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Improving breast cancer sensitivity to paclitaxel by increasing aneuploidy

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Predictive biomarkers for tumor response to neoadjuvant chemotherapy are needed in breast cancer. This study investigates the predictive value of 280 genes encoding proteins that regulate microtubule assembly and function. By analyzing three independent multicenter randomized cohorts of breast cancer patients, we identified 17 genes that are differentially regulated in tumors achieving pathological complete response (pCR) to neoadjuvant chemotherapy. We focused on MTUS1 gene, whose major product ATP3 is a microtubule-associated protein down-regulated in aggressive breast tumors. We show here that low levels of ATP3 are associated with increased pCR rate, pointing to ATP3 as a new predictive biomarker of breast tumor chemosensitivity. Using preclinical models of patient-derived xenografts and 3D models of breast cancer cell lines, we show that low ATP3 levels sensitize tumors to the effects of taxanes but not DNA-damaging agents. ATP3 silencing improves the pro-apoptotic effects of paclitaxel and induces mitotic abnormalities, including centrosome amplification and multipolar spindle formation, which results in chromosome missegregation leading to aneuploidy. As shown by time-lapse videomicroscopy, ATP3 depletion exacerbates cytokinesis failure and mitotic death induced by low doses of paclitaxel. Our results favor a mechanism by which the combination of ATP3 deficiency and paclitaxel treatment induces excessive aneuploidy, which in turn results in elevated cell death. Together, these studies highlight ATP3 as a new important regulator of mitotic integrity and a useful predictive biomarker for a population of chemoresistant breast cancer patients.

MTUS1 | predictive biomarker | taxanes | multipolar spindle | aneuploidy

Introduction
Breast cancer is a leading cause of cancer death among women worldwide. Neoadjuvant chemotherapy, administered before surgery, represents an option for a number of breast cancer patients (1). Pre-operative chemotherapy decreases primary tumor burden, thus facilitating breast conservation (2, 3), and administration of chemotherapy on naïve tumors prior to surgery also provides the opportunity to rapidly measure tumor response and identify breast cancer patients that may gain advantage from the treatment. The achievement of pathological complete response (pCR), characterized by complete eradication of all invasive cancer cells from the breast and axillary lymph nodes, is often considered as a surrogate endpoint for cancer-free survival after neoadjuvant setting, especially in aggressive triple-negative breast tumors (4,5). Clinical parameters, such as estrogen receptor-negative status, high histological grade and high proliferative status have been associated with greater sensitivity to chemotherapy (5,6). However, the proportion of patients who achieve a pCR following preoperative chemotherapy remains low, reaching 15-20% in the whole population and 30-40% in ER-negative tumors (7,8). Considering the rapidly growing area of personalized medicine, the identification of efficient molecular markers that can predict sensitivity to chemotherapy is crucial to select the patients who may benefit from therapy, thereby avoiding unnecessary treatment and associated toxicities for those who remain resistant (9).

The most used regimens in the neoadjuvant setting of breast cancer patients include taxanes and anthracyclines, which combination is associated with improved outcome compared to anthracyclines alone (3). Taxanes (paclitaxel and docetaxel) are microtubule-targeting agents that bind and stabilize microtubules (MT), inducing mitotic arrest and apoptosis (10, 11). At clinically relevant concentrations of the nanomolar range, these drugs suppress MT dynamic instability (11-13) and behave as mitotic poisons that target the mitotic spindle during mitosis, inducing multipolar spindles and centrosomal abnormalities (13). The assembly and dynamics of the mitotic spindle are tightly regulated by a number of MT-associated proteins (MAP) and mitotic kinases (14, 15), suggesting that alterations of MAP expression and/or function in breast tumors may regulate their sensitivity to taxane-based chemotherapy. Gene expression studies indeed identified the MAP tau protein as a predictive biomarker whose down-regulation is associated with increased pCR rate in breast cancer patients (16-21), underlining the interest of studying MT-regulating proteins as new predictors of chemotherapy efficacy.

