

# Phosphorylated mTOR is associated to androgen receptor expression in early triple-negative breast cancer

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**Abstract.** The significance of phosphorylated mTOR (p-mTOR) expression is unknown in triple-negative breast carcinoma (TNBC). The aims of the present study were to assess the expression of p-mTOR in early TNBC and to evaluate possible correlations between androgen receptor (AR) expression, clinicopathological parameters and disease outcome. Between January 2009 and December 2013, all consecutive patients who were diagnosed and completed the treatment of invasive TNBC at our institution were eligible for this analysis. Patients with stage IV disease were excluded. The evaluation of p-mTOR immunohistochemical staining was semi-quantitatively considering both the percentage of positive tumor cells (range, 0-100%) and staining intensity (range, 0-3+). Ninety-eight TNBC patients were included. Approximately 33% of cases were p-mTOR positive and there was no association between positive immunostaining for p-mTOR and DFS (P=0.74) and OS (P=0.81). p-mTOR positivity was associated with small tumor size (P=0.03) and AR expression (P=0.04). High expression of p-mTOR may drive tumor proliferation in almost one third of TNBC. The biological association between mTOR activation and AR pathway suggests that there may exist a subgroup of TNBC in which the combination of both AR antagonism and mTOR inhibition should have a synergistic effect on cell growth and tumor progression.

## Introduction

Triple-negative breast cancers (TNBC) account for approximately 15-25% of all breast cancer. They are characterized

by the lack of expression of estrogen, progesterone receptors (ER/PgR) and human epidermal growth factor receptor 2 (HER2) and by an aggressive clinical course with higher rates of relapse and poor overall survival in metastatic disease (1-3). Treatment options for patients with TNBC are limited due to the absence of hormone receptors and HER2; therefore, cytotoxic chemotherapy currently remains the only available treatment (4).

Given these characteristics, TNBC is a challenge in clinical practice.

Several studies demonstrated that a subgroup of TNBC patients displayed a remarkable sensitivity to chemotherapeutic agents. Between 17 and 58% of TNBC patients have been shown to achieve pathological complete response (pCR) after anthracycline/platinum-based neoadjuvant chemotherapy and these patients had an excellent prognosis. On the contrary, those who failed to achieve a pCR had an exceptionally poor outcome (5,6). Over the past decades there have been several attempts to use genomic data in order to explain the highly variable responses to therapy and clinical outcome of this setting of patients. Recently, genomic analyses have provided additional insights, showing a wide heterogeneity of molecular characteristics of TNBCs by gene expression profile. On this basis, Lehmann *et al* (7) identified six different TNBC subtypes including basal-like (type 1 and 2), immunomodulatory, mesenchymal, mesenchymal stem like and luminal androgen receptor subtype demonstrating the heterogeneity of TNBC.

Ongoing research into the molecular and genetic mechanisms of TNBC tumorigenesis are helping to find out processes involved in local tumor progression and distant metastases.

One of the most important mechanisms involved in the control of neoplastic transformation is the PI3K/Akt/mTOR pathway. The aberrant activation of this cascade seems to be of great importance in breast cancer. In addition, a high activation level of the PI3K/Akt/mTOR pathway has been related to worse prognosis and resistance to conventional chemotherapy.

The mammalian target of rapamycin (mTOR) is an important serine/threonine protein kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family, which functions as an environmental sensor and regulates organismal growth, cell physiology and homeostasis. mTOR is the catalytic

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subunit of two distinct complexes, mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2), which consist of several additional regulatory proteins. The subunit composition of each mTORC dictates its substrate specificity. Main substrates of mTORC1 are S6 kinase 1 (S6KB1) and eIF4E-binding protein 1 (4E-BP1), both implicated in the regulation of mRNA and protein synthesis. mTORC2 controls several members of kinases including Akt and is thereby implicated in the regulation of cell survival, cell cycle progression and anabolism (8,9).

Previous *in vitro* studies showed that phosphorylated mTOR (p-mTOR), the activate form is closely related with the active status of mTOR and the cell proliferative capacity (10,11).

p-mTOR was found more frequently expressed in triple-negative than non-TNBC, suggesting that mTOR may play a crucial role in the molecular biology of TNBC. Besides, emerging preclinical evidence suggested that TNBC cells seem particularly sensitive to mTOR inhibitors, especially the androgen receptor-positive (AR<sup>+</sup>) TNBC cell lines, opening the possibility for the incorporation of target agents in treatments (12-15).

