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Immunohistochemical Assessment of TNFAIP3/A20 Expression Correlates With Early Tumorigenesis in Breast Cancer

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Abstract. *Background/Aim: Limited data exist on the expression pattern of TNFAIP3/A20, as assayed by immunohistochemistry (IHC), in breast cancer tissues. This study aimed to assess A20 expression pattern in breast cancer. Materials and Methods: The expression of A20 was analysed using IHC in 50 breast cancer cases retrieved from the Sharjah Breast Cancer Center at the University Hospital Sharjah, United Arab Emirates. Omics survival data were also used to analyse its association with survival in endocrine-treated subgroups. Results: A20 expression in breast cancer tissues was 'tumor-specific', and as compared to normal tissue areas, its expression was associated with both intensity and extent in early grade 1 ($p<0.0001$) in all molecular subtypes. In addition, using omics survival data from a cohort of 3,520 breast cancer patients, we showed that A20 overexpression associated with lower overall survival rate in the endocrine treated subgroups [hazard ratio (HR)=2.14, 95%CI=1.61-2.82, $p<0.0001$]. Conclusion: A20 can serve as a biomarker for early diagnosis of breast cancers.*

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The TNFα-induced protein 3 (TNFAIP3 also called A20), a ubiquitin-editing enzyme, was originally identified as a protein protecting endothelial cells from TNF-induced cytotoxicity (1, 2). A20 is well-known for its role in attenuating inflammation by restricting nuclear factor kappa B (NF-κB) signaling and cell death (3), thus acting both as anti-inflammatory and antiapoptotic protein. Numerous human genetic reports have indicated that A20 mutations directly correlate with genetic susceptibility to various types of gastric (4) or non-gastric (5) mucosa-associated lymphoid tissue (MALT) lymphomas and in some cases, response to therapy (6). Therefore, A20 may serve as both a diagnostic and prognostic biomarker.

Regarding the diagnostic role of A20, there are limited data on the association of its expression, as assayed by immunohistochemistry (IHC), with tumorigenesis in human cancers and much attention has been given to the role of A20 in both progression (7) and response to chemotherapy (8). In regard to the prognostic use of A20 in breast cancer, a report has shown that A20-overexpressing MCF7 cells exhibit a phenotype of resistance to the tamoxifen pro-apoptotic actions and deregulated bax, bcl2, cyclin D1 proteins expression (6). A recent report has demonstrated that elevated A20 levels in basal breast cancer subtypes promote the metastatic properties of this subtype by inducing expression of epithelial mesenchymal transition (EMT) phenotype markers such as Snail1 and Vimentin (7).

In this study, we explored the clinical utility of TNFAIP3/A20 gene as a biomarker for early diagnosis of breast cancers. We provided evidence that A20 can also serve as a prognostic marker in luminal-estrogen receptor (ER)+ subtypes.

Materials and Methods

A panel of formalin-fixed paraffin-embedded (FFPE) breast cancer samples (n=50) consisting of 26 cases of luminal subtypes, 12 cases of triple positive, 2 cases of HER2 overexpressing and 10 triple negative cases were identified in the University Hospital Sharjah Breast Cancer Care Center clinical database. The samples chosen were from patients with the highest pathological grade the patient had at the time of sampling. Sections were stained with hematoxylin and eosin (H&E) and the reported pathological grade was confirmed by two independent pathologists. The present work was approved by UHS Ethical and Research Committee (Ref. No.: UHSHERCYTOPLASMIC01-28012019). The study was conducted according to the principles of the Declaration of Helsinki.

Immunohistochemical staining. Immunohistochemistry (IHC) for A20 (TNFAIP3) protein was carried out using 4-μm FFPE sections. Antigen retrieval was performed using Bond epitope retrieval solution 2 (EDTA based on pH 9.0) for 20 min prior to incubation with anti-A20 antibody [1:100, Clone EPR2663 ab92324 (Abcam, Cambridge, MA, USA)]. Slides were counterstained with hematoxylin and mounted using DPX. Positive and negative controls were included in all the runs. A20 expression, assessed as cytoplasmic staining.

