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An investigation of 6-Shogaol effects on MCF7 cell lines through a systems biology approach

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Abstract

Introduction: In the literature, to investigate hormonal mechanisms of cell growth of patients with breast cancer (BC), as the second most common cause of death in the world, the researchers frequently used MCF-7 cell lines. And, identifying the functional mechanisms of therapeutics agents as new cancer inhibitors is still unclear.

Methods: We used the NCBI-GEO dataset (GSE36973) to study the effects of 6-Shogaol on MCF-7 cell lines commonly used for more than 45 years in several studies. The pre-processing and post-processing stages were carried out for the target samples to identify the most significant differentially expressed genes between two MCF-7 with and without treated by 6-Shogaol. Furthermore, various analyses, including biological process and molecular function from the DAVID website, the protein–protein interaction (PPI) network, gene-miRNA, gene-transcription factor, gene-drugs, and gene-diseases networks, statistically significant assoications with clinical features and survival rates were conducted.

Results: The initial outcomes revealed thirty significant DEGs. Among which the approach resulted in eleven upregulated and nineteen downregulated genes. Over-expression of *TRADD* and *CREB3L1* and low-expression of *KIF4A* and *PALMD* were substantial in the TNF signaling pathway. Moreover, *hsa-mir-16-5p* and *hsa-mir-124-3p* were inhibitors of breast cancer growth.

Conclusion: The fact that some of genes are associated with survival rates as well as various clinical features including disease stages, it can be deduced that the 6-Shogaol treatment on MCF7 cell lines at the genome level shows inhibition functionalities of the herbal medicine in breast cancer at early stages and pave the way in developing new therapeutic agents.

Keywords: 6-Shogaol, MCF7 cell line, Breast cancer, Systems biology, Survival rate, Clinical features

Introduction

Ginger (*Zingiber officinale Roscoe (*Zingiberaceae*)*) as one of the most frequently used condiments for enhancing the flavor of the food and confectionery industry can also be of traditional herbal medicine covering a diverse spectrum of diseases [1]. Shogaols are a group of natural dehydrated compounds derived from dried ginger rhizomes that have several biological and pharmacological effects such as anti-oxidative, anti-inflammatory, and anti-cancer properties [2]. The variants of Shogaols are related to the length of the alkyl chain, among which 6-Shogaol [1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] is the most potent anti-oxidant [3]. The researchers reported successful therapeutic outcomes of 6-Shogaol for colorectal and breast cancer in some available literature researches [4, 5].

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Breast cancer is the second most common leading cause of death in women globally, according to the WHO cancer reports for 2018 that stand after lung cancer (i.e., https://www.who.int/news-room/fact-sheets/detail/cancer). Various in vitro studies presented the inhibitory activity of 6-Shogaol on blood-borne, colon, breast, and skin carcinoma cells [6]. Additionally, the anti-cancer effect of 6-Shogaol has been reported by Gan et al. for MCF-7 cell lines and animal models [7]. On the other hand, various stages of the breast cancer is of interest of the researchers considering the diagnosis and treatment of the disease at early stages that may beneficial for the patients covering more than 200 studies from Scopus database in 2021. However, the gaps still are available between the computational and biological sciences to understand the state-of-art of molecular functionalities and cellular mechanisms of breast cancer.

The applications of different computational methodologies, among which systems biology and bioinformatics have played vital roles in providing potent biomarkers for diagnosis and prognosis of a specific disease, have frequently utilized on "OMICS" datasets. Various aspects of systems biology approaches may cover the terms metabolomics, genomics, and proteomics. The systems biology techniques can be beneficial for pharmacological sciences. And, the reports showed pharmacological and toxicological effects of compounds assessed for an ailment [8, 9]. For instance, systems pharmacology research revealed the inhibitory effects of three compounds from the ginger family in stroke risks [10]. So, investigating the impact of 6-Shogaol on MCF7 cell lines through the systems biology approach might be of help for those researchers in the field of drug design and discovery to plan for future novel therapeutic agents.

To reach this aim, we have accomplished a thorough systems biology procedure to identify the potential genes differentially expressed between MCF7 cell lines and the corresponding treatment samples with 6-Shogaol. Moreover, the investigation will extend the coverage for an in-depth understanding of involved signaling pathways, while 6-Shogaol is the recommendable treatment for MCF7 cell lines. Finally, the outcomes will be demonstrated and validated through different methodologies in terms of computational, biological aspects, clinical attributes (e.g., TNM stages) and assessment of survival rates.

Materials and methods

Data sources

We downloaded the GSE36973 dataset from The publicly available GEO database of the National Center for Biotechnology Information NCBI-GEO database (i.e., https://www.ncbi.nlm.nih.gov/geo/). Its platform was the GPL6244 [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]. The dataset, comprised 4 MCF-7 cells treated with 10uM 6-Shogaol for ($n=2$; GSM907714 and GSM907715) and control ($n=2$; GSM907712 and GSM907713).

Differentially expressed genes (DEGs) between two types of tissues

The BRB-ArrayTools (v4.6.0, stable version) developed by Dr. Richard Simon and his team was employed to identify significant differentially expressed genes between treated and control MCF-7 cell lines. The built-in preprocessing procedure included disabling the filtering out parameters of the genes, and quantile normalizing of the imported genes as well as finally annotating the genes by "pd.hugene.1.0.st.v1" R package [11]). The "gcrma" R package was the algorithm to determine the target gene expressions based on their probe intensities. Finally, the significant DEGs could be identifiable by comparing the two classes of samples by setting the threshold values of 10,000 and 1 for univariate permutation tests and the fold change.

Gene ontology and functional enrichment analyses

For assessment of GO (gene ontology), biological processes, cellular components, and molecular functions as well as KEGG (Kyoto Encyclopedia of Genes and Genomes) signaling pathway analysis, DAVID v. 6.8 (Database for Annotation, Visualization, and Integrated Discovery) available at http://david.abcc.ncifcrf.gov/summary.jsp was used [12, 13].

Construction of protein–protein interaction, gene-disease, gene-drug networks

The NetworkAnalyst 3.0 freely available at https://www.networkanalyst.ca was used to construct the generic protein–protein interaction (PPI) network using the STRING database among the identified significant DEGs [14, 15]. Moreover, the relationships of target genes with potential drugs and diseases were of the research interest. Finally, we extracted the efficient subnetwork from the first-order constructed PPI network based on Prize-collecting Steiner Forest (PCSF) algorithm, denoted by the minimum network for illustration of the generated figures [16].

Statistical survival analysis of significant genes

For the purpose of exploring the effects of genes on survival rates, the Kaplan–Meier plotter (http://kmplot.com/analysis/) is an online tool that uses $n=4934$ and $n=1880$ patients with relapse-free (RFS) and overall survival (OS) data from the TCGA database on breast cancer [17]. It is possible to determine the relationship between a tumor's unique gene and its patients' overall survival

time using an online KM plotter database built on the TCGA data. The p-value and 95% confidence interval for the hazard ratio were set to 0.05 and 95%, respectively, as the default settings.

Statistical analysis of clinical features

796 breast cancer patients and 96 healthy women are included in the TCGA breast cancer study (TCGA-BRCA). Using the level 3 mRNA sequencing data and clinical information, further analysis into major DEGs in FireBrowse (http://firebrowse.org) was conducted in terms of years to birth, pathological TNM stages, radiation therapy, histological type, number of lymph nodes, and race [18]. The significance level was set at Q values less than 0.3 and p-value less than 0.05 for the three statistical tests of Spearman correlation, Wilcoxon, and Kruskal Wallis.

Results

After pre-processing and post-processing steps performed on the GEO dataset (i.e., GSE36973) using the BRB-ArrayTools, the outcomes showed eleven up-regulated and nineteen down-regulated differentially expressed genes (DEGs) as listed in Table 1 along with their gene expression levels shown in Fig. 1.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8, a functional annotation tool to understand the biological values of genes. The involved BP and MF are GO:0010939 regulation of necrotic cell death dealt with changes in morphological cell volume and GO:0003724 ATP-dependent RNA helicase activity with negative control, respectively. The KEGG pathway enrichment analysis represents the involvement of two up-regulated genes in the TNF signaling pathway. The genes include *TRADD*-Tumor