The aims of the present study were to assess the expression of the activated form p-mTOR in early TNBC and to evaluate possible correlations between immunohistochemical AR expression, clinicopathological parameters and disease outcome.

## Patients and methods

**Eligibility criteria.** Between January 2009 and December 2013, all consecutive patients who were diagnosed and had completed the treatment of invasive TNBC at our institution were eligible for this analysis.

The study obtained the necessary approval by the Department of Medical Oncology, AO Ospedali Riuniti, Ancona. According to Italian legislation, since it was a retrospective study, with no direct patient involvement, ethical approval and patients consent for the study were not required (Official Gazette no. 72 of March 26, 2012). Patients with stage IV disease or with ductal carcinoma *in situ* with or without micro-invasion and patients with lack of information on pathologic or laboratory results were excluded. We analysed several parameters: clinical (age, performance status, type of surgery and adjuvant chemotherapy), pathological (tumor size, grading, necrosis, lymph nodes status, tumor histology, Ki-67 and lympho-vascular invasion) and molecular (AR and p-mTOR).

**Immunohistochemistry.** IHC analysis was performed on formalin-fixed, paraffin-embedded breast cancer tissue. The detection of antigens occurred automatically with Dako PT Link using EnVision<sup>TM</sup> FLEX Target Retrieval Solution High and Low pH (50x) at 98°C.

After treatment with 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase, the sections were incubated with primary antibody: ER (clone 1D5, 1:30; Dako, Carpinteria, CA, USA), PR (clone PgR636, 1:50; Dako), Ki-67 (clone MIB-1, 1:80; Dako), HER2/neu (HercepTest RTU; Dako), AR (clone F39.4.1, 1:60; BioGenex, San Ramon, CA, USA) and phospho-mTOR (Ser2448) (clone 49F9, 1:50; Cell

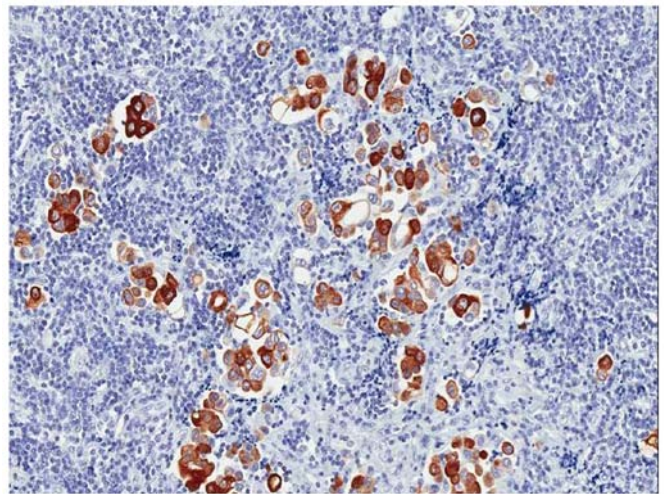


Figure 1. Cytoplasmic immunostaining of p-mTOR in TNBC cells (original magnification, x10).

Signaling Technology Inc., Beverly, MA, USA). The staining was completed using EnVision FLEX<sup>TM</sup>/HRP (Dako) as detection system; 3,3-diaminobenzidine-hydrogen peroxide was used as chromogen. IHC was performed using an autostaining system (Autostainer Link 48; Dako).

For ER, PR, Ki-67 and AR the percentage of positive nuclei was evaluated by counting 5,000 neoplastic cells in different areas of the neoplasia (16).

For Ki-67, the count was performed in the peripheral part of the neoplasia (i.e. the most proliferating part). The staining intensity was not considered. The values were expressed as continuous variable, and ranging from 0 to 100%. Immunostaining for p-mTOR was semi-quantitatively assessed by considering both the percentage of positive neoplastic cells (range, 0-100%), and the strongest staining intensity (range, 0-3+; 0, no staining, 1+, weak, 2+, moderate; 3+, strong) (Fig. 1). Also a 'score of positivity' was calculated by multiplying the value of the percentage of positive neoplastic cells for the value of staining intensity (range, 0-300). HER-2 status was evaluated using a semi-quantitative score (0-3+); patients with 2+ IHC staining for HER2 underwent fluorescence *in situ* hybridization to determine HER2 status (17). The evaluation of the above immunohistochemical staining was carried out, with a double-blind method, by two experienced pathologists, they were not aware of any clinical data of patients, including follow-up and status.

