Potential biomarkers of ductal carcinoma in situ progression

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Abstract

Background Ductal carcinoma in situ is a non-obligate precursor of invasive breast carcinoma and presents a potential risk of over or undertreatment. Finding molecular biomarkers of disease progression could allow for more adequate patient's treatment. Our aim was to identify potentially biomarkers that can predict invasiveness risk.Methods In this epithelial cell-based study archival formalin-fixed paraffin-embedded blocks from six patients diagnosed with invasive lesions (pure invasive ductal carcinoma), six with in-situ lesions (pure ductal carcinoma in situ) , six with synchronous lesions (invasive ductal carcinoma with an in-situ component) and three non-neoplastic breast epithelium tissues were analyzed by gene expression profiling of 770 genes, using the nCounter® PanCancer Pathways panel of NanoString Technologies.Results The results showed that in comparison with non-neoplastic tissue the pure ductal carcinoma in situ was one with the most altered gene expression profile. Comparing pure ductal carcinoma in situ and in-situ component six differentially expressed genes were found, three of them (FGF2 , GAS1 and SFRP1), play a role in cell invasiveness. Importantly, these genes were also differentially expressed between invasive and noninvasive groups and were negatively regulated in later stages of carcinogenesis.Conclusions We propose these three genes (FGF2 , GAS1 and SFRP1) as potential biomarkers of ductal carcinoma in situ progression, suggesting that their downregulation may be involved in the transition of stationary to migrating invasive epithelial cells.

Background

Human carcinogenesis occurs with aberrant accumulation of gene changes, causing oncogene activation and tumor suppressor gene inactivation [1]. Breast cancer (BC) begins as premalignant lesions, progressing to the preinvasive stage of ductal carcinoma *in situ* (DCIS) and culminating as invasive ductal carcinoma (IDC) [2, 3]. DCIS represents 20–25% of newly diagnosed BC and up to 40% can progress to IDC [4]. Gene expression profiling-based studies have shown that distinct stages of progression are evolutionary products of same clonal origin, and that genes conferring invasive growth are disrupted during preinvasive stages [5–9]. Differences among these stages are not clear and there is no consensus as to how gene activation or inactivation alter the course of BC progression.

DCIS is a form of BC where epithelial cells restricted to the ducts exhibit an atypical phenotype [9]. Interestingly, some DCIS lesions progress to IDCs, while others remain unchanged [10]. Finding gene expression patterns that could predict invasive progression would allow us to personalize DCIS treatment to each patient's real needs.

In this study, gene expression profiling was performed in non-neoplastic breast epithelium, pure DCIS, mixed lesions (DCIS-IDC) (IDC with an *in-situ* component) and pure IDCs, aiming to identify molecular predictors of invasive disease risk.

Materials And Methods

Study population

Formalin-fixed paraffin-embedded (FFPE) breast blocks of 3 healthy women were selected as non-neoplastic breast epithelium (control). Specimens with pathological lesions (IDC, DCIS, DCIS-IDC) were obtained from the Department of Pathology of Barretos Cancer Hospital-Sao Paulo, Brazil. Archival FFPE blocks from six patients diagnosed with IDC, six with DCIS and six with IDC with *in-situ* component were selected (Table 1). Cases of IDC and DCIS-IDC were chosen considering the molecular subtype, according St. Gallen consensus [11]. Estrogen receptor (ER), progesterone receptor (PR) and Ki67 expression were determined by immunohistochemical staining, and Her2 expression was determined by immunohistochemistry or fluorescence *in situ* hybridization. Clinical stage was defined by TNM staging [12]. Histological grade was determined as Tavassoli and Devilee [13]. Myriad's hereditary cancer tests were done by Myriad Genetic Laboratories, Inc. (Salt Lake City, Utah, USA) through observations of deleterious mutations, as published by Frank et al. [14]. Selected patients had a mean age of 55 years and were not under risk of hereditary BC, they did not present metastasis and did not receive preoperative systemic treatment.

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study complies with the laws of the country and was This study was approved by Human Research Ethics Committee of Integrated Center of Health Attention-CIAS/UNIMED VITORIA by the protocol number 2.337.052 and by the Human Research Ethics Committee of Barretos Cancer Hospital by the protocol number 1505/2017. Ethics committee regularly authorized the dispensation of consent form.

Include Table 1 Here

RNA extraction

FFPE block 10-µm thick sections were cut onto glass slides and one of them was stained with hematoxylin and eosin (HE). Manual microdissection of epithelial cells was performed using an average of 10 sections, in reference to a HE-stained specimen, isolating the area with, at least, 70% of tumor cells. The DCIS-IDC group undertook microdissection of both IDC and DCIS tissues.

Sample naming is as follows: non-neoplastic breast epithelium - control; pure IDC - IDC_{pure};pure DCIS - $DCIS_{pure}$;IDC of DCIS-IDC group - IDC_{comp} andDCIS of DCIS-IDC group - $DCIS_{comp}$.

RNA was isolated by *RecoverAll™ Total Nucleic Acid Isolation Kit* (Ambion/Life Sciencies, Carlsbad, California, USA), in accordance with manufacturer's recommendations. RNAs were quantified using NanoDrop (ThermoFisher, Waltham, Massachusetts, USA) and Qubit RNA HS Assay kit (ThermoFisher).

Gene expression analysis

Multiplex gene expression analyses were performed at the Molecular Oncology Research Center-Barretos Cancer Hospital by *nCounter® PanCancer Pathways panel* (NanoString Technologies™, Seattle, Washington, USA), which allows the evaluation of 770 genes (730 cancer-related human genes, being 124 driver genes and 606 genes from 13 cancer-associated canonical pathways, and 40 as internal reference loci). An average of 100 ng of RNA was used for hybridization, according to manufacturer's protocol. The system analyses for gene expression digital quantification used was the *nCounter® SPRINT Profiler* (NanoString Technologies™).

Data analysis

Raw counts expression was analyzed using the *nSolver™ Analysis Software* (NanoString Technologies™).

Two-by-two comparisons among control, IDC_{pure}, $DCIS_{pure}$, $DCIS_{comp}$ and IDC_{comp} tissues were performed and differentially expressed genes were selected using expression levels p value ≤ 0.01. Comparisons between the noninvasive group (control and $DCIS_{pure}$) and the invasive group (IDC_{pure}, $DCIS_{comp}$ and IDC_{comp}) were performed. A heatmap comparing the 3 tissues (control, $DCIS_{pure}$ and IDC_{pure}) was made in *nSolver™,* and a Venn diagram was constructed to select genes of interest.

