EFFECTS OF KALIKREIN RELATED PEPTIDASE 6 (KLK6) OVEREXPRESSION IN COLORECTAL CANCER CELLS GROWTH AND VIABILITY

By

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Abstract

Colorectal cancer (CRC) is one of the most common and more life-threatening cancers. Its prognosis improves greatly with early detection, which is why its important to develop biomarkers that can be used to detect the disease in its early stages. KLK6 is a secreted protein commonly overexpressed in CRC due to its effects on cell growth, apoptosis and metastasis. Here we created KLK6 knockouts and heterozygous clones from the SW480 cell line and measured cell growth and viability over multiple days. The knockouts were successfully created and the effectiveness of the CRIPSR silencing was proven by real time PCR to measure mRNA expression, and ELISA to measure KLK6 protein secretion. The heterozygous clones however, showed either normal expression of KLK6, or complete silencing of the gene, verified by the same methods mentioned. The clones which did not express KLK6 showed much slower growth and viability compared to the cell lines which normally expressed the protein.

Introduction

Colorectal cancer

In 2022 in the United States, according to the CDC, colorectal cancer (CRC) ranked third as the most common cancer with the estimated cancer related deaths from this disease reaching 50 000. (1) CRC is a disease in which cells in the colon or rectum grow out of control. It begins as a small polyp on the inner lining of the colon and eventually turns into cancerous tumors. CRC continues to be a medical challenge, since its asymptomatic development and early metastasis makes it very difficult to diagnose and treat.

Prognosis for patients is heavily dependent on stage at diagnosis: when the cancer spreads throughout the inside linings of the colon (Duke's Stage A), the patient has 90% chances of 5-year survival. However, after it spreads beyond the colon, the chances drop to only 5% in Duke's Stage D. Therefore, early diagnosis is vital in treating CRC, which can be facilitated by both invasive and non-invasive means of surveillance. (2)

Ample evidence shows that development of CRC is related to both genetic and environmental factors. Some environmental factors include, abundant red meat consumption, alcohol, smoking, and a sedentary life. Genetic factors are hereditary, like familial adenomatous polyposis (FAP) and hereditary non-polyposis coli (Lynch syndrome), present in 5% of cases. Other genetic factors include inflammatory bowel's disease, diabetes type II, and family history. (3)

Genetics

Colon tumors develop overtime, generating morphological, histological and genetic changes. Benign precancerous tumors are considered a starting point for CRC, from where they can accumulate more genetic changes and be able to invade the bowel wall, local lymph nodes and eventually metastasize to further sites. There are 2 different genetic pathways:

chromosomal instability (CIN), and microsatellite instability (MIS). The former is observed in 65-70% of sporadic cancers and starts with an APC mutation, affecting chromosome segregation during cell division. Loss of APC function triggers a cascade of histological and genetic changes which lead to CRC. The latter starts with a BRAF and a b-catenin mutation, altering growth signal and loss of apoptosis. (4)

Adenomatous polyposis coli (APC):

The APC gene encodes a multifunctional, cytoplasmic protein involved in controlling the Wnt signal transduction pathway since it contains binding sites for b-catenin and Axin proteins. Furthermore, it is associated with the microtubule cytoskeleton via binding to EB1 and IQGAP1 (5), and controls cell adhesion. (6) While all functions mentioned before seem important in regulating and controlling cancer development, the tumor suppressor capabilities from the gene come from its role in regulation of intracellular b-catenin levels. (7) Mutations in the APC gene lead to accumulation of b-catenin inside the cell, activating the T cell factor (TCF) and lymphoid enhance factor (LEF). This result interferes with the Wnt pathway, affecting expression of proteins MYC and cyclin D1, increasing proliferation rate and cycling of cells. (8)

KRAS

Kirsten rat sarcoma viral oncogene homologue (KRAS) is an oncogene with the highest mutation rate among all cancers (30-40%) and highly lethal in CRC. The gene encodes for guanosine-5′-triphosphate (GTP)-binding proteins involved in the RAS/MAPK cell signaling pathway that controls cell growth, cell maturation, and cell death. (9) Mutations inactivate GTPase activity which leaves the protein in a constantly active (GTP-bound) state. This mutation is associated with increased cell proliferation, decreased apoptosis, angiogenesis and increased migration. (10)

Pathways to carcinogenesis

In CRC, the two recognized pathways to carcinogenesis are chromosomal instability pathway, (CIN) with 65-70% of CRC cases, and microsatellite instability pathway (MSI) with 15% of CRC cases. (11)

CIN:

In this pathway tumors develop slowly over decades; somatic cells gain and/or lose whole chromosomes or large parts thereof at elevated rates compared with normal cells, causing aneuploidy, amplifications, insertions, deletions or loss of heterozygosity. This greatly increases the chances of losing tumor suppressor genes and acquiring oncogenes. Different methods like karyotyping, comparative genomic hybridization, and loss of heterozygosity analysis are performed on tumor cells to designate them as CIN positive or negative. (12)

