Testing Gb3(CD77) Expression Level in Cancer Cells

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Abstract: Purpose: Pancreatic cancer and colon cancer are common cancer types that are lethal. This study tries to find the expression pattern of CD77 in cancer cells and investigate whether STxB-SN38 can be used for targeting therapy in Pancreatic cancer and colon cancer. STxB was proved to have high specificity of binding to CD77, and STxB is very efficient at cell killing. Methods: The experiments will use know human cell lines, ATCC and DSMZ. Flow cytometry is used for monitoring cell proliferation and counting. AnnexinV/PI will be used for killing measuring. Possible Results: There are 27 possible results Conclusion: The result of our study will contribute to future clinical trials of Stxs-SN38 targeted therapy. Future studies should focus on eliminating wrong pathways that Stxs-SN38 could kill normal cells. Detecting cancer at an early stage is still an important study to research more.

1 INTRODUCTION

Colon and pancreatic cancer are common cancer types in daily life. Pancreatic cancer is the third most common in the United States. The five-year survival rate for pancreatic cancer was 6% in 2003-2009 and increased to 9% in 2009- 2015 (SEER 2019). Existing methods for treating pancreatic include radiation therapy, ablation or embolization treatments, chemotherapy, targeted therapy, and immunotherapy (SEER 2019). Appropriate therapies are chosen based on the stage of cancer and other factors. Sometimes these treatments are combined to obtain better effects. Colon cancer is the second most common cause of cancer in women and the third most common in men (World Health Organization 2014). It also has the third-highest cancer occurrence and death for people in America (SEER 2019). Types of treatments for colon cancer are the same, but different drugs may be used. One famous drug used for the targeted treatment of colon and pancreatic cancer is irinotecan (IRT). Its analogs of the active metabolite, SN38 have highly increased cytotoxicity than irinotecan (Geyer, Maak, Nitsche, Perl, Novotny, Slotta-Huspenina, Dransart, Holtorf, Johannes, Janssen 2016). Shiga toxins produced by Enterohemorrhagic Escherichia coli (EHEC), one food-borne pathogen, can cause hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (Karmali 1989). Shiga toxins (Stxs) were found to be the first ligands that proceed endocytosis via clathrin-coated vesicles by using glycolipid receptors (CD77) (Malyukova, Murray, Zhu, Boedeker, Kane, Patterson, Peterson, Donowitz, Kovbasnjuk 2009). To trigger the toxic effects of Stxs, translocation of the A1(subunit) fragment into the cytosol at ER is necessary (acewicz, Mobassaleh, Gross, Balasubramanian, Daniel, Raghavan, McCluer, Keusch 1994). However, the un-toxic B subunit of Stxs specifically recognizes and binds its cellular receptor Gb3(CD77) on the plasma membrane, making it a potential tool for targeted therapy. CD77 is one kind of glycolipid. It has been proved that SHIGA toxin conjugate with SN38 can be exploited for targeted therapy of cancer (Geyer, Maak, Nitsche, Perl, Novotny, Slotta-Huspenina, Dransart, Holtorf, Johannes, Janssen 2016). The compound STxB-SN38 requires the receptor Gb3 (CD77) for intracellular uptake leading to a cytotoxic effect (Geyer, Maak, Nitsche, Perl, Novotny, Slotta-Huspenina, Dransart, Holtorf, Johannes, Janssen 2016). Thus, the expression of Gb3 (CD77) is very important and should be investigated more. Until now, Gb3 is reported to have an increased expression on pancreatic and colon cancer cells in humans. However, one paper that used carcinoma as a research target found something interesting. They found that the Gb3 expression falls down dramatically with the increased tumor progression" (Maak, Nitsche, Keller,

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Wolf, Sarr, Thiebaud, Rosenberg, Langer, Kleeff, Friess, et al. 2011). This finding contradicts previous findings. Thus, it is necessary to examine the expression pattern of Gb3 (CD77) of cancer cells. Colon and pancreatic cancer will be tested in this experiment. ATCC and DSMZ cell lines will be used. Use STxB-Cy3 for staining and Shiga without staining for staining control, mix them with cancer cells and then check for fluorescent signal from SHIGA on the cells by FACS. Killing measured by MTT, AnnexinV/PI. The negative control is an isotype-matched antibody for CD77, positive control is a cell line that is already known to express high levels of CD77. I predict the later passage pancreatic cancer and colon cancer cells have increased CD77 and better binding to Shiga and increased killing with Shiga-SN38 compared to earlier passage cells. Measure CD77 by FACS as a function of passage number (cell doublings).

