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Original Research Article

Title: Association between AXL, Hippo transducers and survival outcomes in male breast cancer.

Authors: Anna Di Benedetto ^{1,*}, Marcella Mottolese ^{1,*}, Francesca Sperati ², Cristiana Ercolani ¹, Luigi Di Lauro ³, Laura Pizzuti ³, Patrizia Vici ³, Irene Terrenato ², Abeer M. Shaaban ⁴, Matthew P. Humphries ⁵, Sreekumar Sundara-Rajan ⁵, Maddalena Barba ^{3, 6}, Valerie Speirs ^{5,*}, Ruggero De Maria ⁷, and Marcello Maugeri-Saccà ^{3, 6,*}

¹ Department of Pathology, “Regina Elena” National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy

² Biostatistics-Scientific Direction, “Regina Elena” National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy

³ Division of Medical Oncology 2, “Regina Elena” National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy

⁴ Queen Elizabeth Hospital Birmingham and University of Birmingham, Department of Histopathology, Edgbaston, Birmingham B15 2GW, UK

⁵ Leeds Institute of Cancer and Pathology, Wellcome Trust Brenner Building, University of Leeds, Leeds LS9 7TF, UK.

⁶ Scientific Direction, “Regina Elena” National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy

⁷ Institute of General Pathology, Catholic University of the Sacred Heart, Largo Agostino Gemelli, 10, 00168, Rome

***Corresponding author:** Dr. Marcello Maugeri-Saccà, Division of Medical Oncology B and Scientific Direction, “Regina Elena” National Cancer Institute, Via Elio Chianesi 53, 00144 Rome, Italy. Tel. +39-06-52662724, E-mail: maugeri.marcello@gmail.com; Prof Valerie Speirs, Leeds Institute of Cancer and Pathology, Wellcome Trust Brenner Building, University of Leeds, Leeds LS9 7TF, UK. Tel. 0113 343 8633, e-mail: v.speirs@leeds.ac.uk

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Abstract

Male breast cancer (MBC) is an uncommon malignancy. We have previously reported that the expression of the Hippo transducers TAZ/YAP and their target CTGF was associated with inferior survival in MBC patients. Preclinical evidence demonstrated that Axl is a transcriptional target of TAZ/YAP. Thus, we herein assessed AXL expression to further investigate the significance of active TAZ/YAP-driven transcription in MBC. For this study, 255 MBC samples represented in tissue microarrays were screened for AXL expression, and 116 patients were included. The association between categorical variables was verified by the Pearson's Chi-squared test of independence (2-tailed) or the Fisher Exact test. The relationship between continuous variables was tested with the Pearson's correlation coefficient. The Kaplan-Meier method was used for estimating survival curves, which were compared by log-rank test. Factors potentially impacting 10-year and overall survival were verified in Cox proportional regression models. AXL was positively associated with the TAZ/CTGF and YAP/CTGF phenotypes ($p=0.001$ and $p=0.002$, respectively). Patients with TAZ/CTGF/AXL- or YAP/CTGF/AXL-expressing tumors had inferior survival compared with non-triple-positive patients (log rank $p=0.042$ and $p=0.048$, respectively). The variables TAZ/CTGF/AXL and YAP/CTGF/AXL were adverse factors for 10-year survival in the multivariate Cox models (HR 2.31, 95%CI:1.02-5.22, $p=0.045$, and HR 2.27, 95%CI:1.00-5.13, $p=0.050$). Nearly comparable results were obtained from multivariate analyses of overall survival. The expression pattern of AXL corroborates the idea of the detrimental role of TAZ/YAP activation in MBC. Overall, Hippo-linked biomarkers deserve increased attention in this rare disease.

