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Original Article

Integrative bioinformatics and RNA sequencing based methodology results in the exploration of breast invasive carcinoma biomarkers

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Abstract: Background: Previously reported breast invasive carcinoma (BRIC) biomarkers have compromised utility because of their heterogeneity-specific behaviors. The goal of this study was to find BRIC biomarkers that could be used in spite of the heterogeneity barrier. Methods: Previously reported BRIC-linked hub genes were obtained from the literature via a search technique. A protein-protein interaction (PPI) network of the extracted hub genes was constructed, visualized, and analyzed to explore the top six real hub genes. Following this, real hub genes' expression profiling was carried out using various TCGA data sources and RNA sequencing (RNA-seq) of BT 20 and HMEC cell lines to uncover the tumor-driver roles of the real hub genes. Results: In total, 124 BRIC-linked hub genes were collected from the literature via the search technique. From these collected hub genes, a total of 6 genes, including Centrosomal protein of 55 kDa (CEP55), Kinesin Family Member 2C (KIF2C), kinesin family member 20A (KIF20A), Ribonucleotide Reductase Regulatory Subunit M2 (RRM2), Aurora A Kinase (AURKA), and Protein Regulator of cytokinesis 1 (PRC1) were determined to be the real hub genes. Via expression profiling and validation analyses, we documented the overexpression of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 real hub genes in BRIC patients with different clinical variables. Further correlational analyses showed diverse associations among real hub genes' expression and other important parameters, including promoter methylation status, genetic alteration, overall survival (OS), relapse-free survival (RFS), tumor purity, CD8+ T, CD4+ T immune cell infiltration, and different mutant genes across BRIC samples. Finally, in this work, we investigated several transcription factors (TFS), microRNAs, and therapeutic medicines related to the real hub genes that have great therapeutic potential. Conclusion: In conclusion, we discovered six real hub genes, which may be employed as novel potential biomarkers for BRIC patients with different clinical parameters.

Keywords: BRIC, biomarker, hub genes

Introduction

Breast invasive carcinoma (BRIC) is the most common women's malignancy and leads to a large number of cancer-related deaths every year around the globe [1]. According to recent reports, about 3.45 million cancer cases are annually reported in Europe [1]. As a result of ongoing efforts by researchers, the availability of modern technologies has greatly helped to reduce the BRIC-related mortality rate by identifying reliable potential biomarkers for the timely detection, treatment, and monitoring of prognosis. However, due to the heterogeneity-associated nature of the reported BRIC biomarkers [2], the management of disease in BRIC patients of different cancer stages, races, genders, ages, and subclasses has not been addressed completely and remains a major clinical treatment obstacle [2].

With the help of microarray techniques, various disease-associated differentially expressed genes can be recognized simultaneously [3, 4]. Besides, this technique also enables researchers to carry out a detailed analysis of specified key genes to explore their potential as molecular biomarkers. Gene Expression Omnibus (GEO) database is a free online available microarray and RNA sequencing-based platform maintained by the National Center for Biotechnology Information (NCBI) [5]. This database is one of the most specialized platforms for researchers to submit, re-evaluate, and reanalyze the already submitted microarray datasets for the identification of disease-specific molecular biomarkers [6].

In this work, we re-analyzed multiple GEO datasets to find a few BRIC-associated biomarkers that could be used to overcome the heterogeneity barrier. The current work may be useful in developing a unique system of biomarkers that can be applied to BRIC patients with various clinical characteristics across the heterogeneity-specific barrier.

Methods

Mining of hub genes

Relevant studies that dealt with the BRIC GEO expression datasets, in order to explore hub genes, until June 2022 were searched via "PubMed". For search purposes, the two selected keywords were "Hub genes AND Breast cancer" and "Hub genes AND Breast neoplasia" with the "Research article" filter. By doing so, a total of 108 studies appeared at the end of the search process. Those studies were further shortlisted to only 24 studies that collectively used 31 BRIC GEO datasets. Following the search process, all collected hub genes were compiled into a single pool.

Gene enrichment analysis

The GO "(Gene Ontology)" and KEGG "(Kyoto Encyclopedia of Genes and Genomes)" analyses were performed via the DAVID 9th tool [7]. This is an online platform that is publicly available for GO and KEGG analysis of any given gene list. In these analyses, a p-value <0.05 was regarded as significant.

Real hub genes screening

In this study, STRING "Search Tool for the Retrieval of Interacting Genes/Proteins" [8] analysis was conducted for constructing the protein-protein interaction (PPI) network of the hub genes. Later on, Cytoscape [9] plugin applications, including MCODE and Cytohubba, were used to determine the significant module and the top six real hub genes. Based on the 4 different scoring algorithms, "the maximum neighborhood component (MNC), the density of the maximum neighborhood component (DMNC), the maximal clique centrality (MCC), and the Degree of the Cytohubba" [10], the shared top six genes by these 4 algorithms were selected as real hub genes.

GEPIA-based mRNA expression analysis

The TCGA "(Cancer Genome Atlas)" expression data is used to create gene expression plots based on various pathological factors in the GEPIA "(Gene Expression Profiling Interactive Analysis)" database, a new online web-based tool that enables users to perform interactive and customizable analyses between normal-v-normal cancer samples [11]. In this work, we utilized the TCGA BRIC dataset from GEPIA to analyze real hub genes' expression. The p-value cutoff was selected as 0.05.

mRNA and translation expression validation analysis by other databases

The bc-GenExMiner "(Breast Cancer Gene-Expression Miner)" [12], GENT2 [13], and the UALCAN "(University of ALabama at Birmingham CANcer)" [14] were utilized in this study for the mRNA and translational expression validation of the real hub genes using new independent cohorts of BRIC patients. All these online databases are cancer microarray-based expression analysis platforms, which provide expression analysis results in the form of box plots. Additionally, the UALCAN database was also utilized to measure the expression of real hub genes targeting TFS and miRNAs. The p-value cutoff was selected as 0.05.

Promoter methylation analysis

In this study, the correlations among the real hub genes expression and their promoter me-

thylation levels in BRIC were examined via MEXPRESS through the Pearson correlation method [15]. MEXPRESS database highlight associations among patient clinical information and promoter methylation levels across TCGA datasets. The *p*-value cutoff was selected as 0.05.

cBioPortal analyses

Multidimensional cancer genomic analysis on TCGA cancer datasets is carried out using the cBioPortal, which is an online open-access platform [16]. In this study, this database was used for analyzing genetic mutations in real hub genes across BIRC samples.

Survival analysis

The Kaplan-Meier plotter [17] tool was used to compute the relapse-free survival (RFS) and overall survival (OS) of real hub genes. To do this, BRIC patient samples were divided into 2 different cohorts in accordance with the median expression of the real hub gene (high vs. low). The *p*-value cutoff was selected as 0.05.

Hub genes and immune cells infiltration

The TIMER "(Tumor Immune Estimation Resource TIMER)" database [18] was used in this study to find associations between tumor purity, CD8+ T, CD4+ T immune cell infiltration, and real hub gene expression. A variety of algorithms are used in this database to estimate the abundance of immune cells across different cancers.

TFS-miRNA-mRNA network

To construct the TFS-miRNA-mRNA network, The ENCORI "(Encyclopedia of RNA Interactomes)" and transcriptional regulatory relationships unraveled by sentence-based text-mining (TRRUST) were conducted in the present study [19]. These databases are widely utilized for exploring miRNA-ncRNA and mRNA-miRNA interactions from CLIP-seq interactome data.

MuTarget analysis

The MuTarget [20] analysis was conducted in this study with default setting to identify the mutant genes causing expressional changes in the real hub genes across BRIC. The *p*-value cutoff was selected as 0.05.

Real hub gene associated drugs

The CTD "(Comparative Toxicogenomics Database)" (CTD) database [21] was used in the current work to identify real hub gene-associated different drugs in the current study. Because we believe that the identified real hub genes can be interesting therapeutic targets. This database offers information on drugs that target hub genes from numerous trustworthy sources [21].

In vitro validation of the hub gene expression

Cell lines: One BRIC cell line (BT 20), as well as one normal mammary gland cell line (HMEC) were purchased from the American Type Culture Collection (ATCC, USA) and cultivated in accordance with the manufacturer's instructions.

Total RNA extraction: Total RNA extraction from both BRIC and normal cell lines was done by isopycnic centrifugation as described previously [22]. The extracted RNA was then processed for DNA digestion step of incubation with RNase-free DNase I (Roche, Germany) at 37°C for 15 minutes. The quality of the extracted RNA was checked by a 2100 Bioanalyzer (Agilent Technologies, Germany).

RNA-Seq analysis: RNA samples were sent to Macrogen, Korea company for RNA-seq analysis. Following RNA-seq analysis, the gene expression values of the hub genes were normalized using reads per kilo base million reads (RPKM) and fragments per kilo base million reads (FPKM). The obtained FPKM values against real hub genes in BIRC and normal control cell line were compared to identify differences in the expression level.

Statistics details

For GO and KEGG enrichment analysis, we used Fisher's Exact test for computing statistical difference [23]. Correlational analyses were carried out using the Pearson method. For comparisons, a student t-test was adopted in the current study. All the analyses were carried out in R version 3.6.3 software.

Results

Hub genes collection from the literature

We selected 24 molecular studies that explored hub genes in BRIC GEO datasets. We then

Table 1. Detail of BRIC datasets and hub genes obtained from the literature

BRIC Dataset	samples C/N	Hub genes	Reference
GSE10797	28/5	RPS9, RPL11, RPS14, RPL10A, EPCAM, MELK, KRT8, KRT19,	[59-82]
GSE15852	43/43	KPNA2, ECT2, TPX2, KIF2C, CDCA8, BUB1B, CCNA2, TOP2A,	
GSE92697	26/0	PCNA, CCNB1, CDC20, BIRC5, PHLPP1, UBC, ACACB, TGFB1,	
GSE102484	683/0	ACTB, CASC5, FAM83D, TFAP2C, KIF23, GINS1, CDCA5, CCNE1,	
GSE65212	130/11	KRT16, MYBL2, AGO2, MCM10, TTK, KIF18B, CDKN2A, MME,	
GSE43837	19/0	IGFBP3, CKAP2L, TGM2, ACTA2, PDGFRβ, SUMO1, FYN, CAV1,	
GSE23988	61/0	COL5A1, SKA1, MMP2, CDK1, NDC80, KRT18, STAMBP, JUN,	
GSE20194	230/0	MCM6, FOS, ATF3, STAT1, COL1A1, FN1, TP53, GAPDH, CCND1,	
GSE42568	104/17	HRAS, CAPG, SPI1, LEF1, PBX3, TCF7L2, VCAM1, PLAGL1, PBX1,	
GSE75333	6/3	EGFR, IGF1, LEP, PTEN, FOXO1, FGF2, PPARG, AURKA, IK3CA,	
GSE5847	95/0	CDH1, CDK1, NOTCH1, MAPK14, SRC, HSPA8, ESR1, PPP2CA,	
GSE22035	43/0	RPL4	
GSE3744	47/0	RAC1, KIF20A, RRM2, ASPM, NUSAP1, CEP55	
GSE5764	10/20	PGR, GATA3, ABLIM3, MYC, IL18, CD274, ITGB1, ITGB3, ITGA2B,	
GSE21422	14/5	CXCR4, COL1A2, EGR1, HMOX1, NR3C1, STAT5A, TFF1, FOXA1,	
GSE26910	12/12	HSP90AA1, KIF11, CCNB2, CDKN3, CENPF, PRC1, PTTG1,	
GSE41970	270/59	UBE2C, ZWINT	
GSE8977	7/15		
GSE45827	144/11		
GSE71142	10/0		
GSE86945	100/0		
GSE86946	58/0		
GSE29431	66/0		
GSE65194	167/11		
GSE22093	103/0		
GSE31192	22/0		
GSE9014	123/0		
GSE10780	143/62		
GSE29431	54/12		
GSE61304	59/3		
GSE10810	31/27		
Total = 31	Total = 2908/313	Total = 124	

C = Cancerous, N = Normal.

performed the extraction of hub genes from these studies and pooled these hub genes after normalizing the duplicated hub genes. Ultimately, a pool of 124 hub genes from 31 GEO BRIC datasets containing 2908 BRIC and 313 normal samples was further explored (**Table 1**). Original data (without normalization) can be seen in the Supplementary Material.