2 METHODS AND MATERIALS

2.1 Cell Culture

This experiment will use two known cell lines (ATCC and DSMZ),) Cultured cells in DMEM with FCS (7%), 1% penicillin/streptomycin, and 1% glutamine for 3 weeks. Take another set of cells with the same passage number as the early passage. Pancreatic (DanG and BxPC3) and colorectal (MKN-7，NCI-N87 and HT29) cancer cells will be used. 2.5 µg/mL of STxB-Cy3 will be added as the final concentration.

2.2 Reagents

STxB and STxB coupled with SN38

2.3 Staining

Making a covalent bonding between STxB and fluorophore Cy3. Stain Gb3 on the 3% paraformaldehyde fixed cryosections with STxB-Cy3 for 30 minutes at a final concentration of 10 µg/mL in PBS containing 0.2% BSA (Geyer, Maak, Nitsche, Perl, Novotny, Slotta-Huspenina, Dransart, Holtorf, Johannes, Janssen 2016)

2.4 Flow Cytometry

Seed a total of 2000000 cells on one 10-cm cell culture dish, then harvest and count the cells after 24 hours. Use 20 nmol/L STxB-Cy3 for staining for 15 minutes at 37°C. Use centrifugation for collecting cells. Each experiment is repeated five times.

2.5 Cell Death Measured

AnnexinV/PI a: Add 5 µL Annexin V Alexa Fluor 488 to the target tubes, then incubate in the dark for 15 minutes at room temperature. Add 4 µL of PI that has been diluted 1:10 in 1 x Annexin V binding buffer. Incubate in the dark for 15 minutes at room temperature. Centrifuge samples at 335 x g for 10 minutes. Resuspend cells in 500 µL 1 x Annexin V binding buffer and 500 µL 2% formaldehyde. Add 1 mL 1 x PBS-/-. Centrifuge samples at 425 x g for 8 minutes. Add dd 16 µL of 1:100 diluted RNase A. (Rieger, Aja M et al. 2011) Each experiment is repeated five times.

2.6 Statistical Analysis

Use SPSS for analyzing data.

Table 1: Group A with passage number n (early passage).

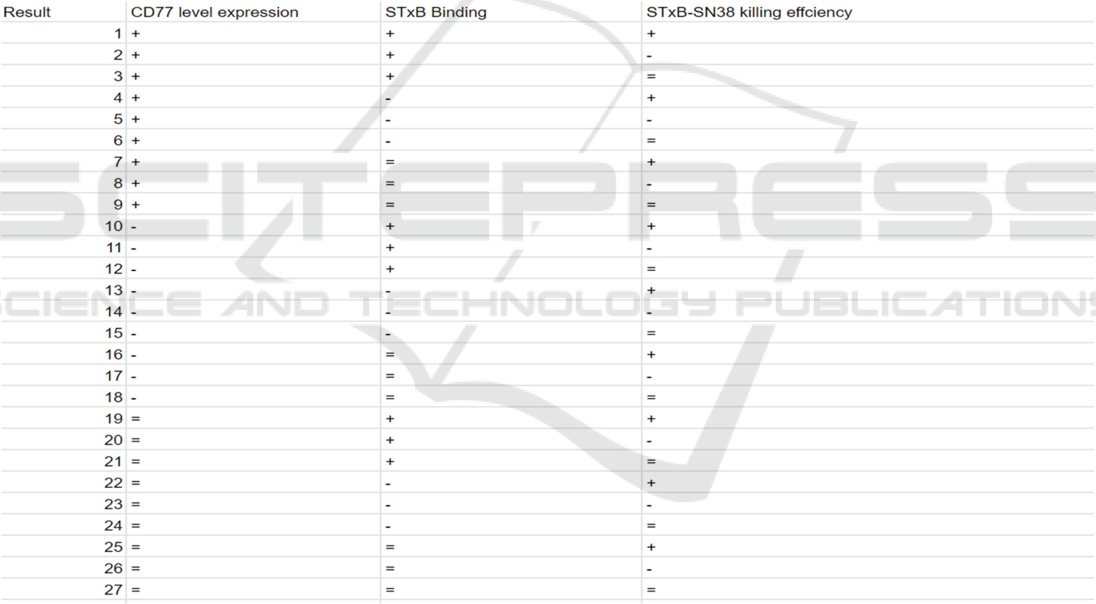
Group	CD77 staining	STxB-Cy3	STx-B-SN38	STx-B-SN38 staining	
1	yes	no	no	no	
2	yes	yes	no	no	
3	no	no	no	no	
4	yes	no	Yes	no	
5	yes	no	yes	yes	

