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Original Article

RNA-seq reveals determinants of sensitivity to chemotherapy drugs in esophageal carcinoma cells

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Abstract: Chemotherapy remains the mainstay of treatment for patients with incurable disease of esophageal carcinoma. Most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy. In present work, the sensitivities of two ESCC cell lines to 9 chemotherapy drugs were identified and the transcriptome of these two cell lines were investigated by RNA-seq, the correlation between the sensitivity to drugs and expression of some genes was attempted to construct. Eca-1 was more resistant to most of the chemotherapy drugs than Eca-109 cell line. RNA-seq results showed that there is dramatic difference in the basal expression between these two ESCC cell lines. Pathway analysis demonstrated that these differentially expressed genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways. The molecules in Gαi signaling (ADCY1 and SSTR3) and actin cytoskeleton signaling (MYH6 and MYH7) were highly expressed in multidrug-resistant Eca-1 cells, which were validated by quantitative PCR. Activation of these two pathways results in the upregulation of downstream signaling, PKA signaling and Src-STAT3, and downregulation of RAF-ERK signaling, which was validated by immunoblotting experiments. Our work proposed that activation of Gαi signaling or actin cytoskeleton signaling may confer ESCC cells resistance to most chemotherapy drugs. Our work might provide potential biomarkers and therapeutic targets for treatment of EC patients.

Keywords: Esophageal carcinoma, chemotherapy drugs, RNA-seq, pathway analysis

Introduction

Esophageal carcinoma (EC) is one of the most virulent malignant diseases with high mortality due to the advanced nature of the disease at presentation. At least 50% of patients present with metastatic cancer and most patients with localized disease will develop metastases despite potentially curative local therapy [1]. It ranks as the sixth leading cause of cancer-related mortality and the eighth most common cancer worldwide [2-4]. EC affects more than 481 000 people worldwide and the incidence is increasing rapidly [5-7]. The prognosis is poor and the overall 5-year survival ranges from 15% to 28% [3, 8, 9]. Surgery and preoperative chemoradiotherapy are optional treatments for patients with resectable tumors to treat both esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). Cytotoxic chemotherapy remains the mainstay of treatment for patients with incurable disease [10]. The most commonly utilized chemotherapy agents are fluoropyrimidine, taxanes (paclitaxel or docetaxel), and platinum compounds. Although both EAC and ESCC are responsive to chemotherapy, the response rates are low [1, 11, 12], especially for patients with advanced diseases [13, 14]. Regarding that most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy.

Previous studies have suggested that several categories of molecules are correlated with the response and/or prognosis of ESCC patients treated with neoadjuvant chemoradiation therapy (CRT): receptor tyrosine kinase (EGFR, MET) [15, 16], tumor suppressors (p53, p21) [17],

cell cycle regulators (Cyclin D1, CDC25B, 14-3-3sigma) [18], DNA repair molecules (p53R2, BRCA1, ERCC1, MLH1) [18-20], cytokines-related (IL6, sIL6R) [21, 22], drug resistance proteins (MRP2) [23], angiogenic factors (VEGF) [18], molecules involved in cell proliferation/invasion/metastasis (Ki-67, COX-2) [18, 24], PI3K/AKT/mTOR signaling molecules (AKT2, mTOR) [9, 25], wnt/β-catenin signaling molecules (PITX2) [26], NOTCH1 signaling molecules (Notch1) [27], and hedgehog signaling molecules (Gli-1) [28]. In addition, several molecules (heat-shock proteins and glucose-regulated proteins, COX7A2, CDK4/6 and Ephrin B3 receptor) [29-32] were supposed to be associated with the sensitivity of EAC cells to chemotherapy. Although so many potential biomarkers for chemotherapy to ESCC and EAC patients have been proposed, few were validated in prospective clinical trials. Furthermore, above biomarkers were mostly deduced through data from DNA microarray, immunohistochemistry, or tissue microarrays. These methods, especially DNA microarray, have many limitations when compared to the next-generation sequencing (NGS) techniques.

RNA-seq (RNA Sequencing), also called "Whole Transcriptome Shotgun Sequencing", is a technology that uses the capabilities of NGS to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time. This facilitates sequencing of the RNA transcripts in cells, providing the ability to look at alternative gene spliced transcripts, post-transcriptional changes, gene fusion, mutations/SNPs and changes in gene expression [33]. In addition to mRNA transcripts, RNA-Seq can look at different populations of RNA to include total RNA, small RNA, such as miRNA, tRNA, and ribosomal profiling [34]. Moreover, RNA-seq is demonstrated to exhibit a much wider dynamic range and greater precision for 97% of expressed genes [35, 36], compared to microarray-based measurements of gene expression.

In this work, two ESCC cell lines were subjected to several chemotherapy drugs to test the sensitivity to these drugs. And then RNA-seq was carried out in the cell lines and the differentially expressed genes were applied to pathway analysis. Then the expression signatures were linked with drug sensitivity, which was validated by qPCR and immunoblotting.

