Title: Gene expression profiling in true interval breast cancer reveals overactivation of mTOR signaling pathway

Authors:
Federico Rojo1,2, Laia Domingo3,4, Maria Sala3,4, Sandra Zazo1, Cristina Chamizo1,
Silvia Menendez2, Oriol Arpi2, Josep Maria Corominas2,5, Rafael Bragado6, Sonia Servitja2,7,
Ignasi Tusquets2,7, Lara Nonell8, Francesc Macià3,4, Juan Martínez9, Ana Rovira2,7, Joan
Albanell2,7,10, Xavier Castells*3,4

Institutional Addresses: 1Pathology Department, IIS-Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain; 2Cancer Research Program, IMIM (Hospital del Mar Medical Research Institute), C/ Dr Aiguader 88, 08003, Barcelona, Spain; 3Epidemiology and Evaluation Department, Hospital del Mar, Pg Marítim 25, 08003 Barcelona, Spain; 4Research network on health services in chronic diseases (REDISSEC); 5Pathology Department, Hospital del Mar, Pg Marítim 25, 08003 Barcelona, Spain; 6Department of Immunology, IIS-Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain; 7Medical Oncology Department, Hospital del Mar, Pg Marítim 25, 08003 Barcelona, Spain; 8Microarray Core Facility (SAM), IMIM (Hospital del Mar Medical Research Institute), C/ Dr Aiguader 88, 08003 Barcelona, Spain; 9Radiology Department, Hospital del Mar, Pg Marítim 25, 08003 Barcelona, Spain; 10Universitat Pompeu Fabra, Pl de la Mercè 10-12, 08002 Barcelona, Spain.

Running title: Gene expression profiling of true interval breast cancers

Key words: interval breast cancer, breast cancer screening, gene expression analysis, mTOR

Financial support

This work was supported by RD12/0036/0051, RD09/0076/0036, RD09/0076/0101 to J Albanell, Instituto de Salud Carlos III-FEDER (PI12/00680, PI12/01552, PI09/01153, PI11/01296 to J Albanell, F Rojo, A Rovira, S Servitja, J Martinez, I Tusquets, R Bragado, S Zazo, X Castells, L Domingo and M Sala), 2009 SGR 321 to J Albanell, 2009 SGR 788 to X Castells and M Sala, CIBER Epidemiología y Salud Pública (CIBERESP) (AE08_004) to M Sala, the Epidemiology
and Public Health Program (IMIM) to M Sala and X Castells, and the "Xarxa de Bancs de tumors" sponsored by Pla Director d’Oncologia de Catalunya (XBTC), to J Albanell, F Rojo, A Rovira. J Albanell and F Rojo are recipients of intensification program ISCIII/FEDER. We thank Fundació Cellex (Barcelona) for a generous donation to the Hospital del Mar Medical Oncology Service.

**Corresponding author:**

Xavier Castells, MD, PhD

Department of Epidemiology and Evaluation, Hospital del Mar.

Passeig Marítim, 25-29, 08003 Barcelona, Spain.

Tel: +0034 932483288; Fax: +0034 932483496

Email: XCastells@parcdesalutmar.cat

**Conflict of interest statement:** There are no conflicts of interests to disclose

**Type of manuscript:** Research article; not invited.

**Word count:** 3986

**Total number of figures and tables:** 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 than screen-detected cancers. However, the worse clinical behavior is not fully understood from a biological 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 in 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 RT-qPCR, in true-interval and screen-detected cancers, respectively.

