The Regulatory Role of NF-κB and Twist1 in Breast Cancer

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ABSTRACT

Breast cancer (BC) is a heterogeneous disease, composed by multiple subtypes with different molecular characteristics and outcomes. In Brazil, this neoplasia is the first cause of cancer death in women, mainly due to late diagnosis, when the possibility of developing metastasis is improved. The high expression of estrogen (ER) and/or progesterone (PR) receptors characterizes the Luminal subtype, whereas gene amplification and/or high expression of Her2 characterizes the Her2 subtype. When none of these three receptors is expressed, the tumor is known as Triple Negative (TN), the most aggressive of all subtypes. The metastatic process depends on the expression of transcription factors (TFs) such as Twist1, Snail, Slug and Sip1, involved with the epithelial-mesenchymal transition (EMT). The aim of our study is to evaluate the role of NF-κB as a regulator of TFs associated with EMT. Through bioinformatics tools, we identified binding sites for NF-κB on. In silico analyzes performed using MetaCore software grouped these genes according to molecular function, revealing numerous correlathe promoter region of these genes, whose regions were confirmed in vitro by chromatin immunoprecipitation. Fusions of the NF-κB binding region and Luciferase gene reporter confirmed the activity of the promoter regions described, confirming the role of NF-κB as regulator of the gene expression of these TFs in aggressive BC cells. In 46 BC samples, the TN group expressed the highest levels of Slug and Sip1 mRNA, compared to Luminal and Her2 subtypes. However, the Her2 group expressed the highest levels of Twist1. Twist1 is described as the master regulator of EMT in BC, although its role in intrinsic subtypes remains to be clarified. The silencing of TWIST1 gene in the HCC-1954 cell line (Her2/neu positive) caused a drastic reduction in its expression, which was confirmed by RT-qPCR and immunoblotting. A large-scale microarray expression analysis through the GeneChip human exon array showed deep molecular changes after Twist1 knockdown, as 141 genes were up-regulated and 190 down-regulatedtions between Twist1 with important biological processes and signaling pathways such as extracellular matrix remodeling, blood coagulation, Th17 signaling, among others. The most differentially expressed genes were confirmed by RT-qPCR. Interleukin (IL) -17 signaling was examined by flow cytometry and immunoblotting for the IL-17RA and Act1 proteins, which act to trigger this signaling. IL-6 and IL-8 levels, which are targets of this signaling, were evaluated by ELISA assays. Both results showed consistent information with the microarray analysis, reporting that Twist1 plays an important role in activating a Th17 profile in the context of breast cancer. Thus, our findings contribute for a better understanding of the participation of NF-κB and Twist1 in breast cancer, especially on Her2 and TN subtypes, demonstrating their potential as targets for the development of new therapies.

RESULTS

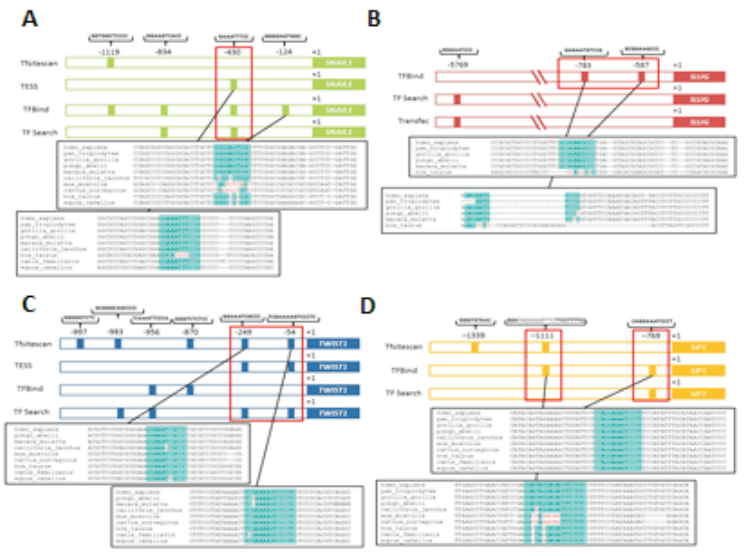

Figura 1. Representative scheme of putative NF-κB binding sites located in the SNAIL1 (A), SLUG (B), TWIST1 (C) and SIP1 (D) promoter regions predicted by Tfsitescan, TESS, TFBind, TFSearch and Transfac bioinformatics tools. +1: transcription start site.

