

CRISPR/Cas9-mediated knockout of the TAM receptor MERTK: a strategy to overcome chemoresistance in bladder cancer

Alina Aimuratova, Molecular Medicine, 2nd year

ORCID iD: 0009-0002-7056-4509

**Submitted in fulfilment of the requirements
for the degree of Master in Molecular Medicine**



Nazarbayev University

School of Medicine

Department of Biomedical sciences

5/1 Kerey and Zhanibek Khandar street,

Astana, Kazakhstan

PI: Marina Kriajevskaia

April 20, 2026

DECLARATION

I hereby declare that the thesis is my original work, and it has been written by me in its entirety. I have acknowledged all the sources of information which have been used in the thesis. This thesis has not been submitted for any degree at any university previously.

Student name:

Alina Aimuratova

ORCID ID:

0009-0002-7056-4509

Signature:

A handwritten signature in black ink, appearing to be 'Alina Aimuratova', written in a cursive style.

Abstract

MERTK is a proto-oncogene with tyrosine kinase from the TAM family, which also include Axl and Tyro3. MERTK is produced on different cell types which include innate and adaptive immune cells as well as platelets and oocytes. MERTK can be activated through binding to its ligands, Gas6 and Protein S. MERTK signaling is involved in a number of events, including platelet aggregation, cytoskeleton reorganization, engulfment, and macrophage clearance of apoptotic cells (efferocytosis).

Recent studies have explored the association between MERTK and many human malignancies and thus suggested this gene as a novel approach for cancer treatment. MERTK activity is most pronounced within the tumor microenvironment as well as in the virus-infected tissues characterized by high externalized phosphatidylserine. MERTK overexpression on tumor cells results in the development of multiple human epithelial cancers. Once MERTK binds to its ligands, it activates many downstream signaling pathways, including PI3K/AKT and MAPK/ERK and STAT, that leads to cell survival and tumor growth and cancer progression. The specific function of MERTK in bladder cancer remains unclear despite its significant effects on human cancer development.

This study focused on using CRISPR/Cas9 to downregulate the gene of MERTK in T24 bladder cancer cells in order to better understand its role in chemoresistance. By designing sgRNAs targeting exon 5 of MERTK, the aim was to transfect bladder cancer cells with sgRNA-containing vectors and confirm gene knockout through detection of deletion events. Overall, this study explored whether downregulation of MERTK expression can make cancer cells more sensitive to treatment, and therefore suggest it as a possible therapeutic target.

Keywords: MERTK, TAM receptor tyrosine kinases, Gas6, Protein S, bladder cancer, chemoresistance, CRISPR/Cas9

Table of Contents

- 1. MERTK as a member of the TAM family receptor tyrosine kinase**
 - 1.1. MERTK Expression
 - 1.2. The role of phosphatidylserine (PtdSer) in MERTK activation
 - 1.3. Physiologic Functions of MERTK
- 2. MERTK signaling in cancer**
 - 2.1. MERTK-Mediated Immune Evasion: Efferocytosis and Checkpoint Regulation
- 3. MERTK in Bladder cancer**
- 4. CRISPR/Cas9 Biology and Its mechanism of action**
- 5. Research Design**
 - 5.1. Hypothesis
 - 5.2. Aim
 - 5.3. Objectives

Materials and Methods

Results

Discussion

Conclusion

References

Introduction

Bladder tumor (BCa) represents the 9th most common tumor type worldwide, with more than 600,000 cases and approximately 230,000 mortality reported in 2022, representing a 3.1% increase from 2020 (Yu et al. 2025, Leslie et al. 2024). The disease shows a pronounced male predominance and exhibits marked heterogeneity in clinical outcomes (Zhou et al., 2025). Approximately $\frac{3}{4}$ part of patients with bladder tumor are diagnosed as non-muscle-invasive disease (NMIBC). However, despite aggressive treatment approaches including transurethral resection and intravesical therapy, 30-50% of NMIBC cases recur within 5 years, and 10-20% progress to the more serious muscle-invasive form (MIBC) (Li et al., 2024). Once the disease progresses to MIBC, treatment becomes more intensive, requiring radical cystectomy and systemic chemotherapy. Unfortunately, survival rates are still low despite these intensive interventions, and about 50% of MIBC patients experience metastatic disease within two years of diagnosis.

The genetic profile of bladder cancer shows extensive molecular variation. The genetic mutations TP53 (49%), PIK3CA (20%), FGFR3 (20%) and RB1 (18%) occur frequently in bladder cancer (Li et al., 2024). The different genetic patterns in tumors lead to unpredictable treatment outcomes and create conditions for drug resistance to develop. Therefore, identifying additional molecular drivers and regulatory mechanisms is essential.

1. MERTK as a member of the TAM family receptor tyrosine kinase (RTK)

MERTK is part of the TAM family of receptor tyrosine kinases, alongside TYRO3 and AXL. The TAM family receptors share structural features and several similar functions to maintain cellular equilibrium and control immune responses as well as tissue restoration. (Graham et al., 2014). The outer segment of these receptors contains a pair of Ig-like domains paired with a pair of fibronectin type III domains which connect to their intracellular tyrosine kinase C-terminal domain (Figure 1).

RTKs of the TAM family can be triggered by two primary physiological ligands, Growth Arrest-Specific 6 (Gas6) and Protein S (ProS). Despite both ligands are specific to the TAM family of RTKs, their binding to these receptors occur with different affinity. While Gas6 is able to activate all of them, it shows the greatest selectivity for Axl. On the other hand, ProS has lower affinity to

Axl, and binds to Tyro3 more strongly than to MERTK. Additionally, the binding affinity of Gas6 to MERTK is higher than that of Protein S, and thus Gas6 functions as the primary activating ligand under most physiological conditions (Lemke, 2013).

TAM family receptors have distinct functions when they are expressed, despite sharing the same phenotype. For instance, aggressive cancer cell lines typically express AXL, which has the highest affinity for its ligand, Gas6, and supports the spread of malignant cells. Moreover, MERTK, that is found mostly on macrophages, is able to suppress immune system function and increase the survival of cancer cells. Furthermore, the expression of TYRO3 in different cell types leads to cancer cell growth and immune system evasion.

The binding of ligands to MERTK triggers protein dimerization which leads to intracellular tyrosine residue autophosphorylation that generates sites for downstream signaling protein binding.

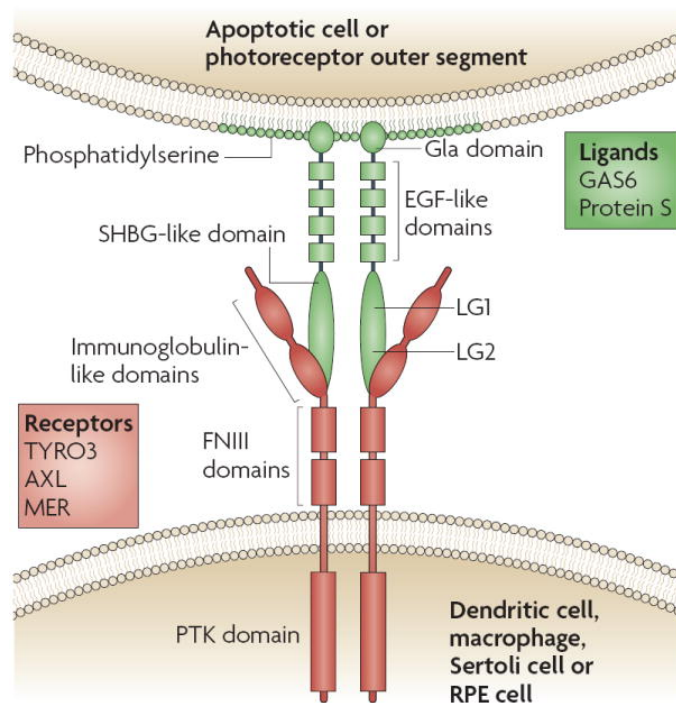


Figure 1. TAM receptors and their ligands. Lemke, G., & Rothlin, C. V. "Immunobiology of the TAM receptors"

1. MERTK expression

MERTK is found on various cells, such as innate and adaptive immune cells as well as platelets and oocytes. (Lahey et al. 2022). Overexpression of MERTK was observed in wide range of human cancers (Lahey et al. 2022).

When compared to nearby normal lung tissue, MERTK expression was elevated in almost 70% of tumours in non-small cell lung cancer (NSCLC) (Yan et al. 2021). A role for MERTK in glioma biology is also supported by the high MERTK expression found in astrocytoma patient samples and corresponding cell lines (Hulse et al., 2020). Another characteristic of breast cancer is elevated MERTK levels; according to Nguyen et al. (2014), about 70% of the tumours that were examined showed noticeably higher protein expression than normal controls.

The characteristic fusion background of the majority of alveolar rhabdomyosarcomas, PAX3–FKHR-transduced cell lines, have been shown to exhibit MERTK induction, and schwannoma tissue has also been shown to exhibit notable overexpression. Higher MERTK mRNA or protein levels have been linked to worse patient outcomes in gastric cancer, whereas high MERTK expression is linked to lower overall and disease-free survival in colorectal cancer (Hulse et al., 2020). Similar trends apply to melanoma: MERTK expression increases in tandem with the severity of the disease, peaking in metastatic lesions and exhibiting negligible expression in benign nevi (Schlegel et al. 2013). Overall, MERTK overexpression is a common characteristic of a variety of cancers and is often linked to more aggressive disease states.

1.2. The role of phosphatidylserine (PtdSer) in MERTK activation

While either ligand can initiate MERTK gene activation alone, optimal receptor binding and activity are achieved when a ligand acts as a bridge to connect MERTK to the lipid phosphatidylserine (PtdSer) (Hulse, et al., 2020). The phospholipid phosphatidylserine (PtdSer) exists primarily in eukaryotic cell membranes. The plasma membrane contains PtdSer in its inner layer during normal cell conditions but apoptotic cells show this phospholipid on their outer surface as shown in Figure 2. Once MERTK is bound to PtdSer through ligand dependent bridging, it promotes the apoptotic cells' removal by the process known as efferocytosis (Hulse, et al., 2020).