MSI:

In this pathway, tumors develop due to a deficient mismatch repair (MMR) system caused by silencing of the MLH1 gene, which encodes for MutL protein. This protein, in complex with MutS, is in charge of recognizing mismatches and insertion/deletion loops on cellular DNA. Other mutations in genes MSH2, MSH3, MSH6, and PMS2 can also disrupt this complex and cause MSI. MMR defects generate an increase in mutation rate, allowing persistence of genome-wide mismatches in repetitive DNA sequences called microsatellites. Tumors generated via the MSI pathway have a better prognosis than CIN, but do not respond to the same chemotherapy. Detection of MSI tumors is done by PCR amplification of specific microsatellite repeats, and immunohistochemical analysis of MMR proteins. (13)

KLK6

Kallikrein-related peptidase 6 (KLK6) is an enzymatically inactive secreted serine protease, which requires cleavage of the five amino acid activation peptides to be active. However, KLK6 is able to activate itself by autoproteolytically cleaving at the internal Arg^{76}-Glu^{77} bond. It has trypsin like activity, and is normally involved in proteolytic cascades and cleaving multiple substances like growth factors, fibronectins, and collagens. (14) KLK6 mRNA copy numbers show high increase in tumors from breast, colon, prostate, and lung cancer, leading to overexpression of KLK6. Transcription levels appear to be controlled by non-CpG island cytosines since, when methylated, they shut down KLK6 expression. (15)

KLK6 role in breaking down fibrinogens and collagens is what gives it a role in tumorigenesis. Degradation of these molecules allows for tumor migration and invasion, and tissue remodeling. Furthermore, KLK6 overexpression causes defects in cell-adhesion and down-regulates E-cadherin protein, which regulates cell interactions and movement (16). In addition to that, apoptosis and angiogenesis pathway seem to also be affected by excess KLK6, since they activate pro-survival pathways. (17) All of these characteristics suggest an oncogenic role for KLK6, specifically by fomenting the epithelial to mesenchymal transition (EMT).

EMT

Epithelial to mesenchymal transition refers to a biological program where epithelial cells lose their identity and acquire a mesenchymal phenotype. This process naturally occurs in the body during wound healing and organism development. Nevertheless, cancer cells can hijack this process to prevent apoptosis, acquire tissue invasiveness, and cancer treatment resistance. This process is closely regulated by transcriptional and genetic factors like SNAIL and TWIST, which inhibit expression of E-cadherin (cell surface marker), promoting the transition. Epithelial cells lose their apical-basal polarity and acquire a migratory phenotype,

commonly seen in mesenchymal cells. This process, highly increases likelihood of metastasis in CRC and is commonly seen in poor prognosis cases. (18)

Specific aims of research

KLK6 is a secreted enzyme, and thus has interesting potential as a biomarker that can be measured by non-invasive means, like blood sampling. Prognosis of CRC improves significantly with earlier diagnosis; Thus, a proper understanding of this protein and its effects on cancer progression are the first steps in developing a possible biomarker which can provide valuable information on the nature of tumors. Also, as mentioned before, KLK6 mRNA and protein are overexpressed in CRC, particularly in metastasized tumors that reached stages III and IV of the disease much faster. (19). With this in mind, the objective of this research is to understand the direct and observable effects of KLK6 on cancer cells. We hypothesize overexpression of KLK6 increases cell growth and survivability. For this, KLK6 knockouts and KLK6 heterozygous cells were created using CRISPR technologies. The cell line utilized was SW480, since it overexpresses KLK6 due to mutations in the APC and KRAS genes. The SW480 cell lines with intact and deleted KLK6 gene were characterized and their behavior related to tumor aggressiveness was measured., Furthermore, heterozygous cell lines were created with the goal of determining if amount of KLK6 expression correlates to the level of tumor aggressiveness. To characterize our samples, we measured mRNA levels in clonally selected cells after CRISPR knockout. And an ELISA was performed on selected clones to measure levels of KLK6 secreted in media.

Methods

Cell lines.

SW480 colon cancer cell line was purchased from the American Type Culture Collection (Rockville, MD). SW480 cells were grown in RPMI supplemented with 10% FBS and 1%

penicillin/streptomycin. KLK6 gene knockout was performed using CRISPR technology and the clones with the knockout of two alleles of KLK6 gene (KLK6 ko #21 and KLK6ko#36) and the clones with knockout of one KLK6 gene allele (KLK6 het#25 and KLK6 het#35) were used for further analyses.