Materials and methods

Cell culture

The ESCC cell lines, Eca-109 and Eca-1, were used in this work. Eca-109 cell line was purchased from China Center for Type Culture Collection, while Eca-1 cell line is a generous gift from Dr. Yao-Qing Yang, Tumor Cell Biology Research Institute of Tongji University, China. These two cell lines were maintained in DMEM medium (Gibco) supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Determination of IC50 dose by MTS assay

Eca-109 and Eca-1 cells (1000 cells each well) were grown in 100 μl of DMEM medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with seven or nine doses of 9 chemotherapy drugs (paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, floxuridine, and cisplatin, epirubicin, and fludarabine) for 120 h. The seven doses were 1/125-, 1/25-, 1/5-, 1-, 5-, 25-, and 125-fold of reference IC50, respectively. The nine doses were 1/100-, 1/31.6-, 1/10-, 1/3.16-, 1-, 3,16-, 10-, 31.6-, and 100-fold of reference IC50, respectively. The reference IC50 doses for paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, floxuridine, and cisplatin, epirubicin, and fludarabine were 0.05, 0.10, 0.005, 0.04, 10, 0.20, 10, 0.2 and 4 μmol/L, respectively. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol. The IC50 calculation was performed with GraphPad Prism 5.0 software.

RNA-seq

Eca-109 and Eca-1 cells (8×10^4) were grown in 2 ml of DMEM medium containing serum per well in a 6-well plate with duplication. All the samples were homogenized with 1 ml Trizol (Invitrogen, Life Technologies) and total RNAs were extracted according to the manufacturer's instruction.

Table 1. Primers used for qPCR validation

gene	forward	reverse
Actb	CACCATGTACCCTGGCATT	GTACTTGCGCTCAGGAGGAG
ADCY1	CGTCCTGCTCCTGCTAGTATTC	AGGCACCCTGGAAAACACT
SSTR3	CCTGCCTTCTTTGGGCTCTA	GCGGTAGGAGAGGAAGCCATA
TRPV6	CCTGCGTGGGATAATCAACA	CGAAGTGAGAACACGCAGTCA
IGFBP5	TGACCGCAAAGGATTCTACAAG	CGTCAACGTACTCCATGCCT
MYH7	CTTTGCTGTTATTGCAGCCATT	AGATGCCAACTTTCCTGTTGC
MYH6	CCAGACGGCACCGAAGAT	TGACATACTCGTTGCCCACTTT
PAX5	ACTTGCTCATCAAGGTGTCAG	TCCTCCAATTACCCCAGGCTT

Preparation of cDNA followed the procedure described in Trapnell et al. [37]. The cDNA library was size-fractionated on a 2% TAE low melt agarose gel (Lonza catalog # 50080), a narrow slice (~2 mm) of the cDNA lane centered at the 300 bp marker was cut. The slice was extracted using the QiaEx II kit (Qiagen catalog # 20021), and the extract was filtered over a Microcon YM-100 microconcentrator (Millipore catalog # 42409) to remove DNA fragments shorter than 100 bps. One-sixth of the filtered sample volume was used as template for 15 cycles of amplification using the paired-end primers and amplification reagents supplied with the Illumina ChIP-Seq genomic DNA prep kit. Each library was loaded into its own single Illumina flow cell lane, producing an average of 14.5 million pairs of 51-mer reads per lane (8.4 million purity filtered read pairs), or nearly 1.5 Gb of total sequence for each sample. Transcripts were assembled from the mapped fragments sorted by reference position.

Quantitative real-time PCR (qPCR)

Total RNA above isolated was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in **Table 1**. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in Eca-109 cells.

Protein isolation and western blotting

Cell pellets were resuspended in 1×SDS loading buffer (1 mmol/L Na_3VO_4, 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), AKT (Santa Cruz, sc8312), p-AKT (Santa Cruz, SC7985-R, pS473), ERK (Abclonal, A0228) and p-ERK (Cell signaling, # 9106S, pT202/204) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

Eca-1 cells display resistance to many cytotoxic drugs compared to Eca-109

Nice chemotherapy drugs were subjected to Eca-109 and Eca-1 cell lines. For each drug, 7 or 9 different doses were used to treat the two cell lines and the IC50 dose was calculated with the aid of GraphPad Prism 5.0 software (**Figure 1A** and **1B**). Interestingly, Eca-1 cells were relatively more resistant to seven cytotoxic drugs (paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, floxuridine, and cisplatin) than Eca-109, while there were no dramatically different sensitivities between these cell lines to epirubicin, Eca-109 was more resistant to fludarabine (drug used in the treatment of hematological malignancies) than Eca-1.

RNA-seq showed that hundreds of genes were differentially expressed between Eca-109 and Eca-1 cell lines and Gαi signaling pathway was activated in Eca-1 cells

Total RNAs from Eca-109 and Eca-1 cells were applied for RNA-seq. The raw data were normalized in a standard distribution, and the basal expression difference was analyzed. The results showed that there were 162 lowly-expressed genes and 186 highly-expressed genes in Eca-1 cells, compared to that in Eca-109 cells. The expression difference was higher than 4-fold for these 348 genes between the two cell lines. The top 40 differentially-expressed genes were list in **Table 2**.

