Gene Expression Profiling in True Interval Breast Cancer Reveals Overactivation of the mTOR Signaling Pathway

Federico Rojo1,3, Laia Domingo5,6, Maria Sala5,6, Sandra Zazo1, Cristina Chamizo1, Silvia Menendez2, Oriol Arpi3, Josep Maria Corominas3,7, Rafael Bragado2, Sonia Servitja3,8, Ignasi Tusquets3,8, Lara Nonell4, Francesc Macià5,6, Juan Martínez9, Ana Rovira3,8, Joan Albanel9,8,10, and Xavier Castells5,6

Abstract

Background: The development and progression of true interval breast cancers (tumors that truly appear after a negative screening mammogram) is known to be different from screen-detected cancers. However, the worse clinical behavior of true interval cancers is not fully understood from a biologic basis. We described the differential patterns of gene expression through microarray analysis in true interval and screen-detected cancers.

Methods: An unsupervised exploratory gene expression profile analysis was performed on 10 samples (true interval cancers = 5; screen-detected cancers = 5) using Affymetrix Human Gene 1.0ST arrays and interpreted by Ingenuity Pathway Analysis. Differential expression of selected genes was confirmed in a validation series of 91 tumors (n = 12; n = 79) by immunohistochemistry and in 24 tumors (n = 8; n = 16) by reverse transcription quantitative PCR (RT-qPCR), in true interval and screen-detected cancers, respectively.

Results: Exploratory gene expression analysis identified 1,060 differentially expressed genes (unadjusted P < 0.05) between study groups. On the basis of biologic implications, four genes were further validated: ceruloplasmin (CP) and ribosomal protein S6 kinase, 70 kDa, polypeptide 2 (RPS6KB2), both upregulated in true interval cancers; and phosphatase and tensin homolog (PTEN) and transforming growth factor beta receptor III (TGFBR3), downregulated in true interval cancers. Their differential expression was confirmed by RT-qPCR and immunohistochemistry, consistent with mTOR pathway overexpression in true interval cancers.

Conclusions: True interval and screen-detected cancers show differential expression profile both at gene and protein levels. The mTOR signaling is significantly upregulated in true interval cancers, suggesting this pathway may mediate their aggressiveness.

Impact: Linking epidemiologic factors and mTOR activation may be the basis for future personalized screening strategies in women at risk of true interval cancers. Cancer Epidemiol Biomarkers Prev; 23(2); 288–99.

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Introduction

The contribution of early detection to the improvement of breast cancer prognostic and survival outcomes, along with progress in radiotherapy, surgery, and systemic treatment modalities, has been largely demonstrated (1–3). The benefit of attending a screening program, however, is reduced for women who experience an interval cancer. Interval cancers manifest clinically between a normal screening result and the following invitation for screening, representing 25% to 30% of cancers detected among screening participants (4). These tumors are an expected part of any screening program and their rate has been recognized as a valid indicator of screening quality. Distinct categories of interval cancer may be distinguished by radiologic findings in the latest screening mammogram, including false negative and true interval breast cancers (5). Although false negatives mainly depend on the radiologist’s skills and occasional misinterpretations of mammograms or additional tests, true interval cancers are related to high growth rate and short sojourn time (6).

There is consensus that interval cancers have a less favorable prognosis than screen-detected cancers (7, 8). Their poor prognosis has been explained as a combination of a delay in diagnosis and more aggressive molecular features. These biologic differences are especially wide for only true interval cancers, as this particular subgroup is
not confounded by screening misinterpretations (6, 9). True interval cancers show increased tumor cell proliferation (6, 10–12) and lower expression of both estrogen receptors (ER) and progesterone receptors (PR; 6, 9, 12, 13) than screen-detected cancers. Some recent studies have found an enrichment of triple negative phenotype among true interval cancers (9, 14, 15). Lately, genetic and epigenetic mechanisms have been related to interval cancers, such as the methylation process of specific genes (16) or the action of growth factors produced in the breast stroma in response to tumoral aggressiveness (17). However, the differences observed in clinical behavior conferring a worse outcome in true interval cancers are not fully understood from a biologic basis. To our knowledge, a complete gene expression profile in true interval compared with screen-detected cancers has not been explored.

