

INVESTIGATION OF ALCAM'S ROLE IN GLIOBLASTOMA SENESENCE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN MATERIALS
SCIENCE AND NANOTECHNOLOGY

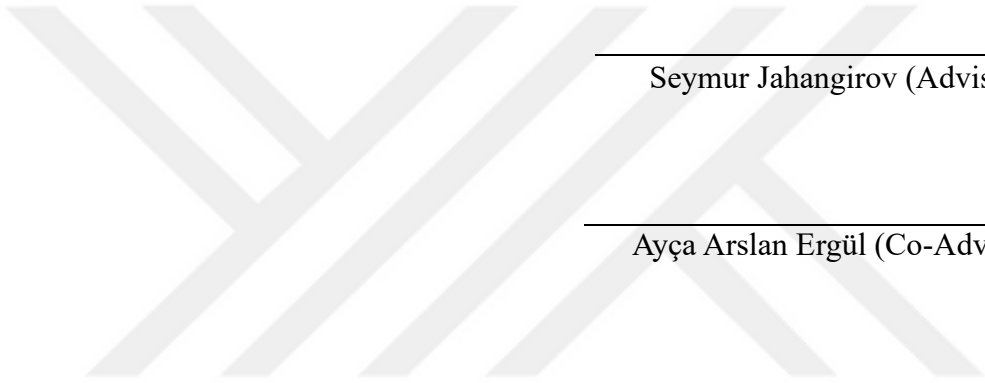
By
Tuba Sena Oğurlu
June 2024

INVESTIGATION OF ALCAM’S ROLE IN GLIOBLASTOMA SENESENCE

By Tuba Sena Oğurlu

June 2024

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.



Seymur Jahangirov (Advisor)

Ayça Arslan Ergül (Co-Advisor)

Çağdaş Devrim Son

Bahar Değirmenci Uzun

Approved for the Graduate School of Engineering and Science:

Orhan Arıkan

Director of Graduate School

ABSTRACT

INVESTIGATION OF ALCAM'S ROLE IN GLIOBLASTOMA SENESENCE

Tuba Sena Oğurlu

M.S. in Materials Science and Nanotechnology

Advisor: Seymur Jahangirov

Co-Advisor: Ayça Arslan Ergül

June 2024

Glioblastoma (GBM) is a type of glioma that is the most common brain tumor in the Central Nervous System. Based on this classification, it is the highest-grade astrocytoma (grade IV), highly aggressive and invasive brain cancer. The primary treatment for GBM involves surgical resection to remove as much of the tumor as possible. This is typically followed by radiotherapy and concurrent temozolomide (TMZ) chemotherapy. Despite these multimodal approaches, the prognosis for GBM patients remains poor, with a median survival of approximately 12-16 months. It is already known that the high invasiveness causes the cancer to reach other tissues in the body. If the cells could migrate to other regions but couldn't proliferate, this would increase the lifespan following the diagnosis. Therefore, one of the most important reasons why these treatments are not effective is the fact that cells can escape senescence induced by TMZ. New genetic tools or targets are needed to help to keep the cells in senescence state or to induce senescence more effectively.

Here, in this study, we wanted to investigate the role of Activated Cell Adhesion Molecule (ALCAM) in GBM senescence. In the literature, it has been shown that GBM patient survival decreases with the increased ALCAM level. Moreover, in patient-derived xenografts, cells with overexpressed soluble ALCAM showed more aggressiveness. On the contrary, when ALCAM was decreased in GBM cell lines, the colony formation ability increased. Therefore, the role of ALCAM in senescence and especially in GBM senescence, if there is, has remained elusive. To shed light on this unclarified relationship,

we used ALCAM targeting small interfering ribonucleic acid (siRNA) and constructed plasmid overexpressing it to manipulate the ALCAM gene level in GBM cells. Expression levels of senescence markers and SA- β gal staining percentage were investigated upon these manipulations. Although significant changes were observed in senescence markers, we cannot say they were consistent or reliable. There is a need for a more comprehensive study to understand the correct relationship. Moreover, conditions like tumor microenvironment, stem cell subpopulation percentage and combinatorial effects should be considered.



Keywords: Glioblastoma, astrocytoma, activated leukocyte cell adhesion molecule (ALCAM), senescence

ÖZET

GLİOBLASTOM SENESENSİNDE ALCAM ROLÜNÜN İNCELENMESİ

Tuba Sena Oğurlu

Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

Danışman: Seymur Jahangirov

Eş-Danışman: Ayça Arslan Ergül

Haziran 2024

Glioblastoma, Merkezi Sinir Sisteminde en yaygın beyin tümörü olan bir glioma türüdür. Bu sınıflandırmaya göre, en yüksek dereceli astrositoma (derece IV), son derece agresif ve invaziv bir beyin kanseridir. Glioblastomanın birincil tedavisi, tümörün mümkün olduğunca büyük bir kısmının çıkarılması amacıyla cerrahi rezeksiyonu içerir. Genellikle bunu radyoterapi ve eş zamanlı temozolomid (TMZ) kemoterapisi izler. Bu multimodal yaklaşımlara rağmen, GBM hastaları için prognoz kötü kalmaktadır ve ortalama hayatta kalma süresi yaklaşık 12-16 aydır. Yüksek invazivliğin, kanserin vücuttaki diğer dokulara ulaşmasına neden olduğu bilinmektedir. Hücreler diğer bölgelere göç edebilir ancak çoğalamazsa, bu teşhisin ardından yaşam süresini artıracaktır. Bu nedenle, bu tedavilerin etkili olmamasının en önemli nedenlerinden biri, hücrelerin TMZ tarafından indüklenen yaşlanmadan kaçabilmesidir. Hücrelerin yaşlanma durumunda kalmasına veya yaşlanmanın daha etkili bir şekilde indüklenmesine yardımcı olacak yeni genetik araçlara veya hedeflere ihtiyaç vardır.

Bu çalışmada, Aktive Edilmiş Hücre Adezyon Molekülü'nün (ALCAM) glioblastoma hücrelerinde yaşlanmadaki rolünü araştırmak istedik. Literatürde, glioblastoma hastası sağkalımının ALCAM seviyesinin artmasıyla azaldığı gösterilmiştir. Ayrıca, hasta kaynaklı ksenograftlarda, çözünebilir ALCAM'i aşırı eksprese eden hücreler daha fazla agresiflik göstermiştir. Aksine, glioblastoma hücre hatlarında ALCAM azaltıldığında, koloni oluşturma yeteneği artmıştır. Bu nedenle, ALCAM'in yaşlanmadaki ve özellikle glioblastoma yaşlanmasındaki rolü, varsa, belirsiz kalmıştır. Bu açıklığa kavuşturulmamış

ilişkiyi aydınlatmak için, ALCAM hedefleyen siRNA kullandık ve glioblastoma hücrelerinde ALCAM gen düzeyini manipüle etmek için onu aşırı eksprese eden plazmid oluşturduk. Bu manipülasyonlar sonrasında yaşlanma belirteçlerinin ekspresyon seviyeleri ve SA- β gal boyama yüzdesi araştırıldı. Yaşlanma belirteçlerinde önemli değişiklikler gözlenmesine rağmen, bunların tutarlı veya ilişkilendirilebilir olduğunu söyleyemeyiz. Doğru ilişkiyi anlamak için daha kapsamlı bir çalışmaya ihtiyaç vardır. Ayrıca, tümör mikroçevresi, kök hücre alt popülasyon yüzdesi ve kombinatorial etkiler gibi koşullar dikkate alınmalıdır.



Anahtar Kelimeler: Glioblastom, astrositom, aktive olmuş lökosit hücre adezyon molekülü (ALCAM), yaşlanma, senesens



To my sister...

Acknowledgements

First of all, I would like to thank my advisor Dr. Seymur Jahangirov for agreeing to be my advisor. I owe a debt of gratitude to my co-advisor Dr. Ayça Arslan Ergül for giving me the opportunity to work together. I am also grateful for the free-thinking environment, her support, generosity, kindness and patience she provided throughout my master's journey. I am happy to be one of the students who grew up confident thanks to the opportunities she offered. I would also like to express my sincere thanks to Dr. Bahar Değirmenci Uzun and Prof. Dr. Çağdaş Devrim Son for their time, consideration and accepting to take part in my thesis defense jury. I would like to acknowledge the help and support of Değirmenci, İnci, Adams and Cevher Lab members.

I would like to thank Elif Akış and Elif Sena Temirci, with whom I shared my master's adventure, for their valuable friendship which I feel lucky to have, support and everything they taught me. I am very grateful to them for making this journey easier. I would especially like to thank Ceylin Çetinkaya for her support in this thesis and friendship, Ayşe Reyhan Kutan for always making me smile, and all the other valuable members of Arslan-Ergül Lab, whose encouragement I always feel. Thanks should also go to İlke Sarı, Eylül Gülşen Yılmaz, Umut Tank and all my other friends whose names I can't mention, for their friendship, support and the help they provided. I am extremely grateful to have Berfin and Yunus in Ankara. They made Ankara my home. Lastly, I would like to express my deepest appreciation to my family and especially my sister for their unwavering support.

Lastly, I would like to thank the TÜBİTAK (Scientific and Technological Research Council of Türkiye) for supporting me throughout my master's studies and for considering me worthy of BİDEB 2210/E scholarship. The experiments carried out in this thesis were funded by TUSEB (Health Institutes of Türkiye) 2022-ACİL-01 grant (no: 22417).

Content

ABSTRACT	iii
ÖZET.....	v
Acknowledgements.....	viii
List of Figures.....	xii
List of Tables	xv
Abbreviations	xvi
1. Introduction.....	1
1.1 Overview of glioblastoma	1
1.1.1 Molecular and cellular characteristics.....	1
1.1.2 Diagnosis.....	2
1.1.3 Etiology, Incidence and Mortality	3
1.1.4 Current treatment strategies and challenges	4
1.2 Cellular Senescence	6
1.2.1 Mechanisms and pathways	6
1.2.2 Senescence markers	7
1.2.3 Senescence in cancer and stem cells: Double Edged Sword	8
1.3 Activated Leukocyte Cell Adhesion Molecule - ALCAM.....	9
1.3.1 Structure, cellular localization and tissue distribution	9
1.3.2 Signaling involving ALCAM, role in cell adhesion, development and cancer.....	10
1.3.3 Soluble form of ALCAM.....	12
1.4 Literature review for ALCAM's role in glioblastoma senescence.....	13
1.4.1 Senescence in glioblastoma	13
1.4.2 ALCAM's role in glioblastoma.....	14
1.4.3 ALCAM's role in senescence	15
1.5 Objectives and Hypotheses	17
2. Materials & Methods.....	18
2.1 Materials.....	18
2.1.1 Cell Lines	18
2.1.2 Cell Culture Materials, Biologic Samples, Chemicals, Reagents and Buffers.....	18
2.1.3 Plasmids	21

2.1.4 Software	21
2.1.5 Enzymes and Master Mixes	21
2.1.6 Primers.....	22
2.1.7 Kits	23
2.1.8 siRNAs	24
2.1.9 Antibodies.....	24
2.1.10 Equipment and Tools	25
2.2 Methods	26
2.2.1 Buffer - Solution preparation	26
2.2.2 Cloning of mammalian ALCAM overexpression plasmid.....	29
2.2.2.1 Amplification of ALCAM sequences	29
2.2.2.2 Double digestion of empty plasmid and PCR products.....	30
2.2.2.3 Construction of final plasmids	31
2.2.2.4 Transformation of ligation products	31
2.2.2.5 Verification of constructed plasmids with colony PCR.....	32
2.2.2.6 Verification of sequence of the constructed plasmids with NGS.	33
2.2.3 ALCAM gene manipulation with siRNA and plasmid transfection	33
2.2.3.1 Cell culture maintenance.....	33
2.2.3.2 Transfection of overexpression plasmids	34
2.2.3.3 Transfection of siRNAs.....	35
2.2.4 Validation of the alterations in ALCAM gene expression	36
2.2.4.1 Measuring ALCAM transcript levels.....	36
2.2.4.2 Checking ALCAM protein levels with immunoblotting.....	37
2.2.4.3 Investigation of ALCAM protein level with immunocytochemistry.....	39
2.2.5 Examining the effect of ALCAM level difference on senescence markers.....	40
2.2.5.1 qRT-PCR for senescence markers.....	40
2.2.5.2 Senescence Associated- β -galactosidase staining.....	41
2.3 Statistical Analysis.....	42

2.3.1 qRT-PCR analysis	42
2.3.2 Immunoblotting analysis.....	43
2.3.3 Immunofluorescence analysis	43
2.3.4 SA β -gal staining analysis.....	44
3. Results	45
3.1 Verification of ALCAM plasmids	45
3.2 Validation of ALCAM gene manipulation in glioblastoma cells	46
3.2.1 ALCAM gene expression changes upon the transfection	46
3.2.2 Changes in ALCAM protein level	52
3.2.2.1 Immunoblotting.....	52
3.2.2.2 Immunofluorescence	57
3.3 Investigation of senescence markers upon the manipulation of ALCAM expression.....	65
3.3.1 qRT-PCR for senescence markers	65
4. Discussion	83
5. Conclusion & Future Aspects	91
Bibliography	92
Appendix.....	101

List of Figures

Figure 3.1. Next Generation Sequencing results	45
Figure 3.2. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in A172 cell line.....	47
Figure 3.3. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in A172 cell line.	47
Figure 3.4. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in LN229 cell line.....	48
Figure 3.5. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in LN229 cell line	49
Figure 3.6. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in T98G cell line.....	49
Figure 3.7. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in T98G cell line	50
Figure 3.8. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in SW1088 cell line	51
Figure 3.9. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in SW1088 cell line.....	52
Figure 3.10. ALCAM variant 1 protein level upon plasmid transfection in A172 cell line.....	53
Figure 3.11. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in A172 cell line.....	53
Figure 3.12. ALCAM variant 1 protein level upon plasmid transfection in LN229 cell line	54
Figure 3.13. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in LN229 cell line.....	54
Figure 3.14. ALCAM variant 1 protein level upon plasmid transfection in T98G cell line	55
Figure 3.15. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in T98G cell line.....	55
Figure 3.16. ALCAM variant 1 protein level upon plasmid transfection in SW1088 cell line	56
Figure 3.17. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in SW1088 cell line.	56

Figure 3.18. Immunofluorescence assay to detect ALCAM signal in A172 cell line transfected with overexpression plasmids	58
Figure 3.19. Immunofluorescence assay to detect ALCAM signal in A172 cell line transfected with ALCAM siRNA.....	59
Figure 3.20. Immunofluorescence assay to detect ALCAM signal in LN229 cell line transfected with overexpression plasmids	60
Figure 3.21. Immunofluorescence assay to detect ALCAM signal in LN229 cell line transfected with ALCAM siRNA.....	61
Figure 3.22. Immunofluorescence assay to detect ALCAM signal in T98G cell line transfected with overexpression plasmids	62
Figure 3.23. Immunofluorescence assay to detect ALCAM signal in T98G cell line transfected with ALCAM siRNA.....	63
Figure 3.24. Immunofluorescence assay to detect ALCAM signal in SW1088 cell line transfected with overexpression plasmids.....	64
Figure 3.25. Immunofluorescence assay to detect ALCAM signal in SW1088 cell line transfected with ALCAM siRNA.....	65
Figure 3.26. Expression changes of senescence markers upon overexpression plasmids' transfection in A172 cell line	66
Figure 3.27. Expression changes of senescence markers in A172 cell line transfected with ALCAM siRNA.....	67
Figure 3.28. Expression changes of senescence markers upon overexpression plasmids' transfection in LN229 cell line	67
Figure 3.29. Expression changes of senescence markers in LN229 cell line transfected with ALCAM siRNA.....	68
Figure 3.30. Expression changes of senescence markers upon overexpression plasmids' transfection in T98G cell line	69
Figure 3.31. Expression changes of senescence markers in T98G cell line transfected with ALCAM siRNA.....	69
Figure 3.32. Expression changes of senescence markers upon overexpression plasmids' transfection in SW1088 cell line.....	70
Figure 3.33. Expression changes of senescence markers in SW1088 cell line transfected with ALCAM siRNA.....	71
Figure 3.34. SA- β -gal staining in A172 cell line upon doxorubicin treatment	72
Figure 3.35. SA- β -gal staining in A172 cell line upon plasmid transfection ..	73
Figure 3.36. SA- β -gal staining in A172 cell line upon siRNA transfection.....	74
Figure 3.37. SA- β -gal staining in LN229 cell line upon temozolomide treatment.....	75

Figure 3.38. SA- β -gal staining in LN229 cell line upon plasmid transfection	76
Figure 3.39. SA- β -gal staining in LN229 cell line upon siRNA transfection..	76
Figure 3.40. SA- β -gal staining in T98G cell line upon temozolomide treatment	77
Figure 3.41. SA- β -gal staining in T98G cell line upon plasmid transfection .	78
Figure 3.42. SA- β -gal staining in T98G cell line upon siRNA transfection...	79
Figure 3.43. SA- β -gal staining in SW1088 cell line upon azacytidine/vidaza treatment.	80
Figure 3.44. SA- β -gal staining in SW1088 cell line upon plasmid transfection	81
Figure 3.45. SA- β -gal staining in SW1088 cell line upon siRNA transfection	82



List of Tables

Table 2.1 List of cell lines used in this study	18
Table 2.2 List of cell culture materials, samples, chemicals and buffers.	18
Table 2.3 Plasmid list	21
Table 2.4 Programs used in this study.....	21
Table 2.5 List of enzymes and master mixes	21
Table 2.6 List of primers.....	22
Table 2.7 List of kits used in this study	23
Table 2.8 List of siRNAs	24
Table 2.9 List of antibodies.....	24
Table 2.10 List of equipment and tools.....	25
Table 2.11 Buffer, solution and medium contents.....	26
Table 2.12 Phusion PCR reaction mixture	30
Table 2.13 Phusion PCR reaction protocol	30
Table 2.14 Colony PCR reaction mix	32
Table 2.15 Colony PCR reaction protocol.....	32
Table 2.16 cDNA conversion protocol.....	36
Table 2.17 GoTaq qRT-PCR mixture for ALCAM variants and GAPDH primers.	37
Table 2.18 GoTaq qRT-PCR protocol.....	37
Table 2.19 GoTaq qRT-PCR mixture for senescence markers.....	41

Abbreviations

AD: Alzheimer's Disease

ADAM17: A Disintegrin and Metalloprotease 17

ALCAM V1: Plasmid with ALCAM Variant 1 sequence

ALCAM V4: Plasmid with ALCAM Variant 4 sequence

ALCAM: Activated Cell Adhesion Molecule

APS: Ammonium Persulfate

BCA: Bicinchoninic Acid

BCL: B-Cell Lymphoma

BSA: Bovine Serum Albumin

CDK: Cyclin-Dependent Kinase

CDKN: Cyclin Dependent Kinase Inhibitor

cDNA: Complementary DNA

CSF: Cerebrospinal Fluid

CYR61: Cysteine-rich angiogenic inducer 61

DAPI: 4',6-Diamidino-2-Phenylindole

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic Acid

EGFR: Epidermal Growth Factor Receptor

FBS: Fetal Bovine Serum

FGF-2: Fibroblast Growth Factor 2

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GBM: Glioblastoma

GFP: Green Fluorescent Protein

GICs: Glioblastoma Initiating Cells

GSC: Glioblastoma Stem Cell

HCl: Hydrochloric Acid

HRP: Horseradish Peroxidase

IDH: Isocitrate Dehydrogenase

KO: Knock out

L1CAM: L1 neuronal Cell Adhesion Molecule

LB: Luria-Bertani

LMNB1: Lamin B1

LT-HSCs: Long-Term Hematopoietic Stem Cells

mAb: Monoclonal Antibody

MCS: Multiple Cloning Site

MGMT: O-6-methylguanine-DNA methyltransferase

MMP: Matrix Metalloproteinases

MRI: Magnetic Resonance Imaging

mRNA: Messenger RNA

mTOR: Mechanistic Target of Rapamycin Kinase

NaCl: Sodium Chloride

NEAA: Non-Essential Amino Acids

NF- κ B: Nuclear Factor kappa B

NGS: Next Generation Sequencing

P/S: Penicillin-Streptomycin

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

PTEN: Phosphatase Tensin Homolog

PVDF: Polyvinylidene difluoride

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

RAGE: Receptor for Advanced Glycation Endproducts

Rb: Retinoblastoma

RIPA: Radio-Immunoprecipitation Assay

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SASP: Senescence-Associated Secretory Phenotype

SA- β -gal: Senescence-associated β -galactosidase

SDS: Sodium Dodecyl Sulfate

siRNA: Small Interfering RNA

ST6GAL1: β -Galactoside α 2,6-sialyltransferase

TAE: Tris-Acetate-EDTA

TBST: Tris-Buffered Saline with Tween 20

TEMED: N,N,N',N'-Tetramethylethylenediamine

TGF- β : Transforming Growth Factor Beta

TMZ: Temozolomide

TNF- α : Tumor Necrosis Factor Alpha

Chapter 1

1. Introduction

1.1 Overview of glioblastoma

Glioblastoma (GBM) is a type of glioma that is the most common brain tumor in the Central Nervous System (CNS). They are classified into 3 groups according to their origins: Oligodendrogliomas, Ependymomas, and Astrocytomas [1]. While Oligodendrogliomas derive from oligodendrocytes, Ependymomas stem from ependymal cells lining the ventricular system in the brain. The last group, Astrocytomas, originate from astrocytes which are particular glial cells supporting the nerves and outnumbering the neurons [2]. The World Health Organization (WHO) classifies astrocytoma according to some features such as atypia, mitosis, endothelial proliferation, and necrosis. Based on this classification, the highest-grade astrocytoma (grade IV) is called GBM. It is a highly aggressive and invasive brain cancer.

1.1.1 Molecular and cellular characteristics

GBM is characterized by a complex pool of genetic alterations and mutations. Loss of function of the tumor suppressor protein p53 is encountered mostly in primary GBM. Deletions or mutations in the Phosphatase and Tensin Homolog (PTEN) gene cause the activation of the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/ Mechanistic Target Of Rapamycin Kinase (mTOR) pathway, promoting cell survival and growth. Another common genetic alteration that results in a constitutively active form that enhances proliferation is seen Epidermal Growth Factor Receptor (EGFR) gene. Loss of Heterozygosity (LOH) on 10q loss constitutes 60% of the GBM and p16 deletion frequency is around 31% [3]. Additionally, mutations in isocitrate dehydrogenase (IDH) 1/2 are more common

in secondary GBMs and are associated with a better prognosis and a distinct metabolic profile [4].

In addition to genetic alterations, epigenetic changes may play a remarkable role in GBM. One common change is the methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter, which silences the DNA repair enzyme MGMT and is associated with a better response to chemotherapy with alkylating agents like TMZ [5]. Additionally, like in every cancer type, aberrant histone modifications, including changes in histone acetylation and methylation patterns, contribute to altered gene expression and chromatin remodeling in GBM cells. These epigenetic modifications influence the behavior and treatment response of GBM [6].

GBM cells show highly invasive behavior, infiltrating into the surrounding brain parenchyma [7]. Key factors contributing to this invasion include matrix metalloproteinases (MMPs), which degrade extracellular matrix components, mediating and facilitating the cell invasion [8]–[10]. Additionally, cell adhesion molecules (CAMs), such as integrins and cadherins, mediate interactions with the extracellular matrix and other cells, further promoting the invasive capabilities of GBM cells [11]–[13].

Another cellular characteristic is that GBM is highly heterogeneous and has subpopulations. One of these subpopulations is known as glioma stem cells (GSCs), exhibit stem cell-like properties, including self-renewal and differentiation potential. These GSCs contribute to tumor heterogeneity, therapy resistance, and recurrence, making them a critical factor in the aggressive nature and poor prognosis of GBM [14].

1.1.2 Diagnosis

Symptoms making clinicians suspicious about GBM are the altered general status of the patient. Epilepsy, intracranial hypertension, motor deficit, loss of body weight and condition, confusion, and difficulty in vision and speech are the clinical features of patients with GBM. After the onset of these presentations, clinicians are to be certain about the status of the person. In line with this purpose, they can do tissue biopsy or use imaging techniques such as magnetic resonance imaging (MRI) and/or computed tomography (CT). The former is a gold standard and usually in a

typical MRI image, infiltrative lesions which have enhanced contrast at the margins indicating blood-brain barrier disruption are seen in the frontal lobe [15]. In addition to diagnosis, with advanced imaging techniques such as perfusion and permeability imaging, it is also possible to determine the grade of the tumor.

The next step is to check molecular markers because for the following treatments, it is critical to distinguish GBM from high-grade astrocytoma. While astrocytomas have mutations in IDH1 or IDH2 gene, this is not the case for GBM. Moreover, Oligodendrocyte Transcription Factor 2 (OLIG2) expression is less in GBM than in astrocytoma. In addition to IDH mutations, MGMT promoter methylation, EGFRvIII amplification, and 1p19q deletion can be counted as predictive biomarkers. However, the heterogeneity of GBM still poses problems[16], [17].

1.1.3 Etiology, Incidence and Mortality

The etiology of GBM has remained elusive. However, there can be several factors increasing to get GBM such as genetic factors and exposure to certain chemicals, carcinogens, and ionizing radiation. These factors can have influence on progression of the disease also.

Since some genetic syndromes such as Li-Fraumeni syndrome, Turcot syndrome, and Lynch syndrome includes genetic alterations in Tumor Protein 53 (TP53), PTEN, and MutS Homolog 2 (MSH2), having them may increase the risk of developing GBM [18]. Independently from having such syndromes, if one has genetic alterations mentioned above again the risk of having GBM increases. These mutations contribute to tumorigenesis by altering cell cycle regulation, promoting cell survival and proliferation, and disrupting cellular metabolism [19].

Environmental and lifestyle factors also play a role in GBM development. Patients who have received radiation therapy for other cancers, particularly in the head and neck region, being at increased risk of developing gliomas. Although there are strong evidence for ionizing radiation as a risk factor, for quantification of the risks there is a need for a more comprehensive studies [20]. In addition to exposure to ionizing radiation, parents being exposed to certain chemicals, such as pesticides, solvents, and polycyclic aromatic hydrocarbons, enable their children to have increased risk of glioma development in the childhood [21].

With its complex etiology which needs more investigation, GBM is the most common primary brain tumor according to the statistical studies held in different countries-regions. It constitutes almost 15% of all brain tumors while the percentage is 45% among the other malignant tumors [22]. When it comes to global incidence of GBM, the average incidence calculated including 6 countries incidence rates is 2,3 per 100,000 person-years [23]. One of the most important factors that enabled us to see these rates has been age. It increases with age. While primary GBM is diagnosed around 65-75 years, secondary GBM which is easier to be handled shows itself around 40 years old patients. In addition to age factor, sex also determines the incidences because male-to-female ratio of approximately 1.6:1[23], [24]. Geography can have influence on the incidence since the incidence in rural areas is less than in the urban counties [22]. Naturally, the underlying reason can be the opportunity to reach healthcare services.

GBM is also known for its poor prognosis. The lifespan following the diagnosis is around 14 months [25]. Several factors influence the prognosis of GBM patients, including age, performance status, extent of surgical resection, molecular markers (e.g., MGMT promoter methylation, IDH1/2 mutation status), and treatment protocols [25]. The primary reasons why GBM is that much lethal are both the invasive nature of GBM which causes deterioration in whole brain and the heterogeneity of the tumor which enable tumor cells to escape from the treatments [26].

GBM remains one of the most challenging cancer types to be treated due to its complex etiology, high incidence, and poor prognosis. Despite advances in understanding the molecular and cellular characteristics of GBM, mortality rates remain high. Continued research is essential to reveal the underlying mechanisms of glioma genesis, improve early detection, and develop more effective therapeutic strategies to enhance patient survival.

1.1.4 Current treatment strategies and challenges

The standard treatment protocol includes maximal surgical removal as much as possible. This is generally followed by radiotherapy and concurrent TMZ chemotherapy [26].

The removal of tumor tissue with surgery has improved a lot, thanks to brain mapping techniques. Modern imaging technologies have enabled precise instrument placement and the identification of critical brain regions to be avoided during surgery. They also determine the tumor margins which maximizes the resected area. After surgery there can be remained cell tissues in the region. Intra-operative MRI and fluorescence imaging further help locating and removing residual cancer cells [26], [27].

The reason why TMZ is most known chemotherapy drug is it can overcome the blood brain barrier which constitutes a drawback for most of the drugs. Moreover, the side effects are comparatively less. It is generally consumed with the radiation therapy. However, the efficiency of TMZ is significantly influenced by the methylation status of the MGMT gene promoter, with patients having a methylated MGMT promoter showing better responses to TMZ. Additionally, other known mechanisms such as the overexpression of EGFR, galectin-1, murine double minute 2 (Mdm2), and mutations in the p53 gene significantly contribute to the development of TMZ resistance [26]–[28]. In addition to TMZ, combinatorial therapy of Vincristine, Procarbazine, and Lomustine, Carmustine, and Methotrexate were used for the treatment of GBM [27].

GBM cells that cannot be surgically removed are targeted with radiation therapy including X-ray photons, gamma photons, and protons. In addition to the increase in damage load in DNA, there is evidence suggesting that radiotherapy can cause reactivation of immune system also [27]. Brachytherapy, which involves placing radioactive sources directly into the tumor bed, is another RT method but comes with significant side effects [29].

As mentioned above, one of the main challenges is the heterogeneity of GBM both inter and intra-tumoral manner. The reproducibility of the results of a treatment method is low. Moreover, glioblastoma initiating cells (GICs), stem cells, are present in the tumor tissue. GICs, identified by markers like CD133, contribute to the recurrence and tumor growth since they have high self-renewal and proliferation capacity. As in other cancer types, blood brain barrier causes drug not to reach to the brain. However, in GBM, the barrier is disrupted and GBM cells can infiltrate very easily and can reach to other regions in the brain. Unfortunately, Blood–Brain Tumor Barrier is formed around the tumor, which prevents drugs from doing their job. In addition, these challenges, the tumor microenvironment with hypoxia and

autophagy makes GBM treatment more complicated. Tumor hypoxia promotes GIC development and activates pathways that enhance therapeutic resistance. Not only in GBM but also in other cancer types, hypoxia-inducible factor 1 (HIF-1) is produced and it has tumorigenic activity under low oxygen conditions, contributing to a poor prognosis. Autophagy also helps GBM cells survive by removing damaged proteins and maintaining cellular homeostasis, thereby again increasing resistance to therapies. Lastly, high DNA repair capacity, such as those mediated by MGMT and Mismatch Repair (MMR), enables GBM cells to repair damage caused by alkylating agents like TMZ [26], [27].

