Screening of differentially expressed genes related to esophageal squamous cell carcinoma and functional analysis with DNA microarrays

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Abstract. The aim of this study was to find disease-associated genes and gene functions in esophageal squamous cell carcinoma (ESCC) with DNA microarrays. We downloaded the gene expression profile GSE20347 from the Gene Expression Omnibus database including 17 ESCC and 17 matched normal adjacent tissue samples. Compared with normal samples, the probe level data were pre-processed and the differentially expressed genes (DEGs) were identified (FDR <0.05, and |logFC|>2) with packages in R language. The selected DEGs were further analyzed with bioinformatic methods. After an interaction network of DEGs was constructed by STRING, we selected the most important hub gene through network topological analysis (including node degree, clustering coefficient and path length) and analyzed functions and pathways of the hub gene network. A total of 538 genes were filtered as DEGs between normal and disease samples, and we selected the gene TSPO as the most important hub gene. Among its interactors, the CTSK gene and the IL8 gene participated in the toll-like receptor signaling pathway which is closely related to tumor occurrence. The TSPO gene and its interactors may affect the cancer-specific gene expression by participating in the toll-like receptor signaling pathway. Our discovery may be useful in investigating the complex interacting mechanisms underlying the disease. However, further experiments are still needed to confirm our result.

Introduction

Esophageal cancer is the eighth most common cancer type and the sixth leading cause of cancer mortality worlwide (1). Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type of esophageal cancer. The disease development is affected by multiple factors, so well-designed multidisciplinary epidemiologic study is needed to examine their roles in ESCC risk (2). A wide range of molecular changes is associated with ESCC, possibly because the esophagus is exposed to many kinds of carcinogens including alcohol and cigarette smoke (3). Despite treatment improvements, such as esophagectomy with lymph node dissection (4) and endoscopic submucosal dissection (ESD) (5,6), the first-year survival rate remains low at 10-13% (7). Thus, searching for new therapies for esophageal cancer is urgent.

Esophageal carcinogenesis (EC) is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. Common genetic alterations associated with ESCC progression are epidermal growth factor receptor (EGFR) and cyclin D1 overexpression, dysregulation of p120 catenin, p16Ink4a and expression of p53 missense mutations (8). Moreover, a study has shown that the significant rise of CEA and AFP in serum has a significance on the early diagnosis and prognosis of esophageal cancer, and they can be used as the auxiliary diagnosis index of esophageal cancer in clinic (9). By using immunohistochemistry, high-mobility group AT-hook2 (HMGA2) which was overexpressed in the panel of ESCC cell lines has been found to be upregulated in ESCC tissues (10). With the development of molecular biology and its wide use in cancer research, many scholars have carried out in-depth research on the pathogeny of esophageal cancer from gene level, and a variety of significant genes were found, as shown above. However, the mechanism of the disease has not been clarified. Thus, further study is imperative.

With the emerging technology of DNA microarray, it is now possible to screen for alterations in the expression of many genes simultaneously. DNA microarray analysis as a global approach is applied to investigate physiological mechanisms in health and disease (11). Gene expression profiling using microarrays is a robust and straightforward way to study the molecular features of different types and subtypes of cancer at a system level. Because the development and progression of cancer are accompanied by complex changes in patterns of gene expression, the DNA microarray technology provides a very useful tool for studying these complex processes.

In this study, we aimed to find disease-associated genes and gene functions in esophageal squamous cell carcinoma (ESCC)

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and to gain more insights into the molecular mechanisms of esophageal cancer. Developing the non-invasive esophageal cancer molecular marker detection may provide evidence for early diagnosis and treatment for esophageal cancer.

Materials and methods

Affymetrix microarray data. We extracted the gene expression profiles from GEO (Gene Expression Omnibus) database (ID: GSE20347) (12), which included 17 ESCC and 17 matched normal adjacent tissue samples. Platform information was GPL571 [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array. Annotation information for all probe sets of ATH1 (25K) is provided by Affymetrix. We downloaded the raw data and the probe annotation files for further analysis.

Data preprocessing. The probe-level data in CEL files were converted into expression measures. Then we imputed missing data with k-nearest neighbor (KNN) algorithm (13) and performed quartile data normalization by the robust multiarray average (RMA) algorithm with defaulted parameters in R affy package (14).

