


# FOXQ1 is Differentially Expressed Across Breast Cancer Subtypes with Low Expression Associated with Poor Overall Survival

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Fahed A Elian<sup>1</sup>  
Ubah Are<sup>1</sup>  
Sunita Ghosh<sup>2,3</sup>  
Paulo Nuin<sup>1</sup>  
Tim Footz<sup>1</sup>  
Todd PW McMullen<sup>4</sup>  
David N Brindley<sup>5,6</sup>  
Michael A Walter<sup>1</sup> 

<sup>1</sup>Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada; <sup>2</sup>Department of Medical Oncology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada; <sup>3</sup>Department of Mathematical and Statistical Sciences, Faculty of Science, University of Alberta, Edmonton, AB, Canada; <sup>4</sup>Department of Surgery, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada; <sup>5</sup>Department of Biochemistry, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada; <sup>6</sup>Cancer Research Institute of Northern Alberta, University of Alberta, Edmonton, AB, Canada

**Purpose:** Forkhead box Q1 (*FOXQ1*) has been shown to contribute to the development and progression of cancers, including ovarian and breast cancer (BC). However, research exploring *FOXQ1* expression, copy number variation (CNV), and prognostic value across different BC subtypes is limited. Our purpose was to evaluate *FOXQ1* mRNA expression, CNV, and prognostic value across BC subtypes.

**Materials and Methods:** We determined *FOXQ1* expression and CNV in BC patient tumors using RT-qPCR and qPCR, respectively. We also analyzed *FOXQ1* expression and CNV in BC cell lines in the CCLE database using K-means clustering. The prognostic value of *FOXQ1* expression in the TCGA-BRCA database was assessed using univariate and multivariate Cox's regression analysis as well as using the online tools OncoLnc, GEPIA, and UALCAN.

**Results:** Our analyses reveal that *FOXQ1* mRNA is differentially expressed between different subtypes of BC and is significantly decreased in luminal BC and HER2 patients when compared to normal breast tissue samples. Furthermore, analysis of BC cell lines showed that *FOXQ1* mRNA expression was independent of CNV. Moreover, patients with low *FOXQ1* mRNA expression had significantly poorer overall survival compared to those with high *FOXQ1* mRNA expression. Finally, low *FOXQ1* expression had a critical impact on the prognostic values of BC patients and was an independent predictor of overall survival when it was adjusted for BC subtypes and to two other FOX genes, *FOXF2* and *FOXMI*.

**Conclusion:** Our study reveals for the first time that *FOXQ1* is differentially expressed across BC subtypes and that low expression of *FOXQ1* is indicative of poor prognosis in patients with BC.

**Keywords:** transcription factors, mRNA expression, copy number variation, predictive marker

## Introduction

Breast cancer (BC) is well known as a highly heterogeneous disease comprising distinct molecular subtypes.<sup>1,2</sup> Each BC subgroup differs from each other in terms of biological characteristics, risk factors, treatment responses, and patient survival outcomes.<sup>1,2</sup> This variability in BC behaviours and characteristics presents major clinical challenges and implications with regard to prognosis and BC management.<sup>3,4</sup> Therefore, much effort has been made to stratify heterogeneous BC subtypes, in order to increase our knowledge of the pathobiology of BC as well as to discover new treatments.<sup>5-8</sup> Typically, BC tumors have been

Correspondence: Michael A Walter  
Department of Medical Genetics, Faculty of Medicine & Dentistry, University of Alberta, 8-32 Medical Sciences Building, Edmonton, AB, T6G 2H7, Canada  
Tel +1 (780) 492-3028  
Email mwalter@ualberta.ca

divided into subtypes according to immunohistochemical (IHC) markers of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), with the combination of differential gene expression.<sup>9</sup> Hence, there are three major subtypes of BC, and these subtypes are luminal (ER positive), triple-negative breast cancer (TNBC), and HER2 BC.<sup>9</sup> Clinicians can determine what therapeutic options are most effective by using well-defined standard IHC markers to identify BC tumor subtype. However, care is now increasingly shifting towards looking at genetic differences to develop more personalized medicine approaches.<sup>10</sup> The Prediction Analysis of Microarray 50 (PAM50) predictor is a multigene signature that stratified traditional pathological classification of BC (TNBC, luminal, HER2) into intrinsic molecular subtypes—luminal A, luminal B, HER2 enriched, normal-like and basal-like (which commonly is TNBC) BC—based on multigene differential expression.<sup>11,12</sup> With this move to precision medicine for cancer treatment, there has been an increasing need to identify new potential biomarkers across the different subtypes of BC.

Forkhead box (FOX) family members are highly conserved transcription factors that have a major role in a plethora of biological functions. These family members have a common DNA-binding domain (DBD) composed of 100 amino acids that is known as the forkhead box or “winged helix” domain.<sup>13</sup> Many FOX transcription factors have been linked to tumorigenesis, where oncogenic and/or tumor-suppressive roles of FOX genes have been suggested in several cancers.<sup>14,15</sup> Members of the FOXA, FOXC, FOXM, FOXO, and FOXP subclasses of FOX proteins, in particular, have direct effects on the initiation, maintenance, progression, and drug resistance of cancers.<sup>16</sup> For example, overexpression of *FOXMI* accelerates the proliferation and progression of prostate cancers in mouse models.<sup>17</sup> The widely studied FOXO proteins are key regulators of tumour suppression, as the simultaneous deletion of *FOXO1*, *FOXO3*, and *FOXO4* alleles in somatic cells invokes thymic lymphomas and systemic haemangiomas in mouse models.<sup>18</sup> Moreover, *FOXF2*, *FOXMI*, *FOXA1*, and *FOXCI* genes were suggested to play critical roles in BC initiation, proliferation, migration, and invasion.<sup>15,19–21</sup> *FOXQ1* is another member of the FOX protein superfamily of transcription factors that regulate the expression of genes necessary for embryonic development, cell proliferation, differentiation, and apoptosis.<sup>22</sup> This single-exon gene is located on

chromosomal region 6p25.3 and its encoded protein is characterized by a distinctive evolutionarily conserved DBD.<sup>23</sup> Functionally, *FOXQ1* plays a role in a diverse range of important biological processes such as angiogenesis, epithelial differentiation, smooth muscle differentiation, mucin secretion, and natural killer cell effector function activation.<sup>13,22,24,25</sup> Moreover, *FOXQ1* has been associated with the aggressive phenotype of cancers such as breast, colorectal, ovarian, and pancreatic cancers, since altered *FOXQ1* expression has been previously detected in human tumor specimens.<sup>26–31</sup> Increasing numbers of studies have examined the role of *FOXQ1* in tumor progression and it has been suggested that suppressing *FOXQ1* expression may decrease invasion and migration in TNBC cell lines.<sup>32,33</sup> *FOXQ1* has also been suggested as potentially being a driving force in the heterogeneity of BC.<sup>6</sup> Thus, additional knowledge of the role of *FOXQ1* in BC could have the potential to improve the diagnosis and treatment of BC tumors. In particular, the role and expression of *FOXQ1* in luminal and HER2 BC needs to be determined.

In this study, we investigated *FOXQ1* mRNA expression and copy number variation (CNV) across BC patient subtypes and cell lines. Furthermore, we assessed the prognostic significance of *FOXQ1* for BC patients. Our results revealed for the first time that *FOXQ1* mRNA is differentially expressed in TNBC, luminal, and HER2 BC patients and cell lines. We found that *FOXQ1* expression is significantly lower in luminal and HER2-positive tumors and that low expression of *FOXQ1* predicts poor overall survival in BC patients. We also found that *FOXQ1* has more copies in tumors from TNBC patients compared to normal breast tissue samples. Interestingly, *FOXQ1* mRNA expression is independent of its CNV in BC cell lines. Our findings highlight that low expression of *FOXQ1* mRNA is associated with significantly poorer survival for different classes of BC.

## Materials and Methods

### Tissue Samples

BC patients' samples were obtained with approval of the University of Alberta Health Research Ethics Board (Pro00018758) with written informed consent, in accordance with the Declaration of Helsinki. The tumor samples from BC patients were collected at surgery and frozen in liquid nitrogen within 20 min of devitalization for further experiments. Normal human breast tissue was obtained from breast

reduction surgery. BC subtypes have been defined using immunohistochemistry (IHC) markers; ER, Estrogen receptor; PR, Progesterone; and HER2, Human Epidermal Receptor (Table 1).