CRISPR

DNA oligonucleotides used for gRNA synthesis were designed using the GeneArt™ CRISPR gRNA Design Tool. Among different candidates, gRNA #1 and #2 were chosen as they target a section from exon 1 to exon 3, of the KLK6 gene in chromosome 7 (position 43824544 - 43832027). The gRNA used was selected due to its capability of binding and disrupting multiple start sections of the KLK6 gene. Furthermore, the gRNAs were synthesized using the GeneArt™ precision gRNA synthesis kit (ThermoFisher, Waltham, MA, USA). CRISPR RNA (crRNA)/trans-activating crRNA (tracrRNA) hybridization and RNP complex formation was done according to the manufacturer's instructions. For transfection, 8 × 104 cells were seeded in 24-well plates and incubated overnight. Following that, cells were transfected with the RNP containing gRNA (125 ng) and recombinant Cas9 nuclease (rCas9; 500 ng) using the CRISPRmax transfection reagent (ThermoFisher). Genomic cleavage efficiency was measured by a PCR-based method using the GeneArt® Genomic Cleavage Detection (GCD) kit. Cells were corrected at 48 h post-transfection and PCR was carried out. Finally, the amplicon was loaded to a 2% agarose gel electrophoresis. The cleavage efficiencies were calculated based on the relative band intensities.

Cell growth curve

Cell counting and passaging procedures

Total RNA extraction from colon cancer cell lines was done 48 hours after subculture using the Qiagen RNA isolation kit. RNA purity was assessed using a Nanodrop spectrophotometer (ND-2000; Thermo

Scientific, Pittsburgh, PA). All RNA samples prepared for experiments exhibited determined 260/280 absorption values between 1.8-2.0.

Quantitative real-time PCR

Total RNA was isolated as described above. Reverse transcription to produce cDNA template was completed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Part #4368814). qPCR was performed using TaqMan® probe (Applied Biosystems, Thermo Fisher Scientific, Inc.) for KLK6 (Hs00160519_ml). 0.2 µg of total RNA was reversed transcribed into cDNA in a 20 µL reaction with random hexamers under thermal condition recommended by the protocol. Real-time PCR amplification was performed with the QuantStudio 3 PCR machine (ThermofisherScientific, Inc.), under the universal thermal cycling conditions recommended by the Assay-on-Demand products protocol. Negative controls without template were included in each plate to monitor potential PCR contamination. The expression of KLK6 was tested in triplicate and each reaction was run in duplicate. To determine the relative expression level of KLK6 gene, the comparative C_T method was used. The C_T value of the target gene was normalized by the endogenous reference β2-microglobin (β2M, FAM (Hs99999907_m1)). The relative expression of each target gene was calculated via the equation $2^{-\Delta C_T}$ where $\Delta C_T = C_{T(\text{target})} - C_{T(\text{endogenous control})}$.

Performed to detect levels of KLK6 mRNA in the clones

Elisa Enzyme-Linked Immunosorbent Assay (ELISA)

KLK6 ELISA. An ELISA kit for the detection of human KLK6 was obtained from Boster Immunoleader (Pleasanton, CA). The assay was performed according to manufacturer's protocol. Conditioned media was collected from SW480 cells and clones. KLK6 antigen levels are expressed as picograms per milliliter of total protein. The plate was read at 490 nm within 30 minutes of assay ending on a Synergy 2 Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Results

CRISPR

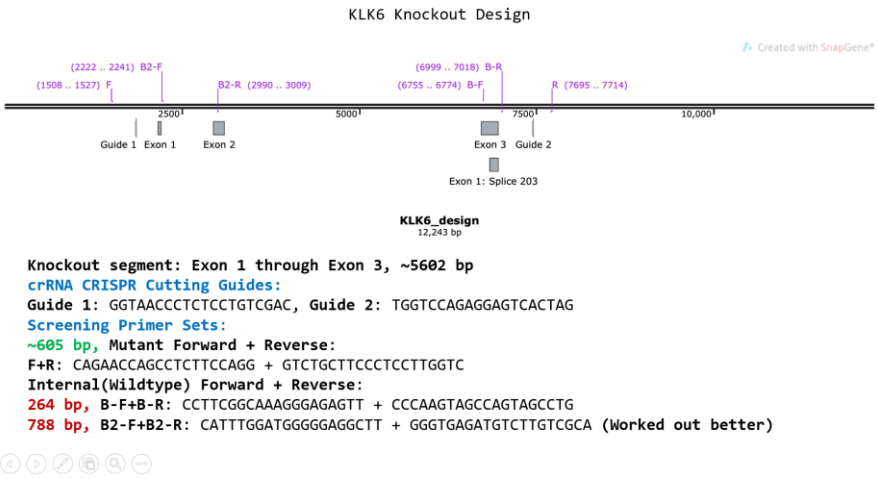
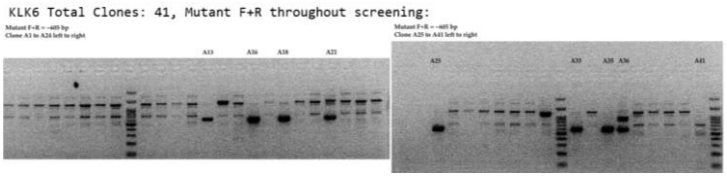


Figure 1: KLK6 knockout design in GeneArt^{TM}. Figure shows the KLK6 gene sequence and highlights important positions like exons and location of gRNA. Furthermore, the figure shows the sequence of the gRNA used and the primers for the wildtype and the mutant.

