Serine 2481-autophosphorylation of mammalian target of rapamycin (mTOR) couples with chromosome condensation and segregation during mitosis: Confocal microscopy characterization and immunohistochemical validation of PP-mTOR^{Ser2481} as a novel high-contrast mitosis marker in breast cancer core biopsies

EUGENI LOPEZ-BONET[1,2*], ALEJANDRO VAZQUEZ-MARTIN[2,3*], MARIA CARMEN PÉREZ-MARTÍNEZ[1,2], CRISTINA OLIVERAS-FERRAROS[2,3], FERRAN PÉREZ-BUENO[1], LUIS BERNADÓ[1,2] and JAVIER A. MENENDEZ[2,3]

[1]Department of Pathology, Dr Josep Trueta University Hospital of Girona; [2]Girona Biomedical Research Institute -IdIBGi; [3]Catalan Institute of Oncology (ICO), Catalonia, Spain

Received August 27, 2009; Accepted October 6, 2009

DOI: 10.3892/ijo_00000481

Abstract. The prognostic abilities of breast cancer gene expression signatures are due mostly to the detection of proliferation activity. One of the strongest, yet simple and well-reproducible proliferation-associated prognostic factors is the mitotic activity index (MAI). However: a) counting mitotic figures is regarded by many histopathologists as cumbersome and time-consuming, and b) most available immunohistochemical markers are much weaker predictors than the MAI. We have investigated the spatio-temporal sub-cellular distribution of the Serine 2481-autophosphorylated form of mTOR (PP-mTOR^{Ser2481}) during the G_1/S-to-M-phase transition both in cultured cancer cells and in cancer tissue specimens. Using a high-resolution, automated confocal high-content imaging system, we observed that mitotic cells notably accumulated a distinct pattern of nuclear and cytoplasmic immunolabelings of PP-mTOR^{Ser2481}. Parallel experiments examining site-specific phosphorylation (i.e., Serine 10 and Serine 28) of the G_2/M marker Histone H3 (PP-H3) revealed that PP-H3$^{Ser10/Ser28}$ staining efficiently detected mitotic cells from prophase until the beginning of anaphase, but not during late anaphase, telophase and cytokinesis. PP-mTOR^{Ser2481} staining associated near and between separating chromosomes not only during early mitotic stages but also to the midzone and to midbody at ana/telophase through cytokinesis. We then evaluated the usefulness of PP-mTOR^{Ser2481} immunostaining for improving the efficiency of mitotic counting using. Anti-PP-mTOR^{Ser2481}-labeled mitotic figures (MFs) were easily seen and permitted a quick identification of mitotic hotspots in formalin-fixed cancer tissues, even at low magnification. Importantly, average mitotic counts were significantly higher when using PP-mTOR^{Ser2481} staining than with the hematoxylin and eosin (H&E) protocol in breast cancer core biopsies. Mitotic count based on PP-mTOR^{Ser2481} immunostaining increased tumor grade by one grade in 2 of 9 breast carcinomas. These findings warrant forthcoming studies to confirm both the accuracy and the prognostic value of PP-mTOR^{Ser2481} as a novel high-contrast immunohistochemical mitosis marker in larger populations of human breast carcinomas.

Correspondence to: Dr Javier A. Menendez, Catalan Institute of Oncology, Girona (ICO-Girona), Dr Josep Trueta University Hospital, Avenida de França s/n, E-17007 Girona, Catalonia, Spain
E-mail: jmenendez@iconcologia.net

*Contributed equally

Key words: mammalian target of rapamycin, cell cycle, mitosis, mitotic marker, Histone H3, rapamycin, breast cancer

Introduction

Recent molecular profiling studies of breast cancer disease have demonstrated the pivotal importance of the genetic makeup of breast tumors. These high-throughput gene expression analyses have shown close associations between molecular portrait of breast carcinomas and tumor biologic and clinical behavior (1-5). Thus, biological processes associated with breast cancer clinical outcome appear to strongly depend on the molecular breast cancer subtypes as originally reported (i.e., basal-like, HER2$^+$, normal-breast like and luminal epithelial/estrogen receptor ER$^+$) (6-9). In line with this renewed molecular taxonomy of breast cancer, several prognostic/predictive gene expression signatures with little overlap in their constituent genes have been identified in the last few years (e.g., ONC-16, NKI-70, EMC-76, NCH-70, CON-52, p53-32, CSR, GGI-128, and CCYC) (10-15). Interestingly, two recent meta-analyses have exposed that the prognostic abilities of breast cancer subtyping and prognosis signatures may largely depend on the detection of proliferation activity (16,17). The largest meta-analysis of

publicly available breast cancer gene expression and clinical data revealed that, when the role of constituent genes of nine prognostic signatures was dissected using gene coexpression modules of key biological breast cancer processes (i.e., proliferation, ER and HER2 signaling), all the prognostic signatures exhibited a similar prognostic performance in the entire dataset comprising 2,833 breast tumors. Interestingly, poor outcomes appeared to mostly act through elevated expression of proliferation genes (16). Similarly, a comprehensive meta-analysis integrating both clinicopathologic and gene expression data identified proliferation as the most important component of several prognostic signatures (14). Therefore, when connecting traditional prognostic factors, gene expression-based breast cancer subtyping, and prognostic signatures, proliferation is the strongest parameter predicting clinical outcome in some breast cancer subtypes (i.e., ER$^+$/HER2) and the common denominator of most prognostic/predictive gene signatures (15).

One of the strongest yet simple and well-reproducible proliferation-associated prognostic factors is the mitotic activity index (MAI) (17-19). However: a) counting mitotic figures is regarded as cumbersome and time-consuming, requiring the experience of trained histopathologists (20,21), and b) most available immunohistochemical markers are much weaker predictors than the MAI (22). In this regard, immunohistochemical detection of Histone H3^{Ser10} phosphoprotein has been recently shown to be a simple and reliable method for quantifying proliferative potential as a mitosis marker in breast cancer patients (23-25). Histone H3 becomes phosphorylated on Serine 10 when the chromosomes condense during prophase and remains phosphorylated until telophase, at which stage it becomes dephosphorylated by specific phosphatases (26-28). Some non-mitotic (interphasic) cells display also labelling with anti-phospho-Histone H3^{Ser10}, a phenomenon that has been related to chromatin relaxation and gene expression (23,29). However, these nuclei can be easily distinguished because of their specific (weak and punctuated) staining pattern and, therefore, they not interfere with the accurate determination of the mitotic index using phospho-Histone H3^{Ser10} staining (23-25).