Although interval cancers are influenced by screening periodicity, the identification of gene expression patterns through gene expression analysis could provide new insights in mechanisms of carcinogenesis and biologic processes that could help to understand and improve cancer prevention and treatment. The aim of this study was to describe the differential patterns of gene expression through microarray analysis in breast cancer detected in a screening program and true interval cancers.

Materials and Methods

Patients and biopsy specimens

The study was conducted at Hospital del Mar (Barcelona, Spain), a publicly funded institution that is running a population-based breast cancer screening program since 1995. A total of 91 histologically confirmed invasive breast cancer diagnosed between 1997 and 2009 were included in the study. Out of them, 79 cases were screen-detected cancers—cancers detected preclinically by screening mammography—and 12 cases were true interval cancers, cancers that were not visible at last screening mammography and that appeared up clinically before the following screening invitation. Details on interval cancer identification and classification process are reported elsewhere (9). All true interval cancers identified in our setting with available tissue sample and reaching the quality criteria were included in the study. We selected a subset of cases from a pool of screen-detected cancers with available tissue sample and also reaching the quality criteria. For both study groups, we ensured that the proportion of the molecular profiles on the basis of ER, PR, and HER2 expression was consistent with those reported in our study population (9).

Five true interval cancers and five screen-detected cancers with available frozen sample for research were selected for microarray processing (discovery set). The validation process of microarray results was extended to 91 samples as follows (validation set): reverse transcription quantitative PCR (RT-qPCR) was carried out on 24 frozen samples (true interval cancers, n = 8; and screen-detected cancers, n = 16), and immunohistochemical validation was performed over all formalin-fixed paraffin-embedded tissues (true interval cancers, n = 12; and screen-detected cancers, n = 79). Figure 1 summarizes the inclusion of tumor samples in the different study phases.

Tumor specimens were retrieved from Hospital del Mar Biobank, which ensures that all patients provide an informed consent to use tumor samples and clinical information for evaluation and scientific research. Immunohistochemical information of ER, PR, HER2, and Ki67 expression was available for all samples. Positivity criteria for ER and PR (SP1 and PgR636 clones, respectively; Dako) was established for >1% of nuclear tumor staining. HER2 was assayed by FISH (Pathvysion; Abbott Molecular). The percentage of proliferation marker Ki67 (MBI clone; Dako) was scored, and high proliferation was considered with a cutoff of 14% or higher.

Clinical and personal data were obtained by consultation of clinical records and hospital registry. Tumor-node-metastasis (TNM) stage was classified using the seventh edition of the American Joint Committee of Cancer.

The study was approved by the Ethics Committee of Parc de Salut Mar and was conducted following the institutional guidelines.

Microarray hybridization and statistical analysis

Purity and integrity of the RNA were assessed by spectrophotometry and nanoelectrophoresis using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and the Nano lad-on-a-chip assay for total eukaryotic RNA using Bioanalyzer 2100 (Agilent Technologies), respectively. Only samples with high purity and integrity were subsequently used in microarray experiments. Microarray expression profiles from a total of 10 patients were obtained using the Affymetrix Human Gene ST 1.0 Arrays (Affymetrix). Briefly, 300 ng of total RNA from each sample was processed, labeled, and hybridized according to the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay (PN 701880 Rev.4). First, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence and was subsequently used as a template to produce many copies of cRNA. A second cycle of cDNA synthesis was performed, in which single-stranded DNA was generated, fragmented, and finally biotin-labeled. Of note, 5.5 μg of the fragmented and biotinylated cDNA was loaded on a Human Gene 1.0 ST Array and hybridized for 16 hours at 45°C and 60 rpm in an Affymetrix GeneChip Oven 645. Following hybridization, the array was washed and stained in the Affymetrix GeneChip Fluidics Station 450. The stained array was scanned using an Affymetrix GeneChip Scanner 3000 7G.