Table 2: Group B with passage number n+10 (late passage).

Group	CD77 staining	STxB-Cy3	STx-B-SN38	STx-B-SN38 staining	
6	yes	no	no	no	
7	yes	yes	no	no	
8	no	no	no	no	
9	yes	no	Yes	no	
10	yes	no	yes	yes	

3 POSSIBLE RESULTS

The table is comparing groups A and B, “+” means the value is higher in a later passage, “-” means the value is lower in a later passage, “=” means the value remains unchanged in a later passage.



Result	CD77 level expression	STxB Binding	STxB-SN38 killing effciency
1	+	+	+
2	+	+	-
3	+	+	=
4	+	-	+
5	+	-	-
6	+	-	=
7	+	=	+
8	+	=	-
9	+	=	=
10	-	+	+
11	-	+	-
12	-	+	=
13	-	-	+
14	-	-	-
15	-	-	=
16	-	=	+
17	-	=	-
18	-	=	=
19	=	+	+
20	=	+	-
21	=	+	=
22	=	-	+
23	=	-	-
24	=	-	=
25	=	=	+
26	=	=	-
27	=	=	=

Figure 1: A table represents all the possible results.

CD77 level expression is compared by group 1 and 6; STxB binding by group 2 and 7; STxB-SN38 killing efficiency by group 4 and 9. Group 3,5,8 and 10 are control groups.

Possible Result 1: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, more cell death (percentage) is observed.

By comparing data from groups A and B, group B has a higher percentage of CD77 and STxB present. Possible Result 2: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, less cell death (percentage) is observed. Possible Result 3: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, the number of cell death (percentage) remains unchanged.

Possible Result 4: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, more cell death (percentage) is observed.

Possible Result 5: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, less cell death (percentage) is observed.

Possible Result 6: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by

STxB-Cy3 decreased, the number of cell death (percentage) remains unchanged.

Possible Result 7: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, more cell death (percentage) is observed.

Possible Result 8: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, less cell death (percentage) is observed.

Possible Result 9: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, the number of cell death (percentage) remains unchanged.

Possible Result 10: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, more cell death (percentage) is observed.

Possible Result 11: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, less cell death (percentage) is observed.

Possible Result 12: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, the number of cell death (percentage) remains unchanged.

Possible Result 13: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, more cell death (percentage) is observed.

Possible Result 14 The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, less cell death (percentage) is observed.

Possible Result 15: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, the number of cell death (percentage) remains unchanged.

Possible Result 16: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, more cell death (percentage) is observed.

Possible Result 17: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, less cell death (percentage) is observed.

Possible Result 18: The CD77 level expression is decreasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, the number of cell death (percentage) remains unchanged.

Possible Result 19: The CD77 level expression is increasing in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, more cell death (percentage) is observed.

Possible Result 20: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, less cell death (percentage) is observed.

Possible Result 21: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 increased, the number of cell death (percentage) remains unchanged.

Possible Result 22: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, more cell death (percentage) is observed.

Possible Result 23: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, less cell death (percentage) is observed.

Possible Result 24: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 decreased, the number of cell death (percentage) remains unchanged.

Possible Result 25 The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, more cell death (percentage) is observed.

Possible Result 26: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, less cell death (percentage) is observed.

Possible Result 27: The CD77 level expression remains unchanged in later passage cancer cells (after one time period of cell doublings), the percentage of cells marked by STxB-Cy3 remains unchanged, the number of cell death (percentage) remains unchanged.

