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The role of Exostosin Glycosyltransferase 1 (EXT1) in Ovarian Cancer

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INTRODUCTION & AIM

Exostosin Glycosyltransferase 1 (EXT1) is a glycosyltransferase involved in the elongation of the saccharide chain of heparan sulfate proteoglycans (HSPGs). HSPGs are important components of the extracellular matrix (ECM) and the cell surface. The integration of results using the PathScore algorithm identified significant enrichment of gluconeogenesis, Heparan Sulfate Glycosaminoglycans (HSGAGs) biosynthesis in refractory patients, indicating a role of ECM remodeling and metabolic flexibility in recurrence. The EXT1 gene, involved in HSPGs biosynthesis, was frequently amplified in TCGA samples, particularly in refractory patients. The ECM profile has been extensively studied in high-grade serous ovarian cancer (HGSOC), but data on the involvement of HPSGs in platinum resistance are still incomplete. This study aims to elucidate how alterations in EXT1-mediated HSPG biosynthesis affect platinum resistance and cancer cell behaviour in HGSOC [1,2].

METHOD

➢ Cell Line Generation and reagents

OVCAR-429 and MDAH-2774 cells were cultured in DMEM supplemented with 10% FBS and 100 units/ml penicillin/streptomycin. Lentiviral particles were prepared using the human EXT1(NM _000127) MISSION shRNA set and mission non-target control transduction virus (SHC002V) (Sigma-Aldrich). The lentiviral particles were packaged into HEK293T cells, transfecting 10 µg sh vector, 10 µg pCMV-deltaR8.91, and 4 µg pCMV-VSVG. Supernatants collected at 24 and 48 hours post-transfection were filtered and used directly for infection cycles with 8 µg/ml polybrene. OVCAR429 transduced cells were selected with 1µg/ml puromycin (Invitrogen). OVCAR-429 control cells (SCR) and two different silenced clones (#995 and #997) were used for experiments. MDAH2774 were transduced with 5ug EXT1 DDK plasmid (Origene) in the presence of Fugene HD and stable expressing clones were selected with antibiotic G418. Heparan sulfate (HS) is synthesized as the glycosaminoglycan component of heparan sulfate proteoglycans (HSPGs). It is expressed on the cell surface of virtually all cell types and basement membranes in mammals. It displays specific interactions with many biologically active proteins and, thus, is involved in many important biological processes. Clone F58-10E4 HS antibody is used to stain cells that react with many types of heparan sulfate proteoglycans. It is essential for the reactivity of 10E4 for N-sulfated glucosamine residues to be present in the structure of heparan sulfate. Its reactivity is eliminated by Pre-treatment with heparinase III (10mU/ml) for 1 hour at 37ºC.

➢ Transcriptomic Analysis

RNA library preparation was manually performed using the Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel (Thermo Fisher Scientific), according to the Ion AmpliSeq Library Kit Plus. Briefly, 10 ng of RNA was reverse transcribed with SuperScript™ Vilo™ cDNA Synthesis Kit (Thermo Fisher). The resulting cDNA was amplified to prepare barcoded libraries using the Ion Xpress™ Barcode Adapters. Barcoded libraries were combined to a final concentration of 50 pM and loaded on Ion 540™ Chips. Sequencing was performed on the Ion GeneStudio S5™ System with Torrent Suite™ Software v5.14.0 (Thermo Fisher Scientific). Differential gene expression (DEG) was analyzed using Transcriptome Analysis Console Software, version 4.0.3.14 (Thermo Fisher), applying the following threshold values: fold change < -1.5 and $> +1.5$, p-value < 0.05, and false discovery rate (FDR) adjusted p-value < 0.15. Gene Set Enrichment analyses were carried out using java software 'GSEA'. GSEA terms associated with an adjusted p-value < 0.05 were considered to be significantly enriched [3-4].

