Analysis of the Effects of GOT1 Knockout in KRAS-mutated Tumor Cells

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Abstract

Recent studies have shown that Pancreatic Ductal Adenocarcinoma arises due to a KRAS-mutated anaerobic pathway, which is regulated by Glutamic-oxaloacetic Transaminase 1 (GOT1). The aim of the study is therefore to examine how certain genes are affected when GOT1 is knocked out. This was performed by extracting RNA from wildtype and GOT1 knockout osteosarcoma cells, cDNA synthesis and analyzing the gene expressions with real-time qPCR. A difference in the gene expressions in GOT1 knockout cells was noted in the genes Glutathione Reductase, Growth Arrest DNA Damage, DNA Damage-inducible Protein and Lactate Dehydrogenase B. Considering that the study is performed on a genetic level, further experiments concentrated on the analogous protein expressions are necessary. Determining the effects and controlling the activity of the regulators in the altered metabolic pathway may in the future result in a potential treatment for pancreatic cancer.

Contents

List of Abbreviations

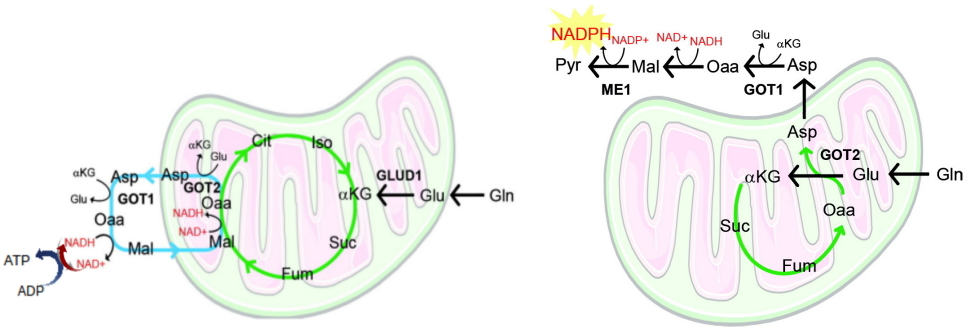
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BiP	Binding Immunoglobin Protein
cDNA	Complementary DNA
CHOP	DNA Damage-inducible Transcript 3
ER	Endoplasmatic Reticulum
GADD	Growth Arrest DNA Damage
GLUD1	Glutamate Dehydrogenase 1
GOT1	Glutamic-oxaloacetic Transaminase 1
GSR	Glutathione Reductas
GSS	Glutathione Synthetase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LDHA	Lactate Dehydrogenase A
LDHB	Lactate Dehydrogenase B
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
OAA	Oxaloacetic Acid
OXPHOS	Oxidative Phosphorylation
PDAC	Pancreatic Ductal Adenocarcinoma
PKM2	Pyruvate Kinase Muscle 2
PPP	Pentase Phosphate Pathway
qPCR	Quantative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
S18	Ribosomal Protein S18
SOD	Superoxide Dismutase
TCA-cycle	Tricarboxylic Acid cycle

1 Introduction

One of the most lethal and insidious cancer forms today is the pancreatic ductal adenocarcinoma (PDAC), with a global death rate of 95-97% within five years after diagnosis [1, 2]. This can be explained by late discovery, which makes surgical resection not attainable, and because of the lack of response to other treatments, as for example chemo- and radiotherapy. This resistance is due to the cellular and molecular features of the pancreatic tumors, each one unique for every patient [3]. Recent studies have given an expanded understanding in the field of the features of the PDAC and this may aid in increasing the survival rate [4].

1.1 Reprogrammed Metabolism

Energy is essential for the cell to sustain its function and existence. There are two different ways to metabolize glucose in the cell, through the aerobic and the anaerobic pathway.



(a) Aerobic pathway (b) Anaerobic pathway

Figure 1: Energy producing pathways in the metabolism [5].

