MAP Kinase and mammalian target of rapamycin are main pathways of gallbladder carcinogenesis: Results from bioinformatic analysis of Next Generation Sequencing data from a hospital-based cohort.

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ABSTRACT

Background: Gallbladder Cancer (GBC) is one of the most common cancers of the biliary tract and the third commonest gastrointestinal (GI) malignancy worldwide. The disease is characterized by the late presentation and poor outcome despite treatment, and hence, newer therapies and targets need to be identified.

Methods: The current study investigated various functionally enriched pathways in GBC pathogenesis involving the genes identified through Next Generation Sequencing (NGS). The Pathway enrichment analysis and Gene Ontology (GO) were carried out after NGS, followed by the construction of the protein-protein interaction (PPI) network to discover associations among the genes.

Results: Of the thirty-three patients with GBC who were screened through next-generation sequencing (NGS), 27somatic mutations were identified. These mutations involved a total of 14 genes. The p53 and KRAS were commonly found to be mutated, while mutations in other genes were seen in one case each, the mean number of mutations were 1.2, and maximum mutation in a single case (eight) was seen in one case. The bioinformatics analysis identified MAP kinase, PI3K-AKT, EGF/EGFR, and Focal Adhesion PI3K-AKT-mTOR signaling pathways and cross-talk between these.

Conclusion: The results suggest that the complex crosstalk between the mTOR, MAPK, and multiple interacting cell signaling cascades can promote GBC

progression, and hence, mTOR - MAPK targeted treatment will be an attractive option.

Keywords: Next Generation Sequencing (NGS), Gene Ontology (GO), Protein-protein interaction network (PPI), Gene Set Enrichment Analysis (GSEA), Signaling network, Disease Ontology (DO) and, Cross-Talk.

Abbreviations: Gene Ontology (GO), Protein-protein interaction network (PPI), WEB-based Gene Set Analysis Toolkit (Webgestalt), Search Tool for the Retrieval of Interacting Genes (STRING), the mammalian target of Rapamycin (mTOR) & the mitogen-activated protein kinase (MAPK) signaling pathways.

INTRODUCTION

Gallbladder cancer (GBC) is a rare malignant neoplasm of the biliary tract and is more prevalent in Asia (1-2). According to GLOBOCAN 2018 data, approximately 1.2% of deaths reported in 2018 were due to GBC (3). It is a relatively rare type of cancer with a poor prognosis with a 5-years survival rate of 10-20%, and a lack of symptoms in its early stages compared to other cancers (4-6). The development of GBC progresses through metaplasia, carcinoma, dysplasia, and invasive malignancy over 5-15 years (7). If GBC is detected earlier and managed effectively, it is completely curable (8). Several internal and external factors are associated with GBC development; of these gallstones (9), various lifestyle-related factors (stress, alcohol, diet, menstrual factors) (10-12), xanthogranulomatous cholecystitis (13), biliary duct infection (14-16), metabolism and lipid peroxidation (17-18), Heavy metals and environmental pollution (19-20) etc., play a crucial role.

Surgery is the primary treatment for early disease, while chemotherapy and radiation are the mainstays in advanced and metastatic GBC (21). The use of other

approaches, such as immunotherapy, hormone therapy, and targeted therapy, is mostly experimental, with a slight improvement in progression-free survival with no benefit in overall survival (22). With the advent of advanced methods such as whole-genome sequencing (WGS) using NGS or microarray platforms, the origin of genomic research has expanded, and newer approaches are being identified (23). This has also helped me understand the molecular mechanisms and prediction of treatment response and outcome.

GBC appears to arise due to undiscovered successive spontaneous mutations involving tumor suppressor genes, oncogenes, genes involved with angiogenesis, cell growth and development, and microsatellite instability (24-29). So far in GBC, about 1281 genetic mutations have been discovered (30). Another recent study exploring the transcriptome identified over 900 differentially expressed gene (31). In this study, thirty-three cases of GBC were screened through NGS for mutational studies, and the results of mutation profiling were analyzed using bioinformatics tools to understand the biological pathways involved in gallbladder carcinogenesis and identify a suitable targeted therapy.

Patients and methods:

A prospective study was carried out between January 2017 to December 2021. After approval from the institute ethics committee, and obtaining a written informed consent, naïve patients with a proven histological diagnosis of gallbladder cancer were included in the study.

Data collection and processing- Comprehensive history and physical examination of the patients were taken, and details were recorded in the preset proforma. Besides hematology and biochemistry, including the tumor marker CA 19-9, an

image-guided biopsy was carried out. CT/MRI/MRCP of the abdomen was carried out to measure the tumor dimensions and stage the disease before initiation of treatment. The tumor tissue was studied for expression of gene mutation by Next-Generation Sequencing. All patients were treated as per standard of care and followed until December 2021.

GO and Pathway enrichment analysis- WEB-based Gene Set Analysis Toolkit (Webgestalt) (32), an online bioinformatics tool that helps to investigate significant enriched Genes and functional pathways, Wikicancer Pathway analysis and Gene Ontology (GO) were performed by using Webgestalt tool (http://www.webgestalt.org).

Network integration and screening of modules- Protein-protein interaction network (PPI) of genes were constructed by using NetworkAnalyst (http://www.networkanalyst.ca.) (33) and Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) (34) based on the confidence scores. Further analysis of involved genes was carried out by constructing a gene-gene interaction network using GeneMania online Tool (35). A signaling network was built among the GBC-specific genes.

Disease ontology- Further, Disease Ontology (DO) analysis was performed through Gene Set Enrichment Analysis (GSEA) (36) as a plugin of the Webgestalt tool. Gene-Associated Disease Interaction network of 14 genes was constructed through NetworkAnalyst.

RESULTS:

A total of 33 cases underwent NGS analysis; among them, mutations were identified in 17 of the patients, and a total of 27 mutations (mean 1.19 SD 1.7,

range 0-8) were identified in 14 genes which were further analyzed (Table 1). The mutations included TP53 (9 cases), KRAS (4 cases), KDR (4 cases), MAP3K1 (4 cases), BRAF (4 cases), PTEN (2 cases), SMAD4 (1 case), NRAS (1 case), CTNNB1 (1 case), EGRF (1 case), PDGFRA (1 case), FBXW7 (1 case), and POLE (1 case) **(Table 1)**.

Functional enrichment analysis- The results of GO enrichment analysis were categorized into three functional categories, i.e. biological processes (BP), molecular function (MF), and cellular components (CC). In the BP, gene enrichment was seen in MAPK cascade, signal transduction by protein phosphorylation, positive regulation of protein phosphorylation, and positive regulation of phosphorylation **(Table 2A)**. In the MF, genes were functionally enriched in transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, protein-containing complex binding, and MAP kinase activity **(Table 2B)**. In the CC, genes were functionally enriched in the membrane raft, membrane micro domain, membrane region, cell junction, receptor complex, and focal adhesion **(Table 2C)**.

Pathway enrichment analysis- Figure 1 shows the ten positive and four negatively related categories according to the false discovery rate (FDR > 0.05). The genes significantly enriched in MAPK, PI3K-AKT, EGF/EGFR, Focal Adhesion, and PI3K-AKT-mTOR signaling pathway. The resulted outcome stipulated that these significant genes were functionally enriched in cancer-associated biological pathways **(Figure 2)**.

GBC-specific genes functional characterization- The Protein-Protein Interaction (PPI) network of 14 significant genes with 14 nodes and 71 edges was constructed using STRING **(Figure 3)**. Further, several hub genes exhibiting co-expression, predicted, and physical and genetic interaction with multiple genes were identified,

and a network was constructed through the GeneMania tool **(Figure 4 and Additional Table 1)**. The NetworkAnalyst Tool built a signaling network through a plugin Signor (https://signor.uniroma2.it) (37) of 13 significant genes, with 503 nodes and 619 edges shown in **(Figure 5)**.

Disease ontology (DO)- Further, DO with FDR (> 0.05) was functionally enriched. The enriched DO showed identified genes to be associated with Melanoma, Gastrointestinal Diseases, Gastrointestinal Neoplasms, Carcinoma and Squamous cells, Nervous System Neoplasms, and Breast Neoplasms **(Figure 6)**. The results of the Gene-Disease associated network of significantly enriched six genes in NetworkAnalyst are presented in **(Additional Figure 1)**.

Cross talk between mTOR/MAPK signaling pathway- High frequency of the mammalian target of Rapamycin (mTOR) & the mitogen-activated protein kinase (MAPK) signaling pathways variation was observed, including PTEN, AKT, TP53, SMAD4, EGFR, and CTNNB1 **(Figure 7)** a pathway of cross-talk between various identified pathways was constructed by data and text mining.

DISCUSSION

Multi-omics characterization of the NGS data from GBC patients identified 14 significant genes and their functional and biological pathways, with MAP kinase and mTOR being the main. Despite recent breakthroughs in surgical procedures and drug development, gallbladder cancer has a dismal long-term prognosis, with a 5-year survival rate ranging from 5% to 13% (38) (39).

Gain-of-function mutations in FGFRs have been described in numerous malignancies, and they play a crucial role in angiogenesis and proliferation (40). To our knowledge, no FGFR3 mutation or amplification has been documented in

gallbladder cancer. Hence, the discovery of Fibroblast Growth Factor Receptor (FGFR3) was a novel result in our investigation.

Moreover, the investigation uncovered that the TP53 family is associated with different mutation in TP53, in most of our cases suggestingthat it acts as a mutagenic driver in GBC. TP53 is the commonest gene studied in the gallbladder and extrahepatic biliary tract cancer. TP53 mutations with or without RAS mutations are reported in up to 50% of gallbladder cancer patients (41). No difference is observed in patients with the anomalous junction of the pancreatic, and biliary duct (42). In some areas, it's higher, while others display lower p53 mutation rates (43). Bolivia reported 50% mutation rates in their patients, all but one patient had a single mutation, while one had three mutations in the same gene. Most of these mutations were on exons 5 and 8 of the gene (44). Eighty single nucleotide variants and 8 indels in 39 genes were identified in their patients with biliary tract cancer, including gallbladder, p53, and KRAS, were the commonest mutations identified in these patients (44). KRAS is a well-known oncogene, commonly mutated in various malignancies (45). Patients with GBC were found to have a mutation in the KRAS gene.

A number of other targets like EGFR, VEGF, BRAF, MAPK, etc., were identified, some of which for the first time. Further, significantly higher identification of mutations in p53 and RAS oncogene signifies that treatment by EGFR antibodies may not be successful in these cases. However, the relatively widespread frequency of MAPK and mTOR signaling pathway mutations (NRAS, BRAF, TP53, AKT, MAPK31, and PTEN) was a remarkable result, opening up possible alternatives for targeted therapy directed against the mTOR pathway. Previous studies have also shown the importance of MAP kinase and mTOR pathways in gallbladder cancer. A study in the gallbladder cancer cell line from typhoid carriers

and an animal model from the same cell line showed mTOR as the main pathway of carcinogenesis, leading authors to suggest targeting of mTOR receptors (46). Further experimental studies demonstrated regression of gallbladder cancer by treatment with mTOR inhibitors (47). This was independent of the typhoid carrier state and was demonstrated to be mediated through PIK3CA/AKT/mTOR pathway (48-52). Although the single-phase I study of docetaxel and temsirolimus was limited by severe myelosuppressive toxicity and failed to meet the objectives (53).

Results of the present study, and bioinformatics show cross-talk between various pathways with mTOR, including the EGFR pathway, p53 pathway, and PIK3CA/AKT pathway, suggesting the need to conduct clinical trials on mTOR inhibitors. The results of this study gives a unique insight into gallbladder carcinogenesis, identifies driver oncogenes, and suggest new therapeutic strategies that need to be tested.