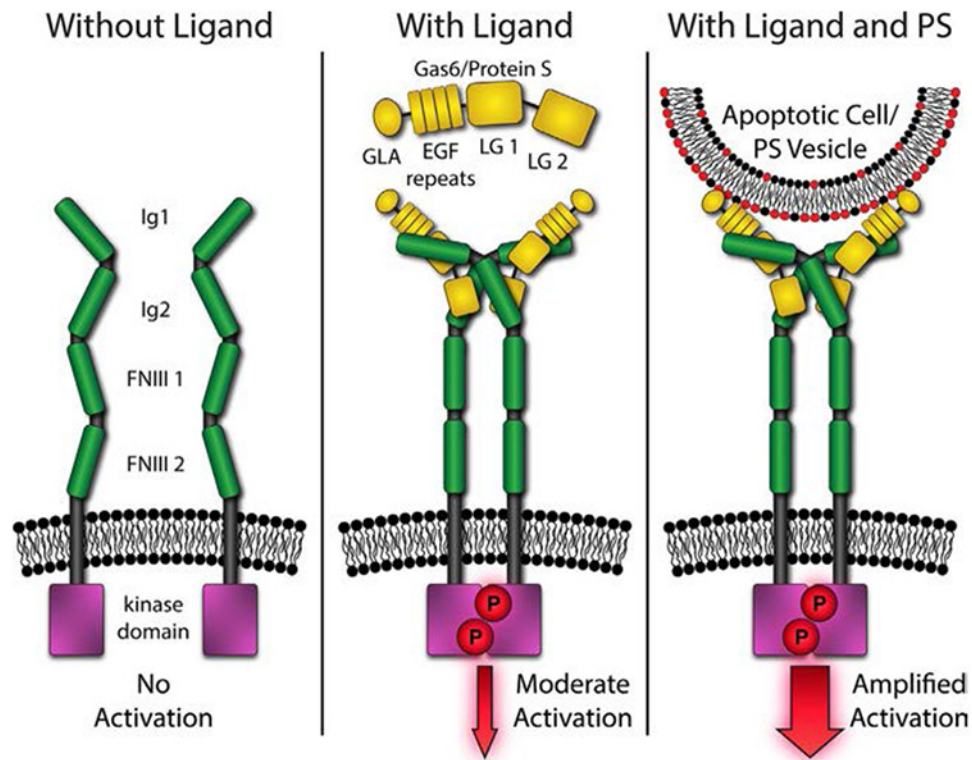


Figure 2. Lahey, K. C. et al. (2022). "MERTK: An emerging target in cancer biology and immuno-oncology".

1.3. Physiologic Functions of MERTK

MERTK performs critical homeostatic functions that include efferocytosis, preventing secondary necrosis and autoimmunity, platelet aggregation and thrombus stabilization as well as regulation of innate immune responses to prevent excessive inflammation (Rothlin et al., 2015).

MERTK's role in efferocytosis is particularly well-characterized. Recognition of PtdSer-expressing apoptotic cells via Gas6/MERTK signaling triggers cytoskeletal reorganization through activation of Rac1 and RhoA GTPases, enabling engulfment of the apoptotic cell (Lemke, 2013). Simultaneously, MERTK activation triggers a shift in the cytokine environment. When MERTK is activated, the expression of immune-checkpoint mediators, including IL-10 and TGF- β , is increased, while pro-inflammatory cytokines production, including IL-12, TNF- α , and IL-1 β , becomes low. This mechanism can be interpreted through the triggering of STAT1 and STAT3 pathways and the inhibition of NF- κ B signaling (Rothlin et al., 2015). Overall, efferocytosis and immunosuppression,

mediated by MERTK, are essential to maintain immune homeostasis, however, in the context of cancer, these mechanisms could result in tumor immune evasion.

2. MERTK signaling in cancer

It has been discovered that MERTK-induced signaling protects cancer cells and also help tumors to evade immune system detection (Lahey et al. 2022). Therefore, MERTK is an attractive target for cancer therapeutics. As earlier described, MERTK activation triggers multiple pathways, including PI3K/AKT, MAPK/ERK, JAK/STAT and YAP/TAZ. These pathways are responsible for cell survival, proliferation, and they also can prevent apoptosis as well as control expression of immune checkpoint proteins (Lahey et al. 2022).

Additionally, another study conducted by Wang et al. in 2024, have discovered that MERTK is able to control the apoptosis of hepatocellular (HCC) cancer cells through ERK/SP1 pathway. Interestingly, AKT and GSK3-beta expression in HCC cells has been increased as the level of MERTK increases (Liu et al. 2020).

Furthermore, overexpression of MERTK directly affects PI3/AKT pathway as well as Myc signaling in triple negative breast cancer (TNBC). Consequently, activation of these pathways in TNBC cells prevented death of apoptotic cells and promoted tumor growth (Lida et al. 2024).

In addition, NF-kB pathway is also activated by MERTK-induced downstream signaling. NF-kB pathway controls inflammatory responses by producing cytokines (Lahey et al. 2022). MERTK regulates NF-kB signaling in macrophages in order to decrease the inflammatory responses.

Furthermore, MERTK directly activates YAP/TAZ pathway, promoting cell proliferation, motility, as well as angiogenesis across various tumor types (Azad et al. 2019).

In melanoma, activation of MERTK caused the phosphorylation of different signaling proteins, such as p38, ERK1/2, GSK3 α/β , Akt, AMPK, STAT5, CHK-2, FAK, and STAT6 (Figure 2). Moreover, MERTK overexpression caused phospho-AKT levels to rise which led to cancer advancement and resistance to therapies (Cummings et al. 2013).

(c)

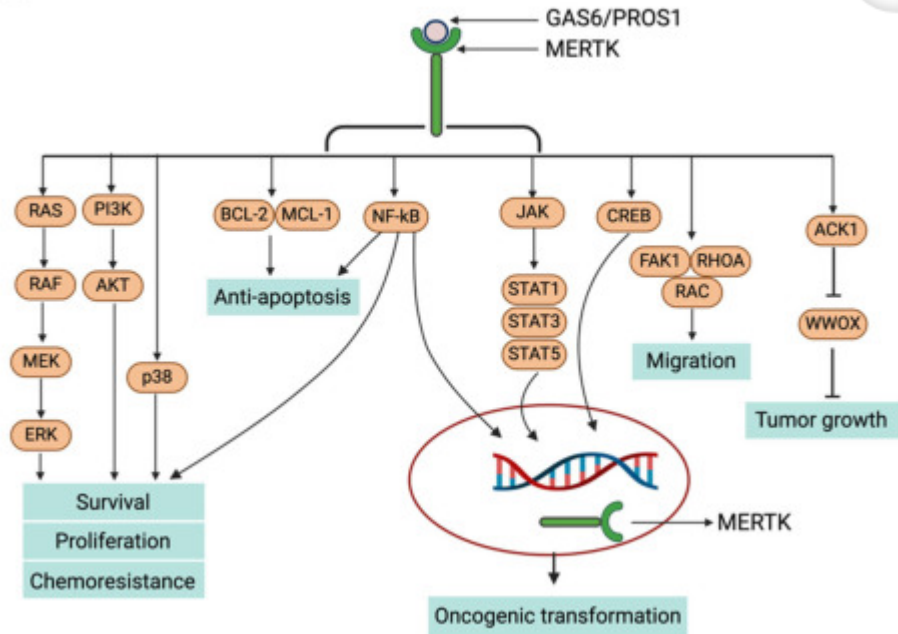


Figure 3. MERTK downstream signaling pathways. Tanim et al., 2024, “International Journal of Molecular Sciences”.

2.1. MERTK-Mediated Immune Evasion: Efferocytosis and Checkpoint Regulation

MERTK functions as a key factor which determines the immunosuppressive nature of tumor microenvironments while simultaneously influencing cancer cell behavior in tumor progression. The expression of MERTK on TAMs leads to efferocytosis of apoptotic tumor cells which produces extensive immunosuppressive effects (Cheng et al., 2024). The process of MERTK-mediated efferocytosis leads TAMs to become M2 polarized cells which express CD163, CD206, arginase-1 while releasing immunosuppressive cytokines including IL-10 and TGF- β . The immunosuppressive activity of M2-polarized TAMs leads to T cell tumor defense suppression through multiple mechanisms.

It has been discovered that efferocytosis causes the increased expression of CD-276 cells on TAMs, that leads to the inhibition of T cell activation and cytotoxic function (Cheng et al., 2024). For example, the clinical observation on bladder cancer patients showed that there is a positive correlation between MERTK expression, TAM infiltration, as well as CD-276 cells expression. The

increase in their level led to the decrease in CD8⁺ T cell infiltration. It can be concluded that MERTK contributes to the immunosuppression of tumor microenvironment. As a consequence, patients with high MERTK/high CD276 expression exhibited significantly worse 5-year overall survival compared to low expressors (Cheng et al., 2024).

Recent studies have discovered another mechanism of MERTK-induced anti-tumor immunity suppression. MERTK regulates PD-1 immune checkpoint components in addition to its effects on TAMs. The PD-1 immune checkpoint system includes PD-1 (programmed cell death protein 1) as its receptor protein and PD-L1 and PD-L2 (programmed cell death 1 ligand 1 and 2) as its two ligands. The PD-1 axis functions as a protective mechanism which regulates persistent inflammation and stops autoimmune reactions. The molecules express themselves through distinct patterns because PD-1 appears on T cells but PD-L1 and PD-L2 exist on different cell types including macrophages and phagocytes (Du et al., 2020). When PD-L1 and PD-L2 binds to receptor protein PD-1, they send “off” signals it inhibits the activation of T-cells, leading to inactive immune system (Cabezón et al. 2015). Thus, this mechanism is broadly used by many cancer types by increasing the expression of PD-1 proteins as well as its ligands to evade T cell anti-tumor immune response.

MERTK activation increases PD-L1 expression on both tumor cells and TAMs through STAT3-dependent transcription (Caetano et al., 2019). Research studies using preclinical models have demonstrated the therapeutic value of this relationship through positive findings. The combination of MERTK inhibition results in a 60-70% decrease of PD-L1 expression while simultaneously improving tumor cell entry for T cells. What's particularly exciting is that combination therapy with MERTK inhibitors and anti-PD-1 antibodies shows synergistic anti-tumor effects, meaning the combined effect is greater than what you'd expect from adding the two treatments together (Caetano et al., 2019; Chen et al., 2023). The efferocytosis signature, comprising genes involved in apoptotic cell recognition and clearance (MERTK, AXL, TYRO3, Gas6, CD36, GULP1), has been identified as an independent prognostic factor in bladder cancer. Yu and colleagues (2025) developed an efferocytosis-related gene signature that stratified 406 bladder cancer patients into high-risk and low-risk groups with significantly different survival outcomes. The process of efferocytosis produces strong immune suppression while it increases checkpoint molecule expression and decreases immunotherapy effectiveness (Yu et al., 2025). Overall, the research demonstrates that

MERTK functions as a key connection point which links cancer development to tumor cell behavior and immune system evasion and treatment resistance.