Gene enrichment analyzes were performed by *FunRich Functional Enrichment Analysis Tool* [15], using the Gene Ontology database. Interaction network analyses were also performed at *FunRich* using FunRich database. The UALCAN [16] was used to evaluate gene expression in BC stages, using only The Cancer Genome Atlas (TCGA) database.

Results

Putative genes involved in DCIS progression

To make a stepwise analysis, we used non-neoplastic epithelial tissue as control (no progression), $DCIS_{pure}$ as stage *in situ* without invasive capacity, $DCIS_{comp}$ as stage *in situ* with invasive capacity and IDC_{pure} lesions as complete progression. We aimed to detect genes involved in the progression of *in situ* lesions to invasive ones.

Eleven comparisons were made two-by-two to obtain the differentially expressed genes (p value ≤ 0.01) (see Additional file 1 - TablesS1-S11). In the comparisons between control and tumor tissues, the greatest differential expression was observed between $DCIS_{pure}$ and control (123 differentially expressed genes - 72 downregulated), and the lowest, between control and IDC_{pure} (66 differentially expressed genes - 46 downregulated). Interestingly, comparing $DCIS_{comp}$ (104 differentially expressed genes) or IDC_{comp} (102 differentially expressed genes) with normal tissue showed more differentially expressed genes than comparing control with IDC_{pure}. The two tissues ($DCIS_{comp}$ and IDC_{comp}) of patients with DCIS-IDC did not present differentially expressed genes between them. IDC_{comp} (probably the progression of its *in-situ* component) was seen to be more distant to the control than IDC_{pure}.

Figure 1 shows the comparison of gene expression between control, $DCIS_{pure}$ and IDC_{pure}. Statistically, the invasive tissue exhibited a more similar profile to control than to the *in-situ* lesions and a comparison between $DCIS_{pure}$ and IDC_{pure} showed only two differentially expressed genes downregulated in the IDC (*CHAD* and *ITGA2)*..

$DCIS_{comp}$ gene expression retains more similarities with IDC_{pure} (2 differentially expressed genes), than with $DCIS_{pure}$ (6 differentially expressed genes) and has a lower similarity with the control (104 differentially expressed genes) (Figure 1 and Additional file 1—Tables S5, S6 and S8), which suggests progressive molecular alterations from $DCIS_{pure}$ to the IDC passing through $DCIS_{comp}$.

The 6 differentially expressed genes found between $DCIS_{pure}$ and $DCIS_{comp}$ (*FGF2, GAS1, IBSP, LAMC3, MAP3K8* and *SFRP1)* may influence progression of *in situ* to invasive lesions. Among them, only *IBSP* is downregulated in noninvasive lesions (Table 2). To verify which genes would have the greatest potential in the acquisition of invasive capacity, a Venn diagram was constructed (Figure 2).

Genes potentially involved in invasiveness should appear in differentially expressed genes between $DCIS_{comp}$ and either $DCIS_{pure}$ or control tissues, whereas, differentially expressed genes between control and $DCIS_{pure}$ may be involved in tumor formation. So, 3 differentially expressed genes (*FGF2, GAS1* and *SFRP1)* that are in common in the comparisons of $DCIS_{pure}$ vs $DCIS_{comp}$ and control vs $DCIS_{comp}$ and not present in the comparison control vs $DCIS_{pure}$, possibly acting in the acquisition of the invasive capacity of $DCIS_{pure}$. In the Venn diagram, these 3 genes are marked with an asterisk.

The comparison between invasive and noninvasive groups shows 53 differentially expressed genes, being 8 upregulated and 45 downregulated in the invasive group, once again supporting the concept that acquisition of invasive capacity is more dependent on loss of gene expression rather than gain. Four of the downregulated genes in invasive group were also differentially expressed between $DCIS_{pure}$ and $DCIS_{comp}$ and the genes most probably involved in the DCIS progression are among them (*FGF2, GAS1* and *SFRP1)* (Table 2).

Include Table 2 Here

Gene functional analysis

Enrichment analysis showed that the main biological processes altered between control and $DCIS_{pure}$ (adjusted p value ≤ 0.01) are related to gene expression regulation, cell proliferation and cell cycle arrest (Figure 3a). For differentially expressed genes between $DCIS_{pure}$ and IDC_{pure}, the most altered processes involve positive regulation of collagen binding, positive regulation of cell projection and substrate-dependent cell migration (p value ≤ 0.01). Comparing invasive and noninvasive groups, the largest changes were seen in cell proliferation and transcription regulation (adjusted p value ≤ 0.01) (Figure 3b). Comparisons between $DCIS_{pure}$ and $DCIS_{comp}$ revealed loci involved in extracellular matrix organization, regulation of angiogenesis, cellular response to growth factor and somatic stem cell maintenance (p value ≤ 0.01) (Table 3). To verify differences between genes potentially involved in DCIS progression (*FGF2, GAS1* and *SFRP1)* and other 3 differentially expressed genes of $DCIS_{pure}$ vs $DCIS_{comp}$ (*LAMC3, MAP3K8* and *IBSP),* enrichment was done separately. In the first analysis, the most altered processes were regulation of angiogenesis, somatic stem cell maintenance, growth factor dependent regulation of satellite cell proliferation and positive regulation of cell fate (p value ≤ 0.01) (Table 3). For the latter 3 genes, there were more changes in extracellular matrix organization, differentiation cell morphogenesis and cell adhesion (p value ≤ 0.01) (Table 3).

Protein-protein interaction (PPI) networks of the 6 differentially expressed genes of $DCIS_{pure}$ vs $DCIS_{comp}$ are shown in Figure 4. In Figure 4a, all interactions are shown and in Figure 4b only the 107 statistically significant interactions were left in the PPI snapshot, showing 3 out of 6 genes (p value ≤ 0.01).

Evaluation of gene expression in normal tissue and in BC stages was made for 3 genes potentially involved in DCIS progression (*FGF2, GAS1* and *SFRP1)* using the TCGA database (Figure 5). Their expressions decrease with the progression of tumor stages, which corroborates our results, when comparing $DCIS_{pure}$ vs $DCIS_{comp}$.

Include Table 3 here

Discussion

Our results show that $DCIS_{pure}$ has the most distinct gene expression profile from normal breast epithelium. We observed two differentially expressed genes between $DCIS_{pure}$ and IDC_{pure} and no differentially expressed genes between $DCIS_{comp}$ and IDC_{comp}. Six differentially expressed genes were found in $DCIS_{pure}$ vs $DCIS_{comp}$, of which 3 were also differentially expressed between control and $DCIS_{comp}$, but not between control and $DCIS_{pure}$. In addition, the same 3 genes (*FGF2, GAS1* and *SFRP1)* showed distinct gene expression profile between noninvasive and invasive groups. Thus, suggesting their involvement in DCIS progression, possibly by acquisition of invasive capacity after downregulation.

