Study of YAP as a prognostic marker for Triple-negative Breast Cancer in Indian Cohort

Submitted by

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Certificate

This is to certify that this dissertation entitled '**Study of YAP as a prognostic marker for Triple-negative Breast Cancer in Indian Cohort**' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents the work carried out by **Dimple Adiwal** at IISER Pune under the supervision of **Dr. Madhura Kulkarni**, Senior Scientist & DBT-Ramalingaswami Fellow, PCCM, Pune during the academic year 2018-2019.

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Declaration

I hereby declare that the matter embodied in the thesis entitled "**Study of YAP as a prognostic marker for Triple-negative Breast Cancer in Indian Cohort**" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of **Dr. Madhura Kulkarni**, Senior Scientist & DBT- Ramalingaswami Fellow, PCCM, Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

Breast carcinoma is one of the most frequently occurring cancers in females, with high proportion of cancer associated deaths in countries like India. The invasive carcinoma is classified into four major molecular subtypes; Luminal A, Luminal B, HER2-positive, and TNBC. The prevalence of TNBC in Indian cohort is found to be up to 31%, which is significantly higher compared to Western populations. It is essential to investigate novel markers to profile this aggressive subset for a better prognosis. One such marker is the Yes-associated protein (YAP). YAP is a co-activator protein that is regulated by the Hippo signaling pathway. Prior studies have shown that YAP protein expression and its target signature expression in breast cancer patients significantly associates with recurrence in breast cancer patients as well as is responsible for epithelial-mesenchymal transition and stemness enrichment of breast epithelial cell lines. This implicates YAP plays a role in breast cancer progression and prognosis and is a potential biomarker. To understand TNBC specific association of YAP with progressive disease characteristics, 50 primary TNBC tissues were evaluated and analyzed for co-relation of YAP expression with clinical features and survival outcomes. YAP was localized in both tumor nuclei, and cytoplasm and its nuclear expression was associated with the presence of lymph node metastasis. Cytoplasmic YAP expression also inversely correlated tumor infiltrating lymphocytes levels. Kaplan-Meier curves revealed that high nuclear to cytoplasmic YAP levels associated with reduced overall survival. These results suggest that further research is needed to suggest that YAP could be a prognostic marker in TNBC patients.

Table of Contents

List of figures

List of tables

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1. Introduction

1.1. Breast Cancer and its Molecular Subtypes

Breast carcinoma is one of the most frequently occurring cancers in females, with a more substantial proportion leading to cancer-associated deaths in countries like India (Bray *et al.*, 2018). Breast cancer incidence in the Indian population is expected to rise to 1.7 million in a population of 1.25 billion by 2035 (Malvia *et al.*, 2017). It comprises of multiple subtypes which have discrete morphologies and clinical implications (Dai *et al.*, 2015). Breast cancers can be comprehensively divided into two main groups: carcinomas and sarcomas. Cancers emerging through epithelial cells and stromal cells of the breast are known as carcinomas and sarcomas, respectively. A majority (90%) of cancers are carcinomas (Vogel, 2017). Carcinomas can be further classified as *in situ* and invasive carcinoma. In *in situ* carcinomas, the atypical clonal proliferation of epithelial cells takes place in the original localized sites and do not invade the breast tissue. The Ductal Carcinoma in Situ (DCIS) and Lobular Carcinoma in Situ (LCIS) are non-invasive breast carcinomas, with about 1.0%-2.6% leading to invasive status (Voduc *et al.*, 2010). *In situ* carcinomas account for approximately 10%-15% of breast carcinomas. In invasive carcinomas, the malignant cells from ducts and lobules infiltrate the breast tissue and continue to grow. These invasive carcinomas can further metastasize to other body parts (Figure 1). Invasive ductal carcinoma (IDC) and invasive lobular carcinomas (ILC) account for approximately 80% and 10-15% of breast carcinomas cases, respectively (Voduc *et al.*, 2010).

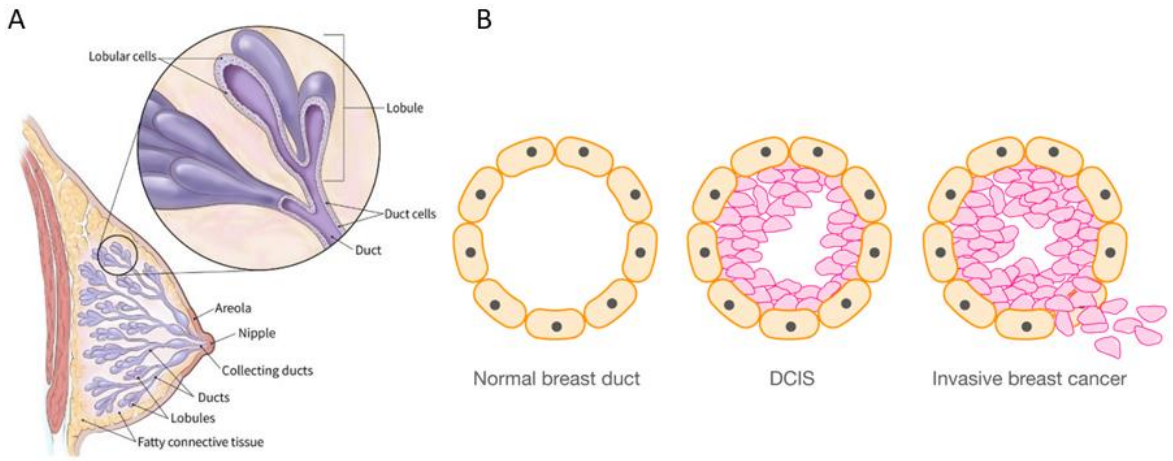

Figure 1. Illustrations of (A) Breast Components: Breasts are made up of lobules, ducts which are surrounded by glandular, fibrous and fatty tissue (B) Normal duct, DCIS and IDC; DCIS: when the cancer cells that have developed in the ducts remain in the ducts, IDC: when the cancer cells that have developed in the ducts invade through the duct walls into the breast tissue. (Society, 2016)

By the advent of molecular expression studies for hormone receptors, the invasive ductal carcinoma is further classified into four major molecular subtypes. The subtypes include Luminal A, Luminal B, HER2-enriched, and TNBC. Luminal A carcinomas are generally estrogen (ER) and progesterone (PR) receptors positive (hormone receptor positive) and HER2 negative; they are typically slow growing. Luminal B carcinomas are hormone receptor positive and can be HER2 positive or HER2 negative, their proliferative index is higher and has a poorer prognosis compared to Luminal A cancers. HER2-enriched carcinomas are hormone receptor-negative and HER2-positive (amplified HER2 gene), their prognosis is poorer as compared to Luminal A and Luminal B cancers. Lastly, TNBCs are characterized by the lack of hormone receptors and HER2-amplification; they are aggressive in nature and are associated with worse prognosis (Telli, 2016;Dai *et al.*, 2015). In Western population, ER-positive, HER2 positive, and Triple negative breast cancers account for approximately 67%, 18%%, and 15% of all invasive ductal breast cancers. In Indian cohort their incidence is around 42%, 27%, and 31%, respectively (Sandhu *et al.*, 2016). The prevalence of TNBC in India is significantly higher compared to the Western population (Thakur, Bordoloi and Kunnumakkara, 2018).

1.2. Clinical management for molecular subtypes of invasive ductal carcinoma

Molecular subtype based on hormone receptor and HER2 expression status is critical in deciding the clinical management (McDonald *et al.*, 2016).

Along with local therapy consisting of surgery and radiotherapy, patients are also treated with systemic therapies depending on the subtype, grade, stage, and other parameters.

ER-positive tumors are frequently treated with endocrine therapies. Hormone therapies aim to starve the cancer cells of estrogen to abrogate cancer cell growth. Treatments such as Selective estrogen-receptor response modulators (SERMs), Luteinizing hormone-releasing hormone agents (LHRHs), aromatase inhibitors, and Estrogen receptor down regulator (ERD) are routinely used for ER-positive patients (Burstein *et al.*, 2016). Along with developing targeted therapies for different subsets of ER-positive patients, gene expression based assay such as Oncotype DX is designed to estimate the likelihood of recurrence, and depending on that score, chemotherapy administration is decided (McVeigh and Kerin, 2017).

For HER2-positive breast cancer, there are targeted therapies with multimodal approaches to inhibit HER2 receptor-mediated intracellular signaling by acting on both the extracellular and intracellular domain of the receptor, ultimately abrogating cell proliferation.Targeted therapies include treatment with Trastuzumab (Herceptin), Ado-trastuzumab emtansine (Kadcyla), and Pertuzumab (Perjeta) (Hudis, 2007; Molina *et al.*, 2001). Treatments (Neratinib and Lapatinib) are also available for reducing the recurrence rates and for the patients who develop metastatic disease and have grown resistant to Trastuzumab (Martin *et al.*, 2017;Geyer *et al.*, 2006).

Treatment options for TNBC patients remain dramatically skewed. The only targeted therapy available is PARPi (olaparib and talazoparib), which are developed recently for 10% to 15% of TNBC patients who have BRCA1/2 germline mutations. PARPi targets this repair process by blocking the catalytic action of PARP, and in tumors with BRCA1/2 mutation where the homologous recombination is dysfunctional, the double-strand breaks can’t be efficiently repaired resulting in cell death of cancer cells (Guney Eskiler *et al.*, 2018;McCann and Hurvitz, 2018). Adjuvant cytotoxic chemotherapy

remains the only therapeutic option for the remaining 85% of TNBC patients (Wahba and El-Hadaad, 2015 ;Lebert *et al.*, 2018).

ER-positive cancers are highly responsive to hormone therapies when combined with chemotherapy. It has been reported that patients with ER-positive tumors have a lower annual risk of recurrence than ER-negative rumors within the first five years of diagnosis. The overall survival rates are higher for ER-positive patients as compared to ER-negative patients (Cuzick, 2019;Pagani *et al.*, 2009). HER2-positive tumors usually respond well to chemotherapy if diagnosed at an early stage. When treated at an early stage and in combination with chemotherapy, Trastuzumab has been reported to reduce the recurrence rate and show better prognosis. The overall survival rate of HER2-positive patients within ten years of diagnosis has been reported to have significantly improved from 75.2% (when treated with chemotherapy alone) to 84% (with treated adjuvantly with Herceptin) (Perez *et al.*, 2014). Early-stage TNBC responds well to chemotherapy (doxorubicin and taxanes) and radiation therapy, but the recurrence rates (38.4%) are high in the first five years following the treatment. If relapsed, the disease generally becomes resistant to chemotherapy (Wahba and El-Hadaad, 2015;Anders *et al.*, 2016;Saw *et al.*, 2019). The survival rates after the recurrence event are significantly low in TNBC as compared to other subtypes. Though highly responsive to NACT, (i.e., high pCR rates, pathological complete response), the overall relapse rates and mortality remain high (Bianchini *et al.*, 2016;Haffty *et al.*, 2006).

With the advent of targeted therapies and long term treatment approaches, the prognosis associated with ER-positive and HER2-positive breast cancers has dramatically improved. Due to a lack of TNBC specific targeted therapies, TNBC patients have poorer prognosis as compared to other subtypes. While there are efficient treatments available for relapse cases in the other two subtypes, TNBC patients are still struggling with the first line of treatments. There is a heightened need to develop TNBC specific targeted therapies for better prognosis (i.e., increased response rates, reduced recurrence rates, and better overall survival).