1.2 Cellular Senescence

Senescence is a state where cells cannot proliferate and grow further but stay metabolically active. The term was used first in 1960s [30]. Although quiescence state has the same type of features but irreversibility on top of that, we will use senescence notion for this type of cell cycle arrest also.

1.2.1 Mechanisms and pathways

Cellular senescence has a complex and multilayered set of reasons. It is kind of a cellular stress response. DNA damage or alterations in DNA are one of these reasons. In each replication, the telomers at the end of each chromosome get shorter. When telomerase enzyme is not active, it cannot compensate for this shortening and cell stops proliferating eventually. In addition to telomere shortening, some environmental sources cause DNA damage such as double strand break, single strand breaks. These damages cause the activation of DNA repair proteins such as ATM and ATR. As a result, tumor suppressor p53 protein gets activated. Activated p53 induces the transcription of p21, a cyclin-dependent kinase (CDK) inhibitor that enforces cell cycle arrest [31]–[34].

Epigenetic changes, including the formation of senescence-associated heterochromatin foci (SAHF), and alterations in chromatin organization, also contribute cells to become senescent cells. Generally, these epigenetic changes are mediated by induction of p16^{INK4a}. It prevents CDK4 and CDK6 from forming a complex, preventing phosphorylation of the retinoblastoma (Rb) protein and cause cell cycle arrest [34].

Senescent cells can also keep the senescent state by autocrine and paracrine signaling. Therefore, the secretion profile of a senescent cell, known as Senescence-Associated Secretory Phenotype (SASP), can reinforce the senescent state. Pro-inflammatory cytokines, chemokines, growth factors, and proteases, all modulate the tissue microenvironment. They are regulated by signaling pathways such as Nuclear Factor Kappa B (NF- κ B) and CCAAT/Enhancer-Binding Protein Beta (C/EBP β) [31], [34].

Additionally, lack of nutrition or other stress factors can hyperactivate rapamycin-mTOR pathway. It has been also shown that inhibition of mTOR by rapamycin causes dysregulation in cytokine production, so SASP. Strong p53 response also cause mTOR inhibition. Since mTOR is responsible for growth, cell cannot grow anymore as result [32], [34].

Lastly, mitochondrial dysfunction and increased production of reactive oxygen species (ROS) are common features of senescent cells and they maintain the senescent state. Therefore, there is a positive feedback loop. Increase in disfunctioning mitochondria and ROS cause DNA damage which at the end cause senescent cells with the pathway mentioned above. Additionally, ROS can modulate SASP, promoting inflammation and reinforcing senescence [33]–[35].

1.2.2 Senescence markers

Senescence-associated β -galactosidase (SA- β -gal) is the most used and a must-have senescence marker. It is based on the enzyme which is inactive in non-senescent cells but active in senescent cells due to increased lysosomal content, since it is present in lysosomes. The presence of SA- β -gal is checked by using a histochemical staining technique, providing a convenient and reliable method for senescence detection. In this chemical assay, X-gal is used as a substrate of β -galactosidase. X-gal is cleaved and at pH 6, this gives the blue color [36], [37].

p16 is encoded by CDKN2A gene which is in INK4A locus. It is a tumor suppressor protein which prevents CDK4 and CDK6 proteins from forming complex which is a check point for cell cycle to continue. Rb protein cannot detach from the E2F since it is not phosphorylated, and cell cycle is arrested. p16 is highly expressed in senescent cells. p16 expression can be induced by various stress

signals, including oxidative stress and DNA damage, reinforcing its role in senescence induction [38], [39].

p21 is also a CDK inhibitor that targets CDK2, which causes cell cycle arrest preventing the progression from G1 to S phase. When tumor suppressor protein p53 is phosphorylated, its level is elevated, which does not leave the E2F free to bind to DNA. The pathway is activated upon DNA damage and other stressors. Detection of p21 is commonly performed using techniques such as western blotting, immunofluorescence, or quantitative Polymerase Chain Reaction (PCR) [38], [40].

Ki67 is known for being a proliferation marker. Its expression is found in cells that have an active cell cycle. It is a negative senescence marker whose expression get elevated during transition from G2 to M phase. The absence of Ki67 in a cell population, combined with the presence of other senescence markers, supports the identification of senescence [41], [42].

Lamin B1 (LMNB1) is a key component of the nuclear lamina, a structure that supports nuclear stability and regulates DNA replication and cell division. During senescence, levels of LMNB1 significantly decrease, which can be detected by techniques such as western blotting or immunofluorescence. The loss of LMNB1 is associated with changes in nuclear shape and function, contributing to anormal nuclear morphology in senescent cells [43].

Cysteine-rich angiogenic inducer 61 (CYR61/CCN1) is associated with migration, adhesion and differentiation behavior in the cell. It is a matricellular protein whose expression is upregulated in senescent cells as part of the SASP. It can even induce senescence via integrin $\alpha 6 \beta 1$ and heparan sulfate proteoglycans in fibroblasts [44] One study suggests that it occurs through p21/p53 proteins [45]. In addition, Kumar et al. (2017) and Xu et al. (2022) observed that CYR61 increases with cell aging.

1.2.3 Senescence in cancer and stem cells: Double Edged Sword

Cellular senescence induction can be considered as a treatment method for tumor. The growth arrest of tumor by induced senescence can even stop migration of the tumor. However, recent studies demonstrate that senescence can be both beneficial or harmful in cancer prognosis [46]. Even these two different outcomes can coexist at the same time.

Senescence can be induced by chemotherapy or by other known treatment methods. In addition to that, Reactivation of oncogene induced senescent mechanism in the cancer cell can prevent cancer evading senescence. The cell cycle arrest in cancer cells can also influence the cells in neighbor with produced SASP cytokines and cell-cell communication. Therefore, this paracrine feature of senescence signaling enable potential cancer cells, premalignant cells to be also targeted. In addition to cell cycle arrest, SASP cytokines recruits the immune cells to the cancer region such as macrophages [46]–[48].

In addition to chemotherapies and know treatments, senolytic drugs are also used as a opposite method. These senolytic drugs aim to reduce the inflammation profile of tumor because senescence induction can create a pro-tumorigenic environment also. SASP which is useful to attract immune cells can suppress the function of these immune cells. It can remodel the tissue, cause stemness, angiogenesis, the increase in migration. Moreover, since the senescent cancer cell is resistant to apoptosis, when it re-enter to the cell cycle, it becomes harder to kill these cells [46]–[48].

1.3 Activated Leukocyte Cell Adhesion Molecule - ALCAM

Activated Leukocyte Cell Adhesion Molecule (ALCAM), also known as CD166 or MEMD, is a transmembrane protein from the immunoglobulin family. It is mostly known for its ability to bind CD6 protein on T cells [49]. The reason of focusing on ALCAM protein is the fact that it is the homolog of alcamb protein in zebrafish [50], of which Erbabba et.al. showed a decrease in the expression in aged zebrafish brains.

1.3.1 Structure, cellular localization and tissue distribution

ALCAM gene is in 3q13.11 location in genome [51]. When one checked the Ensembl website, it is shown that it encodes 4 different ALCAM proteins of which length varies from 583 amino acids (aa) (longest, variant 1) to 344 aa (shortest, variant 4) [52]. Although the variant 1 protein is a transmembrane protein which has 5 immunoglobulin subdomains which are 2 membrane-distal variable and 3 membrane-proximal constant types [50], [53]. While the N-terminal site is

responsible for binding, constant domains enable stabilization of these interactions [54], [55]. Amino acids between 528-549 aa constitute the transmembrane helix structure and the rest of the protein (from 550-583 aa) domain is cytoplasmic and disordered part [56]. It is responsible for the signaling and interaction with cytoskeleton, moreover it offers a docking site for other proteins [57]. Uniprot website shows that the extracellular part of the protein has 9 glycosylation part [56]. When all sites were glycosylated, the protein molecular weight which is calculated as 65 kDa becomes 105 kDa [58].

When Uniprot, Human Protein Atlas and National Center for Biotechnology Information websites for ALCAM were investigated it was seen that brain and lung are the most ALCAM expressing organs, both in messenger RNA (mRNA) and protein level. It is expressed in variety of cell types such as fibroblasts, neurons, pancreatic cells, bone marrow etc. [50]. While ALCAM variant 4 – the soluble form can be seen anywhere inside or outside of the cell, ALCAM variant 1 protein is located in cell membrane. However, it is also possible to see variant 1 inside of the cells thanks to the endomembrane system. It is recycled through endocytosis and can be seen in endosomes [57]. Moreover, the cytoplasmic vs membranous ALCAM ratio can have influence on the cell behavior [59].

The structure of ALCAM allows it to participate in various physiological processes, including immune response, tissue architecture, and cell migration. Distorted expression of ALCAM or disrupted function can be associated with several pathological conditions including developmental disorders, cancer severity, metastasis and prognosis.

1.3.2 Signaling involving ALCAM, role in cell adhesion, development and cancer

It was shown that homophilic interaction of ALCAM is disrupted by usage of cytoskeleton inhibitors [60], which shows there is a connection between actin and ALCAM. In 2014, Tudor and his colleagues found that this connection is mediated by Syntenin-1 and Ezrin proteins. Unfortunately, there is not much reference showing physical interaction through cytoplasmic region of ALCAM yet (there is no known binding motif in cytoplasmic region [53]), except the role in cell matrix. This role in cell matrix affects the cell migration, tumor metastasis, infiltration of the cell through endothelial cells. Although it is not physical, scientist found that β -

Catenin-independent Wnt signaling and Fz3 regulates the Alcam gene expression, probably through the Pax2 and Activating Transcription Factor 2 (ATF2) sites in the ALCAM promoter [61]. In addition to that, a model in which hepatoma-derived growth factor (HDGF) can directly bind to the promoter and cause decrease in expression was proposed to account for ALCAM's role in metastasis (opposing other references). They suggest ALCAM can bind and inhibit the activation of Rho-GTPases Rac1/Cdc42 [62]. In a study it was shown that ALCAM resembles Receptor for Advanced Glycation Endproducts (RAGE) protein. When RAGE is not present, ALCAM can bind to S100B protein and NF- κ B pathway can be activated, which at the end can induce cytokine production and increase migration and proliferation [63], [64].

All these potential signaling result in changes in adhesion behavior through different ways. Same or different cell types can bind to each other by ALCAM-ALCAM binding, which is mediating the tissue integrity and communication between cells. This process is especially essential for immune system to work properly, it helps leukocytes move towards to the inflammation site [53]. CD6 binds to ALCAM in heterophilic interaction [49]. In addition to CD6-ALCAM heterophilic interaction, ALCAM can bind to L1 neuronal cell adhesion molecule (L1CAM), Chl1, and Semaphorins in another cell. This heterophilic interactions are seen to contribute to axonal growth and guidance, emphasizing its multifunctional role in neuronal development [65].

Not only neuronal development but ALCAM have also role in proliferation and other cell type developments. A study showing ALCAM relationship with proliferation demonstrated that Islet-1 positive (ISL1+) cardiac progenitors isolated from embryonic stem cells have ALCAM+ cell population. Genes responsible for proliferation were enriched in these cells [66] Another study shows the role of ALCAM in hematopoietic stem and progenitor cell (HSPC) development [67].

The signaling functions of ALCAM also contribute to its role in cancer. scFv173 antibody was shown as an antibody inhibiting CD166. Usage of this antibody cause inhibition in breast cancer cell invasion in vitro. When the cells treated with this antibody were injected to mouse, the growth of tumor was less in comparison to control group [68]. In 2014, scientists showed that a MMP protein, A Disintegrin and Metalloprotease 17 (ADAM17), cleaves ALCAM as a result of Transforming Growth Factor Beta (TGF- β) signaling in prostate cancer. They demonstrated that

ALCAM shedding is increased in tumor over time. When they decreased the ALCAM level in PC3 cells, these cells could not form metastasis in bone and showed diminished level of proliferation marker, ki67 [69]. When ALCAM was diminished with shALCAM in breast cancer, migration of cancer cells was reduced significantly. Moreover, the tumor volume was reduced even in vivo xenografts. In this study, authors also emphasized on the interaction between Galectin-8 and ALCAM [70]. When the ALCAM was depleted in uterine tumor, it causes a remarkable decrease in tumor size, like migration was attenuated [71]. Lastly, in invasive human pituitary adenomas, miR-148b-3p and miR-152 levels were lower than noninvasive ones. The overexpression of these miRNAs diminished both the proliferation and invasiveness. Since these miRNAs could target ALCAM, ALCAM level was decreased to test if this will show the same effect and in response to that, the proliferation and invasiveness decreased. Unlike downregulation, overexpressed ALCAM induced these features and make the cancer more aggressive [72]. CD166+ pancreas cells formed colonies more efficiently than the CD166- ones. Furthermore, when these cells were injected to the mouse models, the tumor growth of CD166+ pancreas cells were significantly elevated [73].

1.3.3 Soluble form of ALCAM

The reason why we address soluble form of ALCAM separately is it has different roles than the variant 1. Unfortunately, in the literature, some call sALCAM as cleaved part of ALCAM while others consider it as produced by alternative splicing. The latter was first identified in 2004 [74]. It is produced in cells by alternative splicing. It has the first 3 exons common with other ALCAM variants, which are elongated by the 3-4 intron sequence. It corresponds to the first N terminal variable domain. What Ikeda and his colleagues found was the soluble form of ALCAM (sALCAM) increased upon Tumor Necrosis Factor Alpha (TNF- α) treatment, while the variant 1 level did not change. They suggest that sALCAM may have role in inflammation.

In diabetic patients, the ALCAM levels were investigated. sALCAM level was higher in glomeruli and tubules in the kidneys of patients than control groups'. They also found that TGF- β expression is increased by S100B and this increase is associated with ALCAM [75]. Hansen et al., 2014 suggest that the shedding of ALCAM is induced by TGF- β signaling. There can be a positive feedback loop

here. In another study about diabetes, scientist suggest that the decreased shedding of sALCAM can cause deterioration in nephropathy [76]. In breast cancer disease-free survival is associated with high sALCAM [77]. It is even suggested as a marker for severe breast cancer [78]. In melanoma, sALCAM inhibits the formation of ALCAM-ALCAM clusters. It cause also disruption in migration, invasion, MMP2 and L1CAM roles [79]. Retinol epithelium was investigated and it was seen that the sALCAM and ALCAM levels were elevated upon the TNF- α stimulation [80]. Lastly in gastrointestinal cancer, sALCAM was seen to decrease the adhesiveness through the inhibition of SRC kinase which mediates the ALCAM cytoplasmic tail and actin link through ezrin-moesin-radixin [81].

1.4 Literature review for ALCAM's role in glioblastoma senescence

1.4.1 Senescence in glioblastoma

GBM cells can escape senescence thanks to increased telomerase expression [82]. Senescence, like in the other cancer types, can be used as both weapon or can cause deterioration in the progress of astrocytoma [83]. The former case stops the proliferation of cancer cells and prevents them from migration through body while in the latter case, cancer cells can escape from the senescence and reoccur with more aggressive behavior [84].

Known treatments can cause senescence induction in GBM. Chemotherapy ends mostly with accumulation of senescent cells [85]. In 2022, scientist investigated the effect of TMZ treatment on LN229 and A172 cells. Their aim was to find out the reason why TMZ cannot cause apoptosis in GBM cells efficiently. They discovered that TMZ cause senescence induction cells and this is the main pathway of TMZ since, these cells consisted almost 80% of the treated cells [86]. It is also known that this TMZ-caused senescence is dependent on the p21 protein, since senescence couldn't be induced in the cells not expressing p21 [87]. In addition to that, if p21 is required to keep the cells in senescent state or not is not known yet [88]. In 2015, scientists also find a way to increase the efficiency of TMZ in GBM cells. They downregulated the erythropoietin receptor in GBM cells and found that they further induce the senescence and combinatorial treatment is more effective than the only TMZ usage [89].

In addition to TMZ treatment, it seems there are other ways to see senescence related phenotypes in GBM. Radiotherapy, another known treatment method, elevated the senescent cell number to 80% in a cell population [90]. Tumor Treating Field approach caused senescent cell increase in with 5 days of treatment through NF- κ B activation [91]. Another interesting study examined the effect of serum in primary GBM cells (PriGO8A). Serum presence increased senescent cell phenotype in the cell culture and researchers say that, the TGF β pathway has the role in this result [92]. Senescence induction can be achieved with down regulation of inhibitors of senescence. In 2013, scientists showed that exposure to copper sulfate cause induced senescence through the downregulation of Bmi-1 in U87-MG cell line [93]. Protein kinase C ι (PKC ι) that functions in proliferation and migration was depleted and senescence markers were shown in these cells [94].

In all of these studies, we saw that there are too many ways to see induction of senescence in GBM cells or senescent cells already present in patient derived GBM tissues [95]. However, no one can be assured if this has beneficial or harmful results in terms of the fate of GBM patients. While some articles suggest that senolytic drugs can be therapeutic, others support the opposite: senescence induction is more beneficial. Another challenge is patient derived cells and commercially available cell line scan behave differently [92].

1.4.2 ALCAM's role in glioblastoma

There are studies examining ALCAM protein effect on GBM. However, one cannot say they have similar or consistent results. In a study published in 2012, the role of ALCAM protein in GBM cells was investigated, and it was emphasized that ALCAM is directly proportional to the invasive level of the tumor and that cancer patients with high ALCAM levels have a shorter post-diagnosis survival period, making it an important target [96]. In contrast to the study of Kijima et al., 2012, another study did the analysis of glioma related survival risks in The Cancer Genome Atlas (TCGA) GBM datasets. They found that ALCAM has a protective role for patients' survival [97]. In 2012, a comprehensive study investigating the ALCAM protein was carried out in patient derived glioma tissues [98]. They checked both cytoplasmic and membrane ALCAM levels and saw that both ALCAM protein staining was decreased with the increased malignancy. Additionally, they found that there was tendency towards shorter survival in ALCAM-negative GBMs. Furthermore, GBMs with the IDH1-R132H mutation,

which correlates with longer survival [99], showed elevated levels of ALCAM expression compared to those without the mutation. Conversely, all tumors that did not express ALCAM were also devoid of the IDH1-R132H mutation [98].

In the study of Kijima et al., 2012, the subset of ALCAM+CD133+ patient derived GBM cells were enriched with GBM progenitor cells. This subset had much more colony formation ability than the ALCAM-CD133+cell. Moreover, they found that the overexpression of soluble form of ALCAM in xenografts decreased the survival rate in mice transplanted. Based on this information, ALCAM levels were reduced in U87MG and U251 cells, but contrary to expectations, the cells divided more and became more aggressive and invasive [96]. They hypothesized that this may be due to the strong influence of the cancer cell's microenvironment and patient-specific mutations [100]. Then the same group published an abstract in which they said that they used shRNA targeting ALCAM in patient derived GBM cell lines and created xenografts on immunodeficient mouse [101]. They saw the cell motility and invasion in knocked-down cells was less than in the control group. The reason behind this phenotype was not investigated. Moreover, analyses such as cell counting and proliferation rate testing were not conducted, or not mentioned. Somehow, they did not publish any research article about the abstract.

GBM is a heterogeneous cancer and have subpopulations like brain tumor initiating cells (BTIC). Sajina Gc and her team found that ADAM17 level is increased significantly in the cell of which β -Galactoside α 2,6-sialyltransferase (ST6GAL1) was downregulated [102]. It adds sialic acid residues to N-glycosylated proteins like ALCAM. Since ADAM17 which has higher levels of expression in GSCs (U87MG) sheds the ALCAM [103], the ALCAM level was decreased in the ST6GAL1 knocked-down cells. As a result of the knock-down, the tumorigenic potential was decreased [102]. Brain tumor derived mesenchymal stem cells (BT-MSCs) was identified in human GBM biopsies. These cells have CD44+CD9+CD166+ markers and play important role in tumor growth [104]. While in the cultured cells, the ALCAM level is increased, it decreased again when the cells starts to form colonies [104].

1.4.3 ALCAM's role in senescence

In a study conducted by our group in 2020 [105], it was revealed that alcamb protein (the zebrafish homolog of ALCAM) levels are inversely proportional to

aging in the brain. According to this result, ALCAM expression was investigated in human brain Complementary DNAs (cDNAs) obtained from person with Alzheimer's Disease (AD) and the healthy brain. It was shown that the ALCAM level was less in AD brain. Both results belong to the whole brain, therefore there was no cell specific information about ALCAM.

Long-Term Hematopoietic Stem Cells (LT-HSCs) has high expression of ALCAM and it was seen that the level of ALCAM was upregulated with the increased age [106]. It was also shown that when ALCAM was knocked out in LT-HSCs, there was an increase in aging-related gene expression such as Selp, Clu, Cdc42, and Foxo3 [106].

In 2012 scientists investigated the ALCAM expression in the colon mucosa of 20 months and 5 months old rats. They saw that aged rats had higher ALCAM level [107]. They said the reason could be the downregulated miR-9 targeting ALCAM. Blocking EGFR with the inhibitor cetuximab prevented this age-related rise in CD166 levels [107].

In another study, fibroblast growth factor 2 (FGF-2) was investigated to check whether it can induce proliferation of osteoprogenitors obtained from even old individuals. FGF-2 seemed to promote the proliferation of ALCAM⁺ cells in cultures from aged human bone and maintained their numbers in both human and mouse cell cultures. ALCAM expression levels were similar in both young and old cell cultures within each species; however, the response to FGF-2 occurred on different timelines [108].

In addition to Erbaba et al., 2020, ALCAM was encountered in the studies handling one of the aging related diseases, AD, in different populations. Gene expression data from brain tissue show a correlation between ALCAM, ARAP1, Glypican-6 (GPC6), and RNA Binding Fox-1 Homolog 1 (RBFOX1) and brain β -amyloid load [109]. In another study the CD166 level in cerebrospinal fluid (CSF) was less in AD patients in comparison to the normal CSF [110]. In another study, 3q13.11 locus was identified as the region having the genes associated with AD and one of these genes was ALCAM [111]. ALCAM targeting miRNA was also identified in a study conducted in 2019. miR-142 was considered as a non-coding RNA associated with AD. In order to get the function of this miRNA they created knock out (KO) mouse. When the miR-142 is abolished, ALCAM was increased in

this mouse [112]. Lastly, another study showed that ALCAM level is correlated with the cognitive decline, severity of AD [113].

1.5 Objectives and Hypotheses

In this study, we aimed to manipulate both soluble and membrane protein ALCAM in astrocytoma cells 3 of which were GBM cells. We wanted to silence total ALCAM level in cells with ALCAM targeting siRNA usage. In addition to that, we constructed plasmids overexpressing both membrane and soluble form of ALCAM. Since in the literature, we saw they may have different roles, we wanted to analyze their effect separately. Our purpose was to see the senescence marker changes upon the manipulation of ALCAM levels in astrocytoma cells. Our long-term aim was to see whether we can offer a novel target/tool inducing senescence or keeping the cells in senescent state and to be used in GBM treatment so that we can have extended survival time following the diagnosis.

Although there were references both opposing and supporting our hypothesis, we hypothesized that, reduction in ALCAM level may induce senescence or cause decrease in aggressiveness of GBM while overexpression of ALCAM V1 variant may cause more aggressive behavior in GBM and V4 can decrease the proliferation rate of GBM.

Chapter 2

2. Materials & Methods

2.1 Materials

2.1.1 Cell Lines

Table 2.1 List of cell lines used in this study

Cell Line	Type	Passage Number
A172	Glioblastoma (Astrocytoma Grade IV)	<10
LN229	Glioblastoma (Astrocytoma Grade IV)	<20
T98G	Glioblastoma (Astrocytoma Grade IV)	<40
SW1088	Astrocytoma Grade III	<10

2.1.2 Cell Culture Materials, Biologic Samples, Chemicals, Reagents and Buffers

Table 2.2 List of cell culture materials, samples, chemicals and buffers.

Product	Catalog Number	Brand
Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG) with L-glutamine	MCL-002-500ML	Serana
Penicillin-Streptomycin (P/S)	L0022-100	Biowest
MEM Eagle Non-Essential Amino Acids Solution X100 (NEAA)	01-340-1A	Sartorius
Trypsin 0.25% - EDTA 0.02% in HBSS (Trypsin-EDTA)	L0932-100	Biowest

Dimethyl Sulfoxide (DMSO)	F-515	Thermo Fisher Scientific
Fetal Bovine Serum (FBS)	S-FBSP-EU-015	Serana
Dulbecco's Phosphate-buffered saline (DPBS)	D8537-500ML	Sigma-Aldrich
Trypan Blue solution	T8154-100ML	Sigma-Aldrich
Triton X-100	X100-100ML	Sigma-Aldrich
CutSmart Buffer	B7204S	New England Biolabs (NEB)
10X T4 DNA Ligase Buffer	B69	Thermo Scientific
Healthy Brain RNA-cDNA	R1234035-P	Biochain
JM109 <i>E. coli</i> Bacteria	P9751	Promega
Bovine Serum Albumin (BSA)	P6155-100GR	Biowest
Tryptone	1612.00	Condalab
Yeast Extract	92144-500G-F	Sigma-Aldrich
Agar for microbiology	05039-500G	Sigma-Aldrich
Peptone	1616.00	Condalab
Sodium Chloride (NaCl)	969.033.1000-1KG	Isolab
Ethylenediaminetetraacetic acid disodium salt (EDTA disodium salt)	E5134-500G	Sigma-Aldrich
Acetic Acid	901.013.2500	Merck
PageRuler™ Prestained Protein Ladder	26616	Thermo Scientific
Ampicillin Sodium Salt	A9518-5G	Sigma-Aldrich
Kanamycin Sulfate – H ₂ O	26899.02	Serva
Calcium chloride dihydrate	12022-1KG	Sigma-Aldrich
5x DNA Loading Buffer Blue	BIO-37045	Bioline
HyperLadder 1kb	H1-617112A	Bioline
Agarose Tablets	BIO-41027	Bioline
SYBR Safe DNA gel stain	S33102	Invitrogen
Nuclease Free Water	P119E	Promega
Opti-MEM™ Reduced Serum Medium 1X	31985-062	Gibco by Life Technologies
Lipofectamine 3000	L3000-015	Invitrogen by Life Technologies

RiboEx Total RNA Isolation Solution (Trizol)	301-001	GeneAll
Ethanol 96%	920.052	Isolab
siRNA Transfection Reagent	sc-29528	Santra Cruz Biotechnology
siRNA Transfection Medium	sc-36868	Santra Cruz Biotechnology
Formaldehyde 37%	1.04002.2500	Merck
Radio-Immunoprecipitation Assay (RIPA) Buffer	39244.01	Serva
Protease Inhibitor Cocktail	05892970001	Roche
Hydrochloric acid fuming 37% (HCl)	1.00314.2500	Merck
Sodium Hydroxide Pellet (NaOH)	106498	Merck
Trizma base	T1503-1KG	Sigma-Aldrich
Glycine	G7126-1KG	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	28364	Thermo Scientific
SureCast 40% (w/v) Acrylamide – 29:1	HC2040	Invitrogen
SureCast Ammonium Persulfate (APS)	HC2005	Invitrogen
Tween 20	8.22184.0500	Merck
Protein Loading Buffer	426311	BioLegend
Milk Powder	KD4040015	Katkıdünyası
β-Mercaptoethanol	444203	Merck
N,N,N',N'-Tetramethyl ethylenediamine (TEMED)	1.10732.0100	Merck
Methanol	32213-2.5L	Sigma-Aldrich
Iso-propanol	1.09634.2511	Sigma-Aldrich
4',6-diamidino-2-phenylindihydrochloride (DAPI)	D9542-1MG	Sigma-Aldrich
Mounting Medium	ab64230	Abcam
Doxorubicin - Adriamycin	8699511796056	Saba ilaç
Azacytidine - Vidaza	8699538774815	Erkim ilaç
Temozolomide	L01AX03	Koçak Farma

2.1.3 Plasmids

Table 2.3 Plasmid list

Plasmid	Antibiotic Resistance	Tag
pcDNA3.1(+)/myc-His A	Ampicillin	6X His, myc
pEGFP-C1	Kanamycin	EGFP (N-term)

2.1.4 Software

Table 2.4 Programs used in this study

Software	Rights
Geneious	GraphPad Software, LLC
GraphPad Prism	GraphPad Software, LLC
Fiji-ImageJ	- (Free)

2.1.5 Enzymes and Master Mixes

Table 2.5 List of enzymes and master mixes

Enzyme/ Master Mix	Catalog Number	Brand
EcoRI-HF	R3101S	New England Biolabs (NEB)
HindIII-HF	R3104S	New England Biolabs (NEB)
KpnI-HF	R3142S	New England Biolabs (NEB)
GoTaq® qPCR Master Mix	A6001	Promega
iScript Reverse Transcription Supermix for RT-qPCR	1708841	Bio-rad
Phusion High-Fidelity PCR Master Mix with HF Buffer	F531L	Thermo Scientific
2X Taq DNA Polymerase Master Mix RED	A140301	Ampliqon
T4 DNA Ligase 5 Weiss U/ul	EL0011	Thermo Scientific