Analysis of differentially expressed genes (DEGs). The limma package (15) in R was used to identify differentially expressed genes between matched normal adjacent and ESCC tissues. To circumvent the multi-test problem which might induce too many false positive results, the Benjamin and Hochberg (BH) method was used to adjust the raw P-values into false discovery rate (FDR) (16). The FDR <0.05 and |logFC|>2 were used as the cut-off criterion.

Interaction network construction of DEGs. To further analyze these DEGs, we mapped them to the STRING (Search Tool for the Retrieval of Interacting Genes) database (17) to construct interaction network. STRING which is linked to other databases predicted the target gene interaction in use of the gene characteristic and spatial structure. All associations were provided with a probabilistic confidence score, and the pairs with scores were all selected as interactional ones.

Hub protein screening. The node in the interaction network was analyzed to identify the hub protein under the scale-free property of protein-protein interaction (PPI) networks. From previous obtained PPI networks of other species, we have gained knowledge that most of the biological networks are subject to scale-free network property (18), which means in the network, most nodes have only few connections, and those a few nodes which have a large number of connections are the key nodes (the hub). We selected the hub protein through network topology analysis (19) including node degree (20), clustering coefficient (21) and path length (22).

Interaction network construction of the hub gene. The development of many vital activities is achieved by the combination and dissociation of protein. Various important physiological activities and responses to the external and internal environment of cells are based on interactions between proteins to form a signal transduction network system. Therefore, further study of PPI is the necessary premise to understand the vital phenomenon. Osprey software platform is designed for better study of PPI networks and protein complexes. The software itself integrates with BIND (23) and GRID (24) database, involves protein and nucleic acid sequence, and has a collection of more than 50,000 interactions. In our study, Osprey software (25) was applied to identify the interactors of the hub gene to construct an interaction network. DEGs with the same function in the network were annotated through the annotation function of Osprey connected with GO.

Gene Ontology and pathway enrichment analysis. Gene enrichment analysis evaluates differential expression patterns of gene groups with similar or related functions instead of those of individual genes. This approach especially targets gene groups where constituents show subtle but coordinated biological function or property changes by calculating the whole significance of the gene expression changes, which might not be detected by the usual individual gene analysis (26). In our analysis, p-value represents the probability that the gene in a module was randomly endowed with a GO function, and it is usually a standard to assign the module to a function. Smaller p-value is, the more significant biological significance it shows, which proves that the module does not appear randomly, but in order to complete a specific biological function (27). DAVID software, with built-in rich graphical display, clusters the significant gene collections according to their functions, and it has abundant public database links (28). We clustered the DEGs in the interaction network of the selected hub gene. Then DAVID (28,29) was applied to analyze the GO and KEGG pathway enrichment (FDR <0.05).

Results

Analysis of differentially expressed genes. In order to get differentially expressed genes, we obtained publicly available microarray dataset GSE20347 from GEO. After data preprocessing, we identified genes specifically differentially expressed from the standardized data (Fig. 1). Total 538 genes were selected as DEGs at FDR <0.05 and |logFC|>2, including 320 downregulated genes and 218 upregulated genes.

Interaction network construction of DEGs. We mapped the DEGs to the STRING database to construct an interaction network. STRING which is linked to other databases calculated the combined scores of the DEGs in use of the gene characteristic and spatial structure. The interaction network of the DEGs is revealed in Fig. 2.

Hub gene analysis. Based on network topology analysis, we found that the protein interaction network also had scale-free attributes. Three network topology were, respectively, processed with statistical analysis, as shown in Fig. 3. Graph A shows the node degree distribution in the network, and we obtained y = 57.261 x ^ (0.896) (red line in Fig. 3) with the exponential method. The x axis represents the node degrees, which means the number of nodes directly connected with it, and the y axis represents the node number in different degrees. The property of the node degree distribution shows that both loose-connected nodes and close-connected ones exist in the

