Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Inhibition of Yes-Associated Protein-1 (YAP1) Enhances the Response of Invasive Breast Cancer Cells to the Standard Therapy

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Maha Guimei[1,2]
Sana Alrouh[3]
Maha Saber-Ayad[2–4]
Shirin A Hafezi[3]
Arya Vinod[3]
Surendra Rawat[3]
Yazan Wardeh[5]
Tala Mohamad Bakkour[5]
Ahmed Taher El-Serafi[3,6,7]

[1]Department of Pathology, Faculty of Medicine, Alexandria University, Alexandria, Egypt; [2]Clinical Sciences Department, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates; [3]Sharjah Institute of Medical Research, University of Sharjah, Sharjah, United Arab Emirates; [4]Department of Pharmacology, College of Medicine, Cairo University, Cairo, Egypt; [5]College of Medicine, University of Sharjah, Sharjah, United Arab Emirates; [6]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt; [7]Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

Purpose: The deregulation of the Hippo pathway results in translocation ofYes-associated protein-1 *(YAP1)* to the nucleus to exert an oncogenic effect. This effect has been demonstrated in several malignancies, yet, in breast cancer (BC), it remains controversial. The present study aimed to investigate the significance of *YAP1* expression in BC, its relation to cancer stem cells (CSCs), and the effect of its inhibition on tumor cell survival.

Patients and Methods: We evaluated the expression of YAP1 protein and gene using immunohistochemistry (IHC) and RT-qPCR in FFPE tissue from normal and breast cancer cases. We also studied its association with CSC expression (*OCT4, NANOG*, and *SOX2*) and with different clinicopathologic characteristics. Two BC cell lines (MCF7 and MDA-MB -231) were exposed to different concentrations of *YAP1* inhibitor "verteporfin" and cell viability was subsequently assessed.

Results: *YAP1* mRNA was higher in BC compared to the normal breast tissue (p-value=0.040) and was higher in luminal tumors compared to triple-negative breast cancer (TNBC) (p-value= 0.017). Its expression in tumors was significantly associated with the expression of pluripotency markers (*OCT4* and *NANOG*) (p-value= 0.030 and 0.035, respectively) and its inhibition resulted in a significant reduction of CSC expression in both MCF-7 and MDA-MB-231 cells. YAP1 nuclear expression by IHC, which signifies its activation, was more evident in invasive carcinomas compared to normal breast tissue and in-situ foci where the expression was limited to the cytoplasm. The pretreatment of BC cells (MCF7 and MDA-MB-231) with YAP1 inhibitor "verteporfin" resulted in their sensitization to the effect of tamoxifen and doxorubicin, respectively, and significantly decreased tumor cell proliferation and survival.

Conclusion: Our results imply that *YAP1* is highly expressed and activated in BC and its inhibition could represent a possible novel therapeutic strategy that should be further explored and investigated to improve the outcome of breast cancer patients.

Keywords: breast cancer, YAP1, Hippo pathway, cancer stem cells, verteporfin

Introduction

Breast cancer (BC) remains a leading cause of cancer-related deaths among females.[1] The identification of the exact molecular subtype is crucial for prognostic purposes as well as for planning the appropriate therapeutic strategy.[2] Despite the great advances achieved in the field of BC therapy, more personalized therapeutic regimens are still needed in order to improve the outcome and minimize the undesired side effects of currently used medications.

Yes-associated protein-1 *(YAP1)* is the main transcriptional regulator in the Hippo-signaling pathway. This pathway's main function is to regulate organ size

Correspondence: Maha Guimei
Department of Pathology, Faculty of Medicine, Alexandria University, 17 Champollion Street, Alexandria, Egypt
Tel +20 1005384268
Email guimeimaha@gmail.com

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by restricting cell proliferation and enhancing apoptosis.[3] *YAP1*shuttles between the cytoplasm and the nucleus. When Hippo pathway is active, LATS1/2 kinases phosphorylate and sequester *YAP1* in the cytoplasm and prevent its translocation to the nucleus to promote the transcription of Hippo pathway downstream genes that are mostly responsible for cellular proliferation and migration.[4]

The deregulation of the Hippo pathway was reported in several solid malignancies and *YAP1* was shown to play an oncogenic role in tumors like lung, colon, ovary, liver, and prostate cancers.[5,6]

To date, data concerning the exact role of *YAP1* in breast cancer remain largely inconsistent. Conflicting results onto whether *YAP1* acts as an oncogene or as a tumor suppressor gene have been reported in the literature. Studies suggesting an oncogenic effect have demonstrated that *YAP1* overexpression in cell lines was associated with enhanced proliferation,[7] whereas those proposing a tumor suppressor effect observed an increased cell migration in the *YAP1*-downregulated BC cells.[8] This controversy between reported results suggests that the role of *YAP1* in breast cancer may be contextual and may differ according to specific molecular characteristics of the studied cohort. It could also be related to the wide variability in the methods used to detect *YAP1* in these studies.

YAP1 signaling is known to play an important role in promoting embryonic stem cells (ES) and tissue-specific stem cell self-renewal. Recent studies have shown that *YAP1* signaling activated cancer stem cells (CSCs) in the liver leading to enhanced tumor propagation.[9] Cancer stem cells in the breast regulate epithelial–mesenchymal transition (EMT) and their expression is associated with an aggressive tumor pathology and an enhanced metastatic potential.[10] Yet, little is known about the exact relation between of *YAP1* expression and CSCs in Breast cancer.