4 DISCUSSION

Results 10-27 all overturn the hypothesis because the CD77 expression level does not go up for later passage cells. While results 1-9 only supports part of the hypothesis. (The CD77 expression level increase in later number) Result 1: It perfectly supports the hypothesis, which means the later passage cells are presenting more CD77 relative to earlier passage cells, leading to the increased binding of STxB. Thus, more STxB-SN38 kills more cells. This result indicating STxB-SN38 has the potentials to limit cancer grows up as targeted therapy. Moreover, due to its high cytotoxicity, it may replace previous medicine such as Irinotecan. Future experiments are supposed to follow up. Animal research like mice could be done using xenograft. Result 1,14 and 27: In these three experiments, the STxB binding and STxB-SN38 killing efficiency follows the CD77 level. They showed that STxB binding is positively associated with CD77; the killing efficiency is positively associated with STxB binding. For result 27, the CD77 expression level does not show any relationship within later passage cells. It indicates that the CD77 expression level seems not related to the passage number of cells which does not support the hypothesis. For result 14, the CD77 drops in later passage cells. This result opposes that hypothesis. Since much evidence point that colon and Pancreatic cancer have some level of CD77 expression, a further experiment should be followed up. The expression pattern of CD77 could be complicated. The CD77 level possibly increases with the cells grow up and drop while the passage number goes up. The reason might associate with CD77's function as a membrane protein. It may also relate to the gene regulation changes during mitosis.

For results that the STxB binding does not correspond to STxB-SN38 killing efficiency, they can be divided into two groups.

First group are result 2,3,8,11,12,17,20,26. In these results, the killing efficiency of late passage is reduced compared to early passage. In such case, group 4 and 5 in group A and group 9 and 10 in group B should be analyzed deeper. The difference between group 4 and 5 or group 9 and 10 is the STxB-Sn38 staining. By comparing them, it can tell whether the cell pathway of STxB-SN38 changes. If the pathway changes, it is possible that the SN38 group of STxB-SN38 are targeted by another molecule inside the cells and eliminated. These results are unexpected and won't support or deny the hypothesis. The unknown pathway is necessary to study more. If the pathway does not change, then the STxB-SN38 may have dose effects. A high concentration of STxB-SN38 could downregulate the killing efficiency. These results disprove the hypothesis that higher binding of STxB with cells could increase cell killing of STxB-SN38. A new experiment testing the concentration of STxB-SN38 with its highest efficiency could be processed.

Second group are results 4,6,7,13,15,16,22,24,25. In these results, the killing efficiency of late passage is higher compared to early passage. Test if the pathway changes as talked about before in the paper. If the pathway changes, the STxB-SN38 may bind to something unexpected but still trigger the toxic effects and thus kill the cells. Since STxB SN38 lost its high specificity of binding to CD77, the undifferentiated killing of all cells happened and increase the kill numbers. These results are unexpected and won't support or deny the hypothesis. Further experiments could be done by using cells that have low or no expression of CD77 treated with STxB-SN38 for control to see if STxB SN38 lost its specificity. That may reveal another cell pathway of how STxB-SN38 entering the cell. (As mentioned before, STxB requires CD77 to get into cells) If the pathway does not change, the STxB-SN38 may have dose effects as mentioned before. It does not support the hypothesis and more research should be done.

For result 10,11,12,16,17,18,19,20,21, STxB binding is increasing while CD77 is not increasing or remains the same while CD77 is dropping down. This is strong evidence that STxB binds to another receptor protein to get into targeted cells. That makes it harder to let STxB mediate targeted therapy. These results overturn the hypothesis. Later research and study should focus on discovering and investigate the new pathway. Then, the new pathways could be tested (shut down it) to see if they can make the STxB CD77 pathway-specific again.

For results 4,5,6,7,8,9,22,23 and 24, STxB binding is decreasing while CD77 remains the same or increasing. If no huge artificial mistakes are made, then these results

overturn the hypothesis. There might be a new pathway presents as mentioned before. The STxB could also be saturated if the STxB binding remains the same. (result 7,8,9)

5 CONCLUSIONS

Known the expression pattern of CD77 will help a lot in targeted cancer therapy. The result of the study will give a detailed pattern of CD77 expression, which will help further research.

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