After quality control of raw data, these were background-corrected, quantile-normalized, and summarized to a gene level using the robust multichip average (18), obtaining a total of 28,832 transcript clusters, which roughly correspond to genes. Normalized data were then filtered to avoid noise created by nonexpressed
transcript clusters in the condition. Affymetrix annotations (version netaffx 29, human genome 18) were used to summarize data into transcript clusters. Linear Models for Microarray (19), a moderated t statistics model, was used for detecting differentially expressed genes between the tumor samples of study groups. Genes with a P value of <0.01 were selected as significant. The selection of gene candidates for further validation was performed on the basis of statistical and also biologic criteria. A longer list of significant genes with P value <0.05 was selected for functional analysis purposes, over which a biologic filter was then applied using bioinformatics technology to select preferably the genes involved in routes of proliferation, angiogenesis, and apoptosis, or in relevant processes with carcinogenesis and its evolution. We used the tool Ingenuity Pathways Analysis (IPA; Ingenuity Systems) to identify canonical pathways and networks. Data analysis was performed in R (version 2.8.1).

**RT-qPCR**

cDNA was produced using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer’s recommendations for random hexamer primer from 1000 ng of RNA. Specific primers were designed using the Lasergene Software (DNAsStar Inc), the BLAST database search program of the National Centre for Biotechnology Information, and the ProbeFinder software (Roche Diagnostics): phosphatase and tensin homolog deleted on chromosome ten (PTEN; forward primer, 5’-CACACGACGGGAAGACAAG-3’; reverse primer, 5’-CATTITGTCTCTTTTTTAGCATC-3’), transforming growth factor-Beta receptor type III (TGFBR3; forward primer, 5’-CTGAAATCGTGGTGTTTAATTG-3’; reverse primer, 5’-GCTCCATGTTGAAGGTGATG-3’), ceruloplasmin (CP; forward primer, 5’-CTCAGCTGTCAGAATCTAAACC-3’; reverse primer, 5’-GTAGTGTCTAAACATGCTCCTCCC-3’), ribosomal protein S6 kinase, 70 kDa, polypeptide 2 (RPS6KB2; forward primer, 5’-CACCTCGAAGATTATTTGC-3’; reverse

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**Figure 1.** Flowchart with cases included in the study phases, for true interval cancers and screen-detected cancers. Microarray analysis was performed among the discovery set including 5 TIBC and 5 SDBC. The validation process at mRNA and protein level was expanded to a validation series of 24 samples for RT-qPCR analysis (TIBC, n = 8; SDBC, n = 16) and 91 for immunohistochemical analysis (TIBC, n = 12; SDBC, n = 79). Quality criterion in each experimental step is shown. SDBC, screen-detected breast cancer.

TIBC, true interval breast cancers.
primer, 5'-CACAGGGTGCTGAGGATTG-3'), and housekeeping gene *ribosomal protein, large, P0* (RPLP0; forward primer, 5'-GCAGGGTGGCAATGGCC-3'; reverse primer, 5'-CTGGGCAACA TTGGCGACAC-3').

PCRs were carried out on a Light-Cycler480 II (Roche Diagnostics) from 2 μL of cDNA (5 ng/μL). PCR consisted of two steps that included: 10-minute initial incubation at 95°C followed by 45 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The crossing point values were calculated based on the second derivate method. All cDNA samples were tested in triplicate in the same analytical run along with a traceable sample consisted in cDNA from major vault protein (MVP) RNA Human Breast (Stratogene-Agilent). Data were presented as mean ± SEM. The absence of genomic DNA interference for the PCR was controlled using 10 ng RNA for the PCR assay. Products were verified using qPCR and a subsequent 2% agarose gel electrophoresis. A standard curve was generated using serial dilutions of cDNA for all primer pairs and corresponding probes. Efficiency of amplifications was calculated from slope of the standard curves according to the equation: $E = 10^{[(C2 - C1)/C0]}$. Relative quantification was obtained by the Pfaffl ratio method (20), normalized to RPLP0, and expression levels were expressed as an n-fold difference relative to the calibrator sample.

