Development and Characterization of STAT3 Y705H Mutated Breast Cancer Cell Lines for Genetic Validation of the Mechanism of Action of STAT3 Inhibitor TTI-101

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Background:

STAT3 signaling has been shown to play a role in almost all aspects of cancer biology including anti-apoptosis, cell transformation, growth and proliferation, angiogenesis, metastasis and cancer stem cell maintenance. A large amount of clinical and preclinical data in solid and hematological cancers supporting STAT3 as a therapeutic target. As a result, there are a number of STAT3 inhibitors in clinical trials and many more in active development. TTI-101 currently in phase 1 clinical trials is a STAT3 inhibitor that competitively inhibits STAT3 activation by directly targeting the pY-peptide binding site within STAT3's SH2 domain, thus blocking key steps in its activation process, starting with the recruitment to activated cytokine and peptide hormone receptors and consequently; cytokine dependent homodimerization, nuclear translocation and DNA binding. In this work we generated and characterized breast cancer cell lines MDA-MB-468 for validation of mechanism of STAT3 inhibitor TTI-101

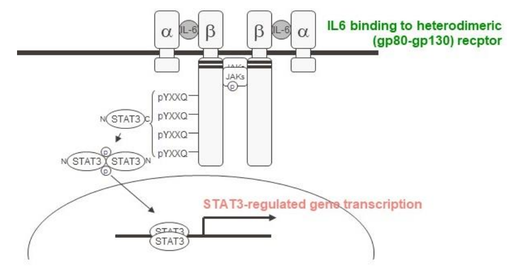

Figure 1. STAT3 Signaling Cascade. The STAT3 signaling pathway is initiated by cytokine binding. Leading to receptor activation, STAT3 recruitment, phosphorylation, dimerization, nuclear localization , DNA binding and ultimately transcriptional activation of specific genes.

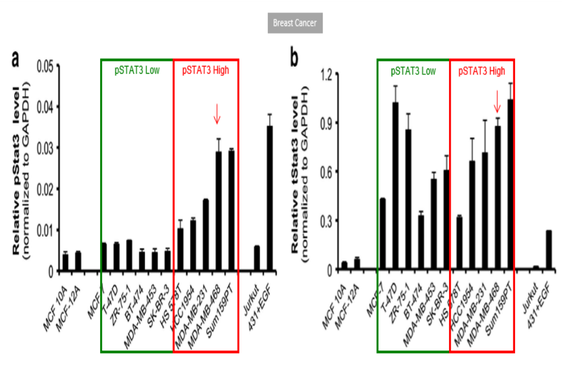

Figure 2. tSTAT3 and pSTAT3 Levels in Breast Cancer Cell Lines. Various cell lines were previously analyzed for tSTAT3 and pSTAT3 activity. The cell line MDA MB 468 shows high levels of both pSTAT3 and tSTAT3, making it a good candidate for further investigation.

Hypothesis:

Our primary hypothesis is that the genetic inactivation of STAT3 by mutating the key Y705 residue to H705 will elicit changes in the transcriptome of breast cancer cell lines similar to those observed with STAT3 inhibitor TTI-101.

Materials and Methods:

15 MDA MB breast cancer cells were cultured in DMEM, 10% FBS at 37° C with an atmosphere of 5% CO_2. Protein expression was analyzed with western blots as well as Luminex Assay. STAT3 activation was also examined using by the TransAM® STAT3 Activation Assay. Immunofluorescence microscopy was used to visualize nuclear translocation. qPCR was utilized to measure the effect of TTI-101 exposure on the expression of STAT3 dependent genes: CCL2, IL8, MUC1, SOC3, and Cyclin D1.

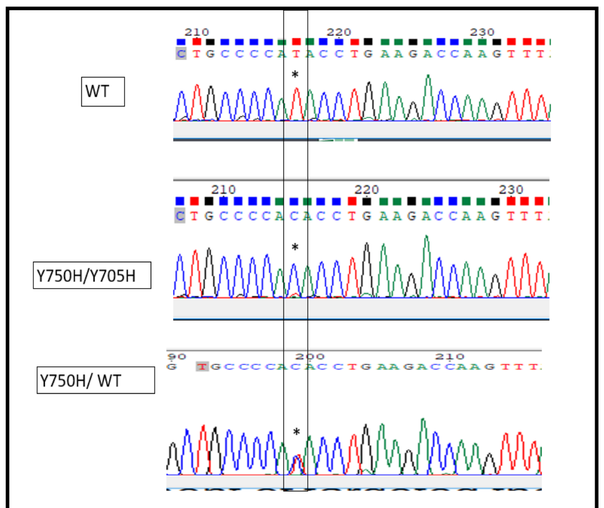

Figure 3. Confirmation of Presence of Y705H Mutation. Chromatogram showing pDNA sequencing results of WT and Y705H cell lines. The Y705H cell line shows a clear mutation from T to C at point 215.