1.3. Recent efforts made to bridge the gap towards TNBC management

Various initiatives are made to overcome the challenges in TNBC treatments. Targeted therapies such as PARPi are approved for TNBC patients with BRCA1/2 mutations. Though the targeted treatment has significantly improved the survival rates of the BRCA subset (12%-15%), the remaining 85% are still struggling (Beniey, Haque and Hassan, 2019). In 2011, six TNBC subtypes displaying characteristic gene expression and ontologies were determined by utilizing gene expression profiles of 587 TNBC cases (Lehmann *et al.*, 2011). They further identified cell lines models specific to TNBC intrinsic subtypes, pharmacologically targeted signaling pathways in those cell lines, and showed that the subtypes were sensitive to different drugs (Lehmann *et al.*, 2011). Tumor infiltrating lymphocytes (TILs) are being investigated to understand their TNBC specific prognostic significance. An independent association was found between the percentage of intratumoral TILs and pCR. Having a high percentage of TILs (>60%) in stroma or tumor were found to have a high rate of pCR (41.7%), whereas only 2% pCR rate was found in tumors lacking TILs (Denkert *et al.*, 2010). Studies are being undertaken to investigate the TNBC specific role of TILs (García-Teijido *et al.*, 2016). Many studies found EGFR, a receptor tyrosine kinase overexpression in TNBC, and considered as a potential therapeutic target (Gumuskaya *et al.*, 2010;Martin *et al.*, 2009). Anti-EGFR treatments are available for colorectal cancer and non-small cell lung cancer (Nakai, Hung and Yamaguchi, 2016). Various clinical trials were conducted for investigating the efficacy of TNBC specific anti-EGFR drugs, but the outcomes have been disappointing by far. So, due to the low overall response rate, the clinical trials have failed (Nakai, Hung and Yamaguchi, 2016). Various tumors use the PD-1 receptor pathway to evade the anti-tumor immune response. High PD-L1 mRNA expression was found in TNBC samples. PD-L1 expression significantly associates with the aggressive clinical parameters (high histologic grade, larger tumor size, and high proliferative index); as a result, studies are being undertaken to explore immunotherapeutic approaches in TNBC (Wu *et al.*, 2018). The clinical perspective of above studies remains disappointing as most of the studies are either under preclinical investigations

or have failed in early trial stages. Novel approaches are necessary to address multiple aspects of TNBC.

1.4. Signaling pathways in TNBC

Various signaling pathways are studied to understand their correlation with TNBC (Minami, Chung and Chang, 2011;Wu *et al.*, 2018;Anders *et al.*, 2016;Pohl *et al.*, 2017). Lehmann et al. reported that TNBC has a characteristic gene expression for the Wnt pathway (Lehmann *et al.*, 2011). A study has shown that the activated Wnt signaling pathway correlated with poorer prognosis and increased metastasis in TNBC patients (N. *et al.*, 2013). Signal transduction pathways such as Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway are being investigated for TNBC specific association through *in vivo* and *in vitro* studies (Wu *et al.*, 2018). One such pathway which has been intensively studied is Hippo signaling. Yes-associated protein (YAP) and TAZ are downstream effectors of the Hippo cascade and have emerged as central determinants of malignancy in various human carcinomas (Lin, Park and Guan, 2018). They are already being investigated for their potential as target in multiple clinical settings (Yuan *et al.*, 2018;Calses *et al.*, 2019 ;Ciamporcero *et al.*, 2016;Santucci *et al.*, 2015).

1.5. YAP and Tumorigenesis

YAP is a co-activator protein that is regulated by the Hippo signaling pathway, as described in Figure 2. Overexpression of YAP is associated with tumor progression, metastasis, and loss of differentiation markers, drug resistance, and poor prognosis in various cancers (Lin, Park and Guan, 2018;Wang *et al.*, 2018;Zhang *et al.*, 2018;Qu *et al.*, 2019;Song *et al.*, 2018).

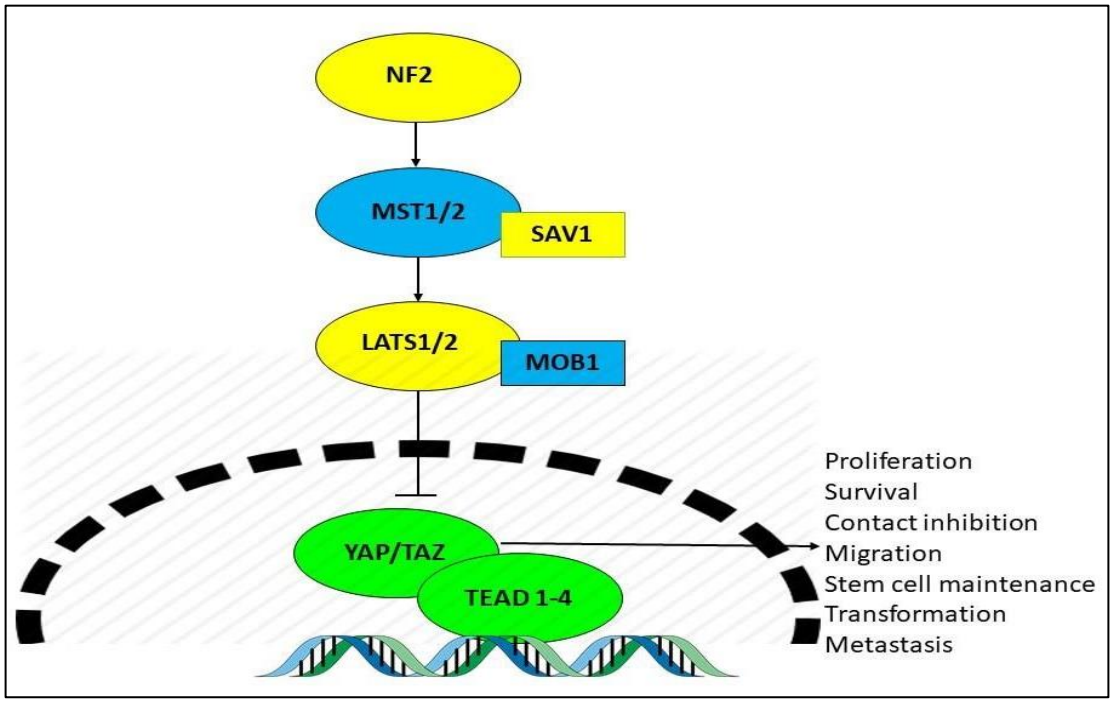

Figure 2. The Hippo Signalling Pathway: MST1/2 and SAV1 form a heterodimeric complex. MST1/2 heterodimeric complex phosphorylates and activates LATS1/2 heterodimeric complex. LATS1/2 complex inactivates YAP by phosphorylating it. Inactivated YAP is then sequestered in the cytoplasm and degraded. Mutation of Hippo components (NF2, SAV1, and LATS1/2) leads to functional impairment of the Hippo signaling pathway, i.e., YAP is translocated to the nucleus. Here it stays active and regulates gene transcription and expression of growth promoters and cell cycle regulators by binding to TEAD protein. YAP and TEAD (shown in green color) are found to be overexpressed in cancers. Adapted from Zhao, Li and Guan, 2010

Abbreviations: YAP: Yes-associated protein, LATS1/2: large tumor suppressor kinases, TAZ: transcriptional activator with PDZbinding domain, MST1/2: a member of the Ste-20 family of protein kinases, NF2: neurofibromin 2, CTGF: connective tissue growth factor, TEAD: Transcriptional enhancer factor.

In hepatocellular carcinoma, overexpression of YAP significantly associates with enhanced tumor aggressiveness and poorer prognosis. In murine liver carcinomas, Yap overexpression is necessary for continued tumor progression (Xu *et al.*, 2009;Zender *et al.*, 2006). Higher levels of YAP expression in pancreatic tumor patient samples is also associated with shortened overall survival (Rozengurt, Sinnett-Smith and Eibl, 2018). In *in vivo* and *in vitro* studies of *Kras* mutants, YAP knockdown leads to the abrogation of growth and proliferation of tumor cells (Zhang *et al.*, 2014). YAP is reported to be overexpressed in approximately 80% of colon cancers (Wang *et al.*, 2013). In Non-small-cell lung cancer (NSCLC), YAP expression is associated with lymph node metastasis and tumor aggressiveness (Wang *et al.*, 2010). In breast carcinomas role of YAP is controversial with two studies that report tumor-suppressive role of YAP while

most studies associate YAP expression with tumor progression in breast cancer and breast epithelial cell lines (Yuan *et al.*, 2008;Jaramillo-Rodríguez *et al.*, 2014;Wang, Su and Ou, 2012;Overholtzer *et al.*, 2006). Multiple immunohistological studies have reported that YAP overexpression significantly correlated with tumor progression, lymph-node positivity, recurrence, and reduced overall survival (Kim, Jung and Koo, 2015;Wang, Su and Ou, 2012;Guo *et al.*, 2019). These studies suggest that carcinogenesis driven by regulation of YAP expression might be determined by the genomic and cellular context of the tumor. Various studies have reported the prognostic significance of YAP with breast cancer subtypes, and survival outcomes, TNBC specific association of YAP, remains to be elucidated (Cao *et al.*, 2017;Kim, Jung and Koo, 2015;Kim, Jung and Koo, 2014).

1.6. The necessity for profiling TNBC in Indian Cohort

In the Indian cohort, significantly high portions (31%) of breast cancer patients are TNBC subtype. While the majority of breast cancers are associated with late age/post-menopausal patients, most of the patients diagnosed with TNBC are young/pre-menopausal women (Sandhu *et al.*, 2016). The relapse rate and mortality rate associated with TNBC are increasing significantly (Thakur, Bordoloi and Kunnumakkara, 2018). Screening TNBC specific reliable prognostic markers is imperative, which may eventually improve the efficiency of anticancer therapy.

In the present study, to investigate association of YAP expression with the TNBC subtype in an Indian cohort, primary TNBC tissues are assessed for nuclear and cytoplasmic YAP expression by immunohistochemistry (IHC). Ki67 (proliferative marker), CD-31 (angiogenic marker), and Vimentin expression (metastatic marker) is assessed as well to confirm the aggressiveness of the tumor. YAP expression scores are analyzed to investigate the association of YAP expression with the clinicopathological data and survival outcomes of the patients.

2. Preliminary data

In a study undertaken by Dr. Madhura Kulkarni (unpublished work), it was reported that YAP associated with high recurrence in breast cancer patients (Figure 3). YAP-signature expression was found to be enriched TNBC subtype (Figure 4).