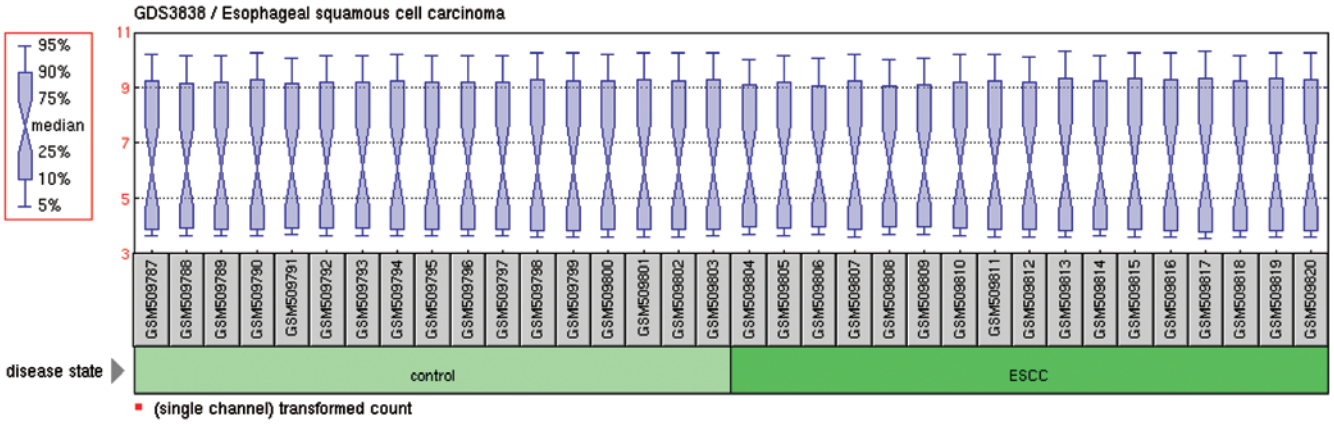

Figure 1. Box graph of the standardized gene expression profile data. The light green columns are adjacent normal samples and the dark green columns are ESCC samples. The concave line in the box is the median of each data group, and the data standardisation degree can be judged by its distribution. That all the concave lines in the figure are almost on the same straight line reveals a good standardization degree.

