"Differential Gene Expression in Breast Cancer"

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Gene expression for assessing tumor biology and biomarker

- **Progression of *in situ* Ductal Carcinoma**
  - Castro NP, Breast Cancer Res. 2008

- **Hereditary Breast Cancer: Influence of BRCA1 mutation in Gene Expression of Triple Negative Tumors**
  - Carraro et al., Journal of Human Genetic – submitted
  - Lisboa, B; Silva F, Pena M et al – submitted
  - Ferreira et al., not published
Progression of *in situ* Ductal Carcinoma (DCIS)

- Ductal carcinoma (80%)
- *In situ* DCIS - 20-30% of all Ductal carcinoma (DC) detected by mammography screening
- Incert evolution (Rapid progression or slow evolution)
- Histologic Classification
  - Comedo or Non-comedo
  - Grade: Low, Medium or High
  - Estrogen/Progesteron Receptor
    - Progride to invasive disease

**Definition of molecular events necessary for the epithelial cells acquire the ability to invade the surround tissue**

- Due to the technological advances in detecting very small or non-palpable lesions, the number of women diagnosed with DCIS and early breast cancer lesions is continuously increasing.
Molecular Divergence of Tumor Epithelial cells

<table>
<thead>
<tr>
<th>Normal (4)</th>
<th>Pure DCIS (5)</th>
<th>In situ component of DCIS-IDC (10)</th>
<th>IDC (10)</th>
</tr>
</thead>
</table>

Laser Microdissection for capturing epithelial cells from tumor lesion
Molecular Divergence: number of differentially expressed genes (DEG) as distance measure (the higher the number of DEG – the more distant the group is allocated)

**Gene Expression Profile**

Normal ≠ PureDCIS ≠ *in situ* component DCIS/IDC ~ IDC

**Morphological Features**

Normal ≠ PureDCIS ~ *in situ* component DCIS/IDC ≠ IDC

Castro *et al.*, Breast Cancer Res. 2008
Two important points

1) From pure DCIS to *in situ* component of DCIS-IDC happen most transcriptional alterations

2) Alterations in gene expression occur before the cells manifest their morphological aspects of invasion
The earliest molecular alterations of epithelial cells

Molecular Difference between cells from intraductal components

Pure DCIS (5)
At least 5 years follow-up

In situ component
DCIS-IDC (10)

Different malignant potential
147 Differentially Expressed Genes

Castro et al., Breast Cancer Res. 2008
Putative genes involved in the progression of *in situ* DCIS

The progression of *in situ* DCIS seems to be markedly characterized by gene downregulation.

### Genes potentially implicated in DCIS progression functionally classified within the biological process category

<table>
<thead>
<tr>
<th>Functional process</th>
<th>Downregulated genes in pure DCIS</th>
<th>Upregulated genes in pure DCIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion and migration</td>
<td>-</td>
<td>AZGP1, C20orf23, C20orf42, CHST10, COL17A1, DGCRC2, GPRR8B, ITGB2, KIF1A, LPXN, NEDD9, PCDH10, PCLKC, PLEKHC1, RGMB</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>CORO1C, CXCL9, IGF5f6, LOX, NCOA4, NMU, SKIL</td>
<td>ARHGAP19, ARHGAP9, C16orf5, C3AR1, CHRNBI1, EPOR, FCGR2B, FCN1, FGFBP1, GIPC1, GPR77, KDR, MAPRE2, PIAS2, RHOU, STK25</td>
</tr>
<tr>
<td>Cell proliferation and apoptosis</td>
<td>NOX4, SULF1</td>
<td>ANAPC13, CDC45L, ERC1, IFT57, RARRES3, REC8L1, SHC1, UTP20</td>
</tr>
<tr>
<td>Transcriptional regulation</td>
<td>MED10, PHTF1</td>
<td>AOF2, ATF2, ETNK2, IRF8, MBD3, MGC21874, SMARCA3, SOX13, TARDBP, ZBTB5</td>
</tr>
<tr>
<td>Metabolism</td>
<td>P4HA1</td>
<td>B4GALT5, BCHE, CA3, CPNE3, CPT1A, DHRS12, FN3K, GBGT1, OSBPL7, PEFD, PITPNM2, UFD1L, ZFP36L1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>CCT5, MARCH8, PTBP2, RAD51AP1</td>
<td>ALMS1, ARFIP1, BOP1, CAMP, CAV1, CIRBP, CLINT1, CLTC1, CTSZ, DHX35, EYA2, FCGR3A, GOSR2, IIMMT, INOC1, KBTBD10, KCTD15, KIAA0664, KPNA6, LSM4, MARK3, MRPS17, NGDN, NUP50, P4HB, PMPCA, POLD3, POMGNT1, PPP2R3A, RABEPK, RPL3, RPL41, RSL1D1, SAMD4A, SLC6A20, SLC9A5, SPOCK2, STX11, SV2B,</td>
</tr>
</tbody>
</table>