Interpretation. All slides were scored semi-quantitatively by two independent pathologists using Allred scoring system to take into account heterogenous staining (9). The Allred score is the sum of the score for IHC intensity (negative=0, weak=1, moderate=2, and strong=3) and extent of positive staining [no stain (0), <1% (1), 10% (2), 11-33% (3), 34-66% (4) and 67-100% (5)]. The score ranges from 0 to 8+ points.

Survival analysis using an omics database. The second aim of this study was to demonstrate the survival outcome of endocrine-treated breast cancer subgroups in relation to A20 expression. To achieve our aim the clinicopathological, survival and RNA-sequencing data of a cohort of 3520 resectable breast cancers from the Sweden Cancerome Analysis Network-Breast (SCAN-B) initiative were used (10). Gene expression data is available through Gene Expression Omnibus (GEO) series GSE96058 (11). The RNA-seq data were normalized using voom method (12). By using the SCAN-B study time-to-event (survival) outcomes and the A20-expression levels data, survival probability was computed for all SCAN-B breast cancer patients in relation to the level of A20 expression using Cox proportional hazard model and the resultant cumulative survival probability curve was plotted. The model was adjusted for age and estrogen receptor (ER)-status in endocrine-treated subgroups.

Statistical analysis. Analysis of the results was performed using R software (v 3.0.2), SPSS Version 26 (IBM Corporation, Chicago, IL, USA) and Graphpad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Categorical variables are presented herein as percentages, and continuous variables are presented as mean±standard deviation (SD). The expression of A20 was used as dichotomous variable, where in our BC cohort a score of 5+ to 8+ was defined as high A20 expression and a score of 0 to 4+ was defined as low A20 expression. In SCAN-B omics database (10, 11), the mean of gene expression levels of A20 across different molecular subtypes (mean±SD of 2.9±0.8) was used to group the cases into high or low A20 gene expression levels (10, 11). We compared characteristics of patients using χ^2 test or Fisher exact test for categorical variables and Student t test for continuous variables. For all analyses, p-values <0.05 were considered significant.

Results

No difference in A20 expression between breast cancer molecular subtypes. There have been mixed reports regarding the pattern of A20 expression in breast cancer molecular subtypes. *In vitro* studies have shown that the levels of A20 expression are higher in ER and/or progesterone receptor (PR)- negative subtypes (6, 7); however, an IHC study of patients' breast tumor tissue showed no difference in A20 levels between ER- and/or PR-negative and positive tumor subtypes (8). In our study, the expression levels of A20 were assayed in breast tumor tissue samples from 50 patients with different breast cancer molecular subtypes (Table I). No difference was observed in A20 expression levels among ER- and/or PR-negative and positive tumor subtypes. There was also no difference in A20 expression levels between the different breast cancer molecular subtypes in the SCAN-B breast cancer cohort (Table II).

Higher A20 expression in breast cancer compared to normal breast tissue. Furthermore, A20 expression levels were assessed in different histological Nottingham grades of cancer both in our and SCAN-B breast cancer cohort, which revealed no differences across these grades. However, as presented in Figure 1, compared to normal breast tissue, there was significantly higher A20 expression in grade 1 (p<0.0001) in all molecular subtypes, suggesting that A20 might be utilized as an early marker for breast cancer detection. This pattern of higher A20 expression in breast cancer compared to normal breast tissue, however, requires further investigations in larger tissue sample series.

Intra-and inter-tumor heterogeneity with regard to A20 expression. Moreover, A20 expression was associated with inter-tumor heterogeneity (Figure 2a-f) in 16 out of 50 cases and intra-tumor heterogeneity in 12 out of 50 cases (Figure 2g).