INTRODUCTION

Male breast cancer (MBC) is a rare disease often arising in elderly men (Ruddy and Winer, 2013). Despite some recent molecular characterization efforts, its biology remains understudied (Piscuoglio, 2016; Johansson 2013; Kornegoor, 2012; Johansson, 2011; Callari, 2011; Johansson, 2012; Fassan, 2009). Indeed, apart from hormone receptors that are frequently expressed (Anderson 2010; Shaaban, 2012), and whose targeting represents the mainstay of medical treatment (Doyen, 2010; Eggemann, 2013; Zagouri, 2013a; Zagouri, 2013b; Di Lauro, 2013; Di Lauro, 2014; Di Lauro, 2015), little is known about the nature of deregulated molecular networks underlying the disease. Coherent with the paucity of data available so far, both in terms of biological processes and therapeutic targets, the European Organization for Research and Treatment (EORTC) has launched an initiative dedicated toward obtaining a better understanding of the disease (EORTC 10085, available at: <http://www.eortc.org/research-groups/breast-cancer-group/ongoing-and-future-projects/>).

Over the past years, an Italy-UK collaboration was set off with the aim of investigating specific oncogenic pathways in MBC, and their relationship with clinical-pathological features and survival outcomes. On the basis of our preclinical experience, the focus was placed on signals connected with cancer stem cells (CSCs), a rare cellular subset whose hallmarks include self-renewal, ability to propagate the tumor in animal models, and resistance to current anticancer therapies (Beck and Blanpain, 2013). The evolutionary conserved Hippo signaling pathway was chosen for proof-of-principle studies, considering that both our group and others demonstrated that TAZ/YAP-mediated gene transcription is associated with the retention/acquisition of stem-like features, self-renewal, epithelial-mesenchymal transition, chemoresistance and metastatic dissemination in breast CSC (BCSC) models (Bartucci, 2015; Cordenonsi, 2011; Xiang, 2014; Chang, 2015; Nandy, 2015). Moreover, our proof-of-concept clinical studies connected the expression of TAZ/YAP to inferior

therapeutic and survival outcomes in female breast cancer patients who received neoadjuvant therapy (Vici, 2014; Vici, 2016).

Hippo is a “two face” pathway, composed by two modules that, in a neoplastic background, elicit opposite biological outcomes (Johnson and Halder, 2014). While core kinases (MST1/2 and LATS1/2) and adaptors (SAV1 and MOB1A/1B) act as tumor suppressors by mediating nuclear exclusion, cytoplasmic retention and proteasomal degradation of the Hippo transducers TAZ/YAP, these, together with their DNA binding platform chiefly consisting in TEAD factors, have been extensively connected with tumor-promoting functions (Johnson and Halder, 2014). Many tumors exhibit defective activation of core Hippo kinases/adaptors, and/or activation of upstream or lateral signals that, albeit not canonically placed within the pathway architecture, can directly activate TAZ/YAP (Piccolo, 2014).

In MBC, we have previously reported that the expression of TAZ/YAP and their established target CTGF conferred inferior survival outcomes (Di Benedetto, 2016a). To provide further insights into the oncogenic role of TAZ/YAP in such an uncommon tumor, we herein assessed AXL, a member of the TAM family of receptor tyrosine kinases (RTKs) (Graham, 2014), as the *Axl* gene is a direct target of the Hippo transcriptional machinery (Azzolin, 2014; Zanconato, 2015). In turn, compelling evidence demonstrated that AXL mediates a variety of oncogenic functions, spanning from cancer cell survival to therapeutic resistance (Graham, 2014), and AXL expression has been reported in a number of hematological and epithelial malignancies (Wu, 2014). Overall, the present study was designed with the goals of i) describing the expression pattern of AXL, and ii) providing further ground on the clinical potential of Hippo-related biomarkers in MBC.

MATERIALS AND METHODS

Study Participants and procedures

For this retrospective analysis samples from 255 histologically confirmed, non-metastatic MBC patients, represented in tissue microarrays (TMAs) (Shaaban, 2012), were immunostained for evaluating the expression of AXL. Eligibility was assessed based on the following criteria: i) complete data on AXL, ii) complete data on TAZ, YAP and CTGF, iii) complete data on the following routine clinical-pathological features: histology, tumor grade, hormone receptors (ER, PgR) and Ki-67, and iv) availability of survival data. On this basis, 116 patients were included. Nodal status was not considered among the inclusion criteria, given that this information was available for 87 patients only. As already specified, tamoxifen was the most commonly administered agent in the adjuvant setting, albeit complete information pertinent to post-surgical therapy were not available (Shaaban, 2012). This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the “Regina Elena” National Cancer Institute of Rome and by the Leeds (East) Research Ethics Committee (06/Q1205/156). Samples were anonymised to the research team and informed consent was not required (Shaaban, 2012). This study adheres to the REMARKS guidelines (McShane; 2006).