**Statistical analysis.** Disease-free survival (DFS) was defined as the interval between the date of diagnosis of TNBC to the first failure (including loco-regional and/or distant relapse, second primary or death). Overall survival (OS) was calculated from the date of diagnosis to the date of the last follow-up visit or death. Patients who were not reported to be dead at the time of the analysis were censored at the date they were last known to be alive. Survival distribution was estimated by the Kaplan-Meier method. Subgroup differences were estimated by Chi-square test. The Cox multivariate proportional hazard regression model was used to evaluate the prognostic factors on disease-free survival

Table I. Baseline characteristics of 98 TNBC patients based on p-mTOR expression.

Characteristics	Total no. of patients (%)	p-mTOR negativity no. of patients (%)	p-mTOR positivity no. of patients (%)	P-value
Age (years)				
≤50	46 (46.9)	31 (31.6)	15 (15.3)	0.83
>50	52 (53.1)	35 (35.8)	17 (17.3)	
Menopausal status				
Yes	45 (45.9)	30 (30.6)	15 (15.3)	0.93
No	53 (54.1)	36 (36.8)	17 (17.3)	
Tumor size				
pT1	56 (57.1)	33 (33.7)	23 (23.4)	<b>0.03</b>
pT2	41 (41.8)	32 (32.6)	9 (9.2)	
pT3-T4	1 (1.1)	1 (1.1)	0 (0)	
Lymph node status (pN)				
pN0	58 (59.1)	41 (41.8)	17 (17.3)	0.52
pN <sup>+</sup>	40 (40.9)	25 (25.6)	15 (15.3)	
Histological type				
Ductal carcinoma	95 (96.9)	64 (65.4)	31 (31.5)	0.21
Lobular carcinoma	1 (1.1)	0	1 (1.1)	
Other	2 (2.0)	2 (2.0)	0 (0)	
Lymphovascular invasion				
No	72 (73.5)	52 (53.1)	20 (20.4)	0.14
Yes	26 (26.5)	14 (14.3)	12 (12.2)	
Necrosis				
No	81 (82.7)	55 (56.2)	26 (26.5)	0.97
Yes	17 (17.3)	11 (11.2)	6 (6.1)	
AR				
Negative	80 (81.6)	58 (59.2)	22 (22.4)	<b>0.04</b>
Positive	18 (18.4)	8 (8.2)	10 (10.2)	
Recurrences				
No	76 (77.6)	52 (53.1)	24 (24.5)	0.87
Yes	22 (22.4)	14 (14.3)	8 (8.1)	
Deaths				
No	81 (82.7)	55 (56.2)	26 (26.5)	0.97
Yes	17 (17.3)	11 (11.2)	6 (6.1)	
Total	98 (100)	66 (67.4)	32 (32.6)	

(DFS) and overall survival (OS). Significant differences in probability of surviving between the data were evaluated by log-rank test. Hazard ratios and 95% confidence intervals (CIs) were estimated from regression coefficients. A significance level of 0.05 was chosen to assess the statistical significance. Statistical analysis was performed with the MedCalc package (MedCalc® v9.4.2.0; MedCalc Software, Ostend, Belgium).

## Results

**Clinicopathological characteristics.** Ninety-eight TNBC patients were included in our analysis. Clinicopathological characteristics are summarized in Table I. Median age was 52

years (range 26-83 years) and the majority of patients (79.6%) underwent breast conservative surgery.

Most patients (57.1%) presented pT1 tumors (up to 2 cm in size). Lymph nodes were disease-positive in 40.9% of cases. Patients (95.9%) received an adjuvant chemotherapy while 12.2% of them underwent neo-adjuvant treatment.

