JOURNAL OF
EMERGING INVESTIGATORS

Article

siRNA-dependent KCNMB2 silencing inhibits lung cancer cell proliferation and promotes cell death

Eunice Jeong[1], Woo Rin Lee[2]

[1] Emma Willard School, Troy, New York

[2] Department of Biological Science, University of Suwon, Wau-ri, Bongdam-eup, Hwaseong, Gyeonggi-do, Republic of Korea

SUMMARY

Non-small cell lung cancer (NSCLC) is one of the most common causes of premature death worldwide. Since NSCLC patients have a high rate of mutation in EGFR and KRAS, a better understanding of the genetic association underlying NSCLC progression is needed. Recent technological advances in gene expression profiling in cancer cells have provided the expression data of thousands of genes. Therefore, we screened hundreds of genes through the Kaplan Meier plotter database to find a novel gene expression pattern associated with lung cancer patient survival. First, we analyzed 3,019 lung cancer patients' data and performed a Kaplan-Meier survival analysis. We found that KCNMB2 upregulation was significantly correlated with poor prognosis in lung cancer patients. Therefore, we hypothesized that KCNMB2 downregulation would inhibit lung cancer cell proliferation. Next, we found that KCNMB2 silencing by small interfering RNA (siRNA) inhibits cell proliferation and promotes apoptosis, suggesting that targeting KCNMB2 may be an effective therapeutic strategy for NSCLC patients.

INTRODUCTION

In 2021, a total of 235,760 new lung cancer cases were diagnosed in the United States, posing an increased susceptibility to death (1). Lung cancers start from the lungs and spread to lymph nodes or other organs (2). Smoking is a widely known factor which increases the risk of developing lung cancer, but it is not the only factor that leads to lung cancers. They are usually grouped into two main types: small cell and non-small cell lung cancer (NSCLC), the latter of which is more common (3). NSCLC patients often show no symptoms in the early cancer stage (3). Also, about 16.7% of metastasized NSCLC patients develop liver metastasis (3). Even though significant advances have been made in targeted agents and immunotherapy in metastatic NSCLC treatment, not all targetable mutations are presented in NSCLC patients. Also, drug resistance can be developed in targeted therapy or immunotherapy. Therefore, identifying new targets and understanding the resistance mechanisms are crucial to overcoming these limitations.

Many diverse cancer gene expression databases provide a rich source of candidate oncogenic drivers of progression and therapeutic target genes (4). They have also been used to classify cancer and predict clinical outcomes. The Kaplan Meier (KM) plotter database provides expression data for 30,000 genes and survival for 25,000 samples from 21 tumor types, including breast, lung, ovarian, and stomach cancer tumors (5). This database provides a tool for meta-analysis-based discovery and validation of survival biomarkers. To identify novel targets for NSCLC, we conducted a meta-analysis of genes specifically overexpressed in NSCLC and associated with decreased patient survival rate.

The most consistently over-expressed gene was Potassium Calcium-Activated Channel Subfamily M Regulatory Beta Subunit 2 (KCNMB2), which encodes an auxiliary beta-subunit that decreases the current activation time for the MaxiK alpha subunit of the MaxiK channel [6]. The MaxiK channel is a large conductance calcium-activated potassium channel. It conducts a large number of potassium ions through the cell membrane and can be activated by electrical currents or by increasing the concentration of Ca^{2+} (7). Overexpression of the MaxiK channel gene has been discovered in several cancers including brain, prostate, and mammary cancers (8). Also, a previous study indicated that MaxiK current may be lost in lung cancer cells that induce multidrug resistance protein 1 (MRP1) (9). Since KCNMB2 may decrease the activation time and current of the MaxiK channel, KCNMB2 may play an important role in MaxiK current loss in lung cancer. However, the precise role of KCNMB2 and MaxiK channel in regulating oncogenesis in lung cancer remains unclear.

Small interfering RNA (siRNA) is artificially synthesized RNA that targets and degrades specific mRNA (9). When the double-stranded siRNA is transfected into cells, it binds onto endoribonuclease Argonaute 2 (Ago2) of the RNA-induced silencing complex (RISC). After Ago2 nicks the passenger strand of siRNA, it is cleaved and removed from the RISC complex, leaving only single-stranded siRNA (guide strand) (10). This leads to Ago2-dependent cleavage of the target mRNA.

