Original Article

Identification of *RRAS* gene related to nasopharyngeal carcinoma based on pathway and network-based analyses

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Background: Nasopharyngeal carcinoma (NPC) is a highly aggressive neoplasm mainly distributed in the eastern and southeastern parts of Asia. NPC has a poor prognosis among head and neck cancers, and molecular-targeted therapies showed limited clinical efficacy.

Methods: We reviewed publications in the PubMed database and extracted genes associated with NPC. The online tool WebGestalt was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for these genes. Next, the two parameters, Jaccard Coefficient (JC) and Overlap Coefficient (OC), were used to analyze the crosstalk of each pair of selected pathways. The new NPC-related genes were predicted by protein-protein interaction (PPI) network combined with hub genes extraction. Western blotting, qRT-PCR, and immunohistochemistry (IHC) were used to detect the expression of candidate genes in NPC cells and tissues, and cellular function assays were used to explore the effects of genes on NPC cells.

Results: A total of 552 genes were identified and used to build an NPC-related gene set (NPCgset). Pathways enriched in KEGG pathway analysis were further used for crosstalk analysis and were grouped into two modules: one was related to the carcinogenesis process, and the other was correlated with the immune response. Eight genes from the NPCgset were selected to build a PPI network, and two hub genes *PIK3CA* and *AKT1* were chosen. Proteins interacting with *PIK3CA* were analyzed; among them, the expression of RRAS was down-regulated in NPC and associated with poor prognosis of NPC patients. Furthermore, RRAS suppressed proliferation, invasion and the epithelial-mesenchymal transformation (EMT) of the HK1 and 5-8F cell lines.

Conclusions: Our study may help to explore the biological processes underlying NPCgset and suggests that RRAS may act as a tumor suppressor gene in NPC.

Keywords: Nasopharyngeal carcinoma (NPC); functional enrichment analysis; pathway crosstalk; hub gene; RRAS

Introduction

Nasopharyngeal carcinoma (NPC), which occurs primarily in the nasopharynx epithelium, is mainly distributed in the eastern and southeastern parts of Asia, south-central Asia, and northern and eastern Africa (1,2). As a highly aggressive neoplasm, the prognosis of NPC patients remains poor, and approximately 15% of patients develop distant metastasis (2).

Genetic predisposition, salted fish consumption, and Epstein-Barr virus (EBV) infection have been proven to be important risk factors for NPC (1,3). Although some biomarkers and signaling pathways have been identified in NPC, it is necessary to conduct a comprehensive analysis of genes that play an important role in NPC carcinogenesis.

RRAS is a Ras-related GTPase (4,5). The human *RRAS* gene is located on chromosome 19q13.3 and was detected by low-stringency hybridization using a v-H-ras probe (6). RRAS was reported to be involved in several cell functions, including the enhancement of integrin function, regulation of cell adhesion, invasion and migration (7-9). The role of RRAS has been reported in breast cancer, colorectal cancer, and melanoma, but it has not been studied in nasopharyngeal cancer (6,7,10).

In this study, we constructed an NPC-related gene set (NPCgset). Kyoto Encyclopedia of Genes and Genomes (KEGG) and pathway crosstalk analyses were used to reveal the signaling pathway modules in NPC. Then, we constructed two protein-protein interaction (PPI) networks to find new genes associated with NPC. Combined with the results of experiments using NPC cell lines and clinical samples, we found that RRAS was down-regulated in NPC and might be a new potential biomarker.

Methods

Identification of NPC-related genes

The online database PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) was retrieved by two independent researchers with the following terms: ("Nasopharyngeal carcinoma") and ("Genotype"[Mesh] OR "Genetic Association Studies"[Mesh]). Next, we reviewed these publications and narrowed the scope of the study by the following criteria: (I) biological experiments must validate genes associated with NPC or have clinical significance; (II) non-coding genes were excluded; (III) genes predicted by bioinformatics methods alone were excluded; (IV) the studies reporting insignificant associations were excluded. The selected genes were used for bioinformatics analysis.

Enrichment analysis for NPC-related genes

GO, and KEGG pathway enrichment analysis was completed by WebGestalt (http://www.webgestalt.org/option.php), which is an online tool that incorporates information from different resources to perform multiple analysis such as GO and KEGG on a given gene list. For both analysis, FDR <0.05 was considered to be significant.

Pathway crosstalk analysis

The candidate pathways obtained from KEGG analysis were used for pathway crosstalk analysis. We introduced two parameters to explore the interaction between each pair of pathways, i.e., the Jaccard Coefficient (JC) = $|(A \cap B)/(A \cup B)|$ and the Overlap Coefficient (OC) = $|A \cap B|/\min(|A|,|B|)$. The average scores of the JC and OC were calculated as grades to rank the pathway pairs. Pathways containing less than ten genes were not included. Pathway pairs with mean values of JC and OC less than 0.5 were removed. The results of pathway crosstalk analysis were visualized using the software Cytoscape.

PPI network construction

The PPI network of candidate genes was constructed using the STRING database (http://string-db.org), and the combined score >0.4 was used as the cut-off criterion. Cytoscape was used to visualize the PPI network, and the hub genes of the network were selected according to the degree that calculated by plugin CytoHubba.

Patients and samples

Thirty-one cases of fresh tissues, including 16 NPC and 15 nasopharyngitis tissues were collected from Sun Yat-sen University Cancer Centre (SYSUCC), and total RNA was extracted for the qRT-PCR assay. Paraffin-embedded specimens from 65 primary NPC patients, including 50 males (77%) and 15 females (23%), were collected for the immunohistochemistry (IHC) assay. The median age of the patients was 45 years with a range from 10 to 66. None of the patients had received radiotherapy or chemotherapy before sampling. The Research Ethics Committee of SYSUCC approved our research, and all patients signed informed consent forms.