Survival outcomes of endocrine-treated subgroups in relation to A20 expression. Previously, an *in vitro* study has reported that A20 expression confers resistance to tamoxifen treatment (6). Therefore, it is not clear what would be the impact of A20 expression in ER- and/or PR-positive tumor subtypes that were on endocrine regimen. Hence, using RNA-seq (11) and survival data from a cohort of 3520 breast cancer patients from the SCAN-B initiative (10), we were able to demonstrate that high levels of A20 are associated with lower overall survival rate in the endocrine treated

Table I. *Main clinical and histopathological features of the patients in relation with A20 expression.*

	A20 low expression n=21	A20 high expression n=29	*p*-Value
Age at diagnosis, years			
mean±SD (range)	51±11 (31-69)	53±13 (30-86)	0.473
Tumor size, mean (SD), cm	2.76 (2)	2.62 (2)	0.820
TNM staging, n (%)			
Stage 1	4 (8)	8 (16)	0.335
Stage 2	10 (20)	10 (20)	
Stage 3	6 (12)	7 (14)	
Nottingham grade, n (%)			
Grade 1	2 (4)	7 (14)	0.124
Grade 2	8 (16)	11 (22)	
Grade 3	11 (22)	11 (22)	
IDC, n (%)	15 (30)	21 (42)	0.139
DCIS, n (%)	12 (24)	11 (22)	0.179
ER, n (%)	15 (30)	22 (44)	0.724
PR, n (%)	15 (30)	20 (40)	0.851
HER2, n (%)	6 (12)	9 (18)	0.851
Ki-67 ≥14%, n (%)	11 (22)	16 (32)	0.845
Molecular subtypes n (%)			
Luminal A	4 (8)	8 (16)	0.419
Luminal B	6(12)	7 (14)	
Triple positive	5 (10)	8 (16)	
HER2-overexpression	1 (2)	1 (2)	
Triple negative	5 (10)	5 (10)	
Axillary lymph nodes n (%)			
No node	6 (12)	12 (24)	0.292
1-3	9 (18)	9 (18)	
4-9	3 (6)	2 (4)	
≥9	2 (4)	3 (6)	
Lymphovascular invasion, n (%)	8 (16)	6 (12)	0.328

DCIS: Ductal carcinoma *in situ*; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; IDC: invasive ductal carcinoma; PR: progesterone receptor; SD: standard deviation.

Table II. *Main clinical and histopathological features of the Sweden Cancerome Analysis Network-Breast (SCAN-B) Initiative patient cohort in relation with A20 expression.*

	A20 low expression n=1407	A20 high expression n=1562	*p*-Value
Age at diagnosis, years			
Mean±SD (range)	64±13 (26-95)	62±13 (24-96)	0.0001
Tumor size, mean, SD, cm	1.987 (1.2)	1.989 (1.2)	0.963
ER, n (%)	1,313 (93)	1,256 (80)	0.0001
PR, n (%)	1,183 (84)	1,127 (72)	0.0001
HER2, n (%)	144 (10)	234 (15)	0.0001
Ki-67 ≥14%, n (%)	343 (24)	449 (29)	0.001
Molecular subtypes*, n (%)			
Normal	88 (6)	111 (7)	0.201
Luminal A	706 (50)	796 (51)	
Luminal B	339 (24)	325 (21)	
HER2-overexpression	129 (9)	165 (11)	
Basal	145 (10)	165 (11)	
Axillary lymph nodes, n (%)			
No node	836 (59)	914 (58)	0.051
1-3	390 (28)	405 (26)	
>4	105 (7)	164 (10)	
Submicro-metastasis	35 (2)	31 (2)	
Chemotherapy, n (%)	488 (35)	703 (45)	0.0001
Endocrine-therapy, n (%)	1,188 (84)	1,109 (71)	0.0001
Overall survival events, n (%)	105 (7)	205 (13)	0.0001
Overall survival days, mean (SD)	1,618 (471)	1,590 (499)	0.130

ER: Estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; SD: standard deviation. *PAM50 molecular subtype classifications.

