doi:10.4149/neo_2018_170507N326

Identification of recurrent risk-related genes and establishment of support vector machine prediction model for gastric cancer

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Received May 7, 2017/ Accepted July 12, 2017

This study sought to investigate genes related to recurrent risk and establish a support vector machine (SVM) classifier for prediction of recurrent risk in gastric cancer (GC). Based on the gene expression profiling dataset GSE26253, feature genes that were significantly associated with survival time and status were screened out. Subsequently, protein-protein interaction (PPI) network was constructed for these feature genes, and genes in this network were optimized using betweenness centrality algorithm in order to identify genes potentially correlated with GC (named as GCGs).

In total, 1202 feature genes were identified to be significantly associated with survival time and status of GC, among which 65 genes were identified as a classifier that was able to recognize recurrence and non-recurrence GC cases with high sensitivity and specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the receiver operating characteristic curve (AUC). Furthermore, the classifier was able to reasonably classify tumor samples in GSE15459 into high and low recurrent risk groups. Among the 65 genes, a set of genes was predicted to have interactions (e.g. *RHOA* interacting with *TGFBR1*, *PRKACA* and *PLCG1*; *TGFBR1* interacting with *TGFBR2*) with each other, and they were found to be involved in some important pathways like MAPK signaling (e.g. *TGFBR1* and *TGFBR2*), adherens junction (e.g. *RHOA*) and apoptosis (e.g. *PRKACA*). The genes in the classifier model may be related to GC recurrence, and the classifier model may contribute to the prediction of recurrent risk in GC.

Key words: gastric cancer, support vector machine, recurrence, gene, network

Gastric cancer (GC) has been existing as the most common cause of cancer death worldwide even since the mid-1990s [1]. Although the mortality has declined over the past several decades, GC still accounts for over 10% of cancer deaths worldwide, especially in Japan, Russia and other countries of the former Soviet Union [2, 3]. High recurrent risk is one of the main causes of the high mortality of GC, which partly results from the histological type, deeper invasion, lymph node metastasis and negative lymph node counts [4].

In the past few years, molecular recurrent risk factors of GC have been revealed. A previous study has reported that multigene methylation in *CHFR* (checkpoint with forkhead and ring finger domains), E-cadherin and *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) are correlated with peritoneal recurrence in GC patients [5]. BMP7 expression is also found to be implicated in tumor recurrence in GC [6]. Furthermore, a set of miRNAs have been reported to be involved in the recurrent risk of GC. Hsa-miR-335 and the combination of hsa-miR-375 and hsa-miR-142-5p have been previously identified as a classifier for recurrent and non-recurrent GC cases [7, 8]. Besides, ectopic expression of miR-196a has been discovered to promote the epithelial-mesenchymal transition (EMT) and migration/invasion capabilities of transfected cells in GC, influencing the recurrence of GC [9]. However, the investigations are still limited to identify the genes that are able to classify the recurrent and non-recurrent GC cases, and more genes involved in the recurrence of GC remain to be discovered.

Support vector machine (SVM) is a novel machine learning method based on statistical learning theory, and it has been used to select genes for cancer classification with expression data [10], such as early detection [11], survival prediction

[12] and classification of cancer types [13]. In this study, in order to identify a gene signature that is able to classify the recurrent and non-recurrent GC cases, SVM algorithm was used to identify recurrent risk-related genes based on two gene expression profiling datasets. The results were expected to enrich the information of molecular mechanisms of GC recurrence, and provide potential novel genes for the prediction of recurrence risk in GC.

Materials and methods

Data acquisition. Gene expression profiling data in the dataset GSE26253 [14] were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) [15]. A total of 432 GC tissue samples from patients who were subjected to curative surgery plus adjuvant chemoradiotherapy were included in this dataset. By comparing the clinical features of all samples, we found that 177 samples were obtained from patients who relapsed after curative resection (named as recurrence samples), and 255 samples were obtained from patients who did not relapse (named as non-recurrence samples). In order to establish the SVM classifier, data of 105 recurrence samples and 155 non-recurrence samples were used as training data, and data of the remaining samples were used as validation data.

Meanwhile, another dataset GSE15459 [16] was also downloaded from GEO, containing the expression data of 200 primary gastric tumor samples. These samples were classified into the groups of high recurrent risk and low recurrent risk using the SVM constructed.

