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Validation of Novel Transcriptional Targets that Underpin CD44-promoted breast cancer cell invasion

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ABSTRACT

Introduction:
Breast cancer (BC) is the most common cancer worldwide, and metastasis is its worst aspect and the first cause of death. Metastasis is a multistep process, where an invasion is a recurring event. The process of BC cell invasion involves three major factors, including cell adhesion molecules (CAM), proteinases and Growth factors.CD44, a family of CAM proteins and the hyaluronic acid (HA) cell surface receptor, acts as cell differentiation, cell migration/invasion and apoptosis regulator.

Rationale:
We have previously established a tetracycline (Tet)-OFF-regulated expression system, both *in vitro and in vivo* (Hill et al, 2006). As a complementary approach, the highly metastatic MDA-MB-231 BC cells expressing high levels of endogenous CD44s (the standard form of CD44), was cultured in the presence and absence of 50 µg/ml of HA. RNA samples were isolated from both cell experimental models, and microarray analysis (12K CHIP from Affymetrix) was applied. More than 200 CD44s transcriptional target genes were identified and were sub-divided into groups of genes based on their function: cell motility, cytoskeletal organization, ability to degrade ECM, and cell survival.

Hypothesis:
Among these 200 identified genes, we selected seven genes (*ICAP-1, KYNU, AHR, SIRT1, SRSF8, PRAD1, and SOD2*) and hypothesized that based on evidence from literature, these genes are potential novel targets of CD44-downstream signaling mediating BC cell invasion.

Specific Aims:
Pursuant to this goal, we proposed the following objectives:

1- Structural validation of *ICAP-1, KYNU, AHR, SIRT1, SRSF8, PRAD1 and SOD2* as novel transcriptional targets of CD44/HA-downstream signaling at both RNA and Protein level using reverse transcription polymerase chain reaction (RT-PCR) and Western Blot respectively.

2-Functional validation of *ICAP-1, KYNU, AHR, SIRT1, SRSF8, PRAD1and SOD2* as novel transcriptional targets that underpin CD44-promoted BC cell migration using wound healing assay after the transfection with siRNA.

LITERATURE REVIEW

Studies stated that CD44 increased BC metastasis into various organs, including , for instance: liver, lung, brain, and bone. It has been reported that the expression of CD44s was suppressed in malignant BC tissues compared to benign breast tissues (McFarlane et al.,2015).
Other studies showed that changing from vCD44 to sCD44 isoforms enhanced the formation of epithelial-mesenchymal transition (EMT). The re-expression of CD44 activated the inhibited EMT phenotype in the CD44-knockdown cells, with decreased expression of epithelial markers and increased expression of mesenchymal markers (Chen, Zhao, Karnad, & Freeman, 2018). In contrast, studies showed that expression of the different CD44 variant isoforms, CD44v3, CD44v4, CD44v6, and CD44v7-8 promoted BC metastasis, produced large tumors, and lowered survival rate (Rizeq et al., 2018), (Mayer et al., 2008).

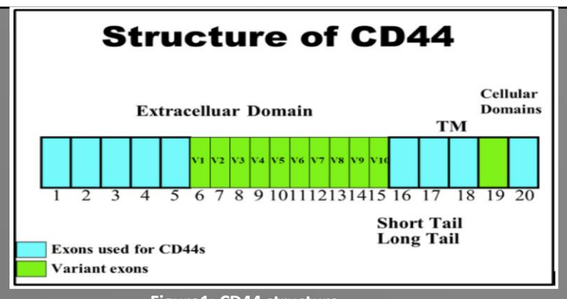

Figure1: CD44 structure

METHODOLOGY

HA Cell Culture
- MDA-231 BC cell line
- control group, the cells were cultured in complete DMEM medium that contains 1% L-Glu, 1% peni./strep., and 10% (FBS).
- treated group were cultured in complete DMEM medium with 100 µg/ml of low molecular weight Hyaluronic acid (HA) for 12 and 24 hrs.

Isolation of RNA samples
- Invitrogen Trizol reagent was used to collect the cell lysate to extract RNA. A nanodrop was used to quantify and identify the concentration and the purity of our RNA. RNA was then stored in -80°C

RT-PCR
- **10 µl RNAto cDNA:** High Capacity cDNA Reverse TranscriptionKit.
- **RT-PCR Optimization:2** µl of 50 ng/µl control cDNA were amplified using 14.5 µl of (AmpliTaq Gold 360 Master Mix kit), 49ºC, 51ºC, 55ºC, 57ºC and 59
- **RT-PCR Process:** 95ºC for 10 minutes. 40 cycles 95ºC for 30 seconds, Tm at 50ºC to 58ºC) for 1 minute each, extension step at 72ºC for 1 minute final extension 72ºC for 10 minutes.