Other studies also have shown that *in-situ* and invasive stages of breast ductal carcinoma are similar in gene expression, suggesting that invasiveness necessary changes are already present in the *in-situ* lesions that will later progress to invasive ones. Therefore, DCIS should acquire enabling gene expression changes much before morphological alterations and invasiveness [5, 17, 18]. In this study, we found only two differentially expressed genes between IDC_{pure} and $DCIS_{pure}$.

Interestingly, the *in-situ* stage ($DCIS_{pure}$) has more molecular differences with control than the invasive stage (IDC_{pure}). But, considering that IDC is the most advanced stage in progression and morphology, we expected greater molecular changes in reference to non-neoplastic tissue.

Our result is probably due to early acquisition of tumor enabling features, which are later followed by minor ones [5]. We believe that IDC loses some of the initial differentially expressed genes, therefore becoming more similar to normal tissue expression wise.

$DCIS_{comp}$ and IDC_{comp} of patients with DCIS-IDC do not have differentially expressed genes between them and are more expression wise distant from control than IDC_{pure}. Initial gene expression changes may remain necessary in DCIS-IDC, since acquisition of invasive potential has not yet been

completed in all cells. In addition, as suggested by Hu et al. [19] and Muggerud et al. [17] many processes involved in DCIS progression may be expression changes in the tumor microenvironment, and not necessarily in tumor cells [20].

We propose that the expression differences between $DCIS_{pure}$ and $DCIS_{comp}$ would identify genes involved in DCIS progression. The 3 differentially expressed genes more likely involved in DCIS progression were *FGF2, GAS1* and *SFPR1,* all downregulated in $DCIS_{comp}$. This fact suggests that progression from $DCIS_{pure}$ to $DCIS_{comp}$ may use silencing mechanisms more often than activating ones.

Our results were able to detect expression changes during the progression of $DCIS_{pure}$ to $DCIS_{comp}$ and, finally, to IDC. Expression differences were larger when comparing $DCIS_{comp}$ vs control (104 differentially expressed genes), decreasing with $DCIS_{comp}$ vs $DCIS_{pure}$ (6 differentially expressed genes) and $DCIS_{comp}$ vs IDC_{pure} (2 differentially expressed genes).

When comparing differentially expressed genes between control and $DCIS_{pure}$, 31% are driver genes, whereas none of the genes that may be involved in DCIS progression or differentially expressed genes between $DCIS_{pure}$ and IDC_{pure} are driver genes, suggesting that major alterations occur at the beginning of carcinogenesis and not at the end.

To confirm gene involvement in invasion, we created two groups: noninvasive group composed of control tissue and $DCIS_{pure}$ and invasive group composed of DCIS-IDC and IDC_{pure}. In this analysis *FGF2, GAS1* and *SFPR1* were downregulated in the invasive group.

Epigenetic alterations may contribute to BC progression by transcriptionally silencing specific tumor suppressor genes [21, 22], which could explain the loss of expression that we observed. DNA hypermethylation of tumor suppressor genes is commonly observed during tumor progression [23, 24] and may be associated with DCIS progression [10]. Studies have shown that a subset of genes are methylated during progression, reinforcing the role of the expression downregulation during BC initiation and/or progression [25–29]. In Conway et al. [30] study, *FGF2* was among the relatively hypermethylated genes in hormone receptor positive, luminal A, or p53 wild-type BCs and Bediaga et al. [31] showed that FGF2 displayed higher hypermethylation levels in the luminal B BC subtype. Lo et al. [32] founded that *SFRP1* gene was frequently hypermethylated in ductal carcinomas. Veeck et al. [33] found a tight correlation between promoter hypermethylation and *SFRP1* downregulation in primary BC tissue. Studies about *GAS1* hypermethylation are seen in lung, pancreatic and prostate cancers [34].

FGF2 gene plays an important role in angiogenesis [35]. It signaling pathway is influenced by crosstalk with integrins [36]. Lower levels of *FGF2* mRNA have been detected in BC, when compared to normal tissues [37]. Low expression also occurs in large tumors, late disease stages, and worse overall and disease-free survival [38, 39]. Pre-clinical studies showed that *FGF2* overexpression can inhibits tumor growth [40, 41]. In addition, many *in vitro* assays have demonstrated a potent inhibitory effect of *FGF2* on BC cells, possibly involving the mitogen activated protein kinase (MAPK) cascade and cell cycle G1/S transition [42–44]. Researchers have suggested that intermediate levels of adhesion between *FGF2* and integrins are needed for optimal cell migration, which could explain the reduction in *FGF2* expression during BC progression [45–47]. Enrichment analysis have shown statistically significant interactions between *FGF2* and MAPK pathway genes and other components of the FGF family. UALCAN analysis has shown an upregulation of *FGF2* in normal tissues, in comparison to primary BC and that *FGF2* downregulation is associated with tumor progression. *FGF2* involvement in BC progression is predominantly related to cell growth regulation [44].

GAS1 gene plays a role in growth suppression inhibiting DNA synthesis [48] and reducing cancer cell line proliferation [49]. In BC cells, Jimenez et al. [50] proposed that this gene can inhibit growth by decreasing angiogenesis. *GAS1* is more expressed in normal tissues, in comparison to primary breast tumor tissue, as seen in TCGA data. Hedgehog (Hh) signaling has been suggested as a critical determinant of tumor progression [51–54]. A progressive increase of Hh expression and Hh pathway activation has been observed from control, DCIS, DCIS with microinvasion and to IDC [55, 56]. GAS1 protein binds Sonic hedgehog (SHH), one of three Hh proteins, and may inhibit Hh signaling [57, 58]. The interaction of *GAS1* with *SHH* was seen but was not statistically significant. The reduction of *GAS1* expression during more advanced BC stages may occur in order to avoid Hh pathway inhibition.

SFRP1 gene is a negative regulator of the Wnt pathway, which is aberrantly activated in BC [33, 59, 60]. SFRPs are cell membrane receptors able to bind Wnt proteins in the extracellular compartment, inhibiting ligand-receptor interaction and signal transduction [61]. Statistically significant interactions of *SFRP1* with Wnt pathway genes were seen and enrichment analysis showed a negative regulation of canonical Wnt receptor signaling pathway. *SFRP1* mRNA is strongly downregulated in BC, having a putative tumor suppressor role. Results of Gauger et al. [62] suggests that loss of *SFRP1* expression allows non-malignant cells to acquire BC tumor features. We saw *SFRP1* downregulation in primary BC in comparison to normal tissue. A lower *SFRP1* expression is seen in invasive lesions.