2.1.6 Primers

Table 2.6 List of primers

Primer Name	Sequence	Tm	Purpose	Targeted Gene/Transcript
AL_V4_V1_HindIII	ATATAT AAGCTT AGGAGGAGGAATAT GGAATCC	65	Cloning	ALCAM all transcripts flanking HindIII region
NS_V1_AL_EcoRI	ATATAT GAATTC CAACTTGGCTTCAG TTTTGTG	63	Cloning	ALCAM V1 flanking EcoRI region
NS_V4_AL_EcoRI	ATATAT GAATTC CTCCTTACTGAACA CCTTGAC	65	Cloning	ALCAM V4 flanking EcoRI region
Pcdna3.1_myc_hisa_F	CGGGACTTTCCAAA ATGTCGT	58	Colony PCR	pcDNA3.1 plasmid before (Multiple Cloning Site) MCS
Pcdna3.1_myc_hisa_R	GGCACCTTCCAGGG TCAAGG	63	Colony PCR	pcDNA3.1 plasmid after MCS
GAPDH_F	TGCACCACCAACTG CTTAGC	59	qRT-PCR	GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)
GAPDH_R	GGCATGGACTGTGG TCATGA	57	qRT-PCR	
cd166_F	ACTTGACGTACCTC AGAATCTCA	55	qRT-PCR	ALCAM all variants

cd166_R	CATCGTCGTACTGCA CACTTT	55	qRT-PCR	
V1-3_F	TCCAGAACACGATG AGGCAGAC	59	qRT-PCR	ALCAM variant 1 and 3
V1-3_R	GTAGACGACACCAG CAACAAGG	58	qRT-PCR	
V4_F	AAGTAGTCTGCAGC AGTGTCAC	57	qRT-PCR	ALCAM variant 4
V4_R	CCTGCTATAGAGACA CTACAGAGAG	56	qRT-PCR	
p21_F	TGTCTTGTACCCTTG TGCCT	56	qRT-PCR	CDKN1A
p21_R	GGCGTTTGGAGTGG TAGAAA	55	qRT-PCR	
p16_F	CCAACGCACCGAAT AGTTACG	56	qRT-PCR	CDKN2A/p1 6INK4a
p16_R	GCGCTGCCCATCATC ATG	57	qRT-PCR	
ki67_F	GAAAGAGTGGCAAC CTGCCTTC	58	qRT-PCR	MKI67
ki67_R	GCACCAAGTTTTACT ACATCTGCC	56	qRT-PCR	
cyr61_F	GGAAAAGGCAGCTC ACTGAAGC	59	qRT-PCR	CYR61/CCN 1
cyr61_R	GGAGATACCAGTTC CACAGGTC	57	qRT-PCR	
lmnb1_F	CCCAAGTGAGGCTG TATAAGGAG	57	qRT-PCR	LMNB1
lmnb1_R	TTCTTCCCTGGCACT GTTGAC	58	qRT-PCR	

2.1.7 Kits

Table 2.7 List of kits used in this study

Kit	Catalog Number	Brand
-----	----------------	-------

Quick-DNA Miniprep Plus Kit	D4068	Zymo Research
Expin Combo GP mini	112-150	GeneAll
Direct-zol RNA Miniprep	R2050	Zymo Research
ZymoPURE II Plasmid Maxiprep Kit	D4202	Zymo Research
Bicinchoninic Acid (BCA) Protein Assay Kit	P011	ABP Biosciences
WesternBright ECL	K-12045-D50	Advansta
Senescence β -Galactosidase Staining Kit	9860S	Cell Signaling Technology

2.1.8 siRNAs

Table 2.8 List of siRNAs

siRNA	Catalog Number	Brand
ALCAM siRNA	sc-43023	Santa Cruz Biotechnology
Control siRNA-A	sc-37007	Santa Cruz Biotechnology

2.1.9 Antibodies

Table 2.9 List of antibodies

Antibody Specific for	Catalog Number	Brand
ALCAM Rb mAb to CD166 [EPR2759(2)]	ab109215	Abcam
Anti-GADPH Mouse Monoclonal Antibody () [clone: FF26A/F9]	649201	Biolegend
Anti- β -Actin Mouse Monoclonal Antibody [C4]	sc-47778	Santa Cruz Biotechnology
GFP (4B10) Mouse mAb	2955	Cell Signaling Technology
Anti-Myc/c-Myc Mouse Monoclonal Antibody [9E10]	sc-40	Santa Cruz Biotechnology

Cy5 AffiniPure™ Donkey Anti-Rabbit IgG (H+L)	711-175-152	Jackson Immuno Research
Cy3 AffiniPure™ Goat Anti-Mouse IgG (H+L)	115-165-003	Jackson Immuno Research
Goat a-mouse IgG(H+L) HRP Conjugate Secondary Antibody	R-0571-500	Advansta
Goat a-rabbit IgG(H+L) HRP Conjugate Secondary Antibody	R-0572-500	Advansta

2.1.10 Equipment and Tools

Table 2.10 List of equipment and tools

Equipment	Model	Brand
CO ₂ Incubator	CCL-170B-8	ESCO
Laminar Flow	Class II Safety Cabinet	Metisafe
Hemocytometer	0640110	Marienfeld-Neubauer
Mr. Frosty Freezing Container	5100-0001	Thermo Fisher Scientific
Waterbath	Bath NB9	Nüve
Otoclave	SteamArt OT 90L	Nüve
Thermal Cycler	C1000 Thermal Cycler	Bio-rad
Shaker Incubator	Innova 44	New Brunswick Scientific
Incubator	INCU-Line	VWR
qRT-PCR Machine	CFX96 Real-Time System	Bio-rad
Nanodrop	NanoDrop 2000 Spectrophotometer	Thermo Scientific
ChemiDoc MP Imaging System	Universal Hood III	Bio-rad
Western-Blot Equipment	Mini Gel Tank, Miniblot NW2000	Invitrogen
Inverted Microscope	Axio Observer A1	Zeiss
Confocal Microscope	SP8 Lase Confocal Microscope	Leica
Inverted Imaging System	15339661	Invitrogen

Ph Meter	SevenCompact	Mettler Toledo
Shaker	VMS-C4 Advanced	VWR
Mini centrifuge	Centrifuge 5418	Eppendorf
Centrifuge	CT6E	VWR
+4 Centrifuge	CT15RE	VWR
Vortex	Pulsing Vortex Mixer	VWR
Rocker	Nutating Mixer	VWR
Power Supply	PowerPac Basic	Bio-rad
Weighing Machine	BJ410C	Precisa
Electrophoretic Gel System	MINICELL EC370M	E-C
Rattler Plating Beads	S1001	Zymo Research
Polyvinylidene difluoride - PVDF membrane	L-08024-001	Advansta
16 mm coverslip	SUP0111600	Marienfeld

2.2 Methods

2.2.1 Buffer - Solution preparation

All the buffers, solutions and mediums used in this thesis were prepared according to Table 2.11.

Table 2.11 Buffer, solution and medium contents

Buffer	Content and Preparation	Purpose
0,5 M EDTA	168,1 gram EDTA disodium salt was dissolved in 800 ml of distilled water. Then the pH was adjusted to 8.0 with HCl 37% and NaOH pellets. The final volume was completed to 1 L.	Gel Electrophoresis
10X TAE – 1X TAE	48,5 gram Tris-base 11,4 ml Acetic Acid 20 ml 0,5M EDTA	Gel Electrophoresis

	-First Tris-base was dissolved in 800 ml of distilled water and other ingredients were added. Then the final volume was completed to 1 L to make 10X TAE (stock). For 1X TAE, 900 ml of distilled water was added to each 100 ml of stock solution.	
LB liquid	5 gram yeast extract 10 gram tryptone 10 gram NaCl -These ingredients were dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes.	Cloning
LB Agar	5 gram yeast extract 10 gram peptone 10 gram NaCl 12 gram Agar -These ingredients were dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes.	Cloning
0,1 M CaCl ₂	1.4701 gram CaCl ₂ .2H ₂ O was dissolved in 100 ml of distilled water. It was autoclaved at 121°C for 15 minutes.	Cloning
10x Protease Inhibitor Solution	1 tablet was dissolved in 5 ml of distilled water. Aliquots were stored at -20°C.	Western blot
10% SDS	10 gram SDS was dissolved in 100 ml of distilled water.	Western blot
10%APS	1 gram of ammonium persulfate was added to 10 ml of water. Aliquots were stored at -20°C.	Western blot
10X TBS – 1X TBS	12 gram Trizma-base 80 gram NaCl - They were dissolved in 800 ml of distilled water. The pH of the solution was adjusted 7.6. Then the solution was completed to 1L for 10X TBS (stock). To make 1X TBS,	Western blot

	100 ml of stock solution was added to 900 ml of water.	
TBS-T	1 ml of Tween-20 was added to 1 L of 1X TBS solution.	Western blot
1,5 M Tris-HCl (pH 8.8)	18,17 gram Trizma-base was added to 50 ml of distilled water. The pH was adjusted to 8.8. Then the solution was completed to 100 ml and stored at +4°C	Western blot
0,5 M Tris-HCl (pH 6.8)	3 gram of Trizma-base was dissolved in 40 ml of distilled water. The pH was adjusted to 6.8 with HCl 37% and NaOH. Then the final volume was completed to 50 ml.	Western blot
10% Resolving gel	4800 µl water 2500 µl 40% Acrylamide 2500 µl 1.5 M Tris-HCl 100 µl 10% SDS 100 µl 10% APS 10 µl TEMED -These ingredients were mixed well and the mixture was poured to gel cassette.	Western blot
4% Stacking gel	3100 µl water 500 µl 40% Acrylamide 1250 µl 0.5 M Tris-HCl 50 µl 10% SDS 50 µl 10% APS 5 µl TEMED	Western blot
10% Milk in TBST	5 gram of milk powder was dissolved in 50 ml of TBS-T	Western blot
Running Buffer	3 gram Tris-base 14,4 gram Glycine 10 ml 10% SDS -They were dissolved in 1 L of water. The solution was stored at +4°C.	Western blot
Transfer Buffer	6 gram Tris-base 28,8 gram Glycine 200 ml of Methanol - Tris and glycine were dissolved in 800 ml water and methanol was added on top that.	Western blot
Stripping Buffer	3,75 gram Glycine	Western blot

	2,5 ml 10% SDS 2,5 ml Tween-20 - They were added to 200 ml of distilled water. The pH was adjusted to 2.2. Then the final volume was completed to 250 ml.	
4% paraformaldehyde (PFA)	37% formaldehyde solution was diluted as 1:3,7. 10% formaldehyde solution includes 4% paraformaldehyde.	Immunocytochemistry
Complete Medium - Medium	5 ml FBS 500 µl NEAA 500 µl P/S 44 ml DMEM with L-glutamine - These ingredients were mixed and stored at +4°C.	Cell culture maintenance
Freezing Medium	900 µl FBS 100 µl DMSO - Cells to be stored can be dissolved in this mixture.	Cell culture maintenance

2.2.2 Cloning of mammalian ALCAM overexpression plasmid

Both ALCAM transcript variant 1 and 4 were constructed. The sequences and the maps of plasmids can be found in Appendix A part.

2.2.2.1 Amplification of ALCAM sequences

ALCAM sequences were amplified from commercially available healthy brain cDNA as a template. The cDNA was diluted to make its concentration as 30 ng/µl. Primer stocks were diluted with nuclease free water as 1:10 for working concentration: 10 µM. For both sequences AL_V4_V1_HindIII primer was used as forward primer, while NS_V1_AL_EcoRI was used for variant 1 and NS_V4_AL_EcoRI was used for variant 4 as reverse primer. The reaction mixtures of total 20 µl were prepared according to Table 2.12 using Phusion Master Mix.

Table 2.12 Phusion PCR reaction mixture

Component	Amount
2x Phusion Master Mix	10 µl
Forward primer	1 µl
Reverse primer	1 µl
Healthy brain cDNA	2 ul
H ₂ O	6 µl

After preparation of reaction components, the tubes were placed into thermal cycler and the reaction was run according to Table 2.13. The extension time was 60 seconds for variant 1 while it was 15 seconds for variant 4.

Table 2.13 Phusion PCR reaction protocol

Cycle step		Temperature (°C)	Time (seconds)
Initial Denaturation		98	30
x35	Denaturation	98	10
	Annealing	63	20
	Extension	72	70-15
Final extension		72	300

For 1.5% of agarose gel, 1 tablet agarose was dissolved in 33 ml TAE buffer, boiled in microwave and 3,3 µl of SYBER SAFE (10000x) was added to the clear agarose liquid. This clear agarose liquid was poured into gel electrophoresis cassette and the comb was placed in order to make wells inside of the gel. After solidification of the gel, agarose is placed into the electrophoresis tank filled with TAE buffer. The PCR products were mixed with 5x loading buffer and loaded into the gel. 3 µl Hyperladder 1 kb was used to determine the band size. The gel was run at 120V for 30 minutes. After running was completed the gel image was taken under Chemidoc MP Imaging System. When the estimated band was seen, the remaining PCR product was purified to 43 µl by Expin Combo GP mini kit and the concentration was measured with Nanodrop.

2.2.2.2 Double digestion of empty plasmid and PCR products

The total volume of purified PCR products ALCAM variant 1, variant 4 and 700 ng empty vector pcDNA3.1(+)/myc-His A were digested with EcoRI-HF and

HindIII-HF enzymes. The reaction mixture was prepared with 1 µl from each restriction enzyme, 5 µl from CutSmart Buffer and the rest was completed to 50 µl by H₂O. The mixture was incubated at 37°C for 2 hours. At the end of 2 hours 1 µl KpnI-HF enzyme was added to prevent self-ligation. After 1 more hour incubation at 37°C, the mixture was kept at 80°C for 20 minutes for deactivation of the enzymes. After the presence of bands were verified, as a last step the digested PCR products and plasmid were purified again.

2.2.2.3 Construction of final plasmids

The last concentrations of both purified and double digested PCR products and empty plasmid were measured and they were put into ligation reaction for 30 minutes at room temperature. 3:1 molar ratio for insert over vector was used for the reaction. 2 µl of T4 DNA Ligase Buffer and 0,2 µl T4 DNA Ligase were used for 20 µl of reaction. The rest was completed with nuclease free water.

2.2.2.4 Transformation of ligation products

Ligation product was transformed immediately following the ligation reaction. In line with this purpose, competent bacteria had been prepared. JM109 bacteria strain was thawed and streaked to agar plate with no anti-biotic content. After overnight incubation at 37°C, a single colony was picked via pipette tip and the tip was immersed into 1 ml of LB broth. The single colony was allowed to be grown at 37°C and inside of the shaker incubator for overnight. On the following day, 0,5 ml of this liquid bacteria was put into 200 ml of LB broth and the flask was placed in a 37°C shaker incubator for 4 more hours. The liquid bacteria were divided into 4 pre-chilled 50 ml of falcon tubes and centrifuged at 5000g for 8 minutes at 4°C. The pellet was dissolved in 10 ml cold 0.1 M CaCl₂ solution and centrifuged again at the same g force. After supernatant was discarded, the pellet was dissolved with 10 ml of 0.1 M CaCl₂ solution, and the bacteria kept on ice for 20 minutes. Bacteria cells were pelleted at 5000g for 10 minutes and the pellet was dissolved in 2 ml of cold CaCl₂ solution. The bacteria were aliquoted into tubes as 100 µl bacteria for each. These aliquots were stored at -80°C.

For heat shock transformation of the ligation product, during ligation reaction, the competent bacteria were taken out from the freezer and thawed on ice. 10 µl of the ligation mixture was added into 100 µl aliquoted bacteria and incubated on ice

for 30 minutes. During this time, the water bath temperature was adjusted to 42°C. After 30 minutes, the bacteria tube was soaked into 42°C and kept there for 45 seconds. The tubes were placed on ice again. All the liquid bacteria is transferred into 800 µl of LB Broth (inside of 50 ml falcon tube) without any antibiotics. The liquid was put into shaker incubator and kept there for 30 minutes at 37°C. As a next step, the 100 µl of this bacteria mixture was spread on LB agar plates with ampicillin (50 ug/ml for each plate) by glass beads.

2.2.2.5 Verification of constructed plasmids with colony PCR

To verify whether the insert is ligated to double digested plasmid or not, colony PCR was performed. After overnight incubation in stable incubator at 37°C, the colonies were checked. 5-10 single colonies were picked via pipette tip and the tips were immersed into 20 µl of water. This bacteria-water mixture was used as a template for the PCR reaction. The mixture was kept at 4°C until the expected bands were verified by colony PCR confirmation. For primers working dilutions of Pcdna3.1_myc_hisa_F and Pcdna3.1_myc_hisa_R were used. The mixture was prepared according to Table 2.14 while protocol in Table 2.15 was used for PCR reaction. Extension time of 170 seconds was applied for variant 1 construct while 60 seconds was chosen for variant 4.

Table 2.14 Colony PCR reaction mix

Component	Amount
2X Taq DNA Polymerase Master Mix RED	10 µl
Forward primer	1 µl
Reverse primer	1 µl
Bacteria-water mixture	2 ul
H ₂ O	6 µl

Table 2.15 Colony PCR reaction protocol

Cycle step		Temperature (°C)	Time (seconds)
Initial Denaturation		95	300
x35	Denaturation	95	25
	Annealing	60	30

	Extension	72	170-60
	Final extension	72	300

2.2.2.6 Verification of sequence of the constructed plasmids with NGS.

The positive colonies were determined and at least 2 of them were chosen for plasmid isolation. All of the remaining water-bacteria mixture was put into 2 ml of LB broth with ampicillin and kept in shaker incubator at 37°C for overnight. Next day, the plasmids were isolated from this 2 ml of bacteria with Quick-DNA Miniprep Plus Kit. 100 ng was sent to relevant company for Next Generation Sequencing (NGS) sequencing. The obtained raw data – fastq files were aligned to the plasmid map in Geneious software. When the whole map has continuous reads aligned to it, the plasmid was considered as confirmed. In order to use the isolated and confirmed plasmids in transfection, we needed more concentrated plasmids with reduced endotoxicity. Therefore, the verified plasmid is transformed to JM109 bacteria again and spread to LB agar with ampicillin for overnight incubation at 37°C. Next day, single cell colonies were immersed into 100 ml LB liquid with ampicillin and the flask was kept at shaker incubator at 37°C for overnight. Next day, maxiprep was carried out with ZymoPURE II Plasmid Maxiprep Kit. After the purification of plasmids, as a last step, endotoxin reduction was carried out not to cause any unwanted response in mammalian cells. The final concentrations were measured with nanodrop, noted and the tubes were stored at -20°C. During nanodrop measurement Absorbance ratios at 260, 280 and 230 nm were considered: 260/280 ratio was expected to be between 1.7 – 2.0 while 260/230 ratio must have been between 2.0 - 2.2. These values were pure DNA values.

2.2.3 ALCAM gene manipulation with siRNA and plasmid transfection

In this study, we used 4 different cell lines. A172, LN229 and T98G are the grade IV astrocytoma cells (GBM) and SW1088 is grade III astrocytoma. These cell lines were transfected with gene manipulating agents. In order to understand the ALCAM role in senescence, we carried out overexpression and downregulation assays in these cell lines with plasmid and siRNA transfection respectively.

2.2.3.1 Cell culture maintenance

The maintenance of the cells was carried out, as in the following. Cell lines in cryotubes stored at -80°C freezer are thawed in water bath and the cell mixture is put into 15 ml falcon tube inside of laminar flow hood where all cell culture work was carried out. In order to dilute the DMSO inside of the freezing medium, 3 ml of complete medium is added for each 1 ml thawed solution. The cell mixture is centrifuged at 300g for 5 minutes to obtain the pellet. Since SW1088 pellet is hard to obtain, the conditions for only SW1088 cell line are 450g for 8 minutes. The supernatant is discarded and according to pellet amount, the cells are dissolved in 5 or 12 ml and seeded to T25 or T75 flasks. The next day flasks are observed under an inverted microscope to check whether cells have been attached to the surface or not. In the second day, the dead cells are removed by simply medium change. During this period, medium in flask was replaced with a fresh one every 2-3 days. When the cells have reached 90% confluency they are passaged. The old medium is removed, and the cells are washed in 1 ml of PBS for each 25 cm^2 . Then the PBS is removed and 1 ml of trypsin for each 25 cm^2 is added to the flask. Cells are incubated at 37°C with trypsin, so that the trypsin becomes activated and detach the cells. After 3-10 minutes, the flask is observed under microscope to see detached cells. If this is not the case, the flask is tapped against a rough object. Since trypsin can be harmful to the cells after some point, it is deactivated with FBS or with a fresh complete medium having FBS (3 ml of complete medium for each 1 ml of trypsin). After several pipetting, the mixture is put to 15 ml falcon. The cell concentration is measured with the help of a hemocytometer. 10 μl of cell solution is mixed with 10 μl of trypan blue and the mixture is dropped on the hemocytometer. After a cover slip is used to spread the mixture on hemocytometer, cells are counted on 10x magnification. The average number for one square is doubled since the dilution factor is 2 and multiplied with 10^4 . The final number was considered as the cell number in 1 ml. For the experiments, the desired cell number is seeded to the wells. The remaining cells are either seeded in the flasks or stored in freezing medium inside of a cryotube with Mr. Frosty freezing container and placed into -80°C freezer.

2.2.3.2 Transfection of overexpression plasmids

Transfection of empty plasmid, V1 and V4 constructs was carried out in 3 biological replicates for each plasmid and for RNA - protein isolation (2 wells for each replicate). Only one well used for immunocytochemistry for each plasmid. For SA- β -gal staining the cells were seeded to 96-well plates. In this part the amounts

were given for 12-well plates only. The cells were seeded to 12-well plates as 1.5×10^5 cell for each well. The next day the medium was replaced with a fresh one and the transfection mixture was prepared. In plasmid transfection Lipofectamine 3000 kit was used. First of all, 2 μ l of Lipofectamine 3000 reagent was diluted in 50 μ l of Opti-MEM in one tube. In another tube 800 ng plasmid in question and 200 ng of pEGFP-C1 plasmid (as transfection control) were diluted in 50 μ l of Opti-MEM. After 2 μ l of P3000 reagent was also added to the second tube, they were mixed by vortexing separately first, then mixed with pipetting in another tube. The mixture was kept at RT for 15 minutes and 100 μ l mixture was added on the wells in a dropwise manner. For each biological replicate the mixture was created separately. The cells were placed in the incubator back. After 24 h following the transfection, transfection efficiency was checked under inverted microscope with blue light (488 nm). It was expected pEGFP-C1 plasmid to give fluorescence signal. For western-blot, immunocytochemistry and SA- β -gal staining assays cells were kept in incubator without changing the medium for 24h more, while they were lysed for RNA isolation.

2.2.3.3 Transfection of siRNAs

A design similar to the plasmid transfection was also used for siRNA transfection. The transfection was carried out according to manufacturer's instructions. 10^5 cells were seeded to 12-well plates, in antibiotic free medium. Next day the transfection mixture was prepared. 3 μ l of siRNA was diluted in 50 μ l siRNA transfection medium (TM). 3 μ l of transfection reagent was also diluted in the same amount of TM. These tubes were first mixed separately by vortexing and then together by pipetting. The 100 μ l of mixture was kept inside of the hood for 30 minutes. At the end of this time, the medium in the wells were removed and cells were washed with the TM. Immediately after this, 400 μ l TM was added to the 100 μ l mixture and the whole 500 μ l was put to each well. This was done for both siRNA-ALCAM and Control-ALCAM. Plates were placed to the incubator again. After 5 hours following the addition of transfection mixture 500 μ l 2X complete medium with antibiotic (10% FBS, 2% NEAA, 2% P/S in DMEM with L-glutamine) was added to each well to prevent serum starvation. Next day the medium on cells was removed with a fresh 1X complete medium. This was considered as the transfection point. Cells put back to incubator was lysed after 24h for RNA isolation while used after 48h for protein isolation, immunocytochemistry and SA- β -gal staining.

2.2.4 Validation of the alterations in ALCAM gene expression

The changes in ALCAM gene expression upon the transfection of cells with gene manipulating agents must have been verified. Therefore, ALCAM level was measured with several methods: Quantitative Real Time Polymerase Chain Reaction (qRT-PCR), western-blot and immunocytochemistry. These were necessary experiments to show the alterations in both mRNA level and protein level.

2.2.4.1 Measuring ALCAM transcript levels

After 24h following the transfection of both plasmid and siRNA, RNA isolation was carried out for each biological replicate with Direct-zol RNA Miniprep kit. The medium in cells was removed and cells were washed with PBS. After PBS removal, 350 µl of Trizol was Isolated RNA concentrations were measured with nanodrop. During nanodrop measurement, the absorbance at 260 nm over absorbance at 280 nm was considered and paid attention to be close to 2.0 for pure RNA solution. Before storing the RNA at -80°C, 800 ng of RNA were taken for cDNA conversion. 4 µl of 5X iScript Reverse Transcription Supermix was added to each tube including RNA. After final volumes were completed to 20 µl, tubes were placed to thermal cycler to run the reaction protocol in Table 2.16.

Table 2.16 cDNA conversion protocol

Step	Time (min)	Temperature (°C)
Priming	5	25
Reverse Transcription	20	46
RT Inactivation	1	95

After cDNA conversion was completed qRT-PCR reaction was set-up. Different primer pairs were used for determining the ALCAM gene expression level. For total ALCAM transcript level detection cd166_F and cd166_R, for ALCAM variant 1-3 detection V1-3_F and V1-3_R, for variant 4 V4_F and V4_R and for housekeeping gene GAPDH_F and GAPDH_R primers were used. These primers were diluted 1:10 for working concentration which is 10 µM. After cDNAs were diluted as 1:3, the reaction mixture was prepared according to Table 2.17. For negative controls

water was used instead of cDNAs in question. Each reaction mixture was put on the plate in duplicates. The reaction was run according to the protocol in 2.18.

Table 2.17 GoTaq qRT-PCR mixture for ALCAM variants and GAPDH primers.

Component	Amount
2x GoTaq® qPCR Master Mix	5 µl
Forward primer	0,25 µl
Reverse primer	0,25 µl
1:3 diluted cDNA	2 µl
H ₂ O	2,5 µl

Table 2.18 GoTaq qRT-PCR protocol

Cycle step		Temperature (°C)	Time (seconds)
Initial Denaturation		95	300
x40	Denaturation	95	25
	Annealing	60	30
	Plate read		
Melt Curve		65-95	5

2.2.4.2 Checking ALCAM protein levels with immunoblotting

After investigating the mRNA levels of ALCAM transcripts, next step was analyzing changes in protein level. In line with this purpose, western-blot assay was carried out for both siRNA and plasmid transfection experiments. 48h after the transfection, the medium on cells were removed and cells were washed with cold PBS. Ice cold RIPA and PI mixture prepared before (72 µl RIPA + 8 µl PI) put in each well placed on ice. After 5 minutes of incubation, the cells were scraped and loaded to microcentrifuge tubes on ice again. Cells were lysed with the help of 1 ml insulin syringe and the viscosity of cells was decreased. The microcentrifuge tubes were placed on ice again and kept there for 30 minutes. During this period of time, cells were mixed by vortexing every 10 minutes. Then cell lysate was centrifuged at 14.000 g for 20 minutes at +4°C. Supernatant was moved to another tube without disturbing the pellet. 9 µl of supernatant was added to 36 µl of RIPA (1:5 dilution) for BCA assay and the rest was stored at -80°C.

BCA Assay was carried out with the standard 2 mg/ml BSA solution of ABP biosciences. Standards were prepared with 1000, 750, 500, 375, 250, 125, 60, 12 µg/ml concentrations and blank as 20 µl final volume. Each well was duplicated as technical replicates. 20 µl of supernatants was added to each well. After all the standards and samples are loaded to the wells, reagent A and B was mixed as 50:1 and making the total volume as 200 µl*(#total well+1). 200 µl of mixture was added to each well. The plate was incubated at 37°C for 40 minutes. After the plate was cooled to RT, the absorbance of each well at 562 nm was measured with microplate reader. Absorbance of each well was corrected according to blank well. Then the standard curve was constituted with absorbance levels corresponding to the known concentrations. The best linear fit formula was obtained. According to the formula each protein's concentration was determined.

The next day, resolving and stacking gels were prepared during the proteins in question were thawed. After resolving gel was loaded to cassette, isopropanol was layered to prevent gel from drying out. When the gel was solidified, the isopropanol was removed and the stacking gel was poured and comb was placed. During the solidification of stacking gel, samples were prepared like following: 5 µl of Loading Buffer, 1,5 µl of β-Mercaptoethanol, 8-20 µg lysates was mixed and the rest was completed with RIPA buffer to 30 µl. These lysates were boiled at 95°C for 5 minutes in thermal cycler. The solidified gels were placed into mini gel tank and the tank was filled with cold running buffer. The boiled lysates were loaded into gel with protein ladder. The samples were run at 90 V until the ladder completely passed to the resolving gel. Then power supply was set to 120 V and running was completed until the ladder reaches to the bottom of the gel. For transfer of the proteins to the WesternBright PVDF-CL membrane, sponges and the Whatman papers were soaked into already prepared transfer buffer for equilibration step. The membrane was activated with 3 minutes incubation in methanol. Then western-blot sandwich was prepared as following: The minus face of transfer apparatus was wetted with transfer buffer and one of the soaked sponges was placed on it. 2 soaked whatman paper were put on top the sponge. Then the gel cassette was opened, the stacking gel part was removed and the whole resolving gel part was soaked into transfer buffer. After 30 seconds, the gel was placed on the whatman papers. The activated PVDF membrane was incubated in transfer buffer for 1 minute and put on top of the gel. Then the other 2 whatman papers were placed on membrane. In order to avoid any bubble formation, a roller was used. After the last sponge was

placed, the sandwich was completed with squeezing the sandwich between minus and plus part (it was also wetted with transfer buffer) of transfer apparatus. It was submerged to transfer buffer inside of the tank. The transfer took 110 minutes at 20 V. After checking if the transfer was successful, the membrane was incubated in 10% milk blocking buffer for 1 hour. At the end of this time membrane was washed with TBS and the primary antibody incubation was carried out at +4°C. Primary antibodies were already prepared in 5% BSA with the following dilutions for each antibody: α -ALCAM: 1/800, α -c-myc: 1/500 (plasmid transfection), α -GFP: 1/1000 (plasmid transfection), α -GAPDH: 1/1000. The next day antibody solutions was put back to falcon tubes and the membrane was washed with TBS-T for 10 minutes and 3 times. Then the secondary antibody incubation was performed at RT for 1 hour. The secondary antibody solutions were already prepared again in 5% BSA for each antibody. The dilutions for both α -Rabbit and α -Mouse (plasmid transfection) were 1/5000. At the end of seconder antibody incubation, the membrane was washed with TBS-T for 4 times for 5 minutes. After all the washing steps were done, the membrane was soaked into TBS. The luminol and peroxide solutions were mixed as 1:1. The membrane was taken out of the TBS solution, the excess TBS solution was removed from the edge of the membrane with the help of a napkin, and the membrane was placed on a transparent plastic. The mixed solution was poured on the membrane, spread and left for 2 minutes, preventing light. The membrane, which was closed again with the help of a transparent plastic, was imaged with the ChemiDoc MP Imaging System.

2.2.4.3 Investigation of ALCAM protein level with immunocytochemistry

The western-blot experiments already gave the protein amount information for ALCAM. However, we also wanted to check the cellular localization of protein, and to see the transient plasmid transfection closer. In line with purpose, we performed immunocytochemistry assay. Different than the other transfection assays, the cells were seeded on 16 mm coverslips in 12-well plates. Coverslips were washed with ethanol 2 times and they were sterilized under UV light for 30 minutes. After final PBS wash they were placed into 12-well plates. Then the cells for transfection was seeded on the coverslips. Both siRNA and plasmid transfection were carried out as mentioned in 2.2.3.2 and 2.2.3.3 parts.

