

RESEARCH ARTICLE

# Transcription Factors and miRNAs Regulate the Mechanism of Drug Resistance in Esophageal Cancer

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**Abstract:** The presence of drug resistance can lead to differences in treatment outcomes among patients using the same drug. Therefore, identification of key biological markers associated with drug resistance can help clinicians to quickly select the appropriate drug to prolong the survival time of patients, and it is important for drug development and detailed study of the drug's mechanism of action. Firstly, we screened different drug resistance and sensitive cell lines in esophageal cancer cell lines to find different drug resistance related genes, and annotated these calculated drug resistance related genes into the transcriptional regulatory network we constructed. The regulatory relationships within this transcriptional regulatory network were all experimentally confirmed and further filtered by real esophageal cancer data to identify drug resistance related modules and key regulators. Thirteen drug resistance-associated modules were identified, each containing 1-6 drug resistance-associated key regulators. Among them, transcription factors SP1, has-miR-21-5p and hsa-miR-1-5p play key regulatory roles in the resistance modules of various drugs, and they regulate drug resistance-associated differentially expressed genes through one-step or multi-step regulatory relationships. Key transcription factors and miRNA in the transcriptional regulatory network that regulate drug resistance-associated genes can be used as potential biomarkers to identify drug resistance to the corresponding drugs in tumor patients.

**Keywords:** Drug resistance, miRNA, Transcription factor, Regulatory network

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## 1. Introduction

Oesophageal cancer is one of the most common and aggressive malignancies. Approximately 400,000 new cases are diagnosed and 300,000 deaths occur worldwide each year<sup>[1]</sup>. According to the National Cancer Institute, the five-year survival rate for patients with oesophageal cancer is only 19.9%. Treatment outcomes for oesophageal cancer have improved considerably in recent years, but the prognosis for oesophageal cancer remains unsatisfactory. Chemotherapy and radiotherapy-based treatments have been used for many patients, and according to a statistical analysis of current medical studies, patients treated with neoadjuvant chemotherapy or combined radiotherapy before surgery for oesophageal cancer have increased survival compared to patients operated on alone<sup>[2]</sup>. However, the approach to patient treatment varies from person to person, and while some patients do well with chemotherapy, others may do less well. This is why it is important to determine which treatments are appropriate for different people, and patients can avoid the harm of side effects that come with trying different ineffective treatments. Therefore, molecular markers that can predict chemotherapy response need to be investigated more to find the appropriate molecular markers to better identify which

drugs are effective for patients and which drugs patients develop resistance to, thus providing new treatments in overcoming potential chemotherapy resistance.

MicroRNAs (miRNAs) are non-coding RNAs that function similarly to transcription factors and regulate the expression of messenger RNAs (mRNAs). miRNAs act as a repressor of gene expression, thereby enabling the regulation of various biological functions. Many studies have now found that miRNAs and transcription factors play a key role in the development of cancer<sup>[3,4]</sup>. Researchers have found that multiple miRNAs are abnormally expressed in patients with oesophageal cancer and normal samples, and these differences in expression directly affect tumour growth, increment and invasion. miR-485-5p can inhibit the proliferation and invasion of oesophageal cancer by decreasing the level of acetylglucosamine transferase, and miR-502 mediates the proliferation of oesophageal cancer cells by promoting AKT phosphorylation<sup>[5,6]</sup>. The aberrant expression of miRNAs and transcription factors has also been found to influence the efficacy of chemotherapy. miR-205 nano preparations can sensitize prostate cancer to chemotherapy, and miR-146a induces resistance by downregulating the expression of CHOP<sup>[7-9]</sup>. There are numerous reports of transcription factors and miRNAs as markers in various cancer studies. miR-590-3p promotes colorectal cancer proliferation by inhibiting WIF1 and DKK1 genes within the Wnt/beta-catenin pathway, and transcription factor ZNF217 is a prognostic biomarker and therapeutic target in breast cancer progression<sup>[10-12]</sup>. However, little research has been done to date on how miRNAs affect drug resistance in oesophageal cancer.

