A Pan-Cancer Analysis of Transcription Factor Forkhead Box O-3: FOXO3

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Primary research

Keywords: FOXO3, FKHRL1, FOXO3a, SIRT1, CREBBP, FKBP5, RERE, SMAD4.

Posted Date: September 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-903061/v1

Abstract

Background: Although available cell - or animal- based evidence supports the relationship between transcription factor FOXO3 and cancers, so far, there has been no pan-cancer analysis of FOXO3.

Methods: We thus first carry on a pan-cancer analysis of FOXO3 across multiple tumors via several online websites based on the datasets of TCGA, including statistical correlations of FOXO3 expression with clinical prognosis, protein phosphorylation, cancer-associated fibroblasts infiltration, tumor mutational burden, and relevant cellular pathway.

Results: FOXO3 was usually as an anti-tumor gene in KIRC, BLCA, CESC, UVM, LUAD and BRCA cancers, while a higher level of FOXO3 may have a certain cancer-promoting effect. As FOXO3 usually played a cancer-promoting role in $ERα^-$ BRCA but in $ERα^+$ BRCA played an anti-tumor role, which may be due to transcription factor ERα translocating FOXO3 to the cytoplasm to weaken the transcription activity of FOXO3. FOXO3 activates autophagy related genes through transcription to maintain the occurrence of basal autophagy when basal autophagy is inhibited. However, FOXO3 can also induce apoptosis through transcription of pro-apoptotic genes if autophagy inhibition persists. The expression of phosphorylated FOXO3 protein at different sites may also affect its transcriptional activity by changing its shuttle between nucleus and cytoplasm. Therefore, When we analyze the functions of FOXO3 in a specific type of cancer, we should combine with the expression of FOXO3, the alteration status of FOXO3, the overall phosphorylation status of FOXO3, the basic autophagy status, the expression levels of ERα, SIRT1, CBP, FKBP5, RERE and SMAD4, and cancer-associated fibroblasts subtype, even consider the *KRAS* mutation, *P53* mutation or non-genetic factors which can influence the functions of cancer-associated fibroblasts in the specific type of cancer.

Conclusions: Our first pan-cancer study has provided a relatively comprehensive understanding of the role of FOXO3 in different tumors, and will give some help and enlightenment to later researchers who study the role of FOXO3 in various cancers.

Background

Gene *FOXO3*, encoding the transcription factor forkhead box O 3 (FOXO3), maps to chromosomal region 6q21 [1], FOXO3, also known as FOXO3a or forehead in rhabdomyosarcoma-like 1 (FKHRL1), is an approximately 71 kDa protein, which has a highly conserved winged-helix forkhead DNA binding domain (FH) (157-237aa), two nuclear localization sites (NLS) (248-250aa, 269-271aa), a nuclear export site (NES) (366-378aa), a KIX-binding domain (433-508aa) and a C-terminal transactivation domain (TAD) (606-644aa) [2]. FH domain is responsible for direct interaction with DNA, which also interacts with ERα to inhibit breast cancer cell proliferation and tumorigenesis [3] and also interacts with P53 to induce apoptosis [4]. NLS domain is necessary for the translocation of FOXO3 from cytoplasm to nucleus, which also mediates the release of FOXO3 from nucleus [5]. TAD domain is required for transactivation of FOXO3 target genes, which also interacts with ERβ [3] and P53 [4].

FOXO3 mediated biological processes are crucial to the health of the whole life, including substrate metabolism, protein turnover, cell survival and cell death. Many regulatory processes, such as phosphorylation, acetylation, ubiquitination, methylation and microRNA (miRNA) binding, can regulate the transcriptional activity of FOXO3. Of particular interest for human health, the subcellular localization of FOXO3 is so important for its activities and functions [6]. Phosphorylation of FOXO3 causes it translocate from the nucleus to the cytoplasm, where it binds to 14–3–3 protein, which prevents it from reentering the nucleus. However, when FOXO3 is in the nucleus, which is mainly involved in the regulation of gene transcription activation [7, 8].

FOXO3 can be used as a tumor suppressor gene to activate the transcription of apoptosis related genes [9], and it can also be used as a tumor promotion gene to activate the transcription of survival related genes [10–12], therefore, we are often puzzled when we analyze FOXO3's function in a specific cancer. In this paper, we first carry on a pan-cancer analysis of FOXO3 including statistical correlations of FOXO3 expression with clinical prognosis, protein phosphorylation, cancer-associated fibroblasts (CAFs) infiltration, tumor mutational burden, and relevant cellular pathway across multiple tumors based on TCGA project, which aids in understanding the role and mechanism of FOXO3 in tumorigenesis in each tumor from the perspective of clinical tumor samples.

Methods

Gene and protein expression analysis

We input FOXO3 in the "Exploration-Gene DE" module of TIMER2 (Tumor immune estimation resource, version 2) (http://timer.cistrome.org/) online search tool to analyze the difference of *FOXO3* gene expression between different tumors and their corresponding normal tissues in TCGA database. Due to the lack of normal tissue data of some tumors in TCGA database, such as cervical squamous cell carcinoma and endocervical adenocarcinoma (TCGA-CESC), skin cutaneous melanoma (TCGA-SKCM), ovarian serous cystadenocarcinoma (TCGA-OV), we input FOXO3 in the "Expression analysis-Expression DIY-Box Plot" module of GEPIA2 (Gene expression profiling interactive analysis, version 2) online tool (http://gepia2.cancer-pku.cn/#index) to obtain the differential expression data of *FOXO3* gene in tumor tissues and corresponding adjacent and normal tissues in TCGA and GTE_X databases under the settings of *p*-value cutoff=0.05, log2FC (fold change) cutoff=1 and match TCGA normal and GTE_X data.

Using the UALCAN online search tool (http://ualcan.path.uab.edu/index.html), the protein expression level of FOXO3 in CPTAC database was analyzed. Select "CPTAC analysis" module on the website of UALCAN, input *FOXO3* gene, and selected "Total-Protein" option to obtain the differential expression data of FOXO3 protein in breast invasive carcinoma (TCGA-BRCA), OV, kidney renal clear cell carcinoma (TCGA-KIRC), lung adenocarcinoma (TCGA-LUAD), colon adenocarcinoma (TCGA-COAD), uterine corpus endometrial carcinoma (TCGA-UCEC) and their corresponding normal tissues.

Correlation analysis between *FOXO3* gene expression and tumor stage

In order to analyze the correlation between *FOXO3* gene expression and tumor stage in different tumors of TCGA database, we also used GEPIA2 tool. We entered the “Expression analysis-Expression DIY-Stage Plot” module, input *FOXO3* gene, selected the target tumor, and then obtained the corresponding data.

Survival analysis

In order to study the relationship between *FOXO3* gene and prognosis, we used GEPIA2 tool again, input gene *FOXO3* in the “Expression analysis-Survival Analysis-Survival Map” module, and selected the “Overall Survival” or “Disease Free Survival” option to obtain the heatmap of correlation between *FOXO3* gene expression and overall survival or disease free survival in all kinds of tumors of TCGA database. According to “cutoff high (50%)” and “cutoff low (50%)”, the tumor cases were divided into high expression group and low expression group of gene *FOXO3*. We used the “Expression analysis-Survival Analysis-Survival Analysis” module of GEPIA2 tool and log-rank test to obtain the data of the correlation between *FOXO3* gene expression and overall survival or disease free survival in each tumor of TCGA database.

Gene alteration analysis

FOXO3 gene alteration was analyzed using the cBioPortal online tool (http://cbioportal.org). We selected the “TCGA pancancer atlas studies” option in the “query” module, input FOXO3 gene, and selected the “cancer types summary” module to get the alteration frequency, mutation type and copy number alteration (CNA) of gene *FOXO3* across all TCGA tumors. By selecting the “mutations” module, we can get the information of *FOXO3* gene mutation site types, mutation sites, tumor samples with detected mutation sites. In order to study the relationship between *FOXO3* gene mutation and clinical prognostic indicators of patients with different types of cancer, we selected the name of the tumor with *FOXO3* gene mutation on cBioPortal website, input *FOXO3* gene, and selected the “comparison/survival” module to obtain the data of the relationship between *FOXO3* gene mutation and clinical prognostic indicators of patients with different types of cancer. Clinical prognostic indicators included overall survival, disease-free survival, disease-specific survival and progression free survival.

Protein phosphorylation analysis

In order to study the phosphorylation level of FOXO3 protein in various cancers. We use the online search web UALCAN to select the "CPTAC analysis" module on the website, input FOXO3 gene, and selected the "phosphoprotein" option to obtain the differential expression data of FOXO3 proteins at different phosphorylation sites in BRCA, OV, KIRC, LUAD, COAD, UCEC and their corresponding normal tissues.

We used UniProt (https://www.uniprot.org/) online tool to find the ID of FOXO3 protein, and then input it into SMART (http://smart.embl-heidelberg.de/smart/show_motifs.pl?ID=C1 S_ Human) online search web to get the schematic diagram of FOXO3 protein domain. We finally annotated the phosphorylation sites and differential expression level of FOXO3 protein in various tumors obtained from UALCAN web on the schematic diagram of FOXO3 protein domain.

