

Co-mutated pathways analysis highlights the coordination mechanism in glioblastoma multiforme

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The mutation of cancer represents a high heterogeneity characteristic, setting a big obstacle in the mechanism study of it. In this study, we explored the distributions of mutated genes in pathways in glioblastoma multiforme (GBM), and constructed networks of co-mutated pathway pairs under the false discovery rate (FDR) control. By comparing the mutation frequencies, a total of 50 mutated genes were screened with the frequency > 3, and TP53, PTEN, and EGFR were the top 3 genes. By KEGG enrichment, 18 pathways of the mutation gene spectrum of GBM were enriched. These pathways were further studied to explore the coordination between pathways, co-mutated pathway pairs, such as mismatch repair/vascular smooth muscle contraction, mismatch repair/long-term depression, mismatch repair/dopaminergic synapse, and TGF-beta signaling pathway/retrograde endocannabinoid signaling pathway were enriched in the network under FDR < 0.01; and cell cycle/p53 signaling was a co-mutated pathway pairs in the network under FDR < 0.05. Meanwhile, the samples overlap levels of enriched pathways were calculated for further confirming of the co-mutated pathway model. By the co-mutated pathway analysis, the coordination mechanism of cancer can be explored, and it may provide basis for the pathogenesis and combined therapy study of cancer.

Key words: co-mutated pathway pairs, gene mutation, glioblastoma multiforme, combined therapy

Cancer is a disease induced by somatic mutations or other molecular alterations in many genes with extreme heterogeneity [1]. The extremely diverse and complex mutational spectrum of cancer has been further revealed by recent high-throughput tumor sequencing studies [2-4]. To elucidate the molecular mechanism of cancer, one important task is to identify the driver mutations, however, it is difficult because genes with low somatic mutation are likely to constitute the vast majority of genes whose mutations contribute to carcinogenesis [5, 6]. Nowadays, cancer candidate genes are mainly identified according to the mutation frequency of individual genes [7, 8]. Nevertheless, individual mutation analysis can provide only limited insights into the carcinogenesis, which may not be able to match that cancer is a progressive disease involving multiple mutations acting in concert [9]. Pathway analysis provides more information about cancer and may help us to understand the mechanisms.

It is widely accepted that pathways often function cooperatively in carcinogenesis [10-12]. For example, based on

large-scale somatic mutation profile of lung adenocarcinoma, Gu *et al* [13] identify many pairs of significantly co-mutated pathways. Mann *et al* [14] conduct a mutagenic screen using sleeping beauty, and the cooperating mutations and pathways in pancreatic adenocarcinoma are revealed. The mutations of genes in different patients could cause equivalent pathway changes to induce cancer [15]. Thus, the identification of cooperation of genes mutation and pathways is of great importance during carcinogenesis [16].

Glioblastoma multiforme (GBM) is the most common and lethal type of brain cancer in humans [3]. Researches show that genes tend to be co-mutated in cancer, and the co-mutated genes are more likely to distribute in different pathways [17, 12]. In the present study, the pathways with co-mutated genes were defined as co-mutated pathway pairs. Then, the pathway distributions of mutated genes of GBM were assessed, and networks of co-mutated pathway pairs were constructed under the control of false discovery rate (FDR) to explore the cooperation of pathways [2].