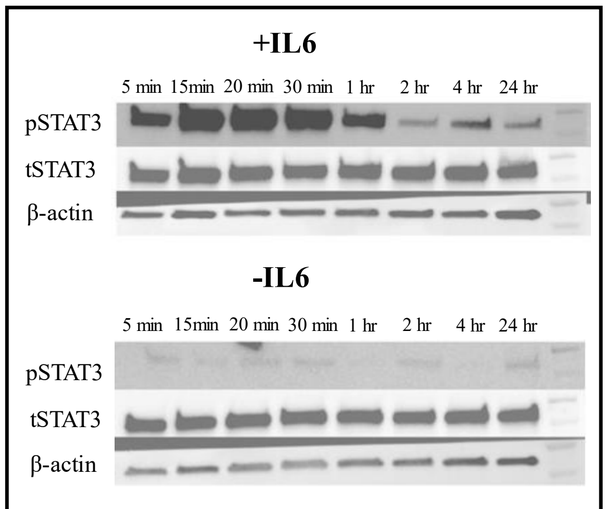

Figure 4. WB of wild type MDA MB 468 Cas9 Control Clone 8 Cells With IL6 Exposure. WT MDA-MB-468 Cells were stimulated with or without IL6 and IL6SR (100ng/ml) at the time points listed above. Protein lysates were analyzed by western blot using antibodies to pSTAT3 and tSTAT3. Cells stimulated with IL6 show an increase in pSTAT3 up until 30 minutes after exposure, where at that point, pSTAT3 decrease. Cells with no IL6 exposure exhibit low, baseline pSTAT3 activity. tSTAT3 stays consistent across all time points regardless of treatment.

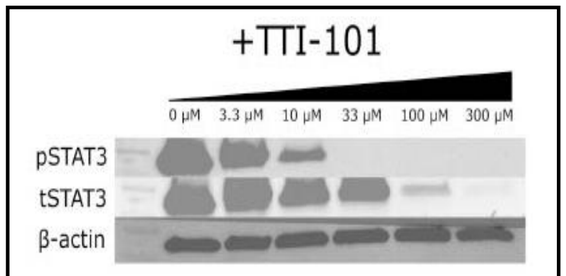

Figure 5. WB of wild type MDA MB 468 Cas9 Control Clone 8 Cells With TTI-101 Treatment. Cells were subject to varying TTI-101 concentrations for 4 hours. They were then stimulated with IL6 for 15 minutes. pSTAT3 expression decreases as the concentration of TTI-101 increases. tSTAT3 expression stays consistent up until 100 µM where there is a sharp decrease in expression.

Results:

CRISPR-Cas9 approach was used to assess the genetic effects of STAT3 blockade. Three different types of MDA MB 468 cell lines were generated; STAT3 knockout (KO), STAT3 Y705H mutants and STAT3 WT controls. The cells were sequenced to confirm changes at the DNA level. Absence of STAT3 protein expression of knockout clones and response to IL6 was confirmed using western blot analysis.

Optimal conditions for IL6 stimulation and TTI-101 treatment were determined using western blot analysis, real time PCR and immunofluorescence. The results show that maximum STAT3 phosphorylation was achieved after 15min of 100ng/ml IL6 treatment. TTI-101 at a concentration of 33uM was shown reduce IL6 dependent phosphorylation by more than 90% after 4Hrs of pretreated. Importantly, levels of tSTAT3 and beta actin remained unchanged at this concentration. Immunofluorescence data showed that Y705H mutated cells prevented STAT3 from translocating to the nucleus in response to IL6 stimulation, showing that nuclear translocation is dependent on phosphorylation at Y705.