Significance
Low levels of ATP3 in breast tumors are associated with increased response to neoadjuvant chemotherapy and ATP3 silencing in breast cancer cells potentiates the effects of paclitaxel, highlighting the importance of this predictive biomarker to select breast cancer patients who are sensitive to taxane-based chemotherapy. ATP3 depletion promotes mitotic abnormalities including centrosome amplification and multipolar spindle formation, which is a source of chromosome segregation errors and aneuploidy. Excessive aneuploidy in ATP3-deficient cells treated with low doses of paclitaxel results in massive cell death.

Reserved for Publication Footnotes
In the present study, we analyzed a panel of 280 genes encoding MT-regulating proteins to evaluate their predictive value as biomarkers of neoadjuvant taxane-based chemotherapy in breast cancer patients. Seventeen genes were identified as being...
differentially expressed in tumors from patients achieving pCR from three independent multicenter randomized breast cancer clinical trials. We focused our interest on candidate tumor suppressor gene MTUS1 (22, 23) that encodes the MT-stabilizing protein ATIP3, previously reported as a prognostic biomarker of breast cancer patient survival (24, 25). We show here that low ATIP3 expression in breast tumors is associated with higher pCR rate. Unexpectedly, ATIP3 deficiency, which is known to increase MT instability (25), improves rather than impairs cancer cell sensitivity to taxanes. Our results favor a model in which ATIP3 depletion sensitizes cancer cells to paclitaxel by increasing centrosome amplification and mitotic abnormalities, leading to massive aneuploidy and cell death.

Results

Gene expression studies identify MTUS1 gene as a predictor of breast tumor response to neoadjuvant chemotherapy

To identify new predictive biomarkers of sensitivity to neoadjuvant chemotherapy in breast cancer, we analyzed a panel of 280 genes encoding MT-regulatory proteins including MAPs and mitotic kinesins. We compared gene expression profiles with clinical data in three independent cohorts (R02, MDA and R04) of 115, 133 and 142 breast cancer patients, respectively (dataset Table S1). We identified a total of 118 genes that were significantly differentially regulated (p < 0.01) in patients who achieved pCR compared to those (NpR) who did not. Among them, 17 were common to all three cohorts (Fig.1A, 1B, Table 1, dataset Table S2). These genes encode structural MAPs that regulate MT stability (MAPT, MTUS1, STMN1), MT-end binding proteins (ASPM, GTSE1, RACGAP1), protein kinases and their regulators (AURKB, MAST4, TPX2) and molecular motors (a total of 8 kinesins) that control mitosis, cytokinesis or intracellular transport. Of note, the MT-stabilizing protein tau encoded by MAPT was previously described as a potent predictor of taxane-based chemotherapy in breast cancer (16-21), therefore validating our gene profiling approach.

Besides MAPT, the MTUS1 gene consistently reached higher fold change and better p value in all three cohorts (Fig. 1B). We thus focused our attention on MTUS1, whose gene product ATIP3 has been identified as a prognostic biomarker of patient survival with potent tumor suppressor effect in breast cancer (24, 25). In each cohort of breast cancer patients examined, MTUS1 Affymetrix probesets intensities were significantly lower in cases with pCR than in those with NpR (Fig.1C, datasets Fig S1-S3). Receiver Operating Curves (ROC) revealed an Area under the curve (AUC) values of 0.717 (p = 0.007), 0.699 (p = 0.0005) and 0.769 (p < 0.0001) in the R02, MDA and R04 cohorts, respectively, indicating that MTUS1 levels predict the response to chemotherapy with good accuracy (Fig 1D, datasets Fig S1-S3).

Tumors were then classified into three groups expressing high, medium and low MTUS1 levels according to heatmap hierarchical clustering (Fig 1E, 1F). In the R02 cohort, the pCR rate was 32% (8/25) in tumors expressing low MTUS1 levels compared to 11.9% (7/59) and 0% (0/31) in those expressing medium and high levels of MTUS1, respectively (Fig 1G), indicating that the subpopulation of low MTUS1-expressing tumors is more prone to achieve complete response. Of note, 100% of tumors with high MTUS1 levels failed to achieve pCR, suggesting that high MTUS1 levels may identify patients unlikely to respond to chemotherapeutic treatment (Fig 1G). Similar results were obtained using the two other cohorts of patients (datasets Fig S2D, S3D).

