Evaluation of CRISPRi/dCas9 knockdown efficacy by qRT-PCR in LN-229 cells

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1. Abstract

Glioblastoma multiforme (GBM) is considered as one of the most common malignant and invasive central nervous system (CNS) tumour. Temozolomide (TMZ) is the only chemotherapeutic alkylating agent demonstrated to have some clinical efficacy in the treatment of GBM along with surgery and radiotherapy. Unfortunately, resistance mechanisms occur, which leads TMZ to work poorly against GBM and therefore the median survival of the patient is only 12 to 15 months. The identification and characterization of genes and pathways involved in chemotherapeutic resistance in GBM has been of long-standing interest to improve the therapies. Therefore, the aim of this thesis was to establish a stable CRISPRi/dCas9 gene knockdown in LN-229 cells. The CRISPRi system was used to generate two knockdown cell lines. The qRT-PCR results showed for gene 1 a knockdown of 96.75% and for gene 2 a knockdown of 79.51%. The knockdown were significant different compared to the control group ($p \leq 0.0001$). These cells have a sufficient knockdown and will be used in the future to analyse their contribution to TMZ. Additionally, a literature search was performed on two additional genes *MPG* and *MSH6*. While higher gene expression of *MPG* seems to be more TMZ resistant compared to the cells with lower gene expression, higher gene expression of *MSH6* as well as lower expression seems to have an effect on TMZ resistance. In both cases the generated adducts by TMZ treatment to induce apoptosis will be either tolerated or not recognized ending in a therapy resistance of the cells. *MPG* as well as *MSH6* seems to be interesting genes for further applications and analysis.

2. Introduction

Glioblastoma multiforme

GBM is the most common malignant primary brain tumour in adults, which are classified as the highest CNS tumour of grade IV by the World Health Organization [1]. A total resection of the tumour can damage brain functions due to the high infiltrative growth pattern of GBM. Therefore, radiation therapy and chemotherapy is often required [1]. The most commonly used alkylating agent for GBM is TMZ. Unfortunately, TMZ resistance can occur due to deoxyribonucleic acid (DNA) repair system or other mechanisms like autophagy, cellular drug efflux etc.[2]. Investigation of genes and pathways involved in resistance mechanism against TMZ in GBM would help to improve the therapies [2].

CRISPR interference

The advantage of clustered regularly interspaced short palindromic repeats interference (CRISPRi) dead CRISPR-associated protein (dCas9) is used to target a specific DNA sequence and performing a gene knockdown. dCas9 has a point mutation on the nuclease domains [3] and the dCas9 is fused to an effector domain such as the Krüppel associated box (KRAB), which repress the transcription of RNA polymerase, promoters binding and splicing of RNA as well as the control of nucleolus function [4]. Due to the properties of the deactivated nucleases combined with the function of KRAB, a gene knockdown is performed, which suppress the gene expression and the help of customized single guide RNA (sgRNA) a sequence specific silencing can be performed with minimal off-target effect [5].

3. Aim and Leading Questions

The goal of this thesis is to establish stable CRISPR knockdown cell lines with two genes for further investigation for the research group using LN-229 as cell line. Additionally, a literature search was performed on two additional genes *MPG* and *MSH6.*

To achieve this goal the following aim and questions were formulated:

1. Could we approve a correctly ligated insert into the pCRISPRi/a backbone by PCR and Sanger sequencing?
2. Does the dCas9 expressing LN-229 cells resulted in a knockdown by transducing them with sgRNA vector and how strong is the knockdown compared to the control group?
3. How are the genes *MPG* and *MSH6* involved in the temozolomide resistance?

4. Material and Methods

- Restriction digestion was performed on plasmid vector pCRISPRi/a-v2 (Addgene #84832) using restriction enzymes BlpI and BstXI.
- The digested plasmid was ligated with the gene of interest and confirmed by colony PCR and Sanger sequencing.
- LN-229 CRISPRi was transduced with the modified pCRISPRi/a-v2 containing the gene of interest was transduced. Transduction rate were determined by using flow cytometry.
- Total RNA from the transduced LN-229 CRISPRi cells were isolated and the gene knockdown were determinated by qRT-PCR

