***NUSAP1* and *KIAA0101* downregulation by neo-adjuvant therapy is associated with better** **therapeutic outcome and survival in breast cancer.**

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# ABSTRACT

**Purpose:** To evaluate whether changes in genomic expression that occur from breast cancer diagnosis to tumor resection after neo-adjuvant chemotherapy (NCT) unveil biomarkers capable to predict therapeutic response and survival.

**Materials and Methods:** Gene expression profiles were determined in tumor samples from 39 BC patients who showed pathological complete response (pCR) or therapeutic failure (no PCR) after NCT (cyclophosphamide-doxorubicin/epirubicin). Based on unsupervised classification analysis of microarray data and Interactome studies, *NUSAP1*, *KIAA0101*, *MME*, and *DST* genes were selected for analyses of NCT response, expression in BC histologic subtypes, and tumor-infiltrating lymphocytes. Finally, correlation analyzes between *NUSAP1* and *KIAA0101* (the most discriminating genes) against disease-free survival (DFS) and overall survival (OS) were performed.

**Results:** A signature constituted by 43 genes stratified pCR and non-pCR patients (FC = 3, FDR *p*-value < 0.0298). Patients achieving pCR showed downregulation of *NUSAP1* and *KIAA0101* in tumor tissues and increased DFS and OS, while overexpression of these genes correlated with poor response and OS. These genes are involved in regulation of the mitotic division.

**Conclusions:** Downregulation of *NUSAP1* and *KIAA0101* after the NCT has a positive impact on tumor response to therapy and patient survival.

**Keywords:** *NUSAP1*, *KIAA0101*, pathological response, neo-adjuvant chemotherapy treatment, survival.

# Introduction

Therapy response and prognosis in breast cancer (BC) are modified by factors like age [1], clinical stage [2], tumor histopathology, and molecular subtypes [3]. Gene expression profiles or genomic signatures performed prior to therapy can provide additional information on tumor biology, and algorithms have been generated to predict risk of relapse and survival and to define the best treatment options [4-6]. This genomic test allows the identification of low-risk biology tumors associated with favorable prognosis and facilitates the therapeutic decision-making for aggressive tumors with poor response to conventional therapies. Genomic signatures can also identify gene expression patterns related to chemo resistance, immune system response, and tumor invasion [7-10].

Comparisons of gene expression analyses of biopsy specimens taken before and after neo-adjuvant chemotherapy treatment (NCT), maybe useful to define tumor molecular adaptations to a specific therapeutic agent or regime [7-10]. The Pathologic complete response (pCR) in BC is defined as the absence of all invasive tumor tissue after completion of NCT cycles [11]. The achievement of pCR after NCT correlates with patient survival [12]. Alternative treatment regimens may improve survival when a pCR is not achieved [13]. Comparisons of the changing patterns of gene signatures in response to chemotherapy may enable predictions of clinical response and prognosis, and sometimes, to recognize new response biomarkers of specific pathways related to treatment resistance and recurrence.

There is no genomic signature to define therapeutic alternatives in patients with incomplete pathological response (non-pCR). Therefore, the identification of gene expression profiles in tumor tissue after NCT that are associated with a good or bad pathological response or survival could facilitate the identification of patients who could benefit from a second-line adjuvant treatment or improve clinical follow-up, as has been shown in some studies assessing pathological response [14]. Review of the biochemical pathways in which these genes are involved could also provide potential therapeutic targets or identify markers for high-risk patients who require closer follow-up.

The aim of this work was to analyze changes in genomic expression in primary BC tumors undergoing NCT and to identify genes associated with prognosis in non-responding patients that could guide new pharmaceutical interventions for a second line of treatment. After validations, our studies show downregulation of *NUSAP1* and *KIAA0101* and overexpression of *MME and DST* in tumor biopsies of patients with pCR after NCT and significant correlations with DFS and OS. *NUSAP1* is involved in cell proliferation and migration, while *KIAA0101* participates in cell cycle control and apoptosis [15, 16]. Overexpression of these genes have been correlated with tumor progression and metastases [17-19]. Downregulation of MME associates with tumor recurrence and metastases [20] while subexpression of DST, a cytoskeletal protein, promotes breast cancer progression, independently of the tumor hormonal status [21].

# Materials and Methods

**Patient population.** BC patients were recruited, consented, and enrolled in the study in the Breast Cancer Center of the San Jose Hospital in Monterrey, Mexico. The Institutional Review Board from the School of Medicine of Tecnologico de Monterrey (CONBIOETICA 19 CEI 011-2016-10-17) authorized the research protocol with the number: P000088-Altru- Pro-CI-CR002. In accordance with the Declaration of Helsinki, informed written consent was obtained from all patients participating in this study. Tissue samples from 54 patients with clinical and/or radiological diagnosis of BC (tumor size > 2 cm and palpable lymph nodes) were collected from July 2011 to October 2014.

**Neo-adjuvant chemotherapy regimens.** Regimens were established according to the clinical stage and the immunohistochemistry (IHC) of the breast tumors by medical oncologists. They consisted of 4 cycles every 3 weeks of either intravenous (IV) cyclophosphamide (500- 1500 mg/m2) and doxorubicin (> 40 mg/m2) or cyclophosphamide (500-1500 mg/m2) and epirubicin (> 60 mg/m2). After receiving any of these schemes, patients received 12 weekly cycles of IV paclitaxel (80 mg/m2) administered in 1 hr. In patients who presented drug toxicity, cycles of carboplatin replaced the drug responsible for the toxicity. Subsequently, surgical resection of the breast was performed in all patients. Some patients received selected adjuvant therapy after NCT, as recommended by the attending oncologist. In such cases, selection of the chemotherapeutic drug was made according to individual patient characteristics and clinical guidelines (i.e.: trastuzumab and tamoxifen).

