

# Screening of Potential Molecular Targets for Proximal, Mid, and Distal Esophageal Cancers

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## Research article

**Keywords:** esophageal cancer, esophageal tumor central location, differentially expressed gene, function analysis

**Posted Date:** June 16th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-34714/v1>

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# Abstract

**Objective:** This study aimed to screen the potential molecular targets for proximal, mid, and distal esophageal cancers.

**Methods:** The clinical data, RNA-seq data, and survival data for TCGA esophageal cancer were retrieved from UCSC Xena database. The samples with clinical information on the esophageal tumor central location were selected. Differential analyses on different groups (mid vs. proximal, distal vs. proximal, and distal vs. mid) were performed. Following the differentially expressed genes (DEGs) in three groups were analyzed, the common genes were subjected to survival analysis, miRNA prediction, and drug-gene analysis. The specific genes were annotated for functional and module analyses.

**Results:** Six common genes, namely *KCNA1*, *CCDC196*, *UNC5CL*, *CYP3A5*, *CA10*, and *REG3A*, were analyzed among three comparison groups. The expression of *CA10* and *REG3A* was significantly correlated in the prognosis of patients with esophageal cancer. The distal vs. mid groups screened 766 specific DEGs, and the mid vs. proximal group screened 99 specific DEGs. Functional analysis showed that the genes of distal vs. mid groups, including *EDN3*, *CGA*, *CCR9*, and *GABRA2*, as well as genes in the mid vs. proximal groups, including *GCGR*, *OXTR*, and *MCHR2*, were significantly enriched in neural functions. In the distal vs. proximal groups, there were 314 specific DEGs, including *CYP2C8* and *OXGR1*.

**Conclusion:** Six common genes may serve as the potential molecular targets for the treatment of all proximal, mid, and distal esophageal cancers.

## Highlights

1. Six common genes might be potential targets for the treatment of esophageal cancers.
2. *CA10* and *REG3A* may be the prognostic factors of esophageal cancer.
3. *EDN3*, *CGA*, *CCR9*, and *GABRA2* may serve as targets during the treatment of distal and mid esophageal cancers.
4. *GCGR*, *OXTR*, and *MCHR2* may be therapeutic targets of mid and proximal esophageal cancers.
5. *CYP2C8* and *OXGR1* might be used as biomarkers to distinguish the distal and proximal esophageal cancers.

## Introduction

Esophageal cancer is a severe malignancy, which has a fatal outcome in the vast majority of cases due to its extremely aggressive nature [1, 2]. Currently, esophageal cancer is the eighth most common incident cancer worldwide, and the sixth leading cause of cancer-related death [3]. Moreover, its incidence is rapidly increasing [4]. Radiotherapy is a commonly used treatment modality in conjunction with chemotherapy as either a definitive or pre-operative treatment for esophageal cancer [5, 6]. Nevertheless,

the optimal choice of therapeutic strategy must be individualized, based on the disease progression and comorbidities of patients [7].

The esophagus is a long tubular organ extending from the neck to the upper abdomen, surrounded by various structures [8, 9]. Esophageal tumors tend to grow and migrate longitudinally along the esophageal wall. Therefore, esophageal cancer is divided into proximal, mid, and distal cancer depending on the tumor position. Generally, surgery plays an important role in achieving locoregional control in patients with mid and distal esophageal cancer [10]. Proximal esophageal cancer is located between the cricopharyngeal muscle and the sternal notch, accounting for less than 5% of all esophageal cancers [11], the management of which remains controversial. If the tumor invades an adjacent organ or has distant metastases, radiotherapy, chemotherapy, and chemoradiotherapy are the therapeutic options regardless of the tumor positions [10]. Recently, the emerging understanding of the molecular mechanisms underlying carcinogenesis provided screen critical molecular targets, which led to the development of drugs that target specific molecular events linked to the progression of many cancers, including esophageal cancer [12]. We speculated that the screening of potential molecular targets in esophageal cancer of different position might guide the treatment of proximal, mid, and distal esophageal cancers.

In this study, we collected the RNA-seq data of esophageal cancer from a public database, and the samples included clinical data of esophageal tumor central location. With analysis of the differentially expressed genes (DEGs) between different tumor positions, we screened several key biomarkers or potential molecular targets for proximal, mid, and distal esophageal cancers.

## **Materials And Methods**

### **Data retrieval and preprocessing**

The clinical data, RNA-seq data, mutation data, and survival data from the Cancer Genome Atlas (TCGA) esophageal cancer were retrieved from the University Of California Santa Cruz, UCSC (UCSC) Xena database. The original gene expression value in TCGA was stored in the form of ENSGXXXXXXXXXXXX.X, which was converted into gene symbol by the R biomaRt package [13]. The data not matching the gene symbol were deleted. In the case that the same gene symbol corresponded to multiple ENSG numbers, the average value of multiple ENSGs was taken as the final expression value of this gene symbol. The clinical expression matrix matched to a gene symbol was extracted according to the gene type, and only the mRNA was extracted for subsequent analysis. Additionally, since the mRNA expressed at low levels may interfere with the results of differential analysis, these mRNAs (that were expressed in at least 1/4th of the samples) were removed.

### **Clinical information processing**

Normal or healthy samples were removed from the esophageal cancer clinical expression profiles, and only tumor samples were selected. We included these esophageal cancers who had never undergone chemotherapy or radiotherapy. The pathological type of esophageal cancer included in the present study

was squamous cell carcinoma. Moreover, from the remaining tumor samples, the samples with clinical information of esophageal tumor central location were included, and the samples without clinical information were excluded. The remaining samples were included in the subsequent analysis, and the corresponding expression profile and survival data of these samples were extracted from the total expression profiles and survival data for differential analysis.