Table 1 List of thirty significant DEGs analyzed from BRB-ArrayTools

ProbeSet	Gene symbol	Gene title	Fold-change	Up/down gene expression
7958051	*ASCL1*	Achaete-scute homolog 1	0.38	Down-regulated
8101904	*ADH7*	Alcohol Dehydrogenase 7	0.95	Down-regulated
7955635	*ARL2BP*	ADP Ribosylation Factor Like GTPase 2 Binding Protein	0.81	Down-regulated
8045768	*ARL6IP6*	ADP Ribosylation Factor Like GTPase 6 Interacting Protein 6	0.76	Down-regulated
8030429	*BCL2L12*	BCL2 Like 12	0.74	Down-regulated
8109572	*C5orf52*	Chromosome 5 Open Reading Frame 52	1.18	Up-regulated
8111234	*CDH12*	Cadherin-12	0.58	Down-regulated
7939642	*CREB3L1*	CAMP Responsive Element Binding Protein 3 Like 1	1.19	Up-regulated
8077712	*CRELD1*	Cysteine Rich With EGF Like Domains 1	1.73	Up-regulated
7917240	*CTBS*	Chitobiase	1.36	Up-regulated
8112260	*DEPDC1B*	DEP Domain Containing 1B	0.75	Down-regulated
7967588	*DHX37*	Probable ATP-dependent RNA helicase	0.88	Down-regulated
8029950	*EHD2*	EH Domain Containing 2	1.12	Up-regulated
8062571	*FAM83D*	Family With Sequence Similarity 83 Member D	0.77	Down-regulated
8168146	*KIF4A*	Kinesin Family Member 4A	0.71	Down-regulated
8024708	*MRPL54*	Mitochondrial Ribosomal Protein L54	1.11	Up-regulated
7915277	*MYCL*	MYCL Proto-Oncogene, BHLH Transcription Factor	1.23	Up-regulated
7911207	*OR2G2*	Olfactory Receptor Family 2 Subfamily G Member 2	1.32	Up-regulated
7903227	*PALMD*	Palmdelphin	0.8	Down-regulated
7928589	*PPIF*	Peptidyl-prolyl cis–trans isomerase F	0.87	Down-regulated
8070330	*PSMG1*	Proteasome Assembly Chaperone 1	0.72	Down-regulated
8041519	*RMDN2*	Regulator Of Microtubule Dynamics 2	0.76	Down-regulated
8112961	*RPS23*	Ribosomal Protein S23	0.82	Down-regulated
7976496	*SERPINA3*	Serpin Family A Member 3	1.77	Up-regulated
7977161	*TDRD9*	ATP-dependent RNA helicase	0.95	Down-regulated
7944554	*TMEM136*	Transmembrane protein 136	0.83	Down-regulated
8069582	*TMPRSS15*	Transmembrane Serine Protease 15	1.26	Up-regulated
8001938	*TRADD*	TNFRSF1A Associated Via Death Domain	1.15	Up-regulated
7957032	*YEATS4*	YEATS Domain Containing 4	0.8	Down-regulated
8151496	*ZNF704*	Zinc Finger Protein 704	0.73	Down-regulated

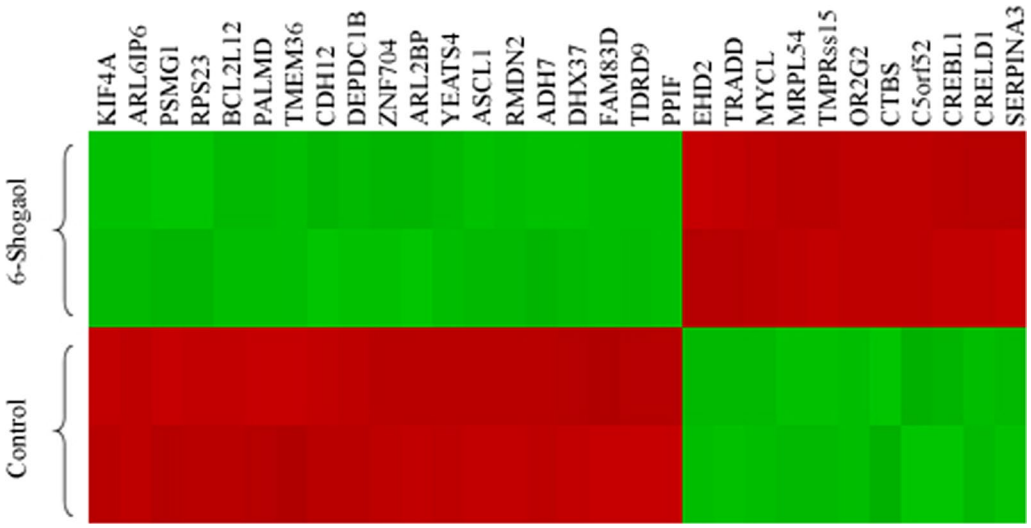


Fig. 1 The gene expression levels for the identified DEGs

necrosis factor receptor type 1-associated DEATH domain and *CREB3L1*-cAMP Responsive Element Binding Protein 3 Like 1.

More details on the pathway is available from https://david.ncifcrf.gov/kegg.jsp?path=hsa04668$TNF%20signaling%20pathway&termId=550028766&source=kegg.

The protein–protein interaction analyzed explicitly for breast cancer tissues proposes that *KIF4A* (Kinesin Family Member 4A) and *PALMD* (Palmdelphin) have been interconnected with other nine genes (i.e., *ARL2BP*, *SHMT2*, *TRADD*, *SH3KBP1*, *NXF1*, *APP*, *ARL6IP6*, *EED*, and *RPS23*) as illustrated in Fig. 2. Moreover, the construction of the miRNA-Gene network (Fig. 3) shows the highest degree of interactions with *hsa-mir-16-5p* and *hsa-mir-124-3p* functioning as suppressors of breast cancer with degree values of 5 and 4, respectively. The network analysis of transcription factors (TFs) and genes in molecular biology reveals the TFs including *MRPL54* (with degree 12), *CREB3L1* (with degree 12), *GATAD2A* (with degree 8), *IRF1* (with degree 9), *ATF1* (with degree 8) are associated with controlling the flow of genetic information from DNA to mRNA (Fig. 4). The construction of a minimized network for gene-disease (Fig. 5) relationships are also indicative of the fact that failure to gain weight and small cell carcinoma of the lung is the most potential illnesses occurring in patients with breast cancer. Finally, the gene-drug network assessment (Fig. 6) demonstrates four potent drugs and chemicals (i.e., 1-Methoxy-2-[2-(2-Methoxy-Ethoxy]-Ethane, 7-AMINO-4-METHYL-CHROMEN-2-ONE, L-Proline, and Cyclosporine) associated directly with down-regulated PPIF gene.

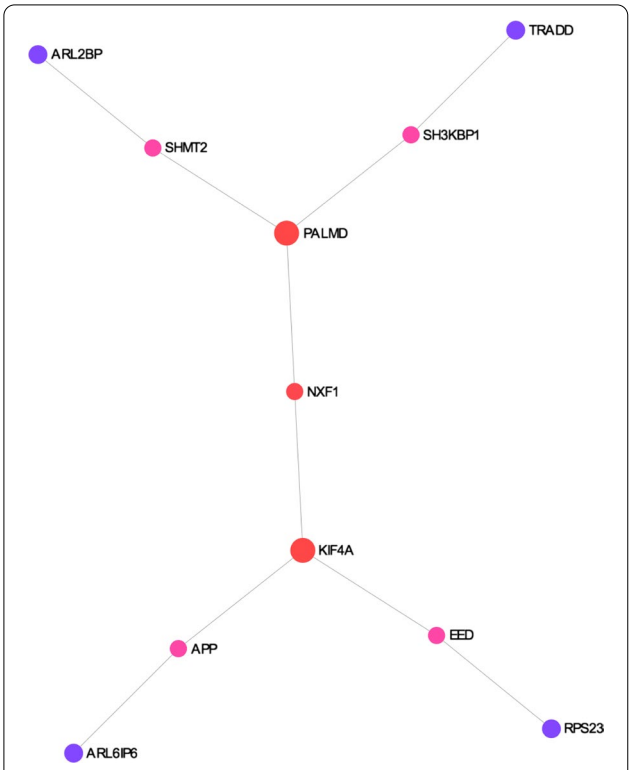


Fig. 2 PPI network analysis for identified significant DEGs. The relationship between blue and red gene encoded proteins is connected by small circles (e.g., NFX1 is between PALMD and KIF4A)

