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Silencing of hnRNP A1 and hnRNP A2/B1 Downregulates the Expression of CD44v6 and CD44v10 Exons in Glioma Cells

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Abstract - *CD44 is a cell surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It undergoes alternative splicing to give rise to variety of transcript variants and isoforms. CD44v6 and CD44v10 alternatively spliced exons of CD44 are evidenced to be linked with the progression of various cancers. The expression of transcripts containing these exons is being used as tumor cell markers in cancers. qRT-PCR analysis for expression of CD44v6 and CD44v10 was performed in various glioma cell lines and effect of knockdown of splice factors was examined. We have found that knockdown of hnRNP A1 and hnRNP A2/B1 led to decrease in expression of CD44v6 and CD44v10 exon. Therefore, these splice factors may positively regulate the expression of CD44v6 and CD44v10.*

Key Words: CD44; CD44v6; CD44v10; glioma; qRT PCR; Alternative splicing; hnRNP A1; hnRNP A2/B1

1. INTRODUCTION

CD44 is a single span transmembrane glycoprotein present on cell surfaces. Depending on the amount of glycosylation, their size may vary from 80-200 kDa. They are involved in cell-cell interactions, cell adhesion and migration [1]. In human CD44 protein is encoded by a single highly conserved gene present on chromosome 11 [2]. The CD44 gene comprises of 19 exons with ten of these (1-5 and 15-19) constant in all spliced transcripts. The standard form CD44s which is present in wide range of normal tissue consists only of the ten constant exons. Whereas the other transcripts contain the constant ten exons and at least one or a combination of other variant exons (6-14) [3]. Exon 1-5 of CD44 encode a CLP/Link domain which functions as docking site for components of extracellular matrix. Hyaluronan, an abundant element of the extracellular matrix (ECM), produced by stromal and cancer cells, is the primary ligand for CD44. Exon 15-19 encode the transmembrane region and the cytoplasmic region of CD44 protein [4]. The cytoplasmic region functions by participating in signaling pathways through interaction with actin cytoskeleton. The region between the transmembrane region and the amino terminal link domain is the stem region. This stem region contains the amino acid sequences encoded by the variant exons of the transcript (Fig. 1.) [5].

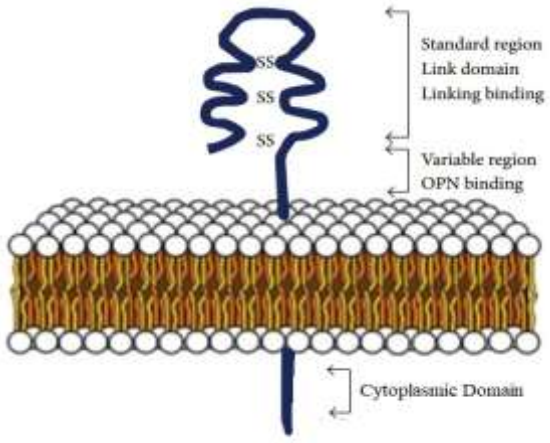

Fig -1: Protein Structure of CD44

CD44v isoforms have additional motifs that confer interactions with ligands in the microenvironment. The variant isoforms may act as co-receptors by binding or sequestering growth factors. Both standard and variant isoforms are present in normal cells. But the level of expression of variant isoforms is relatable with the pathological condition. The expression of variant isoforms is also found to be linked with tumor progression in cancer [6]. Many studies favor that differentiation of Cancer Stem Cells (CSCs) after asymmetrical divisions lead to formation of tumor mass [7]. CD44v6 is noted to have Changed expressions in various cancers. CD44v6 isoforms have been shown to interact with c-Met and its ligand and

thereby altering the signaling to promote cancer cell invasion. In addition, CD44v6 also recruits ERM (ezrin/radixin/moesin) proteins which in turn lead to angiogenesis due to interactions with VEGFR (Vascular Endothelial Growth Factor Receptor). Modulation of these signaling pathways may have an important role in mediating tumorigenic properties of tumor cells leading to tumor progression, metastasis and chemo-resistance [8]. CD44v10 exon when bound to HA induces interactions with Rho-Kinase (ROK) which then phosphorylates IP3 receptors. Phosphorylated IP3 induces internal Ca^{+2} release leading to activation of cytoskeleton and hyaluronan-mediated endothelial cell migration [9].

We have analyzed quantitatively the mRNA expression of CD44v6 and CD44v10 exons by performing qRT-PCR on cDNA samples from three siRNA treated samples of U87 cell lines. SiRNA stands for small interfering RNA, a class of double stranded RNA molecules that operate through RNA interference pathway to degrade mRNA and prevent transcription. The si-RNA used were for 2 splice factors hnRNP A1 and hnRNP A2/B1.