Functional analyses of *FGF2, GAS1* and *SFRP1* suggests a role in DCIS progression, being negative regulators of cell cycle G1/S transition, Hh signaling and the Wnt pathway, respectively. We propose that downregulation favors DCIS progression. Unfortunately, our samples could not be divided into high and low-grade DCIS, nor could we study samples according to cancer molecular subtypes. Studying these groups separately may reveal important events in the DCIS progression.

Conclusions

Understanding BC progression will enable the design of effective strategies for diagnosis and treatment. Progression biomarkers should be able to predict DCIS cases destined to become invasive tumors, therefore allowing for proper monitoring and avoiding overtreatment. Here, we identified 3 progression-specific candidate genes namely *FGF2, GAS1, SFRP1,* downregulated in tissues with invasive capacity. The progression from DCIS to invasive BC is a complex process, being possible that DCIS of distinct molecular phenotypes progress to invasive BC through the acquisition of distinct genetic or epigenetic hits.

Abbreviations

BC: Breast cancer

DCIS: Ductal carcinoma in situ

ER: Estrogen receptor

FFPE: Formalin-fixed paraffin-embedded

HE: Hematoxylin and eosin

Hh: Hedgehog

IDC: Invasive ductal carcinoma

PPI: Protein-protein interaction

PR: Progesterone receptor

QC: Quality Control

SHH: Sonic hedgehog

TCGA: The cancer genome atlas

Declarations

Ethics approval and consent to participate

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study complies with the laws of the country and was approved by Human Research Ethics Committee of Integrated Center of Health Attention-CIAS/UNIMED VITORIA by the protocol number 2.337.052 and by the Human Research Ethics Committee of Barretos Cancer Hospital by the protocol number 1505/2017.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Competing interests

The authors declare that they no conflict of interest.

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Authors' contributions

RSD has made design of the work; acquisition of patient's data; analysis; interpretation of data and drafted the work. ES has made design of the work; acquisition of patient's data; molecular analysis; interpretation of data and revised the work. ACL has made acquisition of patient's data; molecular analysis and revised the work. RACV has made design of the work; acquisition of patient's data making data available from your services as an oncologist; histological analysis; interpretation of data and revised the work. MMCM has made acquisition of patient's data; histological analysis and revised the work. IVVS has made acquisition of patient's data; histological analysis and revised the work. JZP has made acquisition of patient's data making data available from your services as an oncologist and revised the work. LFR have made acquisition of patient's data and histological analysis. NJP have made acquisition of patient's data and revised the work. LPA have made design of the work and acquisition of patient's data. RSR have made interpretation of data and revised the work. EVWS revised the work. LNRA have made acquisition of patient's data. FMG have made acquisition of patient's data. JAS have made acquisition of patient's data. DPV have made acquisition of patient's data. RMR have made acquisition of patient's data; molecular analysis; made its infrastructure available for molecular analysis; interpretation of data and revised the work. IDL have made design of the work; analysis; interpretation of data and revised the work.

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Tables

'able 1. Patients characteristics.

Case ID	Age (years)	Invasive type	Molecular subtype	Histological type	Histological grade	Invasive nuclear grade	DCIS Nuclear grade	DCIS type	ER[f] in DCIS	PR[g] in DCIS	Size (mm)	T[h] stage	N[i] stage	Pathological clinical stage	Myriad test
G1P1	60	IDC[a]	Luminal B Her negativo	IDC	3	3	N	N	N	N	31	T2	N0	IIa	N>50
G1P2	58	IDC	TN[d]	IDC	3	3	N	N	N	N	10	T1b	N0	I	N>50
G1P3	66	IDC	HER2	IDC	3	3	N	N	N	N	33	T2	N1	IIb	N>50
G1P4	62	IDC	Luminal A	IDC	2	2	N	N	N	N	20	T1c	N0	I	N>50
G1P5	43	IDC	Luminal B Her positivo	IDC	1	1	N	N	N	N	40	T2	N0	IIa	N<50
G1P6	42	IDC	Luminal B Her negativo	IDC	2	2	N	N	N	N	20	T1c	N0	I	N<50
G2P1	73	N[b]	N	DCIS[e]	N	N	3	S, C, M, Co	++++	+++	105	Tis	N0	0	N>50
G2P2	47	N	N	DCIS	N	N	3	S, C, M	-	-	50	Tis	N0	0	N<50
G2P3	40	N	N	DCIS	N	N	3	S, Co, C, M	+++	+++	18	Tis	N0	0	N<50
G2P4	50	N	N	DCIS	N	N	3	S, Co	++++	++	30	Tis	N0	0	N>50
G2P5	53	N	N	DCIS	N	N	3	C, M, Co	+	+	60	Tis	N0	0	N>50
G2P6	59	N	N	DCIS	N	N	3	S, M, Co	+	-	20	Tis	N0	0	N>50
G3P1	57	*IDC[c]	TN	IDC-DCIS	3	3	3	S, C, Co	-	-	30	T1c	N0	I	N>50
G3P2	63	*IDC	Her2	IDC-DCIS	3	3	3	S, A, M, Co	-	-	100	T2	N1	IIb	N>50
G3P3	58	*IDC	Luminal B Her negativo	IDC-DCIS	3	3	3	S, C, Co	++++	++	65	T1c	N0	I	N>50
G3P4	63	*IDC	Her2	IDC-DCIS	2	2	3	S, C	-	-	36	T1a	N1	IIa	N>50
G3P5	37	*IDC	Luminal B Her negativo	IDC-DCIS	2	2	2	S, C	++++	+	29	T1a	N1	IIa	N>50
G3P6	50	*IDC	Luminal	IDC-DCIS	3	3	N	S, C,	++++	+	N	T1a	N0	I	N>50

	B Her	Co
	positivo	

S – solid; M – micropapillary; C – cribriform; A – adherent; Co – comedocarcinoma.
ge as defined by TNM staging [12] metastases have always been absent, so the clinical stage is only extrapolated from T and N.
reditary cancer tests were made according Frank et al. [14] - N<50 (family history of hereditary cancer absent and breast cancer diagnosed before 50 years of
50 (family history of hereditary cancer absent and breast cancer diagnosed after 50 years of age).
l grade was obtained according Tavassoli and Devilee [13]. For ER and PR: stained slides were examined as follows: “negative” (-) - absence of brown precipitate
positive samples were labeled as (+) [if there were a few (<10%) scattered cells with precipitate]; (++) [for large areas (10-50%) of positivity] and (+++)
50% to 100% positivity].
ve ductal carcinoma.
lable or absent.
ith *in-situ* component.
- triple negative.
IS - ductal carcinoma *in situ.*
- estrogen receptor.
- progesterone receptor.
tumor.
regional lymph node.

Table 2. DEGs between $DCIS_{pure}$ and $DCIS_{comp}$ and between invasive and noninvasive groups.