## Differential analysis

To investigate the differences in gene expression in esophageal cancer patients with different tumor central locations, edgeR package in R [14] was used to perform differential analyses on different groups (mid vs. proximal, distal vs. proximal and distal vs. mid). Differential analysis was performed based on count value, and the threshold of DEGs for screening was set as  $p\text{-value} < 0.05$  and  $|\log \text{fold change (FC)}| > 0.585$ . The three groups of DEGs obtained were analyzed through Venn diagrams (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

## Analysis of common DEGs from three groups

The expression of common genes was validated in three groups using the Logcpm function in edgeR packet, and the results are shown in boxplots. To investigate the prognostic role of common genes in esophageal cancer, survival analysis was performed using these common genes. The survival analysis was based on the standardized data processed by Logcpm function in edgeR and the TCGA clinical survival data. Based on the standardized data, the optimal cutoff was calculated using the R survminer package [15]. The data values higher than the optimal cutoff were considered as high expression and those lower than the optimal cutoff were considered as low expression. Survival package of R [16] was used for survival analysis, and the gene with  $p < 0.05$  was considered as a significant prognosis-related gene in esophageal cancer.

The R maftools package [17] was used to study the mutations of common genes in esophageal cancer. Statistical analysis was also carried out for common genes. The mutation files were obtained from TCGA Genomic Data Commons (GDC) preprocessed by Mutect.

Additionally, the drug-gene interaction database (DGIdb) [18] was used to analyze the drug-gene interaction pairs of common genes, and the interacting relations were visualized by Cytoscape.

Furthermore, to study the regulatory mechanism of miRNAs in those common genes, miRNA-target interaction was predicted by miRWalk 2.0 [19]. The databases used included miRWalk, Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, Pictar2, PITA, RNA22, RNAhybrid, and Targetscan. The interacting relations, in at least nine databases, were considered as positive relations, and the obtained relations were visualized using Cytoscape.

## Analysis of specific DEGs in three groups

The specific DEGs in three groups were performed for gene ontology biological process (GO-BP) and pathway analyses using Metascape [20]. In addition to functional analysis, Metascape could also

perform protein-protein interaction (PPI) analysis and MCODE [21] subnetwork mining for the input genes. MCODE can calculate the node information of nodes in the network, and the node with the highest score was a seed.

## Results

### Differential analysis

Principal component analysis (PCA) of the original data in three groups showed that except for one sample, the other samples were relatively clustered; thus, the outlier was deleted for subsequent analysis. Differential analysis between groups analyzed 249 (194 upregulated and 55 downregulated) DEGs in the mid vs. proximal groups; 822 (365 upregulated and 457 downregulated) DEGs in the distal vs. proximal groups; 1174 (353 upregulated and 821 downregulated) DEGs in the distal vs. mid groups. The PCA and volcano plot for three comparison groups are shown in Fig. 1.

### Logical analysis of three groups of differential genes

The six common genes among the three comparison groups were analyzed. These genes are potassium voltage-gated channel subfamily A member 1 (*KCNA1*), coiled-coil domain containing 196 (*CCDC196*), unc-5 family C-terminal like (*UNC5CL*), cytochrome P450 family 3 subfamily A member 5 (*CYP3A5*), carbonic anhydrase 10 (*CA10*) and regenerating family member 3 alpha (*REG3A*) (Fig. 2A). The boxplots for six genes are shown in Fig. 2B. Additionally, there were 99 specific genes in the mid vs. proximal groups, 314 specific genes in the distal vs. proximal groups, and 766 specific genes in the distal vs. mid groups.

### Mutation analysis of six common genes in esophageal cancer

In terms of the overall genetic mutations in esophageal cancer, missense mutation was the main variant type in esophageal cancer (Fig. 3A). A missense mutation is a base substitution mutation that results in changes in the amino acid sequence of a polypeptide or the base sequence of functional RNA, most of which have harmful or lethal effects. Analysis of the mutation of six common genes showed that *KCNA1*, *CA10*, and *REG3A* had mutation data in TCGA esophageal cancer, and all the variant types were missense mutations (Fig. 3B).

### Survival analysis of six common genes in esophageal cancer

Survival analysis showed that among the six common genes, *CA10* and *REG3A* had a significant effect on the prognosis of patients with esophageal cancer ( $p < 0.05$ ). Low expression of *CA10* and high expression of *REG3A* had a good prognosis of esophageal cancer (Fig. 4).

# miRNA and drug prediction for six common genes

According to the screening conditions described in the method section, three genes, namely *UNC5CL*, *KCNA1*, and *CA10*, had shown a strong miRNA-target regulatory relation (Fig. 5A). Additionally, three genes, namely *CYP3A5*, *KCNA1*, and *CA10*, were predicted to have drug-gene interactions. Nifedipine had interactions with both *CYP3A5* and *KCNA1*. Guanidine hydrochloride and dalfampridine also had interaction with *KCNA1* (Fig. 5B).