**Tumor sample collection.** Two tissue samples were collected from each patient: a biopsy sample (BS) before NCT paired with tissue after completing the NCT cycles (surgery sample or SS). Thick needle puncture biopsies were obtained using a Bard Magnun 12 Fr gauge Needle (Bard®). Tumor location was marked at diagnosis using the carbon tracking technique [22]. Six to eight tissue cylinders were obtained from each patient. Four samples were used for histopathological analysis and three samples were preserved in RNA-later solution for genomic analysis. The SS were obtained from surgeries for loco regional control (modified radical mastectomy in most of the cases). Tissues were sent to pathology for histopathological and IHC analyses. A 2 x 1 cm piece, marked by the carbon track used during the diagnostic biopsy procedure, was preserved in RNA-later solution for the gene expression analysis.

**IHC and tumor infiltrating lymphocytes (TILs).** Samples were obtained from each patient for hematoxylin‑eosin (H&E) staining and IHC for ER, PR, and HER‑2/neu. The histological grade of the core needle biopsies was obtained prior to neoadjuvant therapy, using the Bloom-Richardson score [23]. The stage of breast cancer was determined according to the American Joint Committee on Cancer (AJCC) [24]. The assessment of the percentage of TILs was performed as per the recommendations of the International TIL Working Group 2014 in breast cancer [25]. Complete methodology for TILs assessment was previously described [26]. IHC for CD3+, CD4+, and CD8+ were also performed inthecoreneedlebiopsies,priortoNCT to define lymphocyte immunophenotypes, followingtheAmericanSocietyofClinicalOncology/CollegeofAmericanPathologistsguidelines [27].

**Treatment Response.** Two pathologists evaluated surgical specimens and assessed tumor response to NCT using the Miller-Payne grading system. For purposes of this study, a grade 5 score in the grading system was considered as pathological complete response (pCR), and the remaining scores (including partial pathological response) were classified as non- pathological complete responses (non-pCR) [28].

**RNA isolation and microarrays analysis (expression profiles).** RNA isolation from BS and SS were prepared using RNeasy Fibrous Tissue Mini Kit (Qiagen, Germantown, MA) following manufacturer's instructions. RNA quality was assessed by capillary electrophoresis using the Experion™ Automated Electrophoresis Station (Bio-Rad, CA, USA). Sample processing, microarray hybridization and gene expression analysis from the selected RNAs was conducted using the GeneChip 3'IVT Express Kit and GeneChip Human Genome U133 plus 2 (Affymetrix, Santa Clara, CA), according to manufacturer's instructions and previously described [29].

**Microarray data processing.** Normalization was performed using RMA (Robust Microarray Analysis) normalization. Samples from five patients were removed because they showed clearly altered profiles compared to the others (abnormal microarray quality controls), leaving 39 patients for this study. Probes with mean expression lower than 3 (in logarithmic scale resulted from RMA) were also removed.

Differential gene expression signature was performed using *t*-test with multiple comparison corrections using the False Discovery Rate method (FDR) [30]. We considered as positive the probes with a significant *p*-value (FDR: *p* < 0.05). These analyses were completed using the free Transcriptome Analysis Console (TAC) 4.0.1 software from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA).

**Gene network.** An interaction analysis of the selected genes was carried out using the online tool STRING: functional protein association networks version 11.0 [31]. The combined score was computed by combining the probabilities from the different evidence channels and corrected for the probability of randomly observing an interaction [32].

**Real-time qPCR validation.** To validate microarray data, four genes were selected based on the results of the microarray differential gene expression and the interactome analyses in SS tissues: two over-expressed (*MME* and *DST*) and two under-expressed (*NUSAP1* and *KIAA0101*) genes. The interactome showed that the selected genes are involved in cell cycle regulation pathways, as will be explained in the results section. *GRAMD1A* was used as an endogenous gene control due to a low variation between samples [29]. Expression analyses were assessed using predesigned hydrolysis probes (*MME*: Hs00153510\_m1, *DST*: Hs00156137\_m1, *NUSAP1*: 150 Hs01006195\_m1, *KIAA0101*: Hs00207134\_m1, *GRAMD1A*: Hs.PT.5840681431) (Thermo- Fisher Scientific). Total RNA aliquots used for microarray assays were analyzed through qPCR using the Quant Studio 3 (Applied Biosystem). *Ct* means for each gene were used for *dCt* (problem minus endogenous), and *2-dCt* analysis was done using calculated *dCt* for all genes. To compare gene expression of pCR and non-pCR groups, the relative expression *2- dCt* was evaluated from qPCR data from all genes after normalization with *GRAMD1A*. Unpaired *T* test with Welch´s correction was used to establish differences (*p-*value < 0.05).

**Disease Free Survival (DFS) and Overall survival (OS).** Comparison of SS gene expression values with disease free survival (DFS) and overall survival (OS) was evaluated in the 39 patients. To evaluate differences in OS, Log-rank (Mantel-Cox) test was used for comparison of Kaplan-Meier survival curves using GraphPad Prism version 6.01 for Windows, GraphPad Software (La Jolla, CA, [www.graphpad.com](http://www.graphpad.com/)). A *p*-value ≤ 0.05 was considered significant in all statistical analyses.

For external validation, Kaplan-Meier Plotter (<http://kmplot.com/)>online database [33] was used to analyze the OS correlated to high versus low gene mRNA expression levels. The Kaplan-Meier Plotter split the BC patient (n = 1,402) samples into two groups according to their median mRNA levels. The Affymetrix probe IDs used for the Kaplan Meier were *KIAA0101/PCLAF* 202503\_s\_at and *NUSAP1* 219978\_s\_at.