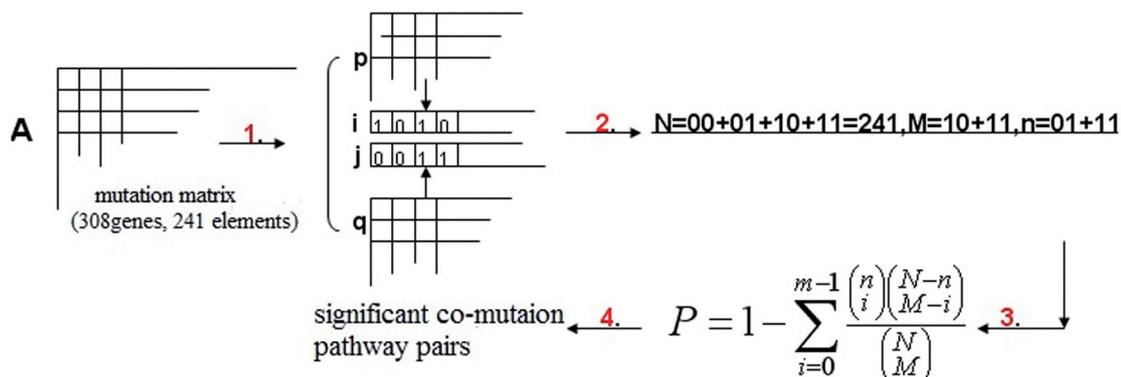


Figure 1. Method used in co-mutated pathway pairs screening

Material and methods

GBM mutation data. The GBM mutation data were downloaded from The Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp/>) database, including 7 batches (Batch1, 2, 3, 4, 6, 7 and 8), 3 testing platforms (BCM_ABI, BI_ABI and WUSM_ABI) and 3 levels. Data from 7 batches and 3 platforms were integrated, and genes with same sense mutation and those not corresponding to Entrez_Gene_Id were removed, to obtain the mutation spectrum of GBM. The mutation spectrum is comprised of 241 mutation samples and 308 mutation genes, with 701 non-same sense mutations. The frequencies of the mutation genes were calculated to screen the genes highly related with GBM.

Pathway enrichment. DAVID (The Database for Annotation, Visualization and Integrated Discovery) provides a comprehensive set of functional annotation tools helping investigators to understand biological meaning behind the large list of genes, of which Fisher is an annotation tool [18]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [19] contains information of how molecules or genes network, displays intracellular biological process by diagrams. Considering that signaling pathways abnormality plays an important role in the proliferation and metastasis, four pathways: genetic information processing, environmental information processing, cellular processes and organismal system of KEGG were chosen for mutation genes pathway enrichment, containing 122 pathways. Fisher was used to identify significant pathways in the four processes with hypergeometric distribution under the false discovery false (FDR) < 0.1.

Scoring Co-mutated pathway pairs. The cooperation between pathways were identified according to the method reported by Gu *et al* [13]. Firstly, the mutation matrix was constructed from the mutation spectrum: 241 samples with 308 mutated genes, the element of samples with mutated genes will be given the value 1, otherwise, the element will be 0; secondly, the [0, 1] vector of interactive pathway pairs was constructed, containing 241 elements. The value 1 represents that mutated genes existed in this pathway, 0 represents no mutated genes

existed; finally, co-mutated pathway pairs were screened by hypergeometric distribution and [0 1] vector according to the FDR value. The method model was diagramed in figure 1.

Co-mutated pathway pairs network construction. In order to explore the interaction between pathways in the occurrence of cancer, the interaction networks of co-mutated pathway pairs were constructed according to FDR values. Meanwhile, the sample overlap levels of the pathways in network were calculated.

Results

Gene mutation frequency. GBM closely related mutation genes were screened by comparing mutation frequency of 308 mutation genes. Genes with frequency not less than 3 are shown in table 1. Tumor suppressor gene TP53, PTEN and proto-oncogene EGFR were the top 3 genes with the highest frequencies. The different frequencies further confirmed the heterogeneity of GBM carcinogenesis. What's more, among all the mutation genes, there were many novel GBM related genes, such as FKBP9, BCL11A, ITPR3, ANK2, ROR2, DDR2, and PSMD13.

Pathway annotation. By DAVID, a total of 18 significant pathways of 308 mutation genes were enriched under FDR < 0.1, as shown in table 2. The nodes of these pathways were divided mainly into several categories: disease, pathways in cancer and Glioma; signaling transduction, ErbB signaling pathway and Phosphatidylinositol signaling system; cell growth and death, p53 signaling pathway; signaling transduction, Focal adhesion and Adherent junction; cell movement, Regulation of actin cytoskeleton; excretory system, Aldosterone-regulated sodium reabsorption. The Glioma pathway and mutation genes enriched in this pathway are shown in figure 2.

Pathways with the higher sample overlap levels may have closer relationship with diseases. The top 30 pathways of the GBM mutation genes listed according to sample overlap levels are shown in table 3. Pathways related with the carcinogenesis of cancer, such as Focal adhesion, MAPK signaling pathway,