## Function and pathway analyses of specific genes

A total of 99 specific genes in the mid vs. proximal groups were enriched in GOs associated with axonemal dynein complex assembly, positive regulation of synaptic transmission, central nervous system neuron differentiation, and the pathway of neuroactive ligand-receptor interaction. The 314 specific genes in the distal vs. proximal groups were enriched in GOs associated with digestion, regulation of neuron differentiation, negative regulation of the Wnt signaling pathway, and regulation of cell cycle checkpoint, as well as pathways of protein digestion and absorption, and sphingolipid metabolism. The 766 genes specific to the distal vs. mid groups were enriched in GO related to cornification, digestion, regulation of intestinal cholesterol absorption, neurotransmitter transport, and pathways of neuroactive ligand-receptor interaction, fat digestion and absorption, and SLC-mediated transmembrane transport (Fig. 6).

## PPI analysis

The PPI network was constructed based on the 99 specific genes in the mid vs. proximal groups, and a subnetwork module, including four genes (glucagon receptor (*GCGR*), oxytocin/neurophysin I prepropeptide (*OXT*), oxytocin receptor (*OXTR*) and melanin-concentrating hormone receptor 2 (*MCHR2*)), was extracted. From the PPI network constructed by 314 specific genes in distal vs. proximal groups, five modules were extracted. Cytochrome P450 family 2 subfamily C member 8 (*CYP2C8*), oxoglutarate receptor 1 (*OXGR1*), ZFP28 zinc finger protein (*ZFP28*), collagen type II alpha 1 chain (*COL2A1*), and urotensin 2 (*UTS2*) were the seed genes. In the PPI network constructed by 766 specific genes in distal vs. mid groups, 11 modules were extracted, and 11 seeds such as endothelin 3 (*EDN3*), glycoprotein hormones, alpha polypeptide (*CGA*), C-C motif chemokine receptor 9 (*CCR9*), gamma-aminobutyric acid type a receptor alpha2 subunit (*GABRA2*), and cholinergic receptor nicotinic alpha 4 subunit (*CHRNA4*) were screened (Fig. 7).

## Discussion

The proximal, mid, and distal esophageal cancers have different biological characteristics. Therefore, surgical and radiation treatment options are usually different. In this study, we selected the DEGs among the three types of esophageal cancers to screen the targeted molecules for the treatment of esophageal cancers. After analysis, six genes were screened to be differentially expressed in all three groups.

Additionally, hundreds of DEGs specific to three comparison groups were also analyzed. These genes may serve as potential therapeutic targets for esophageal cancers.

In this study, six genes were differentially expressed among the three groups. *KCNA1* and *CYP3A5* were regulated by nifedipine, which is the most common kind of calcium channel blocker and is used to treat high blood pressure and angina. Especially, calcium channel blockers were recently reported to be associated with cancer [22]. A previous study has reported that cancer cells that have DNA mismatch repair deficiency are resistant to many cytotoxic drugs. Calcium channel blockers may inhibit the pathways that cause drug-resistance [23]. Thus, we speculated that *KCNA1* and *CYP3A5* might be therapeutic targets of esophageal cancer.

*CA10* and *REG3A* were significantly correlated in the prognosis of patients with esophageal cancer. The *CA10* gene encodes a protein belonging to the carbonic anhydrase family of zinc metalloenzymes. Hypoxia and acidosis are prominent features of many tumors, leading to a different metabolism from normal or healthy cells. Two of the simplest metabolic products, protons, and bicarbonate, are produced by the catalytic activity of the carbonic anhydrase [24]. Carbonic anhydrase IX is considered as a molecular marker that may predict the prognosis of renal cancer [25]. *REG3A* is a member of the *REG* protein family, which has been demonstrated to be involved in the development of some digestive tumors, including gastric cancer, hepatocellular carcinoma, and colorectal cancer [26]. However, its role in esophageal cancer has not yet been reported.

For the other two genes *UNC5CL* and *CCDC196*, *UNC5CL* was found to be regulated by miR-337-3p and miR-512-5p. miR-337 has been reported to distinguish esophageal squamous cell carcinoma patients from healthy controls [27]. Interestingly, a recent study reported that rs10484761 loci, located in a region of ~ 200-kb on chromosome 6p21, near *UNC5CL* gene, was a novel susceptible locus for esophageal squamous cell carcinoma. *CCDC196* belongs to coiled-coil domain-containing proteins that are directly associated with migratory, invasive, and metastatic phenotypes of cancer cells [28–30]. The role of *CCDC196* in esophageal cancer is unknown. Taken together, given the significant differential expression of the *KCNA1*, *CYP3A5*, *CA10*, *REG3A*, *UNC5CL*, and *CCDC196* among three tumor locations, we speculate that these genes might serve as the potential molecular targets for the treatment of all proximal, mid, and distal esophageal cancers. Additionally, *CA10* and *REG3A* might also be potential prognostic factors of esophageal cancer.

In addition to common genes found in the three groups, there were also some genes specific to each position of esophageal cancers. The distal vs. mid groups had the most specific DEGs (766), which suggested that there may exist large differences between the two positions. Mid vs. proximal groups only screened 99 specific DEGs. Interestingly, functional analysis showed that the seed genes of distal vs. mid groups (including *EDN3*, *CGA*, *CCR9*, and *GABRA2*), as well as genes in mid vs. proximal group (such as *GCGR*, *OXTR*, and *MCHR2*), were significantly enriched in neural functions, including neuroactive ligand-receptor interaction, transmission of nerve impulse, and neuronal system. Emerging evidence has suggested that nervous microenvironment plays a critical role in cancer progression [31–33]. Quail et al.

[34] have reported that nervous microenvironment is an important factor in the early stage of invasion and metastasis of pancreatic cancers. Therefore, treatment targeted at neuro-microenvironment may provide a novel strategy to prevent the progression of some cancers.