GO and KEGG analysis

The GO and KEGG enrichment analysis revealed hub the genes that were enriched in different GO and KEGG terms, including "cell division, mitotic nuclear division, and response to drug" biological processes GO terms, and pathways in "cancer, cell cycle, focal adhesion, and proteoglycans in cancer" KEGG terms (**Figure 1** and **Tables 2**, **3**).

Screening of real hub genes

A PPI network of the 124 hub genes was created with the help of STRING. The obtained PPI consisted of 124 nodes and 2110 edges (**Figure 2A**). Then, the MCODE and Cytohubba analyses via Cytoscape software were performed to identify the most significant module in the PPI and a few more closely BRIC relevant genes (real hub genes) via the degree method. The most significant identified module consisted of 43 hub genes (**Figure 2B**), and based on the degree method, the screened six real hub genes were CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 (**Figure 2C** and **Table 4**).

Expression analysis and validation

For analyzing and validating real hub gene expression at the mRNA as well as protein lev-

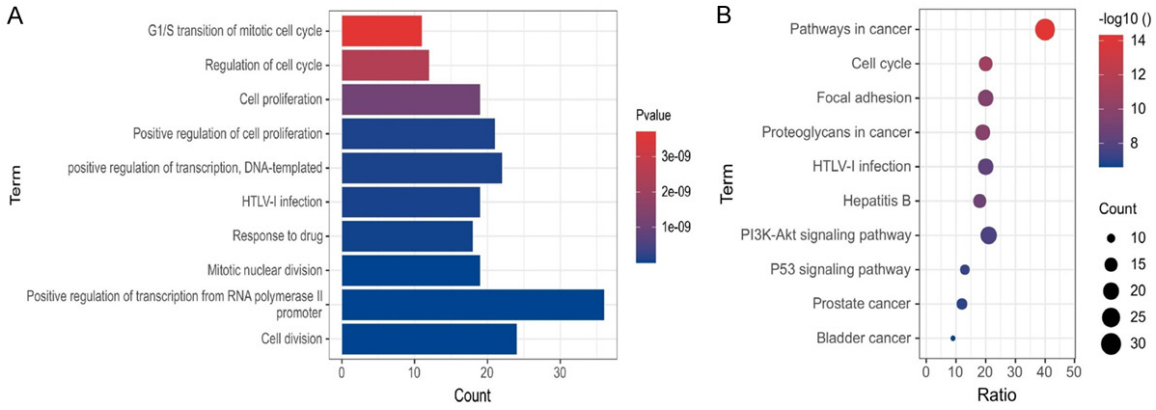


Figure 1. A heatmap representing the GO and KEGG terms across identified hub genes related with BRIC. (A) A heatmap of GO terms across identified hub genes, and (B) a heatmap of KEGG terms across identified hub genes.

Table 2. Details of the GO analysis

Biological process ID	Biological process	Gene count	*P*-value	Gene name
GO:0051301	cell division	24	2.8E-16	UBE2C, CDCA5, CDCA8, BUB1B, KIF11, NR3C1, NDC80, SKA1, ZWINT, AURKA, CCNA2, CDC20, CCNB2, TPX2, CENPF, CCNB1, KIF18B, PTTG1, CCND1, CCNE1, CDK1, BIRC5, KIF2C, FAM83D
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	36	3.5E-16	FOXA1, TOP2A, SPI1, NOTCH1, LEF1, GATA3, NR3C1, FGF2, FOXO1, EGFR, ABLIM3, EPCAM, MYC, PLAGL1, UBC, MYBL2, HRAS, TCF7L2, EGR1, JUN, TFAP2C, TGFB1, CDKN2A, STAT1, IL18, PBX3, IGF1, FOS, MAPK14, ESR1, PBX1, AGO2, PPARG, PGR, TP53, ATF3
GO:0007067	mitotic nuclear division	19	1.3E-13	CDCA5, BUB1B, KIF11, NR3C1, NDC80, SKA1, AURKA, CCNA2, CDC20, ASPM, CCNB2, TPX2, CENPF, PTTG1, CDK1, BIRC5, KIF2C, FAM83D, CEP55
GO:0042493	response to drug	18	4.2E-11	JUN, HSP90AA1, TGFB1, STAT1, SRC, PTEN, GATA3, FOS, ACACB, COL1A1, CENPF, CCNB1, CCND1, CDH1, MYC, CDK1, FYN, PPARG
05166	HTLV-I infection	19	6.7E-11	EGR1, HRAS, STAT5A, TP53, SPI1, CDC20, PTTG1, TGFB1, VCAM1, FOS, CCND1, ATF3, CDKN2A, JUN, PCNA, BUB1B, PDGFRB, MYC, TP53INP1
GO:0045893	positive regulation of transcription, DNA-templated	22	8.1E-11	EGR1, JUN, SPI1, TGFB1, NOTCH1, CDKN2A, STAT1, SRC, LEF1, GATA3, FOS, IGF1, FGF2, ESR1, FOXO1, CCNA2, COL1A1, CCNE1, CDH1, MYC, PPARG, TP53
GO:0008284	positive regulation of cell proliferation	21	8.8E-11	ITGB1, RPS9, TGFB1, NOTCH1, LEF1, PTEN, FN1, TTK, IGF1, FGF2, EGFR, PBX1, CDC20, EPCAM, PRC1, MYC, LEP, BIRC5, STAMBP, HRAS, ATF3
GO:0008283	cell proliferation	19	1.1E-9	TCF7L2, PCNA, SRC, PTEN, BUB1B, MCM10, IGF1, EGFR, TPX2, CENPF, MELK, KRT16, MYC, CDK1, KIF2C, RAC1, FAM83D, HRAS, TP53
GO:0051726	regulation of cell cycle	12	2.5E-9	ITGB1, HSPA8, CCNB2, CENPF, CCNB1, JUN, CCNE1, SRC, LEP, PTEN, MYBL2, FGF2
GO:0000082	G1/S transition of mitotic cell cycle	11	3.7E-9	ITGB1, RRM2, PCNA, CCND1, CCNE1, CDKN2A, CDCA5, CDK1, MCM10, MCM6, CDKN3

els across BRIC patients of different clinicopathological variables, we utilized four different reliable platforms, including GEPIA, bc-GenExMiner, and UALCAN. Taking together the results

Table 3. Details of the KEGG analysis

ID	Pathway	Genes involved	*P*-value	Genes
05200	Pathways in cancer	30	4.9E-15	HRAS, STAT5A, PPARG, SPI1, FOXO1, CDH1, ITGB1, TCF7L2, PTEN, MMP2, TGFB1, CCNE1, FOS, CDKN2A, CXCR4, RAC1, FGF2, MYC, FN1, EGFR, HSP90AA1, TP53, LEF1, IGF1, BIRC5, STAT1, CCND1, JUN, PDGFRB, ITGA2B
04110	Cell cycle	16	1.5E-11	CDK1, TP53, TTK, CDC20, PTTG1, TGFB1, MCM6, CCNB1, CCNE1, CCND1, CDKN2A, CCNB2, PCNA, BUB1B, MYC, CCNA2
04510	Focal adhesion	19	2.8E-10	ACTB, EGFR, HRAS, CAV1, IGF1, ITGB3, PTEN, ITGB1, SRC, COL5A1, CCND1, FYN, JUN, RAC1, COL1A2, PDGFRB, COL1A1, FN1, ITGA2B
05205	Proteoglycans in cancer	18	1.8E-10	ACTB, EGFR, HRAS, CAV1, TP53, ESR1, IGF1, ITGB3, ITGB1, MMP2, SRC, TGFB1, CCND1, MAPK14, RAC1, FGF2, MYC, FN1
05166	HTLV-I infection	19	5.0E-9	EGR1, HRAS, STAT5A, TP53, SPI1, CDC20, PTTG1, TGFB1, VCAM1, FOS, CCND1, ATF3, CDKN2A, JUN, PCNA, BUB1B, PDGFRB, MYC, TP53INP1
05161	Hepatitis B	15	1.6E-9	HRAS, STAT5A, TP53, BIRC5, STAT1, PTEN, SRC, TGFB1, CCNE1, FOS, CCND1, JUN, PCNA, MYC, CCNA2
04151	PI3K-Akt signaling pathway	21	2.0E-8	PHLPP1, EGFR, HRAS, HSP90AA1, TP53, IGF1, ITGB3, PTEN, ITGB1, COL5A1, CCNE1, CCND1, PPP2CA, RAC1, COL1A2, PDGFRB, COL1A1, FGF2, MYC, FN1, ITGA2B
04115	p53 signaling pathway	11	6.0E-8	CCNB1, CCNE1, CDK1, CCND1, CDKN2A, CCNB2, RRM2, TP53, IGF1, IGFBP3, PTEN
05215	Prostate cancer	12	8.9E-8	EGFR, CCNE1, HRAS, CCND1, HSP90AA1, TP53, PDGFRB, FOXO1, LEF1, IGF1, PTEN, TCF7L2
05219	Bladder cancer	9	2.4E-7	EGFR, HRAS, CCND1, CDKN2A, TP53, CDH1, MYC, MMP2, SRC

of expression analysis and validation, we confirmed the significant up-regulation of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 genes at both mRNA and protein levels in BRIC patients with different cancer stages, races, genders, age groups, and menopause status relative to controls (**Figures 3**-**5**).