Immunohistochemistry

TMAs were built from formalin-fixed paraffin-embedded (FFPE) material (Shaaban, 2012). The immunohistochemical assessment of AXL was performed using the polyclonal antibody anti-AXL (HPA037422, Sigma-Aldrich, St. Louis, USA) at the dilution of 1:200. AXL expression was determined both in terms of staining intensity on a four-grade scale (0: negative, 1+: weak, 2+: moderate, 3+: strong) and percentage of tumor-expressing cells (0-100%). Samples were considered positive if $\geq 30\%$ of neoplastic cells exhibited a distinct membranous/cytoplasmic immunoreactivity of any intensity. This cutoff reflected the lowest percentage of AXL-expressing cells observed in this cohort (range 30-90%). Two investigators (ADB and MM) evaluated immunoreactivity. The

modalities used for assessing TAZ, YAP, CTGF, hormone receptors and Ki-67 levels were detailed elsewhere (Shaaban, 2012; Di Benedetto, 2016a).

Statistical analysis

The relationship between categorical variables was assessed with the Pearson's Chi-squared test of independence (2-tailed) or the Fisher Exact test. The Pearson's correlation coefficient was used to establish the correlation between continuous variables. Survival curves were estimated and compared with the Kaplan-Meier method and log-rank test, respectively. Overall survival (OS) was calculated as the time from diagnosis to death due to any cause. For 10-year survival analysis, patients who experienced the outcome of interest after this time point were censored. Potential predictors of 10-year and overall survival were verified in uni- and multivariate Cox proportional regression models. The final multivariate models were built by adjusting for standard clinical-molecular variables (invasive ductal carcinoma (IDC)/invasive lobular carcinoma (ILC) vs other histotypes, G3 vs G1-2, ER+/PgR+ vs other, Ki-67 high vs Ki-67 low) independently on whether these factors were significant at the univariate assessment. Statistical significance was set at $p < 0.05$. Statistical analyses were carried out using SPSS software (SPSS version 21, SPSS Inc., Chicago, IL, USA).

RESULTS

Association between AXL expression and clinical-pathological factors

For this study, 255 MBC samples were screened for the expression of AXL and, based on the aforementioned eligibility criteria, 116 patients were included in the final analysis. Representative immunohistochemical staining is presented in Figure 1. Baseline characteristics of the patients who met the eligibility criteria are summarized in Table 1. As shown in Table 2, we did not observe any significant association between AXL and standard clinical-pathological factors, namely histology, nodal status, tumor grade, hormone receptor status, Ki-67. Conversely, AXL was positively

associated with the TAZ/CTGF and YAP/CTGF phenotypes (chi2 $p=0.001$ and $p=0.002$, respectively, as reported in Table 2). Moreover, a significant positive correlation, in terms of percentage of tumor-expressing cells, was seen between AXL and CTGF (Pearson's correlation coefficient=0.285; $p=0.002$, data available upon request). Overall, these data support our hypothesis of active TAZ/YAP-driven gene transcription in a subset of MBC patients.

Relationship between the TAZ/CTGF/AXL and YAP/CTGF/AXL phenotypes and clinical outcomes