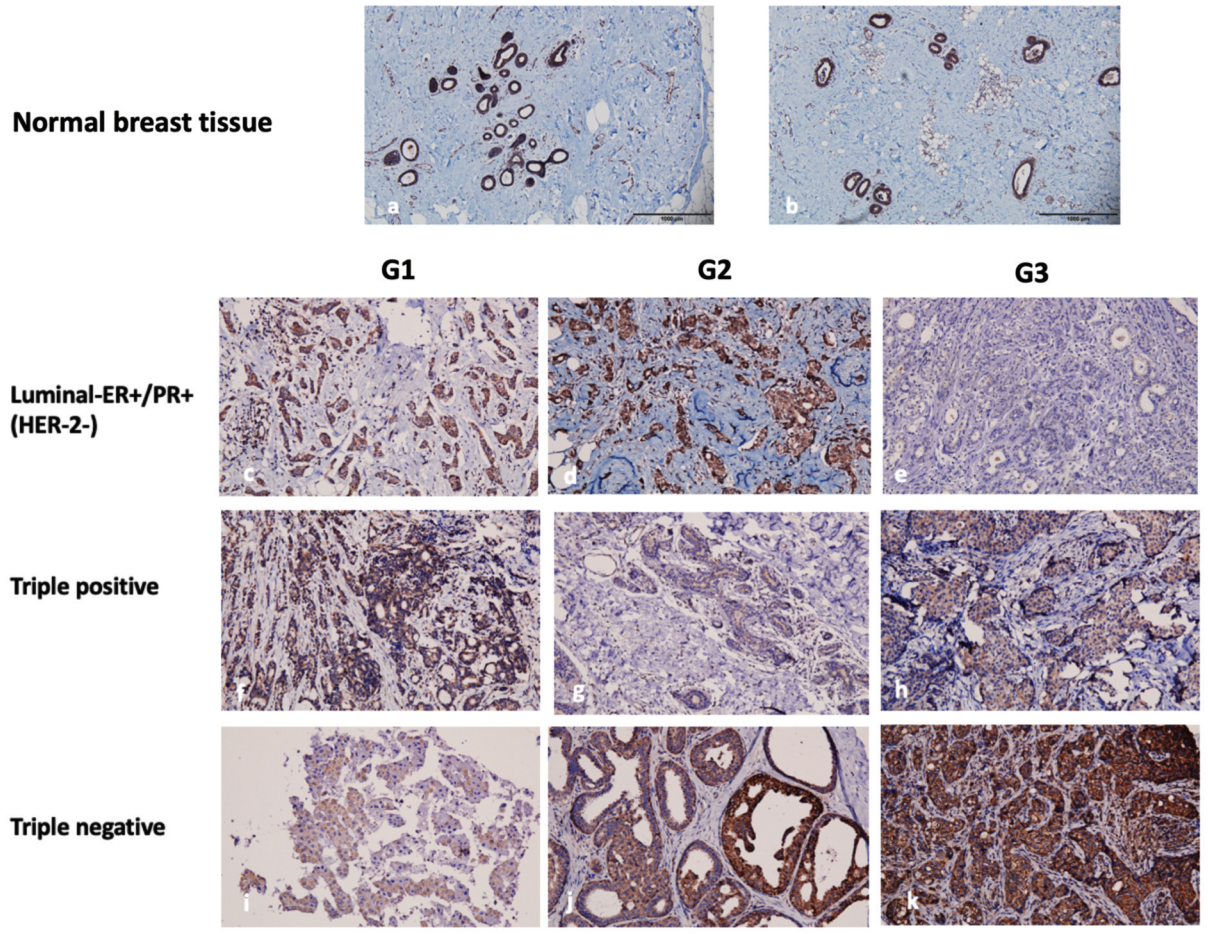
subgroups [hazard ratio (HR)=2.14, 95%CI=1.61-2.82; $p<0.0001$] (Figure 3). These results confirm the previous *in vitro* findings (6) that high A20 expression may confers resistance to endocrine therapy. However, the mechanism requires further investigations.