Immunohistochemistry for protein expression

Immunostaining was performed using 3-μm tissue sections, placed on plus-charged glass slides on a Dako Link platform, as described before (21). Briefly, after deparaffinization, heat antigen retrieval was performed in a pH 9 EDTA-based buffered solution (Dako). Endogenous peroxidase was quenched. Primary antibodies were incubated for 30 minutes at room temperature: anti-CP rabbit monoclonal antibody (mAb) at 1:200, anti-PTEN (clone 6H2.1; Dako) mouse monoclonal antibody (mAb) at 1:100, anti-RPS6KB2 (Sigma-Aldrich) rabbit pAb at 1:80, anti-phospho-RPS6K at Thr389 (clone 108D2; Cell Signaling Technology) rabbit mAb at 1:200, anti-phospho-mTOR at Ser2448 (clone 49F9; Cell Signaling Technology) rabbit mAb at 1:200, anti-phospho-eIF4G at Ser1108 rabbit mAb at 1:50 (Cell Signaling Technology), anti-phospho-4E-BP1 at Thr37/46 (clone 236B4; Cell Signaling Technology) rabbit mAb at 1:200, and anti-phospho-S6 ribosomal protein at Ser235/236 (clone 91B2; Cell Signaling Technology) rabbit mAb at 1:200. Antigen–antibody reaction was detected by incubation with an anti-mouse/rabbit Ig–dextran polymer coupled with peroxidase (Flex+, Dako). Sections were then visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin. All immunohistochemical staining was performed on a Dako Autostainer platform. Antibody sensitivity was calculated in a range of crescent dilutions of primary antibodies from 1:10 to 1:3,000. Specificity was previously confirmed by reverse-phase lysate arrays (21). Sections from the same specimens incubated with normal mouse or rabbit Ig fractions (IR600 and IR750; Dako) instead of primary antibodies were used as negative controls. As positive control, sections of a breast human tumor with a known expression of the markers were stained. Expression of the studied markers was assessed in a blinded fashion by two investigators (F. Rojo and S. Zazo). For CP, PTEN, p-mTOR, p-eIF4G, RPS6KB2, p-RPS6KB2, and p-S6, cytoplasmic staining was required for considering a tumor cell as positive. For p-4EBP1, both cytoplasmic and nuclear staining was considered. A semiquantitative histoscore was calculated for all markers mentioned before, except PTEN. The histoscore was determined by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low%) × 1 + (medium%) × 2 + (high%) × 3, and the results ranged from 0 to 300. PTEN was scored semiquantitatively using the immunoreactive score (IRS), which was calculated as follows: IRS = SI × PP. Staining intensity was defined as 0, negative; 1, weak; 2, moderate; and 3, strong. Positivity percentage was scored as 0, <1%; 1, 1% to 10%; 2, 11% to 50%; 3, 51% to 80%; and 4, >80% to 100% positive cells. PTEN loss was defined as an IRS of 3 or less (22).

Statistical analysis

Statistical analysis was carried out with SPSS version 13.0. A descriptive analysis to compare the distribution of clinicopathologic characteristics between true interval cancers and screen-detected cancers was done using the Fisher exact test. To analyze correlations between expression of markers from immunohistochemistry and RT-qPCR, the Spearman correlation test was conducted. To analyze correlations between markers and phenotypes, the Mann–Whitney *U* test and the Fisher exact test were used. All the statistical tests were two-sided and the level of significance was 0.05. This work was performed in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guideline.

Results and Discussion

Clinicopathologic characteristics in patients with breast cancer

Clinicopathologic characteristics of 91 patients included in the study are shown in Table 1. Some statistically significant differences were observed between both groups. True interval cancers were diagnosed in younger women, and presented a higher proportion of tumors at more advanced stages. Biomarker expression showed statistically significant differences, although true interval cancers expressed less frequently ER, PR, and more cases were HER2+.