## Overall Survival Analysis

Kaplan-Meier curves were generated using the online analysis tools OncoLnc (<http://www.oncolnc.org>)<sup>34</sup> and Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>).<sup>35</sup> OncoLnc and GEPIA use patient survival data and level 3 RNA sequencing expression data of BC tumor samples from the Cancer Genome Atlas (TCGA) invasive breast carcinoma project (BRCA) database (<https://portal.gdc.cancer.gov/projects/TCGA-BRCA>). Patient samples (n=1006) and (n=1055) from TCGA-BRCA were used to generate the Kaplan-Meier (KM) curves in OncoLnc and GEPIA online tools, respectively. Patient samples in OncoLnc and GEPIA were divided into high and low groups based on the median and the quartile of *FOXQ1* expression in BRCA database. The median cut-off divided patients into two groups, high *FOXQ1* expression group are above the 50th percentile, and low *FOXQ1*

expression group are below the 50th percentile. For the quartile cut-off analysis, patients were categorized into two groups, high *FOXQ1* expression group are above the third quartile (higher than 75th percentile), and low *FOXQ1* expression group are below the first quartile (lower than 25th percentile). More details on the mRNA expression data and their normalization can be found in (<http://www.oncolnc.org>).<sup>34</sup> The long-rank test was used for the hypothesis evaluation. In regard to the censorship method, we used right censoring method in which the patients who did not experience the event (death) during the study duration and had no available further follow-up data were censored. The mean age at diagnosis of the BC patients in TCGA-BRCA database is 58.4± 13.2 years and the median follow-up time is 27.7 months where the median follow-up time for the overall survival time to event is 41.8 months and the median follow-up time for the overall survival time to censor is 25.0 months.<sup>36</sup>

Kaplan-Meier survival curves were generated of *FOXQ1* expression in BC subtypes using UALCAN online tool (<http://ualcan.path.uab.edu/index.html>).<sup>37</sup> TCGA-BRCA patient samples were divided into two groups, high and low *FOXQ1* expression groups. Patients with *FOXQ1* transcript per million (TPM) above the third quartile (higher than 75th percentile) were categorized as high *FOXQ1* expression group, while patients with *FOXQ1* TPM below the third quartile (lower than 75th percentile) were categorized as low/medium *FOXQ1* expression group. Subsequently, patients within each group were further stratified into TNBC, luminal, and HER2 BC subtypes. Log rank test was used to generate the *p* value to test statistical significance of survival correlation between BC subtypes and *FOXQ1* expression. UCSC Xena website: <https://xenabrowser.net/><sup>38</sup> was used to generate Kaplan-Meier curves for the TCGA-BRCA data with a cut-off time after 10 years. Log rank test was used to generate the *p* value to test statistical significance of survival correlation.

SPSS version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used to run the Cox's regression hazard model. *p*-value <0.05 was used for statistical significance. We used the TCGA-BRCA data (<https://portal.gdc.cancer.gov/projects/TCGA-BRCA>) to conduct the univariate overall survival of Cox's regression analysis for *FOXQ1* expression. One thousand six TCGA-BRCA patients data with high and low *FOXQ1* expression with respect to the median and quartile cut-off were downloaded from

**Table 1** BC Patient Demographics

Patient	Age	ER_IHC	PR_IHC	HER2_IHC	Tumor Size(cm)
<b>TNBC</b>					
MT2673	43	Neg	Neg	Neg	2.1
MT2112	67	Neg	Neg	Neg	3.5
MT3624	57	Neg	Neg	Neg	1.1
MT3473	52	Neg	Neg	Neg	1.1
MT3800	60	Neg	Neg	Neg	4.1
MT2881	47	Neg	Neg	Neg	7
<b>Luminal BC</b>					
MT3504	55	Pos	Neg	Neg	2.3
MT3874	56	Pos	Neg	Neg	3.5
MT2348	65	Pos	Neg	Neg	1.9
MT3387	51	Pos	Neg	Neg	1.9
MT3193	53	Pos	Neg	Neg	3.2
MT3756	31	Pos	Neg	Neg	1.8
<b>HER2 BC</b>					
MT3866	59	Neg	Neg	Pos	1.4
MT2151	53	Neg	Neg	Pos	0.8
MT2160	59	Neg	Neg	Pos	0.9
GT363	80	Neg	Neg	Pos	5
MT2520	50	Neg	Neg	Pos	NULL
MT2730	74	Neg	Neg	Pos	1.5

**Abbreviations:** ER, estrogen receptor; PR, progesterone; HER2, human epidermal receptor 2; TNBC, triple negative breast cancer; IHC immunohistochemistry; Neg, negative; Pos, positive; cm, centimeters; Null, size of the tissue samples is small (1–3 mm<sup>3</sup>).

OncoLnc (<http://www.oncolnc.org>).<sup>34</sup> OncoLnc includes only patients who have all the necessary clinical data for each cancer such as age, sex, and grade as well as gene expression. More details on TCGA\_BRCA patient data used for *FOXQ1* Cox regression analysis can be found in [Supplementary Table 1](#). Unadjusted and adjusted Cox's regression hazard model was used to determine the association of *FOXQ1*, *FOXF2*, and *FOXMI* with overall survival, more details can be found in [Supplementary Table 2](#).

Finally, we also used TCGA-BRCA data (<https://portal.gdc.cancer.gov/projects/TCGA-BRCA>) to conduct Cox's regression analysis of *FOXQ1* expression adjusted to BC subtypes. One thousand six BC patient data with high and low *FOXQ1* expression with respect to the median were downloaded. Then, we identified each BC patient subtype as TNBC, luminal, and HER2 as previously described in Ref. 39. More details on the latter analysis can be found in [Supplementary Table 3](#).

## RT-qPCR

RT-qPCR was conducted according to the detailed protocol as previously described.<sup>40</sup> Total RNA was extracted from BC patients' tumor samples and human breast normal tissue using the RNAqueous kit (Ambion, Streetsville, ON, Canada) according to the manufacturer's instruction. cDNA was synthesised from 1 µg RNA using Superscript II reverse transcriptase.<sup>41</sup> RT-qPCR assays were performed with a QuantiTect SYBR Green PCR kit (Applied Biosystems Foster City, CA, United States) and analyzed on a LightCycler 96 Real-Time PCR System (Roche Life Science, Penzberg, Germany), at least three times with each reaction in triplicate. mRNA levels were normalized to *TFRC* (selected as an internal control gene) through the  $\Delta\Delta C_t$  method and changes in mRNA levels were described in fold change compared to the control samples. Primers for *FOXQ1* and *TFRC* were designed using the Primer3 software (<http://primer3.ut.ee/>); *FOXQ1*: F: 5'-CG GAGATCAACGAGTACCTCA-3', R: 5'-CAGTCGTTGA GCGAAAGGTT-3'; *TFRC* F: 5'-AACAACAGATTT CGGGAATGC-3', R: 5'-CGTAGGGAGAGAGGAAG TGATA-3'. For statistical analyses, we first compared *FOXQ1* expression in all four groups; normal breast tissue, TNBC, luminal, and HER2, using one-way ANOVA. Then, one-tailed unpaired Student's t-tests and Bonferroni correction were used for multiple comparisons to assess statistical differences.

## Copy Number Variation

*FOXQ1* gene copy number in BC patients was quantified through Real-time qPCR, as previously described<sup>42</sup> to determine if the altered dosage of *FOXQ1* gene affected expression levels in BC tumors. Genomic DNA was extracted from human breast normal tissue and from TNBC, luminal, and HER2 BC patient tumor samples using the EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's instructions. Real-time qPCR was then conducted on the genomic DNA of all samples to measure *FOXQ1* dosage. PCR reactions for normal breast tissue, TNBC, luminal, and HER2 BC patient tumor samples ([Table 1](#)) were analyzed in triplicate. Normal breast tissue samples were used as a control to normalize *FOXQ1* dosage. We used the  $\Delta\Delta C_t$  method as our quantification strategy, with *GJA5* selected as an internal control gene. Average CT values of triplicates were calculated for each sample.  $\Delta C_t$  for each sample was then calculated by subtracting the average CT number of *FOXQ1* from that of *GJA5*. *FOXQ1*: F: 5'-CGGAGATCAACGAGTAC CTCA-3', R: 5'-CAGTCGTTGAGCGAAAGGTT-3'; *GJA5* F: 5'-AGTTCCTCCAGCCAATAGACAGC-3', R: 5'-AAGGCTGAGTAGAGGGAGGAG-3'. We used a two-tailed unpaired Student's t-tests for comparisons of each BC subtype with normal breast tissues to assess statistical differences.