Discussion

In this study, we determined that TNFAIP3/A20 can be an early marker of tumorigenesis in breast cancer as there was a significant correlation of A20 IHC staining with tumorigenesis, whereas in normal tissue areas, A20 IHC staining was negative or significantly lower. We hypothesize that the expression of A20 can be used as a biomarker for early diagnosis of breast cancers.

The role of A20 in human cancers is complicated and is likely tissue specific, since A20 is inactivated in lymphoma by genetic mutations, acts as a tumor suppressor in lymphoid cells, and is oncogenic in solid cancers such as glioma and breast cancer (13, 14). In these cancers, the anti-apoptotic function of A20 impart cancer cells with tumorigenesis (3).Mounting evidence indicates that expression of A20 in breast cancer cells protects them from apoptosis and promotes their proliferation and, in turn, A20 silencing limits their proliferation (15) and increases their susceptibility to the apoptotic effects of chemotherapeutic agents (16). Moreover, in our studied breast cancer cases, A20 IHC staining was associated with inter-and intra-tumor heterogeneity in cases that were showing similar molecular profiles. Thus, considering the A20 role in carcinogenesis (7) and drug resistance (6), the different A20 IHC signals detected between different patient tumor samples, and even within each individual tumor, can predict cancer aggressiveness, implying that in addition to the role of A20 as an IHC marker detecting early tumorigenesis, its expression can also be used as a prognostic marker in breast cancer cases.

There are limited data regarding the A20 IHC staining pattern in human cancers and in particular, breast tumor tissues, whereas much attention has been given to the role of A20 as a tumor-intrinsic factor contributing to both carcinogenesis (7)



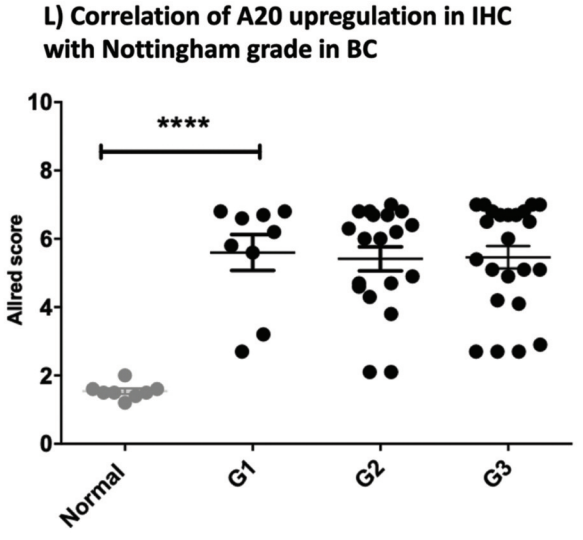


Figure 1. *Correlation analysis of A20 expression (×20) between molecular subtypes and Nottingham histological grade in breast cancer cases. (a and b) normal breast tissues. (c-e) Nottingham grade 1 to 3 in Luminal ER+/PR+ subtypes; (f-h) triple positive subtypes; and (i-k) triple negative subtypes. (L) A20 IHC stationing score in different histological Nottingham grades across all molecular subtypes. Representative data showing that A20 IHC stationing score is significantly higher in early grade 1 tumor compared to normal breast tissue. Sections were stained for A20 (brown) and counterstained with hematoxylin (blue). Statistical comparisons were performed by using t-test. ****p<0.0001. BC: Breast cancer; ER: estrogen receptor; G1; Nottingham grade 1; G2; Nottingham grade 2; G3; Nottingham grade 3; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; PR: progesterone receptor.*