Through this research, we found that KCNMB2 overexpression is associated with decreased survival rate in NSCLC patients. Therefore, we hypothesized that KCNMB2 downregulation would inhibit lung cancer cell proliferation. In this study, we used siRNA to target KCNMB2 and decrease the mRNA expression level of KCNMB2 in A549 lung cancer cells. Knockdown of KCNMB2 by siRNA demonstrated that it is essential for the viability of A549 cells and regulates cancer

cell proliferation. These results demonstrate the importance of using publicly available patient data to uncover novel oncogenic drivers and suggest that KCNMB2 can be used as a therapeutic target in NSCLC.

RESULTS

High KCNMB2 Expression Negatively Correlates with Lung Cancer Progression

To find a novel effect of KCNMB2 on lung cancer progression, we analyzed patient survival rates based on expression level of KCNMB2 in lung cancer patients. To analyze the prognostic value of KCNMB2 expression, we split the patient samples into two groups according to their quantile expression level: high and low expression. Kaplan Meier plot results showed that patients with a higher expression level of KCMNB2 (median survival months = 62) represented a lower survival rate than patients with a lower expression level of KCNMB2 (median survival months = 80) (p = 0.00017, log rank test, **Figure 1**). The survival rate of patients with high KCNMB2 expression decreases faster than patients with low KCNMB2 expression over time.

siRNA Targeting KCNMB2 Significantly Decreased the Expression Level of KCNMB2

Since Kaplan Meier analysis indicates that a high expression level of KCNMB2 may promote lung cancer progression and therefore negatively affect patient survival, we hypothesized that KCNMB2 knockdown might inhibit the cancer cell proliferation. Therefore, we sought to find the optimal concentration of siRNA that would significantly

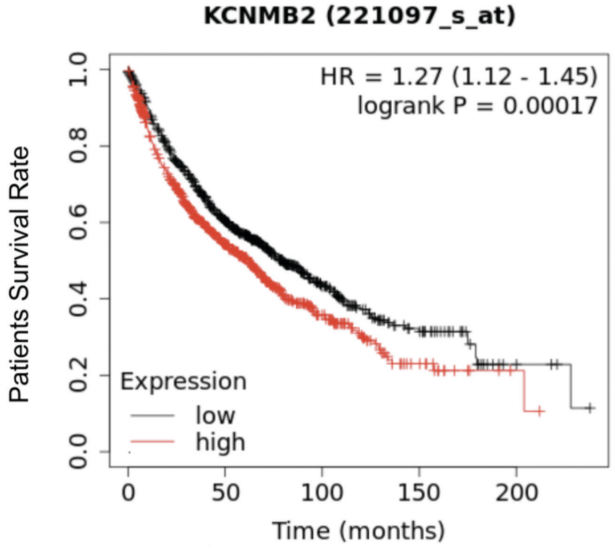

Figure 1: The patient survival rate is lower for lung cancer patients with high KCNMB2 expression levels than low KCNMB2 expression level. Kaplan-Meier plot showing patient survival rate on y-axis and time (month) on x-axis (n = 3019). The prognostic values of the KCNMB2 gene were analyzed by dividing the patient samples into two groups by the median expression of KCNMB2 (cutoff value = 87). The KCNMB2 expression range was 1 to 6612. Log-rank, p = 0.00017.

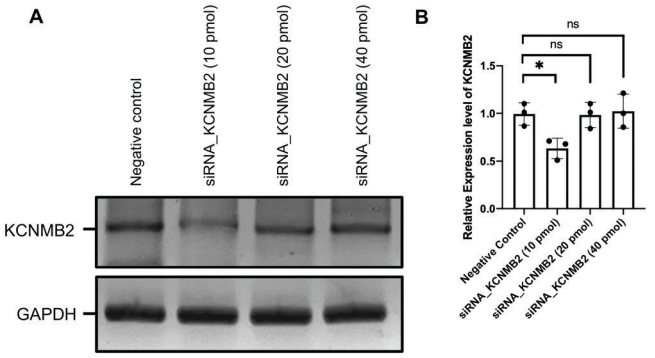

Figure 2: siRNA_KCNMB2 (10 pmol) decreased KCNMB2 expression level in A549 cells. (A) Agarose gel image showing amplified cDNA of KCNMB2 and GAPDH. (B) Bar graph showing mean ± SD relative KCNMB2 expression level to GAPDH. A549 cells were transfected with either negative control condition, or siRNA_KCNMB2 (10, 20, 40 pmol) for 48 hours (n = 2). Unpaired t-test, ns indicates $p > 0.05$, and * indicates $p < 0.05$.