A



B

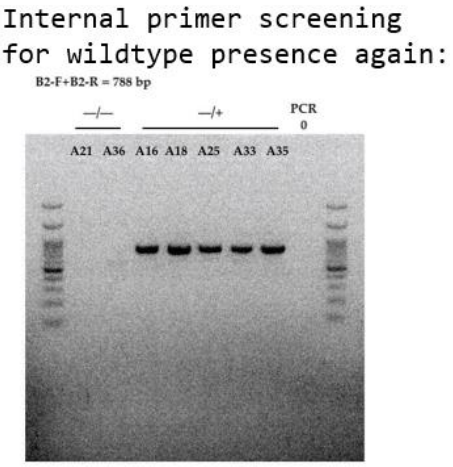


Figure 2: Screening for presence of the KLK6 gene in cells. After removal of the gene with CRISPR, cells were collected and genome was extracted. PCR amplification of KLK6 gene was then performed on the isolated DNA and run in a 2% agar electrophoresis gel. A. Gel showing screening of 41 clones B. Internal primer screening of positive clones. The lanes in the figure are written at the top of the gel and the y axis is used to do size comparison with a ladder.

To compare and analyze the effects of KLK6 expression in CRC cells, KLK6 knockouts and heterozygous clones were created using CRISPR. As observed in Figure 1, the objective was to remove the first 3 exons of the gene by using two gRNA that will flank the sequence. Multiple gRNA testing was performed, the ones showed in figure 1 had the most specificity and highest cleavage efficiency. Furthermore, those sequences were selected as they target a common sequence observed at the start of every KLK6 gene, thus allowing for observable results in different samples. After transfection, cells were collected and PCR was carried out to look for negative and heterozygous clones. Figure 2.A shows the agarose gel with the result form the first PCR screening which recognized the mutant sequences inserted. Clones 21, 36, 16, 18, 25, 33, and 35 had a band around the 788 bp mark instead of 905, thus were selected for internal primer screening. Figure 2.B shows results from a PCR reaction in which the internal primer with a length of 905 bp sequence was amplified. In the figure clones 21 and 36 both show both KLK6 alleles disrupted, as there is no band around the 900 mark. Also, 25 and 35 clones show possible heterozygosity, as there is a band present, meaning one allele at least is intact. These four clones were then selected for further testing.

Quantitative real-time PCR analysis of KLK6 expression

Transcript levels of KLK6 for four different clones obtained from CRISPR were measures by real time PCR to confirm data observed before.

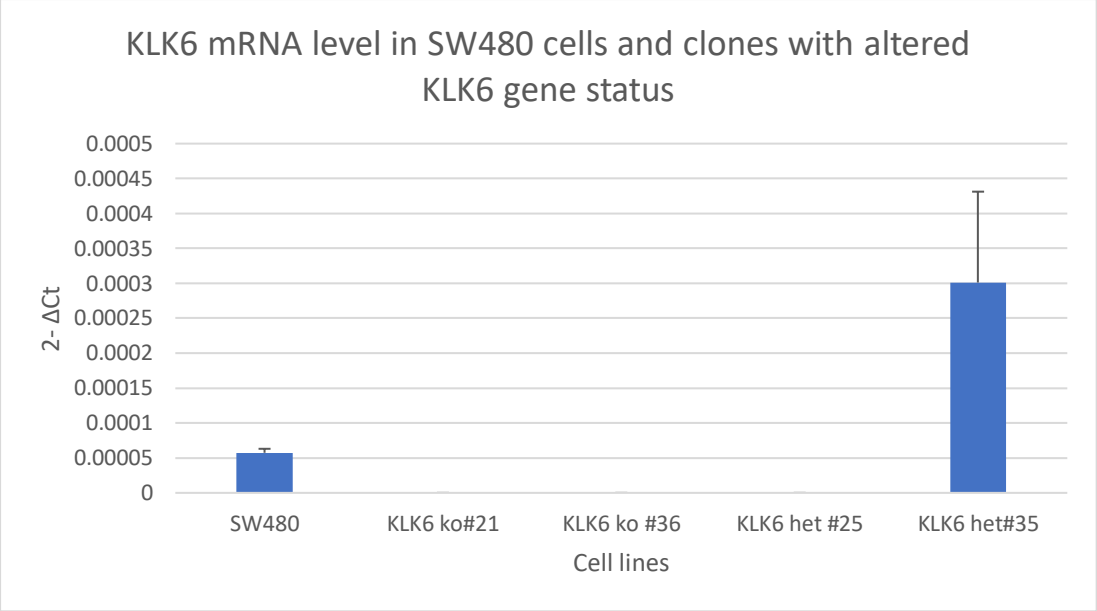


Figure 3: KLK6 mRNA results from quantitative real time PCR performed on the parental cell line and 4 clones obtained after CRISPR. The expression of KLK6 was tested in triplicate and each reaction was run in duplicate. mRNA was extracted and KLK6 was amplified using real-time PCR. Relative expression of KLK6 was measured using the CT method explained before.