Nevertheless, the role of the neuronal system in esophageal cancer has not been fully studied. We speculate that *EDN3*, *CGA*, *CCR9*, and *GABRA2* might serve as potential targets during the treatment of distal and mid esophageal cancers. Additionally, *GCGR*, *OXTR*, and *MCHR2* may be therapeutic targets of mid and proximal esophageal cancers.

In the distal vs. proximal groups, there were 314 specific DEGs, including *CYP2C8* and *OXGR1*. *CYP2C8* encodes a member of the cytochrome P450 superfamily of enzymes, which play essential roles in the metabolism of endogenous and exogenous molecules [35]. The local expression of cytochrome P450s is essential for cancer management since these functionally associated enzymes might be involved in determining the anticancer drug sensitivity [36]. *OXGR1* belongs to the G protein-coupled receptor superfamily, and it is frequently found to be hypermethylated in hepatocellular carcinoma [37]. A recent study revealed that overexpression of *OXGR1* promoted prostate cancer development [38]. Thus, we speculated that *CYP2C8* and *OXGR1* might be used as biomarkers to distinguish between distal and proximal esophageal cancers.

Although the genes screened from our analyses are promising candidate genes, we have not validated these genes through *in vivo* or *in vitro* experiments. Therefore, we will further validate and strengthen our findings by developing animal models and collecting clinical samples.

In conclusion, *KCNA1*, *CYP3A5*, *CA10*, *REG3A*, *UNC5CL*, and *CCDC196* may serve as the potential molecular targets for the treatment of all proximal, mid, and distal esophageal cancers. *CA10* and *REG3A* may be prognostic factors of esophageal cancer. *EDN3*, *CGA*, *CCR9*, and *GABRA2* may serve as potential targets during the treatment of distal and mid esophageal cancers. *GCGR*, *OXTR*, and *MCHR2* may be therapeutic targets of mid and proximal esophageal cancers. *CYP2C8* and *OXGR1* may be used as biomarkers to distinguish between distal and proximal esophageal cancers.

## Declarations

### Ethics approval and consent to participate

Not Applicable.

### Consent for publication

Not applicable.

### Competing Interests

The authors declare that no conflicts of interest exist.



## Funding

This work was supported by the Science Technology Projects of Zhejiang Province (No. 2017C33207).

## Authors' Contributions

All authors participated in the conception and design of the study.

Conceived and drafted the manuscript: Liping Zhong and Zeying Guo;

Analyzed the data: Yuefen Pan and Yurong Chen;

Drew figures: Xiaoyong Zhu;

Wrote the paper: Liping Zhong and Zeying Guo;

Han shuwen takes full responsibility for the work.

All authors read and approved the paper.

## Acknowledgements

The authors gratefully acknowledge the multiple databases, which made the data available.

## Availability of data and materials

The datasets generated during the current study are not publicly available but obtained from corresponding authors on reasonable request.