Table 1. GBM closely related genes by mutation frequency

Gene	Fr	Gene	Fr	Gene	Fr	Gene	Fr	Gene	Fr
TP53 (tumor protein p53)	68	SYNE1 (spectrin repeat containing, nuclear 1)	7	ITGB3 (integrin, beta 3)	4	EPHA7 (ephrin receptor A7)	3	PSMD13 (26S proteasome non-ATPase regulatory)	3
PTEN (phosphatase and tensin homolog)	52	PRKCD (protein kinase C, delta)	6	ITPR3 (inositol 1,4,5-triphosphate receptor 3)	4	FN1 (fibronectin 1)	3	TNFRSF10A (tumor necrosis factor receptor superfamily 10A)	3
EGFR (epidermal growth factor receptor)	36	ST6GAL1 (ST6 beta-galactosamide alpha 2, 6-sialyltransferase)	6	MET (met proto-oncogene)	6	GSTM5 (glutathione S-transferase mu 5)	4	MLL4 (myeloid/lymphoid or mixed-lineage leukemia 4)	3
RB1 (retinoblastoma 1)	16	FKBP9 (FK506 binding protein 9)	6	PDGFRA (platelet derived growth factor receptor alpha)	6	HLA-E (major histocompatibility complex, class I, E)	4	TNK2 (tyrosine kinase, non-receptor, 2)	3
IDH1 (isocitrate dehydrogenase 1)	10	FGFR1 (fibroblast growth factor receptor 1)	5	TEK (tyrosine kinase, endothelial)	5	TNC (tenascin C)	4	MYST4 (MYST histone acetyltransferase monocytic)	3
PIK3C2G (phosphoinositide-3-kinase, class 2, gamma)	10	CHEK2 (checkpoint kinase 2)	5	RHPN2 (rhopilin, Rho GTPase binding protein 2)	5	KIT (tyrosine kinase receptor)	4	PRAME (preferentially expressed antigen in melanoma)	3
PIK3R1 (phosphatidylinositol 3-kinase, regulatory subunit 1)	8	BCL11A (B-cell CLL/lymphoma 11A (zinc finger protein))	5	ANK2 (Ankyrin 2)	5	LGALS3BP (lectin, galactoside-binding, soluble, 3)	3	FBXW7 (F-box and WD repeat domain containing 7)	3
LUM (lumican)	7	COL1A1 (collagen, type I, alpha 1)	4	BRCA2 (breast cancer 2)	4	ROR2 (receptor tyrosine kinase-like orphan 2)	3	ADAMTSL3 (A disintegrin-like and metalloprotease domain with thrombospondin type I motifs-like 3)	3
NF1 (neurofibromin 1)	7	CYP27B1 (cytochrome P450, family 27, subfamily B 1)	4	CDKN2A (cyclin-dependent kinase inhibitor 2A)	4	DDR2 (discoidin domain receptor tyrosine kinase 2)	3	PAPPA2 (pappalysin 2)	3
PIK3CA (phosphoinositide-3-kinase, catalytic A)	7	MSH6 (DNA mismatch repair protein)	4	DOCK1 (dedicator of cytokinesis 1)	4	PIK3CG (phosphoinositide-3-kinase, catalytic)	3	ABCA13 (ATP-binding cassette, sub-family A (ABC1))	3

Fr: frequency

Table 2. Significant pathways of mutation genes

Term	Gene count	P Value	FDR
Pathways in cancer	39	1.87E-12	2.28E-10
Focal adhesion	30	5.26E-12	6.41E-10
Melanoma	15	4.31E-08	5.26E-06
Endometrial cancer	12	6.10E-07	7.44E-05
Glioma	13	6.19E-07	7.55E-05
Prostate cancer	15	8.19E-07	9.99E-05
Non-small cell lung cancer	12	9.13E-07	1.11E-04
Pancreatic cancer	13	2.76E-06	3.36E-04
Colorectal cancer	13	1.44E-05	0.001759
Phosphatidylinositol signaling system	12	2.26E-05	0.002757
Regulation of actin cytoskeleton	20	6.09E-05	0.007398
Aldosterone-regulated sodium reabsorption	8	3.07E-04	0.036717
p53 signaling pathway	10	3.19E-04	0.038183
Bladder cancer	8	3.58E-04	0.042735
ErbB signaling pathway	11	4.78E-04	0.056689
Chronic myeloid leukemia	10	6.69E-04	0.078364
Type II diabetes mellitus	8	7.27E-04	0.084948
Adherens junction	10	8.12E-04	0.094378

FDR: false discovery rate

p53 signaling pathway, Cell cycle, Apoptosis, Wnt signaling pathway, mTOR signaling pathway ranked top in the list, suggesting that these pathways were closely related with the occurrence of GBM.

Co-mutated pathway pairs. Co-mutated pathway pairs cooperated in the GBM process, and they were enriched by hypergeometric distribution. Numbers of the pairs with different FDR values were displayed in table 4.