Cancer-associated fibroblasts infiltration analysis

In order to analyze the relationship between gene *FOXO3* and CAFs infiltration of tumors of TCGA database, we input *FOXO3* gene into the "Immune-Gene" module of TIMER2 website and selected the "Cancer associated fibroblasts" option to obtain the data provided by TIDE, XCELL, MCP-COUNTER and EPIC. We selected the tumor with *P* value were all less than 0.05 in the above four databases, and obtained the scatterplot with the highest Rho value in the above four databases to show the correlation between the gene *FOXO3* and CAFs infiltration.

FOXO3-related gene enrichment analysis

We use the STRING (https://string-db.org/) searcher web, input FOXO3 gene, selected "Homo sapiens" option, and set the following parameters in the "Setting" module: meaning of network edges ("evidence"); active interaction sources ("experiments"); minimum required interaction score ["low confidence (0.150)"]; max number of interactors to show ("no more than 50 interactors" in 1st shell) and network display options ("disable structure previews inside network bubbles"). Finally, 50 experimentally determined FOXO3-binding proteins were screened.

We used GEPIA2 online tool, selected "expression analysis similar gene detection" module, input FOXO3 gene, and set "Top # similar Genes" to 100, then we can get the top 100 FOXO3 expression-correlated genes based on the datasets of all TCGA tumor and normal tissues.

We used the Venn (http://bioinformatics.psb.ugent.be/webtools/Venn/) website to take the intersection of the above two datasets to obtain the common genes and used the "expression analysis correlation analysis" module of GEPIA2 to perform a pairwise gene Pearson correlation analysis of FOXO3 and each of the above common genes. The log2 TPM was applied for the dot plot. The *P*-value and the correlation coefficient (R) were indicated. We also used the "exploration-Gene_Corr" module of TIMER2 to obtain the heatmap data of the above common genes, which contains the partial correlation (cor) and *P*-value in the purity-adjusted Spearman's rank correlation test.

We also used the Metascape (http://metas cape.org) [13] website to perform KEGG and GO molecular function enrichment analysis for the genes in the above two datasets with the settings of $P \leq 0.01$, the minimum count was 3, and the enrichment factor was > 1.5.

Results

Gene and protein expression analysis data

We used TIMER2 online tool to analyze the expression of *FOXO3* gene expression in various tumors in TCGA database. *FOXO3* gene was low expressed in bladder urothelial carcinoma (TCGA-BLCA) ($P < 0.001$), BRCA ($P < 0.001$), CESC ($P < 0.05$), kidney chromophobe (TCGA-KICH) ($P < 0.001$), LUAD ($P < 0.001$), lung squamous cell carcinoma (TCGA-LUSC) ($P < 0.001$), thyroid carcinoma (TCGA-THCA) ($P < 0.001$) and UCEC ($P < 0.01$), while it was high expressed in cholangiocarcinoma (TCGA-CHOL) ($P < 0.05$),

liver hepatocellular carcinoma (TCGA-LIHC) ($P < 0.05$), pheochromocytoma and paraganglioma (TCGA-PCPG) ($P < 0.01$) and stomach adenocarcinoma (TCGA-STAD) ($P < 0.001$) (Figure 1A).

Due to the lack of some normal tissue data in TCGA database, we used GEPIA2 online tool combing with TCGA and GTE_X databases to analyze the expression of *FOXO3* gene in various tumors. *FOXO3* gene was low expressed in CESC ($P < 0.05$), LUAD ($P < 0.05$), OV ($P < 0.05$) and SKCM ($P < 0.05$), but high expressed in acute myeloid leukemia (TCGA-LAML) ($P < 0.05$) and PCPG ($P < 0.05$) (Figure 1B).

We analyzed the expression level of FOXO3 protein in CPTAC database using UALCAN online tool, as shown in figure 1C, FOXO3 protein was low expressed in BRCA ($P < 0.001$), LUAD ($P < 0.001$) and UCEC ($P < 0.001$), but high expressed in KIRC ($P < 0.001$). There was no significant difference in the protein expression level of FOXO3 between normal and COAD or OV tumor tissues.

GEPIA2 online tool was used to analyze the relationship between *FOXO3* gene expression and tumor stage in TCGA database. As shown in figure 1D, the results showed that the expression of *FOXO3* gene in COAD ($P <0.01$), KIRC ($P < 0.05$) and SKCM ($P < 0.05$) tumor tissues was correlated with tumor stage, but there was no correlation in other tumors.

Survival analysis data

The cancer cases were divided into *FOXO3* gene high expression group and *FOXO3* gene low expression group according to the expression level of *FOXO3* gene, we analyzed the correlation between the expression level of *FOXO3* gene and the overall survival or disease-free survival of various tumors recorded in TCGA database using GEPIA2 online tool. As shown in figure 2A, in BLCA (P=0.029), BRCA (P=0.0091) and CESC (P=0.015), high *FOXO3* gene expression was associated with low overall survival. In KIRC (P=1.6e-05) and uveal melanoma (TCGA-UVM) (P=0.00098), high expression of *FOXO3* gene was associated with high overall survival. In BLCA (P=0.015), high *FOXO3* gene expression was associated with low disease-free survival. In UVM (P=5e-04), high expression of *FOXO3* gene was associated with high disease-free survival (Figure 2B).

FOXO3 alteration analysis data

We analyzed the mutations of *FOXO3* gene in different tumor samples of TCGA database, as shown in figure 3A. *FOXO3* gene alteration frequency was the highest in patients with lymphoid neoplasm diffuse large B-cell lymphoma (TCGA-DLBC), more than 10%, and all of them were “copy number deletion”; the mutations of *FOXO3* gene in patients with PCPG were all “copy number deletion”; the mutation frequency of *FOXO3* gene in patients with uterine carcinosarcoma (TCGA-UCS) was about 2%, and all of them were “copy number amplification”; the mutation types of *FOXO3* gene in BRCA, BLCA and CESC tumors all had “copy number deletion”. These results suggest that the low expression of *FOXO3* gene in BRCA, BLCA and CESC tumor tissues may be related to the copy number deletion, and the high expression of *FOXO3* gene in PCPG tumor tissues is not related to the gene mutation. Further analysis revealed the types, sites and case number of the *FOXO3* genetic alteration. Missense mutation was the main type of *FOXO3* gene

mutation, and R250W/Q downstream of FH domain was the most common type of *FOXO3* gene mutation, which can be detected in 1 case of prostate adenocarcinoma (TCGA-PRAD), 2 cases of STAD and 1 case of COAD (Figure 3B). The results also showed that S32 and S253 of FOXO3 did not mutate in all kinds of tumors included in TCGA database. S315F of FOXO3 only occurred in SKCM tumors. The results suggest that S315 of FOXO3 can not be phosphorylated in some SKCM tumors.

We also analyzed the correlation between *FOXO3* gene mutation and clinical survival prognosis of patients with different types of cancer, and the results were shown in figure 3C. Compared with patients without FOXO3 alteration, patients with gene *FOXO3* alteration had lower overall survival rate in adrenocortical carcinoma (TCGA-ACC) (*P*=1.317e-3), BRCA (*P*=0.015) and LUAD (*P*=7.92e-3), but higher overall survival rate in head and neck squamous cell carcinoma (TCGA-HNSC) (*P*=0.0116); Patients with gene *FOXO3* alteration had lower disease-free survival rate in ACC (*P*=0.0241) compared with patients without FOXO3 alteration, but higher in SARC (*P*=0.0252); Patients with gene *FOXO3* alteration had lower disease-specific survival rate in ACC (*P*=0.0183); Patients with gene *FOXO3* alteration had lower progression-free survival rate in UCS (*P*=0.0299).

Protein phosphorylation analysis data

Compared with normal tissues, we used CPTAC online search to analyze the expression of FOXO3 phosphorylation protein at different sites in BRCA, OV, COAD, KIRC, UCEC and LUAD primary tumors and summarized the expression of FOXO3 phosphorylation protein at different sites with the significant difference between normal tissues and primary tumor tissues. The results showed that FOXO3 (S55, S284, S311, T418, T427, S438 and S553) phosphorylation proteins were expressed in BRCA patients; In OV patients, FOXO3 (S284, S299 and T427) phosphorylation proteins were expressed; FOXO3 (S284 and T427) phosphorylation proteins were expressed in the patients with COAD; The expression of S253 and S284 phosphorylation proteins of FOXO3 were detected in KIRC patients; In patients with UCEC, FOXO3 (S253, S284, S311 and S413) phosphorylation proteins were expressed; FOXO3 (S253 and S284) phosphorylation proteins were expressed in LUAD patients (Figure 4A).