Promoter methylation level

Promoter methylation participates in the expression regulation and is closely linked with cancer development and progression [24]. We analyzed the promoter methylation level of the real hub genes in BRIC patients relative to controls via the UALCAN platform. Our results revealed that CEP55, RRM2, and PRC1 were significantly hypomethylated, while KIF2C, KIF20A, and AURKA were significantly hypermethylated in BRIC patients relative to controls (**Figure 6**).

Genetic changes in real hub genes

Information related to genetic alterations and mutational hotspots in the six real hub genes was obtained from three different TCGA BRIC datasets, Breast Invasive Carcinoma (TCGA, firehose legacy) = 1108 samples, Breast Invasive Carcinoma (TCGA, Nature 2012) = 825 samples, and Breast Invasive Carcinoma (TCGA PanCancer Atlas) = 1984 samples, available via the cBioPortal platform. We have observed a varying degree of genetic variation in the real hub genes, out of which AURKA has shown the highest incidence rate (6%) of genetic variations, followed by PRC1, which has shown the second highest genetic variation rate of 2.5%. While other real hub genes, including RRM2, KIF2C, KIF20A, and CEP55, have shown the genetic variation rates of 1.5%, 1.1%, 0.7%, 0.6% in BRIC samples, respectively. In all the real hub genes, the most frequently observed genetic alteration was deep amplification (**Figure 7A**). Additionally, we have also observed that mutations in the CEP55 gene, including the most commonly reported Q446Pfs*6 mutation, lie outside the EABR domain (**Figure 6B**). Similarly, in AURKA, the mutations were also found outside of its most important Pkinase domain (**Figure 6B**). However, on the other side of the coin, the most important domains, including Kinesen of KI2C and KIF-20, and Ribonuc-red-sm and MAP65-ASE1 of AURKA and PRC1, are the major hotspots of the reported mutations (**Figure 7B**).

Survival analysis

Correlations between OS, RFS, and mRNA expression of real hub genes across BRIC

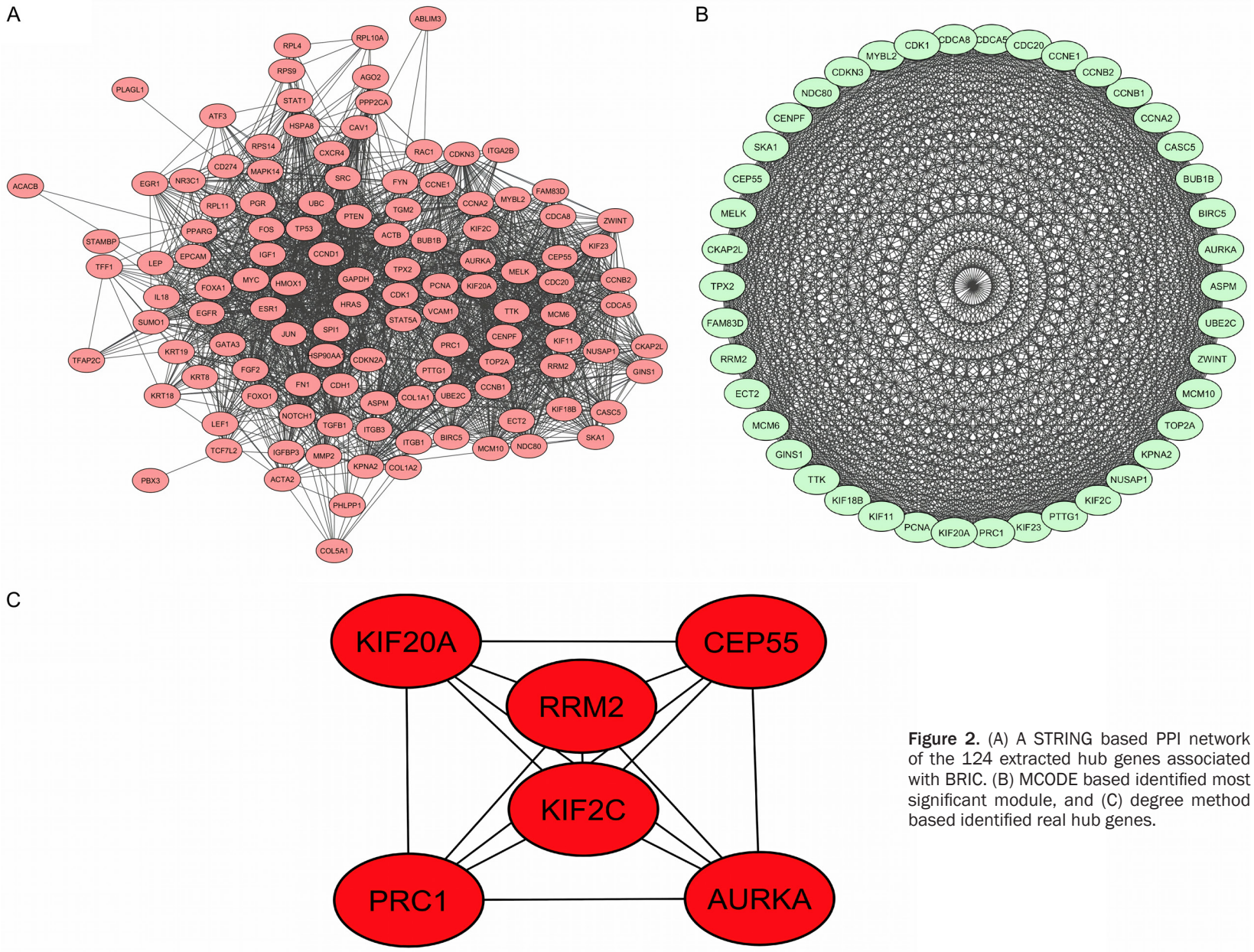


Figure 2. (A) A STRING based PPI network of the 124 extracted hub genes associated with BRIC. (B) MCODE based identified most significant module, and (C) degree method based identified real hub genes.

Table 4. List of real hub genes

Sr. No	Gene	Degree Score	Node count	Centrality score
1	CEP55	41	41	1
2	KIF2C	41	41	1
3	KIF20A	41	41	1
4	RRM2	41	41	1
5	AURKA	41	41	1
6	PRC1	41	41	1

patients were explored via KM plotter. We observed that the higher mRNA expressions of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 were significantly ($P<0.05$) linked with the reduced OS and RFS duration of the BRIC patients, therefore, these genes are supposed to be the good prognostic biomarkers in BRIC patients (**Figure 8**).

Tumor purity, immune cells, and gene expression

Spearman correlations among tumor purity, CD8+ T, and CD4+ T cell infiltration, and real hub gene expression across BRIC were evaluated via TIMER. Results showed notable positive correlations among CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression and tumor purity, and CD8+ T immune cell infiltration level across BRIC (**Figure 9**). Moreover, notable ($P<0.05$) negative correlations among CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression and CD4+ T immune cell infiltration level were also documented across BRIC samples.

Co-expression network

In this study, TRRUST and ENCORI were used to construct the TFS-miRNA-nRNA co-regulatory network. In the obtained network, the total numbers of TFS, miRNAs, and mRNAs were 60, 95, and 6, respectively. In addition, based on the *p*-value for TFS and degree of centrality for miRNAs, we have identified one TF (E2F1) and one miRNA (hsa-mir-16-5p) that target all the 6 real hub genes. Previous studies reported that the PVT1-miR-16-5p/VEGFA/VEGFR1/AKT TFS-miRNA-hub genes axis, and miR-216-5p-Cx43, and miR-16-1-3p/PGK1 miRNA-hub gene axis are the critical modulators of colorectal and BRIC [25]. However, the identified TFS-miRNAs-mRNA co-regulatory network in the current study has highlighted that the E2F1-has-miR-16-5p/CEP55/KIF2C/KIF20A/RRM2/AURKA/PRC1 axis can also be the potential inducer of the BRIC. To further confirm the participation of identified TF and miRNA in BRIC development via up-regulating real hub genes, we further checked the expression of E2F1 and has-mir-16-5p in BRIC patients via UALCAN. In view of our results, a significant up-regulation of E2F1 and hsa-mir-16-5p was also observed in BRIC samples relative to controls. Finally, we suggested that up-regulated E2F1 and has-miR-16-5p may also exert BRIC-inducing effects by overexpressing their target genes, i.e., CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 (**Figure 10**).

Real hub genes-associated mutant genes

To identify crucial mutant genes associated with real hub genes, the MuTarget analysis was conducted to recognize mutant genes correlated with real hub gene expression. We selected the top 3 mutant genes for each real hub gene. As shown in **Figure 11**, the top 3 mutant genes that positively correlate with the expression of each real hub gene are TP53, PIK3CA, and RELN with CEP55, CYFIP1, ZMYM3, and CCDC66 with KIF2C, TP53, DYNC2H1, and FAT3 with KIF20A, TP53, BIRC, and DYNC2H1 with RRM2, TP53, ITSN2, and CFAP44 with AURKA, and TP53, DYNC2H1, and SPTA1 with PRC1.

CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene-associated drugs

To identify relationships among CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 and different therapeutic drugs, a gene-drug interaction network was created with the help of CTD and Cytoscape. The expression of identified real hub gene including CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 can potentially be regulated by a variety of drugs. For example, Allyl sulfide and Arsenic trioxide can elevate the expression level of CEP55 while imetidine and bisphenol A can reduce the KIF2C expression level (**Figure 12**).