Analysis of electrophoretic bands
- CD44 and GAPDH PCR products were run in a 1.5% agarose gel with 3 µl of SYPR green stain. The gel then was set at 120V for 45 minutes.
- ICAP-1A PCR product was run in a 1.5% agarose gel, while KYNU was run in a 2% gel that contains 5 µl of Ethidium bromide dye. The gel then was set at 70V for 30 minutes
- Observed bands on the gel for each gene at 24hrs post-HA treatment were quantified using the ImageJ software and statistically analyzed using Microsoft Excel. two-tailed Student's t-test comparisons (ANOVA). P values less < 0.001 were considered statistically significant

Statistical Analysis

Western Blot
- Transportation of proteins to nitrocellulose membrane by electrophoresis.
- Block for 1 hr. with 5% non-fat milk
- Probing with primary antibodies first overnight.
- secondary antibody for 1 hr.
- Special reagent was added to enable visualization
- CGS device was used for detecting.

SiRNA Cell Culture
- control group, the cells were cultured in complete DMEM medium that contains 1% L-Glu, 1% peni./strep., and 10% (FBS).
- MDA-231 BC cell line
- Treated group, the cells were cultured in complete DMEM medium that contained lipofectamine reagent for 72 hrs.

RESULTS

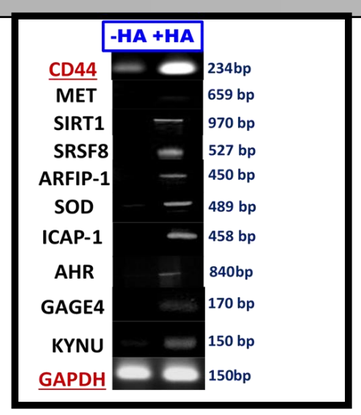

Figure2:RT-PCR gel electrophoresis of CD44 and its potential transcriptional target genes in MDA-231 BC

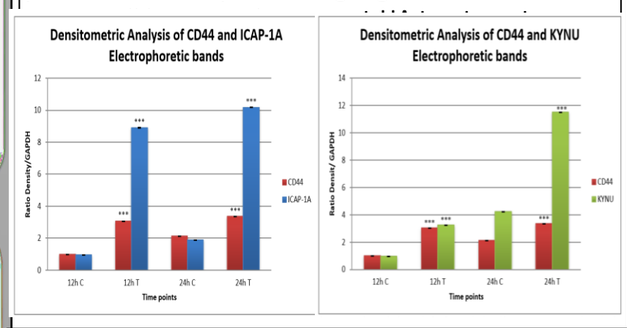

Figure3:Semi-quantitative analysis of CD44 (red) and its target gene ICAP-1A (blue) AND KYNU (green). The difference is considered statistically significant (student's two-tailed t-test, ***P<0.001).

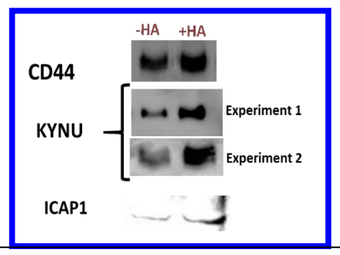

Figure4: Western blot results showing 2 major transcriptional targets of CD44 in MDA-231 BC cell line at 24h post treatment.

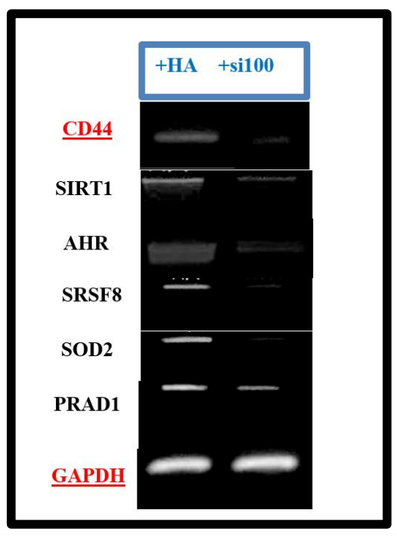

Figure5: RT-PCR gel electrophoresis of CD44 and its potential transcriptional target genes in MDA-231 BC cell line at 72 hours post-siRNA.treatment.