5. Results

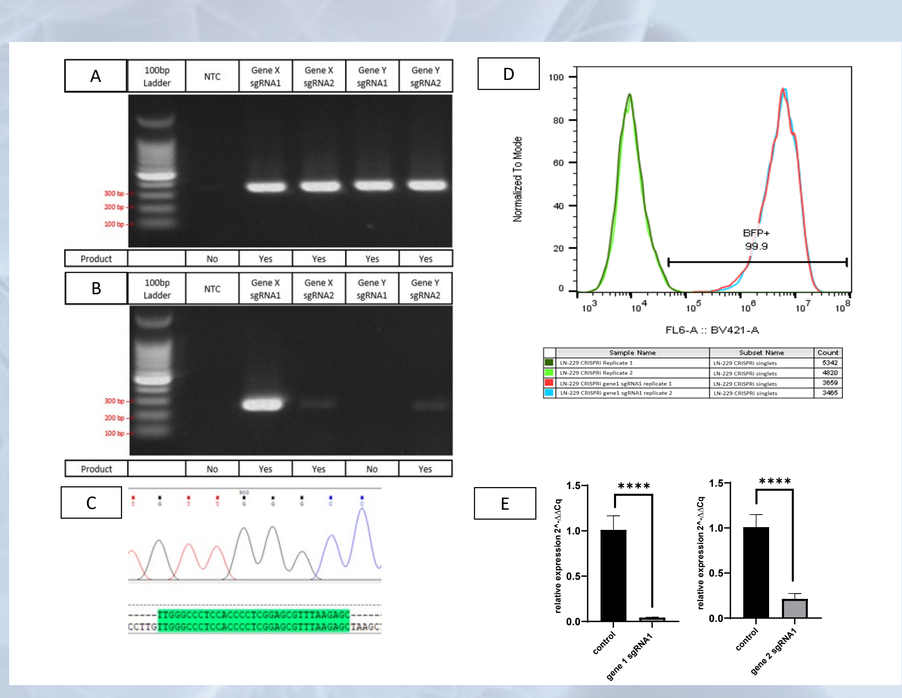

Figure 5.1: **A & B** Representative results of the colony PCR. Colony PCR results for gene 1 sgRNA1 and gene 2 sgRNA1 were same as the result of gene Y sgRNA1. **A**: Primers were designed to target the backbone of the plasmid vector no matter if the insert of the gene of interest is ligated or not. **B:** Primers were designed to target the sequence, which should be removed by the cleavage of the restriction enzymes. **C:** Representative Sanger sequencing results of plasmid vectors. Sanger sequence shows that gene of interest (gene 1 sgRNA1 and gene 2 sgRNA1) was ligated into the backbone of plasmid vector pCRISPRi/a-v2 (Addgene #84832). **D:** Representative flow cytometry result shows the transduction rate. Transduction rate were about 99.9% for gene 1 and gene 2. **E:** The unpaired t-test shows that the relative expression level for both genes are significantly lower than that of the control group. **** $p<0.0001$

The qRT-PCR results showed for gene 1 a knockdown of 96.75% and for gene 2 a knockdown of 79.51%.

6. Discussion

1. The ligated backbone pCRISPRi/a could by verified by PCR and Sanger Sequencing
2. The dCas9 expressing LN-229 resulted in a knockdown of 96.75% respectively 79.51% with a *p*-value of ≤0.0001. Therefore, the generated knockdown cells can be used for further investigation for TMZ resistance analysis.
3. In case of MPG produced adducts by TMZ treatment to induce apoptosis will be removed through the MPG protein, so overexpression leads to resistance against TMZ. In the case of MSH6 overexpression and downregulation of the gene leads to disruption of MutSα formation, which maintain mismatch repair. The adducts will not recognized in this case, which ended up in a survival and more resistant GBM cells.

The production of this knockdown cell lines are just the beginning, but it could be additionally approved by western blots on a protein level. At the end further investigation is needed to approve their involvement in TMZ resistance by TMZ treatment and finally it would be interesting if these genes can be used as a drug targets to treat GBM patients and work with the pharmaceutical industry to invent new drugs.

References

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Figure Glioblastoma multiforme: Retrieved 05.06.2021 from https://theconversation.com/glioblastoma-why-these-brain-cancers-are-so-difficult-to-treat-61376

Figure 5.1 **A-D** – Phour J. (2021) Tumorpathology III, Institute of Pathology, University of Bern, Bern Switzerland

Figure 5.1 **E** – Joseph C. (2021) medi | Center of medical education, Bern Switzerland