant difference in the expression level of circ_0004365 was noted between ESCC and nontumor esophageal tissues (Fig. S2A, B). However, a comparison of the 15 pairs of ESCC tissues obtained before NAC and after NAC showed that the expression of circ_0004365 was significantly upregulated in tissues after NAC (P=0.03, Fig. S2C). Next, we analyzed whether circ_0004365 expression was correlated with the characteristics of ESCC patients (Table III). Accordingly, no significant correlation was observed between circ_0004365 expression level before NAC and cStage and pStage (Fig. 4A-D). Interestingly, the expression of circ_0004365 before NAC was significantly upregulated in patients with poor pathological response (P=0.02, Fig. 4E). Furthermore, patients with advanced pT Stage showed an upregulation of circ_0004365 expression after NAC (P=0.02, Fig. 5A). No significant difference was noted between the expression level of circ_0004365 after NAC and pN and pathological response (Fig. 5B, C).

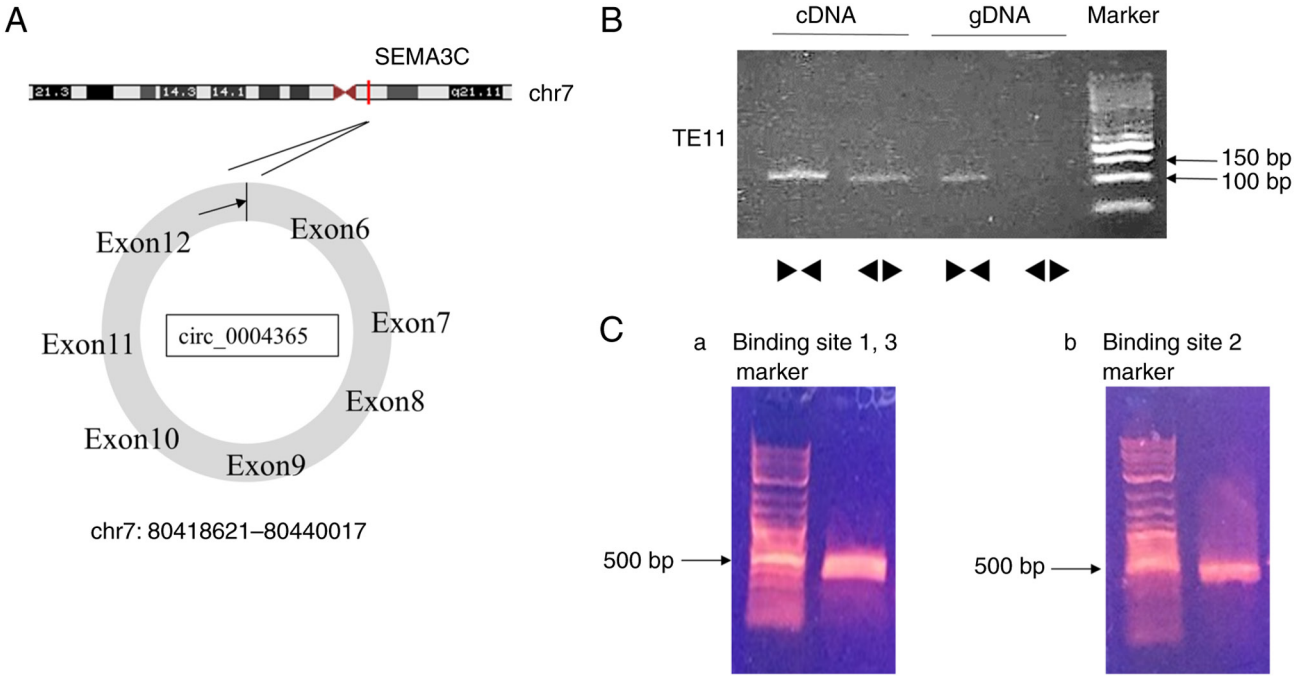

Figure 2. (A) A schematic illustration showing that circ_0004365 is derived from exons 6, 7, 8, 9, 10, 11, and 12 of the SEMA3C gene. (B) RT-qPCR products of TE11 with divergent primers showing circularization of circ_0004365. (Ca) RT-qPCR products with divergent primers showing binding sites 1, 3 of circ_0004365. (Cb) RT-qPCR products with divergent primers showing binding sites 2 of circ_0004365. circRNA, circular RNA; SEMA3C, Semaphorin 3C; cDNA, complementary DNA; gDNA, genomic DNA; RT-qPCR, reverse transcription-quantitative PCR.