## K-Means Clustering

K means clustering analysis was performed to investigate and compare *FOXQ1* CNV and its mRNA expression. In order to conduct K-means clustering, we first identified the ideal number of clusters. Our initial approach was to compare the biological ideal number of clusters, which is 3 clusters, based on cell line annotation and classification; TNBC, luminal, and HER2 (supervised K-means clustering). For the unsupervised ideal number of clusters, we used the elbow method<sup>43</sup> to determine the ideal statistical number of clusters for K-means with the scikit-learn's method of the MinMaxScaler module used with a sum of squares distance and fit between 1 and 15 possible clusters.<sup>43</sup> We then devised Python scripts to identify the cluster sets by applying standard supervised K-means clustering using both number of clusters (supervised and unsupervised) and *FOXQ1* mRNA expression and copy number variation (CNV) as seeds as described.<sup>44</sup>



The mRNA and CNV data of *FOXQ1* were obtained from Cancer Cell Line Encyclopedia online (CCLE) database.<sup>45</sup> K-means clustering performed 1000 iterations using the Euclidean distance and using seeds between 1 and 3 clusters and 1 and 4 clusters. Thirty-six BC cell lines were used for the analysis. Cell lines were grouped as TNBC, luminal, and HER2 BC cell lines as described.<sup>46,47</sup> CCLE CNV data of BC cell lines were obtained using genome-wide human Affymetrix SNP Array 6.0.<sup>45</sup> The mRNA data on the CCLE website were produced by Affymetrix Human Genome U133 Plus 2.0 Array followed by Robust Multiarray Averaging (RMA) method of microarray normalization and were uploaded as log(2) gene expression signal.<sup>45</sup> The log(2) *FOXQ1* expression data for each of the 36 BC cell lines from CCLE were obtained and then scaled to values between 0 and 1 using the equation;

$$Z_i = \{x_i - \min(x)\} / \{\max(x) - \min(x)\}$$

where  $x = (x_1, \dots, x_n)$  producing  $z_i$  as the scaled value for *FOXQ1* mRNA expression in each of the 36 BC cell lines. CNV raw data created by Affymetrix (CEL file) were normalized to copy number estimates using a GenePattern pipeline.<sup>45</sup> 0.5 was used as a cut-off, where cell lines with values above the cut-off were considered as relative high expression of *FOXQ1* cell lines, while cell lines with *FOXQ1* expression below the cut-off were considered as relative low expression of *FOXQ1* cell lines.

## Breast Cancer Patient Publicly Available Dataset

RNAseq data for *FOXQ1* expression in breast cancer patients from the TCGA-BRCA (<https://portal.gdc.cancer.gov/projects/TCGA-BRCA>) were obtained from UCSC Xena website: <https://xenabrowser.net/><sup>38</sup> to generate Boxplots analysis across breast cancer subtypes. One-way ANOVA was applied to generate the  $p$  value to test statistical significance. Microarray data of *FOXQ1* expression in breast cancer patients from the Van't Veer<sup>48</sup> study were accessed using UCSC Xena website: <https://xenabrowser.net/>.<sup>38</sup> Welch's  $t$ -test was applied to generate  $p$  values to test statistical significance.

*FOXQ1* mRNA expression correlation with each of *PDGFRα* and  $\beta$ , *Twist1*, *Zeb2*, and *DACH1* mRNA expression was analyzed in the TCGA-BRCA database using the co-expression analysis tool on cBioPortal.<sup>96,97</sup> Spearman and Pearson were used for

correlation analysis. *FOXQ1*, *PDGFRα* and  $\beta$ , *Twist1*, *Zeb2*, and *DACH1* log2 mRNA values of normalized RSEM RNAseq V2<sup>49</sup> in 994 patient and normal samples were obtained from the PanCanAtlas 2018 of the TCGA-BRCA <https://gdc.cancer.gov/about-data/publications/pancanatlas>.<sup>50</sup>

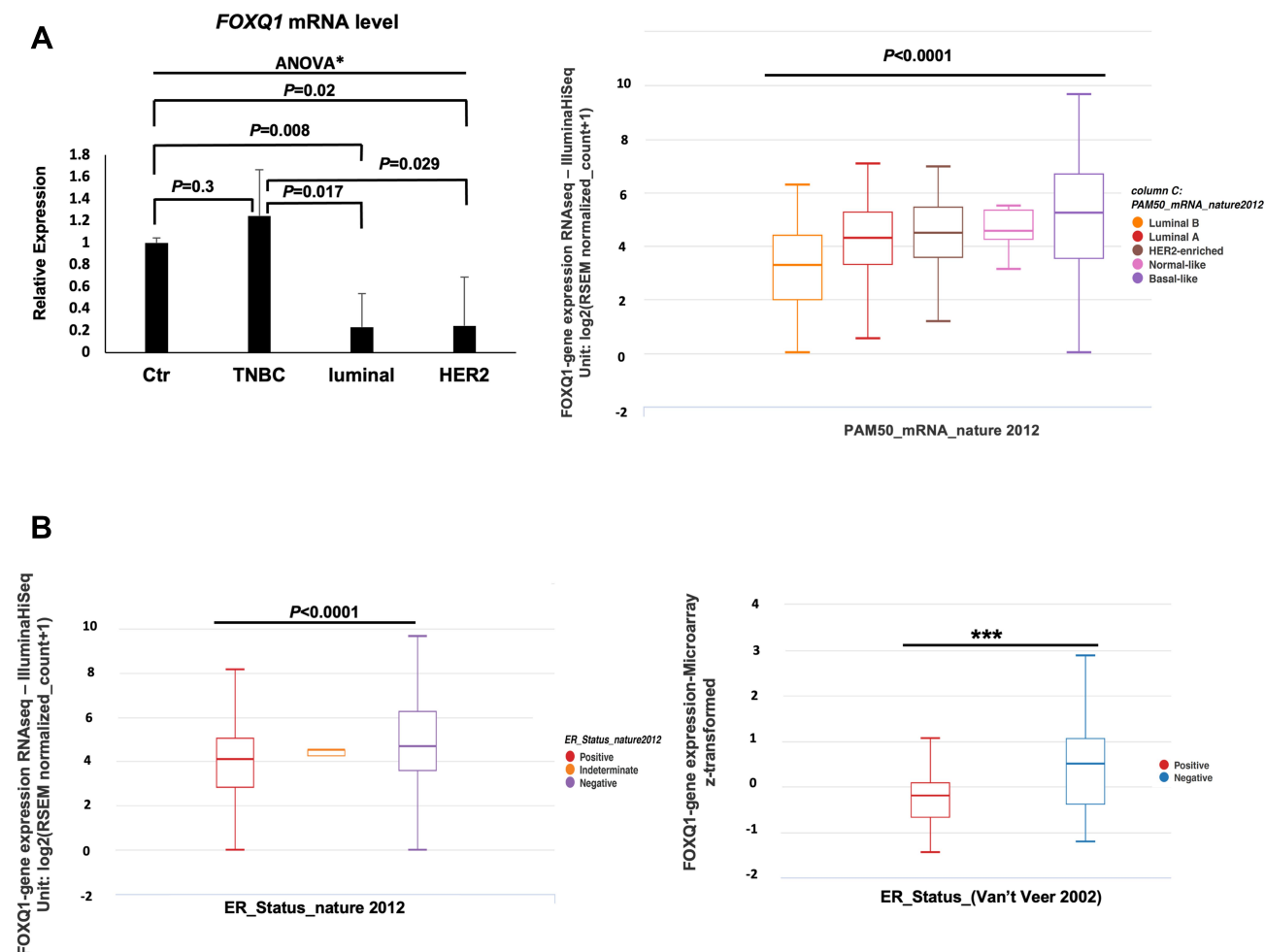
## Results

### *FOXQ1* mRNA Expression Varies Between BC Patient Subtypes

To evaluate *FOXQ1* expression across the different subtypes of BC, RT-qPCR assays were conducted on TNBC, luminal, and HER2 BC patient samples and on normal breast tissue samples (Table 1). One-way ANOVA analysis showed that *FOXQ1* mRNA expression varied between BC patients' subtypes,  $F(30,52) = 3.8$ ,  $p=0.026$ . Furthermore, we found that *FOXQ1* expression was significantly lower in luminal BC when it was compared to normal breast tissue (Figure 1A). The mRNA level of *FOXQ1* was lower in HER2 BC compared to normal breast tissue ( $p=0.02$ ) (Figure 1A). Interestingly, *FOXQ1* mRNA levels were lower in luminal and HER2 BC compared to TNBC ( $p=0.017$  and  $p=0.029$ , respectively) (Figure 1A). We further confirmed our results in an expanded panel of BC patients ( $n = 522$ ) using a publicly available dataset from the Cancer Genome Atlas (Figure 1A). Consistent with our RT-qPCR, we found that the expression of *FOXQ1* mRNA varied across BC patient subtypes (Figure 1A,  $F = 24.64$ ,  $p<0.0001$ ). To substantiate our findings of significant low expression of *FOXQ1* in luminal BC, we further investigated *FOXQ1* expression in a public dataset of BC patients after they were stratified by ER status into ER positive and ER negative. Consistent with our RT-qPCR, we found that the expression of *FOXQ1* was significantly lower in ER-positive BC patients compared to ER-negative BC patients (Figure 1B). These results indicate that *FOXQ1* mRNA levels are differentially expressed among BC patients and that *FOXQ1* could potentially have different roles based on the BC subtype.

