Genetic and Epigenetic Regulation of TOX3 Expression in Breast Cancer

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The SNPs at 16q12, close to the *TOX3* and *CASC16 (LOC643714)* genes, are one of the susceptibility loci identified by GWAS, showing strong evidence for breast cancer association across various populations.

### Previous studies in all populations

| Cytoband | RefSeq Genes | rsid   | ref/risk | Population | Location | Study Type   | Author                          |
|----------|--------------|--------|----------|------------|----------|--------------|---------------------------------|
| 16q12    | *TOX3*, *LOC643714* | **rs3803662** | G/A      | European   | Intron 3  | GWAS         | Easton 2007; Stacey 2007;       |
|          |              | **rs4784227** | C/T      | Asian      | Intron 2  | GWAS         | Long 2010                       |
|          |              | **rs3104793** | T/C      | AA         | Promoter  | Fine-mapping | Ruiz-Narváez                    |
|          |              |         |          | African, AA| Promoter  | Fine-mapping | Olopade group                   |
|          |              | **rs3112572** | G/A      | African, AA| Intron 2  | Fine-mapping | Olopade group                   |

### Women of African Ancestry
Location of SNPs associated with BC

Rs3803662: European
Rs4784227: Asian
Rs3104788, rs3104793: Africans, AA
Tox3 gene

- Trinucleotide repeat (CAG)-containing gene 9 protein (glutamine-rich at C-terminal)
- Involved in bending and unwinding of DNA and alteration of chromatin structure.
- Transcriptional co-activator of the p300/CBP-mediated transcription complex.
- Activates transactivation through cAMP response element (CRE) sites.
- Protects against cell death by inducing anti-apoptotic and repressing pro-apoptotic transcripts.
The mRNA levels TOX3 predict adverse outcome for breast cancer patients.
Figure 1. Increased Expression of TOX3 and CASC16 in luminal breast cancer cell lines.

(A and C) TOX3 and CASC16 expression was assessed in breast cancer cell lines using qRT-PCR relative to expression in normal breast epithelial cell line HMEC (RQ, relative quantity).

(B and D) Expression of TOX3 in luminal cell lines (blue) is statistically significantly higher than expression in basal-like cells (red) and non-malignant breast epithelial cells (green) (**p<0.01).
Figure 2. Expression of TOX3 in breast tumors.
(A to C) Microarray results of TOX3 expression in breast tumors showed a significant difference in TOX3 expression among breast cancer subtypes, with TOX3 expression higher in luminal A, luminal B and Her2 amplified tumors than basal-like tumors and normal breast epithelial tissues. (A) TCGA dataset (p<0.0001); (B) the University of North Carolina (UNC) dataset (p=3.66e-34); (B) (C) the University of Chicago (U of Chicago) dataset (p<0.0001).
Figure 3. eQTL analyses between SNP genotypes and TOX3 expression. (A and B) eQTL analyses were performed in 345 SNPs located 500 kb upstream and 250 kb downstream of the TOX3 gene, using TCGA dataset. A significant association with TOX3 expression was observed in rs3803662 and rs4784227, a risk variant of breast cancer in European [7] and Asian women [8,10], respectively.
Figure 4. Subtype-specific methylation of the TOX3 promoter. (A) UCSC genome browser shows location of CpG islands in the TOX3 promoter at chromosome 16q12. (B) Representative chromatographs in the TOX3 promoter after bisulfite treatment. (C) Methylation levels of four CpG dinucleotides in the promoter region of TOX3 were analyzed using the TCGA HumanMethylation450 Array data from 588 human breast tissues. (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001)
Figure 5. Correlation between the TOX3 promoter methylation and TOX 3 expression in breast tumors. TCGA breast invasive carcinoma gene expression microarray (Agilent 244K, G4502A, version 2013-06-02), and DNA methylation (Illumina Infinium HumanMethylation450 BeadChip, version 2014-05-02) datasets were analyzed. Scatter plot showing correlation between copy number of TOX3 and expression of TOX3 in the breast tumors in four CpG sites.
Figure 6. Copy number of TOX3 in breast cancer. (A) Genomic positions of BAC RP11-132F7 and RP11-748D7 at chromosome 16q12. These clones were selected for homebrewed TOX3 and CASC16 FISH probes, respectively. (B) Representative FISH photomicrographs of TOX3:CEP16 and CASC16:CEP16 in GM14667 (control normal lymphocytes) and ZR-75-30 (luminal) cell lines. (C) TCGA copy number data analysis shows a similar level in copy number of TOX3 between the different breast cancer subtypes in human tumors. (D) Scatter plot showing no correlation between copy number of TOX3 and expression of TOX3 in the breast tumors.
### Table S2. Gene Copy Number (GCN) of TOX3

| Cell Line    | # of cells scored | TOX3 GCN<sup>1</sup> | CEP16 GCN<sup>2</sup> | TOX3:CEP16<sup>3</sup> | Representative clone(s) (TOX3:CEP16, % cells) | TOX3 Amplification | Interpretation<sup>4</sup> |
|--------------|------------------|-----------------------|------------------------|------------------------|-----------------------------------------------|---------------------|---------------------------|
| HMEC         | 60               | 2.0                   | 2.2                    | 1.0                    | 2:2(88%)                                      | No                  | Normal                    |
| ZR7530       | 60               | 4.1                   | 3.9                    | 1.0                    | 4:4(77%)                                      | No                  | Polysomy                  |
| MDAMB175VII  | 60               | 3.1                   | 3.1                    | 1.0                    | 3:3(73%)                                      | No                  | Polysomy                  |
| HCC202       | 60               | 2.0                   | 2.6                    | 0.8                    | 2:3(55%)                                      | No                  | Abnormal, heterogeneity   |
| T47D         | 60               | 2.0                   | 2.8                    | 0.7                    | 2:3(83%)                                      | No                  | Abnormal                  |
| HCC70        | 60               | 6.4                   | 6.3                    | 1.0                    | 6:6(63%)                                      | No                  | Polysomy                  |
| HCC1500      | 60               | 2.0                   | 2.0                    | 1.0                    | 2:2(98%)                                      | No                  | Normal                    |

<sup>1</sup>Mean copy number of gene per cell  
<sup>2</sup>mean copy number of centromere enumeration probe (CEP) per cell;  
<sup>3</sup>mean gene to CEP ratio;  
<sup>4</sup>GCN classification; polysomy, ≥ three copies in >90% of cells
Summary

• We investigated both genetic and epigenetic factors contributing to TOX3 expression in breast tumors and cell lines.
• Expression of TOX3/CASC16 genes is greatly up-regulated in luminal breast cancer compared to basal-like breast cancer or normal breast cells.
• eQTL analysis showed a correlation between genotypes of 16q12 SNPs and expression of TOX3 in breast tumors.
• Bisulfite sequencing of CpG islands in cell lines and analysis of primary breast tumors using TCGA dataset showed significantly lower levels of methylation of the promoter in luminal breast tumors compared to all other subtypes with a significant correlation between expression and methylation of TOX3.
• Flourescent In Situ Hybridization (FISH) revealed polysomy at 16q12 loci in luminal cell lines, but TCGA data showed no correlation between copy number variation and expression of TOX3.
Limitation

• We did not study ER-associated transcription factors (i.e. FOXA1) potentially involved in regulation of TOX3 promoter activity.

• Nevertheless, epigenetic modifications seem significant contributors to drive altered expression of many genes (i.e. miR-29c, TOX3) in the development of breast cancer.