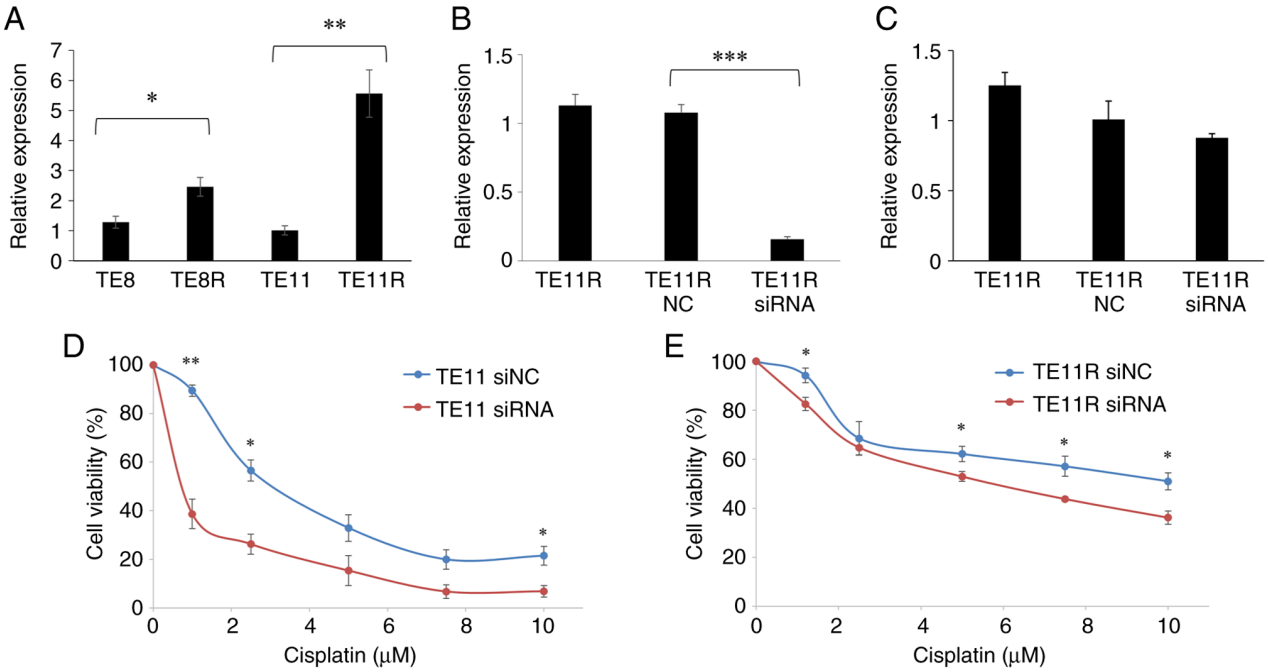

Figure 3. (A) RT-qPCR showing that circ_0004365 was greater in cisplatin-resistant TE11 (TE11R) and TE8 (TE8R) compared with in TE11 and TE8, respectively. (B) RT-qPCR results for circ_0004365 in TE11R cells with or without siRNA treatment. (C) RT-qPCR results for linear transcripts of SEMA3C in TE11R cells with or without siRNA treatment. (D) Relative cell viability of TE11 cells of si-NC-transfected and si-circ0004365-transfected cells in the presence of cisplatin at the indicated concentrations for 48 h. IC50 of TE11 SiNC=2.906574; TE11 siRNA=no data. (E) Relative cell viability of TE11R cells of si-NC-transfected and si-circ0004365-transfected cells in the presence of cisplatin at the indicated concentrations for 48 h. IC50 of TE11R siNC=11.07173; TE11R siRNA=5.747184. *P<0.05, **P<0.01, ***P<0.001. NC, negative control; IC50, half maximal inhibitory concentration; RT-qPCR, reverse transcription-quantitative PCR; circRNA, circular RNA; siRNA, short interfering RNA.