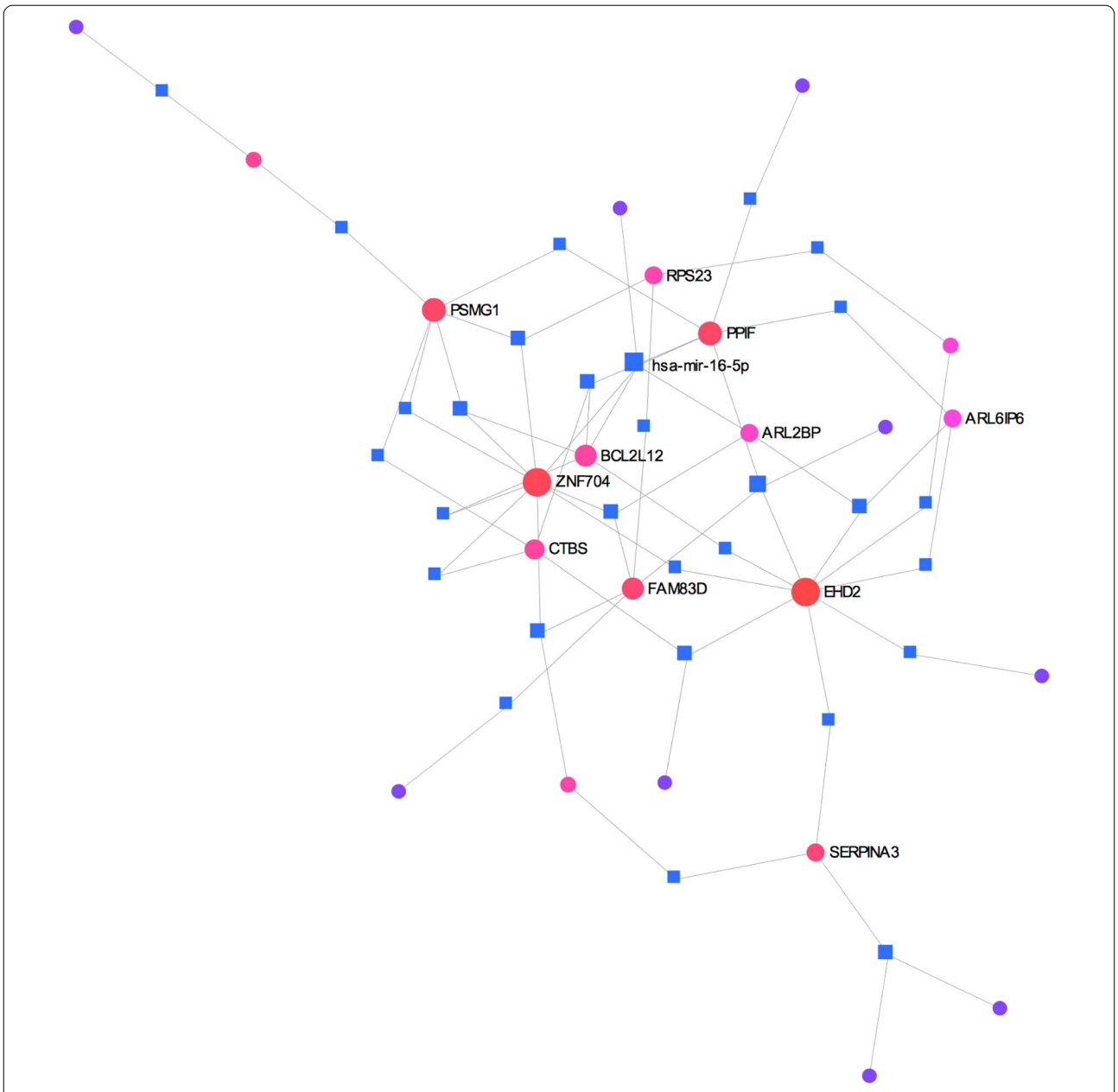


Fig. 3 miRNA-gene network analysis for identified significant DEGs. The identified DEGs are illustrated by circles and associated mi-RNAs are presented by squares

The survival analyses identified one and seven upregulated as well as seven and fiffteen downregulated genes for OS and RFS, respectively (illustrated in Fig. 7 (OS) and Fig. 8 (RFS)) that are statistically significant.

According to Table 2 and considering the DEGs identified from the 6-Shogaol treatment on MCF7 cell line as well as the statistical analysis of TCGA-BRCA, 8 and 5 DEGs are found to be associated with older and younger patients, respectively. Additionally, total of 7 significant genes are asscoiated with pathological T stage of the breast carcinoma; on the other hand, 4 genes are involved in pathological N stage and no genes are covered for the M stage. This may be indicative of the fact that 6-shogaol can be only efficient at the early stages of the disease.

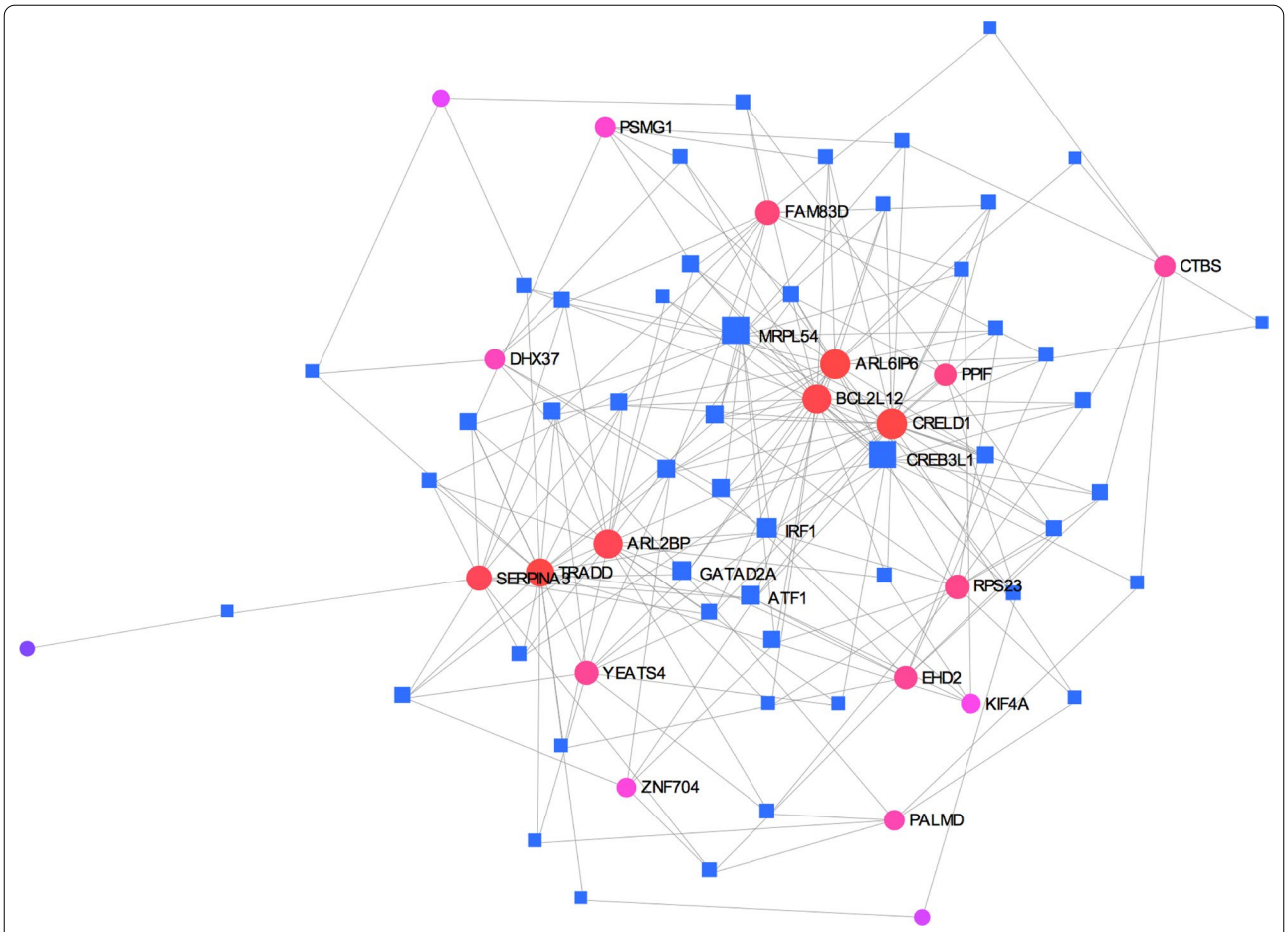


Fig. 4 Gene-TFs network analysis for identified significant DEGs The identified DEGs are illustrated by circles and associated transcription factors are presented by squares

Furthermore, 3 genes are found to be significant for radiation therapy and 2 genes are related to the higher number of lymph nodes.

Discussion

The inhibition of breast cancer among women is an important and challenging issue in the world. Different types of treatments were proposed and tested by outstanding researchers, among which 6-Shogaol showed its effectiveness in inhibiting the breast cancer cell invasion [1, 4, 19]. So, integrated bioinformatics investigation on effects of 6-Shogaol on MCF7 cell lines became the aim of the current research to provide an in-depth systems biology insights in the field. The primary purpose of studies available in the literature is frequently on identifying the significant biomarker genes to be used in diagnosis, prognosis, and designing drugs for the specific diseases, here, we studied 6-Shogaol structure's effect on MCF7 cell lines. Significant DEGs between MCF7 cell lines treated and untreated by 6-Shogaol have roles in inhibiting the active constituent of ginger on the expression of different genes. Tumor necrosis factor (TNF), also known as TNF-α and a cytokine, has a critical role in several cellular functions such as apoptosis, cell survival, and tumorigenesis progression and inhibition [20, 21].

The expression levels of some proteins, such as *TRADD* may play a vital role in inducing apoptosis in M cells of MCF7 cell lines [22]. Although the mechanism of *TRADD* in various types of cancers is still unclear, however, the downregulation of *TRADD* has been reported in cancers, including acute myeloid

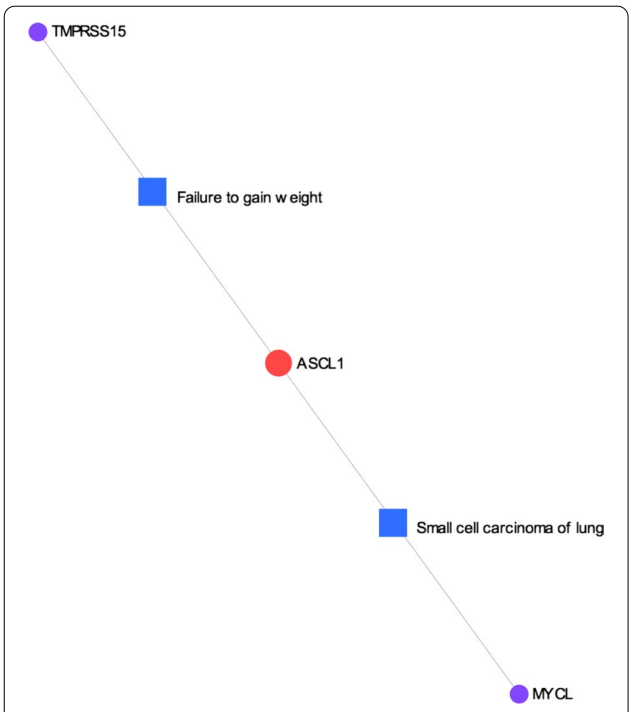


Fig. 5 Gene-disease network analysis for identified significant DEGs. The identified DEGs are illustrated by circles and associated diseases are presented by squares

leukemia (AML) exact pathogenesis of leukemia by inducing TNF signaling pathway [7]. The previous studies have also shown the low expression level of *CREB3L1* in specifically higher grade tumors in breast cancer [23, 24]. Moreover, the upregulation of *CREB3L1* frequently exists in small and medium-grade tumors [24]. So, the upregulation outcome of these proteins through 6-Shogaol treatment for MCF7 cell lines will be evidence for its effectiveness in treating the disease. The PPI minimized network analysis for mammary breast tissue revealed the downregulated *KIF1A* had significant effects in suppressing the activity of PARP-1 (poly (ADP-ribose) polymerase-1) while patients with breast cancer used doxorubicin treatment [25]. Additionally, the expression level of *PALMD* in grade III breast cancer tumor was significantly upregulated in comparison to normal tissues, and the downregulation of *PALMD* could be an indicative of the suppressive effect of 6-shogaol on MCF7 cell lines [26]. The outcomes derived from the construction of the miRNA-gene network have shown that the downregulated *miR-16* has remained conserved among many types of cancer, including breast cancer [27]. Some research reports presented overexpression of *miR-16-5p* may act as an inhibitor of breast cancer