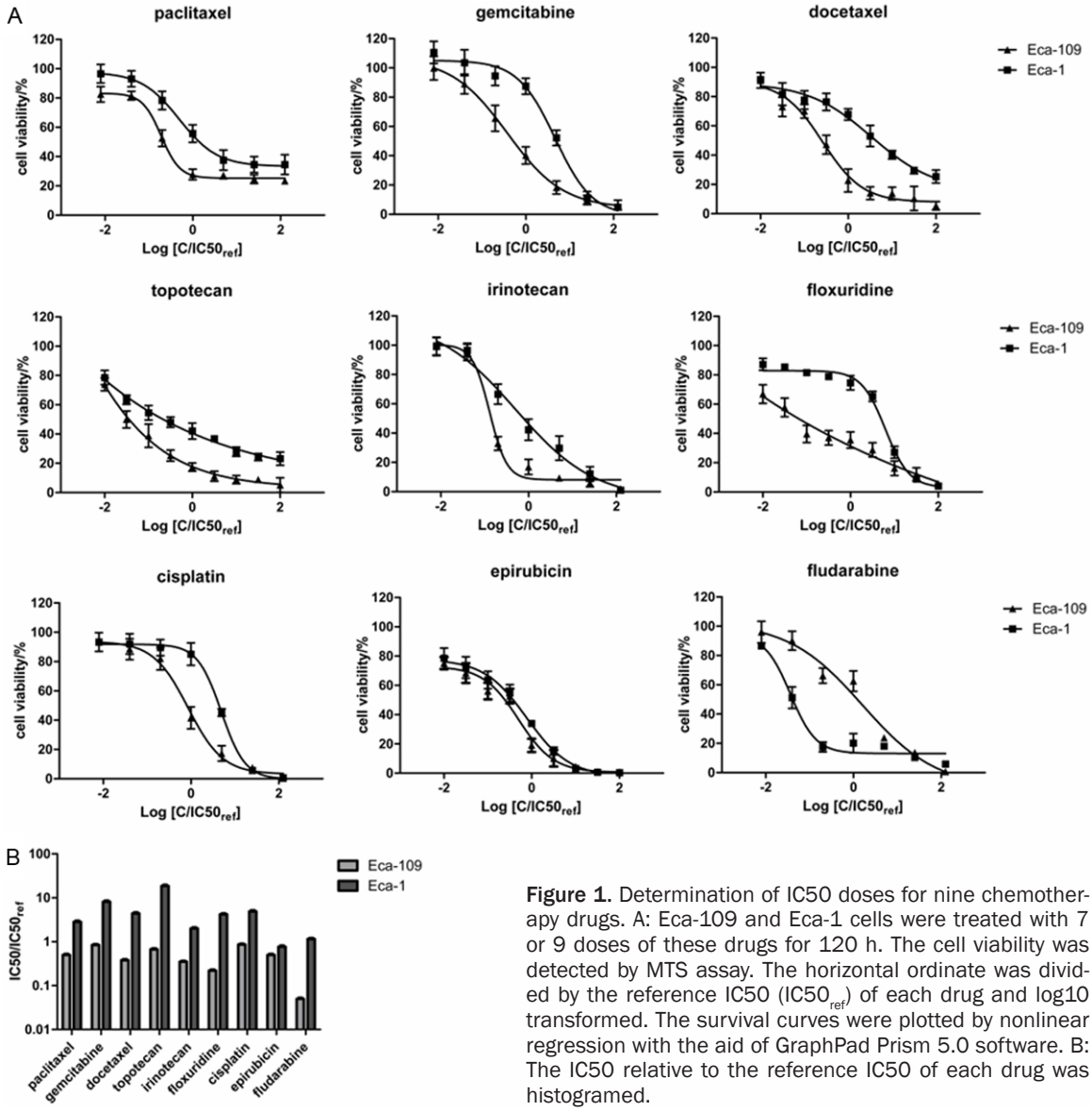

Figure 1. Determination of IC50 doses for nine chemotherapy drugs. A: Eca-109 and Eca-1 cells were treated with 7 or 9 doses of these drugs for 120 h. The cell viability was detected by MTS assay. The horizontal ordinate was divided by the reference IC50 ($IC50_{ref}$) of each drug and log10 transformed. The survival curves were plotted by nonlinear regression with the aid of GraphPad Prism 5.0 software. B: The IC50 relative to the reference IC50 of each drug was histogramed.