### Low Expression of *FOXQ1* is Associated with Poorer Overall Survival in Breast Cancer Patients

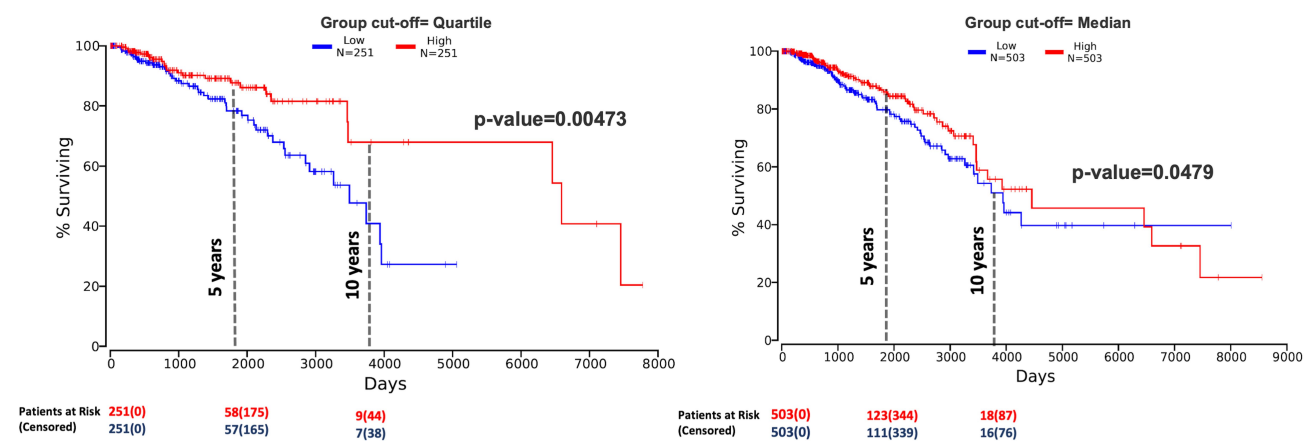
Kaplan-Meier curves were performed on the TCGA-BRCA dataset using the OncoLnc,<sup>34</sup> GEPIA,<sup>35</sup> and UALCAN<sup>37</sup> online tools to investigate whether *FOXQ1* mRNA expression is prognostic for life expectancy. BC patients were divided into



**Figure 1** FOXQ1 is differentially expressed across breast cancer subtypes and is under-expressed in luminal and HER2 breast cancer patient samples. **(A)** Left, qPCR experiments were conducted to measure FOXQ1 mRNA levels in normal breast tissue (Ctr, n=6) acquired from reduction mammoplasties, TNBC (n=6), luminal (n=6), and HER2 (n=6) BC patient samples. One-way ANOVA analysis was used to assess statistical difference in all groups (\*F(30,52) = 3.8, p=0.026 followed by unpaired t-tests and Bonferroni corrections were used for multiple comparisons.  $\alpha = 0.05$ , adjusted  $\alpha = 0.0125$ . The  $\Delta\Delta CT$  method was used for analysis and TFRC was used as a reference gene. Error bars represent standard error of the mean (SEM). **(A)** Right, box plots showing FOXQ1 expression across BC intrinsic subtypes (PAM50). FOXQ1 RNAseq data in BC subtypes from the TCGA-BRCA data<sup>95</sup> were used. Luminal B (orange), n = 127, Minimum = 0.00, Q1 = 2.0, Median = 3.29, Q3 = 4.40, Maximum = 6.26; Luminal A (red), n = 231, Minimum = 0.527, Q1 = 3.31, Median = 4.32, Q3 = 5.26, Maximum = 7.08; HER2-enriched (brown), n = 58, Minimum = 1.17, Q1 = 3.58, Median = 4.50, Q3 = 5.44, Maximum = 6.94; Normal-like (pink), n = 8, Minimum = 3.13, Q1 = 4.25, Median = 4.58, Q3 = 5.35, Maximum = 5.49; Basal-like (purple), n = 98, Minimum = 0.00, Q1 = 3.54, Median = 5.25, Q3 = 6.68, Maximum = 9.65. One-way ANOVA was used for statistical significance (F = 21.25, p<0.0001). FOXQ1 expression is expressed in RSEM (RNA-seq by expectation-Maximization) format. **(B)** Left, box plot of FOXQ1 RNAseq data in BC patients stratified by ER status. ER-positive (red), n = 601, Minimum = 0.00, Q1 = 2.82, Median = 4.12, Q3 = 5.05, Maximum = 8.13; ER-intermediate (orange), n = 2, Minimum = 4.24, Q1 = 4.24, Median = 4.50, Q3 = 4.50, Maximum = 4.50; ER-negative (blue), n = 179, Minimum = 0.00, Q1 = 3.58, Median = 4.68, Q3 = 6.26, Maximum = 9.65. One-way ANOVA was used for statistical significance (F = 24.64, p<0.0001). **(B)** Right, box plot of microarray data of FOXQ1 expression in BC patients stratified by ER status from the Van't Veer et al<sup>48</sup> study. ER-positive (red), n = 71, Minimum = -1.46, Q1 = -0.67, Median = -0.20, Q3 = 0.08, Maximum = 1.06; ER-negative (blue), n = 46, Minimum = -1.22, Q1 = -0.38, Median = 0.5, Q3 = 1.06, Maximum = 2.87. Welch's t-test was used for statistical significance (\*\*\*p<0.0001). Whiskers represent minimum and maximum values. The TCGA-BRCA<sup>95</sup> and the Van't Veer et al<sup>48</sup> data were accessed using UCSC XENA cancer genome browser: <https://xenabrowser.net/>.

two risk groups with high and low FOXQ1 expression levels with respect to the quartile and the median used as the cut-off values. BC patients with low FOXQ1 mRNA expression had significantly shorter overall survival time compared to those with high FOXQ1 mRNA expression (Figure 2, p=0.00473 and p=0.0479) and (Supplementary Figure 1, p=0.0087 and p=0.042), and after 10 years follow-up (Supplementary Figure 2).

In addition, a univariate Cox's regression analysis was conducted for overall survival on the TCGA-BRCA patient data with respect to the expression of FOXQ1. The results of the univariate Cox's regression analysis in Table 2 indicate that BC patients who have high FOXQ1 expression ( $\geq 50$ th percentile) were significantly associated with 29% lower risk of death as compared to BC patients who have low FOXQ1 expression (p=0.048). Further



**Figure 2** Low *FOXQ1* expression predicts poor overall survival in BC patient. Kaplan-Meier (KM) analysis identified low and high-risk BC patient groups based upon significant differences of *FOXQ1* mRNA in BRCA-TCGA database. BC patients were divided with high and low *FOXQ1* expression levels with respect to the quartile and the median used as the cut-off value. This graph was generated using the bioinformatics online tool OncoLnc (<http://www.oncolnc.org>)<sup>34</sup> and then modified to include the number of patients at risk at 0, 5 and 10 years.

analysis was conducted, comparing the highest quartile and the lowest quartile of *FOXQ1* expression. The results indicate that *FOXQ1* high expression ( $\geq 75$ th percentile) was significantly associated with about 51% lower risk of BC patient's death as compared to the low *FOXQ1* expression (below 25th percentile) with a *p*-value of 0.006 (Table 2).

Furthermore, using UALCAN online tool, we examined the correlation of *FOXQ1* expression and BC subtypes by dividing patients into two groups; high and low/medium *FOXQ1* expression cohorts (Figure 3). Kaplan-Meier curves indicate that patients with low/medium *FOXQ1* expression had significantly shorter overall survival time than those with high expression of *FOXQ1* (Figure 3, *p*=0.011). Subsequently, using the TCGA-BRCA data, we conducted Cox's regression analysis for *FOXQ1* expression stratified by BC subtype as well as for *FOXQ1* expression adjusted for BC subtype (Table 3). For HER2 subtype, high *FOXQ1* expression was significantly associated with about 73% lower risk of death as compared to low *FOXQ1* expression (Table 3, *p*=0.024). The same trend was observed for luminal and TNBC subtypes indicating that high *FOXQ1* expression showed a reduced risk of death by 19% and

32%, respectively. However, these associations were not statistically significant (Table 3).

When adjusting for BC subtype, high *FOXQ1* expression showed a 32% lower risk of death (Table 3, *p*=0.036). The unadjusted analysis from Table 2 and adjusted analysis from Table 3 indicate that *FOXQ1* is an independent predictor of overall survival in BC patients. Together, these results demonstrate that low expression of *FOXQ1* predicts poor overall survival in patients with BC.