CONCLUSION

The study reports the results of DNA sequencing and demonstrated 14 key genes in gallbladder carcinogenesis, including P53, RAS, EGFR, MAP3K1, PTEN, etc. The analysis also demonstrates that the mTOR and MAPK signaling networks were major pathways in gallbladder carcinogenesis. We suggest that the complex crosstalk between the mTOR, MAPK, and multiple interacting cell signaling cascades promotes gallbladder carcinogenesis by activating cell division. This suggests that mTOR inhibitors are an attractive option in the treatment of treatment gallbladder cancer, and this needs to be tested in clinical trials.

Authors Contribution

MR: Conduct of the study, bioinformatics analysis and interpretation and preparation of the draft manuscript

VJC: Collection of the data, design of study, interpretation of results and preparation of manuscript

RP: data collection, interpretation of results and preparation of manuscript

MS: Interpretation of pathological and molecular results, preparation of manuscript

MP: concept and design, interpretation of results, Editing of the final manuscript

All authors read and approved final manuscript for publication

Conflict of Interest

The authors declare there are no conflicts of interest

Ethical approval and Consent

The study was approved by the Institute Ethics committee vide approval letter no Dean/EC/2020/2045 dated 18.7.2020 written informed consent was taken from all patients participating the study

Consent to Publish

Not applicable

Funding

None

Acknowledgement

None

REFERENCES

1. Mehrotra R, Tulsyan S, Hussain S, Mittal B, Singh Saluja S, Singh S, Tanwar P, Khan A, Javle M, Hassan MM, Pant S, De Aretxabala X, Sirohi B, Rajaraman P, Kaur T, Rath GK. Genetic landscape of gallbladder cancer: Global overview. Mutat Res Rev Mutat Res. 2018 Oct-Dec;778:61-71. doi: 10.1016/j.mrrev.2018.08.003. Epub 2018 Aug 23. PMID: 30454684..
2. Jin H, Cui M. Gene silencing of heparanase results in suppression of invasion and migration of gallbladder carcinoma cells. Biosci Biotechnol Biochem. 2018 Jul;82(7):1116-1122. doi: 10.1080/09168451.2018.1456316. Epub 2018 Mar 29. PMID: 29598788..
3. Rawla P, Sunkara T, Thandra KC, Barsouk A. Epidemiology of gallbladder cancer. Clin Exp Hepatol. 2019 May;5(2):93-102. doi: 10.5114/ceh.2019.85166. Epub 2019 May 23. PMID: 31501784; PMCID: PMC6728871..
4. Nemunaitis JM, Brown-Glabeman U, Soares H, Belmonte J, Liem B, Nir I, Phuoc V, Gullapalli RR. Gallbladder cancer: review of a rare orphan gastrointestinal cancer with a focus on populations of New Mexico. BMC Cancer. 2018 Jun 18;18(1):665. doi: 10.1186/s12885-018-4575-3. PMID: 29914418; PMCID: PMC6006713..
5. Akhtar J, Priya R, Jain V, Sakhuja P, Agarwal AK, Goyal S, Polisetty RV, Sirdeshmukh R, Kar S, Gautam P. Immunoproteomics approach revealed elevated autoantibody levels against ANXA1 in early stage gallbladder carcinoma. BMC Cancer. 2020 Dec 1;20(1):1175. doi: 10.1186/s12885-020-07676-6. PMID: 33261560; PMCID: PMC7709428.

6. Horsley-Silva JL, Rodriguez EA, Franco DL, Lindor KD. An update on cancer risk and surveillance in primary sclerosing cholangitis. Liver Int. 2017 Aug;37(8):1103-1109. doi: 10.1111/liv.13354. Epub 2017 Jan 28. PMID: 28028930.

7. Espinoza JA, Bizama C, García P, Ferreccio C, Javle M, Miquel JF, Koshiol J, Roa JC. The inflammatory inception of gallbladder cancer. Biochim Biophys Acta. 2016 Apr;1865(2):245-54. doi: 10.1016/j.bbcan.2016.03.004. Epub 2016 Mar 12. PMID: 26980625; PMCID: PMC6287912.

8. Kanthan R, Senger JL, Ahmed S, Kanthan SC. Gallbladder Cancer in the 21st Century. J Oncol. 2015;2015:967472. doi: 10.1155/2015/967472. Epub 2015 Sep 1. PMID: 26421012; PMCID: PMC4569807.

9. Espinoza JA, Bizama C, García P, Ferreccio C, Javle M, Miquel JF, Koshiol J, Roa JC. The inflammatory inception of gallbladder cancer. Biochim Biophys Acta. 2016 Apr;1865(2):245-54. doi: 10.1016/j.bbcan.2016.03.004. Epub 2016 Mar 12. PMID: 26980625; PMCID: PMC6287912.

10. Pandey M, Singh S, Shukla VK. Diet and gallbladder cancer: A case-control study. Eur J Cancer Prevention 2002; 11: 365-8. PMID: 12195163

11. Pandey M, Singh S, Shukla VK. Life-style, parity, Menstrual and reproductive factors and gallbladder cancer. Eur J Cancer Prev 2003; 12: 269-72. PMID: 12883378

12. Pandey M. Risk factors for gallbladder cancer a reappraisal. Eur J Cancer Prev 2003; 12: 15-24. PMID: 12548106

13. Dixit VK, Prakash A, Gupta A, **Pandey M**, Kumar M, Gautam A, Shukla VK. Xanthogranulomatous cholecystitis. *Dig Dis Science* 1998; **43(5)**: 940-2. PMID: 9590403

14. Shukla VK, Singh H, **Pandey M**, Upadhyaya SK, Nath G. Carcinoma of the gallbladder is it a sequel of typhoid? *Dig Dis Sci* 2000; **45**: 900-3. PMID: 10795752

15. Pandey M, Shukla M. Helicobacter species are associated with possible increase in risk of hepatobiliary cancers. *Surgical Oncology* 2009; 18:51-56 PMID: 18715780

16. **Pandey M**, Mishra RR, Dixit R, Jaiswal R, Shukla M, Nath G. Helicobacter bilis in human gallbladder cancer: Results of a case control study and meta analysis. Asia Pacific journal of Epidemiology and prevention 2010; 11: 343-47.PMID: 20843113

17. **Pandey M**, Shukla VK. Fatty acids, biliary bile acids, lipid peroxidation products and gallbladder cancer: A hypothesis. *European J Cancer Prevention* 2000; **9**:165-71. PMID: 10954255

18. **Pandey M**, Shukla VK, Singh S, Roy SK, Rao BR. Biliary lipid peroxidation products in gallbladder cancer: increased peroxidation or biliary stasis?. *Eur J Cancer Prev* 2000; **9**: 417-22. PMID: 11201680

19. Shukla VK, Arya NC, Pitale A, **Pandey M**, Dixit VK, Reddy CD, Gautam A. Metallothionein expression in carcinoma of the gallbladder. *Histopathology* 1998; **33**: 154-7. PMID: 9762548

20. **Pandey M:** Environmental pollutants in gallbladder cancer. J Surg Oncol 2006; 93(8):640-3 PMID: 16724354. 10.1002/jso.20531

21. Turgeon MK, Maithel SK. Cholangiocarcinoma: a site-specific update on the current state of surgical management and multi-modality therapy.

Chin Clin Oncol. 2020 Feb;9(1):4. doi: 10.21037/cco.2019.08.09. Epub 2019 Sep 2. PMID: 31500433; PMCID: PMC7186525.

22. Zheng Q, Wu C, Ye H, Xu Z, Ji Y, Rao J, Lu L, Zhu Y, Cheng F. Analysis of the efficacy and prognostic factors of PD-1 inhibitors in advanced gallbladder cancer. Ann Transl Med. 2021 Oct;9(20):1568. doi: 10.21037/atm-21-4747. PMID: 34790774; PMCID: PMC8576663.

23. Roy N, Kshattry M, Mandal S, Jolly MK, Bhattacharyya DK, Barah P. An Integrative Systems Biology Approach Identifies Molecular Signatures Associated with Gallbladder Cancer Pathogenesis. J Clin Med. 2021 Aug 10;10(16):3520. doi: 10.3390/jcm10163520. PMID: 34441816; PMCID: PMC8397040.

24. Mishra SK, Kumari N, Krishnani N. Molecular pathogenesis of gallbladder cancer: An update. Mutat Res. 2019 Nov;816-818:111674. doi: 10.1016/j.mrfmmm.2019.111674. Epub 2019 Jul 6. PMID: 31330366.

25. Priya R, Pandey M, Shukla VK. Biomarkers in carcinoma of the gallbladder. *Expert opinion on medical diagnosis* 2008; 2:511-26.

26. Dixit R, Shukla VK, Pandey M. Molecular alterations in gallbladder cancer. World Journal of Pathology 2012, 1:7

27. Dixit R, Singh G, Pandey M, Basu S, Bhartiya SK, Singh KK, Shukla VK. Association of Methylenetetrahydrafolate Reductase Gene Polymorphism (MTHFR) in Patients with Gallbladder Cancer. J Gastrointest Cancer. 2016 Mar;47(1):55-60. doi: 10.1007/s12029-015-9794-0.

28. Srivastava V, Patel B, Kumar M, Shukla M, Pandey M. Cyclin D1, retinoblastoma and p 16 protein expression in carcinoma of the

346 gallbladder. Asia Pacific J Cancer Prev Asian Pac J Cancer Prev. 2013;
347 14(5):2711-5
348 29. Dixit R, Pandey M, Tripathi SK, Dwivedi AN, Shukla VK. Comparative
349 Analysis of Mutational Profile of Sonic hedgehog Gene in Gallbladder
350 Cancer. Dig Dis Sci. 2017 Mar;62(3):708-714. doi: 10.1007/s10620-016-
351 4438-1. Epub 2017 Jan 5. PMID: 28058596
352 30. .Maurya SK, Tewari M, Mishra RR, Shukla HS. Genetic aberrations in
353 gallbladder cancer. Surg Oncol. 2012 Mar;21(1):37-43. doi:
354 10.1016/j.suronc.2010.09.003. Epub 2010 Sep 29. PMID: 20880699.
355 31. Dixit R, Pandey M, Tripathi SK, Dwivedi AND, Shukla VK. Genetic
356 mutational analysis of β-catenin gene affecting GSK-3β phosphorylation
357 plays a role in gallbladder carcinogenesis: Results from a case control
358 study. Cancer Treatment and Research Communications, 2020;
359 23:100173 doi.org/10.1016/j.ctarc.2020.100173 PMID: 32344182
360 32. Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: gene set
361 analysis toolkit with revamped UIs and APIs. Nucleic Acids Res. 2019
362 Jul 2;47(W1):W199-W205. doi: 10.1093/nar/gkz401. PMID: 31114916;
363 PMCID: PMC6602449.
364 33. Xia J, Gill EE, Hancock RE. NetworkAnalyst for statistical, visual and
365 network-based meta-analysis of gene expression data. Nat Protoc. 2015
366 Jun;10(6):823-44. doi: 10.1038/nprot.2015.052. Epub 2015 May 7.
367 PMID: 25950236.
368 34. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J,
369 Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV.
370 STRING v11: protein-protein association networks with increased
371 coverage, supporting functional discovery in genome-wide experimental