In this study, we integrated and identified cell lines that were sensitive and resistant to different drugs, and found a collection of genes that played key roles in sensitive and resistant cell lines. After that, we integrated multiple databases of transcription factors and miRNA regulating target genes, and constructed a transcription regulatory network containing transcription factors and miRNA. These regulatory relationships were confirmed by experiments. In order to determine whether these regulatory relationships were truly activated in oesophageal cancer, we used data from oesophageal cancer patients to calculate correlations for each pair of relationships within the network, obtaining pairs of relationships that were significantly associated in oesophageal cancer, and the screened networks were further analysed. The differentially expressed genes obtained from differential analysis by using hyporesistance and sensitive cell lines under the effect of different drugs were screened for drug-specific regulatory modules to fundamentally find out which transcription factors or miRNAs regulate those genes that affect drug resistance-related cell lines, and these transcription factors and miRNAs may become markers for new drug

resistance prediction for subsequent drug development and patient treatment.

## 2. Materials and methods

### 2.1. Identification of sensitive and resistant cell lines

Expression data and IC50 values following drug action were obtained for all esophageal cancer cell lines from the Genomics Database of Cancer Drug Sensitivity (GDSC)<sup>[11]</sup>. All cell lines treated with each drug were then classified separately into sensitive and resistant cell lines. One drug acts on multiple cell lines, and the IC50 value of each cell line is different. The mean and standard deviation of the IC50 values of different cell lines under the action of the same drug were calculated to determine the classification criteria. Cell lines with IC50 values greater than the mean plus 0.8 times the standard deviation were classified as drug-resistant and those with IC50 values less than the mean minus 0.8 times the standard deviation were classified as sensitive. To ensure the statistical accuracy of the subsequent calculations, the number of both drug-resistant and sensitive cell lines was required to be no less than five, and both sensitive and drug-resistant cell lines were identified based on the two measures.

### 2.2. Collection of genes significantly associated with drug resistance

Differential expression was calculated by identifying multiple groups of drug-associated sensitive and resistant cell lines, using a *t* test with a threshold of  $P < 0.01$  for significant results, and a collection of drug resistance-associated genes for each group is obtained. Correlation calculations were carried out for categorically completed cell lines with all 13 drugs to obtain 13 sets of differentially expressed genes.

### 2.3. Construction of a transcriptional regulatory network for esophageal cancer

In order to identify which miRNAs and transcription factors regulate drug resistance and functionally relevant genes, experimentally confirmed transcriptional regulatory relationships were collated from multiple databases. Relation pairs of miRNA regulating target genes were obtained from miRTarBase<sup>[13]</sup> and TarBase<sup>[14]</sup>, transcription factor-to-target gene and miRNA regulatory relationships are from TRANSFAC<sup>[15]</sup>. A separate transcription factor-to-miRNA regulatory database TransmiR is also integrated<sup>[16]</sup>, and all regulatory relationships were experimentally confirmed, yielding a total of 5,265 regulatory pairs and 2,772 nodes within the initial transcriptional regulatory network.

The mRNA and miRNA expression data for esophageal cancer were downloaded from the TCGA

database<sup>[17]</sup>, and the miRNA names of the downloaded data were first annotated as the mature miRNAs names, and the ENSG names of genes were annotated as standard gene abbreviations. Correlations were calculated for each pair of relationships within the network using the obtained esophageal cancer data, and Pearson correlation was chosen as the method of calculation, with a correlation threshold of  $P < 0.05$ . The screened network is referred to as the esophageal cancer-specific transcriptional regulatory network. The remaining 2,445 regulatory pairs and 1,612 nodes were included in the network.

#### 2.4. Drug resistance related module mining

In order to obtain the transcriptional regulatory modules associated with various drug resistance, differentially expressed genes calculated from sensitive and resistant cell lines were put into the network. Based on the theory of connected guilt, and to find the key genes that regulate the seed genes within the network as much as possible, this study used the differential genes as seed genes and performed an extended step search for their associated regulatory genes to obtain the drug-specific regulatory network. The network is not a fully connected network, but consists of several separated components. As genes with a higher degree of node play an important role in the network, the largest component of the module is selected here. The largest component of the module contains the most core genes and the greatest number of nodes compared to other components, i.e. more information, and finally the rest of the scattered points are eliminated. This module is called the drug resistance module of the drug of interest.