Because some kinase-active forms of the mammalian target of rapamycin (mTOR) appears to act as mitotic survival checkpoints (30,31) we herein explored the spatio-temporal sub-cellular distribution of the Serine 2481-autophosphorylated form of mTOR (PP-mTOR^{Ser2481}) during the G_1/S-to-M-phase transition. To definitely established whether PP-mTOR^{Ser24812} specifically marks the M-phase of cell cycle, we took advantage of a high-resolution, automated confocal imaging system, we concurrently monitored the spatio-temporal sub-cellular distribution of the G_2/M-associated site-specific phosphorylation (i.e., Ser10 and Ser28) of Histone H3 (26,32,33) and that of the Serine 2481-autophosphorylated form of mTOR in cultured cancer cells. Second, we have tested the utility of PP-mTOR^{Ser2481} immunolabelling as an aid to the rapid, reliable, and objective quantification of cellular proliferation in human cancer tissues.

Materials and methods

Cell lines and culture conditions. HeLa human cervical cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely grown in improved MEM (IMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 U/ml)/streptomycin (100 μg/ml), and 2 mM L-glutamine. Cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

Reagents. The following antibodies were used in this study, mouse mAb anti-phospho-Histone H3 (Ser10) [clone 6G3 phospho-Histone H3 (Ser10) mouse mAb, Cell Signaling Technology, Cat. no. 97065]; rabbit polyclonal anti-phospho-mTOR (Ser2481) antibody (no. 2974, Cell Signaling Technology, Inc.) and, Alexa Fluor® 488 Rat anti-Histone H3 (pS28) (Clone HTA28, BD Pharmingen™, material no. 558610). Phospho-Histone H3 (Ser10) (6G3) mouse mAb detects endogenous levels of Histone H3 only when phosphorylated at Serine 10. The antibody does not cross-react with other phosphorylated histones or acetylated histone H3. Phospho-mTOR (Ser2481) antibody (no. 2974, Cell Signaling Technology, Inc.) detects endogenous levels of mTOR only when phosphorylated at Serine 2481. The HTA28 monoclonal antibody reacts with Histone H3 phosphorylated at Serine 28 in its N-terminal tail. It does not recognize the unphosphorylated Histone H3 protein.

Immunofluorescence staining and high-content confocal imaging. Cells were seeded at approximately 5,000 cells/well in 96-well clear bottom imaging tissue culture plates (Becton-Dickinson Biosciences, San Jose, CA, USA) optimized for automated imaging applications. Triton® X-100 permeabilization and blocking, primary antibody staining, secondary antibody staining using Alexa Fluor 488/594 goat anti-rabbit/mouse IgGs (Invitrogen, Probes, Eugene, OR, USA) and counterstaining (using Hoechst 33258, Invitrogen) were performed following BD Biosciences protocols. Images were captured in different channels for Alexa Fluor 488 (pseudo-colored green), Alexa Fluor 594 (pseudo-colored red) and Hoechst 33258 (pseudo-colored blue) on a BD Pathway™ 855 Bioimager System (Becton-Dickinson Biosciences) with x20 or x40 objectives (NA 075 Olympus). Merging images, confocal Z stack acquisition and 3D visualization were obtained according to the Recommended Assay Procedure using BD Attovision™ software.

Tumors. Paraffin-embedded blocks of 22 malignant tumors were retrieved from the routine files of the Department of Pathology, Dr Josep Trueta University Hospital of Girona (Catalonia, Spain). Tumor types are shown in Table I. Of 12 breast carcinomas, 3 were mastectomies and 9 represented core biopsies. The remaining tumor samples were excisional biopsies.

Immunohistochemistry. Immunohistochemical analyses were performed on formalin-fixed, paraffin-embedded block tissues from the cancer tissue collection in our Department of Pathology, Dr Josep Trueta University Hospital of Girona including core biopsies and surgical specimens. Tumor tissues were analyzed for PP-mTOR^{Ser2481} state using standardized immunohistochemical techniques and robotic autostainers (Dako Cytomation, Inc.). Briefly, a 3-μm section of the cancer tissue

Table I. Tumor types analyzed.

Tumor localization	Diagnosis	Tumor samples	No. of cases
Endometrium	Carcinosarcoma	EB	1
	Endometrioid carcinoma	EB	2
Ovary	High grade serous carcinoma	EB	1
Cervix	Squamous carcinoma *in situ*	EB	1
	Grade III squamous carcinoma	EB	1
Kidney	Sarcomatoid renal cell carcinoma	EB	1
Urinary bladder	High grade urothelial carcinoma	EB	1
Testis	Embrionary carcinoma	EB	1
Breast	Invasive ductal carcinoma	EB	3
	Ductal carcinoma *in situ*	EB	1
	Invasive ductal carcinoma	CB	9
Total			22

EB, excisional biopsies; CB, core biopsies.

was placed on positively charged glass microscope slides. Deparaffinization involved incubation of the slides in xylene followed by graded alcohol series in a routine manner. The PP-mTOR^{Ser2481} expression state was evaluated using heat-induced epitope retrieval (HIER) followed by incubation with 1:50 dilution of the phospho-mTOR (Ser2481) antibody (no. 2974; Cell Signaling Technology, Inc.). Binding results were visualized with the Envision^{+} system (Dako Cytomation), which uses a horseradish peroxidase-labelled polymer that is conjugated with secondary antibodies. Diaminobenzidine was used as the chromogen.

Counting of mitotic figures (MFs). The definition of observable mitotic figures (MFs) in the H&E staining protocol was adopted according to van Diest *et al* (20) and comprised the following criteria: a) absence of nuclear membrane signifying the end of prophase, b) presence of condensed chromosomes, either clotted (beginning metaphase), arranged in a plane (metaphase or anaphase) or in separate clots (telophase), the latter counted as one MF. Hypercromatic nuclei, fragmented chromatin and apoptotic nuclei were ignored.