The purpose of measuring mRNA levels was to corroborate the information obtained after CRISPR. As a control, KLK6 expression in SW480 cells was measured. The clones 21 and 36 KO both show no mRNA expression, aligning with our previous results. For the heterozygous cell lines het 25 also showed no mRNA expression. However, het 35 had extremely high levels of KLK6 mRNA, showing a 6-fold increase compared to the parental cell line.

Analysis of secreted KLK6 protein

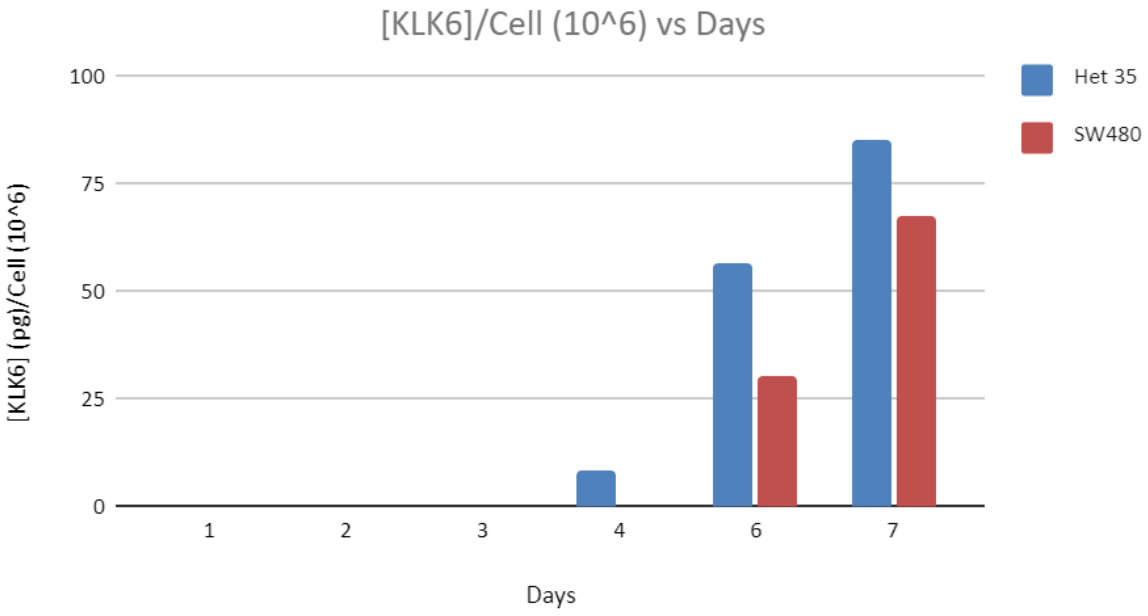


Figure 4: Results from ELISA analysis of KLK6 secreted protein per cell in each cell line over the course of 6 days. Cells were cultivated in a 6-well plate then conditioned media was collected each day and cells were counted. An ELISA kit for the detection of human KLK6 was used to measure levels of KLK6 in each sample.

After measuring mRNA levels, ELISA was performed to confirm the KLK6 protein levels being secreted. For KO 21 and 36 there was no KLK6 detected in any of the samples. In addition to that, het 25 also had no detectable levels of KLK6 protein in media. On the other hand, het 35 showed expression of KLK6 protein, and once again, higher than the parental cells line by a considerable amount. As observed in Figure 4, het 35 and SW480 cells only started secreting KLK6 after the first 4 days, while the other samples did not secrete KLK6 throughout the whole experiment. However, by day 6 and 7 the amount of KLK6 expressed by cell in het 35 and the parental cell line increased substantially.

Effect of KLK6 gene knockout on cell growth.