And then these 348 genes were applied to Ingenuity Pathway Analysis (IPA) online software. The results showed that these genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways (**Figure 2A**). In Gαi signaling pathway, the downstream PKA and Src-STAT3 signaling were predicted to be activated, while the ERK signaling was predicted to be suppressed in Eca-1 cell line (**Figure 2B**). These predictions were deduced basing on the highly expression of four genes (ADCY1, CNR1, PTGER3 and SSTR3) and the low expression of CHRM2 in Eca-1 cells, compared to that in Eca-109 cells.

qPCR validation demonstrated that molecules in Gαi signaling and actin cytoskeleton signaling were highly expressed in Eca-1 cells

To validate the RNA-seq data, expression of 7 genes was investigated in Eca-109 and Eca-1 cells by qPCR assay. The relative expression of these 7 genes in Eca-1 was log2 transformed and plotted (**Figure 3**). The change folds varied to some extent between the RNA-seq data and qPCR data, however, expression trends of most of genes were consistent between two data sets except that of two genes, MYH7 and MYH6. RNA-seq data showed that MYH7 and MYH6 were expressed at a low level in Eca-1, while the qPCR data demonstrated that MYH7 and

Table 2. Top 40 differentially expressed genes between Eca-109 and Eca-1 cells

Gene	Fold Change (Eca-1 vs Eca-109)	Gene	Fold Change (Eca-1 vs Eca-109)
KRT18P1	0.08	CCDC144NL	7.35
AC097639.8.1	0.09	RP11-203M5.6.1	7.60
RP11-55L3.2.1	0.10	RP11-450H5.2.1	7.91
MPPED2	0.10	MKRN4P	8.03
CTD-2089024.1.1	0.11	RP11-173E2.1.1	8.16
RP11-405A12.1.1	0.11	AC104843.3.1	8.24
SCDP1	0.11	PRKRIRP1	8.31
RP3-342P20.2.1	0.12	RP11-392P7.1.1	8.39
RP11-64K12.1.1	0.12	WBP11P1	8.48
RP11-21I10.2.1	0.12	AC006026.9.1	8.57
HERC2P5	0.13	RP11-297L17.6.1	8.69
API5P1	0.13	ST13P18	8.79
RBMXP2	0.13	FTH1P12	8.93
RP13-395E19.2.1	0.13	ST13P5	9.07
RP11-798L4.1.1	0.13	TAF1L	9.53
RP13-98N21.2.1	0.14	SUCLA2P1	9.89
RP13-98N21.3.1	0.14	CTB-33G10.1.1	10.18
ESRRAP1	0.14	RP11-244F12.1.1	10.49
AC139452.2.1	0.14	KCNA7	12.56
GLULP4	0.14	USP8P1	18.25

MYH6 were highly expressed in Eca-1 cells. Expression trends of 71% (5/7) genes in RNA-seq data were validated by qPCR, suggesting that the RNA-seq data were reliable for further analysis. ADCY1, SSTR3, TRPV6, IGFBP5, MYH7 and MYH6 were validated to be highly expressed, while PAX5 was validated to be low expressed in Eca-1 cells.

Western blotting experiments showed that AKT/ERK signaling was activated in Eca-109 cells

Then the most famous signaling molecules essential for cell growth and survival, AKT and ERK, were investigated in Eca-109 and Eca-1 cells. Interestingly, the phosphorylation of AKT and ERK was activated in Eca-109, compared with that in Eca-1.

Discussion

Chemotherapy remains the mainstay of treatment for patients with incurable disease of esophageal carcinoma. Most patients respond poorly to chemotherapy, it is necessary to figure out biomarkers for chemotherapy sensitivity or resistance to perform the individualized therapy.

In present work, the sensitivities of two ESCC cell lines to 9 chemotherapy drugs were identified and the transcriptome of these two cell lines were investigated by RNA-seq, the correlation between the sensitivity to drugs and expression of some genes was attempted to construct.

In drug tests, we found that Eca-1 was relatively resistant to seven most commonly used cytotoxic drugs (paclitaxel, gemcitabine, docetaxel, topotecan, irinotecan, floxuridine, and cisplatin), compared to Eca-109 cell line. Therefore, Eca-1 cell line can be considered as a multidrug-resistant ESCC cell line. In addition, Eca-1 was more sensitive to fludarabine (drug used in the treatment of hematological malignancies) than Eca-109, suggesting that some drugs used in treatment of other cancers may have good antitumor activity in partial ESCC patients. This indicates that a high throughput drug screen in more ESCC cell lines might make us find more potential effective drugs for part of patients.

RNA-seq results showed that dramatic difference existed in the basal expression between these two cell lines. Pathway analysis demonstrated that these differentially expressed genes were mainly enriched in Gαi signaling, calcium signaling, cAMP-mediated signaling, G-protein coupled receptor signaling and actin cytoskeleton signaling pathways. Gαi signaling pathway has been proposed to mediate the signal transduction from Hedgehog signaling to NF-κB signaling [38, 39], cross-talk to EGFR signaling pathway [40], and regulate cancer cell proliferation by Src-STAT3 signaling [41]. In our data, Gαi signaling was predicted to be activated in Eca-1 cells mainly based on the highly expression of ADCY1 and SSTR3, which was validated by qPCR (**Figure 3**). Due to the highly expression of ADCY1, downstream PKA and Src-STAT3 signaling were predicted to be activated whereas the RAF-ERK signaling were pre-