FOXO3 (S284) (*P*=1.9e-2) and FOXO3 (S311) (*P*=1.9e-3) phosphorylation proteins were highly expressed in BRCA patients; FOXO3 (S284) (*P*=2.7e-5) phosphorylation protein was low expressed in OV patients; FOXO3 (T427) (*P*=6.3e-7) phosphorylation protein was low expressed in COAD patients; FOXO3 (S253) (*P*=6.3e-27) and FOXO3 (S284) (*P*=2.3e-10) phosphorylation proteins were highly expressed in KIRC patients; FOXO3 (S253) (*P*=2.9e-2) and FOXO3 (S413) (*P*=4.9e-8) phosphorylation proteins were low expressed in patients with UCEC; FOXO3 (S253) (*P*=2.7e-7) phosphorylation protein was low expressed in LUAD patients; FOXO3 (S284) (*P*=6.5e-7) phosphorylation protein was highly expressed in LUAD patients (Figure 4B).

Cancer-associated fibroblasts infiltration analysis data

we used the EPIC, MCPCOUNTER, XCELL and TIDE algorithms to investigate the potential relationship between the infiltration level of CAFs and *FOXO3* gene expression in diverse cancer types of TCGA. After

a series of analyses, we observed a statistical positive correlation of gene *FOXO3* expression and the estimated infiltration value of CAFs for the tumors of COAD, HNSC, HNSC-HPV-, LIHC, LUAD, MESO, OV, PAAD, SARC and TGCT (Figure 5A). The scatterplot data with the highest Rho value in the four databases of each tumor was shown in figure 5B.

Enrichment analysis of FOXO3-related partners

To further investigate the molecular mechanism of FOXO3 in tumorigenesis, we screened out the top 50 experimentally determined FOXO3-binding proteins and the top 100 FOXO3 expression-correlated genes for a series of pathway enrichment analyses. The interaction network of the top 50 experimentally determined FOXO3-binding proteins was shown in figure 6A. An intersection analysis of the above two datasets was performed and obtained five common genes: SIRT1, CREBBP, FKBP5, RERE and SMAD4, as shown in figure 6B. We used the GEPIA2 tool to combine all tumor expression data of TCGA and obtained the above five genes that correlated with FOXO3 expression, The gene *FOXO3* expression level was positively correlated with that of SIRT1 (P<0.001) (R=0.49), CREBBP (P<0.001) (R=0.5), FKBP5 (P<0.001) (R=0.51), RERE (P<0.001) (R=0.52) and SMAD4 (P<0.001) (R=0.53) (Figure 6C). The corresponding heatmap data also showed a positive correlation between FOXO3 and the above five genes in all the cancer types (Figure 6D). KEGG enrichment analysis showed that these genes of the above two datasets were mainly involved in the regulation of FOXO signaling pathway, cell cycle, PI3K-Akt signaling pathway, thyroid hormone signaling pathway and Chagas disease (American trypanosomiasis) (Figure 7A, B, Table S1). Molecular function analysis of GO enrichment analysis showed that these genes mainly played roles in transcription factor binding, protein C-terminus binding, enzyme binding, p53 binding, protein serine/threonine kinase activity and tau protein binding (Figure 7C, D, Table S2).

Discussion

In this paper, we found that high expression of FOXO3 in KIRC tissues in Fig. 1C, and the KIRC patients with the high expression of FOXO3 had the increased overall survival rate in Fig. 2A, suggesting that FOXO3 had an anti-tumor effect in KIRC. Wei Zhai et al. also found that lncRNA-URRCC was highly expressed in KIRC tissues and URRCC enhanced the expression of EGFL7 to activate P-AKT signaling, which suppressed FOXO3 activation to promote KIRC cell proliferation and invasion [14]. Mi-Ae Kang et al. also identified that interleukin 4 receptor alpha (IL4Rα)-positivity and co-expression of IL4Rα and interleukin 13 receptor subunit alpha 1 (IL13Rα1) were the independent indicators of shorter cancer-specific survival and relapse-free survival in 199 KIRC patients, and silencing IL4Rα or Janus kinase 2 (JAK2), a well-known down-stream tyrosine kinase under the heterodimeric receptor complex of IL4Rα and IL13Rα1, induced the activation of FOXO3 in KIRC cells [15]. Wenyuan Nie et al. also found that patients with KIRC with an increased miR122 level in tumor tissues had a shortened metastasisfree survival and miR-122 also promoted KIRC cell proliferation and invasion through suppressing FOXO3 activation [16]. The expression of phosphofructokinase-M (PFKM) was lower in KIRC tumor tissues than that in the adjacent normal tissues, which was related to the poor overall survival of KIRC patients, FOXO3 can inhibited the growth, migration and invasion of KIRC cells through increasing the expression

of PFKM [17]. Hejia Zhu et al. also found that miR-362-3p was frequently down-regulated in human KIRC tissues, and overexpression of miR-362-3p in KIRC cells significantly suppressed the proliferation, cell cycle and motility through inhibiting SP1/AKT/FOXO3 signaling [18]. The loss of FOXO3 induced epithelial-mesenchymal transition (EMT) of KIRC cells by upregulating snail family transcriptional repressor (SNAIL1), which promoted tumor cells metastasis [19]. All the above experimental results are consistent with our conjecture, which proves that FOXO3 does play a role as a tumor suppressor in KIRC. However, we still have a limited understanding of its regulation mechanisms in KIRC, which needs further exploration.

As FOXO3 was low expressed in BRCA at both mRNA and protein levels in Fig. 1A and 1C, and high expression of *FOXO3* gene in BRCA tissues was directly associated with the low overall survival rate in Fig. 2A, suggesting that FOXO3 at a higher level maybe have a cancer-promoting effect in BRCA. Jie Chen et al. found that nuclear FOXO3 was positively associated with lymph node, poor prognosis and P-Akt expression in BRCA, activated Akt failed to inactivate FOXO3 and re-localize it to the cytoplasm, and nuclear-targeted FOXO3 did not induce cell death or cell cycle arrest. Sustained nuclear FOXO3 expression in BRCA might lead to cancer progression and develop into an invasive phenotype [20]. Another report had also provided a theoretical basis for the above hypothesis, over-expressed FOXO3 can induce Caveolin-1 (Cav1) expression to decrease motility, invasiveness, and anchorage-independent growth in estrogen receptor α-positive ($ERα^+$) breast cancer cells, while over-expressed FOXO3 played the opposite effects in ERα-silenced cells and in ERα-negative ($ERα^-$) cell lines. The nuclear localization staining of FOXO3 was positively correlated with the degree of tumor invasion in $ERα^-$ breast cancer cells, on the contrary, the correlation between the nuclear localization staining of FOXO3 and the degree of tumor invasion was negative in $ERα^+$ breast cancer cells [21]. since it was well known that the expression of ERα had been reported to reduce the metastatic potential of breast cancer cells, and which was closely related to the decrease of motility and invasiveness of $ERα^-$ breast cancer cells [22]. All the above results inferred that FOXO3 as an oncogene triggers some targeted genes in the nucleus of $ERα^-$ breast cancer cells, but when FOXO3 works together with other transcription factors, such as ERα, the transcription activity FOXO3 was weakened, and FOXO3 was mainly translocated to cytoplasm. Further studies also found that activation FOXO3 in the nucleus can promote the transcription of ERα in breast cancer cells [23, 24], which may be the other supplementary mechanism for the cancer inhibition function of FOXO3 in the $ERα^+$ breast cell lines. In this paper, we also found that the BRCA patients with FOXO3 alteration had the low overall survival, and the main type of gene alteration was amplification, combined with the above conjecture that FOXO3 at a higher level maybe have a cancer-promotion effect in BRCA, we infer that this amplification of FOXO3 in BRCA may promote its transactivation to perform its cancer-promotion functions.

We also found that FOXO3 was low expressed in BLCA and CESC at mRNA level in Fig. 1A, high expression of *FOXO3* gene in BLCA tissues was directly associated with the low overall survival rate or disease free survival in Fig. 2, and high expression of *FOXO3* gene in CESC tissues was directly associated with the low overall survival rate in Fig. 2A, suggesting that FOXO3 may be a tumor

suppressor gene, which at a higher level also has a cancer-promoting effect in CESC and BLCA. Hyun-Jung Kim et al. discovered that BKM120, a pan-PI3K inhibitor, treatment led to the nuclear accumulation of FOXO3 in PIK3CA-mutant CESC Caski cells, which showed a synergistic effect of BKM120 and autophagy inhibitor hydroxychloroquine (HCQ) and the strong induction of autophagy, and in which autophagy inhibition improved the anti-tumor efficacy of PI3K inhibitor [25]. FOXO3, as reported in all the available literature above, played an inhibitory role in CESC [26–28]. we also found that FOXO3 was a tumor suppressor gene via inducing cell cycle arrest and apoptosis, and there was no evidence that FOXO3 played a role in promoting the progression of BLCA [29–35] after reading all the available literature. Therefore, whether FOXO3 at a higher level having a cancer-promoting effect in CESC and BLCA needs our further studies.