➢ Migration and Time Lapse Microscopy

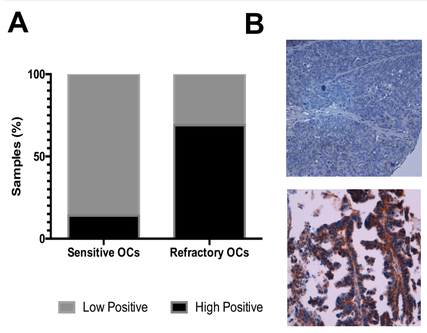
Cells were plated in a 6-well Multiwell Plate. Wounds were made in the cell monolayer with a 200ul pipette tip, and images were taken at regular intervals to track cell migration and quantify migration rate every 10 minutes for 24 and 72 hours using a Thunder Dmi8 (Leica Microsystems) equipped with a camera. Cell motility and migration were tracked using image analysis software to assess the effects of EXT1 manipulation.

➢ MTT Assay

Cells were seeded at 5000 cells/well in triplicate and assessed daily using the MTT assay. After incubation with MTT reagent (5 µg/mL, Sigma-Aldrich) at 37° C for 3 hours, formazan, indicating cell viability, was solubilized with 100 µL Isopropanol per well. Formazan production was measured using a microplate reader (Multiskan FC) at 570 nm, with 690 nm as the reference wavelength. The assay was performed on OVCAR-429 clones (SCR, #995, #997) treated with Oxaliplatin (30µM) for 48 and 72 hours. Similarly, EXT1 overexpressed MDAH-2774 clones (PC-DNA3 #9 and EXT1 #4) were treated with Oxaliplatin (25µM, 50µM) for the same duration.

RESULTS & DISCUSSION

EXT1 PROTEIN IN OVARIAN CANCER TISSUES



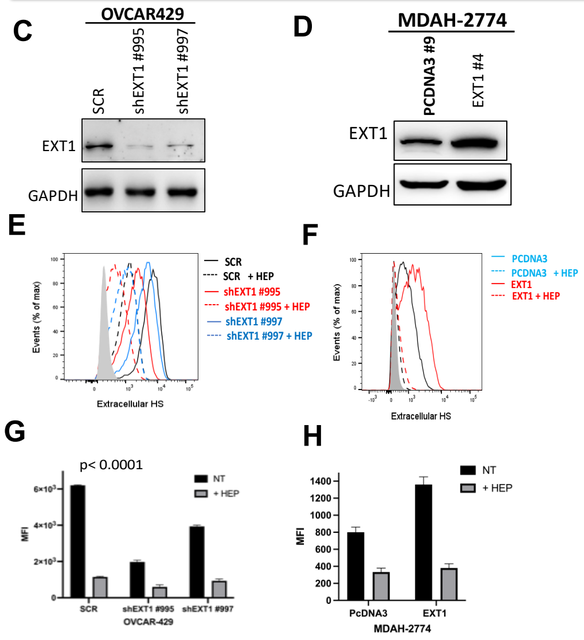
Since EXT1 resulted the most frequently amplified gene of TCGA in refractory patients, we investigated the levels of EXT1 protein by Immunohistochemistry on a Tissue MicroArray: EXT1 expression was high in 69% of Refractory patients respect to 14% of Sensitive patients This result prompted us to further investigate the role of EXT1, as a biomarker for therapy response.

Protein levels of EXT1 in OCs.

Panel A. The level of EXT1 protein was assayed by IHC in Refractory (N=13) and Sensitive patients (N=21) ($p<0.005$).

Panel B Representative immunostainings of EXT1 in low positive (upper panel) and high positive tumors (lower panel) (Magn. 20x).

EXT1 MODULATES THE EXPRESSION OF HEPARAN SULFATES

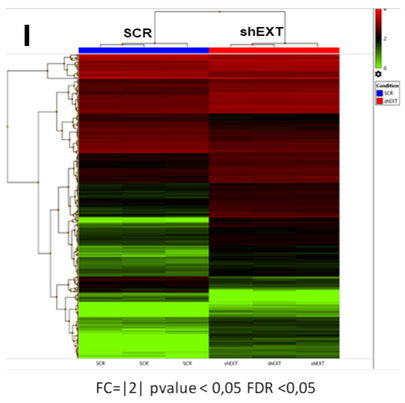


EXT1 silencing in the OVCAR-429 cell line is shown in **Panel C**, while EXT1 expression in MDAH-2774 is shown in **Panel D.** The plot in **Panel E** shows the surface expression of HS in OVCAR429 shEXT1 #995 (**red** lines) and shEXT1 #997 (**blue** lines) compared to the control SCR cells (**black** lines), while **Panel F** shows the staining of heparan sulphates in MDAH-2774 control cells (**sky blue** lines) and EXT1 overexpressing cells (**red** lines). The dashed lines represent cells treated with heparanase. The grey solid peak represents cells stained with the secondary antibody used as a control.