Experimental in vitro validation of the hub gene expression and methylation status

In this work, by performing RNA-seq analysis of one BRIC cell line (BT 20), as well as one normal mammary gland cell line (HMEC), the expres-

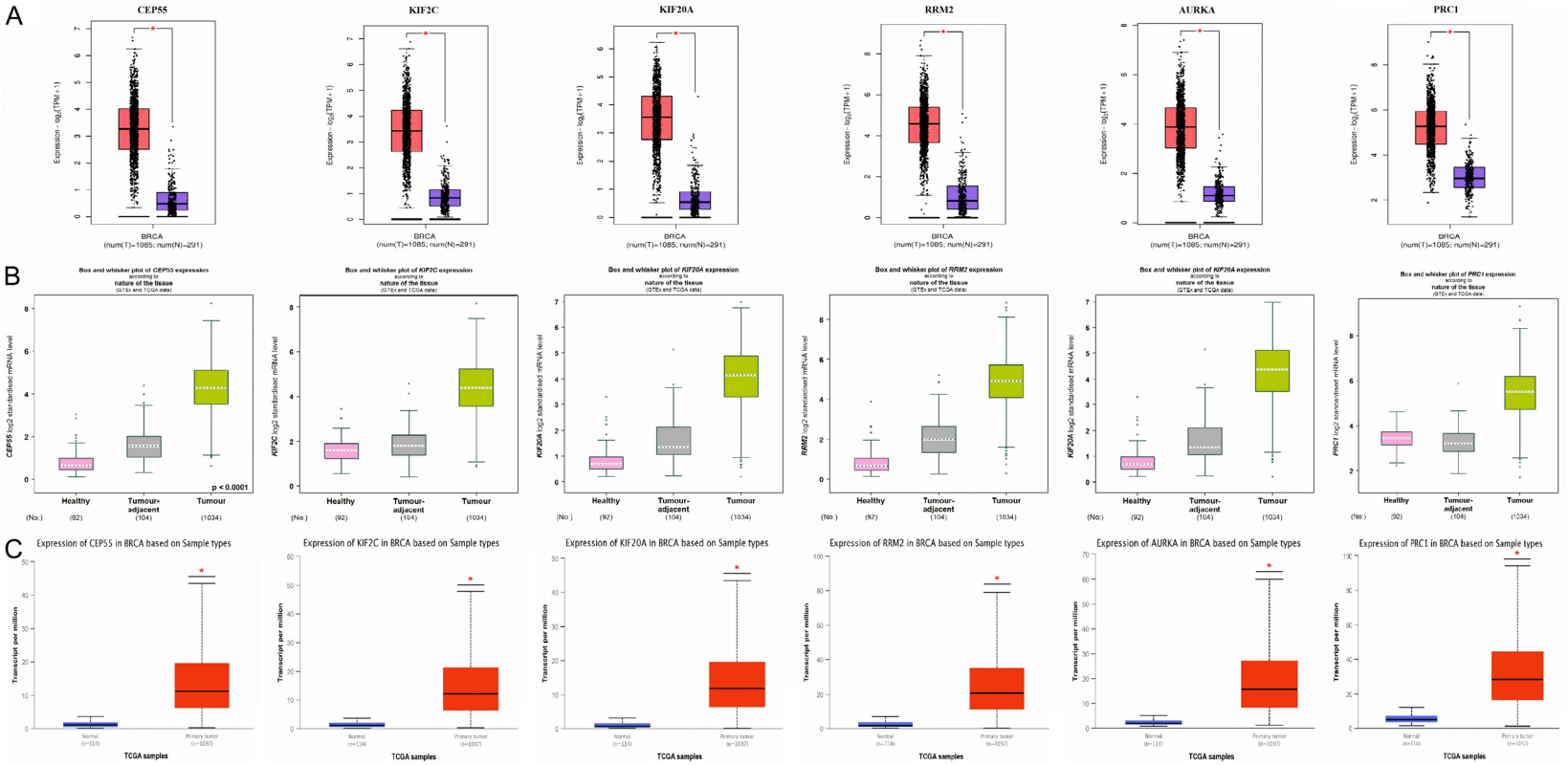


Figure 3. Transcription expression of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 in control and BRIC samples via GEPIA, bc-GenExMiner, and UALCAN databases. (A) Via GEPIA, (B) via bc-GenExMiner, and (C) via UALCAN.

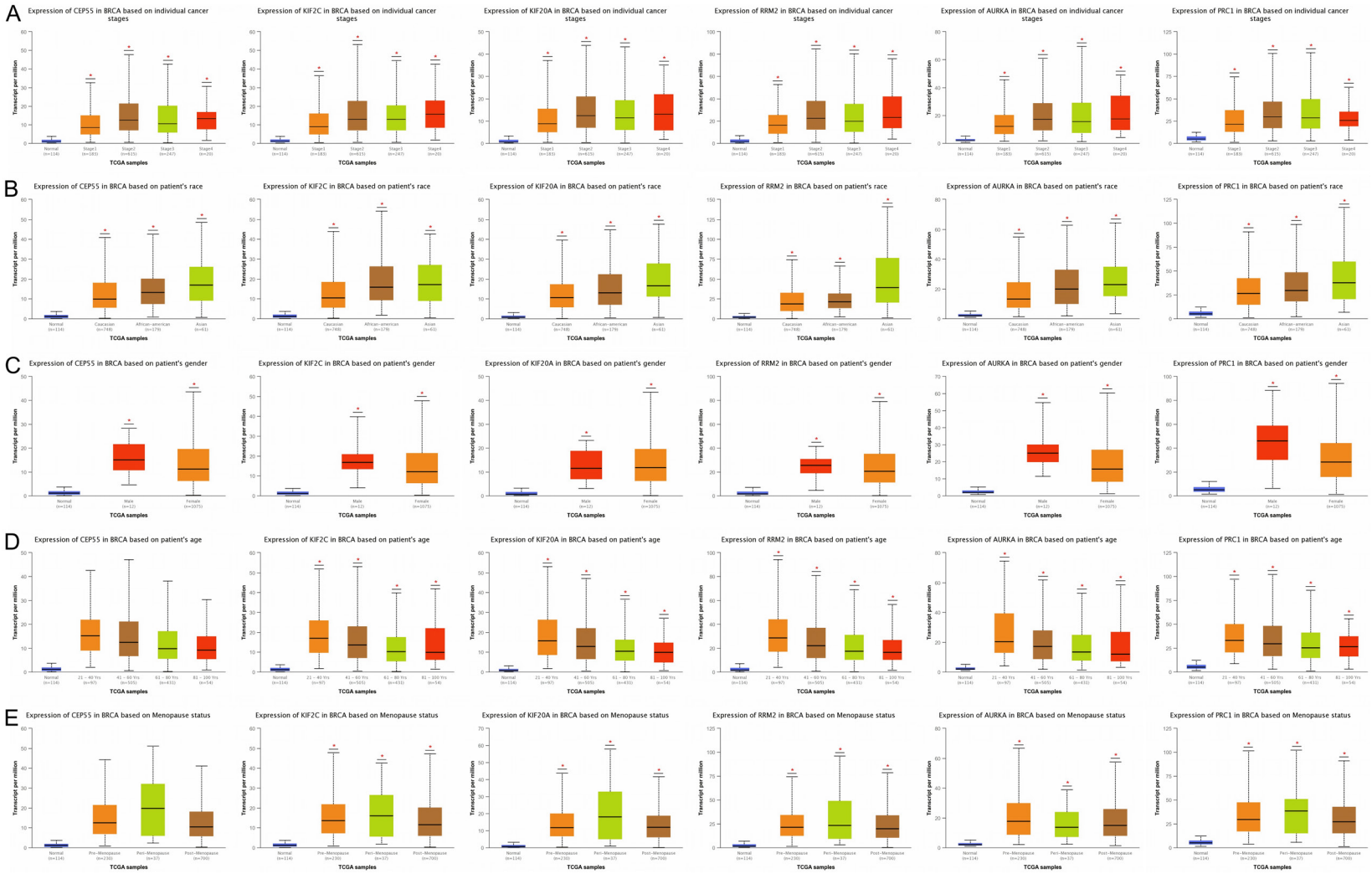


Figure 4. Transcription expression of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 across BRIC patients of different clinicopathological features. (A) Expression across different cancer stages, (B) Expression across different races, (C) Expression across different genders, (D) Expression across different age groups and, (E) Expression across different menopause statuses.

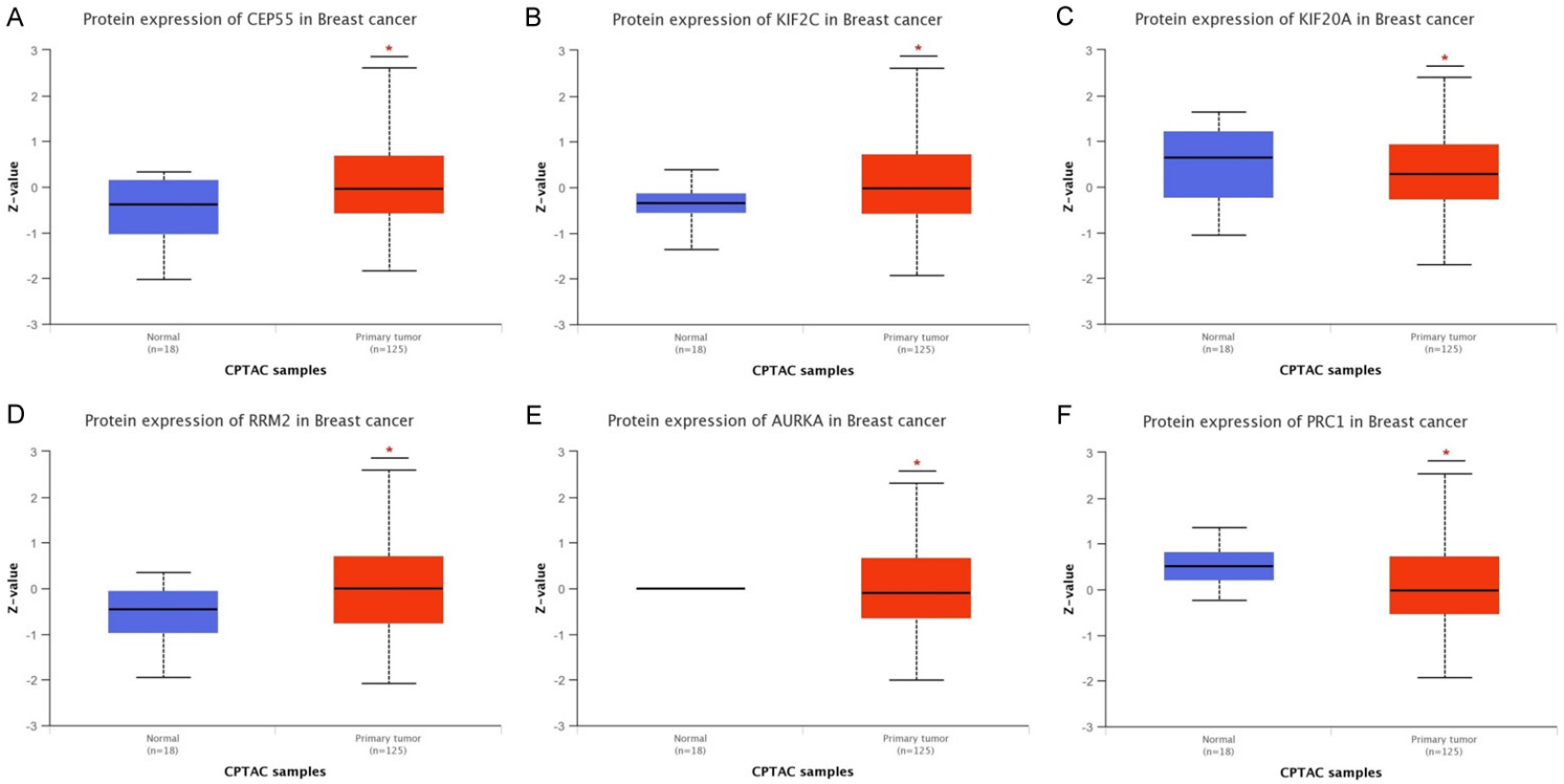


Figure 5. Translation expression of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 in BRIC patients relative to controls. (A) CEP55, (B) KIF2C, (C) KIF20A, (D) RRM2, (E) AURKA, and (F) PRC1.