Patients whose tumors had a triple-positive phenotype (TAZ/CTGF/AXL or YAP/CTGF/AXL) experienced shorter survival compared with their negative counterparts (log rank $p=0.042$ and $p=0.048$, respectively. Figure 2, panel A and B), analogously to the TAZ/CTGF and YAP/CTGF models that we previously connected with inferior survival outcomes. Results from the univariate regression analyses, presented in Table 3, performed for identifying variables impacting 10-year survival confirmed that the TAZ/CTGF/AXL and YAP/CTGF/AXL phenotypes are adverse prognostic indicators (HR 2.37, 95%CI: 1.05-5.34, $p=0.038$, and HR 2.31, 95%CI:1.02-5.22, $p=0.044$, respectively). The multivariate Cox models (Table 3) confirmed these data (TAZ/CTGF/AXL: HR 2.31, 95%CI: 1.02-5.22, $p=0.045$. YAP/CTGF/AXL: HR 2.27, 95%CI:1.00-5.13, $p=0.050$). Nearly comparable results, even though with a trend toward statistical significance, were obtained in the multivariate Cox models for overall survival (TAZ/CTGF/AXL: HR 2.19, 95%CI: 0.97-4.94, $p=0.058$. YAP/CTGF/AXL: HR 2.16, 95%CI:0.96-4.87, $p=0.063$) (Table 4).

DISCUSSION

The present study aimed at providing further evidence supporting the negative prognostic significance of active TAZ/YAP-mediated gene transcription in MBC patients. To this end, 116 MBC samples, previously characterized for the expression of TAZ, YAP and CTGF (Di Benedetto, 2016a), were immunostained for AXL, another established target of the Hippo transducers. The message conveyed by the present study is that: i) AXL expression is significantly associated with the TAZ/CTGF and YAP/CTGF phenotypes, and positively correlated with CTGF, an established TAZ/YAP target gene, ii) patients whose tumors harbored the TAZ/CTGF/AXL and YAP/CTGF/AXL molecular backgrounds had adverse survival outcomes compared with patients whose tumors did not show molecular evidence of TAZ/YAP activation, and iii) the prognostic significance of the triple-positive model herein investigated was comparable to that of the previously described TAZ/CTGF and YAP/CTGF models, further enforcing the hypothesis that deregulation of the Hippo machinery may represent an important source of oncogenic stimuli in MBC.

We acknowledge that, being retrospective in nature, our study has some limitations, chiefly the fact that we were unable to consider cancer-specific events owing to the lack of complete information related to the cause of death, as discussed elsewhere (Di Benedetto, 2016a). Nevertheless, ten-year survival is a suitable endpoint to overcome this drawback, when considering that MBC is a disease of elderly men and with a long natural history, comparable to that of luminal-type breast cancer arising in post-menopausal women (Ruddy and Winer, 2013). Overall, our analyses at an intermediate time point (10-year survival) conveyed the message that deregulated Hippo pathway is involved in the genesis of MBC, analogously to earlier preclinical and clinical reports in female breast cancer (Maugeri-Saccà, 2015).

Next, our interest towards the Hippo pathway in MBC extends beyond the analysis of key pathway components. Indeed, we are currently analyzing other pathways intersecting the Hippo cascade at various levels. Bearing in mind the number of molecular signals interacting with the Hippo cascade (Piccolo, 2014), we focused our attention on the following: i) the mevalonate pathway and, ii) the DNA damage repair (DDR) machinery. Evidence of metabolic control of the Hippo pathway stemmed from a high-throughput screening of Food and Drug Administration (FDA)-approved compounds performed in the attempt of identifying TAZ/YAP modulators through a drug repositioning approach. This screening identified both hydrophilic and lipophilic statins, a class of widely prescribed cholesterol-lowering medications, as the most potent TAZ/YAP modulators. Mechanistically, it was demonstrated that the mevalonate pathway promotes TAZ/YAP activation via the production of geranylgeranyl pyrophosphate (GGPP), which is essential for correct membrane localization, and consequently activation, of Rho GTPases (Sorrentino, 2014). These, in turn, inhibit TAZ/YAP phosphorylation independently on the activity of core Hippo pathway kinases (LATS1/2). Interestingly, the effects of statins were reproduced with the administration of nitrogen-containing bisphosphonates, such as zoledronic acid, a class of compounds that inhibit farnesyl pyrophosphate synthase, another key enzyme in the mevalonate cascade (Sorrentino, 2014). Overall, the preclinically documented metabolic control of TAZ/YAP operated by the mevalonate pathway, together with the association between obesity and MBC, makes the mevalonate pathway an attractive therapeutic target in MBC (Humphries, 2015). Our results indicates that 3-Hydroxy-3-methylglutarylcoenzyme A reductase (HMG-CoAR), the rate-limiting enzyme of the mevalonate cascade and the target of statins (Buhaescu and Izzedine, 2007), is significantly co-expressed with Hippo transducers, suggesting that the mevalonate pathway regulates the activity of TAZ/YAP (Di Benedetto, 2016b). When reanalyzing this association in light of the data on AXL herein presented, we confirmed that HMG-CoAR is positively associated with both the TAZ/CTGF/AXL and YAP/CTGF/AXL phenotypes (Fisher Exact test $p=0.024$ and $p=0.014$, respectively). Regarding the DDR, a wave of preclinical evidence connected key