48h after the transfection, cells on coverslip were washed with cold PBS. Then they were fixed in 4% PFA for 10 minutes. Cells were washed with cold PBS 3

times for 5 minutes. For permeabilization step, cells were incubated in 0.1% Triton-X in PBS for 3 minutes. After cells were washed again, blocking was carried out with incubation in 10% BSA for 1 hour. Blocking solution was removed and without washing the membrane, primary antibody solution was put on the cells. The incubation was held at +4°C for overnight in wet chamber. Immunofluorescence antibody dilutions were as following for each antibody: α -ALCAM: 1/300, α -c-myc: 1/250 (only used for plasmid transfection). Next day, the antibody solutions were removed and washed 3 times with PBS. Cells were incubated in secondary antibody solution for 1 hour at RT in dark chamber. The secondary antibody dilutions were 1/1000 for both α -Rabbit Cy5 and α -Mouse Cy3 (only used for plasmid transfection). Then cells were washed again 3 times for 5 minutes and counterstaining was performed with DAPI 1 μ g/ml. DAPI incubation took 3 minutes and washed with tap water 4 times for 5 minutes. While the coverslip was still in PBS, slide and mounting medium were prepared. 10 μ l mounting medium was enough for 16 mm coverslip. After waiting for 5 minutes, the coverslips were sealed with nail polish. Then slides were imaged under confocal microscope either under 63x magnification or 40x.

2.2.5 Examining the effect of ALCAM level difference on senescence markers

Cells of which ALCAM level was manipulated with both siRNA targeting ALCAM mRNAs and plasmid overexpressing ALCAM variant 1 and 4 transcripts became ready for further investigation. As mentioned in the introduction part, for senescence markers Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*), *CDKN1A*, marker of proliferation Ki67 (*MDA6*), *CYR61* and *LMNB1* gene transcripts were chosen. Their expression level was determined with qRT-PCR analysis. In addition to them, a must-have marker SA- β -gal staining was also performed.

2.2.5.1 qRT-PCR for senescence markers

Transfection and RNA isolation were already performed for part 2.2.4. Therefore, the same cDNAs (stored at +4) were used for investigation of senescence markers in qRT-PCR. Although the reaction protocol was same (Table 2.18) for every gene, reaction mixture was different since the primer concentration was different (Table 2.19). GAPDH was used again as a housekeeping gene. Each reaction was set with technical duplicates. For each gene amplicon different primer

sets were used: for p21, p21_F and p21_R; for p16, p16_F and p16_R, for *MKI67*, ki67_F and ki67_R; for *CYR61*, cyr61_F and cyr61_R, for *LMNB1*, lmn1_F and lmn1_R.

Table 2.19 GoTaq qRT-PCR mixture for senescence markers

Component	Amount (p16)	Amount (p21)	Amount (CYR61)	Amount (ki67)	Amount (LMNB1)
2x GoTaq® qPCR Master Mix	5 µl				
Forward primer	0,45 µl	0,3 µl	0,35 µl	0,4 µl	0,25 µl
Reverse primer	0,45 µl	0,3 µl	0,35 µl	0,4 µl	0,25 µl
1:3 diluted cDNA	2 µl				
H ₂ O	2,1 µl	2,4 µl	2,3 µl	2,2 µl	2,5 µl

2.2.5.2 Senescence Associated-β-galactosidase staining

To be able to make a correct assessment, checking only gene expression levels for senescence-related genes was not enough. One of the most common senescence assays is SAβ-gal staining. It was done for both plasmid and siRNA transfection again.

Although it is a well-known assay, it is important to have a positive control since the pH can have significant effects, it has to be around ~ pH 6. Literature review was conducted to find the true positive controls. As a result, doxorubicin was used for A172, temozolomide for LN229 and T98G while azacitidine for SW1088. Each drug's stock solution was prepared as 20 µM for doxorubicin and azacitidine and as 15 mM for temozolomide and stored at -20°C. 5.10² cells were seeded to 96-well plates. Next day complete mediums including drugs were prepared with 15 nM final concentration for adriamycin and azacitidine and 50 µM for temozolomide. The mediums were replaced with these drug including mediums. After 24h, the mediums were replaced with fresh mediums. For cells to recover, they were kept for 5 days. At the end of this time, the cells taken out off incubator for staining protocol. As to experimental groups, for plasmid and siRNA transfection 2.10³ cells were seeded to 96-well plate in triplicates for each group. Next day, the transfection

was carried out as mentioned before with the adjusted amounts for 100 µl final volume in each well. After 48h following the transfection, the staining protocol was carried out.

Senescence β -Galactosidase Staining Kit from CST was used for SA β -gal staining. The mediums of cells were thrown away and cells were washed with PBS. PBS was discarded and cells were fixed with 1X fixative solution for 15 minutes. During this time staining solution was prepared as follows: 93 µl 1X Staining solution, 1 µl Solution A, 1 µl Solution B and 5 µl 20 mg/ml X-gal dissolved in DMSO/DMF. Fixative solution was removed, and cells were washed 2 times with PBS. The staining solution was poured to the wells and cells were kept at 37°C. Next day staining was confirmed with inverted imaging system under 20x magnification.

2.3 Statistical Analysis

We designed our experiments in such a way that there were always 3 replicates and duplicates for each replicates in qRT-PCR experiments. We used GraphPad Prism to create our graphs. Since in every case we compared only 2 groups we used student's t-test. The reason why we chose unpaired test rather than the paired is we created all three replicates at the same day even though the master mixes given to them was prepared independently. P-values or significance levels were shown in the figures. For significance levels “*” was used meaning that “*” stands for $p \leq 0.05$; “**” stands for $p \leq 0.01$; “***” stands for $p \leq 0.001$ and “****” stands for $p \leq 0.0001$.

2.3.1 qRT-PCR analysis

qRT-PCR analysis was done with the $2^{-\Delta\Delta C_t}$ formula. First, the average of the Cq/Ct values obtained from the technical replicates of each biological replicate was taken. These values were then subtracted from the Cq value of the corresponding GAPDH. This was called normalization to the housekeeping gene and was ΔC_t . Then, the mean of the control group ΔC_t s was subtracted from each ΔC_t value for normalization according to the control-untreated group. This value was called $\Delta\Delta C_t$. Finally, $\Delta\Delta C_t$ values were multiplied by -1 and written as a the power of 2. The results were written as 3 separate lines for t-test analysis. Since the geometric

mean assures that every value contributes to the mean proportionally, it was used in qPCR analysis, graphs were drawn with geometric mean and geometric standard deviation. If there is an outlier it wasn't included in the analysis. Since the analysis was unpaired, the "corresponding" control or experimental group wasn't removed from the analysis.

2.3.2 Immunoblotting analysis

In addition to qRT-PCR, we also did western blot analysis. Fiji-ImageJ software was used in line with this purpose. First of all, the western blot images were arranged in such a way that all the bands were horizontally aligned with zero angle and the band edges are clear as much as it was possible. This was done in the default pictures apps. Then the image was uploaded to ImageJ and a rectangle was drawn having the bands in question (ALCAM, GFP (Green Fluorescent Protein)) inside of it. The rectangle was used to select the first lane. Then the housekeeping bands were selected as next lane with another rectangle having the same sizes. Then lines were plotted and with wand tool the areas for each band were calculated. Each value for ALCAM or GFP band was normalized by dividing them to corresponding GAPDH value. Then all the normalized values were divided into the mean of control group normalized values. Therefore, the control was considered as 1.

2.3.3 Immunofluorescence analysis

Confocal microscopy images were stored both as merged and with different channels. There only 2 channel for siRNA transfection Cy5 (ALCAM) and DAPI while it was 3 for plasmid transfection Cy5, Cy3 (c-myc) and DAPI. Every channel coming from the same image was uploaded to ImageJ separately. Their type was adjusted as 8 bit and image became inverted. As a next step, the threshold was determined. Then the area was measured. The same process was done for both channels standing for ALCAM and DAPI. The ALCAM values were divided to DAPI values coming from the same image. Then all of the values were normalized according to the means of control group again. The graphs were drawn with GraphPad Prism again using means with SD values.

2.3.4 SA β -gal staining analysis

Most time-consuming analysis was SA β -gal staining analysis. It required both total cell number and the number of blue-stained cells. Since the microscope where the SA β -gal staining results were imaged had no feature to show DAPI signal, we had to count the numbers by eye. To make it true, 2 people counted the cells without knowing from which experiment the image came from. Moreover, AI-based tools was also used just to see the differences are proportional. Then the average values for each replicate were noted. The percentages was calculated as $100 * (\text{Blue-Stained Cell Number} / \text{Total Cell Number})$. This time the values were not adjusted based on the mean percentage value of the control group. GraphPad Prism was used again to show the percentages using the mean with SD values.

Chapter 3

3. Results

3.1 Verification of ALCAM plasmids

In order to overexpress the ALCAM gene in GBM cells, we produced constructs with ALCAM V1 and V4 sequence with 6x His and c-myc tag in frame. Since colony PCR result (see Appendix B: Figure B.1) doesn't give any sequence information, we needed to send our plasmid for sequencing. We have obtained the fastq files and after alignment in Geneious software we have seen that there is only one nucleotide difference at 1478th bp as T>C (Figure 3.1) in V1 construct (978th bp for protein coding). To check if this nucleotide change affects the protein sequence or not, we separated the sequence into codons and saw that the codon change become GCT > GCC, which is still Alanine. We aligned the transformed protein sequence to the reference protein sequence and it was seen that there is no difference in the protein sequence (not shown).



Figure 3.1. Next Generation Sequencing results. Constructed variants were sent to the company and the reads from NGS data were aligned to the reference gene. Each read aligned to the reference were shown at the below the construct map. It was seen that V1 construct had T>C nucleotide change at the 1478th bp of the constructed plasmid, which corresponds to 978th bp of the protein coding sequence.

3.2 Validation of ALCAM gene manipulation in glioblastoma cells

Before analyzing senescence marker differences upon ALCAM gene manipulation, we had to verify that these tools work in both mRNA and protein level. We used 4 different cell lines for our purpose. 3 of them were GBM cell lines: A172, LN229 and T98G, while one of them was astrocytoma cell line: SW1088. In these cell lines, we transfected cells with the plasmid coding for ALCAM Variant 1 (V1), Variant 4 (V4) to overexpress the corresponding proteins in cells and with empty pcDNA3.1 myc-His A as control group. In addition to overexpression, we transfected the cells with siRNA targeting all ALCAM variants. As a control group, control siRNA targeting no transcripts in human cells was used.

3.2.1 ALCAM gene expression changes upon the transfection

Cells seeded to 12-well plates were transfected with gene manipulating agents and at the end of 24h, the total RNA of the cells was isolated. Following the cDNA conversion, qRT-PCR experiments were set up.

In A172 cell line transfected with plasmids (Figure 3.2), transfection of both ALCAM constructs increased total ALCAM transcript level significantly. While V1 construct increased variant 1-3 level, it didn't change the variant 4 level. V4 construct increased variant 4 level, however it also caused a not significant but noteworthy increase in variant 1-3 expression.

Then cells were transfected with ALCAM targeting siRNA (Figure 3.3). It was shown that siRNA-ALCAM significantly declined both the total ALCAM variant and variant 1-3 levels. Although siRNA attenuated the variant 4 expression, the decrease was not significant.

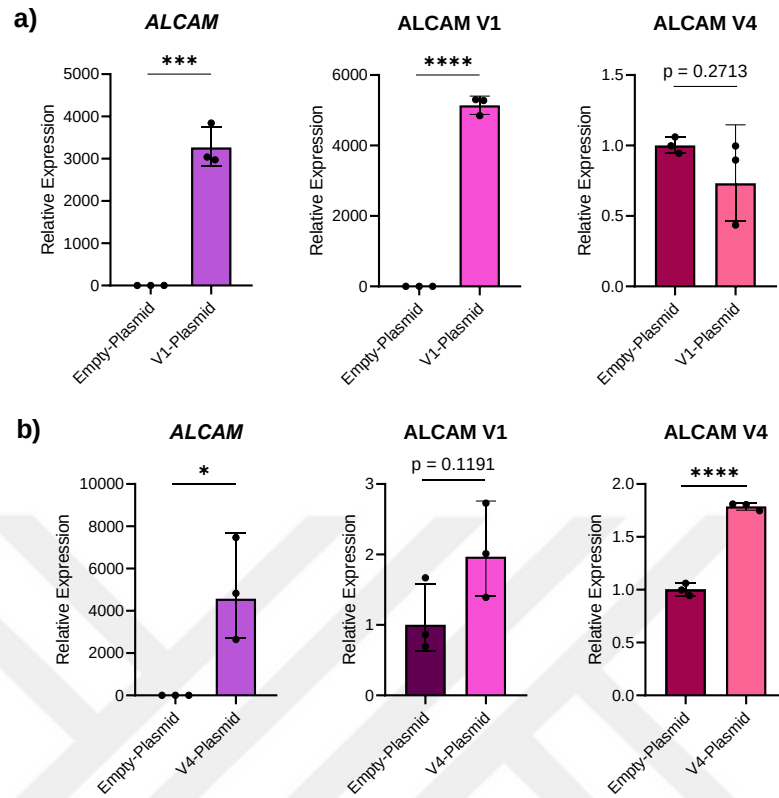


Figure 3.2. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in A172 cell line. Total ALCAM, ALCAM variant 1-3 and ALCAM variant 4 expression levels were measured upon (a) V1 and (b) V4 transfection in A172 cell line. Transfection was carried out in triplicates and the qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes in ALCAM variants were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

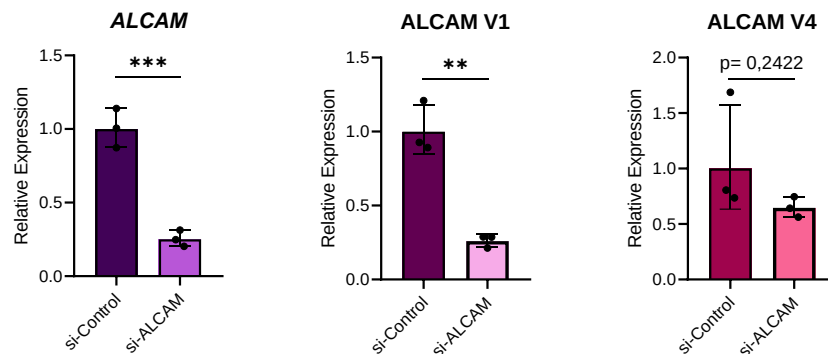


Figure 3.3. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in A172 cell line. Expression levels of different ALCAM variants were measured upon siRNA transfection in A172 cell line. Transfection was done in triplicates and qRT-PCR was carried out in duplicates. GAPDH was used as control gene and $2^{-\Delta\Delta C_t}$ formula was used for the calculation of the fold

changes. Unpaired student t-test was used for the statistical analysis (**, $p \leq 0.01$; ***, $p \leq 0.001$).

LN229 cell was also transfected with overexpression plasmids (Figure 3.4). It has been seen that both V1 and V4 constructs elevated the total ALCAM variants and variant 1-3 levels significantly. While there were rise also in variant 4 level upon the transfection of both plasmids, they were not significant. ALCAM level in LN229 cells was tried to be decreased via siRNA (Figure 3.5). While the siRNA pool targeting all ALCAM variants could not decrease the variant 4 level, variant 1-3 and the total transcript expression was increased significantly.

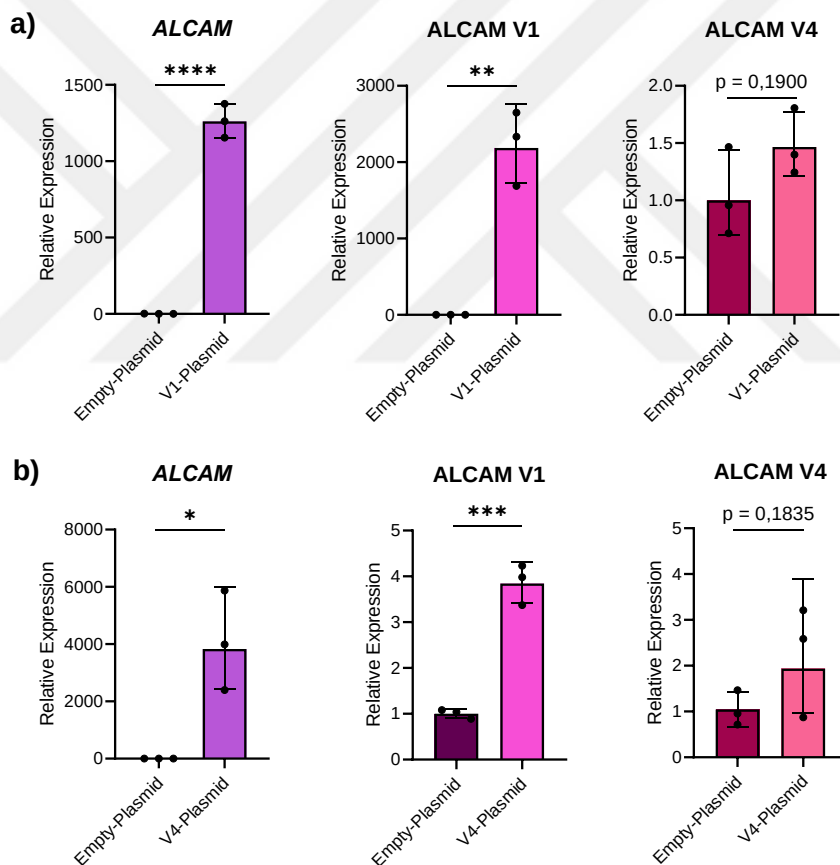


Figure 3.4. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in LN229 cell line. Different ALCAM levels were investigated upon (a) V1 and (b) V4 transfection in LN229 cell line. 3 biological and 2 technical replicates were used for transfection and qRT-PCR respectively. GAPDH was the housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

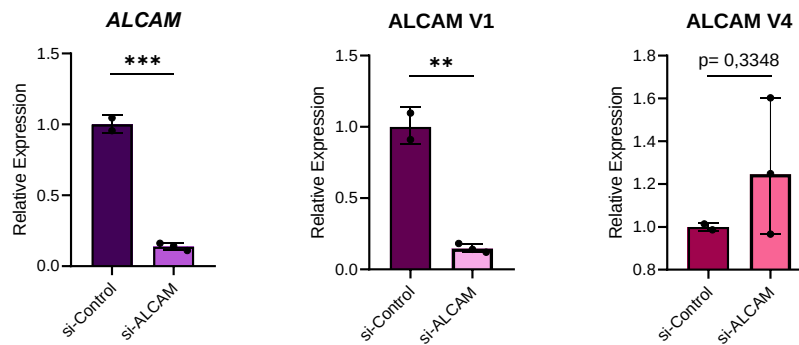


Figure 3.5. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in LN229 cell line. Expression levels of different ALCAM variants were measured upon siRNA transfection in LN229 cell line. Transfection was done in triplicates and qRT-PCR was carried out in duplicates. However, one of the biological replicates transfected with siRNA-ALCAM was discarded since it was an outlier. GAPDH was the housekeeping gene to calculate ΔC_t and the relative expressions (fold change) were calculated with $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (**, $p \leq 0.01$; ***, $p \leq 0.001$).

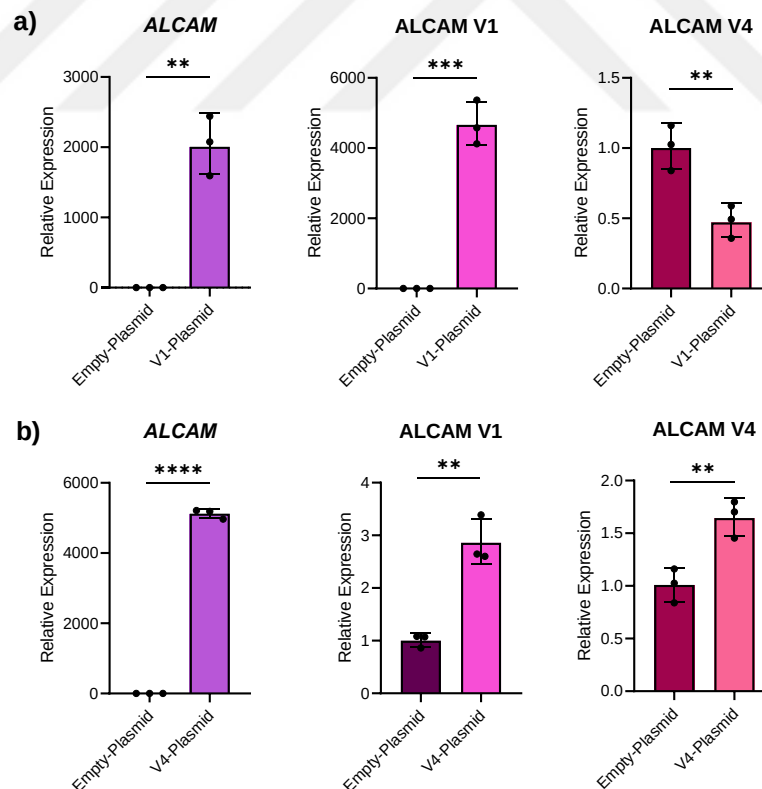


Figure 3.6. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in T98G cell line. (a) V1 and (b) V4 transfection were carried out in T98 cells. Primers amplifying different ALCAM variants were used for qRT-PCR analysis. 3 biological replicates and 2 technical

replicates were used for each experimental and control group. Housekeeping gene was GAPDH and the relative expression levels were calculated with the $2^{-\Delta\Delta C_t}$ formula. Statistical analysis was done with unpaired student t-test (**, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Another plasmid transfected cell line was T98G (Figure 3.6). While both plasmids caused statistically significant effects on each variant's expression, the effect of V1 plasmid on variant 4 level was the opposite of common pattern. It caused a decrease in variant 4 level while other variants' levels increased by the given plasmids.

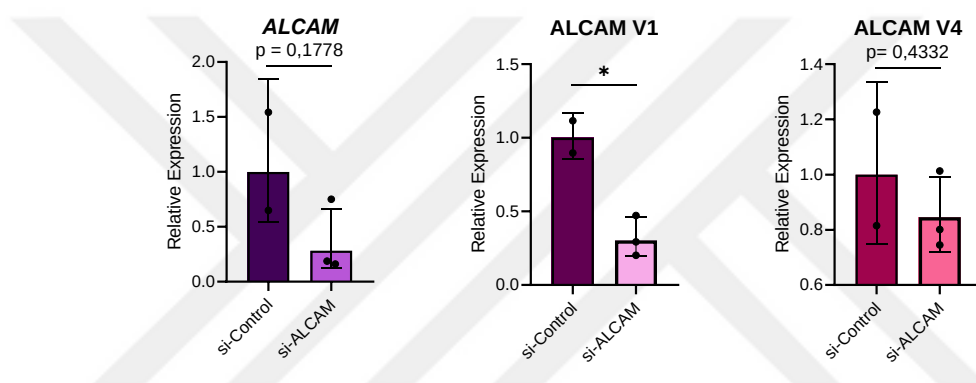


Figure 3.7. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in T98G cell line. siRNA targeting all ALCAM variants was given to the T98G cells. Although the transfection was done in triplicates, since there was an outlier in qRT-PCR results, one of them was excluded from the analysis in experimental group. qRT-PCR was carried out again in 2 technical replicates. GAPDH was the housekeeping gene and $2^{-\Delta\Delta C_t}$ formula was used for fold change. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$).

When it comes to downregulation of ALCAM protein (Figure 3.7) in T98G cells, one can say that siRNA pool could target all the variants and diminish the ALCAM transcript levels in cells. However, only the decrease in variant 1 level was statistically significant.

In order to see the effect of aggressiveness level of cancer cells, SW1088 was used as an astrocytoma grade III cell line. It was also transfected with the two constructs and the changes in ALCAM transcript levels were measured separately (Figure 3.8). In SW1088, compared to the control groups, V1 plasmid elevated both total and variant 1-3 levels while it had no effect on variant 4. Although there was increase in the both total variant level and variant 4 upon V4 transfection, rise in

the variant 4 level was not significant. It could not also give consistent results for variant 1 level, therefore there was no change. In terms of knocking down, one could not detect any remarkable decrease in the ALCAM level upon siRNA transfection (Figure 3.9).

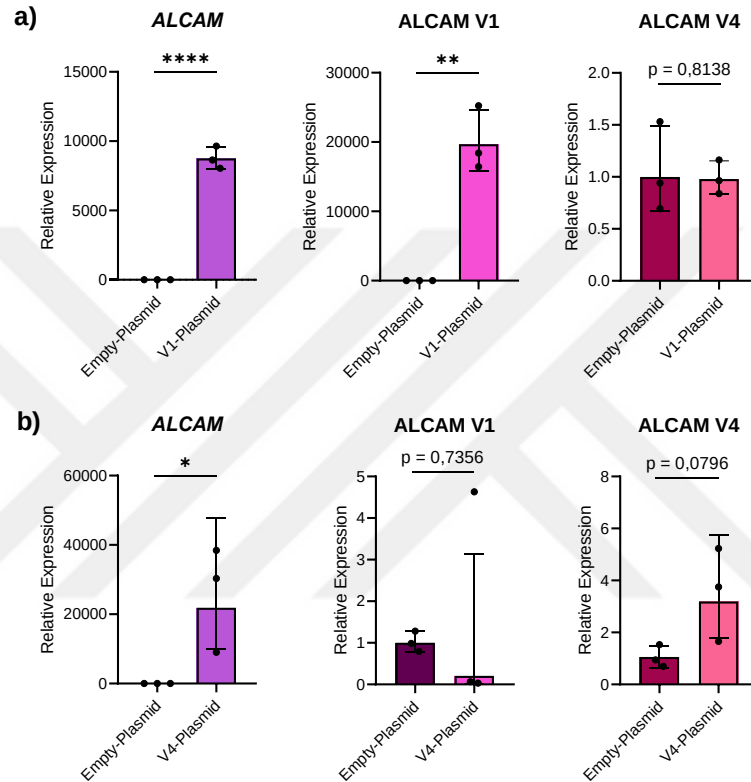


Figure 3.8. Expression changes of ALCAM transcript variants upon overexpression plasmids' transfection in SW1088 cell line. SW1088 cells were transfected with (a) V1 and (b) V4. Different variant levels were measured: total, variant 1-3 and variant 4. 3 biological replicates and 2 technical replicates were used for each experimental and control group. GAPDH was the housekeeping gene. Relative expression levels were calculated with the $2^{-\Delta\Delta C_t}$ formula. Statistical analysis was done with unpaired student t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$).

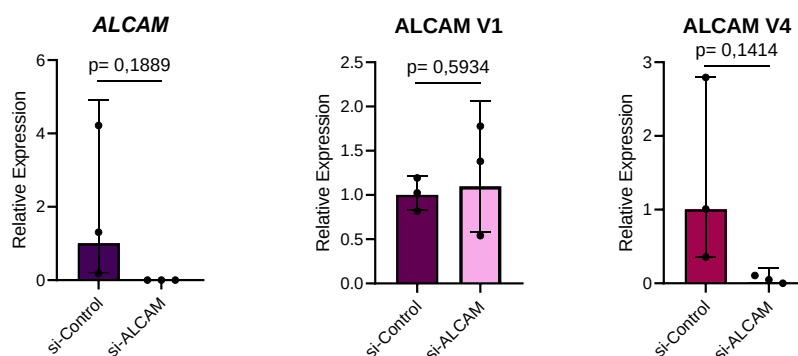


Figure 3.9. Expression changes of ALCAM transcript variants upon siRNA-ALCAM transfection in SW1088 cell line. SW1088 cells were transfected with ALCAM targeting siRNA. Different variant levels were measured, total, variant 1-3 and variant 4. Transfections were performed in triplicates and qPCR was done with duplicates for each replicate. GAPDH was the housekeeping gene. Relative expression levels were calculated with the $2^{-\Delta\Delta C_t}$ formula. Statistical analysis was done with unpaired student t-test.

3.2.2 Changes in ALCAM protein level

In addition to qRT-PCR results, we wanted to check the protein level differences upon the transfection of gene manipulating agents. In line with this purpose, we did western-blot assays. Since the cellular localization (soluble vs membrane ALCAM) can be important in the aggressiveness manner, we performed immune fluorescence assay. This also gave us the information of increase or decrease in ALCAM signal.

3.2.2.1 Immunoblotting

At the end of the 48h following the transfection of agents, we obtained the whole cell lysate and measured to protein levels. After running and separating the proteins in whole lysate according to their sizes, proteins were transferred to the membranes on which we detected the proteins of interest by chemiluminescence. To be able to verify if there is a decrease or increase ALCAM level, anti-ALCAM antibody was used while anti-GAPDH as loading control. In addition to that, to learn the transfection efficiency, we used anti-GFP antibody and anti-c-myc antibody to see the plasmid tags (see Appendix D). In general the ALCAM variant 4 protein could not be detected on the membrane (not shown).

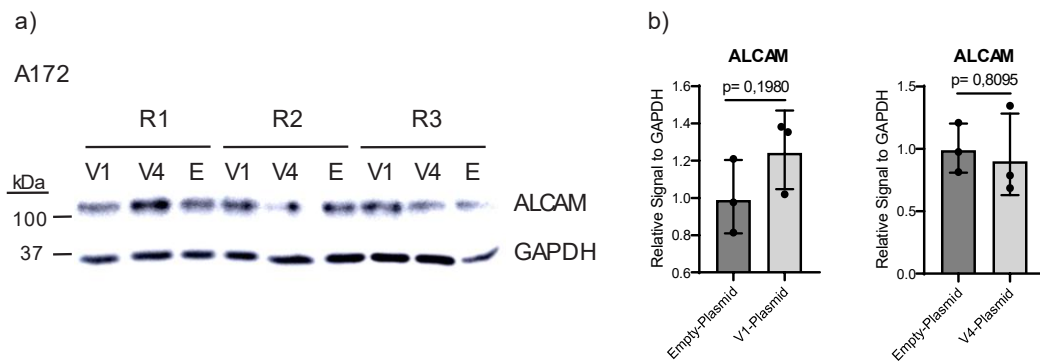


Figure 3.10. ALCAM variant 1 protein level upon plasmid transfection in A172 cell line. (a) Western-blot image for ALCAM detection in plasmid given A172 cells and (b) statistical analysis. Whole cell lysates were loaded to the gel in triplicates for each transfection. GAPDH was used as the housekeeping gene. Relative protein levels were calculated in ImageJ software as ALCAM/GAPDH signal. Statistical analysis was done with unpaired student t-test.