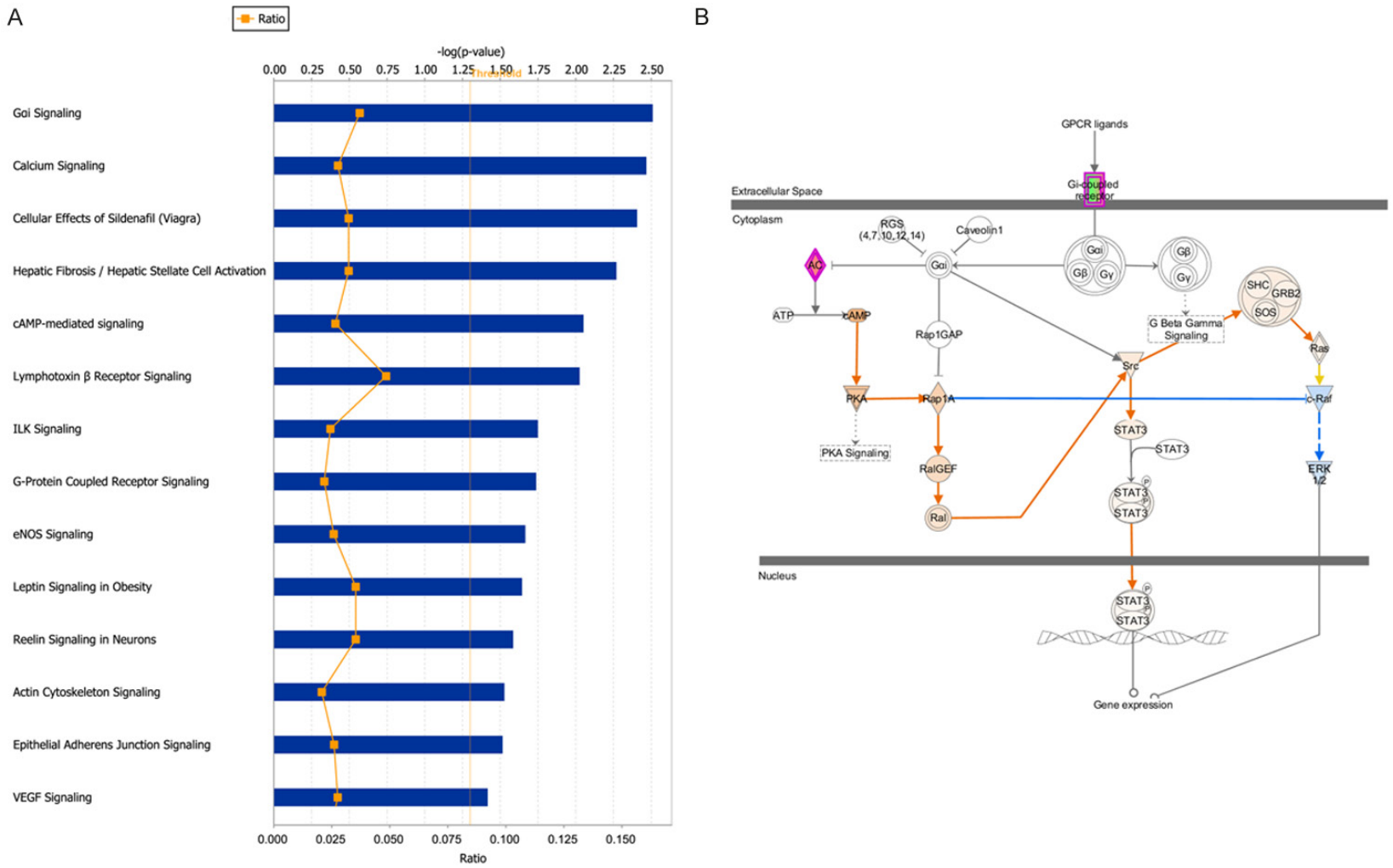

Figure 2. Ingenuity pathway analysis (IPA) for those differentially expressed genes between Eca-109 and Eca-1 cells. A: The most significant canonical pathways in which the differentially expressed genes were enriched. The 348 differentially expressed genes (expression difference >4-fold) were applied to Ingenuity Pathway analysis (IPA) software, and the most significant canonical pathways were shown. B: Gαi signaling pathway was predicted to be activated in multidrug-resistant Eca-1 cells.

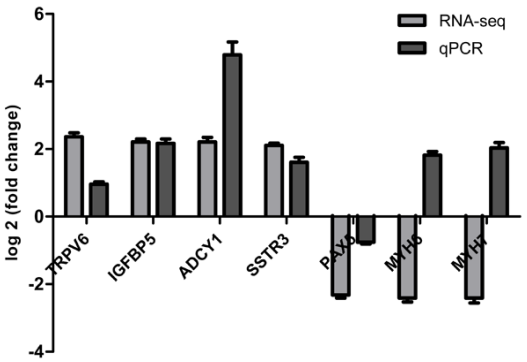

Figure 3. qPCR validation for RNA-seq data. The fold change of expression in multidrug-resistant ESCC cell line (Eca-1) was calculated relative to multidrug-sensitive cell line (Eca-109), the error bar represents the standard deviation (SD). The fold change was log2 transformed, so the gene whose value of log2 (fold change) was higher than zero, was highly expressed in multidrug-resistant EC cell line.

dicted to be inactivated (**Figure 2B**). The latter prediction was validated by immunoblotting experiments (**Figure 4**), ERK and AKT signaling was actually in lower activity in Eca-1 cells. These suggested that Gαi signaling status may be potential signature for sensitivity to chemotherapy drugs. This hypothesis warrants further functional validation in more EC cell lines and tissues from EC patients.