orchestrators of the DDR network, namely ATM and ATR, with the Hippo pathway (Pefani DE and O'Neill, 2016). In a greater detail, Hippo pathway kinases are targeted by the ATM/Chk2 and ATR/Chk1 pathways, thus participating in an array of interconnected biological processes spanning from cell-cycle checkpoint activation and replication fork stability to DNA repair and apoptosis (Pefani DE and O'Neill, 2016). The molecular cooperation between the Hippo pathway and the molecular network safeguarding genome integrity is further enforced when considering the interactions between Hippo pathway components and p53. Indeed, it was demonstrated that LATS2 stabilizes p53 (Ganem NJ, 2014), and that LATS1/2 silencing shifted p53 function toward an oncogenic (gain-of-function), mutant-like state (Furth N, 2015). Moreover, YAP physically interacts with mutant p53 proteins promoting the aberrant expression of cell cycle-related genes (Di Agostino S, 2016). Our group initiated characterization of the ATM-Chk2 and ATR-Chk1 axes in MBC, with the aim of providing novel information on DNA repair-linked biomarkers and their connection with the Hippo machinery and metabolic avenues.

We believe that our efforts toward achieving a better comprehension of the molecular basis of MBC potentially hold important therapeutic implications. Indeed, inhibition of the Hippo transducers TAZ/YAP was preclinically obtained with various Food and Drug Administration- (FDA) approved compounds, such as statins, bisphosphonates, verteporfin, dobutamine, metformin, and dasatinib (Sorrentino, 2014; Frangou, 2014; Rosenbluh, 2012; Liu-Chittenden, 2012; Yu, 2014; Bao, 2011; Wang, 2014; DeRan, 2014). In our opinion, agents targeting the mevalonate pathway (e.g. statins and nitrogen-containing bisphosphonates) and glucose-lowering compounds (e.g. metformin) are those for which it is plausible gathering retrospective, hypothesis-generating data. Indeed, a non-negligible fraction of MBC patients supposedly received these treatments for co-existing medical conditions, such as elevated cholesterol levels, osteoporosis and diabetes, since these comorbidities are fairly common in elderly men. Reconsidering survival outcomes of MBC patients in light of the

use of these agents, target expression, and activation of the TAZ/YAP-mediated oncogenic program is a strategy to be pursued.

In summary, by analyzing AXL, an established target of TAZ/YAP widely exploited in the preclinical setting as a proxy of their activation, we herein provided further evidence to the detrimental role of activated Hippo transducers in MBC. Furthermore, ongoing investigations will shortly help elucidate the connection between Hippo and other oncogenic signals.

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Conflicts of interest

All authors disclose no conflict of interest.

Author contributions

VS, RDM and MM-S conceived the study and designed the study. ADB, MM, CE, AMS carried out molecular pathology analyses. FS, IT and MB performed statistical analyses. LDL, LP, PV, MPH and SS-R acquired the data related to clinical-pathological features and therapeutic outcomes. MM-S wrote the final version of the manuscript. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Figure Legends

Figure 1: Representative examples of immunohistochemical expression of AXL, TAZ, YAP and CTGF in MBC samples. Panels a-b-c show a sample with cytoplasmic AXL expression (a) cytoplasmic/nuclear TAZ (b) and cytoplasmic CTGF (c). In panels d-f, a tumor case with cytoplasmic AXL expression (d), cytoplasmic/nuclear YAP (e) and cytoplasmic CTGF (f). Scale bar 30 μm . Magnification 40x. Inset magnification 10x.