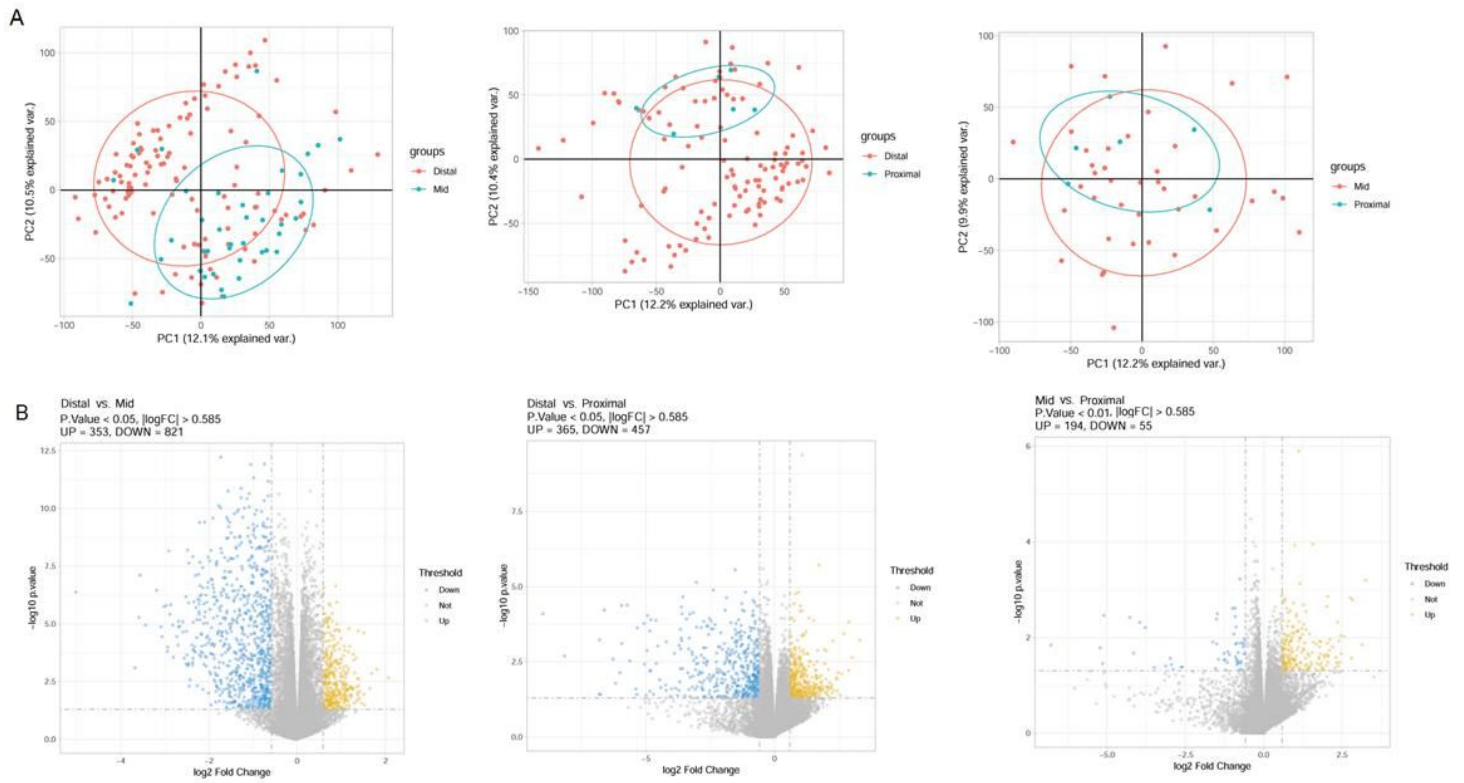
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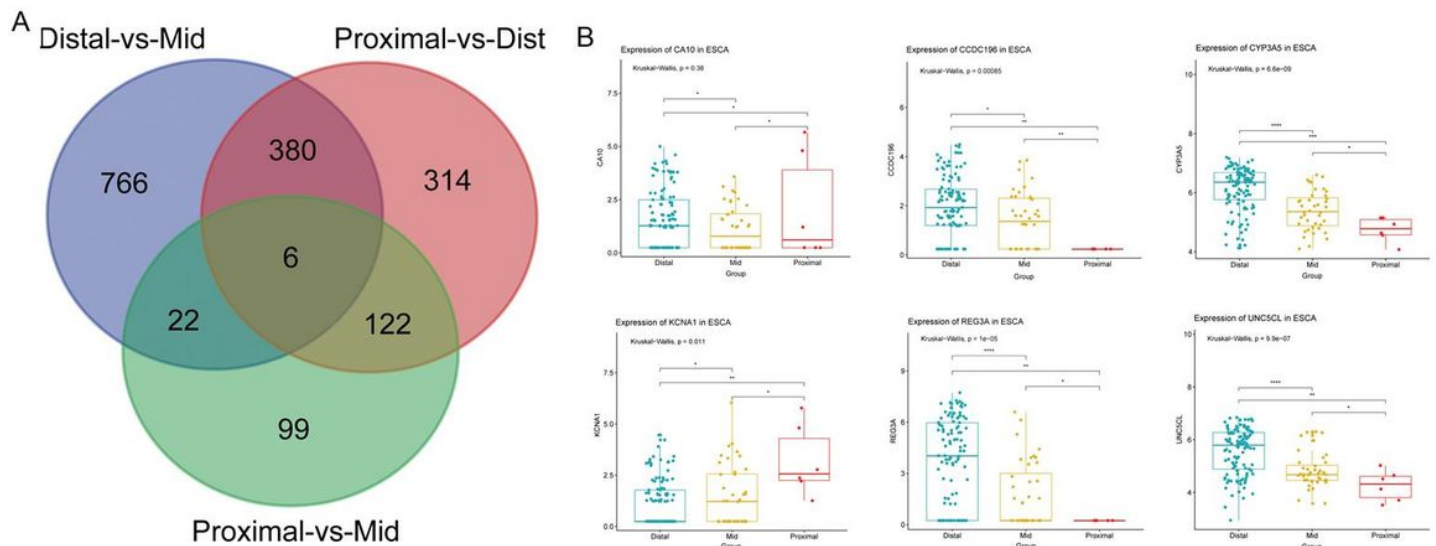
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## Figures