High expression of *FOXO3* gene in UVM tissues was also found directly associated with the high overall survival rate and disease free survival rate in Fig. 2, suggesting that FOXO3 had an anti-tumor effect in UVM. Ming Bai et al. had identified that microRNA-194 inhibited human melanoma cell growth via down-regulating PI3K/AKT/FOXO3 [36]. Pyruvate dehydrogenase kinase 1 (PDK1) was reported to have an important function in melanoma development and progression in part through inhibiting the transcriptional activation of FOXO3 [37]. Zhen Dong et al. also found that FOXO3aSIRT6 regulatory axis inhibited tumor cell proliferation in melanoma [38]. Xin Yu et al. identified that NOVA alternative splicing regulator 1 (NOVA1) acted as an oncogene in melanoma partly through down-regulating FOXO3 expression [38]. Another report also found that inhibition of FOXO3 by IGF-1 via the PI3K/Akt pathway had an important role in IGF-1 induced proliferation and invasion of UVM cells [40]. Teng Yu et al. also reported that mammalian sterile 20-like kinase 1 (MST1) activation by curcumin mediated JNK activation, FOXO3 nuclear translocation and apoptosis in melanoma cells [41]. Another report also found the pristimerin-induced melanoma cell death via inhibiting PI3K/Akt/FOXO3 signalling pathway [42]. Fengxia Yan also found that FOXO3 suppressed melanoma development by increasing the expression of Bcl2like protein 11 (BCL2L11) and cyclindependent kinase inhibitor 1B (CDKN1B) [43]. By consulting the existing literature, we found that FOXO3 did play an anti-tumor role in UVM.

As FOXO3 can induce autophagy through transcription of autophagy related gene: DEPP in human neuroblastoma cancer cells [10], LC3Ⅱ in human hepatoma, breast and osteosarcoma cancer cells [11, 12], and FOXO3 was also a substrate for basal autophagic degradation, when autophagy was continuously inhibited, the expression level of FOXO3 was increased, and which was regulated translocating to the nucleus so as to increase its activity where it can activate target gene, a pro-apoptotic gene BBC3/PUMA [9]. All above suggests that FOXO3 seems to play an important role in the steady-state feedback of correcting autophagy perturbations. When basal autophagy is inhibited, FOXO3 activates autophagy related genes through transcription to maintain the occurrence of basal autophagy, however, if autophagy inhibition persists, FOXO3 can also induce apoptosis through transcription of pro-apoptotic genes. Therefore, we can understand why FOXO3 acts as a tumor suppressor in some cancers but a tumor promotion factor in other cancers, which may be related to the status of basic autophagy in different cancers.

In Fig. 1A and 1C, the mRNA and protein levels of FOXO3 were low expressed in LUAD tissues, the overall survival of the group with FOXO3 alteration was decreased compared with the group without FOXO3 alteration and the main type of alteration of FOXO3 were mutation and deep deletion in Fig. 3. We speculated that FOXO3 seemed to be a tumor suppressor gene in LUAD and FOXO3 alteration in LUAD will destroy its cancer inhibition function. Fang Yang et al. had identified that carfilzomib (CFZ), a second generation proteasome inhibitor, inhibited the growth of LUAD cells through increasing the transactivation of FOXO3 to promote Gadd45a expression [44]. Another report also found that HCQ-induced upregulation and nuclear translocation of FOXO3 was involved in the antitumor effects of HCQ in LUAD cells [45]. Oliver R Mikse et al. also found that FOXO3 was a suppressor of LUAD carcinogenesis, a role frequently lost through gene deletion [46]. All the above data show that our deduction was very reliable, of course, a large number of experiments were needed in order to prove this.

Transcription factor FOXO3 mainly regulates a variety of cellular processes, including apoptosis, cell cycle progression, autophagy, DNA damage and tumorigenesis, through targeting the expression of effector genes in cancer [2]. Therefore, the subcellular location of FOXO3 is so important for its function. A Brunet et al. in 1999 had identified that survival factors can induce Akt activation to phosphorylate FOXO3 at T32, S253 and S315 sites to promote FOXO3 binding with 14-3-3 proteins, which facilitated FOXO3 translocation to cytoplasm and inhibited apoptosis [7]. Serum- and glucocorticoid-inducible kinases (SGKs) appeared to phosphorylate FOXO3 at T32 and S315 sites to promote FOXO3 translocation to cytoplasm and suppress FOXO3-dependent transcription, including cell cycle arrest and apoptosis [47]. In response to oxidative stress, MST1 directly phosphorylated FOXO3 at Ser207, resulting in its translocation to the nucleus and activation of FOXO3 [48, 49]. AMPK directly phosphorylated FOXO3 at six serine/threonine residues (Thr179, Ser399, Ser413, Ser555, Ser588, and Ser626), which are different from the AKT or SGK phosphorylation sites, resulting in transactivation of FOXO3 without affecting FOXO3 nuclear localization [49, 50]. Deleption of gamma-glutamylcyclotransferase (GGCT) inhibiting cell growth via increasing FOXO3 phosphorylation at ser413 in PC3 human prostate cancer and A172 glioblastoma cells may be closely related to up-regulating the nuclear protein levels of FOXO3, a transcriptional factor involved in tumor suppression [51]. 3-bromopyruvate, a potent inhibitor of glycolysis, triggered apoptosis through increasing AMPK-mediated phosphorylation of FOXO3 at Ser413 and then promoting its transactivation in HCT116 human colon cancer cells [52]. In the non-tumorigenic mammary epithelial cell line MCF-10A, high glucocorticoid levels immediately activated FOXO3 and SGK-1. Following FOXO3 transcription increased, the protein was phosphorylated at T32 leading to its inactivation. Treatment with AMPK activated stimulation triggered phosphorylation of FOXO3 by AMPK at Ser 413, thereby counteracting inactivation of FOXO3 by SGK-1, which induced the transcriptional activation of LKB1 inhibiting cell cycle and tumor progression [53]. All the above results indicated that increasing the protein level of phosphorylated FOXO3 at Ser253, Thr32 and Ser315 inhibited its transcriptional activation while increasing phosphorylated FOXO3 at Ser413, Thr179, Ser399, Ser555, Ser588, Ser626 and Ser207 sites inversely promoted its transcriptional activation, which suggests that the expression of phosphorylated FOXO3 protein at different sites may affect its transcriptional activity by changing its shuttle between nucleus and cytoplasm. Although we found that the expression level of

FOXO3 phosphorylation at Ser284 in BRCA, OV, LUAD and KIRC, the expression level of FOXO3 phosphorylation at Ser253 in KIRC, LUAD and UCEC, the expression level of FOXO3 phosphorylation at Ser311 in BRCA, the expression level of FOXO3 phosphorylation at T427 in COAD, and the expression level of FOXO3 phosphorylation at Ser413 in UCEC all had significant differences compared with that in the corresponding normal tissues. However, we still have not found any reports about the sublocation and function of these phosphorylated FOXO3 protein at the above phosphorylation sites in the corresponding cancer, which need our further studies in the future.

CAFs are cells negative for epithelial, endothelial and leukocyte markers with an elongated morphology and lacking the mutations found within cancer cells, which are a key component of the tumor microenvironment with diverse functions, such as depositing and remodeling the extracellular matrix (ECM), which promotes cancer invasion and metastasis via increasing the stiffness of tumor tissue, triggers pro-survival and pro-proliferation signaling in cancer cells and impedes the migration of infiltrating leukocytes to decrease immune surveillance of tumors. IL-6, CXC-chemokine ligand 9 (CXCL9) and TGFβ, the secretome of CAFs, also has immunosuppressive functions in reducing T cell responses. Recently, we have also found that CAFs not only have the above cancer pro-tumorigenic effects, but also have some antitumorigenic effects via immune enhancement. Since CAFs have different subtypes in different cancers, which have heterogeneity, *KRAS* mutation or different *P53* mutational status or non-genetic factors can also influence the functions of CAFs [54]. All the above indicate that understanding the subtypes of CAFs infiltrating in different cancer cells, the correlation between gene *FOXO3* expression and CAFs infiltration of different tumors, and *KRAS* mutation or different *P53* mutational status or non-genetic factors which can influence the functions of CAFs are so important for analyzing the tumor inhibition or promotion role of FOXO3 in various cancers.