Figure 2: Kaplan-Meier survival curves of overall survival regarding: (A) TAZ/CTGF/AXL and (B) YAP/CTGF/AXL (N=116).

Table 1: Baseline characteristics of MBC patients characterized for the expression of AXL (N=116)

Characteristics		N (%)
Age at diagnosis	Median (min-max) [IQ range]	67 (34-88) [59-75]
Histology	IDC/ILC	101 (87.1)
	Other	15 (12.9)
Grade	G1-2	57 (49.1)
	G3	59 (50.9)
Nodal status	Positive	50 (43.1)
	Negative	37 (31.9)
	Unknown	29 (25.0)
Hormone Receptors	Positive (ER+/PgR+)	99 (85.3)
	Other*	17 (14.7)
Ki-67	High ($\geq 14\%$)	51 (44.0)
	Low ($< 14\%$)	65 (56.0)
TAZ/CTGF	Positive	43 (37.1)
	Negative	73 (62.9)
YAP/CTGF	Positive	48 (41.4)
	Negative	68 (58.6)
AXL	Positive	37 (31.9)
	Negative	79 (68.1)

Abbreviations: ER: estrogen receptor, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma, PgR: progesterone receptor.

* ER+/PgR- (N=15), ER-/PgR- (N=2)

Table 2: Association between AXL, clinical-pathological factors and expression of the TAZ/CTGF and YAP/CTGF phenotypes.

		AXL		Chi2 Test p-value
		Positive N(%)	Negative N(%)	
Histology	IDC/ILC	32 (31.7)	69 (68.3)	0.999*
	Other	5 (33.3)	10 (66.7)	
Grade	G1-2	14 (24.6)	43 (75.4)	0.096
	G3	23 (39.0)	36 (61.0)	
Nodal status	Positive	18 (36.0)	32 (64.0)	0.666
	Negative	15 (40.5)	22 (59.5)	
Hormone Receptors	ER+/PgR+	31 (31.3)	68 (68.7)	0.745
	Other	6 (35.3)	11 (64.7)	
Ki-67	High	17 (33.3)	34 (66.7)	0.769
	Low	20 (30.8)	45 (69.2)	
TAZ/CTGF	Positive	22 (51.2)	21 (48.8)	0.001
	Negative	15 (20.5)	58 (79.5)	
YAP/CTGF	Positive	23 (47.9)	25 (52.1)	0.002
	Negative	14 (20.6)	54 (79.4)	

Abbreviations: ER: estrogen receptor, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma, PgR: progesterone receptor.

* Fisher's Exact Test

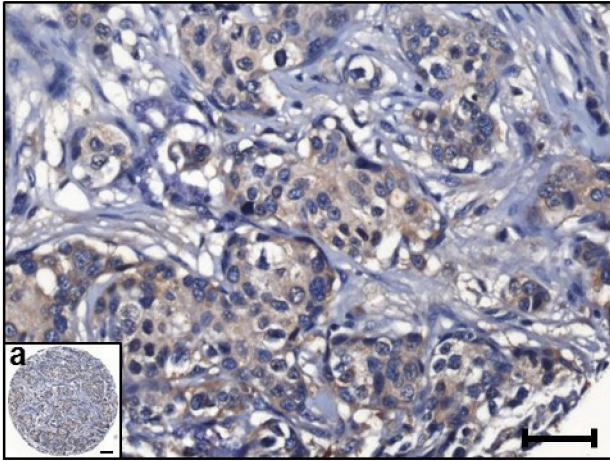
Table 3: Uni- and multivariate Cox regression models for 10-year survival (N=116)