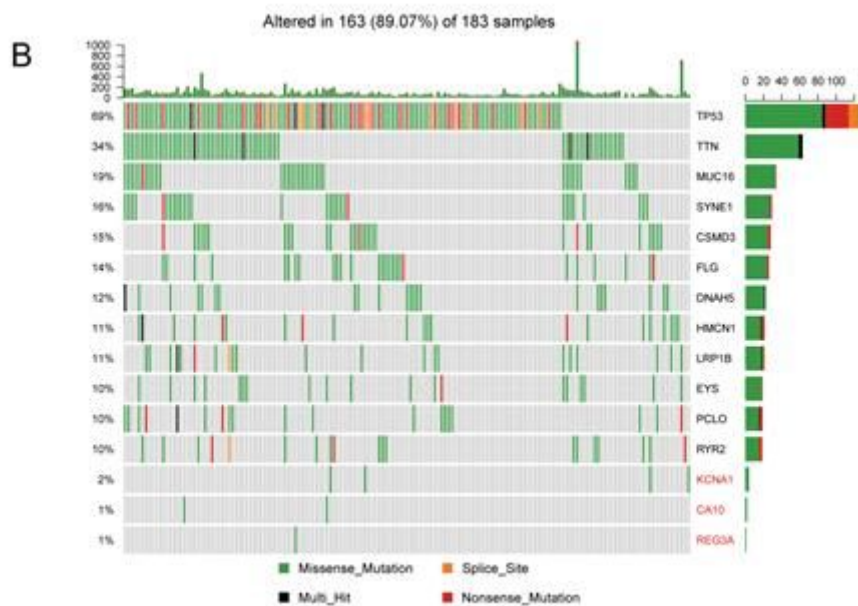
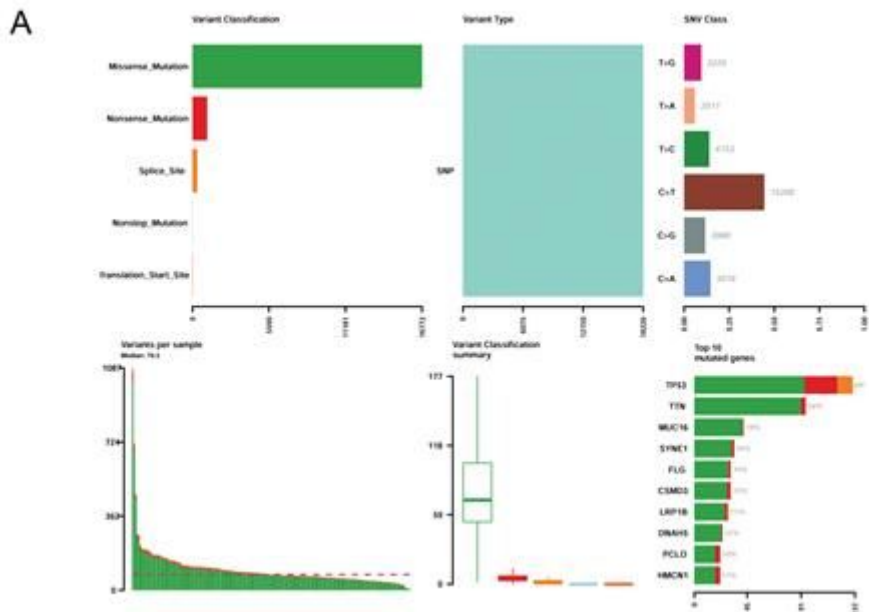
**Figure 1**

The principal component analysis (PCA) (A) and a volcano plot (B) for three comparison groups. In volcano plots, yellow represents upregulated genes, and blue represents downregulated genes.



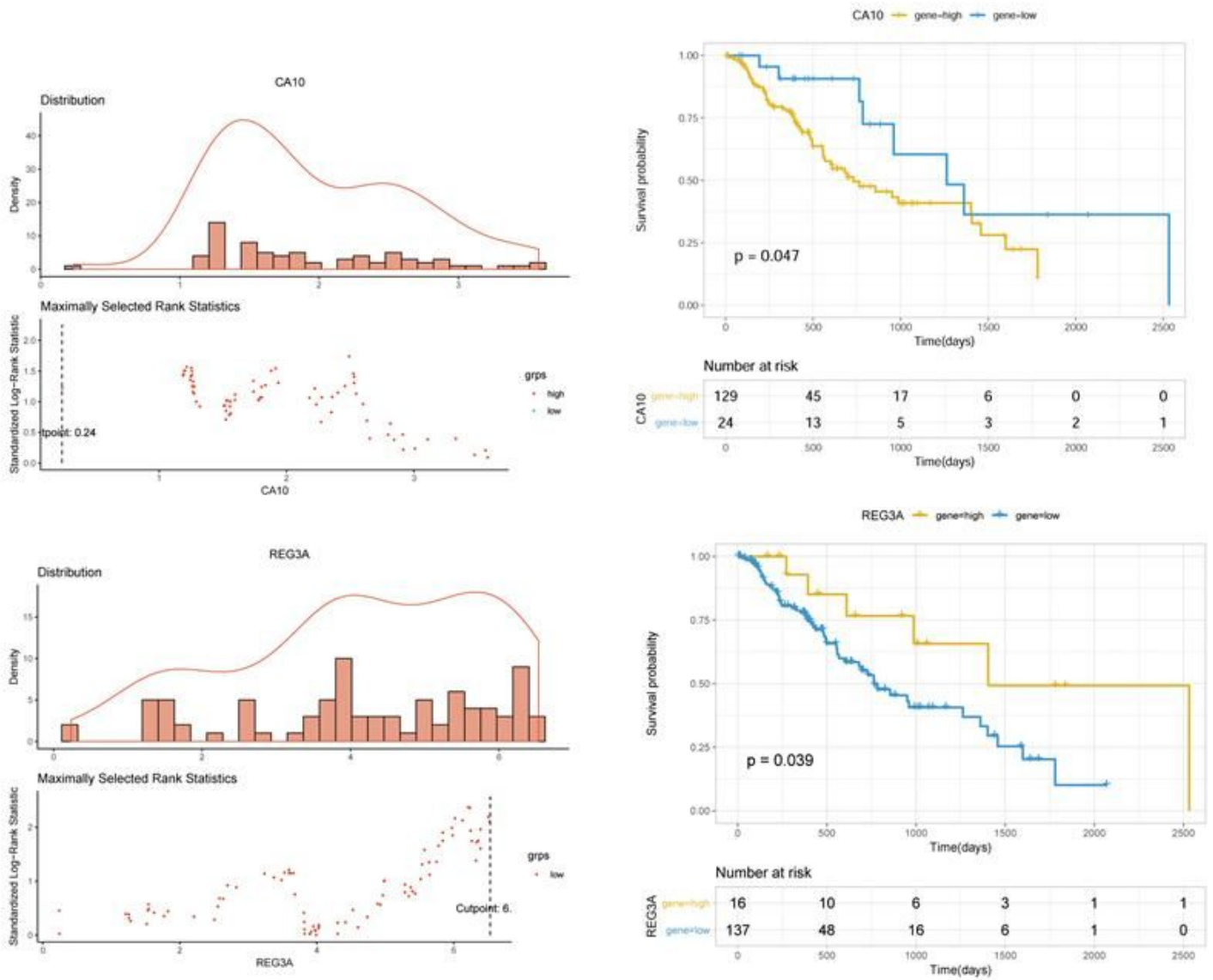
**Figure 2**

A: Logical analysis results of three groups of differentially expressed genes. B: The boxplots of six differentially expressed genes in three different positions of esophageal cancers.