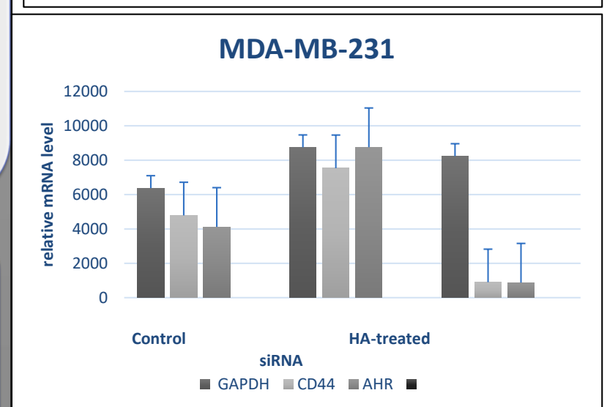

Figure6: Semi-quantitative analysis of CD44 and its target AHR. The difference is considered statistically significant (student's two-tailed t-test, ***P<0.001).

DISCUSSION

According to our results the specified transcriptional targets of CD44/HA are proven structurally by using RT-PCR and Western Bot. Literature review also supports our hypothesis as ICAP-1 activate KRIT1 leading to the activation of NOTCH signalling which in return will activate PI3K/AKT pathway resulting in tumor progression and survival (Stroeken et al., 2006). Similarly KYNU, AHR and SIRT1 interfere in the PI3K/AKT pathway leading to tumor proliferation and metastasis (Ci et al., 2019)(Ye et al., 2018) (Jin, X., Wei, Y., et.al. 2018).

According to (Liu Z, He Q, et.al 2015) SOD2 has the capability to promote both migration and invasion via its interaction with C-MYC protein. Furthermore, PRAD1 which is also known as CCND1 was found to interact with MAPK pathway transforming human embryonic lung fibroblast into malignant tumor.

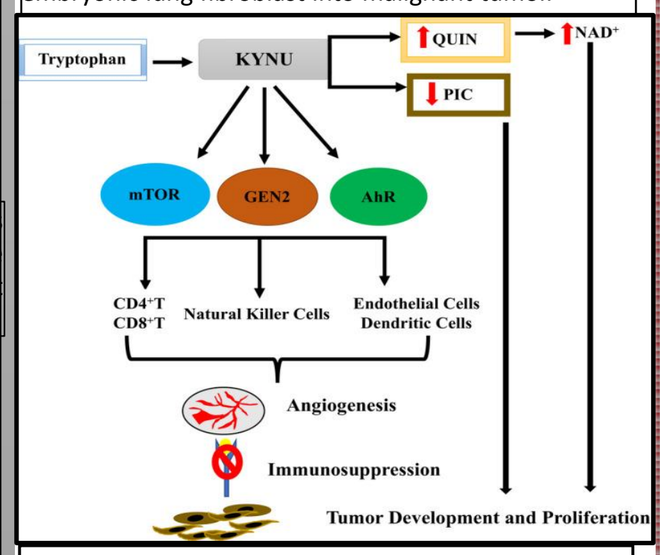

Figure7: KYNU pathways associated with tumor development (Al-Mansoob, M., Ouhtit, A. 2021)

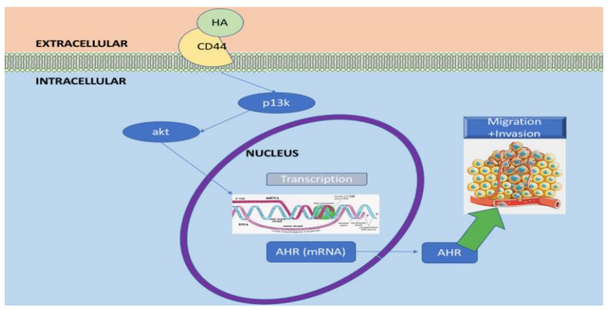

Figure8: AHR mechanisms that promote invasion

CONCLUSION

In conclusion, using semi-quantitative RT-PCR, western blot and siRNA transfection analysis, our study confirmed that the specified targets are potential novel HA/CD44 transcriptional target.
Unfortunately, because of several restrictions, due to COVID pandemic, a number of experiments were not completed.
To further validate these genes, additional in vitro experiments using RT-PCR and western blot analyses on samples isolated from BC cells treated with CD44 siRNA scenario are required. Additional experiments using various other BC cells lines, including normal epithelial breast cells are also needed to validate our results.

REFERENCES

- Hill, A., Mcfarlane, S., Mulligan, K., Gillespie, H., Draffin, J. E., Trimble, A.,Waugh, D. J. J. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene*, 25(45), 6079–6091. doi: 10.1038/sj.onc.1209628
- Al-Mansoob, M., Gupta, I., Stefan Rusyniak, R., & Ouhtit, A. (2021). KYNU, a novel potential target that underpins CD44-promoted breast tumour cell invasion. *Journal of cellular and molecular medicine*, *25*(5), 2309–2314. https://doi.org/10.1111/jcmm.16296