		Univariate Cox regression model		Multivariate Cox regression model		Multivariate Cox regression model	
		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Histology	IDC/ILC vs other	1.20 (0.36-3.96)	0.762	1.00 (0.30-3.34)	0.994	1.02 (0.30-3.42)	0.977
Grade	G3 vs G1-2	1.84 (0.89-3.80)	0.099	1.69 (0.79-3.60)	0.175	1.70 (0.80-3.63)	0.169
Hormone Receptors	ER⁺/PgR⁺ vs other	0.66 (0.28-1.54)	0.335	0.71 (0.29-1.71)	0.447	0.71 (0.29-1.71)	0.448
Ki-67	High vs Low	1.18 (0.58-2.39)	0.655	1.13 (0.54-2.38)	0.737	1.13 (0.54-2.36)	0.753
TAZ/CTGF/AXL	TAZ/CTGF/AXL vs other	2.37 (1.05-5.34)	0.038	2.31 (1.02-5.22)	0.045		
YAP/CTGF/AXL	YAP/CTGF/AXL vs other	2.31 (1.02-5.22)	0.044			2.27 (1.00-5.13)	0.050

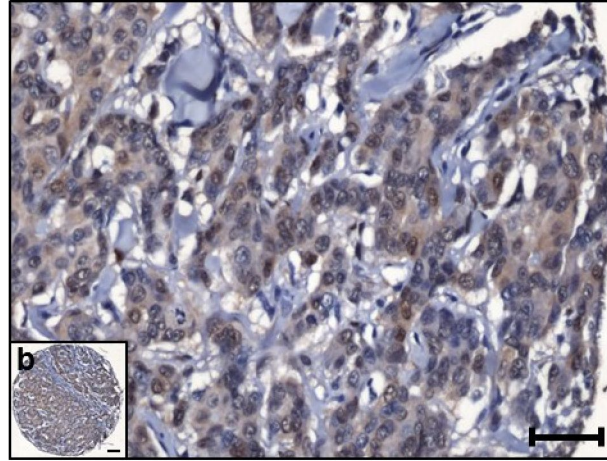
Table 4: Univariate and multivariate Cox regression models for overall survival (N=116).

		Univariate Cox regression model		Multivariate Cox regression model		Multivariate Cox regression model	
		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Histology	IDC/ILC vs other	1.30 (0.40-4.29)	0.662	1.11 (0.33-3.70)	0.871	1.13 (0.34-3.77)	0.848
Grade	G3 vs G1-2	1.94 (0.94-3.97)	0.071	1.85 (0.88-3.91)	0.106	1.86 (0.88-3.93)	0.103
Hormone Receptors	ER⁺/PgR⁺ vs other	0.76 (0.32-1.76)	0.519	0.85 (0.35-2.04)	0.718	0.85 (0.35-2.04)	0.719
Ki-67	High vs Low	1.09 (0.54-2.18)	0.805	1.01 (0.49-2.08)	0.982	1.00 (0.48-2.07)	0.995
TAZ/CTGF/AXL	TAZ/CTGF/AXL vs other	2.26 (1.01-5.08)	0.048	2.19 (0.97-4.94)	0.058		
YAP/CTGF/AXL	YAP/CTGF/AXL vs other	2.21 (0.98-4.97)	0.054			2.16 (0.96-4.87)	0.063

