Research On Correlation Of Notch Signaling Pathway In The Prognosis Of Breast Cancer

The cancer stem cell (CSC) hypothesis is regarded as a reasonable explanation for the rapid multiplication of cells in the disease. They are self-sufficient cells which seem to have increase tumorigenicity and self-renewal, which allow them to expand more rapidly than normal cells. While the topic of the CSC is debated and not yet completely understood, it is clear that cancer cells are supported by a number of signaling mechanisms which may be paracrine, like Wnt/beta-Catenin signaling, or by cell to cell signal, such as Notch.

The Wnt (Wingless/Integrated) signaling pathway is an evolutionarily conserved signaling pathway. It plays a role in processes vital for survival like embryonic development, tissue regeneration in high turnover organs like the skin, gut and in the development of pluripotent stem cells like hematopoietic stem cells, but also in tumorigenesis. In the canonical pathway, the absence of Wnt ligands leads to the phosphorylation of the beta catenin by GSK3, which leads to the beta catenin being degraded by the proteasomes. When Wnt ligands are present, they bind to the Frizzled (Fzd) receptors, recruit kinases and the Dishevelled (Dvl) protein, which inhibits the degradation of beta catenin. This results in translocation of beta catenin to the nucleus, initiating replication. Almost 50% of breast cancers, including triple negative breast cancer, have been shown to have increased beta catenin levels due to active Wnt signaling caused by overexpressed Wnt signaling receptors and molecules. Thus, regulating the canonical Wnt signaling pathway for controlling cancer proliferation has become a major focus.

The Notch signaling pathway consists of four Notch receptors, named Notch, which are activated by the binding of one of their five major ligands, Delta-like ligand (DLL1), Delta-like ligand (DLL3), Delta-like ligand 4 (DLL4), Jagged-1 (JAG1) and Jagged-2 (JAG2) from an adjacent cell. This leads to ?-secretase induced cleavage of Notch Intracellular Domain (NICD), which is translocated into the nucleus, interacts with CSL using the Mastermind (MAM) co-activator and initiates transcription. Notch signaling was first found to play a role in cancer in acute lymphoblastic leukemias, following which it was found to play a role in numerous other cancers, including breast cancer.

Numerous studies have been done studying the correlation of Notch signaling pathway in the prognosis of breast cancer. Notch also interacts with other signaling pathways like the Wnt pathway, which further increases its usefulness for cancer cells. Thus, inhibition of Notch signaling has been tested to block the pathway and abrogate the multiplication of cancer cells by a variety of methods including antibodies 18, ?-secretase inhibitors.

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This study used three Wnt signaling inhibitory proteins, namely, Wnt Inhibitory Factor-1 (WIF1), Secreted Frizzled Related Protein 2 (SFRP2) and Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1), and one Notch signaling protein, Jagged-1 (Jag1), for being expressed by the adeno-associated viral vectors serotype 6 (AAV-6) as decoys to block the signaling in vitro in triple negative breast cancer cells. A decoy involves the use of the binding domain of a receptor/protein, without the signalling apparatus. This acts as an inhibitor by binding the ligand and preventing it from binding to the actual receptor. However, there are certain drawbacks in the use of decoys like the expression of decoy proteins as a single domain. To address this problem, collagen domains of C1qTNF3 were used, which trimerize and help increase the expression levels of the decoys.

Methods

1. Molecular cloning: cDNA of human DKK1, WIF1, JAG1 and SFRP2 were ordered from DNASU (Arizona). The plasmids were digested using restriction enzymes. PCR products of above genes and AAV vector CTR1 (containing the Inverted terminal repeat/ITR sequence) were digested with the restriction enzymes BamH1 and EcoR1, and purified using gel extraction. Ligation products were then transformed into DH5? competent cells. Glycerol stocks were made and frozen for each of the constructs. Minipreps using QIAGEN miniprep kit were performed and verified by digestion and sequencing. Maxipreps were performed using QIAGEN maxiprep kits and glycerol stocks from the miniprep samples.
2. Transfection: HEK293T cells were used for transfection. They were cultured in Dulbeco's Modified Eagle Medium (DMEM) (ThermoFisher, Waltham, MA) containing 10% fetal bovine serum (FBS) and 1% PenStrep a day prior, to obtain ~80% confluency of cells at time of the transfection. Polyethylenimine (PEI) was used for transfecting the cells and the plate was incubated at 37°C overnight. The media was changed after 16 hours. The cells and conditioned media were collected after 24 hours. The cells were resuspended using 500 µl Phosphate Buffer Saline (PBS). The cells were lysed using sonication, with amplitude of 70 for 12 seconds in 1 second pulses.
3. Western blot: Cell lysate sample were prepared with reducing agent. Conditioned media were prepared both with and without reducing agent. SDS-PAGE was performed with 10% Bis-Tris gel and MOPS buffer. The proteins were transferred to a PVDF membrane at 100V for 50 minutes. The membrane was then blocked using 0. 5% Casein-PBS buffer solution to block the non-specific sites. 2 µl of mouse anti-FLAG antibody M2 at 1/2000 dilution was incubated with the membrane for 1 hour at room temperature. The membrane was then washed once using 100 ml of TBS-TWEEN 20 buffer and 0. 01% Sodium dodecyl sulphate (SDS), followed by two washes using only TBS-TWEEN20 buffer. This was followed by incubation with 1/5000 diluted AlexaFluor 680 goat anti-mouse antibody in 50 ml of PBS-casein buffer with 0. 01% SDS. The membrane was

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developed using Li-Cor Odyssey.