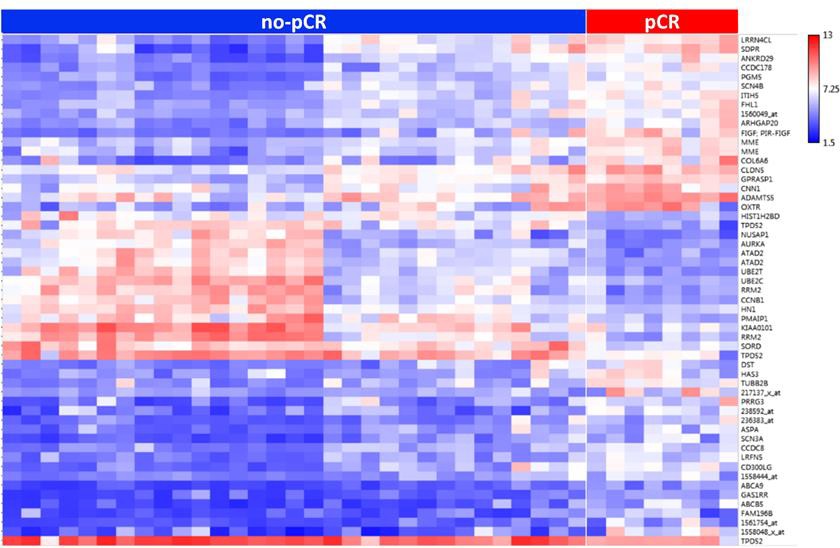
# Results

**Patients.** Fifty-four patients were enrolled in the study, but only 44-paired samples (BS and SS) satisfied the RNA quality and quantity standards needed for the microarray analysis. After this, five sets of samples were eliminated because failed to achieve quality standards, leaving 39 patient sample sets for the final analyses. Demographic and clinical characteristics of the patients are described in Table 1. Only 8 (20.5%) out of the 39 patients reached pCR, according to the Miller-Payne grading system.

**Table 1.** Patient demographic and clinic characteristics.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **All patients (n = 39)** | | **pCR (n = 8) (20.5%)** | | **non-pCR (n = 31) (71.5%)** | | ***p* value** |
| **Age at diagnosis (years)** | 48 | 26 to 63 | 47 | 38 to 57 | 48 | 26 to 63 | 0.73 |
| **BMI (body mass index, kg/m2)** | 28.21 | 20.78 to 39.65 | 28.4 | 20.78 to 39.65 | 28.17 | 24.84 to 33.07 | 0.88 |
| <25 | 8 | 21.0% | 1 | 12.5% | 7 | 22.6% |  |
| >25 | 28 | 72.0% | 6 | 75.0% | 22 | 71.0% |  |
| No Data | 3 | 7.7% | 1 | 12.5% | 2 | 6.4% |  |
| **Menopause status** |  |  |  |  |  |  |  |
| Pre | 21 | 54.0% | 5 | 62.5% | 16 | 51.6% | 0.88 |
| Post | 18 | 46.0% | 3 | 37.5% | 15 | 41.4% |  |
| **Family history** |  |  |  |  |  |  |  |
| Yes | 19 | 49.0% | 3 | 37.5% | 16 | 51.6% | 0.75 |
| No | 20 | 51.0% | 5 | 62.5% | 15 | 41.4% |  |
| **Diabetes mellitus** |  |  |  |  |  |  |  |
| Yes | 2 | 5.0% | 0 | 0.0% | 2 | 6.5% | 0.87 |
| No | 37 | 95.0% | 8 | 100.0% | 29 | 93.5% |  |
| **Number of children** |  |  | 3.625 |  | 3.161 |  | 0.44 |
| Nulliparous | 4 | 10.0% | 0 | 0.0% | 4 | 12.9% | 0.22 |
| 1 or 2 | 12 | 31.0% | 2 | 25.0% | 10 | 32.3% |  |
| >3 | 23 | 59.0% | 6 | 75.0% | 17 | 54.8% |  |
| **Lactation** |  |  |  |  |  |  |  |
| Yes | 16 | 41.0% | 3 | 37.5% | 13 | 41.9% | 0.97 |
| No | 11 | 28.0% | 2 | 25.0% | 9 | 29.0% |  |
| No data | 12 |  |  |  |  |  |  |
| **Smoking** |  |  |  |  |  |  |  |
| Yes | 5 | 13.0% | 2 | 25.0% | 3 | 9.7% | 0.25 |
| No | 34 | 87.0% | 6 | 75.0% | 28 | 90.3% |  |
|  |  |  |  |  |  |  |  |
| **Clinical stage** |  |  |  |  |  |  |  |
| I | 1 | 3.0% | 1 | 12.5% | 0 | 0.0% | 0.82 |
| II | 19 | 49.0% | 2 | 25.0% | 17 | 54.8% |  |
| III | 19 | 49.0% | 5 | 62.5% | 14 | 45.2% |  |
| **TNM classification** |  |  |  |  |  |  |  |
| T1 | 1 | 2.6% | 1 | 12.5% | 0 | 0.0% | 0.93 |
| T2 | 18 | 46.2% | 1 | 12.5% | 17 | 54.8% |  |
| T3 | 11 | 28.2% | 4 | 50.0% | 7 | 22.6% |  |
| T4 | 9 | 23.1% | 2 | 25.0% | 7 | 22.6% |  |
| N0 | 7 | 17.9% | 2 | 25.0% | 5 | 16.1% |  |
| N1 | 23 | 59.0% | 4 | 50.0% | 19 | 61.3% |  |
| N2 | 9 | 23.1% | 2 | 25.0% | 7 | 22.6% |  |
| M0 | 39 | 100.0% | 8 | 100.0% | 31 | 100.0% |  |
| **IHC markers** |  |  |  |  |  |  |  |
| ER+ | 16 | 41.0% | 2 | 25.0% | 14 | 45.2% | 0.30 |
| ER- | 23 | 59.0% | 6 | 75.0% | 17 | 54.8% |  |
| PR + | 17 | 43.6% | 2 | 25.0% | 15 | 48.4% | 0.23 |
| PR- | 22 | 56.4% | 6 | 75.0% | 16 | 51.6% |  |
| HER2+ | 7 | 17.9% | 5 | 62.5% | 2 | 6.5% | 0.0017\*\* |
| HER2 - | 32 | 82.1% | 3 | 37.5% | 29 | 93.5% |  |
| ki67 | 15.4 | 5 to 70 | 17.14 | 5 to 50 | 14.92 | 2 to 70 | 0.76 |
| **Molecular subtype** |  |  |  |  |  |  |  |
| Luminal A | 10 | 25.6% | 1 | 12.5% | 9 | 29.0% | 0.51 |
| Luminal B | 6 | 15.4% | 0 | 0.0% | 6 | 19.4% |  |
| HER2+ | 7 | 17.9% | 5 | 62.5% | 2 | 6.5% |  |
| Triple negative | 16 | 41.0% | 2 | 25.0% | 14 | 45.2% |  |
| ***NUSAP1*(BS)** |  |  |  |  |  |  |  |
| Overexpressed | 17 | 43.6% | 2 | 25.0% | 15 | 48.4% | 0.426 |
| Underexpressed | 22 | 56.4% | 6 | 75.0% | 16 | 51.6% |  |
| ***KIAA0101*(BS)** |  |  |  |  |  |  |  |
| Overexpressed | 18 | 46.2% | 4 | 50.0% | 14 | 45.2% | >0.9999 |
| Underexpressed | 21 | 53.8% | 4 | 50.0% | 17 | 54.8% |  |
| ***NUSAP1*(SS)** |  |  |  |  |  |  |  |
| Overexpressed | 12 | 30.8% | 0 | 0.0% | 12 | 38.7% | 0.0417\* |
| Underexpressed | 27 | 69.2% | 8 | 100.0% | 19 | 61.3% |  |
| ***KIAA0101*(SS)** |  |  |  |  |  |  |  |
| Overexpressed | 28 | 71.8% | 1 | 12.5% | 24 | 77.4% | 0.0003\*\*\* |
| Underexpressed | 11 | 28.2% | 7 | 87.5% | 4 | 12.9% |  |