Together, these data suggested that increased circ_0004365 expression in ESCC patients was correlated with resistance to chemotherapy, including cisplatin.

Analysis of the regulatory network of circRNAs, miRNAs, and mRNAs. Biological prediction and analysis predicted that circ_0004365 binds 33 miRNAs to regulate the expression of

Table III. The clinical characteristics of patients with esophageal squamous cell carcinoma.

Characteristic	Cases, n (%) Tissues before NAC	Tissues after NAC
Sex		
Male	38 (81)	28 (72)
Female	9 (19)	11 (18)
Ethnicity		
Asian	47	39
Others	0	0
Tumor location		
Ut	5 (11)	3 (8)
Mt	21 (45)	16 (41)
Lt	21 (45)	20 (51)
Histology		
Poor	0	7 (18)
Well/moderate	42 (89)	29 (74)
Not evaluable	5 (11)	3 (8)
cT[a]		
1	4 (9)	1 (3)
2	8 (17)	5 (13)
3	28 (60)	27 (69)
4	7 (15)	6 (14)
cN[a]		
0	11 (23)	7 (18)
1	24 (51)	18 (46)
2	11 (23)	14 (36)
3	1 (2)	0
cM[a]		
0	40 (85)	31 (79)
1	7 (15)	8 (21)
cStage[a]		
I	7 (15)	4 (10)
II	7 (15)	5 (13)
III	26 (55)	22 (56)
IV	7 (15)	8 (21)
Preoperative chemotherapy		
Present	34 (72)	39
Absent	13 (28)	0
Operation		
Absent	5 (11)	3 (8)
Present	42 (89)	36 (92)

[a]UICC 7th (21). Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; DCF, docetaxel, cisplatin, and 5-fluorouracil; NAC, neoadjuvant chemotherapy.

187 mRNAs (Table SII), and the ceRNA network, circRNAs, miRNAs, and mRNAs network was visualized (Fig. 6). CircInteractome (26) predicted that miR-503 had the most binding sites for circ_0004365 (Fig. S3A). The sequence of 3 miRNA-binding sites of circ_0004365 and miR-503 is shown in Fig. S3B. We designed 2 pairs of divergent primers to specifically target the binding sites (Table SI). The one targets binding site 1 and 3 continuously and the other targets binding site 2. RT-PCR products with divergent primers showed binding 3 sites of circ_0004365 (Fig. 2C a, b). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that hsa04390: Hippo signaling pathway, hsa04650: Natural killer cell mediated cytotoxicity, hsa04623: Cytosolic DNA-sensing pathway, and hsa04514: Cell adhesion molecules were enriched (Table SIII).

Discussion

Cisplatin is among the most predominant drugs for chemotherapy (27). Several studies have shown that preoperative chemotherapy including cisplatin followed by surgical resection improves survival from ESCC (4). However, the prognosis of ESCC still remains poor, with it ranking sixth in terms of cancer deaths worldwide (1). As such, elucidating the mechanisms causing resistance to cisplatin in ESCC could improve the prognosis of ESCC patients. The present study initially clarified the role of circ_0004365 in ESCC. In line with this, we performed RNA-seq analysis, with CIRCexproler showing that several circRNAs were significantly upregulated in cisplatin-resistant TE11 cells than in parental TE11. Among those validated by RT-qPCR, circ_0004365 was greater in cisplatin-resistant TE11 cells than in parental TE11 cells. This increased expression pattern in cisplatin-resistant cells was also observed in cisplatin-resistant TE8 cells. Knockdown of circ_0004365 by siRNA enhanced cisplatin sensitivity in TE11 cells and cisplatin-resistant TE11 cells, suggesting that circ_0004365 may play an important role in the development of ESCC resistance to cisplatin.