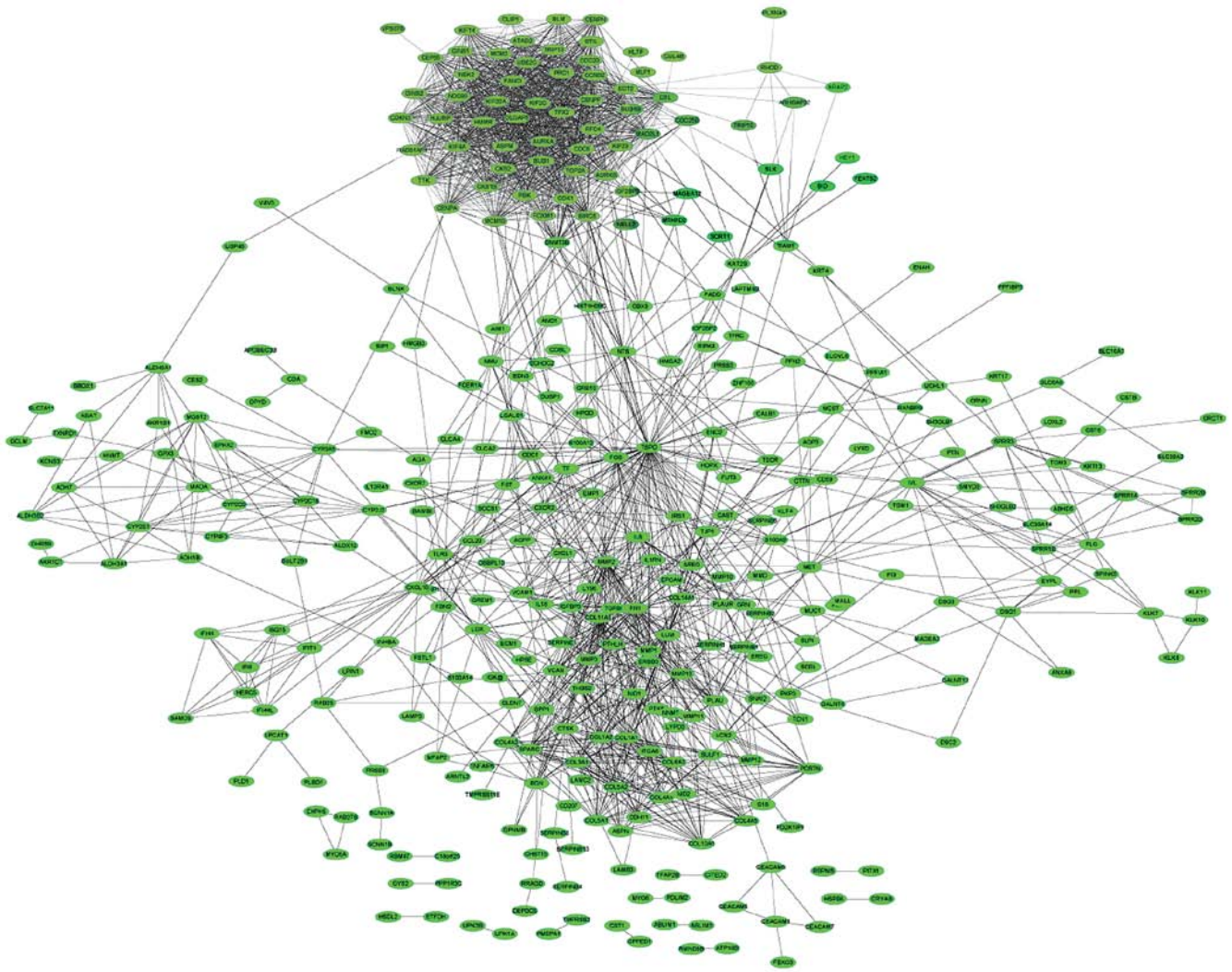

Figure 2. Interaction network of DEGs.

network. Graph B shows the clustering coefficient distribution in the network, and the clustering coefficient is able to display the aggregation degree of nodes. It is seen in the graph that the distribution range of the clustering coefficient is [0, 1]. The nodes with high clustering coefficient are in the minority, and the clustering coefficient distributions mostly locate in the