As seen in Figure 1a, the aerobic pathway uses the oxidative phosphorylation (OXPHOS) in the mitochondria as an energy provider [4]. In the final step of

the OXPHOS, which depends on the conversion of Nicotinamide Adenine Dinucleotide (NAD^+) to NADH, Adenosine Diphosphate (ADP) is converted to Adenosine Triphosphate (ATP), and the energy is provided to the cell through the energy-carrying ATP [5]. Responsible for this cycle is the enzyme Glutamate Dehydrogenase 1 (GLUD1), which plays a part in providing the mitochondria with glutamine. The anaerobic pathway, Figure 1b, uses the noncanonical pentase phosphate pathway (PPP) [6]. The PPP metabolizes glutamine with a method contrasting to the classical mitochondrial pathway, which is by providing the cell with a different kind of energy carrier, the reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) [5].

Healthy cells uses OXPHOS as an energy-providing pathway, since it is more efficient compared to the anaerobic pathway. Recent studies have shown that the metabolism in PDAC is rewired to the anaerobic pathway [7]. By forcing the cell to use this specific metabolism, the NADPH production dominates in PDAC [8]. What seperates these two kinds of energy carrying molecules is their function in the cell. NADPH is, as opposed to ATP, primarily used for cell proliferation [5], and when it is dominating the PDAC instead of ATP, the cell division happens more rapidly [6]. Furthermore, the PPP increases the cell's dependency on glutamine [5]. It has been shown that the Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) is liable for this reciprocated metabolism in PDAC [4, 5, 9]. Linked to KRAS in altering the metabolism pathway is the enzyme Glutamic-oxaloacetic Transaminase 1 (GOT1) [8].

1.2 GOT1 in Healthy Cells and PDAC Cells

GOT1 is an enzyme in the cytoplasm that plays a role in several of the metabolic pathways [10]. Its main function is to convert glutamine-derived Aspartic Acid (ASP) into Oxaloacetic acid (OAA) [4]. In healthy cells (using the OXPHOS) GOT1 is active in the conversion to sustain the NAD^+ and the NADH circulation seen in Figure 1a. However, in PDAC cells, GOT1 operates in the PPP for the NADPH production to regulate the cellular redox balance, consequently controlling the NADPH/$NADP^+$ ratio [5]. This enzyme has a central part in supporting the cell with energy and is therefore fundamental for the cell's survival in PDAC, since the KRAS transcriptionally represses GLUD1 while activating GOT1 [4, 8]. This is believed to happen in order to maintain the cellular redox homeostasis [5].

1.3 Oxidative and ER Stress in Tumor Cells

A redox imbalance is termed as oxidative stress [11]. The redox homeostasis in the cell is a balance between the amount of antioxidants as reactive oxygen species (ROS) and the velocity with which the cell's biological system detoxifies the oxidants and repair the damages [12]. In healthy cells, the ROS production is regulated by the tricarboxylic acid cycle (TCA-cycle), as a consequence of the glutamine catabolism, and, ROS can therefore alter the redox balance[13]. Weinberg et al have, among others, indicated that mitochondrial ROS increase are induced by mutated KRAS, to control the cellular proliferation [6]. When the regular energy metabolism pathway is inactive, the cells are more vulnerable to ROS. ROS is a regulator of the cell cycle progression and is essentially required for tumorgenesis [14]. Mitochondrial metabolism is chiefly utilized to provide the resources, ROS,

for a hasty cell division. Consequently, a cellular oxidative stress can cause cell death or adaption; the cells that do not succeed to adapt will therefore die or fail to divide [13].

Cell death can also happen due to endoplasmatic reticulum (ER) stress, which too can be caused by an imbalance in the redox homoestasis. When the normal functions in the ER are disturbed, it will lead to a stress response called the unfolded protein response. This response is designed to compensate for eventual damage, and is responsible for restoring the homeostasis in the ER. However, if the functions are restrained for a longer period, the stress response will trigger apoptosis [15].

1.4 Aim of the Study

Due to the connection between KRAS, GOT1 and the altered metabolism, it is of interest to disrupt the pathway that is active in tumor cells, without damaging healthy cells. The aim of this study is to analyze the gene expressions of nine different genes, Lactate Dehydrogenase A (LDHA), Lactate Dehydrogenase B (LDHB), Pyruvate Kinase Muscle 2 (PKM2), Glutathione Reductas (GSR), Glutathione Synthetase (GSS), Superoxide Dismutase (SOD), DNA damage-inducible Transcript 3 (CHOP), Binding Immunoglobulin Protein (BiP) and Growth Arrest DNA Damage (GADD). LDHA, LDHB and PKM2 are part of the metabolism [16, 17, 18], GSR, GSS and SOD respond to oxidative stress [19, 20, 21] and BiP, CHOP and GADD respond to stress in the ER [24, 22, 23]. The goal is to study how the metabolic pathways are affected by the knocked out GOT1. It is also of