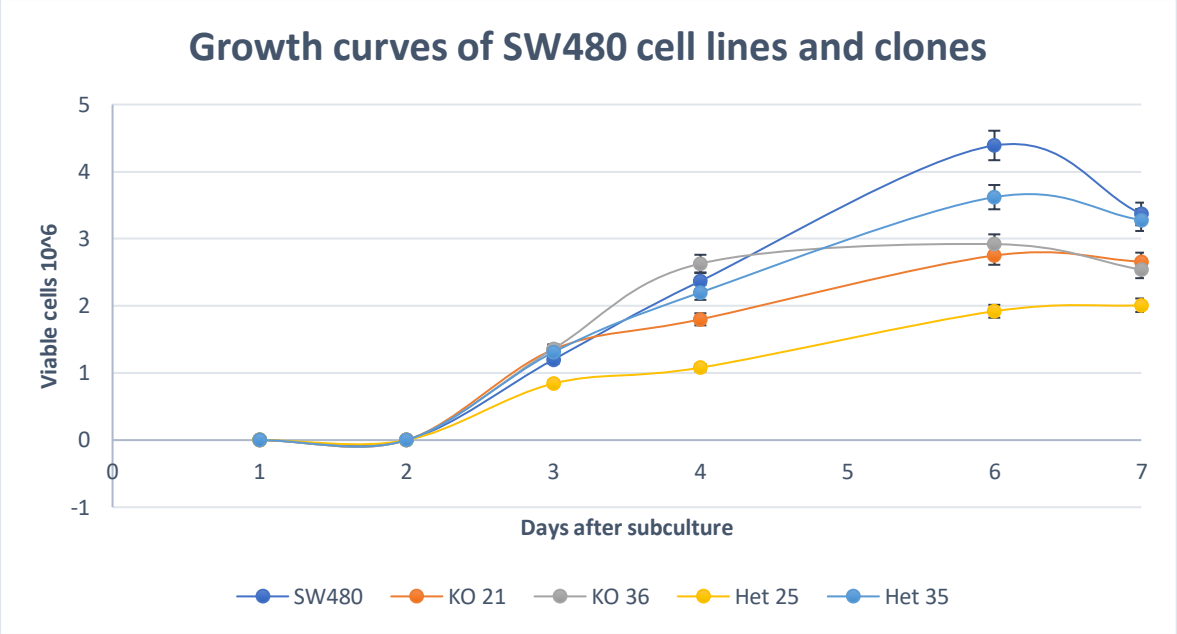


Figure 5: Cell growth of each cell line over 6 days. Cells were cultivated in 6-well plates and counted each day. Viable cells were graphed.

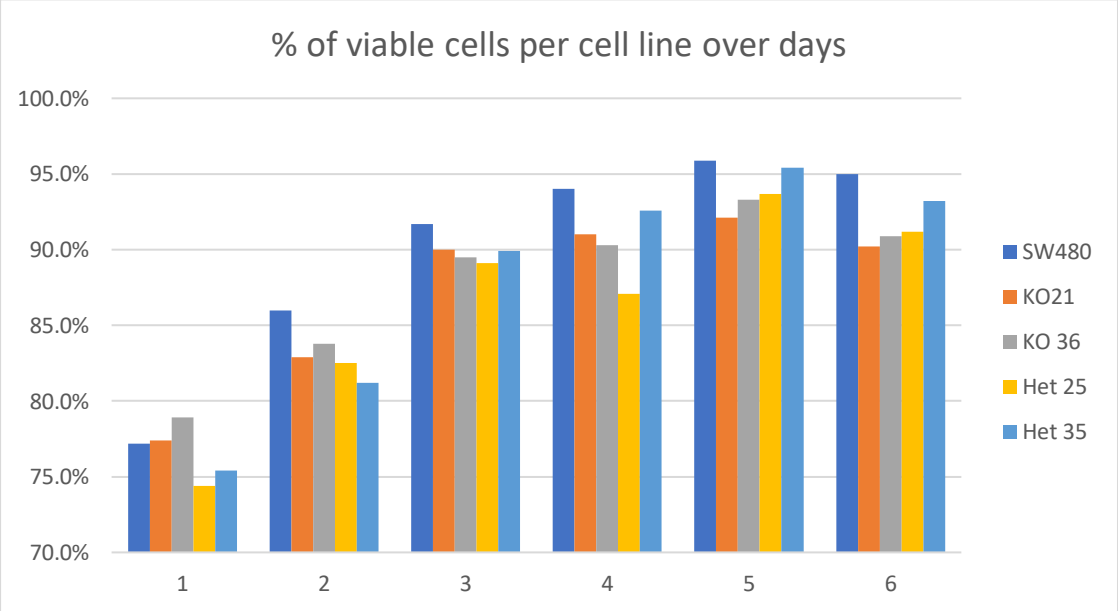


Figure 6: % of viable cells in each cell line over 6 days. Data obtained from the same experiment.

Finally, after measuring KLK6 expression in each cell line, effect of KLK6 in cell growth and % viability was measured. Figure 5 shows SW480 cells reached a much higher total number of cells than all of the other cell lines. This was somewhat expected, since the parental cell line expressed KLK6 protein normally, thus increasing cell growth. Both of the knockouts had significantly fewer cells than the parental cell line by day 6, showing less

growth. The only point where a KO 36 cell growth was over SW480 was at day 4, which could have happened due to high initial number of cells in KO 36. Both knockouts however plateau faster than SW480, as expected since they did not express KLK6. Similarly, het 25, which expressed no KLK6 protein, had the lowest cell growth out of all the samples and plateau at day 7 at the lowest number of cells in all samples. Het 35 had a very similar growth curve to KO 21, and by day 7 cells were still growing, but at a slower rate to the parental cell line.