Co-mutated pathways network. Under the level of FDR < 0.01, 22 co-mutated pathway pairs were chosen, and a co-mutated pathways network was constructed based on these 22 pairs (figure 3) to help to elucidate the interactions between pathways. Mismatch repair, TGF-beta signaling pathway are the two hub pathways with the highest degrees (connection numbers of pathways). There were 10 and 5 pathways connected with mismatch repair pathway and TGF-beta signaling pathway respectively. Pathways related with Nervous system, such as Dopaminergic synapse, signaling Retrograde endocannabinoid, Long-term depression and Glutamatergic synapse were also mapped in this network,

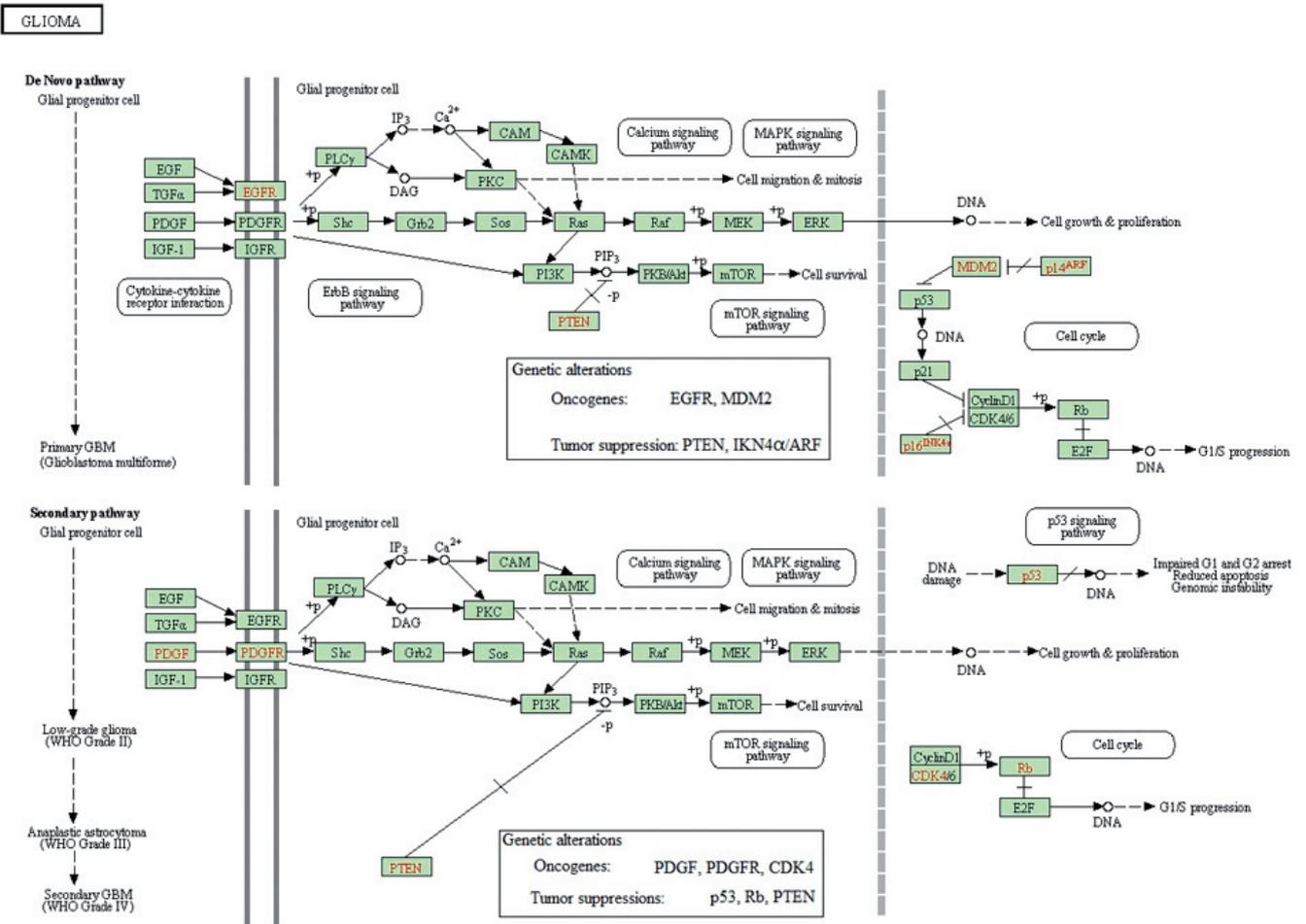


Figure 2. Glioma pathway. The red genes are mutation genes enriched in this way.