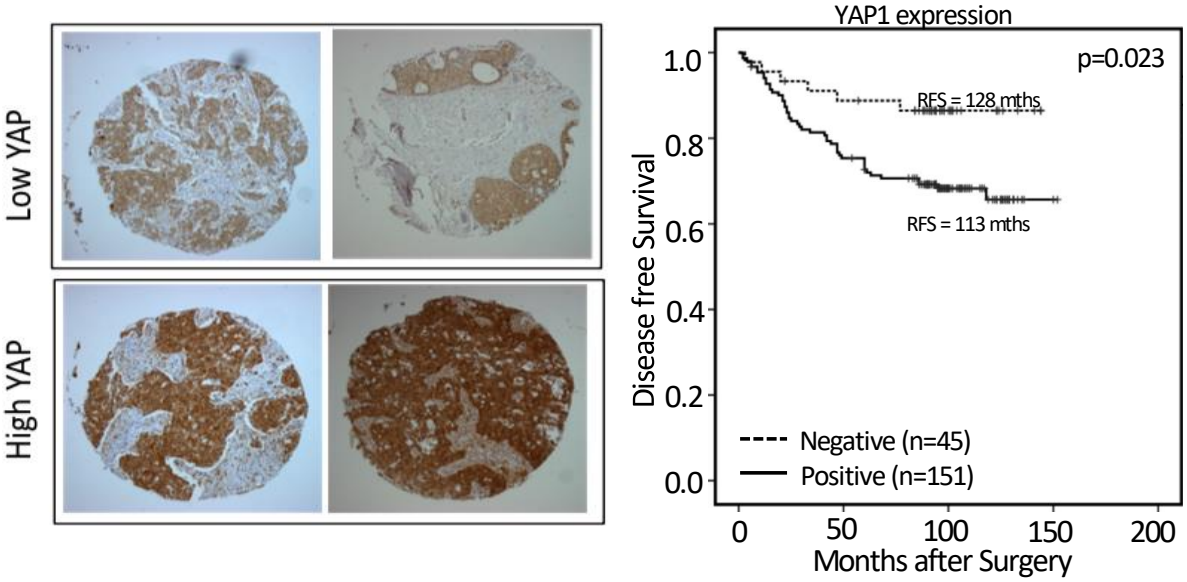

Figure 3. TMA analysis of breast cancer patient samples. Representative TMA images for YAP expression in breast cancer biopsies (left image). Univariate analysis of disease-free survival (Kaplan-Meier method) with respect to YAP1 expression scores in 248 breast cancer patient biopsies using the Log-rank (MantelCox) statistical test. DFS was calculated from the date of surgery until the date of recurrence. The presence of YAP significantly associates with decreased Disease-free Survival (right image)

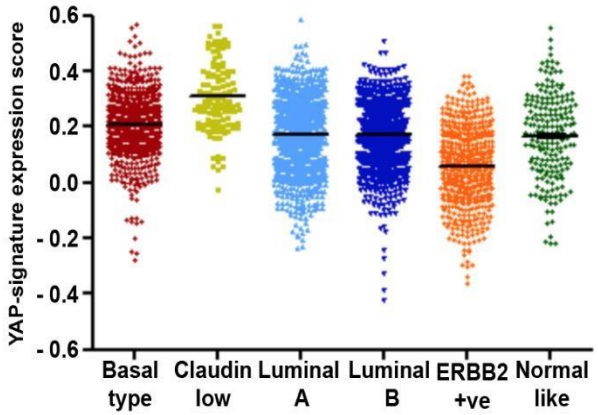

Figure 4. YAP signature for individual patient expression data plotted in a box-whisker plot for each of the molecular subtypes in 26 breast cancer cohorts from Gene Expression Omnibus (GEO) and ArrayExpress. (each gray dot represents individual patient, n=3992)

3. Aim and Objectives

The aim of this study is to test the prognostic significance of YAP with aggressive features of TNBC.

Objectives:

1. Standardise immunochemistry (IHC) protocol for the newly setup laboratory
2. Investigate association of YAP expression with clinicopathological parameters of TNBC patients
3. Investigate association of YAP with patient survival outcomes
4. Investigate association of YAP expression with other molecular markers
5. Investigate association of YAP expression with pathological complete respone

4. Material and Methods

4.1. Patient Data Collection

Patient data and tissue blocks were collected following ethical approval (dated 21st July 2018 and IECHR/VB/2018/016) and guidelines.

After obtaining consent from patients diagnosed with breast cancer and undergoing treatment at Prashanti Cancer Care Mission, their medical history was retrieved. All the information from the time of diagnosis until the treatment and follow-ups were obtained and curated according to their diagnosis, and the treatment received.

The tissue blocks were then transferred from Prashanti Cancer Care Mission Clinic to the IISER Pune lab following ethical approval (IECHRIVB/2018/016) and guidelines.

4.2. Data Sorting

Once the curated patient data was received, the database was sorted such that only TNBC primary tissue was selected.

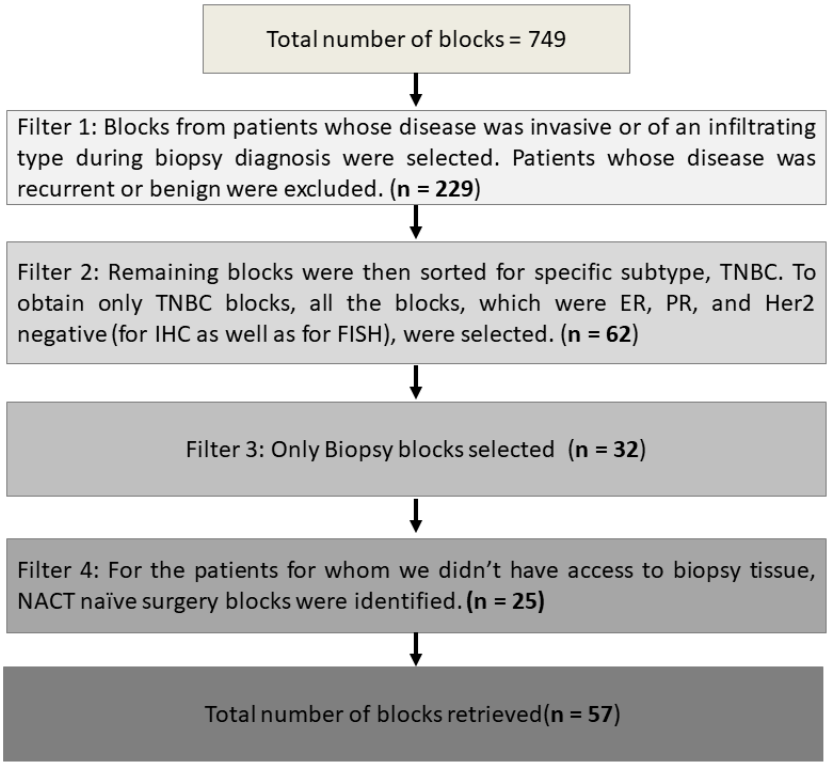

Figure 5. Schematic of steps involved in sorting TNBC primary tissue blocks. 32 biopsy blocks and 25 surgery blocks were procured from PCCM

The database consisted of information for samples from 749 patients as of 2nd April 2019. Data sorting steps are summarised in Figure 5.

Thus, 57 TNBC primary tumor blocks were identified. 32 of them were biopsy samples, while 25 were NACT naïve surgery samples.

4.3. H&E Staining

Primary breast tumor tissue blocks were procured, sectioned, and followed through H&E to know the optimal tumor percentage prior to IHC. Blocks were sectioned at 4 µm using microtome (Leica RM2255 Fully Automated Rotary Microtome). Sections were deparaffinized in a hot air oven at 62°C (overnight). Once deparaffinized tissues were rehydrated as follows:

a) Xylene: two washes 15 minutes each
b) 100% Ethanol: one wash for 3 minutes
c) 90% Ethanol: one wash for 3 minutes
d) 70% Ethanol: one wash for 3 minutes
e) 50% Ethanol: one wash for 3 minutes
f) Distilled water: one wash for 3 minutes

After rehydration, the slides were transferred to the humidifying chamber. The slides were incubated with 100 -200 µl of ready to use filtered Hematoxylin for 10 minutes at room temperature. Each slide was washed under running tap water, with tissue side away from the running water until sufficient de-staining was attained. Slides were dipped in a jar containing 1% acid alcohol to fix the stain for 5 seconds. Slides were then washed in running tap water for 10-15 minutes. After washing, the slides were incubated with a 0.5% eosin working solution for 1 minute.

The tissues were then dehydrated using the following steps.

a) 70% Ethanol: one wash for 1 minute
b) 90% Ethanol: one wash for 1 minute
c) 100% Ethanol: one wash for 1 minute
d) Air Dry for 10 minutes
e) Xylene: two washes 15 minutes each

After dehydration, slides were mounted in DPX.

4.4. Immunohistochemistry

After HnE staining IHC was performed for YAP (nuclear and cytoplasmic), Ki67, Vimentin, and CD31.

Blocks were sectioned at 3 µm thickness using microtome (Leica RM2255 Fully Automated Rotary Microtome). Paraffin-embedded sections were deparaffinized in a hot air oven at 62°C (overnight). Once deparaffinized tissues were rehydrated as follows:

a. Xylene: two washes 15 minutes each

b.100% Ethanol: one wash for 3 minutes

c.90% Ethanol: one wash for 3 minutes

d.70% Ethanol: one wash for 3 minutes

Distilled water: one wash for 3 minutes

After rehydration, using a PAP pen (BIOCARE Medical), a hydrophobic barrier around the tissue was drawn. Tissues were treated with 100µl-200µl 0.3% hydrogen peroxide (Thermo Scientific) and incubated for 30 minutes in a humidifying chamber to block non-specific background staining due to the presence of endogenous peroxidases. The slides were then washed by immersing the slides for 5 minutes with TBST buffer (0.05% Tween 20 in 1% TBS). Heat-Induced Epitope Retrieval (HIER) using Multi-Epitope Retrieval System was performed. The slides were heated in 1x citrate buffer (Thermo Scientific 5x) (pH 6.0 for YAP) and 10mM Tris-EDTA (pH 9 for CD31, Ki-67, and Vimentin) for 20 minutes at 100°C. After retrieval, slides were allowed to cool in a buffer and washed by placing in TBST buffer for 5 minutes. The tissues were then treated with 100µl-200µl Protein block (Thermo Scientific) and incubated for 30 minutes in a humidifying chamber. The slides were then washed by immersing the slides with TBST buffer for 5 minutes. The slides were then incubated with 100µl-150µl primary antibody for the required time and temperature as given in the antibody guideline (Table 1).

After incubation, slides were washed by immersing with TBST buffer, four washes for 5 minutes each. Slides were then incubated with 100-200µl of Primary Antibody Amplifier Quanto(Thermo Scientific) for 10 minutes at room temperature. After incubation, slides

were washed by immersing with TBST buffer, four washes for 5 minutes each followed by 10 minutes incubation with HRP-polymer Quanto (Thermo Science).

Antibody	Dilution	Incubation time	Incubation Temperature
YAP (Rabbit monoclonal)	1:200	Overnight	4 °C
CD31 (DAKO Monoclonal Mouse Anti-Human CD31, Endothelial Cell Clone JC70A)	Ready to use	60 min	25 °C
Vimentin (DAKO Monoclonal Mouse Anti-Vimentin, Clone V9)	Ready to use	60 min	25 °C
Ki67 (DAKO Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1)	Ready to use	60 min	25 °C

Table 2 : Primary Antibodies used
YAP (1:200), CD31 (RTU), Vimentin (RTU) and Ki67 (RTU)
RTU: Ready to use

Then the slides were rinsed with by immersing with TBST buffer, four washes for 5 minutes. 100-200µl 3,3'-diaminobenzidine (DAB Quanto Chromogen, 30 µl of DAB in 1ml DAB Substrate) was added and incubated for 10 seconds. The slides were placed in a water bath for 5 minutes, followed by counterstaining with Hematoxylin (1:1).

The tissues were then dehydrated using the following steps.

a.70% Ethanol: one wash for 1 minute

b.90% Ethanol: one wash for 1 minute

c.100% Ethanol: one wash for 1 minute

d.Air Dry for 10 minutes

e.Xylene: two washes 15 minutes each

After dehydration, slides were mounted in DPX.