**Results:** Exploratory gene expression analysis identified 1060 differentially expressed genes (unadjusted p<0.05) between study groups. Based on biological implications, four genes were further validated: ceruloplasmin (CP) and ribosomal protein S6 kinase, 70kDa, 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 epidemiological factors and mTOR activation may be the basis for future personalized screening strategies in women at risk of true-interval cancers.
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 25-30% 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 radiological findings in the latest screening mammogram, including false negative and true interval breast cancers (5). While 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 biological differences are especially wide considering only true interval cancers, since 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 (ER) and progesterone receptors (PR) (6,9,12,13) than screen-detected cancers. Some recent works 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 biological 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 biological 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 has run 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 pre-clinically 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 the 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). Validation process of microarray results was extended to 91 samples as follows (validation set): Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) was carried out in 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 tumors 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, Glostrup, DK) was established for >1% of nuclear tumor staining. HER2 was
assayed by Fluorescence in situ Hybridization (Pathvysion, Abbott Molecular, Des Plaines, IL). The percentage of proliferation marker Ki67 (MIB1 clone, Dako) was scored, and high proliferation was considered with a cut-off of 14% or higher.

Clinical and personal data were obtained from consultation of clinical records and hospital registry. Tumor-node-metastasis (TNM) stage was classified using 7th American Joint Committee of Cancer.

The study was approved by the Ethics Committee of Parc de Salut Mar and was conducted following 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, Wilmington, DE) and the Nano lab-on-a-chip assay for total eukaryotic RNA using Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), respectively. Only samples with high purity and integrity were subsequently used in microarray experiments. Microarray expression profiles from a total of ten patients were obtained using the Affymetrix Human Gene ST 1.0 arrays (Affymetrix, Santa Clara, CA). Briefly, 300ng 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). Firstly, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence and 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. 5.5µg of the fragmented and biotinylated cDNA was loaded on a Human Gene 1.0 ST array and hybridized for 16 hours at 45ºC 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, it was background corrected, quantile-normalized and summarized to a gene-level using the robust multi-chip average (18) obtaining a total of 28832 transcript clusters, which roughly correspond to genes. Normalized data was then filtered to
avoid noise created by non-expressed 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<0.01 were selected as significant. The selection of genes candidates for further validation was performed on the base of statistical and also biological criteria. A longer list significant genes with p-value<0.05 was selected for functional analysis purposes, over which a biological filter was then applied using bioinformatics technologies to select preferably the genes involved in routes of the proliferation, angiogenesis and apoptosis, or in relevant processes with the 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, Madison, WI) according to manufacturer’s recommendations for random hexamer primer from 1000ng of RNA. Specific primers were designed using the Lasergene Software (DNASTar Inc, Madison, WI), 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’-CACACGACGGGAAGACAG-3’; reverse primer, 5’-CATTCTTCCTTCTTTTAGCATC-3’), *transforming growth factor-Beta receptor type III* (*TGFBR3*) (forward primer, 5’-CTGAAATCGTGGTGTATTTATG-3’; reverse primer, 5’-GCTTCTACATGGTGTGATG-3’), *ceruloplasmin* (*CP*) (forward primer, 5’-CTCAGGCTGTCAGAATCTAAACC-3’; reverse primer, 5’-GTAGTGTCAAACCATGCTTTCCC-3’), *ribosomal protein S6 kinase, 70kDa, polypeptide 2* (*RPS6KB2*) (forward primer, 5’-CACCTCGAAGATTTATTGGC-3’; reverse primer, 5’-CACAGGTGTCTGAGGATTTG-3’) and housekeeping gene *ribosomal protein, large, P0* (*RPLP0*) (forward primer, 5’-GCAGGTGTTCGACAATGGC-3’; reverse primer, 5’-CTGGCAACACTTGCGGACAC-3’).