3. MERTK in Bladder cancer

Although the functional role of MERTK is well described in leukemia, glioblastoma, and melanoma, its specific mechanisms in bladder cancer remain less well defined. Current evidence supports that MERTK is overexpressed in approximately 55-70% of bladder cancer patients, with significantly higher expression in muscle-invasive disease as compared to non-muscle-invasive disease (Yu et al., 2025; Cheng et al., 2024).

Clinical assessment of 156 patients also demonstrated that elevation of MERTK may result in more aggressive tumor phenotypes and a greater incidence of lymph node metastasis and therefore highlighting MERTK as a candidate activator of tumor progression (Cheng et al., 2024). Experimental data support these observations and show that MERTK actively promotes the proliferation, migration, and invasion of bladder cancer cells. Particularly, a decrease of MERTK expression in T24 and UMUC3 bladder cancer cell lines resulted in significant changes: proliferation was decreased by 35-50%, migration reduced by 60%, and invasion through Matrigel reduced by 70% (Cheng et al., 2024). It is seen that these effects are through critical signaling pathways, as decreased MERTK expression caused phosphorylation of AKT and ERK to be decreased. That means MERTK is a key factor in how cancer cells survive and evade the immune system.

MERTK is critical to cancer cells and their environments. To understand MERTK and evaluate the effects of knockout, the gene must be completely "turned off". The CRISPR/Cas9 system allows for precision genome editing, which is one of the most powerful methods at our disposal, enabling us to examine the activity of MERTK in the context of cancer cells with unmatched precision.

4. CRISPR/Cas9 Biology and Its mechanism of action

To investigate the functional role of MERTK in bladder cancer progression and therapy resistance, the CRISPR/Cas9 system was selected as the primary tool for genomic modification. It's a tool that was originally discovered as a sort of "immune system" for bacteria, helping them defend themselves against viruses by "remembering" and cutting up viral DNA (Hernandez, F. J., 2025). Today, researchers have adapted this natural defense mechanism into a highly precise way to edit or completely remove specific genes in human cells.

The CRISPR/Cas9 system operates through a specialized ribonucleoprotein complex consisting of the Cas9 nuclease and a synthetic single-guide RNA (sgRNA). The sgRNA is engineered to contain a 20-nucleotide sequence that is complementary to a specific target locus within the MERTK gene (Han et al., 2020). Upon introduction into the bladder cancer cell lines, the sgRNA directs the Cas9 protein to the target site, where it recognizes a Protospacer Adjacent Motif (PAM) sequence (Allemailem et al., 2023). Once positioned, the Cas9 nuclease induces a double-strand break (DSB) at the precise genomic coordinates. This disruption triggers the cell's endogenous DNA repair machinery, specifically the Non-Homologous End Joining (NHEJ) pathway. Because NHEJ is inherently error-prone, it frequently introduces small insertions or deletions at the repair site (Li et al., 2022). These mutations often result in a frameshift that prematurely terminates protein translation, and thus effectively silencing the gene.

A primary reason for choosing CRISPR/Cas9 over other common methods, such as small interfering RNA (siRNA), is because CRISPR/Cas9 method gives more stable and irreversible effect (Li et al., 2022). As it was previously described, MERTK is highly overexpressed in aggressive bladder cancer subtypes, and thus a temporary "knockdown" may not be sufficient to overcome its oncogenic effects or to observe long-term changes in chemotherapy sensitivity. A CRISPR-mediated knockout ensures that the MERTK receptor is entirely absent throughout all subsequent experimental stages, providing a clearer understanding of how its removal impacts cell behavior over time.

Research design

While the oncogenic role of MERTK is well-established in several other malignancies, its specific impact on the progression and treatment resistance of bladder cancer remains largely unexplored. MERTK is particularly significant because it correlates with muscle-invasive disease and acts as a primary mediator of tumor aggressiveness and metastasis. Beyond its functional role in proliferation and immune evasion, MERTK also actively changes the tumor microenvironment by creating an immunosuppressive niche that helps the cancer evade the body's natural defense. Because of this dual role, MERTK represents a critical therapeutic vulnerability. By using a CRISPR/Cas9 knockout approach, this research aimed to determine if downregulation of MERTK expression can disrupt these survival mechanisms and sensitize resistant bladder cancer cells to chemotherapy.

5.1 Hypothesis:

CRISPR/Cas9-mediated knockout of MerTK will change the cancer cells behavior and make them more sensitive to chemotherapy.

5.2 Aim:

To perform a targeted deletion in MerTK using paired sgRNAs that cause double-strand breaks, resulting in a functional knockout of the MerTK receptor.

5.3 Objectives:

- Construction of an sgRNA-containing vector that targets MerTK;
- Transfection of T24 bladder cancer cells;
- Isolation of single-cell clones with deletion and identifying successful clones;

Materials and Methods

Objective 1: Construction of an sgRNA-containing vector that targets MerTK

Designing guide sequences for region of interest

A crucial step in my research was choosing the right single-guide RNAs, or sgRNAs, to guide the Cas9 protein to the MERTK gene. My aim was to completely knock out the gene using a "double-cut" approach and make sure it wasn't functional anymore, which meant I had to find a part of the gene that was vital for the protein to work. After looking at the different versions of the gene and the structure of the MERTK receptor, I decided to target **Exon 5**. This was a key decision, as it would determine the success of my knockout. By carefully selecting the right sgRNAs and targeting the right part of the gene, I hoped to achieve a complete and functional knockout of the MERTK gene.

I selected Exon 5 for a few key reasons. First, it's an early exon that's always included in the mRNA versions of the MERTK gene. By removing this exon, I could be sure that the knockout would affect the protein, no matter which version the bladder cancer cells were trying to make. Second, Exon 5 is important for the receptor's ability to bind to its ligands. If I could delete this exon, it wouldn't just lower the protein levels, but it would also cause a frameshift mutation. This would introduce a premature stop signal, telling the cell to stop making the MERTK protein altogether. Once I had my target exon in mind, I used a tool called the *Genome Browser* to zoom in on the MERTK gene and find the exact DNA sequences. To make a double-cut deletion, I needed to design four special guides, called sgRNAs. I had to consider a few technical factors to make sure these guides would work well and be safe. The most important factor was the Protospacer Adjacent Motif, or PAM site. Each guide needs to be followed by a special sequence, "NGG", in the DNA. Without this PAM site, the Cas9 enzyme isn't capable to produce a deletion. After finding candidate sequences with the correct PAM sites, I had to evaluate their off-target scores. This was a crucial step, because I didn't want my CRISPR system to accidentally cut other parts of the genome, which could lead to unwanted effects. I used a tool called the MIT Guide Specificity Score, and only selected guides that had very high specificity scores. I also looked at the GC content of the sequences, aiming for a range between 40% to 60%. Optimal guide RNA binding requires a balanced GC content; too little causes weak targeting, while too much leads to excessive stability or inhibitory secondary structures that reduce editing efficiency. Another detail I considered was the distance between the two guides. I

wanted the deletion to be large enough to be easily detectable by a PCR screening later on, but not so large that it would be difficult for the cell to repair the break. By placing my guides about 150 to 200 base pairs apart, I ensured that the resulting deletion would create a clear shift on an agarose gel. This would make it easier to detect the deletion and confirm that the knockout was successful. In this experiment, I used sgRNAs that were previously designed by a former master's student, Aruzhan Anuarbekova, to evaluate whether these sgRNAs are capable to produce double cut in the MERTK gene.

Plasmid Digestion

After designing sgRNA-oligonucleotides, I started preparing the pRG2 plasmid backbone. I used BsaI-HF restriction enzyme to cut the circular plasmid DNA at two sites, downstream of the U6 promoter. For the digestion reaction, 3 ug of pRG2 plasmid DNA (at a concentration of 1000 ng/uL) was combined with 2 uL of BsaI-HF enzyme, 3 uL of 10X CutSmart Buffer, and 22 uL of nuclease-free water, resulting in a total reaction volume of 30 uL. This mixture was then incubated at 37°C for 1.5 hour. In order to inactivate the enzyme, the incubation at 80°C for 20 min was used. The digestion of the plasmid was confirmed using agarose gel electrophoresis in 1xTAE buffer. I prepared 1% agarose gel containing ethidium bromide, and loaded 1 ul of digested plasmid, alongside an undigested circular plasmid as a control to verify successful digestion.

Purification of pRG2 vector

After confirming the successful digestion of the pRG2 plasmid, the linearized backbone DNA (vector) was extracted using the QIAquick Gel Extraction Kit (QIAGEN, #28704). The purification began by carefully excising the DNA band at approximately 2400 bp from 1% agarose gel using a sterile scalpel under a UV transilluminator. I made sure to minimize the amount of extra agarose and limited the exposure to UV light in order to prevent any potential damage. The gel slice was then placed in a microcentrifuge tube and all other steps were performed according the manufacturer's protocol. Finally, the purified vector was eluted, and subsequently used for the ligation of the target sgRNA sequences.

Insertion of sgRNA sequences into the pRG2 vector

In order to perform ligation of pRG2 vector with desired sgRNA sequences, the annealing reaction was prepared by mixing 1 ul of each sense and antisense strands at a concentration of 100 pmol/ul with 23 ul of 1xCutSmart buffer. The annealing was performed using the following thermocycler conditions: an initial denaturation at 95°C for 3 min, followed by a gradual decrease in temperature at a rate of 2°C/sec from 95°C to 85°C, and then at 0.1°C/sec from 85°C to 25°C, with a final hold at 4°C.

Promega T4 DNA ligase (#M1801) was used in order to insert the annealed gRNA into the pRG2 vector. The ligation mixture consisted of 1 ul of annealed gRNA, 0.5 ul of T4 DNA ligase, 1.5 ul of pRG2 vector, 1 ul of ligation buffer, and 6 ul of nuclease-free water, resulting in a total volume of 10 ul. As a negative control, the mixture was prepared without insert, adding just water. The mixtures were then incubated at room temperature for 3 hours.

Bacterial transformation

The ligation products were transformed into chemically competent *E. coli* TOP10 cells using a standard heat shock protocol. Briefly, 0.5 ul of the ligation mixture was added to 10 ul of competent cells and gently mixed. The cells were incubated on ice for 30 minutes, followed by a heat shock at 42°C for 1 min. After heat shock, cells were returned to ice immediately for 5 minutes, and 90 ul of pre-warmed LB broth was added at a ratio of 1:9. The mixture was then incubated at 37°C for 30 minutes to allow recovery. Finally, transformed cells were plated onto LB agar plates supplemented with ampicillin antibiotic (100ug/mL). Two volumes (10 ul and 90 ul) were used to ensure optimal colony distribution. Plates were incubated overnight at 37°C. To evaluate transformation efficiency, both positive and negative controls were included.

