The EuroBiotech Journal
RESEARCH ARTICLE
Medical Biotechnology

Networking *Salt Inducible Kinase 1* Regulatory Perturbations on Type 2 Diabetes- Breast Cancer Co-Morbidity Associated Molecular Bridge

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Abstract

Type 2 diabetes mellitus (T2DM) is associated with a 16% elevated risk of breast cancer (BC). However, the underlying molecular mechanisms are yet to be fully understood. T2DM and BC are multifactorial and polygenic in nature, hence it is plausible an interplay between various signalling pathways be wired into the co-morbidity program. *Salt inducible kinase 1 (SIK1)* was previously validated in silico as a hub gene for T2DM-BC molecular crosstalk. To probe into its functional niche within the co-diseasome, this study constructed and subjected *SIK1* associated regulome to network modelling. Gene mutations, and transcription factors (TF), hub proteins and microRNA (miRNA) associated with *SIK1* and its protein-protein interactions (PPIs) were extracted from MuTarget and EnrichR, respectively. TF-miRNA regulatory network iteration was studied on Cytoscape, to identify SIK1 associated 143 PPIs. Interestingly, these were enriched for KEGG pathways *PI3K-AKT* signalling, and pathways in cancer. Furthermore, ClinVar disease terms particularly included T2DM and BC, highlighting their potential implication in co-morbidity. Top hub genes included *TP53, EP300, AKT1, CREB1, HIF1A, EGFR, SMARCA4, HDAC2, NFKB1* and *HDAC5*. Prospective studies on potentiating these hub genes particularly *TP53*, in context to *SIK1* molecular dynamics may provide further insights into the molecular links tying T2DM to BC.

Keywords: *Salt inducible kinase 1;* type 2 diabetes mellitus; breast cancer; regulome; hub genes

Introduction

Diabetes mellitus is a cluster of chronic metabolic disorders characterized by hyperglycemia. This can be accounted by a lack of insulin secretion, its inaction or a combination of both. In case of former, an autoimmunity mediated destruction of insulin secreting pancreatic beta cells forms the molecular basis for type 1 diabetes mellitus (T1DM) [1]. On the other hand, type 2 diabetes mellitus (T2DM), which accounts for 90-95% of the reported diabetic cases, progresses through insulin resistance leading to increased blood glucose levels and compensatory hyperinsulinemia [2]. Further complications in the form of long-term multi-organ dysfunctions arise due to prolonged hyperglycemic exposures. These include vascular complications such as nephropathy, retinopathy, neuropathy, cardiovascular disease and cancer [3].

Diabetes and cancer are both complex, chronic diseases posing global health challenges with their ever increasing incidences [4]. At a prevalence of 10.5% globally and 17.1% in Pakistan, T2DM is deemed as the "silent killer" [5], [6], [7]. Global statistics also place cancer amongst the leading causes of death worldwide with up to 20 million new cases and 9.7 million deaths in 2022 [8]. To further aggravate the health burden, an association between T2DM and increased risk of several types of cancer has been reported. In particular, surmounting systematic evidence supports T2DM patients are at a 16% elevated risk of breast cancer (BC) as compared to individuals without T2DM, particularly rising to 38% in case of triple negative breast cancer (TNBC) [9], [10], [11].

Although, the exact underlying mechanisms sponsoring this causative association are yet to be fully deciphered, several hypotheses have been proposed. Research supports that the interplay between hyperglycemia, hypoxia, adiposity, hormonal imbalances and

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DOI: 10.2478/ebtj-2025-0008

chronic inflammation potentially plays a central role in T2DM-BC crosstalk [12]. The equation is further complicated with the multi-factorial interactions between genetics and environmental factors including but not limited to diet, age, obesity, and physical activity.

Decoding the molecular bases for T2DM-BC association may potentiate precise therapeutic targeting in cases of T2DM induced BC and T2DM incident in BC patients. Furthermore biomarker discovery may also enable co-morbidity associated risk modification, early diagnosis and prediction of prognostic outcome. To this end previous insilico studies have identified potential key genes associated with T2DM and BC [13], [14].

Of particular interest, the starting point of this study is *Salt inducible kinase 1* (*SIK1)*, a hub gene previously validated in silico for T2DM-BC crosstalk [13]. The study reported several other key genes common between T2DM and BC based on the differentially expressed hub gene analyses of disease specific transcriptomic data including but not limited to *tumor protein (TP53)*, and interleukins. However, only *SIK1* was also found to be differentially expressed in a co-morbid patient. This highlighted the potential for further investigating *SIK1* as a biomarker for T2DM-BC association.

SIK1 is one of the three serine threonine kinases (*SIKs*) belonging to the *adenosine monophosphate activated protein kinases (AMPK)* superfamily [15]. *SIKs* have previously been implicated in tumorigenesis, particularly associated with metabolic reprogramming [16]. In particular, *SIK1* is implicated in various physiologies including mineral balance, gluconeogenesis, and associated dysregulations such as diabetes [17]. Furthermore, Metformin, an *AMPK* agonist, routinely prescribed drug for T2DM, was reported to restore suppressed *SIK1* expression under hyperglycemic conditions. In case of BC, *SIK1* is crucial for p53 activation, subsequently leading to anoikis of tumour cells whereas its loss of function was associated with tumour metastasis [18], [19].

Based on these findings, the potential role of *SIK1* in mediating inter pathway crosstalk and disease-disease interactions is emergent, however, there is further need to understand in depth, its context specifically to T2DM-BC co-morbid state. Hence, this study employed a systems biology approach to model *SIK1* regulome and identify potential hub genes implicated in these diseases, particularly, on the T2DM-BC axis. Here, four levels of regulation are analysed including mutational profiles (in) directly modulating *SIK1* expression, miRNA, transcription factors (TF) and protein-protein interaction (PPI) hub proteins associated with *SIK1* and its 1st shell of interacting proteins.

The aim of this study design was to construct *SIK1* associated regulome and assess its functional niche, particularly in context to mechanisms associating with T2DM-BC signalling and specifically potentiating T2DM induced BC and BC complicated with T2DM. Understanding the role of *SIK1* in metabolic homeostasis, its disruption and corresponding origination of disease, particularly in context to T2DM and BC may provide a therapeutic window for targeting the T2DM-BC association, and hence address a global health concern.

Materials-Methods

This study employed a computational systems biology approach to evaluate the implication of SIK1 in T2DM-BC molecular crosstalk. Figure 1 summarizes the methodology adopted.

2.1. Mutation Analysis

Mutation analysis was performed for *SIK1* on MuTarget (https://www.mutarget.com/) which utilized TGCA breast cancer expression data from 979 patients. This web portal enables the identification of mutational targets particularly contributing towards novel potential biomarker and therapeutic target discovery [20]. Significant gene perturbations affecting *SIK1* expression in BC were extracted through performing the target run.

2.2. Interacting Proteins Extraction

Protein-protein interactions (PPIs) for *SIK1* were determined through STRING v12.0 (https://string-db.org/). This database integrates information on physical and functional interactions between proteins based on various sources including but not limited to scientific literature based text mining, and predictions based on databases and experiments showing interaction or co-expression [21]. These interactions are curated and then scored on the basis of confidence (low to very high). Top ten pairs (1st shell) at a confidence score of 0.4 were visualized.

2.3. Transcription Factor, Hub Proteins and miRNA Association Analysis

Transcription factors (TF) enriched for *SIK1* and its top ten interacting proteins (*SIK1*- interacting signature) were determined from functional enrichment tool EnrichR (https://maayanlab.cloud/Enrichr/), using the embedded Transcription Factor PPIs database generated data. EnrichR integrates up to 102 gene set libraries which enable gene enrichment analyses for a wide array of ontologies, pathways, phenotypes and other categories [22], [23]. Reported PPI hub genes were also extracted. The miRNAs corresponding to the *SIK1* and its interacting signature were also retrieved from EnrichR (outsourced from miRTarbase). The target genes for each of the miRNA were determined at miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php). This repository provides large scale data for miRNA-target gene interactions, sourced from biological experiments [24].

2.4. Protein-Protein Network Construction

All significant mutated genes, associated TFs, hub proteins and target genes of miRNA identified were composed as a gene set and submitted into STRING interface along with *SIK1* and SIK1- interacting signature. The protein-protein interactions (PPI) network was generated at a confidence score of 0.4.