Cell lines and transfections

The human NPC cell lines 5-8F, 6-10B and HK1 were maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, China), C666-1 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, China), and they were all supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, South America). NP69, an immortalized nasopharyngeal epithelium cell line, was grown in defined K-SFM medium supplemented with EGF and bovine pituitary extract (Gibco, Thermo Fisher Scientific, USA). These cell lines were cultured in an atmosphere of 5% CO_2 at 37 ℃. siRNA duplexes against RRAS and the negative control (NC) were purchased from GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen, USA) as described previously (11).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, USA) following the manufacturer's protocol as described previously (11). The primer sequences for the qRT-PCR assays were as follows: RRAS sense: 5'-TGTCTGACTACGACCCCACT-3'; RRAS anti-sense: 5'-GCCCACCTCGTTGAAACTCT-3'; IRS2 sense: 5'-GCCACCATCGTGAAAGAGTGA-3'; IRS2 anti-sense: 5'-CCATCCGGGAACAAGGGAAA-3'; GAPDH sense: 5'-CTCCTCCTGTTCGACAGTCAGC-3'; GAPDH anti-sense: 5'-CCCAATACGACCAAATCCGTT-3'.

IHC staining

The IHC assay was performed using GTVision III Detection System/Mo &Rb [Gene Tech (Shanghai) Co., Ltd., China], following the manufacturer's instruction, and the results were scored by two evaluators independently. The percentage of positive cells was graded as 0 (≤10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%), and the intensities were graded as negative [0], weakly positive [1], moderately positive [2] and strongly positive [3]. The multiplied intensity score and percentage score was used as the total IHC score.

Western blotting

Proteins were harvested from cell lines by the Whole Cell Lysis Assay Kit (Nanjing KeyGen Biotech Co.,

Ltd., Nanjing, China) according to the manufacturer's instructions. The concentration of total protein was quantified by the BCA assay kit (Beyotime Institute of Biotechnology) and Gen5 Software (CHS 2.06; BioTek Instruments, Winooski, VT, USA). 10% SDS-PAGE and PVDF membranes (GE Healthcare Life Sciences, UK) were used to separate and transfer the protein. Next, the membranes were incubated with primary antibodies, including E-cadherin (1:1,000; CST; cat. no. 3195), N-cadherin (1:1,000; CST; cat. no. 13116), Vimentin (1:1,000; CST; cat. no. 5741), RRAS (Proteintech Group Inc.; cat. no. 27457-1-AP) and GAPDH (1:1,000; CST; cat. no. 5174) at 4 ℃ overnight. Secondary antibodies (HRP-linked anti-rabbit IgG; 1:5,000; CST, cat. no. 7074) were subsequently incubated with the membranes for 1 hour at room temperature. The protein signals were detected using an electrochemiluminescence (ECL) system (Tanon, China).

CCK8 proliferation assay

The CCK8 Cell Counting Kit (JingXin Biological Technology, Guangzhou, China) was used to detect cell proliferation according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (SpectraMax® M5 Multi-Mode Microplate Reader; Molecular Devices LLC, Sunnyvale, CA, USA).

Colony formation assays

HK1 cells with different treatments were seeded in six-well plates (1,000 cells per well) and were cultured for two weeks. Colonies were fixed with methanol for 10 min and then were stained with 0.5% crystal violet (Weijia Biology Science and Technology Co., Ltd) for 10 min. The colonies were then counted under a dissection microscope.

Invasion assays

Cells were counted and diluted with serum-free RPMI-1640 medium to 5×10^4 cells/200 μL. Next, the cell suspensions were added to Matrigel-coated chambers (BD Biosciences), which were inserted into a 24-well plate with 10% FBS in RPMI-1640 medium (700 μL/well). The chambers were removed 36 hours later, and the cells left in the upper chambers were swabbed. The invasive cells in five random fields per well were counted under the microscope.
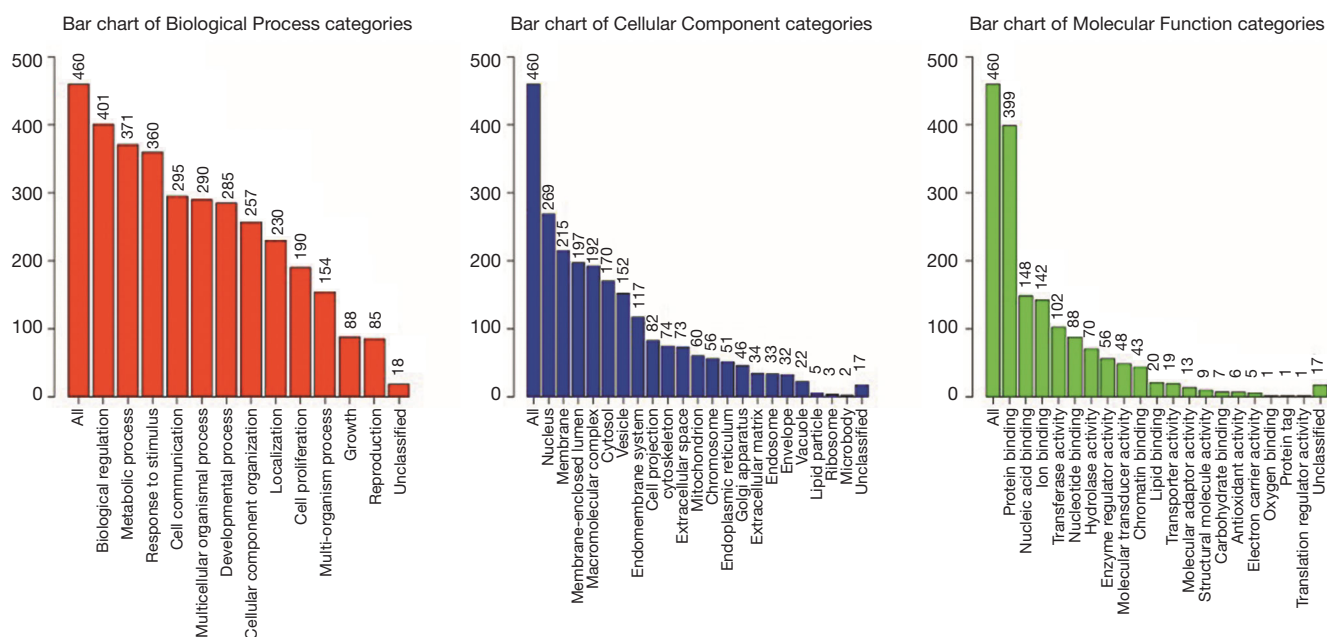
Figure 1 Significantly enriched GO terms. GO, Gene Ontology.