## Relation of *FOXQ1* Copy Number in BC Subtypes to mRNA Levels

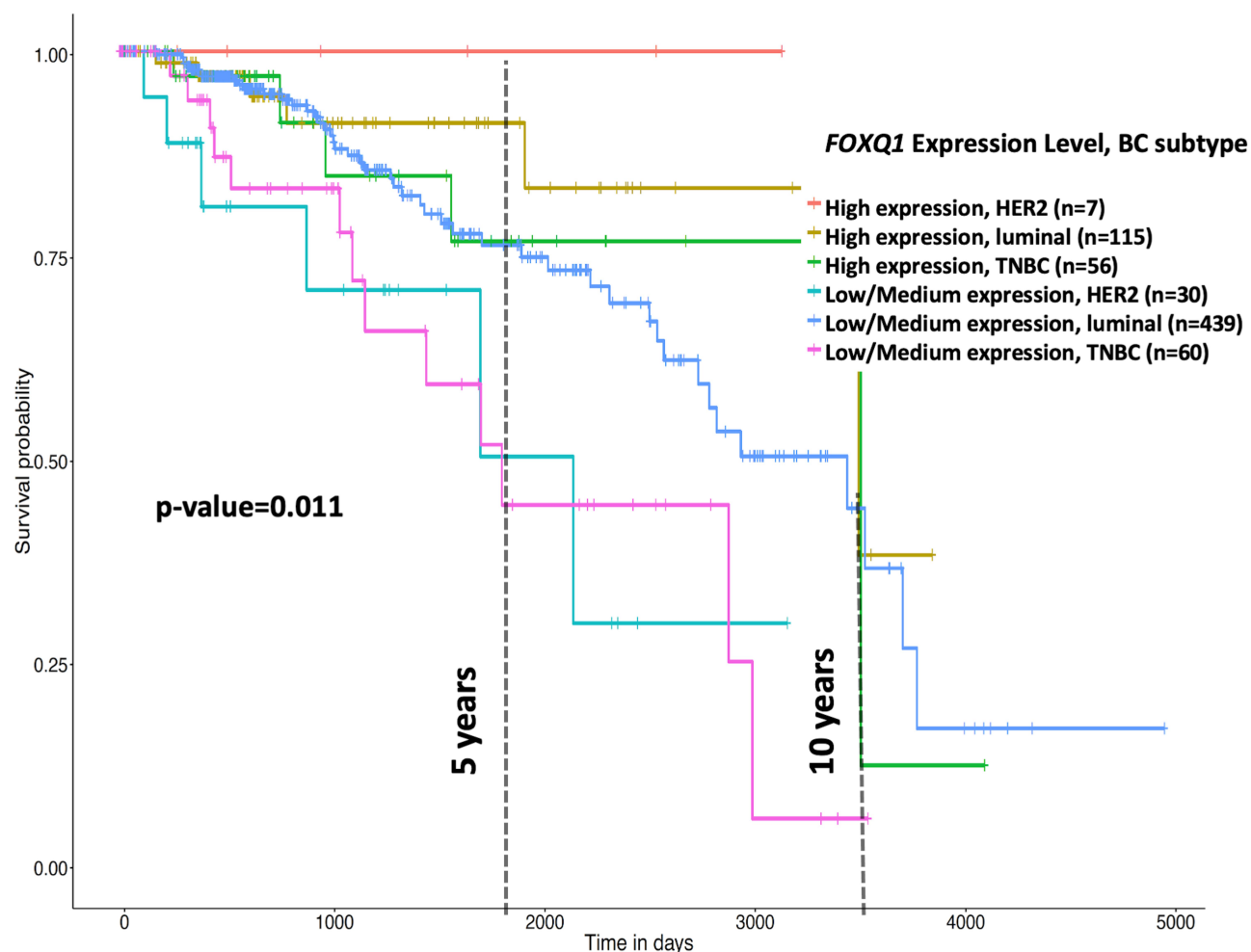
It has recently been suggested that CNV correlates with gene expression in BC cell lines and patient tissue.<sup>51</sup> To determine *FOXQ1* CNV in the subtypes of BC patients, qPCR assays were conducted on TNBC, luminal, and HER2 BC patient DNA samples (Table 1) and on normal breast tissue DNA samples. The number of copies of *FOXQ1* was significantly higher in TNBC patients compared to normal breast tissue *p*=0.03 (Figure 4). Although *FOXQ1* appeared to have a trend for more copies in HER2 BC patients, this did not reach statistical significance (Figure 4, *p*=0.08).

**Table 2** Hazard Ratios from the Univariate Cox's Regression Analysis for the TCGA-BRCA Database

Variable	Median Cut-Off				Quartile Cut-Off			
	$\beta$ Estimate	HR	95% CI	<i>p</i> -value	$\beta$ Estimate	HR	95% CI	<i>p</i> -value
<i>FOXQ1</i> expression	-0.344	0.709	(0.504–0.999)	<b>0.049</b>	-0.712	0.49	(0.296–0.812)	<b>0.006</b>

**Note:** Bold values are statistically significant.

**Abbreviations:** HR, hazard ratio; CI, confidence intervals;  $\beta$  estimate, coefficient.



**Figure 3** Low *FOXQ1* expression predicts poor overall survival in BC patient subtypes. Kaplan-Meier (KM) analysis of the effect of high and low/medium *FOXQ1* expression on overall survival of HER2, luminal, and TNBC patients shows a cumulative significance of  $p=0.011$ . This graph was generated and modified using the bioinformatics online tool UALCAN online tool (<http://ualcan.path.uab.edu/index.html>)<sup>37</sup>.

Unsupervised and supervised K-means clustering analyses (Table 4 and Figure 5) were performed to identify clusters of BC cell lines as well as to investigate any associations between *FOXQ1* mRNA expression and gene copy number. Unsupervised K-means clustering identified 4 clusters among BC cell lines for *FOXQ1* mRNA compared to their copy number (Table 4 and Figure 5A).

We found some cell lines clusters that have similar copy number (orange cluster vs red cluster, Figure 5A) but different mRNA expression (Figure 5A). We also found similar results after we conducted supervised K-means clustering, where some cell line clusters had high and/or low *FOXQ1* mRNA expression but similar ranges of variation in relative copy number (Figure 5B). For the supervised K-means clustering, we chose 3 as the number of clusters to correspond to the 3 different

subtypes of BC; TNBC, luminal, and HER2 (see K-means clustering in method's section). Interestingly, unsupervised and supervised K-means clustering analyses suggest that the steady state level of *FOXQ1* mRNA expression appears to be independent of gene copy number in BC cell lines. Thus, *FOXQ1* mRNA expression levels are not correlated with *FOXQ1* CNV in BC subtypes. However, consistent with our results where *FOXQ1* expression was lower in luminal and HER2 BC patient tumors (Figure 1), we found that *FOXQ1* expression was low in all luminal and HER2 cell lines (Figure 5A and B). Similar to our observation of increased expression of *FOXQ1* in TNBC patient tumors compared to luminal and HER2 (Figure 1), we found that *FOXQ1* has elevated expression in some TNBC cell lines as compared to luminal and HER2 BC cell lines (Figure 5A and B). Interestingly, we found numerous TNBC cell lines cluster



**Table 3** Cox's Regression Analysis for *FOXQ1* Expression Based on Median Cut-off Stratified by BC Subtypes and Adjusted for BC Subtypes

Variables	$\beta$ Estimate	HR	95% CI	p-value
<b>HER2 subtype (n=77)</b> <i>FOXQ1</i> (Low Expression) High Expression	-1.31	0.27	(0.09–0.84)	<b>0.024</b>
<b>Luminal subtype (n=707)</b> <i>FOXQ1</i> (Low Expression) High Expression	-1.21	0.81	(0.53–1.25)	0.341
<b>TNBC subtype (n=175)</b> <i>FOXQ1</i> (Low Expression) High Expression	-0.39	0.68	(0.28–1.62)	0.383
<b>Adjusted for BC subtypes</b> <i>FOXQ1</i> (Low Expression) High Expression	-0.39	0.68	(0.47–0.98)	<b>0.036</b>

**Notes:** Bold values are statistically significant. The reference category is presented in the parentheses.

**Abbreviations:** HR, hazard ratio; CI, confidence intervals;  $\beta$  estimate, coefficient.

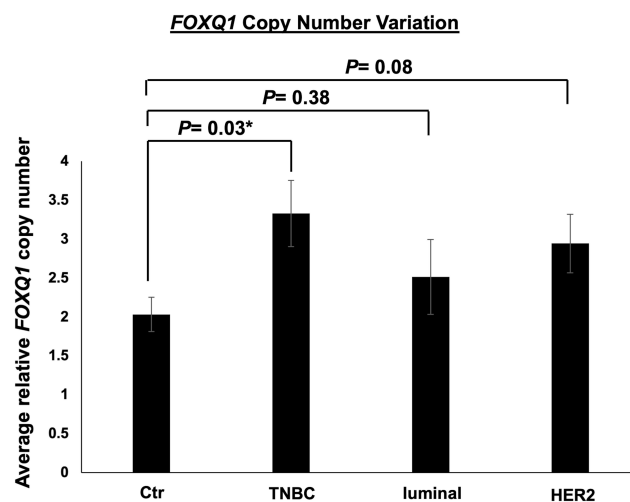
with other luminal and/or HER2 cell lines and have low *FOXQ1* expression. This difference of *FOXQ1* mRNA expression within the TNBC classification might be attributed to the distinct subgroups (basal A and basal B) that exists within TNBC cell lines.<sup>47</sup>