knock down the expression level of KCNMB2. We analyzed the knockdown effect of three different concentrations of siRNA targeting KCNMB2 (siRNA_KCNMB2) **(Figure 2)**. We analyzed the band intensity of KCNMB2 and GAPDH and used GAPDH as a normalizing factor to quantify the expression level of KCNMB2. 10 pmol of siRNA_KCNMB2 transfection significantly decreased the relative expression level of KCNMB2 compared to the negative control, which was transfected with scrambled siRNA that does not target any human genes (p = 0.045, unpaired t-test, **Figure 2**). Transfection with 20 or 40 pmol of siRNA_KCNMB2 did not significantly decrease the expression level of KCNMB2 (p = 0.999 and p = 0.992 respectively, unpaired t-test, **Figure 2**). In conclusion, 10 pmol was the most efficient concentration for KCNMB2 knockdown in A549 lung cancer cells. Hence, 10 pmol of siRNA concentration was used for the next experiments.

KCNMB Knockdown Decreased Lung Cancer Cell Proliferation

Cell proliferation is how quickly a cancer cell copies its DNA and divides into two cells indicating faster-growing cancer cells are more aggressive. Therefore, we inhibited the expression of KCNMB2 to test its effect on the proliferation of NSCLC tumor cells. Based on the Kaplan Meire analysis, we hypothesized that KCNMB2 knockdown would decrease proliferation compared to the negative control. We used PrestoBlue™ assay to measure cell proliferation. When added to cells, the PrestoBlue™ reagent (resazurin) is modified by the reducing environment of the viable cell and turns red (resorufin) in color. This color change can be detected using absorbance measurements (570 nm/600 nm). Cell media in the KCNMB2 knockdown significantly decreased the absorbance measurements compared to negative control indicating that the KCNMB2 knockdown inhibited cell proliferation (p = 0.0001, unpaired t-test, **Figure 3**). Overall,

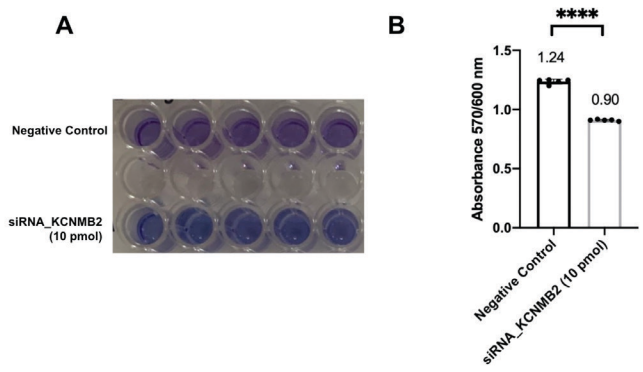

Figure 3: KCNMB2 knockdown decreased cancer cell proliferation. (A) Image of 96 well plate with negative control and siRNA_KCNMB2 transfected samples with Prestoblue reagent. (B) Quantification of cancer cell proliferation by measuring absorbance and calculating the ratio (570 nm / 600 nm) on microplate spectrometry (n = 4). A549 lung cancer cells were grown after transfection either for 48 hours. Unpaired *t*-test, **** indicates $p<0.0001$.

these results indicate that KCNMB2 knockdown decreases cell proliferation.

KCNMB2 Knockdown Increased Lung Cancer Cell Death

Since cancer cell proliferation can be affected by cell death, we analyzed the concentration of live and dead lung cancer cells after siRNA_KCNMB2 transfection to find the effect of the KCNMB2 knockdown on cell death. The Acridine Orange/Propidium Iodide double staining method was used to determine the number of live and dead cells in negative control and siRNA_KCNMB2 transfected cells. The green (live) and red (dead) fluorescence cells represent live and dead cells **(Figure 4)**. Both negative control and KCNMB2 knockdown samples contained both green and red cells. However, KCNMB2 knockdown cells showed fewer live and dead cells than negative control cells in qualitative analysis