PCR reactions were carried out on a Light-Cycler480 II (Roche Diagnostics) from 2µl of cDNA (5ng/µl). PCR conditions consisted of two steps that included: 10min an initial incubation at 95ºC followed by 45 cycles of 15sec at 95ºC and 30sec at 60ºC. The crossing point values were calculated based on 2nd derivate method. All cDNA samples were tested in triplicate in the same analytical run along with a traceable sample consisted in cDNA from MVP TM RNA Human Breast (Stratagene-Agilent, La Jolla, CA). Data were presented as mean +/- standard error of the mean. The absence of genomic DNA interference for the PCR was controlled using 10ng and RNA for the PCR assay. Products were verified by no-signal by qPCR and subsequent 2% agarose gel electrophoresis. A standard curve was generated using serial dilutions of cDNA for all primer pairs and corresponding probes. Efficiencies of amplifications were calculated from slope of the standard curves according to the equation: E =10[-1/slope]. 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 3µm tissue sections, placed on plus charged glass slides in a Dako Link platform, as described before (21). Briefly, after deparaffinization, heat antigen retrieval was performed in pH9 EDTA-based buffered solution (Dako). Endogenous peroxidase was quenched. Primary antibodies were incubated for 30 minutes at room temperature: anti-CP rabbit polyclonal antibody (pAb) (Abcam, Cambridge, MA) at 1:25, anti-PTEN (clone 6H2.1, Dako) mouse monoclonal antibody (mAb) at 1:100, anti-RPS6KB2 (Sigma-Aldrich, St. Louis, MO) rabbit pAb at 1:80, anti-phospho-RPS6K at Thr389 (clone 108D2, Cell Signaling, CS, Danvers, MA) rabbit mAb at 1:200, anti-phospho-mTOR at Ser2448 (clone 49F9, CS) rabbit mAb at 1:100, anti-phospho-eIF4G at Ser1108 rabbit pAb at 1:50 (CS), anti-phospho-4E-BP1 at Thr37/46 (clone 236B4, CS) rabbit mAb at 1:200 and anti-phospho-S6 ribosomal protein at Ser235/236 (clone 91B2, CS) 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 were performed in a Dako Autostainer platform. Antibody sensitivity was calculated in a range of crescent dilutions of primary antibodies from 1:10 to 1:3000. 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 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 (FR and SZ). 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 %) x 1 + (medium %) x 2 + (high %) x 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 x PP. Staining intensity was defined as...
0=negative, 1=weak, 2=moderate, and 3=strong. Positivity percentage was scored as 0<1%; 1=1-10%; 2=11-50%; 3=51-80%; and 4>80-100% positive cells. PTEN loss was defined as an IRS of 3 or less (22).

Statistics

Statistical analysis was carried out with SPSS version 13.0. A descriptive analysis to compare distribution of clinicopathological characteristics between true interval cancers and screen-detected cancers was done using Fisher’s exact test. To analyze correlations between expression of markers from immunohistochemistry and RT-qPRC, Spearman correlation test was calculated. To analyze correlations between markers and phenotypes, Mann-Whitney U test and Fisher’s 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 Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guideline.
RESULTS AND DISCUSSION

Clinicopathological characteristics in breast cancer patients

Clinicopathological characteristics of 91 patients included in the study are shown in Table 1. Some statistically significant differences were observed among both groups. True interval cancers were diagnosed in younger women, and presented a higher proportion of tumors at more advances stages. Biomarker expression did not show statistically significant differences, although true interval cancers expressed less frequently ER, PR, and more cases were HER2+.

The present study has some limitation, the first one being the sample size. Cases come 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’ difficult the availability of cases reaching the quality standards for a genomic study but enables to focus on the most aggressive subset of interval cancers.

Identification of genes differentially expressed in true interval cancers vs 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 (Figure 2A). Subsequent differential expression analysis revealed a total of 1060 significantly differentially expressed genes (unadjusted p<0.05) between the study groups (Supplementary Table 1) (GEO accession number: GSE47108). Four genes were selected within the top 10 differential expressed genes, based on the referenced biological 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 while 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 also has 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 mammalian target of rapamycin (mTOR) (30). Upregulation of 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 RPS6K (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 RPS6K 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 the phosphoinositide 3-kinase (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 down-expressed gene was TGFBR3. It is a member of a superfamily of cytokines involved in regulating and mediating a variety of normal and pathological processes, including wound healing, fibrosis and cancer progression (39). Most cell types express three different sizes of TGF-Beta receptors: Type I (53kD), Type II (70-85kD), and Type III (250-350kD). 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 TGFBR3
receptor regulates migration (40). Loss of TGFBR3 is a frequent genetic event during human breast cancer development. However, the effects of TGFBR3 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 biological replicates in this study. Differential expression of CP, RPS6KB2, PTEN, and TGFBR3 mRNA between both set of tumor samples was confirmed by normalized to normal breast tissue RT-qPCR in the same specimens (TGFR3, p=0.018, R²=0.83; CP, p<0.001, R²=0.79; PTEN, p=0.002, R²=0.48; and RPS6KB2, p=0.006, R²=0.25) (Figure 2B).