	DEGs	Gene	t statistic	*p* value	FC[a]
$DCIS_{pure}$ vs $DCIS_{comp}$	Downregulated in $DCIS_{pure}$	*IBSP*	-3.86	8,00E-03	-3.4
	Upregulated in $DCIS_{pure}$	***FGF2***	**4.16**	**4,00E-03**	**1.5**
		GAS1	**3.6**	**7,00E-03**	**2.67**
		LAMC3	4.11	2,00E-03	2.06
		MAP3K8	3.49	8,00E-03	1.91
		SFRP1	**4.75**	**1,00E-03**	**2,61**
Noninvasive vs invasive group	Downregulated in noninvasive group	*ARID2*	-3.42	4,00E-03	-1.52
		BCL2L1	-3.17	6,00E-03	-1.7
		BMP8A	-4.11	4,00E-04	-2.28
		CCNB1	-3.12	5,00E-03	-1.95
		CDC25C	-3.16	4,00E-03	-1.91
		OSM	-3.11	5,00E-03	-2.23
		UTY	-3.1	5,00E-03	-1.97
		WHSC1	-3.26	6,00E-03	-1.46
	Upregulated in noninvasive group	*AXIN2*	3	8,00E-03	2.14
		CNTFR	2.95	9,00E-03	2.4
		COL6A6	3.26	7,00E-03	3.75
		DKK1	2.79	1,00E-02	2.02
		DTX1	2.82	9,00E-03	1.76
		EFNA5	3.21	4,00E-03	1.71
		FGF10	3.26	3,00E-03	3.03
		FGF2	**3.73**	**2,00E-03**	**2.91**
		FGF7	3.71	1,00E-03	2.72
		FOS	2.8	1,00E-02	2.6
		FZD7	3.3	4,00E-03	2.09
		GAS1	**3.67**	**1,00E-03**	**2.5**
		GLI3	3.11	5,00E-03	1.73
		GRIA3	3.7	2,00E-03	2.88
		IGF1	4.25	3,00E-04	2.72
		IRS1	3.28	6,00E-03	2.05
		ITGA9	3.55	2,00E-03	2.04
		ITGB8	3.47	2,00E-03	2.83
		JAK1	4.45	1,00E-04	1.32
		JUN	3.19	7,00E-03	2.51
		KLF4	3.78	1,00E-03	2.4
		LAMB3	2.9	1,00E-02	2.47
		LAMC2	2.9	1,00E-02	2.14
		LEPR	4.43	3,00E-04	3.08
		LIFR	3	7,00E-03	2.16
		MAP3K8	2.98	8,00E-03	1.56
		MET	4.09	4,00E-04	1.99
		NGFR	3.48	2,00E-03	2.49
		NTRK2	3.32	4,00E-03	4.17
		PDGFRA	3.85	1,00E-03	2.01
		PLD1	4.14	3,00E-04	1.81
		PRKCA	3.7	1,00E-03	1.89
		PROM1	2.89	9,00E-03	3.68
		RELN	3.05	9,00E-03	3.25
		SFRP1	**4.01**	**5,00E-04**	**5.79**
		SOX17	3.84	8,00E-04	2.41
		SOX9	3.01	6,00E-03	2.63
		SRPY1	3.73	1,00E-03	2.15
		SRPY2	3.47	3,00E-03	2.16
		TCF7L1	3.23	4,00E-03	2.15
		TGFBR2	3.9	7,00E-04	2.24
		THEM4	2.86	9,00E-03	1.6
		TNN	3.47	2,00E-03	2.64
		TSC1	2.83	9,00E-03	1.35
		TSPAN7	3.61	1,00E-03	2.4

In bold are the genes potentially involved in DCIS progression.
FC - fold change.
DEGs -differentially expressed genes.
$DCIS_{comp}$ - DCIS as component.

$DCIS_{pure}$ - pure DCIS.

Table 3. Top 10 biological process of DEGs between $DCIS_{comp}$ and $DCIS_{pure}$ and comparisons with control tissue.

$DCIS_{pure}$ vs $DCIS_{comp}$ DEGs		
Biological Process	**Genes in database**	***p*-value (Ht)[a]**
Extracellular matrix organization	*FGF2, LAMC3, IBSP*	2,59E-05
Regulation of angiogenesis	*FGF2, SFRP1*	4,37E-05
Cellular response to growth factor stimulus	*IBSP, SFRP*	8,87E-05
Somatic stem cell maintenance	*FGF2, SFRP1*	1,88E-04
Growth factor dependent regulation of satellite cell proliferation	*FGF2*	3,36E-04
Positive regulation of cell fate specification	*FGF2*	3,36E-04
Stromal-epithelial cell signaling involved in prostate gland development	*SFRP1*	3,36E-04
Convergent extension involved in somitogenesis	*SFRP1*	3,36E-04
Negative regulation of canonical Wnt receptor signaling pathway	*SFRP1*	3,36E-04
Osteoblast differentiation	*IBSP, SFRP1*	4,69E-04
$DCIS_{pure}$ vs $DCIS_{comp}$ DEGs, excluding control vs $DCIS_{comp}$ and control vs $DCIS_{pure}$ DEGs		
Biological Process	**Genes in database**	***p*-value (Ht)**
Extracellular matrix organization	*LAMC3, IBSP*	3,62E-04
Cell morphogenesis involved in differentiation	*LAMC3*	6,73E-04
Cell adhesion	*LAMC3, IBSP*	2,00E-03
Astrocyte development	*LAMC3*	2,86E-03
Stress-activated MAPK cascade	*MAP3K8*	4,54E-03
Bone mineralization	*IBSP*	6,04E-03
Positive regulation of cell adhesion	*IBSP*	7,22E-03
Cellular response to growth factor stimulus	*IBSP*	7,38E-03
Activation of MAPKK activity	*MAP3K8*	8,22E-03
Stress-activated protein kinase signaling cascade	*MAP3K8*	8,56E-03
$DCIS_{pure}$ vs $DCIS_{comp}$ DEGs, including control vs $DCIS_{comp}$ and excluding control vs $DCIS_{pure}$ DEGs		
Biological Process	**Genes in database**	***p*-value (Ht)**
Regulation of angiogenesis	*FGF2, SFRP1*	8,76E-06
Somatic stem cell maintenance	*FGF2, SFRP1*	3,79E-05
Growth factor dependent regulation of satellite cell proliferation	*FGF2*	1,68E-04
Positive regulation of cell fate specification	*FGF2*	1,68E-04
Stromal-epithelial cell signaling involved in prostate gland development	*SFRP1*	1,68E-04
Convergent extension involved in somitogenesis	*SFRP1*	1,68E-04
Negative regulation of canonical Wnt receptor signaling pathway involved in controlling type B pancreatic cell proliferation	*SFRP1*	1,68E-04
Negative regulation of bone remodeling	*SFRP1*	3,36E-04
Neural crest cell fate commitment	*SFRP1*	3,36E-04
GO:1904956	*SFRP1*	3,36E-04

- hypergeometric test.