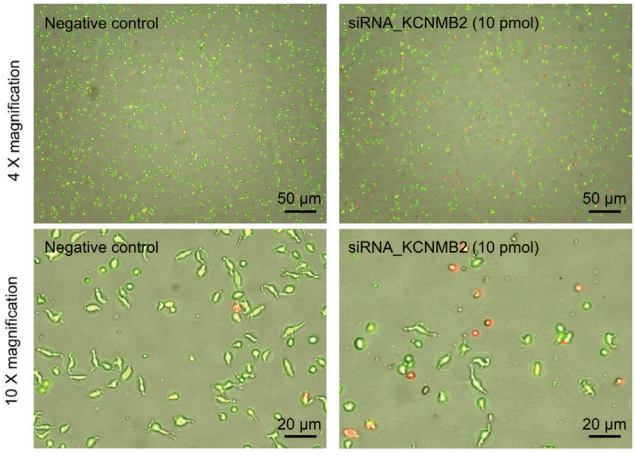

Figure 4: siRNA_KCNMB2 (10 pmol) transfection decreased the number of live cells (green) and increased cell death (red). Cell images showing live (green) and dead (red) cells. The images were taken in 4x, and 10x magnification after transfected A549 cells were grown for 48 hours.

	Live cell concentration (cells/ml)	Dead cell concentration (cells/ml)	Total cell concentration (cells/ml)	Viability % (live/total)
Negative control	2.40×10^6	3.69×10^4	2.44×10^6	98.5%
siRNA_KCNMB2	1.34×10^6	2.32×10^5	1.57×10^6	85.2%

Table 1: Comparison of live and dead cell concentration between negative control and siRNA_KCNMB2 transfected cells. These values were measured singularly from each sample.

(Figure 4). KCNMB2 knockdown cells showed lower viability (85.2%) compared to the negative control (98.5%) in a single measurement of cell count data **(Table 1)**. Even though the same number of cells were prepared in both conditions before transfection, the total number of cells, including live and dead cells, was less in KCNMB2 knockdown cells than in negative control **(Table 1)**. In conclusion, KCNMB2 knockdown increased lung cancer cell death.

DISCUSSION

Identifying novel targets for NSCLC is crucial for both NSCLC diagnosis and treatment. For targeted cancer therapies, novel molecular targets and biomarkers provide a promising direction in drug developments and treatments. Therefore, we conducted a meta-analysis of genes specifically overexpressed in NSCLC and associated with decreased patient survival rate. A group of NSCLC patients with high KCNMB2 expression showed a lower survival rate compared to a group of patients with low KCNMB2 expression. This suggests that high expression of KCNMB2 could increase lung cancer progression. We found that KCNMB2 knockdown by siRNA decreased KCNMB2 mRNA expression and decreased cancer cell proliferation by inducing cell death. This indicates that KCNMB2 may function as an oncogene in lung cancer and that KCNMB2 may be a potential target gene for the patients who have high expression of KCNMB2.

Previous studies showed that overexpression of the Maxik channel protein is associated with cancer cell growth and invasion, suggesting that KCNMB2 overexpression is associated with increased cancer progression, since KCNMB2 e encodes the beta subunit of MaxiK channel (11,12). Malignancy of human gliomas correlates with MaxiK channel overexpression, which induces glioma cell growth (11). The high conductance MaxiK channel also plays an important role in the survival of breast cancer patients. High expression of MaxiK channel was found to increase breast cancer cell proliferation and invasion (12). However, the role of MaxiK channel on NSCLC is unclear. Therefore, we speculated that KCNMB2 overexpression may activate the conductance of MaxiK channel and decrease survival rate of cancer patients by promoting the cancer growth.

Several limitations for this research should be addressed in future experiments. We only analyzed the effect of KCNMB2 knockdown on cancer cell proliferation and cell death. Since numerous factors are involved in the multi-step process of cancer progression, such as cell migration, invasion,

metastasis, and reprogramming of cell metabolism, additional experiments evaluating multiple functional properties of KCNMB2 should be performed. Using more complex cell models would allow for the verification of the function of KCNMB2. Moreover, using an animal model, the cancer cell could be tested by inhibiting the proliferation of KCNMB2 and if the proliferation rate declines in live animals. Also, the effect of KCNMB2 knockdown on normal lung cells and tissues was not investigated. Therefore, future experiments testing the side effects of targeting KCNMB2 in patients should be also tested. The cell counting staining method cannot detect the late stage of cell death because propidium iodide only stains the DNA inside the nucleus. The late stage of cell death is characterized by DNA fragmentation after nuclear condensation; therefore, red cells only represent the early stage of cell death. Therefore, there is a possibility that late-stage dead cells were undetectable because they no longer had a nucleus which makes it impossible to be stained as red **(Figure 4)**. Therefore, the percentage of dead cells measured in KCNMB2 siRNA transfected cells could be underestimated.