Isolation of sgRNA-pRG2 plasmid using MiniPrep kit

On the next day, single bacterial colonies obtained after transformation were inoculated in 2 mL pre-warmed LB broth supplemented with ampicillin antibiotic (100ug/mL) in a 15 mL conical tube, and incubated at 37°C overnight with shaking at 300 rpm. After 16 hours, this overnight culture was collected into a 2mL microcentrifuge tube, and centrifuged at 1700xg for 10 minutes. The Eurogene Plasmid MiniPrep Color kit (#BC121S) was used to isolate the pRG2 plasmid with inserted gRNA,

and all the following steps were performed according to the manufacturer's protocol. Subsequently, isolated plasmids were collected in a 1.5 mL microcentrifuge tube, and their concentrations were measured using NanoDrop™ 2000/2000c Spectrophotometers. In order to check whether the insertion worked, isolated plasmids were analyzed by agarose gel electrophoresis using 1% agarose gel in 1xTAE buffer. Samples were mixed with loading dye, and loaded into the wells, alongside a 1kB DNA ladder, original pRG2 plasmid, and the negative control to compare their mobility and confirm successful insertion. Finally, samples were stored at -20°C until further use.

Sanger Sequencing

After confirming the same mobility across all samples, the presence of the desired sgRNA insert was analyzed using Sanger sequencing analysis with the BrilliantDye™ Terminator v3.1. For each sequencing reaction, 150 ng of sgRNA-plasmid DNA was mixed with 1.5 ul of 5x Sequencing buffer, 1 ul of primer, 1 ul of 2.5x sequencing dye, and 5.5 ul of nuclease-free water, resulting in a total volume of 10 ul. Two sets of reactions were prepared using forward and reverse primers at a concentration of 5 pmol/ul. The mixture then was placed in a thermocycler machine with the following conditions: 96°C for 45 sec, followed by 25x cycles of {96°C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min}, and final hold at 4°C. Meanwhile, a precipitation solution using sodium acetate was prepared by mixing 37.5 ul of 3M NaAc with 782.5 ul of 96% ethanol and 180 ul of nuclease-free water. After thermal cycling, the sequencing products were transferred to 1.5 mL microcentrifuge tubes, and mixed with 50 ul of NaAc solution to remove excess salts, fluorescent dyes, and incorporated nucleotides. Samples were incubated at room temperature for 30 minutes, and then centrifuged at 13,200 x g at 4°C for 30 minutes. The supernatant was carefully removed, and the pellet was washed with 75% ethanol, followed by centrifugation under the same conditions. After removing the residual ethanol, the pellet was air-dried at 38-40°C for 15 minutes, and resuspended in 10 ul of Hi-Di formamide (Applied Biosystems, #4311320). Samples were incubated at room temperature for 20 min, followed by a denaturation at 95°C for 2 min using the thermomixer, and incubated on ice for 5 minutes. Finally, samples were delivered to NCB for subsequent sequence analysis.

Isolation of sgRNA-pRG2 plasmid using MaxiPrep kit

After confirming the presence of the correct insert in the plasmid backbone, large-scale plasmid isolation was performed using the ZymoPURE™ II Plasmid Maxiprep Kit (ZymoResearch, #D4202). The procedure began with preparation of a starter culture, where colonies previously used for Miniprep isolation were inoculated into 2 mL of pre-warmed LB broth supplemented with 2 ul of ampicillin antibiotic (100 ug/mL) in a 15 mL conical tube, and incubated at 37°C for 8 hours with shaking at 300 rpm. For overnight culture, 300 ul of the starter culture was transferred into 150 mL of pre-warmed LB broth containing 150 ul ampicillin antibiotic (100 ug/mL) in a 1 L flask, and incubated overnight under the same conditions. The next day, the bacterial culture was harvested and transferred into 50 mL conical tubes, and centrifuged at 3,400 x g for 10 minutes. Subsequent plasmid purification steps, including cell lysis, neutralization, DNA binding, washing, and elution , were carried out according to the manufacturer's protocol. The isolated plasmid DNA was collected into 1.5 mL microcentrifuge tubes, and its concentration and purity were determined using a Nanodrop spectrophotometer. The isolated plasmids were subsequently verified by Sanger sequencing, and samples were delivered to NCB for analysis.

Objective 2: Transfection of T24 bladder cancer cells

Cell culture and Expansion

In this research project, T24 human bladder cancer cells were used for all cell culture experiments. Initially, frozen T24 cells were rapidly thawed in a 37°C water bath, and transferred to a 15 mL conical tube containing 6 mL of pre-warmed complete growth medium to remove residual dimethyl sulfoxide (DMSO). Complete growth medium was prepared in advance supplementing Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, and 1% non-essential amino acids. After resuspending cells in growth medium, cells were centrifuged at 300 x g for 5 minutes, and the resulting supernatant was discarded. The cell pellet was resuspended in 5 mL complete growth medium, and seeded into a T25 culture flask. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

After 48 hours, when cells reached approximately 80% confluency, they were passaged into a T75 culture flask. The culture medium was aspirated carefully, and cells were washed with phosphate-buffered saline (PBS). Cells were then detached using 1 mL of Trypsin-EDTA and incubated at

37°C for 5 minutes until detachment was observed under the microscope. In order to neutralize trypsin activity, 2 mL of complete growth medium (in a ratio of 1:2) was added. The cell suspension was collected then into a new 15 mL conical tube and centrifuged at 300 x g for 5 minutes, and the resulting supernatant was discarded. The cell pellet was resuspended in 1 mL of complete growth medium, and then transferred into a T75 culture flask containing pre-warmed 14 mL of complete growth medium. Cells were then incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours.

Cells splitting

After 24 hours, cells were examined under the microscope, and were observed to reach approximately 95-98% confluency. Therefore, cells were splitted and passaged into a new flask in order to proceed to cell transfection. Following detachment as described above, the cell pellet was resuspended in 2 mL of complete growth medium. Cell concentration was determined using Trypan blue. Briefly, 10 µL of the cell suspension was mixed with 10 µL of Trypan blue, and the mixture was loaded onto a hemocytometer for counting. The cell density was estimated to be approximately 6.2×10^6 cells/mL. Based on this concentration, a 1:5 dilution was prepared. Specifically, 400 µL of the cell suspension was transferred into a new T75 culture flask containing pre-warmed complete growth medium. Cells were then incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO₂.

Seeding cells into a 6-well plate to asses transfection efficiency

When the cells reached approximately 75 - 80% confluency, they were seeded into a 6-well plate at a density of 200,000 cells per well in order to evaluate transfection efficiency. Transfection was performed using Lipofectamine 3000 according to the manufacturer's protocol. After 24 hours, when the cells reached 65 - 70% confluency with a viability of approximately 85 - 90%, cells were undergone transfection. First of all, the culture medium was replaced with 2 mL of fresh medium per well, and continued incubation. For transfection, two tubes were prepared. In Tube A, 125 uL of Opti-MEM was mixed with 5 uL of Lipofectamine 3000. In Tube B, 125 uL of Opti-MEM was combined with 1.5 ug of plasmid DNA isolated from Maxiprep (0.5 ug of each Cas9, either two from dels1-dels4) + 1 ug eGFP and 5 µL of P3000 enhancer reagent. Then the mixture of Tube B

were added to Tube A. The mixture was gently mixed and incubated at room temperature for 15 minutes to allow complex formation. After incubation, the transfection mixture was added dropwise to each well of the 6-well plate to ensure even distribution. The wells were arranged as follows: Well 1 contained transfection reagents with eGFP only (positive control for transfection efficiency); Well 2 contained Dels1 + Dels2 + Cas9 + eGFP; Well 3 contained Dels2 + Dels4 + Cas9 + eGFP; Well 4 contained Dels3 + Dels1 + Cas9 + eGFP; Well 5 contained Dels4 + Dels1 + Cas9 + eGFP; and Well 6 contained untransfected cells (negative control). The plate was then incubated at 37 °C in a CO₂ incubator for 48 hours.

Transfection

After assessing the transfection efficiency, the main transfection experiment was carried out to create a double-cut in exon 5 of the MERTK gene using two sgRNAs. Cells were seeded into a 6-well plate at a density of 200,000 cells per well. After 24 hours, when the cells reached around 75 - 80% confluency, transfection reagents were added following the same protocol described above. In this experiment, 3 ug of plasmid DNA isolated from Maxiprep (1 ug of each Cas9, either two from dels1-dels4) was used to improve transfection efficiency. The wells were arranged as follows: Well 1 contained transfection reagents with eGFP only (positive control); Well 2 contained Dels1 + Dels2 + Cas9; Well 3 contained Dels2 + Dels4 + Cas9; Well 4 contained Dels3 + Dels1 + Cas9; Well 5 contained Dels4 + Dels1 + Cas9; and Well 6 contained untransfected cells (negative control). After adding the transfection mixture, the plate was incubated at 37 °C in a CO₂ incubator for 48 hours.

Deletion detection in the transfected cells

After 48 hours of incubation, cells were collected to verify deletions. First, the plate was observed under a microscope to assess cell health and confluency. The medium was then removed, and cells were washed with 2 mL of PBS. Next, 250 uL of trypsin was added to detach the cells, followed by incubation for 2 minutes at 37 °C. Once partially detached, the cells were resuspended in 1 mL of complete medium and transferred into 1.5 mL tubes. The samples were centrifuged at 300 × g for 5 minutes, after which the supernatant was discarded. The cell pellet was then resuspended in 30 uL of PBS and mixed thoroughly.

Genomic DNA was prepared using a Proteinase K - based lysis method. The cell suspension was transferred into PCR tubes, and the thermocycler was set as follows: 5 minutes at 95 °C, 20 minutes at 65 °C, 5 minutes at 95 °C, and then held at 16 °C. While the samples were at 16 °C, 3 uL of Proteinase K (1 mg/mL) was added at a 1:10 ratio, and the samples were returned to incubation. The resulting lysate was then used directly for PCR amplification. The PCR reaction mixture consisted of 10 uL KOD Hot Start enzyme mix, 1 uL of forward primer and 1 uL of reverse primer (10 ng/uL each), 2 uL of lysate, and 6 uL of nuclease-free water. The PCR program was set as follows: initial denaturation at 95 °C for 3 minutes; followed by 41 cycles of 95 °C for 30 seconds, 59 °C for 1 minute, and 70 °C for 1 minute; with a final extension at 70 °C for 3 minutes and a final hold at 4 °C. Finally, the PCR products were analyzed using 1.5% agarose gel electrophoresis, run at 80 V for 45 minutes.