**Gene expression profile analysis.** The following comparisons were made between BS and SS gene expression data in pCR and non-pCR patients to assess the gene expression modifications induced by NCT: pCR-BS vs pCR-SS (Supplementary Figure 1), non-pCR-BS vs non-pCR-SS (Supplementary Figure 2), pCR-BS vs non-pCR-BS (Supplementary Figure 3), and pCR-SS vs non-pCR-SS (Figure 1). Of these comparisons, significant non-supervised sample clustering (pCR vs non-pCR) was only achieved in the last one, in SS (Figure 1).



**Figure 1. Non-supervised sample clustering (heat map) of differential gene expression in SS (pCR vs non-pCR).** Blue areas represent low gene expression, while red represents high gene expression. The top row separates the non-pCR (blue) and pCR (red) patients. Each column represents a different sample and each row, a single probe. Official gene or probe symbols are displayed at the right-side margin.

From the comparisons of gene expression profiles, sets of genes were generated with which Venn diagrams and interactome networks were constructed to facilitate the selection of candidate genes for survival analyses. The first comparison assessed changes induced by NCT in samples of patients achieving pCR (BS = 8 vs SS = 8). A profile of 21 probes representing 14 genes was found (FC = +3, FDR *p*-value < 0.05) (Figure 2A). Three genes were overexpressed (*TOP2A, RRM2*, and *CDKN3*) and eleven were downregulated (*EGR2, ADAMTS5, JUN, APOLD1, DUSP1, CYR61, ATF3, EGR1, PTGS2, RGS1,* and *FOSB*) (Supplementary Figure 1). These genes were enriched in pathways regulating cell death, apoptosis, morphogenesis, and circadian processes (Figure 2A). Likewise, the study of the non-pCR subset of patients (BS = 31 vs SS = 31) identified four overexpressed genes in SS biopsies (*NAMPT, DUSP1, RGS1*, and *FOS*) (FC = + 3, FDR *p-*value < 0.05) (Figure 2B and Supplementary Figure 2). Gene Network by STRING added 10 nodes (proteins) to show a network around the 4 main genes on this gene profile. Pathways enriched by these genes were associated to response to ionizing radiation, metal ions, lipids, organ nitrogen compounds, and drugs (Figure 2B). Comparisons between pCR and non-pCR were made in the BS (Figure 2C and Supplementary Figure 3). Immunologic pathways were enriched in this comparison (IgG binding, and MHC class II receptor activity), but these genes barely interacted.