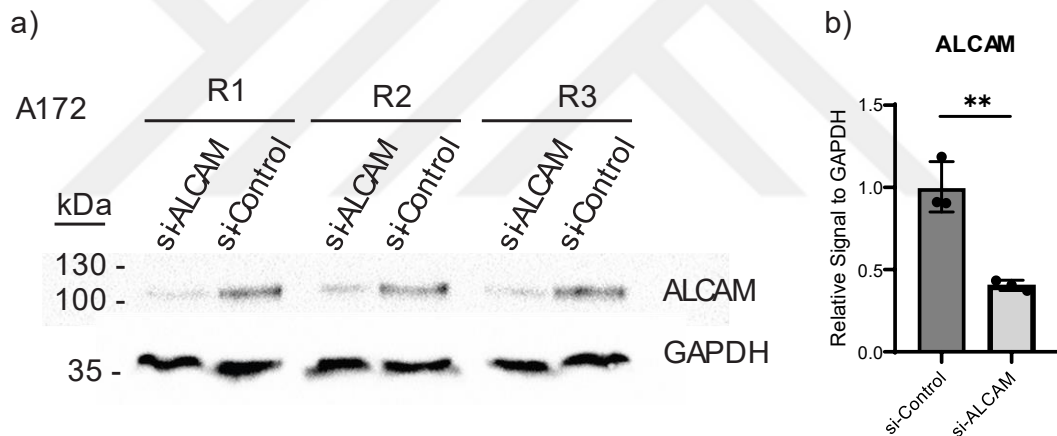


Figure 3.11. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in A172 cell line. (a) Western-blot image for ALCAM detection in A172 cells transfected with ALCAM and control siRNA and (b) statistical analysis. Whole cell lysates were loaded to the gel in triplicates for each transfection. GAPDH was used as the housekeeping gene. Relative protein levels were calculated in ImageJ software as ALCAM/GAPDH signal. Statistical analysis was done with unpaired student t-test. (**, $p \leq 0.01$).

A172 cell lysates (Figure 3.10) showed that V1 plasmid caused an increase in ALCAM level, which is not statistically significant while V4 had no influence. While the plasmids didn't show any remarkable effect, siRNA caused decrease in protein levels, which can be detected even by eye (Figure 3.11). Statistically speaking, ALCAM level is decreased in A172 cells with 2 level of significance.

In the membrane transferred with LN229 cell lysates, no increase was detected for ALCAM protein upon plasmid transfections (Figure 3.12). After normalization with GAPDH levels, the same result, there is no change in protein levels, was shown with t-test. In contrast to overexpression, decreasing ALCAM level with siRNA worked in protein level (Figure 3.13). The protein level in cells transfected with siRNA pool was diminished significantly.

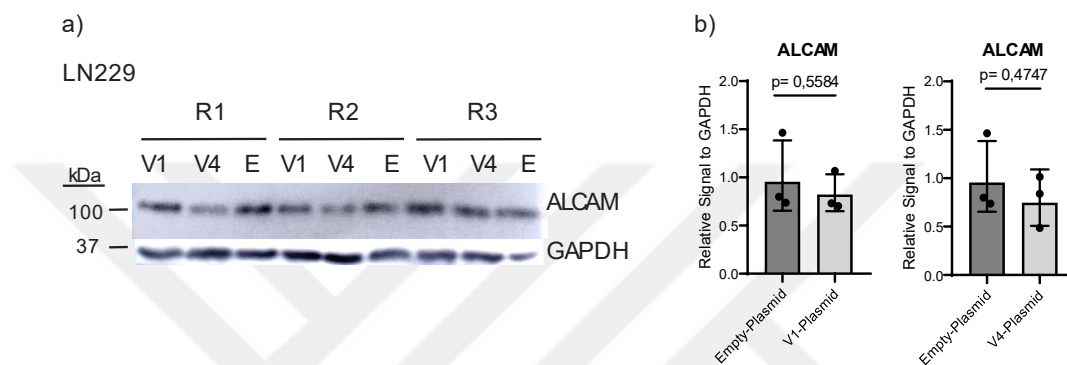


Figure 3.12. ALCAM variant 1 protein level upon plasmid transfection in LN229 cell line. (a) Western blot image was taken to detect ALCAM in LN229 cells transfected with plasmids, and (b) statistical analysis was conducted. Whole cell lysates were loaded onto the gel in triplicates for each transfection. GAPDH served as the housekeeping gene. Relative protein levels were quantified using ImageJ software by calculating the ALCAM to GAPDH signal ratio. Statistical analysis was performed using an unpaired student's t-test.

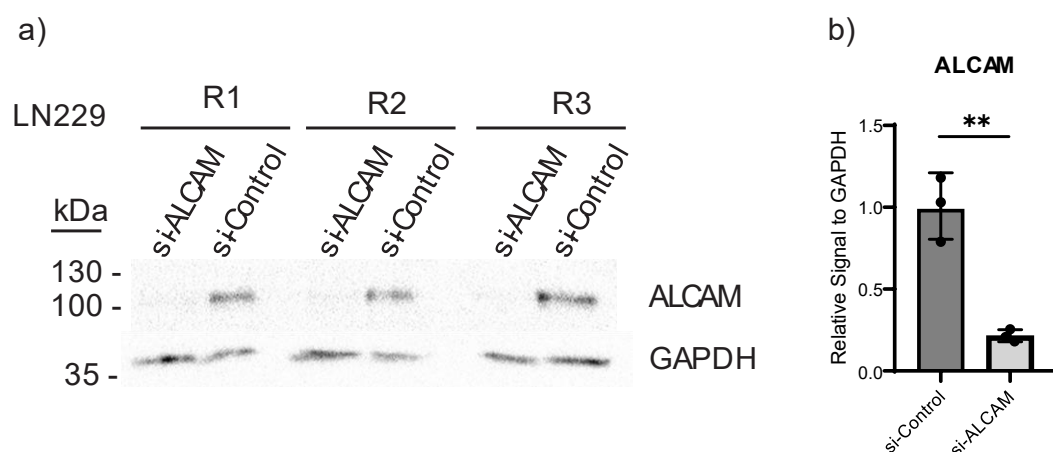


Figure 3.13. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in LN229 cell line. a) Western blot image was to detect ALCAM in LN229 cells transfected with ALCAM and control siRNA, and (b) statistical analysis was conducted. Whole cell lysates were loaded onto the gel in triplicates for each

transfection. Housekeeping gene was GAPDH. Relative protein levels were quantified using ImageJ software by calculating the ALCAM to GAPDH signal ratio. Statistical analysis was performed using an unpaired student's t-test (** $p \leq 0.01$).

In terms of overexpression of ALCAM protein, both V1 and V4 plasmids increased the protein level in T98G cells (3.14). These elevations were also significant after normalization with GAPDH control. siRNA agent also diminished the ALCAM level and this decrease was also statistically very significant (3.15).

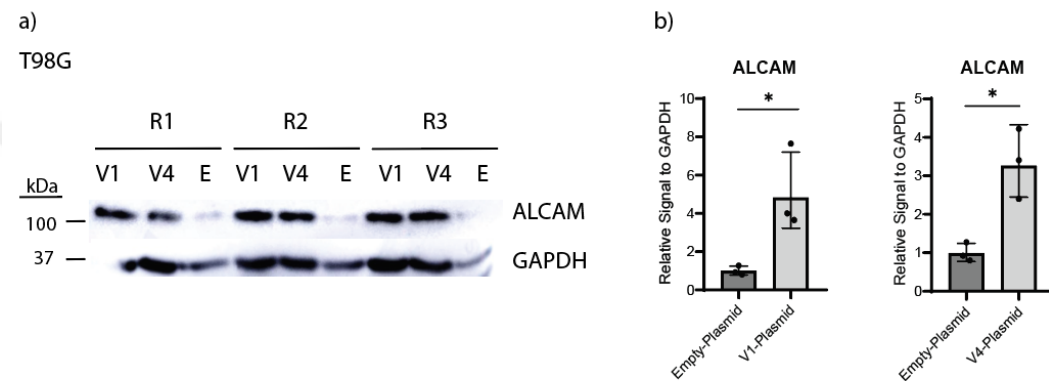


Figure 3.14. ALCAM variant 1 protein level upon plasmid transfection in T98G cell line. (a) Western blot image was taken to detect ALCAM in T98G cells transfected with plasmids, and (b) statistical analysis was conducted. Whole cell lysates were loaded onto the gel in triplicates for each transfection. GAPDH served as the housekeeping gene. Relative protein levels were quantified using ImageJ software by calculating the ALCAM to GAPDH signal ratio. Statistical analysis was performed using an unpaired student's t-test.

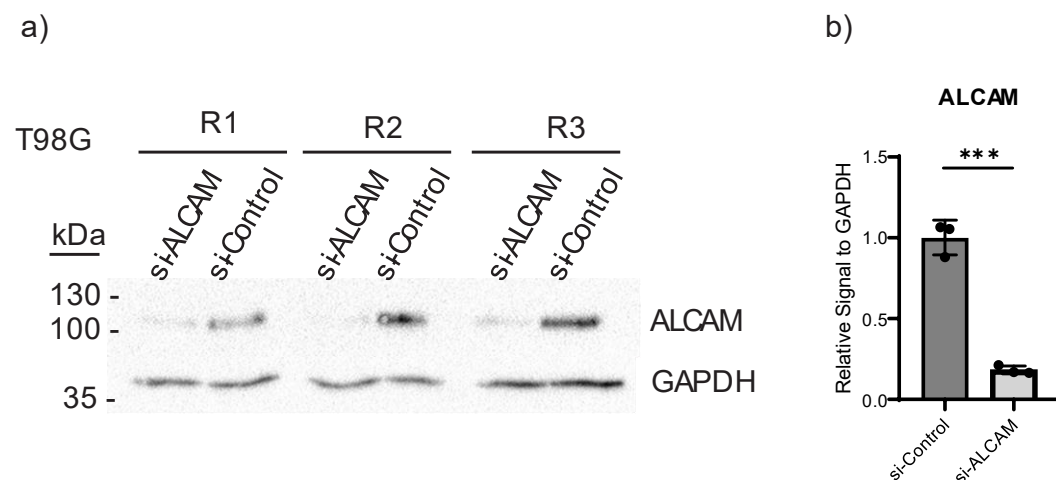


Figure 3.15. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in T98G cell line. ALCAM level in T98G cell lysates transfected with plasmids was shown in a) western blot image and the (b) statistical analysis was conducted. For

each transcription pair, all the triplicates were loaded. Since housekeeping gene was GAPDH, relative protein levels were quantified using ImageJ software by calculating the ALCAM/GAPDH signal. Statistical analysis was performed using an unpaired student's t-test ($*p \leq 0.05$).

SW1088 cells did not show any significant change upon both transfection of plasmids (Figure 3.16) and siRNA pool. Although the ALCAM protein amount was decreased in siRNA (Figure 3.17), we can not say they were significant changes.

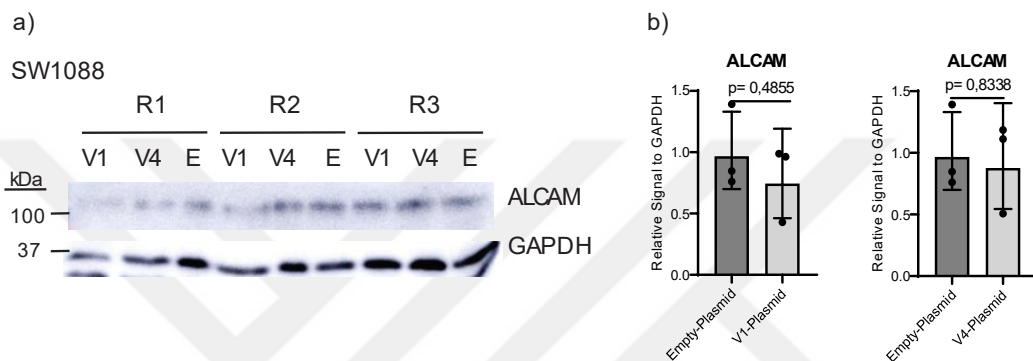


Figure 3.16. ALCAM variant 1 protein level upon plasmid transfection in SW1088 cell line. (a) Western blot image was taken to detect ALCAM in SW1088 cells transfected with plasmids, and (b) statistical analysis was conducted. Whole cell lysates were loaded onto the gel in triplicates for each transfection. GAPDH served as the housekeeping gene. Relative protein levels were quantified using ImageJ software by calculating the ALCAM to GAPDH signal ratio. Statistical analysis was performed using an unpaired student's t-test.

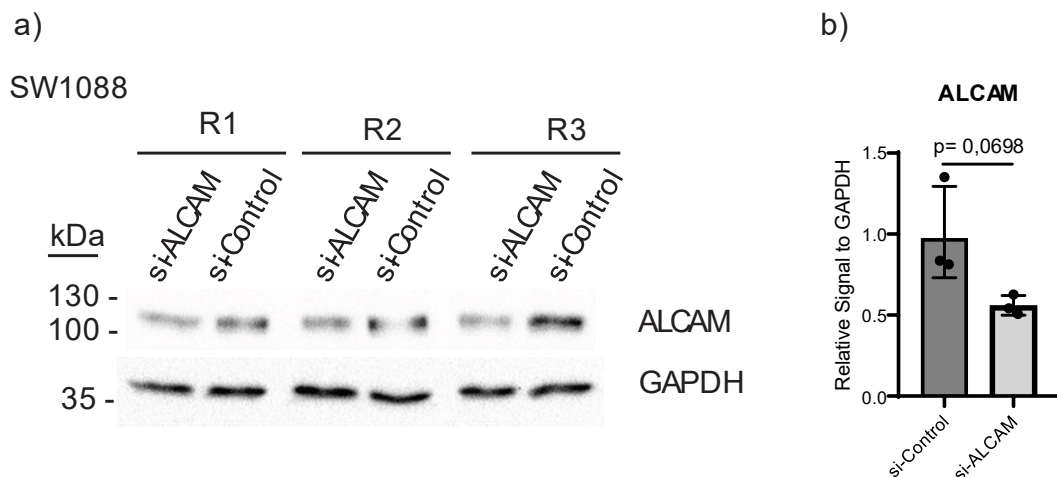


Figure 3.17. ALCAM variant 1 protein level upon ALCAM-siRNA transfection in SW1088 cell line. ALCAM level in SW1088 cell lysates transfected with plasmids was shown in a) western blot image and the (b) statistical analysis was conducted. For each transcription pair, all the triplicates were loaded. Since housekeeping gene was GAPDH, relative protein levels were quantified using

ImageJ software by calculating the ALCAM/GAPDH signal. Statistical analysis was performed using an unpaired student's t-test.

3.2.2.2 Immunofluorescence

In order to both detect the location of ALCAM proteins inside of the cell and to check whether there change in protein level also, we performed immunofluorescence assay. After 48h following the transfection, coverslips having seeded cells on them were washed and incubated inside of antibody solutions. DAPI staining was used for counterstaining, anti-ALCAM antibody was used for ALCAM detection and c-myc protein to see the transfected cells.

In RNA level investigation part, pEGFP-C1 plasmid was also transfected together with the constructs to check the transfection efficiency and for A172, it was so low that the camera could not detect the signal. Therefore, there was no remarkable increase in ALCAM level of the cells given with V1 constructs in comparison to the cells with empty vector (Figure 3.18). Moreover, c-myc did not give strong signal showing that the cells took the constructs. Although V4 increased the ALCAM level, the change was not significant at all. In the cells transfected with siRNA, the difference was not visible by eye, while the signal analysis showed that siRNA-ALCAM pool diminished the ALCAM proteins significantly (Figure 3.19).

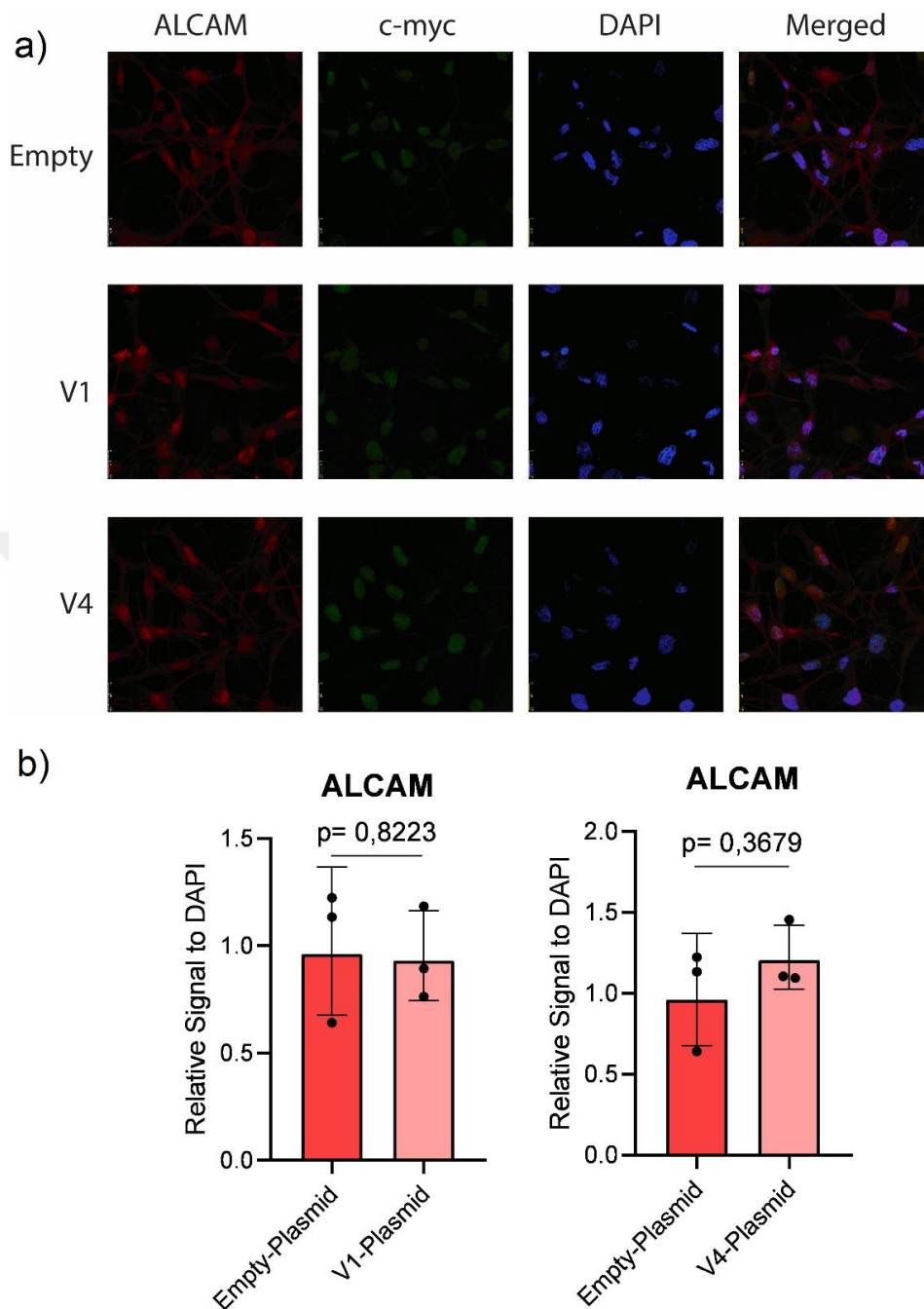


Figure 3.18. Immunofluorescence assay to detect ALCAM signal in A172 cell line transfected with overexpression plasmids. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from A172 cells transfected with plasmids, and (b) statistical analysis including other replicates (see Appendix E). c-myc signal was not considered in analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

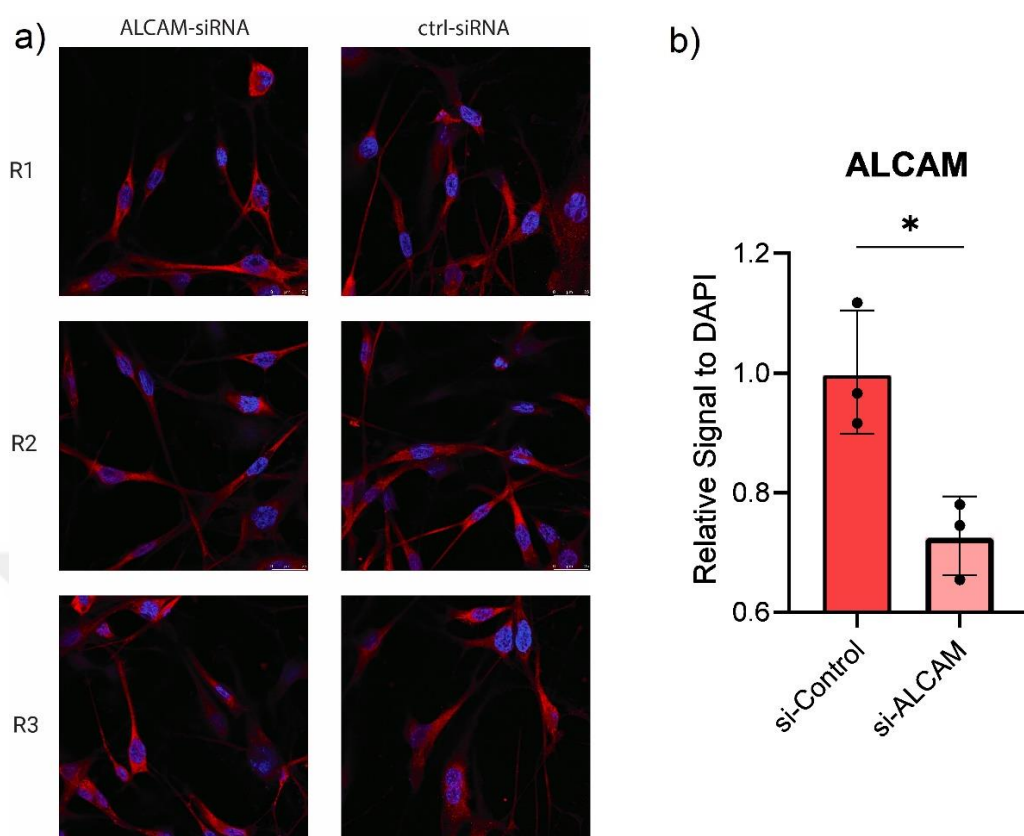


Figure 3.19. Immunofluorescence assay to detect ALCAM signal in A172 cell line transfected with ALCAM siRNA. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from siRNA transfected A172 cell line, and (b) statistical analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

In LN229 cells, unlike A172, the transfection could be detected with c-myc antibody in the cells given with the V1 and V4 constructs (Figure 3.20). Both V1 and V4 constructs increased the signal in transfected cells. Especially the cells transfected with V4 constructs showed remarkable rise in the signal while that was not statistically significant yet. For knocking-down, siRNA transfection showed that the ALCAM signal coming from the cells was decreased (Figure 3.21), however, the analysis showed that this is not significant due to variance in the signal.

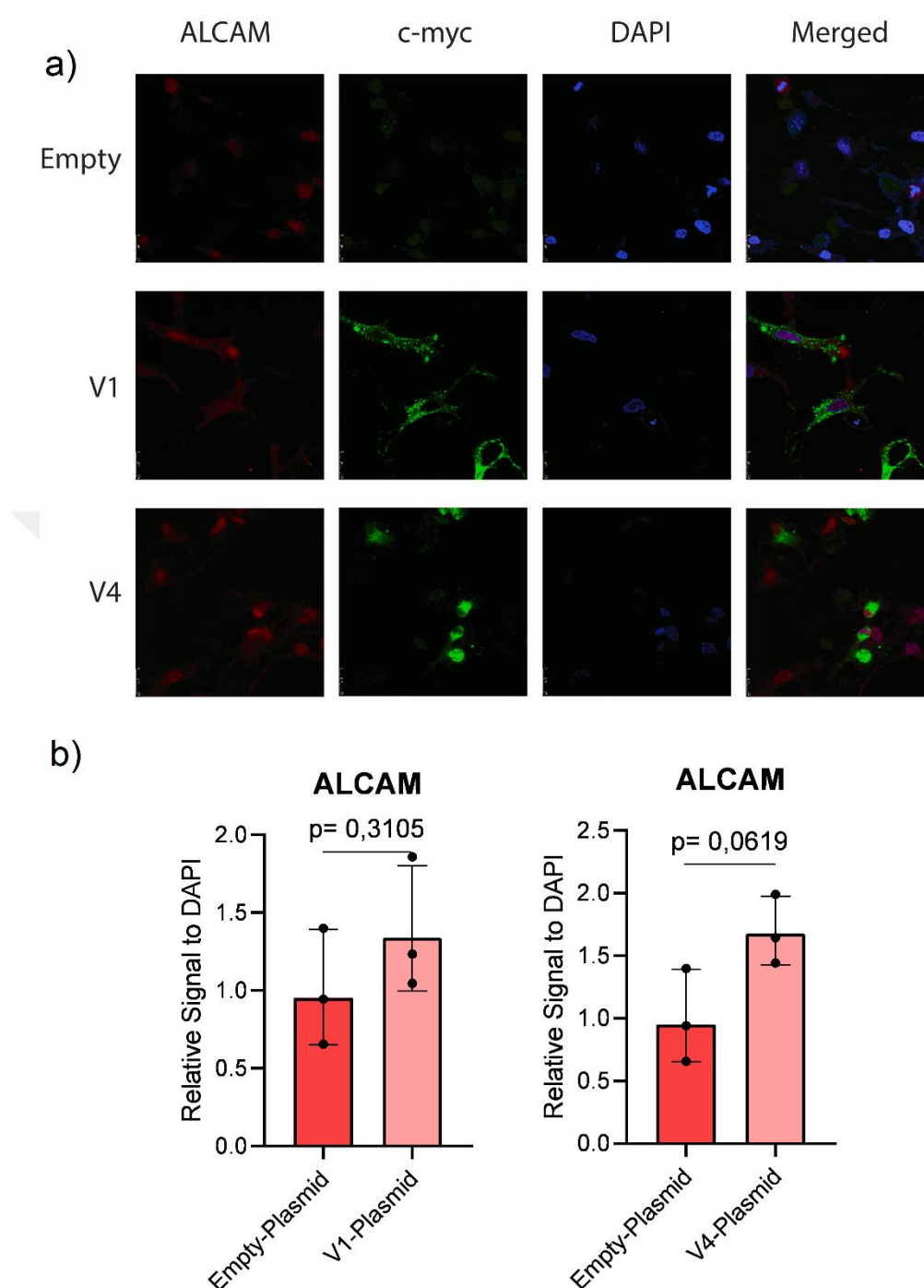


Figure 3.20. Immunofluorescence assay to detect ALCAM signal in LN229 cell line transfected with overexpression plasmids. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from LN229 cells transfected with plasmids, and (b) statistical analysis including other replicates (see Appendix E). c-myc signal was not considered in analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

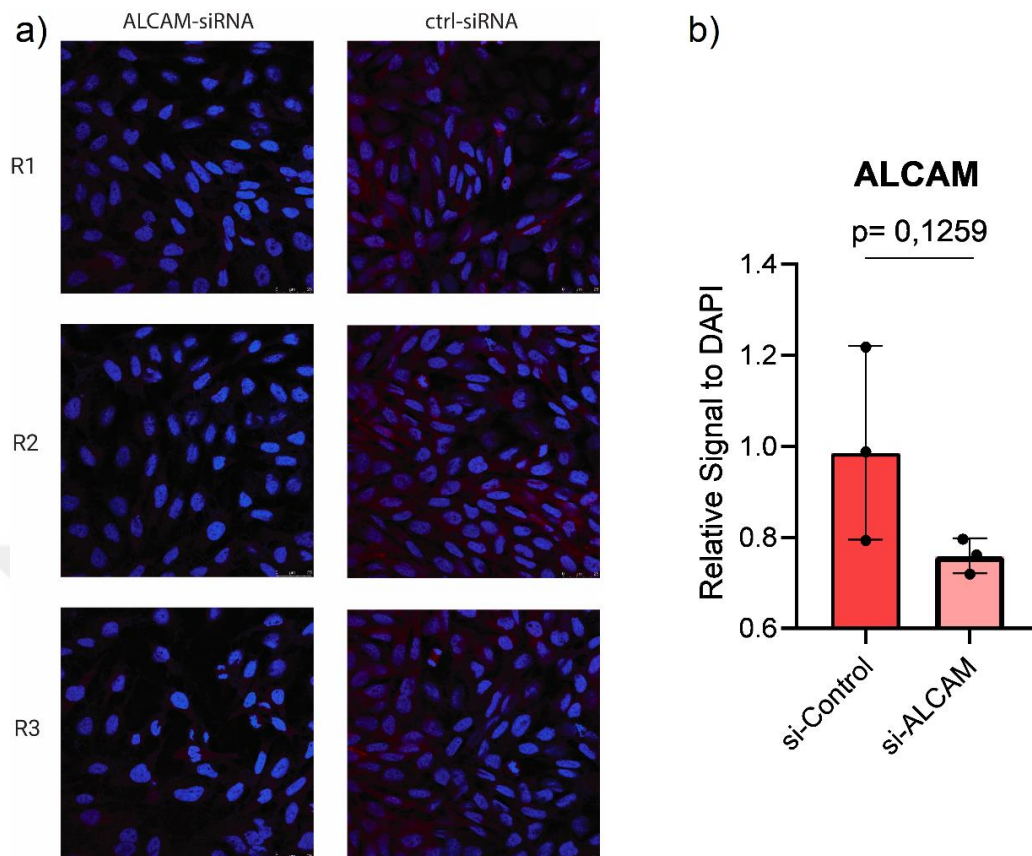


Figure 3.21. Immunofluorescence assay to detect ALCAM signal in LN229 cell line transfected with ALCAM siRNA. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from siRNA transfected LN229 cell line, and (b) statistical analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

Like in the LN229 cells, the transfection was confirmed with c-myc signal in T98G cells transfected with both of the constructs (Figure 3.22). Although it is hard to see the ALCAM signal by eye, the statistical analysis showed that there were increases upon the construct transfection, however they were again not significant. When it comes to downregulation of ALCAM (Figure 3.23), ALCAM signal was diminished while we cannot say this decrease has p value less than 0.5.