The graph in **Panel G** shows the mean fluorescence intensity (MFI) of HS staining of OVCAR-429 and its derivatives, in **Panel H** the staining intensities in MDAH-2774.

A reduction of heparan sulfates at the cell surface is observed in OVCAR-429 in which EXT1 expression was suppressed, while increased levels are observed in overexpressing cells (MDAH-EXT1). Treatment with heparinase (HEP) restores the heparan sulphates level of control cells.

CHANGES IN HEPARAN SULFATES AFFECTS GENE EXPRESSION IN OVARIAN CANCER CELLS

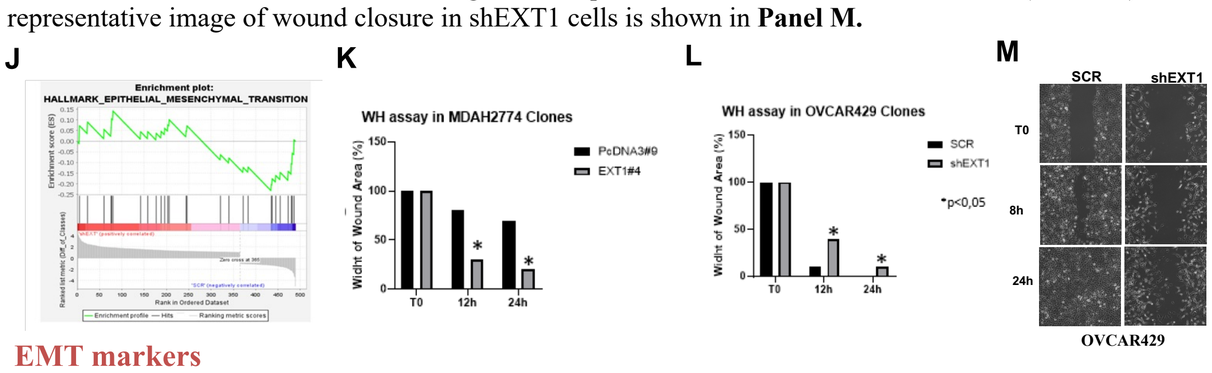


Gene	FC UP
DHRS3	22,12
CP	20,63
TNFRSF9	18,87
CDH6	16,31
IL33	14,78
CSF3	14,7
CSF2	11,05
IDO1	9,99
KCNK3	9,59
TNFAIP6	9,36
Gene	**FC Down**
LCN2	-29,96
CACNA2D1	-9,96
EDN1	-9,55
TAGLN	-8,25
TGFB2	-5,17
EXT1	-4,99
DYSF	-4,97
NGF	-4,69
KCNJ8	-4,61
THBS1	-4,54

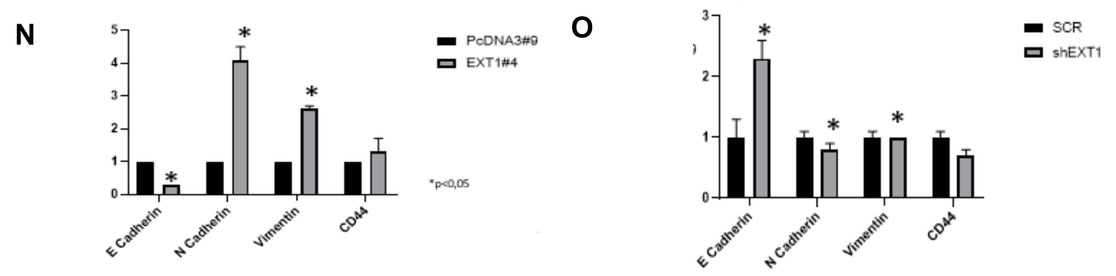
We performed a gene expression analysis of cells in which EXT1 was knocked down (shEXT1) vs control cells (SCR).Our transcriptomic analysis revealed that cells with reduced EXT1 expression (shEXT1) exhibited differential expression of 323 genes, of which 241 genes upregulated and 82 genes downregulated. The top 10 upregulated and downregulated differentially expressed genes in shEXT1 cells are displayed in the **Panel I.**