372 datasets. Nucleic Acids Res. 2019 Jan 8;47(D1):D607-D613. doi:
373 10.1093/nar/gky1131. PMID: 30476243; PMCID: PMC6323986.
374 35. Franz M, Rodriguez H, Lopes C, Zuberi K, Montojo J, Bader GD,
375 Morris Q. GeneMANIA update 2018. Nucleic Acids Res. 2018 Jul
376 2;46(W1):W60-W64. doi: 10.1093/nar/gky311. PMID: 29912392;
377 PMCID: PMC6030815.
378 36. Korotkevich G, Sukhov V, Budin N, Shpak B, Artyomov MN,
379 Sergushichev A. Fast gene set enrichment analysis. BioRxiv, 2021:
380 060012. https://www.biorxiv.org/content/10.1101/060012v3
381 37. Lo Surdo P, Calderone A, Cesareni G, Perfetto L. SIGNOR: A Database
382 of Causal Relationships Between Biological Entities-A Short Guide to
383 Searching and Browsing. Curr Protoc Bioinformatics. 2017 Jun
384 27;58:8.23.1-8.23.16. doi: 10.1002/cpbi.28. PMID: 28654729.
385 38. Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome.
386 Clin Epidemiol. 2014 Mar 7;6:99-109. doi: 10.2147/CLEP.S37357.
387 PMID: 24634588; PMCID: PMC3952897.
388 39. Dasari BVM, Ionescu MI, Pawlik TM, Hodson J, Sutcliffe RP, Roberts
389 KJ, Muiesan P, Isaac J, Marudanayagam R, Mirza DF. Outcomes of
390 surgical resection of gallbladder cancer in patients presenting with
391 jaundice: A systematic review and meta-analysis. J Surg Oncol. 2018
392 Sep;118(3):477-485. doi: 10.1002/jso.25186. PMID: 30259519.
393 40. Haugsten EM, Wiedlocha A, Olsnes S, Wesche J. Roles of fibroblast
394 growth factor receptors in carcinogenesis. Mol Cancer Res. 2010
395 Nov;8(11):1439-52. doi: 10.1158/1541-7786.MCR-10-0168. Epub 2010
396 Oct 13. PMID: 21047773.
397 41. Espinoza JA, Bizama C, García P, Ferreccio C, Javle M, Miquel JF,
398 Koshiol J, Roa JC. The inflammatory inception of gallbladder cancer.

399 Biochim Biophys Acta. 2016 Apr;1865(2):245-54. doi:
400 10.1016/j.bbcan.2016.03.004. Epub 2016 Mar 12. PMID: 26980625;
401 PMCID: PMC6287912.
402 42. Chao TC, Wang CS, Jan YY, Chen HM, Chen MF. Carcinogenesis in
403 the biliary system associated with APDJ. J Hepatobiliary Pancreat Surg.
404 1999;6(3):218-22. doi: 10.1007/s005340050110. PMID: 10526055.
405 43. Asai T, Loza E, Roig GV, Ajioka Y, Tsuchiya Y, Yamamoto M,
406 Nakamura K. High frequency of TP53 but not K-ras gene mutations in
407 Bolivian patients with gallbladder cancer. Asian Pac J Cancer Prev.
408 2014;15(13):5449-54. doi: 10.7314/apjcp.2014.15.13.5449. PMID:
409 25041017.
410 44. Hirata K, Kuwatani M, Suda G, Ishikawa M, Sugiura R, Kato S,
411 Kawakubo K, Sakamoto N. A Novel Approach for the Genetic Analysis
412 of Biliary Tract Cancer Specimens Obtained Through Endoscopic
413 Ultrasound-Guided Fine Needle Aspiration Using Targeted Amplicon
414 Sequencing. Clin Transl Gastroenterol. 2019 Mar;10(3):e00022. doi:
415 10.14309/ctg.0000000000000022. PMID: 30908307; PMCID:
416 PMC6445609.
417 45. Liu P, Wang Y, Li X. Targeting the untargetable KRAS in cancer
418 therapy. Acta Pharm Sin B. 2019 Sep;9(5):871-879. doi:
419 10.1016/j.apsb.2019.03.002. Epub 2019 Mar 6. PMID: 31649840;
420 PMCID: PMC6804475.
421 46. Scanu T, Spaapen RM, Bakker JM, Pratap CB, Wu LE, Hofland I,
422 Broeks A, Shukla VK, Kumar M, Janssen H, Song JY, Neefjes-Borst EA,
423 te Riele H, Holden DW, Nath G, Neefjes J. Salmonella Manipulation of
424 Host Signaling Pathways Provokes Cellular Transformation Associated
425 with Gallbladder Carcinoma. Cell Host Microbe. 2015 Jun 10;17(6):763-

74. doi: 10.1016/j.chom.2015.05.002. Epub 2015 May 28. PMID: 26028364.

47. Mohri D, Ijichi H, Miyabayashi K, Takahashi R, Kudo Y, Sasaki T, Asaoka Y, Tanaka Y, Ikenoue T, Tateishi K, Tada M, Isayama H, Koike K. A potent therapeutics for gallbladder cancer by combinatorial inhibition of the MAPK and mTOR signaling networks. J Gastroenterol. 2016 Jul;51(7):711-21. doi: 10.1007/s00535-015-1145-1. Epub 2015 Nov 27. PMID: 26614007.

48. Chen K, Zhu P, Chen W, Luo K, Shi XJ, Zhai W. Melatonin inhibits proliferation, migration, and invasion by inducing ROS-mediated apoptosis via suppression of the PI3K/Akt/mTOR signaling pathway in gallbladder cancer cells. Aging (Albany NY). 2021 Sep 27;13(18):22502-22515. doi: 10.18632/aging.203561. Epub 2021 Sep 27. PMID: 34580235; PMCID: PMC8507264.

49. Yang D, Chen T, Zhan M, Xu S, Yin X, Liu Q, Chen W, Zhang Y, Liu D, Yan J, Huang Q, Wang J. Modulation of mTOR and epigenetic pathways as therapeutics in gallbladder cancer. Mol Ther Oncolytics. 2020 Dec 3;20:59-70. doi: 10.1016/j.omto.2020.11.007. PMID: 33575471; PMCID: PMC7851494.

50. Wencong M, Jinghan W, Yong Y, Jianyang A, Bin L, Qingbao C, Chen L, Xiaoqing J. FOXK1 Promotes Proliferation and Metastasis of Gallbladder Cancer by Activating AKT/mTOR Signaling Pathway. Front Oncol. 2020 Apr 17;10:545. doi: 10.3389/fonc.2020.00545. PMID: 32363163; PMCID: PMC7180204.

51. Zong H, Yin B, Zhou H, Cai D, Ma B, Xiang Y. Inhibition of mTOR pathway attenuates migration and invasion of gallbladder cancer via

EMT inhibition. Mol Biol Rep. 2014 Jul;41(7):4507-12. doi: 10.1007/s11033-014-3321-4. Epub 2014 Mar 13. PMID: 24623408.

52. Yang P, Javle M, Pang F, Zhao W, Abdel-Wahab R, Chen X, Meric-Bernstam F, Chen H, Borad MJ, Liu Y, Zou C, Mu S, Xing Y, Wang K, Peng C, Che X. Somatic genetic aberrations in gallbladder cancer: comparison between Chinese and US patients. Hepatobiliary Surg Nutr. 2019 Dec;8(6):604-614. doi: 10.21037/hbsn.2019.04.11. PMID: 31929987; PMCID: PMC6943012.

53. Amin M, Gao F, Terrero G, Picus J, Wang-Gillam A, Suresh R, Ma C, Tan B, Baggstrom M, Naughton MJ, Trull L, Belanger S, Fracasso PM, Lockhart AC. Phase I Study of Docetaxel and Temsirolimus in Refractory Solid Tumors. Am J Clin Oncol. 2021 Sep 1;44(9):443-448. doi: 10.1097/COC.0000000000000852. PMID: 34310349.

Legends for figures-

Figure 1 - Pathway Analysis: Wikicancer Pathway: 10 positive related categories and 4 negative related categories are identified as enriched categories.

Figure 2 – Signaling Pathway Network diagram

Figure 3 – Protein-Protein Interaction network of 14 genes was constructed in STRING

Figure 4 – Gene-Gene Interaction network of 14 genes was constructed in GeneMania

Figure 5: Functional characterization of genes through Network Analyst tool

Figure 6 – Disease Ontology of 14 genes constructed through Webgestalt

Figure 7: Showing the crosstalk between various pathways identified through bioinformatic analysis of NGS data in gallbladder cancer.

Table 1 – List of 14 significant genes.

Gene-Name	Description	UniProt _Id	Score
TP53	tumor protein p53	P04637	79.46
KRAS	KRAS proto-oncogene, GTPase	P01116	106.48
EGFR	epidermal growth factor receptor	P00533	167.6
KDR	kinase insert domain receptor	P35968	64.13
NRAS	NRAS proto-oncogene, GTPase	P01111	76.05
MAP3K1	mitogen-activated protein kinase kinase kinase 1	Q13233	58.84
BRAF	B-Raf proto-oncogene, serine/threonine kinase	P15056	116.32
PTEN	phosphatase and tensin homolog	P60484	191.07
CTNNB1	catenin beta 1	P35222	87.09
FGFR3	fibroblast growth factor receptor 3	P22607	99.14
PDGFRA	platelet derived growth factor receptor alpha	P16234	101.17
FBXW7	F-box and WD repeat domain containing 7	Q969H0	66.7
POLE	DNA polymerase epsilon, catalytic subunit	Q07864	42.19
SMAD4	SMAD family member 4	Q13485	87.93

Table 2(A) – G0: BIOLOGICAL PROCESS

FDR; false discovery rate

Gene Set	Description	Size	Expect	Ratio	P-Value	FDR
GO:0000165	MAPK cascade	896	0.75276	15.941	0.000000000000044853	0.00000000023567
GO:00230	signal transduction by	907	0.76200	15.748	0.000000000000	0.00000000

14	protein phosphorylation				051847	023567
GO:0001934	positive regulation of protein phosphorylation	982	0.82501	14.545	0.00000000000013411	0.00000000040641
GO:0042327	positive regulation of phosphorylation	1028	0.86366	13.894	0.00000000000023181	0.00000000052686
GO:0043408	regulation of MAPK cascade	745	0.62590	17.575	0.00000000000042732	0.00000000006536
GO:0010562	positive regulation of phosphorus metabolic process	1097	0.92163	13.020	0.00000000000042732	0.00000000006536
GO:0045937	positive regulation of phosphate metabolic process	1097	0.92163	13.020	0.00000000000050326	0.00000000006536
GO:0043410	positive regulation of MAPK cascade	546	0.45871	21.800	0.00000000000117	0.00000000013295
GO:0031401	positive regulation of protein modification process	1190	0.99976	12.003	0.00000000000013278	0.00000000013413
GO:0006468	protein phosphorylation	1860	1.5627	8.3192	0.00000000000050494	0.00000000045904

484

485 **Table 2(B) - GO: CELLULAR COMPONENT**

486 FDR; false discovery rate

Gene Set	Description	Size	Expect	Ratio	P-Value	FDR
GO:0045121	membrane raft	311	0.22981	17.406	0.000060379	0.025307
GO:0098857	membrane	312	0.23055	17.350	0.000061138	0.025307

	microdomain					
GO:0098589	membrane region	324	0.23941	16.708	0.000070799	0.025307
GO:0030054	cell junction	1268	0.93696	6.4037	0.00015284	0.025307
GO:0043235	receptor complex	396	0.29262	13.670	0.00015387	0.025307
GO:0005925	focal adhesion	404	0.29853	13.399	0.00016619	0.025307
GO:0005924	cell-substrate adherens junction	407	0.30074	13.300	0.00017099	0.025307
GO:0030055	cell-substrate junction	411	0.30370	13.171	0.00017754	0.025307
GO:0090575	RNA polymerase II transcription factor complex	159	0.11749	25.534	0.00019384	0.025307
GO:0044798	nuclear transcription factor complex	196	0.14483	20.714	0.00035868	0.042145