Due to its proposed oncogenic effect, YAP1 has been regarded as a potential target for therapy. Verteporfin (VP) is an FDA-approved photosensitizer that is used in the treatment of macular degeneration.[11] It has recently been recognized as a *YAP1* antagonist, capable without light activation, of disrupting *YAP1–TEAD* interaction and thus downregulating the transcription of downstream proto-oncogenes such as *c-myc*, *Axl*, and *survivin*.[12,13] The effect of *YAP1* inhibition using VP has been investigated in hepatocellular carcinoma, retinoblastoma as well as in ovarian tumors where it resulted in inhibition of tumor cell proliferation by suppressing *YAP1* activity.[12–14] However, this effect has not been investigated in BC.

In this study, we investigated the significance of *YAP1* expression in breast cancer and its relation to CSC expression and other clinicopathological parameters of the tumors. Furthermore, we explored the effect of *YAP1* inhibition using "verteporfin" on breast cancer tumor cell survival and proliferation.

Patients and Methods

Study Population

The present study was conducted on 23 formalin-fixed paraffin-embedded (FFPE) specimens from patients diagnosed with Invasive ductal carcinoma, NST. The study comprised five cases of luminal A, 12 cases of luminal B cases and 6 cases triple-negative breast cancer (TNBC). Five samples of normal breast tissues were used as control. Invasive lobular carcinomas and other special type breast carcinomas were not included in the current study. Specimens were obtained from the archives of the pathology department, Alexandria University. All patients had undergone surgical tumor resections whether total mastectomies or local conservative resections followed by chemotherapy ± hormonal therapy and were followed up for a mean period of 5 years after completion of treatment. Patient clinicopathological characteristics including patient age, tumor grade, TNM stage and lymphovascular (LV) invasion as well as follow-up data were retrieved from the records of the Pathology and Oncology departments. Data concerning estrogen and progesterone receptor expression (ER and PR), human epidermal growth receptor-2 (HER2), and Ki67 expression were also retrieved from patient records (Table 1).

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Alexandria University (Alexandria, Egypt). Written informed consent was obtained from the patients according to the Helsinki declaration.

YAP1 Immunohistochemistry (IHC)

Representative 4 µm thick tumor sections were immunohistochemically stained according to previously described protocol.[15] Antigen retrieval was done by boiling in a Tris/EDTA (pH 9.0) for 20 minutes. Anti-active YAP1 Rabbit monoclonal antibody was used at a dilution of 1:2000 (ab205270, Abcam, Cambridge, UK). Rabbit-specific HRP/DAB (ABC) IHC Detection Kit (ab64261,

Table 1 Clinicopathologic Characteristics of the Studied Tumors

Characteristics	N	%
Age (mean ± SD), years	48.22 ± 11.59	
Clinical stage (n=23)		
II (Low)	10	43
III (High)	13	57
Grade (n=23)		
G1-G2 (Low)	16	70
G3 (High)	7	30
Ki-67 (n=21)		
<14%	4	19
≥14%	17	81
Molecular subtype (n=23)		
Luminal A	5	22
Luminal B	12	52
Triple-Negative	6	26
Perinodal fat infiltration (n=23)		
Positive	14	61
Negative	9	39
LV invasion (n=23)		
Positive	14	61
Negative	9	39
in situ component (n=23)		
Positive	9	39
Negative	14	61

Abcam, Cambridge, UK) was used following the manufacture's protocol. Positive and negative control slides were included in all the runs. Prostate tissue was used as a positive control for YAP1. The immunohistochemical interpretation of YAP1 expression was performed by a research associate (SR) and a pathologist (MG). Because activation of YAP1 leads to its translocation to the nucleus, positive staining was considered only when YAP1 was strongly expressed in more than 20% of the tumor cell nuclei.[16] All cytoplasmic staining or minimal nuclear staining were considered negative.

RNA Extraction and RT-qPCR

Total RNA was extracted from FFPE specimens using FFPE RNA purification kit (cat. 25300, NORGEN BIOTEK, Thorold, ON, Canada) following the manufacture's protocol. This was followed by Reverse transcription into complementary DNA (cDNA) using TruScript First Strand cDNA Synthesis Kit (cat. 54,420, NORGEN BIOTEK, Thorold, ON, Canada).

The reaction mix was based on GoTaq qPCR Master Mix (cat. A6002, Promega, Madison, Wisconsin, Unites States) in a final volume of 20µL. The thermal cycler Rotor-Gene Q (QIAGEN, Hilden, Germany) was used according to the following conditions: 95°C for 15 minutes followed by 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 30 seconds. The threshold cycle value (CT) of each gene was normalized against the CT value of the housekeeping gene (GAPDH). The fold change was determined as $2^{-\Delta\Delta Ct}$. Fold change was calculated with reference to control samples. Each sample was tested in triplicates. The primers used are listed in (Table 2).

Cell Culture

MDA-MB-231 and MCF-7 breast cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, Missouri, Unites States) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, St. Louis, Missouri, Unites States) at 37°C in a humidified chamber with 5% CO2.