Statistical analysis

Statistical analysis was conducted using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Repeated measures ANOVA were used to analyze the results of CCK8. Student's *t*-test tested the differences between the two groups in other cell function experiments. Kaplan-Meier analysis and the log-rank test were used to explore the relationship between RRAS expression and overall survival (OS) or progression-free survival (PFS). χ^2-test analyzed the correlation between RRAS expression and clinicopathological features of NPC patients. P values less than 0.05 were considered to indicate statistical significance. The Cox regression analysis was used to analyze the association between the expression of RRAS and OS after adjusting for known important clinicopathologic features, and P values less than 0.1 were considered to indicate statistical significance.
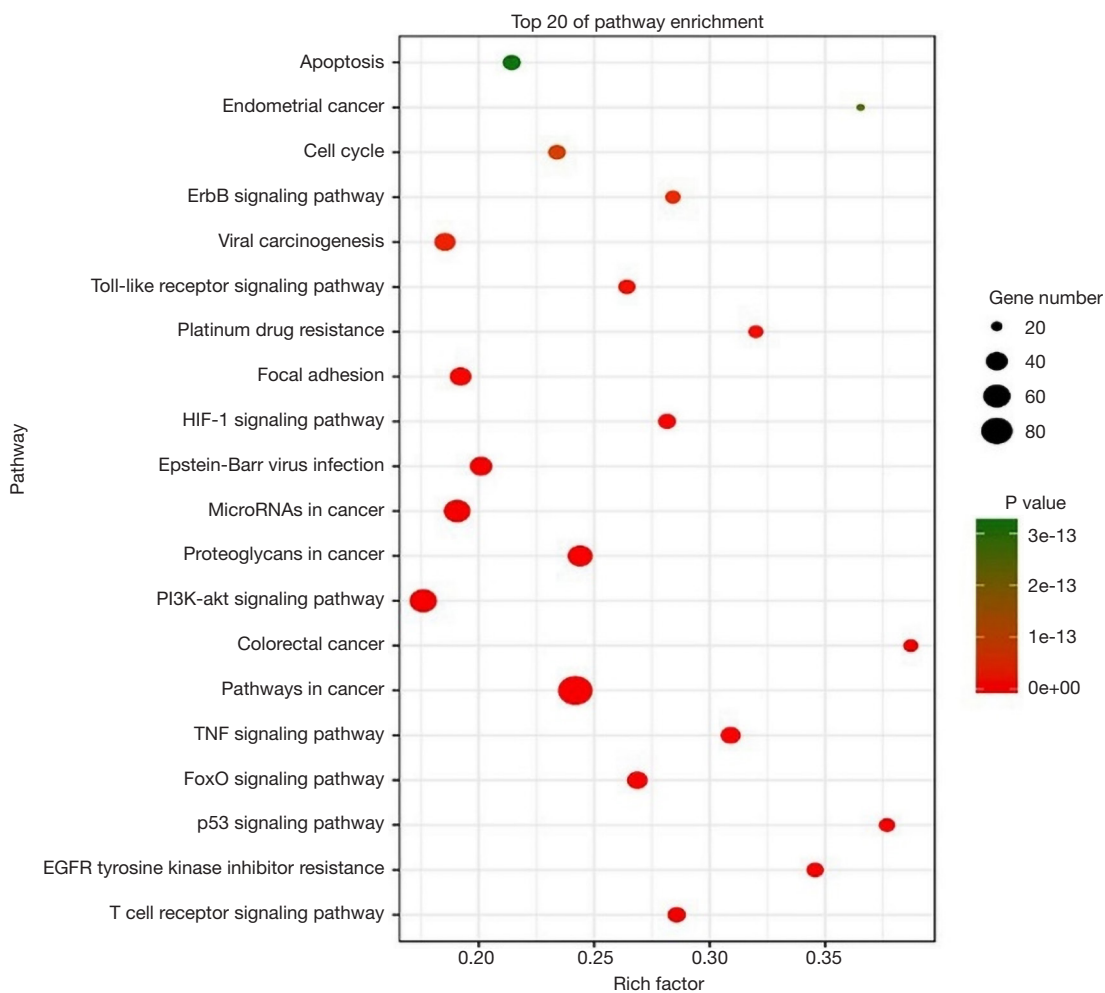
Results

Identification of genes associated with NPC

By October 10, 2018, 2,799 publications were retrieved in the PubMed database, and 531 of them were selected after a review of the abstracts. We reviewed these publications for details and an NPC-related gene set (NPCgset) with 552 members from 514 reports was constructed. The details of all genes in the gene set are provided in http://fp.amegroups.cn/cms/tcr.2019.04.04-1.pdf.

Gene Ontology (GO) and pathway enrichment analysis in the NPCgset

Our NPCgset contained 552 user IDs, in which 431 were annotated to the selected GO functional categories and in the reference gene list, and these genes were used for GO analysis (*Figure 1*). The details of the top 39 GO terms are shown in *Table S1*. GO terms related to cell proliferation (e.g., epithelial cell proliferation and positive regulation of cell cycle), and migration (e.g., cell-substrate adhesion and regulation of cell-cell adhesion) have been shown to play an important role in the progression of NPC. In the KEGG pathway enrichment analysis, 127 significantly enrichment pathways were found. The top 20 pathways are shown in *Figure 2*. Among them, EGFR tyrosine kinase inhibitor resistance and EBV infection deserve particular attention, because EGFR-targeted therapy has been introduced in NPC treatment (12) and EBV infection is a special risk factor for NPC (13). Other pathways, including tumor-associated biological processes (e.g., p53 signaling pathway, microRNAs in cancer, and pathways in cancer) and pathways related to the immune response (e.g., T-cell

**Figure 2** Significantly enriched KEGG pathways of NPCgset. KEGG, Kyoto Encyclopedia of Genes and Genomes.

receptor signaling pathway and Toll-like receptor signaling pathway) were also significantly enriched in the NPCgset. These results suggested that our NPCgset is relatively reliable and is competent for the following studies.