487

488 **Table 2(C) - GO: MOLECULAR FUNCTION**

489 FDR; false discovery rate

Gene Set	Description	Size	Expect	Ratio	P-Value	FDR
GO:0004714	transmembrane receptor protein tyrosine kinase activity	64	0.053756	74.411	0.00000019221	0.00035199
GO:0019199	transmembrane receptor protein kinase activity	81	0.068035	58.794	0.0000004992	0.00035199
GO:0044877	protein-containing complex binding	1062	0.89201	8.9685	0.00000056259	0.00035199
GO:0004709	MAP kinase kinase	27	0.022678	132.29	0.0000013635	0.00053512

	kinase activity					
GO:0042802	identical protein binding	1696	1.4245	6.3179	0.0000014255	0.00053512
GO:0035639	purine ribonucleoside triphosphate binding	1786	1.5001	5.9995	0.0000022127	0.00066600
GO:0032555	purine ribonucleotide binding	1850	1.5539	5.7920	0.0000029824	0.00066600
GO:0017076	purine nucleotide binding	1865	1.5665	5.7454	0.0000031934	0.00066600
GO:0032553	ribonucleotide binding	1865	1.5665	5.7454	0.0000031934	0.00066600
GO:0019838	growth factor binding	138	0.11591	34.509	0.0000042221	0.00079249

Figure 1 - PATHWAY ANALYSIS: WIKICANCER PATHWAY

10 positive related categories and **4 negative related** categories are identified as enriched categories.

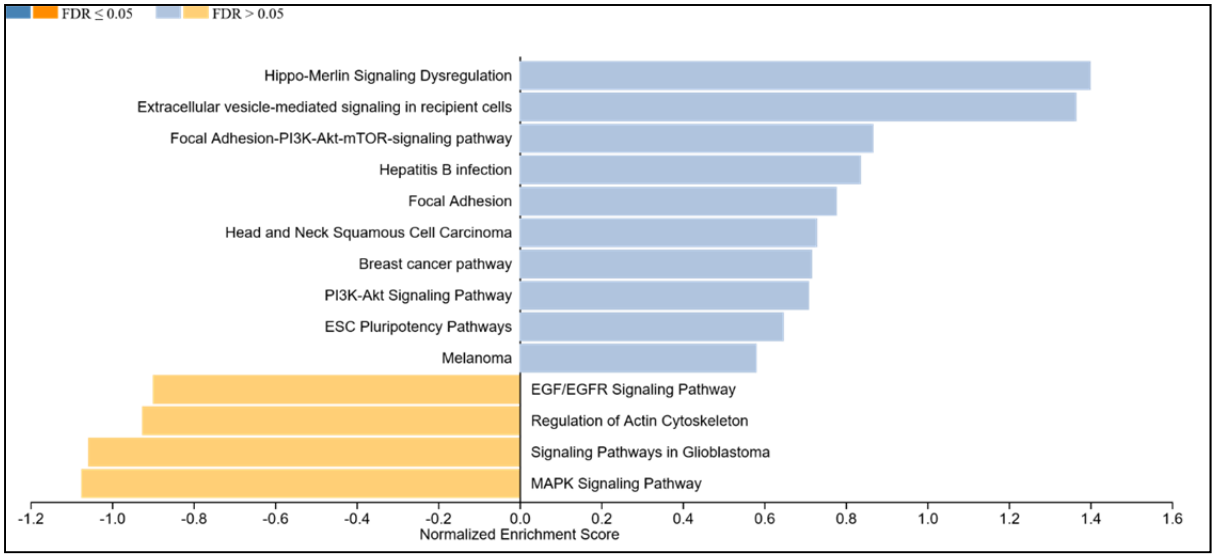

Figure 2 - Pathway Network

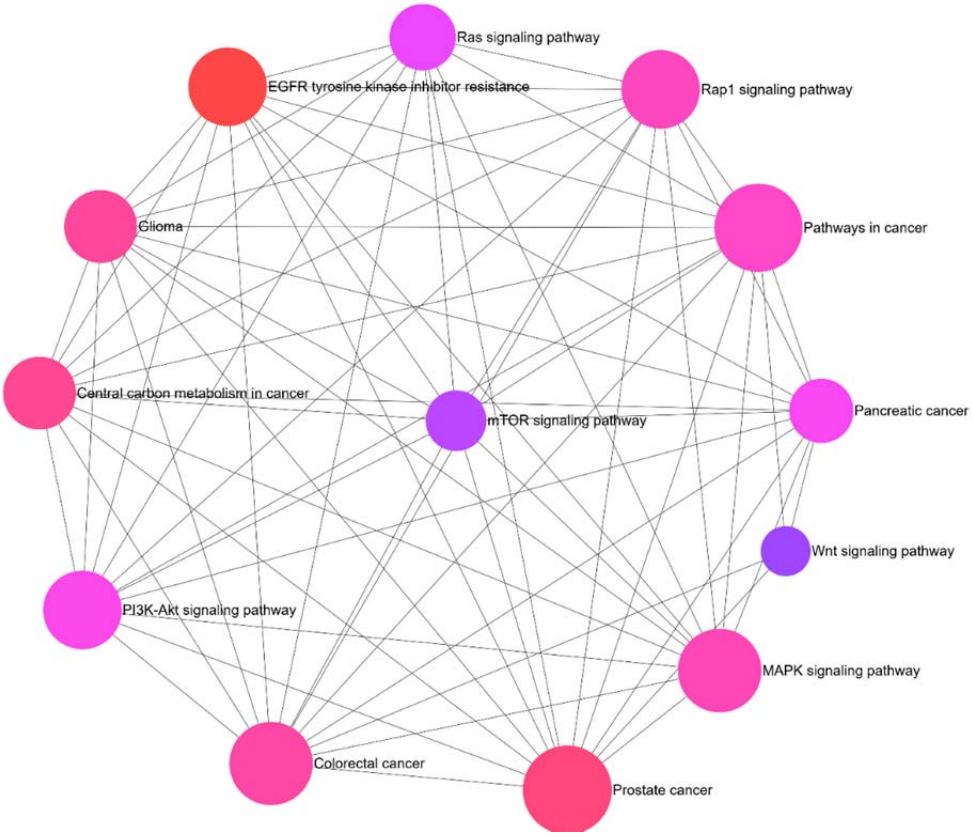

Figure 3 – Protein-Protein Interaction network of 14 genes was constructed in STRING

Known Interactions - curated databases – light blue; experimentally determined - pink

Predicted Interactions - gene neighborhood – green; gene fusions – red; gene co-occurrence – dark blue

Others - Text-mining – yellow; co-expression – black; protein homology – grey

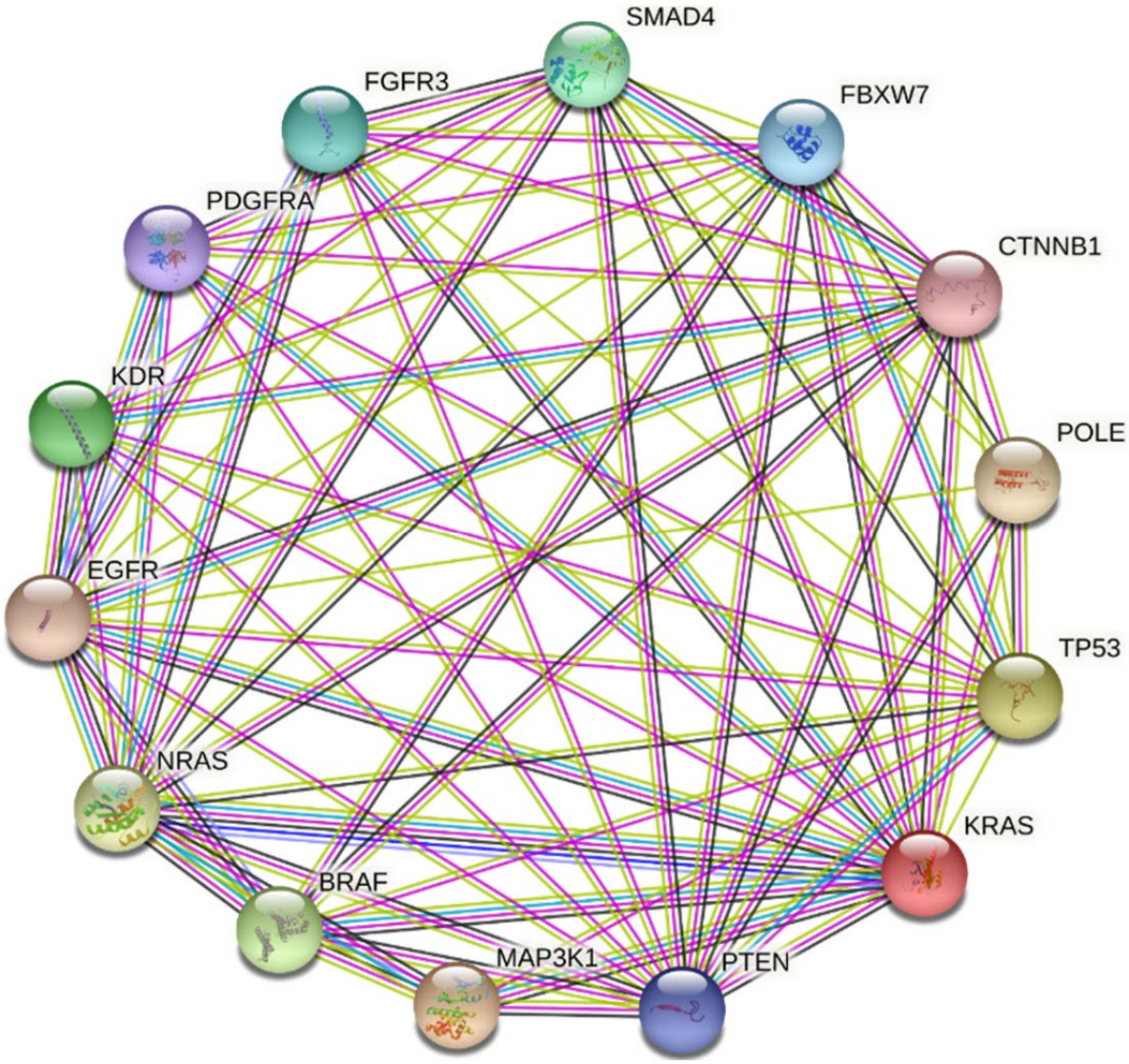

Figure 4 – Gene-Gene Interaction network of 14 genes was constructed in GeneMania

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534 Colored edges represented the interaction between the genes; physical interaction- pink; genetic interaction- green;
535 predicted- orange; co-expression- purple; shared protein domain- grey; pathway- light blue.