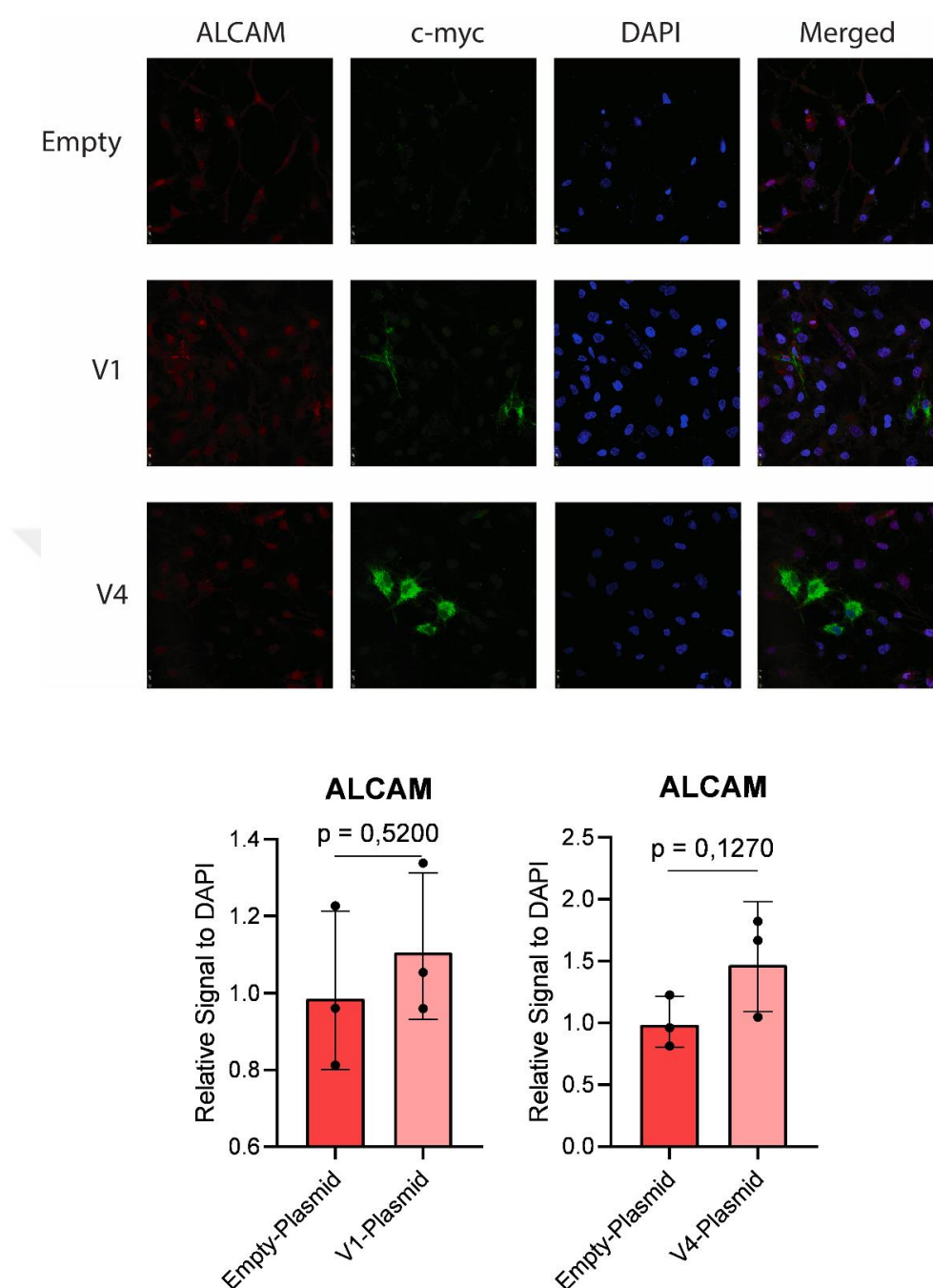


Figure 3.22. Immunofluorescence assay to detect ALCAM signal in T98G cell line transfected with overexpression plasmids. (a) Confocal image was taken under 40x magnification to detect ALCAM signal coming from T98G cells transfected with plasmids, and (b) statistical analysis including other replicates (see Appendix E). c-myc signal was not considered in analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

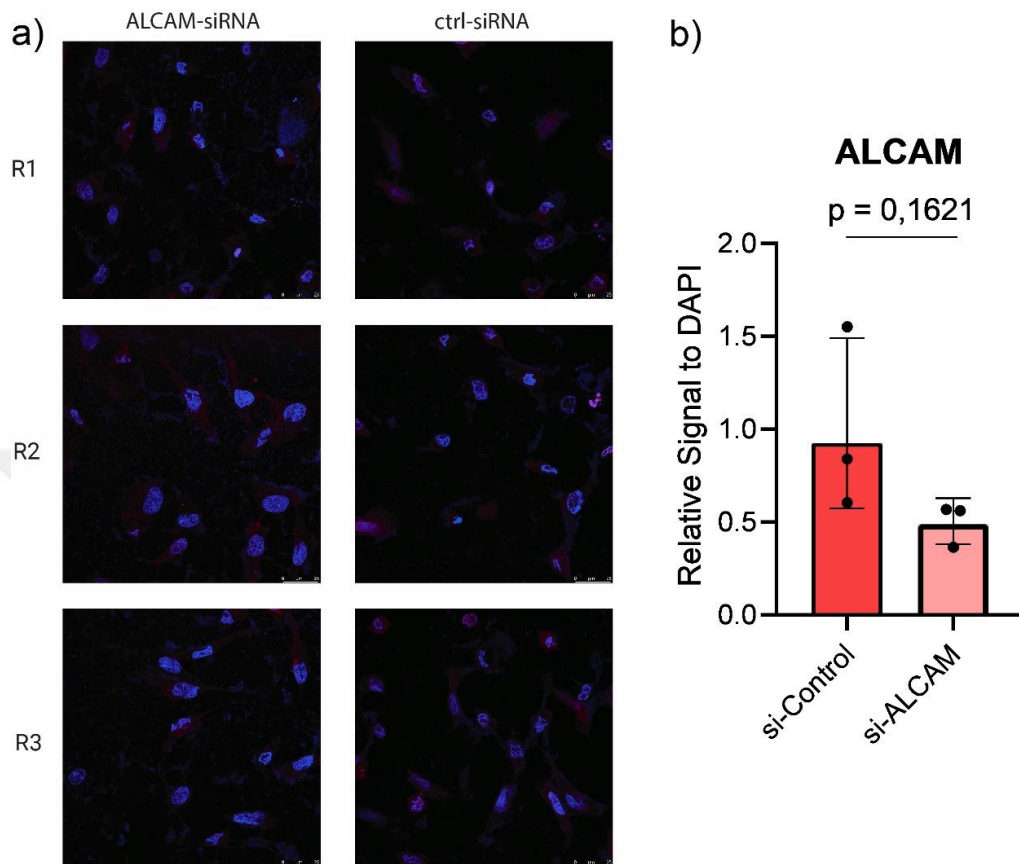


Figure 3.23. Immunofluorescence assay to detect ALCAM signal in T98G cell line transfected with ALCAM siRNA. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from siRNA transfected T98G cell line, and (b) statistical analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

Lastly, we checked the ALCAM signal in SW1088 cells. The strongest signal for c-myc tag came from the SWO88 cells (Figure 3.24). For ALCAM signal, cells given with both V1 and V4 constructs showed statistically significant increase. Although, there was decrease upon the siRNA transfection, it was not statistically remarkable (Figure 3.25).

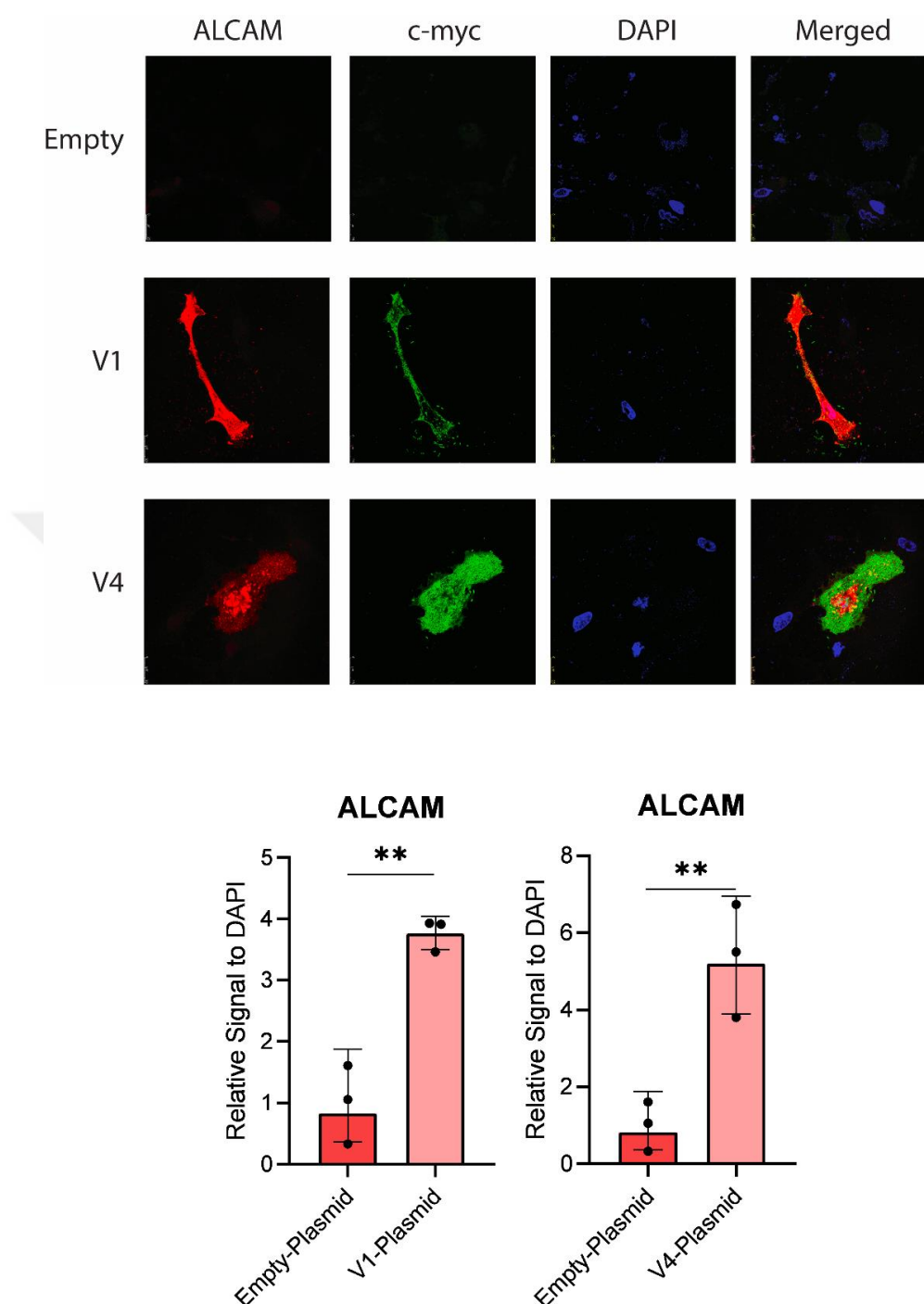


Figure 3.24. Immunofluorescence assay to detect ALCAM signal in SW1088 cell line transfected with overexpression plasmids. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from SW1088 cells transfected with plasmids, and (b) statistical analysis including other replicates (see Appendix E). c-myc signal was not considered in analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test (**, $p \leq 0.01$).

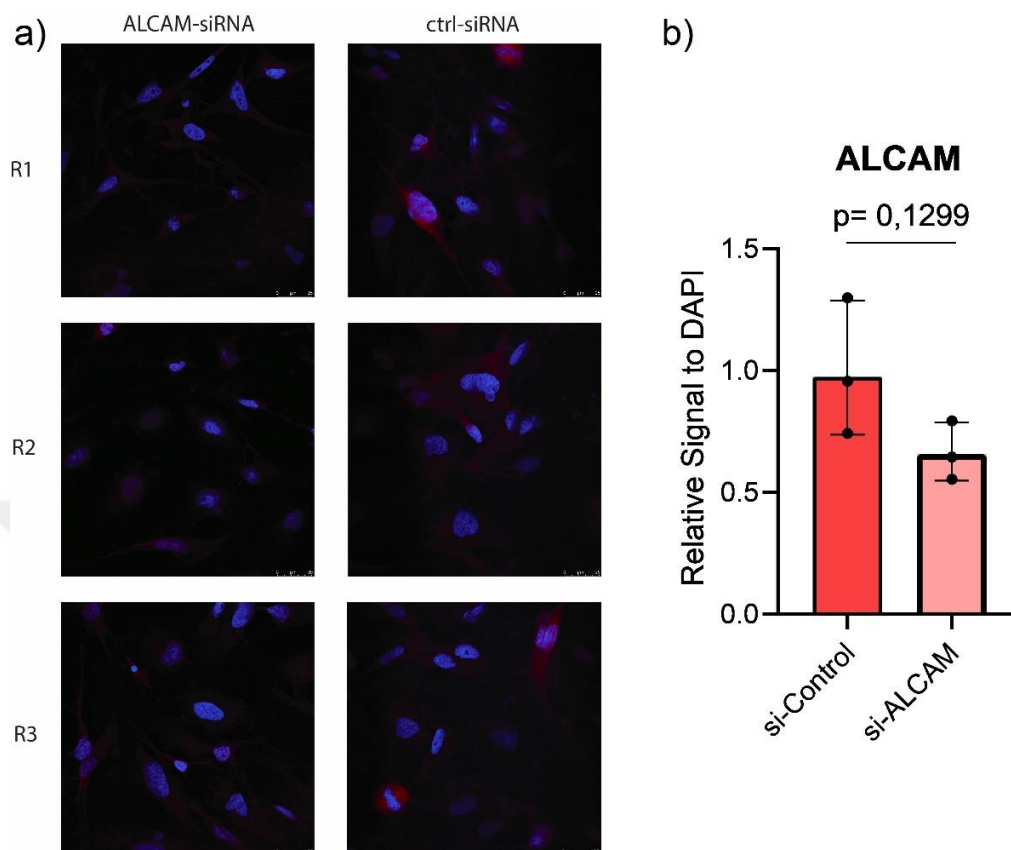


Figure 3.25. Immunofluorescence assay to detect ALCAM signal in SW1088 cell line transfected with ALCAM siRNA. (a) Confocal image was taken under 63x magnification to detect ALCAM signal coming from siRNA transfected SW1088 cell line, and (b) statistical analysis. DAPI was used as counterstaining. Relative signals were quantified using ImageJ software by calculating the ALCAM to DAPI signal ratio. Statistical analysis was performed using an unpaired student's t-test.

3.3 Investigation of senescence markers upon the manipulation of ALCAM expression.

After the analysis of the efficiency of the gene manipulating agent, both senescence markers' gene expression levels and SA- β -gal staining were investigated.

3.3.1 qRT-PCR for senescence markers

For qRT-PCR analysis, p21, p16, ki67, Lamin B1 were chosen as the most known markers, while CYR61 level was investigated since there were some clues

in the literature. As a housekeeping gene GAPDH was chosen for normalization. We could not get any signal from p16 most of the time (while the primers were working in HEK cells (see Appendix E: Figure E.1), therefore we did not include p16 in the result part.

Although there were changes in A172 cells when they are transfected with constructs, none of these differences were significant (Figure 3.26). This was also valid for the downregulation part. There were no remarkable differences in the expression of these genes (Figure 3.27).

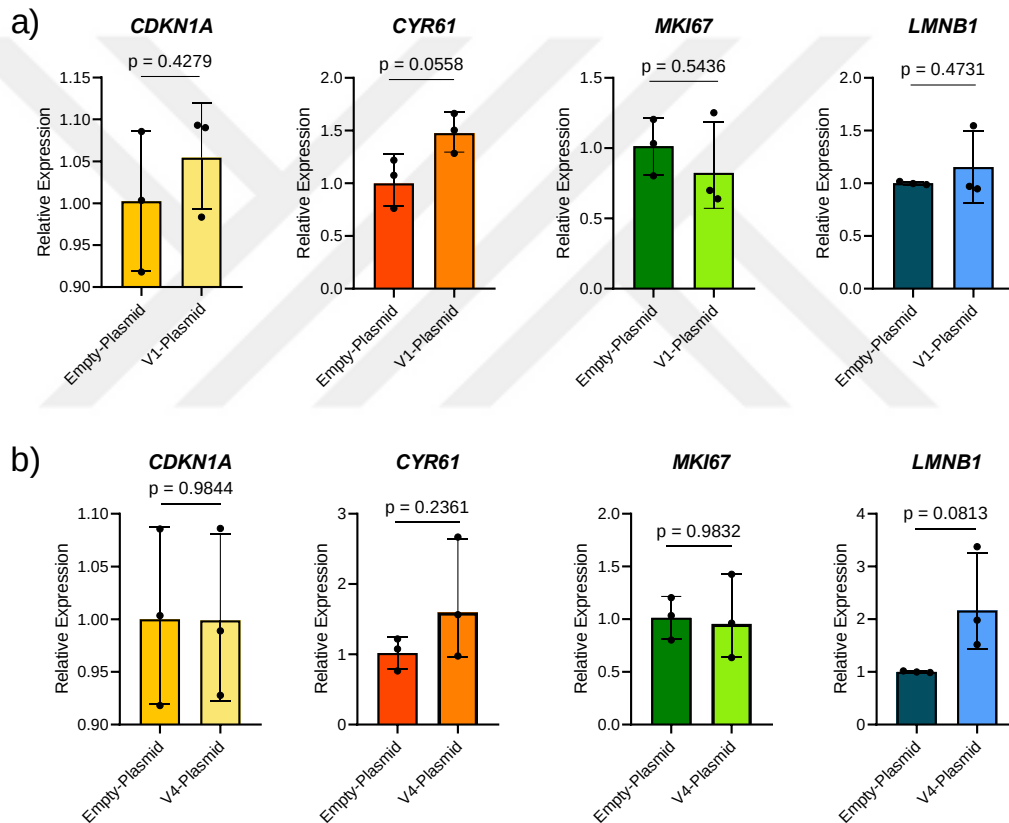


Figure 3.26. Expression changes of senescence markers upon overexpression plasmids' transfection in A172 cell line. Senescence marker expressions were measured upon (a) V1 and (b) V4 transfection in A172 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis.

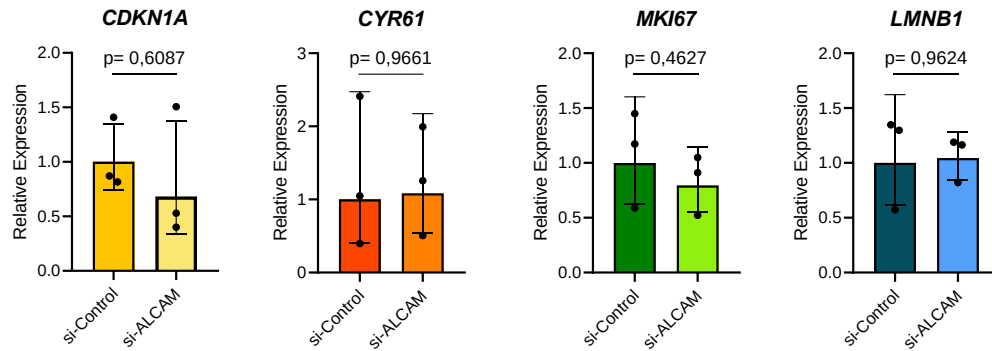


Figure 3.27. Expression changes of senescence markers in A172 cell line transfected with ALCAM siRNA. qRT-PCR results for senescence markers upon the siRNA transfection in A172 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis.

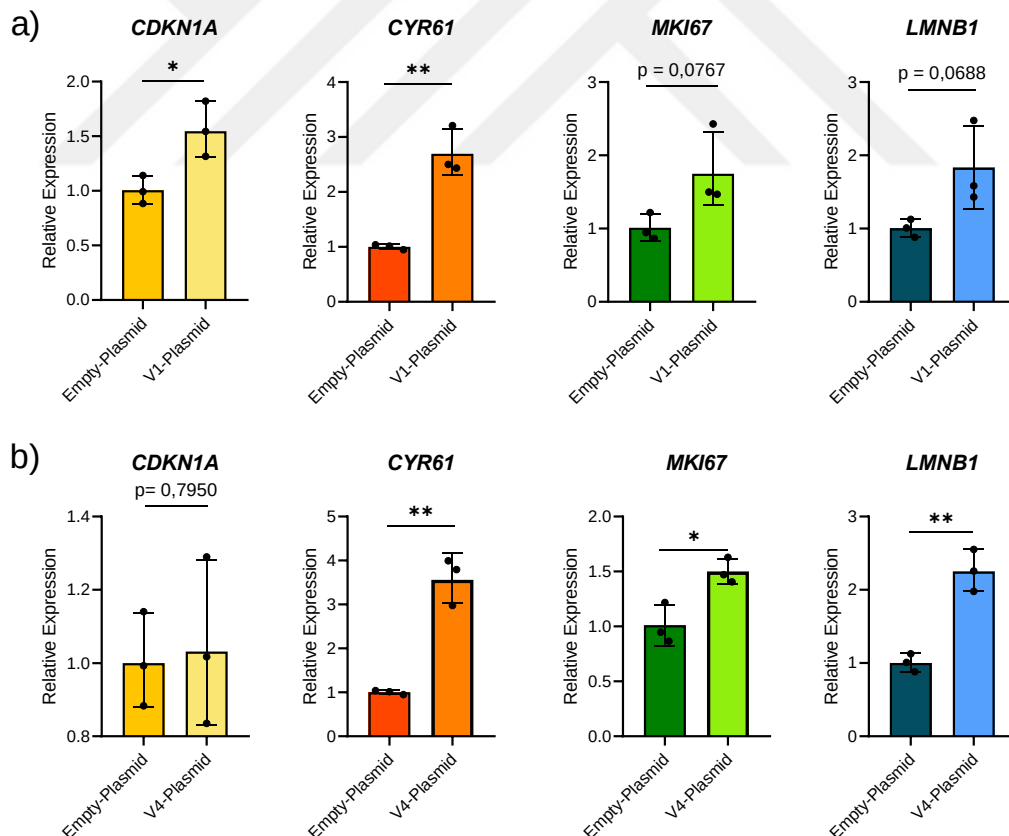


Figure 3.28. Expression changes of senescence markers upon overexpression plasmids' transfection in LN229 cell line. Senescence marker expressions were measured upon (a) V1 and (b) V4 transfection in LN229 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was

chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$; **, $p \leq 0.01$).

In LN299 cells (Figure 3.28), V1 construct increased the p21 expression (CDKN1A mRNA) significantly while V4 did not change the p21 level. Meanwhile, both V1 and V4 constructs elevated the CYR61 levels, only V4 increased the LMNB1 and ki67 mRNA levels. V1 also increased expression of the ki67 and LMNB1, however, these changes were not significant. As to downregulation of ALCAM (Figure 3.29), transfection of siRNA did not change any gene's expression.

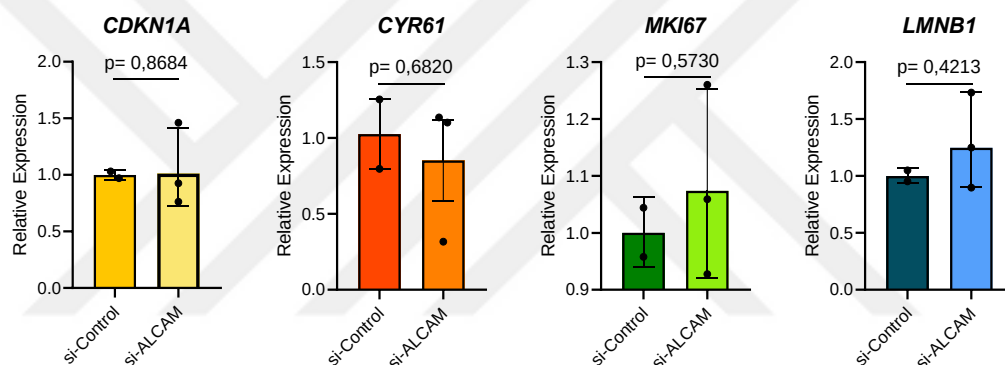


Figure 3.29. Expression changes of senescence markers in LN229 cell line transfected with ALCAM siRNA. qRT-PCR results for senescence markers upon the siRNA transfection in LN229 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis.

The last GBM cell line was T98G (Figure 3.30). The V1 transfected T98G cells showed significant increase in p21 level, while V4 did not cause any changes in p21. In CYR61 expression, lower levels was observed in cells transfected with V1, while higher levels were seen in the cells with V4. However, these remarkable changes were not statistically significant. Lastly, both V1 and V4 constructs increased the LMNB1 levels significantly. In the downregulation part (Figure 3.31), only the increase in CYR61 level was observed as significant. While the ki67 increase upon ALCAM downregulation was also observed as remarkable, the change was not statistically significant again.

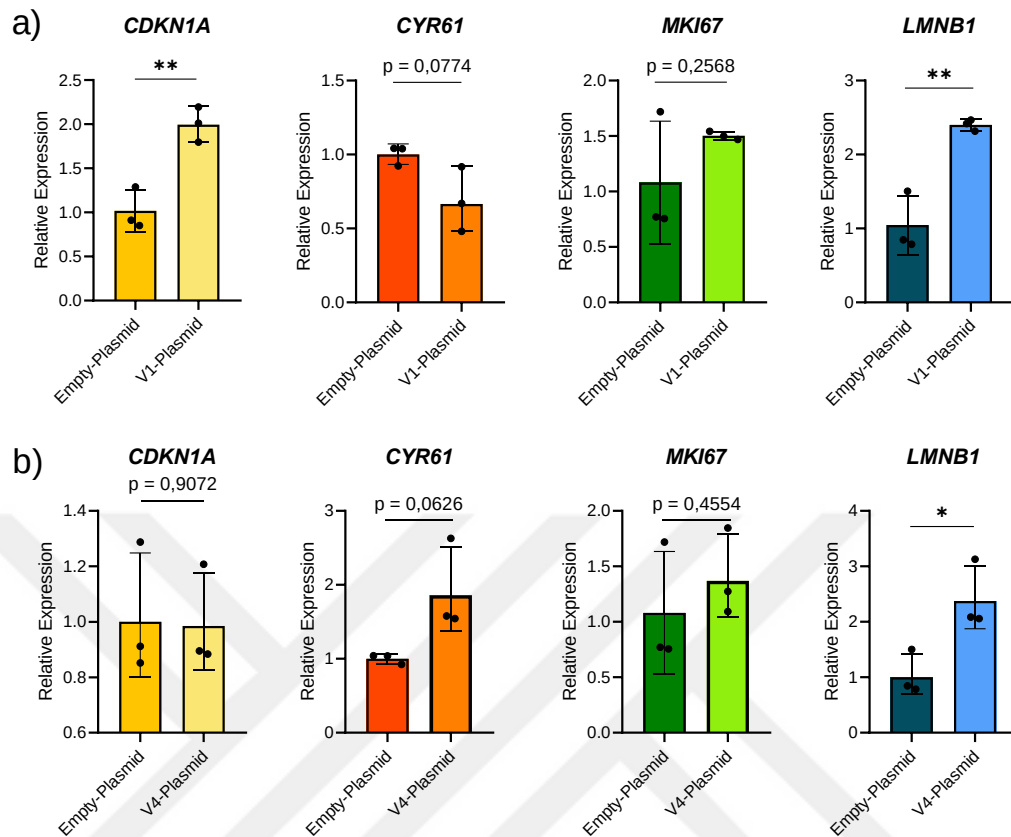


Figure 3.30. Expression changes of senescence markers upon overexpression plasmids' transfection in T98G cell line. Senescence marker expressions were measured upon (a) V1 and (b) V4 transfection in T98G cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$; **, $p \leq 0.01$).

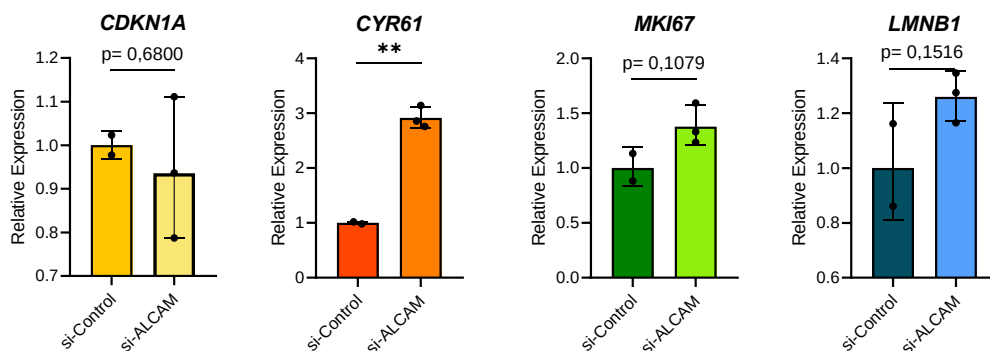


Figure 3.31. Expression changes of senescence markers in T98G cell line transfected with ALCAM siRNA. qRT-PCR results for senescence markers upon the siRNA transfection in T98G cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene

and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (**, $p \leq 0.01$).

SW1088 cell line (Figure 3.32) representing the astrocytoma cells showed opposite results in changes of p21 expression for each of the constructs. While V1 increased the p21 expression significantly, V4 decreased it. V4 plasmid also decreased CYR61 level, while there were no changes upon V1 transfection in SW1088 cells. No change was observed in ki67 expression. While there was increase in LMNB1 in the cells transfected with V1, the change was not significant like in the cells transfected with V4. Variation was so high in siRNA transfection (Figure 3.33). No information could be obtained from downregulation of ALCAM about senescence state of SW1088.

