In Vitro Investigation of Tributyrin as a Master Regulator in Keloid Pathogenesis

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Abstract

Objective: We previously uncovered four master regulators through Ingenuity Pathway Analysis software's Causal Network Analysis (CAN) (QIAGEN, Redwood City, CA, USA) that were hypothesized to have a driver role in keloid methylation gene targets and influence keloid pathogenesis. The objective is to investigate the role of tributyrin as a master regulator and to assess its influence on modulating the expression patterns of four downstream statistically significant keloid genes *in vitro*. We expect that the expression levels of the four gene targets of tributyrin will be consistent with the hypothetical experiment indicated in CNA, where *VAMP5*, *GALNT3*, *PPP1R13*-α are overexpressed and *TNS1* is under expressed.

Materials and Methods: Tributyrin (Sigma-Aldrich, St. Louis, MO, USA), was used in an methyltetrazolium (MTT) cell proliferation assay (American Type Culture Collection, Manassas, VA, USA) to determine its IC50 dosage for in vitro studies using keloid cell lines. The expression levels of keloid specific downstream genes *VAMP5*, *TNS1, GALNT3*, *PPP1R13*-α in the tributyrin causal network, will be assessed using quantitative reverse transcriptase PCR (qRTPCR) for verification based on the hypothetical experiment produced by CNA. Additional genes investigated were *COL1, COL3, AND FGF2*.

Results: The MTT assay, concentrations of 5 mM, 2.5 mM, 1 mM, 0.5 mM, 0.25 mM, 0.1 mM for 24, 48 and 72 hours, demonstrated that the IC50 for tributyrin was at 48 hours with a concentration of 1 mM. One sample t-tests for keloid effects with no drug resulted in lower expression for COL3a1 and FGF2 (p=0.042 and p=0.013, respectively). With tributyrin treatment, there was lower expression for COL3a1, FGF2 (p=0.003, p=0.01) and a significant increase PPP1R13L (p=0.016) in the keloid cells. Gene expression with tributyrin there was higher for FGF2, PPP1R13L and TNS1 (p=0.034, p=0.006 and p=0.006, respectively).

Conclusion: Tributyrin's influence in modulating the expression of the downstream keloid specific genes would be one step in dissecting out mechanistically its role in keloid pathogenesis. Our hypothesis did not necessary reflect our in vivo results possibly due to small number of cell lines.

Introduction

Keloids are benign fibroproliferative tumors that extend beyond the original wound.[1] Keloids affect 11 million patients in the developed world[2,3] predominantly darker skinned individuals, including black and Hispanic.[4] They are found in family groups and parallelism in identical twins has been found.[5] The incidence varies from 4.5% to 16%.[6] However, some studies suggest lower incidence in the head and neck area after surgery.[7] Clinical outcomes for the treatment of keloids are disappointing. There are high recurrence rates adding to the psychological stress from cosmetic deformities.[8,9] Additionally, quality of life is impaired because of associated loss of function, pruritus and pain.[10] Surgical excision of keloids has a 50-100% recurrence.[11] While surgery with adjuvant therapy such as steroid injection, silicone therapy, pressure therapy, radiotherapy, and anticancer drugs have a recurrence rate of up to 50%.[5]

The failure of adjuvant therapies underscores the heterogeneity of keloid formation and the lack of current treatment to account for its complexity. With the increased focus on epigenetics, where changes in gene functioning/expression is not a result of changes in DNA sequence, many researchers are interested in its contribution to the pathogenesis of disease.[12,13] One epigenetic factor is methylation. Methylation is a natural stop sign that causes halting of gene repression (Figure 1).[14,15] These factors can activate or repress gene expression, and they appear to be instrumental in cancer tumorigenesis.[16]

We previously reported uncovering four master regulators found through Causal Network Analysis program of QIAGEN's Ingenuity Pathway Analysis (QIAGEN, Redwood City, CA, USA).[17] Per the program, these master regulators, tributyrin used for this study, have a driver role in keloid pathogenesis (Sigma-Aldrich, St. Louis, MO, USA). We sought to investigate tributyrin at the cellular and molecular level.