Data preprocessing. The downloaded raw gene expression data were conducted the background correction, quantile normalization, probe summarization and log2-transformation using Robust microarray analysis (RMA) package [17]. The probes that corresponded to the multiple genes were removed, while the mean expression value of multiple probes that corresponded to one gene was defined as the final expression value of the gene.

Identification of feature genes. Based on the recurrence and survival information of all samples, survival analysis was performed using the Surv function in survival package of R [18] . Meanwhile, using the expression value of the total gene set as a variable, Cox regression analysis was performed using the coxph function in survival package [19], in order to screen feature genes that were significantly associated with survival time and status. Here, |coefficient| >1 and p-value <0.05 were set as the cut-off criteria.

Construction of protein-protein interaction (PPI) network. PPIs of the above identified feature genes were searched in the Human Protein Reference Database (HPRD, http://www.hprd.org/), a database with the PPI information of human genes. The genes in HPRD that interacted with at least 5 feature genes were collected along with the interactions to construct the PPI network of feature genes, which was visualized by the Cytoscape software (http://www.cytoscape.org/) [20]. In the network, gene (or protein) was presented by node, while the interactions were presented by lines. The degree of a node was the number of nodes that are interacting with it.

Optimization of feature genes in the PPI network. In order to identify the feature genes that significantly correlated with GC (named as GCGs), betweenness centrality (BC) algorithm [21] was applied to calculate the significance of nodes in the PPI network. The formula was as follows:

$$C_{B(v)} = \sum_{t \neq v \neq \mu \in V} \frac{\sigma_{st(v)}}{\sigma_{st}}$$

where σ_{st} represents the number of the shortest routes from s to t; $\sigma_{st(v)}$ represents the number of nodes (v) in the shortest routes from s to t. The $C_{B(v)}$ value was from 0 to 1, and bigger $C_{B(v)}$ value means higher significance of the node. The top 100 genes with higher $C_{B(v)}$ value were chosen for further analysis.

Two-way clustering analysis between expressions of the top 100 genes and tissue samples was performed. Pearson Correlation Coefficient was calculated to compare the expression similarity between two samples, after that the expression similarity matrix of samples was generated. Both, two-way clustering and expression similarity matrix were visualized by heatmap software [22].

Establishment of classification model using SVM. Based on expression of GCGs, a support vector mechanism (SVM) was constructed to classify the tissue samples by calculating the probability with which samples belonged to a certain category. Five indexes including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the receiver operating characteristic (ROC) curve (AUC) were used to evaluate the classification efficacy.

Pathway enrichment analysis. The GCGs were mapped to pathway terms in the KEGG PATHWAY database (http://www.kegg.jp/kegg/pathway.html). The p-value of each pathway term was calculated by the Fisher's exact test. The calculation formula is as follows:

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the total number of genes enriched in all pathway terms; n is the number of GCGs enriched in all pathway terms; M is the number of genes in a certain pathway term; and m is the number of GCGs in a certain pathway term.

Results

Identified feature genes. Cox regression analysis identified a total of 1202 feature genes that were significantly associated with survival time and status. Among them, 619 genes were negatively associated with survival time, and 583 genes were positively correlated with survival time.