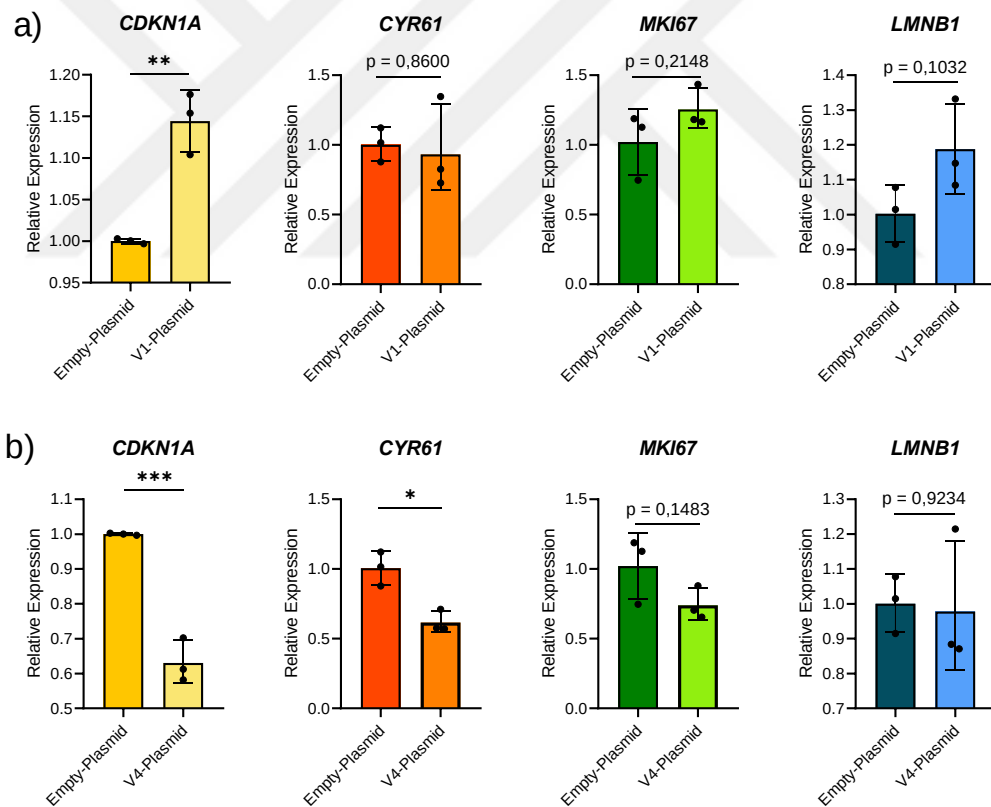


Figure 3.32. Expression changes of senescence markers upon overexpression plasmids' transfection in SW1088 cell line. Senescence marker expressions were measured upon (a) V1 and (b) V4 transfection in SW1088 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

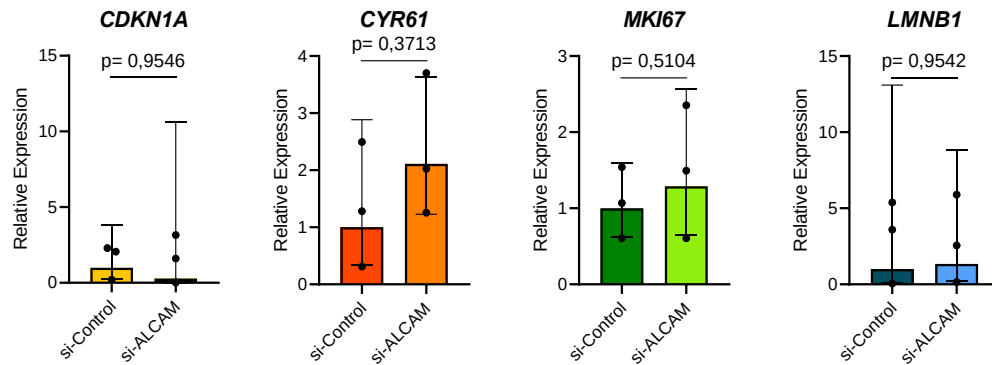


Figure 3.33. Expression changes of senescence markers in SW1088 cell line transfected with ALCAM siRNA. qRT-PCR results for senescence markers upon the siRNA transfection in SW1088 cell line. qRT-PCR was designed with 2 technical replicates for each biological replicate. GAPDH was chosen as housekeeping gene and the fold changes were calculated with the $2^{-\Delta\Delta C_t}$ formula. Unpaired student t-test was used for the statistical analysis.

3.3.2 SA- β -gal staining

Lastly, SA- β -gal staining was carried out 48h after the transfection of ALCAM level manipulating agents. The fixed cells were incubated in staining solution for overnight and the next day cells were observed under microscope at 20x magnification. The percentage of blue cells over total cells were calculated. In order to verify whether the SA- β -gal kit was working or not, Doxorubicin for A172, Azacytidine-Vidaza for SW1088 and Temazolomide for LN229 and T98G cell lines were used.

Doxorubicin treatment in A172 cells (Figure 3.34) cause increase in blue cell percentage with significancy. Given V1 plasmid increased the variant 1 level (Figure 3.35), however there was decrease in blue stained cell percentage in A172 cells. This decrease had the p value of 0,1488, which is not statistically significant. When it comes to downregulation of ALCAM, we see that ALCAM siRNA did not cause any change in SA- β -gal staining.

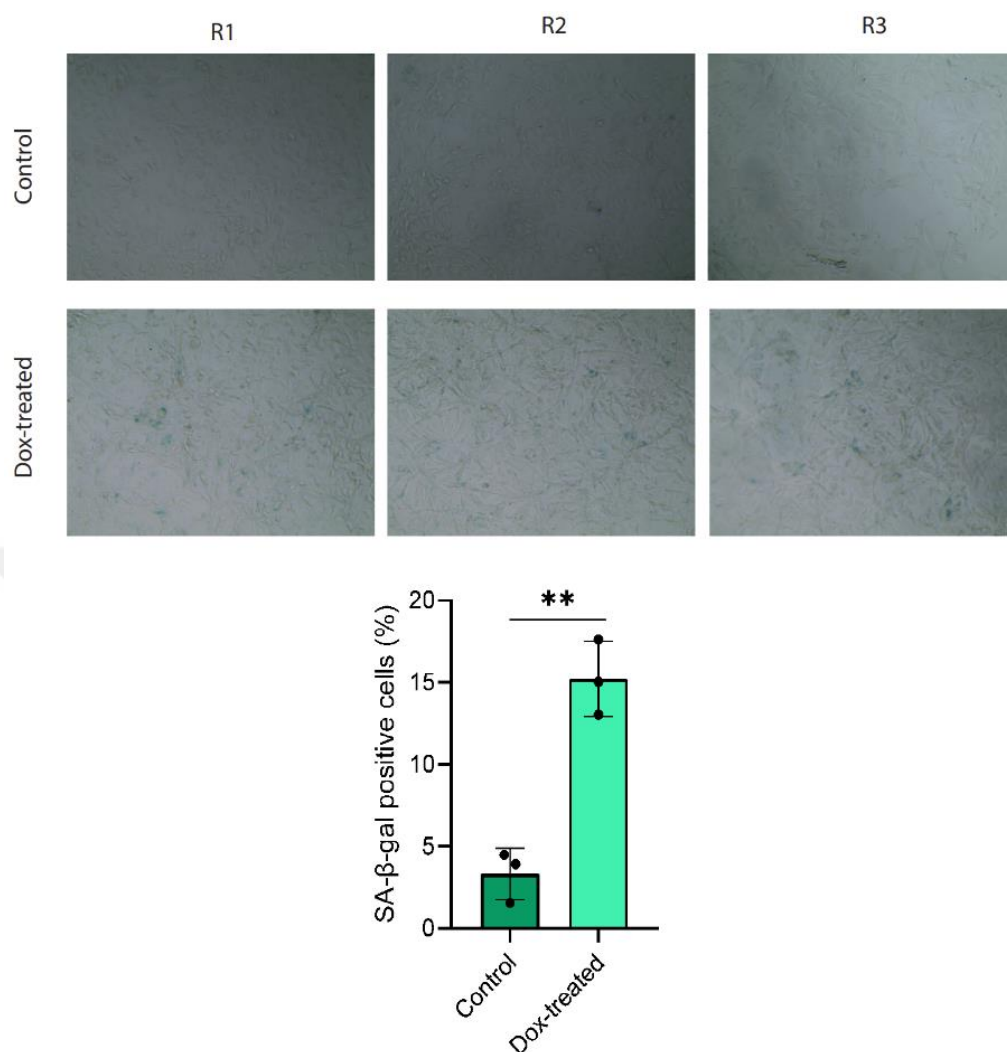


Figure 3.34. SA-β-gal staining in A172 cell line upon doxorubicin treatment. (a) Staining of doxorubicin treated and control cells as positive control of staining kit and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test (**, $p \leq 0.01$).

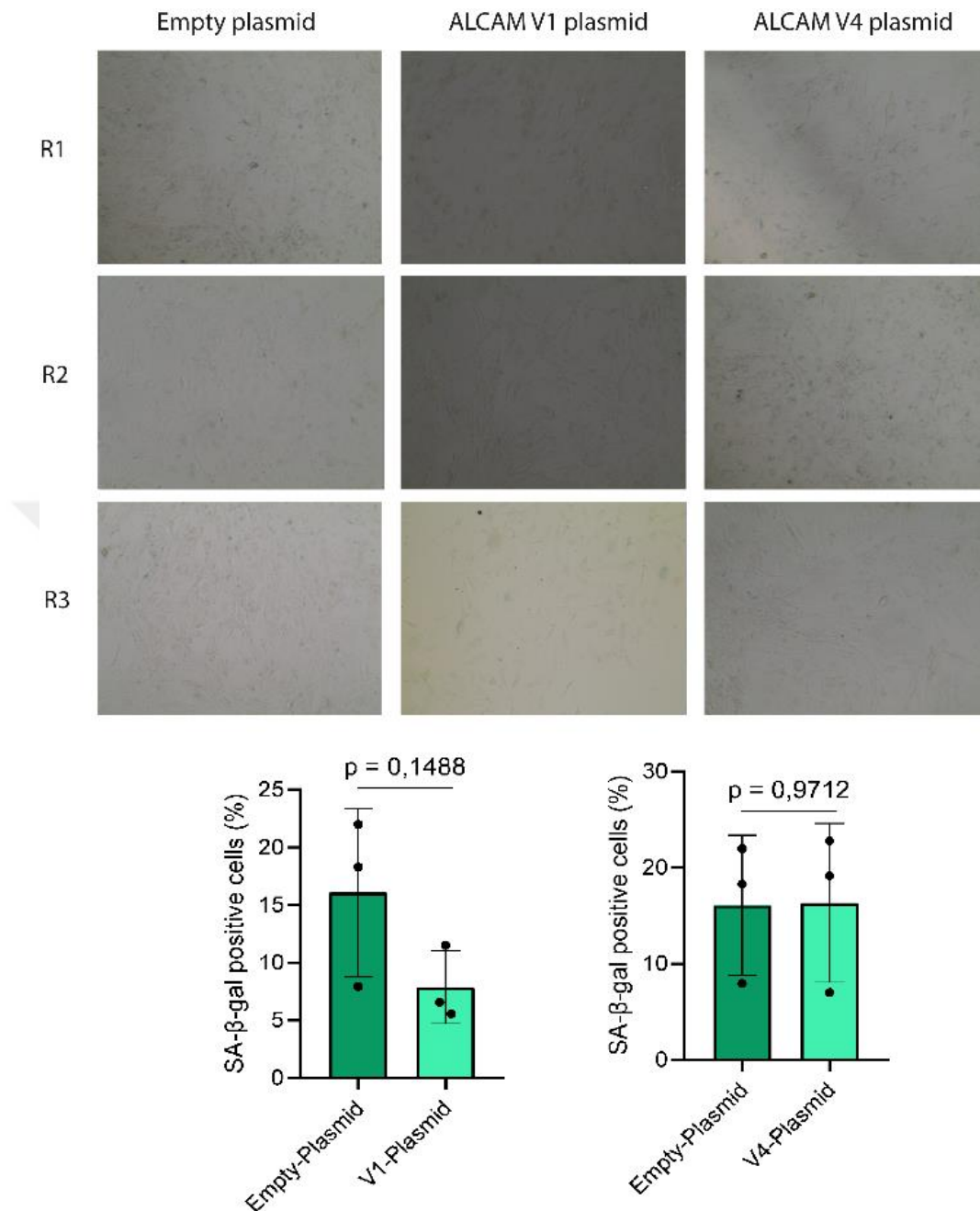


Figure 3.35. SA-β-gal staining in A172 cell line upon plasmid transfection. a) Staining image of A172 cells transfected with overexpression plasmids and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

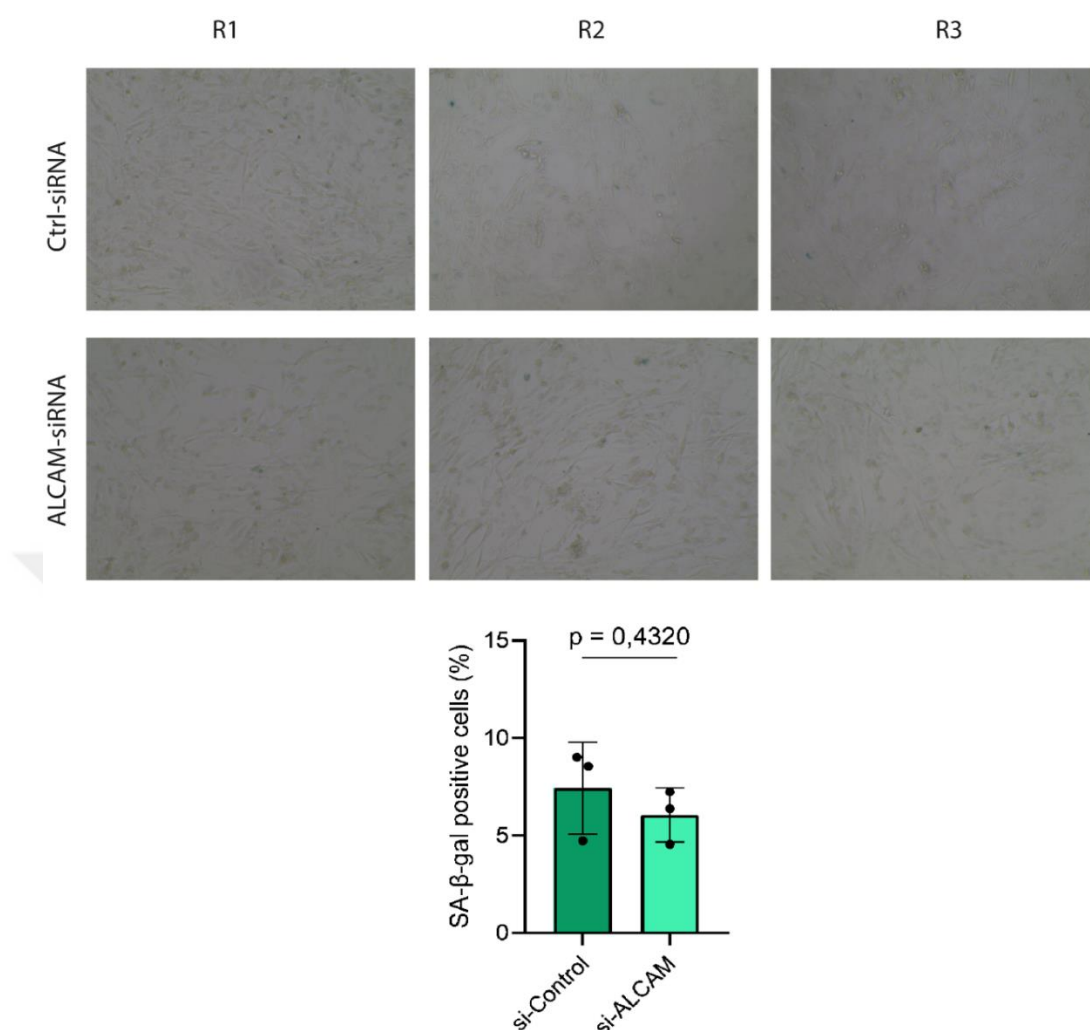


Figure 3.36. SA-β-gal staining in A172 cell line upon siRNA transfection. (a) Staining image of A172 cells transfected with ALCAM and control siRNA and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

LN229 cell line was the only cell line we observed no blue stained cell in the transfected wells (Figure 3.38, Figure 3.39). Although the variance in blue-stained cell upon temozolomide treatment was not small, since there was no stained cell in the control well at all, the increase was significant (Figure 3.37)

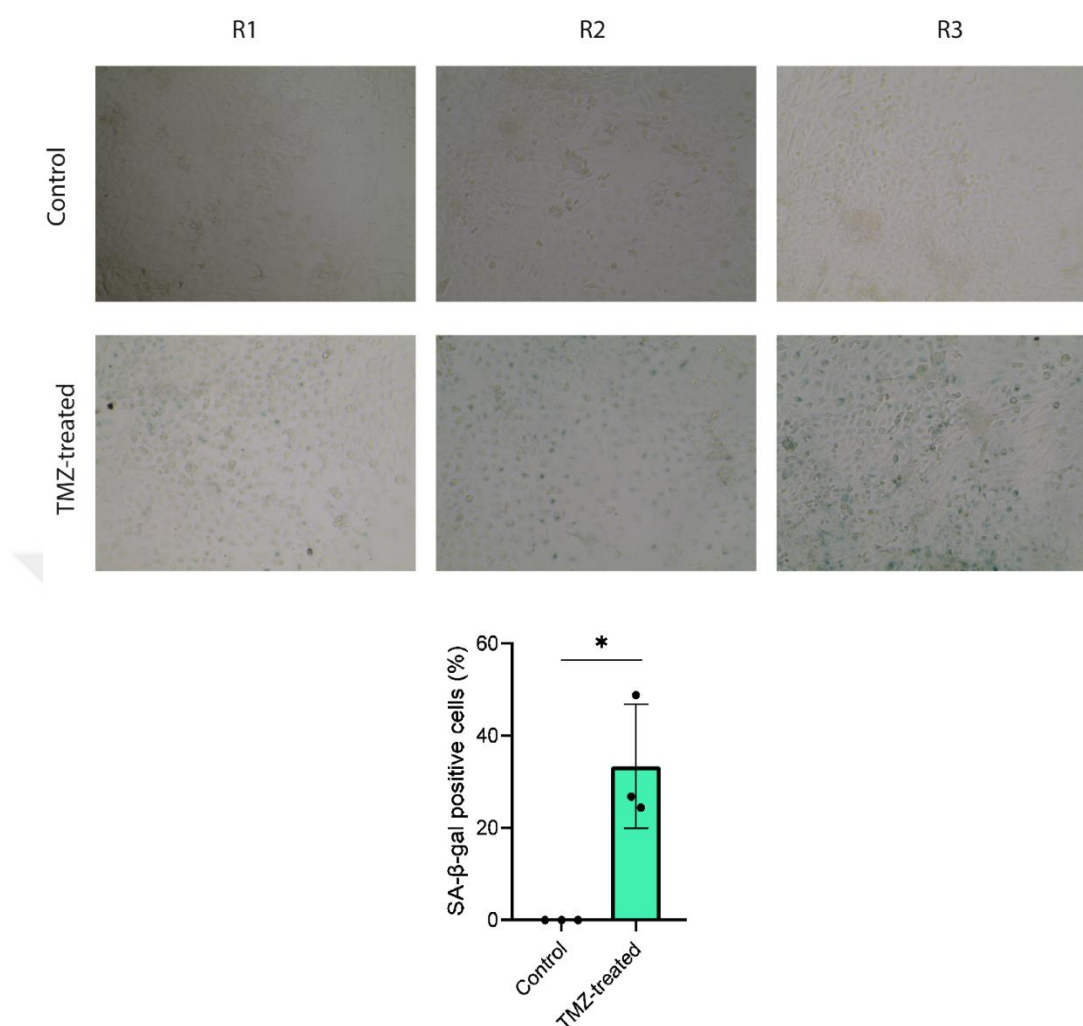


Figure 3.37. SA-β-gal staining in LN229 cell line upon temozolomide treatment. (a) Staining of temozolomide treated and control cells as positive control of staining kit and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test (*, $p \leq 0.05$).

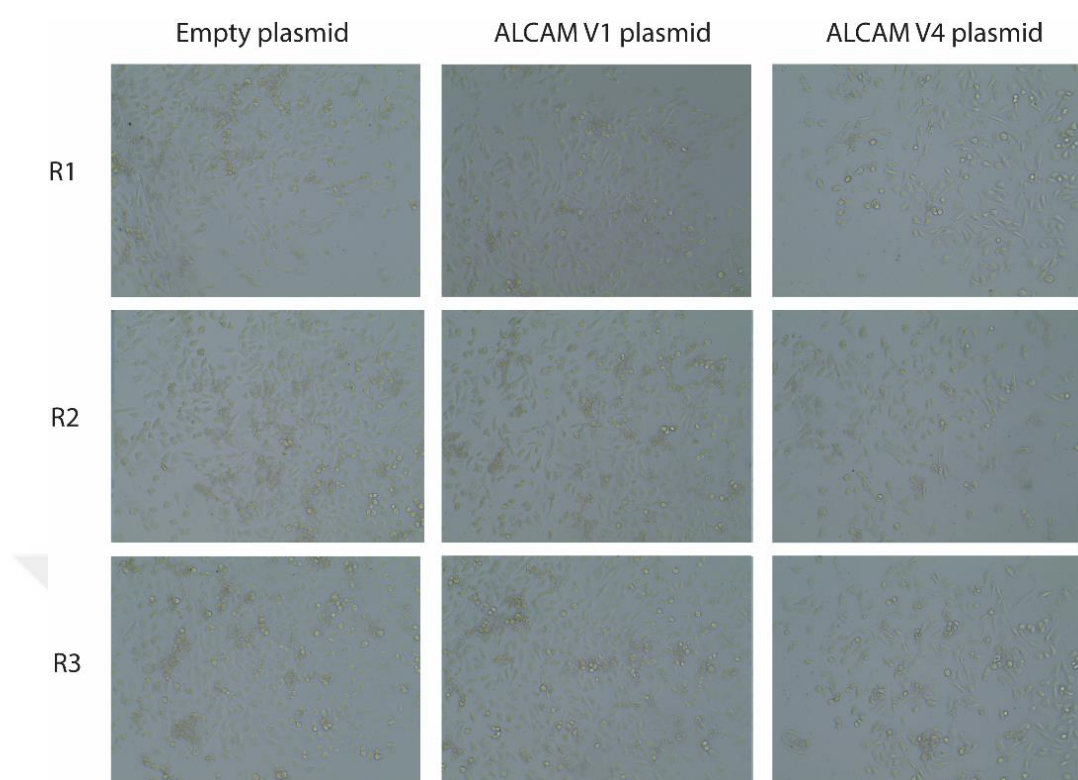


Figure 3.38. SA- β -gal staining in LN229 cell line upon plasmid transfection. (a) Staining image of LN229 cells transfected with overexpression plasmids and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

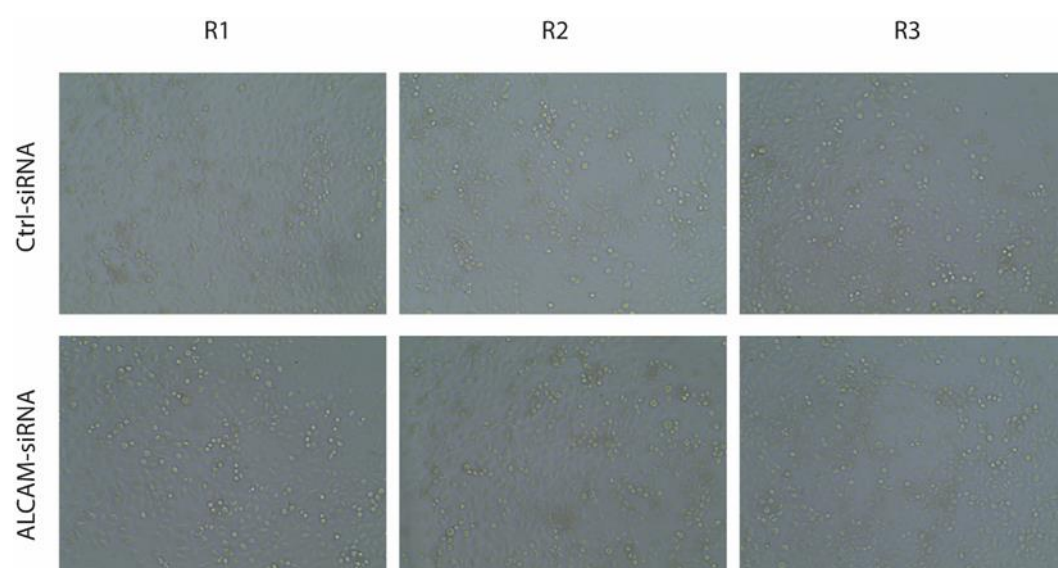


Figure 3.39. SA- β -gal staining in LN229 cell line upon siRNA transfection. (a) Staining image of A172 cells transfected with ALCAM and control siRNA and (b) the statistical analysis was shown. Both blue stained and total cell numbers were

counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

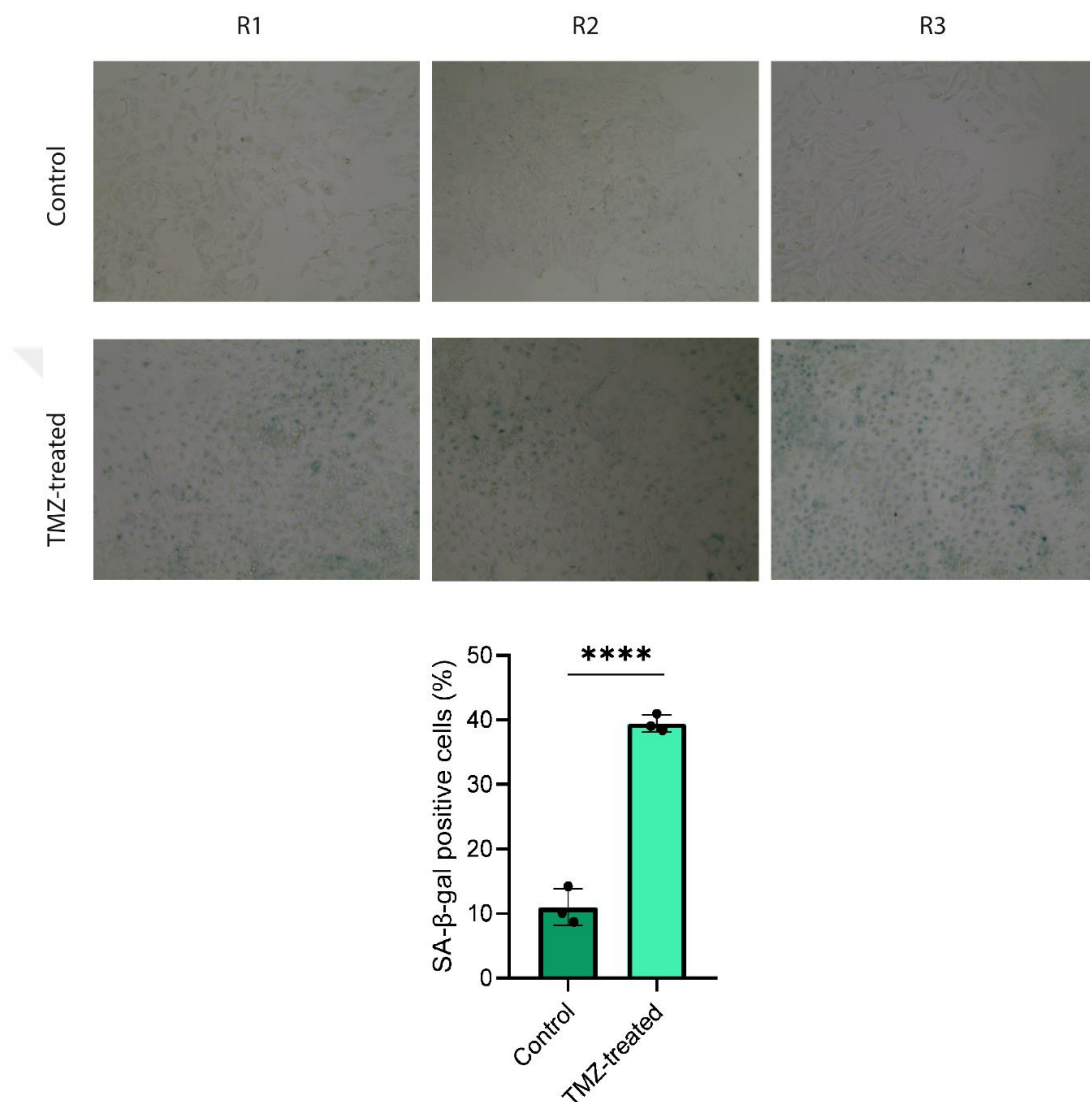


Figure 3.40. SA-β-gal staining in T98G cell line upon temozolomide treatment. (a) Staining of temozolomide treated and control cells as positive control of staining kit and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test (****, $p \leq 0.0001$).

The cell stained most was T98G. Temazolomide caused a significant increase in the percentage of SA-β-gal positive T98G cells (Figure 3.40). In addition to Temazolomide positive control, V1 construct also increased blue cells in SA-β-gal

staining (Figure 3.41). However, the V4 plasmid and siRNA did not change the difference of staining percentage between treated cells and controls (Figure 3.42).