Univariate logistic regression analysis including age, hormone receptors, HER2, tumor grade, stage, nodal status and MTUS1 level identified hormonal receptors ER (OR 4.46; CI[1.4-14.2])

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* Entered as continuous variable (probeset 212096_s_at values)

Univariate and multivariate analysis are shown on the left and right, respectively.
Fig. 2. A- Left: Immunohistochemistry performed on human breast cancer xenograft sections of a Tissue Microarray (TMA) using anti-MTUS1 monoclonal antibody. Shown are representative photographs of tumors expressing low (left) or high (right) levels of ATIP3. A bar represents 500 μm. Insets are shown in the bottom with a bar representing 50 μm. Right: Correlation between ATIP3 mRNA expression level (by qPCR) and immunohistochemistry score (IHC score). B- Scattered dot plot of ATIP3 (qPCR) mRNA expression level in HBCx treated with Docetaxel (DTX) (left panel) or with Anthracyclind-Cyclophosphamide (AC) (right panel). Tumors are classified according to their response to drug treatment. R indicates resistance and S indicates sensitivity to the treatment. Numbers of samples are under brackets. *p<0.05. C- Percentage of responsive HBCx according to ATIP3 level. Tumors were subdivided into groups expressing high-ATIP3 versus low-ATIP3 levels based on the median value of ATIP3 measured by real-time RT-PCR. *p<0.05. D- Dose response curves of SUM52PE spheroids expressing (shCtrl) or not (shATIP3) ATIP3 and treated with increasing concentrations of Paclitaxel (PTX). E- SUM52PE spheroids expressing (shCtrl) or not (shATIP3) ATIP3 were treated for 6 days with 50nM Paclitaxel (PTX) or 100nM Doxorubicin (DOXO) and photographed. Picture represents one spheroid of the quadruplicate. Spheroid area were measured and results are plotted in the histogram on the right. ****p<0.0001 F- Representative photographs of SUM52PE spheroids expressing endogenous ATIP3 (shCtrl) or silenced by shRNA (shATIP3) treated with 50nM of PTX for 72hrs prior to staining with TUNEL reagent (green) and DAPI (blue). Quantification of apoptosis, measured as percent of TUNEL-positive cells, is shown on the right. Obj x20, scale bar 100 μm. **p<0.01 G- Western Blot analysis of PARP cleavage in SUM52PE spheroid expressing (shCtrl) or not (shATIP3) ATIP3 treated for 72hrs with increasing concentrations of PTX. Vinculin (Vinc) is used as internal loading control. Quantification is shown below.

Table 2. dataset Table S3. In multivariate analysis, ER (OR 4.66; CI[1.46-14.81]) and MTUS1 (OR 12.16; CI[3.88-38.07]) were identified as independent predictors of pCR (Table 2, dataset Table S3). These results indicate that low MTUS1 status may be used to identify patients with high response rates. Notably, 32% of low-MTUS1 tumors were associated with pCR, compared to 25% for ER-negative tumors. Combining ER status and low MTUS1 levels further increased the pCR rate from 7% to 25% among ER-positive tumors, and 25% to 39% among ER-negative tumors of the R02 cohort (Fig 1H).

Similar results were obtained with the two other series of patients (datasets Fig. S2E, S3E). Real time RT-PCR analysis performed in a panel of 106 breast tumors of the R02 cohort using 3 different pairs of oligonucleotides showed significant correlation between ATIP3 mRNA levels and MTUS1 Affymetrix probe set intensities.
Fig. 4. A. FACS analysis of DNA content of HeLa cells transfected with scramble or ATIP3-directed siRNA prior treatment with SnM PTX for 18h. Shown is the proportion of cells in each cell cycle fraction. B. Proportion of HCT116 metaphase spreads containing abnormal numbers of chromosomes. Inset shows a representative image of metaphase spread and corresponding number of chromosomes is indicated in the upper left corner of the image. C. Percentage of aneuploid cells among HCT116 cells silenced (siATIP3) or not (siCtrl) for ATIP3 and treated or not with SnM PTX for 18hrs. Number of metaphase spreads analyzed in 3 independent experiments is shown under brackets. *p<0.05, **p<0.01D. Proportion of tumors from the Curie (Fig 1I, datasets Fig S1D-S1F). Together, these results indicate that low ATIP3 mRNA levels predict breast tumor response to chemotherapy.