GENE ONTOLOGY ENRICHMENT ANALYSIS

Gene Ontology enrichment analysis highlighted that one of the most significantly ($p < 0.05$) downregulated categories in the shEXT1 cells was the Epithelial to Mesenchimal Transition (EMT) **(Panel J).** On this basis we performed a migration assay using Time Lapse Microscopy. We found that EXT1 overexpressing cells had almost closed the wound after 12 hours (**Panel K**). In contrast, OVCAR-429 cells in which EXT-1 was downregulated required 24 hours to close the wound (**Panel L**). A representative image of wound closure in shEXT1 cells is shown in **Panel M.**

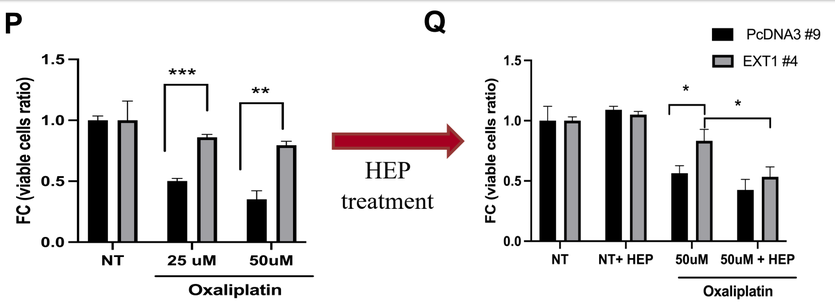


The mRNA levels of EMT markers in the overexpressing cells MDAH-2774 (**Panel N**) and the EXT1 depleted cells, OVCAR-429 (**Panel O**) were assessed to analyze the migratory capacity of cells.

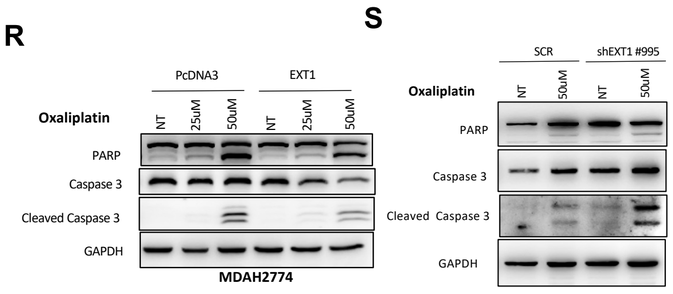


EXT1 AFFECTS DRUG SENSITIVITY IN OVARIAN CANCER CELLS

Overexpressing cells MDAH-2774 EXT1 #4 (**Panel P**) are more viable following treatment with increasing concentrations of Oxaliplatin compared to PcDNA-3 #9. Heparanase treatment was able to restore sensitivity to platinum drugs in EXT1 clones.



Western Blot analysis of apoptotic markers, such as Caspase 3 and PARP (**Panel Q**), showed that they were less cleaved in EXT1 overexpressing clones. On the Contrary, OVCAR-429 shEXT1 cells were more sensitive to treatment with 50uM Oxaliplatin **(Panel S),** according to the cleavage level of Caspase 3.



CONCLUSION

- Elevated EXT1 protein levels in refractory patients compared to sensitive patients, suggesting its potential as a biomarker for response to therapy.
- Ext-1 encodes an enzyme required for chain elongation and synthesis of HS. HSPGs interact with many proteins, including growth factors, chemokines, and structural proteins of the extracellular matrix, to influence cell growth and the response of cells to the tumor microenvironment.
- Ext-1 influences the migration rate of cancer cells and resistance to platinum drugs.

FUTURE WORK / REFERENCES

- Further analysis will shed light on the role of EXT1 in ovarian cancer tumorigenesis and drug resistance in order to identify new putative cancer biomarkers and therapeutic targets.

References
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