Results

Objective 1: Construction of an sgRNA-containing vector that targets MerTK

The design of sgRNAs

In total, 4 sgRNA sequences were designed. Their main function is to guide Cas9 to perform deletion in Exon 5 of the MerTK gene.

Table 1. Sense and antisense strands of guide RNAs.

Name	Location	Sequence	Specificity score (MIT)	Efficiency score (Doench et al. 2016)
MERTK E5dels1	chr2:111965201- 11196 5223	5' – CACCGATGGCGGTCTTCAGTTGTG - 3'	84	78%

MERTK E5dela1		5' – AAACCACAACCTGAAGACCGCCATC - 3'		
MERTK E5dels2	chr2:111965256- 11196 5278	5' – CACCGGAGTGCAGATCAACATCAA - 3'	81	91%
MERTK E5dela2		5' – AAACTTGATGTTGATCTGCACTCC - 3'		
MERTK E5dels3	chr2:111965304- 11196 5326	5' – CACCGCATGTTCTGGGAGCTGGTG - 3'	54	63%
MERTK E5dela3		5' – AAACCACCAGCTCCCAGAACATGC - 3'		
MERTK E5dels4	chr2:111965419- 11196 5441	5' – CACCGGGGAACCCTGACACCAAAG - 3'	75	92%
MERTK E5dela4		5' – AAACCTTTGGTGTGTCAGGGTTCCCC - 3'		

In total, 4 pair of sgRNAs have been constructed as followings:

1. Combination of MERTK E5dels1 with E5dels2 comes with a deletion of **55** base pairs;
2. Combination of MERTK E5dels1 with E5dels3 comes with a deletion of **104** base pairs;
3. Combination of MERTK E5dels2 with E5dels4 comes with a deletion of **163** base pairs;
4. Combination of MERTK E5dels1 with E5dels4 comes with a deletion of **218** base pairs;

Plasmid Digestion

In order to digest the pRG2plasmid, *BsaI-HF* restriction enzyme was used, and gel electrophoresis analysis showed two fragments, a larger fragment at approximately 2460 base pairs and a smaller fragment at 670 base pairs. The vector was then extracted from the gel, and purified to the final concentration of 47.8 ng/ul.

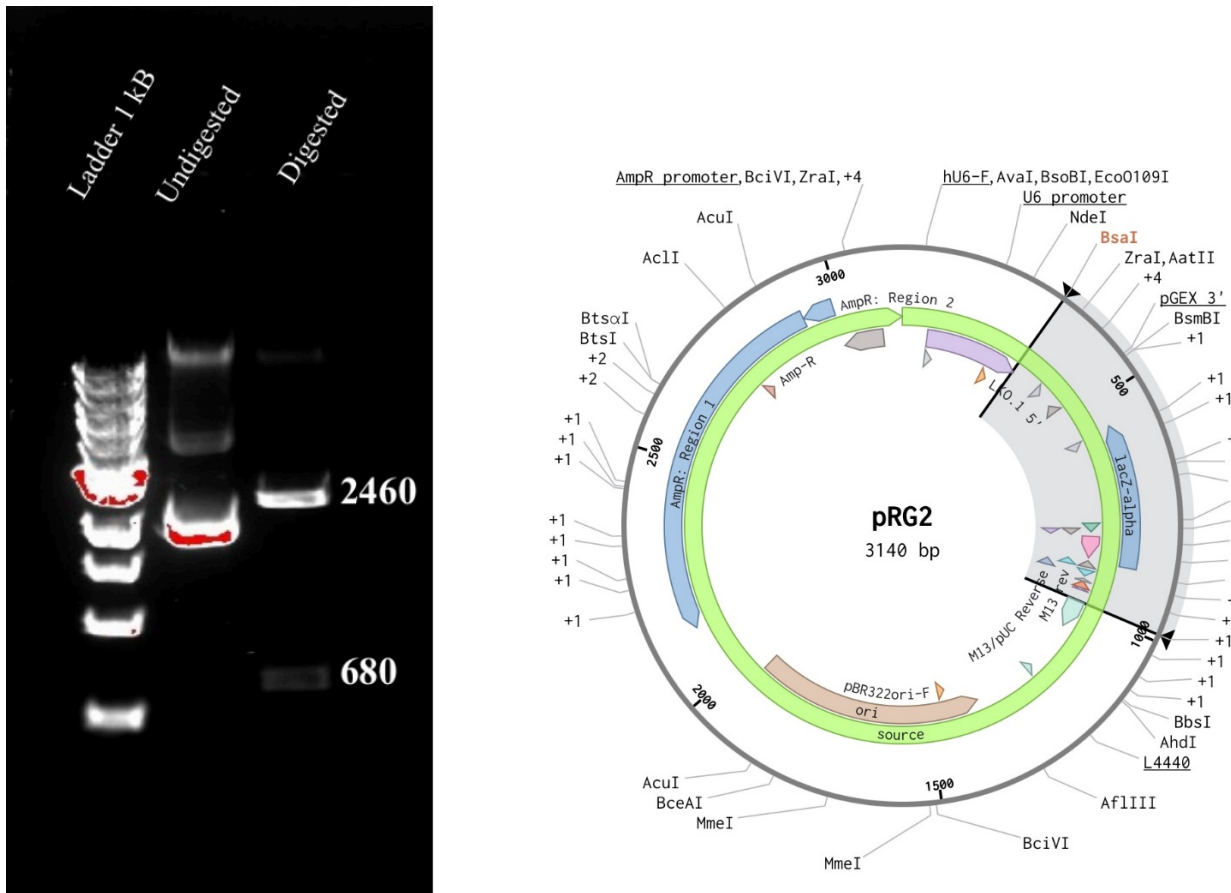


Figure 4. a) *pRG2* plasmids digestion after gel electrophoresis. b) Plasmid map (Benchling)

Insertion of sgRNA sequences into the pRG2 vector

In order to dilute sense and antisense strands to 100 ng/ul, the following formula was used:

$$C_{stock} V_{stock} = C_{final} V_{final}$$

1. For MerTK E5dels1, the stock concentration was **247.8 ng/uL**. To prepare a working solution of 20 uL volume and 100 ng/uL concentration, the dilution was calculated using the same formula:

$$247.8 \text{ ng/uL} \times V_{\text{stock}} = 100 \text{ ng/uL} \times 20 \text{ ul}$$

$$V_{\text{stock}} = 8.07 \text{ ul}$$

Thus, 8.07 uL of the stock solution was taken, and 11.93 uL of water was added, so the total volume to be 20 ul. The same calculation was applied to all other sgRNA sequences to prepare working solutions with the desired concentration.

The ligate was transformed into chemically competent *E. coli* TOP10 cells, following to the protocol described in Materials part, and the next morning the colonies were counted.

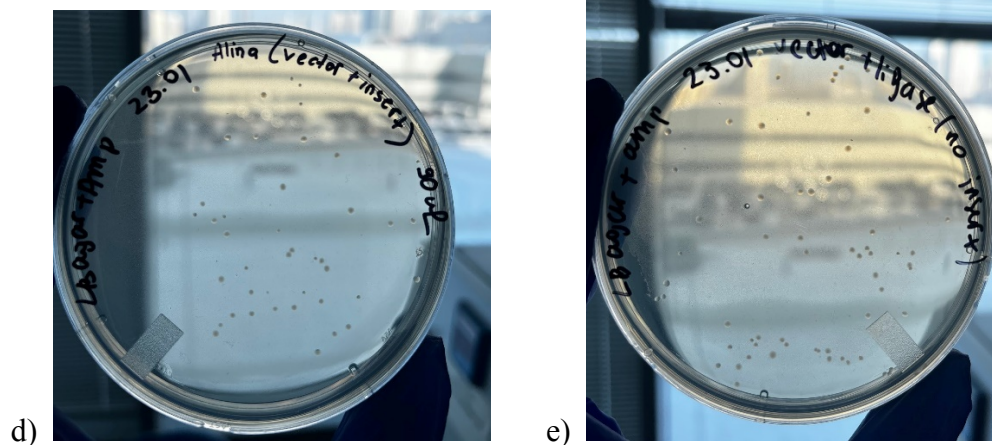
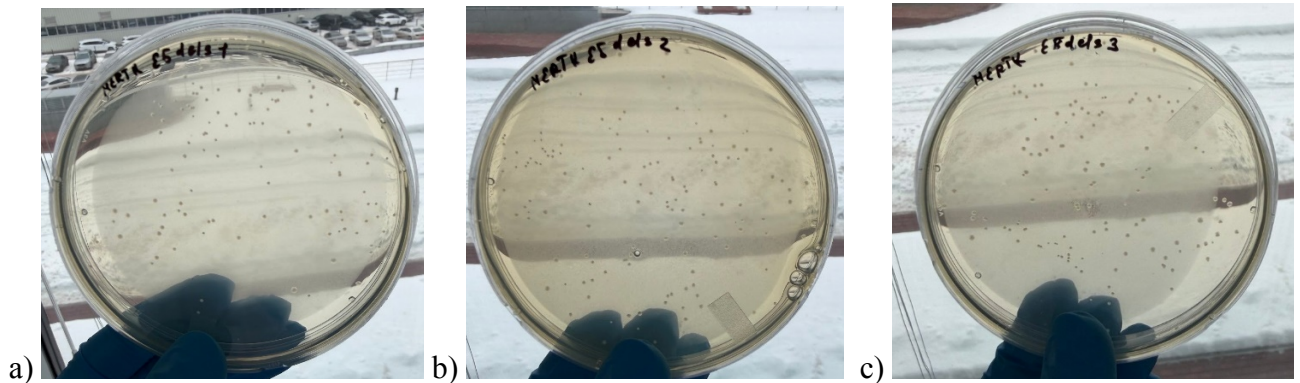


Figure 5. Transformed colonies: a) pRG2 plasmid with inserted MERTK E5dels1 sgRNA. b) pRG2 plasmid with inserted MERTK E5dels2 sgRNA. c) pRG2 plasmid with inserted MERTK E5dels3 sgRNA. d) pRG2 plasmid with inserted MERTK E5dels4 sgRNA. e) Negative control for insertion - pRG2 plasmid with water.