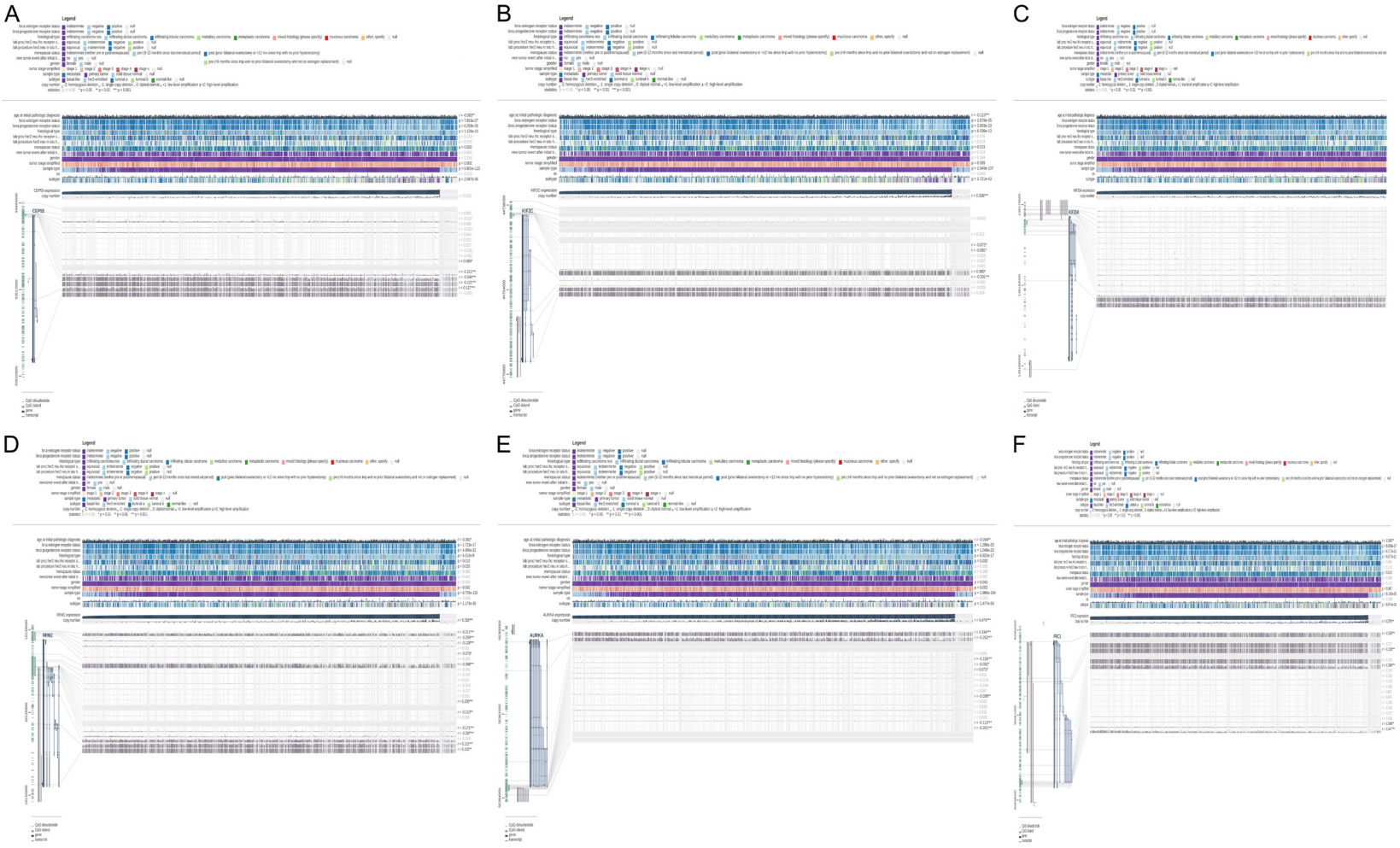


Figure 6. CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 promoter methylation level in control and BRIC patients. (A) CEP55, (B) KIF2C, (C) KIF20A, (D) RRM2, (E) AURKA, and (F) PRC1.

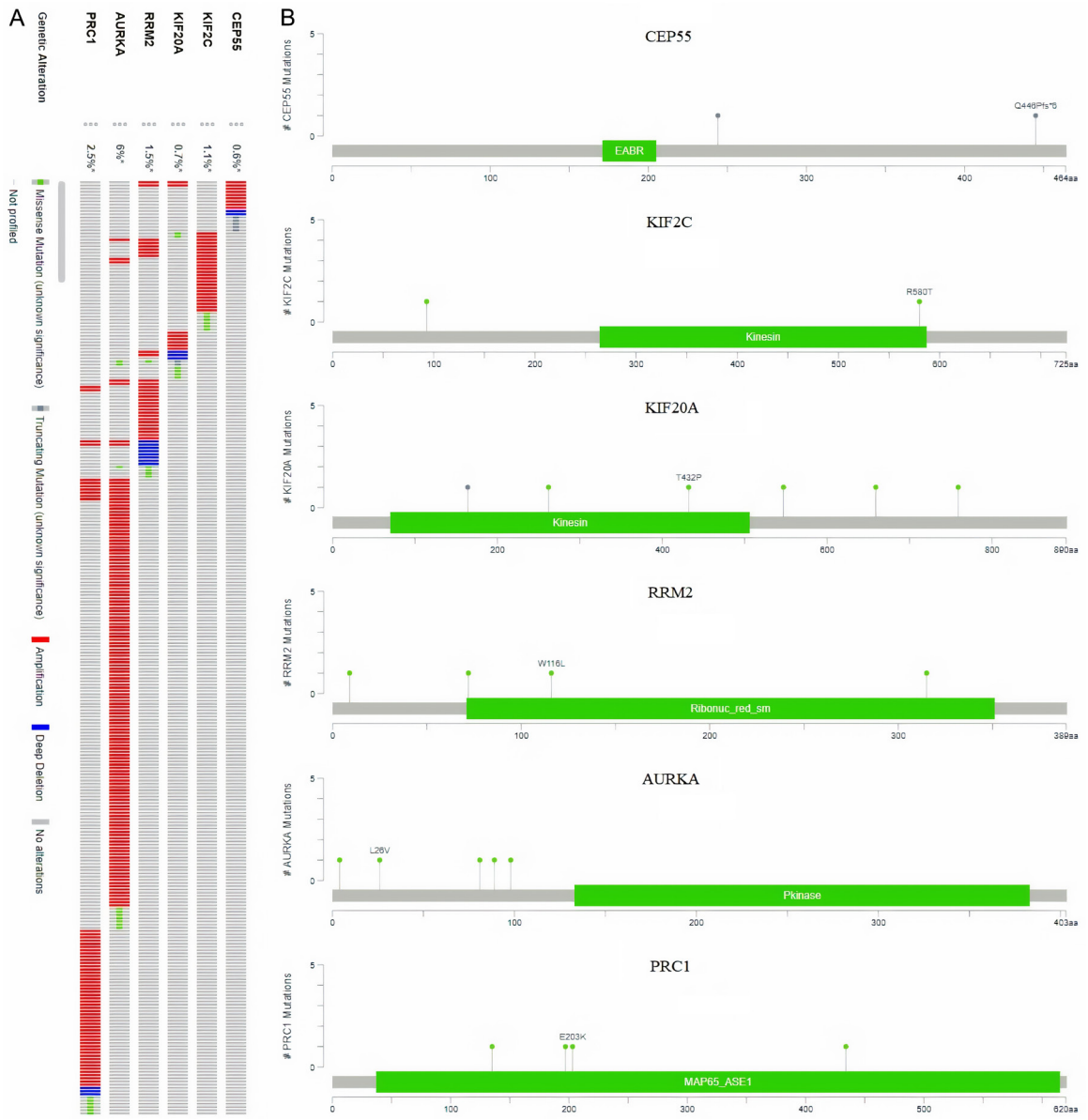


Figure 7. Frequencies of the genetic alterations and mutational hotspots identification CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 across BRIC samples. (A) A view of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene-associated genetic alterations frequencies in BRIC samples, and (B) A view of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene-associated mutational hotspots in BRIC samples.

sion levels of the hub gene were validated. The expression levels of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 genes were validated using FPKM values. The FPKM is a quantitative value with widespread use in RNA-seq analysis. As shown in **Figure 13**, it was noticed that hub genes express in both normal and BRIC cell lines, and FPKM values of the hub genes were notably higher in BRIC cell line (BT 20) as compared to a normal cell line (HMEC) (**Figure 13**).

Discussion

This study was launched to discover the BRIC biomarkers that could be employed over the heterogeneity barrier. To do so, initially we extracted 124 hub genes from the literature. Later, the creation of a PPI network of the hub genes and a significant module identification from this network have helped us to obtain six real hub genes, including CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1.