All H&E sections were carefully examined (magnification, x400) and the one showing the highest proliferation, usually in the peripheral growing areas of the excisional biopsies, was selected for assessment of mitotic activity. Starting from the subjectively most mitotically active area of the tumor and moving between consecutive fields, approximately 10 consecutive high power fields (HPF) were counted with an Olympus BH-2 microscope. In core biopsies samples without apparent area of highest mitotic activity, counting of MFs started at the edge of the cylinder. Once starting in an area, additional fields were selected randomly. No attempts were made to maximize the counting by selecting those fields with a higher number. Mitotic counts were performed without knowledge of the results of the PP-mTOR^{Ser2481}-based immunohistochemical staining. PP-mTOR^{Ser2481}-positive MFs were counted in the same way as in H&E-stained sections and indicated as number of labeled MFs per 10 HPF. Mitotic Ser^{2481}-phosphorylation of mTOR begins just prior to prophase. Because nuclei at this stage would not be identified as MFs on conventional H&E staining, PP-mTOR^{Ser2481}-positive nuclei in the presence of nuclear membrane were excluded from quantification. Because the MF would be higher in tumors with abundant cytoplasm, PP-mTOR^{Ser2481}-positive cytoplasm was only accepted as MF if condensed chromosomes were clearly recognizable in the same cell.

Statistical analysis. Statistical analysis was carried out with XLSTAT (Addinsoft™). Wilcoxon's paired test was carried out to compare the mitotic figure count assessed with H&E staining and PP-mTOR^{Ser2481} immunolabelling in 10 consecutive fields (magnification, x400) of the 22 surgical specimens (excisional biopsies) and core biopsies. Spearman's correlation was used to assess the relationship between variables. For all analyses, a $P<0.05$ was considered to be significant.

Results

Confocal microscopy analyses reveal that Serine 2481-autophosphorylation of mTOR couples with chromosome condensation and segregation during mitosis. First, we investigated the spatio-temporal sub-cellular geography of mTOR Ser^{2481} phosphorylation during the M-phase of cell cycle in asynchronous growing HeLa cells. We performed double immunofluorescence staining of phospho-Ser^{2481} mTOR with mitosis-specific phosphorylated forms of Histone H3. In mitotic cells, Histone H3 is phosphorylated at Ser^{10} (26) and Ser^{28} (32,33). There is tight correlation between Histone H3 phosphorylation and mitotic chromosome condensation initiating during early prophase (32-35) and metaphase chromosomes are always found to be heavily phosphorylated for Histone H3 (33,34,36). Accordingly, immunolabeling

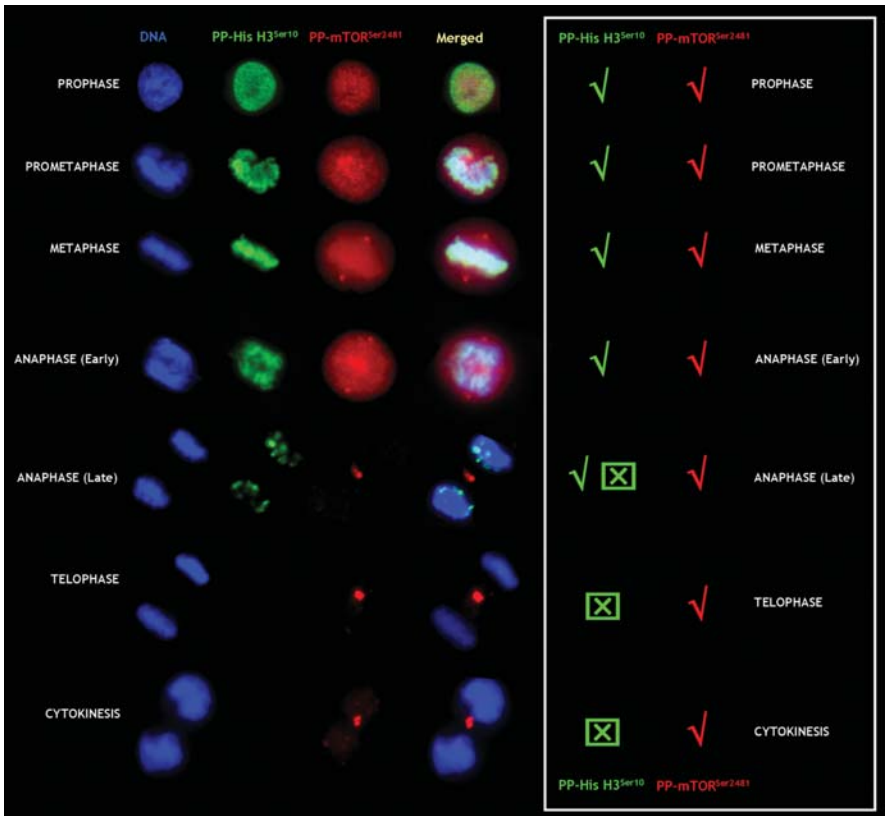

Figure 1. Spatio-temporal relationship between PP-mTOR^{Ser2481} and PP-Histone H3^{Ser10} during mitosis and cytokinesis. Asynchronous growing HeLa cells were fixed and stained as described in Materials and methods. Figure shows representative portions of cell dividing-containing images captured in different channels for PP-mTOR^{Ser2481} (red), PP-Histone H3^{Ser10} (green) and Hoechst 33258 (blue) with a x40 objective and merged on BD Pathway 855 Bioimager System using BD Attovision software.