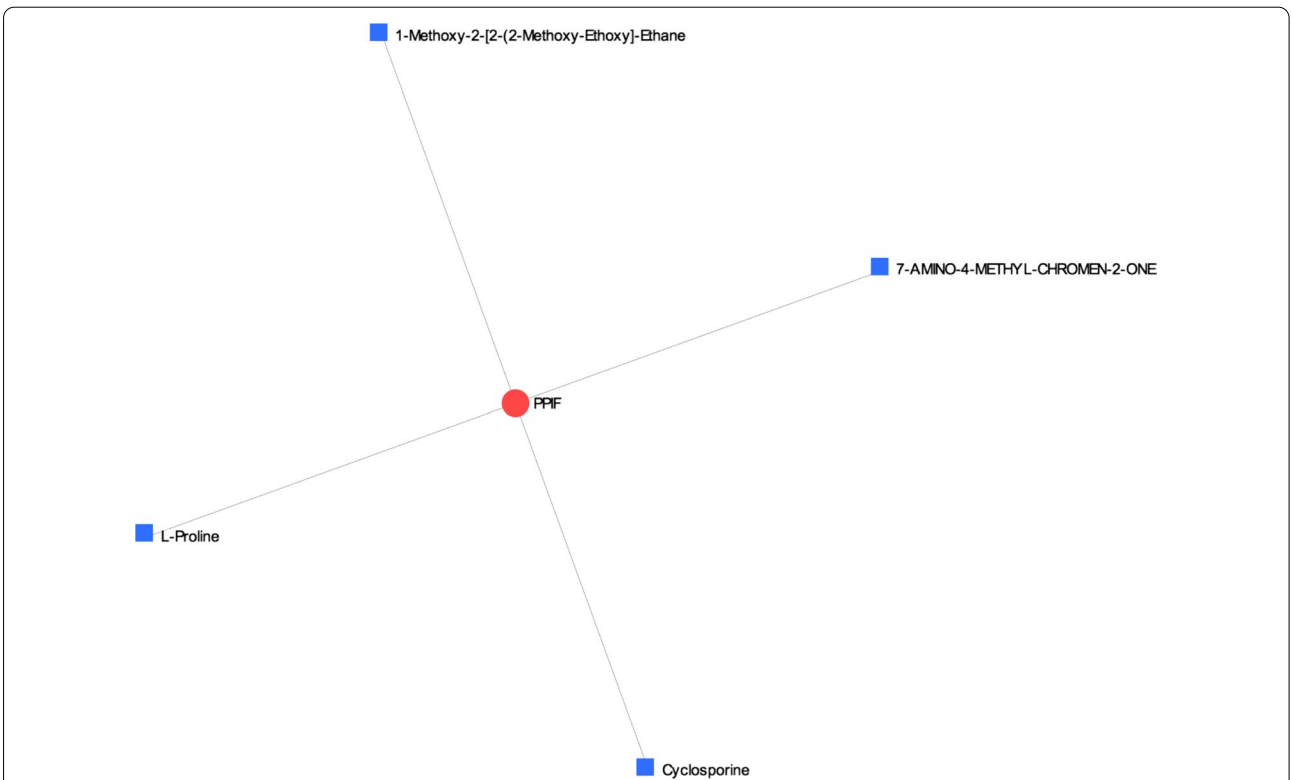


Fig. 6 Gene-drug network analysis for identified significant DEGs. The identified DEGs are illustrated by circles and associated chemicals are presented by squares

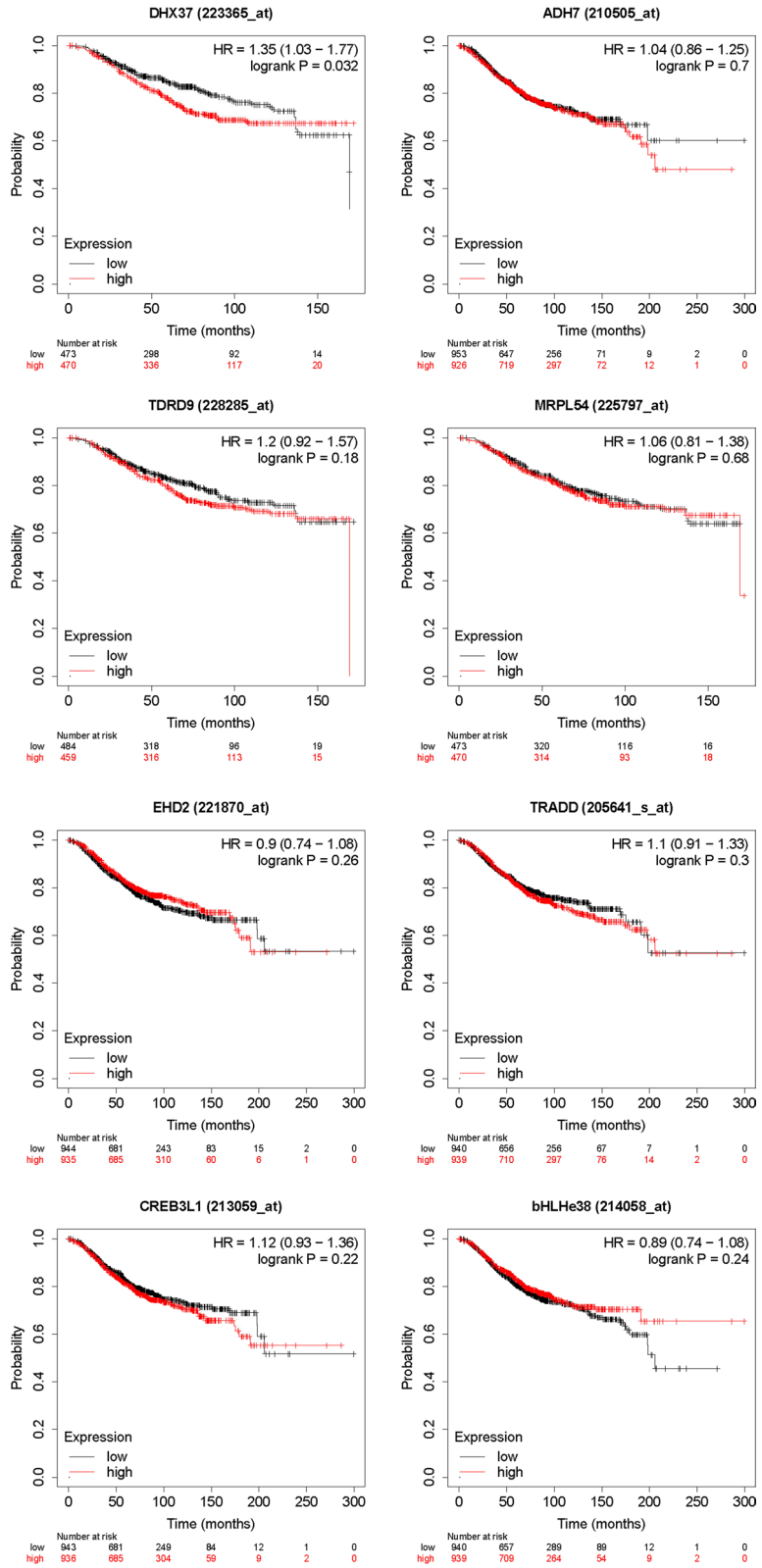
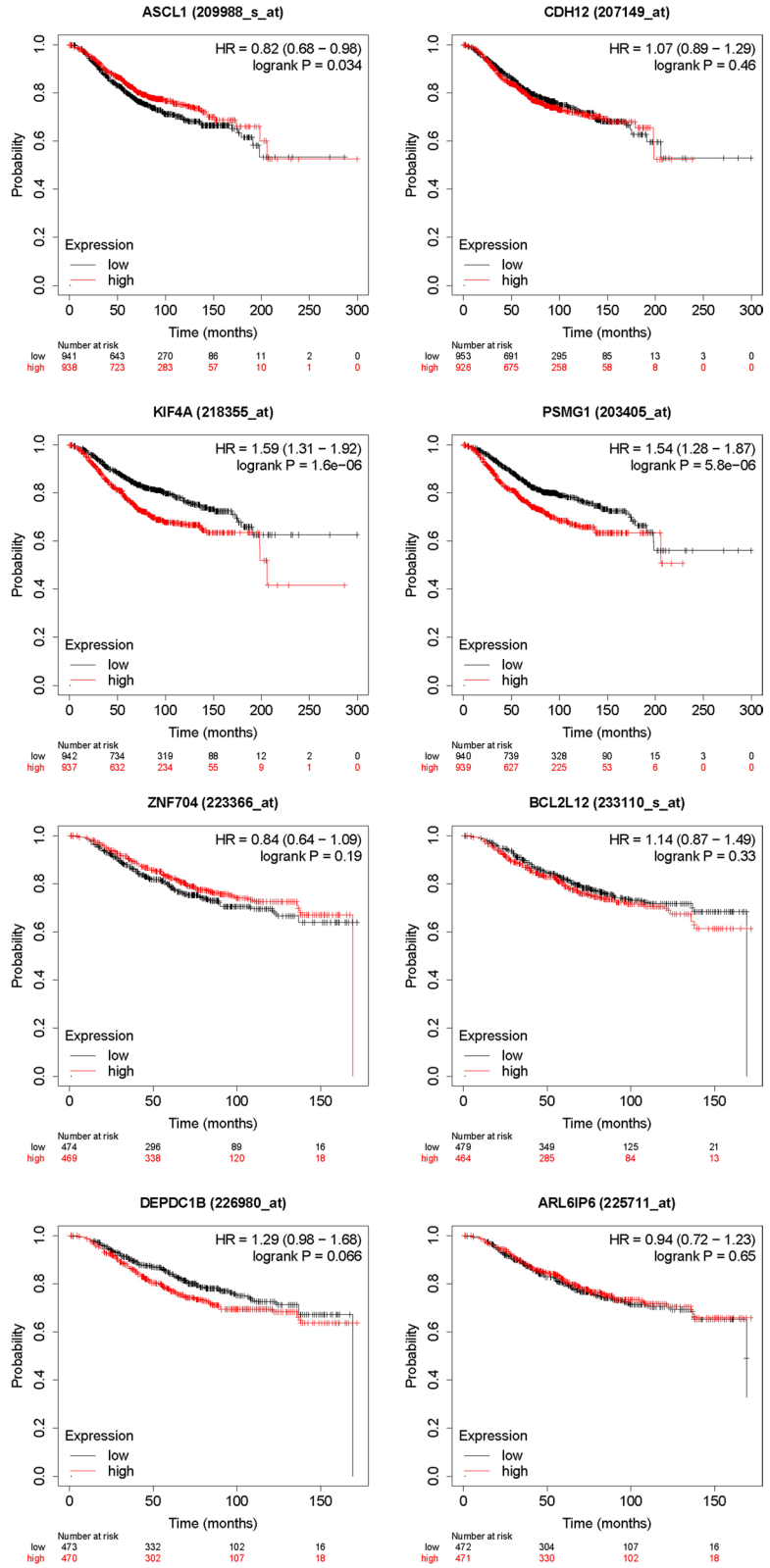