#### 2.5. Functional enrichment analysis and analysis of drug action-related pathways

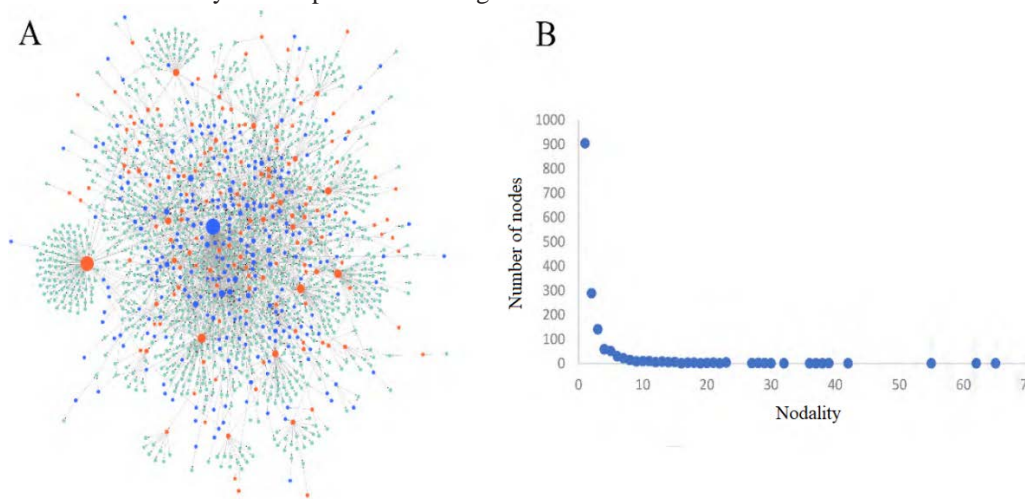
The functional enrichment analysis was performed using

the online website tool DAVID (Database for Annotation, Visualization, and Integrated Discovery)<sup>[5]</sup>, which eliminates miRNAs from the module and uses all transcription factors and target genes in the module for enrichment analysis of GO\_BP functions and KEGG pathways.

### 3. Analysis of results

#### 3.1. Esophageal cancer-specific transcriptional regulatory network

By integrating the regulatory relationships of miRNAs and transcription factors on target genes from multiple databases, a transcriptional regulatory network was constructed in which all relationships were experimentally confirmed. As many genes are specifically expressed in different tissues, we wanted to validate the regulatory relationships by using real esophageal cancer data, and downloaded the tumor data of 173 esophageal cancer patients from TCGA. The correlation of all regulatory relationship pairs was calculated using Pearson correlation. After calculation, the relationship pairs with significant  $p$  value were selected as the relationship pairs that can exercise specific regulatory functions in esophageal cancer. The network formed by these pairs is called the esophageal cancer-specific transcriptional regulatory network. Based on this network, we can explore the regulatory relationships of certain important gene within the network, and further investigate whether the gene's ability to perform its function is due to the regulation of other genes, or to find out whether the altered expression value of the gene is due to the regulation of other genes from another perspective. The construction of such an esophageal cancer-specific network facilitated subsequent studies of the regulatory mechanisms associated with key regulators (see **Figure 1**).



**Figure 1.** The transcriptional regulatory network of characteristic esophageal cancer.

Note: (A) Orange dots within the network are miRNAs, blue dots are transcription factors and green dots are target genes; (B) Degree distribution of nodes within the network.

### 3.2. Esophageal cancer drug resistance module

By differentiating the esophageal cancer cell lines that were subjected to drug action, cell lines with different drug resistance capabilities under the action of the same drug were distinguished, and differentially expressed genes

were calculated for resistant and sensitive cell lines under the action of the same drug, which caused the cell lines to develop a drug resistance response during drug action, thus making the drug less effective. A total of 13 esophageal cancer drugs were screened that met the conditions for analysis (see **Table 1**).