Figure 2. Relative luciferase activity in MDA-MB-231 cells transfected with pGL3-plasmid containing the SLUG (A), TWIST1 (B) and SIP1 (C) promoter regions. DHMEQ: dehydroxymethylepoxyquinomicin, the specific NF-kB/p65 inhibitor.

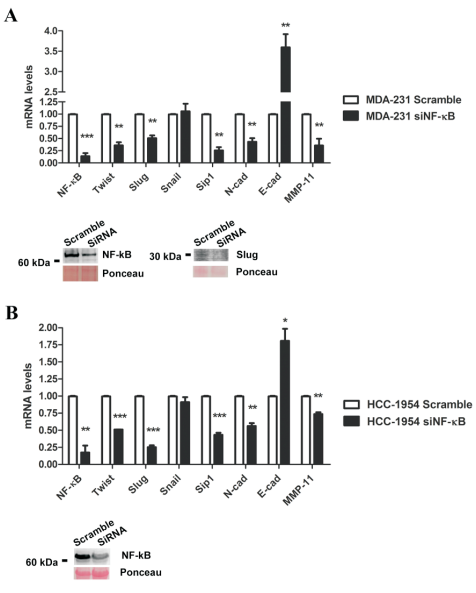

Figure 3. Relative expression of EMT-phenotype markers after NF-κB/p65 silencing. The mRNA levels were assessed in MDA-MB-231 (A) and HCC-1954 (B) cells.

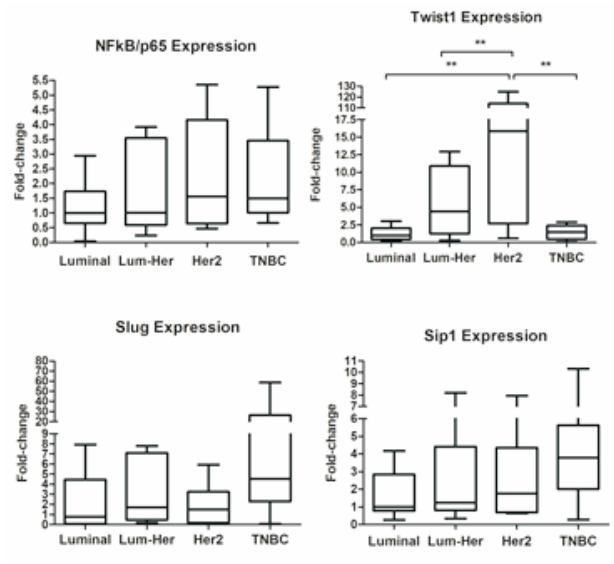

Figure 4. Box-plot graphs showing gene expression for NF-kB/p65, Twist1, Slug and Sip1 in Luminal, Luminal-Her2, Her-2 and Triple-negative (TNBC) breast cancer subtypes. Median and range of mRNA values are shown. Expression was normalized by ACTB and GAPDH mRNA levels and Ct was calculated vs. Luminal median.

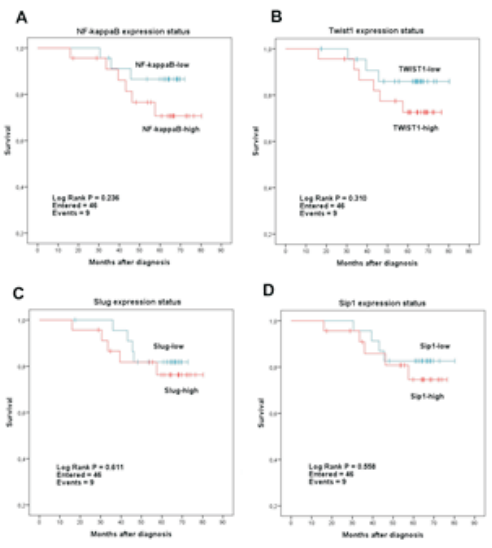

Figure 5. Kaplan-Meier's survival analysis in breast cancer patients according to the expression of NF-κB/p65 (A), Twist1 (B), Slug (C) and Sip1 (D).