The mean follow-up time was 4.7 years (0.65-8.3 years). The median DFS was 4.9 years (range, 0.18-8.35) and the median OS was 5.1 years (range, 0.65-8.3). All tumors were grade 3 and with a high proliferating index (Ki-67 >20%). Lympho-vascular invasion and necrosis were reported in 26 (26.5%) and 17 cases (17.3), respectively. The androgen receptor expression was reported in 18 cases (18.4%) and the p-mTOR was positive in 32 cases (32.6%). P-mTOR was

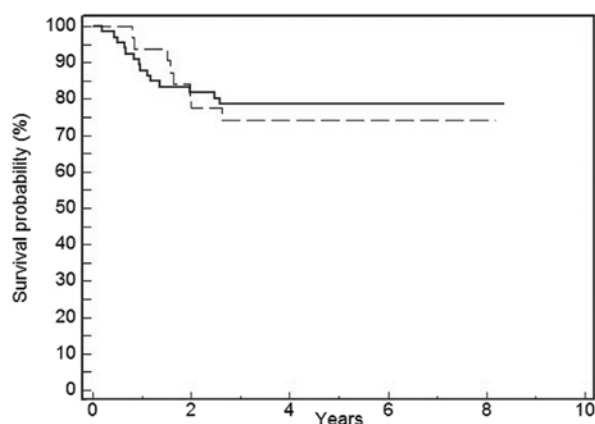


Figure 2. Kaplan-Meier DFS curves for TNBC patients according to p-mTOR positivity (continuous line) and p-mTOR negativity (dashed line).

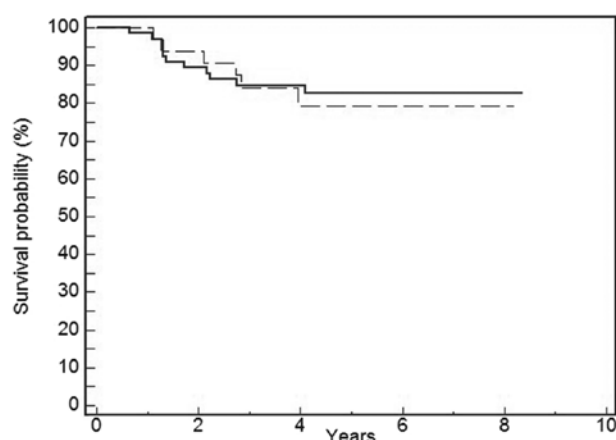


Figure 3. Kaplan-Meier OS curves for TNBC patients according to p-mTOR positivity (continuous line) and p-mTOR negativity (dashed line).

located exclusively in the cytoplasm and its expression did not correlate with any of the following clinicopathological features investigated (Table I). Notably, p-mTOR positivity was associated with small tumor size ( $P=0.03$ ) and AR expression ( $P=0.04$ ).

Univariate survival analysis revealed that positive immunostaining for p-mTOR was not associated with DFS ( $P=0.74$ ) (Fig. 2) and OS ( $P=0.81$ ) (Fig. 3). Tumor size ( $P=0.03$ ) and lymph node involvement ( $P=0.03$ ) were significantly related to worse DFS and OS (Tables II and III).

Multivariate analysis confirmed that tumor size was the only significant independent prognostic variable influencing both DFS and OS ( $P=0.05$  and  $P=0.03$ , respectively) while lymph node involvement influenced only OS ( $P=0.05$ ).

## Discussion

The PI3K/Akt/mTOR pathway regulates several cellular functions such as cell growth, survival and proliferation, characterizing tumorigenesis as well as tumor progression (18). In breast cancer, a high activation level of the PI3K/Akt/mTOR pathway has been related to resistance to conventional chemo and endocrine therapy. The recent BOLERO-2 trial comparing everolimus plus exemestane vs. placebo plus exemestane in

Table II. Univariate and multivariate analysis of sample features and DFS.

Parameters	Univariate analysis	Multivariate analysis		
	P-value	HR	95% CI	P-value
Age (years)	0.91			
≤50 vs. >50				
Tumor size (cm)	0.03			
≤2 vs. >2		2.39	0.97-5.86	0.05
Lymph node status	<b>0.04</b>			
pN0 vs. pN <sup>+</sup>		2.25	0.96-5.25	0.06
Lympho-vascular invasion	0.18			
Negative vs. positive				
Necrosis	0.52			
Negative vs. positive				
AR expression	0.49			
≤10 vs. >10				
p-mTOR	0.74			
Negative vs. positive				

Table III. Univariate and multivariate analysis of sample features and OS.

Parameters	Univariate analysis	Multivariate analysis		
	P-value	HR	95% CI	P-value
Age (years)	0.64			
≤50 vs. >50				
Tumor size (cm)	<b>0.01</b>			
≤2 vs. >2		3.48	1.13-10.67	<b>0.03</b>
Lymph node status	<b>0.02</b>	2.63	0.97-7.12	<b>0.05</b>
pN0 vs. pN <sup>+</sup>				
Lympho-vascular invasion	0.11			
Negative vs. positive				
Necrosis	0.14			
Negative vs. positive				
AR expression	0.39			
≤10 vs. >10				
p-mTOR	0.95			
Negative vs. positive				

women with resistance to no-steroidal aromatase inhibitors demonstrated a 6-month improvement in progression-free survival leading to the approval for the treatment of ER-positive metastatic breast cancer.