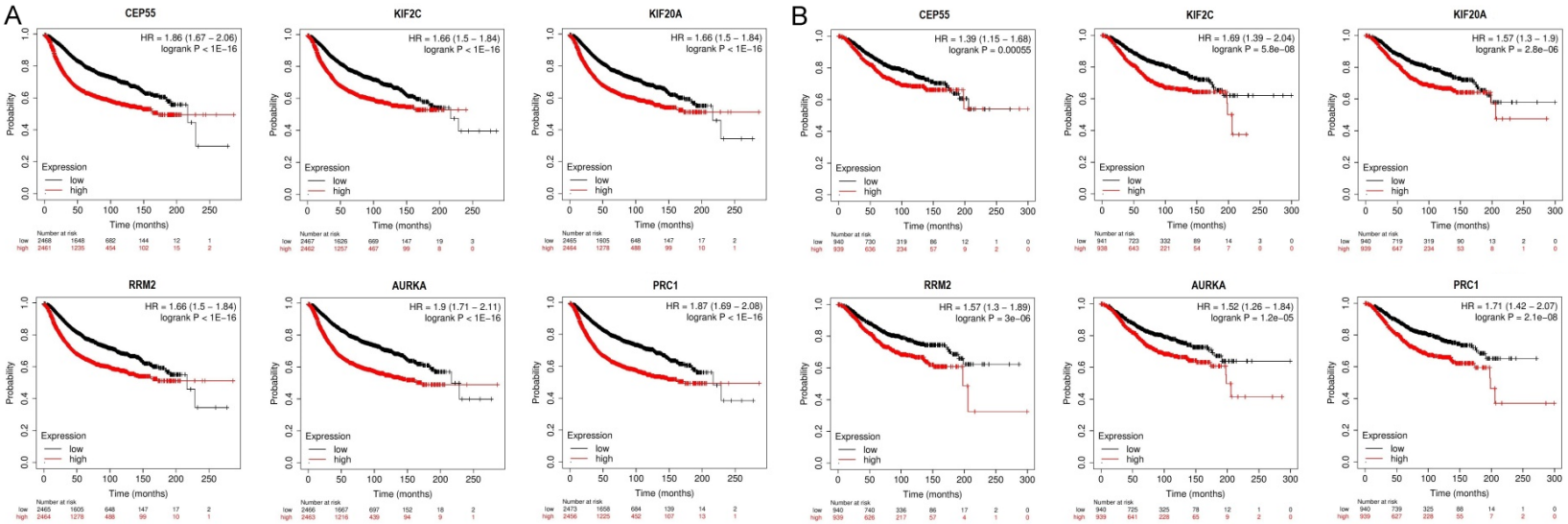
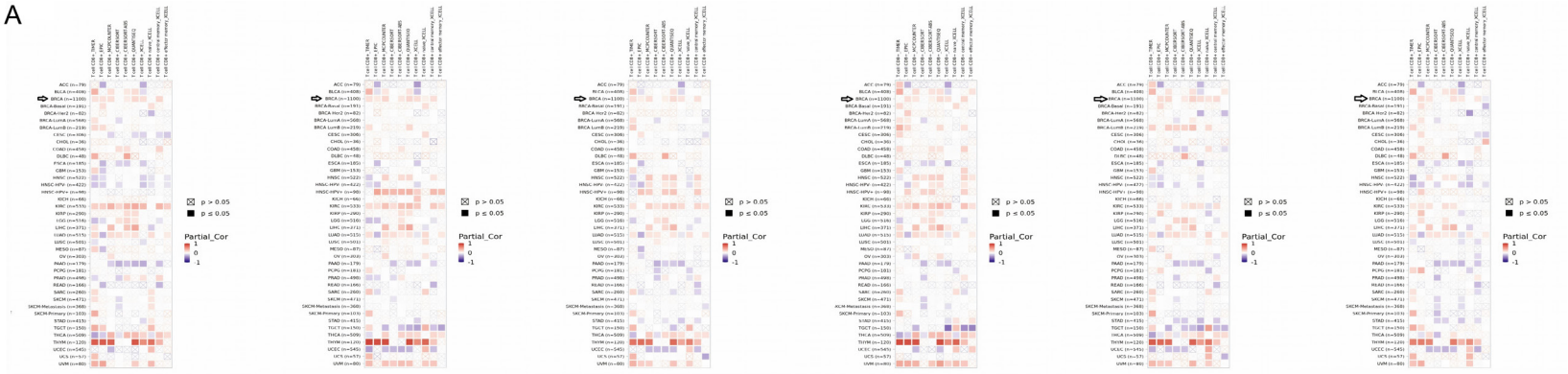


Figure 8. The prognostic information of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 in BRIC patients via KM plotter. (A) The calculated RFS values of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1, and (B) The calculated OS values of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1. Blue color indicates this low expression while red color indicates the high expression of a gene.



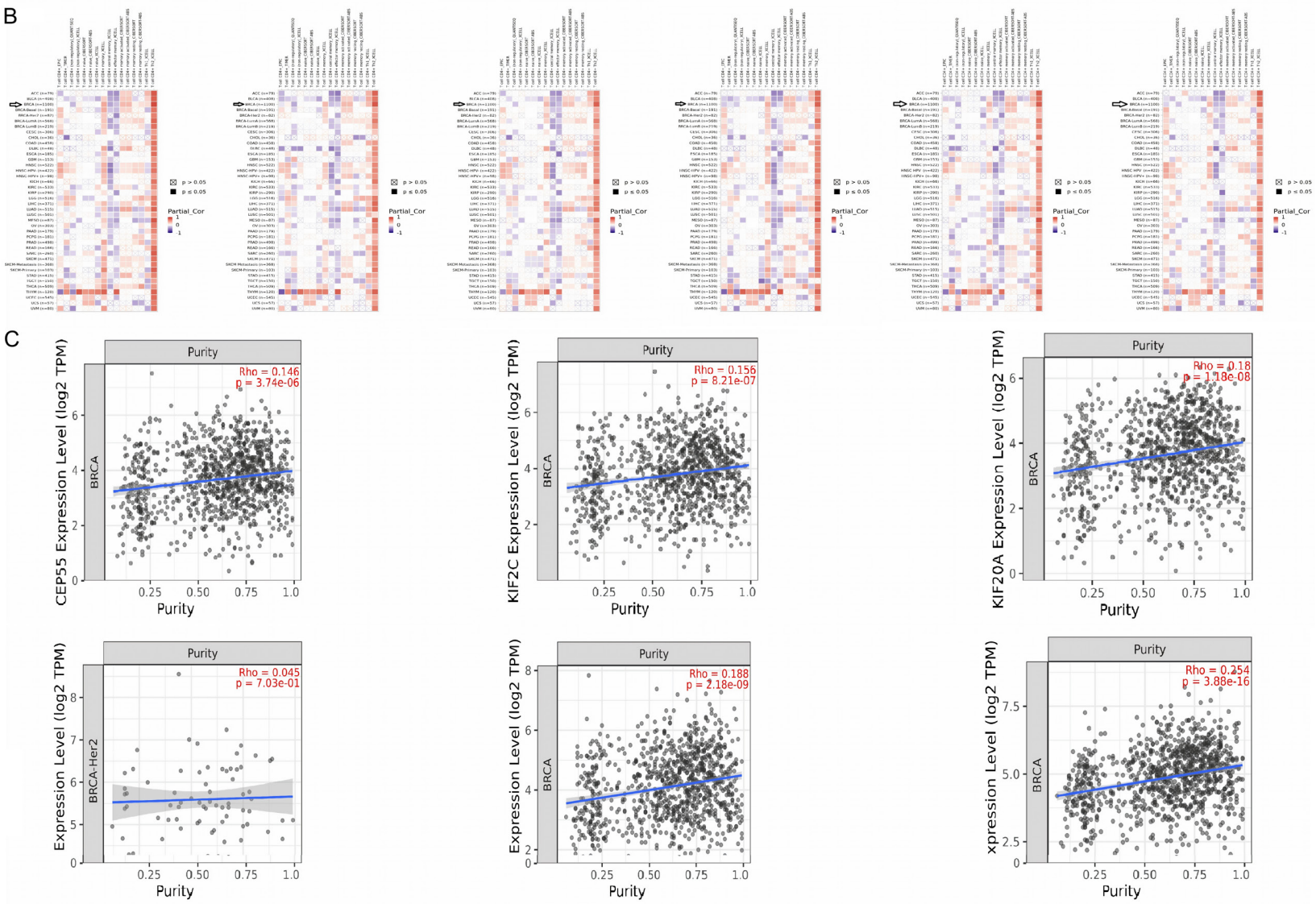


Figure 9. TIMER based Spearman correlational analysis between tumor purity, CD8+ T immune cells infiltration, CD4+ T immune cells infiltration, and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression across BRIC samples. (A) Between CD8+ T immune cells infiltration and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1gene expression, (B) Between CD4+ T immune cells infiltration and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression, and (C) Between tumor purity and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression.

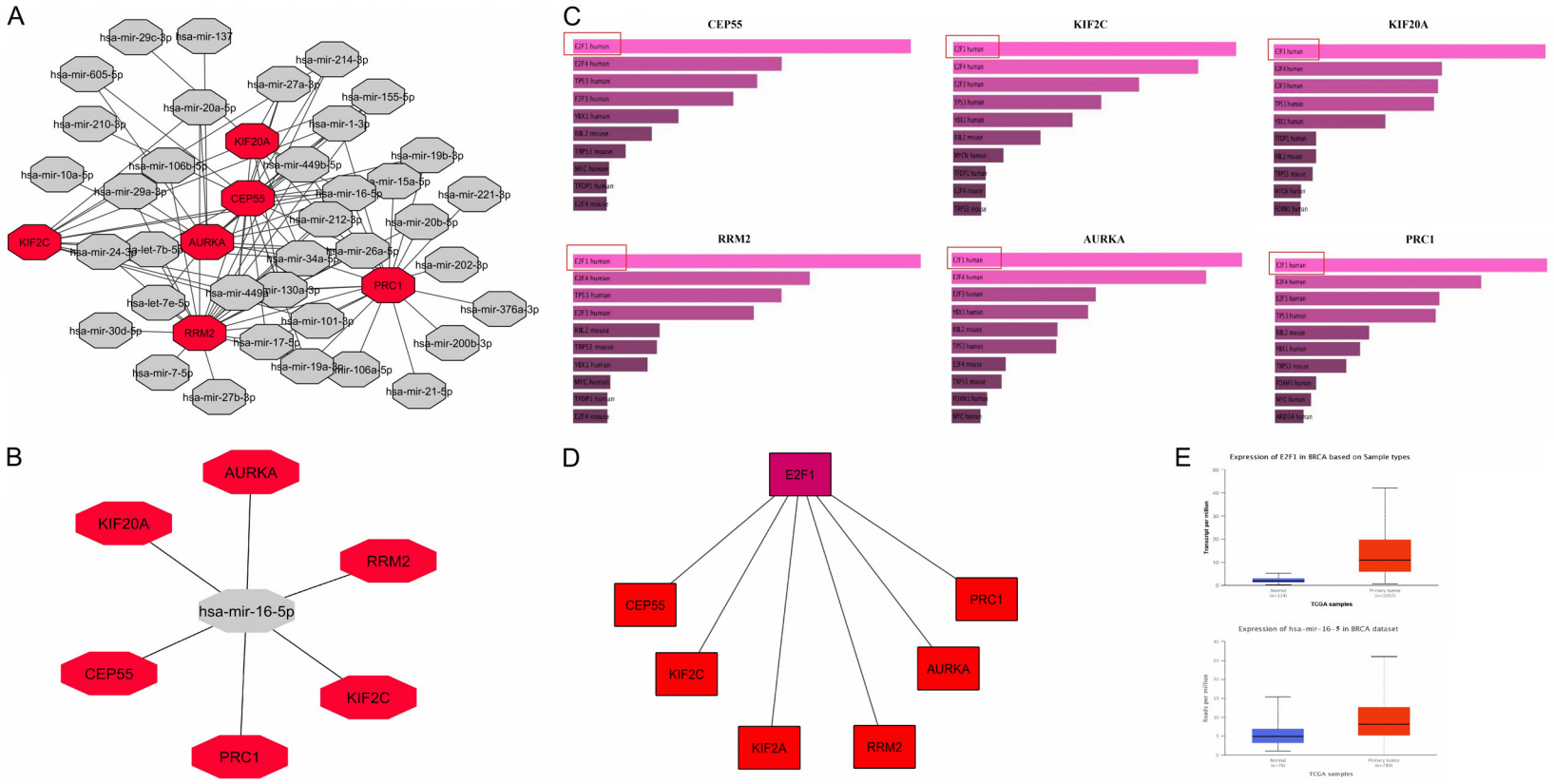


Figure 10. Identification of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 targeted potential TFS, miRNAs, and their expression analysis in BRIC. (A) A network of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 targeted miRNAs, (B) A network of has-mir-16-5p miRNA and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1, (C) CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene targeted TFS, (D) A network of E2F1 and CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1, (E) expression analysis of the E2F1 and hsa-mir-16-5p in BRIC samples paired with controls. The red nodes represent the real hub gene, grey nodes represent the miRNAs, while purple node represent the TFS.