Table 4. Amount of bacterial colonies after incubation at 37°C overnight

Plate	Number of colonies
pRG2 + E5dels1	120
pRG2 + E5dels2	138
pRG2 + E5dels3	119
pRG2 + E5dels4	48
pRG2 + water	54

Isolation of sgRNA-pRG2 plasmid using MiniPrep kit

pRG2 plasmid DNA with inserted gRNAs was then isolated via miniprep as it was mentioned in the Materials and Methods part. The concentrations ranged between 36.8 ng/ul to 52.6 ng/ul. Isolated plasmid DNA was then resolved on 1% agarose gel, which showed a strong signal at the expected size, confirming the presence of the insert and plasmid. Additionally, the electrophoresis revealed no additional bands or smearings, that also corresponds to high purity of isolated DNA.

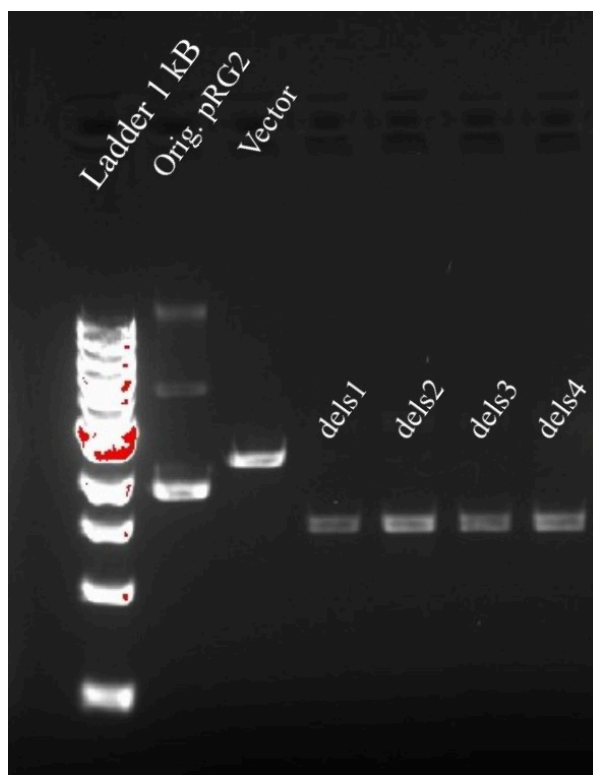


Figure 6. Verification of pRG2 plasmid clones after miniprep using 1% gel electrophoresis. 1: 1 kb Ladder. 2: undigested pRG2(-GG). 3: digested pRG2 vector backbone. 4-7: pRG2 plasmids containing four different sgRNA inserts, respectively.

Table 5. The Purity and Volume of MiniPrep isolated Plasmids

Plasmid name	Concentration (ng/ul)
pRG2 - E5dels1	39.6
pRG2 - E5dels2	42.5
pRG2 - E5dels3	36.8
pRG2 - E5dels4	52.6

The isolated plasmids containing sgRNA inserts migrated lower than the vector backbone on the gel. This difference is primarily due to the smaller size of the inserted fragment, as the sgRNA sequence

(~25 bp) is significantly shorter than the original fragment it replaced. Consequently, the reduced plasmid size results in faster migration during gel electrophoresis, leading to a lower position on the gel.

Isolation of sgRNA-pRG2 plasmid using MaxiPrep kit

The positive clones containing the sgRNAs were then selected for plasmid isolation using the ZymoPURE Plasmid Maxiprep Kit (Zymo Research, #D4202) to obtain higher quantities of DNA. After isolation, the DNA concentration was measured using Nanodrop spectrophotometry. In order to verify the integrity of isolated plasmids, samples were resolved on 1% agarose gel. It showed distinct bands corresponding to the expected plasmid size, confirming successful plasmid isolation. No significant degradation or contamination was observed.

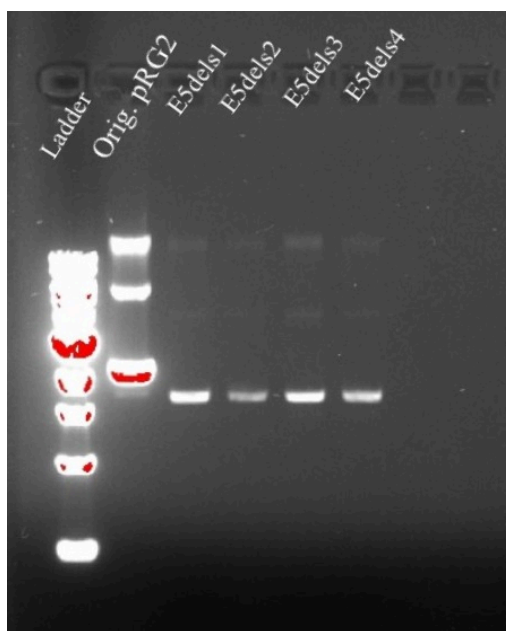


Figure 7. Verification of pRG2 plasmid clones after maxiprep using 1% gel electrophoresis. 1: 1 kb Ladder. 2: undigested pRG2(-GG). 3-6: pRG2 plasmids containing four different sgRNA inserts, respectively.

Table 6. The Purity and Volume of MaxiPrep isolated Plasmids

Plasmid name	Concentration (ng/ul)	V (ul)
pRG2 - E5dels1	256.8	450
pRG2 - E5dels2	342.5	500
pRG2 - E5dels3	387.9	500
pRG2 - E5dels4	308.4	500

Sanger Sequencing

Sequencing results confirmed successful sgRNA cloning, and that the inserted sequences are correct. Alignment of the obtained sequences with the expected sequence demonstrated correct integration of the sgRNA without mutations in the target region. The sequencing reads showed high similarity to the expected sequence, which indicates successful cloning. No significant insertions, deletions, or frame shifts were observed.



Figure 7. Sequencing analysis: MERTK E5dels1 with #413 forward primer (5 pmol/ul). The upper sequence represents the expected sequence, while the lower sequence corresponds to the obtained sequencing result.

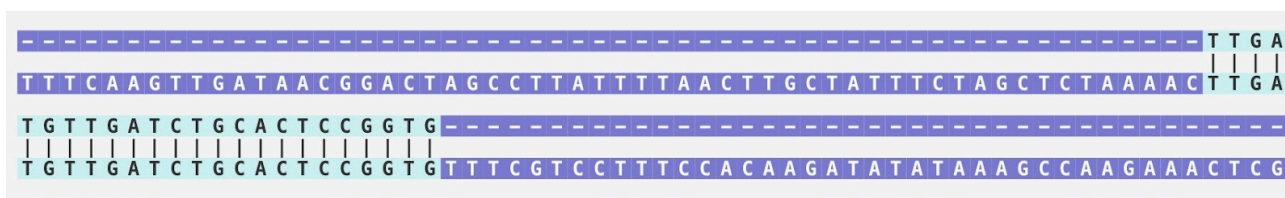


Figure 8. Sequencing analysis: MERTK E5dels2 with #319 reverse primer (5 pmol/ul).

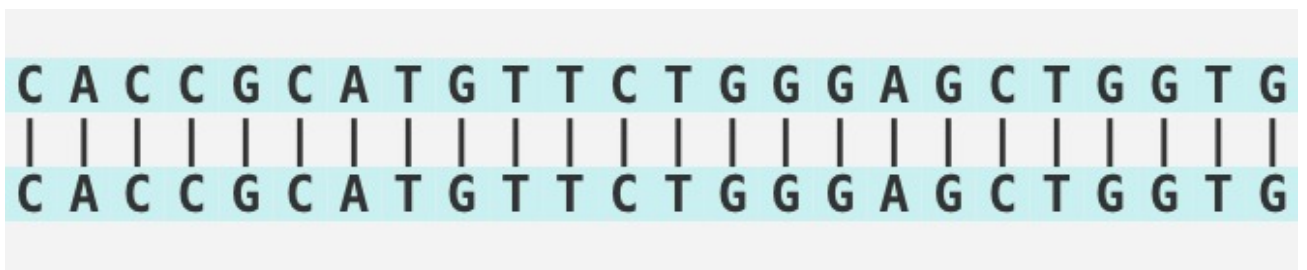


Figure 9. Sequencing analysis: MERTK E5dels3 with #413 forward primer (5 pmol/ul).



Figure 10. Sequencing analysis: MERTK E5dels4 with #413 forward primer (5 pmol/ul).

Objective 2: Transfection of T24 bladder cancer cells

Seeding cells into a 6-well plate to assess transfection efficiency

T24 cells were analyzed 72 hours after transfection. Weak eGFP fluorescence was observed in most wells, indicating low transfection efficiency. Only a small number of cells showed visible fluorescence, and the signal was consistent across all wells.

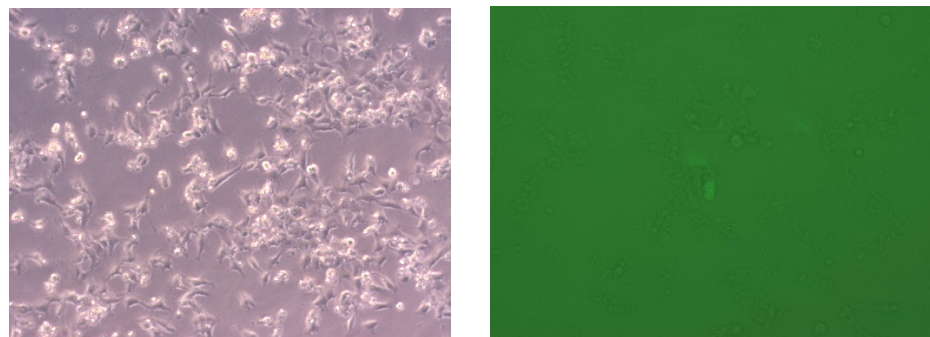


Figure 11. T24 cells transfected with eGFP protein alone after 72 hours.

Initially, 500 ng of each plasmid isolated from Maxiprep, along with 500 ng of Cas9 plasmid, were used for transfection. However, the observed transfection efficiency was low, and the viability decreased to approximately 25%. To address this, the amount of plasmid DNA was increased to 1

ug, while the amount of eGFP protein was kept unchanged. In addition, a high level of cell death was observed after 72 hours. Therefore, the post-transfection incubation time was reduced to 48-50 hours.

Transfection

After optimization of the transfection conditions, a slight improvement in transfection efficiency was observed across all wells. After 48 hours, microscopic examination of the cells was performed, and a viable cell counts were higher than in the previous conditions. In addition, the number of fluorescent cells increased, indicating improved transfection efficiency.