**Figure 3**

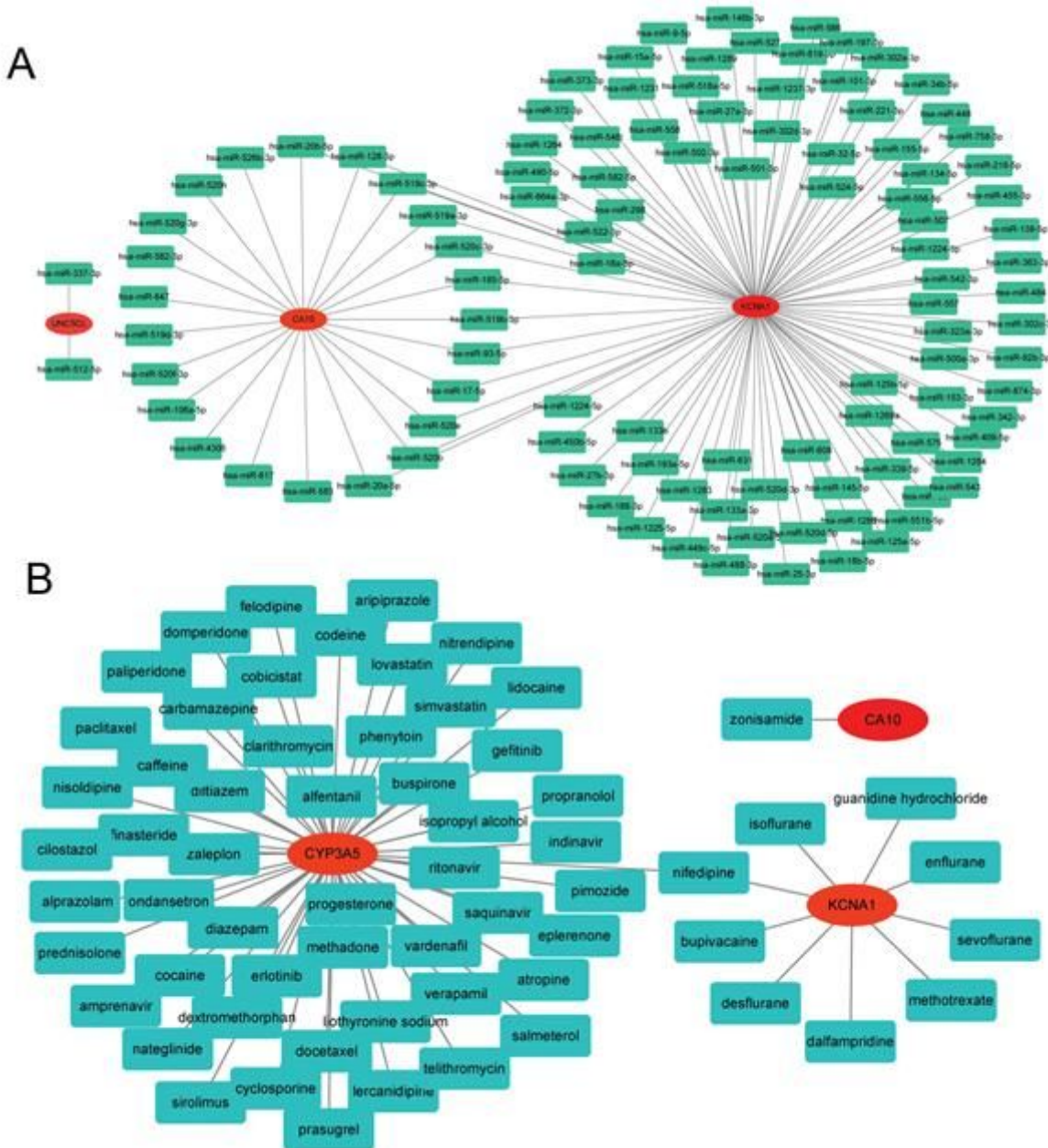
A: Overall genetic mutations in esophageal cancer. B: Analysis of mutations of six genes in esophageal cancer. Red represents the significantly differentially expressed genes, and black represents the top 10 mutations in esophageal cancers.



