THOR: A DNA methylation-based marker for breast cancer management

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BACKGROUND

- Breast cancer (BC) is the most common cancer and a leading cause of death among women worldwide. Given BC heterogeneity and clinical variability, the identification of biomarkers that could detect BC in an early disease stage and predict tumor behavior is an important yet unmet need.
- Limitless self-renewal is a crucial process in cancer, which is essentially attained through telomerase activation in BC by human Telomerase Reverse Transcriptase (h*TERT*) expression. Genetic and epigenetic events were shown to regulate h*TERT* and to have clinical implications in h*TERT* activation-dependent cancers.
- One of the mechanisms associated with h*TERT* expression in cancer is the hypermethylation of a specific region in the h*TERT* promoter defined by our group as *TERT* Hypermethylated Oncological Region (THOR).

HYPOTHESIS

- THOR can have an impact on h*TERT* upregulation and be a biomarker of malignancy and patient outcome in BC.
- This region could be used as well as a therapeutic target for this disease.

RESEARCH APPROACH

- Aim 1: Characterization of THOR region in normal breast tissue.
- Aim 2: THOR methylation and h*TERT* expression analysis for the breast invasive carcinoma cohort using The Cancer Genome Atlas (TCGA) database.
- Aim 3: THOR methylation analysis and h*TERT* expression in a BC validation cohort.

METHODS

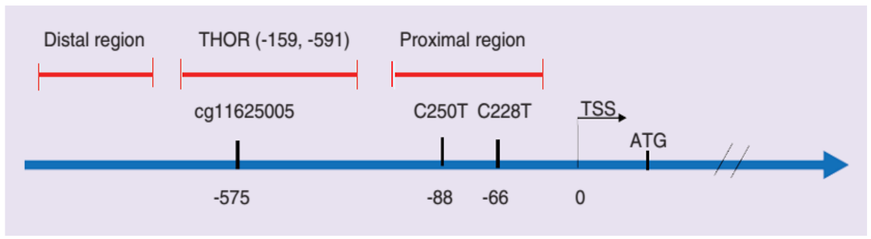

Figure 1. Schematic representation of h*TERT* promoter. THOR, localizes between –159 and –591 base pairs from the TSS. The full extent of THOR is 433 bp (chr5:1295321-1295753) and comprises 52 CpG sites. The position of the probe cg11625005 used to evaluate THOR methylation status is shown (chr5:1295737, hg19 assembly). Known *TERT* promoter mutations C250T and C228T (chr5:1295250 and chr5:1295228, respectively) are also indicated. The scheme is not scaled. THOR: TERT hypermethylated oncologic region; TSS: Transcription start site.

Aim 1:

Epigenetic characterization of THOR region → NIH Roadmap Epigenomics Project
- DNA methylation analysis (MeDIP-Seq)
- Histone marks (ChIP-seq)
- Chromatin state (ChromHMM)

Aim 2:

TCGA Breast invasive carcinoma cohort (n=841)
- THOR status → Illumina HumanMethylation 450K array dataset
- h*TERT* expression → Illumina HiSeq 2000 RNA Sequencing dataset

Aim 3:

Validation Cohort (n=241) (CHAlgarve, Portugal)
- **THOR status** → Bisulfite pyrosequencing
- **h*TERT* expression** → Droplet digital PCR (ddPCR)