Pathway crosstalk analysis

To remove the interference of redundant information and make a more concise understanding of the interactions between the pathways extracted from enrichment analysis, we introduced pathway crosstalk analysis, which has been reported previously (14). The theoretical basis of the method was that if two pathways shared a proportion of a gene set, they were considered to have crosstalk (15). Among the pathways enriched in KEGG analysis, 51

pathways were closely related to NPC according to previous studies (http://fp.amegroups.cn/cms/tcr.2019.04.04-2.pdf), of which 32 met our criteria and were used to build the network. According to our crosstalk, two representative models were identified, and pathways involved in each model shared more common features (*Figure 3*). One module was mainly related to the carcinogenesis process, including pathways in cancer, proteoglycans in cancer and VEGF signaling pathway. The other module contained the Toll-like receptor signaling pathway, T-cell receptor signaling pathway and so on, which presented an obvious correlation with the immune system. The interaction of two pathways, EGFR tyrosine kinase inhibitor resistance and choline metabolism in cancer, acted as the main connection between the two models.
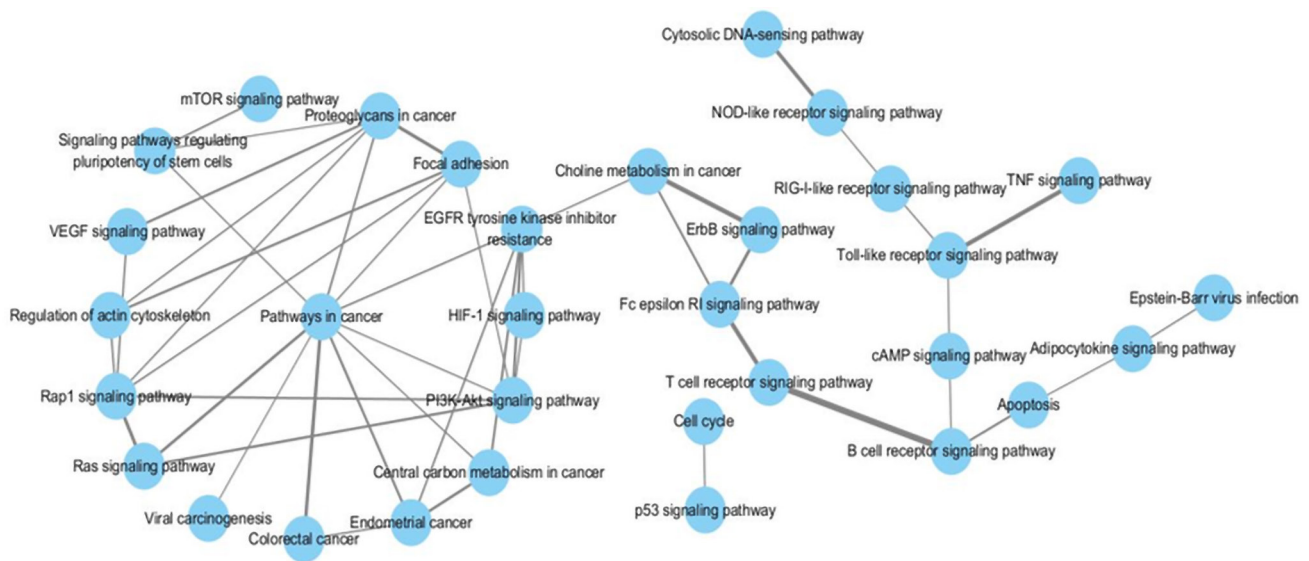
Figure 3 Pathway crosstalk among NPCgset-enriched pathways. Nodes represent pathways, and edges represent crosstalk between pathways. Edge-width corresponds to the score of the specific pathway pair; larger edge-width indicates a higher score.
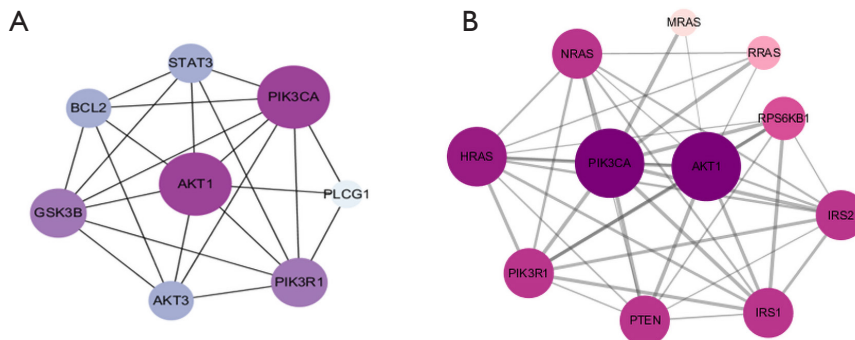

Figure 4 Protein-protein interaction analysis. (A) Protein-protein interaction network constructed with the eight duplicate genes and hub gene identification, and darker color and larger size indicate a higher degree; (B) protein-protein interaction network of proteins associated with *PIK3CA*. Darker color and larger size indicate a higher degree.

Table 1 Connectivity degree of genes

Gene symbol	Degree
PLCG1	3
GSK3B	6
STAT3	5
AKT3	5
BCL2	5
PIK3R1	6
PIK3CA	7
AKT1	7

PPI analysis

Since EBV infection and EGFR tyrosine kinase inhibitor resistance has been proven to be important in the treatment of NPC, our study first focused on these two pathways. Eight duplicate genes in the two pathways were extracted, i.e., *AKT3*, *AKT1*, *GSK3B*, *PIK3CA*, *PIK3R1*, *PLCG1*, *BCL2*, and *STAT3*. The PPI network of these eight genes was obtained using STRING (*Figure 4A*), and the connectivity degrees were calculated by CytoHubba (*Table 1*). Among them, *PIK3CA* and *AKT1* with the highest degrees were chosen as hub genes. Proteins that interact with *PIK3CA* were predicted in STRING