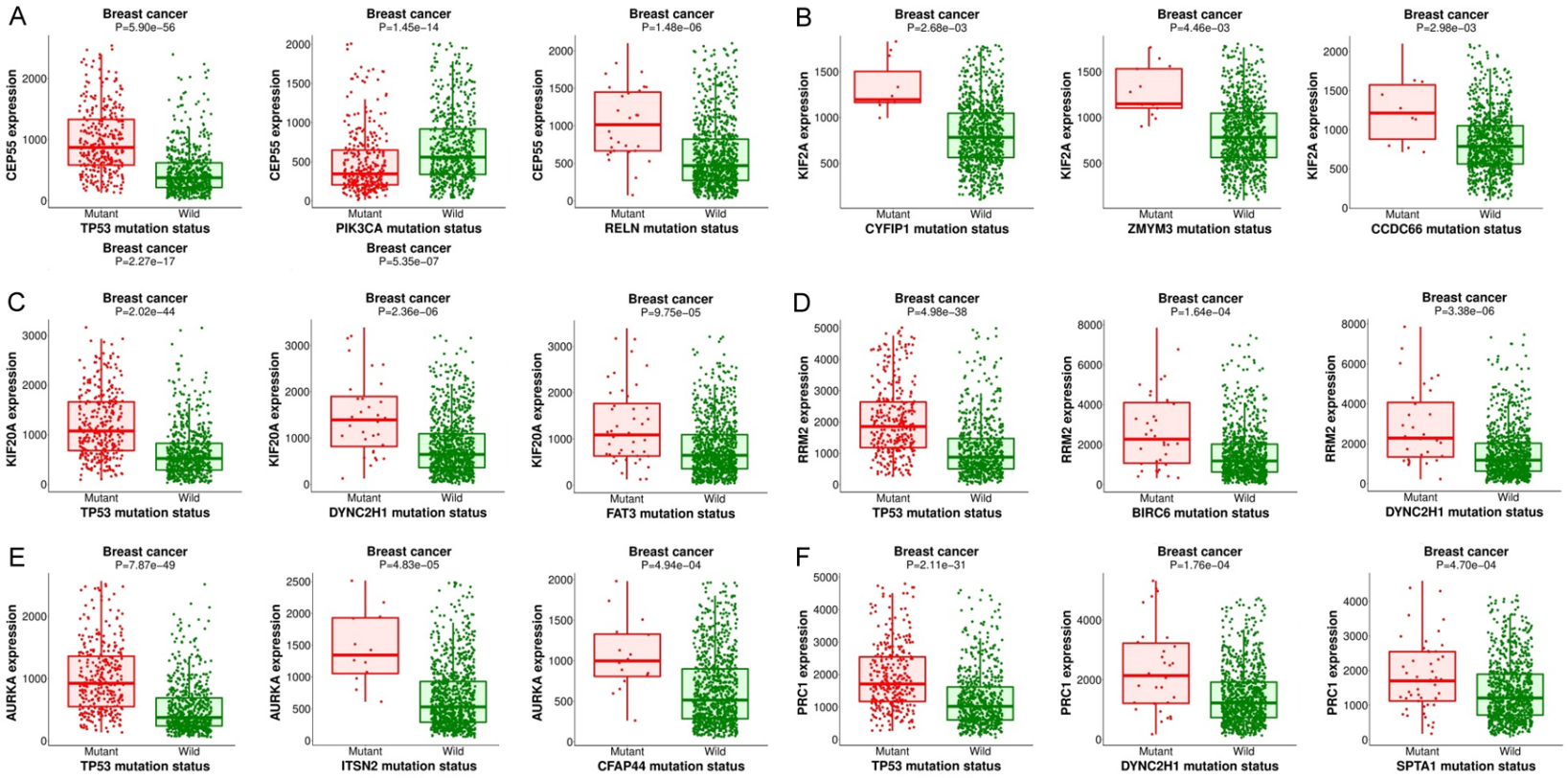


Figure 11. CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 positively correlated mutant genes in BRIC from MuTarget. (A) Top 3 correlated genes with CEP55, (B) Top 3 correlated genes with KIF2C, (C) Top 3 correlated genes with KIF20A, (D) Top 3 correlated genes with RRM2, (E) Top 3 correlated genes with AURKA, and (F) Top 3 correlated genes with PRC1.

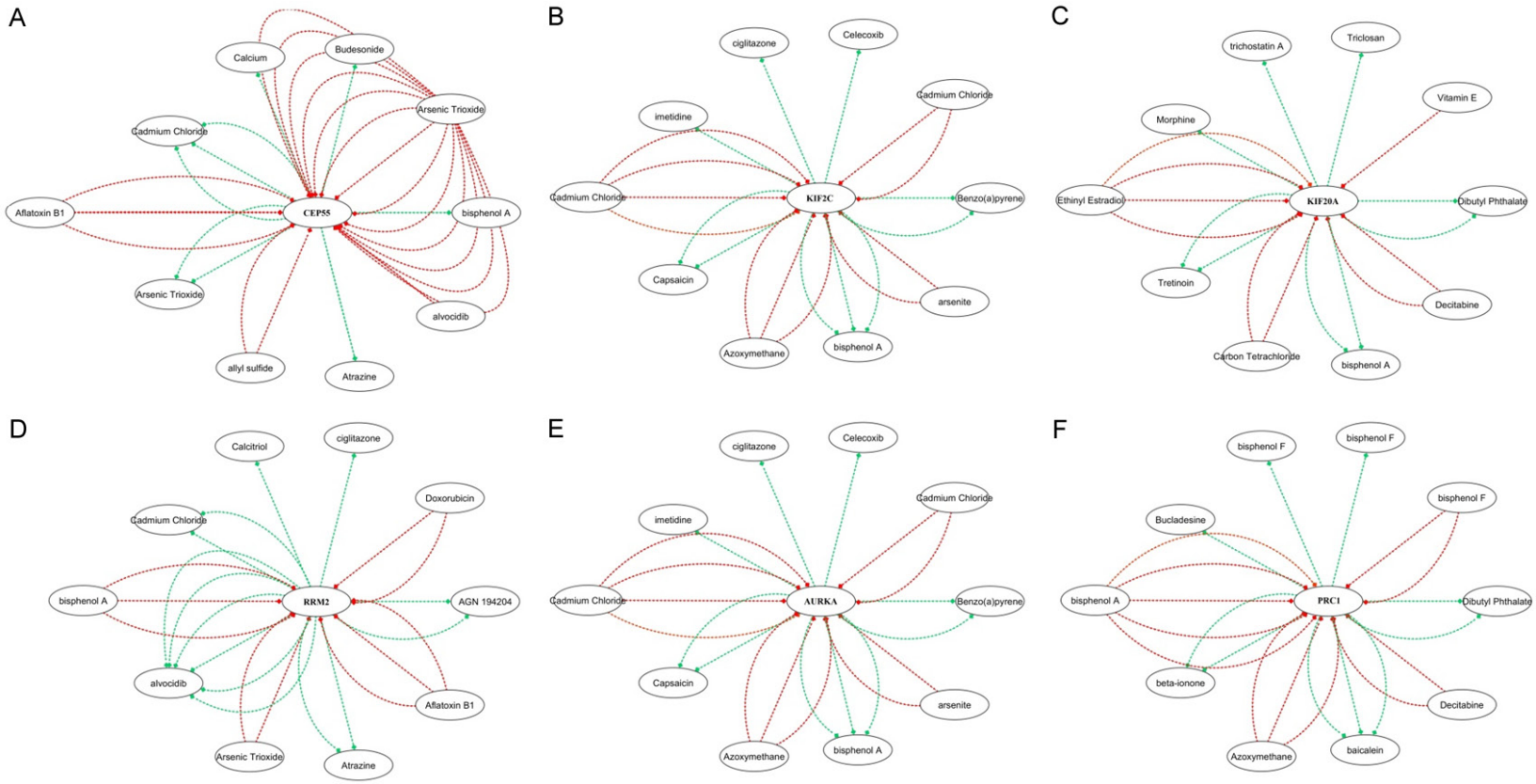


Figure 12. Gene-drug interaction network of the CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1. (A) CEP55, (B) KIF2C, (C) KIF20A, (D) RRM2, (E) AURKA, and (F) PRC1. Red arrows: drugs that increase the real hub genes expression, Green arrows: drug that decrease the real hub genes expression while the numbers of arrows represent the supported numbers of studies by literature.

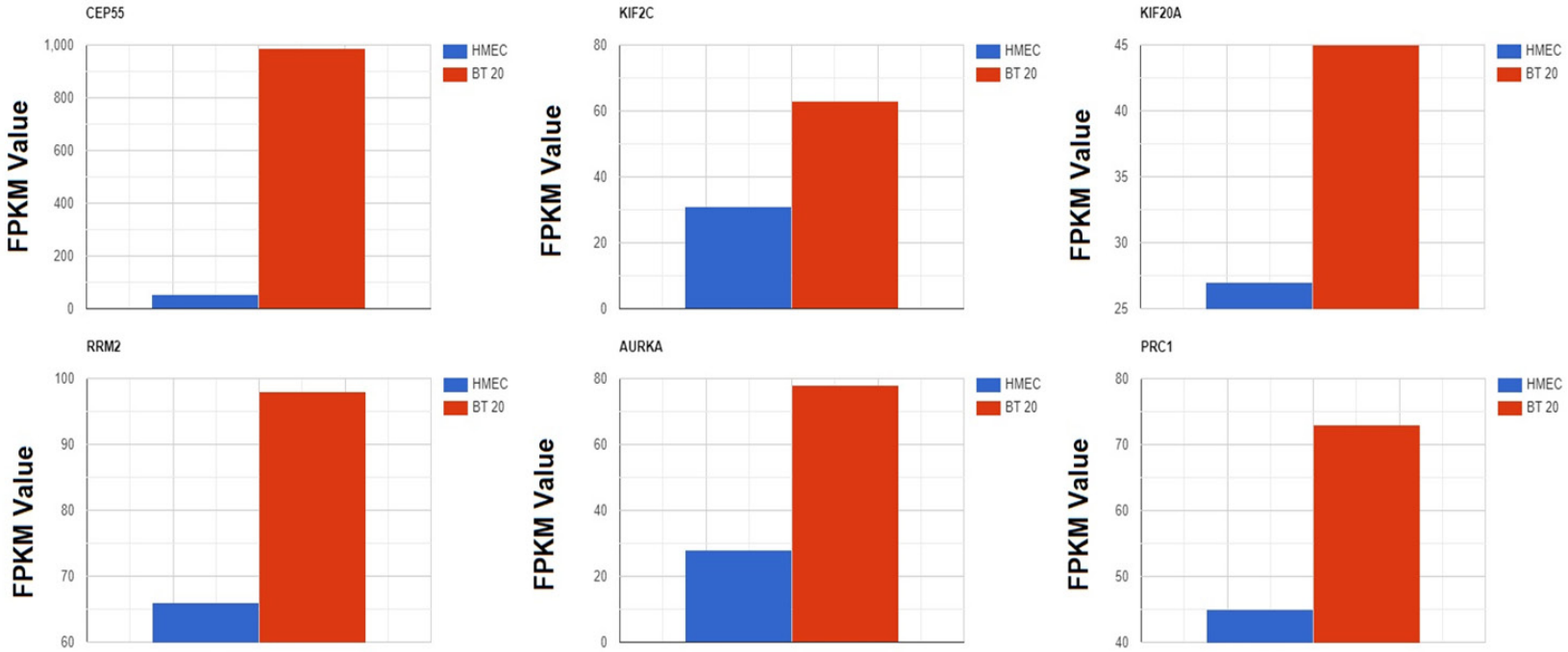


Figure 13. Validating CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 gene expression using BT 20 and HMEC cell lines via RNA-seq analysis.