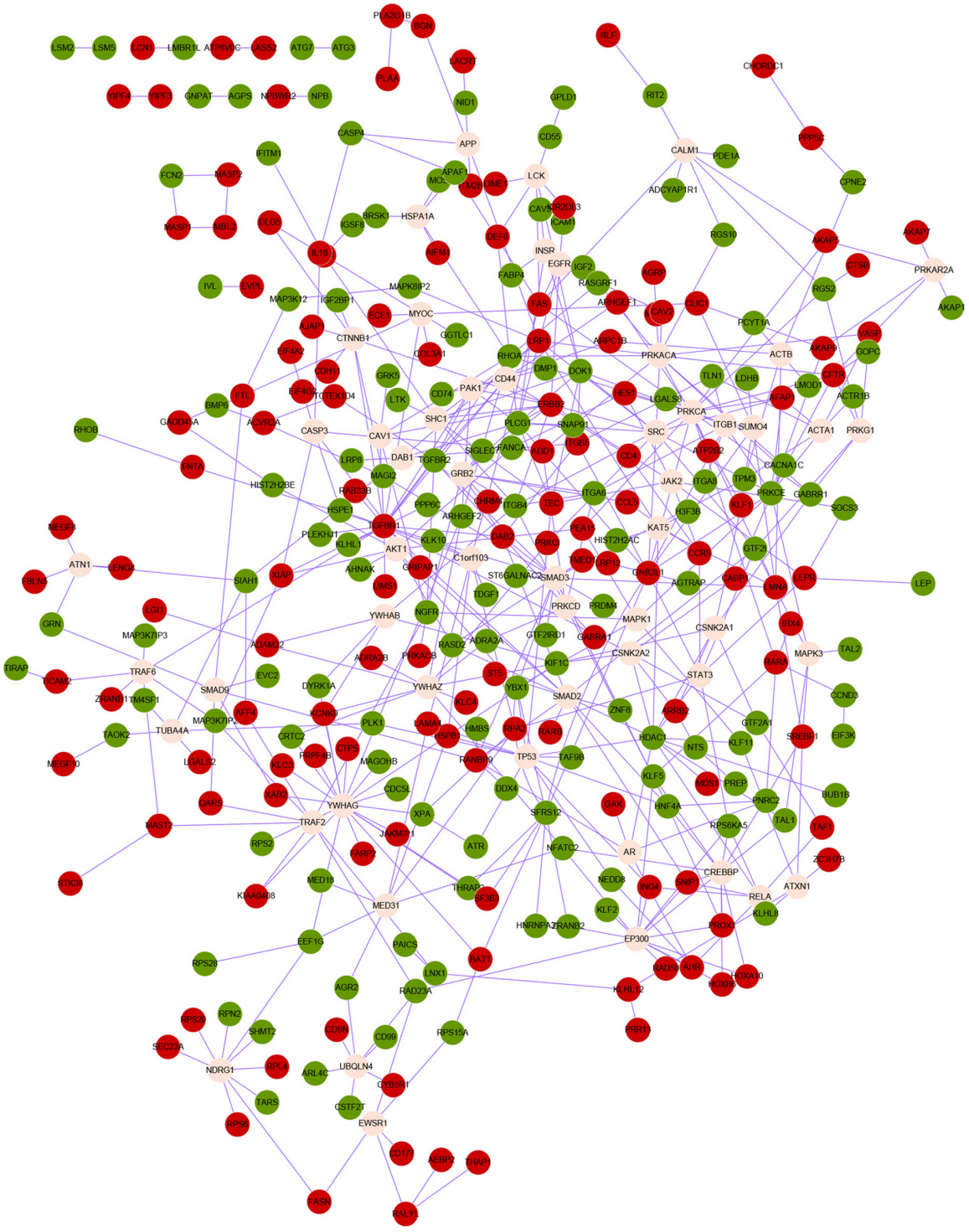

Figure 1. The protein-protein interaction network of feature genes. Red nodes represent the feature genes that are positively correlated with survival time; green nodes represent genes that are negatively associated with survival time; light pink nodes represent the genes that interact with at least 5 feature genes in the Human Protein Reference Database.

Analysis of PPI network. The PPI network of feature genes was comprised of 334 nodes (genes) and 519 lines (interactions) (Figure 1). In the network, most of the nodes had low degrees, and only 15 nodes had a degree of no less than 10, such as *TGFBR1* (degree=20), *YWHAG* (degree=18), *PRKCA* (degree=15) and *SMAD3* (degree=15). Among these