### *FOXQ1* Expression is an Independent Predictor of Overall Survival in BC Patients When Adjusted to *FOXF2* and *FOXMI* Expression

We also investigated the overall survival of *FOXQ1* adjusted for *FOXF2* and *FOXMI* expression. We evaluated *FOXF2* and *FOXMI* genes due to their critical role in BC initiation, proliferation, migration, and invasion.<sup>19,20,52</sup>

TCGA-BRCA patients were divided into low and high expressing *FOXF2*, *FOXMI*, and *FOXQ1* groups using the medians as cut-off values, followed by a univariate and multivariate Cox's regression overall survival analysis. *FOXMI* high expression was associated with 1.5 times higher risk of BC patient death as compared to *FOXMI* low expression values ( $p=0.013$ ; Table 5). Adjusted Cox's regression model with *FOXQ1*, *FOXMI*, and *FOXF2* expression levels indicated that *FOXQ1* and *FOXMI* were significantly associated with overall survival (Table 5;  $p=0.05$  and  $p=0.011$ , respectively). The results indicate that *FOXQ1* and *FOXMI* were independent predictors of overall survival in BC patients. Having high expression of *FOXQ1* was associated with 32% lower risk for overall

survival when adjusted for *FOXF2* and *FOXMI* ( $p=0.05$ ; Table 5). Higher expression of *FOXMI* was associated with 1.6 times higher risk of death when adjusted for *FOXQ1* and *FOXF2* expressions, this association was statistically significant ( $p=0.011$ ; Table 5). Our results show that *FOXQ1* expression correlates inversely with overall survival in BC patients and that *FOXQ1* expression is an independent predictor of BC overall survival when



**Figure 4** *FOXQ1* has more copies in TNBC compared to control samples. qPCR experiments were conducted on genomic DNA to measure *FOXQ1* dosage in TNBC, luminal, and HER2 BC patient tumor tissues. The  $\Delta\text{CT}$  method was used for analysis and *GJA5* was used as a reference gene. *FOXQ1* dosage was calculated using  $2^{[\Delta\text{CT sample} - \Delta\text{CT control}]}$ . Control (Ctrl) samples are normal breast tissue samples acquired from reduction mammoplasties. Ctrl (n=6), TNBC (n=6), luminal (n=6), and HER2 (n=6). Two-tailed unpaired Student's t-tests were used for comparisons of each BC subtype with normal breast tissue to assess statistical differences. Error bars represent standard error of the mean (SEM). \* $p<0.05$ .

**Table 4** BC Cell Line Names and Types That Were Used for Standard K-Means Clustering

BC Cell Line Subtype	FOXQ1 Relative Expression	FOXQ1 Relative CNV	Unsupervised K-Means Clusters	Supervised K-Means Clusters
<b>TNBC</b>				
CAL-148	0.046847	-0.1		
DU4475	0.071856	-0.1		
CAL-51	0.047552	0		
HCC1395	0.093519	0.41		
BT-549	0.036633	0.04		
HCC1806	1	0.49		
MDA-MB-231	0.71962	0.41		
CAL-120	0.550194	0.29		
CAL-851	0.63156	0.28		
HDQ-PI	0.797992	0.20		
HCC38	0.344311	0.51		
Hs 578T	0.373547	0.24		
HCC1187	0.327932	0.3		
HCC1143	0.686685	-0.15		
HCC70	0.713632	-0.09		
HCC1937	0.775097	-0.48		
MDA-MB-436	0.551427	-0.33		
MDA-MB-157	0.037161	0.02		
HCC2157	0.058471	-0.82		
BT-20	0.057591	-0.67		
<b>Luminal</b>				
CAMA-1	0.031349	0.08		
MDA-MB-415	0.070447	-0.16		
EFM-192A	0.034519	0.18		
BT-483	0.068158	-0.05		
KPL-1	0.095104	-0.15		
MCF-7	0.024833	0		
MDA-MB-175	0.435717	-0.24		
MDA-MB-134	0	-0.01		
HCC1428	0.054773	-0.48		
<b>HER2</b>				
MDA-MB-453	0.023248	-0.07		

(Continued)

Table 4 (Continued).

BC Cell Line Subtype	FOXQ1 Relative Expression	FOXQ1 Relative CNV	Unsupervised K-Means Clusters	Supervised K-Means Clusters
HCC1569	0.016555	-0.08		
HCC2218	0.027474	-0.03		
HCC202	0.012328	0.14		
HCC1954	0.441881	0.09		
JMT-1	0.447693	-0.22		
AU565	0.054773	-0.54		

**Notes:** Cell line types were sorted as described in.<sup>46,47</sup> The mRNA expression and CNV of *FOXQ1* in 36 BC cell lines were obtained from CCLE online database.<sup>45</sup> Cell lines with no defined subtypes were not considered for consistency. Four clusters were used for unsupervised K-means clustering, while 3 clusters were used for supervised clustering. For supervised and unsupervised clustering, cell lines within each column that have the same color are grouped together. These clusters and data are plotted in Figure 5.

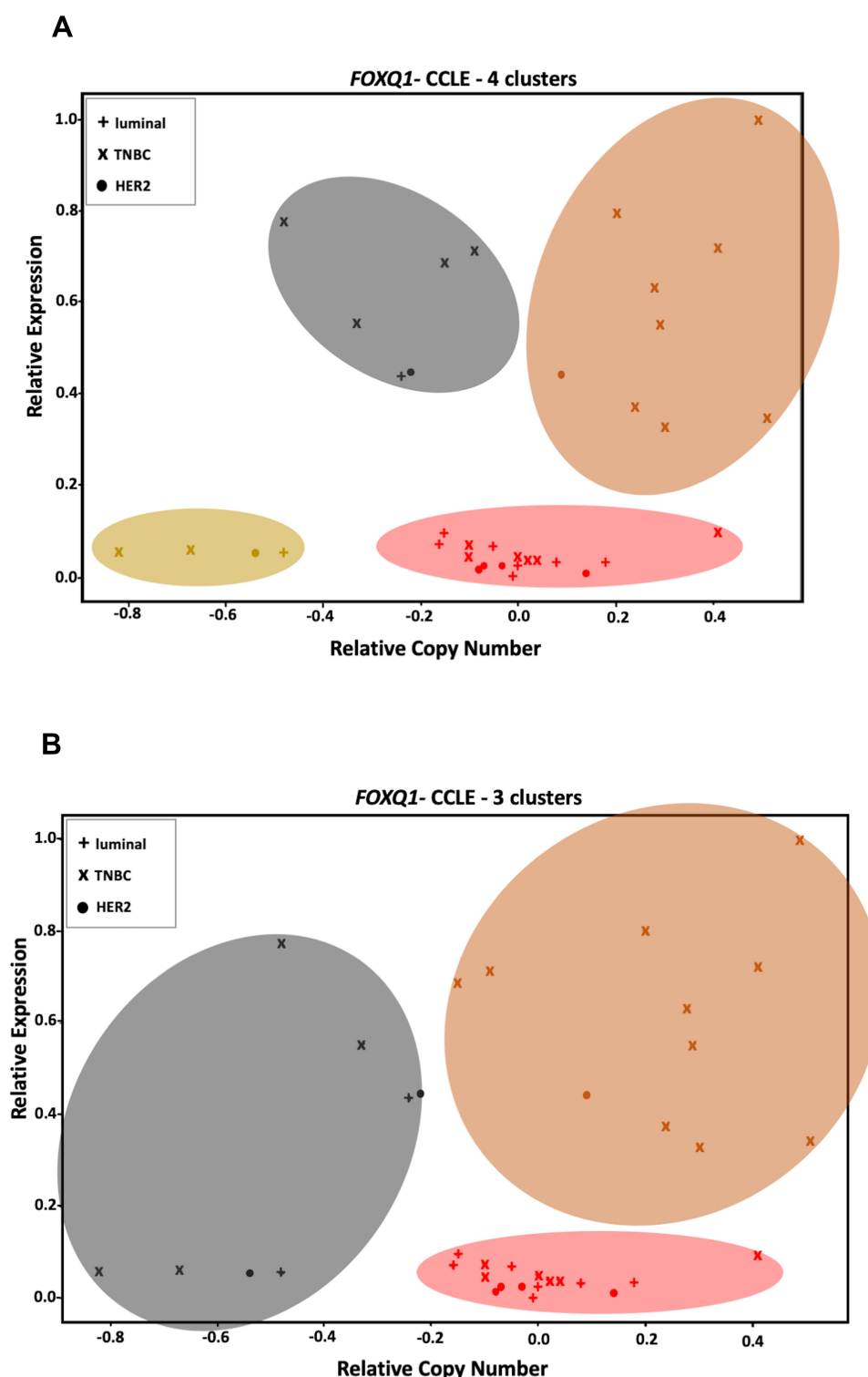
adjusted to the expression of *FOXF2* and *FOXMI* in BC patients.