According to our information, the present report's initial goal was to analyze the association between circ_0004365 and cancer drugs. Exons 6, 7, 8, 9, 10, 11, and 12 of the *SEMA3C* gene were used to create circ_0004365. *SEMA3C* is reportedly an oncogene, and it has been shown in certain studies that high levels of SEMA3C expression are associated with cancer development and poor prognosis in several cancers, such as breast, prostate, gastric, liver, pancreatic, and lung cancers (28). It was shown that SEMA3C is overexpressed in 85% of glioblastomas and helps to maintain glioma cancer stem-like cell self-renewal and drive tumor progression by promoting Wnt signaling (29,30). In the present study, the expression of SEMA3C in TE11R was not affected by knocking down circ_0004365 expression. There may be no association between SEMA3C expression and circ_0004365 expression so that we didn't quantify SEMA3C expression in ESCC tissues.

A novel class of noncoding RNAs, circRNAs are expected to be biomarkers of disease owing to their universality, stability, conservatism, and specificity (7-11). Studies have already reported that some circRNAs are potential diagnostic and prognostic markers in patients with ESCC. Xu *et al* demonstrated that that hsa-circ_0000654 expression in ESCC tissues was significantly higher compared to adjacent nontumor tissues and that high circ_0000654 expression was notably correlated with the higher T stage and local lymph