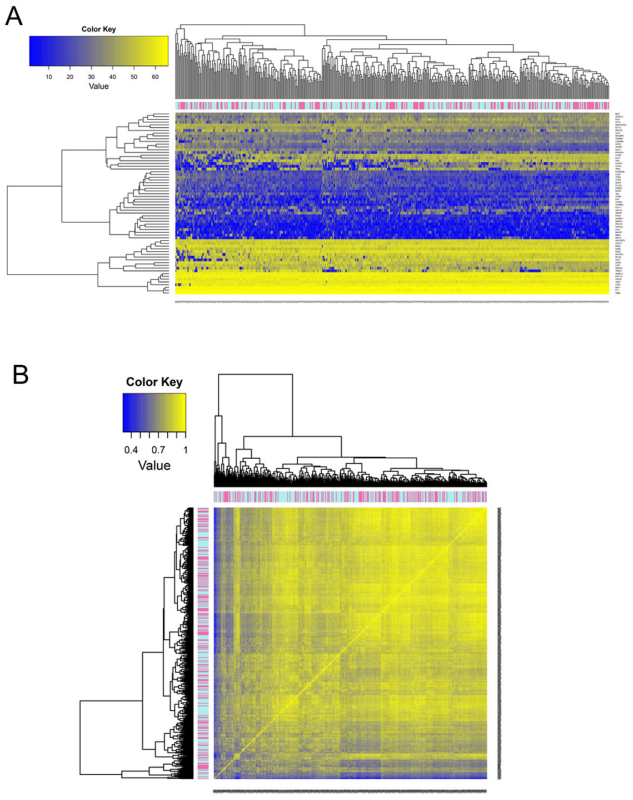

Figure 2. The tree diagram displaying two-way clustering of the 65 feature genes in recurrence and non-recurrence samples (A), and the expression similarity matrix displaying a higher expression similarity of the samples (B). The higher the expression similarity is, the closer to yellow the color is. By contrast, the color is closer to blue. Red bars represent the samples with better prognosis, and the cyan bars represent the samples with worse prognosis.

genes, *TGFBR1* interacted with *TGFBR2*; *RHOA* interacted with *TGFBR1*, *PRKACA* and *PLCG1*.

Identification of GCGs. In order to find out the potential GC-related genes, BC algorithm was used to calculate the significance of genes in the PPI network. In the top 100 genes with high significance, 65 were feature genes, which were identified as GCGs. The top 10 ones (e.g. *TGFBR1*, *MBL2* and *MASP1*) are shown in Table S1. The remaining 35 genes among the top 100 genes were the ones that were interacting with at least 5 feature genes in the PPI network.

According to the two-way clustering, the 65 GCGs were clustered into three classes (Figure 2A). Besides, expression similarity matrix showed that the majority of samples exhibited a high expression similarity (Figure 2B).

Sample classification using the GCGs. Based on the expression of the 65 GCGs, the established SVM classifier was able to identify 146 non-recurrence samples from the 150 in the training set (94.19%), and 94 recurrence samples from the 105 ones (89.52%). The classification accuracy reached 92.31%.