## Discussion

*FOXQ1* plays an important role in BC tumor re-initiation,<sup>53</sup> stemness and chemoresistance,<sup>54</sup> epithelial-mesenchymal transition (EMT), invasion, and metastasis.<sup>28</sup> Increasing evidence supports the hypothesis that *FOXQ1* is a biomarker for cancer prognosis and diagnosis. Indeed, high levels of *FOXQ1* expression can predict poor overall survival in hepatocellular carcinoma, gastric, colorectal, pancreatic, and non-small cell lung cancers.<sup>29,55-57</sup> However, the prognostic value of *FOXQ1* in BC is not well known. In particular, the expression of *FOXQ1* in other subtypes of BC, specifically luminal and HER2 BC, is not well studied. Thus, a molecular understanding of the mechanism of altered expression of *FOXQ1* warrants further investigation. In this study, we report for the first time that *FOXQ1* mRNA is differentially expressed across different types of BC patients and cell lines. In contrast to the earlier studies of a limited number of BC cell lines,<sup>28,30,32,58</sup> our studies of an expanded panel of thirty-six BC cell lines show that *FOXQ1* has low expression in many TNBC cell lines and in all luminal, HER2 BC patients and cell lines. We also show that this low expression of *FOXQ1* is associated with poor prognosis in BC subtypes and that *FOXQ1* expression is an independent predictor of overall survival in BC patients.

In order to understand *FOXQ1* expression across BC subtypes, we investigated *FOXQ1* expression in BC tumor tissues (Table 1). *FOXQ1* expression across BC subtypes was analyzed in TNBC, luminal, and HER2 tumor tissues (Figure 1A). Two previous papers<sup>6,30</sup> studied *FOXQ1* expression data of BC tumors from the TCGA-BRCA database and

found an overexpression of *FOXQ1* in TNBC compared with luminal BC<sup>30</sup> and with HER2 BC.<sup>6</sup> Similarly, it was suggested that *FOXQ1* is overexpressed in BC tumor tissue compared with normal adjacent tissue;<sup>32</sup> however, it was not clear what BC subtypes were used for this analysis. Consistent with these studies, we found that *FOXQ1* mRNA levels were higher in TNBC tumor tissues when compared with luminal BC tumor tissues ( $p=0.017$ ) (Figure 1A). As well, *FOXQ1* mRNA levels were higher in TNBC compared with HER2 BC ( $p=0.029$ ) (Figure 1A). Importantly, we reveal for the first time that *FOXQ1* is expressed at lower levels in luminal BC ( $p=0.008$ ) and in HER2 BC ( $p=0.029$ ) when each subtype was compared with unmatched normal breast tissue (Figure 1A). A limitation of our RT-qPCR analysis was the lack of available samples of matched normal breast tissue. However, our RT-qPCR analysis results were substantiated by our analyses of publicly available data from the Cancer Genome Atlas (Figure 1A and B). Interestingly, we found lower expression of *FOXQ1* in luminal B than in luminal A (Figure 1A). Luminal B has been suggested to have higher levels of proliferation and cell cycle markers genes, but lower luminal markers compared to luminal A.<sup>59-61</sup> In addition, luminal A has a good prognosis compared to luminal B, where the latter has a higher recurrence rate, lower overall survival rate, and more invasive and proliferative cancers.<sup>62,63</sup> Whether *FOXQ1* has a subtype-specific tumor-suppressive role in luminal BC, in particular in luminal B similar to its neighbour gene, *FOXCI*,<sup>64</sup> has yet to be determined. Nevertheless, our results reveal that significant differences in *FOXQ1* expression occur in BC subtypes, with high *FOXQ1* expression in TNBC and low *FOXQ1* expression in luminal and HER2.



**Figure 5** *FOXQ1* expression is independent of its CNV in BC cell lines. (A) unsupervised and (B) supervised K-means clustering analyses show different clusters of BC cell lines that have similar ranges of CNV but different *FOXQ1* expression (the orange cluster vs the red cluster). Unsupervised clustering identified an extra cluster in BC cell lines compared to supervised clustering (the olive cluster, lower left).

We further investigated the impact of *FOXQ1* expression on BC patients' overall survival. Using the OncoLnc,<sup>34</sup> and GEPIA<sup>35</sup> online tools, overall survival curves identified two

risk groups with high and low *FOXQ1* mRNA expression levels (Figure 2 and [Supplementary Figures 1 and 2](#)). We found that BC patients with low *FOXQ1* expression had



**Table 5** Cox's Regression Analysis for *FOXQ1*, *FOXF2*, and *FOXM1* Expression Levels from the TCGA-BRCA dataset Based on Median Cut-off

	Univariate				Multivariate			
Variables	$\beta$ Estimate	HR	95% CI	p-value	$\beta$ Estimate	HR	95%	p-value
<i>FOXQ1</i> expression	-0.344	0.709	(0.504–0.999)	<b>0.049</b>	-0.389	0.678	(0.459–1.001)	<b>0.05</b>
<i>FOXF2</i> expression	-0.221	0.802	(0.570–1.127)	0.204	0.062	1.064	(0.716–1.583)	0.758
<i>FOXM1</i> expression	-0.437	1.548	(1.096–2.188)	<b>0.013</b>	0.465	1.591	(1.112–2.278)	<b>0.01</b>

**Note:** Bold values are statistically significant.

**Abbreviations:** HR, hazard ratio; CI, confidence intervals;  $\beta$  estimate, coefficient.

significantly shorter overall survival than BC patients with high *FOXQ1* expression (Figure 2, and Supplementary Figures 1 and 2). Univariate Cox regression analysis for *FOXQ1* expression, where BC patients who have high *FOXQ1* expression were found to be significantly associated with lower risk of death as compared to BC patients who have low *FOXQ1* expression (Table 2). Kaplan-Meier curves also showed that TNBC, luminal, and HER2 BC patients with low *FOXQ1* expression have significantly poorer overall survival compared to TNBC, luminal, and HER2 BC patients with high *FOXQ1* expression (Figure 3). When adjusting for BC subtype, high *FOXQ1* expression showed significantly lower risk of death (Table 3) indicating that *FOXQ1* is an independent predictor of overall survival in BC patients. This suggests that low expression of *FOXQ1* in BC patients is significantly correlated with poor prognosis.

Importantly, we found *FOXQ1* has lower expression in HER2 BC patient tissues (Figure 1A) and that HER2 BC patients with low *FOXQ1* expression had significantly shorter overall survival compared to HER2 BC patients with high *FOXQ1* expression (Table 3, Figure 3). This is an interesting result giving that HER2 BC, in comparison to other BC subtypes, is normally known to respond well to numerous targeted therapies that favourably impact patients overall survival.<sup>65</sup> On the other hand, TNBC lacks targeted therapy and is considered to have a clinically aggressive phenotype compared to other BC subtypes.<sup>66</sup> Although TNBC patient tissue had higher *FOXQ1* mRNA expression compared to luminal and HER2 (Figure 1A), those TNBC patients with low expression of *FOXQ1* appeared to have poorer overall survival compared to those with high *FOXQ1* expression (Table 3 and Figure 3). This suggests that, clinically and case-by-case, BC patients in the TNBC cohort who have low *FOXQ1* expression could have poorer clinical outcomes. Further studies that focus on stratifying the TNBC subtype

further using gene signatures and cancer stages could reveal better understating of *FOXQ1* expression in TNBC.

Intriguingly, while it has been reported that high *FOXQ1* expression predicts poor overall survival in hepatocellular carcinoma, gastric, colorectal, pancreatic, and non-small cell lung cancers,<sup>29,55–57</sup> we found that high expression of *FOXQ1* favourably impacts overall survival in BC (Tables 2 and 3), suggesting a dual roles of *FOXQ1* across human cancers. Dual role of *FOXQ1* and other FOX genes in cancers has been reported before. For instance, it was suggested that the ectopic expression of *FOXQ1* promotes tumour growth in colorectal cancer by inhibiting apoptosis.<sup>29</sup> At the same time, *FOXQ1* over-expression did not impact BC cell survival and/or proliferation in the highly metastatic 4T1 mouse cell line and it induced cell death in epithelial BC cell lines, MCF7 (labeled as luminal BC cell line) and BT-20 (labeled as TNBC cell line)<sup>28</sup> supporting a tumor-suppressive role of *FOXQ1*. High expression of *FOXF2* enhanced EMT, migration and invasion of lung cancer cells<sup>67</sup> and in TNBC,<sup>68</sup> which directly contrasts other reports where low expression of *FOXF2*-induced EMT and was associated with poor overall survival in BC.<sup>19</sup> Furthermore, high expression of *FOXA1* was associated with poor overall survival in prostate cancer,<sup>69</sup> while high expression of the same protein favourably impacted BC prognosis.<sup>70,71</sup> In addition, *FOXA1* activated luminal genes and repressed basal genes in ER-positive and ER-negative BC cells, respectively,<sup>72</sup> indicating different roles of *FOXA1* in different BC subtypes. This further highlights the complex and differing roles of FOX proteins in cancer. Our data also contrast the findings of Qiao et al who suggested that overall survival was significantly poorer in BC patients with high *FOXQ1* expression.<sup>58</sup> Qiao et al<sup>58</sup> used the van de Vijver cohort to generate Kaplan-Meier plots of their overall survival analysis but the number of patients within that cohort who had high and low levels of *FOXQ1* was not indicated.<sup>58</sup> Moreover, Van de Vijver et al<sup>73</sup> included microarray data of 295 BC tumors, some