**Figure 4**

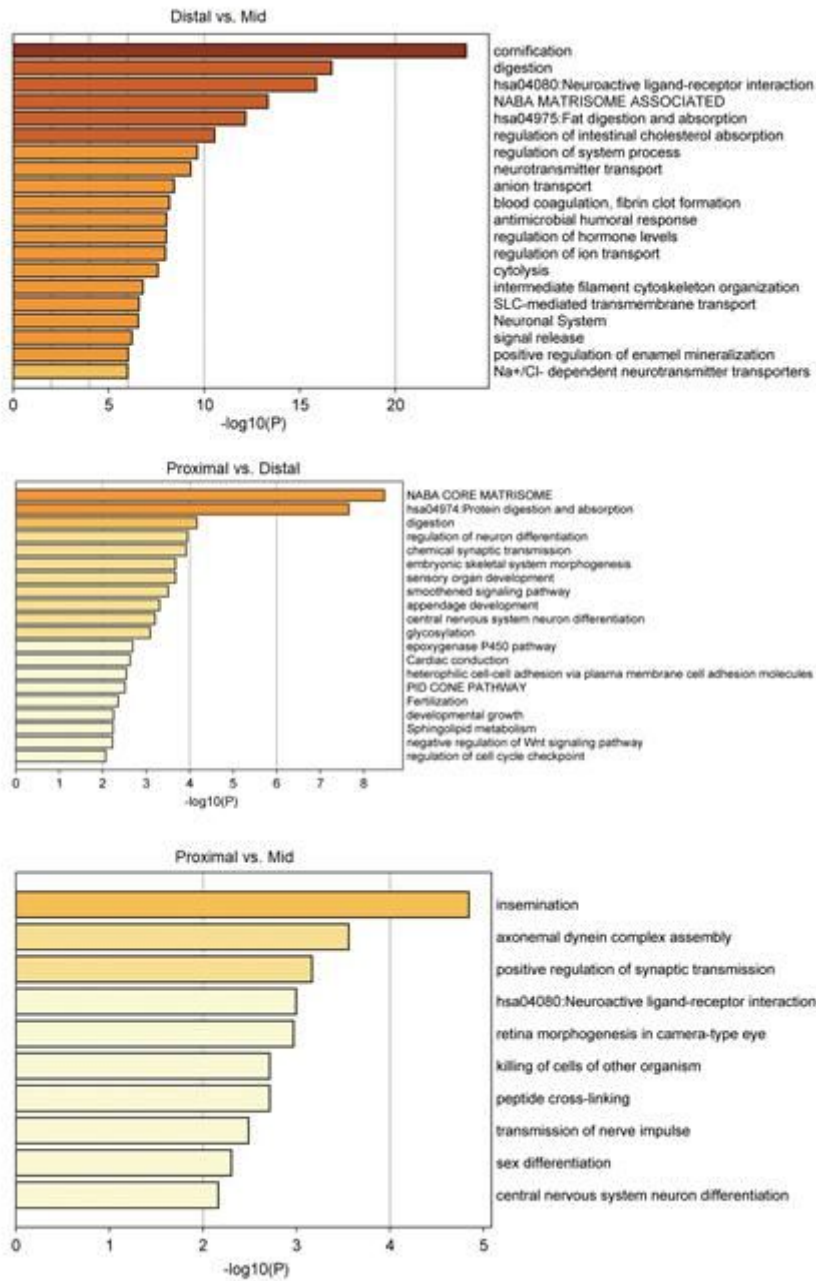
Survival analysis results of CA10 and REG3A in esophageal cancer. The panel on the left is the optimal cutoff value calculated by the survminer, and the panel on the right is the result of survival analysis based on the optimal cutoff.





**Figure 5**

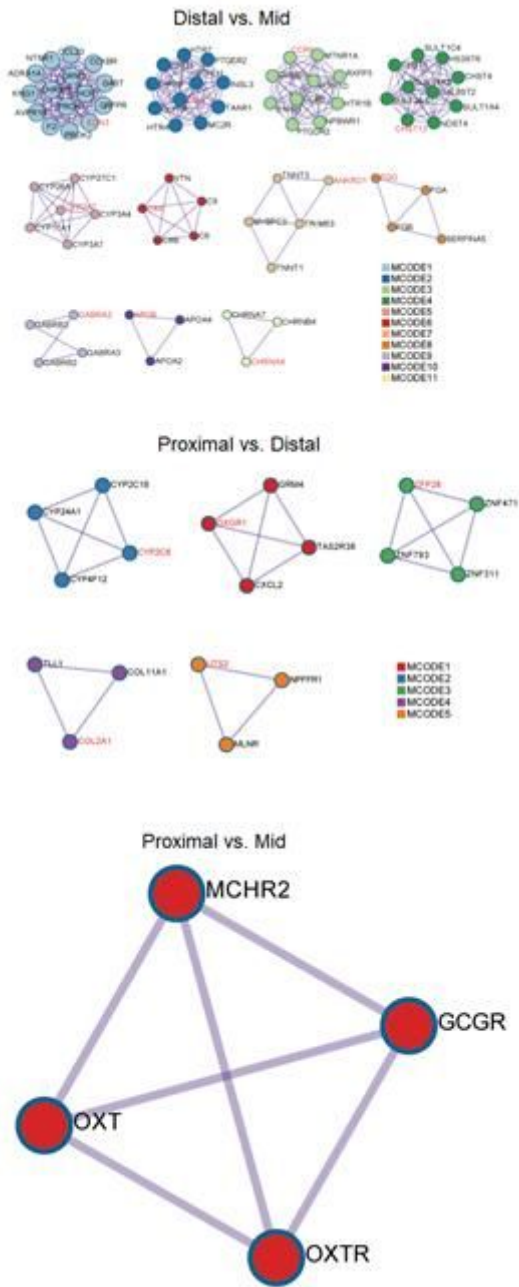
A: miRNA-target prediction results from miRWalk 2.0. Red represents mRNA, and the green represents miRNA. B: Drug-gene prediction results of six common genes. Red and blue represent genes and drugs, respectively.



**Figure 6**

The biological processes and pathways enriched by differentially expressed genes in three comparison groups.





**Figure 7**

The sub-network modules were extracted from protein-protein interaction networks. Gene in red represents the seed gene.