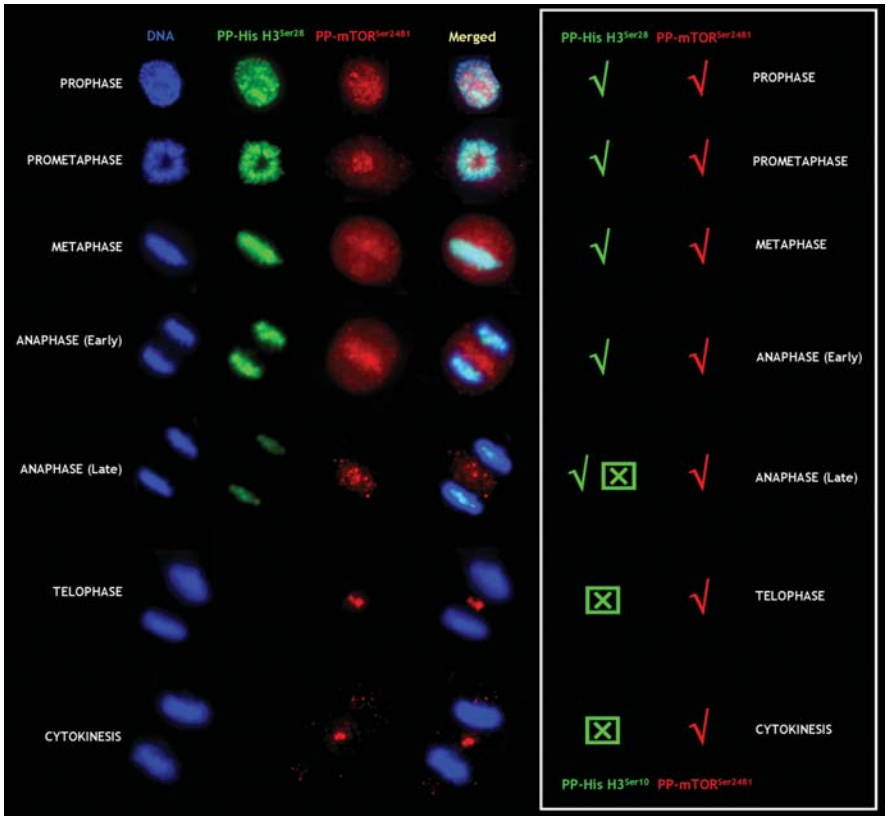

Figure 2. Spatio-temporal relationship between PP-mTOR^{Ser2481} and PP-Histone H3^{Ser28} during mitosis and cytokinesis. Asynchronous growing HeLa cells were fixed and stained as described in Materials and methods. Figure shows representative portions of cell dividing-containing images captured in different channels for PP-mTOR^{Ser2481} (red), PP-Histone H3^{Ser28} (green) and Hoechst 33258 (blue) with a x40 objective and merged on BD Pathway 855 Bioimager System using BD Attovision software.

with the G_2/M markers phospho-Ser[10] Histone H3 (Fig. 1) and phospho-Ser[28] Histone H3 (Fig. 2) became up-regulated in mitotic cells from the end of G_2-phase until the beginning of anaphase, with a weak positivity remaining at late anaphase that became undetectable at telophase and during cytokinesis.

In prophase cells, identified by the appearance of chromosome condensation, PP-mTOR^{Ser2481} staining could be seen throughout the nucleus (Figs. 1 and 2). Although this faint punctuate staining seemed to be distributed along the arms of the chromosomes, there was no evident co-localization between PP-mTOR^{Ser2481} and Ser[10]- and Ser[28] phosphorylated forms of Histone H3. PP-mTOR^{Ser2481} reactivity was somewhat prominent at centromere-like structures and both the number of these structures and their staining intensity with PP-mTOR^{Ser2481} correlated with the degree of chromatin condensation. However, we failed to observe a co-localization between PP-mTOR^{Ser2481} with the centromere proteins INCENP and aurora-B (data not shown). In general, there was a trend towards a decrease in general chromosomal staining as the cell progressed towards metaphase. When phospho-Ser[10] Histone H3 and phospho-Ser[28] Histone H3 were confined to the metaphase plate, PP-mTOR^{Ser2481} was most prominent as a broad cytoplasmic staining. In some cases, PP-mTOR^{Ser2481} appeared to accumulate in some extent as discrete centrosomal-like foci at the spindle poles (Figs. 1 and 2). Chromosome-associated reactivity of PP-mTOR^{Ser2481} was not longer detected from the onset of anaphase when chromatids are pulled apart and start migrating towards the poles. Rather, PP-mTOR^{Ser2481} reactivity was then detected in association with the mitotic spindle, particularly in the region of the stembodies at the spindle equator. This localization of PP-mTOR^{Ser2481} persisted during the furrowing process. Thus, at the completion of telophase, prominent staining of PP-mTOR^{Ser2481} was apparent as doublet on either side of the midbody within the intracellular bridge. The detection of PP-mTOR^{Ser2481} to the center of the midbody at the cleavage furrow area reached its maximum during early telophase and, with time, the intensity decreased at the junction between the two daughter cells. Concurrent with this progressive loss of PP-mTOR^{Ser2481} at the intercellular bridge was an increase in reactivity within the reforming nuclei (Figs. 1 and 2).

Immunohistochemical staining with an anti-PP-mTOR^{Ser2481} antibody allows rapid and precise determination of mitotic activity in breast cancer core biopsies. Second, we tested if PP-mTOR^{Ser2481}-based immunohistochemical staining could objectively detect and quantitate the mitotic fraction in human cancer tissues. All mitotic figures assessed by H&E-based standard histology were strongly labelled with the anti-PP-mTOR^{Ser2481} antibody in breast cancer core biopsies. Indeed, PP-mTOR^{Ser2481} staining permitted quick identification of the area of highest mitotic activity for subsequent counting in human cancer tissues, even at low magnification (Fig. 3). Mitotic figures from prophase through metaphase, anaphase, and telophase, could be easily identified with PP-mTOR^{Ser2481} immunohistochemical staining (Fig. 4). Furthermore, we confirmed that cells displaying morphological features of apoptosis or necrosis were negative for PP-mTOR^{Ser2481}, thus suggesting that mitotic count based on PP-mTOR^{Ser2481} immunohistochemistry could be more accurate than the determined on

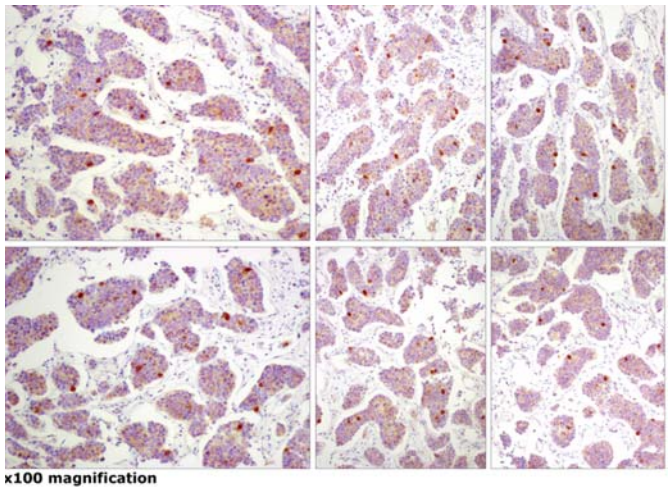

Figure 3. Easy identification of mitoses in PP-mTOR^{Ser2481}-stained sections of an infiltrating ductal breast carcinoma. Representative fields of a breast cancer core biopsy are shown (original magnification, x100).