The CEP55 gene encodes a 55 kDa protein consisting of 464 amino acids, which is initially described as a midbody-related protein. CEP55 is a key regulator of physical cytokinesis [26]. The overexpression of CEP55 was earlier linked to the pathogenesis and poor prognosis of lung [27], oral [28], cervical [29], breast cancers [28], and osteosarcoma [30]. In addition, CEP55 knockdown was also found to inhibit tumor cell proliferation [31]. In sum, the CEP55 up-regulation can result in disordered cytokinesis and lead to the enhancement in multinucleated cells, which is a key phenomenon of tumorigenesis [32].

The KIF2C belongs to the kinesin-13 family, which plays key roles in cell cycle regulation and progression [33]. Thus, KIF2C is supposed to be involved in tumorigenesis. However, the KIF2C role in tumorigenesis has not been explored deeply so far. Previously, *Nakamura et al.* have shown that KIF2C overexpression is linked to nodal metastasis and poor prognosis in gastric cancer [34]. *Wei et al.* have revealed the significant up-regulation of KIF2C in hepatocellular carcinoma promoting cell proliferation, cell migration, cell invasion, and metastasis [35]. Moreover, a study by *Abdel-Fatah et al.* has also reported the overexpression of KIF2C across BRIC patients and associated this overexpression with unfavorable clinicopathological variables [36].

KIF20A is another important member of the kinesin-13 family and is involved in chromosome segregation and mitosis. Previous studies have implicated KIF20A overexpression in different human cancers including pancreatic cancer [37], bladder cancer [38], gastric cancer [39], head and neck cancer [40], lung cancer [41], melanoma [42], and breast cancer [43]. However, to date, only a few studies have explored the KIF20A role in breast cancer. Moreover, a decreased level of KIF20A has also been documented in pancreatic ductal adenocarcinoma [44].

Ribonucleotide reductase (RR) is a key enzyme, mainly involved in DNA replication and repair processes [45]. RRM2 is an important subunit of RR, and it has recently gained much attention in cancer research because of its significant dysregulation in different human cancers, including BRIC [46]. It was earlier reported that cancer patients with overexpressed RRM2 suffer from poor prognoses and tumor recurrence in different cancers such as BRIC, lung cancer, and cancers of the colorectum and crevices [47]. Furthermore, it is also observed that RRM2 overexpression enhances BRIC cell proliferation and inhibits apoptosis [48]. However, the mechanisms behind the involvement of RRM2 in BRIC development and progression are not completely understood.

AURKA is a member of the serine/threonine kinase family, which is very important for activating cell division processes through mitosis regulation [49]. Apart from these functions, when AURKA is differentially expressed, it could act as an oncogene and participate in cancer development and progression [49]. The aberrant expression of AURKA across human cancers was previously reported by various studies. For example, the overexpression of AURKA was revealed in colon, breast, and lung cancer patients [50].

The PRC1 gene, also known as MAP65, is the substrate of cyclin-dependent kinases (CDKs). PRC1 up-regulation has already been seen in different human cancers, including the cancers of the breast [51], bladder [52], and kidney [53]. Additionally, a study by *Kanehira et al.* has reported that knockdown of PRC1 using siRNA can inhibit the proliferation of breast cancer cells [52]. Recently, a study by *Chen et al.* explored that PRC1 promotes metastasis and tumorigenesis of hepatocellular carcinoma by dysregulating the Wnt signaling pathway [54]. In our study, we found that CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1 were significantly overexpressed in BRIC patients with diverse clinical parameters compared to non-cancer samples. Taken together, the expression profiling of CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1, it was spectated that overexpression of these real hub genes may serve as potential biomarkers of BRIC regardless of different clinical parameters.

Moreover, the identified real hub genes were found to be altered in a minor proportion of the BRIC patients. Additionally, it was also explored in the current study that genetic mutations can alter amino acids at different positions in the resultant proteins from the real hub genes. Furthermore, this study document-

ed significant negative correlations among real hub gene promoter methylation levels and expressions. This study also revealed that real hub gene overexpression was associated with worse prognosis of BRIC patients.

We also documented correlations among tumor purity, CD8+ T, CD4+ T immune cell infiltration levels, and the expression of real hub genes across BRIC. Results showed that real hub genes have positive correlations with the tumor purity in BRIC, which further confirmed that a higher proportion of tumor cells in BRIC is linked with the real hub gene overexpression. The CD8+ T and CD4+ T immune cells are the core constituents of immunotherapy [55]. Earlier, *Trojan et al.* in their trial study successfully used CD8+ T immune and CD4+ T immune cells infiltration levels for the immunotherapy of LSCC patients [56]. In the current study, our results showed positive correlations among real hub genes (CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1) expressions at mRNA level and CD8+ T immune cells infiltration in BRIC. In addition to this, in the current study, we also explored the significant negative correlations among real hub genes (CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1) expressions at the mRNA level and CD4+ T immune cell infiltration in BRIC. Collectively, the observed correlations shed light on the new possible aspects of the real hub genes in BRIC tumorigenesis by regulating CD8+ T and CD4+ T immune cells. To the best of our knowledge, we are the first to explore such correlations among real hub genes (CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1) and CD8+ T and CD4+ T immune cells across BRIC.

By constructing a TFS-miRNA-real hub genes co-regulatory network, it was observed that one TF (E2F1) and one miRNA (miR-16-5p) target all six real hub genes for regulating their expressions. It is noted by previous studies that PVT1-miR-16-5p/VEGFA/VEGFR1/AKT TFS-miRNA-hub genes axis, and miR-216-5p-Cx43, miR-16-1-3p/PGK1 miRNA-hub genes axis are the critical modulators of colorectal cancer and BRIC [25, 57, 58]. In view of our results, we suggested that E2F1-has-miR-16-5p/CEP-55/KIF2C/KIF20A/RRM2/AURKA/PRC1 TFS-miRNAsmRNA co-regulatory networks can also be used as novel therapeutic targets for treating BRIC.

Conclusion

This detailed, systematic study has led us to the identification of six real hub genes (CEP55, KIF2C, KIF20A, RRM2, AURKA, and PRC1), that may be utilized as a novel diagnostic and prognostic biomarkers, and therapeutic targets for the precise treatment of BRIC.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. List of the BRIC-associated hub genes extracted from previous studies

Datasets	Name of hub genes	No. hub genes	Reference
GSE10797	RPS9, RPL11, RPS14, RPL10A	4	[1-24]
GSE15852 GSE92697	EPCAM, MELK, KRT8, KRT19, KPNA2, ECT2	6	
GSE102484	TPX2, KIF2C, CDCA8, BUB1B, and CCNA2	5	
GSE65212	TOP2A, PCNA, CCNB1, CDC20, BIRC5, CCNA2	6	
GSE43837	PHLPP1, UBC, ACACB, TGFB1, ACTB	5	
GSE102484	CASC5, CKAP2L, FAM83D, KIF18B, KIF23, SKA1, GINS1, CDCA5, MCM6	9	
GSE20194 GSE23988	CCNE1, KRT16, MYBL2	3	
GSE42568	AGO2, CDC20, CDCA5, MCM10, MYBL2, TTK	6	
GSE75333	CDKN2A, MME, PBX1, IGFBP3, TFAP2C, VCAM1, KRT18, TGM2, ACTA2, STAMBP	10	
GSE5847	PDGFRβ, SUMO1, COL1A1, FYN, CAV1, COL5A1, MMP2	7	
GSE22035 GSE3744 GSE5764 GSE21422 GSE26910	TOP2A, BIRC5, CDK1, CCNB1, NDC80	5	
GSE26910 GSE10797	JUN, FOS, ATF3, STAT1, COL1A1, FN1	6	
GSE41970	TP53, GAPDH, CCND1, HRAS, PCNA	5	
GSE8977	CAPG, TP53INP1, SPI1, LEF1, PBX3, TCF7L2, PLAGL1, EGFR	8	
GSE21422 GSE42568 GSE45827	IGF1, LEP, KIF11, PTEN, FOXO1, FGF2, CCNB1, PPARG, AURKA, IK3CA, CDH1, CDK1	15	
GSE71142	NOTCH1 and MAPK14	2	
GSE86945 GSE86946 GSE102088	HSP90AA1, SRC, HSPA8, ESR1, ACTB, PPP2CA, RPL4	7	
GSE29431 GSE45827 GSE65194	CCNB1, RAC1, TOP2A, KIF20A, RRM2, ASPM, NUSAP1, BIRC5, BUB1B, CEP55	10	
GSE22093 GSE23988	PGR, ESR1, GATA3, ABLIM3	4	
GSE31192	FOS, MYC, ACTA2, IL18, CD274	5	
GSE9014	ITGB1, ITGB3, ITGA2B, CXCR4	4	
GSE10780	FOS, COL1A2, EGR1, HMOX1, GATA3, CDK1, NR3C1, PPARG, STAT5A, TFF1, FOXA1	11	
GSE21422 GSE29431 GSE42568 GSE61304	CDK1, CCNA2, TOP2A, CCNB1, KIF11, MELK	6	
GSE10810	AURKA, BIRC5, BUB1B, CCNB1, CCNB2, CDC20, CDK1, CDKN3, CENPF, PRC1, PTTG1, TOP2A, TPX2, UBE2C, ZWINT	15	

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