2. METHODOLOGY

2.1 SiRNA Transfection

A day after the plating of glioma cells, they were transfected with siRNA against hnRNP A1 andhnRNP A2/B1 as per Santa Cruz Inc. protocol (Santa Cruz Inc.). After 48 hours from transfection, lysates were made with Triton X-100 buffer.

2.2 Isolation of RNA from siRNA Treated Cell Lines

U87 are glioblastoma cell line used in brain cancer research. Total RNA from three siRNA treated U87 cells were used. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used for extraction of total RNA, and the first-strand cDNA was generated using random hexamer and superscript II (Invitrogen, Carlsbad, CA, USA).

2.3 Primer Design and Synthesis

Primers for qRT-PCR were designed using CD44 mRNA sequences from NCBI accession no. NM_000610.3. To design primers the nucleotide sequence of CD44v3, CD44v6 and CD44v10 was uploaded in Primer-Blast [10]. From the primer blast results, the primer having suitable GC content and Tm was chosen. For efficient amplification in qRT-PCR cycles the amplicon length of 100bp to 200bp was chosen. The primer only showing predicted amplicons on intended target i.e. having high specificity was chosen. Table-1 shows the primers used for qRT-PCR analysis and their respective amplicon size. All the primers used for qRT-PCR analysis were synthesized by IDT (Integrated DNA Technologies).

Table -1: Primer sequences

Primer		***Sequences 5' to 3'***	***Length***	***Amplicon***
GAPDH	Forward	AACGGGAAGCTTG TCATCAATGGAAA	26	194bp
	Reverse	GCATCAGCAGAGG GGGCAGAG	21	
CD44v6	Forward	AACGGAAGAAAC AGCTACCA	21	100bp
	Reverse	CCCTGTTGTCGAA TGGGAGT	20	
CD44v10	Forward	AGTGAAAGGAGC AGCACTTCA	21	131bp
	Reverse	ACATCATTCCTAT TGGTAGCAGGG	24	

2.4 Quantitative Real-Time PCR

Quantitative Real-Time PCR uses fluorescent reporters to determine absolute amount of a known sequence in a sample. In qRT-PCR, DNA amplification is monitored at each cycle of PCR. When the DNA is in the log linear phase of amplification, the amount of fluorescence increases above the background. A positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. EvaGreen® Supermix- Bio-Rad was used for SYBR Assay with cDNA and primers in 7300 thermocycler (Applied Biosystems). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene served as an internal control. Thermal cycling consisted of a warm-up step of 2 min at 50°C and initial denaturation at 95°C for 10 m, followed by 40 cycles of each polymerase chain reaction (PCR) step: 95°C for 15 s and 60°C for 1 m.

2.5 Gel Electrophoresis

After quantitative real-time PCR products were run on a 2% agarose gel (Fig. 2.). TE (Tris EDTA) buffer was used for gel electrophoresis. 2% agarose was added to TE buffer and solution was heated till it became transparent. Then, it was left to cool down till it became bearable to touch. 5 micro-liter of EtBr (Ethidium Bromide) was added to the solution. The solution was then poured to the casting tray, attached with the comb and left for solidification. After solidification the gel was transferred to the buffer tank and DNA samples were loaded in the wells. The gel was run at 100V for one hour and the bands were analyzed in Gel Documentation Apparatus.

3. RESULTS

In order to compare expression levels of CD44v6 in all the siRNA treated cells, the n-fold values were calculated from cycle-threshold values (Chart-1). The expression of CD44v6 was the highest in non-knockdown(control) cells, while knock down of splice factors resulted in lower expression of exon v6. Similar results were obtained with CD44v10 exon (Chart-2), with non-knockdown cells having the highest expression.