Paraffin Embedding of Breast Cancer Cell Lines

One million of MCF-7 and MDA-MB-231 cells were collected in a microcentrifuge tube and allowed for centrifugation at 500 g for 6 minutes. Then, 30µL of thrombin and 50µL of plasma were added to the cell pellet. Cells were briefly vortexed, then incubated at 37°C for 15 min to allow for clot formation. Cell clots were then wrapped in a filter paper and transferred to a cassette for processing using Excelsior™ AS Tissue Processor (Thermo Fisher Scientific) that uses the following reagents: 10%

Table 2 Primer Sequence for *YAP1, NANOG, OCT4*and *SOX-2*

Gene	Primer Sequence (5′-3′)
GAPDH	F: CCAGGTGGTCTCCTCTGACTTC R: CATACCAGGAAATGAGCTTGACA
YAP1	F: ACCCACAGCTCAGCATCTTC R: GCTGTGACGTTCATCTGGGA
NANOG	F: AGTCCCAAAGGCAAACAACCCACTTC R: TGCTGGAGGCTGAGGTATTTCTGTCTC
OCT4	F: ACATCAAAGCTCTGCAGAAAGAACT R: CTGAATACCTTCCCAAATAGAACCC
SOX2	F: GGGAAATGGGAGGGGTGCAAAAGAGG R: TTGCGTGAGTGTGGATGGGGATTGGTG

Formalin, 15 minutes for fixation, then the following each for 1 hour: 10% Formalin, 75% Alcohol, 90% Alcohol, 95% Alcohol, three changes of 100% Alcohol, three changes 100% Xylene, heated paraffin wax, and then another two changes of paraffin wax each for 30 minutes. Tissues were then embedded in paraffin and FFPE blocks were prepared and stored in room temperature.

Cell Viability MTT Assay

Cells were seeded in 96-well microtiter plates (104 cells/well) overnight. Five micrometers of verteporfin was tested alone and/or in combination with different doses of tamoxifen and doxorubicin for 24 hours. Then, cells were incubated with either tamoxifen alone and/or doxorubicin alone (0.01, 0.05, 0.1, 0.5, 1, 5 and 10 uM) or in combination with 5uM of verteporfin for 24 hours. For viability, a volume 200 µL of Methylthiazolyldiphenyl-tetrazolium bromide, MTT (CAT #M5655) was added to each well to detect cell viability. Then, cells were incubated in a humidified incubator at 5% CO2 at 37°C for 2 hours. Two hundred-microliter Dimethyl sulfoxide (CAT #276855) (DMSO) well was used to dissolve MTT product and the absorbance was read at 570 nm using a plate reader. All concentrations were tested in triplicates. Experiments were carried out 3 times.

Western Blotting

The cell culture dishes were placed on ice. Cell lysates were made using 1% triton Lysis buffer (TLB) cocktail supplemented with PMSF, 100mM NaVo3, 1M NaF, and Protease inhibitor cocktail. Subsequently, the lysates were centrifuged at 14000g for 20 min at 4°C. Then, protein concentration was determined using the Thermo Scientific Pierce BCA Protein Assay Kit. The cell lysates (10µg of protein per lane) were diluted in 1X Laemelli's buffer solution, at 95°C for 5 min. The proteins were then loaded into SDS-PAGE (10% resolving gel, and 3% stacking gel). Immunoblotting was performed by probing proteins transferred onto nitrocellulose membranes using a Trans-Blot® TurboTM Blotting system (Bio-Rad) according to the standard transfer protocol. The membrane was blocked for 1hr at room temperature with 5% of Bovine Serum Albumin (BSA) in Tween Tris-Buffered saline (TTBS). Followed by incubation with the primary antibody overnight at 4°C on a shaker and then with secondary anti-mouse IgG (1/1000) antibody for 1 hour at room temperature. The blots were then visualized with enhanced chemiluminescence (ECL) Kit and imaged using ChemidocTM Touch Imaging System (Bio-Rad). The housekeeping protein β –actin was used to normalize the levels of protein detected by confirming that protein loading is the same across the gel. A loading control, β –actin (Primary antibody, rabbit) level was detected using anti-rabbit secondary antibody. The following primary antibodies from the R&D system and Cell signaling were used: Anti-YAP1 1;1000 mouse monoclonal antibody (Cat. # MAB8094, R&D system), B-actin 1;1000 Rabbit antibody (cell signaling). Secondary antibodies: Anti-rabbit (1/1000) for B-actin, Anti-mouse for YAP1 (1/1000) were used.

Statistical Analysis

Statistical analyses were done using SPSS version 23. Student's t and Chi-Square tests were used for continuous and categorical variables and the Mann–Whitney *U*-test for non-normally distributed samples. Correlation between two quantitative continuous variables was estimated by Spearman's rho. Values of $P < 0.05$ were considered statistically significant.

CompuSyn software was used to detect drug interaction. It is based on the Chou-Talalay method for quantitative drug combination applying the median-effect equation. The output is expressed as a combination index (CI), where CI<1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively.

Results

YAP1 Nuclear Expression in Invasive Tumors of Luminal Type

YAP1 cytoplasmic expression was noted in 92% of tumors whereas nuclear expression, which theoretically signifies *YAP1* activation, was only demonstrated in 67% of the cases. All cases showing positive nuclear expression consistently showed high mRNA levels by qPCR. We also noted marked heterogeneity in YAP1 protein expression within tumors, with areas showing strong nuclear staining and other areas showing total negativity, tumors were considered positive when more than 20% of the cells showed strong nuclear YAP1 expression.