RESULTS

Roadmap:THOR is localized in a repressive chromatin region

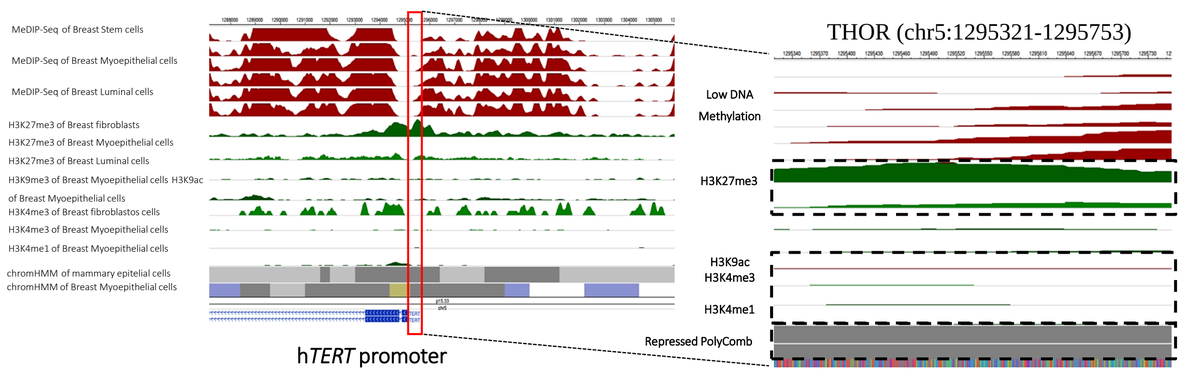

Figure 2. THOR is localized in a repressive chromatin region in normal breast cells. According with MeDIP-Seq data, THOR is hypomethylated in the different normal breast cells analyzed. ChIP-Seq data evidence enrichment of histone repressive marks (H3K27me3 (green peaks)) and low recruitment of active histone marks (H3K9ac, H3K4me1 and H3K4me3) in normal cells. ChromHMM classified THOR as a repressed polycomb region (grey color).

TCGA: cg11625005 methylation distinguishes normal from malignant breast tissue

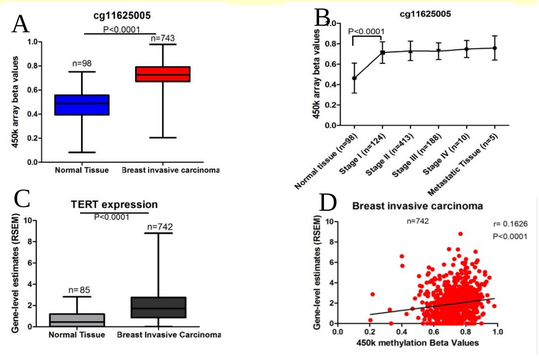

Figure 3. cg11625005 (within THOR) distinguishes normal from malignant breast tissue and is positively correlated with h*TERT* expression in breast carcinoma. Breast invasive carcinoma show higher THOR methylation (cg11625005) when compared to benign breast tissue (p<0,0001) (A) and allows the differentiation of benign tissue from cancer from the earliest disease stage (B). h*TERT* is differentially expressed in benign and malignant tissue (p<0,0001) (C) and it is positively correlated with THOR methylation status (D).

Validation Cohort: THOR differentiate benign tissue from breast cancer from the earliest stage of disease

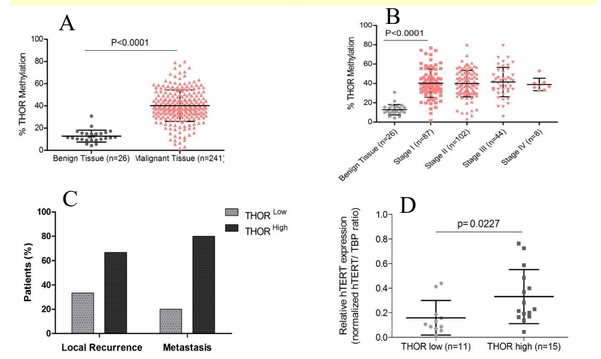

Figure 4. THOR region is specifically hypermethylated in malignant breast tissues. Pyrosequencing analysis reveals higher THOR methylation in malignant breast tissue when compared to healthy benign tissue (p<0.0001) (A). Levels of THOR methylation are significantly higher between any disease stage and benign tissue (p< 0.0001) (B). A higher percentage of patients with THOR ^{High} is observed amongst patients that had local recurrence (66,7% vs 33,3%) and metastasis (80% vs 20%) (C). h*TERT* is more expressed in patients with higher THOR levels (D). The cut-off value of 30,86% with an area under the ROC curve equal to 0,9574 and p< 0.0001 (100% specificity and 78.84% sensitivity) was used.