The present study has some limitations; the first is the sample size. Cases were obtained from a single screening program and were diagnosed and treated in a single hospital. This reduces the sample size but ensures the quality and homogeneity of the data. In addition, restricting interval cases to “true interval cancers” makes the availability of cases reaching the quality standards for a
genomic study difficult but enables one to focus on the most aggressive subset of interval cancers.

Identification of genes differentially expressed in true interval cancers versus screen-detected cancers

The global transcriptome analysis between true interval cancers and screen-detected cancers revealed a good segregation of the arrays in their respective classes based on expression values. Two major clusters were seen with this analysis, one containing all of true interval cancer samples, and the other containing all of screen-detected cancer samples (Fig. 2A). Subsequent differential expression analysis revealed a total of 1,060 significantly differentially expressed genes (unadjusted \( P < 0.05 \)) between the study groups (Supplementary Table S1; GEO accession number: GSE47108). Four genes were selected within

![Table 1. Clinicopathologic characteristics of the series of 91 patients with breast cancer, including screen-detected and true interval cancers](image)

\(^{a}\)The Fisher exact two-sided test.
the top 10 differential expressed genes, based on the referenced biologic processes and molecular functions related with breast cancer in the literature, CP, RPS6KB2, PTEN, and TGFBR3 for further investigations. CP and RPS6KB2 were upregulated in true interval cancers, whereas PTEN and TGFBR3 were downregulated.

Briefly, CP encodes a metalloprotein that binds most of the copper in plasma and is involved in the peroxidation of Fe(II) transferrin to Fe(III) transferrin. CP has been linked to the invasive phenotypes in a breast cancer model (23) and has also been proposed as a diagnostic tumor marker in the follow-up of patients with breast cancer (24, 25) and in other tumor types, such as renal cell carcinoma (26) and hepatocellular carcinoma (27, 28).

Another interesting gene overexpressed in true interval cancer was RPS6KB2, which is a member of the ribosomal S6 kinase family of serine/threonine kinases. It is involved in protein synthesis and required for cell proliferation (29). RPS6KB2 is phosphorylated and activated by mTOR (30). Upregulation of phosphoinositide 3-kinase (PI3K) signaling by several mechanisms results in an increase in AKT activity, which leads to the activation of mTOR. The increased activity of mTOR drives the subsequent activation of its effectors, including 4E-BP1 and RPS6KB (31). The phosphorylated and activated forms of RPS6K and 4E-BP1 cooperatively promote translational upregulation of the proteins needed for protein synthesis and cell-cycle progression mediated by the S6 ribosomal protein and the
eukaryotic translation initiation factor 4G (eIF4G). Overexpression of RP56K has been reported in several types of tumors (31, 32), including breast cancer (33). PTEN is a dual-specificity protein phosphatase and an important negative regulator of cell growth and survival. The main substrates of PTEN are inositol phospholipids generated by the activation of PI3K (34), and thus is a major negative regulator of the PI3K/Akt signaling pathway (35). Among other functions, PTEN also regulates p53 protein levels and activity (36), and is involved in G protein–coupled signaling during chemotaxis (37). PTEN is among the most commonly mutated genes in a broad range of human cancers, including breast cancer (38).

Another downexpressed gene was TGFR3. It is a member of a superfamily of cytokines involved in regulating and mediating a variety of normal and pathologic processes, including wound healing, fibrosis, and cancer progression (39). Most cell types express three different sizes of TGF-Beta receptors: type I (53 kD), type II (70–85 kD), and type III (250–350 kD). The type III receptor (or betaglycan), is a transmembrane proteoglycan with a large extracellular domain and a 43–amino acid residue cytoplasmic domain that binds TGF-Beta2. The TGFB3 receptor regulates migration (40). Loss of TGFR3 is a frequent genetic event during human breast cancer development. However, the effects of TGFB3 receptor on migration, invasion, and tumor progression are not confined to breast cancer (41), but have also been demonstrated in non–small cell lung, ovarian, pancreatic, and prostate cancer models (42).