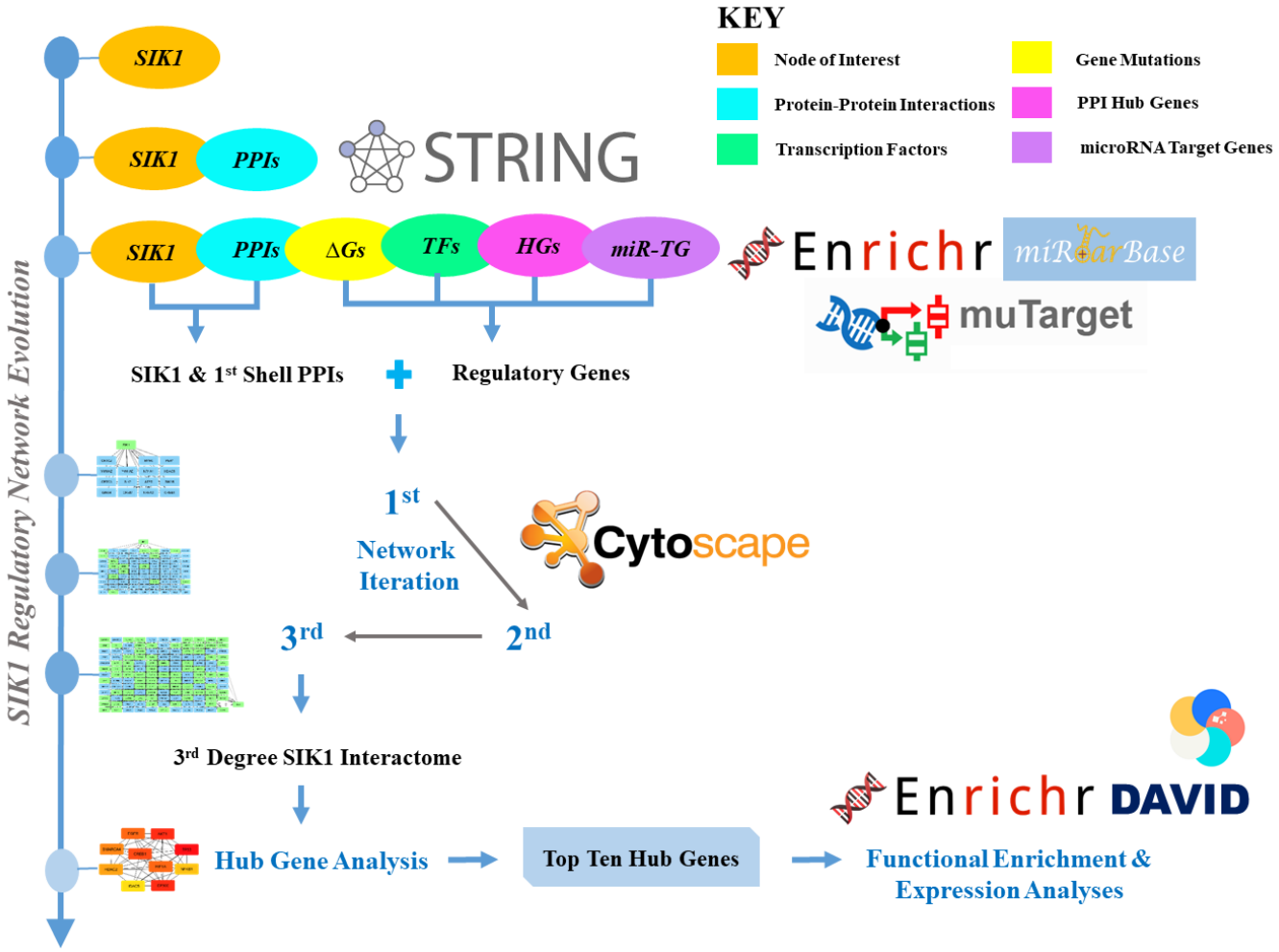

Figure 1. Study Methodology Flow Diagram. The figure depicts the bioinformatics pipeline adopted to construct and analyse SIK1's regulome and identify its hub genes. The directionality of the timeline represents first, the expanse of the regulatory network under study through its construction and then its narrowing down to the top ten genes as the emergent molecular crux through hub gene analyses.

2.5. Network Iteration and Hub Gene Analysis

The PPIs were imported into Cytoscape version 3.10.1, and analysed with the 'network analyser' tool. Cytoscape is an open source software that provides visualization and analysis of complex networks [25]. Cytohubba plugin version 0.1 was applied to iterate the network with SIK1 input as the node of interest. 1st, 2nd and 3rd stage interacting nodes and their networks were recorded. The 3rd stage (iterated) interaction network was further subjected to hub gene analysis. Topological parameter of degree was assessed to determine network centrality.

2.6. Functional Enrichment Analyses

The gene ontology particularly for biological processes was performed on EnrichR. Pathway enrichment was also studied through integrated KEGG database. DAVID software (https://david.ncifcrf.gov/) was utilized to generate KEGG map for the most enriched pathway. DAVID is another bioinformatics tool for gene set enrichment analyses and particularly enables the visualization of KEGG pathways, a feature not available on EnrichR [26].

2.7. Hallmarks of Cancer Overrepresentation Analysis

The association of the top ten hub genes with hallmarks of cancer was evaluated at Cancer Hallmarks (https://cancerhallmarks.com/). This is a web tool embedded with Kaplan Meir plotter, which integrates expression data from over 21 types of cancer and determines gene expression association with survival outcomes [27], [28]. The cancer hallmarks analysis utilizes an integrated gene set of 6763 candidate hallmark genes derived from seven resources to determine hallmarks of cancer enrichment for input genes. It also provides an additional resource i.e. a core set of genes n= 1574 that are common between two or more resources. The latter was utilized to elucidate on the representation of hub genes in various hallmarks of cancer.

2.8. Hub Gene Expression Validation

The expression of each of the hub genes was validated through previously studied and analysed datasets including GSE29231 (T2DM), GSE70905 (BC) and GSE150586 (co-morbid patients) [13]. The selection criteria for studying differential expression included adjusted $p < 0.05$ and log $|FC| \geq 1$.

Results

3.1. Target to Mutation Backtracking

A total of 81 significant gene mutations were found to be affecting *SIK1* expression (custom threshold set at p value cut off= 0.05 and fold change cut off = 1.44) with greater than 1% prevalence [20]. Top ten genes are tabulated and graphed, Table 1 and Figure 2.

Table 1. Mutation Targets for *SIK1*. Top ten significant mutations are listed along with the effect on *SIK1* expression in terms of up-regulation (↑) down-regulation (↓) and fold change (FC).

#	Gene	Effect on *SIK1* Expression	FC (Mutant/Wild-Type)
1	*TTC21B*	↓	33.33
2	*MAP3K13*	↓	6.25
3	*ANKRD12*	↓	5.56
4	*KMT2B*	↓	4.00
5	*SALL1*	↑	4.86
6	*TANC2*	↓	7.69
7	*DNAH17*	↓	2.70
8	*UPF3B*	↓	10.00
9	*MYH14*	↓	7.14
10	*BAZ2B*	↓	3.85

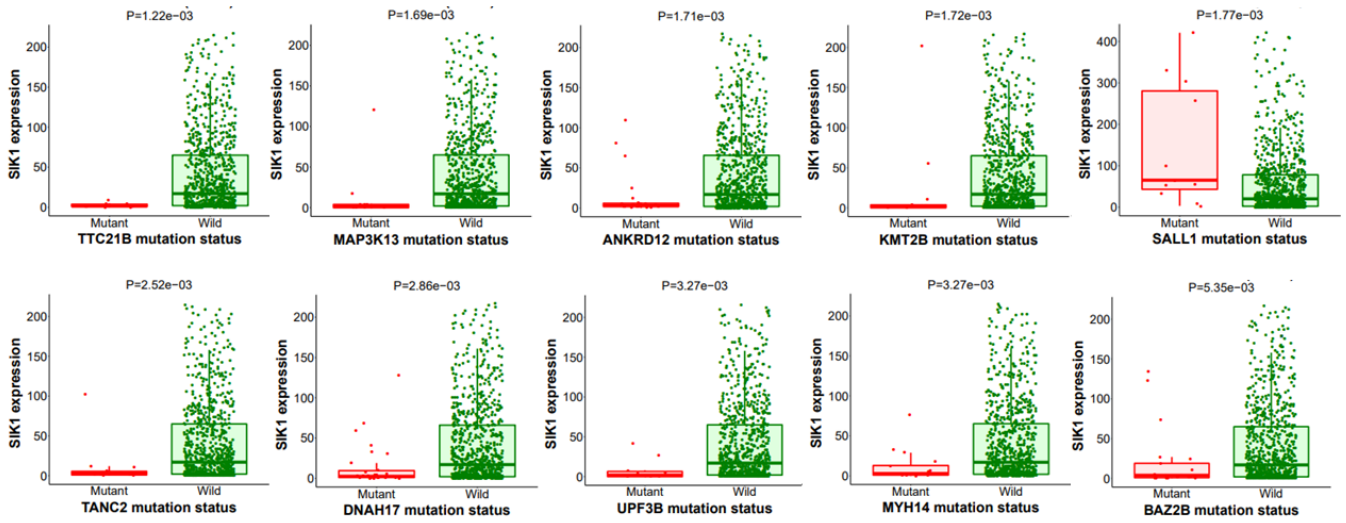

Figure 2. *SIK1* Expression in Mutated Gene Profiles. Statistical significance is represented for each graph