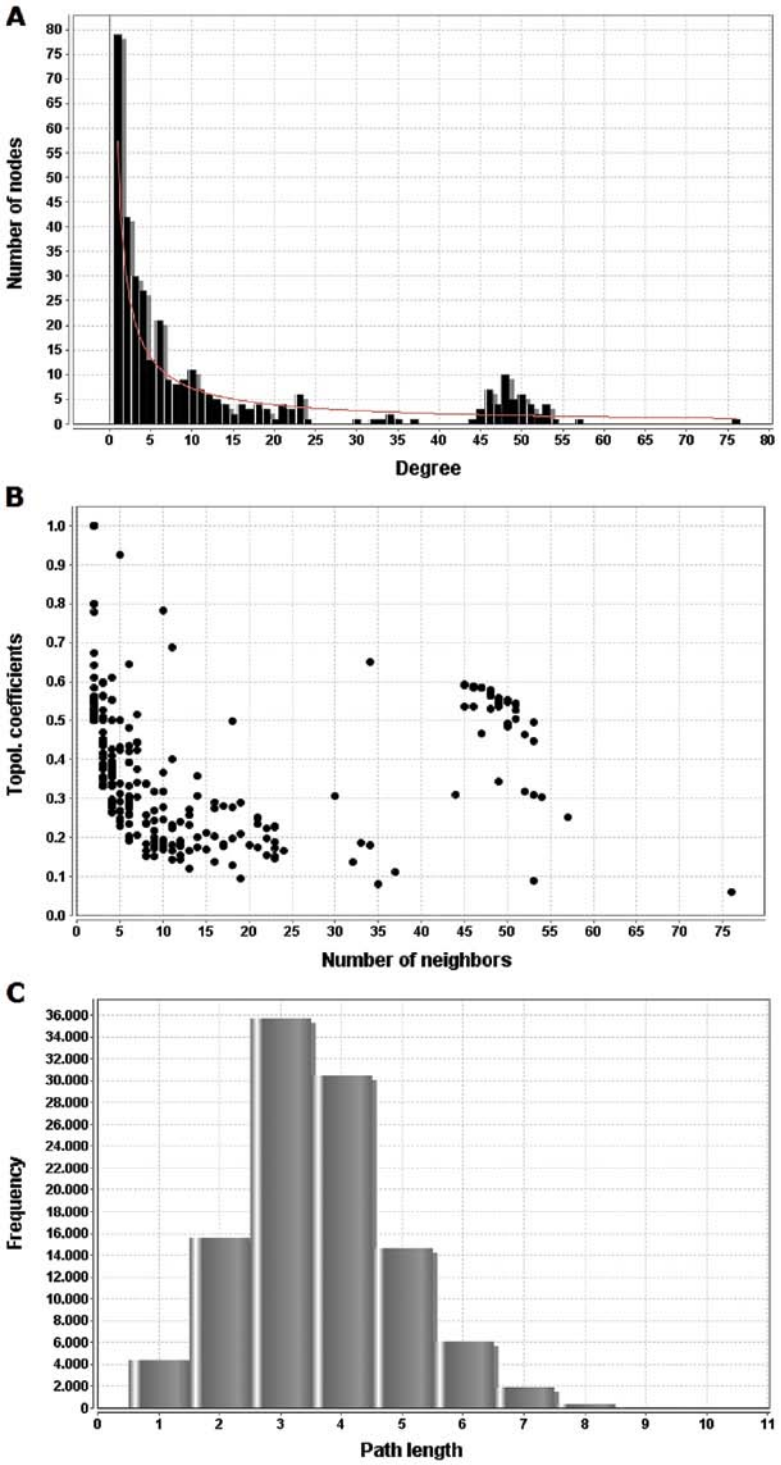

Figure 3. The topology analysis graph of the DEG network. (A) The node degree distribution graph of the network. (B) The clustering coefficient distribution of the network. (C) The path length distribution graph of the network.

area of <0.5. Graph C shows the path length of the network, indicating that the length of most paths was about at 3-4. After analyzing the three topology property parameters of the network, we can conclude the small world effect of network which is subject to scale-free property. Table I shows the degree of distribution of the top ten nodes in the network. We could see that a high degree of the node is relative to the high clustering coefficient, and the highest degree node is the translocator protein (TSPO).

Hub gene network analysis. Osprey software was used to identify the hub genes and the interactors of gene TSPO from all the DEGs, and to construct PPI network. DEGs with the same function in the network were annotated through the annotation function of Osprey connected with GO. As shown in Fig. 4, different colors represent different GO annotation nodes.

Gene Ontology and pathway enrichment analysis. To gain further insights into the functions of genes in our interaction

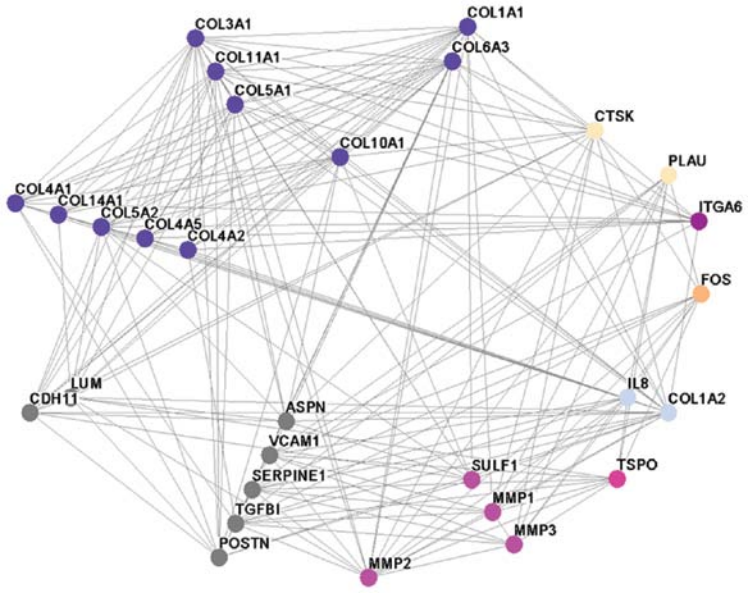

Figure 4. The interaction network of the hub gene TSPO. Different colors represent different GO annotation nodes, and the nodes with the same color have the same GO annotation.