**Table 1.** The number of sensitive resistant cell lines in 13 drugs

Drug name in English	Drug name in Chinese	Number of sensitive cell lines	Number of drug-resistant cell lines
Trametinib	曲美替尼	8	13
Topotecan	拓扑替康	9	18
Teniposide	替尼泊苷	8	19
Sabutoclax	-	15	12
Podophyllotoxin bromide	-	8	5
MK-1775	-	6	12
Gemcitabine	吉西他滨	8	12
Epirubicin	表柔比星	17	5
Dasatinib	达沙替尼	7	13
Dactolisib	-	20	5
Camptothecin	喜树碱	12	10
AZD7762	-	10	11
Afatinib	阿法替尼	7	19

Ten of these drugs, including Trametinib, Topotecan, AZD7762 and Afatinib, have been shown to be used in the treatment of patients with esophageal cancer<sup>[18-26]</sup>, while the remaining three drugs, Teniposide, Dasatinib and Dactolisib, have also been studied for their potential value in the treatment of esophageal cancer and some differences in sensitivity have been found between patients using the drugs<sup>[27-29]</sup>. Differential expression analyses were performed for each drug, and a collection of 13 differentially expressed genes was obtained. We found that some genes were differentially expressed at high frequencies in the two groups of samples in the presence of different drugs, and that these genes may influence the resistance of multiple drugs in esophageal cancer. In particular, three genes, FANCD2OS, GUCY2F and HES7, were analyzed and found to be significantly differentially expressed in six different drugs. A literature search also revealed that mutations in these genes were significantly associated with the development of multiple cancers and affected the prognosis of patients, as confirmed in previous studies and trials<sup>[30-33]</sup>. Continued investigation of these drug resistance-associated genes will reveal whether the poorer prognosis of patients with mutations in these drug resistance-associated genes is due to resistance to therapeutic agents.

To find out whether these drug resistance-associated genes are regulated by other genes, or whether they rely on regulating the expression of other genes so as to actually achieve drug resistance in patients, the differentially expressed genes were mapped within an esophageal cancer-specific regulatory network, and the set

of genes closest to the input seed genes was extracted from within the network, and the screened maximal linkage network was extracted as the regulatory module specific to the various drugs in esophageal cancer. The maximal linkage components within the network were selected to retain both the key regulatory factors within the network and the differentially expressed genes associated with drug resistance in esophageal cancer as much as possible, thus enabling continued analysis of the function of the modules as well as extraction of the regulatory relationships at each step to find the key regulatory factors. Based on the volume approach above, 13 drug resistance modules for esophageal cancer were eventually mined (see **Figure 2**).

Because each drug resistance gene is different, the size of the modules also varies. Within most modules, drug resistance-associated genes were found to be located downstream of the module, meaning that the expression of these drug resistance-associated genes is regulated by a range of transcription factors and miRNAs, and alterations in one or more key regulators upstream may lead to the emergence of aberrant expression of drug resistance-associated genes.