**Gene differential expression by quantitative RT-qPCR in additional true interval and screen-detected cancer patients**

Then, we aimed to validate the gene expression-based subclasses observed in the discovery set in an expanded series (n=24) by RT-qPCR for the four selected genes, including fresh-RNA from the same specimens used for Affymetrix analysis (n=10) and a set of additional samples (n=14) (Figure 1). Notably, the expression of three of the selected genes significantly discriminated true interval cancers vs screen-detected cancers in this expanded series (CP, p=0.032; PTEN, p=0.020; and RPS6KB2, p=0.019), but not TGFR3 (p=0.120) (Figure 3A). Both microarray data and RT-qPCR results showed that CP and RPS6KB2 were significantly upregulated in true interval cancers when compared to screen-detected cancers. The fold change of CP, RPS6KB2 and PTEN by RT-qPCR for both groups of tumors were consistent with microarray data. CP showed an expression of 36.1-folds in true interval cancers vs 1.7-folds in screen-detected cancers. RPS6KB2 expression in true interval cancers was 2.0-folds vs 0.8-folds in screen-detected cancers. In contrast, PTEN was downregulated by 0.8-folds in true interval cancers vs 1.9-folds expression in screen-detected cancers. In the case of TGFBR3, the expression of true interval cancers relative to normal breast was 0.4-folds, whereas in screen-detected cancers it was 0.6-folds.
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 RPS6KB2) in the series of 24 patients. The analysis revealed a significant correlation between PTEN protein expression, measured by IRS, and PTEN mRNA transcripts (p=0.005, $R^2=0.67$); and between CP protein Histoscore and gene expression (p=0.011, $R^2=0.63$) and RPS6KB2 protein Histoscore and gene expression (p=0.011, $R^2=0.48$) (Figure 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 RPS6KB2). 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 (Figure 4A). True interval cases showed higher CP expression compared with screen-detected cases (mean Histoscore CP, 189 ± 24 (standard deviation) vs 49 ± 38, respectively; p=0.002) (Figure 4B). CP overexpression was detected in 38 (41.8%) of cases, and was significantly higher in true interval cancers (n=12, 100%) compared with screen-detected cancers (n=26, 32.9%) (p<0.001), and confirming expected expression predicted by the microarray analysis. PTEN expression was detected in the cytoplasm in both normal and tumor cells (Figure 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 cases (n=11, 91.7%) compared with patients with screen-detected cancers (n=21, 26.6%), (p=0.001) (Figure 4B). RPS6KB2 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 (Figure 4A). True interval cancers showed higher RPS6KB2 expression compared with screen-detected cancers (mean Histoscore, 242 ± 34 vs 114 ± 54, respectively;
RPS6KB2 overexpression was significantly more pronounced in true interval (n=12, 100%) compared with screen-detected cancers (n=23, 29.1%), (p<0.001).

Overactivation of mTOR pathway in true interval cancers

RPS6KB2 and PTEN are strongly associated with activation of 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 (Figure 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 vs screen-detected cancers.

Expression of p-mTOR in true interval cancers was superior than observed in screen-detected cancers (mean Histoscore, 204 ± 20 vs 56 ± 35, respectively; p<0.001) (Figure 5B). Overexpression levels of p-mTOR was detected in 100% of true interval cancers, but only in 25 (31.6%) of screen-detected 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) (Figure 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) (Figure 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). Same differential pattern was seen for phosphorylated S6, which 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 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). We and others have previously reported the relevance of the mTOR in breast and other tumor types, showing that this pathway activation is associated with poor outcome in breast, ovarian and prostate cancer (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 mTOR pathway associated with true interval cancers might also open new scenarios in predicting prognosis in breast cancer patients, and the availability of biological agents against mTOR strengthens the clinical relevance of this work. Further studies with larger sets of patients are needed to verify the overactivation of 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 supports clinical trials with these agents in women with true interval cancers. Furthermore, it would be important to link epidemiological factors and mTOR activation, which may be the basis for future personalized screening strategies for women at risk of true interval cancers.
List of abbreviations