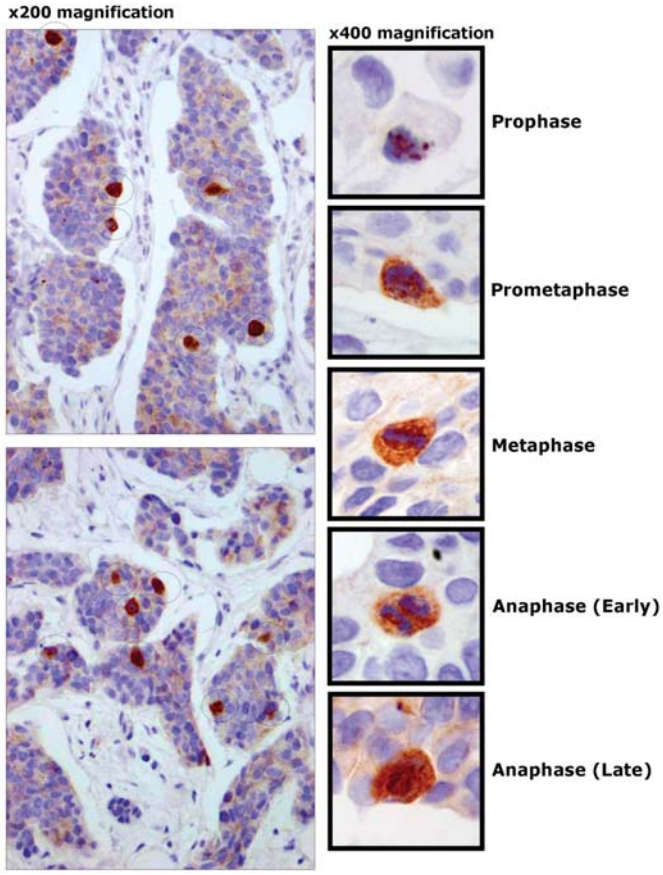

Figure 4. Easy and rapid identification of mitotic phases in the PP-mTOR^{Ser2481} staining of breast cancer biopsies. Examples of breast cancer cells in prophase, prometaphase, metaphase and early separation of daughter cells during anaphase in the PP-mTOR^{Ser2481} staining of human breast cancer core biopsies are shown (original magnification: left panels, x200; right panels x4000).

H&E staining. Hence, eye-catching PP-mTOR^{Ser2481}-positive spots were particularly useful when trying to detect mitotic cells in high-grade breast carcinomas with dense cellularity and numerous apoptotic or necrotic cells (data not shown).

Third, we finally compared mitotic counts in PP-mTOR^{Ser2481}-stained and H&E-stained paraffin-embedded

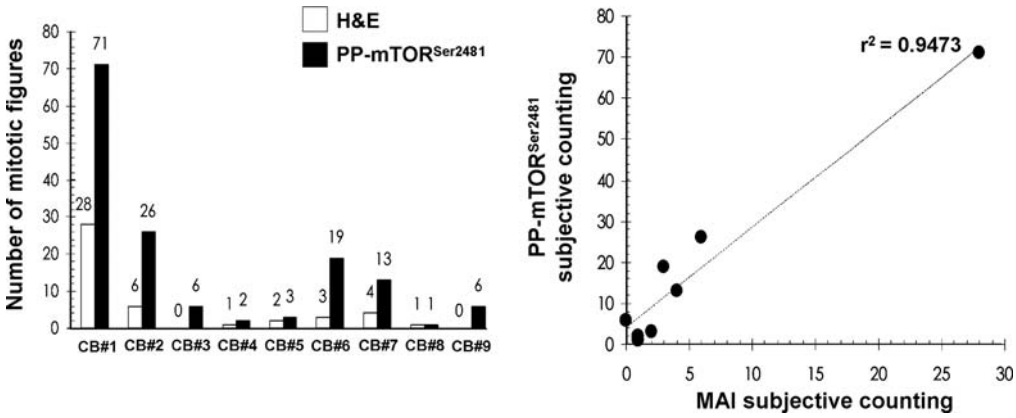

Figure 5. Improvement of mitosis counts by applying PP-mTOR^{Ser2481} staining in breast cancer core biopsies. Average number of mitotic figures per tumor in 9 different breast cancer core biopsies depending on the staining method used (left). Scatter plot showing correlation of H&E-based MAI subjective counting and the PP-mTOR^{Ser2481} index in breast cancer core biopsies (right).

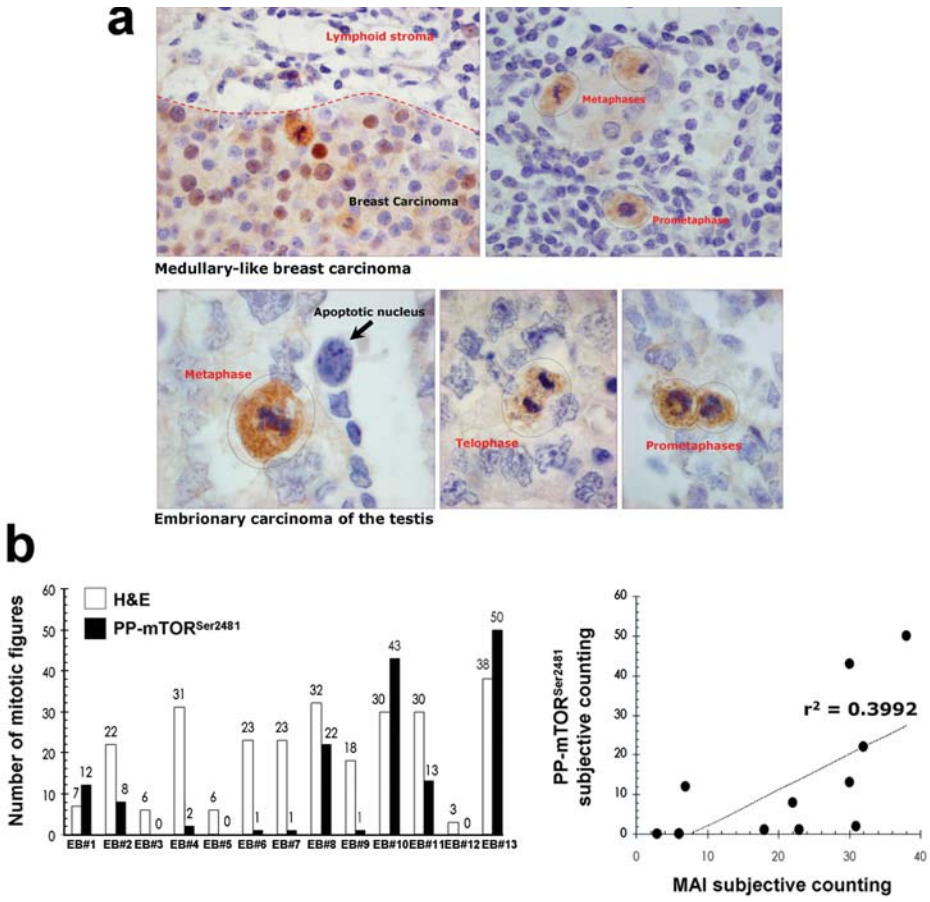

Figure 6. (a) Unsatisfactory PP-mTOR^{Ser2481} staining in excisional biopsies. (b) Detrimental of mitosis counting by applying PP-mTOR^{Ser2481} staining in excisional biopsies. Average number of mitotic figures per tumor in 13 different excisional biopsies depending on the staining method used (left). Scatter plot showing correlation of H&E-based MAI subjective counting and the PP-mTOR^{Ser2481} index in excisional biopsies (right).