Materials and Methods

In a pilot study of 6 keloid and 6 normal tissue samples, we identified 152 genes that were differentially methylated between keloid and normal using the Illumina 450K beadchip methylation platform (Illumina, San Diego, CA, USA).[14] The top 10 statistically genes (false discovery rate < 0.015) were identified as *VAMP5*, *ACTR3C*, *GALNT3*, *KCNAB2*, *LRRC61*, *SCML4*, *SYNGR1*, *TNS1*, *PLEKHG5*, *PPP1R13*-α. These 10 genes were then uploaded into QIAGEN's Causal Network Analysis, resulting in 4 master regulators.[17] Thus a network was created with the genes being the downstream products and the master regulator being the upstream control. The keloid specific downstream genes of the tributyrin causal network were: *VAMP5*, *TNS1, GALNT3*, *PPP1R13*-α. Additional genes investigated were *COL1, COL3, and FGF2.*

As a first step, the IC50 (the concentration at which 50% of inhibition is observed) and cell proliferation were determined for tributyrin using methyltetrazolium (MTT)-based assays kit, each with incremental concentrations (0.1mM, 0.25mM, 0.5mM, 1 mM) at 24, 48 and 72 hours (American Type Culture Collection, Manassas, VA, USA). MTT assays were conducted according manufacturer protocols. All experiments were repeated 3 times. The IC50 dose will was used to treat keloid cell lines.

cDNA from total RNA will be obtained using QuantiTect Rev Kit (QIAgene) (QIAGEN, Valencia, CA, USA). The QuantiTect Reverse Transcription Kit provides a fast and convenient procedure for efficient reverse transcription and effective genomic DNA removal.

Each PCR reaction will be set up in a 20-µl final volume containing 2 µl cDNA from each sample. qRT-PCR conducted in triplicate using a TaqMan Universal PCR master mix (Applied Biosystems) in 96-well plates in a 7900HT Sequence detector (Applied Biosystems). Gene expression levels will be normalized to GAPDH (reference gene) expression in each sample.

Results

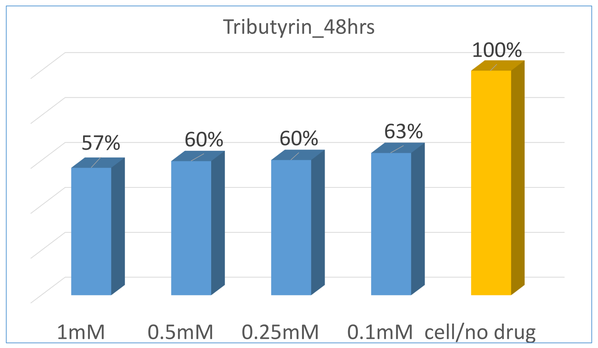

Figure 1: MTT Assay, demonstrating IC50.

gene	mean_diff	STD	paired_t-stat	pValue
COL1a1	0.28	0.35	1.40	0.295
COL3a1	-0.59	0.22	-4.71	0.042
FGF2	-0.64	0.13	-8.60	0.013
GALNT3	0.42	0.61	1.19	0.355
PPP1R13L	0.64	1.43	0.78	0.519
TNS1	-0.05	0.41	-0.19	0.864
VAMP5	0.05	0.35	0.26	0.820

Table 1: Expression of genes without drug, one sample T-tests, keloid versus normal cells.

gene	mean_diff	STD	paired_t-stat	pValue
COL1a1	3.65	1.51	4.18	0.053
COL3a1	-0.89	0.08	-18.78	0.003
FGF2	-0.42	0.07	-9.97	0.010
GALNT3	0.29	0.98	0.51	0.661
PPP1R13L	6.91	1.51	7.92	0.016
TNS1	0.83	0.40	3.61	0.069
VAMP5	-0.11	0.13	-1.44	0.285

Table 2: Expression of genes with drug, one sample T-tests, keloid versus normal cells.