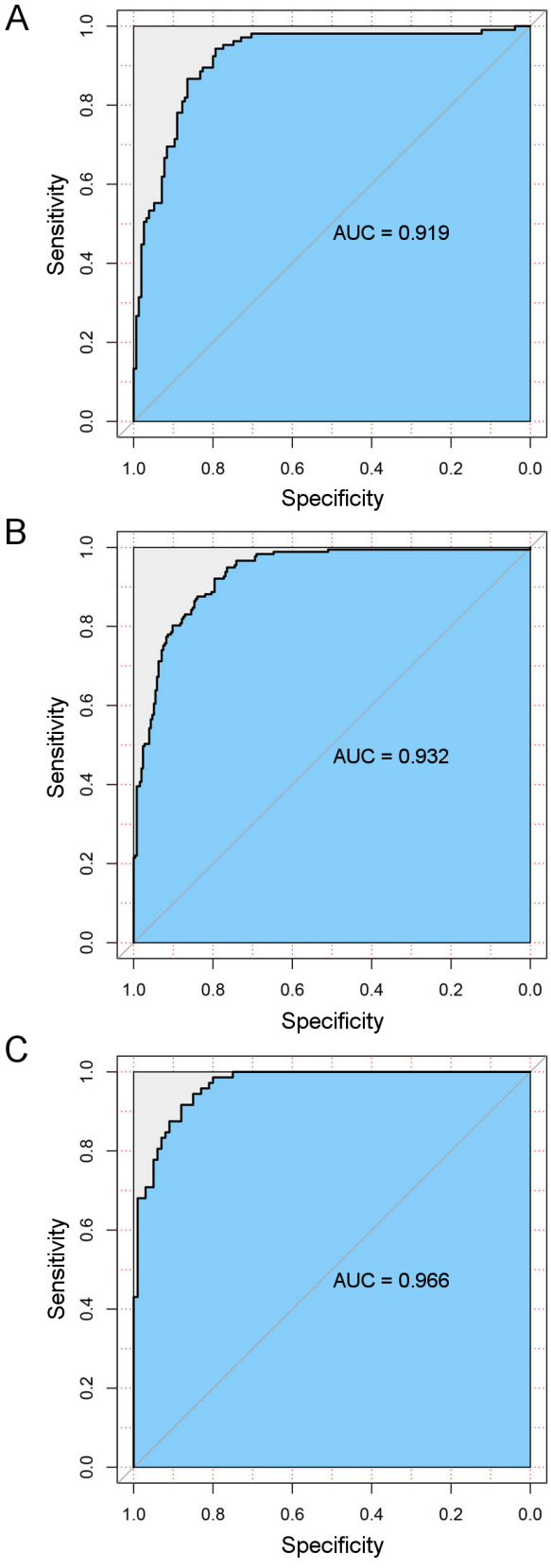

Figure 3. The AUC of training set, testing set and combined set.

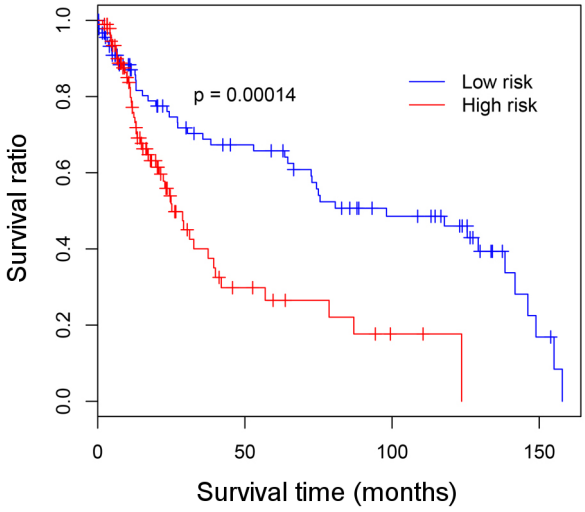

Figure 4. Survival curve with high and low recurrent risk classified by the SVM classifier.

In order to verify the repeatability and portability of the established SVM classifier, the remaining 72 recurrence samples and 100 non-recurrence samples in the validation set were classified by the SVM classifier. The 69 recurrence samples and 96 non-recurrence samples were identified and the accuracy was 95.93%. Collectively, the SVM classifier was able to identify 163 recurrence samples (94+69) and 242 non-recurrence samples, and the total accuracy reached 93.75% (405/432).

Additionally, in order to evaluate the classification results, five indexes (sensitivity, specificity, PPV, NPV and AUC) were assessed. The total sensitivity and specificity reached 95.0% and 97.2%, respectively. Meanwhile, the total PPV and NPV was 96.1% and 96.5%, respectively (Table S2). The AUC of training set and testing set was 91.9% and 93.2%, respectively (Figure 3A and B), and the total AUC was 96.6% (Figure 3C). These results indicate that the established SVM classifier has a reliable classification function.

Validation of classification effect of the SVM classifier. Another dataset GSE15459 was used to validate the classification effect of the SVM classifier, which contained 200 GC samples. After excluding 8 samples for failed quality control, 192 GC samples were included for further analysis. The groups of high recurrent risk and low recurrent risk in this dataset were classified by the SVM classifier. A total of 102 samples were recognized as high recurrent risk samples, while 90 samples were defined as low recurrent risk samples. Survival curve showed that the survival ratio of the samples with high recurrent risk was significantly lower than that of samples with low recurrent risk (p=0.00014, Figure 4). This result suggests that the SVM classifier is able to reasonably classify tumor samples according to recurrent risk, which is a critical factor for survival time of GC patients.

Enrichment analysis of the 65 GCGs. In order to reveal the potential functions of the 65 GCGs, the KEGG pathway enrichment analysis of the GCGs was performed. In total, 11 pathways were significantly enriched by the 65 GCGs. A set of 11 GCGs were associated with the MAPK (mitogen-activated protein kinase) signaling pathway, such as *TGFBR1* and *TGFBR2*. Other GCGs were significantly implicated in the pathways like adherens junction (e.g. *RHOA*, *TGFBR1* and *TGFBR2*) and apoptosis (e.g. *PRKACA*) (Table S3).