sections according to the Scarff-Bloom-Richardson histoprognostic grading (37) modified by Elston and Ellis (38), in a pilot study including 9 breast cancer core biopsies (Fig. 5). A strong correlation was found between the two methods. Importantly, 8 of the 9 invasive ductal breast carcinomas showed a higher number of PP-mTOR^{Ser2481}-positive mitotic cells compared with H&E staining. Thus, average mitotic counts were significantly higher when using PP-mTOR^{Ser2481} labeling (mean 16, range 1-71) than with the H&E staining protocol (mean=5; range 0-28) in breast cancer core biopsies ($p=0.011$; Wilcoxon's non-parametric paired test). Moreover, when the global histoprognostic score was re-evaluated on the basis of phospho-Ser^{2481} mTOR staining in this small sample of breast carcinomas, it was shifted from grade 2 to grade 3 in two cases. Whereas the phospho-mTOR (Ser2481) antibody (no. 2974, Cell Signaling Technology, Inc.) worked very well on core needle biopsies, the staining results were largely unsatisfactory in a majority of excisional biopsies including

lumpectomy specimens (Fig. 6a). Indeed, average mitotic counts were significantly lower when using PP-mTOR^{Ser2481} labeling (mean 12, range 0-50) than with the H&E staining protocol (mean 21, range 3-38) in large specimens (p=0.036, Wilcoxon's non-parametric paired test) (Fig. 6b).

Discussion

In breast carcinomas, the mitotic count is of paramount importance in determining the prognosis and is an integral part of the widely used Elston-Ellis modification of Scarff-Bloom-Richardson grading system (37), also known as the Nottingham combined histological grade or Nottingham grading system (38). Because all the mitotic phases are well discernible by using the H&E staining protocol, some authors have suggested that immunohistochemical detection of mitoses is not necessary in breast cancer as mitotic counts are highly reproducible if adhering to a strict protocol (20). However, mitotic figures are rarely encountered, due to the short duration of the M-phase in comparison to the other phases of the cell cycle, and their detection requires high magnification thus making the screening of large tissue areas tedious and time-consuming. Moreover, when the demonstration of the M-phase is crucial, none of the most available immunohistochemical proliferation markers [e.g., cyclin D1, proliferating cell nuclear antigen (PCNA), Ki67] assesses the actual rate of cell division. Indeed, when using methods aimed to reveal DNA synthesis we should consider that this process might occur also prior to apoptosis or might indicate DNA repair.

Some kinase-active forms of the mammalian target of rapamycin (mTOR), a master integrator of cell growth and division in response to cell energy state, nutrient status, and growth factor stimulation (39-41), unexpectedly appear to act as mitotic survival checkpoints. Yaba *et al* have revealed that PP-mTOR^{Ser2448} is present at very high levels during the M-phase in ovarian granulosa cells (30). They further reported that PP-mTOR^{Ser2448}, but not total mTOR, was enriched on or near the mitotic spindle and also near the contractile ring during cytokinesis. Phosphorylation at Thr421/Ser424 of phospho-S6 kinase, a downstream effector of mTOR, has been also shown to be strongly up-regulated during mitosis in tissues of rats and mice (42). Using tissue sections of human carcinomas and cultured cancer cells we recently revealed that the active form of the α catalytic subunit of AMPK (PP-AMPKα^{Thr172}), a pivotal upstream regulator of mTOR activity, is specifically increased in mitotic cells, with immunopositive PP-AMPKα^{Thr172} condensations displaying a dynamic distribution through all stages of mitosis *in vivo* (43,44). Our group has also revealed that PP-mTOR^{Ser2481}, but not total mTOR, appears to accumulate within nuclear dots displaying dynamic expression during the M-phase. The number of immunopositive condensations of PP-mTOR^{Ser2481} directly related with the percentage of mitotic cells, which suggested that this autophosphorylated form of mTOR may directly control cancer cell proliferation through its previously unrecognized capacity to interact with the mitotic apparatus (31). We now present evidence that Serine 2481-autophosphorylation of mTOR couples with chromosome condensation and segregation during mitosis, which allows rapid and accurate identification of mitotic figures not only in cultured cancer cells but also in breast cancer biopsies. Our current findings strongly suggest that PP-mTOR^{Ser2481}-based immunohistochemical staining could be used as a novel high-contrast mitosis marker: a) PP-mTOR^{Ser2481} showed an enriched expression that correlated strongly and specifically with the mitotic status of cultured cancer cells, b) on the basis of double-immunofluorescence staining and confocal microscopy analyses, all mitotic figures positively marked with the Ser10-/Ser28-phosphorylated G_2/M marker Histone H3 also co-expressed high levels of PP-mTOR^{Ser2481}, c) whereas PP-Histone H3$^{Ser10/Ser28}$ efficiently detected cells at the onset of mitosis (prophase, metaphase, weaker at the beginning of anaphase, but not during late anaphase, telophase and cytokinesis), PP-mTOR^{Ser2481} expression was seen in all stages of mitosis, with the phosphoprotein showing either an adjacent pattern to condensed chromatin (in early stages of mitosis) or a tight localization to the region of the mitotic spindle and midbody within the intracellular bridge (in late stages of mitosis and cytokinesis), d) PP-mTOR^{Ser2481}-based immunolabelling of paraffin-embedded tissue sections allowed for high-quality, reproducible detection of mitotic figures in human cancer tissues, e) there was a very good correlation between the mitotic figure counts performed on traditional H&E-stained tissue sections and those performed on anti-PP-mTOR^{Ser2481}-immunostained tissue sections of breast cancer biopsies, f) PP-mTOR^{Ser2481} labelling significantly improves the mitotic count assessed by standard H&E sections in breast cancer core biopsies and, as a consequence, the histoprognostic grade of breast carcinomas could be upgraded in a significant proportion of cases.