As we obtained the results that FOXO3 bound with and had a positive correlation with SIRT1, CREBBP (CBP), FKBP5, RERE or SMAD4 at mRNA level in many types of cancer, which drew our attentions to the relationship between FOXO3 functions and the expression of SIRT1, CREBBP, FKBP5, RERE and SMAD4. After consulting the literature, we found lots of important information as follows: SIRT1 exerted inhibitory activities on chemoresistance and cancer stem cells (CSCs) properties in gastric cancer through the activations of FOXO3 and AMPK. AMPK promoted nuclear translocation of FOXO3 and enhanced its transcriptional activities. In addition, FOXO3 increased the expression level and activation of AMPK by directly binding to its promoter and activating the transcription of AMPK [55]. SIRT1 also increased FOXO3 deacetylation and lapatinib sensitivity in HER2-positive breast cancer [56]. The elevation of SIRT1 and SKP2 expression was responsible for the downregulation of FOXO3 protein levels through promoting SKP2-mediated FOXO3 ubiquitination in malignant PC3 and DU145 prostate cells [57]. SIRT1 deficiency in Bladder cancer cells could suppress cell viability by inducing cell cycle arrest possibly via FOXO3-related pathways [33]. All the above suggest that SIRT1 in cancer cells seems to influence the phosphorylation, deacetylating and ubiquitination modification status of FOXO3, which has an effect on the transcriptional activation of FOXO3 to drive different functions. CBP promoted the transcriptional activation of FOXO3 through binding with FOXO3 via KIX domain [58], which suggests that CBP acts as a coactivator of the transcription factor FOXO3 to increase the transcriptional activity of FOXO3. FKBP5

increased the activation of autophagy in the oxygen and glucose deprivation and reoxygenation model via downregulating P-AKT (Ser473)/P-FOXO3 (Ser253) signaling pathway to increase ischemia and reperfusion injury [59], which infers that FKBP5 maybe have an effect on the function of FOXO3 in cancers through regulating the phosphorylation state of FOXO3. G Fu and C Peng in 2011 identified that growth differentiation factor (NODAL), a member of the transforming growth factor-β superfamily, promoted cyclin G2 (CCNG2) transcription to suppress ovarian cancer cell proliferation by upregulating FOXO3 expression, inhibiting FOXO3 phosphorylation and enhancing its synergistic interaction with SMADs, and knockdown of SMAD4 blocked the activity of FOXO3 [60], which infers that SMAD4, another transcription factor, can influence the transactivation of FOXO3 through binding with FOXO3. Although we did not find that RERE can influence the transactivation of FOXO3, we found that RERE usually co-localized with the other transcription factor in the nucleus [61], which infers that RERE maybe influence the transactivation of FOXO3 in the cancer cell nucleus.

Conclusions

In this paper, we found that FOXO3 was usually as an anti-tumor gene in KIRC, BLCA, CESC, UVM, LUAD and BRCA cancers, while a higher level of FOXO3 may have a certain cancer-promoting effect. As FOXO3 usually played a cancer-promoting role in $ERα^-$ BRCA but in $ERα^+$ BRCA played an anti-tumor role, which may be due to transcription factor ERα translocating FOXO3 to the cytoplasm to weaken the transcription activity of FOXO3.

We also known that whether FOXO3 played a role as an oncogene or anti-tumor gene in different cancers, which may be related to the different status of basic autophagy in different cancers, in which FOXO3 activates autophagy related genes through transcription to maintain the occurrence of basal autophagy when basal autophagy is inhibited. However, FOXO3 can also induce apoptosis through transcription of pro-apoptotic genes if autophagy inhibition persists.

The expression of phosphorylated FOXO3 protein at different sites may affect its transcriptional activity by changing its shuttle between nucleus and cytoplasm. In short, when we analyze the functions of FOXO3 in a specific type of cancer, we should combine with the expression of FOXO3, the alteration status of FOXO3, the overall phosphorylation status of FOXO3, the basic autophagy status, the expression levels of ERα, SIRT1, CBP, FKBP5, RERE and SMAD4, and cancer-associated fibroblasts subtype, even consider the *KRAS* mutation, *P53* mutation or non-genetic factors which can influence the functions of CAFs in the specific type of cancer.

List Of Abbreviations

FOXO3
forkhead box O-3; FKHRL1:forehead in rhabdomyosarcoma-like 1; FH:forkhead DNA binding domain; NLS:nuclear localization sites; NLS:nuclear localization sites; NES:nuclear export site; TAD:transactivation domain; miRNA:microRNA; CAFs:cancer-associated fibroblasts; TCGA-CESC:cervical squamous cell

carcinoma and endocervical adenocarcinoma; TCGA-SKCM:skin cutaneous melanoma; TCGA-OV:ovarian serous cystadenocarcinoma; TCGA-BRCA:breast invasive carcinoma; TCGA-KIRC:kidney renal clear cell carcinoma; TCGA-LUAD; TCGA-COAD:colon adenocarcinoma; TCGA-UCEC:uterine corpus endometrial carcinoma; CNA:copy number alteration; R:correlation coefficient; TCGA-BLCA:bladder urothelial carcinoma; TCGA-KICH:kidney chromophobe; TCGA-LUSC:lung squamous cell carcinoma; TCGA-THCA:thyroid carcinoma; TCGA-CHOL:cholangiocarcinoma; TCGA-LIHC:liver hepatocellular carcinoma; TCGA-PCPG:pheochromocytoma and paraganglioma; TCGA-STAD:stomach adenocarcinoma; TCGA-LAML:acute myeloid leukemia; TCGA-UVM:uveal melanoma; TCGA-DLBC:lymphoid neoplasm diffuse large B-cell lymphoma; TCGA-UCS:uterine carcinosarcoma; TCGA-ACC:adrenocortical carcinoma; TCGA-HNSC:head and neck squamous cell carcinoma; IL4Rα:interleukin 4 receptor alpha; IL13Rα1:interleukin 13 receptor subunit alpha 1; JAK2:Janus kinase 2; PFKM:phosphofructokinase-M; EMT:epithelial-mesenchymal transition; SNAIL1:snail family transcriptional repressor; Cav1:Caveolin-1; $ERα^{+}$:estrogen receptor α-positive; $ERα^{-}$:$ERα^{-}$negative; HCQ:hydroxychloroquine; PDK1:Pyruvate dehydrogenase kinase 1; NOVA1:NOVA alternative splicing regulator 1; MST1:mammalian sterile 20-like kinase 1; BCL2L11:Bcl2like protein 11; CDKN1B:cyclindependent kinase inhibitor 1B; CFZ:carfilzomib; SGKs:serum- and glucocorticoid-inducible kinases; GGCT:gamma-glutamylcyclotransferase; ECM:extracellular matrix; CXCL9:CXC-chemokine ligand 9; CBP:CREBBP; CSCs:cancer stem cells; NODAL:growth differentiation factor; CCNG2:cyclin G2.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by the National Natural Science Foundation of China (No.81872125), Outstanding Scientific Fund of Shengjing Hospital (NO.201704), Science and technology research project of education department of Liaoning province (No.LK201632).

Authors' contributions

Wei Sun was responsible for confirming the topic. Guiyang Cai was responsible for writing the first draft of this article. Jiacheng Li was responsible for graph makeup optimization. Wei Sun and Qing Yang contributed to furtherly editing and polishing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