A significant non-supervised sample clustering was only achieved in the last comparison of SS group (Figure 1 and Figure 2D). This pCR (*n* = 8) vs non-pCR (*n* = 31) comparison revealed a profile of 55 probes corresponding to 43 genes (FC = 3, FDR *p-*value < 0.0298) (Figure 1D). Thirty genes were over-expressed in patients with pCR (*ABCA9, ABCB5, ADAMTS5, ANKRD29, ARHGAP20, ASPA, CCDC178, CCDC8, CD300LG, CLDN5, CNN1, COL6A6, DST, FAM196B, FHL1, FIGF; PIR-FIGF, GAS1RR, GPRASP1, HAS3, ITIH5, LRFN5, LRRN4CL, MME, OXTR, PGM5, PRRG3, SCN3A, SCN4B, SDPR*, and *TUBB2B*), while thirteen were down-regulated (*ATAD2, AURKA, CCNB1, HIST1H2BD, HN1, KIAA0101, NUSAP1, PMAIP1, RRM2, SORD, TPD52, UBE2C*, and *UBE2T*). Three and five proteins of this gene profile were enriched for the mitotic nuclear division and for the anaphase promoting complex-dependent catabolic process.

|  |
| --- |
| A |
| B |
| C |
| D |

**Figure 2. Comparisons between BS, SS and pathological responses.** For each component in the figure, intersections of differentially expressed gene sets are shown in Venn diagrams on the left side and protein interaction networks are shown on the right side. Gene ontology (GO) tables are shown at the bottom. **A.** Genetic profiles for BS (green) and SS (red) in pCR patients showing differential expression of 14 genes involved in cell death, morphogenesis, and circadian processes. **B.** Genetic profiles for BS (yellow) and SS (blue) in non-CPR patients, showing four overexpressed genes involved in response to ionizing radiation, metal ions, lipids, and drugs. **C.** Genetic profiles for pCR and non-pCR patients in BS showing 21 differentially expressed genes involved in the immune response. **D.** Gene profiles for pCR and non-pCR patients in SS, showing 43 differentially expressed genes involved in the regulation of mitotic division. BS=Biopsy sample (pretreatment); SS= Surgical sample (post treatment); pCR= pathological complete response; non-pCR= without complete pathological response. PPI: *p*-value of protein-protein interaction enrichment.

**Gene Network.** The online tool “STRING: functional protein association networks version 11.0” [31] was used to investigate interactions among genes identified as differentially expressed in each comparison. The value of the interaction network was significant (PPI enrichment *p-*value: 2.26E-10), meaning that these proteins have more interactions among themselves than what would be expected from a random set of proteins of similar size drawn from the genome (Figure 2). According to the protein interaction analysis, *AURKA, CCDC8, CCNB1, NUSAP1*, and *UBE2C* are involved in the regulation of nuclear division during mitosis, while *AURKA, CCNB1*, and *UBE2C* genes are part of the anaphase promoter complex dependent catabolic process.

# qPCR Validation. Four genes (*DST, MME, NUSAP1* and *KIAA0101*/*PCLAF*) were selected to validate the microarrays by qPCR as explained in the Methods section. The remaining DNA from the tissue sample was scarce, therefore only 31 samples (pCR=5, non-pCR=26) out of 39 had enough quality and quantity of total RNA to perform qPCR validation analysis. Supplementary Figure 4 shows the box plot of *NUSAP1* and *KIAA0101* expression by qPCR (4A and 4B) and by microarray (4C and 4D). A similar analysis was made for *DST* and *MME* expression by qPCR (4E and 4F) and by microarray (4G and 4H). This qPCR analysis corroborated the expression patterns of these differentially expressed genes.

***NUSAP1* and *KIAA0101* gene expression.** In the subset of patients achieving pCR, *NUSAP1* and *KIAA0101* gene expressions were higher in the BS compared to the SS (Two way- ANOVA, F = 22.12, *p-*value = 0.0053) (Figure 3A and 3C), while there was no significant difference in the expression values in the non-pCR groups (Two way-ANOVA, F = 1.246, *p*- value = 0.2739) (Figure 3B and 3D). It is important to note that the expression of *NUSAP1* after NCT was significantly higher in luminal B tumors compared to the rest of the histological subtypes*.* (F test = 4.88, *p* value = 0.0063) (Supplementary Figure 5).

|  |  |
| --- | --- |
| **A** | **B** |
| **C** | **D** |

**Figure 3. *NUSAP1* and *KIAA0101* gene expression based on microarrays data.** **A** and **B**: *NUSAP1* gene expression in BS and SS in pCR (A) and non-pCR (B) patients, respectively. **C** and **D**: *KIAA0101* gene expression in BS and SS in pCR (C) and non-pCR (D) patients, respectively. Blue lines and triangles: triple negative molecular subtype; red lines and squares: luminal A/B molecular subtype; green lines and circles: HER2 molecular subtypes. Two way-ANOVA was performed and *p-*value < 0.05 was considered as significant.

**Expression of *NUSAP1* and *KIAA0101* and response to treatment.** Response to NCT was considered the primary response variable and was evaluated using the Miller-Payne grading system. The association between the *NUSAP1* and *KIAA0101* genes with response to treatment was tested. The expression of *NUSAP1* and *KIAA0101* after NCT are inversely associated with pCR, implying that the downregulation of these genes has a favorable effect for the patient, as showed in Table 2 (*NUSAP1*: OR = 0.0, 95% IC= 0.00-0.99, *p* = 0.0417\*; *KIAA0101*: OR = 0.02, 95% IC = 0.002-0.243, *p* = 0.0003\*\*\*).