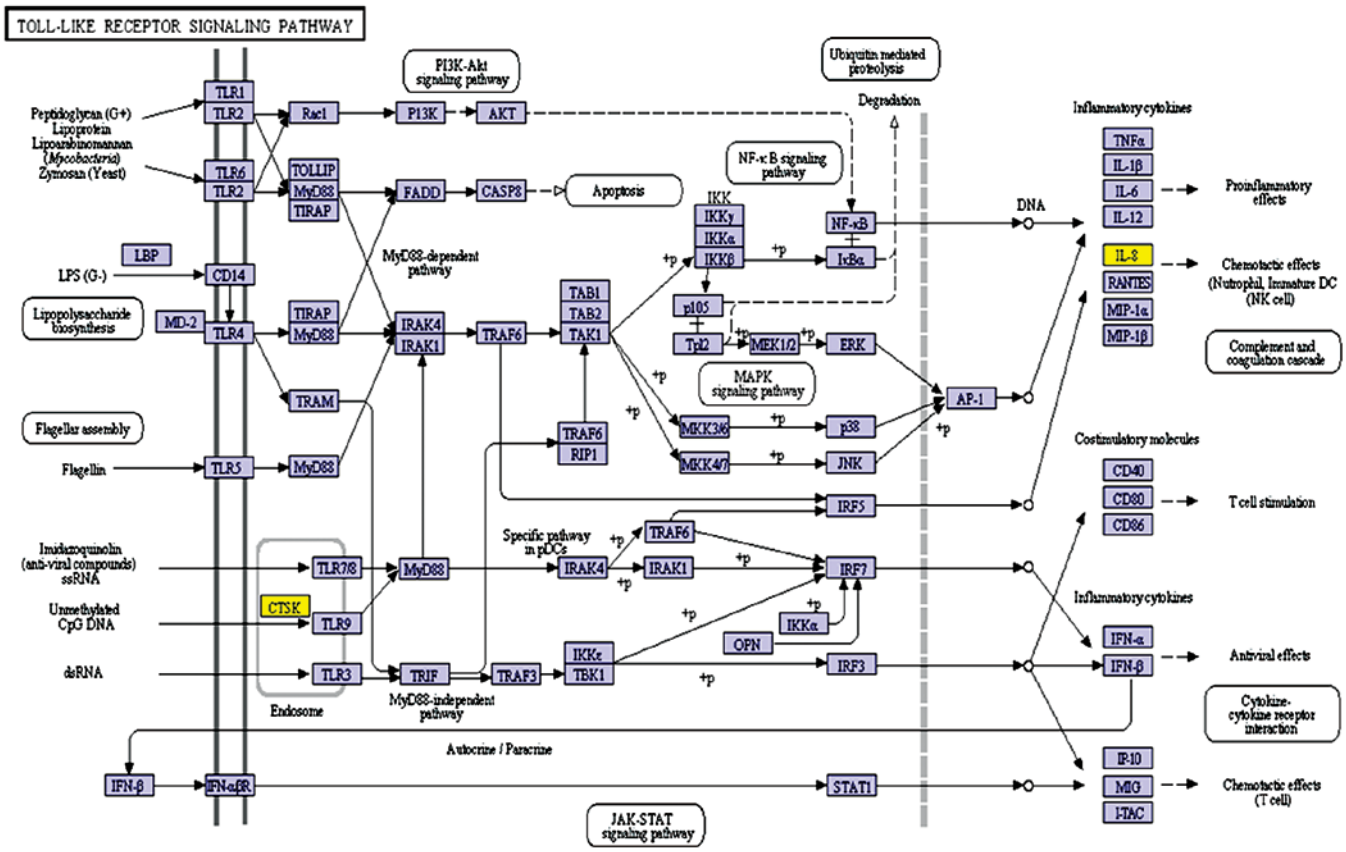

Figure 5. The toll-like receptor signaling pathway. The yellow nodes represent the DEGs in the interaction network of the hub gene.

network, we used DAVID to analyze the significantly enriched GO functional nodes in the network of TSPO, and we finally obtained 12 significant nodes (Table II). Fig. 5 shows the toll-like receptor signaling pathway (FDR <0.05) in which the DEGs

Table I. Degree of distribution of the top ten nodes in the network.

Gene ID	Degree	Clustering coefficient
TSPO	76	0.9631579
BIRC5	57	0.69924812
CDK1	54	0.77987421
BUB1	53	0.81132075
FN1	53	0.20391872
PBK	53	0.80769231
RFC4	53	0.80043541
AURKB	52	0.78733032
CDC6	52	0.83785822
CENPF	51	0.87215686

in the TSPO network participated. In our study, cathepsin K (CTSK) and interleukin 8 (IL8), which were interacted with TSPO, participated in this pathway.