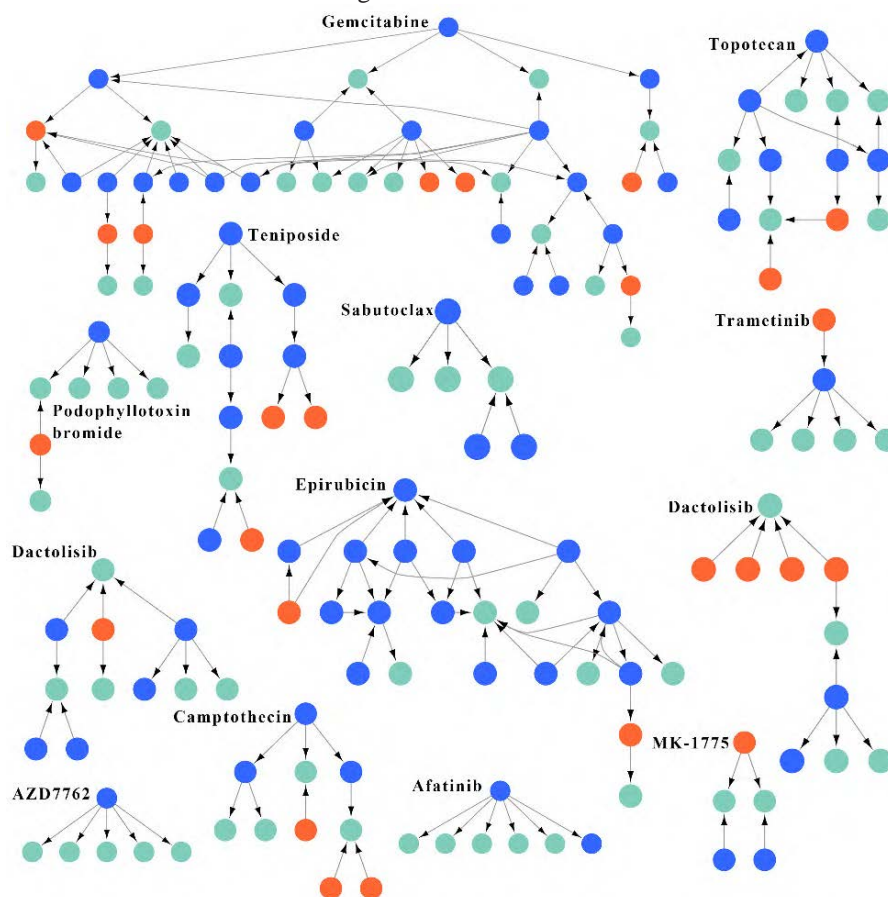
We analyzed the function of each module after mining. We used DAVID, an online functional annotation tool, and found that many of the modules were enriched for functions related to cell proliferation and angiogenesis. Interestingly, we also found that both CRHBP and TP53 in the gemcitabine module are genes related to cell response to drugs, the enriched results of the genes in the drug related module.

### 3.3. The impact of key regulatory genes on drug resistance

In this study, 13 drugs were specifically analyzed and all resistance-associated genes were found to occur downstream of the modules, and detailed analyses were carried out for the corresponding modules, using the topotecan module as an example (see **Figure 4**).

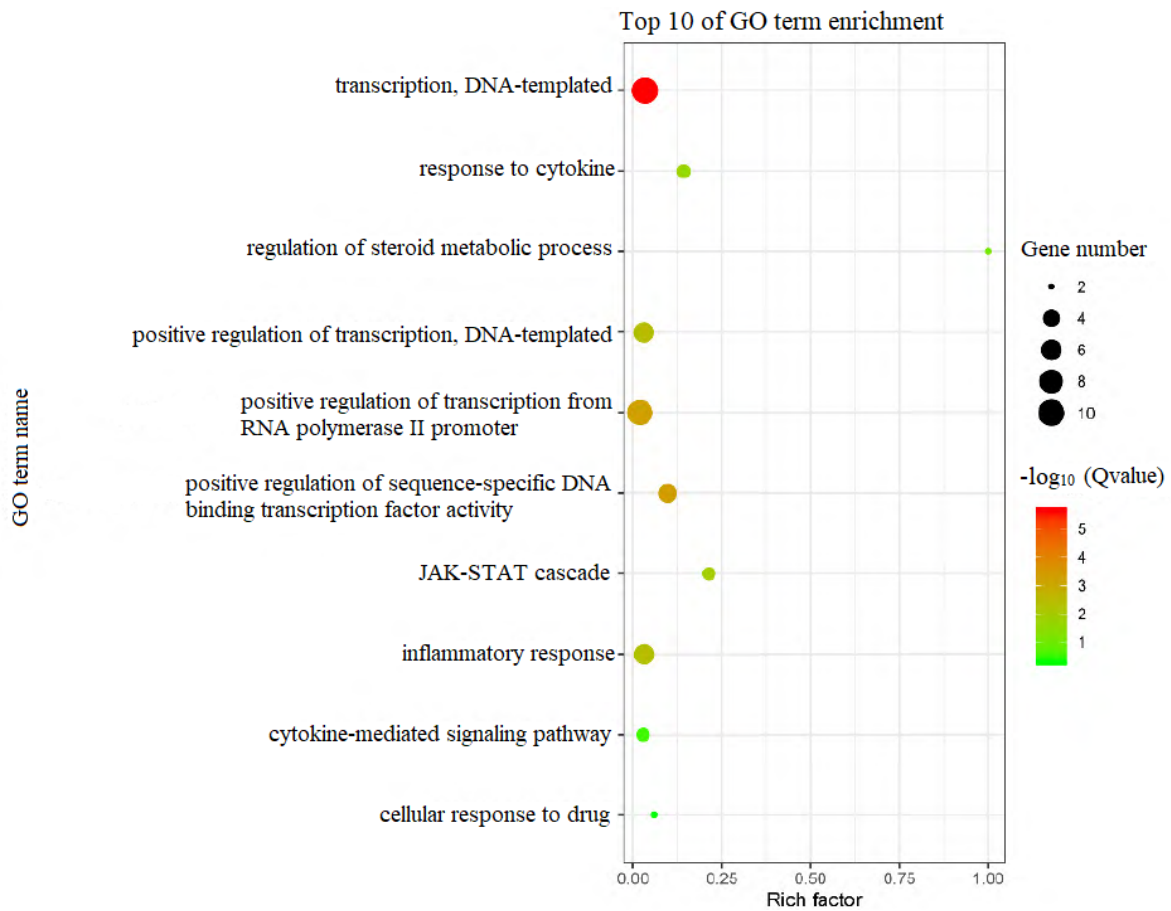
The yellow dots within the module are differentially expressed drug resistance-associated genes, and then in our study of these differentially expressed genes we found that genes on the leaf nodes within the module, represented by the CSF1 gene, have been found to be significantly associated with drug resistance in many studies<sup>[35]</sup> and that their exercise of function is also regulated due to the miR-130 miRNA family of genes<sup>[36]</sup>, which is consistent with our finding of regulatory relationships within the module. We then further analyzed the most upstream transcription factors and miRNAs within the network. The most upstream gene within this module is SP1, which can regulate the corresponding pathways through altered expression to enable drug resistance in tumor patients, including the MAPK signaling pathway<sup>[37]</sup>. RELA gene in MAPK pathway is also regulated by SP1 in the module to further regulate