**Table 2.**  ***NUSAP1* and *KIAA0101* and response to treatment**

|  |  |  |
| --- | --- | --- |
|  | ***NUSAP1*** | ***KIAA0101*** |
| **OR** | 0.00 | 0.02 |
| **95% IC** | 0.000 to 0.9915 | 0.002068 to 0.2430 |
| **p value** | 0.0417\* | 0.0003\*\*\* |
| **Sensitivity** | 0.00 | 0.13 |
| **95% IC** | 0.000 to 0.3244 | 0.6412 to 47.09% |
| **Specificity** | 0.61 | 0.14 |
| **95% IC** | 0.4382 to 0.7627 | 5.699 to 31.49% |
| **Positive Predictive Value** | 0.00 | 0.04 |
| **95% IC** | 0.000 to 0.2425 | 0.2052 to 19.54% |
| **Negative Predictive Value** | 0.70 | 0.36 |
| **95% IC** | 0.5152 to 0.8415 | 15.17 to 64.62% |
| **Likelihood Ratio** | 0.00 | 0.15 |

**Tumor infiltrating lymphocytes.**  *NUSAP1* and *KIAA0101* expression versuslymphocyte immunophenotypes and TILs were evaluated priortoNCT (Table 3). There were no correlations among TILsand *NUSAP1* and *KIAA0101* expression levels (*NUSAP1*: r = 0.10, 95% IC = -0.39 - 0.25, *p* = 0.65; KIAA0101: r = -0.07, 95% IC = 0.85 - 0.96, *p* = 0.54). Representative images of TILs evaluation are shown in Supplementary figure 6.

**Table 3.** **TILs correlation**

|  |  |  |  |
| --- | --- | --- | --- |
| **TILs correlation** |  |  |  |
| **N=39** | **r** | **95% IC** | ***p value*** |
| ***NUSAP1* and TILS%** | 0.10 | -0.39 to 0.25 | 0.65 |
| ***KIAA0101* and TILS%** | -0.07 | -0.22 to 0.40 | 0.54 |
| ***NUSAP1* and *KIA0101*** | 0.92 | 0.85 to 0.96 | 2.22 E-16 |

**Disease Free Survival (DFS) and Overall Survival (OS).** Patients were followed up for 46.5 months in average (SD = 20.34, range 5.1-79.2 months). Supplementary Figure 7 shows that Her2+ patients have better OS, although significance levels were not reached (*p* value = 0.0732). *NUSAP1* and *KIAA0101* expression patterns were compared against tumor relapse for DFS and death due to BC for OS. Regarding DFS, the number of relapses was significantly higher in patients with overexpression of *NUSAP1* in the surgical sample (SS) (38%, Log-rank Mantel-Cox test, Chi square = 4.665, *p-*value = 0.0308) (Figure 4A). Likewise, higher levels of *NUSAP1* gene expression in the SS (84 to 50%) were also associated with decreased OS (Log-rank Mantel-Cox test, Chi square = 5.198, *p-*value = 0.0226) (Figure 4C). Similarly, *KIAA0101* gene overexpression negatively affected OS, with a reduction from 80 to 71% (Log-rank Mantel-Cox test, Chi square = 0.4024, *p*-value = 0.5258 (Figure 4B and D). Comparisons of gene expression patterns from BS failed to classify responders and no responders. OS results were replicated by analyzing public data on 1,402 patients from the Kaplan-Meier Plotter website ([http://kmplot.com](http://kmplot.com/)). Low levels of *NUSAP1* and *KIAA0101* were associated with greater OS (Log-rank HR = 1.82 CI = 1.46-2.26 *p-*value = 6.2e-08 and Log-rank HR = 1.47 CI=1.19-1.82 *p-*value=0.00039) (Figures 4E and 4F, respectively).

|  |  |
| --- | --- |
| **A** | **B** |
| **C** | **D** |
| **E** | **F** |

**Figure 4. Disease free survival (DFS) and overall survival (OS) against *NUSAP1* and *KIAA0101* gene expression profiles.** **A and B:** DFS curves considering *NUSAP1* and *KIAA0101* gene expression profiles after NCT (SS), respectively. Blue lines: under expression, red lines: overexpression. **C and D:** OS curves considering *NUSAP1* and *KIAA0101* gene expression profiles after NCT (SS), respectively. Blue lines: under expression, red lines: overexpression. **E and F:** OS curves considering *NUSAP1* and *KIAA0101* expression profiles from the Kaplan-Meier Plotter website (http://kmplot.com), respectively. Black lines: under expression, red lines: overexpression.

# Discussion

Omics technologies, in particular global gene expression analyses, have had a great impact on the understanding of BC biology, the classification of pathological subtypes, the design of prognostic algorithms, and most importantly, the discovery and implementation of new and more effective therapies to control this disease [34]. All these advances have positioned BC as one of the archetypal entities in Precision Medicine. Improvements in the selection of therapies based on the different molecular subtypes of BC, have yields higher DFS and prolonged OS. However, after a certain time of treatment, a high proportion of patients who do not fully respond to the assigned therapy has been observed. Therefore, just as it is important to choose the appropriate therapeutic regimen at the beginning of treatment, it is also a challenge to define the most appropriate therapies beyond the first line, especially in pre-treated patients [35]. Analysis of the molecular response to NCT may offer an opportunity to define prognoses and alternative therapies in patients with BC diagnosis [12].

In this work, we studied gene expression profiles obtained through unsupervised cluster analysis of BS and SS tissues in patients with PCR and non-PCR after NCT. After analyzing the different expression profiles, when combining sample types (BS or SS) and the response or not to NCT, the only analysis that classified pCR from non-pCR patients was the comparison of expression profiles in SS tissues. This analysis unveiled 30 overexpressed and 13 underexpressed genes in treated tumors (Supplementary Table 1). These genes are mainly involved in the regulation of nuclear division during mitosis, such as the catabolic process dependent on the anaphase promoter complex. Deregulated gene expression of some of these genes has been reported in BC. For example, *CCNB1, KIAA0101, NUSAP1, RRM2, UBE2C*, and *UBE2T* alterations are part of a gene signature identified in BC tumorigenic process in young women from the Middle East [36]. Moreover, *CCNB1*, *RRM2*, and *UBE2C* genes are included in the PAM50 signature as elements for the molecular classification of BC lesions [37]. However, there are no reports of a genetic signature to predict BC response after NCT.