Low ATIP3 expression in breast tumors and cancer cells increases sensitivity to taxanes but not DNA-targeting agents

Pre-clinical studies were undertaken using patient-derived xenografts (26) to confirm and extend our results obtained on breast cancer patients. Twenty-two models of human breast cancer xenografts grown in mice (HBCx) were exposed to either Docetaxel (DTX) or Anthracycline plus Cyclophosphamide (AC). ATIP3 expression levels in HBCx were evaluated by real time RT-PCR and validated by IHC (Fig 2A, dataset Table S4). ATIP3 mRNA levels were then plotted according to sensitivity (S) or resistance (R) of tumors to DTX and AC treatment. As shown in Fig 2B, DTX-sensitive xenografts displayed significantly lower ATIP3 levels than DTX-resistant ones (median expression value 0.14 versus 0.33). No significant difference in ATIP3 level was observed between AC-sensitive and AC-resistant HBCx (Fig 2B), indicating that ATIP3 levels are associated with the response to the microtubule-targeting agent DTX rather than to DNA-targeting drugs. Accordingly, the response rate to DTX was significantly higher in low-ATIP3 compared to high-ATIP3 expressing HBCx (70% vs 16.7%) whereas the response rate to AC remained similar in both groups of tumors (80% vs 75%) (Fig 2C).

We then investigated the consequence of ATIP3 depletion on breast cancer cell viability upon exposure to chemotherapeutic agents. ATIP3 silencing in SUM52-PE cells (dataset Fig S4A) markedly improved the cytotoxic effects of both docetaxel (DTX) and paclitaxel (PTX) (29) but had no effect on the cellular response to doxorubicin (Fig 2E). Similar results were obtained in HCC1143 and MDA-MB-231 breast cancer cells treated with DTX and PTX, respectively (datasets Fig S4E, S4F), confirming that ATIP3 deficiency sensitizes breast cancer cells to taxanes but not to DNA-targeting drugs.

Taxanes are mitotic poisons that arrest cells in mitosis and trigger apoptosis. Multicellular spheroids treated with clinically relevant doses of PTX (13) were arrested in mitosis, a phenotype that was further increased in ATIP3-deficient cells (dataset Fig S5A). ATIP3-deficiency also increased the percentage of cells undergoing apoptosis following treatment with low doses of PTX (Fig 2F, dataset Fig S5B). According to molecular markers of apoptosis, such as cleavage of PARP (Fig 2G) and of caspase-3 (dataset Fig S5C) as well as decline in anti-apoptotic protein Mcl-1 levels (dataset Fig S5D), were elevated upon PTX treatment in ATIP3-deficient compared to ATIP3-proficient spheroids. Thus, ATIP3 deficiency improves the mitotic and pro-apoptotic effects of taxanes.

PTX-induced mitotic defects are increased in ATIP3-deficient cells

To get insight into the mechanism by which ATIP3 depletion sensitizes cancer cells to the effects of PTX, we examined the consequences of ATIP3 silencing on mitosis. HeLa cells were used as a reference model in these experiments because they express endogenous ATIP3 and are more suitable than SUM52 cells for cell imaging. As shown in Fig 3A, ATIP3 depletion induced the formation of multipolar spindles (18%) and raised from 55% to almost 100% the percentage of multipolar cells in the presence of low doses of PTX. ATIP3 depletion also markedly increased the number of spindle poles formed upon PTX treatment, with a significant fraction of spindles showing more than 5 poles (Fig 3B), some of them beingacentromial (Fig 3C), indicating excessive mitotic abnormalities. The increased number of spindle poles was mainly due to centrosome amplification which was markedly increased when combining ATIP3-silencing and PTX treatment (Fig 3D). Accordingly, ATIP3 silencing was associated with supernumerary centrosomes and the number of centrosomes per mitotic cell was further increased in ATIP3-deficient cells upon PTX treatment (Fig 3E, dataset Fig S6A). More than half of supernumerary centrosomes contained either one or no centriole (dataset Fig S6B), underlining major centrosomal defects. Together these results indicate that ATIP3 silencing induces centrosome amplification leading to multipolar spindles, a phenotype that is amplified upon PTX treatment.