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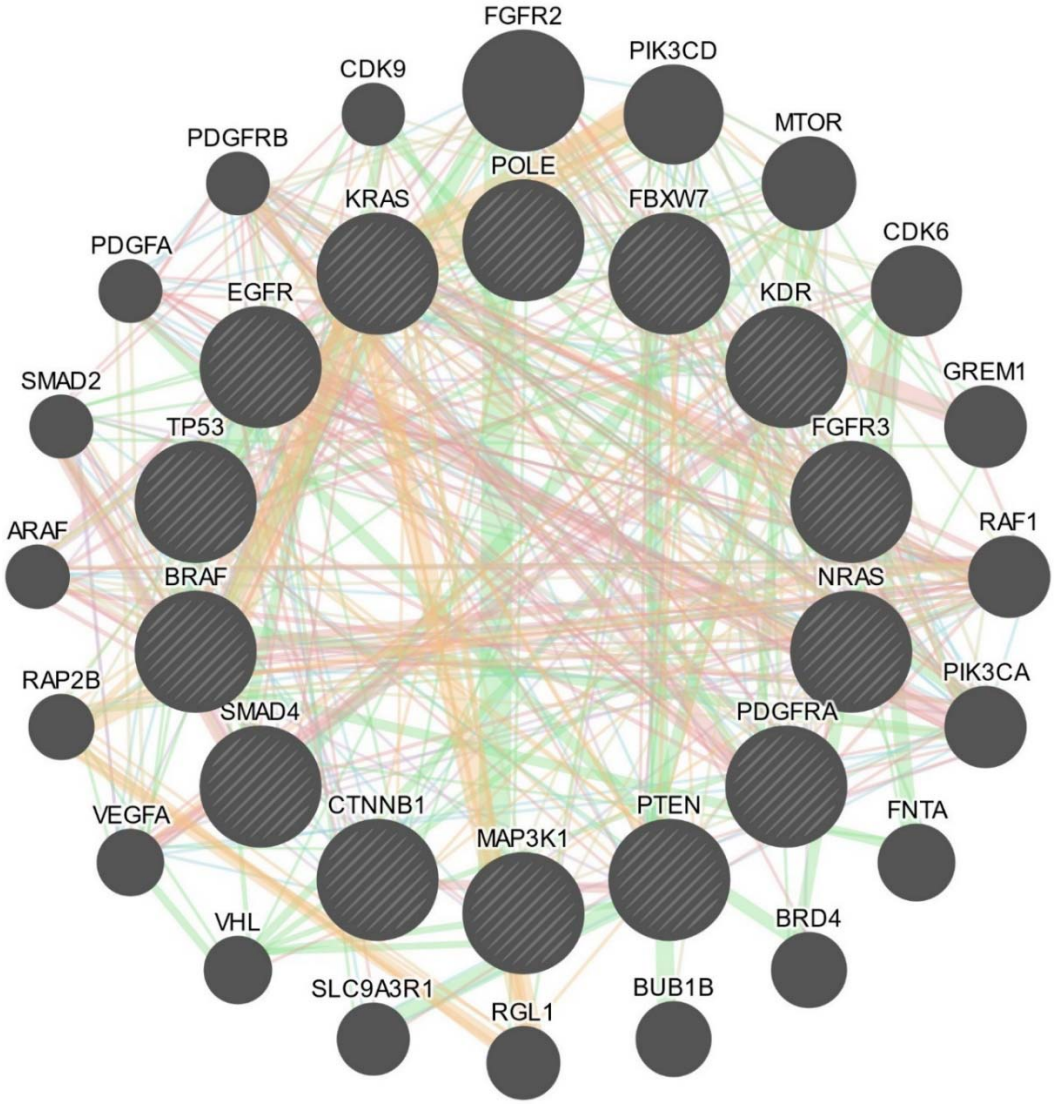

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Figure 5 - Signaling network

Genes (circle light green), complex (Circle light green), pink (circle dark), chemical (square light blue), protein family (square orange), small molecule (square light pink), stimulus (square blue-green), and phenotype (square yellow).

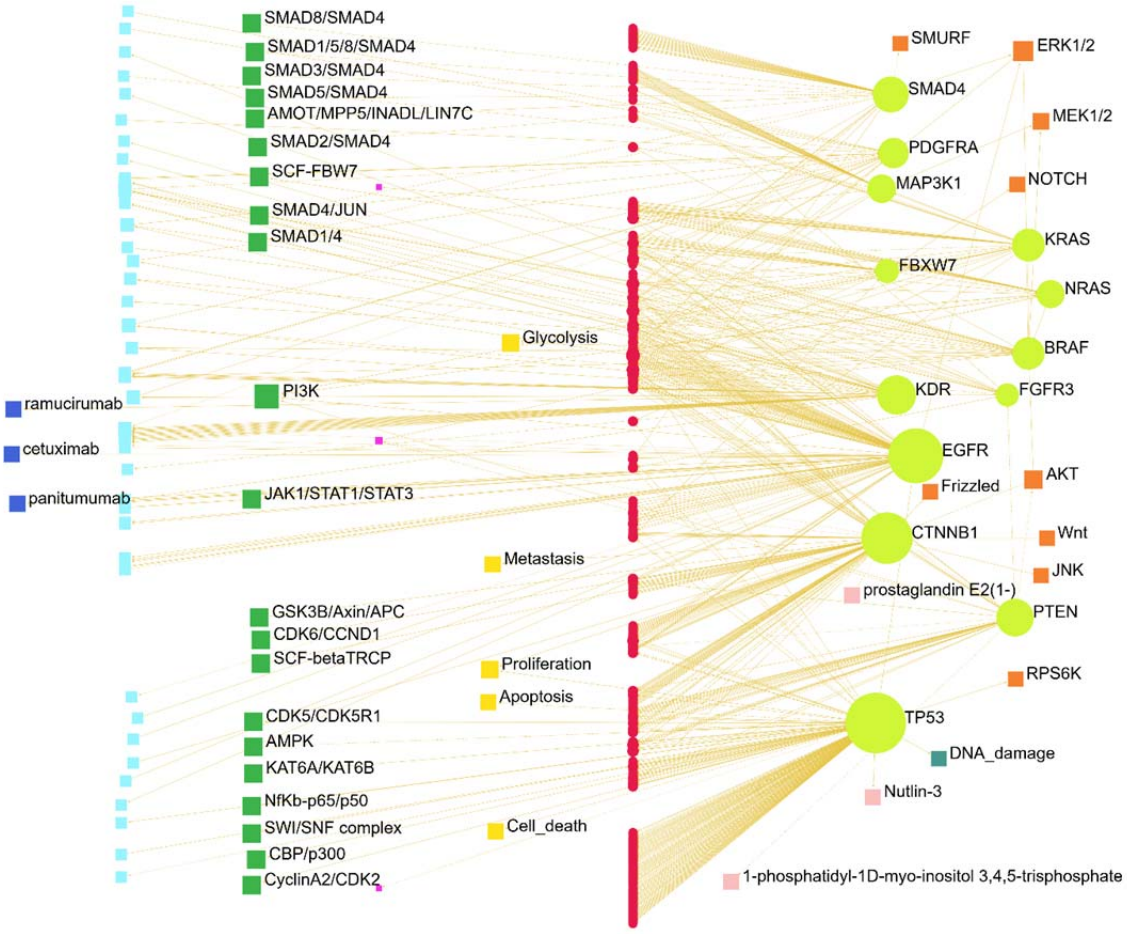

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Figure 6 – Disease Ontology of 14 genes was constructed through Webgestalt

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10 positive related categories and **7 negative related** categories are identified as enriched categories.

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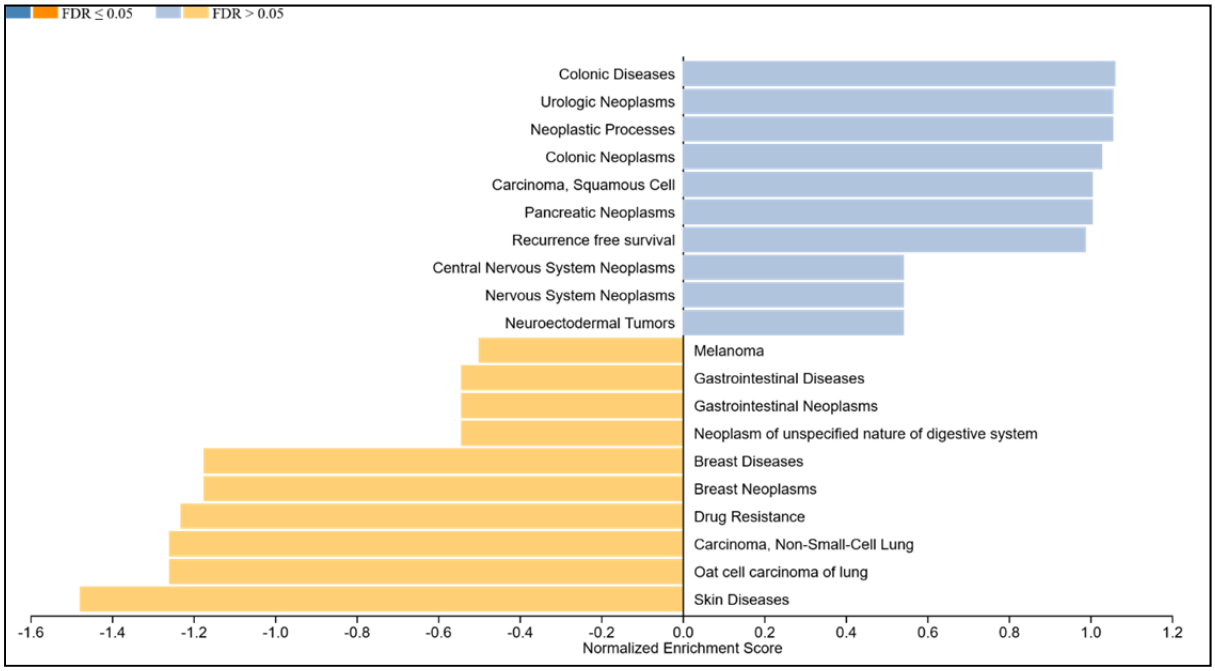

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Figure 7 - Crosstalk among associated pathways

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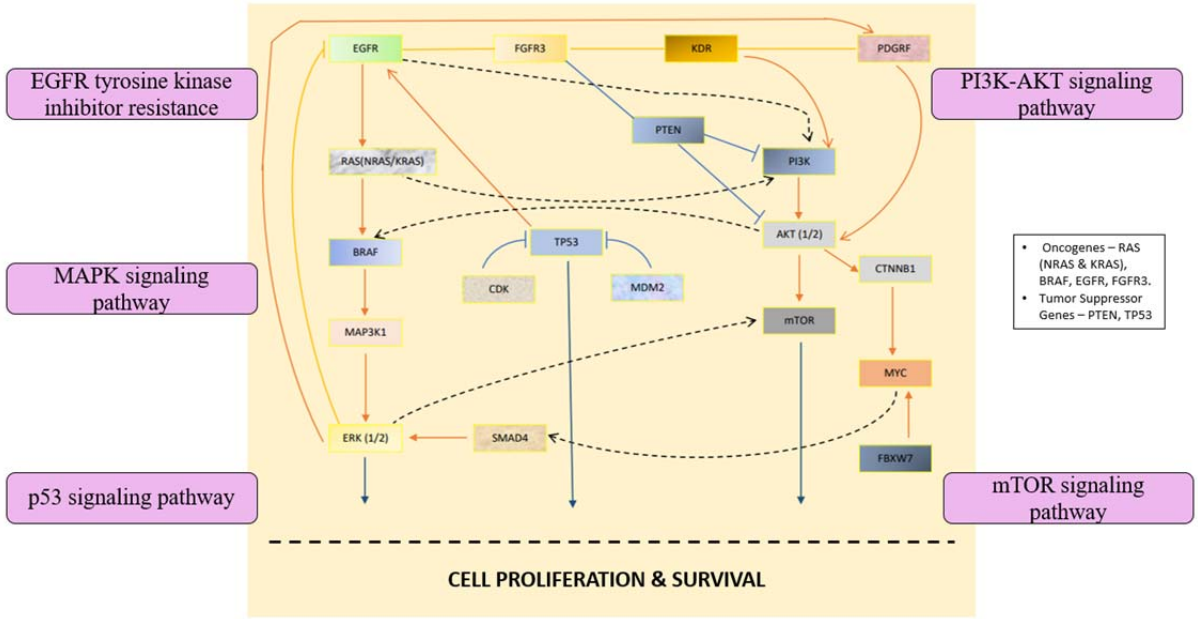

Additional Figure 1 – Gene-Associated disease Interaction network of 14 genes was constructed through NetworkAnalyst.