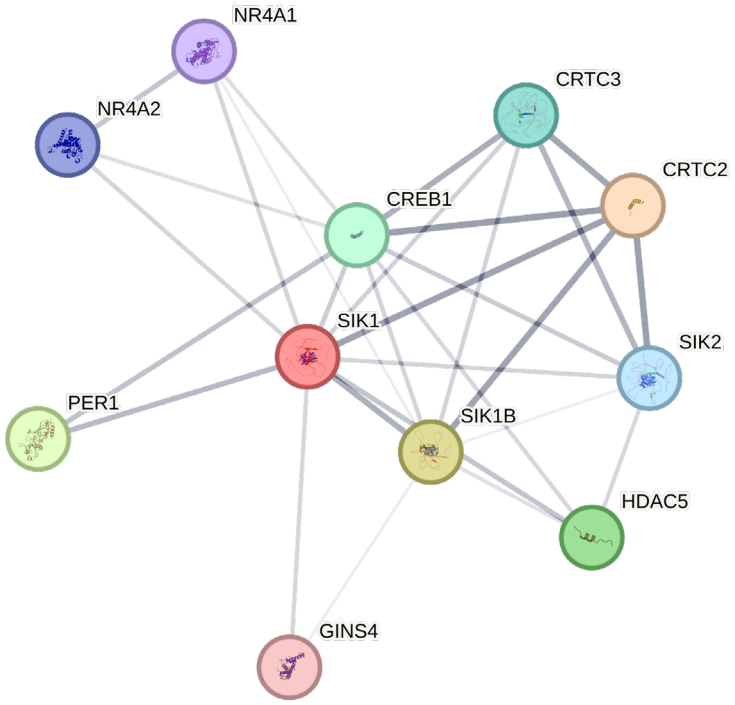

Figure 3. *SIK1* Interacting Protein Network. Circles represent nodes (proteins) and the lines connecting them represent edges (protein-protein interaction). The thickness of the line is indicative of the strength of supporting data.

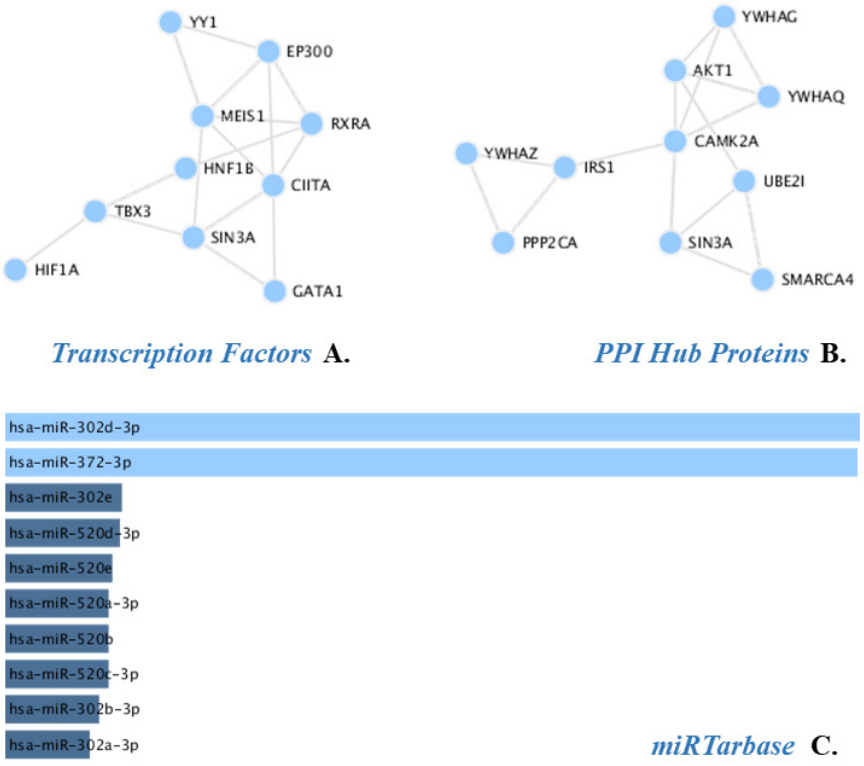

Figure 4. *SIK1* associated Regulome Determination through Functional Enrichment Analyses. The figure displays top ten enrichment terms for categories ($p < 0.05$): A- Transcription factors, B- Protein- protein interaction hub proteins and C- microRNA (miR).

Table 2. Gene/Protein Composition of *SIK1*'s Regulation Dataset and their Functional Niche. The query terms include *SIK1* and its top ten interacting proteins. Transcription factors (TF), protein- protein interaction (PPI) hub proteins and microRNA (miRNA) are listed in respective columns. The target genes corresponding to each of the miRNAs are also included.

Query	TF	PPI Hubs	miRNA	Target Genes
SIK1	*HIF1A*	*YWHAZ*	hsa-miR-302d-3p	*TRPS1; MBNL2; KLF13; VEGFA ERBB4; NR4A2; LEFTY1; LEFTY2; CDK2; CCND2; AKT1*
CREB1	*MEIS1*	*AKT1*	hsa-miR-372-3p	*WEE1; TRPS1; MBNL2; KLF13; CDKN1A; LATS2; ERBB4; NR4A2; VEGFA; TGFBR2 RHOC; NFIB; CDK2; CCNA1; RECK; TNFAIP1 DKK1; BTG1; LEFTY1; TXNIP; ATAD2*
SIK1B	*SIN3A*	*YWHAG*	hsa-miR-302e	-
HDAC5	*CIITA*	*SMARCA4*	hsa-miR-520d-3p	*EPHA2*
SIK2	*GATA1*	*IRS1*	hsa-miR-520e	*CD46; PFKP; MAP3K14; MAP4K4*
CRTC2	*EP300*	*CAMK2A*	hsa-miR-520a-3p	*CDKN1A; PFKP; WEE1; HOXD8*
CRTC3	*YY1*	*SIN3A*	hsa-miR-520b	*CDKN1A; MICA; LAMTOR5; IL8 MAP3K2; CCND1; CD46; PFKP*
NR4A1	*RXRA*	*PPP2CA*	hsa-miR-520c-3p	*APP; CD44; MTOR; SIRT1; GPC3; MICA*
NR4A2	*TBX3*	*YWHAQ*	hsa-miR-302b-3p	*CCND2; BMI1; TGFBR2; RHOC; AKT1; HDAC4; EGFR; ERBB4*
PER1	*HNF1B*	*UBE2I*	hsa-miR-302a-3p	*CDK4; CCND1; CDKN1A; LEFTY1; LEFTY2 DAZAP2; SLAIN1; TOB2; NR2F2; AKT1; TAC1; CDK2; BMI1*
GINS4	*TP53*	*YWHAE*	hsa-miR-302c-3p	-
	TRIM28		hsa-miR-373-3p	-
	SMARCA4		hsa-miR-17-5p	-
	NR3C1		hsa-miR-93-5p	-
	NFKB1		hsa-miR-6776-3p	-
	DACH1		hsa-miR-34a-3p	-
	POLE		hsa-miR-519d-3p	-
	ZBTB7B		hsa-miR-1224-5p	-
	MTF2		hsa-miR-20b-5p	-
	BCOR		hsa-miR-28-5p	-
	GATA3		hsa-miR-20a-5p	-
	GATA2		hsa-miR-106b-5p	-
	CLOCK		hsa-miR-6165	-
	NEUROD1			
	KLF4			
	SMAD3			
	ATF1			
	NR0B1			
	HDAC2			
	PDX1			
	TBL1XR1			
	MEF2A			
	REST			
	CREM			
	RUNX3			
	IKZF1			
	MITF			
	NFYA			
	RCOR1			
	ATF3			
	MEF2C			
	NFATC1			

3.2. Determination of SIK1 Interactome

Top ten *SIK1*-interacting proteins were extracted at confidence level 0.4, as its interacting signature, as shown in Figure 3. The network is comprised of 11 nodes, with 29 edges, and an average degree of 5.27 (PPI enrichment p value of 2.16e-06).

3.3. Transcription Factor, Hub Proteins and miRNA Identification

Transcription factors (TF) PPIs showed 42 statistically significant TFs associated with *SIK1* and its interacting proteins ($p < 0.05$), as depicted in Figure 4A (top ten most significantly enriched TFs at $p < 0.05$). Furthermore, 11 significant PPI hub proteins were found ($p < 0.05$), top ten of which are visualized in Figure 4B.

Figure 4C displays the top ten statistically significant miRNAs including but not limited to hsa-miR-302d-3p, hsa-miR-372-3p and hsa-miR-302e. A total of 25 miRNA were found to be statistically significant ($p < 0.05$), and of these the 23 human miRNA were utilized subsequently. Next, gene targets corresponding to each miRNA were searched, and those with strong evidence were shortlisted. Hence, target genes for 9 miRNA were found and proceeded further with.

Table 2 displays the TFs, PPI hub proteins and target genes corresponding to each of the 9 statistically significant miRNAs.