We then examined the consequences of ATIP3 silencing and PTX treatment on cell fate at the single cell level using time-lapse videomicroscopy (Fig 3F, datasets movies 1-4). ATIP3 silencing increased the time in mitosis (Fig 3G) and induced the formation
of multipolar cells that were able to divide, giving rise to 2 or 3 viable daughter cells containing several nuclei (Fig 3H, 3I, dataset movie 2). PTX at low dose induced a majority (79%) of cells with multipolar spindles, among which 14% were unable to divide. These cells either died in mitosis or underwent cytokinesis failure, giving rise to groups of multinucleated cells that ultimately died during the following division (Fig 3H, 3I, dataset movie 3). ATIP3-silencing combined with low dose of PTX induced massive (95%) formation of multipolar cells, 41% of which died during the first division from cytokinesis failure or mitotic death (Fig 3H, 3I, dataset movie 4). Thus, ATIP3 silencing exacerbates mitotic abnormalities and subsequent cell death induced by PTX treatment.

ATIP3 depletion is associated with increased aneuploidy

The formation of multipolar spindles is a source of chromosome missegregation and aneuploidy. To test this hypothesis, we analyzed cellular DNA content by flow cytometry to assess DNA ploidy in HeLa cells treated or not with low doses of PTX. In line with previous studies (11, 13), treatment with PTX at the nanomolar range resulted in a hypodiploid (<2N) population of cells (Fig 4A, dataset Fig S7A). This population of aneuploid cells disappeared at higher concentrations of PTX (100 nM) when cells were arrested in G2/M (dataset Fig S7B) and was negative for Annexin-V labelling (dataset Fig S7C), excluding the possibility that these cells may be apoptotic. In ATIP3 depleted cells, hypodiploidy could not be detected by flow cytometry procedures in the absence of treatment (Fig 4A). However, following PTX exposure, the population of hypodiploid cells was raised from 12% in control cells to 25% in ATIP3-deficient cells, indicating that ATIP3-silencing increases PTX-induced aneuploidy.

Aneuploidy was also evaluated by counting chromosome number per cell, in metaphase chromosome spreads of HCT116 cells, used as a reference cellular model in these studies because they are nearly diploid and chromosomally stable. PTX treatment significantly increased the incidence of both hypodiploid (<40 chromosomes) and hyperdiploid (>50 chromosomes) cells and ATIP3 silencing further increased aneuploidy (Fig 4B, 4C).

To assess the clinical relevance of our findings, we analyzed a series of 88 breast cancer patients in which ploidy had been evaluated. Tumors were grouped according to low- and high-

MTUS1 level using heatmap classification as reported (24, 28) and compared with ploidy status (dataset Table S5). As shown in Fig 4D, 65% of low-MTUS1 tumors were found to be aneuploid compared to 42% of high-MTUS1 tumors. Since aneuploidy often results from chromosomal instability (CIN), we investigated whether MTUS1 levels may also be associated with CIN. A "CIN25 signature", comprising a panel of 25 differentially regulated genes has previously been reported in breast cancer (29). We therefore classified breast tumors as high-CIN or low-CIN based on the "CIN25 signature" and compared the signature with MTUS1 levels. As shown in Fig 4E and dataset Fig S8, 40% of breast tumors with high-MTUS1 levels were classified as high-CIN compared to 60% of low-MTUS1 tumors. Together, these results confirm the notion that breast tumors expressing low levels of ATIP3 are more prone to chromosomal instability and aneuploidy.