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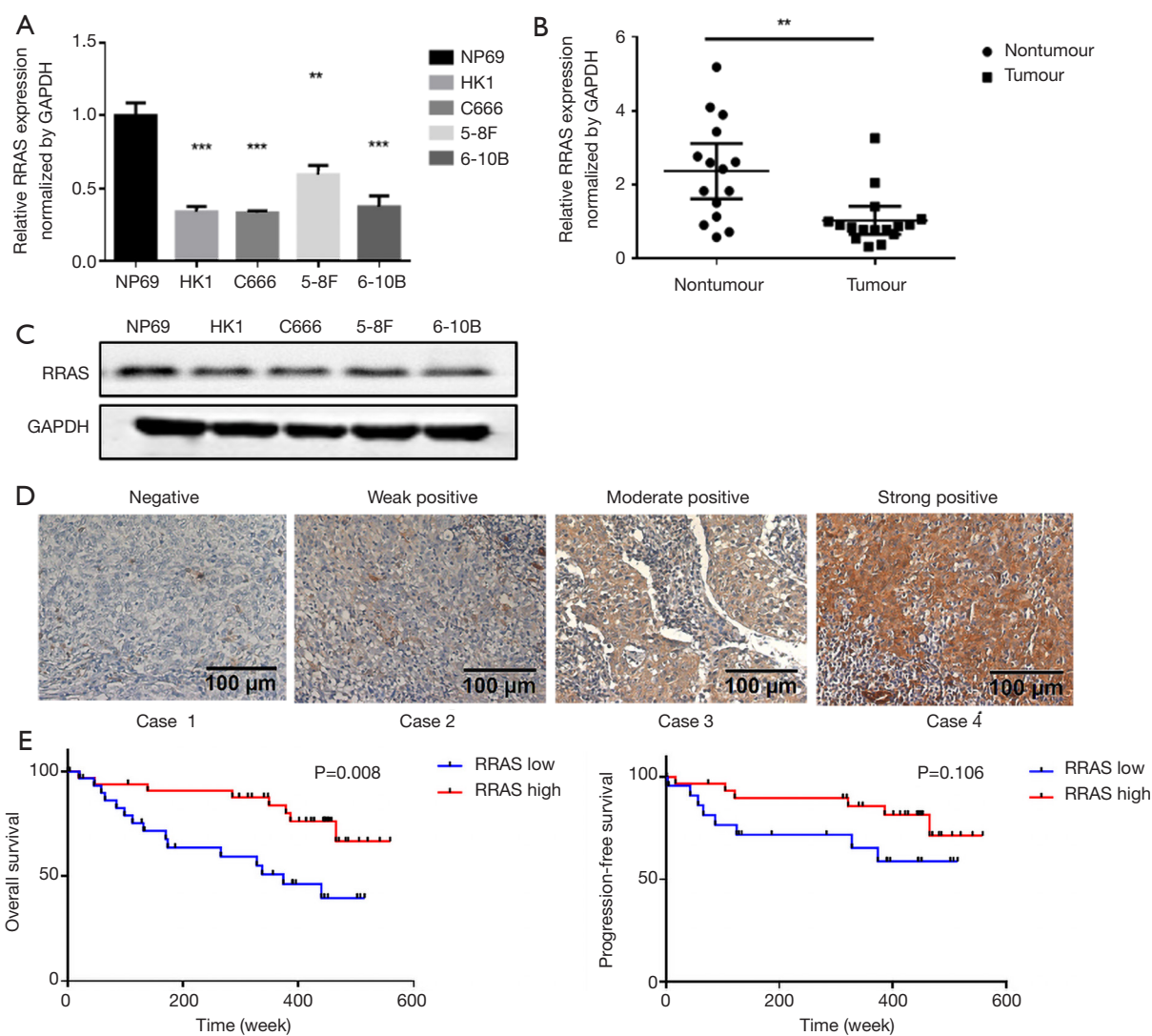
Figure 5 RRAS is down-regulated in NPC and correlates with poor prognosis. (A) Expression level of RRAS mRNA in nasopharyngeal cell lines and immortalized epithelial cell line NP69; (B) the expression level of RRAS mRNA in 16 NPC tissues and 15 noncancerous nasopharyngeal tissues; (C) the expression level of RRAS protein in NPC cell lines and the immortalized epithelial cell line NP69 was analyzed by western blot; (D) representative immunohistochemical staining of RRAS protein in NPC specimens; (E) Kaplan-Meier survival analysis indicated that patients with high expression of RRAS had a longer overall survival (OS), but had no significant correlation with progression-free survival (PFS). P<0.05 was regarded as statistically significant. **, P<0.01; ***, P<0.001. NPC, nasopharyngeal carcinoma.

(*Figure 4B*), most of which were included in our NPCgset except RRAS and IRS2, which have not been reported in NPC. To confirm that the RRAS and IRS2 proteins interacted with *PIK3CA* play an important role in NPC carcinogenesis, we conducted the following experiment.

RRAS is down-regulated in NPC tissues and cells

The mRNA expression levels of RRAS and IRS2 were evaluated by qRT-PCR in NPC cell lines (HK1, C666, 5-8F, 6-10B), immortalized nasopharyngeal epithelial cell line NP69, fresh NPC tissues, and nasopharyngitis tissues. The results demonstrated that the expression of IRS2 mRNA showed no significant difference in NPC cell lines and tissues compared with the control (*Figure S1*). However, RRAS mRNA expression was lower in NPC cell lines (*Figure 5A*) and significantly decreased in NPC tissues compared with non-tumor tissues (*Figure 5B*). Moreover, the lower protein

Table 2 Association of RRAS expression with clinicopathological features of NPC

Variable	No.	RRAS expression		P value (χ^2-test)
		Low (n=32)	High (n=33)	
Gender				0.341
Female	15	9 (60.0)	6 (40.0)	
Male	50	23 (46.0)	27 (54.0)	
Age				0.108
<45	30	18 (60.0)	12 (40.0)	
≥45	35	14 (40.0)	21 (60.0)	
EA/IgA				0.388
<1:20	31	17 (54.8)	14 (45.2)	
≥1:20	34	15 (44.1)	19 (55.9)	
VCA/IgA				0.953
<1:160	12	6 (50.0)	6 (50.0)	
≥1:160	53	26 (49.1)	27 (50.9)	
Clinical staging				0.019*
I–III	34	12 (35.3)	22 (64.7)	
VI	31	20 (64.5)	11 (35.5)	
Distant metastasis				0.082
No	47	20 (42.6)	27 (57.4)	
Yes	18	12 (66.7)	6 (33.3)	