4.5. Scoring of immunostained tissue sections

Scoring was done by a certified pathologist Dr. Anirudha Puntambekar, Ruby Hall Clinic, Pune. YAP expression was scored based on staining intensity and the percentage of regions that are positive for nuclear or cytoplasmic expression. Percent expression was then binned as follows: 0 (none), 1 (<10%), 2 (10–50%) and 3 (>50%). Intensity is scored and then binned as follows: 0 (none), 1 (light), 2 (moderate) and 3 (intense). A final composite score was calculated as the product of intensity and percent expression, and the composite score lied within the range of 0 to 9. ROC was determined, and hence the scores were binned as follows, the YAP sections that scored greater than or equal to 6 were regarded as high YAP expression, and the sections that scored less than six were regarded as low YAP expression. Ratio of nuclear to cytoplasmic YAP was calculated by dividing percentage of positive nuclear YAP expression by percentage of positive cytoplasmic YAP expression in tumor regions. Ki-67 was scored high for percent tumor nuclei expression ≥25% (Duffy *et al.*, 2017). CD31 and Vimentin were considered positive if the tumor cells exhibited immunostaining. Representative images and scoring procedures for Ki-67, Vimentin, YAP, and CD31 expression are shown in Figures 8,9,10 and 11, respectively.

4.6. Statistical Analysis

All the statistical analysis was carried out using the Statistical Package for Social Science v. 21 software (SPSS Inc., Chicago, IL, USA). The associations between categorical variables (eg., age of diagnosis, menopausal status, tumor grade, tumor size) were assessed using Pearson's chi-squared test (χ^2), and Fisher's exact test. Overall survival and Disease-free survival analyses were analyzed using the Kaplan-Meier method, and log-rank tests. Cox's proportional hazard regression test was used to identify factors affecting recurrence free survival. A Pearson's r was computed to assess the relationship between YAP and other expression markers included in this study. The reported P values are two sided and *p*-value of < 0.05 was considered statistically significant.

5. Results

5.1. Standardization of IHC protocol

To standardize that IHC protocol in a newly set-up laboratory, we selected two tissue samples for whom Ki67 % expression was already reported by a pathologist. With a NORDIQ certified Ki67 antibody (DAKO Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1), we tested expression of Ki67 in those two tissue samples, the expression scores as validated by a pathologist, tally with the reported scores, as shown in Figure 6).

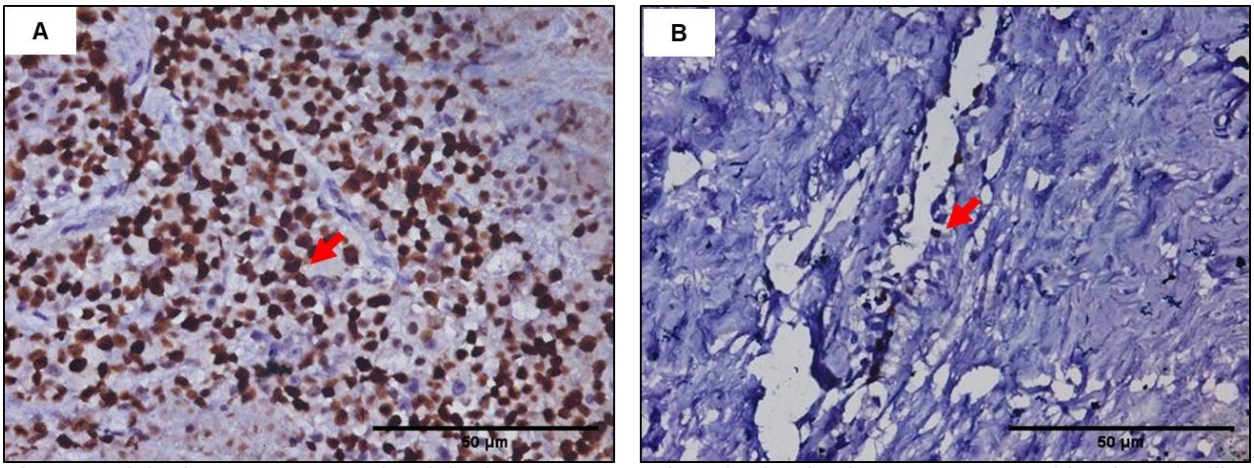

Figure 6: (A) Ki67 80% expression (B) Ki67 3% expression obtained using Mantra at Perkin Elmer facility at 40x magnification.

For positive tissue control, benign breast tumor was stained with YAP, Vimentin, CD31, and Ki67 (Figure 7), and the results were positive. No primary control (aka secondary antibody only control) was run simultaneously to determine nonspecific binding (Figure 7).

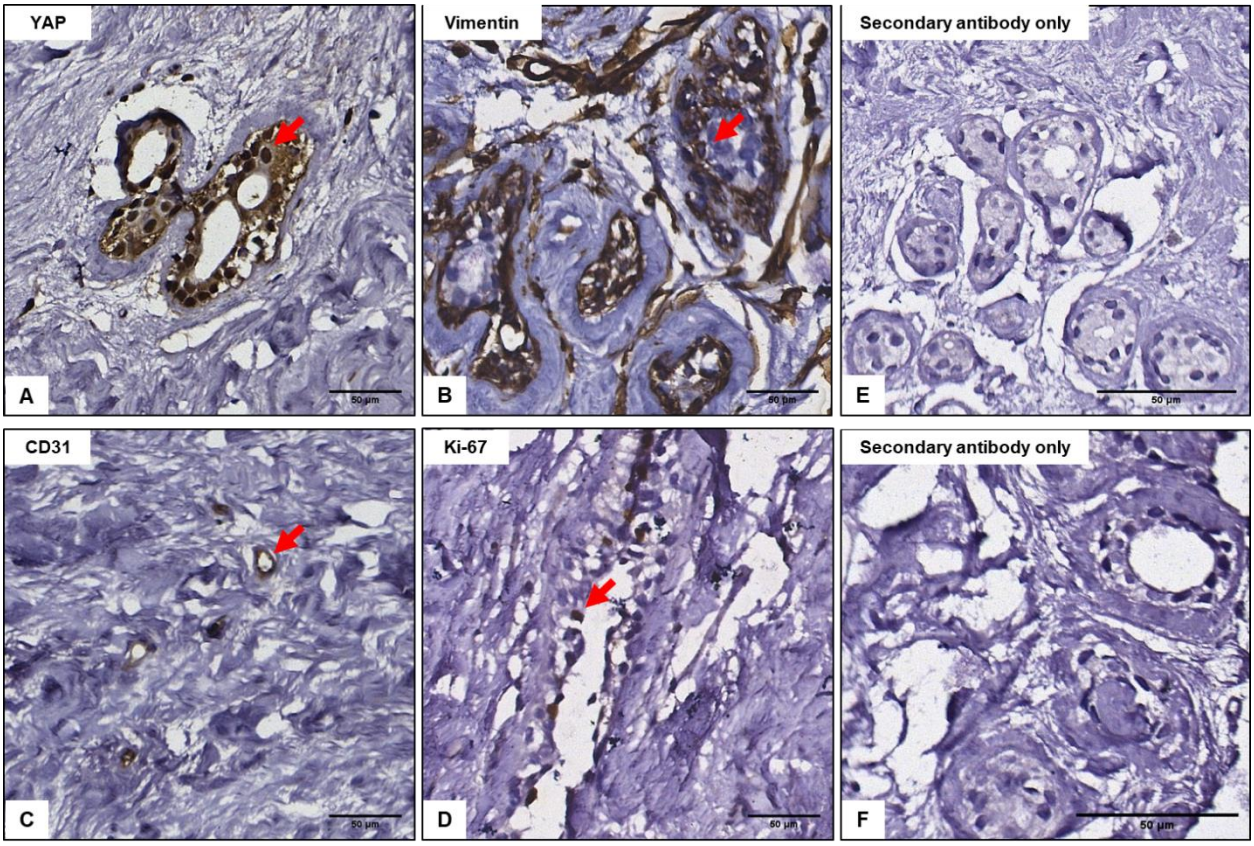

Figure 7: Standardization Images : (A) YAP primary control, (B) Vimentin primary control, (C) CD31 primary control, (D) Ki67 primary control (E, F) No primary control for tumor tissue at 40x magnification.

As no false positives were detected, the assay was confirmed to be working correctly. The clones of the CD31, Vimentin, and Ki-67 antibodies used are certified and tested by NORDIQ. Optra Scans-15 HD model was used to image the above sections.

5.2. Representative Images

Figure 8(A) is representative of high Ki-67 expression. Percent Ki-67 expression in (A) is 40%. As the expression level is ≥25%, it is regarded as high.

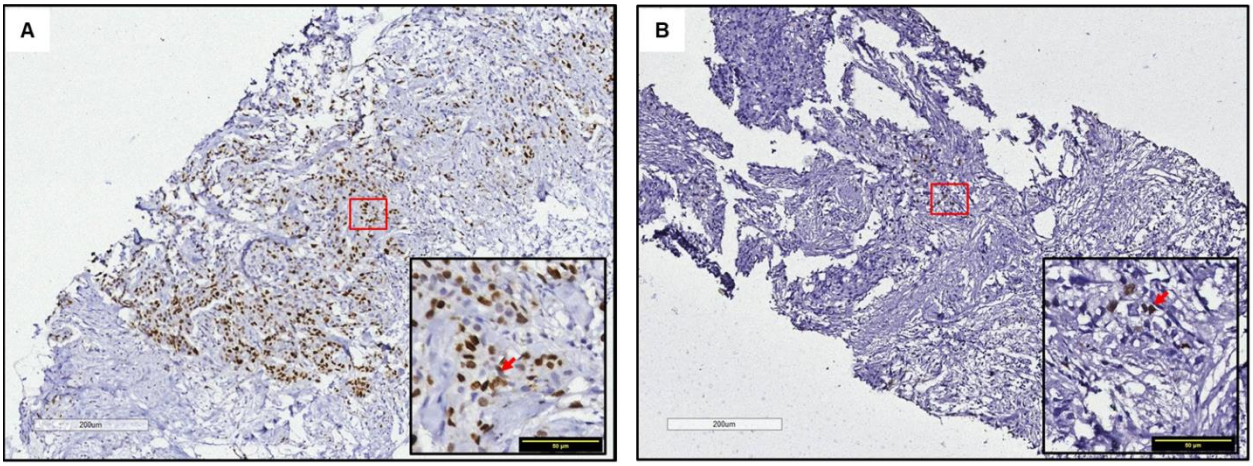

Figure 8: Representative images of Ki67 expression at 10X and 40X, respectively. Imaged on Optra Scans-15 HD model

Figure 8(B) is representative of low Ki-67 expression. Percent Ki-67 expression in (B) is 10%. As the expression level is <25%, it is regarded as low expression.

Figure 9(A) is representative of positive Vimentin expression as Vimentin is expressed in the tumor cytoplasm.

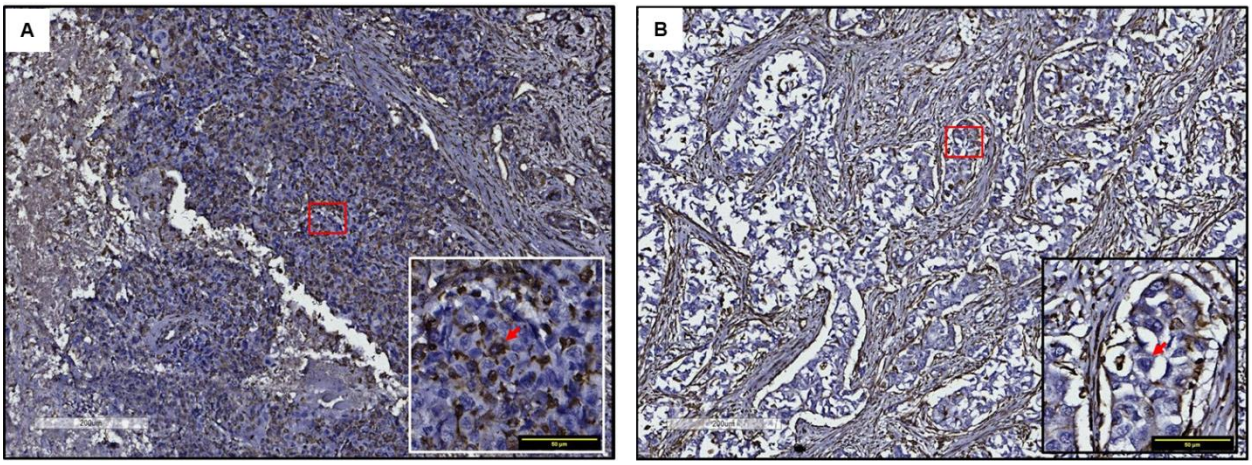

Figure 9: Representative images of Vimentin expression at 10X and 40X, respectively. Imaged on Optra Scans-15 HD model

9(B) is representative of absence Vimentin expression as Vimentin is absent in the tumor cytoplasm.