Compared to past studies regarding KCNMB2, we discovered that a high expression level of KCNMB2 significantly correlates lung cancer patient survival rate. Moreover, we demonstrated that targeting KCNMB2 by siRNA inhibited lung cancer cell proliferation by inducing cell death. Overall, the knockdown of KCNMB2 may have the potential to be used as a therapeutic target for lung cancer therapy.

MATERIALS AND METHODS

KM Plot Survival Analysis

Kaplan-Meier plotter (KM plotter) was used to identify gene expression patterns that correlated with patient survival (13). Gene expression data from 3019 lung cancer patients were downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), European Genome-phenome Archive (EGA) (https://ega-archive.org/), and The Cancer Genome Atlas (TCGA) (http://tcga-data.nci.nih.gov). The cutoff value of gene expression was chosen as the median to split the patient samples into two groups based on KCNMB2 expression. The cutoff value used in this analysis was 87 (the expression range of KCNMB2 was 1 to 6612). The Kaplan-Meier survival plot was used to compare the survival of the two lung cancer patient groups, and 95% confidence intervals and log-rank p-values were calculated.

Cell Culture and siRNA Transfection

A549, non-small cell lung cancer cells, were purchased from Korea Cell Line Bank. The cells were maintained in RPMI1640 (Gibco) cell culture media. RPMI1640 was supplemented with 10% fetal bovine serum (FBS) and 1 % penicillin and streptomycin. 0.3 x 106 A549 cells were seeded in a 24-well plate. Control siRNA, scrambled siRNA that does not target any human gene, and siRNA targeting KCNMB2 (Bioneer, Cat # 10242-1) were mixed with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Cat # LMRNA015) and incubated for 5 minutes. 10 pmol, 20 pmol, and 40 pmol concentrations of siRNA targeting KCNMB2 mixtures were prepared. Then, the cells were incubated for 48 hours.

Total RNA Extraction from Cultured Cells

Total RNA was extracted using the AccuPrep® Universal RNA Extraction Kit (Bioneer, Cat. # K-3140). The total RNA was extracted according to the manufacturer's instructions.

cDNA Synthesis

The following steps were used to synthesize cDNA from extracted RNA. A total 10 µL of cDNA was synthesized with TOPscript™ Reverse Transcriptase (Enzynomics, Cat # RT002S). The following reverse transcription reaction was performed: 1) 25 °C for 10 minutes, 2) 42 °C for 1 hour, 3) 95 °C for 5 minutes, 4) 4 °C for 10 minutes.

Polymerase Chain Reaction

The following primer sequences were used to amplify KCNMB2 cDNA (153 bp): Forward primer: 5′ - GAGGACCGAGCTATTCTCCTG - 3′ Reverse primer: 5′ - TGTTTCCGTGATGGACGCATT - 3′. For GAPDH amplification (101 bp), the following primer sequences were used: Forward primer: 5′ - ACAACTTTGGTATCGTGGAAGG - 3′ Reverse primer: 5′ - GCCATCACGCCACAGTTTC - 3′. The following steps were used to amplify KCNMB2 and GAPDH gene using a PCR machine (Biorad): 1) 95 °C for 3.5 minutes, 2) 60 °C for 30 seconds, 3) 72 °C for 20 seconds, 4) 34 cycles of: I) 95 °C for 30 seconds, II) 60 °C for 30 seconds, III) 72 °C for 5.2 seconds; 5) 12 °C for 10 minutes.

Agarose Gel Electrophoresis

1.5% agarose gel was prepared with Tris-Borate EDTA buffer. RedSafe™ (Intronbio, Cat # 21141), which is a DNA staining solution, was added to the heated solution and was mixed well by swirling. After the gel was solidified, the amplified samples of KCNMB2 and GAPDH were transferred into individual wells, and the gel was run at 100 volts for 25 minutes. DNA bands were visualized on a blue light illuminator. Densitometry used from each cDNA amplified band of agarose gels was analyzed using by ImageJ program (ver 1.53k15). The band intensity of GAPDH was used to normalize KCNMB2 expression level.

Prestoblue Assay

After cells were transfected with siRNA and incubated for 48 h in a 96-well plate, Prestoblue cell viability reagent (Invitrogen, Cat # A13261) was added directly to cells in culture medium. The cells were incubated for 30 minutes at 37 °C. Both 570 and 600 nm absorbance was measured using a microspectrometer (Biotek). 570 nm values were normalized to the 600 nm values for each well. The normalized 570 nm absorbance was plotted on the graph.