Out of all the cases showing YAP1 nuclear positivity, 86.6% were of luminal type expressing estrogen and/or progesterone receptors and only 13.3% of the cases belonged to the TNBC category. Yet, YAP1 nuclear expression did not show any statistically significant

Table 3 Nuclear Expression of YAP1 in Relation to Ki67%, ER, PR, Her2 and Tumor Molecular Subtype

Variables	Nuclear Expression of YAP1				P-value
	Negative		Positive		
	N	%	N	%	
ki-67					0.658
< 14%	1	25	1	12.5	
≥ 14%	3	75	7	87.5	
ER status					1.00
Negative	1	20	1	14	
Positive	4	80	6	86	
PR status					0.236
Negative	2	50	1	12.5	
Positive	2	50	7	87.5	
Her-2 status					0.491
Negative	4	100	5	62.5	
Positive	0	0	3	37.5	
Molecular subtype					0.583
Luminal (A&B)	3	75	7	86	
Triple negative	1	25	1	14	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor.

correlation with independent expression of ER, PR, HER2, or Ki67% or with tumor molecular subtype (Table 3).

In the foci of Ductal carcinoma in situ (DCIS), YAP1 IHC expression was mainly cytoplasmic with no evidence of nuclear staining. Whereas in normal breast tissue, YAP1 expression was only limited to the myoepithelial cells with faint cytoplasmic staining in luminal epithelial cells (Figure 1A-D).

YAP1 mRNA Expression is Higher in BC and is Associated with Stemness

The expression of *YAP1* mRNA was significantly higher in BC compared to the normal breast (p=0.040, Mann–Whitney *U*-test) (supplementary Figure 1) and was higher in Luminal tumors compared to TNBC (p=0.017, *t*-test). (Figure 2) There was no statistically significant correlation between *YAP1* mRNA expression and patient age, tumor grade, TNM stage, perinodal fat infiltration, lymphovascular invasion nor with patient clinical outcome after the follow-up period.

In BC tissues, the expression of CSCs *(OCT4, NANOG, SOX2)* was significantly higher compared to normal breast tissue (Figure 3A). The expression of *OCT4* and *NANOG* was significantly associated with higher *YAP1* expression in tumors (p-value= 0.030 and 0.035, respectively, Spearman's Rho test). Stem cell markers showed a significant association with features of aggressiveness; *OCT4*demonstrated a significant association with a high proliferation index (Ki-67% ≥14%) (p=0.010, *t*-test), and *SOX2* was associated with lymphovascular invasion (p=0.005, *t*-test). (Table 4)

Treatment of MCF-7 cells with *YAP1* inhibitor "VP", at a dose of 5uM, resulted in a significant reduction of *YAP1* mRNA (p=0.04) and a significant reduction in expression of pluripotency markers; *SOX2* and *NANOG* (p-value= 0.008 and 0.005, respectively). (Figure 3B)

Whereas in MDA-MB-231 cells, a similar reduction in mRNA expression of *YAP1* and *SOX2* was observed, yet, it did not reach statistical significance. Moreover, the expression of *NANOG* was increased in VP-treated cells compared to untreated cells (p=0.03). (Figure 3C)

The present study did not demonstrate a significant difference between the mRNA expression of *YAP1* in the two studied breast cancer cell lines (MCF-7 and MDA-MB -231) (p=0.09). And on the protein level, the IHC study showed that YAP1 was equally expressed in both nuclei and cytoplasm in both cell lines (Figure 4A-C).

YAP1 Inhibition by Verteporfin Sensitizes BC Cells to Standard Therapy

First, we confirmed the inhibitory effect of verteporfin on *YAP1* expression in both MCF7 and MDA-MB-231 cell lines. Then, we explored the effect of verteporfin treatment alone at several doses on tumor cell viability. For MDA-MB231 cells, IC_{50} was 3.986 µM calculated by GraphPad Prism8. For MCF7 cells, the maximum inhibition of proliferation was only 28% (so no IC_{50} was calculated). (Figure 5A-C)

After that, we evaluated the effect of pre-treatment of cells with different doses of verteporfin (either 2 or 5 uM) followed after 24 hours by treatment with Tamoxifen or Doxorubicin. We compared the effect of VP pretreatment on cell viability with the effect of using tamoxifen or Doxorubicin as a single treatment. We found that pretreatment with verteporfin at a dose of (5 µM) for 24 hours followed by tamoxifen in the case of MCF7 cells caused the sensitization of the pre-treated cells and resulted in a significant reduction of proliferation at all doses of tamoxifen compared to untreated cells ($p < 0.05$). Similar results were obtained upon pretreatment of MDA-MB231 cells with doxorubicin. The interaction between