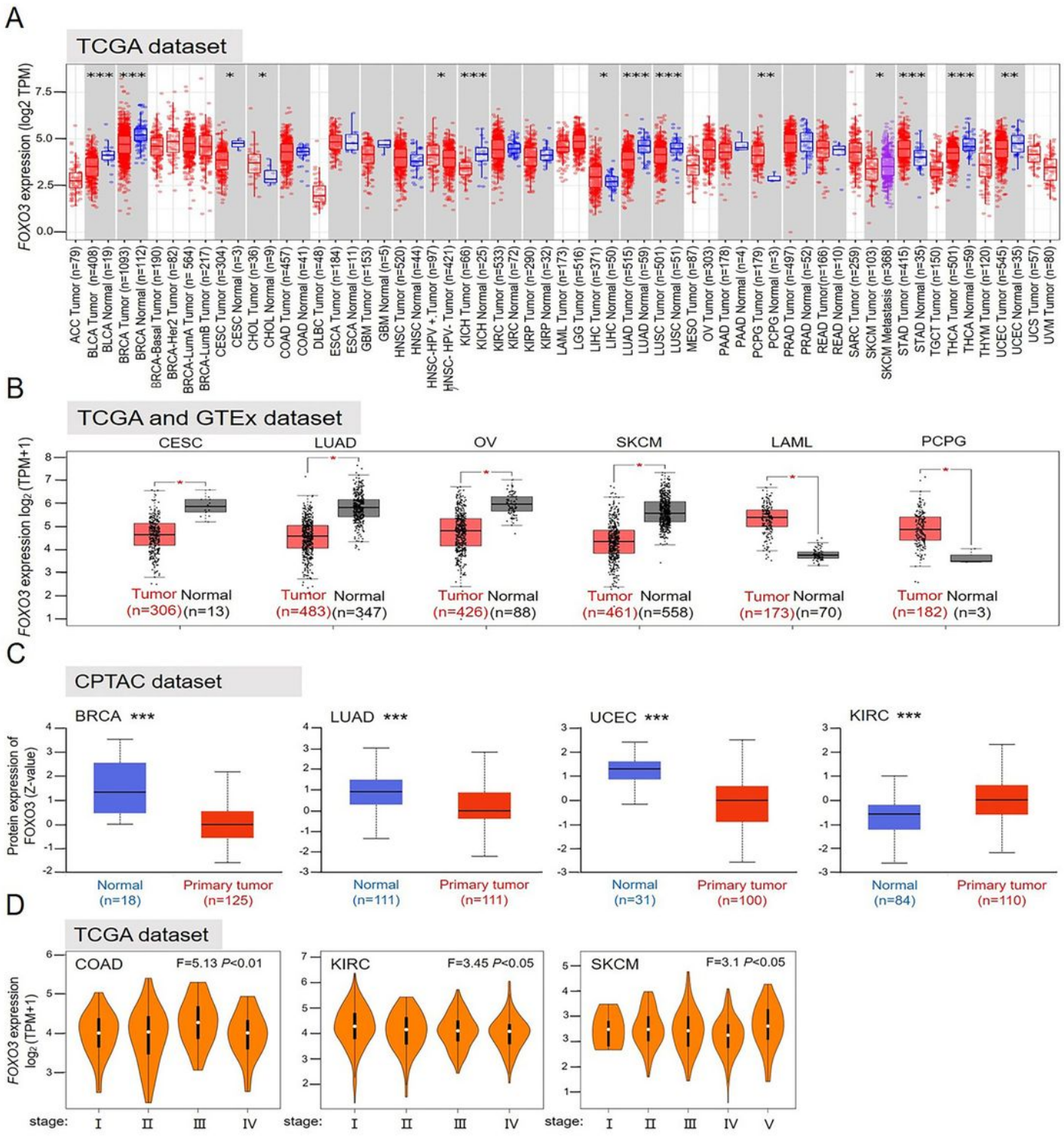

Figure 1

FOXO3 expression in different tumors and the association between FOXO3 expression and pathological stages. (A)The expression status of the FOXO3 gene in different cancers or specific cancer subtypes was analyzed through TIMER2, * P<0.05; ** P<0.01; *** P<0.001. (B) The box plot data of the FOXO3 gene in different cancers or specific cancer subtypes was analyzed through GEPIA2, * P <0.05. (C) Based on the CPTAC dataset, we analyzed the expression level of FOXO3 total protein between normal tissue and

primary tissue of BRCA, OV, KIRC, LUAD, COAD, and UCEC, *** P<0.001. (D) Based on the TCGA data, the expression levels of the FOXO3 gene were analyzed associated with the main pathological stages (stage I, II, III, and IV) of COAD, KIRC and SKCM, Log2 (TPM+1) was applied for log-scale.

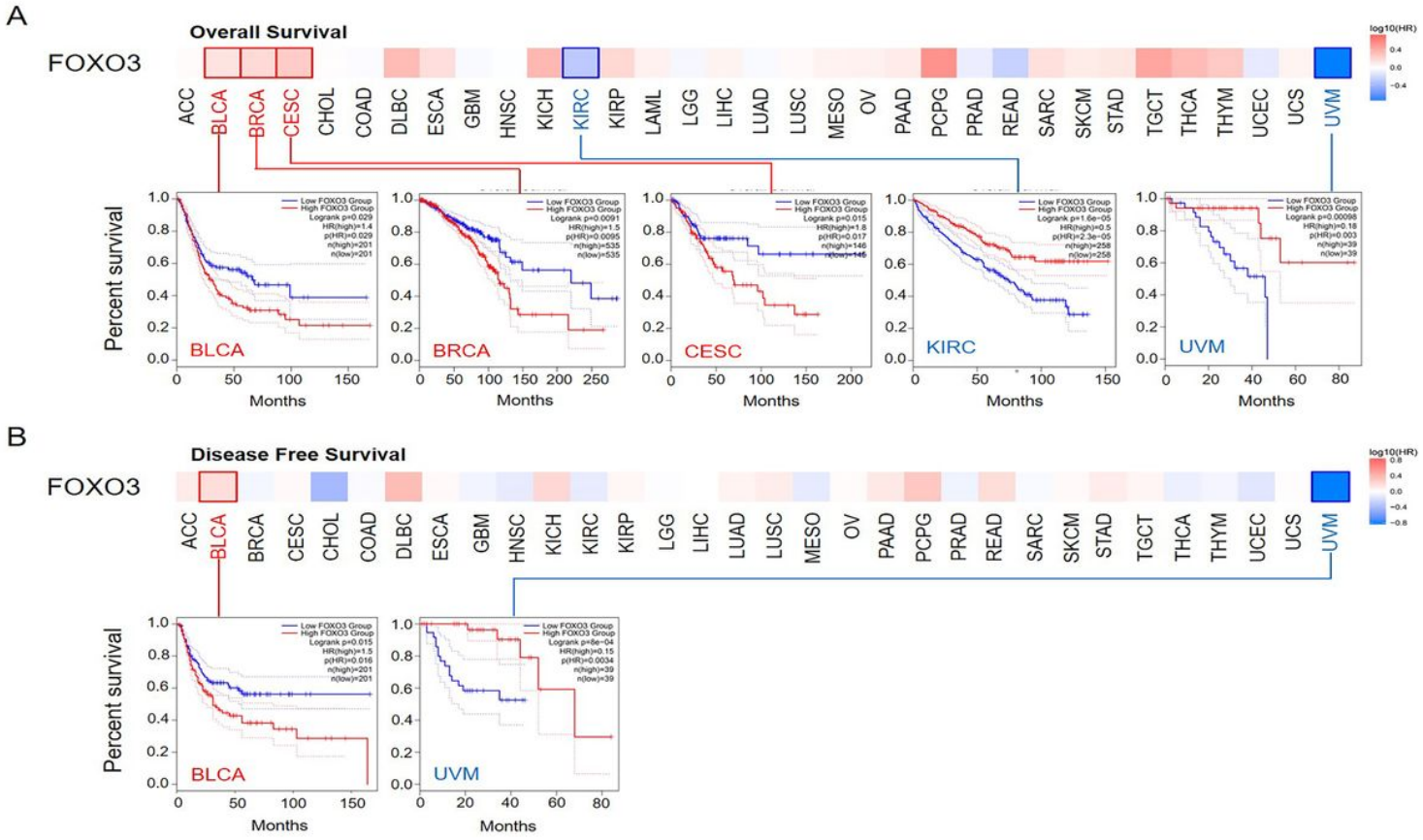

Figure 2

Correlation between FOXO3 expression and survival prognosis of cancers in TCGA. We used the GEPIA2 tool to perform overall survival (A) and disease-free survival (B) analyses of different tumors in TCGA by FOXO3 gene expression. The survival map and Kaplan-Meier curves with positive results were given.

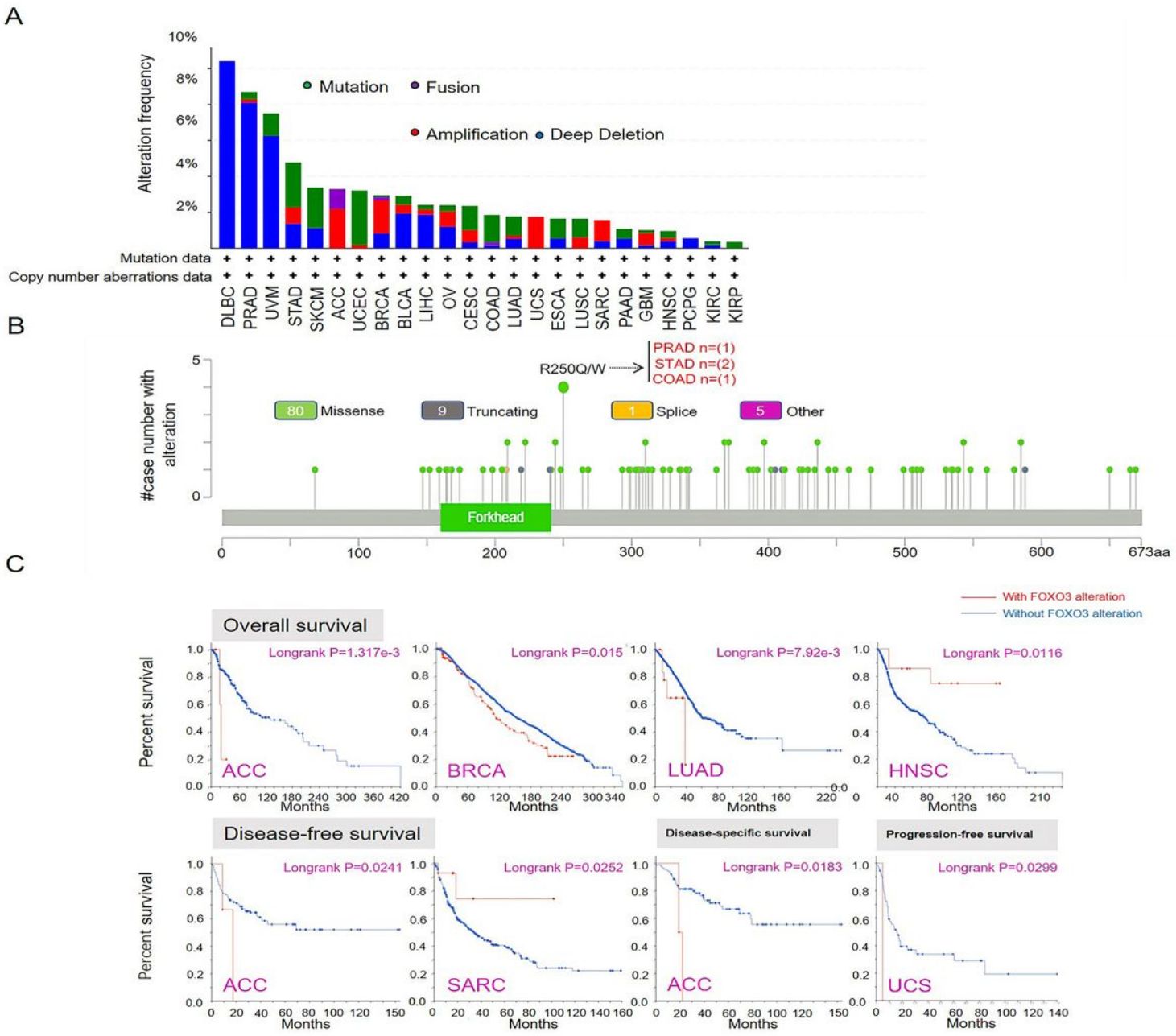

Figure 3

Alteration feature of FOXO3 gene in different tumors of TCGA. We analyzed the alteration features of FOXO3 gene for all the TCGA tumors using the cBioPortal tool. The alteration frequency with mutation type (A) and mutation site (B) were displayed. We also displayed the correlation between mutation status and overall, disease-specific, disease-free or progression-free survival of all the TCGA tumors which had significant difference (C) using the cBioPortal tool.