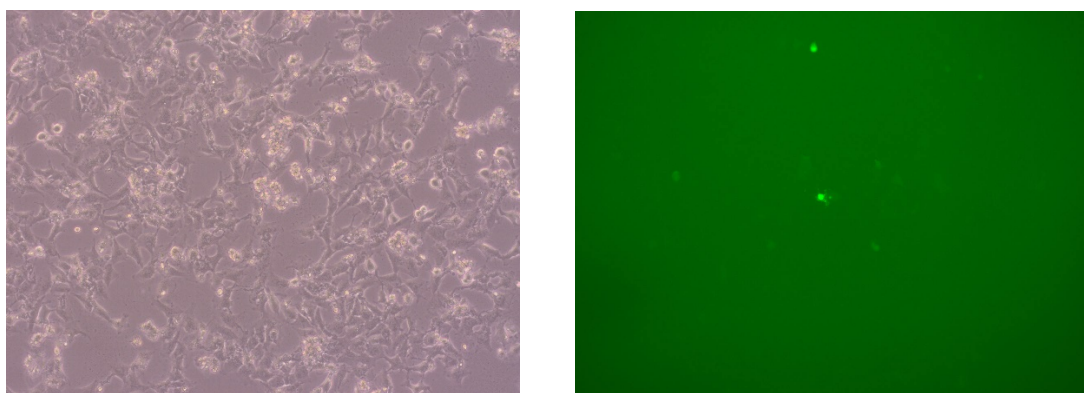


Figure 12. T24 cells transfected with eGFP protein alone after 48 hours

Deletion detection in the transfected cells

Once the cells were successfully transfected, gel electrophoresis was performed to analyze the deletion. The protocols for cell collection and PCR setup are outlined above in materials part. The chosen primers demonstrated high specificity for the plasmid constructs, and both sets showed good performance; however, primer set 1 was used for further analysis. Gel electrophoresis results indicated that most sgRNA combinations were effective in generating the expected deletions. However, no clear result was obtained for E5dels1+ E5dels3 condition, likely due to loss of cells during the washing step, resulting in a very low amount of DNA template for PCR. Despite this, based on the successful results from other sgRNA combinations, it can be assumed that this pair may also be functional. Among all conditions, E5dels1+ E5dels4 combination showed the strongest deletion signal. A clear deleted amplicon was observed, suggesting highly efficient editing in this group.

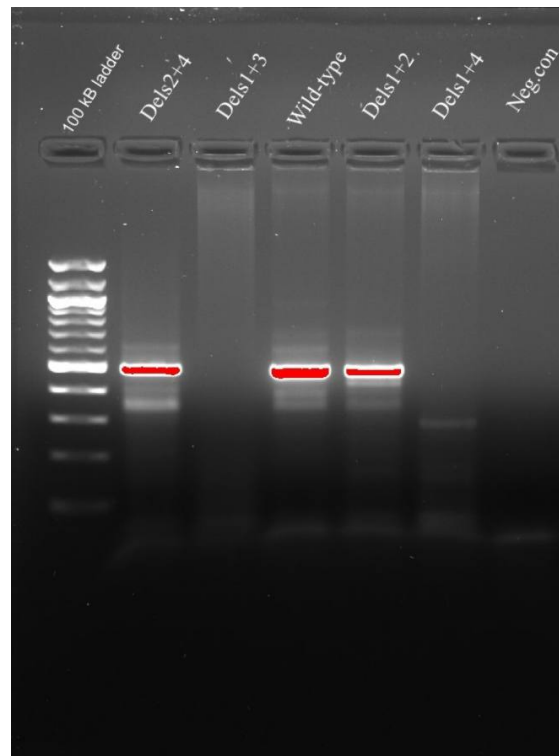


Figure 13. Analysis of transfected cell amplicons via electrophoresis. 1: 100 kb ladder; 2: E5dels2+E5dels4 sgRNAs; 3: E5dels1+E5dels3 sgRNAs; 4: Untransfected cells; 5: E5dels1+E5dels2 sgRNAs; 6: E5dels1+E5dels4 sgRNAs; 7: Negative control (water instead of lysate)

The band size of untransfected cells is approximately 500 base pairs. When E5dels2 and E5dels4 are combined, they produce a deletion of 163 base pairs, resulting in an expected band at around 337 base pairs. In comparison, the combination of E5dels1 and E5dels2 generates a smaller deletion of 55 base pairs, and gel electrophoresis showed a strong signal at 445 base pairs, indicating that some cells successfully underwent gene editing. Finally, the combination of E5dels1 and E5dels4 leads to a larger deletion of 218 base pairs, with a corresponding strong band at 282 base pairs, suggesting efficient editing in the collected cells. In some samples, two bands were observed, where one is at 500 base pairs, and another is at the lower expected size. This result indicates that the vast majority of the cell population remains unedited, with only a small proportion successfully edited. It can be explained by incomplete or low efficiency of transfection across the cells.

Discussion and Conclusion

Overall, the experiment could be thought as successful, since the primary goal of obtaining double-cut deletion at Exon 5 in MERTK gene has been accomplished. The designed sgRNAs were successfully constructed, inserted into the plasmid, and verified through sequencing, which confirms that they can be used for further experiments targeting the MERTK gene. Additionally, the primers designed for PCR amplification were also effective, as they allowed detection of deletion products. However, due to time limitations, it was not possible to proceed with seeding cells into a 96-well plate or to isolate single-cell clones with a confirmed MERTK knockout. Therefore, while a full functional knockout model was not established, the current results demonstrate that the designed system is suitable for future screening and identification of successful knockout clones.

The design of sgRNAs targeting exon 5 was an important part of this study. Choosing an early exon that is present in all transcript variants increases the chance of generating a functional knockout. In addition, the use of a double-cut strategy allowed the creation of deletions of different sizes, which can lead to frameshift mutations and complete loss of protein function. The fact that deletion amplicons were eventually detected in PCR confirms that gene editing did occur, even if the efficiency was relatively low.

During performing Sanger sequencing analysis, multiple challenges had occurred. One of them involved unexpected results, where extra nucleotides and missing bases were present in the analyzed sequences. Another issue encountered a sequencing chromatogram, where overlapping and low peaks were observed. All the steps during the experiment were performed according to the protocol, and all measurements were carefully prepared. The concentrations of DNA and primers were also optimized, which suggested that the issue was not related to sample preparation. After repeating the sequencing several times, it was assumed that the problem could be related to sample delivery, as the samples were previously transported using courier services. Even though the samples were properly sealed using parafilm and ice packs, the sequencing results remained inconsistent with the expected sequences. To address this issue, the delivery process was handled personally to ensure better sample stability. After this change, the sequencing results showed high similarity to the expected sequences, confirming that the problem was most likely related to transportation conditions.

However, resolving this issue required additional time, which limited the time available for downstream cell culture experiments.

One of the challenges involved cell viability during transfection. After 72 hours of post-transfection incubation, cells showed very low viability. As it was expected, PCR analysis gave negative results, indicating high toxicity of Lipofectamine-3000 to T24 cells. Based on assumption that this bladder cancer cell line is fast-growing and high toxicity of transfection reagents, the incubation time was reduced to 48 hours. This optimization positively affected on the cell viability and resulted in more acceptable cell survival. In addition to decreasing the incubation time, the standard protocol of changing the medium after 20 hours of post-transfection incubation was followed, that allowed more cells to survive and enabled successful detection of deletion products.

Moreover, the efficiency of transfection itself is considered as a major challenge during this experiment. Despite increasing the amount of plasmid DNA, transfection efficiency remained low, and only a handful of cells (approximately 5-6 cells per field of view) transfected successfully. This low efficiency probably limited detection of deletion amplicons and the edited gene in the cells, because only a small proportion of cells could be found to contain the edited gene. After multiple rounds of optimization, including adjusting reagent ratios and incubation conditions, deletion amplicons were eventually detected, confirming that gene editing had occurred. But this low efficiency emphasizes further optimization, to enhance transfection conditions, is necessary.

In summary, while several technical challenges were encountered during the experiment, including issues with sequencing, cell viability, and transfection efficiency, these problems were identified and partially resolved. The successful detection of deletion amplicons confirms that the CRISPR/Cas9 system was functional and capable of targeting the MERTK gene. Although further work is required to improve efficiency and generate stable knockout clones, the results provide a strong foundation for future experiments.

References

- Allemailem, K. S., Alsahli, M. A., Almatroudi, A., Alrumaihi, F., Al Abdulmonem, W., Moawad, A. A., Alwanian, W. M., Almansour, N. M., Rahmani, A. H., & Khan, A. A. (2023). Innovative strategies of reprogramming immune system cells by targeting CRISPR/Cas9-based genome-editing tools: A new era of cancer management. *International Journal of Nanomedicine*, 18, 5531–5559. <https://doi.org/10.2147/IJN.S424872>
- Azad, T., Nouri, K., van Rensburg, H. J., Maritan, S. M., Wu, L., Hao, Y., Montminy, T., Yu, J., Khanal, P., Mulligan, L. M., & Yang, X. (2019). A gain-of-functional screen identifies the Hippo pathway as a central mediator of receptor tyrosine kinases during tumorigenesis. *Oncogene*, 39(334-355). <https://doi.org/10.1038/s41388-019-0713-7>
- Baralle, F. E., & Giudice, J. (2017). Alternative splicing as a regulator of development and tissue identity. *Nature Reviews Molecular Cell Biology*, 18(7), 437-451. <https://doi.org/10.1038/nrm.2017.27>
- Branchford, B. R., Stalker, T. J., Law, L., Acevedo, G., Sather, S., Brzezinski, C., Wilson, K. M., Minson, K., Lee-Sherick, A. B., Davizon-Castillo, P., Ng, C., Zhang, W., Neeves, K. B., Lentz, S. R., Wang, X., Frye, S. V., Earp, H. S. III, DeRyckere, D., Brass, L. F., Graham, D. K., & Di Paola, J. A. (2018). The small molecule MERTK inhibitor UNC2025 decreases platelet activation and prevents thrombosis. *Journal of Thrombosis and Haemostasis*, 16(2), 352-363. <https://doi.org/10.1111/jth.13875>
- Cabezón, R., Carrera-Silva, E. A., Flórez-Grau, G., Errasti, A. E., Calderón-Gómez, E., Lozano, J. J., España, C., Ricart, E., Panés, J., Rothlin, C. V., & Benítez-Ribas, D. (2015). MERTK as negative regulator of human T cell activation. *Journal of Leukocyte Biology*, 97(4), 751–760. <https://doi.org/10.1189/jlb.3A0714-334R>
- Caetano, M. da S., Younes, A. I., Barsoumian, H. B., Quigley, M., Menon, H., Gao, C., Spires, T., Reilly, T. P., Cadena, A. P., Cushman, T. R., Schoenhals, J. E., Li, A., Nguyen, Q.-N., Cortez, M. A., & Welsh, J. W. (2019). Triple therapy with MerTK and PD1 inhibition plus radiotherapy promotes abscopal antitumor immune responses. *Clinical Cancer Research*, 25(24), 7576-7584. <https://doi.org/10.1158/1078-0432.CCR-19-0795>
- Chen, K. V. M., De Groot, A. E., Mendez, S. A., Mallin, M. M., Amend, S. R., & Pienta, K. J. (2023). Targeting MerTK decreases efferocytosis and increases anti-tumor immune infiltrate in prostate cancer. *Medical Oncology*, 40(10), Article 284. <https://doi.org/10.1007/s12032-023-02153-z>
- Cheng, M., Chen, S., Li, K., Wang, G., Xiong, G., Ling, R., Zhang, C., Zhang, Z., Han, H., Chen, Z., Wang, X., Liang, Y., Tian, G., Zhou, R., Zhu, Y., Ma, J., Liu, J., Lin, S., Xu, H., Chen, D., Li, Y., & Peng, L. (2024). CD276-dependent efferocytosis by tumor-associated macrophages promotes immune evasion in bladder cancer. *Nature Communications*, 15, 2818. <https://doi.org/10.1038/s41467-024-42865-9>
- Cummings, C.T., Deryckere, D., Earp, H.S., Graham, D.K. (2013) Molecular pathways: MERTK signaling in cancer. *Clin Cancer Res.* 19(19):5275-80. DOI: 10.1158/1078-0432.CCR-12-1451