Previous *in vitro* studies showed that p-mTOR correlated with the activation of mTOR and an increase in prolifer-

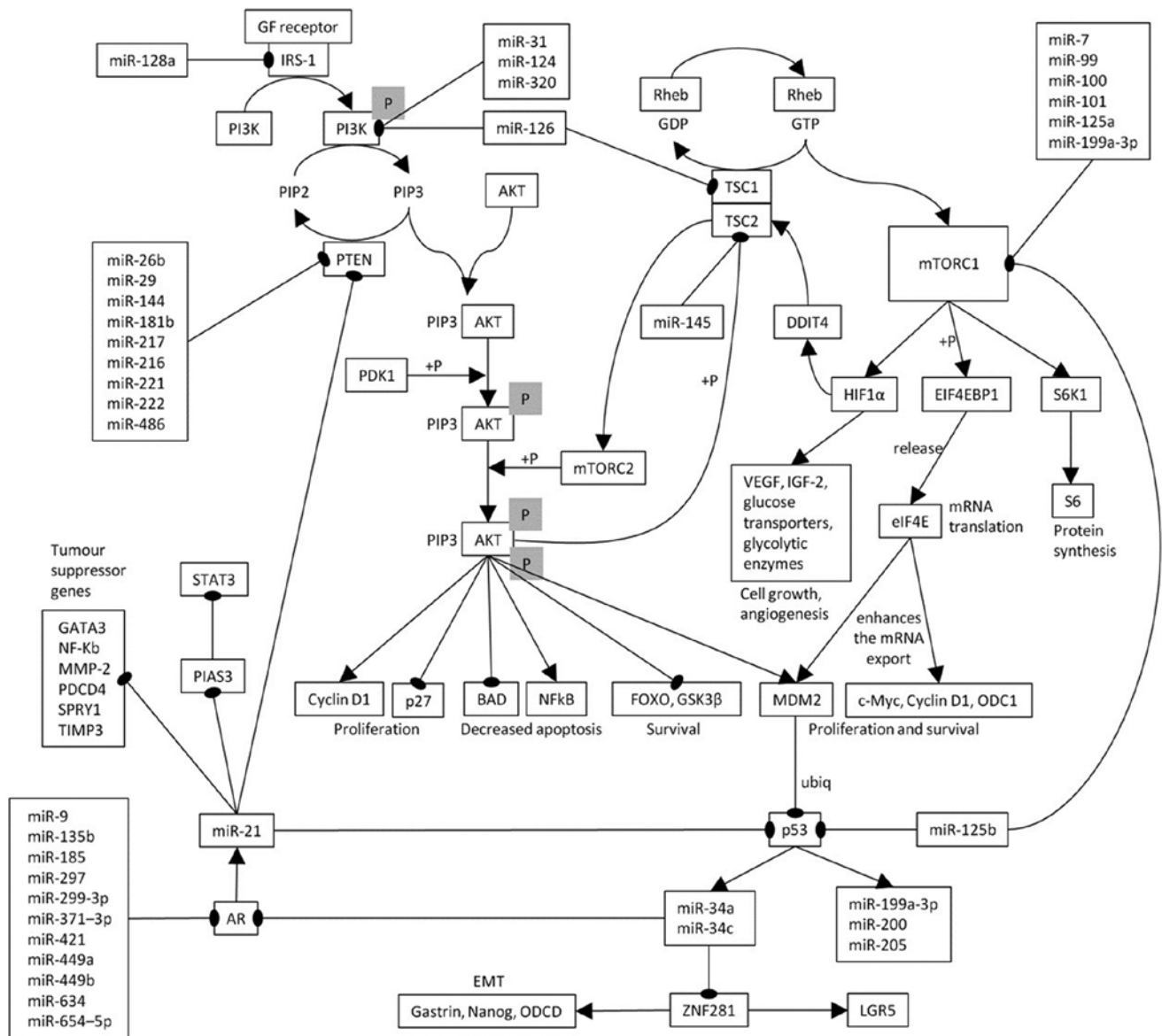


Figure 4. The pathway that links mTORC1 to AR is shown. Arrows, activation, the connectors ending with thick point represent inhibition. RPS6KB1, ribosomal protein S6 kinase, 70 kDa, polypeptide; 1 RPS6, ribosomal protein S6; EIF4EBP1, eukaryotic translation initiation factor 4E binding protein 1; EIF4E, eukaryotic translation initiation factor 4E; HIF1A, hypoxia inducible factor 1,  $\alpha$  subunit; VEGF, vascular endothelial growth factor genes; DDIT4, DNA-damage-inducible transcript 4; 14-3-3; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; NBN, nibrin; MDM2, MDM2 proto-oncogene; E3, ubiquitin protein ligase; ODC1, ornithine decarboxylase 1; AR, androgen receptor; PIAS3, protein inhibitor of activated STAT3; PTEN, phosphatase and tensin homolog; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; ubiq, ubiquitination; +P, phosphorylation.

eration (10,19). In the present study, the high expression of the active form of mTOR (p-mTOR) was present in almost one third of TNBC, suggesting that aberrant activation of the PI3K/Akt/mTOR may drive tumor proliferation in this subtype of breast cancer. Although there are no therapeutic evidence using mTOR inhibitors in TNBC, these data may open new therapeutic scenarios, also suggested by *in vitro* and *in vivo* assays, in which mTOR inhibitors demonstrated anti-tumor activity in TNBC respectively in cell lines and mouse xenograft models (18,20,21) indicating mTOR as the potential target.

Three reports have previously been published on the expression of p-mTOR in breast cancer; Bose *et al* (22) found increased levels of p-mTOR in high grade vs. low grade

cancers. However, Zhou *et al* (13) found no relationship between p-mTOR and tumor grade. Recently Walsh *et al* (12) showed a high expression of p-mTOR in ~36% of triple-negative breast carcinomas; this result is consistent with the present study. Furthermore, they revealed that p-mTOR was significantly more frequently expressed in triple-negative than non-triple negative diseases, suggesting that inhibitors directed against this protein may be effective in at least some patients affected by this subtype of breast cancer.