Figure 10(A) is representative of low nuclear and low cytoplasmic YAP expression. It was scored as follows, NYAP percent expression:90% (bin:3), NYAP intensity :1, NYAP Composite Score: 3(Low), CYAP percent expression:10% (bin:1), CYAP intensity :1, CYAP Composite Score : 1(Low).

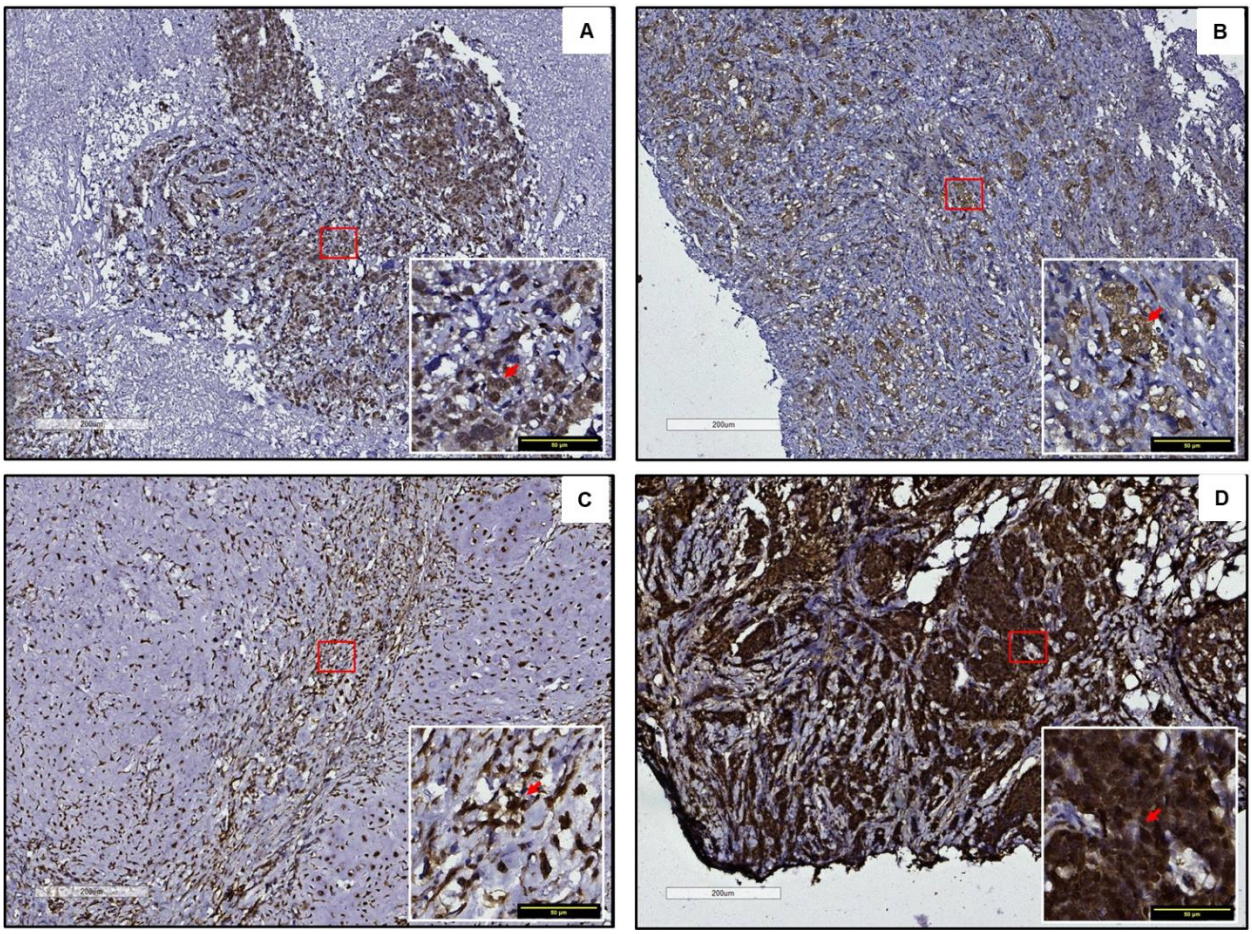

Figure 10: Representative images of nuclear and cytoplasmic YAP expression at 10X and 40X, respectively. Imaged on Optra Scans-15 HD model
(A) Low Nuclear and Low Cytoplasmic expression (B) Low Nuclear and High Cytoplasmic expression (C) High Nuclear and Low Cytoplasmic expression (D) High Nuclear and High Cytoplasmic expression; Nuclear YAP : NYAP, Cytoplasmic YAP: CYAP

Figure 10(B) is representative of low nuclear and high cytoplasmic YAP expression. It was scored as follows, NYAP percent expression:40% (bin:2), NYAP intensity :1, NYAP Composite Score :2(Low), CYAP percent expression:70% (bin:3), CYAP intensity :2, CYAP Composite Score : 6(High). Figure 10(C) is representative of high nuclear and low cytoplasmic YAP expression. It was scored as follows, NYAP percent expression:90% (bin:3), NYAP intensity :3, NYAP Composite Score: 9(High), CYAP percent expression:10% (bin:1), CYAP intensity :1, CYAP Composite Score : 1(Low). Figure 10(D) is representative of high nuclear and high cytoplasmic YAP expression. It

was scored as follows, NYAP percent expression:90% (bin:3), NYAP intensity :3, NYAP Composite Score :9(High), CYAP percent expression:90% (bin:3), CYAP intensity :3, CYAP Composite Score : 9(High).

Figure 11(A) is representative of positive CD31 expression as CD31 is expressed in the tumor cytoplasm.

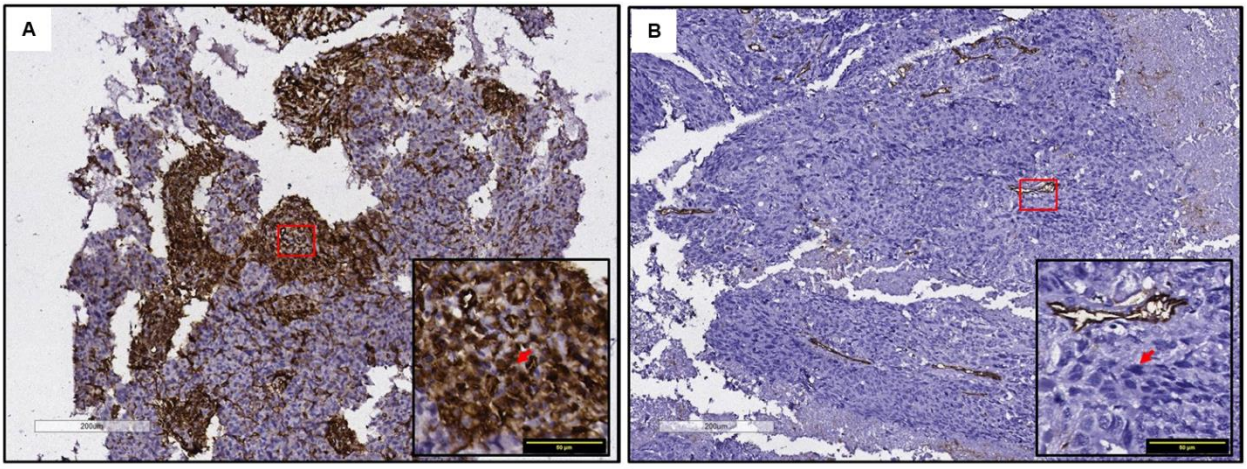

Figure 11: Representative images of CD31 expression at 10X and 40X, respectively. Imaged on Optra Scans-15 HD model

11(B) is representative of absence CD31 expression as CD31 is absent in the tumor cytoplasm.

5.3. Tissue processing and scoring summary

57 TNBC tissue blocks were retrieved from PCCM. Some tissues were lost during IHC staining process at antigen retrieval step. Successfully processed tissues were scored by Dr. Anirudha Puntambekar, Ruby Hall Clinic, Pune (Table 2).

No. of TNBC blocks received from PCCM	57
No. of blocks processed	57
No. of tissues stained and scored by Dr. Anirudh for YAP	54
No. of tissues stained and scored by Dr. Anirudh for Ki67	50
No. of tissues stained and scored by Dr. Anirudh for CD31	53
No. of tissues stained and scored by Dr. Anirudh for Vim	49

Table 2: Tissue processing summary

Antibody	Expression Score	
Nuclear YAP	High	Low
n = 54	40	14
Cytoplasmic YAP	High	Low
n = 54	40	14
Ki67	High	Low
n = 50	42	8
CD31	Yes	No
n = 53	25	28
Vimentin	Yes	No
n = 49	34	15

Table 3: Scoring summary

40 tissues expressed high nuclear and cytoplasmic YAP expression (Table 3). 14 tissues expressed low nuclear and cytoplasmic YAP expression. 38 tissues had high Ki67 expression. CD31 was positive in 25 tissues. Vimentin was positive in 34 tissues.

5.4. Patient Characteristics

Characteristics	No. of case of examination (%)
Ethnicity	Indian
Age (n = 53)	
≤ 50	31(58.4)
> 50	22(41.5)
nav = 1	
Menopausal Status (n = 48)	
Pre	20(41.6)
Post	28(58.3)
nav = 6	
Histology Grade (n = 52)	
High	29(55.7)
Low	23(44.2)
nav = 2	
LVI Status (n = 44)	
Positive	13(29.5)
Negative	31(70.4)
nav = 10	
Clinical Tumor Size (n = 49)	
cT1	7(14.2)
cT2	38(77.5)
cT3	1(2.04)
cT4	1(2.04)
nav = 5	
Clinical LN status (n=43)	
Positive	27(62.7)
Negative	16(37.2)
nav = 11	
pTNM Stage (n = 33)	
I	4 (12.1)
II	25 (75.7)
III	4 (12.1)
nav = 21	

54 TNBC patients included in this study had a median age of 49 years (range: 37- 61 years). At the time of diagnosis, 58.3% (28/48) and 41.6% (20/48) of the patients were post-menopausal and pre-menopausal, respectively (Table 4). 62.7% (27/43) of the patients were found to harbor lymph node positivity. Lymphovascular invasion was present in 29.5% (13/44) of the patients. 55.7% (29/52) and 44.2% (23/52) had high grade (grade = 3) and low grade (grade < 3) tumors, respectively. 77.5% (38/49) patients had tumor size ranging from 20mm to 50mm (cT2). According to the American Joint Committee on cancer staging system grouping criteria, 12.1% (4/33) of the patients had stage I, 75.7% (25/33) had stage II, and 12.1% (4/33) had stage III disease, respectively. The patients were followed up for a mean of 29 ± 25 months, and the last follow-up occurred in September 2019.