Live and Dead Cell Count Analysis

LUNA-FL fluorescent cell counter device (Logos Biosystem, Cat # L20001) was used to measure the number and the viability of cells. 18 µl of the cell sample was mixed with 2 µl Acridine Orange/Propidium Iodide Stain (Logos Biosystem, Cat # cat # F23001). Then, 10-12 µl of the mixed cell suspension were added into a PhotonSlide™. If acridine orange and propidium iodide were stained, acridine orange fluorescence is mostly quenched by propidium iodide showing green fluorescence. Therefore, the live cells were stained with green fluorescence by acridine orange, and dead cells were stained by propidium iodide with red fluorescence. LUNA-FL™ software (version 3.0) was used to count the cells.

Statistical Test

All statistical tests were performed using the Prism 8 program (GraphPad). Log-rank and unpaired t-tests were used to analyze the statistical significance of the data. *p*-values less than 0.05 were considered statistically significant.

ACKNOWLEDGMENTS

E.J. would like to thank her parents for their constant support and belief in me. E.J. genuinely appreciates the passionate love from her father and the wise guidance from her mother.

Received: March 3, 2022
Accepted: June 13, 2022
Published: November 1, 2022

REFERENCES

1. Siegel, Rebecca L *et al.* “Cancer Statistics, 2021.” *CA: a cancer journal for clinicians* vol. 71,1 (2021): 7-33. doi:10.3322/caac.21654
2. Thai, Alesha A., *et al.* “Lung Cancer.” *The Lancet*, vol. 398, no. 10299, Aug. 2021, pp. 535–54, doi:10.1016/S0140-6736(21)00312-3.
3. Clark, Shane B., and Saud Alsubait. “Non Small Cell Lung Cancer.” *StatPearls*, StatPearls Publishing, 2022.
4. Chen, Ron, *et al.* “A Meta-Analysis of Lung Cancer Gene Expression Identifies PTK7 as a Survival Gene in Lung Adenocarcinoma.” *Cancer Research*, vol. 74, no. 10, May 2014, pp. 2892–902, doi:10.1158/0008-5472.CAN-13-2775.
5. Gyorffy, Balázs, *et al.* “Implementing an Online Tool for Genome-Wide Validation of Survival-Associated Biomarkers in Ovarian-Cancer Using Microarray Data from 1287 Patients.” *Endocrine-Related Cancer*, vol. 19, no. 2, Apr. 2012, pp. 197–208, doi:10.1530/ERC-11-0329.
6. Guntur, Divya, *et al.* “Revisiting the Large-Conductance Calcium-Activated Potassium (BKCa) Channels in the Pulmonary Circulation.” *Biomolecules*, vol. 11, no. 11, Nov. 2021, doi:10.3390/biom11111629.
7. Toro, Ligia, *et al.* “MaxiK Channel and Cell Signalling.” *Pflugers Archiv: European Journal of Physiology*, vol. 466, no. 5, May 2014, pp. 875–86, doi:10.1007/s00424-013-1359-0.
8. Ge, Lisheng, *et al.* “Big Potassium (BK) Ion Channels in Biology, Disease and Possible Targets for Cancer Immunotherapy.” *International Immunopharmacology*, vol. 22, no. 2, Oct. 2014, pp. 427–43, doi:10.1016/j.intimp.2014.06.040.
9. Scherr, Michaela, *et al.* “Gene Silencing Mediated by Small Interfering RNAs in Mammalian Cells.” *Current Medicinal Chemistry*, vol. 10, no. 3, Feb. 2003, pp. 245–56.
10. Dana, Hassan, *et al.* “Molecular Mechanisms and Biological Functions of SiRNA.” *International Journal of Biomedical Science*: IJBS, vol. 13, no. 2, June 2017, pp. 48–57.
11. Abdullaev, Iskandar F., *et al.* “Calcium-Activated Potassium Channels BK and IK1 Are Functionally Expressed in Human Gliomas but Do Not Regulate Cell Proliferation.” *Plos One*, vol. 5, no. 8, Aug. 2010, p. e12304, doi:10.1371/journal.pone.0012304.
12. Goda, Amira A., *et al.* “The Maxi-K (BK) Channel Antagonist Penitrem A as a Novel Breast Cancer-Targeted Therapeutic.” *Marine Drugs*, vol. 16, no. 5, May 2018, doi:10.3390/md16050157.
13. Lánczky, András, and Balázs Győrffy. “Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation.” *Journal of medical Internet research* vol. 23,7 e27633. 26 Jul. 2021, doi:10.2196/27633