3.4. PPI Network Construction

The regulome of *SIK1* was constructed on STRING software. This included the gene of interest itself, 10 *SIK1*-interacting proteins (1st shell), 81 genes which associated with affecting *SIK1* expression through their mutations, 42 significantly enriched TF terms, 11 PPI hub genes, and 76 target genes for identified miRNA. These computed to 221 genes and were

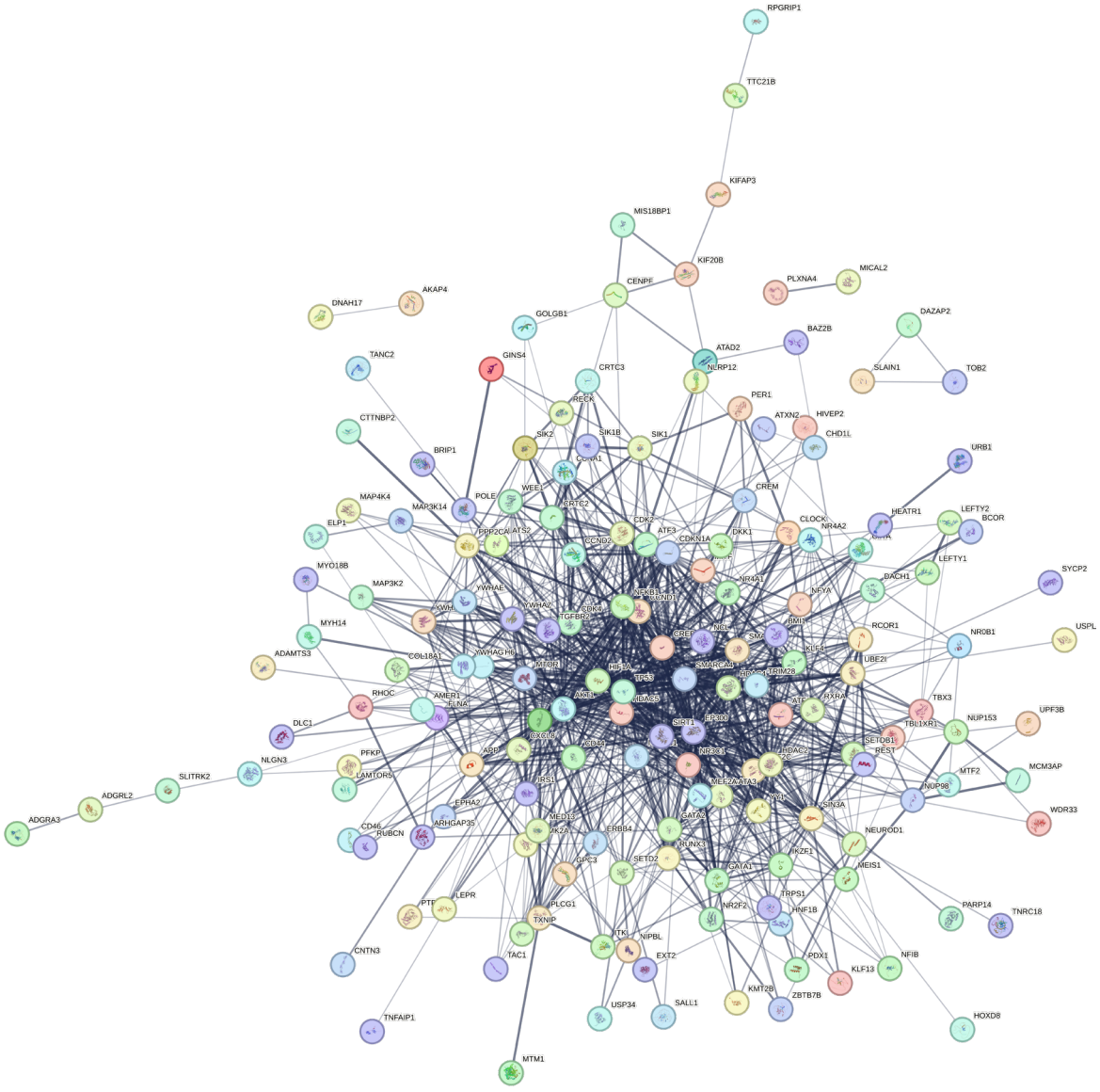

Figure 5. Protein- Protein Interaction Network for *SIK1* associated Genes. Circles represent nodes (proteins) and the lines connecting them represent edges (protein-protein interaction). The thickness of the line is indicative of the strength of supporting data (default setting for confidence score).

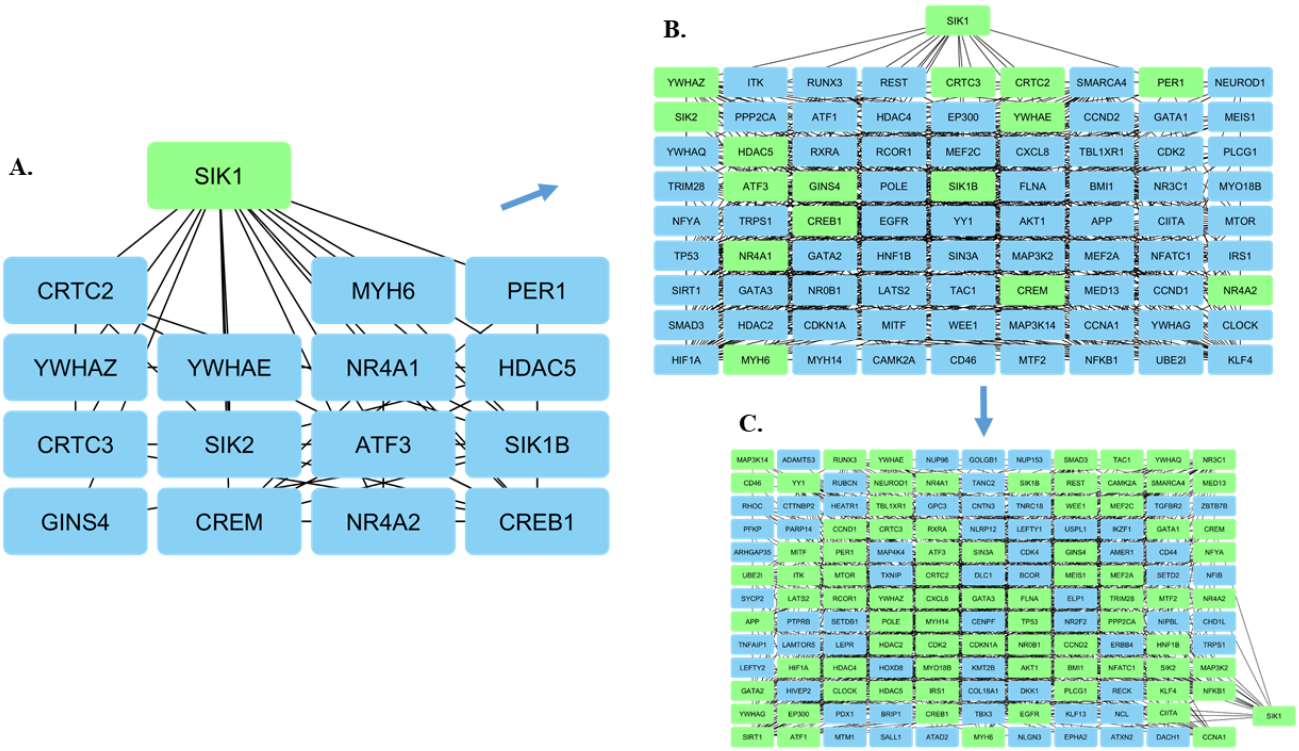

Figure 6. *SIK1* Regulatory Network Iteration. A: 1st stage nodes (n=15); B: 2nd stage nodes (n=81); C: 3rd stage nodes (n=143). Green boxes represent query nodes at each iteration and blue boxes the added layer of interacting proteins. The lines represent protein-protein interactions.

queried into STRING to generate protein-protein interaction (PIN) network as shown in Figure 5.

The network comprised of 189 nodes connected by 1090 edges and with an average node degree of 11.5 (p < 1.0e-16). Repetitions within the input gene list were removed. Interaction based on sources included text mining, experiments, data-

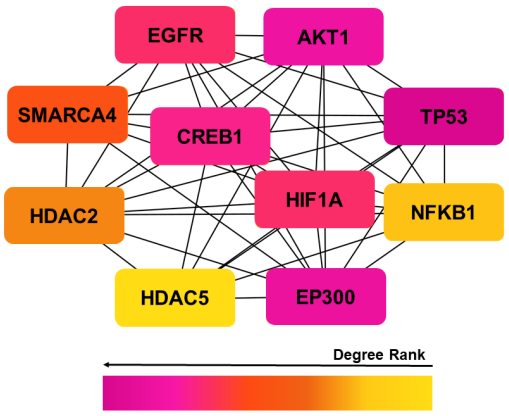

Figure 7. Hub Gene Analysis. Boxes represent hub genes. The lines represent protein-protein interactions. The colour intensity from yellow to pink depicts increasing degree rank for the nodes.