Table 4: Clinical characteristics of patients

Abbreviations: nav: patient information for the parameter under consideration not available; Histology Grade ≥ 3: High, Grade < 3 : Low; LVI: Lymphovascular Invasion; Clinical Tumor Size, cT1: T≤ 20 mm, cT2: 20mm<T≤50mm, cT3: T>50mm, cT4: Tumor has grown into chest wall and/or skin; LN: Lymph node; TNM : Tumor Node Metastasis

5.5. Association of YAP expression with clinicopathological parameters of TNBC patients

YAP expression was detected in both the nuclei and cytoplasm of tumor cells (Figure 10). Overall high nuclear and cytoplasmic YAP expression was observed in tumor cells of 74% (40/54) tissues. Associations of nuclear YAP and cytoplasmic YAP expression with clinicopathological parameters was compared using Pearson χ2 and Fisher's exact test (Table 5). Nuclear YAP expression significantly correlated with the presence of lymph node metastasis (P=0.033). Cytoplasmic YAP expression associated with tumor infiltrating lymphocytes (TILs) (P=0.082), although it was not statistically significant. No significant association was observed between YAP expression and other clinical parameters.

Characteristics		n	Cytoplasmic YAP Expression		*P*	Nuclear YAP Expression		*P*
			High n(%)	Low n(%)		High n(%)	Low n(%)	
Ethnicity	Indian	n= 54						
Age at Diagnosis (years)	Age ≤ 50 = 31	n =53	23 (43.4)	8 (15.1)	0.797	22 (41.5)	9 (17)	0.608
	Age > 50 = 22		17 (32.1)	5 (9.4)		17 (32.1)	5 (9.4)	
Menopausal Status	Pre = 20	n =48	16 (33.3)	4 (8.3)	0.904	14 (29.2)	6 (12.5)	0.701
	Post = 28		22 (45.8)	6 (12.5)		21 (43.8)	7 (14.6)	
Histology Grade	High = 29	n = 52	21 (40.4)	8 (15.4)	0.629	21 (40.4)	8 (15.4)	0.904
	Low = 23		18 (34.6)	5 (9.6)		17 (32.7)	6 (11.5)	
LVI Status	Positive = 13	n = 44	8 (18.2)	5 (11.4)	0.131*	10 (22.7)	3 (6.8)	1*
	Negative = 31		26 (59.1)	5 (11.4)		25 (56.8)	6 (13.6)	
Clinical Tumor Size	cT1 = 7	n=45	4 (8.9)	3 (6.7)	0.362*	5 (11.1)	2 (4.4)	1*
	cT2 = 38		29 (64.4)	9 (20)		28 (62.2)	10 (22.2)	
Clinical LN Status	Positive = 27	n = 43	18 (41.9)	9 (20.9)	0.484*	17 (39.5)	10 (23.3)	**0.033***
	Negative = 16		13 (30.2)	3 (7)		15 (34.9)	1 (2.3)	
TILs Status	>50% = 16	n = 50	9 (18)	7(14)	**0.082***	12 (24)	4 (8)	1*
	≤ 50% = 34		28 (56)	6 (12)		26 (52)	8 (16)	

Table 5: Association of nuclear and cytoplasmic YAP expression with clinicopathological parameters of TNBC patients
YAP expression was assessed for association with Age, Menopausal status, Histology grade, LVI status, Clinical tumor size, Clinical lymph node positivity and tumor infiltrating lymphocytes (TILs)
* *P* based on Fisher's exact for * marked parameters

More than 40% of high nuclear and cytoplasmic YAP expression was observed in patients whose age was less than or equal to 50 (Table 5). A similar distribution was observed in 58.3% of postmenopausal patients. 40% of high grade tumors expressed

high nuclear and cytoplasmic YAP. 29.5% of the patients with lymphovascular invasion had 18.2% of high cytoplasmic YAP and 22.7% of high nuclear YAP. More than 60% of cT2 tumors expressed high cytoplasmic and nucleic YAP. Around 40% of lymph node positive tumors expressed high cytoplasmic and nucleic YAP. Low cytoplasmic and low nuclear YAP expression was observed in patients with TILs level more than 50%. In patients with TILs level less than or equal to 50% high YAP expression (52% nuclear YAP, 56% cytoplasmic YAP) was observed.

5.6. Prognostic significance of YAP expression in TNBC

Kaplan-Meier curves and a log-rank test was used to analyze overall survival and disease free survival outcomes with respect to cytoplasmic and nuclear YAP expression (Table 10).

Overall Survival (OS)

Overall Survival (OS) was calculated from the date of biopsy/diagnosis to the last follow up date (Figure 12). Event is considered ~~only~~ when death has occurred due to cancer. Out of 54 patients, diagnosis date, and follow up information was available for 52 patients (Table 6). Average follow up time concerning overall survival is 2±2 years and median follow up time is 2 years (Table 7).

Follow up for OS	Months	Years
Minimum Follow up Time	0.00 (n=1)	0.00
Maximum Follow up Time	129.00 (n=1)	11
Average Follow up Time	29± 25	2± 2
Median Follow up Time	24	2
Mode	6	0.5

Table 6: Patient Summary
One patient failed to follow up. Fifty-one patients have more than zero months of follow up.

Patient Follow up Status	
Number of patients with greater than 0 months for overall survival	N = 51
Number of patients with 0 months follow-up for overall survival	N = 1
Number of patients who no biopsy date	N = 2
Total	N = 54

Table 7: Follow up Time for OS
Minimum follow up time is zero months for one patient. Maximum follow up time is one hundred and twenty nine months (11 years) for one patient. The average follow up time for OS is 29±25 months.

In each tissue both nuclear and cytoplasmic YAP expression was assessed. OS was assessed for high and low YAP expression based on composite scores. Out of 52 patients, primary tumors of 13 presented with low nuclear YAP while 39 presented with high nuclear YAP expression (Figure 12A).

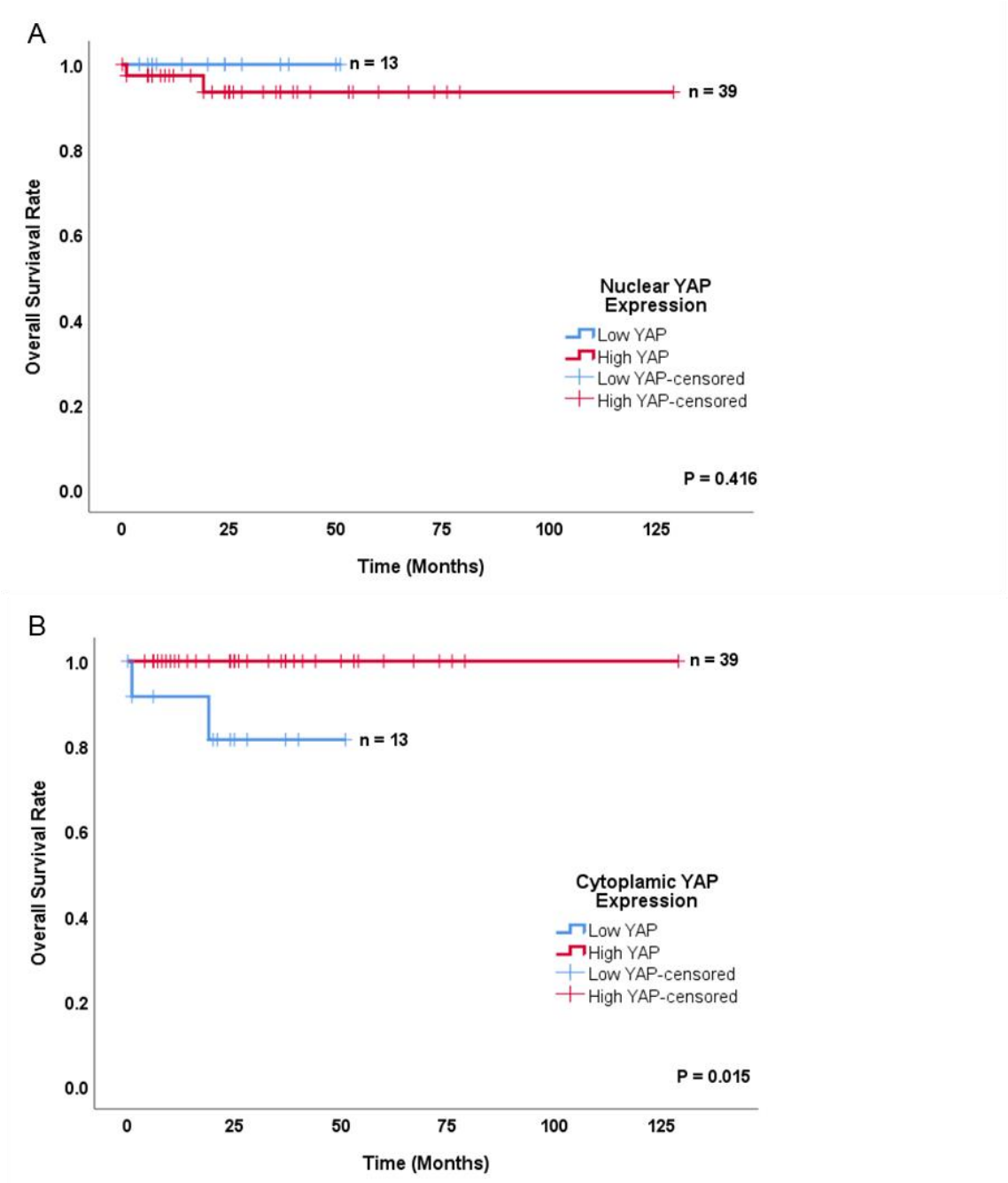

Figure 12: Overall Survival curve of TNBC patients with **(A)** nuclear YAP as a risk factor. Two events are observed in the group with high nuclear YAP expression **(B)** cytoplasmic YAP as a risk factor. Two events are observed in the group with low cytoplasmic YAP expression.

Two events have occurred; in both events, the patients had high nuclear YAP expression. However, nuclear YAP expression wasn't significantly associated with OS (*P* = 0.416, Figure 12A). Out of 52 patients, primary tumors of 13 presented low cytoplasmic YAP while 39 presented high cytoplasmic YAP expression (Figure 12B). Two events have occurred; in both events, the tumors have low cytoplasmic YAP expression. Low cytoplasmic YAP expression significantly correlated with reduced OS in patients (*P* = 0.015, Figure 12B).

Since nuclear YAP is transcriptionally active form of YAP, in order to test if higher nuclear compared to cytoplasmic expression of YAP matted towards survival outcomes, ratio of nuclear to cytoplasmic expression of YAP percent was calculated . The ratios were binned into three categories, for higher, lower, or equal expression of nuclear YAP with respect to cytoplasmic YAP. Ratio of < 0.90 was regarded as low nuclear to cytoplasmic YAP percent. 0.90 ≤ Ratio (r) ≤ 1.0 was regarded as equal nuclear to cytoplasmic YAP percent. Ratio > 1.0 was regarded as high nuclear to cytoplasmic YAP percent. OS was then analyzed with respect to these three bins (*P* = 0.007, Figure 12C).