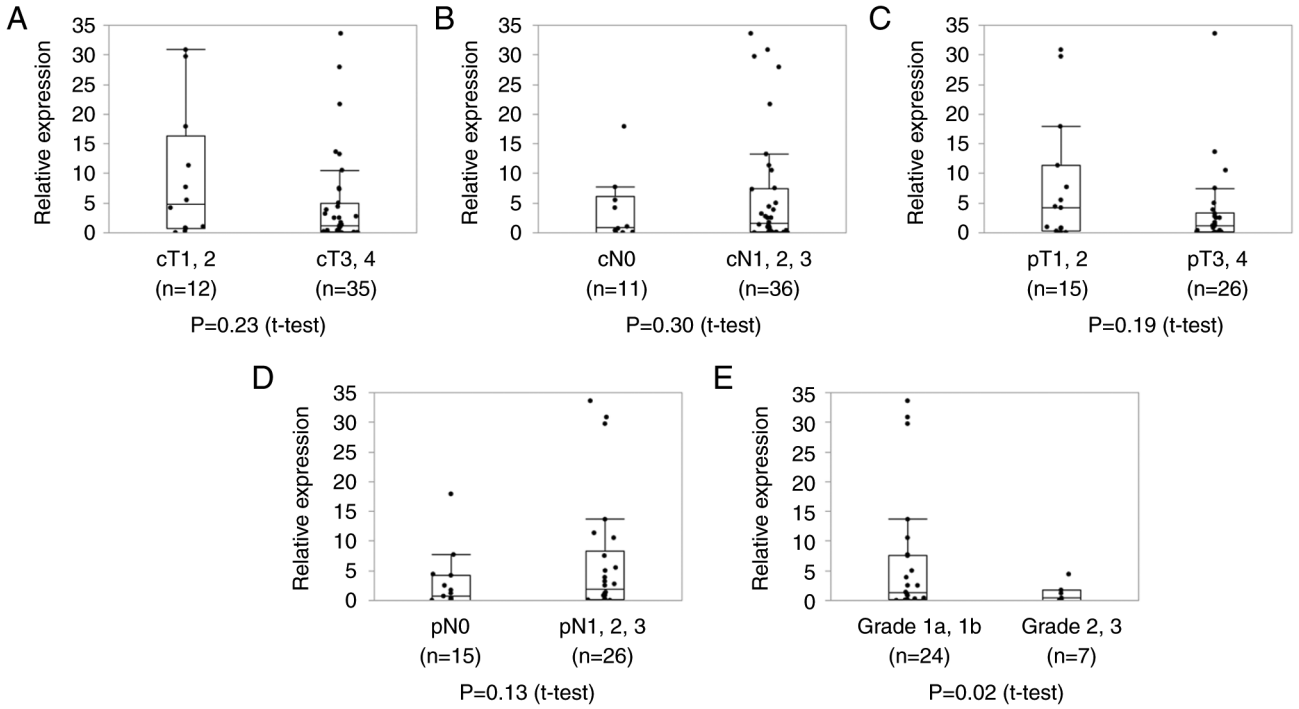

Figure 4. Relative expression of circ_0004365 in esophageal squamous cell carcinoma tissues before neoadjuvant chemotherapy and its correlation with clinical characteristics, (A) cT stage, (B) cN stage, (C) pT stage, (D) pN Stage and (E) pathological response. circ, circular RNA.

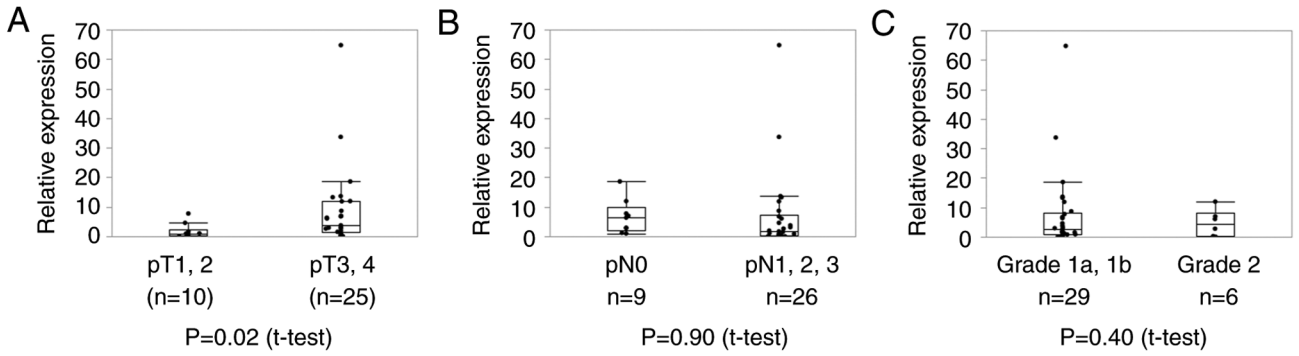

Figure 5. Relative expression of circ_0004365 in esophageal squamous cell carcinoma tissues after neoadjuvant chemotherapy and its association with clinical characteristics, (A) pT stage, (B) pN stage and (C) pathological response. circ, circular RNA.

node metastasis (31). Shi Y reported that hsa-circ_0006168 expression level was notably greater in ESCC tissues than in matched normal tissues and that high hsa_circ_0006168 expression was markedly correlated with the TNM stage and lymph node metastasis of ESCC patients (32). Furthermore, more and more *in vitro* studies have reported that some circRNAs can regulate the proliferation, migration, invasion, apoptosis, cell cycle, and epithelial-mesenchymal transition (EMT) (31-35). Evidence accumulated to date have shown that the expression of some circRNAs in ESCC are certainly associated with the progression of ESCC; however, only a handful reports have clinically demonstrated the value of circRNAs as biomarkers. For instance, Hu *et al* reported (36) that the plasma levels of circGSK3β were reduced after surgery and that circGSK3β levels were much higher in patients with recurrence/metastasis 10 months after surgery than in those without recurrence/metastasis. This suggests that plasma circGSK3β level may be a valuable clinical predictor of ESCC. The present study also demonstrated the possible utility of circRNA as a clinical predictor of cisplatin sensitivity in ESCC. Evidence has shown that circ_0004365 expression before NAC was significantly upregulated in patients with poor pathological response. This suggests that the upregulation of circ_0004365 may predict the sensitivity to cisplatin in ESCC. Furthermore, a significantly upregulation of circ_0004365 expression after NAC was observed in patients with advanced pT Stage. This suggests that cisplatin-resistant patients might still have numerous tumor cells remaining given that the expression of circ_0004365 was still upregulated after NAC. Taken together, these results suggest that increased circ_0004365 expression in ESCC patients may be correlated with resistance to cisplatin and that circ_0004365 could potentially be used as a clinical biomarker or a therapeutic target.

