Epigenetic Regulation of hTERT Expression in Breast Cancer Cells: Analysis of Methylation Status, Expression Levels, and Apoptosis with HDACi and Calpeptin Combination Therapy

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Abstract
The hTERT gene encodes the catalytic subunit of human telomerase reverse transcriptase and is required for telomerase activity. Telomerase is turned off in differentiated somatic cells but is re-expressed in the vast majority of cancer cells, allowing for sustained cellular division and growth. Because there are two CpG islands upstream of hTERT, suggesting a role for methylation in the control of this gene’s transcription, we looked at the correlation between expression levels and methylation status of the hTERT gene in breast cancer cells. Further, we used a combination drug treatment of HDAC inhibitors (HDACi) and calpeptin to determine the treatment’s effects on methylation status and expression of hTERT. Though the drug treatment caused decreased expression of the hTERT gene, the methylation status remained unaffected, indicating other mechanisms of control in the expression of telomerase. Because telomerase inhibition has been shown to cause apoptosis in cancer cells and expression of telomerase in somatic cells leads to apoptotic resistance, we looked at the effect of HDACi on apoptosis. We found increased expression of both intrinsic and extrinsic pro-apoptotic genes with this combination treatment.

Background
- hTERT has two upstream CpG Islands located at -4600bp to -4300bp and -900bp into exon 2 (Fig. 1).
- Studies suggest that increased telomerase expression in cancer is correlated with hypermethylation of the upstream CpG island.
- CCCTC-binding factor (CTCF) binds to repress gene silencing.

We hypothesized that demethylation with HDACi would allow binding of CTCF and inhibition of hTERT.

Figure 1: hTERT Promoter Region and CpG Islands

- For expression analysis, total RNA was prepared, converted to cDNA, and qPCR performed with an actin control. Despite an unchanged methylation status, both MDA-231 and MCF-7 cell lines demonstrated decreased hTERT expression with the drug treatments (Fig. 5).
- Our lab previously demonstrated an increase in apoptosis and cell-cycle inhibition in breast cancer cell lines MCF-7 and MDA-231 following treatment with calpeptin, a calpain protease inhibitor. (Fig. 3).

Figure 2: Methylation and Gene Expression
A. Current Theory in Epigenetics:
- CpG methylation leads to gene silencing; demethylation leads to gene expression.

B. hTERT Regulation Hypothesis:
- hTERT methylation leads to gene expression; demethylation leads to gene silencing.

Figure 3: Calpain-inhibitor-induced Apoptosis

Objectives
- To understand how the methylation status of the CpG island upstream of the hTERT gene correlates with telomerase expression.
- To determine if a combination therapy of HDACi and calpeptin leads to down-regulation of telomerase expression.
- To see how this combination therapy affects the expression of apoptotic genes.

Results
- Cell lines MDA-231 (ER-, PR-, HER2-) and MCF-7 (ER+, PR+, HER2+) were treated with HDACi (SB or SAHA) and/or calpeptin.
- Genomic DNA was isolated from treated and untreated cells, followed by bisulfite treatment and methylation-specific PCR.
- Gel analysis and sequencing indicate methylation upstream and downstream of the transcription start site in both cell lines (Fig. 4).
- Methylation levels remained unchanged following all drug treatments.

Figure 4A: Methylation Levels of CpG islands in MDA-231 (blue) and MCF-7 (green) as measured by bisulfite treatment and methylation-specific PCR. The right graph represents the Methylation levels at CpG positions -634bp, -582bp, -1233bp, and -1365bp.

Figure 4B: Location of CpG Methylation

For expression analysis, total RNA was prepared, converted to cDNA, and qPCR performed with an actin control. Despite an unchanged methylation status, both MDA-231 and MCF-7 cell lines demonstrated decreased hTERT expression with the drug treatments (Fig. 5).

Figure 5a: Expression Levels of Control and Treated MDA-231 Cells

Figure 5b: Expression Levels of Control and Treated MCF-7 Cells

Conclusions
- Treatment with HDACi and calpeptin decreases expression of hTERT and increases expression of known pro-apoptotic genes in multiple breast cancer cell lines, including one that is triple-negative.
- Methylation does not seem to play a role in the regulation of telomerase. Because other genes are being demethylated by this drug treatment while telomerase remains unaffected, other proteins may be involved in the regulation of demethylation of those genes. For example, accessory proteins may bring demethylating enzymes to only certain locations in the genome.
- Given the changes in hTERT expression with HDACi, it is possible that another mechanism like histone acetylation or histone methylation is regulating telomerase expression and is being altered by our drug combination (Fig. 7).
- Further investigation, such as by CHIP analysis to look at histone acetylation and methylation, is required to better understand the way by which HDACi alters telomerase expression.

Figure 7: Proposed Model of the Effects of HDACi/Calpeptin Drug Treatment