Actin cytoskeleton signaling has been associated with the sensitivity of cancer cells to anti-microtubule drugs, such as paclitaxel, docetaxel and vinorelbine [42-44]. In our work, RNA-seq data suggested that actin cytoskeleton signaling was suppressed in Eca-1 cells, based mainly on the lower expression of MYH6 and MYH7. However, subsequent qPCR results showed that MYH6 and MYH7 were actually highly expressed in Eca-1. Therefore, the actin cytoskeleton signaling should be activated in Eca-1 cells. This demonstrates that although RNA-seq has been proposed to be relative more accurate than DNA microarray [35, 36], further validation by qPCR is essential before the data have been used to deduce some conclusions.

Collectively, our work proposed that activation of Gαi signaling or actin cytoskeleton signaling may confer ESCC cells resistance to most chemotherapy drugs. Our work might provide potential biomarkers and therapeutic targets for treatment of EC patients.

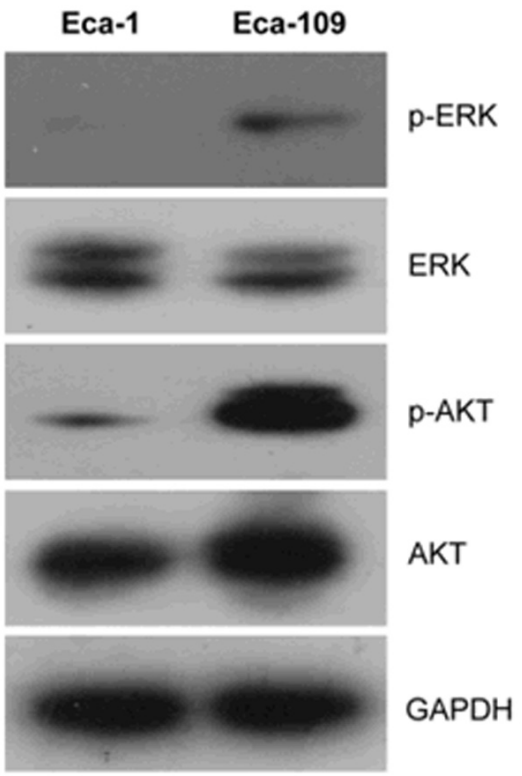

Figure 4. Immunoblotting of AKT/ERK for two ESCC cell lines. Total proteins from Eca-1 and Eca-109 cells were subjected to SDS-PAGE and blotted onto PVDF membrane. And then the protein expression of AKT, p-AKT, ERK and p-ERK in the two cell lines was examined.

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Disclosure of conflict of interest

We have no conflict of interest to declare.

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References

[1] Enzinger PC, Ilson DH, Kelsen DP. Chemotherapy in esophageal cancer. Semin Oncol 1999; 26: 12-20.

[2] Lao-Sirieix P, Fitzgerald RC. Screening for oesophageal cancer. Nat Rev Clin Oncol 2012; 9: 278-287.