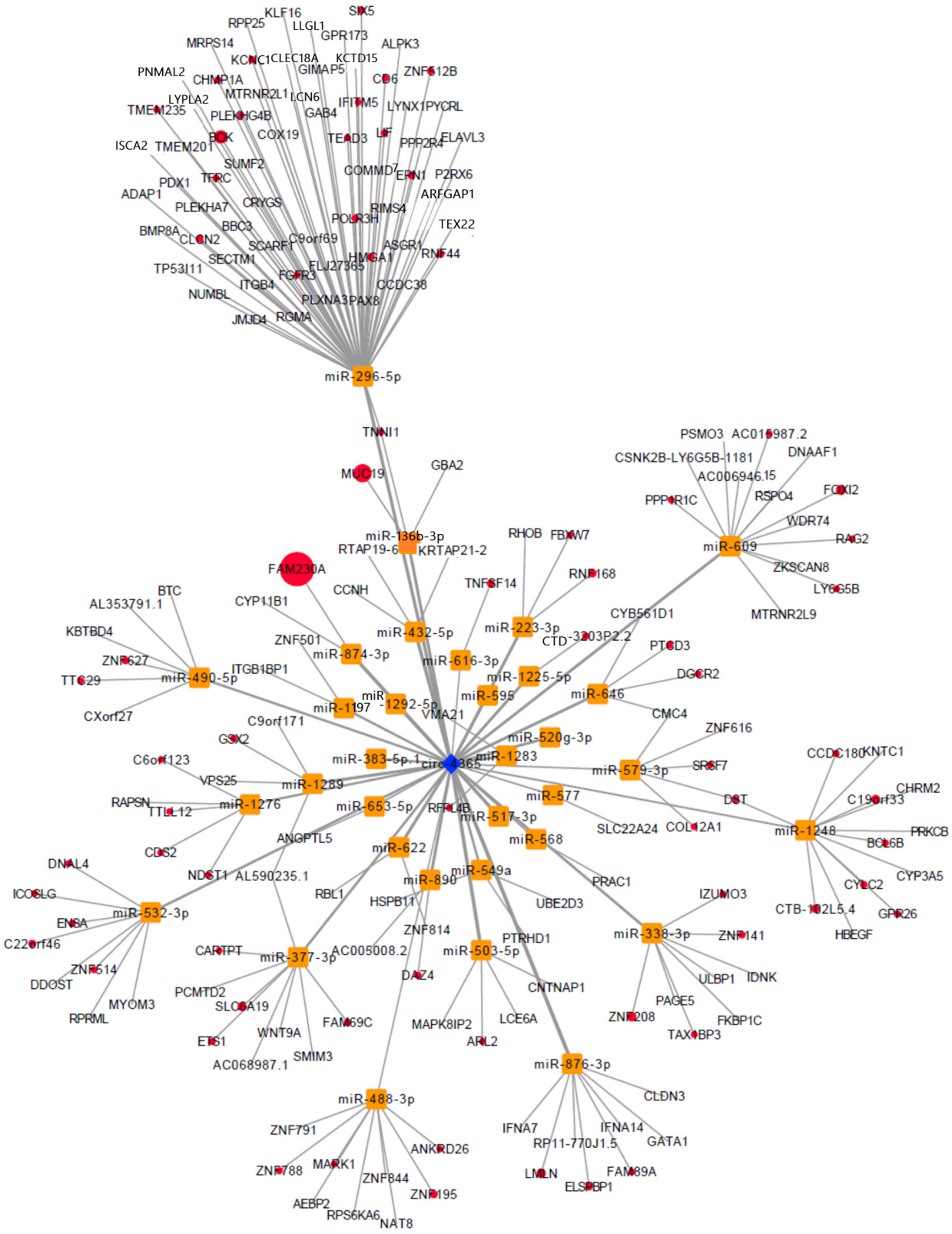

Figure 6. circRNAs-miRNAs-mRNA network visualized using Cytoscape. circRNA, circular RNA; miRNA, microRNA.