suggesting the important roles of nervous system dysfunction in GBM carcinogenesis. It can be also seen from figure 2, that ErbB signaling pathway/Adherens junction (FDR=0.0095), Cytokine-cytokine receptor interaction/Regulation of actin cytoskeleton (FDR=0.0032), Jak-STAT signaling pathway/Natural killer cell mediated cytotoxicity (FDR=0.0032) were co-mutated pathway pairs, even though their degrees were low. Meanwhile, the sample overlap levels of the pathways in the network were calculated (table 5). Regulation of actin cytoskeleton, ErbB signaling pathway and Cytokine receptor interaction were the top 3 pathways, they have close relationship with GBM.

Under the level of FDR < 0.05, 132 co-mutated pathway pairs were chosen, and a co-mutated pathways network was constructed based on these pairs (figure 4). Cell cycle/p53 signaling pathway (FDR=0.04433) was an important pair in the network. Furthermore, cytokine-cytokine receptor interaction-MAPK signaling pathway (FDR=0.06957), and Wnt signaling pathway-Neurotrophin signaling pathway had the trend to co-mutate (FDR=0.05858).

Discussion

Cancer is a progressive disease caused by the mutations of various genes in different pathways [13]. To fully elucidate the mechanisms of the carcinogenesis, studies on pathway and genes collaboration are very necessary. In this study, we assessed the mutation frequencies of 308 mutation genes of GBM, and then conducted a pathway enrichment of all the genes. The co-mutated pathway pairs were screened with the control of FDR, and two interaction networks of co-mutated pathway pairs were constructed under the FDR value 0.05 and 0.01.

TP53, PTEN, and EGFR are the top three genes with high mutation frequency. TP53 is undoubtedly the most influential genetic factor related to the occurrence of the human brain tumors [20]. The type and distribution of TP53 mutations differs between GBM subtypes [21]. EGFR amplification and mutation are invariably expressed in a heterogeneous manner in GBM [22], and the genomic alterations of EGFR play a crucial role in pathogenesis of GBM [23]. Decreased PTEN expression has been shown in GBM [24-26].