interest to see if the disruption of GOT1 could enable a way to kill the tumor cell due to energy fatigue. To achieve this, the gene expressions of the different genes will be compared between GOT1 knockout and wild type osteosarcoma cells. Osteosarcoma cells can be used since a KRAS mutation is established, and therefore respond the same way as PDAC when GOT1 is knocked out. Ribosomal protein S18 (S18) will be used for the normalization of the genes in the comparisions. Depending on the results of the analysis, this could be a development to future treatment of the malignant disease.

2 Method

The study was performed using RNA extraction, complementary DNA (cDNA) synthesis and real-time quantative polymerase chain reaction (qPCR). All the solutions and reactions were placed on ice during the procedures and vortexed to get homogenous solutions.

2.1 RNA Extraction

RNA extraction was performed using Qiagen's RNeasy Mini Kit, Purification of Total RNA from Animal Cells using Spin Technology [25]. The cells used were from already developed osteosarcoma cell lines, grown in monolayer, one GOT1 knockout cell line and one with GOT1. The cells were pipetted up and down in the tubes they were in five times before 350 µL of 70% ethanol was added to the cells. Then they were pipetted again and 700 µL of the solution was transferred to a RNeasy spin column placed in a 2 ml collection tube, and centrifuged at 10 000 rpm for 15 s. 700 µL Buffer RW1 was added to the column and centrifuged at 10

000 rpm for 15 s before adding 500 µL of Buffer RPE to the column, which was then centrifuged at 10 000 for 2 min. The flow-through was discarded after every centrifugation. The spin column was moved to a new 2 ml collection tube, and centrifuged at 13 000 rpm for 1 min. The column was placed in a 1.5 ml collection tube and 40 µL of RNase-free water was added directly to the filter of the spin column, and was centrifuged at 10 000 rpm for 1 min. In total 18 RNA samples were extracted, 9 wild type and 9 GOT1 knockout samples. The absorbance and concentration of the RNA were measured using the NanoDrop Spectrophotometer ND-1000, the data can be found in Appendix A.

2.2 cDNA Synthesis

Complementary DNA (cDNA) was synthesised from the RNA templates, using the High Capacity cDNA Reverse Trancription Kit (Applied Biosystems) [26]. A mastermix was made, see Table 1, and divided into 20 tubes. The different RNA samples were added seperately, 2.5 µL in each tube. Two cDNA samples were used as a control, and water was added instead of RNA templates. 20 µL of DNA synthesis master mix was prepared for each sample. The reverse transcription of the samples were performed in a 2720 Thermal Cycler (Applied Biosystems) [27] in 25°C for 10 min, 37°C for 120 min and 85°C for 5 min.

Table 1: Components and amounts of the master mix for the cDNA synthesis.

Components	**Amount 1 Reaction [µL]**	**Total Amount [µL]**
10X RT Buffer	2	40
25X dNTP mix	0.8	16
10X RT Random	2	40
MultiScribe™ Reverse Transcriptase	1	20
Nuclease-free H_2O	11.7	234
RNA	2.5	-
Total	20	350

2.3 Real-time qPCR

Firstly primers ordered from Sigma-Aldrich, with sequences coding for the studied genes, see Appendix B, were diluted to 10 µM . This was performed by adding 90 µL of water and 10 µL of each primer into a tube, which then was centrifuged. The primers were then freezed for future usage.

The qPCR was performed using KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal(Kapa Biosystems) [28]. 10 µL of PCR master mix was prepared for 20 reactions for each gene, see Table 2. The master mix and the different cDNAs, 1 µL in each well, were then distributed into three 96-well plates, with one negative control with water instead of cDNA of each gene. The plate was centrifuged for

1 min with 1 000 rpm and analyzed in the 7500 Fast Real Time PCR System. The relative amount of target, normalized to the endogenous reference (S18) and relative to another reference (the first sample of each specific gene) were calculated with

$$\text{Relative} \quad \text{quantification} = 2^{-\Delta\Delta(CT)},$$

where the CT value is the cycle threshold.