[3] Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. Lancet 2013; 381: 400-412.
[4] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-2917.
[5] Schweigert M, Dubecz A, Stein HJ. Oesophageal cancer--an overview. Nat Rev Gastroenterol Hepatol 2013; 10: 230-244.
[6] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009; 59: 225-249.
[7] Lepage C, Rachet B, Jooste V, Faivre J, Coleman MP. Continuing rapid increase in esophageal adenocarcinoma in England and Wales. Am J Gastroenterol 2008; 103: 2694-2699.
[8] Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med 2003; 349: 2241-2252.
[9] Hildebrandt MA, Yang H, Hung MC, Izzo JG, Huang M, Lin J, Ajani JA, Wu X. Genetic variations in the PI3K/PTEN/AKT/mTOR pathway are associated with clinical outcomes in esophageal cancer patients treated with chemoradiotherapy. J Clin Oncol 2009; 27: 857-871.
[10] Strong VE, D'Amico TA, Kleinberg L, Ajani J. Impact of the 7th Edition AJCC staging classification on the NCCN clinical practice guidelines in oncology for gastric and esophageal cancers. J Natl Compr Canc Netw 2013; 11: 60-66.
[11] Ilson DH, Saltz L, Enzinger P, Huang Y, Kornblith A, Gollub M, O'Reilly E, Schwartz G, DeGroff J, Gonzalez G, Kelsen DP. Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. J Clin Oncol 1999; 17: 3270-3275.
[12] Webb A, Cunningham D, Scarffe JH, Harper P, Norman A, Joffe JK, Hughes M, Mansi J, Findlay M, Hill A, Oates J, Nicolson M, Hickish T, O'Brien M, Iveson T, Watson M, Underhill C, Wardley A, Meehan M. Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. J Clin Oncol 1997; 15: 261-267.
[13] Iwase H, Shimada M, Tsuzuki T, Hirashima N, Okeya M, Hibino Y, Ryuge N, Yokoi M, Kida Y, Kuno T, Tanaka Y, Kato B, Esaki M, Urata N, Kato E. Concurrent chemoradiotherapy with a novel fluoropyrimidine, S-1, and cisplatin for locally advanced esophageal cancer: long-term results of a phase II trial. Oncology 2013; 84: 342-349.
[14] Wang J, Chang J, Yu H, Wu X, Wang H, Li W, Ji D, Peng W. A phase II study of oxaliplatin in combination with leucovorin and fluorouracil as first-line chemotherapy in patients with metastatic squamous cell carcinoma of esophagus. Cancer Chemother Pharmacol 2013; 71: 905-911.
[15] Yamamoto Y, Yamai H, Seike J, Yoshida T, Takechi H, Furukita Y, Kajiura K, Minato T, Bando Y, Tangoku A. Prognosis of esophageal squamous cell carcinoma in patients positive for human epidermal growth factor receptor family can be improved by initial chemotherapy with docetaxel, fluorouracil, and cisplatin. Ann Surg Oncol 2012; 19: 757-765.
[16] Lennerz JK, Kwak EL, Ackerman A, Michael M, Fox SB, Bergethon K, Lauwers GY, Christensen JG, Wilner KD, Haber DA, Salgia R, Bang YJ, Clark JW, Solomon BJ, Iafrate AJ. MET amplification identifies a small and aggressive subgroup of esophagogastric adenocarcinoma with evidence of responsiveness to crizotinib. J Clin Oncol 2011; 29: 4803-4810.
[17] Zhang SS, Huang QY, Yang H, Xie X, Luo KJ, Wen J, Cai XL, Yang F, Hu Y, Fu JH. Correlation of p53 status with the response to chemotherapy-based treatment in esophageal cancer: a meta-analysis. Ann Surg Oncol 2013; 20: 2419-2427.
[18] Okumura H, Uchikado Y, Setoyama T, Matsumoto M, Owaki T, Ishigami S, Natsugoe S. Biomarkers for predicting the response of esophageal squamous cell carcinoma to neoadjuvant chemoradiation therapy. Surg Today 2014; 44: 421-8.
[19] Gao Y, Zhu J, Zhang X, Wu Q, Jiang S, Liu Y, Hu Z, Liu B, Chen X. BRCA1 mRNA expression as a predictive and prognostic marker in advanced esophageal squamous cell carcinoma treated with cisplatin- or docetaxel-based chemotherapy/chemoradiotherapy. PLoS One 2013; 8: e52589.
[20] Fareed KR, Al-Attar A, Soomro IN, Kaye PV, Patel J, Lobo DN, Parsons SL, Madhusudan S. Tumour regression and ERCC1 nuclear protein expression predict clinical outcome in patients with gastro-oesophageal cancer treated with neoadjuvant chemotherapy. Br J Cancer 2010; 102: 1600-1607.
[21] Yoneda M, Fujiwara H, Furutani A, Ikai A, Tada H, Shiozaki A, Komatsu S, Kubota T, Ichikawa D, Okamoto K, Konishi H, Murayama Y, Kuriu Y, Ikoma H, Nakanishi M, Ochiai T, Otsuji E. Prognostic impact of tumor IL-6 expression after preoperative chemoradiotherapy in patients with advanced esophageal squamous cell carcinoma. Anticancer Res 2013; 33: 2699-2705.
[22] Makuuchi Y, Honda K, Osaka Y, Kato K, Kojima T, Daiko H, Igaki H, Ito Y, Hoshino S, Tachibana S, Watanabe T, Furuta K, Sekine S, Umaki T, Watabe Y, Miura N, Ono M, Tsuchida A, Yamada T. Soluble interleukin-6 receptor is a serum biomarker for the response of esophageal car-