Fig. 7 The KM plot analyese for the identififfed genes along with their p-value for overall survival rate (note: the other name for AACT is SERPINA3 as listed in the figure and no OS data were available for OR2G2 and C5orf52 genes)



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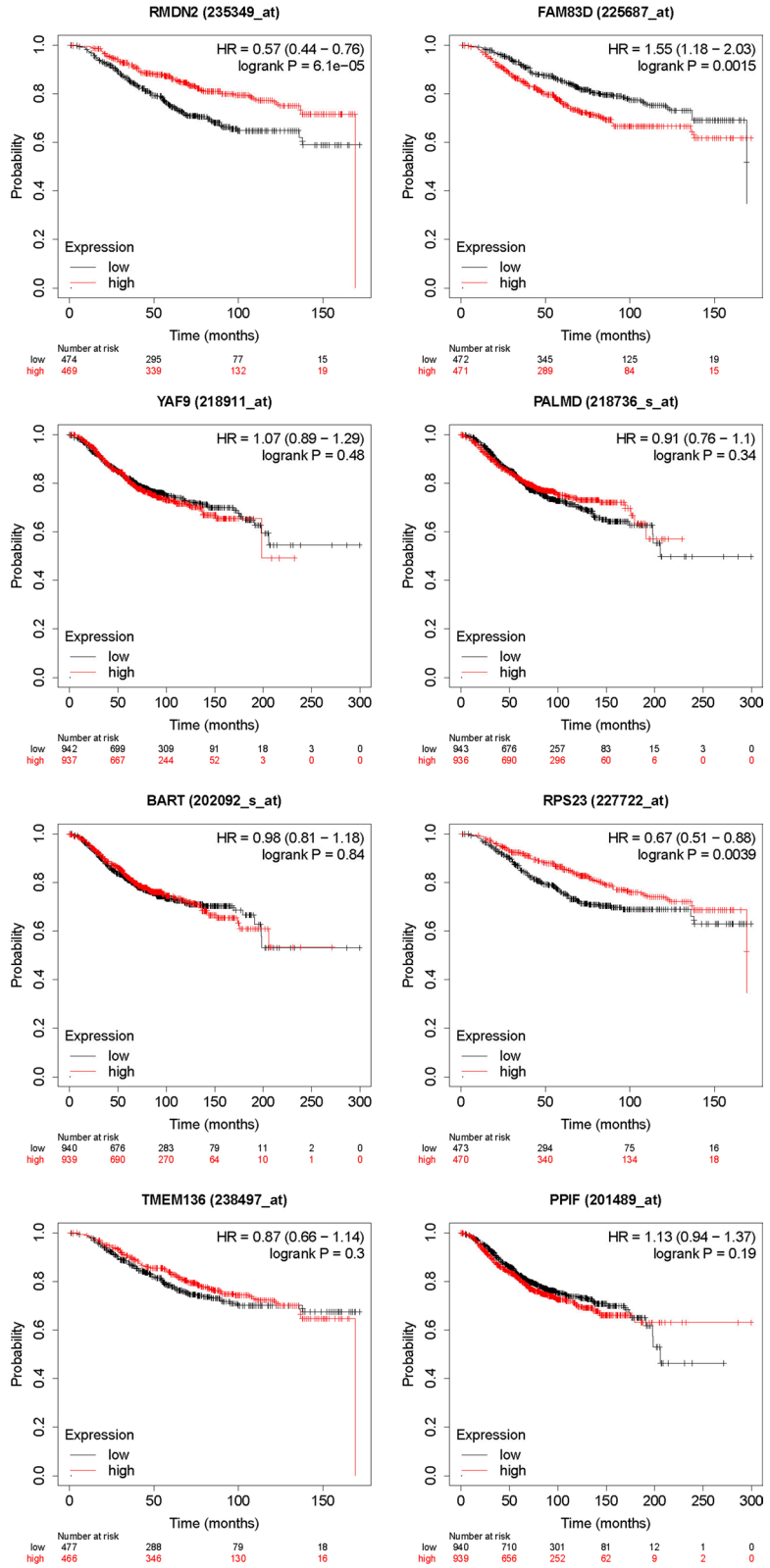

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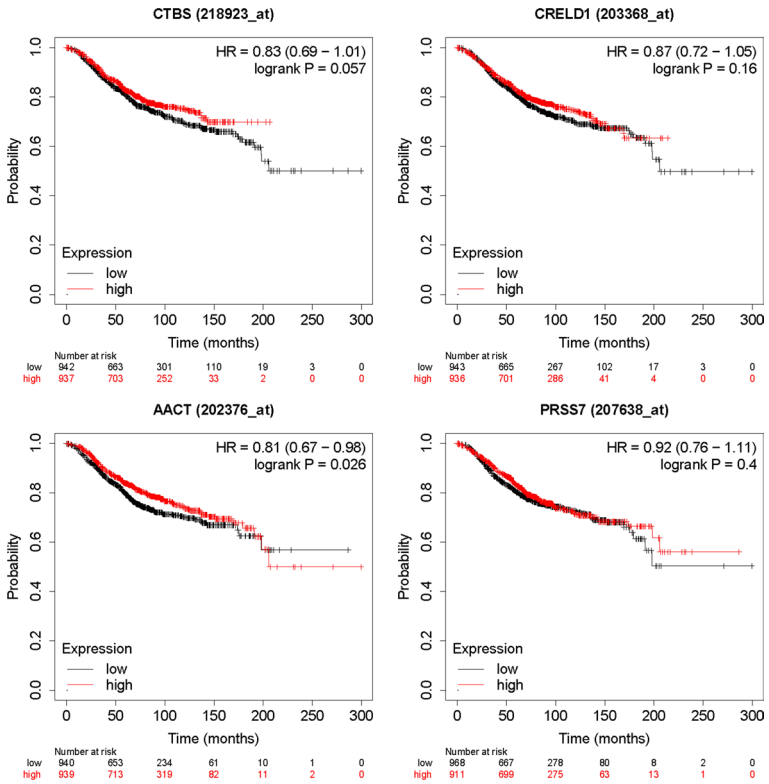


Fig. 7 continued

progression and development, and hence, may play a vital role in the design of novel drugs and therapeutics for patients with breast carcinoma [28, 29]. Similarly, downregulated *miR-124-3p* involved in various types of cancer, has frequently been reported in the literature; however, the in-depth mechanisms of this gene are still unclear in breast cancer development [30]. And, various researchers reported the inhibitory role of the *miR-124-3p* gene in breast and gastric cancers progression [31].

Regarding the weight gain, some studies hinted on the fact that the survivors of breast cancer were the major problem in women following their chemotherapy treatment [32, 33]. Additionally, the outcomes of the clinical literature experiments emphasized the direct relationship between small cell lung carcinoma and breast cancer (i.e., breast metastases), demonstrating two case reports for 38- and 66-year-old women [34, 35]. The literature studies confirmed the outcomes of gene-drug network construction for their effective treatments against breast cancer. These work through carbonic anhydrase IX and XII inhibitors, targeting progesterone metabolism, and downregulation of *PKM2* [36–38]. However, 1-Ethoxy-2-(2-methoxy ethoxy)ethane was also an inhibitor of the Hepatitis C virus (from PubChem registered Patent: US9085587), which might play a role in preventing breast cancer growth as well.