Gene-Disease associated network of significantly enriched 6 genes was constructed in NetworkAnalyst. Here, colored circular dots represent genes whereas a blue-colored square box represents associated diseases.

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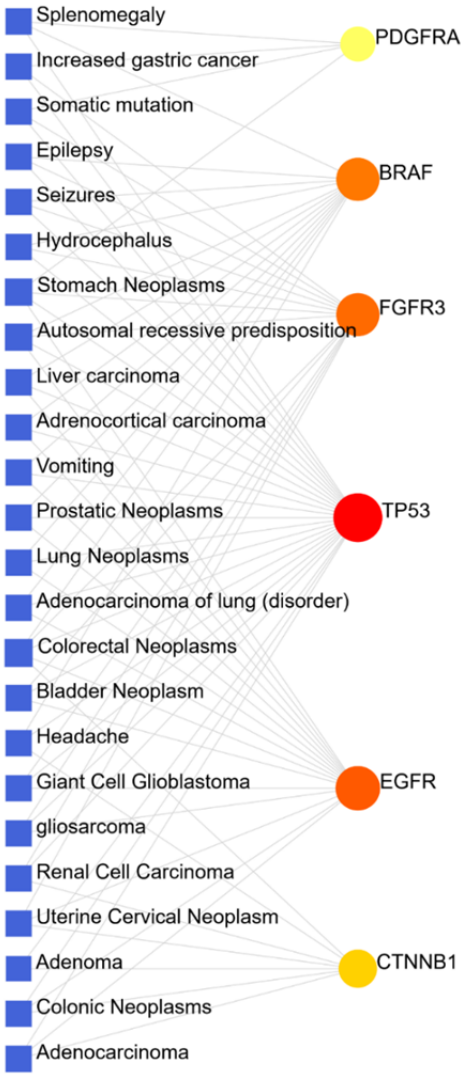

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573 **Additional Table 1 – Genemania Network**

Group	Name	Weight	Reference
Physical Interactions	Li-Fu-2017	0.1534091	The OncoPPi network of cancer-focused protein-protein interactions to inform biological insights and therapeutic strategies. Li et al. (2017). Nat Commun
Physical Interactions	IREF-bhf-ucl	0.083547061	
Physical Interactions	Huttlin-Harper-2017	0.061949513	Architecture of the human interactome defines protein communities and disease networks. Huttlin et al. (2017). Nature
Physical Interactions	Kennedy-Kolch-2020 A	0.054815514	Extensive rewiring of the EGFR network in colorectal cancer cells expressing transforming levels of KRAS^{G13D}. Kennedy et al. (2020). Nat Commun
Physical Interactions	Barrios-Rodiles-	0.044266821	High-throughput mapping of a dynamic signaling network in mammalian cells.

	Wrana-2005		Barrios-Rodiles et al. (2005). Science
Physical Interactions	IREF-dip	0.014490396	
Physical Interactions	Lim-Zoghbi-2006	0.006780127	A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. Lim et al. (2006). Cell
Physical Interactions	IREF-mint	0.006441559	
Physical Interactions	Vastrik-Stein-2007	0.003195357	Reactome: a knowledge base of biologic pathways and processes. Vastrik et al. (2007). Genome Biol
Physical Interactions	IREF-reactome	0.003195357	
Predicted	I2D-Formstecher-Daviet-2005-Head-Fly2Human	0.157594586	Protein interaction mapping: a Drosophila case study. Formstecher et al. (2005). Genome Res
Predicted	I2D-Formstecher-Daviet-2005-Embryo-Fly2Human	0.055660286	Protein interaction mapping: a Drosophila case study. Formstecher et al. (2005). Genome Res
Predicted	I2D-BioGRID-Mouse2Human	0.019817572	BioGRID: a general repository for interaction datasets. Stark et al. (2006). Nucleic Acids Res
Predicted	I2D-MINT-Mouse2Human	0.019571411	MINT: a Molecular INTeraction database. Zanzoni et al. (2002). FEBS Lett
Predicted	I2D-IntAct-Fly2Human	0.016090701	The IntAct molecular interaction database in 2010. Aranda et al. (2010). Nucleic Acids Res
Predicted	Wu-Stein-2010	0.013103625	A human functional protein interaction network and its application to cancer data analysis. Wu et al. (2010). Genome Biol
Predicted	I2D-MINT-Fly2Human	0.004700681	MINT: a Molecular INTeraction database. Zanzoni et al. (2002). FEBS Lett
Co-expression	Chen-Brown-2002	0.05778729	Gene expression patterns in human liver cancers. Chen et al. (2002). Mol Biol Cell
Co-expression	Boldrick-Relman-2002	0.039628511	Stereotyped and specific gene expression programs in human innate immune responses to bacteria. Boldrick et al. (2002). Proc Natl Acad Sci U S A
Co-expression	Wang-Maris-2006	0.028184098	Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. Wang et al. (2006). Cancer Res
Co-expression	Rosenwald-Staudt-2001	0.023532352	Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. Rosenwald et al. (2001). J Exp Med
Co-expression	Alizadeh-Staudt-2000	0.017085698	Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Alizadeh et al. (2000). Nature
Co-expression	Rieger-Chu-2004	0.007378505	Toxicity from radiation therapy associated with abnormal transcriptional responses to DNA damage. Rieger et al. (2004). Proc Natl Acad Sci U S A
Co-expression	Jiang-de Kok-2017	0.005616687	Omics-based identification of the combined effects of idiosyncratic drugs and inflammatory cytokines on the development of drug-induced liver injury. Jiang et al. (2017). Toxicol Appl Pharmacol
Genetic Interactions	BIOGRID-SMALL-SCALE-STUDIES	0.0257526	
Genetic Interactions	Luo-Elledge-2009	0.022631469	A genome-wide RNAi screen identifies multiple synthetic lethal interactions with

			the Ras oncogene. Luo et al. (2009). Cell
Genetic Interactions	Lin-Smith-2010	0.018636898	A genome-wide map of human genetic interactions inferred from radiation hybrid genotypes. Lin et al. (2010). Genome Res
Genetic Interactions	Martin-Elledge-2017	0.018508272	A Role for Mitochondrial Translation in Promotion of Viability in K-Ras Mutant Cells. Martin et al. (2017). Cell Rep
Shared protein domains	INTERPRO	0.005217705	
Shared protein domains	PFAM	0.004710743	
Pathway	Wu-Stein-2010	0.006699506	A human functional protein interaction network and its application to cancer data analysis. Wu et al. (2010). Genome Biol