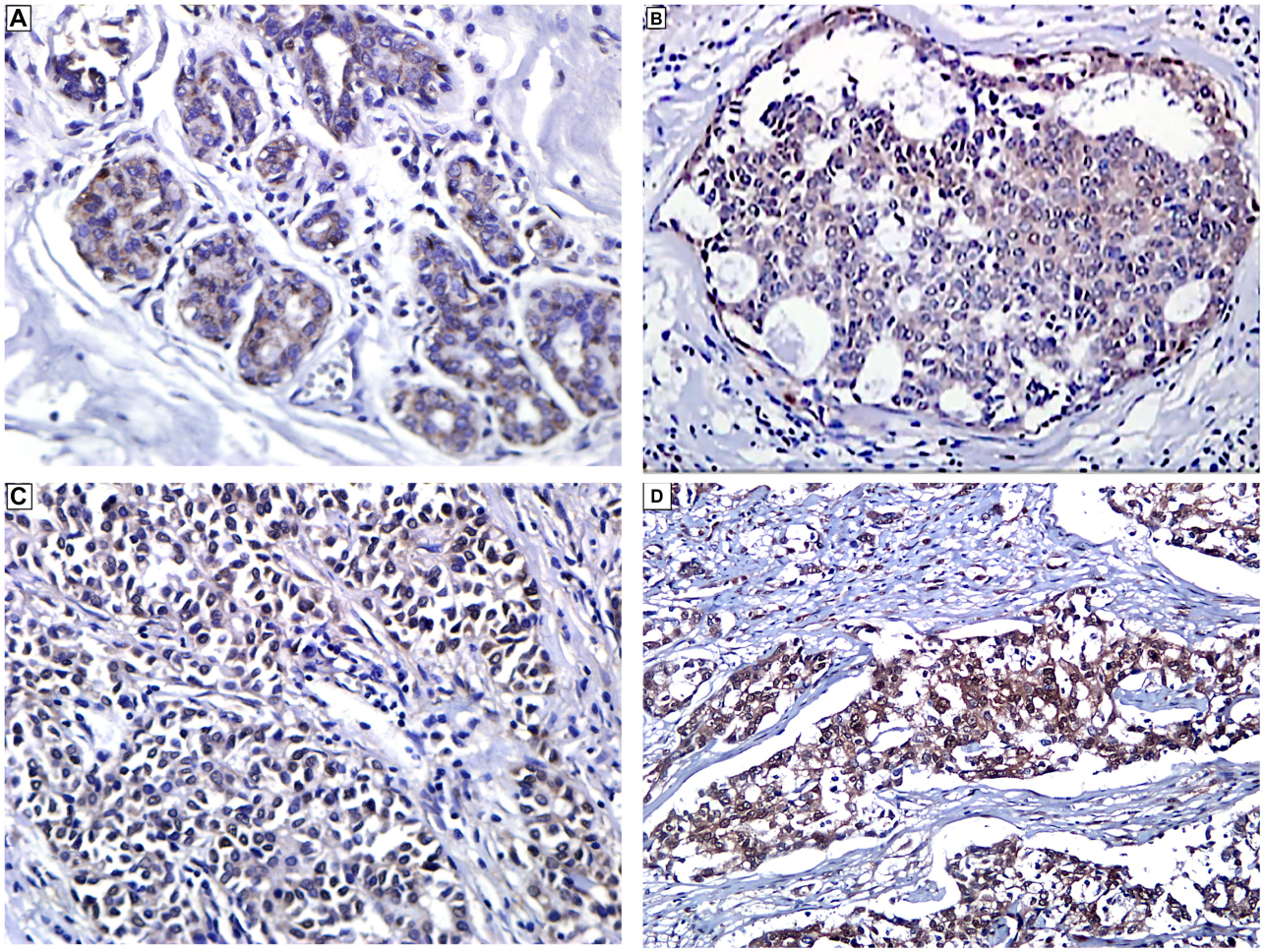

Figure 1 Immunohistochemical expression of YAP1 in breast cancer. (**A**) Normal breast acini showing expression of YAP1 in myoepithelial cells and cytoplasmic expression in luminal cells (Immunoperoxidase, x400). (**B**) Ductal carcinoma in-situ (DCIS) showing cytoplasmic expression of YAP1 in ductal epithelial cells (Immunoperoxidase, x200). (**C** and **D**) Invasive ductal carcinoma, NST (luminal type) showing cytoplasmic and nuclear expression of YAP1 in tumor cells (Immunoperoxidase, x200).

verteporfin and tamoxifen in MCF7 or verteporfin and doxorubicin in MDA-MB231 was calculated through observing the effect at all tested concentrations of each drug as well as the combinations. Combining verteporfin with either tamoxifen or doxorubicin resulted in a CI <1, indicating synergism (Figure 6A and B).

Discussion

The inactivation of the Hippo pathway with the resultant *YAP1* translocation to the nucleus is known to exert an oncogenic effect in many tumors.[4] However, data concerning the exact role of *YAP1* in breast cancer are still far from being conclusive. The present study demonstrated a significantly higher mRNA levels as well as predominantly nuclear protein expression of YAP1 in invasive breast cancers compared to normal breast tissue. We also demonstrated a significantly higher *YAP1* expression in luminal compared to TNBC.

The observed higher mRNA levels of *YAP1* together with the nuclear protein expression in tumors provide good evidence for *YAP1* activation in BC compared to normal breast.[4] These results are following studies that have previously suggested an oncogenic role for *YAP1* in BC; One study has shown that animals transplanted with *YAP1*-overexpressing cells had an enhanced tumor growth[7] and another study demonstrated that YAP1 nuclear expression was higher in brain metastasis and was associated with shorter overall survival in BC patients.[17] On the other hand, an opposite effect was suggested by Yuan et al who showed that *YAP1* expression was lost in tumors compared to normal tissues, and its knockdown increased invasiveness and reduced tumor response to treatment.[8] The observed discrepancy between the results of these studies can be attributed to the different methods used to evaluate *YAP1* expression as well as the different types of