- Dvinge, H., Kim, E., Abdel-Wahab, O., & Bradley, R. K. (2016). RNA splicing factors as oncoproteins and tumour suppressors. *Nature Reviews Cancer*, 16(7), 413-430. <https://doi.org/10.1038/nrc.2016.51>
- Graham, D. K., DeRyckere, D., Davies, K. D., & Earp, H. S. (2014). The TAM family: Phosphatidylserine sensing receptor tyrosine kinases gone awry in cancer. *Nature Reviews Cancer*, 14(12), 769-785. <https://doi.org/10.1038/nrc3847>
- Han, H. A., Pang, J. K. S., & Soh, B. S. (2020). Mitigating off-target effects in CRISPR/Cas9-mediated in vivo gene editing. *Journal of Molecular Medicine (Berlin)*, 98(5), 615–632. <https://doi.org/10.1007/s00109-020-01893-z>
- Hernandez, F. J. (2025). Nucleases: From primitive immune defenders to modern biotechnology tools. *Immunology*, 174(3), 279–286. <https://doi.org/10.1111/imm.13884>
- Huelse, J. M., Fridlyand, D. M., Earp, S., DeRyckere, D., & Graham, D. K. (2020). MERTK in cancer therapy: Targeting the receptor tyrosine kinase in tumor cells and the immune system. *Pharmacology & Therapeutics*, 213, 107577. <https://doi.org/10.1016/j.pharmthera.2020.107577>
- Iida, M., Crossman, B. E., Kostecki, K. L., Glitchev, C. E., Kranjac, C. A., Crow, M. T., Adams, J. M., Liu, P., Ong, I., Yang, D. T., Kang, I., Salgia, R., & Wheeler, D. L. (2024). MerTK drives proliferation and metastatic potential in triple-negative breast cancer. *International Journal of Molecular Sciences*, 25(10), 5109. <https://doi.org/10.3390/ijms25105109>
- Lahey, K. C., Gadiyar, V., Hill, A., Desind, S., Wang, Z., Davra, V., Patel, R., Zaman, A., Calianese, D., & Birge, R. B. (2022). MERTK: An emerging target in cancer biology and immunoncology. *International Review of Cell and Molecular Biology*, 368, 35–59. <https://doi.org/10.1016/bs.ircmb.2022.04.004>
- Lahey, K. C., Varsanyi, C., Wang, Z., Aquib, A., Gadiyar, V., Rodrigues, A. A., Pucella, R., Desind, S., Davra, V., Calianese, D. C., Liu, D., Cho, J. H., Kotenko, S. V., De Lorenzo, M. S., & Birge, R. B. (2024). Regulation of MerTK surface expression via ADAM17 and γ -secretase proteolytic processing. *International Journal of Molecular Sciences*, 25(8), 4404. <https://doi.org/10.3390/ijms25084404>
- Lemke, G. (2013). Biology of the TAM receptors. *Cold Spring Harbor Perspectives in Biology*, 5(11), a009076. <https://doi.org/10.1101/cshperspect.a009076>
- Lemke, G., & Rothlin, C. V. (2008). Immunobiology of the TAM receptors. *Nature Reviews Immunology*, 8(5), 327-336. <https://doi.org/10.1038/nri2303>
- Li, C., Du, Y., Zhang, T., Wang, H., Hou, Z., Zhang, Y., Cui, W., & Chen, W. (2022). "Genetic scissors" CRISPR/Cas9 genome editing cutting-edge biocarrier technology for bone and cartilage repair. *Bioactive Materials*, 22, 254–273. <https://doi.org/10.1016/j.bioactmat.2022.09.026>
- Leslie, S. W., Sajjad, H., & Agarwal, S. (2024). Bladder cancer. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK536923/>
- Li, J., Jin, K., Hu, X., & Ding, X. (2024). Molecular mechanisms and therapeutic strategies for bladder cancer. *Frontiers in Oncology*, 14, 1234567. <https://doi.org/10.3389/fonc.2024.1234567>

- Linger, R. M., Cohen, R. A., Cummings, C. T., Sather, S., Migdall-Wilson, J., Middleton, D. H., Lu, X., Barón, A. E., Franklin, W. A., Merrick, D. T., Jedlicka, P., DeRyckere, D., Heasley, L. E., & Graham, D. K. (2013). Mer or Axl receptor tyrosine kinase inhibition promotes apoptosis, blocks growth and enhances chemosensitivity of human non-small cell lung cancer. *Oncogene*, 32(29), 3420-3431. <https://doi.org/10.1038/onc.2012.355>
- Liu, H., Wang, S., Zhang, Z., Jie, J., Song, L., & Hua, S. (2020). Diagnostic value of soluble form of Mer tyrosine kinase (sMerTK) in tuberculous pleural effusion and malignant pleural effusion. *BioMed Research International*, 2020, Article 1496935. <https://doi.org/10.1155/2020/1496935>
- Liu, H., Wang, S., Zhang, Z., Jie, J., Song, L., & Hua, S. (2020). Diagnostic value of soluble form of Mer tyrosine kinase (sMerTK) in tuberculous pleural effusion and malignant pleural effusion. *BioMed Research International*, 2020, Article 1496935. <https://doi.org/10.1155/2020/1496935>
- Meng, L., Zhu, X., Ji, X., Wang, B., Zhang, H., Zhang, G., Xue, Y., & Wang, C. (2025). Advances in the immunological microenvironment and immunotherapy of bladder cancer. *Frontiers in Immunology*, 16, 1609871. <https://doi.org/10.3389/fimmu.2025.1609871>
- Nguyen, K. Q., Tsou, W. I., Calarese, D. A., Kimani, S. G., Singh, S., Hsieh, S., Liu, Y., Lu, B., Wu, Y., Garforth, S. J., Almo, S. C., Kotenko, S. V., Birge, R. B., & Debebe, Z. (2014). Overexpression of MERTK receptor tyrosine kinase in epithelial cancer cells drives efferocytosis in a gain-of-function capacity. *Journal of Biological Chemistry*, 289(37), 25737-25749. <https://doi.org/10.1074/jbc.M114.570838>
- Rothlin, C. V., Carrera-Silva, E. A., Bosurgi, L., & Ghosh, S. (2015). TAM receptor signaling in immune homeostasis. *Annual Review of Immunology*, 33, 355-391. <https://doi.org/10.1146/annurev-immunol-032414-112103>
- Sather, S., Kenyon, K. D., Lefkowitz, J. B., Liang, X., Varnum, B. C., Henson, P. M., & Graham, D. K. (2007). A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. *Blood*, 109(3), 1026-1033. <https://doi.org/10.1182/blood-2006-05-021634>
- Schlegel, J., Sambade, M. J., Sather, S., Moschos, S. J., Tan, A. C., Wings, A., DeRyckere, D., Carson, W. E., Trembath, D. G., Tentler, J. J., Eckhardt, S. G., Kuan, P. F., Hamilton, R. L., Duncan, L. M., Miller, C. R., Nikolaishvili-Feinberg, N., Midkiff, B. R., Liu, J., Zhang, W., Yang, C., Wang, X., Frye, S. V., Earp, H. S., Shields, J. M., & Graham, D. K. (2013). MERTK receptor tyrosine kinase is a therapeutic target in melanoma. *Journal of Clinical Investigation*, 123(5), 2257-2267. <https://doi.org/10.1172/JCI67816>
- Tanim, K. M., Holtzhausen, A., Thapa, A., Hulse, J. M., Graham, D. K., & Earp, H. S. (2024). MERTK inhibition as a targeted novel cancer therapy. *International Journal of Molecular Sciences*, 25(14), 7660. <https://doi.org/10.3390/ijms25147660>
- Thorp, E., Vaisar, T., Subramanian, M., Mautner, L., Blobel, C., & Tabas, I. (2011). Shedding of the Mer tyrosine kinase receptor is mediated by ADAM17 protein through a pathway involving reactive oxygen species, protein kinase C δ , and p38 mitogen-activated protein kinase (MAPK). *Journal of Biological Chemistry*, 286(38), P33335-P33344. <https://doi.org/10.1074/jbc.M111.264694>

- Wang, D., Zhang, W., Chen, L., Zhang, Y., & Li, X. (2024). Disruption of MerTK increases the efficacy of checkpoint inhibitors by enhancing ferroptosis and immune response in hepatocellular carcinoma. *Cell Reports Medicine*, 5(5), 101415. <https://doi.org/10.1016/j.xcrm.2024.101415>
- Yan, D., Hulse, J.M., Kireev, D.T., Zikang C., Luxiao G., Subir., Xiaodong F., Stephen V. B., Madhusmita S., Frank R., Suresh S.O., Taofeek E., H. Shelton D., Deborah G., Douglas K. (2022). MERTK activation drives osimertinib resistance in EGFR-mutant non-small cell lung cancer. *The Journal of Clinical Investigation*, 15, 132. DOI: <https://doi.org/10.1172/JCI150517>
- Zhou, Y., Zhang, H., Yan, H., Han, P., & Liu, Y. (2025). Immune landscape and prognostic significance of gene expression profiles in bladder cancer: Insights from immune cell infiltration and risk modeling. *Iranian Journal of Allergy, Asthma and Immunology*, 24(4), 519-532. <https://doi.org/10.18502/ijaai.v24i4.19132>