*P<0.05. NPC, nasopharyngeal carcinoma.

level of RRAS in NPC cell lines than in NP69 was also confirmed by western blotting (*Figure 5C*). Next, RRAS expression was evaluated in 65 paraffin-embedded NPC specimens by IHC assay. Positive expression of RRAS protein was detected on the cytoplasm and cell membrane. The IHC scores for RRAS in these NPC tissues ranged from 0 to 12, and the median score was 4 (*Figure 5D*). The median value of IHC score (≥4) was used as a cut-off value. Lower expression of RRAS was significantly related to advanced clinical stages (P=0.019) (*Table 2*). No significant differences were found between RRAS expression and other clinicopathological features. Higher expression of RRAS was associated with longer OS of patients (P=0.008) (*Figure 5E*). Multivariate analysis showed that the down-regulation of RRAS (RRAS expression: low *vs.* high; HR =2.244, 95% CI: 0.925–5.441, P=0.074) and distant metastasis (distant metastasis: no *vs.* yes; HR =0.146, 95% CI: 0.059–0.364, P<0.001) were significantly associated with a shorter OS (*Table 3*). Our results indicated that RRAS might be associated with the prognosis of NPC patients.

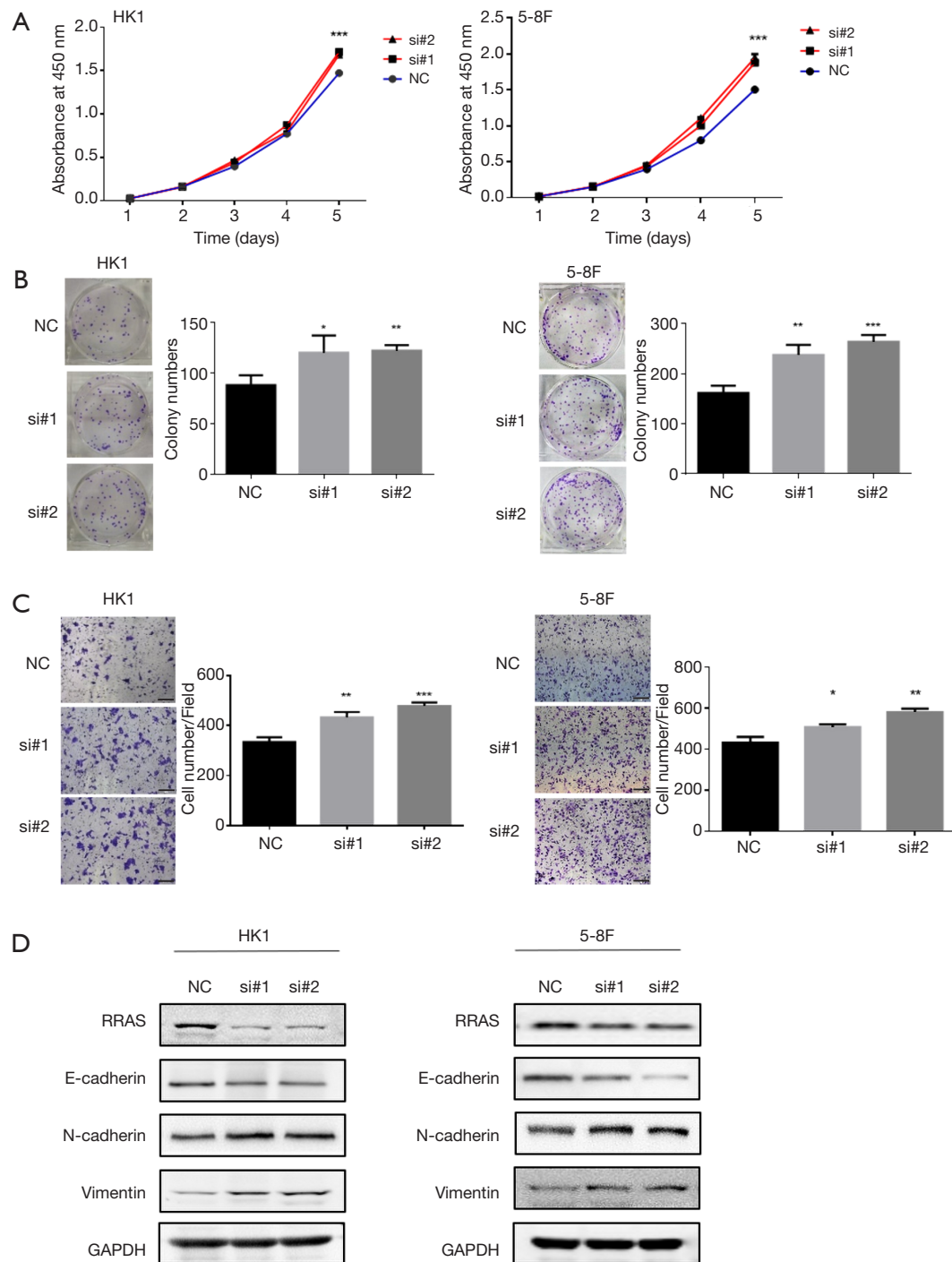### *RRAS knockdown promotes cell proliferation and invasion*

To explore the role of RRAS in NPC cells, RRAS mRNA knockdown was mediated by two specific siRNA oligos. RRAS knockdown significantly promoted the growth rate, colony formation and invasive ability of HK1 and 5-8F cells (*Figure 6A,B,C*). The depletion of RRAS was verified by

Table 3 Univariate and multivariate analyses on clinicopathologic features of NPC patients

Factors	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	P	HR (95 % CI)	P
Age: <45 *vs.* ≥45	0.570 (0.240–1.353)	0.203	–	–
Gender: female *vs.* male	0.976 (0.361–2.641)	0.962	–	–
EA/IgA: <1:20 *vs.* ≥1:20	0.928 (0.407–2.119)	0.86	–	–
VCA/IgA: <1:160 *vs.* ≥1:160	0.899 (0.304–2.659)	0.0847	–	–
Clinical staging: I–III *vs.* VI	0.307 (0.129–0.727)	0.007**	–	–
Distant metastasis: no *vs.* yes	0.126 (0.051–0.309)	<0.001***	0.146 (0.059–0.364)	<0.001***
RRAS expression: low *vs.* high	3.045 (1.282–7.230)	0.012*	2.244 (0.925–5.441)	0.074[#]

[#], P<0.1; *, P<0.05; **, P<0.01; ***, P<0.001. NPC, nasopharyngeal carcinoma.