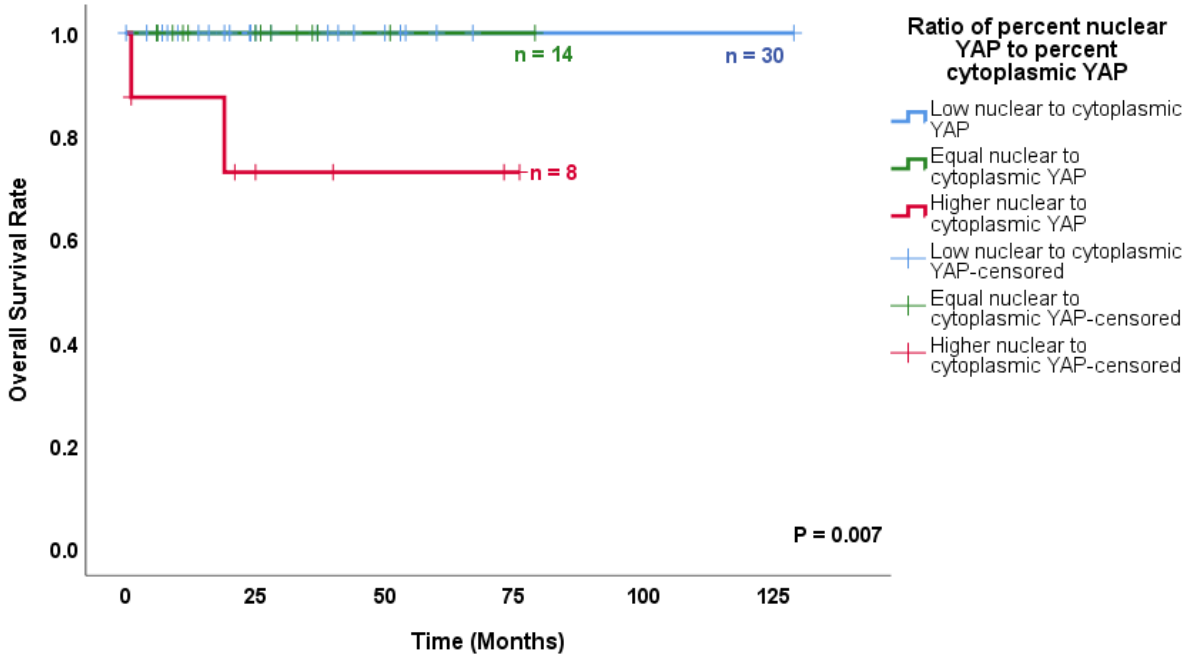

Figure 12C: Overall Survival curve of TNBC patients with percent nuclear YAP to percent cytoplasmic YAP as a risk factor plotted using Kaplan-Meier model. Two events are observed in the group with high nuclear to cytoplasmic YAP percent.

Out of 52 patients, 30 patients had low nuclear to cytoplasmic YAP expression, 14 patients had equal nuclear to cytoplasmic YAP expression, and 8 patients had high nuclear to cytoplasmic YAP expression. Two events that occurred had high nuclear to cytoplasmic YAP expression ratio. Higher nuclear YAP expression compared to that of cytoplasmic YAP expression significantly correlated with decreased OS in patients (*P* = 0.007, Figure 12C).

Disease-free Survival (DFS)

Disease-free survival (DFS) is calculated since the time patient is disease free (i.e., surgery in this particular cohort), till the time of local or distance recurrence (Figure 13). Event is considered when the disease recurred. Out of 54 patients, surgery date was available for 50 patients (Table 8). Average follow up time concerning disease free survival is 2±2 years and median follow up time is 1.6 years (Table 9).

Patient Follow up Status	
Number of patients with greater than 0 months for DSF	N = 43
Number of patients with 0 months follow-up for DFS	N = 7
Number of patients with no surgery date	N = 4
Total	N = 54

Table 8: Patient Summary
Seven patients failed to follow up. Forty-three patients have more than zero months of follow up.

Follow up Time for DFS	Months	Years
Minimum Follow up Time	0 (n=7)	0
Maximum Follow up Time	129 (n=1)	11
Average Follow up Time	26± 26	2±2
Median Follow up Time	19	1.6
Mode	0	0

Table 9: Follow up Time for DFS
Minimum follow up time is zero months for seven patients. Maximum follow up time is one hundred and twenty nine months (11 years) for one patient. The average follow up time for DFS is 26±26 months.

Out of 50 patients, primary tumors of 12 presented with low nuclear YAP expression, while 38 present with high YAP expression in tumor nuclei (Figure 13A). All five recurrence events have occurred in patients with high nuclear YAP expression.

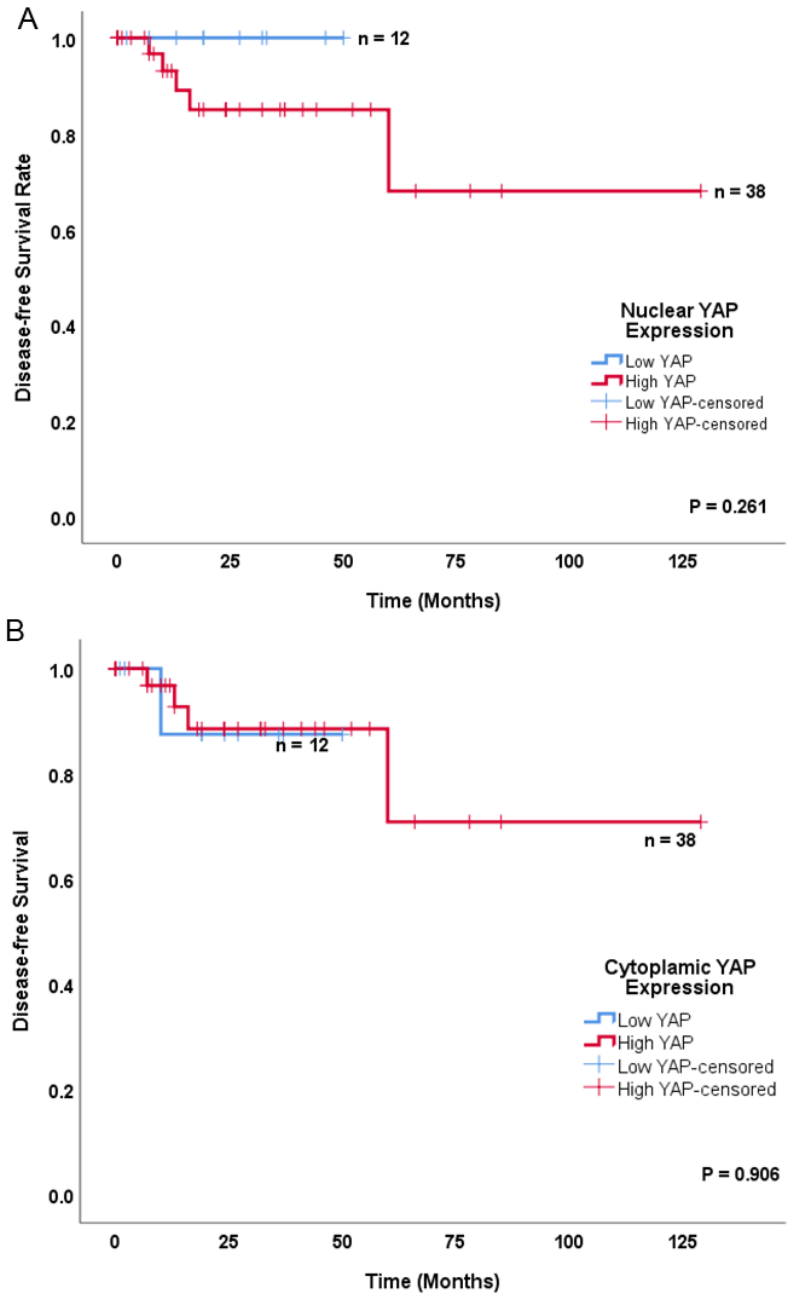

Figure 12: Disease-free Survival curve of TNBC patients with **(A)** nuclear YAP as a risk factor. All five events are observed in the group with high nuclear YAP expression **(B)** cytoplasmic YAP as a risk factor Four events are observed in the group with high cytoplasmic YAP expression. One event is observed in the group with low cytoplasmic YAP expression.

Out of 50 patients, primary tumors of 12 present with low cytoplasmic YAP expression, while 38 present with high YAP expression in tumor cytoplasm (Figure 13B). Out of five recurrence events, 4 events have occurred in the patients with high cytoplasmic YAP expression, and one event is observed in the patient with low cytoplasmic YAP. However, no significant association was found between nuclear YAP expression, cytoplasmic expression and DFS (*P*=0.261, Figure 12A; *P*=0.906, Figure 12B).

DFS was analysed for ratio of percent nuclear YAP to percent cytoplasmic YAP expression as well (Figure 12C).

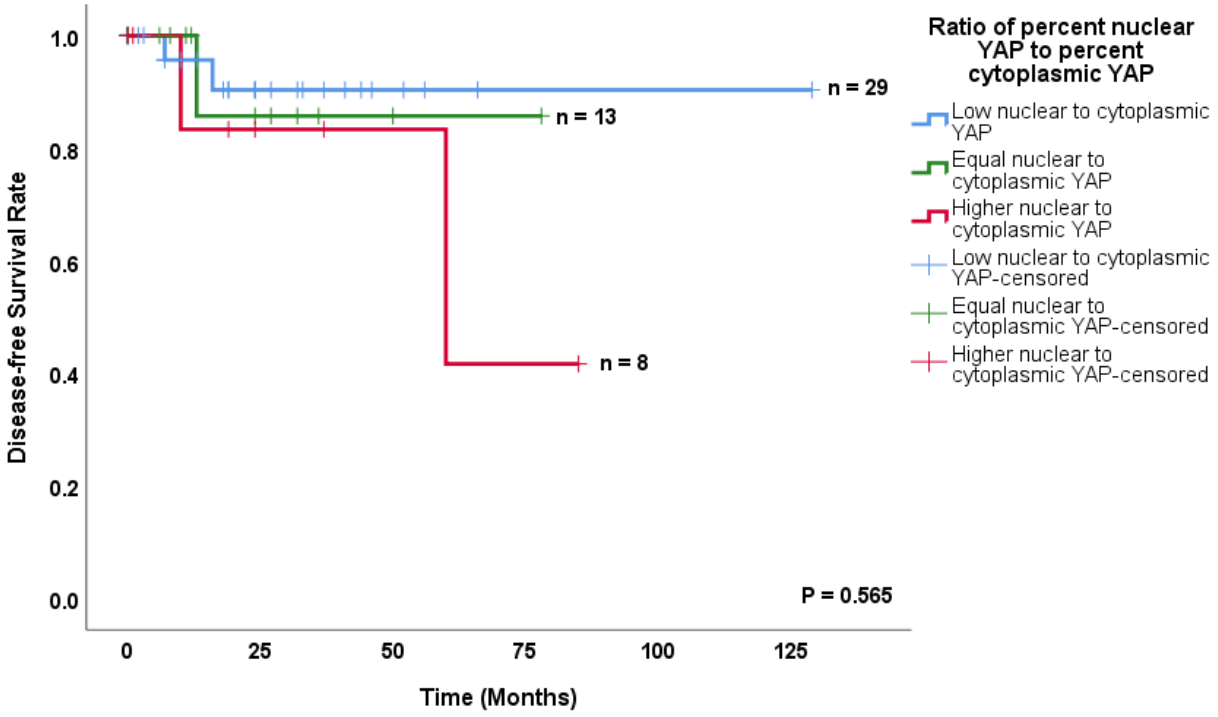

Figure 12C: Disease-free Survival curve of TNBC patients with percent nuclear YAP to percent cytoplasmic YAP as a risk factor plotted using Kaplan-Meier model. Two events had occurred in the group with low nuclear to cytoplasmic YAP, one event had occurred in the group with equal nuclear to cytoplasmic YAP and two events occurred in the group with high nuclear to cytoplasmic YAP**.**

Out of 50 patients, 29 patients had low nuclear to cytoplasmic YAP expression with two events, 13 patients had equal nuclear to cytoplasmic YAP expression with one event, and 8 patients had high nuclear to cytoplasmic YAP expression with two events. However, no significant association was found between nuclear to cytoplasmic YAP percent with recurrence free survival (*P* = 0.565, Figure 12C).