Genes belonged to more than one biological process were assigned in a hierarchical manner in the following order: cell adhesion and migration; signal transduction; cell proliferation and apoptosis; transcriptional regulation; and metabolism. Those with no classification in the five categories were classified as miscellaneous. DCIS, ductal carcinoma *in situ*. 
Validation in independent Sample set
(epithelial cells captured by laser – Ferreira et al., Diagn Mol Pathol, 2010)

- 61 genes: 32 (52.4%) in concordance with microarray results (Fold change ≥ 2; p < 0.01)

- 30 out of 32 genes upregulated in pure DCIS
- 2 out of 32 genes downregulated in pure DCIS
ANAPC13 mRNA and protein

- Chromosome 3q 22.2.
- Encodes a component of Anaphase complex (subunit 13) (APC/C) – cell cycle.
- Highly conserved among the species

Protein: cytoplasmic and nuclear staining – 74 amino acids

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure DCIS</td>
<td>25 (69,50)</td>
<td>11 (30,50)</td>
<td>0,02</td>
</tr>
<tr>
<td>in situ component of DCIS-IDC</td>
<td>11 (40,80)</td>
<td>16 (59,20)</td>
<td></td>
</tr>
</tbody>
</table>
ANAPC13 expression along tumor progression

mRNA level – epithelial cells

Protein level

DCIS: 41 specimens
In situ component of DCIS-IDC: 36 specimens
IDC: 187 specimens

Sens-Abuázar et al., Translational Oncology. 2012
ANAPC13 as Biomarker

For progression of pure DCIS

<table>
<thead>
<tr>
<th>Frequency and Intensity</th>
<th>Negative</th>
<th>Positive</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/o progression</td>
<td>7 (35,00)</td>
<td>13 (65,00)</td>
<td>0,18</td>
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<tr>
<td>with progression</td>
<td>5 (71,40)</td>
<td>2 (28,60)</td>
<td></td>
</tr>
</tbody>
</table>

For prognosis in Invasive Ductal carcinoma (IDC)

Survival curves based on ANAPC13
Kaplan Meier curve

- 187 Invasive Ductal Carcinoma

- Overall survival: p-value=0.004
- Disease free survival: p-value=0.04
ANAPC13 is an independent prognostic factor in invasive breast cancer

- Women diagnosed with ANAPC13 negative tumor has twice the risk of dying from the disease than patients with positive tumor

Sens-Abuázar et al., Translational Oncology. 2012
**ANAPC13 expression** versus genomic instability

- Participation in chromatides separation in cell division

qRT-PCR in 42 IDC cases

Gains and losses according to ANAPC13 expression level

- High expression
- Low expression

**copy number alterations (CNAs)**

Sens-Abuázar *et al*., Translational Oncology. 2012
Gene expression modulated by **ANAPC13** expression level

- MCF7: Tumorigenic human breast cell lines
- ANAPC13 sense and antisense ORF were inserted into pCDNA3.1/myc-His vector
  - cDNA microarray platform G4851A 8X60K (Agilent®) (ANAPC13 expression: low, medium and high level)
  - Short Time-series Expression Miner (STEM)

![Graphs showing gene expression modulation](image)

- Enrichment of Cell Cycle-related Biological Processes

<table>
<thead>
<tr>
<th>Profile A Category Name</th>
<th>#Genes Assigned</th>
<th>#Genes Expected</th>
<th>#Genes Enriched</th>
<th>p-value</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromosome</td>
<td>104</td>
<td>31.0</td>
<td>6.5</td>
<td>+24.5</td>
<td>5.3E-15</td>
</tr>
<tr>
<td>chromosomal part</td>
<td>92</td>
<td>28.0</td>
<td>5.7</td>
<td>+22.3</td>
<td>8.2E-14</td>
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<tr>
<td>cell cycle</td>
<td>209</td>
<td>42.0</td>
<td>13.0</td>
<td>+29.0</td>
<td>1.2E-13</td>
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<tr>
<td>organelle organization</td>
<td>225</td>
<td>42.0</td>
<td>14.0</td>
<td>+28.0</td>
<td>1.9E-12</td>
</tr>
<tr>
<td>M phase</td>
<td>105</td>
<td>23.0</td>
<td>6.5</td>
<td>+21.5</td>
<td>3.3E-12</td>
</tr>
</tbody>
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<th>#Genes Enriched</th>
<th>p-value</th>
<th>Corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell cycle process</td>
<td>154</td>
<td>18.0</td>
<td>3.1</td>
<td>+14.9</td>
<td>2.6E-9</td>
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<tr>
<td>cell cycle phase</td>
<td>127</td>
<td>16.0</td>
<td>2.6</td>
<td>+13.4</td>
<td>7.0E-9</td>
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<tr>
<td>cell cycle</td>
<td>209</td>
<td>20.0</td>
<td>4.2</td>
<td>+15.8</td>
<td>1.2E-8</td>
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<tr>
<td>nuclear</td>
<td>125</td>
<td>14.0</td>
<td>3.5</td>
<td>+14.0</td>
<td>3.9E-9</td>
</tr>
</tbody>
</table>
ANAPC13 expression level interferes in Cell Proliferation Rate