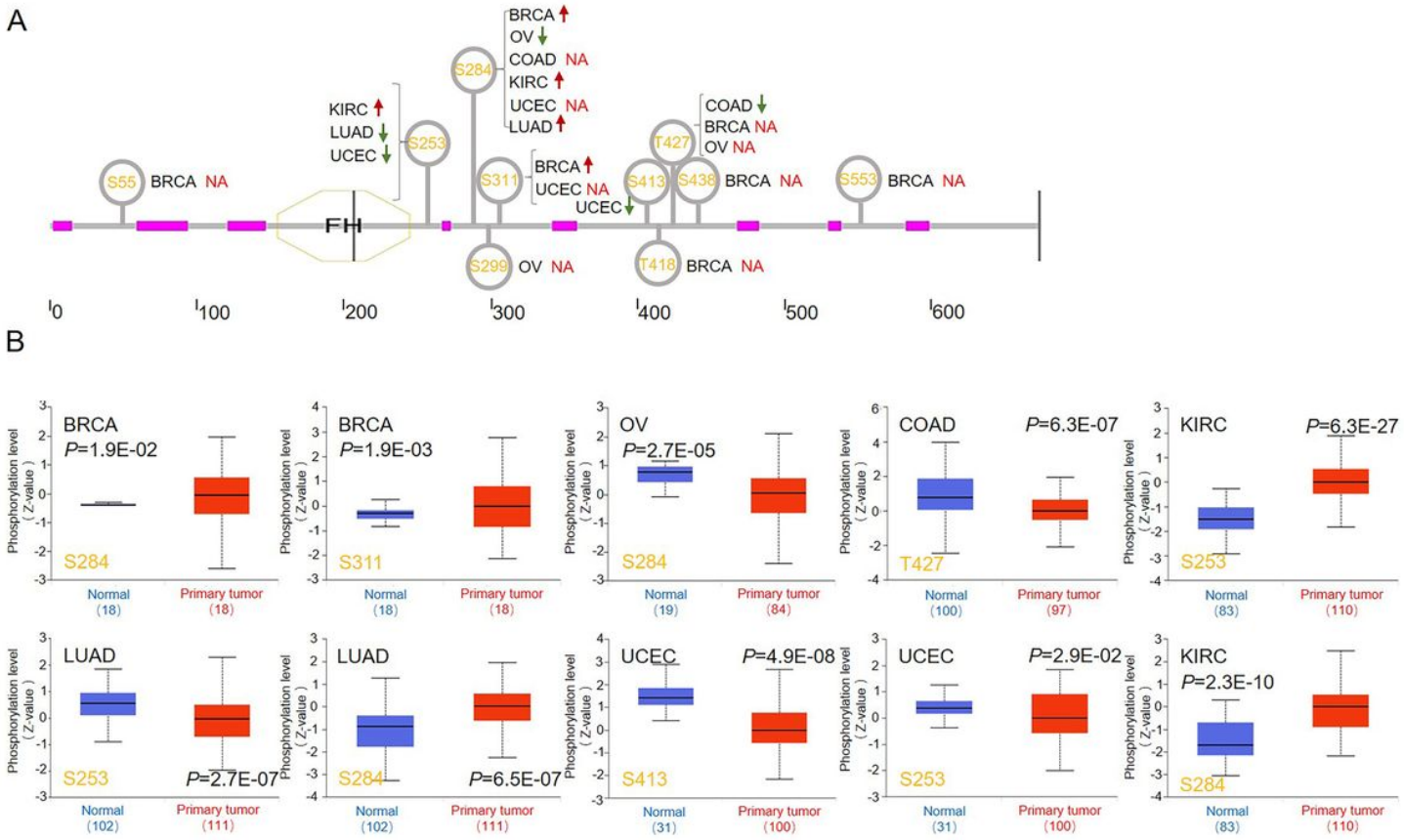

Figure 4

Phosphorylation protein analysis of FOXO3 in different tumors. Based on the CPTAC dataset, we analyzed the expression level of FOXO3 phosphoprotein at different sites between normal tissues and primary tissues of BRCA, OV, KIRC, LUAD, COAD, UCEC via the UALCAN. The phosphoprotein sites with positive, negative and unrelated results were displayed in the schematic diagram of FOXO3 protein (A) and which had significant difference were displayed in the form of the box plots for different cancers, including BRCA, OV, COAD, KIRC, UCEC and LUAD (B).

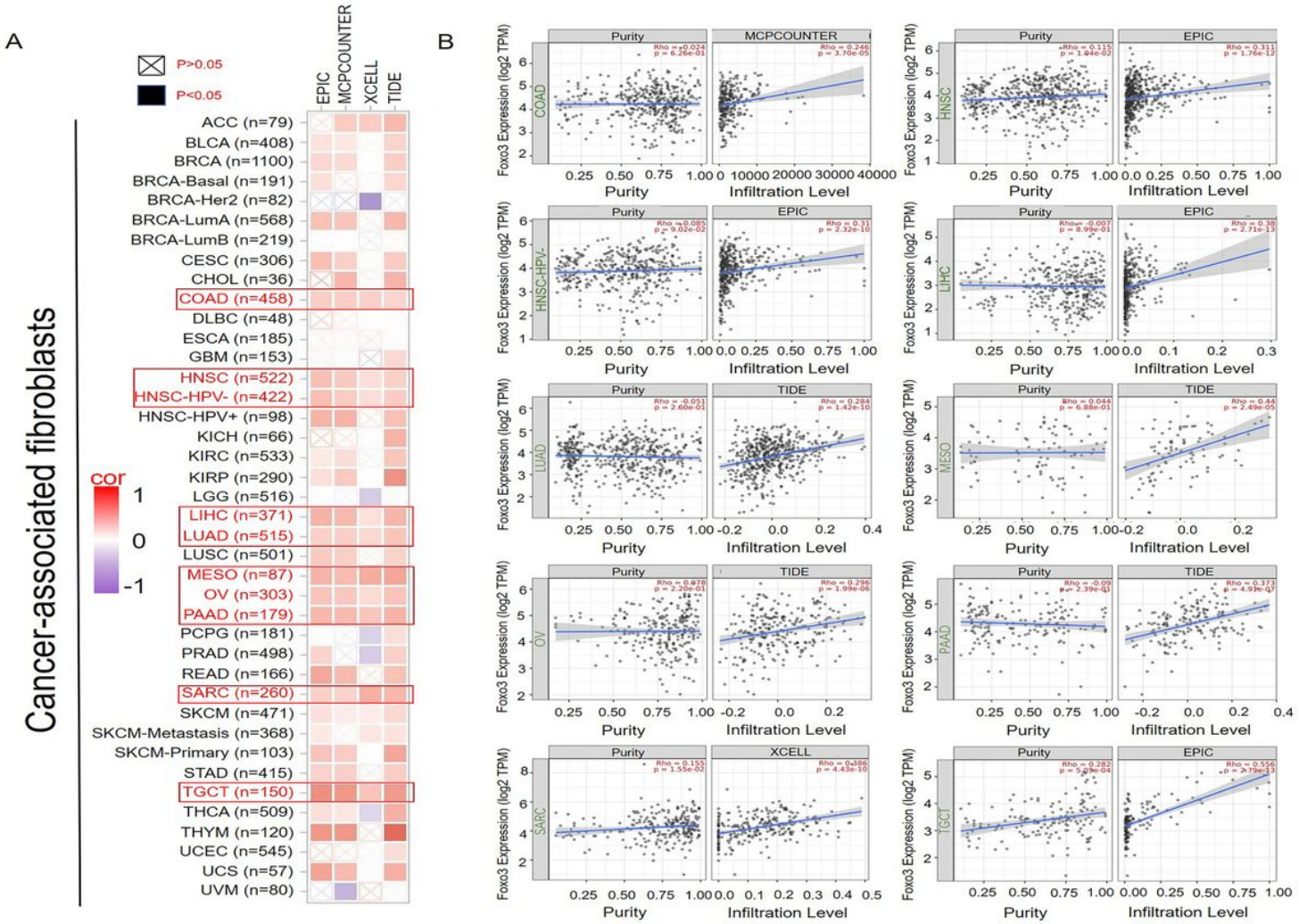

Figure 5

Correlation analysis between FOXO3 gene expression and immune infiltration of cancer-associated fibroblasts. Four different algorithms were used to explore the potential correlation between the expression level of the FOXO3 gene and the infiltration level of cancer-associated fibroblasts across all types of cancer in TCGA.