4. AAV/minivirus production: DNA of DKK1, WIF1, JAG1 and SFRP2 were co-transfected with AAV6 capsid helper plasmid into HEK293T cells at 70% confluency in a six well plate. Polyethylenimine was used to transfect a total of 2. 7µg of DNA, 1. 8 µg of the helper plasmid (containing Rep and Cap sequences for AAV6) and 0. 9 µg of the protein DNA onto HEK293T cells. The supernatant, which contains the minivirus was collected after 72 hours.
5. Transduction: For the in vitro triple negative breast cancer (TNBC) experiment, MDA-MB231 cancer cells were used. The cells were grown using DMEM with 10% FBS. Each well contained HEK293T cells with 2 mls of DMEM. Transduction of the virus was performed in six well plates. 100 µl of minivirus was added to the cells. They were then incubated for 48 hours following which 1 ml of the media was removed and replaced with 1 ml of fresh media for each well. The plate was then incubated for another 48 hours.

Results

1. Cloning: All the genes (DKK1, WIF1, JAG1, SFRP2) were successfully cloned into the CTR1 vector. The ligation of the desired genes of interest were confirmed using digestion and sequencing (data not shown). The vectors were then transfected into the HEK293T cells for studying the expression levels of each of the proteins. Western blot was performed to study the same, in cell lysate as well as media. Clear, observable overexpression of the desired proteins, namely, DKK1, WIF1, JAG1 and SFRP2 were seen in both, the HEK293T cell lysate and the reduced media. The calculated molecular weights were 44 kDa for DKK1, 57 kDa for WIF1, 65 kDa for JAG1 and 50 kDa for SFRP2. The cell lysates and the media exhibit overexpression of all the proteins due to trimerization of the collagen domain.
2. Minivirus production: Since the desired proteins were being overexpressed, miniviruses were generated for each of the desired proteins. The supernatant of each well should contain the miniviruses for each protein. The supernatant was collected in 1. 5 ml microcentrifugation tube.
3. Cancer cell transduction: The miniviruses for each of the proteins, DKK1, WIF1, JAG1 and SFRP2 were used to transduce into the MDA-MB231 triple negative breast cancer cells. The level of expression of the proteins was assessed using western blots. No observable expression of proteins was seen from the MDA-MB231 cancer cells. The transduction of the minivirus will be repeated.

Discussion

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The role of signaling pathways like Wnt and Notch in tumorigenesis has been widely studied. DKK1, WIF1 and SFRP2, all inhibit the Wnt signalling pathways. DKK1 binds to the LRP6 co-receptor while WIF1 and SFRP2 both bind to the Wnt at different sites 20. JAG1 on the other hand, is presented to the Notch receptors by an adjoining cell. Hence, in this study, based on the aforementioned information, AAV vectors were used to deliver genes coding for different inhibitors and ligands, as decoys for binding with the receptors in the Wnt and Notch pathways on the triple negative breast cancer cells. AAV serotype 6 was used as the vector as it has been shown to have better transduction in the MDA-MB231 cells. The desired genes were subcloned into a CTR1 vector, containing the ITR sequences. The collagen domain was used to increase the expression of the proteins. Since the collagen domain is capable of trimerizing, it was incorporated into the vector. This was further verified by overexpression in the HEK293T cells. The plasmids for each protein was then co-transfected into HEK293T cells with the helper plasmid containing the Rep and Cap sequences for AAV6. While the major gene modification is happening within the cells, there is a limited release of virions into the media by the HEK293T cells. This is termed as "minivirus". The minivirus for each protein was collected and used to transduce the MDA-MB231 triple negative breast cancer cells.

The next step in the study is to determine if the miniviruses can induce release of the proteins. To study this, western blots on the supernatant collected from the transduced cancer cells. This will be followed by binding assays and functional activity assays to check the affinity and effectivity of the proteins in blocking the signalling pathways. The final goala is to deliver decoy proteins in a non cell autonomous fashion to the tumor microenvironment. Also, we wish to deliver a cocktail of the decoy proteins and find efficient combinations for the same, in order to effectively inhibit cancer signaling. While numerous strides have been made in the field of cancer research, including checkpoint blockade, neoantigen studies and CAR-T cells, there is no cure for it because of the plethora of tactics used by cancer to avoid being cleared.

Signalling pathways are one such tactic for cancer genesis and evasion, since blocking just one pathway is ineffective in treating cancer. Thus, creating decoys for blocking multiple signalling pathways is essential and could play a major role in cancer therapy in the future.

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