Figure 6 RRAS knockdown promotes cell proliferation, invasion, and EMT. (A) CCK8 assays showed that RRAS knockdown significantly promotes the proliferation of HK1 and 5-8F cells compared with the controls; (B) colony formation assays showed that RRAS knockdown significantly promotes the growth of HK1 and 5-8F cells compared with the controls; (C) the invasion ability of HK1 and 5-8F cells were significantly enhanced after RRAS depletion (sclar bar =200 μm); (D) the knockdown efficiency of RRAS in HK1 and 5-8F cells were confirmed by Western blot, and knockdown of RRAS decreased the expression of E-cadherin and increased the expression of N-cadherin, vimentin. $P<0.05$ was regarded as statistically significant. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. EMT, epithelial-mesenchymal transformation.

western blotting (*Figure 6D*). Moreover, RRAS inhibition increased the expression of N-cadherin and Vimentin and decreased E-cadherin expression in NPC cells (*Figure 6D*). The results indicated that RRAS might suppress proliferation, invasion and EMT phenotype of NPC cells.

Discussion

In this study, we constructed a systematic framework of NPC-related biochemical processes by extracting the genes associated with NPC from publications and detecting the interaction of these genes using enrichment and network analyses. Then, we successfully predicted a new NPC related gene RRAS. The GO enrichment analysis of the NPCgset identified the biological processes related to NPC, and most of them were consistent with previous studies. For example, genes related to the cell-cell adhesion, epithelial cell proliferation, positive regulation of the cell cycle and kinase activity have been extensively studied in NPC (3,11,16). However, some new terms enriched in our analysis remains to be explored, such as the response to steroid hormone. The role of steroid hormones has been studied in several cancers, including breast cancer, prostate cancer and bladder cancer (17-19). Dai *et al.* (20) found that steroid hormone metabolomics can be a promising biomarker tool for early HCC. The NPC incidence rate in men are two to three times more than in women (1); in addition to the differences in living habits, the response to steroid hormone might be associated with this phenomenon.

To eliminate redundant information in pathway crosstalk analysis, pathways such as dilated cardiomyopathy and amoebiasis were excepted, and two main modules were constructed. One module was mainly related to carcinogenesis. The PI3K-Akt signaling pathway, MAPK signaling pathway, VEGF signaling pathway, and mTOR signaling pathway in this module has been reported to be important in NPC (21-24). The pathways in the other module were mainly associated with the immune response, suggesting that both innate immunity and specific immunity contribute to NPC.

We found that EGFR tyrosine kinase inhibitor resistance and choline metabolism in cancer act as a bridge between the two modules. EGFR was reported to be expressed in more than 85% of NPC patients (25) and predicts a worse prognosis (26). However, Hsu *et al.* (27) reported that the effect of inhibiting EGFR activation in NPC alone was not satisfactory. Therefore, it is of great value to explore the mechanism of EGFR inhibitor resistance. Next, we focused on EBV infection because it is perhaps one of the most

extensively studied risk factors of NPC (24).

Two hub genes *PIK3CA* and *AKT1* in PPI network were all proven to be associated with NPC (26). Because more studies on *AKT1* have been reported in NPC, we focused on *PIK3CA*. Among the proteins that interact with *PIK3CA*, two of them (RRAS and IRS2) has not been reported in NPC until now. Others such as HRAS, NRAS, IRS1, PI3KR1 (P85), and S6K have been included in our NPCgset. MRAS was shown to bind to RPeL43, which was reported to be a potential marker of NPC (28).

RRAS was selected in our further study because it is down-regulated in NPC cell lines and tissues. The role of RRAS is controversial in different types of tumors. RRAS is overexpressed in a subset of gastric cancers by DNA demethylation and promotes cell survival (29). RRAS also act as an oncogene in the tumor growth of cervical epithelial cells and melanoma (5,7). However, Song *et al.* (6) reported that RRAS was reduced in breast cancer tissues and play a role as a tumor suppressor. Komatsu and Ruoslahti (9) also reported that RRAS suppressed intimal hyperplasia and tumor angiogenesis. The effects of RRAS may vary in different tumor types and stages. Up to now, the expression profiles and functions of RRAS in NPC has not been reported. In the present study, we confirmed that RRAS is down-regulated in NPC and might inhibit NPC proliferation and invasion. Our results indicate a tumor suppressor role of RRAS in NPC.

Different bioinformatics strategies have been reported in several studies to identify candidate genes associated with NPC. Hui *et al.* (30) performed a comprehensive analysis of methylation profiling and gene expression profiling to identify potentially critical differentially methylated genes in NPC. Wang *et al.* (31) analyzed the online dataset to find differentially expressed genes, and then GO, and KEGG analyses and the PPI network were applied to identify candidate genes related to EBV-associated NPC. We introduced a new strategy reported by Hu *et al.* (14) that has not been applied in NPC. Because the software GenRev is not available now, we adjusted our method to identify genes that interact with hub genes identified through pathway crosstalk and PPI analysis.

Our strategy provided a more targeted method that can eliminate the interference of much redundant information and has been proven to be feasible by biological experiments. It can be extended to analyze other pathways in our NPC pathway crosstalk module, and can also be applied to other diseases. However, there are still some limitations to our study. First, we only retrieved literature published on

PubMed by October 10, 2018. Genes reported in some unpublished studies or those published after October 2018 were not included in our NPCgset. Therefore, our gene set needs to be constantly improved and updated. Second, although we have developed a series of criteria to select genes included in our study, false positives are still inevitable based on the uneven quality of the studies. Additionally, the results removed from our NPCgset according to our criteria might be proven to be associated with NPC in further studies. Third, in our pathway crosstalk analysis, the cell cycle and p53 signaling pathway were included but showed weaker crosstalk than other pathways involved in the two modules according to our algorithm; more studies are needed to identify potential connections between these pathways.