Four genes were selected for qPCR validation analyses based on the results of the differential gene expression study and SS interactome analysis: two over-expressed (*MME* and *DST*) and two under-expressed (*NUSAP1* and *KIAA0101*). These validation studies corroborated the expression patterns observed in the microarray analyses. The gene expression levels of *NUSAP1* and *KIAA0101* were more discriminating in the qPCR analyzes and for this reason, they were chosen to perform the DSF and OS studies (Supplementary Figure 4).

DSF and OS studies based on expression levels in SS demonstrated that low *NUSAP1* expression was associated with better DFS. Similarly, underexpression of *NUSAP1* and *KIAA0101* were associated with increased OS (Figure 4 A-F).

The most important observation of this study is that the pCR achieved with the NCT regimens (cyclophosphamide/doxorubicin or cyclophosphamide/epirubicin) is associated with a significant decrease in the gene expression levels of *NUSAP1* and *KIAA0101*. This is consistent with the fact that the expression of these genes is involved in cell division processes and may modulate cancer progression, as will be discussed below. As reported, this clinical response presupposes better DFS and OS [11]. Furthermore, higher expression levels of these same genes in the tumor biopsy before treatment (BS) were associated to worse survival, indicating that these genes are potential predictors of survival in diagnostic biopsies.

The study suggests that the Her2+ subtype responds favorably to NCT (p value = 0.017) and that the luminal B subtype responds poorly, with no observed significant difference. Profile expression patterns of *NUSAP1* and *KIAA1010* genes in different molecular subtypes of BC after NCT showed that *NUSAP1* was overexpressed in luminal B tumors compared to luminal A, Her2+, and triple negative subtypes (Figure 3 and Supplementary Figure 5). Colak, et al. reported overexpression of *NUSAP1* and *KIAA0101* in ductal *in situ* carcinoma and invasive ductal carcinoma when compared to normal age-matched controls [36]. TILs have been reported to modulate the NCT response in breast cancer [38]. In this study, no correlations were observed between TIL counts and the expression of *NUSAP1* and *KIAA0101* in BS tissues from patients with PCR and without PCR.

The protein codified by *KIAA0101* (*PCLAF* or PCNA Clamp Associated Factor) is a PCNA binding protein which acts as a regulator of the number of centrosomes and is involved in DNA repair during DNA replication [15]. Overexpressed *KIAA0101* has been also associated with a decreased survival in BC patients [15], but not with the pathological response to NCT. *NUSAP1* gene expression levels showed a remarkable inverse correlation with survival (Fig 4A, 4C and 4D). This gene encodes for the nucleolar spindle associated protein that binds to chromatin and microtubules and is critical for the cytokinesis spindle assembly during mitosis [16]. *NUSAP1* overexpression has been reported in bladder, cervix, colon, glioblastoma, liver, lung, oral squamous cell carcinoma, prostrate, kidney, and breast cancers [39-43], and multiple studies have correlated its overexpression with poor prognosis [15, 40, 41, 44-48]. Zhang et al. demonstrated that down-regulation of *NUSAP1* suppressed proliferation, migration, and invasion of MCF7 cells by disturbing the regulation of *CDK1* and *DLGAP5* and increased susceptibility to epirubicin [41]. Our findings are similar to those of Qiu et al., who reported higher *NUSAP1* expression in tumor than in adjacent healthy tissue and an inverse correlation between *NUSAP1* expression and OS in BC patients. These findings were corroborated in a BALB/c-nu mouse model in which they determined the involvement of *NUSAP1* in tumor proliferation, migration, and invasion [18]. Finally, *NUSAP1* has been proposed as a carcinogenic element whose overexpression would help tumor progression in Triple Negative Breast Cancer (TNBC) cells, participating in the epithelial-mesenchymal transition and the Wnt/β-catenin pathways [17].

Our findings, together with those previously reported, indicate that these two genes may be prognostic genetic markers in BC, but at the same time, potential therapeutic targets. NUSAP1 and PCLAF (KIAA0101) proteins are involved in BCRA1-mediated DNA repair. NUSAP1 increases BRCA1 protein expression [49], while PCLAF regulates the number of centrosomes by interacting with BRCA1 [15]. Since the biological role of *NUSAP1* and *KIAA0101* genes involves cell cycle pathways, patients with elevated transcription levels of these genes may benefit from chemotherapy drugs interfering with BRCA1, such as platinum derivatives. *NUSAP1* overexpression could also be treated with galiellalactone, a fungal metabolite with antitumor and anti-inflammatory properties. Galiellalactone downregulates *NUSAP1* in DU145 by targeting the NF-κB and STAT3 pathways, inducing cell cycle arrest [50]. Another option to target *NUSAP1* overexpression is the antitumor compound isopicrinin, isolated from *Rhazya stricta*, an inhibitor of the microtubule assembly [51].

# Finally, decreased expression of *NUSAP1* seems to sensitize osteosarcoma cells to taxol, since NUSAP1 interacts with the SUMO E3 ligase complex, contributing to the adequate chromosomal segregation [52]. *NUSAP1* knockdown has been observed to potentiate paclitaxel-induced apoptosis in oral squamous cell carcinoma [53].