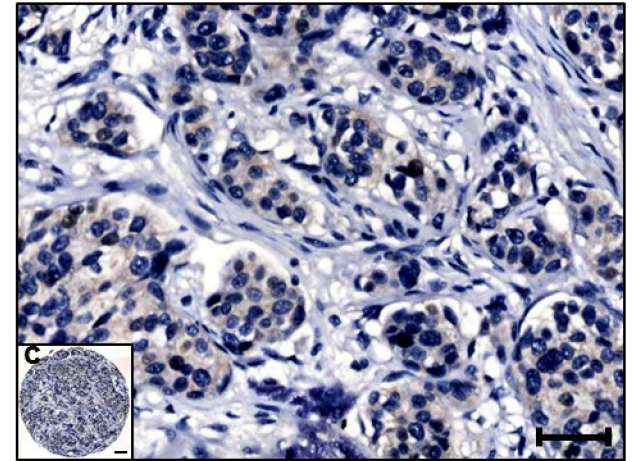
AXL



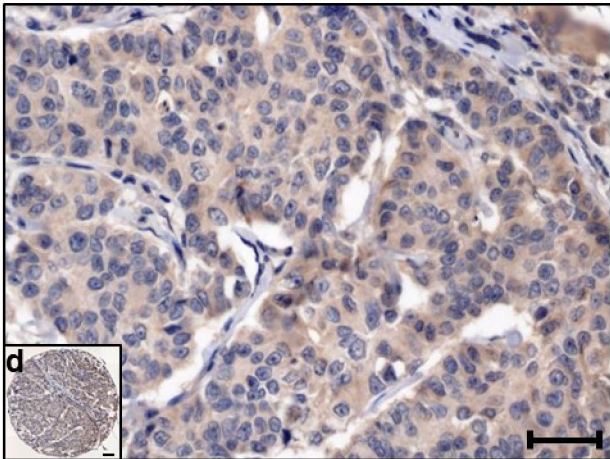
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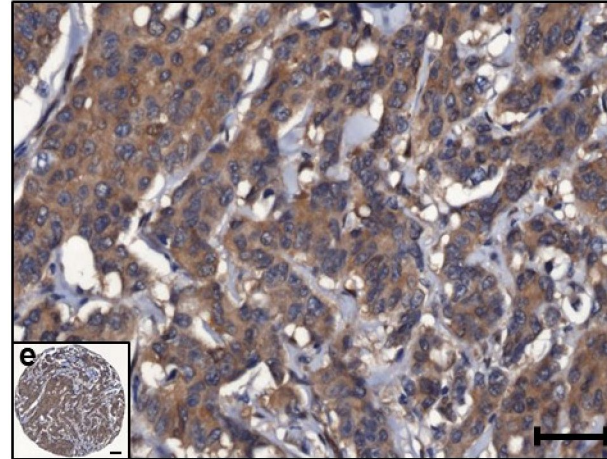
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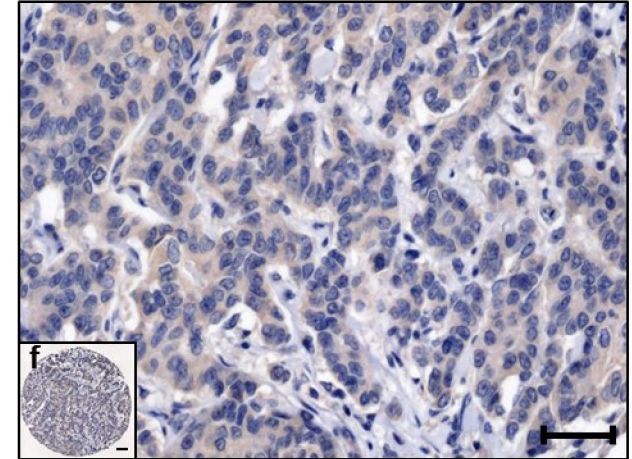
AXL



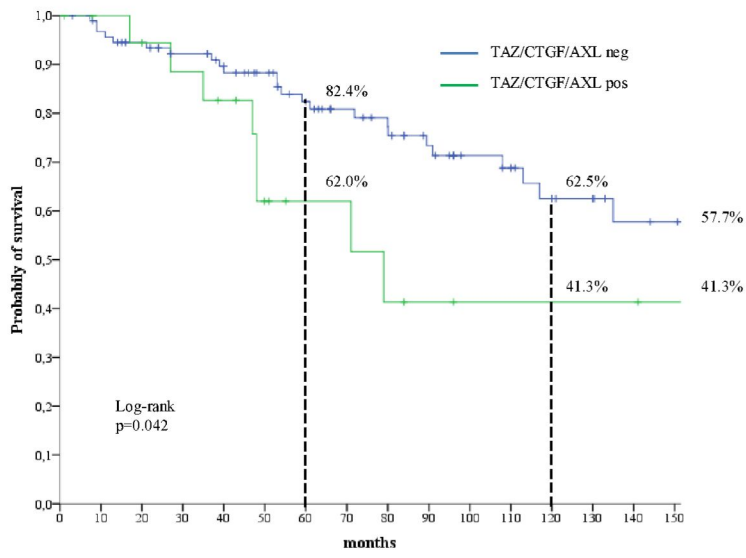
YAP



CTGF



A



B