Cell number
xCSELLigence System monitors cellular events in real time

ANAPC13 overexpressing cells
ANAPC13 downregulating cells
MOCK2
MCF7

Glucose Uptake assay

Nakahata, A, Ricca T; not published
Summary

1) The most dramatic changes in gene expression profile of epithelial cells occur in the transition from pure DCIS to in situ component of DCIS-IDC during the course of breast tumor progression.

2) Gene expression program for invasion is established in epithelial cells before morphological manifestation

3) ANAPC13 is potential biomarker for pure DCIS and IDC of the breast

4) ANAPC13 expression level modulated the cell proliferation rate

5) Loss of ANAPC13 is associated with genomic instability
Perspectives

- Microenvironmental role in the progression of DCIS
  - myoepithelial cells (MEC)
    - involved in the maintenance of the basement membrane
  - surrounding fibroblast

Mutation Profile – Pre-invasive lesions (distinct malignant potential – Exome sequencing)
Hereditary Breast Cancer: Influence of \textit{BRCA1} mutation in Gene Expression of Triple Negative (TN) Tumors

- Hereditary BC (HBC) is an autosomal dominant disease
  - germ line mutations in \textit{BRCA1} and \textit{BRCA2} genes
  - higher risk of developing breast and ovarian cancer
  - (HBOC - Hereditary Breast and Ovarian Cancer syndrome)

- 240 women screened for \textit{BRCA1} and \textit{BRCA2} genes
  - Point mutations and indels – Gene sequencing
  - Chromosomal rearrangements- MLPA and CGH
    - (~ 25% mutation rate)
Identification of a gene signature of \textit{BRCA1}/\textit{BRCA2} associated tumors

- Fifty-four patients under 35 years old (median age of 31 years old - range 22-35),
- Agilent platform
  - 9 \textit{BRCA1}/2 associated and 23 \textit{BRCA1}/2 negative tumors
  - 48 differentially expressed genes

- Up-regulated genes in \textit{BRCA1}/2 associated tumors - DNA repairs and mitotic cell cycle-related processes
- Up-regulated genes in \textit{BRCA1}/2 negative tumors - cell signaling and metabolic pathway-related processes

Distinct mechanisms is involved in triggering tumorigenesis in \textit{BRCA1} associated and negative tumors

Carraro et al., submitted
**BRCA1** mutation status and its relation with tumor subtype and familial history

- Young patients diagnosed with TN tumors – 46% mutation rate in **BRCA1** gene (6 out of 13)

- Young patients diagnosed with TN tumors and with positive familial history – 72% mutation rate in **BRCA1** gene (5 out of 7)

Brazilian young patients with TN tumor is fair mandatory for the **BRCA1** mutation screening

**BRCA1** mutation triggers a significant proportion of TN tumors

Carraro et al., submitted
Triple negative breast cancer (TNBC)

- TNBC- ER/PR, HER2 negative
- Very aggressive Breast Tumor subtype

\textbf{RNA-seq (whole transcriptome from tumor and normal adjacent tissues) in SOLID platform}

- \textit{BRCA1} associated tumor [non-sense mutation - R1751X (e20)] – deleterious
- \textit{BRCA1 unclassified variant (UV) associated tumor} [missense mutation - Q356R (e11)] – no deleterious
- \textit{BRCA1/BRCA2} negative tumor (wild type patient)
Summary

• Distinct mechanisms might be involved in triggering tumorigenesis in BRCA1 associated and negative tumors
• Germ line mutation in BRCA1 gene can have high prevalence in negative TN tumors in Brazilian young patients

Perspectives

• Definition of BRCA1 mutation prevalence in TNBC
  – High-throughput screening of BRCA1 gene in HR(-) tumors to establish the prevalence of somatic and germline BRCA1-mutations
    • Barcode approach based on Carraro et al., PLoS One, 2011
      – (ROCHE-454 Junior)
• Association with clinical characteristic and drug response
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