Validation Cohort: THOR is an early stage candidate biomarker

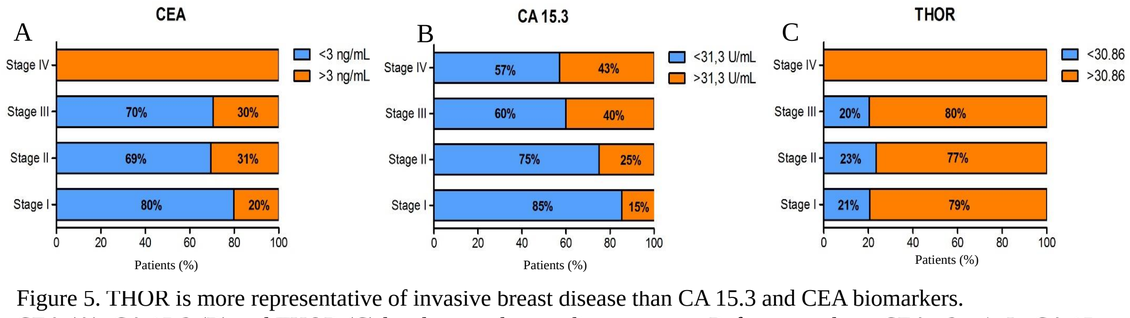

Figure 5. THOR is more representative of invasive breast disease than CA 15.3 and CEA biomarkers.
CEA (A), CA 15-3 (B) and THOR (C) levels according to disease stages. Reference values: CEA <3ng/mL; CA 15-3 <31.3 U/mL and THOR <30.86% (AUC: 0.9574, p< 0.0001 with 100% specificity and 78.84% sensibility).

CONCLUSIONS

- THOR is localized in a repressive chromatin region, which suggests that THOR may lead to increased h*TERT* expression in BC by blocking the binding of transcriptional repressors.
- In both TCGA and CHAlgarve cohorts, THOR was significantly hypermethylated in malignant breast tissue when compared to healthy tissue (p<0.0001).
- In the validation cohort, patients with THOR^{High} showed increasing levels of h*TERT* expression (p=0.0227; CHAlgarve cohort).
- THOR was able to differentiate cancer from normal tissue from the earliest stage of disease, which evidence its potential as a candidate biomarker for early BC detection.

FUTURE DIRECTIONS

Targeted THOR demethylation using CRISPR-dCas9

- CRISPR/dCas9 plasmids expressing both dCas9-TET1 (DNA demethylase) and the specific gRNA targeting THOR region will be transfected into BC cell lines.

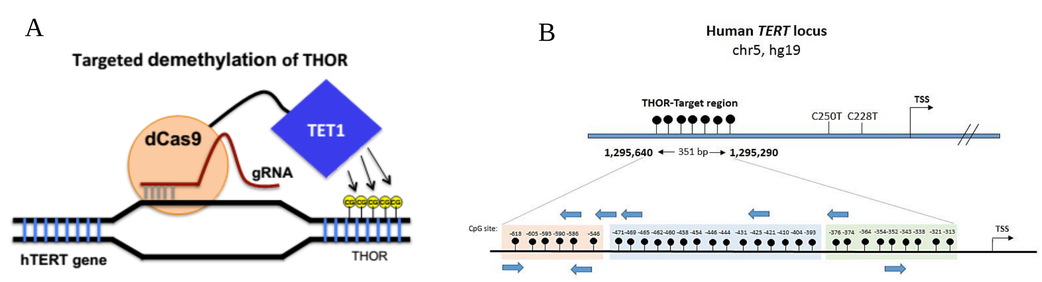

Figure 6 –THOR demethylation using CRISPR/dCas9. Schematic representation of the CRISPR-Cas9 modified system to specifically demethylate THOR region. (A). Eight sgRNAs targeting THOR are shown in blue color, with the arrows point toward the PAM sequence. CpG sites are represented as dot blots. 9 CpG sites proximal to TSS are highlighted in light green (Fragment A2); 15 CpGs located in the mid THOR are highlighted in light blue (Fragment A3) and 6 CpGs correspondent to UTSS region are highlighted in light orange (Fragment A4) (B).

- We expect to induce targeted THOR demethylation in breast cancer cell lines and consequently supress h*TERT* expression.

Therapeutic target

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