# In summary, our studies show significant results of downregulation of *NUSAP1* and *KIAA0101* and overexpression of *MME* and *DST* in SS, predicting pCR. BS data do not reach significance, but this correlation is also registered. On the contrary, the data suggest that overexpression of *NUSAP1* and *KIAA0101* are associated with decreased DFS. This information could be useful to implement a second line of treatment or more aggressive regimes.

# It is important to highlight some limitations of this study. The first is that the sample is small, but the NCT schemes and sampling were standardized for most study participants. The sample also has an overrepresentation of TNBC because the NCT program prioritizes patients with this tumor subtype.

# Conclusions

Downregulation of *NUSAP1* and *KIAA0101* in SS after NCT are associated with favorable therapeutic response and prognosis in BC. Overexpression of these two genes opens the possibilities for personalized therapies for patients who do not respond adequately to NCT.

**Data Availability.** The dataset generated and analyzed during the current study can be available from the corresponding author on reasonable request.

# Declaration of interests. None.

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# Authors' contributions.

Gerardo I. Magallanes-Garza: Conceptualization, Methodology, Investigation, Writing - Original Draft, and Writing - Review & Editing, and Visualization; Sandra K. Santuario-Facio: Conceptualization, Methodology, Investigation, Writing - Original Draft, and Writing - Review & Editing, and Visualization; Arlina F. Varela-Varela: Validation and Investigation; Servando Cardona-Huerta: Conceptualization, Investigation, Writing – Review & Editing; Pablo Ruiz-Flores: Validation and Investigation; Jorge Haro-Santa-Cruz: Validation and Investigation; Yadira X. Perez-Paramo: Validation and Investigation. Gabriela S. Gomez-Macias: Validation and Investigation; Daniel Davila-Gonzalez: Data Curation and Visualization; Javier Valero-Gomez: Validation and Investigation; Gissela Borreo-Soto: Validation and Investigation; Augusto Rojas-Martinez: Writing – Original Draft, and Writing - Review & Editing, and Visualization; Rocio Ortiz-Lopez: Conceptualization, Methodology, Writing - Original Draft, and Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

**Ethics approval and consent to participate.** The Institutional Review Board from the School of Medicine of Tecnologico de Monterrey (CONBIOETICA 19 CEI 011-2016-10-17) authorized the research protocol with the number: P000088-Altru-Pro-CI-CR002. In accordance with the Declaration of Helsinki, informed written consent was obtained from all patients participating in this study.

# Supplementary Material

Supplementary material shows three Heatmaps of comparisons with groups of samples (BS and SS). Supplementary materials also include qPCR validations plots and expression levels of *NUSAP1* according to the molecular subtype after the NCT. Finally, a supplementary table contains the differently expressed probes in pCR and non-pCR surgical samples.

**Supplementary Figure 1.** Heatmap of pCR patients (BS vs SS). Blue areas: low gene expression, red areas: high gene expression. Top row: Blue is for SS and red for BS tissues Each column represents a patient sample and each row, a single probe. Official symbols for gene or probe identification are displayed in the right side.

**Supplementary Figure 2.** Heatmap of non-pCR patients (BS vs SS). Blue areas: low gene expression and red areas, high gene expression. Top row: BS (blue) and SS (red). Each column represents a different sample, and each row is for a single probe. Official symbols for gene or probe identification are displayed in the right side.

**Supplementary Figure 3.** BS tissue comparisons between non-pCR (n = 31) and pCR (n = 8). Profile of 30 probes representing 21 genes (11 over and 10 sub expressed in pCR, Fold Change: +2, *p*-val: ≤ 0.01). Blue areas: low gene expression, red areas: high gene expression. Top row. Blue: non-pCR patients and red: pCR patients. Each column represents a different patient sample and each row, a single probe. Official symbols for gene or probe identification are displayed in the right margin.

**Supplementary Figure 4.** Validation by qPCR (*NUSAP1, KIAA0101, DST,* and *MME*). A and B represent expression of *NUSAP1* and *KIAA0101* analyzed by qPCR, respectively. C and D represent expression levels of *NUSAP1* and *KIAA0101,* respectively, according to the expression signal after normalization with RMA from the microarray data. E and F represent expression of *DST* and *MME* analyzed by qPCR, respectively. G and H represent expression levels of *DST* and *MME*, respectively, according to the expression signal after normalization with RMA from the microarray data. The red lines represent the non-pCR patient group and the blue lines, the pCR group. The unpaired *t* test with Welch's correction was used for comparisons.

**Supplementary Figure 5.** Expression levels of *NUSAP1* according to the molecular subtype after the NCT (SS). LA = Luminal A, LB = Luminal B, TN = Triple Negative. One-way ANOVA and Holm-Sidak's multiple comparisons test were used for comparisons.

**Supplementary Figure 6.** Microscopic evaluation of TILs (A) Low TILs 10X. Fibrous stroma is observed between the tumor cells, with little lymphoplasmacytic infiltrate in a percentage of 5%. (B) Moderate TILs 10X. Moderate lymphoplasmacytic infiltrate is seen in the tumoral stroma in a percentage of 30%. (C) High TILs 10X. Dense lymphoplasmacytic infiltrate observed in the stroma between the neoplastic cells in the upper left area in a percentage of 80%.

**Supplementary Figure 7.** Overall survival according to the molecular subtype after the NCT (SS). LA = Luminal A, LB = Luminal B, TN = Triple Negative. Log-rank (Mantel-Cox) test were used for comparisons.

# Supplementary Table 1. Probes differentially expressed in pCR and non-pCR surgical samples.

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