In our results, 32.6% of cases were p-mTOR positive and its high expression was not related to the considered clinicopathological features neither to DFS and OS at the univariate and multivariate survival analysis. However, in patients who had small tumor size and early stage, p-mTOR

positivity was significantly higher and that is consistent with a previous study (13). Of interest, the present investigation is the first clinical retrospective study showing the strong correlation between p-mTOR immunostaining and AR positivity in a subgroup of TNBC. It is consistent with other reports and it confirms microarray analysis recently conducted on TNBC (23).

A spectrum of somatic mutations have been discovered in TNBC; mutations in PIK3CA (10.2%), the gene that encodes the p110 $\alpha$  catalytic subunit of phosphatidylinositol-3 kinase (PI3K) are the most common. Lehmann *et al* (23,24) observed that all AR-positive (AR<sup>+</sup>) TNBC cell lines contain the PIK3CA mutation (H1047R) and are highly sensitive to the PI3K/mTOR inhibitor NVP-BEZ235, suggesting that combination of AR antagonism and PI3K inhibition may have a synergistic effect on AR<sup>+</sup> TNBC cell growth. Collectively, these findings are similar with other reports (1) and consistent with observations that hormonally responsive cancers, such as those expressing ER and AR are more likely to acquire PIK3CA mutations (24).

Up to date, in BC AR expression and relation with the PI3K pathway were studied in cell lines or xenograft models and the exact mechanism of action of AR in TNBC is still controversial (1,24-26). Due to the uncertain biological significance of the relationship between mTOR and AR, we defined the regulation pathway linking mTOR with AR by means of literature analysis and referring to Reactome ([www.reactome.org](http://www.reactome.org)) and KEGG databases (<http://www.genome.jp/kegg/pathway.html>).