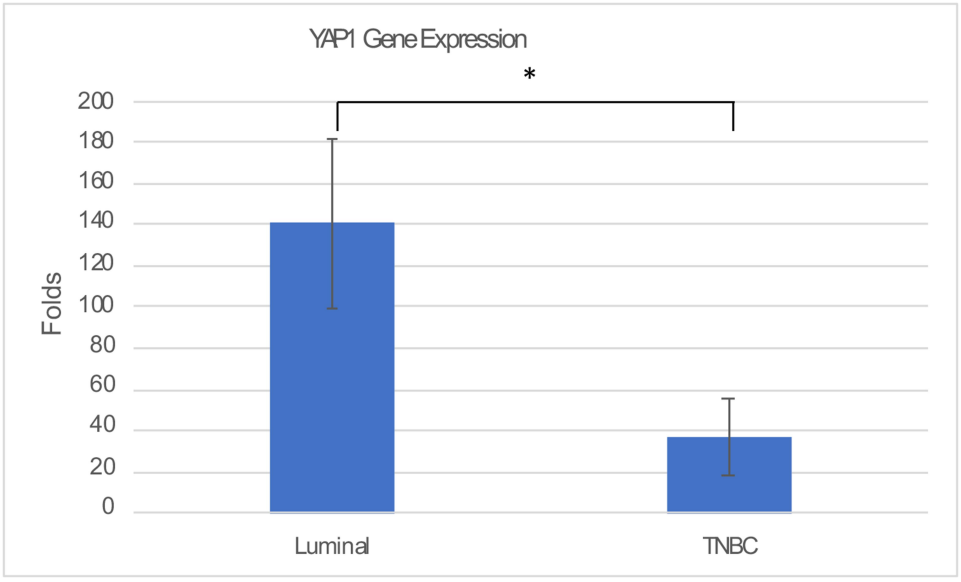

Figure 2 *YAP1* mRNA expression in the different molecular subtypes of breast cancer. qRT-PCR analysis showing significant increase in average *YAP1* mRNA in luminal compared to TNBC (*P = 0.017).

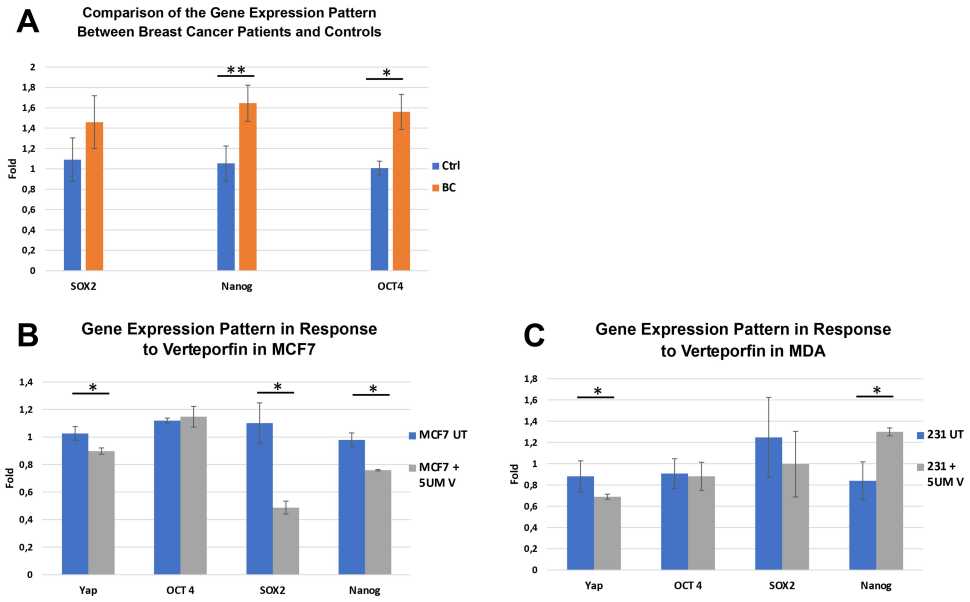

Figure 3 (**A**) *NANOG, OCT4* and *SOX2* mRNA expression in breast cancer and control. (**A**) Transcript levels of *NANOG, OCT4*and *SOX2* in breast cancer tissues and normal breast tissues quantified by qRT-PCR and presented as fold induction showing significant increase in *NANOG* and *OCT4* in Breast cancer. *P < 0.05, **P < 0.01. (**B**) MCF-7 cells showing significant reduction in mRNA expression of *YAP1*, *SOX2* and *NANOG* in response to VP treatment. *P < 0.05. (**C**) MDA-MB-231 cells showing reduction in expression of *YAP1* and *SOX2* and increase in *NANOG* in response to VP treatment. *P < 0.05.

antibodies being used. Hence, in our study, we opted to use an antibody that is specifically directed against "active YAP1", we combined the detection of both mRNA and protein to confirm our results and we used both human BC tissue as well as BC cell lines.

In the present study, *YAP1* expression was significantly higher in hormone receptor-positive tumors (luminal) compared to TNBC. This was evident both on the mRNA level and less evident on the protein level. Previous studies have also shown reduced *YAP1* expression in breast cancers lacking ER and PR expression[18] as well as increased nuclear YAP1 expression in PR positive tumors.[19] Thus, our data further emphasize the existence of a positive correlation between hormone receptors and

Table 4 Stem Cell Marker Expression in Breast Cancer in Relation to the Different Clinicopathological Characteristics of the Tumors

Variables	SOX2				NANOG				OCT4			
	N	%	Mean	P-value	N	%	Mean	P-value	N	%	Mean	P value
Ki67												
<14%	3	20	1.34	0.386	3	20	1.63	0.967	4	25	2.28	0.010*
>14%	12	80	0.94		12	80	1.65		12	75	1.32	
Perinodal fat												
Negative	7	50	1.54	0.914	7	50	1.63	0.891	7	47	0.72	0.465
Positive	7	50	1.48		7	50	1.69		8	53	0.69	
LV invasion												
Negative	5	33	2.3	0.005*	5	33	1.65	0.973	5	31	1.69	0.639
Positive	10	67	0.99		10	67	1.64		11	69	1.50	
In situ												
Negative	8	53	1.52	0.806	8	53	1.63	0.902	8	50	1.45	0.529
Positive	7	47	1.39		7	47	1.67		8	50	1.67	
Grade												
G1-G2	9	60	1.60	0.513	9	60	1.38	0.060	10	62.5	1.53	0.859
G3	6	40	1.24		6	40	2.05		6	37.5	1.60	
TNM stage												
Low	5	33	1.91	0.232	5	33	1.35	0.253	6	37.5	1.71	0.514
High	10	67	1.23		10	67	1.79		10	62.5	1.47	

Note: * P < 0.05 were considered statistically significant.