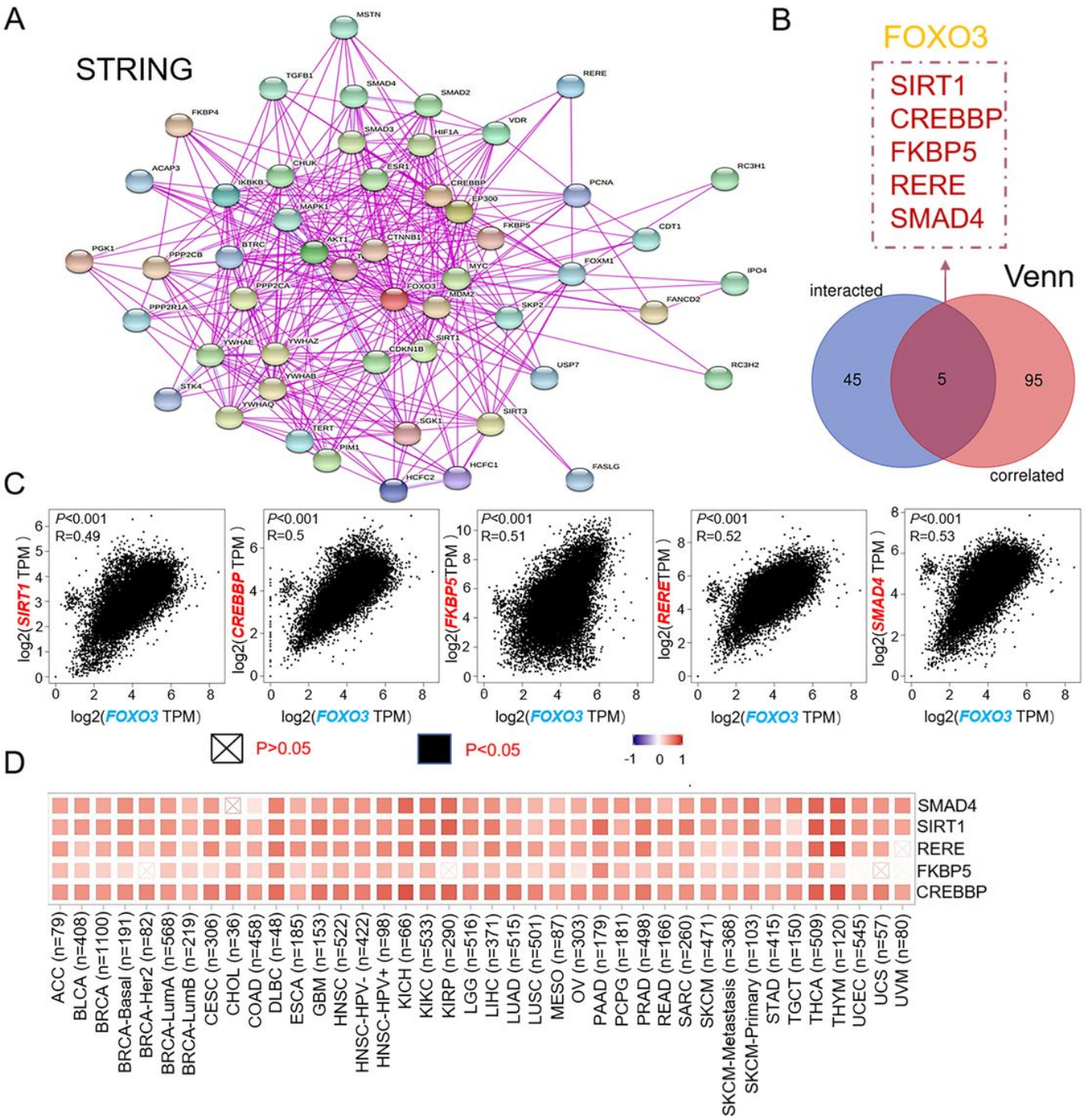

Figure 6

An analysis of FOXO3-related gene. (A) We first obtained the available experimentally determined FOXO3-binding proteins using the STRING tool. (B) Using the GEPIA2 approach, we also obtained the top 100 FOXO3-correlated genes in TCGA projects, an intersection analysis of the FOXO3-binding and correlated genes was conducted. (C) Using the GEPIA2 approach, we also analyzed the expression correlation between FOXO3 and above 5 selected targeting genes, including SIRT1, CREBBP, FKBP5, RERE and SMAD4. (D) The corresponding heatmap data in the detailed cancer types were displayed.

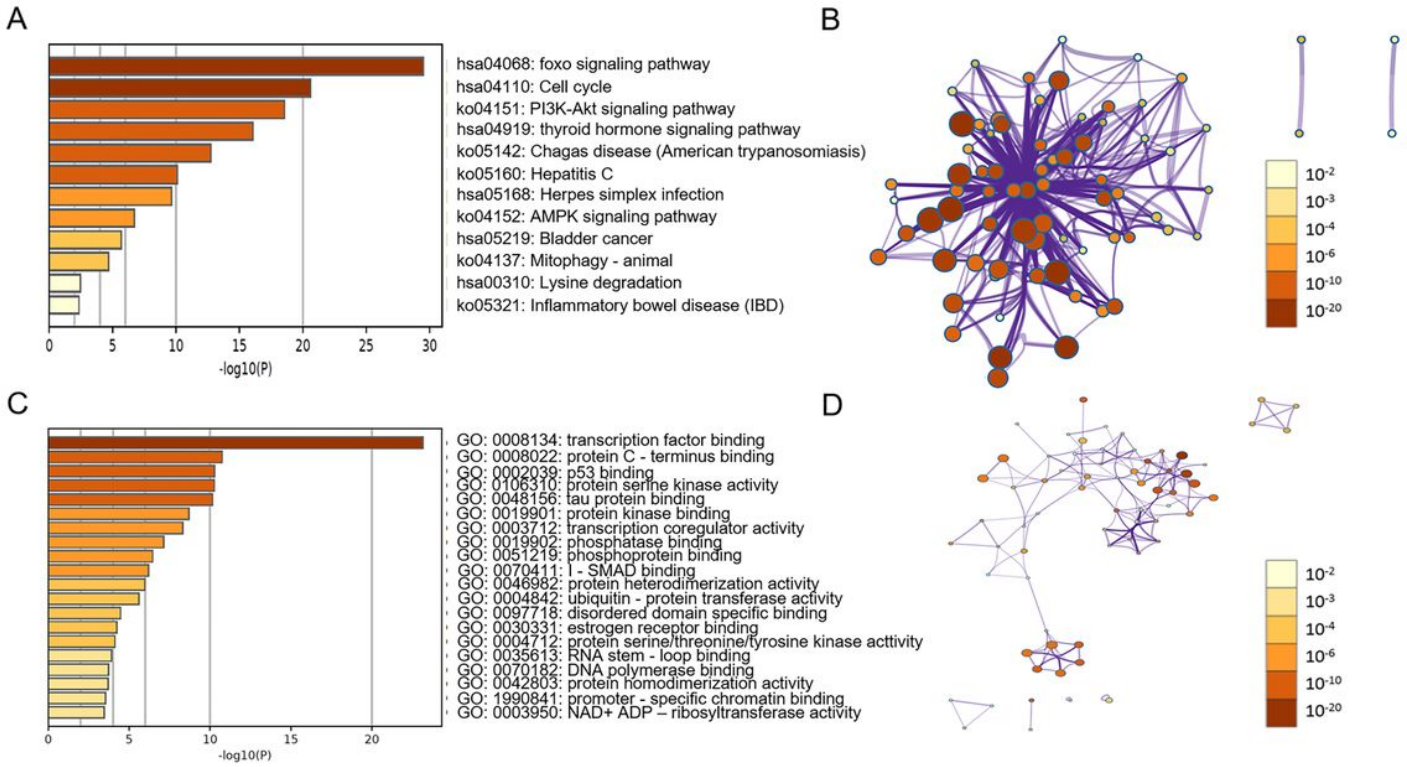

Figure 7

FOXO3-related gene enrichment analysis. (A, B) The KEGG pathways of the FOXO3-binding and correlated genes were enriched through bar graphs and networks. (C, D) The molecular functions of the FOXO3-binding and correlated genes were enriched through bar graphs and networks.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.xlsx