Altogether, in the current study, the effects of 6-Shogaol in inhibiting the cell metastasis in MCF7 cell lines were proposed using the systems biology technique. Furthermore, one may deduce that 6-Shogaol can be successful in treating patients with breast cancer, specifically at early stages of the disease, as a beneficial therapeutic agent that can be the target of assessment in future randomized clinical trials.

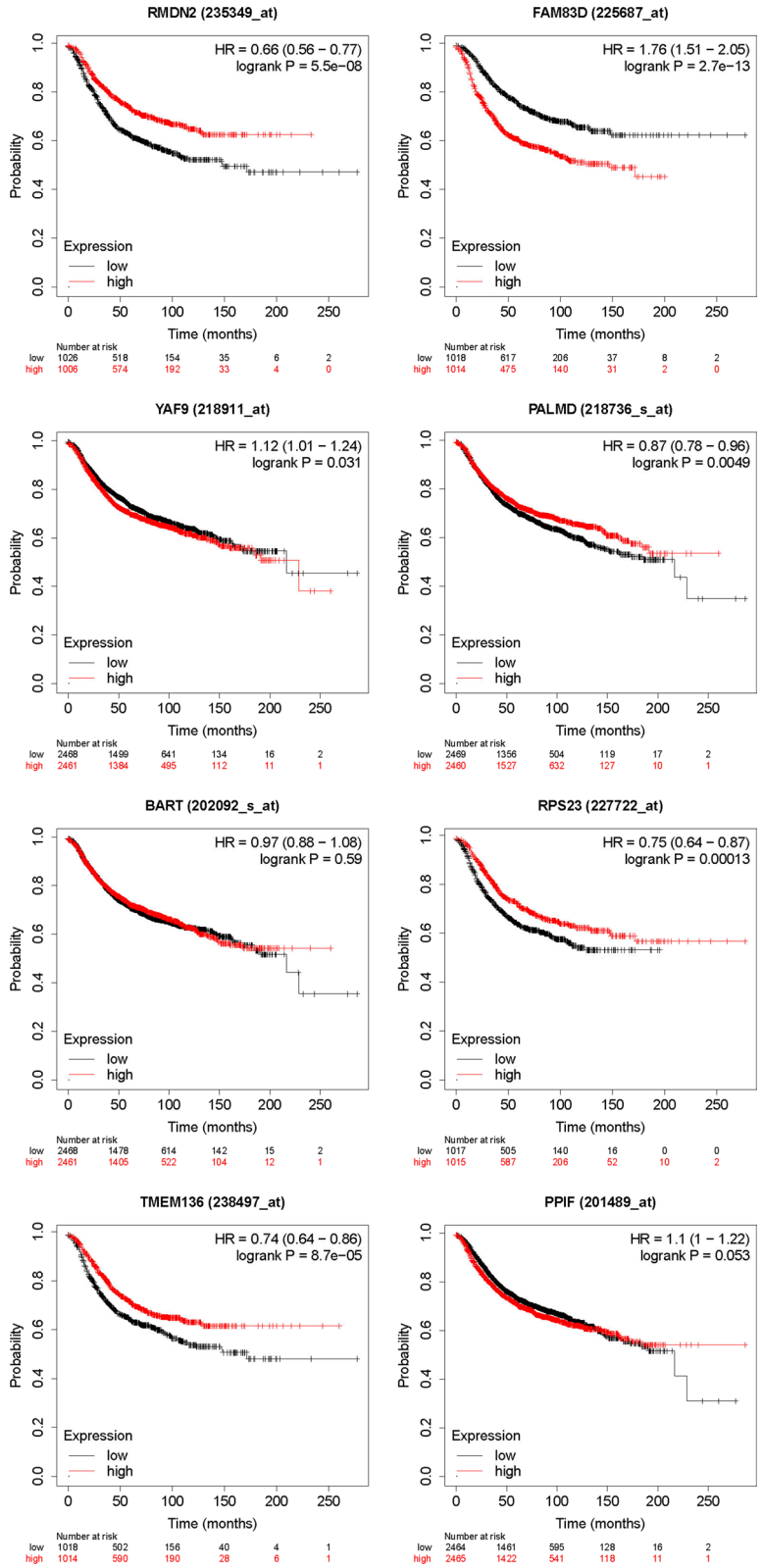
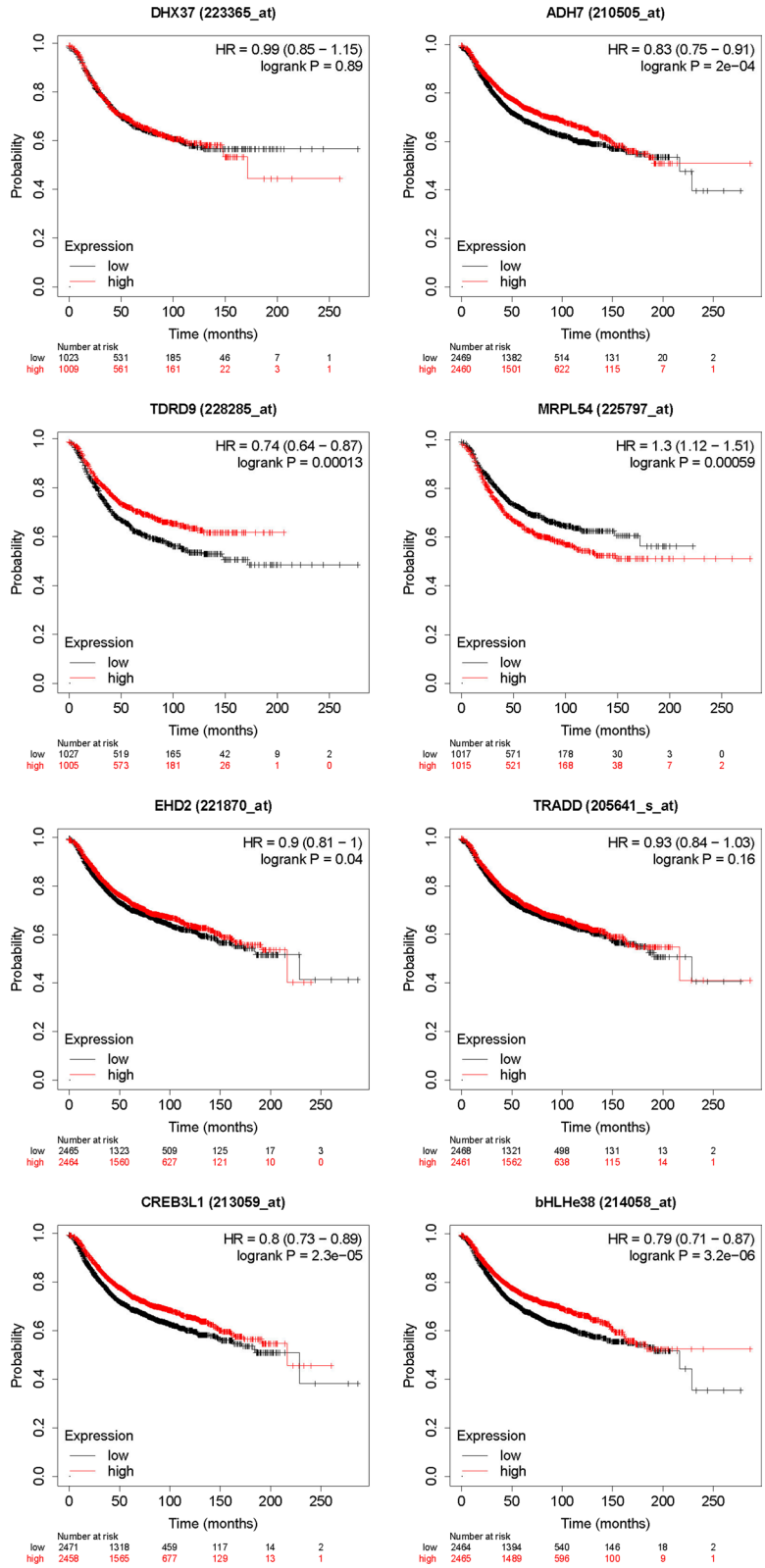


Fig. 8 The KM plot analyese for the identififfed genes along with their p-value for relapse free survival rate (note: the other names for the genes are listed as YAF9 (YEATS4), bHLHe38 (MYCL), SERPINA3 (AACT), TMPRSS15 (PRSS7) and no RFS data were available for OR2G2 and C5orf52 genes)