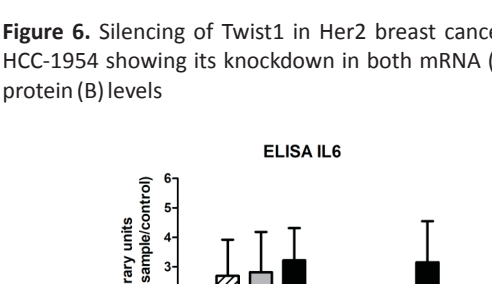

Figure 6. Silencing of Twist1 in Her2 breast cancer cells HCC-1954 showing its knockdown in both mRNA (A) and protein (B) levels

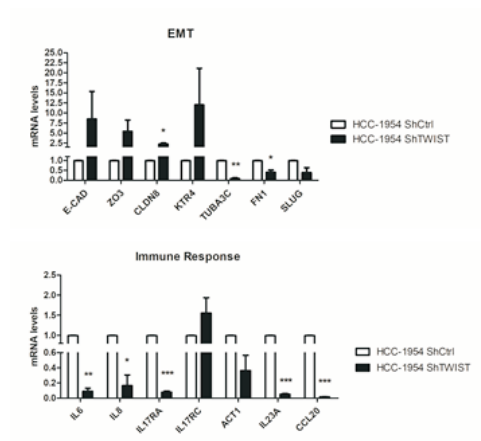

Figure 8. Confirmation of altered transcripts in global expression by quantitative RT-qPCR.

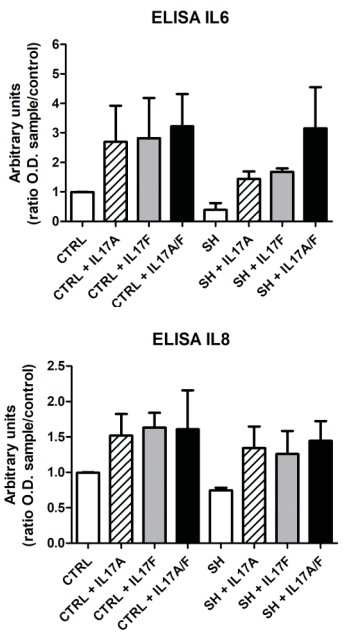

Figure 9. Levels of secreted IL-6 and IL-8 in the supernatant of HCC-1954 ShCTRL (CTRL) and ShTWIST cells that were treated with 50 ng/ml of recombinant factors IL-17A or IL-17F or treated with both (25 ng/ml each).

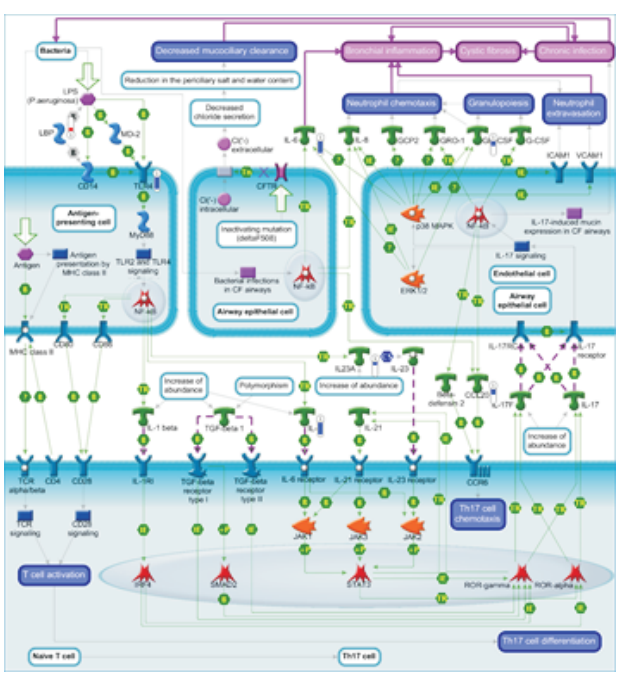

Figure 7. *Th17-mediated Immune Response* was the second signaling more altered in consequence of Twist1 silencing. The relative expression data of the genes identified in the study are visualized on the map through a thermometer in blue (for down-regulation) or red (for up-regularion).

Projeto Gráfico: Setor de Edição e Informação Técnico-Científica / INCA