other drug resistance-related genes. We have also identified two miRNAs within the network that are located at the leaf nodes, hsa-miR-9-5p and hsa-miR-125a-5p, which are more likely to function directly to enable the development of drug resistance in the body. A search of recent studies has revealed that hsa-miR-9-5p plays an important role in post-transcriptional regulation by altering DNA topoisomerase II $\alpha$  to cause drug resistance in the corresponding cell line<sup>[38]</sup>. We have found that genes located upstream can be regulated through a series of regulatory relationships to progressively regulate drug resistance-related genes, while genes located at the root node can directly respond to the development of drug resistance in the organism, but often a single gene abnormality is not sufficient to accurately determine whether the organism has developed drug resistance. This is why it is important to identify upstream genes that regulate one or more drug resistance-related genes and perform important functions, and this study suggests that these upstream genes may become new biomarkers for future drug resistance studies. By analyzing the modules, this study identified key regulatory genes within the different drug modules that could be potential biomarkers (see **Table 2**).



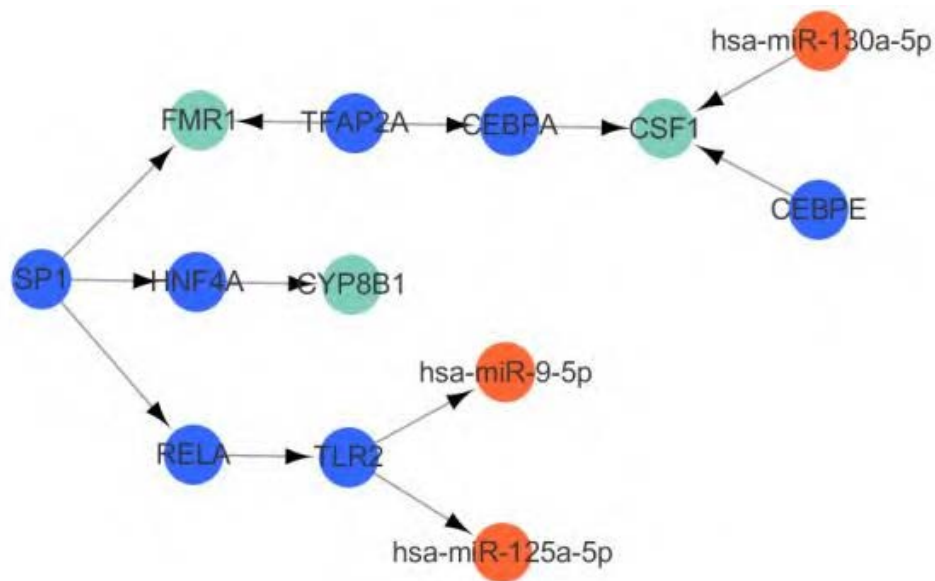
**Figure 2.** The 13 drugs resistance-related characteristic modules.