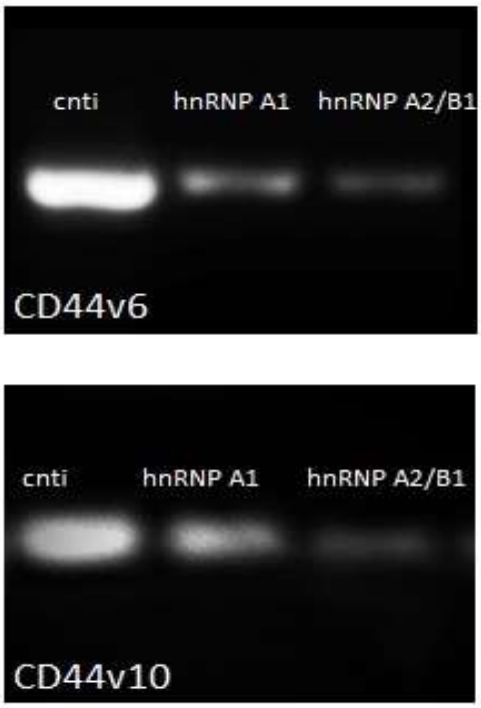

Fig -2: Images of gel after quantitative real-time PCR amplification of CD44v6 and CD44v10 exons from control and hnRNP A1 and hnRNP A2/B1 siRNA treated cells.

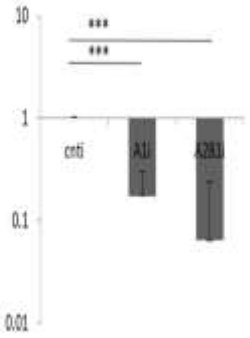

Chart -1: Graphical representation of n-fold of CD44v6 exon in control, hnRNPA1 and hnRNP A2/B1 silenced samples

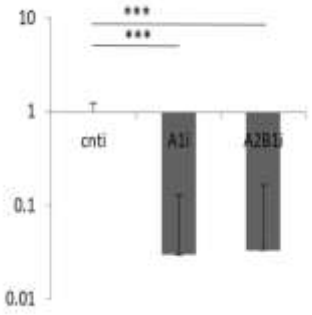

Chart -2: Graphical representation of n-fold of CD44v10 exon in control, hnRNPA1 and hnRNP A2/B1 silenced samples

4. CONCLUSION

It was observed that knockdown of splice factors hnRNP A1 andhnRNP A2/B1 resulted in decrease in expression of CD44 variant exons. When these splice factors were not silenced the expression of CD44v6 and CD44v10 was highest. Therefore, these splice factors may be responsible for expression of these variant exons.

REFERENCES

[1] Ponta, Helmut, Larry Sherman, and Peter A. Herrlich. "CD44: from adhesion molecules to signalling regulators." Nature reviews Molecular cell biology 4.1 (2003): 33-45.

[1] U GONTHERT. CD44: A Multitude of Isoforms with Diverse Functions. Current Topics in Microbiology and Immunology, Vol. 184 © Springer-Verlag Berlin' Heidelberg 1993

[2] Chen Chen, Shujie Zhao, Anand Karnad, and James W. Freeman. The biology and role of CD44 in cancer progression: therapeutic implications. Journal of Hematology & Oncology (2018) 11:64

[3] Bajorath, J□gen. "Structure and function of CD44: characteristic molecular features and analysis of the hyaluronan binding site." Mammalian Carbohydrate Recognition Systems. Springer, Berlin, Heidelberg, 2001. 85-103.

[4] Thapa, Ranjeeta, and George D. Wilson. "The importance of CD44 as a stem cell biomarker and therapeutic target in cancer." Stem cells international 2016 (2016). [6] Nawwaf S. Basakran, BMSc, MSBs. CD44 as a potential diagnostic tumor marker. Saudi Med J 2015; Vol. 36 (3): 273-279

[5] D. G. Tang. Understanding cancer stem cell heterogeneity and plasticity. Cell Research, vol. 22, no. 3, pp. 457–472, 2012.

[6] A. Kikuchi. Regulation of beta-catenin signaling in the Wnt pathway. Biochem. Biophys. Res. Commun. 268 (2000) 243–248.

[7] Jijiwa, Habibe Demir, Snehalata Gupta, Crystal Leung, Kaushal Joshi1, Nicholas Orozco3, Tiffany Huang, Vedat O. Yildiz, Ichiyo Shibahara, Jason A. de Jesus, William H. Yong, Paul S. Mischel, Soledad Fernandez, Harley I. Kornblum, Ichiro Nakano. CD44v6 Regulates Growth of Brain Tumor Stem Cells Partially through the AKT-Mediated Pathway. PLOS ONE Published: September 6, 2011. https://doi.org/10.1371/journal.pone.0024217

[8] Patrick A. Singleton, Lilly Y.W. Bourguignon. CD44v10 interaction with Rho-kinase (ROK) activates inositol 1,4,5-triphosphate (IP3) receptor-mediated Ca2+ signaling during hyaluronan (HA)-induced endothelial cell migration. Cell Motility and the Cytoskeleton 53:293–316 (2002)

[9] Ye, Jian, et al. "Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction." BMC bioinformatics 13.1 (2012): 134.