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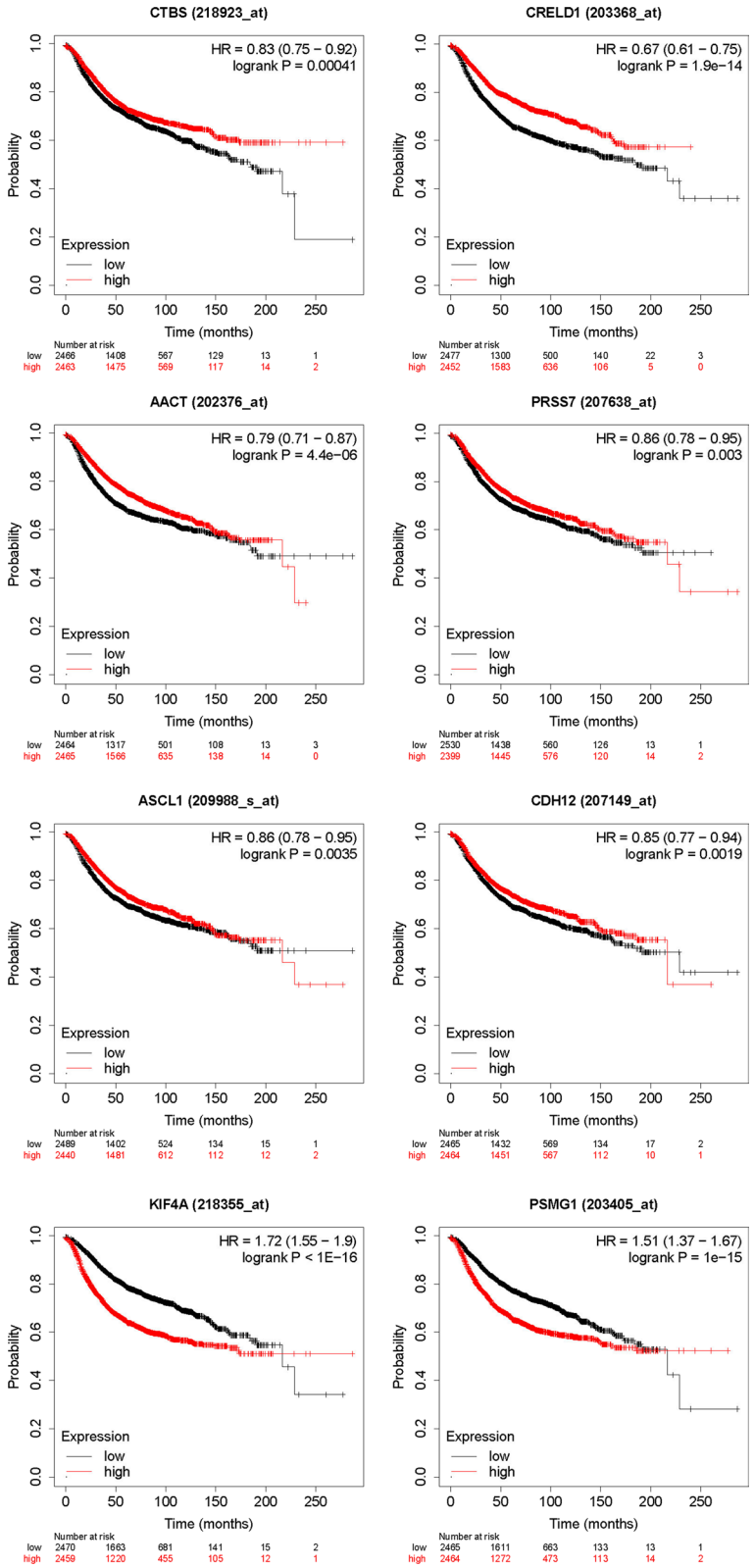

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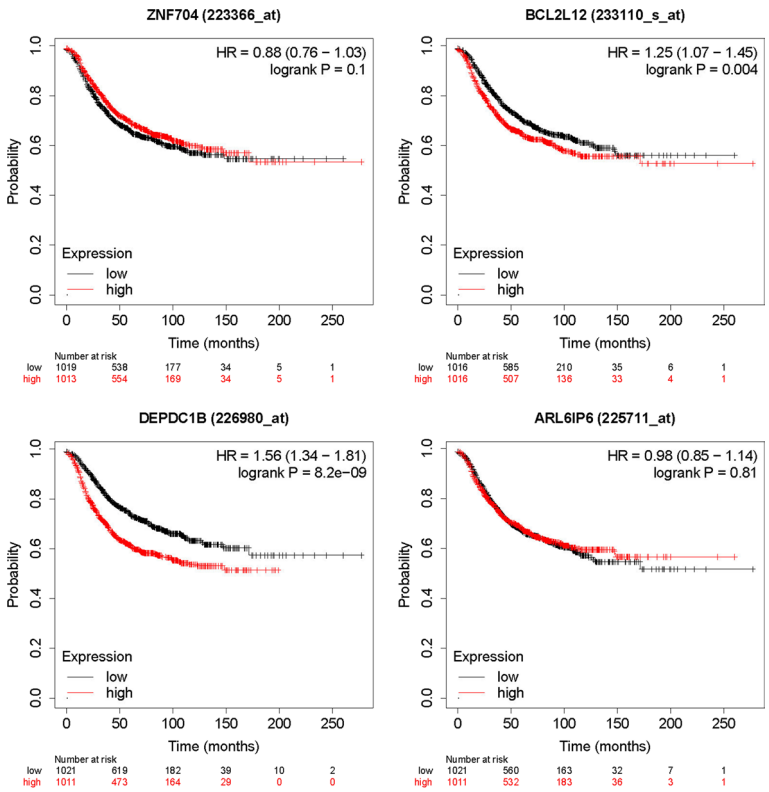

Fig. 8 continued

Conclusion

Breast cancer is the cause of the death of a large number of women in the world. Determining adequate treatment for BC is a critical problem of drug design and discovery. 6-Shogaol, as one of the potential constituent of ginger (*Zingiber officinale*), plays a role in breast cancer inhibition, cell apoptosis, and anticancer and anti-proliferative activities. In this study, we used systems biology method to demonstrate the 6-Shogaol effect on MCF-7 cell line and provide a computational way for validating the *in-vitro* results of the literature even for those without experimental confirmations. 6-Shogaol results in high expression of *TRADD* and *CREB3L1in* inducing the TNF-α signaling pathway as one of the essential paths of eliminating various types of tumors. Also, downregulation levels of *KIF1A* and *PALMD* are indicative of the anti-cancer effects of 6-Shogaol on MCF-7 cells. Additionally, 6-Shogaol could epigenetically affect the miRNA dysregulation. Moreover, the relationship between identified DEGs and clinical features are statistically verified and the OS and RFS survival rates are statistically analysed for the significant genes affected through the 6-Shogaol treatment on MCF7 cell line. This research indicates that the function of 6-Shogaol herbal medicine can be similar to anticancer drugs and also can be a potential component of anti-cancer drugs for treating breast cancer specifically at early stages of the cancer development.

Table 2 The outcomes of FireBrowse for assessment of clinical features versus mRNA genes

VariableName	PATHOLOGY_T_STAGE			PATHOLOGY_N_STAGE			PATHOLOGY_M_STAGE		
ResultType	SpearmanCorr	corrP	Q	SpearmanCorr	corrP	Q	Wilcoxon test P	Q	AUC
ASCL1	0.0562	0.1987	0.473	−0.006	0.8925	0.965	0.5857	0.999	0.5413
CDH12	0.0614	0.1603	0.425	0.022	0.6187	0.849	0.5555	0.999	0.5447
ADH7	−0.048	0.2725	0.557	−0.0226	0.6081	0.843	0.7523	0.999	0.524
ARL2BP	−0.0316	0.4704	0.722	0.0797	0.07081	0.338	0.3758	0.999	0.5671
ARL6IP6	−0.0764	0.08062	0.303	−0.0204	0.6442	0.862	0.3298	0.999	0.5739
BCL2L12	0.0855	0.05035	0.241	0.0276	0.5319	0.801	0.7442	0.999	0.5248
C5orf52	Not available	Not available	Not available	Not available	Not available	Not available	Not available	Not available	Not available
CREB3L1	−0.0431	0.325	0.608	0.1092	0.01319	0.151	0.3107	0.999	0.5768
CRELD1	0.0318	0.4681	0.721	−0.0278	0.5295	0.799	0.7828	0.999	0.5209
CTBS	−0.1253	0.004059	0.0699	−0.1071	0.01499	0.162	0.4891	0.999	0.5525
DEPDC1B	0.1125	0.009939	0.107	0.0155	0.7254	0.901	0.4444	0.999	0.558
DHX37	0.1048	0.01639	0.139	0.0424	0.3366	0.667	0.3918	0.999	0.5649
EHD2	−0.0543	0.2143	0.492	0.0489	0.2684	0.605	0.8616	0.999	0.5133
FAM83D	0.1393	0.001387	0.0436	0.0753	0.08782	0.373	0.2772	0.999	0.5824
KIF4A	0.182	2.77E−05	0.00861	0.0662	0.1334	0.447	0.1619	0.999	0.606
MRPL54	0.0342	0.4352	0.695	0.0484	0.2727	0.609	0.4138	0.999	0.562
MYCL1	0.069	0.1148	0.359	−0.029	0.5108	0.788	0.5585	0.999	0.5444
OR2G2	0.1089	0.0126	0.121	0.0649	0.1412	0.458	0.5839	0.999	0.5415
PALMD	−0.0751	0.08602	0.311	−0.0586	0.1839	0.514	0.4686	0.999	0.5549
PPIF	−0.0128	0.7698	0.904	0.012	0.785	0.925	0.147	0.999	0.6099
PSMG1(DSCR2)	0.0416	0.3423	0.624	−0.0381	0.3888	0.709	0.8784	0.999	0.5116
RMDN2(FAM82A)	−0.1211	0.005494	0.0803	−0.0259	0.5572	0.816	0.3933	0.999	0.5647
RPS23	−0.0542	0.2151	0.493	−0.0387	0.3809	0.702	0.01789	0.999	0.6794
SERPINA3	−0.0569	0.1938	0.467	−0.0669	0.1295	0.441	0.3289	0.999	0.574
TDRD9	−0.0371	0.3969	0.667	0.0776	0.07846	0.356	0.4551	0.999	0.5566
TMEM136	−0.0733	0.09355	0.324	−0.1036	0.01865	0.179	0.654	0.999	0.534
TMPRSS15(PRSS7)	0.0734	0.09308	0.323	0.071	0.1077	0.409	0.932	0.999	0.5065
TRADD	0.0769	0.07856	0.299	0.1267	0.003973	0.0906	0.187	0.999	0.6
YEATS4	0.0117	0.7893	0.913	−0.0594	0.1786	0.508	0.9511	0.999	0.5047
ZNF704	0.0012	0.9775	0.992	0.0525	0.2341	0.569	0.5741	0.999	0.5426