Although we detected approximately three times as many M-phase cells with PP-mTOR^{Ser2481} immunolabelling than with H&E, we should acknowledge that this significantly higher sensitivity of PP-mTOR^{Ser2481}-based immunohistochemistry for mitotic figures solely occurs in breast cancer core biopsies but not in excisional ones. PP-mTOR^{Ser2481} staining was interpretable in few breast cancer lumpectomies and mastectomies and most excisional cases were excluded from evaluation. Since phosphorylation of proteins is rapidly reversible, it is likely that fixation needs to be carried out quickly after tissue sampling. Thus, inappropriate fixation of the tumor tissue within large specimens is most probably the cause for our poor results with PP-mTOR^{Ser2481} staining in excisional biopsies. Accordingly, immunohistochemical detection of mitotic figures using PP-Histone H3^{Ser10} and PP-Histone^{Ser28} has previously been shown to be sensitive to delayed or elongated fixation (33,45). Moreover, it is obvious that the improvement of reproducibility of mitosis counts by applying PP-mTOR^{Ser2481}-based immunohistochemistry could not be determined in this preliminary study with overall low numbers of each tumor subtype. Also, the exact influence of PP-mTOR^{Ser2481} staining for the determination of changes in prognosis prediction in breast cancer subtypes due higher sensitivity of this novel mitotic marker remains to be established on a larger number of breast cancer samples.

As accurately indicated by Skaland *et al* (25), mitotic counts strictly following the proper WHO count procedure (46) are not well liked by many pathologists and implementation of a worldwide general quality control system is not realistic. Moreover, suboptimal tissue fixation has been also

demonstrated to impact adversely on ability to assess mitotic frequency resulting in a systematic downgrading of a proportion of breast cancer cases. This results in a significant reduction in the proportion of breast cancer cases assigned to grade 3 with resultant increase in grade 2 cases. In this regard, a breakthrough has been the demonstrated correlation between phosphorylation of nuclear histone H3, mitotic counts, and also prognosis in a homogeneous group of node-negative invasive breast cancers under 55 years and long follow-up (24). Using PP-Histone H3^{Ser10} counting by digital analysis, the same group recently validated the prognostic value of proliferation measured by PP-Histone H3^{Ser10} in a large series of invasive lymph node-negative breast cancer patients less than 71 years old (25). Our current findings revealing for the first time that PP-mTOR^{Ser2481} immunolabelling may represent a simple and reliable method for quantifying proliferative potential as a mitosis marker in breast cancer core biopsies, further support the notion that 'application of an accurate high-contrast immunohistochemical mitosis marker would have the potential to make mitosis counting much faster, easier to assess, potentially well-reproducible, more accurate, and suitable for highly accurate counting by automated digital image analysis' (25). A study is currently underway in our laboratory to confirm both the accuracy and the prognostic value of PP-mTOR^{Ser2481} as a novel high-contrast immunohistochemical mitosis marker in larger populations of human breast carcinomas.

Acknowledgments

A. Vazquez-Martin is the recipient of a 'Sara Borrell' post-doctoral contract [CD08/00283, Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria (FIS), Spain]. J.A. Menendez is the recipient of a Basic, Clinical and Translational Research Award (BCTR0600894) from the Susan G. Komen Breast Cancer Foundation (TX, USA). This study was supported in part by Instituto de Salud Carlos III [Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria (FIS), Grants CP05-00090, PI06-0778 and RD06-0020-0028 to J.A. Menendez]. J.A. Menendez was also supported by a Grant from the Fundación Científica de la Asociación Española Contra el Cáncer (AECC, Spain).

References

1. Van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R and Friend SH: Gene expression profiling predicts clinical outcome of breast cancer. Nature 415: 530-536, 2002.
2. Van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH and Bernards R: A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347: 1999-2009, 2002.
3. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL and Liu ET: Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci USA 100: 10393-10398, 2003.
4. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, van't Veer LJ and Perou CM: Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 355: 560-569, 2006.
5. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, Sawyer LR, Wu J, Liu Y, Nanda R, Tretiakova M, Ruiz Orrico A, Dreher D, Palazzo JP, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush JF, Ellis MJ, Olopade OI, Bernard PS and Perou CM: The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7: 96, 2006.
6. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO and Botstein D: Molecular portraits of human breast tumours. Nature 406: 747-752, 2000.
7. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P and Børresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98: 10869-10874, 2001.
8. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Børresen-Dale AL and Botstein D: Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 100: 8418-8423, 2003.
9. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET and Bergh J: An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci USA 102: 13550-13555, 2005.
10. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, Nordgren H, Farmer P, Praz V, Haibe-Kains B, Desmedt C, Larsimont D, Cardoso F, Peterse H, Nuyten D, Buyse M, van de Vijver MJ, Bergh J, Piccart M and Delorenzi M: Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst 98: 262-272, 2006.
11. Chang HY, Nuyten DS, Sneddon JB, Hastie T, Tibshirani R, Sørlie T, Dai H, He YD, van't Veer LJ, Bartelink H, van de Rijn M, Brown PO and van de Vijver MJ: Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 102: 3738-3743, 2005.
12. Buyse M, Loi S, van't Veer L, Viale G, Delorenzi M, Glas AM, d'Assignies MS, Bergh J, Lidereau R, Ellis P, Harris A, Bogaerts J, Therasse P, Floore A, Amakrane M, Piette F, Rutgers E, Sotiriou C, Cardoso F and Piccart MJ: TRANSBIG Consortium. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst 98: 1183-1192, 2006.
13. Bogaerts J, Cardoso F, Buyse M, Braga S, Loi S, Harrison JA, Bines J, Mook S, Decker N, Ravdin P, Therasse P, Rutgers E, van't Veer LJ and Piccart M: TRANSBIG consortium. Gene signature evaluation as a prognostic tool: challenges in the design of the MINDACT trial. Nat Clin Pract Oncol 3: 540-551, 2006.
14. Desmedt C, Sotiriou C and Piccart-Gebhart MJ: Development and validation of gene expression profile signatures in early-stage breast cancer. Cancer Invest 27: 1-10, 2009.
15. Sotiriou C and Pusztai L: Gene-expression signatures in breast cancer. N Engl J Med 360: 790-800, 2009.
16. Wirapati P, Sotiriou C, Kunkel S, Farmer P, Pradervand S, Haibe-Kains B, Desmedt C, Ignatiadis M, Sengstag T, Schütz F, Goldstein DR, Piccart M and Delorenzi M: Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res 10: R65, 2008.
17. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, Delorenzi M, Piccart M and Sotiriou C: Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 14: 5158-5165, 2008.
18. Baak JP, van Diest PJ, Janssen EA, Gudlaugsson E, Voorhorst FJ, van der Wall E and Vermorken JB: Collaborators of multicenter morphometric mammary carcinoma project (MMMCP). Proliferation accurately identifies the high-risk patients among small, low-grade, lymph node-negative invasive breast cancers. Ann Oncol 19: 649-654, 2008.
19. Baak JP, van Diest PJ, Voorhorst FJ, van der Wall E, Beex LV, Vermorken JB and Janssen EA: Prospective multicenter validation of the independent prognostic value of the mitotic activity index in lymph node-negative breast cancer patients younger than 55 years. J Clin Oncol 23: 5993-6001, 2005.