YAP1 activity in BC. This relation can be explained by the ability of *YAP1* to modulate the ligand-dependent transcriptional activity of ER and PR via its WW domain-binding protein (WBP-2),[20] or by the fact that *YAP1/TEAD4* act as cofactors that bind to ER-bound enhancers, induce ER target genes and enhance the Estrogen-induced oncogenic growth.[21] This interesting observation certainly warrants an in-depth investigation in order to elucidate the exact underlying mechanistic pathways linking *YAP1* to hormone receptors and further explore the possibility of using *YAP1* as an actionable therapeutic target in luminal tumors.

The ability of malignant cells to initiate tumors is known to be highly dependent on self-renewal and stem cell-like properties. These properties are orchestrated by embryonic antigens like (*OCT4, NANOG*, and *SOX-2*), and are controlled by signaling through various pathways including the Hippo pathway.[22,23] Chemotherapeutic agents target only the non-CSCs population within tumors and leaves behind a CSC-rich tumor environment responsible for drug resistance, metastasis, and recurrence, which are the major causes of cancer mortality.[24,25] This, in turn, has led to a search for novel therapeutic strategies that can target or inhibit the CSC-generating pathways. In the

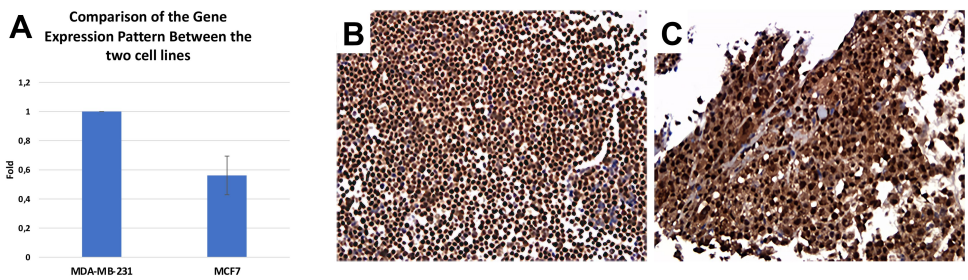

Figure 4 YAP1 expression in MCF-7 and MDA-MB-231 breast cancer cell line. (**A**) qRT-PCR analysis showing no significant difference in *YAP1* expression between MCF-7 and MDA-MB-231 breast cancer cell lines. (**B, C**) Immunohistochemical expression of YAP1 in cytoplasm and nucleus in both MCF-7 (**B**) and MDA-MB-231 (**C**) cells (Immunoperoxidase, x200).

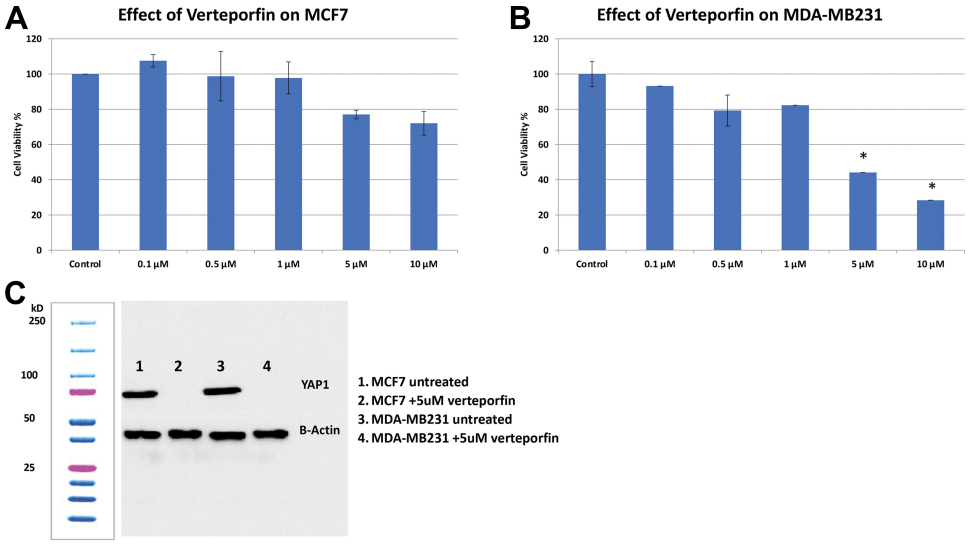

Figure 5 The effect of verteporfin single treatment. (**A**) MTT assay showing the effect of single treatment of MCF-7 cell lines with verteporfin for 24 hours at doses of 0.1, 0.5, 1, 5 and 10 uM (*= p-value <0.0001). (**B**) MTT assay showing the effect of single treatment of MDA-MB231 cell lines with verteporfin for 24 hours at doses of 0.1, 0.5, 1, 5 and 10 uM (*= p-value <0.0001). (**C**) Western Blotting showing the effect of VP treatment on YAP1 protein expression in both MCF-7 and MDA-MB-231 cell lines (YAP1 1;1000 mouse monoclonal antibody (Cat. # MAB8094, R&D system), B-actin 1;1000 Rabbit antibody (cell signaling), secondary antibodies: Anti-rabbit (1/1000) for B-actin, Anti-mouse for YAP1). A specific band was detected for YAP1 at approximately 70–75 kDa, band for B-actin was detected at approximately 42–45 kDa.