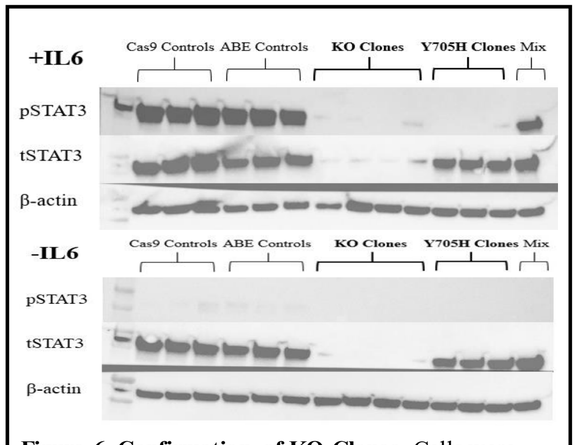

Figure 6. Confirmation of KO Clones. Cells were exposed to IL6 for 15 minutes. STAT3 KO clones, as shown in bolded brackets, show no pSTAT3 or tSTAT3 expression. The Y705H clones show no pSTAT3 activity, while maintaining their tSTAT3 activity.

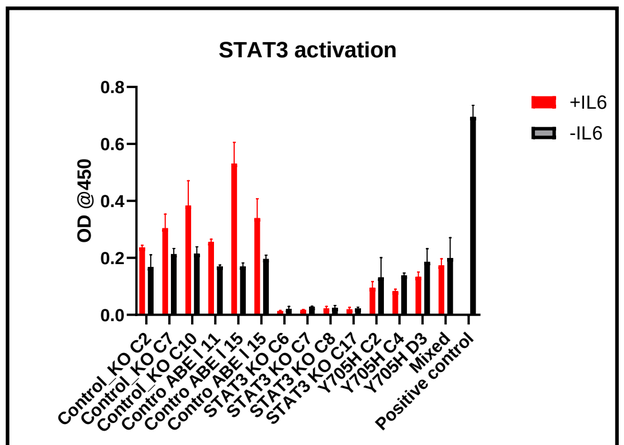

Figure 7. STAT3 Activation Assay. Cells were exposed to IL6 and measured for their STAT3 activation (DNA binding activity). KO and Y705H clones show significantly less STAT3 activation than the controls when exposed to IL6. (Red vs corresponding black bars)

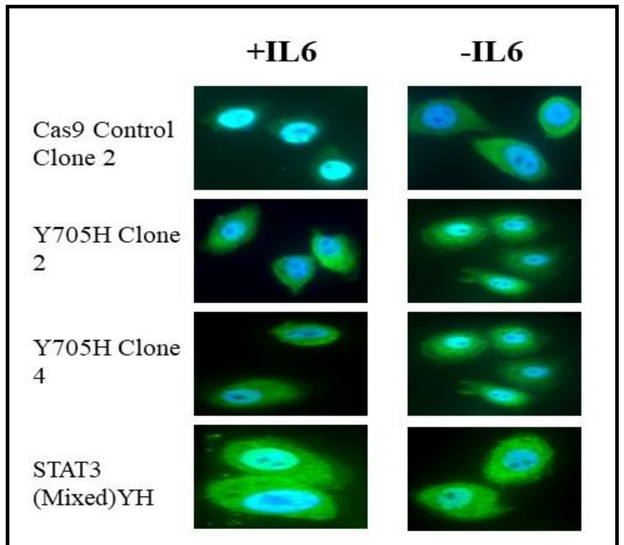

Figure 8. Immunofluorescence Microscopy of Y705H Mutants. All cell lines were exposed to IL6 for 15 minutes and fixed with 4% paraformaldehyde. In all of the Y705H mutants, STAT3 does not translocate to the nucleus, as depicted in green, when exposed to IL6. The control clone shows STAT3 once translocated to the nucleus.

Conclusions

Optimal experimental treatment conditions of IL6 exposure length and TTI-101 concentration were identified. These conditions will guide future experiments to further investigate the impact of TTI-101 on MDA MB 468 cells. The KO Clone identities were confirmed and can be used in larger experiments. Y705H mutants were found to behave similar to KO cells upon exposure. Although, future experiments are needed to determine the exact changes ongoing in the cells.

References

1) Bharadwaj U, et. alTargeting Janus Kinases and Signal Transducer and Activator of Transcription 3 to Treat Inflammation, Fibrosis, and Cancer: Rationale, Progress, and Caution. Pharmacol Rev. 2020 Apr;72(2):486-526.
2) Xu X, Kasembeli MM, Jiang X, Tweardy BJ, Tweardy DJ (2009) Chemical Probes that Competitively and Selectively Inhibit Stat3 Activation.
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4) Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science. 1994 Apr 1;264(5155):95-8.