Table 2: Components and amounts of the master mix for qPCR.

Components	**Amount 1 Reaction [µL]**	**Total Amount [µL]**
KAPA SYBR Fast qPCR Master Mix Universal	5	100
Forward Primer	0.2	4
Reverse Primer	0.2	4
ROX LOW	0.2	4
Water	3.4	68
Template (cDNA)	1	-
Total	10	**180**

2.4 Statistical test

The mean values and the standard deviations were calculated. A two-tailed Student's T-test was performed for every comparision.

3 Results

The results are presented in Figure 2-8. Figure 2, 3, 4 and 5 show the gene expressions of the different genes with all samples included. Figure 6, 7 and 8 represent the gene expressions with six chosen samples, which had non-contaminated RNA. The results are presented in seperate bar graphs, which represents the relative quantification of the studied gene in wild type and GOT1 knockout osteosarcoma cells. The error bars serve as the standard deviations. With all samples included, GOT1 showed significant difference, see Figure 2, and LDHB, CHOP and GADD showed tendency to a difference, see Figure 3b, Figure 4b and Figure 4c. GSR showed tendency to a difference after contaminated RNA samples omitted, see Figure 8a. LDHA, PKM2, GSS, SOD and BiP showed no significant difference. As seen in Figure 2, there is no considerable gene expression of GOT1 in the GOT1 knockout cell. Of the genes studied in the metabolism, LDHB showed a reduced gene expression, as seen in Figure 3b and 6b. In the comparisions of the ER stress related genes, both CHOP and GADD showed an increased gene expression in the GOT1 knockout cells with all the samples included, as seen in Figure 4b and 4c. However, in the comparisions without the contaminated RNA, only GADD showed a difference in expression, see Figure 7c. Of the genes studied responding to oxidative stress, GSR showed an increased expression only in the comparisions with the non-contaminated RNA, see Figure 8a.

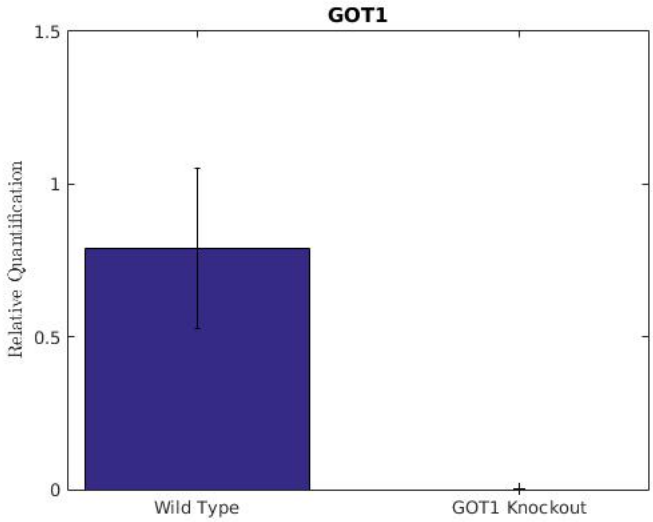
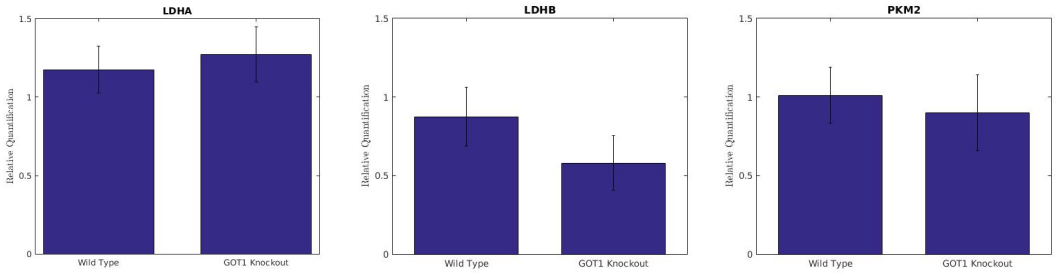
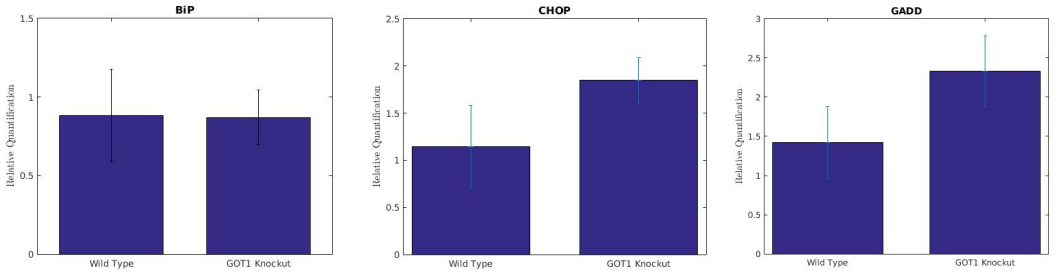