cinoma to neoadjuvant chemoradiotherapy. Cancer Sci 2013; 104: 1045-1051.
[23] Yamasaki M, Makino T, Masuzawa T, Kurokawa Y, Miyata H, Takiguchi S, Nakajima K, Fujiwara Y, Matsuura N, Mori M, Doki Y. Role of multidrug resistance protein 2 (MRP2) in chemoresistance and clinical outcome in oesophageal squamous cell carcinoma. Br J Cancer 2011; 104: 707-713.
[24] Akutsu Y, Hanari N, Yusup G, Komatsu-Akimoto A, Ikeda N, Mori M, Yoneyama Y, Endo S, Miyazawa Y, Matsubara H. COX2 expression predicts resistance to chemoradiotherapy in esophageal squamous cell carcinoma. Ann Surg Oncol 2011; 18: 2946-2951.
[25] Li SH, Huang EY, Lu HI, Huang WT, Yen CC, Huang WC, Chen CH. Phosphorylated mammalian target of rapamycin expression is associated with the response to chemoradiotherapy in patients with esophageal squamous cell carcinoma. J Thorac Cardiovasc Surg 2012; 144: 1352-1359, 1359.e1351.
[26] Zhang JX, Tong ZT, Yang L, Wang F, Chai HP, Zhang F, Xie MR, Zhang AL, Wu LM, Hong H, Yin L, Wang H, Wang HY, Zhao Y. PITX2: a promising predictive biomarker of patients' prognosis and chemoradioresistance in esophageal squamous cell carcinoma. Int J Cancer 2013; 132: 2567-2577.
[27] Liu J, Fan H, Ma Y, Liang D, Huang R, Wang J, Zhou F, Kan Q, Ming L, Li H, Giercksky KE, Nesland JM, Suo Z. Notch1 is a 5-fluorouracil resistant and poor survival marker in human esophagus squamous cell carcinomas. PLoS One 2013; 8: e56141.
[28] Yoshikawa R, Nakano Y, Tao L, Koishi K, Matsumoto T, Sasako M, Tsujimura T, Hashimoto-Tamaoki T, Fujiwara Y. Hedgehog signal activation in oesophageal cancer patients undergoing neoadjuvant chemoradiotherapy. Br J Cancer 2008; 98: 1670-1674.
[29] Slotta-Huspenina J, Wolff C, Drecoll E, Feith M, Bettstetter M, Malinowsky K, Bauer L, Becker K, Ott K, Hofler H, Becker KF, Langer R. A specific expression profile of heat-shock proteins and glucose-regulated proteins is associated with response to neoadjuvant chemotherapy in oesophageal adenocarcinomas. Br J Cancer 2013; 109: 370-378.
[30] Aichler M, Elsner M, Ludyga N, Feuchtinger A, Zangen V, Maier SK, Balluff B, Schone C, Hierber L, Braselmann H, Meding S, Rauser S, Zischka H, Aubele M, Schmitt M, Feith M, Hauck SM, Ueffing M, Langer R, Kuster B, Zitzelsberger H, Hofler H, Walch AK. Clinical response to chemotherapy in oesophageal adenocarcinoma patients is linked to defects in mitochondria. J Pathol 2013; 230: 410-419.
[31] Ismail A, Bandla S, Reveiller M, Toia L, Zhou Z, Gooding WE, Kalatskaya I, Stein L, D'Souza M, Litle VR, Peters JH, Pennathur A, Luketich JD, Godfrey TE. Early G(1) cyclin-dependent kinases as prognostic markers and potential therapeutic targets in esophageal adenocarcinoma. Clin Cancer Res 2011; 17: 4513-4522.
[32] Schauer M, Janssen KP, Rimkus C, Raggi M, Feith M, Friess H, Theisen J. Microarray-based response prediction in esophageal adenocarcinoma. Clin Cancer Res 2010; 16: 330-337.
[33] Maher CA, Kumar-Sinha C, Cao X, Kalyana-Sundaram S, Han B, Jing X, Sam L, Barrette T, Palanisamy N, Chinnaiyan AM. Transcriptome sequencing to detect gene fusions in cancer. Nature 2009; 458: 97-101.
[34] Ingolia NT, Brar GA, Rouskin S, McGeachy AM, Weissman JS. The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. Nat Protoc 2012; 7: 1534-1550.
[35] Berger MF, Levin JZ, Vijayendran K, Sivachenko A, Adiconis X, Maguire J, Johnson LA, Robinson J, Verhaak RG, Sougnez C, Onofrio RC, Ziaugra L, Cibulskis K, Laine E, Barretina J, Winckler W, Fisher DE, Getz G, Meyerson M, Jaffe DB, Gabriel SB, Lander ES, Dummer R, Gnirke A, Nusbaum C, Garraway LA. Integrative analysis of the melanoma transcriptome. Genome Res 2010; 20: 413-427.
[36] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009; 10: 57-63.
[37] Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 2010; 28: 511-515.
[38] Ogden SK, Fei DL, Schilling NS, Ahmed YF, Hwa J, Robbins DJ. G protein Galphai functions immediately downstream of Smoothened in Hedgehog signalling. Nature 2008; 456: 967-970.
[39] Qu C, Liu Y, Kunkalla K, Singh RR, Blonska M, Lin X, Agarwal NK, Vega F. Trimeric G protein-CARMA1 axis links smoothened, the hedgehog receptor transducer, to NF-kappaB activation in diffuse large B-cell lymphoma. Blood 2013; 121: 4718-4728.
[40] Ghosh P, Beas AO, Bornheimer SJ, Garcia-Marcos M, Forry EP, Johannson C, Ear J, Jung BH, Cabrera B, Carethers JM, Farquhar MG. A G{alpha}i-GIV molecular complex binds epidermal growth factor receptor and determines whether cells migrate or proliferate. Mol Biol Cell 2010; 21: 2338-2354.
[41] Ram PT, Iyengar R. G protein coupled receptor signaling through the Src and Stat3 pathway: role in proliferation and transformation. Oncogene 2001; 20: 1601-1606.

[42] Crown J, O'Leary M. The taxanes: an update. Lancet 2000; 355: 1176-1178.
[43] Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004; 4: 253-265.
[44] Kavallaris M. Microtubules and resistance to tubulin-binding agents. Nat Rev Cancer 2010; 10: 194-204.