ER: estrogen receptor; PR: progesterone receptor; RT-qPCR: reverse transcription quantitative polymerase chain reaction; TNM stage: tumor-node-metastasis stage; IPA: Ingenuity pathway analysis; PTEN: phosphatase and tensin homolog deleted on chromosome ten; CP: ceruloplasmin; RPS6KB2: ribosomal protein S6 kinase, 70kDa, polypeptide 2; TGFBR3: transforming growth factor-Beta receptor type III; RPLP0: ribosomal protein, large, P0; IRS: immunoreactive score; RSK: ribosomal S6 kinase; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; eIF4G: eucaryotic translation initiation factor 4G
References


Table 1. Clinicopathological characteristics of the series of 91 breast cancer patients, including screen-detected cancers and true interval cancers.

<table>
<thead>
<tr>
<th></th>
<th>Screen-detected cancers</th>
<th>True interval cancers</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median (range)</td>
<td>60.4 (50-68)</td>
<td>58.7 (52-68)</td>
<td>0.017</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>5 (7.1)</td>
<td>4 (36.6)</td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>65 (92.7)</td>
<td>7 (36.4)</td>
<td></td>
</tr>
<tr>
<td>Family history of breast cancer</td>
<td></td>
<td></td>
<td>0.047</td>
</tr>
<tr>
<td>No</td>
<td>67 (94.4)</td>
<td>8 (72.7)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (5.6)</td>
<td>3 (27.3)</td>
<td></td>
</tr>
<tr>
<td>TNM Stage</td>
<td></td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>I</td>
<td>40 (55.6)</td>
<td>3 (25.0)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>26 (36.1)</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3 (4.2)</td>
<td>2 (16.7)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0 (0)</td>
<td>1 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td>0.406</td>
</tr>
<tr>
<td>Ductal</td>
<td>61 (80.3)</td>
<td>12 (100)</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>11 (14.5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>4 (5.2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td>0.192</td>
</tr>
<tr>
<td>1</td>
<td>27 (43.5)</td>
<td>2 (18.2)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24 (38.7)</td>
<td>5 (45.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11 (17.7)</td>
<td>4 (36.4)</td>
<td></td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td>0.193</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (11.4)</td>
<td>3 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>70 (88.6)</td>
<td>9 (75.0)</td>
<td></td>
</tr>
<tr>
<td>PR status</td>
<td></td>
<td></td>
<td>0.332</td>
</tr>
<tr>
<td>Negative</td>
<td>26 (32.9)</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>53 (67.1)</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
<td></td>
<td>0.085</td>
</tr>
<tr>
<td>Negative</td>
<td>69 (87.3)</td>
<td>8 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (12.7)</td>
<td>4 (33.3)</td>
<td></td>
</tr>
<tr>
<td>P53 status</td>
<td></td>
<td></td>
<td>0.999</td>
</tr>
<tr>
<td>Negative</td>
<td>64 (83.1)</td>
<td>10 (83.3)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>13 (16.9)</td>
<td>2 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Proliferation (Ki67)</td>
<td>Low (&lt;14%)</td>
<td>High (&gt;14%)</td>
<td>0.039</td>
</tr>
<tr>
<td>proliferation</td>
<td>55 (69.7)</td>
<td>24 (30.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Phenotype</td>
<td>Luminal A</td>
<td>Luminal B</td>
<td>0.336</td>
</tr>
<tr>
<td></td>
<td>51 (64.6)</td>
<td>16 (20.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (50.0)</td>
<td>2 (16.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Her 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (12.7)</td>
<td>3 (25.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triple Negative</td>
<td>2 (2.5)</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

*Fisher's exact two-sided test
Legends for figures

Figure 1. Flowchart with cases included in the study phases, for true interval cancers and screen-detected cancers.