Over the past 50 years, cisplatin has become a common chemotherapeutic drug for numerous tumors, including ESCC and testicular, lung, bladder, ovarian, and liver cancers, among others (31,37). However, resistance to cisplatin often develops with continuous application. In line with this, the mechanism of cisplatin resistance has been widely studied to improve the prognosis of the patients with various cancers. There also have been several reports indicating the correlation between circRNAs and cisplatin resistance. Zou FW reported that circ_001275 was upregulated in cisplatin-resistant esophageal cancer tissues and

cell lines and that circRNA_001275 promoted the proliferation of cisplatin-resistant cells (38). Chang *et al* found that circ_0007142 was increased in cisplatin-resistant ESCC and that silencing of circ_0007142 enhanced cisplatin sensitivity in ESCC (39).

With the development of high-throughput sequencing technology and broad use of bioinformatics analysis, more and more circular RNAs and their regulatory downstream have been predicted and validated. In the present study, we constructed a circRNA-miR-mRNA network in ESCC using bioinformatics analysis. The bioinformatic database CircInteractome (26) predicted that miR-503 had the most binding sites for circ_0004365, which RT-PCR products with divergent primers showed. Visualization of the circRNA-miR-mRNA network also revealed the interaction between circ_0004365 and miR-503. Interestingly, Qiu T reported that miR-503 regulated the resistance of non-small cell lung cancer cells to cisplatin and cell apoptosis, at least in part, by targeting Bcl-2 (40). Evidence has suggested that circ_0004365 could possibly influence cisplatin resistance through downstream processing as may be corroborated by the aforementioned results, which showed that circ_0004365 has binding sites with miR-503 for sponging that may affect the cisplatin resistance. KEGG analysis demonstrated that BBC3, TEAD3, WNT9A, LLGL1, and BMP8A genes were enriched in the Hippo signaling pathway. It has been reported that Hippo signaling pathway is frequently mutated in ESCC. Song *et al* previously reported that the Hippo signaling pathway plays an important regulatory role in the development of esophageal cancer (41). Furthermore, Zhou *et al* demonstrated that Hippo signaling pathway transcription factor YAP as a molecular target for arsenic induced synthetic effects with cisplatin treatment in ESCC (42).

More and more studies have revealed the biological mechanism of circRNAs as competitive endogenous noncoding RNAs, not only for sponging miR but also for regulating parental gene expression, transcriptional translation, and protein modification (7,9,12,14,43-45). Future studies should analyze the mechanisms by which circ_0004365 influences cisplatin resistance. Furthermore, there is a need to evaluate a larger number of samples and long-term prognosis such as survival and recurrence.

In conclusion, we identified a novel circ_0004365 derived from SEMA3C via RNA-seq. Our findings suggested that circ_0004365 may play an important role in the cisplatin resistance of ESCC, which may provide new insights into its use as a potential biomarker and therapeutic target for patients with cisplatin-resistant ESCC.

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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. Nucleotide sequence data reported are available in the DDBJ Sequenced Read Archive under the accession number PRJDB16229.

Authors' contributions

MoY and KT conceived and designed the present study. MoY conducted the majority of the molecular and cellular experiments. MoY, KT, MaY, KoY, TM, TS, KaY, TT, YK, KN, HE and YD contributed to data acquisition and analysis. KeY, HM and YO performed bioinformatics, statistical analysis and interpretation of the data. MoY and KT were major contributors in writing the manuscript and confirm the authenticity of all the raw data. All authors provided supervision of the manuscript, and read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Human Ethics Review Committee of the Osaka University School of Medicine (approval no. 16305-4) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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