Table 3. Regulatory Niche of Hub Genes. Transcription factor (TF); PPI (protein-protein interaction); miRNA (microRNA)

TF	PPI Hub	miRNA-Target	Mutation	Interaction
TP53; SMARCA4; EP300; HIF1A; HDAC2; NFKB1	AKT1; SMARCA4	*AKT1; EGFR*	-	*CREB1; HDAC5*

bases and co-expression (confidence score 0.4).

3.5. Protein-Protein Interaction Network Analysis

The network was imported into Cytoscape v3.10.2. Network analysis showed nodes to be 165, edges at 2180 and average node degree to be 13.73. Cytohubba plugin was applied. *SIK1* was entered as node of interest. 1st stage nodes with shortest paths included 15 genes-*PER1, CREB1, YWHAZ, SIK2, NR4A2, CRTC3, NR4A1, CRTC2, YWHAE, MYH6, SIK1B, GINS4, HDAC5, ATF3, CREM* directly interacting with *SIK1*, as shown in Figure 6A.

2nd stage consisted of 66 additional nodes while 3rd degree nodes included 62 added proteins. The network was reanalysed to give 143 nodes with 1063 edges, average node degree to be 14.85. Hub gene analysis according to degree algorithm was performed on these nodes and their edges to obtain the top ten hub genes, represented in Figure 7.

These included *TP53, EP300, AKT1, CREB1, HIF1A, EGFR, SMARCA4, HDAC2, NFKB1* and *HDAC5*. Regulatory niche of each of these is tabulated, Table 3.

3.6. Pathway Enrichment of the Network Vs Hub Genes

The 143- gene list was submitted to EnrichR to study their functional enrichment. The biological terms of statistical significance ($p << 0.05$) associated with transcription ($p < 0.05$ for all terms) and gene expression (p = 5.68e-08), as presented in

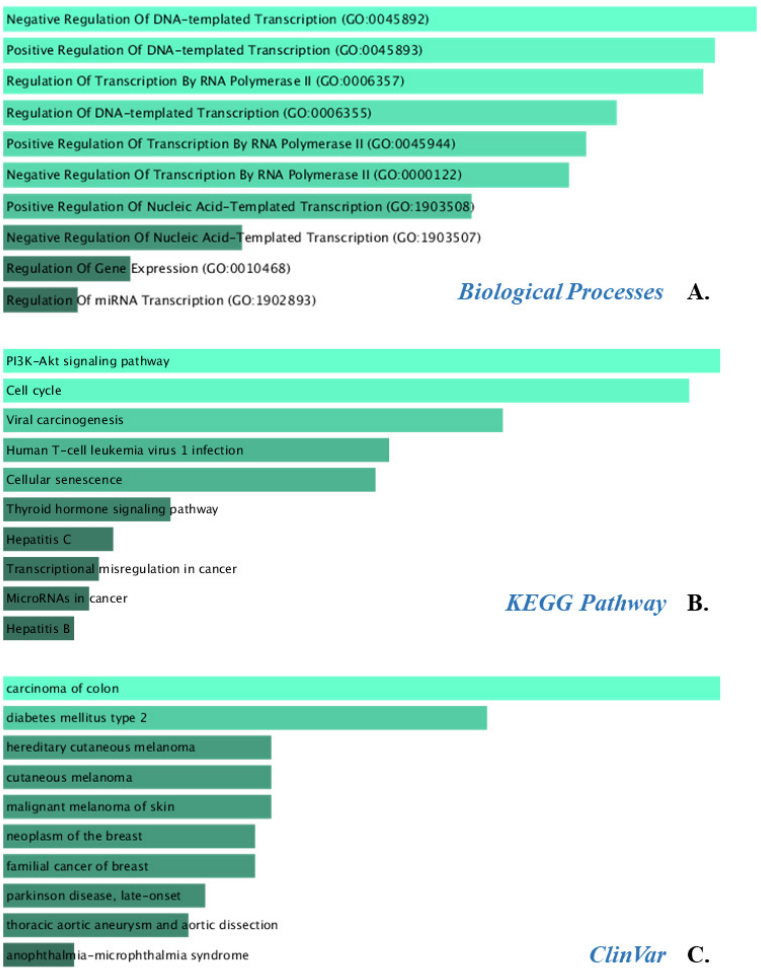

Figure 8. Functional Enrichment Analysis for 143 Node Network. Top significant terms are depicted for **A**- Biological processes; **B**- KEGG pathway and **C**- ClinVar.

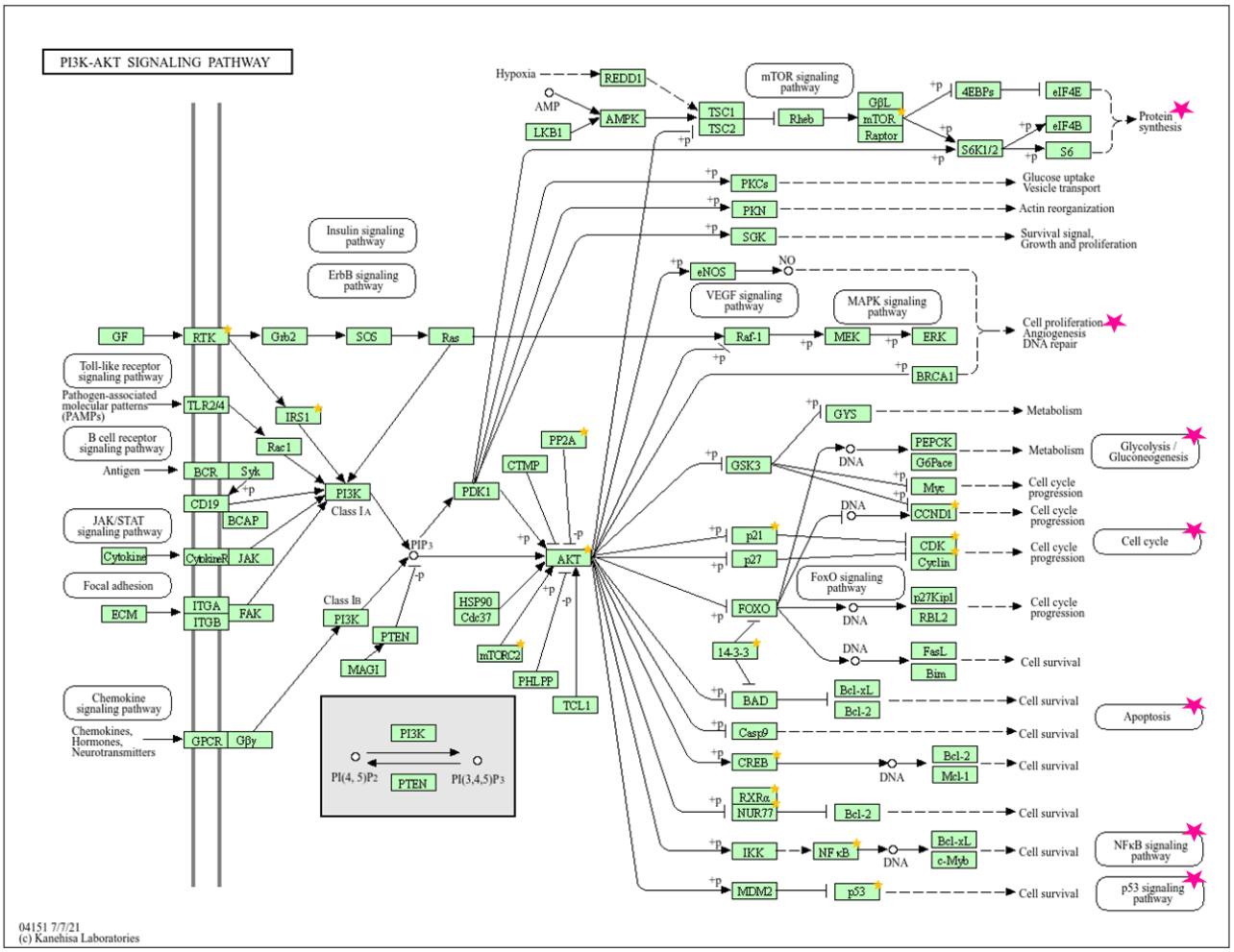

Figure 9. KEGG Map for PI3K-AKT Pathway. The implicated genes are indicated with yellow star and the affected characteristic mechanisms with a pink star.

Figure 8.

The KEGG terms of particular interest included *PI3K-AKT* signalling (p = 9.02e-15), cell cycle (p = 1.27e-14), and transcriptional misregulations (p = 8.16e-12) and miRNA in cancer (p = 9.09e-12). The implication of the hub genes in diseases and phenotypes were assessed with ClinVar. Interestingly, T2DM (p = 4.49e-05) and BC related terms (neoplasm- p =9.23e-04) were amongst the most significantly enriched terms.