True interval breast cancers: TIBC; Screen-detected breast cancer: SDBC

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; and SDBC, n=16) and 91 for immunohistochemical analysis (TIBC, n=12 and SDBC, n=79). Quality criterion in each experimental step is shown.

Figure 2. Discovery of differential gene expression between true interval cancers and screen-detected cancers (n=10).

True interval breast cancers: TIBC; Screen-detected breast cancer: SDBC;

A. Unsupervised hierarchical gene expression clustering reveals a differential pattern between two groups of patients at <0.05 significance level and logRatio >1.2 (n=1060 genes), using Affymetrix Human Gene 1.0 ST arrays. B. Validation of gene expression differences between true interval and screen-detected cancers by RT-qPCR, confirming significant upregulation of CP (ceruloplasmin) and RPS6KB2 (ribosomal protein S6 kinase, 70kDa, polypeptide 2) in true interval cancers, and PTEN (phosphatase and tensin homolog) and TGFBR3 (transforming growth factor-Beta receptor type III) in screen-detected cancers.

Figure 3. Differential expression of candidate genes between true interval cancers and screen-detected cancers in an expanded validation series (n=24).

True interval breast cancers: TIBC; Screen-detected breast cancer: SDBC

A. Significant upregulation of CP and RPS6KB2 and downregulation of PTEN in true interval cancers, confirmed by RT-qPCR. TGFBR3 differential expression was not confirmed in this expanded series. B. Significant correlations of expression at protein (immunohistochemistry) and mRNA level (RT-qPCR) for CP, RPS6KB2 and PTEN in the 24 cases.
Figure 4. Differential expression of CP, RPS6KB2 and PTEN between true interval cancers and screen-detected cancers at protein level.

True interval breast cancers: TIBC; Screen-detected breast cancer: SDBC; Histoscore: Hscore

A. Representative expression differences for CP, RPS6KB2 and PTEN in true interval cancers and screen-detected cancers in the complete series (n=91). All markers were mainly detected in the cytoplasm of tumor cells. B. Overexpression of CP and RPS6KB2 was significantly detected in true interval vs screen-detected cancers. In contrast, downregulation of PTEN expression was significantly associated with screen-detected cancers.

Figure 5. Overactivation of mTOR signaling pathway in true interval cancers.

True interval breast cancers: TIBC; Screen-detected breast cancer: SDBC; Histoscore: Hscore

A. Representative expression differences for phosphorylated mTOR, 4E-BP1, elF-4G, RPS6K and S6 between true interval vs screen-detected cancers, indicating activation of the marker. Cytoplasmic expression was detected in all markers; in addition, nuclear staining was observed for p-4E-BP1. B. Overexpression of phosphorylated forms of proteins and mTOR pathway overactivation were significantly associated with the true interval cancers.
Fig. 4

A

<table>
<thead>
<tr>
<th></th>
<th>CP</th>
<th>PTEN</th>
<th>RPS6KB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDBC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIBC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

- **CP**
  - SDBC: 150 ± 10
  - TIBC: 200 ± 15 (p = 0.002)

- **RPS6KB2**
  - SDBC: 100 ± 5
  - TIBC: 250 ± 15 (p = 0.006)

- **PTEN**
  - SDBC: 20%
  - TIBC: 50%
  - PTEN positive: IRS = 6-12
  - PTEN negative: IRS = 0-3 (p = 0.001)
Gene expression profiling in true interval breast cancer reveals overactivation of mTOR signaling pathway

Federico Rojo, Laia Domingo, Maria Sala, et al.

Cancer Epidemiol Biomarkers Prev. Published OnlineFirst December 17, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1055-9965.EPI-13-0761

Supplementary Material
Access the most recent supplemental material at:
http://cebp.aacrjournals.org/content/suppl/2013/12/18/1055-9965.EPI-13-0761.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.