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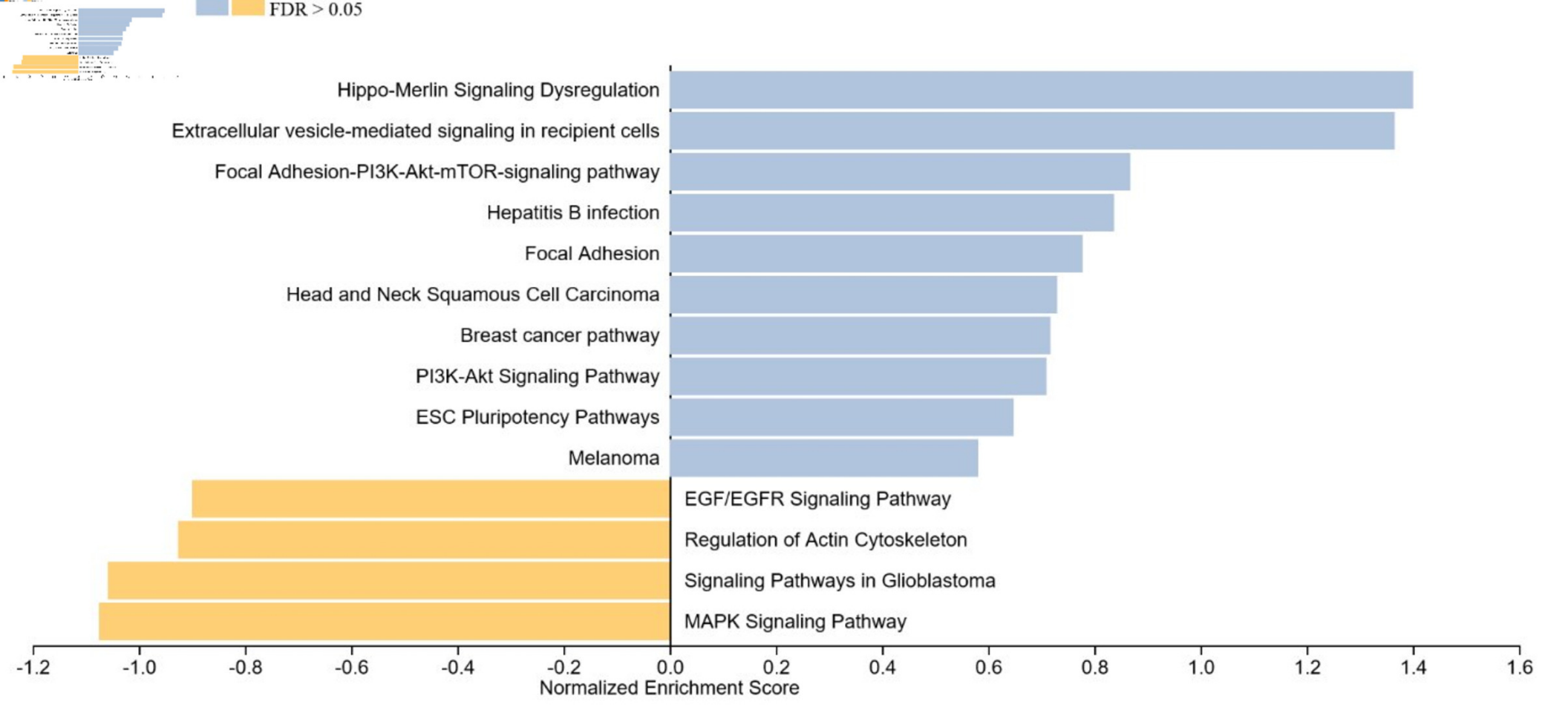
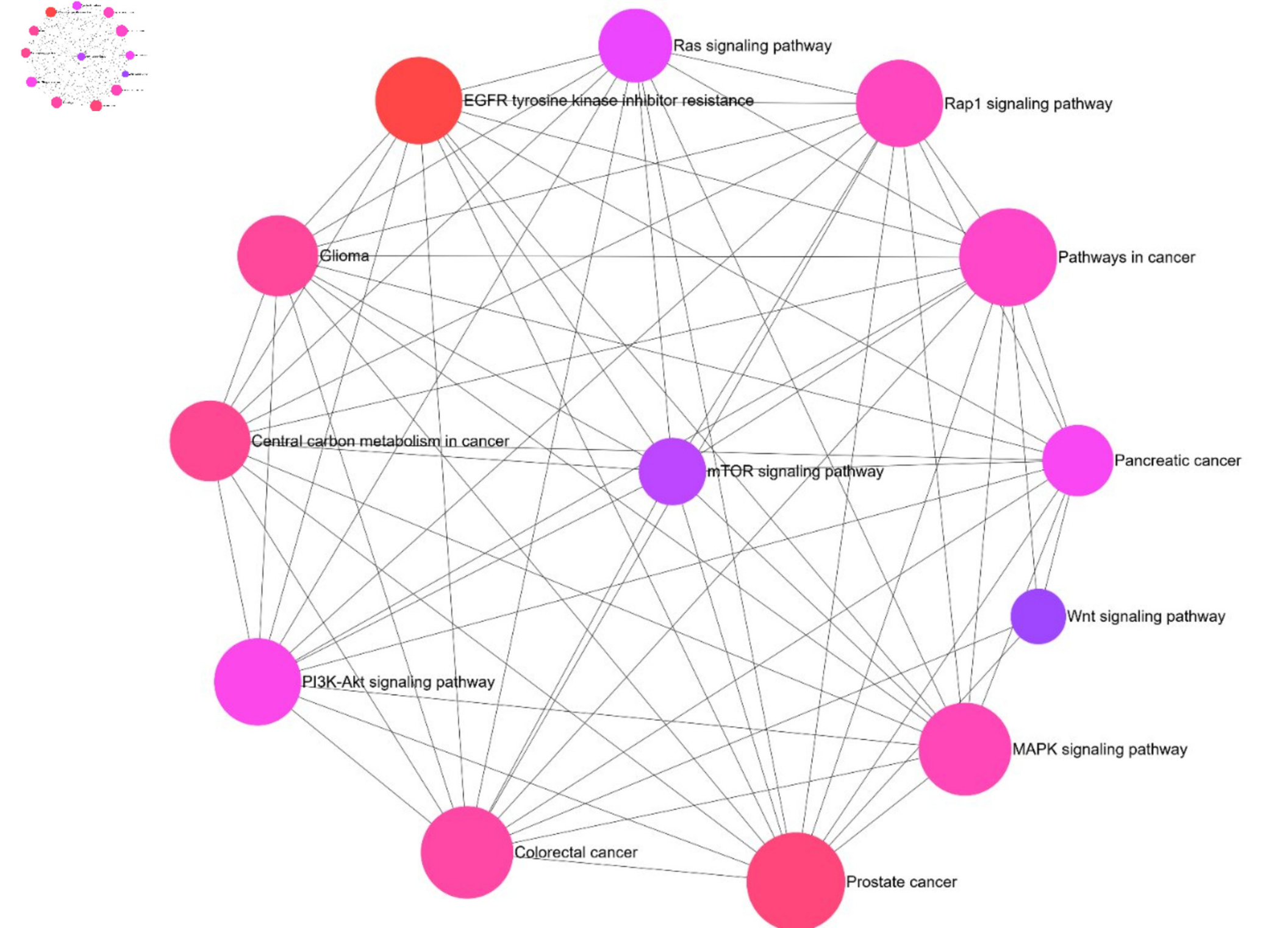
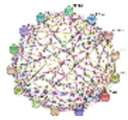
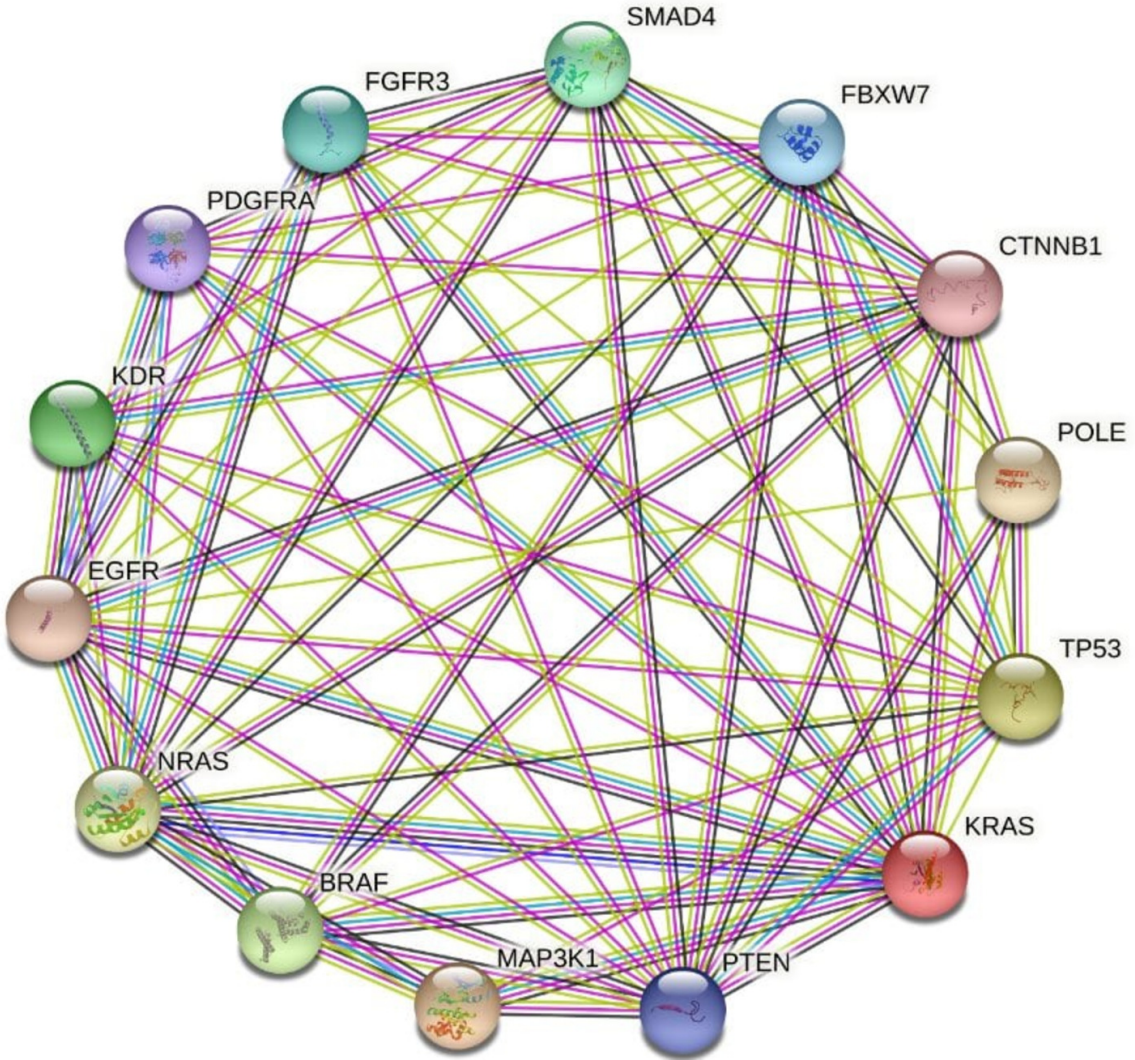

FDR > 0.05
Hippo-Merlin Signaling Dysregulation
Extracellular vesicle-mediated signaling in recipient cells
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway
Hepatitis B infection
Focal Adhesion
Head and Neck Squamous Cell Carcinoma
Breast cancer pathway
PI3K-Akt Signaling Pathway
ESC Pluripotency Pathways
Melanoma
EGF/EGFR Signaling Pathway
Regulation of Actin Cytoskeleton
Signaling Pathways in Glioblastoma
MAPK Signaling Pathway
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Normalized Enrichment Score


Ras signaling pathway
EGFR tyrosine kinase inhibitor resistance
Rap1 signaling pathway
Glioma
Pathways in cancer
Central carbon metabolism in cancer
mTOR signaling pathway
Pancreatic cancer
Wnt signaling pathway
PI3K-Akt signaling pathway
MAPK signaling pathway
Colorectal cancer
Prostate cancer

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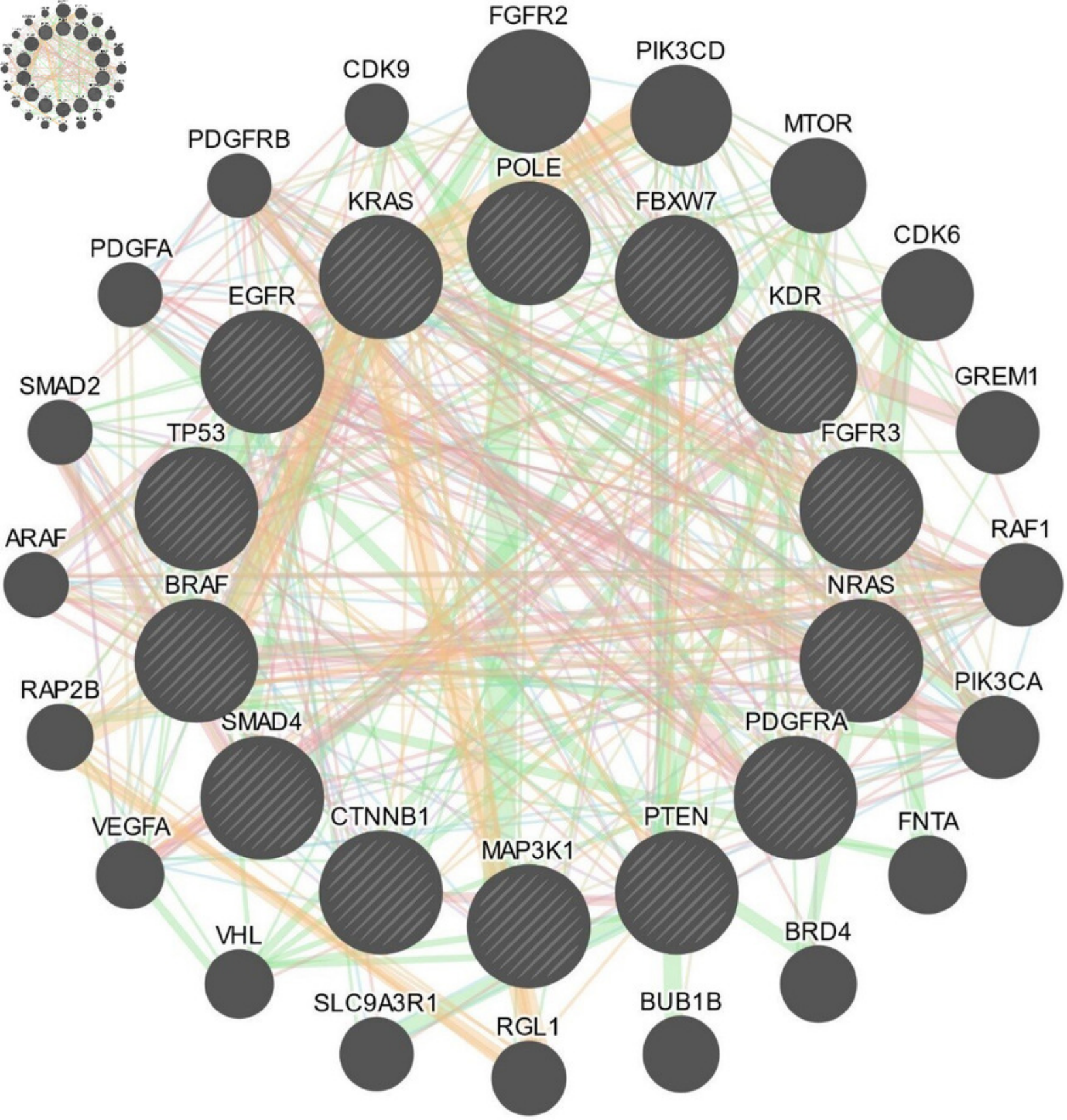
PDGFRA
CTNNB1
KDR
POLE
EGFR
TP53
NRAS
KRAS
BRAF
MAP3K1
PTEN