In figure 6 the percent of viable cells per cell line over 6 days is shown. This graph was made to better evaluate cell growth and survival over multiple days. From day 2, SW480 had the highest percent of viable cells in all samples, while het 35 followed second from day 3 to day 6. Both KO 21 and 36 and het 25 had similar values throughout the experiment, as they reached a much lower percent of viable cells by the end of the experiment.

Discussion

KLK6 silencing, transcription levels and KLK6 secretion

After CRISPR, DNA from cells was extracted and amplified using PCR. The objective of this part was to obtain the KLK6 – clones and the heterozygous clones. Looking at Figure 2B, it suggests the clones 21 and 36 do not express any KLK6 gene as there is no distinguishable bar around the 900 bp mark, section amplified by PCR. For clones 35 and 25, Figure 2A shows one allele was disrupted, as they contain the inserted sequence from PCR. However, in Figure 2B they have a bar present at the 900 bp mark, meaning the other allele is functional, suggesting they could be heterozygous clones. mRNA levels were measured via real time PCR to further confirm the data. Figure 3 shows the levels of mRNA in each clone and both KO 21 and 36 show no transcription of KLK6, which aligns with our previous data. However, the heterozygous clones show non-expected data. The het 25 clone shows no transcription of KLK6, even though it has at least one allele intact in its DNA as observed in

Figure 2B. On the other hand, the clone het 35 shows high levels of mRNA, even higher than the parental cell line. To verify the obtained data, secreted KLK6 in media was measured via an ELISA and the results are shown in Figure 4. The graph show that KLK6 secretion correlates to the mRNA levels observed before, since only the parental cell line and the het 35 clone secrete KLK6. Based on all this information we can confidently say the 21 and 36 clones have a completely silenced KLK6 gene. In het 25, the transcription and secretion of KLK6 results suggest it also has a silenced gene in both alleles, even though the results from CRISPR do not align with this. A possible explanation could be that although CRISPR technology is a precise tool, the mechanisms to repair the DNA breaks in the cell can be faulty, and can result in mistakes in nucleotide sequences. This would lead to a lack of expression even with an allele unaffected by CRISPR. In the case of the het 35 clone, it was confirmed only one allele was functional, nevertheless KLK6 is expressed at even higher levels compared to parental cell line. This could be due to elevated synthesis of KLK6 from the intact allele who evoked a compensatory mechanism as the other allele was disrupted. This mechanism has been seen before in mice and other animals, and occurs as other gene or genes functionally compensate for the loss-of-function genotypes, restoring normal function. (20)

Cell growth and viability

From the characterized cell clones, cell growth over 6 days was measured and showed in Figure 5. From the graph the knockouts can be compared to the parental cell line. SW480 cells have the highest growth out of all the clones, while both knockouts showed lower cell growth at all points, except for KO 36 at day 4. This could be due to a high initial titer of cells which escalated fast, but plateaued at a lower cell count. Furthermore, het 35 shows the same results, as it had a higher cell number at each data point except for KO 36 day 4. Het 25 has the lowest cell growth out of all the clones, supporting our hypothesis. Considering these

results KLK6 has some influence in cell growth over time, as cell clones with overexpressed KLK6 had a higher cell count than clones with silenced KLK6 gene. The higher cell growth could be attributed to survival pathways activated by the excess KLK6

In terms of viability Figure 6 shows results from the same experiment as Figure 5. Looking at the data, SW480 cell line has the highest %viability than all the other cell clones in all data points from day 2, and het 35 has the second highest viability. The knockouts and het 25 all have a lower viability, with no clear difference between the 3. This once again suggests KLK6 has a role in cell viability which could be due to apoptosis prevention and activation of survival pathways.

Conclusions

From the obtained results it can be concluded that knockout KLK6 clones were obtained. This was proven by real time PCR and ELISA, measuring both mRNA, and secreted protein. On the other hand, the heterozygous clones were not successfully isolated, since they either expressed KLK6 normally (het 35), or showed silencing of both alleles (het 25). The former could have happened due to unspecific binding of the gRNA, causing the enzymes to not cleave the sequence whatsoever. In the case of het 25, the lack of expression could have happened due to the DNA repair mechanism in the cell being faulty, resulting in mistakes in nucleotide sequences. This would lead to a lack of expression even with an allele unaffected by CRISPR. From the cell growth and percent viability experiments, it was determined that KLK6 has some effect in both cases, as cell clones expressing KLK6 had faster cell growth and more viable cells. It has been hypothesized that KLK6 activates survival pathways and can prevent apoptosis in cells, which the results in this paper corroborate. Further investigation must be performed in order to better characterize the effects of KLK6 in cells. An invasion assay may prove useful in determining the effects of KLK6 in tumor

aggressiveness and metastasis. Also, more research needs to be performed to better understand the interactions between KLK6 and other related genes, more specifically those which may be able to restore function after allele silencing.