Discussion

In the present study, a total of 1202 feature genes that were significantly associated with survival time and status of GC were identified. A 65 gene classifier was obtained by constructing the PPI network of feature genes and conducting the BC algorithm to recognize the recurrence and non-recurrence GC cases. The established gene classifier had a high sensitivity and specificity, PPV, NPV and AUC. Additionally, according to the KEGG pathway enrichment analysis of the 65 GCGs, genes were significantly associated with 11 pathways like MAPK signaling pathway, adherens junction and apoptosis.

In the PPI network, *TGFBR1* had the highest degree (n=20), and it interacted with genes like *TGFBR2* and *RHOA*, both of which were predicted to be related to the pathway of adherens junction. Both *TGFBR1* and *TGFBR2* encode receptors of transforming growth factor beta (TGF-β), and the receptor/ligand complex phosphorylates proteins which regulate the transcription of genes related to cell proliferation [23]. A recent study reported *TGFBR2* as a cancer driver gene in diffuse GC, and *TGFBR2* knockdown promotes tumor metastasis and invasion, which increases the metastatic recurrence risk [24]. In recurrent breast tumors, mutations of TGFBR2 have been detected [25]. Besides, reduced *TGFBR2* expression results in intrahepatic metastasis and shorter recurrence-free survival in hepatocellular carcinoma [26]. These studies indicate the negative association of *TGFBR2* with cancer recurrence. Although there is no other evidence to prove the relationship between *TGFBR1* and GC recurrence, genetic variations of *TGFBR1* have been found to be associated with risk of aggressive prostate cancer and biochemical recurrence [27], as well as the risk and prognosis of muscle-invasive bladder tumors [28]. Therefore, we speculated that *TGFBR1* may be also relevant to GC recurrence. Furthermore, both *TGFBR1* and *TGFBR2* were predicted to be related to the MAPK signaling pathway. The associations of these two genes with the MAPK signaling pathway have been previously reported [29]. Besides, *TGFBR1*6A* variant has been found to enhance MCF-7 breast cancer cell migration and invasion through RhoA and MAPK pathway activation [30]. Collectively, *TGFBR1* and *TGFBR2* may influence the recurrence of GC by interrupting the MAPK signaling pathway.

RhoA (Ras homolog family member A) protein is a member of the Rho family of small GTPases and is involved

in tumor cell proliferation and metastasis [31]. In human GC cells, overexpression of *RhoA* is highly correlated with aggressive lymph node metastasis and poorer survival, and inhibition of *RhoA* expression effectively decreases the cell invasiveness [32]. Study has discovered that *RhoA* activation is able to facilitate lysophosphatidic acid-induced cell migration and invasion in GC [33]. As a result, *RhoA* may participate in the GC recurrence, along with *TGFBR1* and *TGFBR2*.

Beside the interaction with *TGFBR1* and *TGFBR2*, *RhoA* also interacted with other GCGs, such as *PRKACA* and *PLCG1* in the PPI network. *PRKACA* encodes one of the catalytic subunits of protein kinase A and participates in anti-apoptotic signaling [34], which supports the result that *PRKACA* was enriched in the apoptosis pathway. High expression of *PLCG1* (Phospholipase C, gamma 1) has been previously found to be associated with a worse clinical outcome in terms of incidence of distant metastases in breast cancer [35]. Although there is no any experimental evidence to prove the correlations of *PRKACA* and *PLCG1* with GC recurrence, we speculated that they might be related to GC recurrence via its interaction with *RhoA*.

In conclusion, using SVM, 65 genes significantly associated with survival time and status of GC were identified as a classifier that was able to recognize recurrence and non-recurrence GC cases. Among those genes, a set of genes was predicted to have interactions (e.g. *RhoA* interacting with *TGFBR1*, *PRKACA* and *PLCG1*; *TGFBR1* interacting with *TGFBR2*) and be involved in pathways like MAPK signaling (e.g. *TGFBR1* and *TGFBR2*), adherens junction (e.g. *RhoA*) and apoptosis (e.g. *PRKACA*). Additionally, *TGFBR1*, *PRKACA* and *PLCG1* were firstly found to be potentially correlated with GC recurrence, which will be further confirmed in animal models. The classification role of the SVM classifier will also be validated in the clinic in our future study.

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