The relationship between AR expression and the mTOR pathway could be explained by inherent biological data from literature analysis (Fig. 4). It should be noted that miR-21 and miR-34a, key elements of our pathway, were shown to be expressed in TNBC (20,26,27,28). In fact, there are many modifier pathways, specifically GTPase activating proteins TSC1 and TSC2 are negative regulators of mTORC1 and positive regulators of mTORC2 (29). These proteins act on Rheb GTPase hydrolyzing the GTP converting it to Rheb-GDP complex. Rheb is activated bound to GTP and turned off when bound with GDP, so respectively triggering or defusing mTORC1. Activated mTORC1, by phosphorylating some downstream effectors, gives rise to an increased protein translation and autophagy inhibition. In particular, it activates RPS6KB1 kinase that, by phosphorylating RPS6, induces cell growth and proliferation. Moreover, mTORC1 phosphorylates EIF4EBP1 that releases eIF4E so activating the translation of various mRNAs including HIF1A. This is the  $\alpha$  subunit of a transcription factor that, in response to hypoxia, activates transcription of genes involved in regulation of erythropoiesis, angiogenesis, vascular tone, matrix metabolism, glucose metabolism, cell proliferation and survival, apoptosis (VEGF, IGF-2 and EPO) (30). Moreover, HIF1A induces DDIT4 transcription that, through the dissociation of 14-3-3 inhibitory protein from TSC2 (31), activates TSC2 that in turn inhibits mTORC1. eIF4E not only plays a key role in translation, but also in the nucleus where it promotes the export of specific mRNAs, as c-myc, Mdm2, NBN, ornithine decarboxylase (ODC1), and cyclin D1, that support proliferative and survival signalling pathways (32-34). MDM2, by ubiquitination, leads to p53 transcription factor degradation by the proteasome and, in turn, to miR-34 family repression, the latter is a direct

transcriptional target of p53 (35) and the miR-34 silencing is frequent in several tumors, including breast cancer, and correlates with metastasis and poor survival (36). Since miR-34a/c can regulate AR (37), miR-34 repression causes lack of AR inhibition. AR promotes miR-21 transcription by some androgen responsive elements (AREs) in miR-21 promoter (in particular, three binding sites are known: ARE1 and ARE2/3 (38,39). The oncogene miR-21 indirectly suppresses p53 with a feedback loop mechanism (40) and downregulates some tumor suppressor genes, including PIAS3 (41), giving rise also to STAT3 upregulation. Furthermore, miR-21 blocks PTEN (42-44), the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that dephosphorylates PIP3 to PIP2 negatively regulating AKT/mTORC1 signalling pathway. This pathway analyses demonstrates a correlation between activated mTORC1 and AR expression, of which the key elements are p53, miR-21 and miR-34. Therefore, low level of activated mTORC1 yields low AR expression.

It seems that some microRNAs are deregulated in TNBC. Particularly, miR-21 and miR-34a were significantly overexpressed in breast cancer, but miR-21 was significantly overexpressed in TNBC vs. non-TNBC, moreover, it seems to be associated to occurrence of lymph node metastases (27).

Other authors showed that miR-185 was strongly downregulated in TNBC tissues and its ectopic expression suppressed tumor proliferation, directly targeting DNMT1 and E2F6 (45). We can suggest that miR-185 acts also by suppressing AR. Another study showed miR-126 downregulation (46) and, according to our pattern, this should increase AKT/mTORC1 activation by targeting PI3K but, on the contrary, reducing mTORC1 by its target TSC2. Instead miR-145 downregulation (46) should ameliorate prognosis by TSC2 restoration and so mTORC1 reduction. It is interesting that miR-101 and miR-125a were associated to metastasis (46) instead we show that they should block mTORC1. The evidence that miR-31 downregulation causes an enhancement in metastasis (47) can be explained because it is an AKT inhibitor. The highly migratory and metastatic characteristics of TNBC with low miR-200 family expression (48) can be due to the inferred p53 low levels. The fact that miR-205 is downregulated but miR-200a/b/c is upregulated (46) cannot be realized by our pathway. Certainly their regulation is much more complex than that shown here and much remains to be clarified. However, this pathway can be useful also to plan new experiments.

All these complex results and analyses suggested that a high expression of the active form of mTOR (p-mTOR) and consequently an aberrant activation of the PI3K/Akt/mTOR pathway may drive tumor proliferation and that is true for almost one third of TNBC. Currently, TNBC is a subset of breast cancer with no available targeted therapies and hence adverse clinical outcome. Those findings could provide important information as to the potential opportunity for novel targeted and personalized treatment for these women but further translational investigations regarding the therapeutic efficacy of mTOR inhibitors in TNBC are required. Our analysis also confirms the biological association between mTOR activation and AR pathway, suggesting that may exist a subgroup of TNBC in which the combination of both AR antagonism and mTOR inhibition should have a synergistic effect on cell growth and tumor progression.

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