References

1. Centers for Disease Control and Prevention. (2023). *Basic information about colorectal cancer*. Centers for Disease Control and Prevention. Retrieved April 13, 2023, from https://www.cdc.gov/cancer/colorectal/basic_info/index.htm
2. de la Chapelle, A. Genetic predisposition to colorectal cancer. Nat Rev Cancer 4, 769–780 (2004). https://doi.org/10.1038/nrc1453
3. Board, P.D.Q.C.G.E., Genetics of Colorectal Cancer (PDQ(R)): Health Professional Version, in PDQ Cancer Information Summaries. 2002, National Cancer Institute (US): Bethesda (MD).
4. Simon K. (2016). Colorectal cancer development and advances in screening. Clinical interventions in aging, 11, 967–976. https://doi.org/10.2147/CIA.S109285
5. Munemitsu, S., Souza, B., Müller, O., Albert, I., Rubinfeld, B., & Polakis, P. (1994). The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. Cancer research, 54(14), 3676-3681.
6. Fodde R. (2002). The APC gene in colorectal cancer. European journal of cancer (Oxford, England : 1990), 38(7), 867–871. https://doi.org/10.1016/s0959-8049(02)00040-0
7. Korinek, V., Barker, N., Morin, P. J., Van Wichen, D., De Weger, R., Kinzler, K. W., ... & Clevers, H. (1997). Constitutive transcriptional activation by a β-catenin-Tcf complex in APC−/− colon carcinoma. Science, 275(5307), 1784-1787.
8. Risio, M., Coverlizza, S., Ferrari, A., Candelaresi, G. L., & Rossini, F. P. (1988). Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. Gastroenterology, 94(4), 899-906
9. Arrington AK, Heinrich EL, Lee W, Duldulao M, Patel S, Sanchez J, Garcia-Aguilar J, Kim J. Prognostic and Predictive Roles of KRAS Mutation in Colorectal Cancer. International Journal of Molecular Sciences. 2012; 13(10):12153-12168. https://doi.org/10.3390/ijms131012153
10. Xiuli Liu, MD, PhD, Maureen Jakubowski, MT, Jennifer L. Hunt, MD, *KRAS* Gene Mutation in Colorectal Cancer Is Correlated With Increased Proliferation and Spontaneous Apoptosis, *American Journal of Clinical Pathology*, Volume 135, Issue 2, February 2011, Pages 245–252, https://doi.org/10.1309/AJCP7FO2VAXIVSTP
11. Nguyen, L. H., Goel, A., & Chung, D. C. (2020). Pathways of Colorectal Carcinogenesis. Gastroenterology, 158(2), 291–302. https://doi.org/10.1053/j.gastro.2019.08.059
12. Pino, M. S., & Chung, D. C. (2010). The chromosomal instability pathway in colon cancer. Gastroenterology, 138(6), 2059–2072. https://doi.org/10.1053/j.gastro.2009.12.065
13. Vilar, E., Gruber, S. Microsatellite instability in colorectal cancer—the stable evidence. Nat Rev Clin Oncol 7, 153–162 (2010). https://doi.org/10.1038/nrclinonc.2009.237
14. Ghosh MC, Grass L, Soosaipillai A, Sotiropoulou G, Diamandis EP. Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol 2004;25:193 – 9

15. Pampalakis G, Arampatzidou M, Amoutzias G, Kossida S, Sotiropoulou G. Identifi cation and analysis of mammalian klk6 orthologue genes for prediction of physiological substrates. Comput Biol Chem 2008;32:111 – 21.
16. Rezze GG, Fregnani JH, Duprat J, Landman G. Cell adhesion and communication proteins are differentially expressed in melanoma progression model. Hum Pathol 2011;42:409 – 18.
17. Scarisbrick IA, Epstein B, Cloud BA, Yoon H, Wu J, Renner DN, et al. Functional role of kallikrein 6 in regulating immune cell survival. PLoS One 2011;6:e18376.
18. Georgakopoulos-Soares, I., Chartoumpekis, D. V., Kyriazopoulou, V., & Zaravinos, A. (2020). EMT Factors and Metabolic Pathways in Cancer. *Frontiers in oncology*, *10*, 499. https://doi.org/10.3389/fonc.2020.00499
19. Pandey, R., Zhou, M., Chen, Y., Darmoul, D., Kisiel, C. C., Nfonsam, V. N., & Ignatenko, N. A. (2021). Molecular Pathways Associated with Kallikrein 6 Overexpression in Colorectal Cancer. *Genes*, *12*(5), 749. https://doi.org/10.3390/genes12050749
20. Lam, E. W., Glassford, J., Banerji, L., Thomas, N. S., Sicinski, P., & Klaus, G. G. (2000). Cyclin D3 compensates for loss of cyclin D2 in mouse B-lymphocytes activated via the antigen receptor and CD40. *The Journal of biological chemistry*, *275*(5), 3479–3484. https://doi.org/10.1074/jbc.275.5.3479