The maximum overlap was found for the terms "*PI3K-AKT* pathways" and "pathways in cancer" (not in the top ten but significant term, p = 1.00e-09) with 22 and 21 genes implicated with them, respectively. These included *YWHAE, CRTC2, CDKN1A, IRS1, YWHAZ, NFKB1, EGFR, MTOR, PPP2CA, NR4A1, CCND2, CREB1, RXRA, CCND1, YWHAQ, CDK4, ERBB4, CDK2, AKT1, TP53, YWHAG*, and *EPHA2* for the former term. In case of the latter, the implicated genes include *CDKN1A, HDAC2, SMAD3, CXCL8, CAMK2A, MITF, HIF1A, NFKB1, EGFR, MTOR, TGFBR2, CCNA1, CCND2, RXRA, CCND1, CDK4, CDK2, AKT1, EP300, PLCG1*, and *TP53*.

These terms were depicted on KEGG pathway maps to elucidate on the role of each of the genes, generated on DAVID, Figure 9 and Supplementary Information Appendix (Figure 12).

The KEGG map for *PI3K-AKT* pathway was retrieved with the 22 genes given as input in DAVID, p value = 4.1e-30 (adjusted p = 6.9e-28, Benjamini correction). It showed the role of specific implicated genes in *PI3K-AKT* pathway mediated protein synthesis, glycolysis/gluconeogenesis, cell cycle, apoptosis and crosstalk with other pathways such as those regulated by *NFKB* and *p53*.

Furthermore, the map in supplementary Figure 12 depicts the pathways in cancer, regulated by the listed genes, resulting in cancer phenotypic characteristics such as proliferation, evading apoptosis, genome instability, sustained angiogenesis, insensitivity to anti-growth signals and response to chemotherapy, p = 2.9e-25 (adj. p = 5.1e-23, Benjamini).

The functional enrichment analysis was repeated for the top ten hub genes to study the molecular and clinical implication of *SIK1*- regulatory network evolution. The most statistically significant enrichment terms are shown in Figure 10.

For BP, these were positive regulation of transcription (p << 0.05 for all three terms) and miRNA processes (p = 1.79e-11). KEGG enriched terms included pathways in cancer (p = 1.00e-09), *HIF1* signalling (p =1.08e-09) and miRNA in cancer (p = 2.63e-09). The ClinVar showed these ten hub genes to associ-

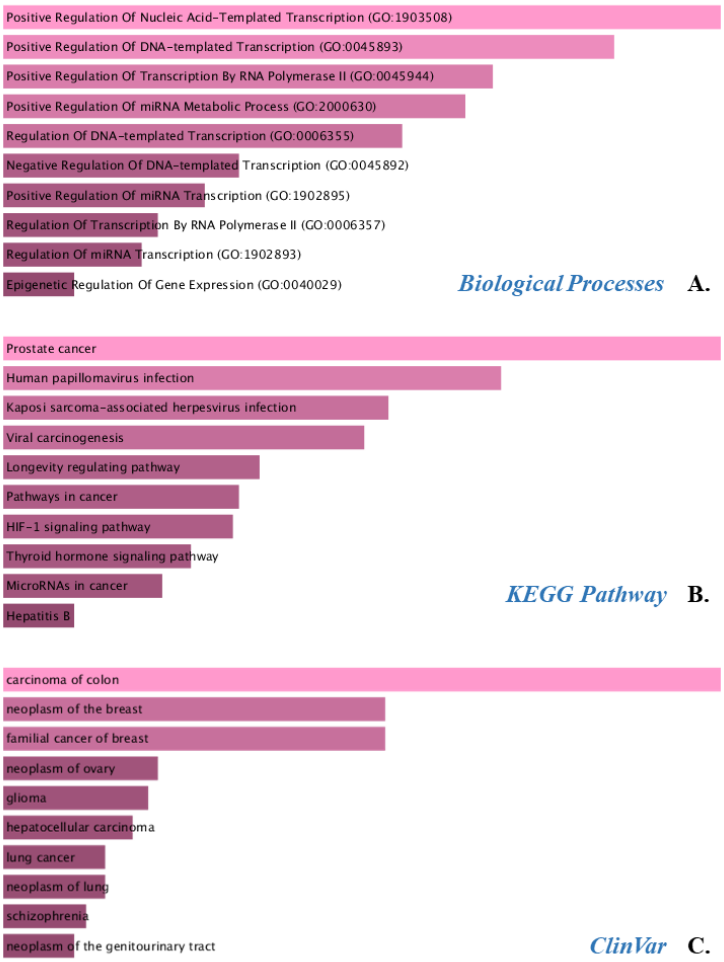

Figure 10. Functional Enrichment Analyses for Top Ten Hub Genes. Top significant terms are depicted for A- Biological processes; B- KEGG pathway and C- ClinVar.

ate with several types of cancer including breast neoplasm (p = 7.85e-05).

3.7. Over-Representation of Hallmarks of Cancer

The genes were found to be implicated in all of the ten hallmarks with statistical significance adjusted $p < 0.05$, with the exception of genome instability, Figure 11.

The overrepresentation of hallmarks of cancer terms is assessed by studying the overlap between the cancer hallmarks associated gene lists fed into the analysis. To enhance validity, the core hallmark genes set (with 1574 genes derived from at least two sources) was employed.

3.8. Expression Analysis of Hub Genes

The expression of each of the hub genes was determined, as detailed in Table 4.

Only TP53 was found to be commonly up-regulated in both T2DM and BC. However, its expression was not found in the validation dataset for co-morbid patients.

Discussion

In this study, the *SIK1* regulome was constructed and analysed to identify its molecular crux in terms of hub genes. The significance of this approach is appended to elucidating the potential implication of these critical players in type 2 diabetes mellitus-breast cancer (T2DM-BC) crosstalk. Networking the dynamics of molecular mechanisms underlying normal physiologies not only provide insight into possible perturbations leading to the development and progression of pathogenicity, but also potentiate the discovery of switch genes as candidate targets for diagnostic, prognostic, predictive and therapeutic targets [29]. For the first part i.e the construction of the *SIK1* regulome, gene mutations associating with up/down regulation of *SIK1* expression were determined through MuTarget. This online tool allows for the determination of target gene expression in gene mutated profiles [20]. The changes in gene expression is determined after performing differential gene expression analysis on RNA-sequencing data derived from solid cancers, in this case, BC, with Mann-Whitney U test for statistical significance. The top ten genes are tabulated in Table 1. With a total

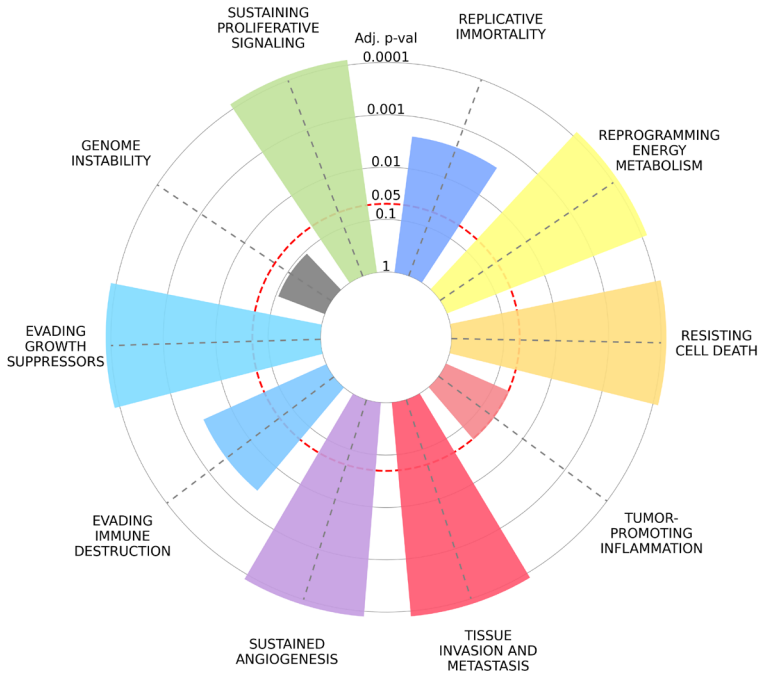

Figure 11. Hallmarks of Cancer Overrepresentation Analysis. The core set of hallmark cancer genes on the tool was utilized.

Table 4 Differential Expression of Hub Genes. ↑: Up- regulation; ↓: down- regulation and -: not differentially expressed/ not found. Exp: expression; F.C: fold change.