FGFR2
PIK3CD
CDK9
MTOR
PDGFRB
POLE
KRAS
FBXW7
CDK6
PDGFA
EGFR
KDR
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TP53
FGFR3
RAF1
ARAF
BRAF
NRAS
PIK3CA
RAP2B
SMAD4
PDGFRA
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VEGFA
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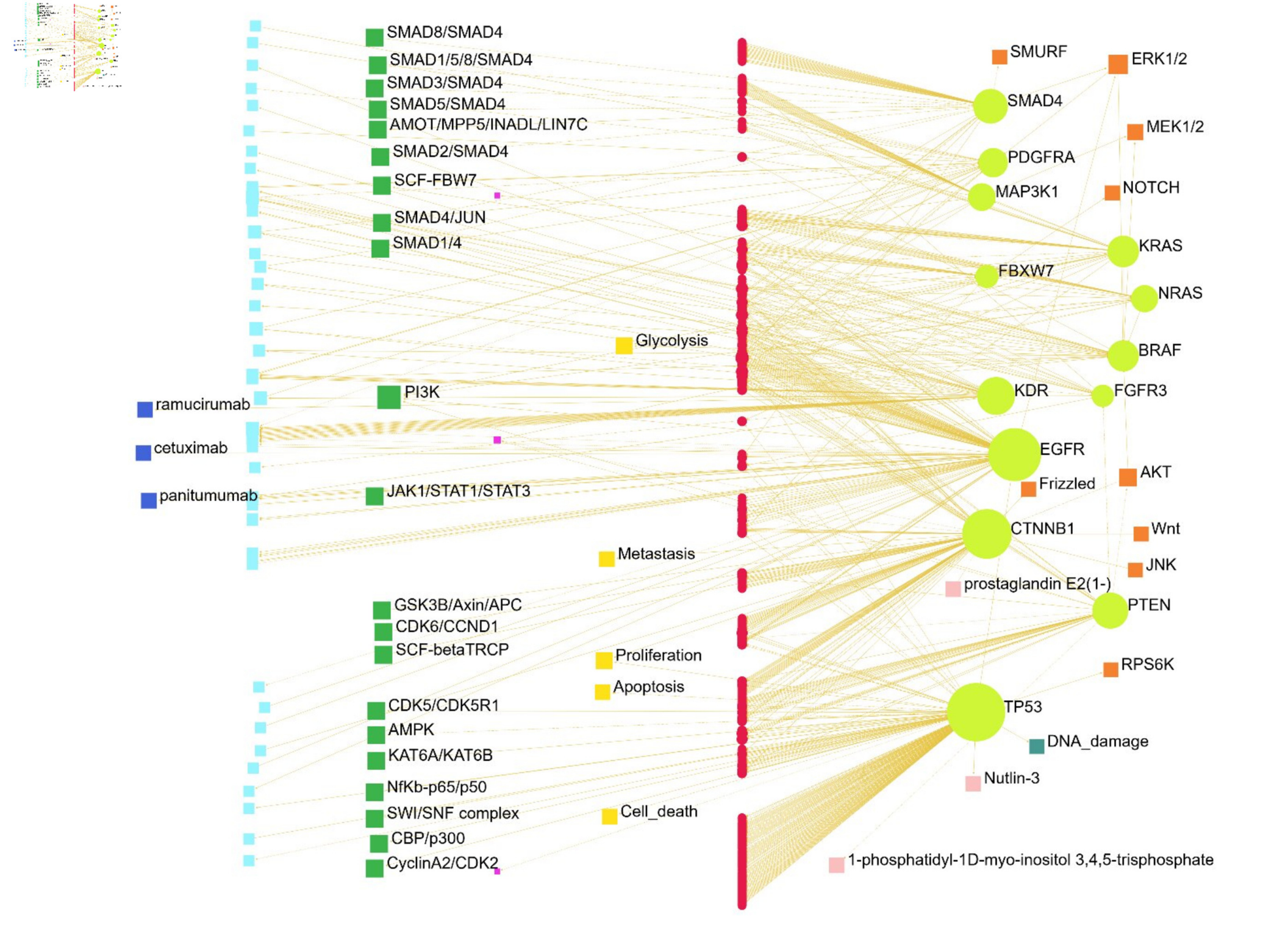
PTEN
MAP3K1
BRD4
VHL
SLC9A3R1
BUB1B
RGL1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SMAD1/5/8/SMAD4
SMAD3/SMAD4
SMAD5/SMAD4
AMOT/MPP5/INADL/LIN7C
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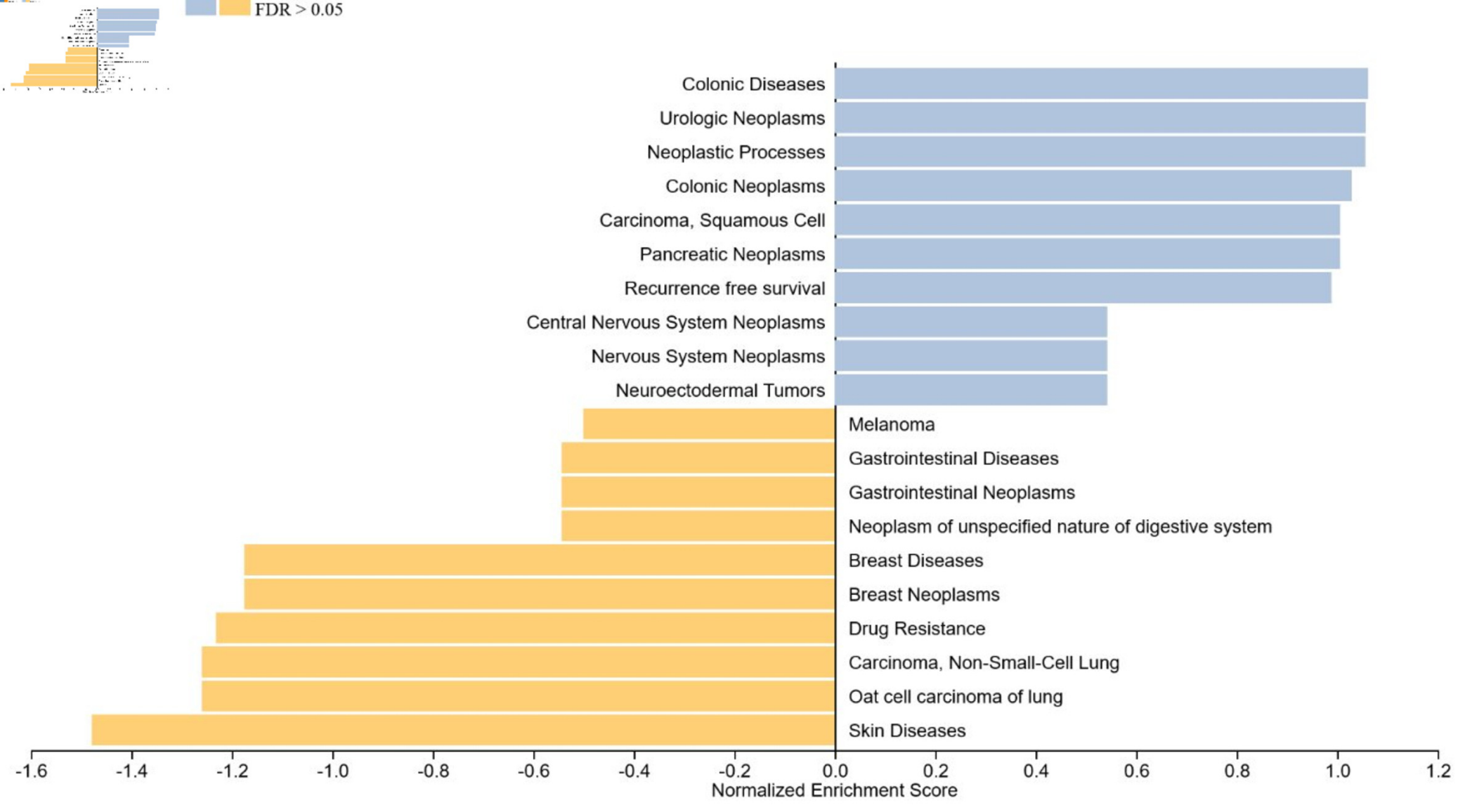
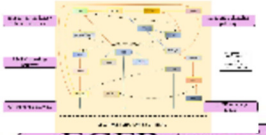
SCF-FBW7
SMAD4/JUN
SMAD1/4
Glycolysis
PI3K
ramucirumab
cetuximab
panitumumab
JAK1/STAT1/STAT3
Metastasis
GSK3B/Axin/APC
CDK6/CCND1
SCF-betaTRCP
Proliferation
Apoptosis
CDK5/CDK5R1
AMPK
KAT6A/KAT6B
NfKb-p65/p50
SWI/SNF complex
CBP/p300
CyclinA2/CDK2
Cell_death
SMURF
ERK1/2
SMAD4
MEK1/2
PDGFRA
MAP3K1
NOTCH
KRAS
FBXW7
NRAS
BRAF
KDR
FGFR3
EGFR
AKT
Frizzled
CTNNB1
Wnt
JNK
prostaglandin E2(1-)
PTEN
RPS6K
TP53
DNA_damage
Nutlin-3
1-phosphatidyl-1D-myo-inositol 3,4,5-trisphosphate


FDR > 0.05
Colonic Diseases
Urologic Neoplasms
Neoplastic Processes
Colonic Neoplasms
Carcinoma, Squamous Cell
Pancreatic Neoplasms
Recurrence free survival
Central Nervous System Neoplasms
Nervous System Neoplasms
Neuroectodermal Tumors
Melanoma
Gastrointestinal Diseases
Gastrointestinal Neoplasms
Neoplasm of unspecified nature of digestive system
Breast Diseases
Breast Neoplasms
Drug Resistance
Carcinoma, Non-Small-Cell Lung
Oat cell carcinoma of lung
Skin Diseases
-1.6
-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Normalized Enrichment Score



EGFR tyrosine kinase inhibitor resistance

MAPK signaling pathway

p53 signaling pathway

PI3K-AKT signaling pathway

mTOR signaling pathway

EGFR
FGFR3
KDR
PDGRF
PTEN
PI3K
RAS(NRAS/KRAS)
AKT (1/2)
BRAF
TP53
CDK
MDM2
CTNNB1
MAP3K1
mTOR
MYC
ERK (1/2)
SMAD4
FBXW7

- Oncogenes – RAS (NRAS & KRAS), BRAF, EGFR, FGFR3.
- Tumor Suppressor Genes – PTEN, TP53

CELL PROLIFERATION & SURVIVAL


Splenomegaly
Increased gastric cancer
Somatic mutation
Epilepsy
Seizures
Hydrocephalus
Stomach Neoplasms
Autosomal recessive predisposition
Liver carcinoma
Adrenocortical carcinoma
Vomiting
Prostatic Neoplasms
Lung Neoplasms
Adenocarcinoma of lung (disorder)
Colorectal Neoplasms
Bladder Neoplasm
Headache
Giant Cell Glioblastoma
gliosarcoma
Renal Cell Carcinoma
Uterine Cervical Neoplasm
Adenoma
Colonic Neoplasms
Adenocarcinoma
PDGFRA
BRAF
FGFR3
TP53
EGFR
CTNNB1