20. Van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH and Kwee WS: Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 23: 603-607, 1992.
21. Molenaar WM, Plaat BE, Berends ER and te Meerman GJ: Observer reliability in assessment of mitotic activity and MIB-1-determined proliferation rate in pediatric sarcomas. Ann Diagn Pathol 4: 228-235, 2000.
22. Van Diest PJ, van der Wall E and Baak JP: Prognostic value of proliferation in invasive breast cancer: a review. J Clin Pathol 57: 675-681, 2004.
23. Bossard C, Jarry A, Colombeix C, Bach-Ngohou K, Moreau A, Loussouarn D, Mosnier JF and Laboisse CL: Phosphohistone H3 labelling for histoprognostic grading of breast adenocarcinomas and computer-assisted determination of mitotic index. J Clin Pathol 59: 706-710, 2006.
24. Skaland I, Janssen EA, Gudlaugsson E, Klos J, Kjellevold KH, Søiland H and Baak JP: Phosphohistone H3 expression has much stronger prognostic value than classical prognosticators in invasive lymph node-negative breast cancer patients less than 55 years of age. Mod Pathol 20: 1307-1315, 2007.
25. Skaland I, Janssen EA, Gudlaugsson E, Klos J, Kjellevold KH, Søiland H and Baak JP: Validating the prognostic value of proliferation measured by Phosphohistone H3 (PPH3) in invasive lymph node-negative breast cancer patients less than 71 years of age. Breast Cancer Res Treat 114: 39-45, 2009.
26. Pauslon JR and Taylor SS: Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. J Biol Chem 257: 6064-6072, 1982.
27. Sauvé DM, Anderson HJ, Ray JM, James WM and Roberge M: Phosphorylation-induced rearrangement of the histone H3 NH2-terminal domain during mitotic chromosome condensation. J Cell Biol 145: 225-235, 1999.
28. Hans F and Dimitrov S: Histone H3 phosphorylation and cell division. Oncogene 20: 3021-3027, 2001.
29. Prigent C and Dimitrov S: Phosphorylation of serine 10 in histone H3, what for? J Cell Sci 116: 3677-3685, 2003.
30. Yaba A, Bianchi V, Borini A and Johnson J: A putative mitotic checkpoint dependent on mTOR function controls cell proliferation and survival in ovarian granulosa cells. Reprod Sci 15: 128-138, 2008.
31. Vazquez-Martin A, Oliveras-Ferraros C, Bernadó L, López-Bonet E and Menendez JA: The serine 2481-autophosphorylated form of mammalian Target Of Rapamycin (mTOR) is localized to midzone and midbody in dividing cancer cells. Biochem Biophys Res Commun 380: 638-643, 2009.
32. Goto H, Tomono Y, Ajiro K, Kosako H, Fujita M, Sakurai M, Okawa K, Iwamatsu A, Okigaki T, Takahashi T and Inagaki M: Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J Biol Chem 274: 25543-25549, 1999.
33. Hirata A, Inada K, Tsukamoto T, Sakai H, Mizoshita T, Yanai T, Masegi T, Goto H, Inagaki M and Tatematsu M: Characterization of a monoclonal antibody, HTA28, recognizing a histone H3 phosphorylation site as a useful marker of M-phase cells. J Histochem Cytochem 52: 1503-1509, 2004.
34. Hendzel MJ, Wei Y, Mancini MA, van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP and Allis CD: Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106: 348-360, 1997.
35. Wei Y, Mizzen CA, Cook RG, Gorovsky MA and Allis CD: Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena. Proc Natl Acad Sci USA 95: 7480-7484, 1998.
36. Juan G, Traganos F, James WM, Ray JM, Roberge M, Sauve DM, Anderson H and Darzynkiewicz Z: Histone H3 phosphorylation and expression of cyclins A and B1 measured in individual cells during their progression through G2 and mitosis. Cytometry 32: 71-77, 1998.
37. Bloom HJ and Richardson WW: Histological grading and prognosis in breast cancer: A study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 11: 359-377, 1957.
38. Elston CW and Ellis IO: Pathological prognostic factors in breast cancer I: The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. Histopathology 19: 403-410, 1991.
39. Bjornsti MA and Houghton PJ: The TOR pathway: a target for cancer therapy. Nat Rev Cancer 4: 335-348, 2004.
40. Wullschleger S, Loewith R and Hall MN: TOR signaling in growth and metabolism. Cell 124: 471-484, 2006.
41. Sabatini DM: mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 6: 729-734, 2006.
42. Schmidt T, Wahl P, Wüthrich RP, Vogetseder A, Picard N, Kaissling B and Le Hir M: Immunolocalization of phospho-S6 kinases: a new way to detect mitosis in tissue sections and in cell culture. Histochem Cell Biol 127: 123-129, 2007.
43. Vazquez-Martin A, López-Bonet E, Oliveras-Ferraros C, Pérez-Martínez MC, Bernadó L and Menendez JA: Mitotic kinase dynamics of the active form of AMPK (phospho-AMPK alphaThr172) in human cancer cells. Cell Cycle 8: 788-791, 2009.
44. Vazquez-Martin A, Oliveras-Ferraros C and Menendez JA: The active form of the metabolic sensor AMP-activated protein kinase α (AMPKα) directly binds the mitotic apparatus and travels from centrosomes to the spindle midzone during mitosis and cytokinesis. Cell Cycle 8: 2385-2398, 2009.
45. Tapia C, Kutzner H, Mentzel T, Savic S, Baumhoer D and Glatz K: Two mitosis-specific antibodies, MPM-2 and phosphohistone H3 (Ser28), allow rapid and precise determination of mitotic activity. Am J Surg Pathol 30: 83-89, 2006.
46. Tavassoli FA and Devilee P: Pathology and Genetics: Tumours of the Breast and Female Genital Organs. WHO Classification of Tumor Series. IARC Press, Lyon, pp9-112, 2003.