#	Hub Gene	Exp. in T2DM (F.C)	Exp. in BC (F.C)	Exp. in T2DM-BC
1	*TP53*	↑ (1.22)	↑ (1.20)	-
2	*EP300*	↑ (1.54)	-	-
3	*AKT1*	-	-	-
4	*CREB1*	-	-	-
5	*HIF1A*	-	↓ (-1.23); ↑ (1.26)	-
6	*EGFR*	↑ (1.76)	↓(-1.54)	-
7	*SMARCA4*	-	↓ (-1.41)	-
8	*HDAC2*	-	-	-
9	*NFKB1*	-	↑ (2.50)	-
10	*HDAC5*	-	↓ (-1.96)	-

of 81 mutations implicated in modifying *SIK1* expression, all of these resulted in down-regulation of *SIK1* expression with the exception of *SALL1, USPL1, NIPBL, RPGRIP1, BZRAP1, PTCHD3, FBN3, NRK* and *MICAL2*, which elevated *SIK1* expression. *TTC21B* disrupted *SIK1* most drastically with a fold change of -33.33 (in 10 mutants compared to 969 wild-types). While the molecular function of *Tetratricopeptide Repeat Domain 21B (TTC21B*) protein is mostly unknown, it has been associated with several types of cancers [30].

SIK1's interacting partners were also extracted from STRING software, based on sources including text mining, databases, experiments and co-expression. This was important so as to identify the proteins that associated with SIK1 and hence would be potentially implicated in its functional niche. Also the generation of a gene set was required for the subsequent gene set enrichment (GSEA). Hence, this set of ten genes including *SIK1B and SIK2*, along with gene of interest i.e. *SIK1*, were then utilized as input on EnrichR to determine the statistically significant ($p < 0.05$) enriched terms for categories such as transcription factors, reported hub proteins and miRNA. EnrichR also outputs adjusted *p* values calculated through Benjamini-Hochberg multiple testing correction to account for false positives.

Interestingly, the enriched terms included genes such as *hy-*

poxia inducible factor 1 alpha (*HIF1A*- adjusted p = 0.00287), which serves as the gateway to cancer and has been previously reviewed for its role in T2DM-BC crosstalk [12]. Furthermore, *AKT1 (*adjusted p = 0.0280), also known as *protein kinase B (PKB)* and *nuclear factor kappa beta 1 (NFKB1*- adjusted p value = 0.0380) have been found to be critical for immunometabolism associated with complex diseases including T2DM, TNBC, and osteoarthritis (OA) in insilico [31]. Additionally, while *SIK1* has been previously implicated in the T2DM-BC molecular crosstalk in silico, it is worth mentioning that it was also found to be an up-regulated key gene for OA [32], potentiating its role in the immuno-metabolic origin of disease on the T2DM-OA-BC axis. However further research is warranted to elucidate on all of these genes and *SIK1* in particular, and their role in disease-disease interactions.

In case of microRNA (miRNA), the target genes corresponding to each of the miRNA were also derived; some targeted multiple genes, while for some miRNA, gene targets were not found. The former were subsequently included and the resulting gene set was utilized for the regulatory network construction. miRNA establish a critical tier of regulation within a cellular system. Previously a miRNA signature common to both T2DM and BC was reported [33], however there was no overlap with the miRNAs under study. Yet, miR-34a was found to be implicated in both T2DM and BC in a recent study [34]. This miRNA is involved beta cell function [35] and also targets *HDAC1/7* to regulate cell survival and resistance to therapy [36]. Moreover, miRNA hsa-miR-302b-3p's (adjusted p = 0.00371) target gene- *epidermal growth factor receptor (EGFR)* was also previously implicated in the T2DM-OA-TNBC association [31].

The generated gene set was subjected to subsequent network iteration and hub gene analyses to identify the centrality of the constructed regulome. For this, the PPIs were studied on STRING and imported to Cytoscape for further analysis. Since the node of interest is SIK1, the aim was to determine layer by layer, its interactive proteins and their interactions in the generated network. The 1st degree interactors included 15 other nodes such as YWHAZ, CREM, MYH6 and ATF3, which were not included in the interactions derived from STRING as the top ten PPIs initially. The 2nd degree interactors included nodes such as CCND2, EP300 CXCL8, TP53, MAP3K2, IRS1, HIF1A, CD46 and NFKB1, amongst others. The 3rd iteration added nodes including but not limited to CD44, TNFAIP1, LEPR, and ERBB4. Next, these were studied through applying the Cytohubba plugin v0.1 after analysing the network. The top ten hub genes based on the degree algorithm were obtained. This method computes the number of connections each node has, which has been positively correlated with the essentiality of the gene within the network [37]. It is interesting to note and affirm that the top ten hub genes were iterated as output in the first two analysis runs, in line with their centrality within the network. These hub genes included *AKT1, NFKB1 and HIF1A,* highlighted beforehand, and also *TP53, a* crucial gene for both T2DM and BC [13]. The iterations were limited to three so as to maintain a focus on SIK1 as the node of interest.

Functional enrichment analyses of the regulome showed its implication in transcription, *PI3K-AKT* pathway (adj. p = 3.32e-06), cell cycle (adj. p = 9.28e-05), and T2DM and cancer specific terms particularly including breast cancer (adj p = 1.33e-04), reflecting at the potential relevance of this regulome in T2DM-BC crosstalk. Other significant terms also include choline metabolism (adj. p = 5.45e-05), glucagon signalling (adj. p = 6.91e-05), insulin resistance (adj. p = 6.92e-05), *AGE-RAGE* signalling (adj. p = 0.00228), osteoclast differentiation (adj. p = 9.75e-05), *TNF* signalling (adj. p = 7.53e-05), and adipocytokine signalling pathway (adj. p =0.00119). In particular, 22 key genes associated with the *PI3K-AKT* pathway and 21 genes were included in various hallmarks of cancer, as mapped in Figure 9 and Figure 12 (Supplementary).

In further support of this, the hub genes also associated with transcription (adj. p values << 0.05), and cancer (adj. p values << 0.05), *HIF1* signalling (adj. p = 2.33e-08), *PI3K-AKT* pathway (adj. p = 3.32e-06), insulin resistance (adj. p = 6.92e-05), *AGE-RAGE* signalling in diabetic complications (adj. p =0.00228), and metabolic (choline, central carbon -adj. p values << 0.05) and inflammatory pathways (*TNF*, T cell and B cell signalling-adj. p values << 0.05). Again, the maximum overlap of seven hub genes (*HDAC2, EP300, AKT1, TP53, HIF1A, EGFR* and *NFKB1*) was found for the pathways in cancer (adj. p value = 2.33e-08), Figure 10. For *PI3K-AKT* pathway, *CREB1, AKT1, TP53, EGFR* and *NFKB1* were associated. Interestingly, both these pathways implicated *AKT1, TP53, EGFR* and *NFKB1.*

To further study the role of these hub genes in cancer development, the cancer hallmarks analysis was performed. As shown in Figure 11, overrepresentation for nine hallmarks of cancer is depicted, at statistical significance adjusted p <0.05 (marked by red dotted line). Cancer hallmarks analysis corrects for multiple hypothesis testing by computing false discovery rate (FDR) using the Benjamini-Hochberg method [38]. Maximum overlap of 8 genes was found for sustaining proliferative signalling and for resisting cell death (7 genes). In particular, reprogrammed energy metabolism hallmark overlapped with 4 genes; including *TP53* and *HIF1A* and tumour promoting inflammation only with *NFKB1*. While *TP53* mediated genome instability was also shown in Figure 9, this was not supported further in the hallmarks enrichment analysis, hence further study is required.

The expression of these hub genes in T2DM, BC and T2DM-BC microarray expression was also studied. Only *TP53* emerged as a hub gene of interest depicting common expression pattern in both diseases (adjusted p values < 0.05 using the Benjamini-Hochberg method applied previously at GEO2R). However its expression was not found in co-morbid patients. The lack of availability of other datasets derived from co-morbid patients provide a challenge to further analyse the expression of these hub genes in association to co-morbidity. Smaller sample size, particularly of T2DM dataset also pose a limitation. This warrants further study and analysis of expression data from

T2DM, BC and co-morbid patients on a larger scale to bridge the molecular gaps further in associating T2DM with BC.

In support of this study's findings, previous studies report the role of *SIK1* in breast carcinogenesis, particularly implicating the *PI3K-AKT* axis [39]. AKT was found to phosphorylate SIK1, leading to the loss of its tumour suppressing activities and hence contributing to tumorigenesis in breast cells. Furthermore, SIK1 also shown to directly interact with and increase the transcriptional activity of p53, mediating oxidative phosphorylation and inhibiting aerobic glycolysis and cell growth [40]. Whereas, *SIK3* isoform was shown to promote aerobic glycolysis, indicating at the isoform specific roles of SIKs in breast carcinogenesis. The implication of SIKs in metabolic homeostasis and its consequences in terms of tumorigenesis have also been explored, with *SIK1* emerging as a favourable prognostic factor for breast cancer [16]. In case of diabetes, *SIK1* attenuates insulin secretion while *SIK2* promotes it, elucidating on their contrasting roles in metabolic regulations [41]. Besides its anti-proliferative metabolic adapter niche, SIK1 also tunes into anti-inflammatory characteristics during early phases of tumour development. It inhibits pro-inflammatory cytokines and promotes anti-inflammatory cytokines. This not only reflects on its role as a critical node in signalling networking, mediator and regulator governing crosstalk between various signalling pathways [42], but also highlights its potential on the immuno-metabolic axis, as a target for personalised regulatory cancer strategies, particularly addressing the T2DM-BC association. In addition, its role in other diseases with overlapping immuno-metabolic features is also reported. *SIK1* plays a crucial role in osteoblast differentiation, emerging as a key gene for osteoarthritis [43].