Gs - differentially expressed genes.

IS_{comp} - DCIS as component.

IS_{pure} - pure DCIS.

Figures

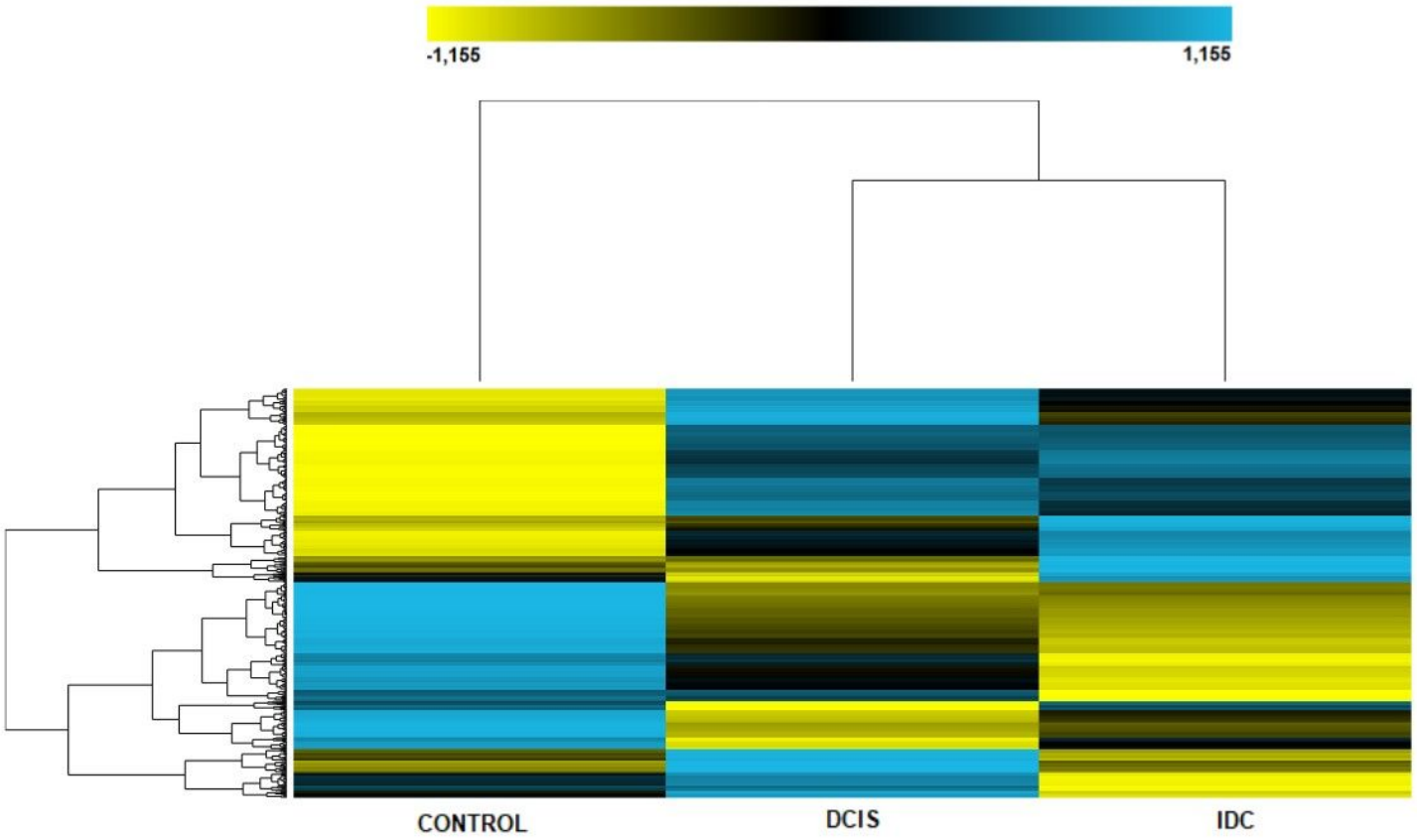

Figure 1

Hierarchical clustering of 730 genes and its gene expressions. Genes of nCounter® PanCancer Pathways panel. Gene expressions are in non-neoplastic (control), ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) tissues. Agglomerative clustering was made in nSolver™ Analysis Software. Individual genes are arranged in rows and samples' groups in columns. Color scale is shown above the figure.