of which were lymph-node/ER-positive and some of which were lymph-node/ER-negative. OncoLnc,<sup>34</sup> GEPIA,<sup>35</sup> and UALCAN,<sup>37</sup> on the other hand, use up-to-date RNA sequencing data of the TCGA-BRCA database. In our study, we applied OncoLnc and GEPIA (respectively) to obtain Kaplan-Meier curves on BC tumors (n=1006 and n=1055), with high (n=503 and n=524), and low (n=503 and n=531) levels of *FOXQ1* expression (Figure 2 and Supplementary Figure 1). The TCGA-BRCA data were also used to perform the adjusted and unadjusted Cox regression (see methods and Supplementary Tables 1, 2, and 3). The differences in numbers of BC tumor tissue samples, BC subtypes, and assays used could explain why we obtained different clinical outcomes of *FOXQ1* expression compared to Qiao et al<sup>58</sup>.

We also measured *FOXQ1* CNV in BC samples (Table 1) to determine if altered copy number could underlie the differences in mRNA expression levels in TNBC, luminal, and HER2 tumors (Figure 4). Although there are numerous studies that have identified an association between copy number alteration and altered gene expression, how CNVs alter gene expression in BC is not well understood.<sup>51,74,75</sup> We found that *FOXQ1* copy number is significantly amplified in TNBC compared with normal breast tissue (Figure 4). In contrast, no significant changes in *FOXQ1* copy number were found in luminal and HER2 BC compared to control samples (Figure 4). To explore the possible ramifications of these findings further, we performed K-means clustering to investigate and compare *FOXQ1* CNV and its mRNA expression. Our K-means clustering analyses took two approaches; unsupervised (Figure 5A) and supervised (Figure 5B). For the supervised K-means clustering, we determined the biological ideal number of clusters based on BC cell lines classification, TNBC, luminal, and HER2. For the unsupervised K-means, we used the elbow method<sup>43</sup> to identify the ideal statistical number of clusters. For both methods, the numbers of clusters and the *FOXQ1* expression and CNV were used as seeds. We found that some clusters have high copy number with low mRNA expression, while other clusters have high mRNA expression with no CNV (Figure 5A and B). Intriguingly, this suggests that *FOXQ1* expression is independent from *FOXQ1* copy number in BC cell lines.

Several interesting patterns, however, emerge from our K-means clustering analysis. Our supervised analysis investigated if *FOXQ1* expression and CNV correlate within a -specific BC subtype more than others. Intriguingly, while it was expected that TNBC cell lines would cluster together based on *FOXQ1* expression and CNV (Figure 5B), we found some TNBC cell lines also cluster with HER2 and

with HER2 and luminal BC cell lines (red and black clusters, Figure 5B). Furthermore, our unsupervised analysis identified 4 clusters (Table 4 and Figure 5A), an extra cluster compared to the supervised method (Table 4 and Figure 5B). This suggests that *FOXQ1* expression and CNV could potentially identify a sub-population of BC within TNBC, HER2, and luminal BC cell lines. This supports the findings of Yang et al, which suggest that *FOXQ1* expression could drive the heterogeneity of BC subtypes.<sup>6</sup> Consistent with our findings of low levels of *FOXQ1* expression in luminal and HER2 BC patient tumors (Figure 1), we found *FOXQ1* mRNA to be low in all luminal and HER2 BC cell lines (Figure 5A and B). Interestingly, while other studies reported *FOXQ1* overexpression in TNBC cell lines,<sup>28,32</sup> our results show that *FOXQ1* has low expression in many TNBC cell lines (Figure 5A and B), but *FOXQ1* is overexpressed in some TNBC cell lines (Figure 5A and B). The difference in *FOXQ1* overexpression across TNBC cell lines is striking. It has been previously suggested that, on the basis of molecular features and morphology and invasion potential, TNBC might be further subdivided into basal A and basal B types.<sup>47</sup> Basal A has a less differentiated and more epithelial-like morphology, whereas basal B has a more mesenchymal-like morphology which is more invasive.<sup>47</sup> Basal B has additionally been characterized as exhibiting more stem-cell-like characteristics ("stemness"), as cells of this subgroup have been found to possess the CD44<sup>+</sup>/CD24<sup>-</sup> low phenotype<sup>47</sup> normally associated with mammary cancer stem cells (CSCs).<sup>76,77</sup> Moreover, a role of *FOXQ1* in stemness,<sup>54,78</sup> and EMT<sup>28</sup> has been reported. This could provide an explanation of why *FOXQ1* is overexpressed in certain TNBC cell lines rather than other *FOXQ1* TNBC cell lines (Figure 5A and B). Further classification of TNBC cell lines into basal A and B could provide a clearer picture of the different consequences of *FOXQ1* expression between these two TNBC subtypes.

Very few direct targets of FOXQ1 are known. These genes may be positively regulated by FOXQ1 such as *PDGFRα* and  $\beta$ , *Twist1* and *Zeb2*<sup>54</sup> or negatively regulated by FOXQ1 such as *DACH1* gene.<sup>30</sup> Consistent with these studies, we found in preliminary studies of BC gene expression databases a significant positive correlation between *FOXQ1* and *PDGFRα* or  $\beta$ , *Twist1* and *Zeb2* and a significant negative correlation between *FOXQ1* and *DACH1* (Supplementary Figure 3). While it has been suggested that FOXQ1 regulates these genes that play roles in BC stemness and EMT, there is no specific transcriptional signature that has been proposed for FOXQ1 in either development or cancer. The underlying mechanisms altering *FOXQ1* expression in BC subtypes, as

well remain elusive. This is not surprising given the lack of knowledge on the regulatory machinery controlling the expression of transcription factors such as *FOXQ1*. The actual number of genes regulated by *FOXQ1* is unknown but could number in the thousands based upon similar analyses of the direct targets of the related *FOXC1* transcription factor.<sup>79</sup> Thus, while a “transcriptional addiction in cancer” has been proposed,<sup>80</sup> it remains a challenge to precisely identify in cancer how and when transcription factors are themselves regulated and activated, and then to understand the target genes regulated by the activity of these transcription factors in normal and disease states.<sup>81</sup> Epigenetics, gene function redundancy, tissue specificity, predicting enhancer-promoter regions, and the role of coactivators are a few among many other challenges to studying transcription factors in cancer.<sup>81</sup> Future studies that reveal the regulation of *FOXQ1* expression, together with the identification of direct transcriptional targets of *FOXQ1*, might reveal how *FOXQ1* expression is controlled and the consequences of its dysregulation of this regulation in BC.

Our research, and that from other groups,<sup>29,55–58</sup> clearly demonstrate that *FOXQ1* is another member of the FOX class of transcription factors with a key emerging role in cancer.<sup>14,15</sup> Previous studies have investigated the role of *FOXQ1* expression, regulation, and function in relation to the expression of *FOXF2* and *FOXMI* genes, function and roles in BC and other diseases. Our findings that *FOXQ1* is an independent predictor of overall survival in BC when adjusted to *FOXMI* and *FOXF2* expressions highlight the prognostic significance of *FOXQ1* expression in BC patients (Table 5). *FOXQ1* and *FOXMI* expression levels have previously been shown to have prognostic value in colorectal cancer patients.<sup>56</sup> Moreover, a recent study suggested that *FOXQ1* expression is negatively regulated by *FOXF2* in BC cells.<sup>82</sup> *FOXQ1*, *FOXF2*, and *FOXC1* are all located within a 300 kb region of chromosome 6, physically linking in close proximity three FOX transcription factors with substantial roles in BC,<sup>15,19,83,84</sup> development,<sup>13,24,25,85–87</sup> and in several diseases.<sup>88–94</sup> Our results suggest that *FOXQ1* expression in BC patients could have significant prognostic value for survival from BC.

## Conclusion

In our study, we show that *FOXQ1* is differentially expressed in BC subtypes. Low expression of *FOXQ1* predicts poor overall survival in BC and is an independent prognostic predictor. Moreover, *FOXQ1* CNV alterations are not associated with its expression in BC.

Understanding the consequences of *FOXQ1* expression in BC subtypes could have clinical value.

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## Disclosure

Todd PW McMullen is a consultant with Eli-Lilly and Ipsen. The authors report no other potential conflicts of interest for this work.

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