Conclusions

In our study, we extracted genes associated with NPC from publications in the PubMed database and constructed an NPCgset. GO, and KEGG pathway enrichment analysis was used to study the characteristics of genes in the NPCgset. Pathway Crosstalk Analysis showed that the pathways involved in the NPCgset could be summarized into two models: one module was related to the carcinogenesis process, and the other was correlated with the immune response. Furthermore, we constructed two PPI networks to search for new candidate genes potentially related to NPC. Finally, we found that RRAS was down-regulated in NPC, and the knockdown of RRAS promoted cell proliferation and invasion.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.04.04). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Our research was approved by the Research Ethics Committee of Sun Yat-sen University Cancer Center (No. GZR2015-062), and all patients signed informed consent forms for publication of this manuscript and any accompanying images.

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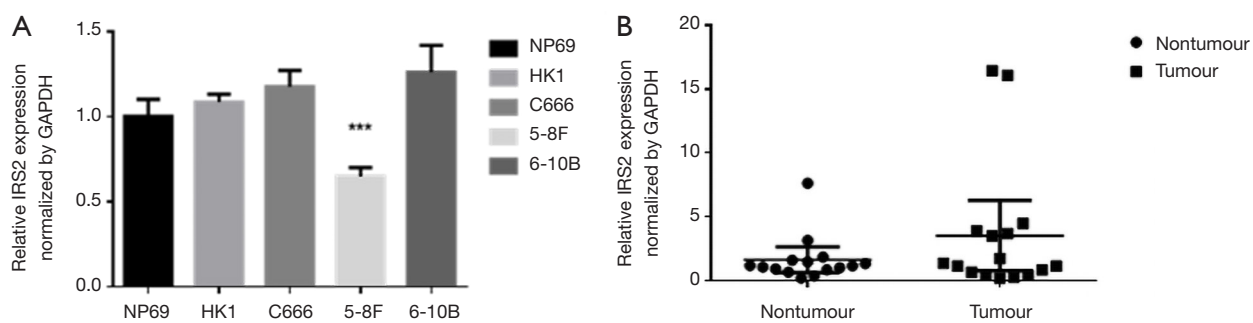
Figure S1 Expression level of IRS2 mRNA in NPC cell lines and tissues. (A) Expression level of IRS2 mRNA in nasopharyngeal cell lines and immortalized epithelial cell line NP69; (B) expression level of IRS2 mRNA in 16 NPC tissues and 15 noncancerous nasopharyngeal tissues. NPC, nasopharyngeal carcinoma.

Table S1 Gene ontology biological process terms enriched in NPC-related genes

Gene set	Description	No. of genes	P value	FDR
GO:0001667	Ameboidal-type cell migration	46	0	0
GO:0002521	Leukocyte differentiation	53	0	0
GO:0006979	Response to oxidative stress	53	0	0
GO:0007050	Cell cycle arrest	38	0	0
GO:0007159	Leukocyte cell-cell adhesion	64	0	0
GO:0007346	Regulation of mitotic cell cycle	67	0	0
GO:0007568	Aging	52	0	0
GO:0009314	Response to radiation	60	0	0
GO:0009612	Response to mechanical stimulus	35	0	0
GO:0009617	Response to bacterium	61	0	0
GO:0010035	Response to inorganic substance	57	0	0
GO:0022407	Regulation of cell-cell adhesion	53	0	0
GO:0033002	Muscle cell proliferation	39	0	0
GO:0033674	Positive regulation of kinase activity	57	0	0
GO:0040017	Positive regulation of locomotion	60	0	0
GO:0042493	Response to drug	58	0	0
GO:0044770	Cell cycle phase transition	62	0	0
GO:0045785	Positive regulation of cell adhesion	57	0	0
GO:0045787	Positive regulation of cell cycle	48	0	0
GO:0048514	Blood vessel morphogenesis	66	0	0
GO:0048545	Response to steroid hormone	48	0	0
GO:0048732	Gland development	59	0	0
GO:0050673	Epithelial cell proliferation	56	0	0
GO:0050900	Leukocyte migration	53	0	0
GO:0051052	Regulation of DNA metabolic process	48	0	0
GO:0051090	Regulation of sequence-specific DNA binding transcription factor activity	48	0	0
GO:0051098	Regulation of binding	45	0	0
GO:0051272	Positive regulation of cellular component movement	58	0	0
GO:0070482	Response to oxygen levels	55	0	0
GO:0070997	Neuron death	49	0	0
GO:0071216	Cellular response to biotic stimulus	38	0	0
GO:0071417	Cellular response to organonitrogen compound	55	0	0
GO:0071900	Regulation of protein serine/threonine kinase activity	57	0	0
GO:0090130	Tissue migration	41	0	0
GO:0097191	Extrinsic apoptotic signaling pathway	42	0	0
GO:0097193	Intrinsic apoptotic signaling pathway	41	0	0
GO:2001233	Regulation of apoptotic signaling pathway	55	0	0
GO:0031589	Cell-substrate adhesion	42	1.11E–16	2.23E–15
GO:0007369	Gastrulation	32	2.22E–16	4.34E–15
GO:0048144	Fibroblast proliferation	23	4.44E–16	8.46E–15
GO:0018212	Peptidyl-tyrosine modification	45	6.66E–16	1.24E–14
GO:0071559	Response to transforming growth factor beta	34	1.78E–15	3.22E–14
GO:0001101	Response to acid chemical	41	2.44E–15	4.23E–14
GO:0071214	Cellular response to abiotic stimulus	39	2.44E–15	4.23E–14
GO:0002764	Immune response-regulating signaling pathway	50	3.33E–15	5.64E–14
GO:0031349	Positive regulation of defense response	46	4.22E–15	6.99E–14
GO:1901652	Response to peptide	48	7.55E–15	1.22E–13
GO:0072331	Signal transduction by p53 class mediator	36	1.02E–14	1.62E–13
GO:0001819	Positive regulation of cytokine production	44	1.19E–14	1.85E–13