The standard deviations for each gene between study groups were small (below 1.50), indicating that the gene expression is tightly clustered around the mean of 5 sets of biologic replicates in this study. Differential expression of CP, RP56K2, PTEN, and TGFB3 mRNA between both sets of tumor samples was confirmed by normalized to normal breast tissue by RT-qPCR in the same specimens sets of tumor samples was confirmed by normalized to

gene expression (0.8-fold in true interval cancers versus 1.9-fold expression in screen-detected cancers. In contrast, PTEN was downregulated by 0.8-fold in true interval cancers versus 1.9-fold expression in screen-detected cancers. In the case of TGFBR3, the expression of true interval cancers relative to normal breast was 0.4-fold, whereas in screen-detected cancers it was 0.6-fold.

Protein and mRNA expression levels were also correlated for the three genes that demonstrated a differential profile between both groups of tumors (CP, PTEN, and RP56K2) in the series of 24 patients. The analysis revealed a significant correlation between PTEN protein expression, measured by IRR, and PTEN mRNA transcripts (P = 0.005; R² = 0.67); CP protein histoscore and gene expression (P = 0.011; R² = 0.63); and RP56K2 protein histoscore and gene expression (P = 0.011; R² = 0.48; Fig. 3B).

Validation of protein differential expression in true interval cancers and screen-detected cancers

Protein expression in the complete series of 91 cases was determined by immunohistochemistry for the three genes (CP, PTEN, and RP56K2). Overexpression for each marker was defined on the basis of median of expression in tumor cells across complete series as threshold. CP expression was mainly detected in the cytoplasm of tumor cells, showing a heterogeneous distribution across tumor section. Faint CP expression was also observed in stromal cells (i.e., fibroblasts and endothelial cells), but not in lymphocytes. Normal breast diffusely expressed weak CP (Fig. 4A). True interval cases showed higher CP expression compared with screen-detected cases [mean histoscore CP, 189 ± 24 (SD) vs. 49 ± 38, respectively; P = 0.002; Fig. 4B]. CP overexpression was detected in 38 (41.8%) cases, and was significantly higher in true interval cancers (n = 12; 100%) compared with screen-detected cancers (n = 26; 32.9%; P < 0.001), confirming expected expression predicted by the microarray analysis. PTEN expression was detected in the cytoplasm in both normal and tumor cells (Fig. 4A). PTEN loss of expression was observed in 32 (35.2%) cases, defined as IRS negativity positive criteria (0–3 scores). Patients with true interval cancers significantly showed higher proportion of PTEN loss of expression (n = 11; 91.7%) compared with patients with screen-detected cancers (n = 21; 26.6%; P = 0.001; Fig. 4B). RP56K2 protein expression was observed predominantly in the cytoplasm of tumor cells, showing a heterogeneous distribution across the tissue section. Weak and focal expression was also present in stromal cells (Fig. 4A). True interval cancers showed higher RP56K2 expression compared with screen-detected cancers (mean histoscore, 242 ± 34 vs. 114 ± 54, respectively; P = 0.006; Fig. 4B). RP56K2 overexpression was significantly more pronounced in true interval (n = 12; 100%) than in screen-detected cancers (n = 23; 29.1%; P < 0.001).
Overactivation of the mTOR pathway in true interval cancers

RPS6KB2 and PTEN are strongly associated with the activation of the mTOR signaling pathway in cancer. Therefore, we planned to further study the activation of the mTOR cascade, assaying the expression of phosphorylated forms of mTOR, RPS6K, eIF4G, 4E-BP1, and S6 protein in our series (Fig. 5A). Cytoplasmic expression for p-mTOR, p-RPS6K, p-eIF4G, and p-S6 was demonstrated in tumor cells, showing an important variation of staining intensities between cases. p-4E-BP1 was detected both in nucleus and cytoplasm of tumor cells. A significant overexpression of p-mTOR pathway factors was demonstrated in true interval cancers versus screen-detected cancers.