Note: The orange dots in the network are miRNAs, the blue dots are transcription factors and the green dots are target genes.



**Figure 3.** Results of gene enrichment analysis in Gemcitabine module.

Note: Larger nodes indicate that the function contains more genes in the module and the redder the color the more significant the result.



**Figure 4.** The characteristic module of Topotecan.

Note: The orange dots in the network are miRNAs, the blue dots are transcription factors and the green dots are target genes.

**Table 2.** The potentially key regulators in 13 drug modules

Drug name in English	Potential biomarkers
Trametinib	hsa-miR-200b-5p
Topotecan	hsa-miR-130a-5p, SP1, CEBPE
Teniposide	NONO, SP1, hsa-miR-1-5p
Sabutoclax	GTF3A, SP1, TFAP4
Podophyllotoxin bromide	ETV4, hsa-miR-204-5p
MK-1775	ZFHX3, hsa-miR-1-5p, REST
Gemcitabine	CEBPB, CEBPE, E2F4, RFX1, TFAP2A, hsa-miR-146a-5p, hsa-miR-16-5p
Epirubicin	FOXA1, IRF2, SP1, hsa-miR-192-5p, hsa-miR-21-5p
Dasatinib	CEBPB, SMAD3, USF1, USF2, hsa-miR-204-5p
Dactolisib	SMAD3, hsa-miR-204-5p
Camptothecin	SP1, hsa-miR-1-5p, hsa-miR-204-5p, hsa-miR-21-5p
AZD7762	NFE2L2
Afatinib	NFIC

#### 4. Conclusion

Drug resistance is an important cause of cancer treatment failure. Some studies have found that some transcription factors and miRNAs can affect the production of drug resistance, but most of these studies are aimed at the genes directly related to drug resistance, and there is still a lack of research on why the expression of these genes is abnormal. In this paper, we constructed an esophageal cancer-specific transcriptional regulatory network, and based on this network, we uncovered 13 drug resistance modules that act on esophageal cancer cell lines. In addition, subsequent studies identified key regulators upstream of the transcriptional regulatory pathway, including SP1 and hsa-miR-21-5p and hsa-miR-1-5p, which may alter the expression of drug resistance genes in one or more steps, leading to the development of drug resistance in patients. The key regulators within each module can be used as potential biomarkers to detect the suitability of a patient for a particular drug. The current differential analysis of drug resistant and sensitive samples was done based on different esophageal cancer cell lines, and in the future, if sufficient data are available, the accuracy of the results could be further enhanced by using population data from samples with more complex expression patterns of different cancer subtypes for analysis. The regulatory relationships analyzed in the current study were exclusively derived from a database that collated experimentally confirmed relationships. As the study continues to progress and potential regulatory relationships may not have been identified by researchers, these regulatory relationships used now may not be complete and may miss some of the true pairs of regulatory relationships that exist, resulting in a reduced degree of network connectivity, and some real regulatory relationships will be omitted when selecting the largest connected component of the network. If the problem of false positive rate can be solved well, subsequent studies can use software predicted regulatory pairs instead of real

regulatory pairs, which will greatly increase the size of the transcriptional regulatory network and eventually enrich the knowledge mined.

#### Conflicts of interest

There are no conflicts of interest regarding the publication of this paper.

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