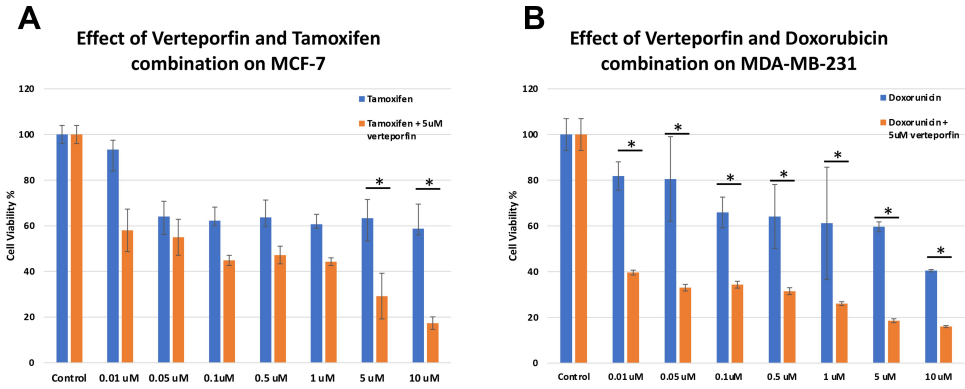

Figure 6 Cell viability with and without pre-treatment with verteporfin. (**A**) Effect of combined treatment with verteporfin and tamoxifen on the viability MCF-7 cells tested by MTT. The cells were treated with Tamoxifen (Tam) alone, at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 uM, and with the same doses of Tamoxifen after 24-hour pre-treatment with verteporfin (VP) at a dose of 5 uM. Statistical difference was tested using ANOVA test and posthoc Tukey Kramer. (*= p-value <0.0001). (**B**) Effect of combined treatment with verteporfin and doxorubicin on the viability of MDA-MB-231 cells tested by MTT. The cells were treated with Doxorubicin (Dox) alone, at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 uM, and with the same doses of doxorubicin after 24-hour pre-treatment with verteporfin (VP) at a dose of 5 uM. Statistical difference was tested using ANOVA test and posthoc Tukey Kramer. (*= p-value <0.0001).

present study, we demonstrated a significantly higher expression of stem cell markers (*OCT4* and *NANOG*) in BC compared to normal breast and a significant association between the expression of those markers and *YAP1* was identified. This association between *YAP1*and stemness has been demonstrated in several tumors. However, the exact CSC-specific regulatory mechanisms have not been fully investigated. In urinary bladder carcinomas, *SOX2* was associated with *YAP1* expression and contributed to the accumulation of urothelial CSCs.[26] In lung cancer cells, *NANOG* and *OCT4* expression were downregulated in spheroids silenced for *YAP1/TAZ*,[27] and in Non-small cell lung cancer cells (NSCLC), *YAP1* was found to interact with *OCT4* in order to induce self-renewal in vitro.[28] As for breast cancer, one recent study has shown that attenuation of YAP1 nuclear accumulation was associated with decreased expression of stemness markers.[29]

In the present study, we demonstrated that MCF-7 cells expressed high levels of *YAP1* mRNA and that inhibition of *YAP1* in these cells, using VP, was associated with a significant reduction in the expression stemness markers

(*SOX2* and *NANOG*). This, may in turn, suggest that targeting *YAP1* could represent a potential novel method for reducing CSCs in hormone-dependent BC cells.

As for MDA-MB-231 cells, although they expressed high levels of *YAP1* mRNA, VP treatment resulted in a reduction of *SOX2*expression that was not as significant as well as an increased *NANOG* expression in VP-treated cells compared to untreated cells. This latter unexpected finding together with the less evident reduction in stem cell markers in MDA-MB-231 cells can be attributed to the high PD-L1 expression in these cells. PD-L1 is known to regulate *OCT4* and *NANOG* and has a direct effect on sustaining the stemness of CSCs in a *PI3K/AKT*-dependent manner.[30]

Since the results of the present study mostly favored a tumor-promoting effect for *YAP1* in BC, we decided to explore the effect of *YAP1* inhibition using verteporfin (VP) on tumor cell survival and proliferation. We observed that adding the *YAP1* inhibitor, verteporfin (VP) to Tamoxifen, or Doxorubicin resulted in a significantly reduced survival of both MCF-7 and MDA-MB231 cells, respectively, by exerting a synergistic effect to the anticancer activity of these commonly used drugs. The anticancer effect of VP has been recently explored in many tumors; In bladder cancer cells (UMUC-3 and 5637 cells), VP dramatically inhibited cancer cell invasion properties[31] and in retinoblastoma cell lines (Y79 and WERI), it inhibited the growth, proliferation, and viability of the tumor cells in a dose-dependent manner.[13] Although not demonstrated in our study, VP is suggested to exert this effect by increasing the levels of a *YAP1* chaperon protein, 14-3-3σ thus retaining *YAP1* in the cytoplasm and targeting it for degradation in the proteasome.[32]

Conclusion

Although the findings in our study remain riddled with many open questions, and despite the small number of cases examined in the study, the available data provide sufficient evidence to pinpoint *YAP1* as a prime candidate for the development of anti-cancer treatments and suggest that its inhibition using VP, or any other inhibitory technique, could represent a promising strategy that may result in more effective personalization of BC treatment regimens.

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Disclosure

The authors declare no conflicts of interest.

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