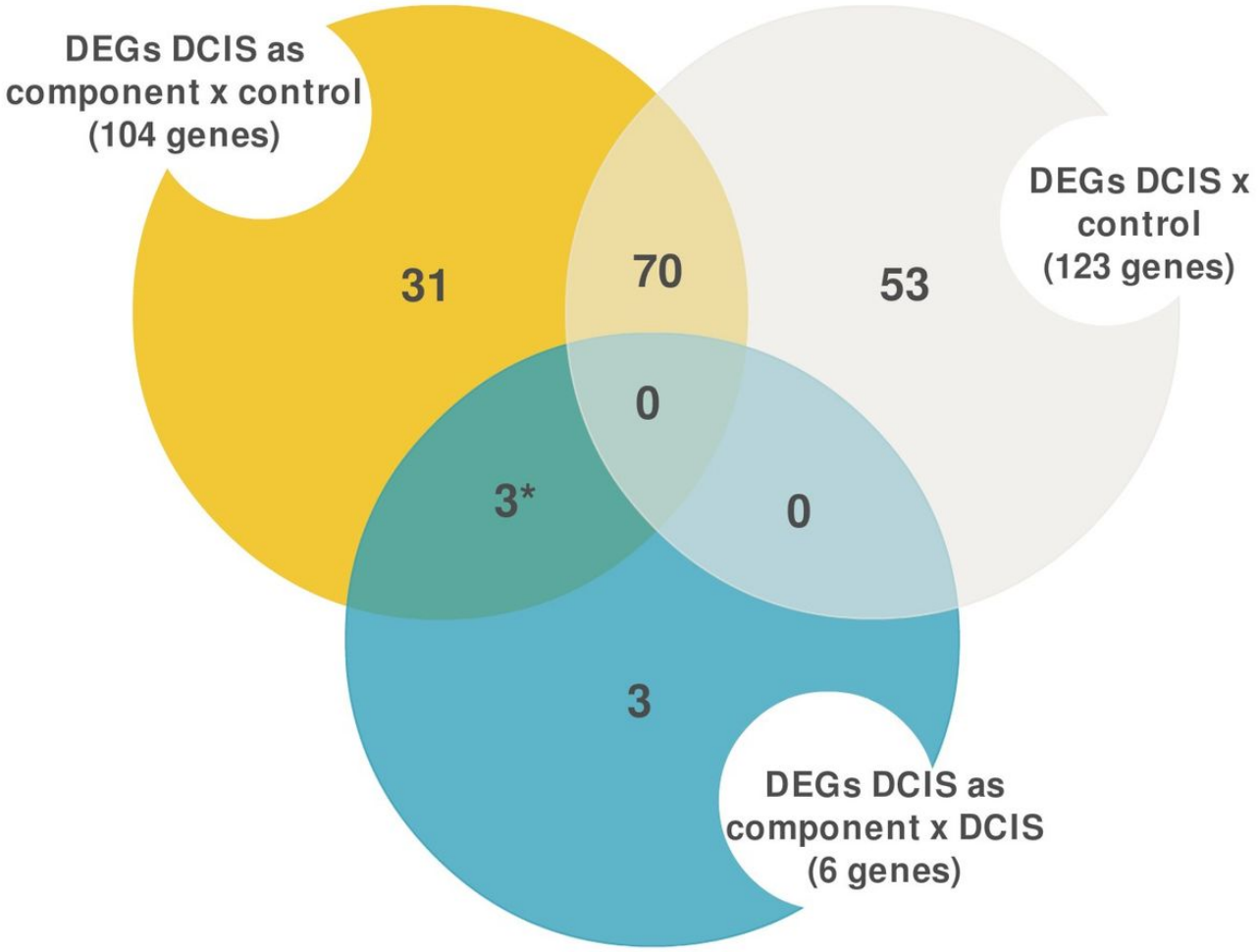

Figure 2

Putative genes involved in ductal carcinoma in situ (DCIS) progression. Venn diagram depicting the common and distinct genes in each comparison. Genes most likely involved in the acquisition of pure DCIS invasive capacity are marked with an asterisk. DEGs - Differentially expressed genes.

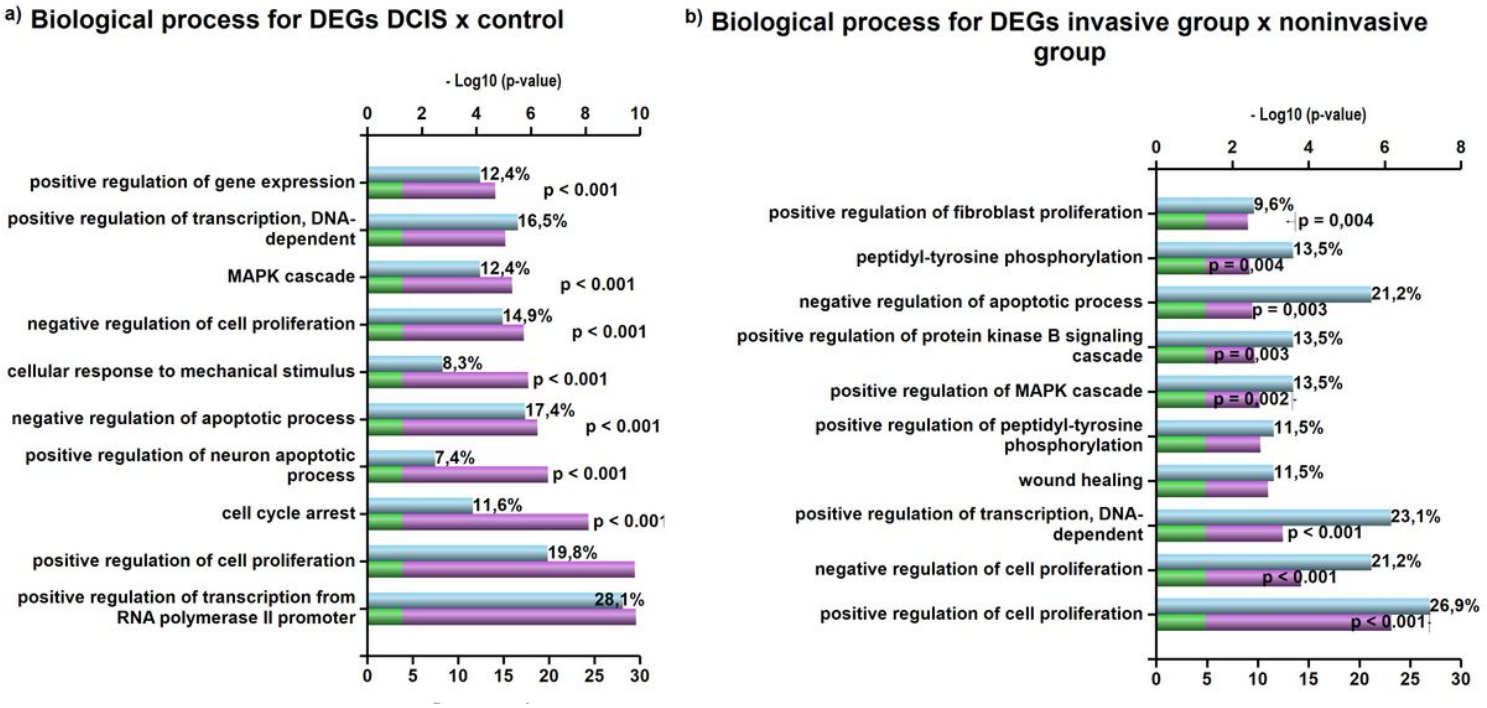

Figure 3

Top 10 Biological Processes for differentially expressed genes. Comparisons are between: a) Control vs DCIS and b) noninvasive vs invasive groups. Gene enrichment analyzes were performed by FunRich Functional Enrichment Analysis Tool, using the Gene Ontology database.