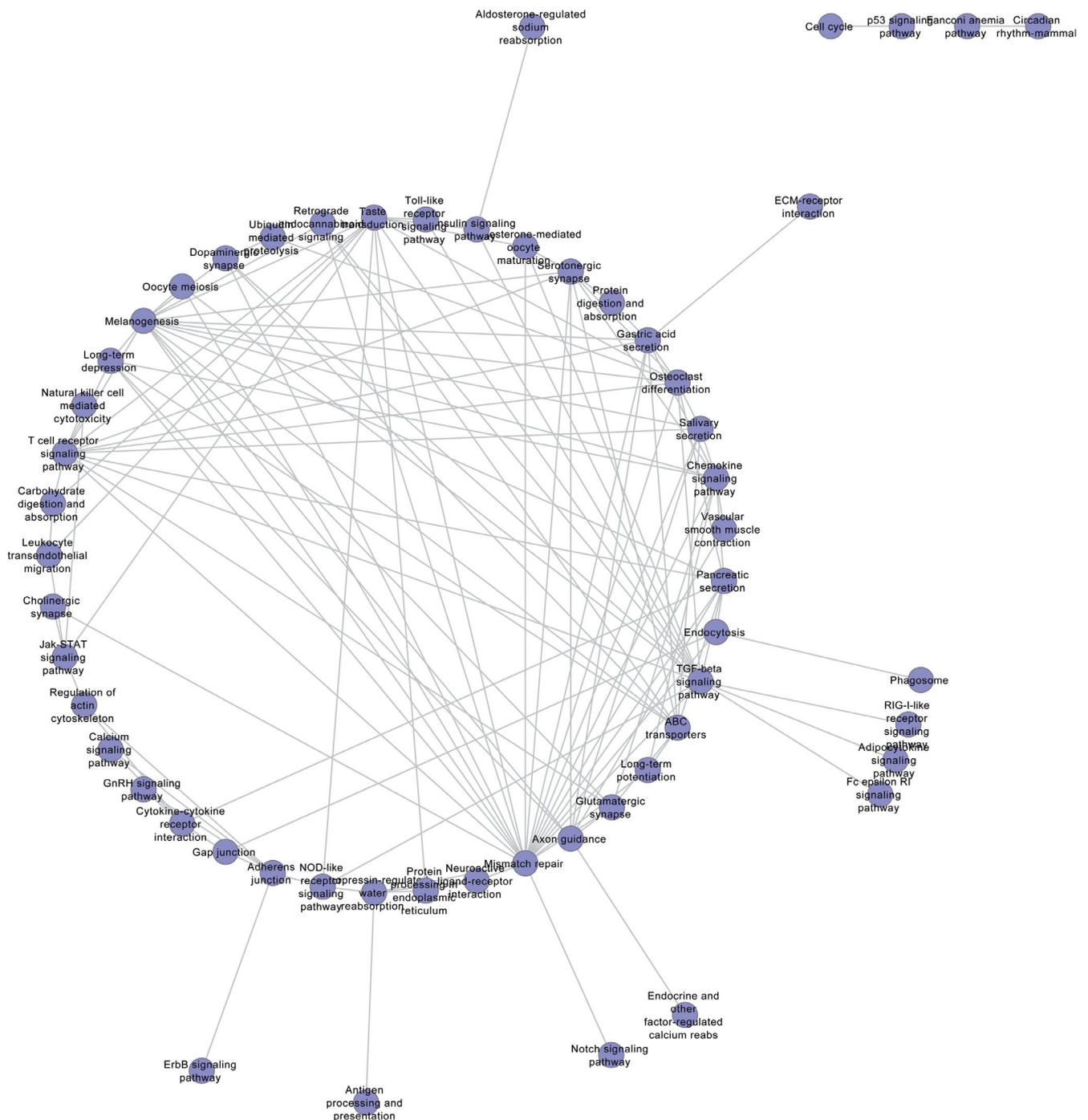


Figure 3. Interaction networks of co-mutated pathways under false discovery rate < 0.01 . The nodes represent pathways, and the edges between pathways represent their co-mutated relationship.

Mismatch repair was a hub pathway in the network under $FDR < 0.01$. We deduced that genes mutation in the hub pathway of mismatch repair can increase the mismatch mutation in the samples, which can sequentially increase the co-mutation in GBM, and thus the risk of GBM increases. DNA mismatch

repair targets errors generated during DNA replication, and plays an essential role in maintaining genomic fidelity and stability [27]. Mismatch repair may go awry in GBM [28]. Reduced expression of mismatch repair proteins (MSH) is characteristic of GBM recurring after current standards of