Gene	mean_diff	paired_t_statistic	pValue
COL1a1	10.11	3.84	0.062
COL3a1	-0.90	-3.09	0.091
FGF2	0.67	5.26	0.034
GALNT3	-0.39	-0.54	0.644
PPP1R13L	18.80	13.01	0.006
TNS1	2.64	12.87	0.006
VAMP5	-0.48	-1.25	0.339

Table 3: comparisons of gene expression for keloid cultures with and without treatment

Materials & Methods Cont.

Assays are run in triplicate to avoid inter-assay variability. Data are evaluated using the comparative CT (ΔΔCt) method for calculating relative quantitation of gene expression.18,19 A 2-fold or greater change in mRNA expression will be considered as biologically significant. Post-treatment expression levels of VAMP5, TNS1, GALNT3, PPP1R13-α, COL1, COL3, and FGF2, will be determined by qRTPCR.

Gene expression data for eleven genes from three pairs of samples (one keloid and one normal) from each of three keloid patients was analyzed. The gene expression levels were observed under two conditions: no treatment and treatment with tributyrin. The outcome of main interest for the analysis was the difference in gene expression with and without drug treatment (normalized relative to the normal samples). The gene expression was represented by an RQ value (relative quantitation normalization) using the formula: $RQ=2^{-\Delta\Delta CT}$, where ΔΔCT is the "delta delta CT" value.

Three analyses were undertaken. The first two used one sample (or paired) t-tests to test for keloid and drug effects, respectively for each gene's expression levels. [Note: for the keloid effect tests the null hypothesis is that the keloid RQ is equal to the normal tissue value of 1.0.] The third analysis used one way analysis of variance (ANOVA) to and two sample t-tests to test for differences among the drug effects.

Discussion

1. Tributyrin, prodrug of butyrin, is isolated from natural products such as butter. It decreases human fibroblast proliferation, fibrosis and promotes apoptosis.[20-24]
2. In the literature, the role of *VAMP5* is not understood in keloid formation.[25-28]
3. *PPP1R13L* is involved in promotion of DNA repair, apoptosis, and cell proliferation regulation. Mutations/polymorphisms result in cancer. Overexpression results in P53 inhibition.[14-15, 29-31]
4. *GALNT3* is involved in calcium regulation. Mutations result in decreased or aberrant FGF23, which causes increased renal tubular reabsorption causing hyperphosphatemia, hypercalcemia, and high vitamin D. No relation to keloids or fibroblast has been found in the literature.[32-35]
5. TNS1 protein mediates myofibroblast-mediated matrix formation. A knockout of TNS1 disruption of TGF-β induced myoblast differentiation and reduced extracellular matrix contraction.[36-37]

Conclusion

Currently, there are no markers to predict or guide keloid therapy. As previously described, the failure of adjuvant therapies, despite being based on current clinical, histological, or molecular observations supports the need for better understanding of the pathogenesis of keloid formation. Furthermore, it underscores the heterogeneity of keloid formation and the lack of current treatment to take account of its complexity. By applying our knowledge of epigenetics, keloid specific therapies may be identified because of growing understanding of the molecular pathogenesis of keloids. Keloid-specific methylation markers have the potential to serve as novel treatment targets for keloids. Furthermore, pathway analysis of epigenetic profiles will guide our understanding of epigenetically derived data and lead to novel hypothesis for keloid pathogenesis and treatment. It's not only important to discover one gene, but finding the whole network of players is important, because it expands our potential targets for keloid therapy.[20] This someday may result in patient focused multimodal therapy. Specifically, the outcomes from this project would be a major step forward towards translational research on keloids. The methods and concepts described could change how keloids are treated and prevented in the future.

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