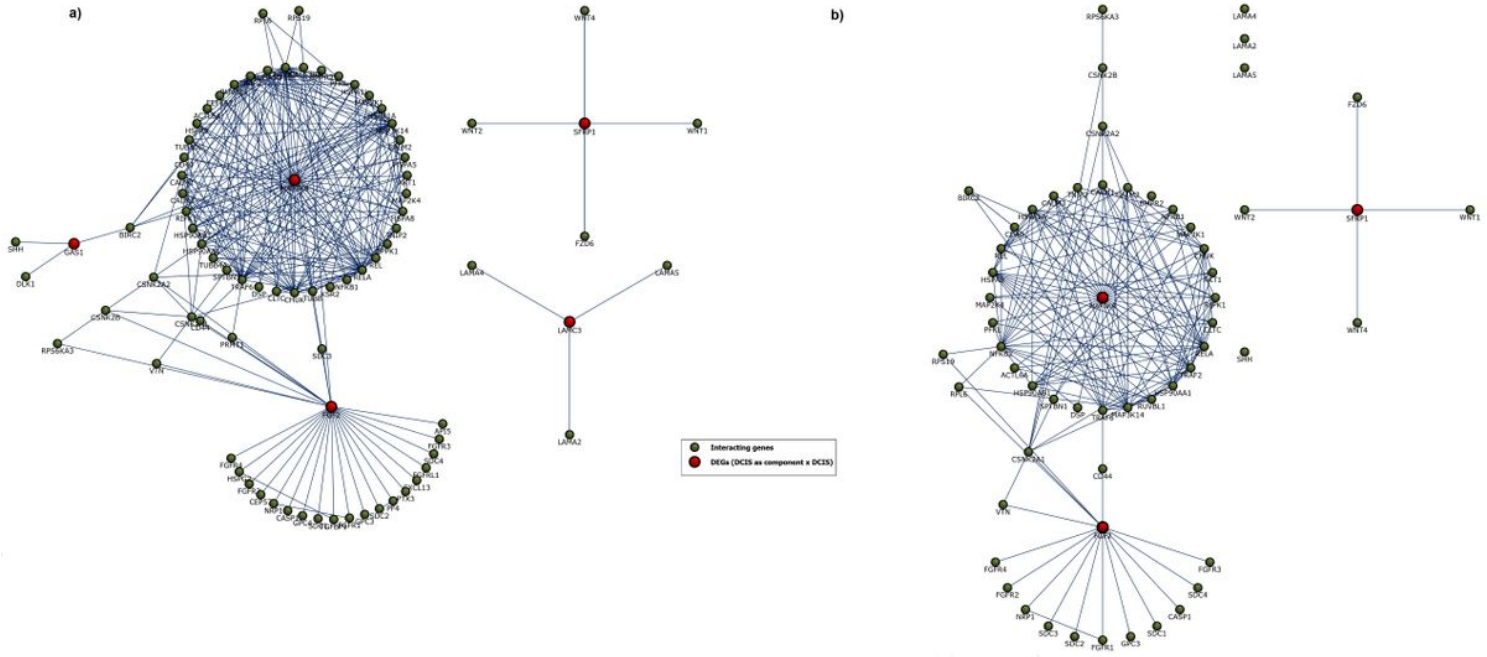

Figure 4

Snapshot of protein-protein interaction networks. Networks are made with the 6 differentially expressed genes between ductal carcinoma in situ as component (DCIScomp) and pure DCIS (DCISpure). Interaction diagram was generated using FunRich Functional Enrichment Analysis Tool and FunRich database. a) Network diagram with all annotated interactions. b) Network diagram illustrating the 107 statistically significant interactions (p value ≤ 0.01).

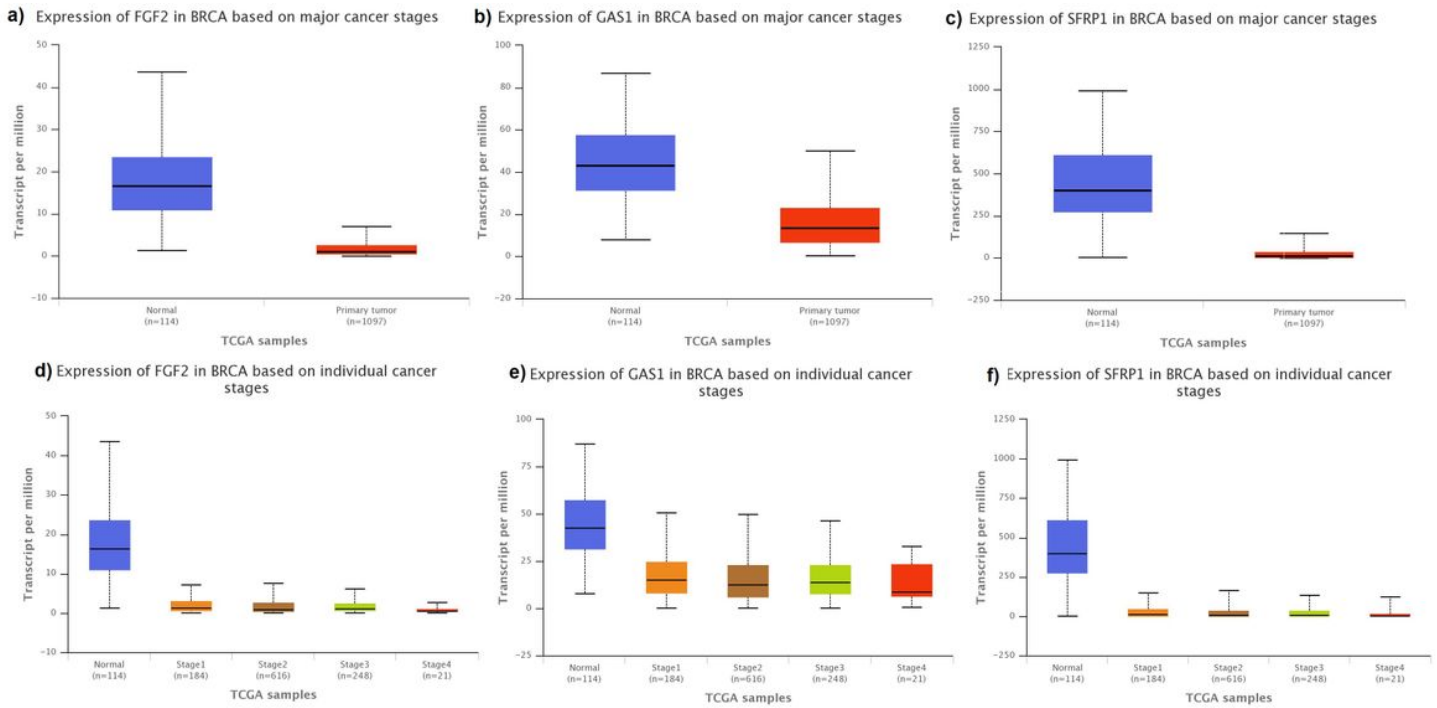

Figure 5

Comparisons of FGF2, GAS1 and SFRP1 expressions. Comparisons are made between normal tissue and primary tumor and among breast cancer (BC) progression stages. This data was generated online in UALCAN website based on the The Cancer Genome Atlas database. a) Expression of FGF2 in normal tissue and primary tumor. b) Expression of GAS1 in normal tissue and primary tumor. c) Expression of SFRP1 in normal tissue and primary tumor. d) Expression of FGF2 in BC stages. e) Expression of GAS1 in BC stages. f) Expression of SFRP1 in BC stages.