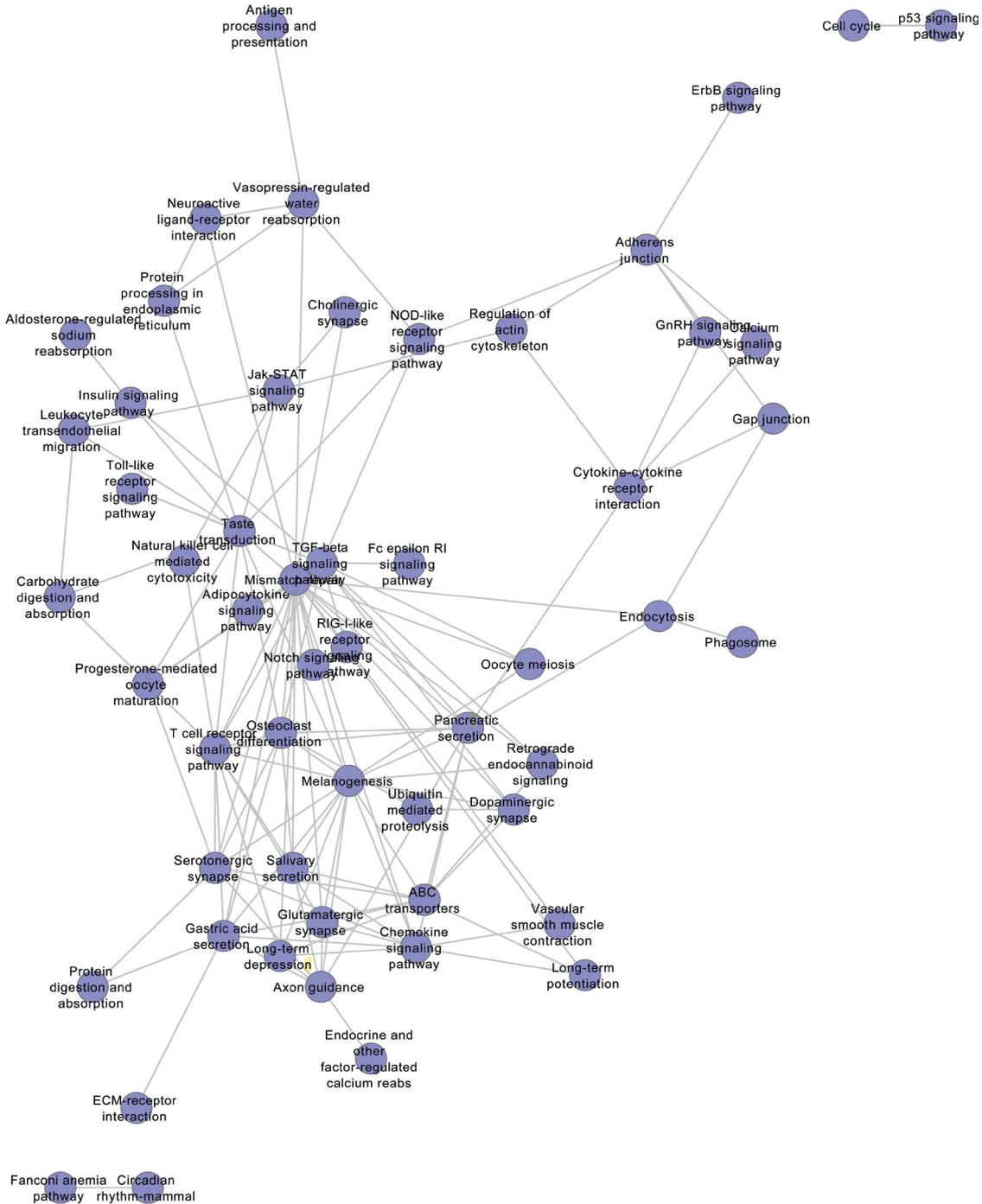


Figure 4. Interaction networks of co-mutated pathways under false discovery rate < 0.05. The nodes represent pathways, and the edges between pathways represent their co-mutated relationship.

care, and MSH6 mutations are reported in three glioblastoma specimens [29]. Vascular smooth muscle contraction pathway, long-term depression pathway, and Glutamatergic synapse were co-mutated pathways of mismatch repair. Vascular smooth muscle contraction pathway is involved in the regula-

tion of the blood flow and pressure. By gene set enrichment and meta-analysis, vascular smooth muscle contraction and long-term depression pathways are enriched to be closely related with the set of imported glioma genes [30]. PRKCB is associated with the vascular smooth muscle contraction and

Table 3. Top 30 pathways according to samples overlap levels

name	Gene count	Mutation number	Sample size
Pathways in cancer	325	39	155
Prostate cancer	89	15	148
Melanoma	71	15	146
Glioma	65	13	144
Endometrial cancer	52	12	139
Small cell lung cancer	84	7	119
Pancreatic cancer	70	13	118
Non-small cell lung cancer	54	12	117
Focal adhesion	200	30	116
MAPK signaling pathway	268	20	116
p53 signaling pathway	68	10	112
Hepatitis C	131	10	110
Bladder cancer	42	8	106
HTLV-I infection	263	22	105
Epstein-Barr virus infection	203	15	98
Neurotrophin signaling pathway	125	11	93
Chronic myeloid leukemia	73	10	92
Cell cycle	124	11	85
Colorectal cancer	62	9	85
Measles	136	10	85
Apoptosis	87	7	82
Phosphatidylinositol signaling system	81	12	82
Transcriptional misregulation in cancer	180	15	82
Herpes simplex infection	184	9	77
Wnt signaling pathway	151	11	77
Basal cell carcinoma	55	7	75
Inositol phosphate metabolism	61	7	72
Huntington disease	184	3	71
mTOR signaling pathway	64	8	70
Regulation of actin cytoskeleton	212	20	70

Table 4. Numbers of co-mutated pathway pairs under different false discovery rate (FDR)