Overall survival and disease free survival rates were also analyzed for Ki67, CD31 and Vimentin expression and are summarized in Table 10.

Markers	OS		DFS	
	Log-rank	*p*	Log-rank	*p*
Nuclear YAP (High vs Low)	0.662	0.416	1.262	0.261
Cytoplasmic YAP (High vs Low)	5.885	0.015	0.014	0.906
Ki67 (>25% vs ≤25%)	2.126	0.145	0.758	0.384
Nuclear to Cytoplasmic YAP (High vs Equal vs low)	9.821	0.007	1.143	0.565
CD31 (positive vs negative)	0.081	0.776	0.39	0.533
Vimentin (positive vs negative)	0.348	0.555	0.092	0.762

Table 10: Association of Ki67, CD31, Vimentin, and YAP with survival outcomes

Ki67, CD31, Vimentin expression did not significantly associate with survival outcomes.

Cox-regression analysis was done to assess whether any clinical parameters, along with YAP expression (nuclear, cytoplasmic, and nuclear to cytoplasmic YAP ratio), had contribution towards disease free survival rates.

A Risk Factors	HR	95.0% CI for HR		P
		Lower	Upper	
Nuclear YAP expression	1.216	0.762	1.939	0.412
Tumor Grade	2.643	0.329	21.250	0.361
Lymphnode Positivity	1.528	0.214	10.891	0.672
Age at diagnosis	1.149	0.978	1.351	0.091
Menopausal Status	0.068	0.001	4.199	0.201

B Risk Factors	HR	95.0% CI for HR		P
		Lower	Upper	
Cytoplasmic YAP expression	0.871	0.593	1.278	0.479
Tumor Grade	1.804	0.262	12.436	0.549
Lymphnode Positivity	2.016	0.252	16.127	0.509
Age at diagnosis	1.099	0.948	1.274	0.212
Menopausal Status	0.255	0.006	11.054	0.478

C Risk Factors	HR	95.0% CI for HR		P
		Lower	Upper	
Ratio of Nuclear YAP to Cytoplasmic YAP	3.439	1.056	11.203	**0.040**
Tumor Grade	1.244	0.132	11.735	0.849
Lymphnode Positivity	1.675	0.165	16.959	0.662
Age at diagnosis	1.255	1.009	1.560	**0.041**
Menopausal Status	0.153	0.000	266.54	0.622

Table 11: COX analysis of DFS for TNBC patients with clinical parameters and A) nuclear YAP expression B) Cytoplasmic YAP expression and C) Nuclear to cytoplasmic YAP ratio as risk factors

Abbreviation: HR: Hazard Ratio

It was observed that nuclear YAP expression showed association towards increased risk of recurrence (Table A, *P* = 0.551) while, cytoplasmic YAP expression showed association towards lowered risk of recurrence (Table B, *P* = 0.448). However, recurrence risk wasn't affected significantly when these factors were compared independently.

Whereas, when ratio of nuclear to cytoplasmic YAP percentage expression was assessed with clinical parameters, recurrence risk was significantly affected (Table C, *P* = 0.010). Ratio of nuclear to cytoplasmic YAP expression significantly associated with

increased recurrence risk (HR = 3.439, p = 0.040). With increasing age the recurrence risk also increased significantly (HR = 1.255, p = 0.041). Other factors like tumor grade, lymph node positivity, and menopausal status did not contribute significantly towards the risk of recurrence.

SPSS couldn't perform Cox regression for OS as only two events were present, and information regarding lymphnode positivity and tumor grade concerning those two events was not available during the time of analysis.

5.7. Association of YAP expression with other molecular markers

		Ki67 % expression	Vimentin % expression	CD31 % expression	TILs %
Nuclear YAP expression	Pearson's r	-0.029	0.106	-0.123	-0.052
	p	0.841	0.467	0.378	0.721
	N	50	49	53	50
Cytoplasmic YAP expression	Pearson's r	-0.063	0.050	-0.172	-.334
	p	0.662	0.732	0.217	**0.018**
	N	50	49	53	50
Percent Nuclear YAP to Percent Cytoplasmic YAP ratio	Pearson's r	-0.159	0.137	-0.112	-0.020
	p	0.270	0.347	0.425	0.891
	N	50	49	53	50

Table 12: Association of YAP with clinical markers and TILs using Pearson's r

High Ki-67 expression was observed in 84% (42/50) of the tissues. CD31 and Vimentin expression was assessed and found positive in 50% (25/50) and 69.3% (34/49) of the tissues, respectively (Table 3). A Pearson's r was computed to assess for any correlation between these markers and YAP (Table 12). YAP expression (nuclear YAP composite score, cytoplasmic YAP composite score and ratio of percent nuclear to cytoplasmic YAP), Ki67, CD31, Vimentin, and TILs were considered.

Trend of negative association was observed between TILs levels and YAP expression at all the three levels, nuclear, cytoplasmic, and nuclear to cytoplasmic. However, only cytoplasmic YAP composite scores showed significant negative association. No significant association was found between YAP and other markers.

5.8. Association of YAP expression with patient response to treatment

To investigate the possible co-relation of YAP expression with response to Neoadjuvant chemotherapy (NACT), association of YAP expression with pathological complete response (pCR) status was assessed (Table 13). Pathological complete response is when tumor responds to NACT and pathologically no residual tumor is found in the surgery tissue. Out of 54 patients in our cohort, 14 patients had received NACT. 6 of 14 patients achieved pathological complete response (pCR) after the

pCR status		Cytoplasmic YAP Expression		*P*	Nuclear YAP Expression		*P*
		High n(%)	Low n(%)		High n(%)	Low n(%)	
pCR = 6	n = 14	6 (49.2)	0	0.473	2 (14.3)	4 (28.6)	0.592
No pCR = 8		6 (49.2)	2 (14.3)		5 (35.7)	3 (21.4)	

Table 13: Association of nuclear and cytoplasmic YAP expression with pCR status

treatment while 8 of them showed no pCR (Table 13). For high cytoplasmic YAP expression equal distribution is observed for patients who had complete pathological response or patients who didn't have complete pathological response. In case of nuclear YAP (35.7%) who didn't achieve pCR have high nuclear YAP expression and patients (28.6%) who achieved pCR have low nuclear YAP expression. Although not significant, a trend towards high expression of nuclear YAP is observed in absence of pCR (Table 13).

6. Discussion

Prognostic utility of YAP in breast cancer remains controversial. A few studies identify YAP to be associated with disease progression in breast cancer (Kim, Jung and Koo, 2014; Kim, Jung and Koo, 2015;min Kim *et al.*, 2015), while two studies show loss of YAP expression to be associated with breast cancer progression and poor prognosis (Yuan *et al.*, 2008;Tufail *et al.*, 2012).

To confirm prognostic value of YAP in TNBC, both cytoplasmic and nuclear expression of YAP were assessed for any association with patient outcomes. In overall survival (OS), decreased OS was found in patients with high nuclear YAP expression and low cytoplasmic YAP expression. Nuclear or cytoplasmic localization of YAP at cellular level, showed opposite outcomes, i.e. reduced survival in patients with high nuclear YAP expression while better survival in patients with high cytoplasmic YAP expression.

YAP being a co-activator protein, its nuclear expression is required for activation of genes that are involved in aggressive propagation of the disease, like EMT and stem-ness associated genes (Lin, Park and Guan, 2018;Maugeri-Saccà *et al.*, 2015). To find out if the worse outcome translated with respect to transcriptionally active form of YAP, i.e., higher nuclear expression of YAP, ratio of its nuclear to cytoplasmic expression was computed and assessed for OS and DFS outcomes. It was found that high nuclear to cytoplasmic YAP expression affected the survival outcomes negatively. Thus, perhaps, having only high nuclear YAP or low cytoplasmic YAP isn't enough to speculate overall survival but having high nuclear YAP to cytoplasmic YAP ratio indeed significantly associates with reduced overall survival.

The finding that having high nuclear to cytoplasmic YAP ratio independently predicted OS but not DFS might be attributable to small and insufficient DFS specific follow up . But in COX – regression analysis, together with clinical parameters (tumor grade, lymphnode positivity, and age at diagnosis), nuclear to cytoplasmic YAP ratio was found to associate with reduced DFS. Moreover, though other parameters (tumor grade, lymphnode positivity) associated with reduced DFS, the association wasn't significant while, ratio of percentage of nuclear to cytoplasmic YAP was found to be an independent factor to increase recurrence risk in the cohort. These results suggest that

having high transcriptionally active YAP with respect to cytoplasmic YAP resulted in decreased overall survival and increased recurrence risk.

CD31, Vimentin and Ki67 also didn't show significant association with survival outcomes.

Overexpression of nuclear YAP has been reported in multiple cancers (Cao *et al.*, 2017b;Misra and Irvine, 2018). Consistent with other studies, our study shows high YAP expression in 74% of TNBC tissues. Aggressive cancers display high proliferative index (Richardsen *et al.*, 2017;Inwald *et al.*, 2013;Duffy *et al.*, 2017), which is confirmed by high percentage of Ki67 expression. In our cohort, 84% of the tissues expressed high Ki67 expression confirming high proliferative index in TNBC tissues. Metastatic potential of various cancers is associated with Vimentin expression (Richardson *et al.*, 2018 ;Anders and Carey, 2009;Liu *et al.*, 2016). In this study 69.3% of TNBC tissues were found to be positive for Vimentin expression. 47.2% of the tissues were positive for CD31 which is an established marker of angiogenesis (Majchrzak *et al.*, 2013;Basilio-De-Oliveira and Pannain, 2015) and high expression of CD31 was positively associated with higher stage, confirming angiogenic induction in higher stage tumors. Incidences of higher percentages of these markers (Ki-67, CD31 and Vimentin) in the tissues confirm that out cohorts aligns with the known fact that TNBC is indeed an aggressive disease.

Patient characteristics were assessed for their association with YAP expression. TNBC is often associated with high risk of lymph-node(LN) metastasis (Al-Mahmood *et al.*, 2018). Higher expression of nuclear YAP expression was significantly associated with LN positivity suggesting its potential role in promoting LN metastasis. However, additional *in vitro* studies are required to understand its mechanistic involvement. High nuclear and cytoplasmic YAP was found in 40.4% of high grade tumors, 63.3% of tumors with size 20mm to 50mm. But, no significant association was found between these parameters and YAP expression; this might be attributable to the small sample size.

YAP expression was also assessed for its association with expression of CD31, Vimentin, Ki67 and TILs. Higher TILs percentages have been associated with better prognosis in many cancer studies (Stanton and Disis, 2016;Salgado *et al.*, 2015;García-Teijido *et al.*, 2016). In this study, negative association was found between TILs levels

and YAP expression (nuclear, cytoplasmic and nuclear to cytoplasmic YAP ratio) suggesting that having high TILs might lead to lower YAP expression. No significant association was found between expression of YAP and other markers (Ki67, CD31 and Vimentin).

This study demonstrates potential role of YAP as a prognostic marker of survival outcomes of TNBC pateints. By far no study has investigated the effect of nuclear to cytoplasmic YAP ratio on survival outcomes. Overexpression of nuclear YAP was associated with aggressive parameter such as lymphnode positivity in TNBC. However, there are some limitations and challenges in this study. As this is a first translational study in India to assess the association of YAP in TNBC patient samples, collecting patient samples, and maintaining follow-up remains challenging. Even in a small cohort YAP turns out to be a potential candidate as biomarker therefore, additional studies in bigger cohorts are necessary to investigate prognostic value of YAP in TNBC.

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