Expression of p-mTOR in true interval cancers was superior to the expression observed in screen-detected cancers (mean histoscore, 204 ± 20 vs. 56 ± 35, respectively; *P* < 0.001; Fig. 5B). Overexpression levels of p-mTOR was detected in 100% of true interval cancers, but only in 25 (31.6%) of screen-detected breast cancers (*P* < 0.001). Enhanced expression of the phosphorylated form of RPS6K was also more frequently observed in true interval cancers (mean histoscore, 211 ± 33 vs. 82 ± 50, respectively; *P* = 0.001; Fig. 5B). Overexpression of p-RPS6K was detected in all studied cases of true interval cancers.
(n = 12; 100%), but only in 32.9% (n = 26) of screen-detected cancers (P < 0.001). Similar findings were observed for p-eIF4G; higher expression was seen in true interval compared with screen-detected cancers (mean histoscore, 208 ± 39 vs. 88 ± 49, respectively; P < 0.001; Fig. 5B), and overexpression was present in a higher proportion of true interval cancers (n = 12; 100%) compared with screen-detected cancers (n = 22; 27.8%; P < 0.001). The same differential pattern was seen for phosphorylated S6, whose expression was more pronounced in true interval cancers (mean histoscore, 130 ± 44 vs. 34 ± 29, respectively; P < 0.001). This p-S6 was detected at overexpressing levels in all true interval cancers (n = 12; 100%), but only in 35.4% (n = 28) of screen-detected cancers (P < 0.001). Finally, phosphorylation of 4E-BP1 was also more frequent in true interval cancers (mean histoscore, 208 ± 39 vs. 88 ± 49, respectively; P = 0.038) and overexpression level was fully associated with true interval (n = 12; 100%), compared with screen-detected cancers (n = 20, 25.3%; P < 0.001). Overall, these data indicate that mTOR pathway activation is more frequent in true interval cancers, demonstrating significant higher levels of expression of phosphorylated mTOR, RPS6k, S6, 4E-BP1, and eIF-4G compared with screen-detected cancers. This is in concordance with their more aggressive clinical behavior, when compared with screen-detected cancer (6). Others and we have previously reported the relevance of mTOR in breast and other tumor types, showing that this pathway activation is associated with poorly outcome in breast, ovarian, and prostate cancers (21, 43–45).

Conclusions

True interval breast cancers biologically differ from screen-detected cancers, both at gene and protein levels. Our data provide evidence for deregulated PI3K and mTOR signaling accompanied by the overexpression of CP in true interval cancers. Overactivation of the mTOR pathway associated with true interval cancers might also open new scenarios in predicting prognosis in patients.
with breast cancer, and the availability of biologic agents against mTOR strengthens the clinical relevance of this work. Further studies with larger sets of patients are needed to verify the overactivation of the mTOR pathway in this subset of tumors. True interval cancers may represent a subpopulation of breast cancers that may be particularly sensitive to PI3K/Akt-mTOR inhibition and our data support clinical trials with these agents in women with true interval cancers. Furthermore, it would be important to link epidemiologic factors and mTOR activation, which may be the basis for future personalized screening strategies for women at risk of true interval cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: F. Rojo, M. Sala, J.M. Corominas, S. Servitja, I. Tusquets, J. Albanell, X. Castells


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Rojo, L. Domingo, M. Sala, S. Zazo, C. Chamizo, J.M. Corominas, R. Bragado, S. Servitja, F. Macià, J. Martínez, X. Castells

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Rojo, S. Zazo, C. Chamizo, R. Bragado, S. Servitja, I. Tusquets, L. Nonell, J. Albanell

Writing, review, and/or revision of the manuscript: F. Rojo, L. Domingo, M. Sala, S. Zazo, C. Chamizo, O. Arpi, S. Servitja, I. Tusquets, L. Nonell, F. Macià, J. Martínez, A. Rovira, J. Albanell, X. Castells

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Rojo, L. Domingo, J.M. Corominas, J. Martínez

Study supervision: F. Rojo, M. Sala, X. Castells

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