Figure 2: Gene expression in wild type and GOT1 knockout cells of GOT1.



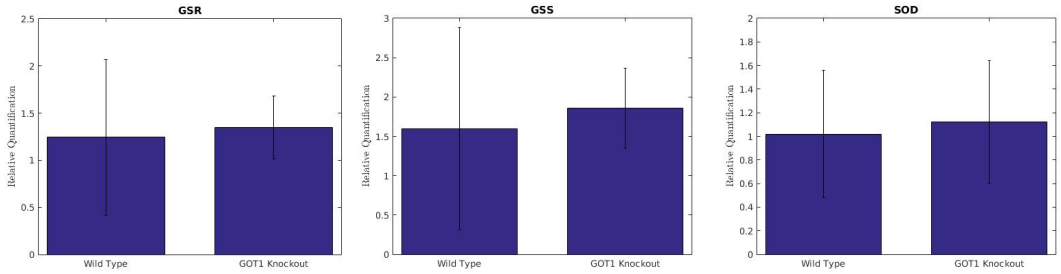
(a) LDHA (b) LDHB (c) PKM2

Figure 3: Gene expressions in wild type and GOT1 knockout cells of genes in the metabolism, analyzed with all samples.



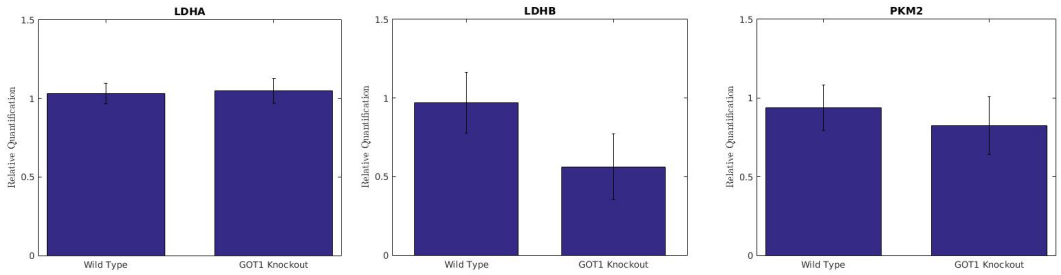
(a) BiP (b) CHOP (c) GADD

Figure 4: Gene expression in wild type and GOT1 knockout cells of genes responding to ER stress, analyzed with all samples.



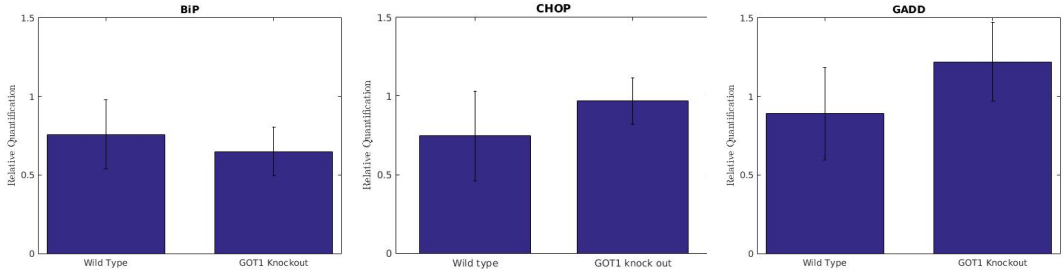
(a) GSR (b) GSS (c) SOD

Figure 5: Gene expression in wild type and GOT1 knockout cells of genes responding to oxidative stress, analyzed with all samples.