While the findings of this study further reiterate network based evidence on the potential implication of *SIK1* genes in T2DM-BC interplay particularly on the *AKT-p53* axis, however, the insilico nature of this study presents limitations and further experimentation is necessary for validation.

Conclusion

Type 2 diabetes mellitus (T2DM) patients are at a moderate yet significantly elevated risk of developing breast cancer (BC). In the event of co-morbidity, further complexities present challenges for disease diagnosis, prognosis and therapeutics. This study adopted a bioinformatics pipeline for the construction of *salt inducible kinase 1* (SIK1) regulome and its subsequent hub gene analysis to identify the most critical nodes potentially implicated in T2DM-BC molecular crosstalk. Identifying *tumor protein (TP53)* as a significant hub gene and implicated in the enriched *PI3K-AKT* pathway, associates closely with the functional niche of *SIK1* within this network. Hence, the findings of this study unravel the role of *SIK1* and its regulatory network in context to the interplay between T2DM and BC molecular mechanisms of pathogenesis. It also presents further grounds for an integrated in silico and in vitro approach to potentiate biomarker discovery for targeting T2DM-BC association. However, validation through experimentation is crucial to further decode the molecular events leading to breast carcinogenesis in T2DM patients and onset of T2DM in patients with BC.

Abbreviations

T2DM *Type 2 diabetes mellitus;* ***BC*** *Breast cancer;* ***SIK*** *Salt inducible kinase;* ***TP53*** *Tumour protein 53;* ***PER1*** *Period circadian protein homolog 1;* ***CREB1*** *cAMP-response element binding protein 1;* ***YWHA*** *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase;* ***NR4A*** *Nuclear orphan receptor 4A;* ***CRTC*** *CREB-regulated transcription coactivator;* ***MYH6*** *Myosin heavy chain 6;* ***GINS4*** *Go-Ichi-Ni-San (GINS) complex subunit 4;* ***HDAC*** *Histone deacetylase;* ***ATF3*** *Activating transcription factor 3;* ***CREM*** *cAMP responsive element modulator;* ***EP300*** *Adenovirus early region 1A associated protein p300;* ***AKT*** *Ak strain transforming;* ***HIF1A*** *Hypoxia inducible factor 1 alpha;* ***SMARCA4*** *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4;* ***NFKB1*** *Nuclear factor (NF)-kappa B p105 subunit;* ***PI3K*** *Phosphatidylinositol-3 kinase;* ***CDKN1A*** *Cyclin dependent kinase inhibitor 1A;* ***IRS1*** *Insulin receptor substrate 1;* ***EGFR*** *Epidermal growth factor receptor;* ***MTOR*** *Mechanistic target of rapamycin;* ***PPP2CA*** *Protein phosphatase 2 catalytic subunit alpha;* ***CCN*** *Cyclin;* ***CDK4*** *Cyclin-dependent kinase 4;* ***ERBB4*** *Erb-B2 receptor tyrosine kinase 4;* ***EPHA2*** *Ephrin type-A receptor 2;* ***SMAD3*** *Mothers against decapentaplegic homolog 3;* ***CXCL8*** *C-X-C Motif chemokine ligand 8;* ***CAMK2A*** *Calcium/calmodulin dependent protein kinase II alpha;* ***MITF*** *Microphthalmia-associated transcription factor;* ***TGFBR2*** *Transforming growth factor beta receptor 2;* ***RXRA*** *Retinoid X receptor alpha;* ***PLCG1*** *Phospholipase C gamma 1;* ***SALL1*** *Spalt like transcription factor 1;* ***USPL1*** *Ubiquitin specific peptidase like 1;* ***NIPBL*** *Nipped-B-like protein;* ***RPGRIP1*** *Retinitis pigmentosa GTPase regulator-interacting protein 1;* ***BZRAP1*** *Peripheral-type benzodiazepine receptor-associated protein 1;* ***PTCHD3*** *Patched domain containing 3;* ***FBN3*** *Fibrillin 3;* ***NRK*** *Nik related kinase;* ***MICAL2*** *Microtubule associated monooxygenase, calponin and LIM domain containing 2;* ***TTC21B*** *Tetratricopeptide Repeat Domain 21B;* ***MAP3K2*** *Mitogen-activated protein kinase kinase kinase 2;* ***CD44*** *Cluster of differentiation 44;* ***TNFAIP1*** *Tumor necrosis factor alpha-induced protein 1;* ***LEPR*** *Leptin receptor;* ***R(AGE) Receptor of*** *(Advanced glycosylation end-product*

Conflict of Interest

The authors declare that there are no competing interests.

Ethical Review and Approval

Not applicable.

Acknowledgements

Not applicable.

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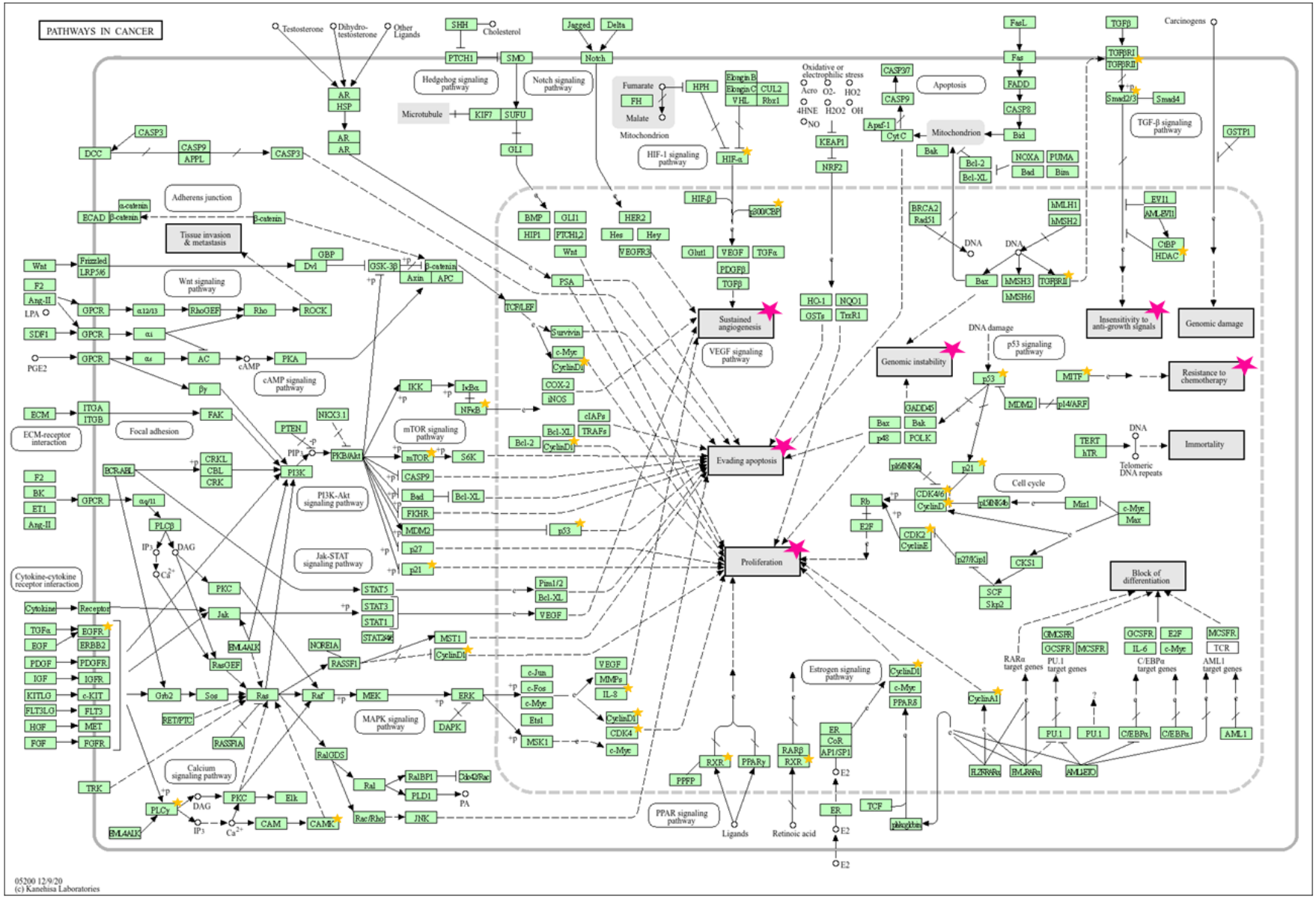

Figure 12. KEGG Map for Pathways in Cancer. The hub genes are indicated with yellow star and the affected cancer characteristic phenotypes with a pink star.