VariableName	YEARS_TO_BIRTH			RADIATION_THERAPY			NUMBER_OF_LYMPH_NODES		
ResultType	SpearmanCorr	corrP	Q	Wilcoxon test P	Q	AUC	SpearmanCorr	corrP	Q
ASCL1	0.0896	0.04105	0.123	0.08574	0.537	0.546	−0.0592	0.2332	0.665
ADH7	0.0174	0.6914	0.815	0.5353	0.892	0.5166	−0.0654	0.1873	0.623
ARL2BP	−0.0165	0.708	0.826	0.7883	0.955	0.5072	0.1254	0.01124	0.237
ARL6IP6	−0.0721	0.1003	0.232	0.7802	0.953	0.5075	−0.0017	0.9723	0.994
BCL2L12	−0.1129	0.009983	0.0448	0.7943	0.956	0.507	0.0109	0.8255	0.959
C5orf52	Not available	Not available	Not available	Not available	Not available	Not available	Not available	Not available	Not available
CDH12	−0.0389	0.3757	0.559	0.4698	0.866	0.5194	0.0411	0.4072	0.783
CREB3L1	2.00E−04	0.9972	0.999	0.6371	0.918	0.5126	0.1392	0.004862	0.176
CRELD1	0.1039	0.01773	0.0676	8.06E−05	0.0913	0.6056	−0.0209	0.6733	0.904
CTBS	0.0348	0.4282	0.606	0.005811	0.223	0.5739	−0.0467	0.347	0.748
DEPDC1B	−0.0971	0.02679	0.0911	0.7703	0.952	0.5078	0.0194	0.6958	0.912
DHX37	−0.071	0.1059	0.241	0.1473	0.627	0.5388	0.0246	0.6208	0.884
EHD2	−0.0776	0.07715	0.193	0.9618	0.993	0.5013	0.0841	0.08976	0.488
FAM83D	−0.1004	0.02206	0.079	0.8784	0.979	0.5041	0.0528	0.287	0.708
KIF4A	−0.1073	0.0144	0.0579	0.879	0.98	0.5041	0.0482	0.3311	0.737
MRPL54	0.1897	1.33E−05	0.000391	0.3667	0.811	0.5242	0.1126	0.02294	0.303
MYCL1	0.1018	0.02028	0.0745	0.153	0.635	0.5383	−0.0501	0.3129	0.728

Table 2 (continued)

VariableName	YEARS_TO_BIRTH			RADIATION_THERAPY			NUMBER_OF_LYMPH_NODES		
ResultType	SpearmanCorr	corrP	Q	Wilcoxon test P	Q	AUC	SpearmanCorr	corrP	Q
OR2G2	0.0974	0.02636	0.09	0.007625	0.24	0.5715	0.061	0.2186	0.653
PALMD	− 0.1313	0.002711	0.0177	0.7352	0.945	0.5091	− 0.0345	0.4871	0.825
PPIF	− 0.0446	0.3103	0.497	0.22	0.709	0.5329	− 0.0111	0.8231	0.958
PSMG1(DSCR2)	− 0.0767	0.08072	0.199	0.402	0.83	0.5225	− 0.0683	0.1683	0.601
RMDN2(FAM82A)	0.0162	0.7131	0.829	0.2962	0.767	0.528	− 0.049	0.3231	0.732
RPS23	0.0925	0.03492	0.11	0.5689	0.902	0.5153	− 0.0195	0.6949	0.911
SERPINA3	0.1289	0.003228	0.02	0.6112	0.912	0.5136	− 0.0241	0.628	0.887
TDRD9	− 0.0249	0.5703	0.725	0.08365	0.535	0.5464	0.0368	0.4585	0.81
TMEM136	0.0473	0.2819	0.467	0.8048	0.958	0.5066	− 0.0819	0.09837	0.498
TMPRSS15(PRSS7)	0.015	0.7329	0.842	0.4832	0.871	0.5188	0.0547	0.2702	0.696
TRADD	− 0.0593	0.1768	0.341	0.5873	0.906	0.5146	0.1027	0.03812	0.36
YEATS4	0.0419	0.3408	0.527	0.9355	0.988	0.5022	− 0.0165	0.7396	0.929
ZNF704	0.1834	2.57E−05	0.000633	0.643	0.919	0.5124	− 3.00E−04	0.9945	0.998

VariableName	HISTOLOGICAL_TYPE		RACE	
ResultType	Kruskal_ Wallis_P	Q	Kruskal_ Wallis_P	Q
ASCL1	0.6302	0.715	0.4264	0.706
CDH12	0.287	0.445	0.004113	0.108
KIF4A	5.29E−05	0.00132	0.00361	0.103
PSMG1(DSCR2)	6.92E−05	0.00159	0.251	0.593
ZNF704	0.6903	0.759	0.05046	0.312
BCL2L12	0.002162	0.0182	0.09876	0.41
DEPDC1B	0.001027	0.0108	0.04954	0.31
ARL6IP6	0.1319	0.281	0.9639	0.982
RMDN2(FAM82A)	0.0006258	0.00758	0.01507	0.192
FAM83D	7.92E−07	7.97E−05	0.06783	0.353
YEATS4	0.6501	0.729	0.6305	0.806
PALMD	0.001206	0.0121	0.003709	0.104
ARL2BP	0.8425	0.874	0.5415	0.762
RPS23	0.004951	0.0321	3.05E−05	0.00937
TMEM136	0.246	0.406	0.01884	0.209
PPIF	0.7755	0.823	0.4352	0.711
DHX37	0.1737	0.333	0.2396	0.582
ADH7	0.4253	0.563	0.1695	0.509
TDRD9	0.004103	0.0283	0.008456	0.149
MRPL54	0.8367	0.87	0.9014	0.949
EHD2	0.001054	0.011	0.9842	0.992
TRADD	0.06848	0.188	0.7823	0.885
C5orf52	Not available	Not available	Not available	Not available
CREB3L1	0.3843	0.53	0.7106	0.849
MYCL1	0.1923	0.352	0.7082	0.848
TMPRSS15(PRSS7)	0.6754	0.747	0.005527	0.122
OR2G2	0.1753	0.334	0.002666	0.09
CTBS	0.8408	0.873	0.9667	0.983
CRELD1	0.1945	0.355	0.2027	0.544
SERPINA3	0.1757	0.335	0.06304	0.343

Abbreviations
ASCL1: Achaete-scute homolog 1; ADH7: Alcohol Dehydrogenase 7; ARL2BP: ADP Ribosylation Factor Like GTPase 2 Binding Protein; ARL6IP6: ADP Ribosylation Factor Like GTPase 6 Interacting Protein 6; BCL2L12: BCL2 Like 12; BP: Biological process; CC: Cellular component; C5orf52: Chromosome 5 Open Reading Frame 52; CDH12: Cadherin-12; CREB3L1: CAMP Responsive Element Binding Protein 3 Like 1; CRELD1: Cysteine Rich With EGF Like Domains 1; CTBS: Chitobiase; DAVID: Database for Annotation, Visualization, and Integrated Discovery; DEG: Differentially Expressed Genes; DEPDC1B: DEP Domain Containing 1B; DHX37: Probable ATP-dependent RNA helicase; EHD2: EH Domain Containing 2; FAM83D: Family With Sequence Similarity 83 Member D; GEO: Gene Expression Omnibus; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; KIF4A: Kinesin Family Member 4A; KM: Kaplan–Meier; MF: Molecular functions; MRPL54: Mitochondrial Ribosomal Protein L54; MYCL: MYCL Proto-Oncogene, BHLH Transcription Factor; NCBI: National Center for Biotechnology Information; OR2G2: Olfactory Receptor Family 2 Subfamily G Member 2; OS: Overall survival rate; PALMD: Palmdelphin; PCSF: Prize-collecting Steiner Forest; PPIF: Peptidyl-prolyl cis–trans isomerase F; PPI: Protein–protein interaction; PSMG1: Proteasome Assembly Chaperone 1; RFS: Relapse-free survival rate; RMDN2: Regulator of Microtubule Dynamics 2; RPS23: Ribosomal Protein S23; SERPINA3: Serpin Family A Member 3; TDRD9: ATP-dependent RNA helicase; TMEM136: Transmembrane protein 136; TMPRSS15: Transmembrane Serine Protease 15; TRADD: TNFRSF1A Associated Via Death Domain; WHO: World Health Organization; YEATS4: YEATS Domain Containing 4; ZNF704: Zinc Finger Protein 704.

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Authors' contributions
BS and SA—Conceptualization; EA, BS and SA—Data curation; EA and SA—Formal analysis; EA and BS—Investigation; EA, BS and SA—Methodology; BS—Project administration; BS and SA—Supervision; EA, BS and SA—Roles/Writing—original draft; EA, BS and SA—Writing—review & editing. All authors read and approved the final manuscript.

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