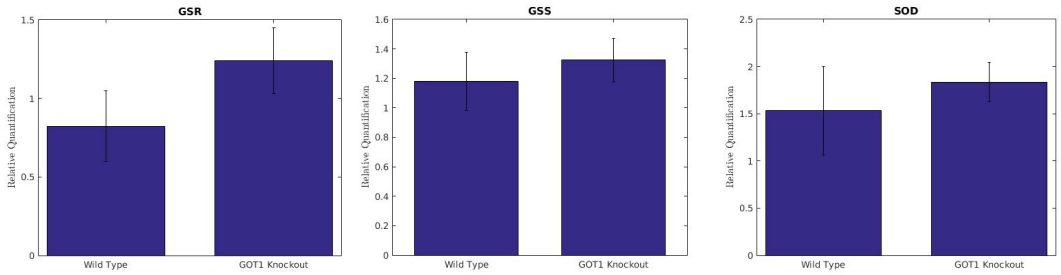
(a) LDHA (b) LDHB (c) PKM2

Figure 6: Gene expressions in wild type and GOT1 knockout cells of genes in the metabolism with non-contaminated RNA samples.



(a) BiP (b) CHOP (c) GADD

Figure 7: Gene expression in wild type and GOT1 knockout cells of genes responding to ER stress with non-contaminated RNA samples.



(a) GSR (b) GSS (c) SOD

Figure 8: Gene expression in wild type and GOT1 knockout cells of genes responding to oxidative stress with non-contaminated RNA samples.

4 Discussion

The aim of this study was to compare the gene expressions measured with real-time qPCR. Considering the value of the t-test in the GOT1 comparision, p=0,00000018, it was statistically separated from the coincidence, and a significant reduction can be concluded. This means that conclusions can be drawn with assurance that GOT1 is knocked out in all the other studied genes as well, which is essential for further analysis.

The reduced gene expression in LDHB could indicate that a knockout of the GOT1 gene causes a dysfuncionality in the metabolic pathway. Since the gene coding for LDHB shows less activity, the disruption probably slows down or block the PPP. The decelerated or suspended pathway in PDAC, or in osteosarcoma cells as in this case, results consequently in less energy in the cell. Limited energy will engender either slower cell proliferation, completely prohibited cell division or even cell apoptosis. These outcomes could be possible in the rest of the genes that showed a difference in gene expression in the GOT1 knockout cells. A reason to the in-

creased expression of the stress related gene GADD, could be boosted ER stress and the increased expression of GSR is probably due to more oxidative stress in the cell. These conclusions can be drawn since the genes are more active in the GOT1 knockout cells compared to the wild type osteosarcoma cells, and happen due to the environmental changes in the genes when GOT1 is knocked out, which causes the cells to stress. If the ER and oxidative stress is established long enough, this will eventually lead to slower or blocked cell proliferation, or apoptosis. This would in a larger picture impede continued tumor growth.

Based on the results, regulation of GOT1 could theoretically restrain the tumor. However, only the mRNA levels of different genes in GOT1 knockout cell lines were measured, and not their analogous proteins. It must be taken into consideration that an affected gene does not necessarily mean a difference in protein expression, as gene expressions can be regulated by translational, post-transcriptional and protein degeneration [29]. In order to further analyze and determine the effects of GOT1 knockout, it is essential to study the protein expressions of the genes of interest in these cell lines.

However, these results are not completely credible. For the RNA to be useful in studies, the samples must be of a certain quality. The general requirement is set to a 260/280 nm ratio value of at least 2.0, and a 260/230 ratio value also of at least 2.0. If they are below a value of 2.0, the samples are probably contaminated with for example proteins or phenol [30]. As seen in Appendix A, the majority of the samples had a 260/230 ratio value below the set value. Consequently, to be able to carry out the experiment, the adequant 260/230 ratio value was set to 1.8,

which included only three samples in each cell line. Hence, the quantity of samples was very low and the assurance of the results can therefore not be guaranteed. Additionally, considering the low quality of the RNA samples, the comparisions with all samples included could be misleading as well, since the gene expressions could be affected by contamination. The comparisions have a relatively high standard deviation, in addition to overlapping between the different cell lines, which even further add to the uncertainty. The high standard deviations in these comparisions, are due to the samples developed in different cell cultures, which enables the possibility of the gene reacting differently in variated samples under the same circumstances. The large number of omitted RNA, due to possible contaminations, is therefore the largest source of error in this study.