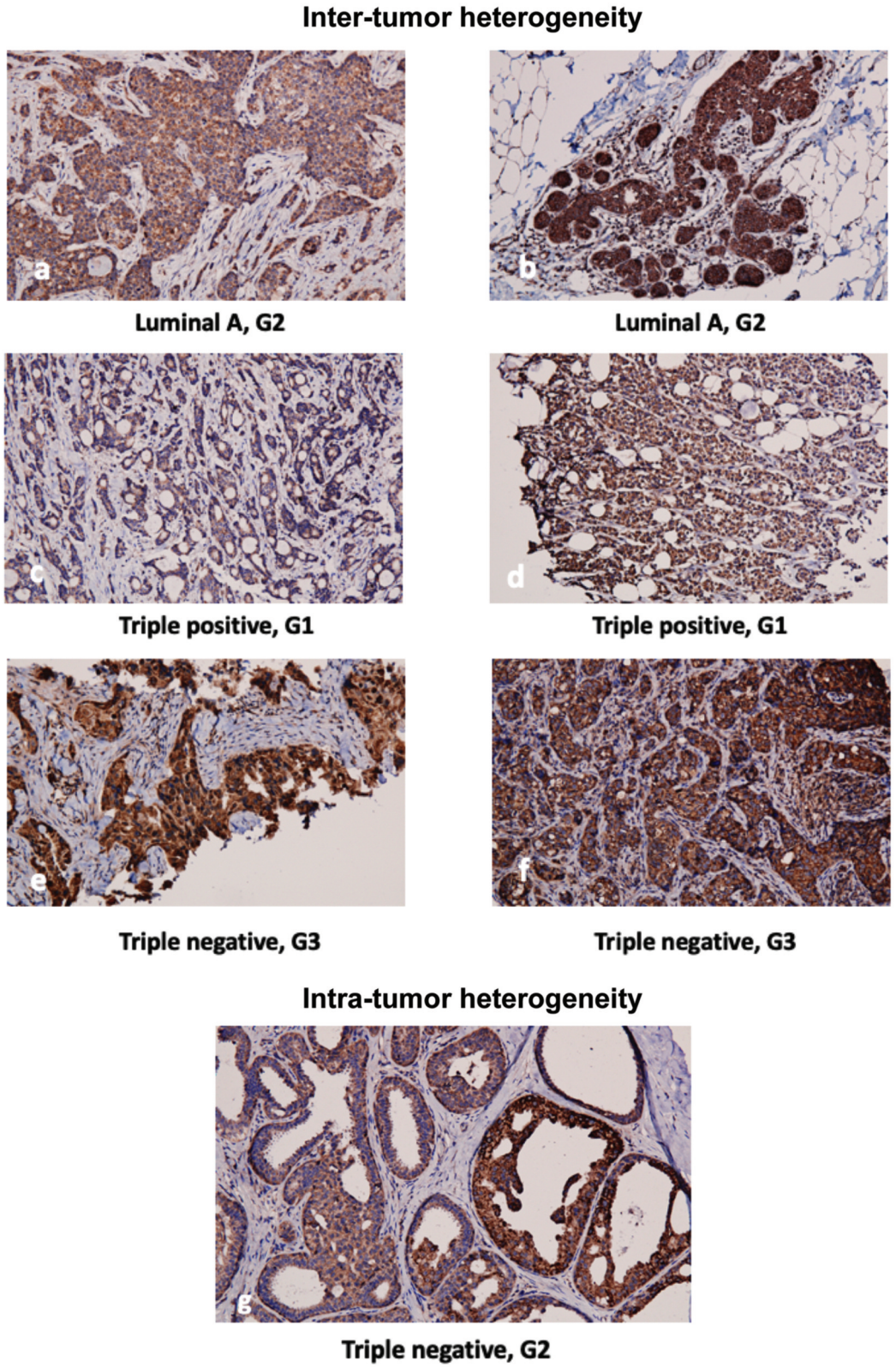


Figure 2. *Inter-tumor and intra-tumor heterogeneity regarding A20 expression (×20) in breast cancer cases. Inter-tumor heterogeneity in A20 expression is shown in sections (a) to (f), and intra-tumor heterogeneity in A20 expression is shown in section (g). Sections were stained for A20 (brown) and counterstained with hematoxylin (blue). G1: Nottingham grade 1; G2: Nottingham grade 2; G3: Nottingham grade 3.*