Discussion

Based on transcriptional profiling of three independent cohorts of breast cancer patients treated with taxane-based neoadjuvant chemotherapy, we show here for the first time that microtubule-associated protein ATIP3 is an independent predictive biomarker of the response to treatment. Low levels of ATIP3 were significantly more frequent in breast tumors that achieved pCR (38%) compared with those that did not respond to treatment, suggesting that low ATIP3 expression may be used as a marker to identify breast cancers that are highly sensitive to taxane-containing chemotherapy. Importantly, in all three cohorts analyzed, 98% to 100% of tumors expressing high levels of ATIP3 failed to achieve pCR, indicating that ATIP3 may also be a useful biomarker to select patients unlikely to respond to conventional chemotherapy, which is of clinical importance to limit toxicity and side effects of ineffective treatments. Low ATIP3 levels predict the response to neoadjuvant chemotherapy even better than ER-negative status and can further identify responders among ER-negative tumors. This finding is of particular interest for triple-negative breast tumors (TNBC) for which chemotherapy remains the unique therapeutic option (30). Further validation in adjuvant trials is warranted to firmly establish the value of ATIP3 as a predictive biomarker in clinical practice in breast cancer. It will be interesting to broaden our study to other types of malignancies, such as prostate, lung or ovarian cancer, where taxanes are frequently used.

Pre-clinical studies performed on breast cancer patients-derived xenografts and in 3D-models of multicellular spheroids further allowed us to investigate the predictive value of ATIP3 in the response to taxanes compared to anthracyclines and showed that low levels of ATIP3 are associated with high sensitivity to docetaxel, with no impact on the response to DNA-targeting agents. Accordingly, ATIP3-silencing sensitizes cancer cells to low doses of paclitaxel and potentiates the well-known effects of the drug on mitotic arrest and apoptosis. Although consistent with data from breast cancer patients, this was an unexpected result given that ATIP3 silencing increases MT dynamics (25), which is opposite to the MT-stabilizing effects of taxanes.

Results presented here indicate that silencing of the MT-stabilizing protein ATIP3 induces multiple mitotic abnormalities that mimic those induced by PTX. We propose a mechanism in which ATIP3 silencing, by causing centrosome amplification and multipolar spindle formation, amplifies the effects of taxanes and thereby exacerbates mitotic abnormalities, chromosome segregation errors, CIN features and aneuploidy, ultimately leading to cell death in response to treatment.

It has been widely shown that centrosome amplification, leading to spindle multipolarity and subsequent chromosome missegregation and aneuploidy, promotes tumor initiation and progression (31-33). Centrosome amplification is also associated with worse clinical outcome in breast cancer (34, 35). In this context, our findings that ATIP3 deficiency induces centrosome amplification are consistent with previous observations that low ATIP3 levels in breast tumors are associated with poor patient prognosis (25). Strikingly, our data also indicate that combining ATIP3 deficiency and PTX treatment causes excessive centrosome amplification and aneuploidy, which in turn triggers massive cell death in mitosis. This is in line with previous observations that increasing chromosome missegregation and aneuploidy beyond a critical threshold leads to cancer cell death and tumor suppression (36, 37), and supports our clinical results showing higher pCR for ATIP3-deficient breast cancer patients treated with taxane-based chemotherapy.

In conclusion, while the consequences of centrosome amplification and CIN for therapeutic responses in cancer patients still remain a matter of debate (38), our data emphasize for the first time the link between centrosome amplification and increased pCR rates for breast tumors. Our results highlight ATIP3 as a novel predictive biomarker to select a population of breast cancer patients who are likely to benefit from taxane-based chemotherapy and open the way to new therapeutic strategies based on increasing centrosomal alterations to achieve chemosensitivity.

Materials and Methods

Studies using cohorts of patients and patient-derived xenografts were reviewed by ethical committees and approved by institutional review boards. All patients signed an informed consent for voluntary participation in the trial. Details on patients and samples, clinical data and gene profiling are provided in Supporting Materials and Methods. These also describe RNA

Acknowledgements
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