It is of interest to develop further studies concentrated on the genes that, if not definitely showed a significant difference, at least showed a tendency to be affected by the knocked out GOT1. To secure the effects, it would be necessary to analyze both on a genetic level and a protein level, as well as with more RNA pure samples with duplicates or triplicates of each sample. Future research also need to be targeted on how healthy cells are affected by GOT1 knockout, so a blocking of the gene does not damage more than it helps. This study contributes to highlight the possibilities of a future cure for pancreatic cancer. With further studies in the field, a treatment that is targeting this area is possible, and would eventually hopefully decrease the high death rate in PDAC.

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6 Appendix

A Measurements of RNA Concentration and Absorbance with NanoDrop Spectrophotometer

Table 3: RNA concentration and absorbance.

Sample	Concentration[ng/µL]	A260 [nm]	A280 [nm]	260/280	260/230
WT1	461,76	11,544	5,583	2,07	1,29
WT2	454,44	11,361	5,592	2,03	2,08
WT3	470,89	11,772	5,708	2,06	1,98
WT4	678,16	16,954	7,993	2,12	1,44
WT5	775,70	19,392	9,156	2,12	1,31
WT6	549,37	13,734	6,381	2,15	1,69
WT7	515,20	12,880	6,044	2,13	1,87
WT8	499,00	12,475	5,878	2,12	1,64
WT9	516,17	12,904	6,041	2,14	0,99
GOT1	431,22	10,781	5,190	2,08	1,88
GOT2	393,29	9,832	4,686	2,10	2,09
GOT3	395,56	9,889	4,740	2,09	1,21
GOT4	426,20	10,655	5,179	2,06	1,86
GOT5	580,13	14,503	6,512	2,23	1,93
GOT6	512,38	12,809	5,827	2,20	0,98
GOT7	456,61	11,415	5,526	2,07	1,93
GOT8	507,38	12,685	5,843	2,17	1,41
GOT9	450,71	11,268	5,392	2,09	1,46

B Gene and Primer Sequences for qPCR

BiP

Forward: CTCAACATGGATCTGTTCCG

Reverse: CCAGTTGCTGAATCTTTGGA

Full gen sequence can be found at CCSD [31].

CHOP

Forward: TCCTGGAAATGAAGAGGAAGA

Reverse: CAGGGAGCTCTGACTGGAAT

Full gen sequence can be found at CCSD [32].

GADD

Forward: TCCGAGTGGCCATCTATGTA

Reverse: AGGGTCCGGATCATGAGTAG

Full gen sequence can be found at CCSD [33].

GOT1

Forward: AGTCTTTGCCGAGGTTCCG

Reverse: GTGCGATATGCTCCCACTC

Full gen sequence can be found at CCSD [34].

GSR

Forward: ACTTGCGTGAATGTTGGATG

Reverse: ACCCTCACAACTTGGAAAGC

Full gen sequence can be found at CCSD [35].

GSS

Forward: AAGCCTATGCTGTGCAGATG

Reverse: TTTGATGGTGCTGGAAAGAG

Full gen sequence can be found at CCSD [36].

LDHA

Forward: ATGGAGATTCCAGTGTGCCT

Reverse: TGGGTGCAGAGTCTTCAGAG

Full gen sequence can be found at CCSD [37].

LDHB

Forward: GAGGCAACAGTTCCAAACAA

Reverse: AGCCAGAGACTTTCCCAGAA

Full gen sequence can be found at CCSD [38].

PKM2

Forward: GGCATCTGATGTCCATGAAG

Reverse: TTTCATCAAACCTCCGAACC

Full gen sequence can be found at CCSD [39].

S18

Forward: TCACTGAGGATGAGGTGGAA

Reverse: GCTTGTTGTCCAGACCATTG

Full gen sequence can be found at CCSD [40].

SOD

Forward: TGAAGAGAGGCATGTTGGAG

Reverse: ATGATGCAATGGTCTCCTGA

Full gen sequence can be found at CCSD [41].