Discussion

Esophageal carcinogenesis (EC) is a multi-stage process and features high rates of mortality and morbidity. Therefore, there is an urgent need to explore the mechanism of EC, and develop an effective prevention strategy. Our study aimed to find specific expression genes and gene functions in esophageal squamous cell carcinoma with a group of ESCC tissue microarray expression data. After network analysis of DEGs, gene TSPO was selected as the hub gene by using network topology analysis. Among its interactors, gene CTSK and IL8 participated in toll-like receptor signaling pathway which is closely related to tumor occurrence.

The TSPO, which was previously called peripheral-type benzodiazepine receptor (PBR), is an 18-kDa protein with 5 transmembrane domains (30). TSPO plays important roles in steroidogenesis, cell proliferation (in both normal and cancerous cells) and apoptosis (31), and its overexpression has been shown to correlate with tumorigenicity in various human malignancies such as brain, breast, colon, and prostate cancer (32). The TSPO has been suggested to interact with a mitochondrial protein complex, and summarized as mitochondrial membrane permeability transition pore (MPTP), which is considered to regulate the mitochondrial membrane potential (33). In addition, it has been found that the mechanism whereby TSPO initiates the mitochondrial apoptosis cascade includes oxidation of cardiolipins i.e. ROS generation at mitochondrial levels (34,35). A recent study shows that TSPO can modulate the activity of this Fo proton pump in the inner mitochondrial membrane (36). From these studies it is hypothesized that enhanced TSPO levels present a mechanism directed to cause programmed cell death, a mechanism that apparently functions to its potential in established cancers (32,34,36,37). Toll-like receptor (Toll-like receptors, TLRs) is a homologous toll protein that has survived the evolution from fruit flies to human. The toll protein plays a key role in the innate immunity process to defend against microbial invasion by identifying pathogen-associated molecular patterns (PAMPs) (38,39), and mediates tumor immunotherapy through TLRs signaling pathway (40). Human esophageal epithelial cells can sense endogenous danger signals, in part through TLR3 signaling (41). Cathepsin K (CTSK) is a novel and unique lysosomal enzyme, for unlike other lysosomal enzymes which show wide tissue distribution and expression, it is highly cell and tissue specific (42). Recently, it was shown that CTSK affected the innate immune response by compromising TLR9 signaling (43) and TLR7-dependent Th17 polarization (44). In addition, interleukin-8 (IL8) is a chemokine that has an autocrine or paracrine tumor-promoting role and significant potential as a prognostic or predictive cancer biomarker (45). Using the cytokine IL8 as a physiological read-out of the inflammatory response, researchers have found that TLR3 is the most functional of the expressed TLRs in both primary and immortalized esophageal epithelial cell lines (46), and suppression of TLR3 signaling leads to reduced IL-8 induction in esophageal epithelial cells (41).

Table II. Gene Ontology enrichment terms in the interaction network of the hub gene.

GO-ID	Description	FDR	Count
30198	Extracellular matrix organization	1.70E-14	11
43062	Extracellular structure organization	8.28E-13	11
1501	Skeletal system development	2.88E-08	10
32501	Multicellular organismal process	1.56E-07	24
48731	System development	1.88E-07	19
48856	Anatomical structure development	7.57E-07	19
7275	Multicellular organismal development	0.000003314	19
48513	Organ development	6.20E-06	15
32502	Developmental process	0.000011089	19
9888	Tissue development	0.000020948	10
9653	Anatomical structure morphogenesis	0.00018728	11
16043	Cellular component organization	0.0059098	13

CTSK can be induced after TLR activation (47), and squamous cell carcinoma (SCC) cells upregulated fibroblastic CTSK expression more potently than normal keratinocytes, which was mainly attributable to SCC-derived IL-1α and IL8 (48). The interaction of gene TSPO and its interactors (CTSK and IL8) may affect the cancer specific gene expression by participating in TLRs signaling pathway.

Reasonable use of chip technology not only makes the analysis of large amounts of biological information quick and easy, but also is helpful to find the key molecular markers associated with esophageal tumor biological behavior accurately. Our discovery may be useful in investigating the complex interacting mechanisms underlying the disease, and provides a new clue and the direction for the early diagnosis of esophageal cancer and treatment. However, further experiments are still needed to confirm our results.

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