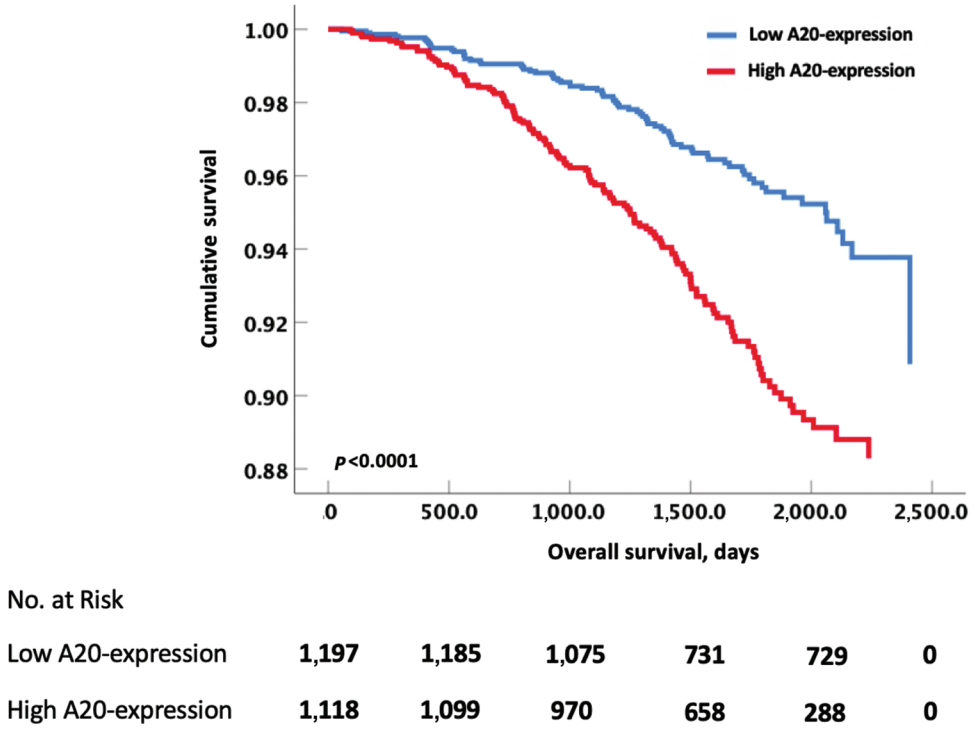


Figure 3. *Survival outcome of endocrine treatment in relation to A20 expression. Presented data shows that high A20-expression breast cancer was associated with lower overall survival compared to low A20-expression breast cancer subgroups. Cox proportional model was adjusted for age and estrogen receptor-status, p<0.0001.*

and the response to endocrine or chemotherapy (6, 8). In this study, we used the clinicopathological and RNA-seq data of 3,520 SCAN-B breast tumor tissues (11), and were able to demonstrate that A20 overexpression was associated with lower response to endocrine therapy and negatively impacted survival in patients with breast cancer.

Detection and treatment of breast cancer at the earliest stage are critical for patient's survival (17). This investigation demonstrated a novel gene as a breast tumor marker for early diagnosis of tumorigenesis in breast cancer cases, however, the findings of this study need to be validated in a larger well-characterized breast-cancer cohort.

Conclusion

In conclusion, A20 is strongly correlated with early-stage breast cancer and may be a potentially useful IHC marker to detect low-grade early tumors across all molecular subtypes. Its expression also correlates with lower response to endocrine therapy in luminal-ER+ subtypes.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

FSSA: Conceptualization, data curation, formal analysis, investigation, methodology, validation, writing – original draft, writing – review & editing. NAK: Data curation, writing – original draft, writing – review & editing. IMT: Data curation, formal analysis, investigation, methodology, writing review & editing. NSSA: Conceptualization, formal analysis, writing – original draft, writing – review & editing. SR: IHC of the A20. MJ: Data curation, formal analysis, investigation, methodology. KS: Formal analysis, writing – original draft, writing – review & editing. RH: Conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft, writing – review & editing. RB: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft, writing – review & editing.

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