	FDR<=0.01	FDR<=0.05	FDR<=0.1	FDR<=0.2
Numbers	22	132	295	680

Table 5. Sample overlap levels of hub pathways in the network

name	number	name	number
Mismatch repair	5	Dopaminergic synapse	12
RIG-I-like receptor signaling pathway	5	Vascular smooth muscle contraction	14
Taste transduction	6	T cell receptor signaling pathway	21
Pancreatic secretion	7	Progesterone-mediated oocyte maturation	22
Salivary secretion	8	Toll-like receptor signaling pathway	22
Serotonergic synapse	8	Jak-STAT signaling pathway	23
Gastric acid secretion	9	Natural killer cell mediated cytotoxicity	27
Melanogenesis	9	Chemokine signaling pathway	32
TGF-beta signaling pathway	9	Adherens junction	51
Glutamatergic synapse	10	Cytokine-cytokine receptor interaction	54
Long-term depression	10	ErbB signaling pathway	57
Retrograde endocannabinoid signaling	11	Regulation of actin cytoskeleton	70

long term depression pathway in glioma [30], and is a gene with a higher lifetime death hazard in male GBM patients than in females [31]. So, we induce that MSH6 in mismatch repair pathway and PRKCB in vascular smooth muscle contraction and long term depression pathway co-mutated, causing the collaboration of these pathways in the carcinogenesis of GBM. The neurotransmitter glutamate is produced and released from glioma cells as a byproduct of glutathione synthesis, glioblastoma tumors release glutamate to enhance their highly malignant behavior, and in fact, the invasive nature of gliomas enhanced by glutamate release is one of the most important limitations to effective disease control [32].

TGF-beta signaling pathway was another hub pathway in the network under $FDR < 0.01$, and retrograde endocannabinoid signaling was one co-mutated pathway of it. TGF-beta signaling pathway acts as an oncogenic factor in GBM and is considered a therapeutic target [33]. TGF-beta signaling pathway is a unique therapeutic target compared to the other commonly studied signaling pathways in GBM in that it may represent an important crosslink to various intracellular processes [34]. TGF-beta can function as a tumor suppression gene in GBM, and can alter collagen synthesis, cell adhesion and invasiveness in gliomas [35, 36]. Endocannabinoids functions as retrograde signaling molecular at synapses throughout the brain [37], and plays an anti-proliferative effect upon rat C6 glioma cells [38]. Cannabinoids have displayed a great potency in reducing glioma tumor growth either in vitro or in animal experimental model, and a pilot clinical trial on patients with GBM demonstrate that cannabinoids have good safety profile and remarkable antitumor effects[39]. The impaired tumor growth of GBM in presence of cannabinoids may be involved with the activation of cannabinoids 2 (CB2), and the expression and distribution of CB2 receptors are abundant in GBM [40]. The research of Gardner et al suggests that TGF-beta actively regulates lymphocyte CB2 receptor expression in an autocrine and paracrine manner [41]. Similarly, TGF-beta signaling pathway and retrograde endocannabinoid signaling pathway may be co-mutated through the co-mutation of TGF-beta and CB2 in GBM. Cytokine-cytokine receptor interaction pathway was related to the tumorigenesis and signaling transduction in the progress of

glioma [42]. The actin cytoskeleton is a dynamic structure that plays a fundamental role in diverse process in all eukaryotic cells [43]. The regulation of actin cytoskeleton pathway and cytokine-cytokine receptor interaction pathway were enriched in the co-mutated pathway network in this study, suggesting that they may play a cooperating work in the carcinogenesis of GBM.

The p53 signaling pathway exerts an important role in glioma pathogenesis [44], and it is an inactivating mutation of p53 signaling pathway in GBM. The type and distribution of p53 may differ between GBM subtypes [34]. MAPK signaling pathway is involved in GBM cell migration and proliferation [45], and it is one changed pathway in glioma [30]. Thus p53 signaling pathway and MAPK signaling pathway are changed in GBM. However, they were not mapped in the network of co-mutated pathway pairs, suggesting that not all the carcinogenesis close related pathways will co-mutate with other pathways.

Nevertheless, in the network under FDR < 0.05, cell cycle/p53 signaling pathway was found to co-mutate. The p53 pathway, which mutates inactively in GBM [34], is most intensely implicated in cell cycling regulation during times of cell repair and cell growth, and the mutation of p53 will cause the impaired G1 and G2 arrest [34].

Judged from the two networks of co-mutated pathway pairs, some enriched pathways co-mutated with other pathways in monotonous model such as Cell cycle-p53 signaling pathway, Cytokine-cytokine receptor interaction-MAPK signaling pathway, that is, the connection degrees of the hub pathways were usually not so high.

By the co-mutated pathway analysis, the coordination mechanism of cancer can be explored, which may provide basis for the pathogenesis and combined therapy study of cancer. The pathways changed in GBM and their co-mutated relationships can be detected by the method used in this study.

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