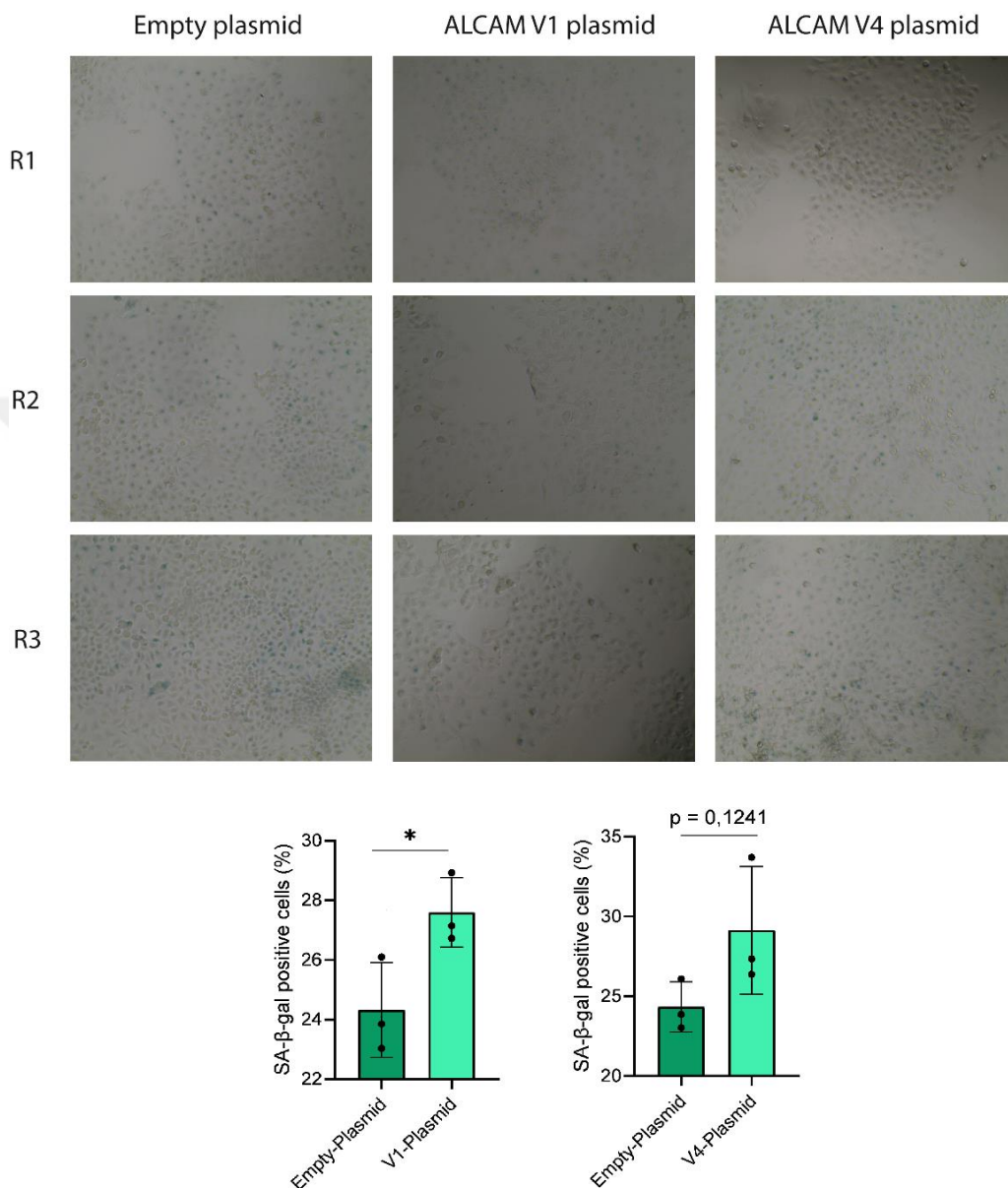


Figure 3.41. SA-β-gal staining in T98G cell line upon plasmid transfection. a) Staining image of T98G cells transfected with overexpression plasmids and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test (*, $p \leq 0.05$).

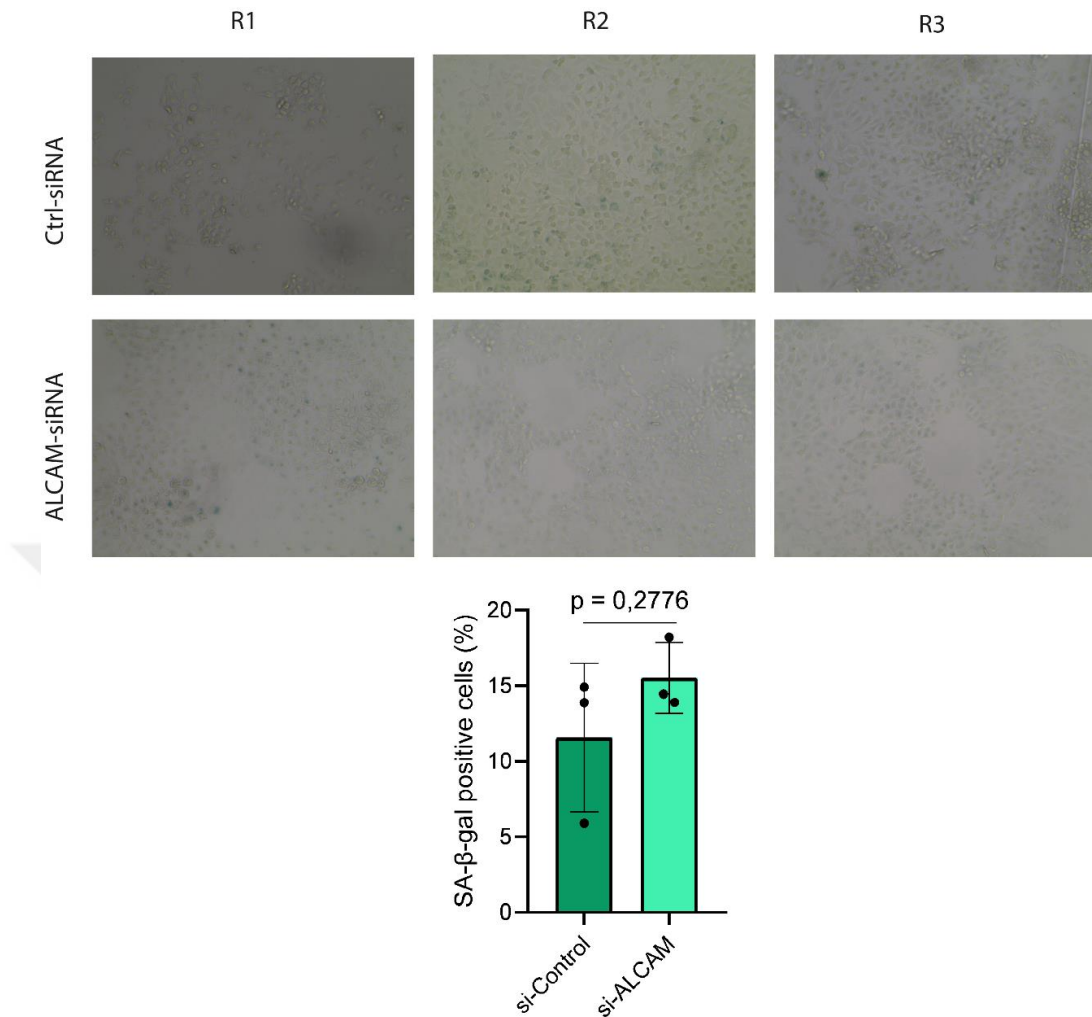


Figure 3.42. SA-β-gal staining in T98G cell line upon siRNA transfection. (a) Staining image of T98G cells transfected with ALCAM and control siRNA and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

The only cell line did not show significance for positive control was SW1088 (Figure 3.43). The increase in blue stained cell number was not significant. While V4 caused a highly remarkable increase in SA-β-gal staining ($p = 0,0522$), the difference was not statistically significant (Figure 3.44). Plasmids used to overexpress ALCAM variants did not cause any difference in ALCAM level of SW1088 (Figure 3.44).

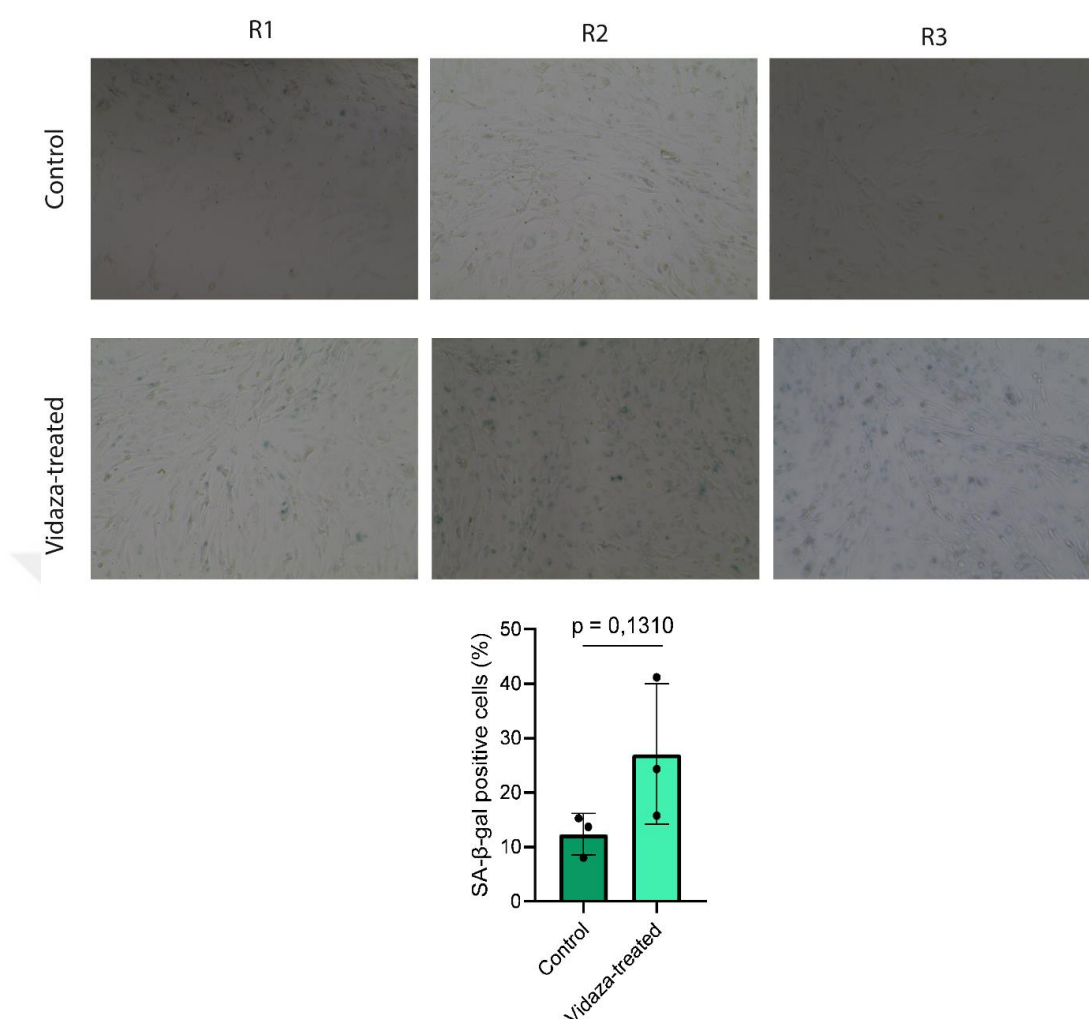


Figure 3.43. SA-β-gal staining in SW1088 cell line upon azacytidine/vidaza treatment. (a) Staining of azacytidine treated and control cells as positive control of staining kit and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

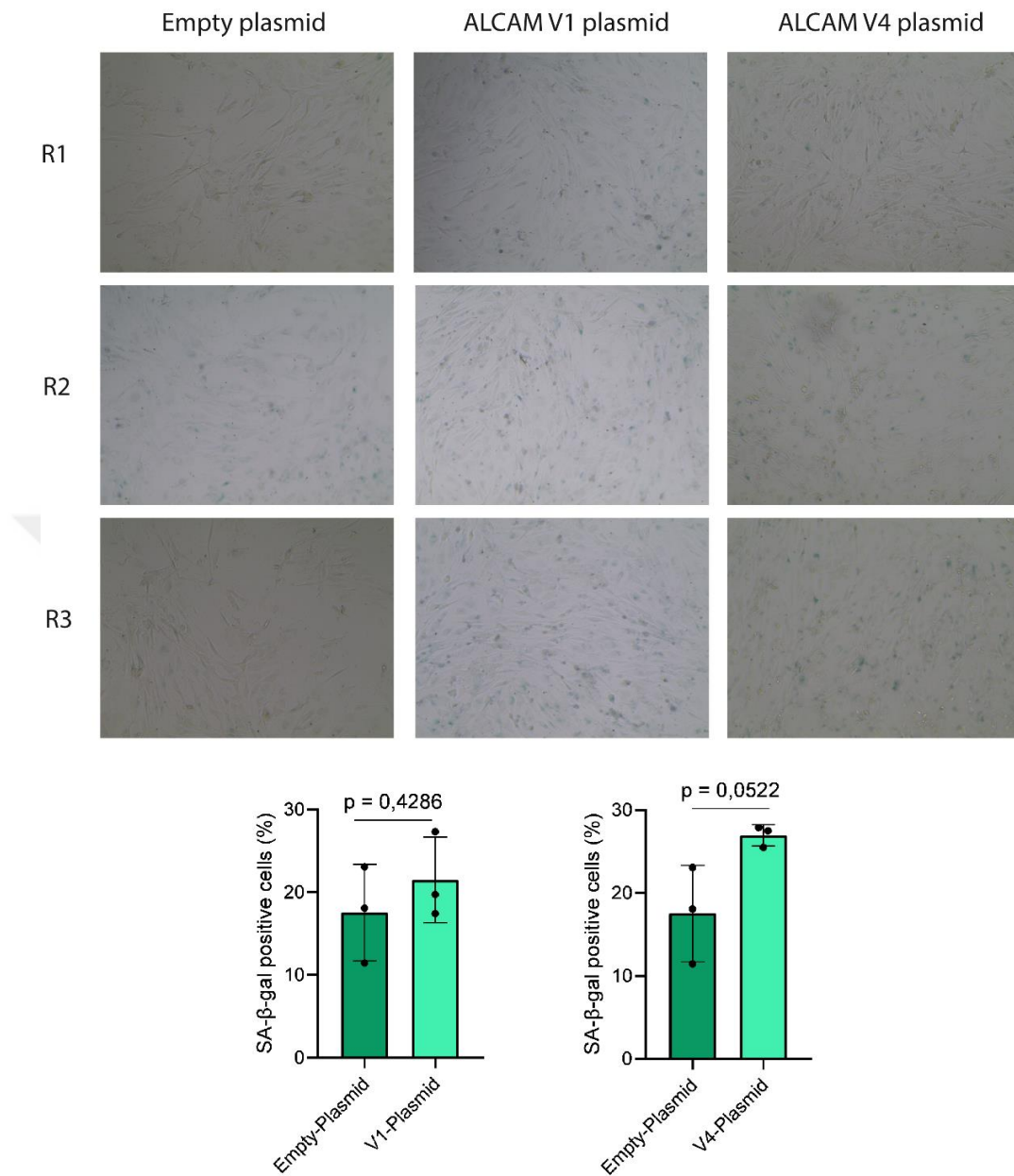


Figure 3.44. SA-β-gal staining in SW1088 cell line upon plasmid transfection. a) Staining image of SW1088 cells transfected with overexpression plasmids and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

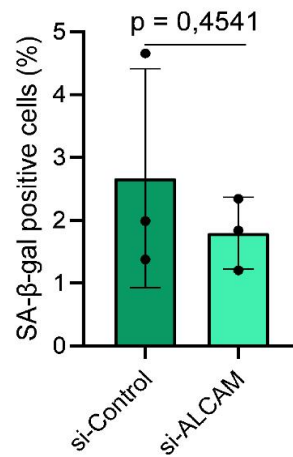
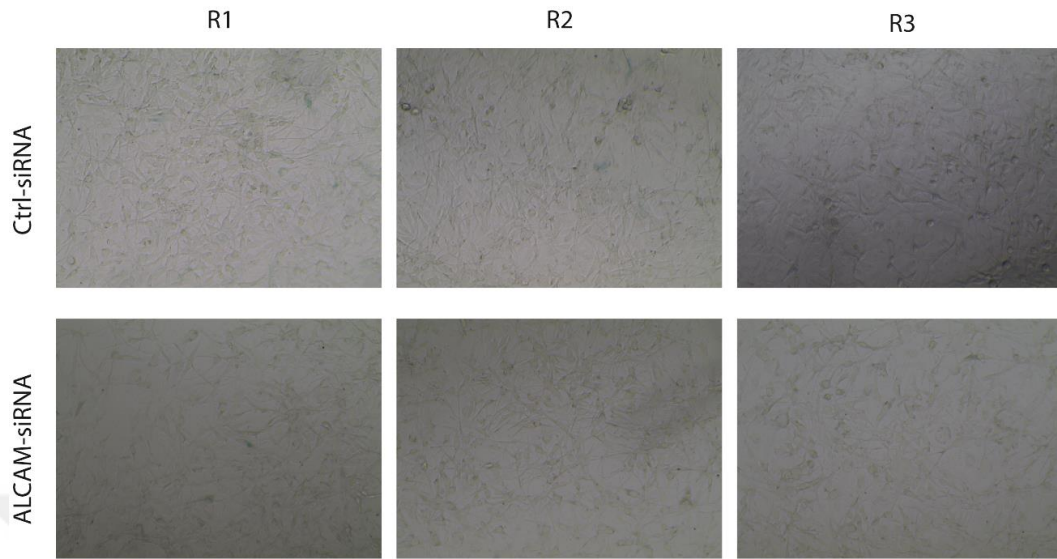


Figure 3.45. SA-β-gal staining in SW1088 cell line upon siRNA transfection. (a) Staining image of SW1088 cells transfected with ALCAM and control siRNA and (b) the statistical analysis was shown. Both blue stained and total cell numbers were counted twice by 2 different people, blind analysis was carried out by $100 \times (\# \text{Blue cell} / \# \text{Total cell})$. Statistical analysis was performed using an unpaired student's t-test.

Chapter 4

4. Discussion

GBMs are the most aggressive brain cancer which has short duration of survival time following diagnosis. The most significant reason of this result is the fact that GBM reappears after interventions such as surgery. Therefore, therapies mean to alleviate the situation of the patient and to prolong the survival time. Since cancer cells can evade senescence, restarting the senescence state in them is a well-established method for treatment.

In our previous study [105], we demonstrated that ALCAM may have a critical role in human brain aging, since we saw a decrease in *alcamb* gene (homolog of ALCAM) in zebrafish brains with increasing age. Additionally, ALCAM level was less in AD brain in comparison to healthy human brain. In line with this finding, we checked the literature and found clues that made us hypothesize that it may be possible to induce senescence by knocking-down ALCAM in GBM cells.

First, GBM has the mechanism, high telomerase expression, to escape senescence [82]. Therefore, senescence induction can be used to stop the growth of tumor. There are methods to induce senescence in GBM such as Radiotherapy [90], Tumor Treating Field [91], or manipulation of some gene expressions [93], [94]. TMZ treatment, a well-known treatment, also causes senescence in GBM cells [85]. Others even suggested that this is the main pathway of TMZ [86]. However, it was seen that TMZ-induced senescence can be escaped and these cells can re-enter to cell cycle [86]

In addition to the study of Erbaba et. al., 2020, which is our starting point, we found some clues in literature to speculate that ALCAM may have a role in senescence. Age phenotype in LT-HSCs was increased when they used ALCAM KO organism [106]. In osteoprogenitors, when the age phenotype was tried to be decreased, ALCAM level was increased in these treated cells [108]. There were

also studies showing the link between less ALCAM level and age-related neurodegenerative disease AD, although there is no direct association with senescence [112].

There were also studies showing the relationship between ALCAM and invasion, proliferation, inflammation and growth state, which we can consider as suggestions supporting that downregulation of ALCAM expression would be suitable for GBM cells. ALCAM may have role in cytokine production [64]. ALCAM inhibition or knocking down cause reduction in tumor volume, decrease in proliferation markers, less metastasis and migration [68]–[72]. ALCAM can be associated with proliferation, colony formation [66],[73]. When ALCAM level is increased, the cancer become more aggressive also [72].

In addition to these supporting references, there were studies opposing or not supporting our hypothesis. In some studies, ALCAM level was found much more in aged individuals or associated with age phenotype [106], [107]. In aging related disease AD, ALCAM was associated with AD and AD severity [109]–[111], [113]. In the study, miR-142 KO mouse was used, in addition to Alcam, Tgfbr1 gene was also upregulated, which is associated with senescence induction [112], [114]. In pancreas cancer cells, the CD166- and CD166+ cells were compared, and it was seen that the former's ability to invade and migrate was higher than the latter's [73].

When it comes to ALCAM mediated senescence induction in GBM, we cannot say there were direct and consistent evidence. Kijima et al. published an article in 2012 in which they demonstrated that the lifespan of patients with higher ALCAM level was less than the patients with lower ALCAM level (<60). They first analyzed patient derived GSCs with ALCAM+CD133+ markers. It was also shown that ALCAM presence increased the colony formation ability. They increased the soluble ALCAM level and saw that the tumor prognosis deteriorated. On the contrary of expectations, when ALCAM level is decreased in commercially available GBM cell lines, they became more aggressive. All these results made ALCAM an important target. They did not perform any senescence related assays [96]. When ST6GAL1 adding sialic acid to ALCAM is decreased, it increases the ADAM17 level [102]. In response to that ALCAM level is decreased and GBM initiating cells become less aggressive and invasive [102]. Again, in GSC subpopulation having CD44+CD9+CD166+ markers is seen as important for the tumor growth [104].

There were articles which are about ALCAM in GBM and not supporting our idea also. Wang et al. in 2022 analyzed TCGA data and found that ALCAM protein protect patients in the manner of survival. Allmendinger et al., 2012 showed that total ALCAM level was decreased with the increased aggressive nature of GBM and ALCAM+ GBM enable patients to survive longer. Again, the result of another study showed that ALCAM expression was associated with longer survival [98], [99].

The association of ALCAM with aging in human cells has not been directly demonstrated before. One of our goals was to be the first study to demonstrate this. Our long-term goal was to increase life expectancy and quality of life after diagnosis in patients with GBM. If the barriers to brain cancer treatment such as the blood-brain barrier and cancer heterogeneity are removed with advancing technology, every study in literature will become even more meaningful. In line with these clues and purposes, we manipulated ALCAM level in GBM cell lines using both ALCAM targeting siRNA and ALCAM overexpressing plasmid. We transfected these agents to the cells and showed if they worked as they were supposed to do.

In A172 cell line, transfection of plasmids was not efficient when the cells were observed under microscope. There were very few EGFP signal. However, when the mRNA level of ALCAM variant were investigated, there was increase in ALCAM level in total. Moreover, when variant 1 was given only variant 1 level was increased and same is valid for variant 4 also. However, we can see the same efficiency in protein level, both in confocal and western-blot images the ALCAM level increase was not that much persuasive. Therefore, in plasmid transfection, we can not see the true effect of ALCAM overexpression. It only contributes through mRNA interaction. One could claim that the overexpressed protein could aggregate inside of the cell, but the confocal images oppose that idea. When it comes to downregulation of ALCAM, we saw that both total ALCAM and variant 1 level decreased significantly. Moreover, we see the same effect in protein level with immune assays. The only doubtful part is variant 4. We cannot make sure (not only in this cell line but also in all) whether we can see the true ALCAM variant 4 changes or not since we could not detect the protein in immunoblotting. During optimization of ALCAM antibody, we used SH-SY5Y cells as positive control with 25 µg protein. When we closed the 105 kDa band and image the membrane again

we could detect the other variants (see Appendix G: Figure G.1). However, in these lysates we could not detect. The reason can be the normal or low level in non-positive control cell lysates, the transfer time (we increased the transfer time since we changed the buffer content) or both for the siRNA transfection case. In general, we can say siRNA worked while plasmid could not fully fulfill its job, which probably due to low efficiency.

In LN229 cells, the plasmid efficiency was seen under microscope (see Appendix C). We also get increased level of total ALCAM upon plasmid transfection. The only interesting part was there was also increase in variant 1 upon variant 4 transfection. There can be a positive feedback loop to see this effect in LN229. Unfortunately, variant 4 plasmid was not that efficient since no significant change could be detected in mRNA level. When it comes to translation of the mRNAs transcribed from these plasmids, one cannot claim that the plasmids cause an increase in protein level. Neither western blot assay nor confocal image showed the increase significantly (still considerable for confocal). Unlike plasmid transfection, siRNA had significant influence both in mRNA manner and protein (western-blot). Interestingly the ALCAM variant 4 level increased but that was not significant also. During transcription of variant 1 mRNA, siRNA start to target it while still the transcription continues. Then this situation can change the splicing situation. Overall, this siRNA pool could not decrease the ALCAM variant 4 efficiently in all cell lines. In brief, siRNA works efficiently, while plasmid results are suspicious.

The last GBM cell line was T98G. Plasmid transfection was successful. It could increase the ALCAM level in general. Again, there was a positive feed back loop since given V4 increased variant 1 level. The only result needed a speculation was the fact that V1 given T98G cells decreased variant 4 expression. Somehow, splicing proteins must have worked more efficiently when the ALCAM variant was given. Western-blot results demonstrated that plasmid also worked in protein level. Although the increase shown in immunocytochemistry was not significant, the western-blot result was persuasive. Downregulation in ALCAM level was only significant for variant 1 and 3. The immunocytochemistry signal for ALCAM decreased upon siRNA transfection but that was not statistically significant. However, the western-blot result showed a significant decrease in ALCAM level. In brief one can say, in T98G both plasmid and siRNA transfection worked in the way they were supposed to do.

The only astrocytoma cell line was SW1088. The EGFP signal was remarkable. ALCAM V1 works efficiently for total and variant 1 ALCAM. ALCAM V4 increased variant 4 level remarkably, however that was not significant. Unfortunately, there was no increase in western-blot and since immunocytochemistry analysis can be biased we can not make sure if the increase in ALCAM proteins were enough to cause a difference. Both mRNA and protein difference were seen in siRNA transfection, they were not significant. In brief, plasmid transfection was more efficient than the siRNA transfection.

As a next step, senescence markers were investigated. All the senescence markers except the p16 were detected. The reason why we could not detect p16 in these cell lines can be the low or no expression at all. For instance Cellosaurus says T98G and LN229 have CDKN2A deletion. Prior to experiments the Depmap and Cellosaurus platforms had been checked and we could not find any clue about the presence of p16 deletion in A172 and SW1088. Since we first started our experiments with A172 and SW1088 cell lines, we performed qPCR analysis for T98G and LN229, although we did not expect any signal. However, after seeing no signal not only in these cells but also in A172 and SW1088 we have deepened our search about the mutations in them. We found that these 2 cell lines also have the CDKN2A deletion (One can check the The Brain Cancer Cell Panel (ATCC® TCP-1017™)).

A172 cells did not demonstrate any significant difference in qPCR results of senescence markers. Still, there were considerable differences in CYR61 protein upon variant 1 increase and LMNB1 protein upon variant 4 increase. These findings normally suggest that the senescence phenotype increases, which opposes our hypothesis. However, SA- β -gal staining did not support this idea. Yet, we already said plasmid transfection did not work efficiently in A172, so the results do not give any information. When it comes to downregulation of ALCAM, it did not change any marker in A172.

LN229 cells showed a significant increase in CYR61 protein upon both variant 1 and 4 increase in the cells. In addition to increase in CYR61, p21 level was also increased upon variant 1 elevation. These results support the opposite of our hypothesis, which says this cell can be in senescent stage, while the ki67 and LMNB1 levels demonstrate the cells become much more aggressive and

mechanosensitive. The downregulation of ALCAM did not give any information to be speculated on. Unfortunately, the must have marker SA- β -gal staining did not work efficiently in LN229.

The cell line in which both plasmid and siRNA transfection worked most effectively was T98G. LMNB1 increase supports our hypothesis while p21 does not. Like in LN229, the variant 1 increase caused p21 level increase. Moreover, the staining result also say that increase in variant 1 cause senescence. When the manipulated signaling was CYR61 related pathway, the variant 4 and 1 acted differently in patient derived glioblastoma cells (GSE29384). In this microarray assay carried out in glioblastoma, the variant 4 and 1 levels changed in opposite directions upon the CYR61 protein. The CYR61 changes in T98G cells were not statistically significant but considerable still and it resembles the same result of glioblastoma cells. While they can be interpreted as supporting elements for the hypothesis, change in response to variant 4 increase contradicts with the literature findings like soluble form of ALCAM cause aggressiveness in cancer. The only significant change in ALCAM silencing was in CYR61 mRNA. However, the silencing of ALCAM also elevated the CYR61 mRNA level, which supports our hypothesis that senescence phenotype increases with ALCAM reduction.

The last cell line was SW1088. The results which may pave the way for other projects is the p21 level change in response to plasmid transfection. While V4 decreased the p21 level, V1 did the opposite. These results say that soluble form of ALCAM can help cells to escape senescence, while membrane form increases the change of being a senescent cell. CYR61 decrease upon the V4 transfection supports the idea that soluble ALCAM decreases senescent cell number in the population. Surprisingly, these results disagree with the staining result. SA- β -gal staining of cells given with V4 was considerably much more than the empty plasmid given cells, indicating the senescence is induced in cells with variant 4 overexpression. Since the variation in siRNA results was too high, none of them give any information.

To summarize, the results were not consistent both among different cell lines and in the same cell line. ALCAM level change causes alterations in GBM cells. However, whether these changes are related to senescence or not is suspicious. The reason why we could not get consistent results can be the heterogeneity of GBM,

the lack of true tumor microenvironment, or need for further experiments to explain the meaning of the results.

GBM is highly heterogeneous tumor. In vitro studies using commercially available cell lines has less heterogeneity than the real tumors [115], but still they are also heterogeneous [116]. Both cancer lines and the tumor has cancer stem cells and most of the references we considered to hypothesize that ALCAM reduction can induce senescence was in GSCs. Since we have not separated the cells with flow-cytometry, the reason why we see results disagreeing with each other even in the same cell line can be heterogeneity. In addition to cancer stem cells, patient derived cells have not only tumor or cancer stem cells but also T cells having CD6 receptor of the ligand CD166, macrophages and fibroblasts [117]. At least to see the effect of ALCAM receptor, one can carry out co-culture of T cells and manipulated GBM cells.

Another factor is the microenvironment. The best example which can account for this potential reason is the study of Kijima et al. They analyzed patient derived tumors, suggested ALCAM reduction can enhance the prognosis in GBM, then they used cell lines, and they did in vivo experiments. While the hypothesis they had did not come true in in vitro study, it did in in vivo study. Since we did not create true tumor microenvironment, or we did not perform any co-culture. One can carry out xenografts to create a similar microenvironment for the cells. The first plan was also to inject the manipulated cells to zebrafish embryos to see at least the migration of them through organism. However, since the results were not consistent, we did not want to sacrifice any zebrafish.

In order to understand the effect of ALCAM change we need to perform further analysis. To see invasion, one can perform matrigel transfer assay. To make sure colony forming ability colony formation assay should be performed. As mentioned above, senescence marker variance can be improved. H2AX (γ H2AX) and phosphorylated p53 are DNA damage markers. Although ALCAM probably do not create any DNA damage they could still give additional information. As mentioned above, SASP genes' expression levels could give more comprehensive information. Apoptosis could be measured with BCL protein expressions.

The reason why we see ALCAM is lower in older brains or in Alzheimer's brains in our starting point [105] could be explained by examination of the whole brain. It

is not the astrocytes where glioblastoma comes from, but perhaps the decreasing number of neurons in Alzheimer's and old age may have caused these results. Such a speculation is quite plausible given the high expression of ALCAM in neurons [65]. Moreover, to be able to confirm that the decrease in ALCAM is the reason of senescence rather than the result of aging, we need much more evidence. For curiosity, we also checked the ALCAM level in A172 cells treated by doxorubicin, however there was no difference between the dox treated and control cells (see Appendix H: Figure H.1).



Chapter 5

5. Conclusion & Future Aspects

Our study aimed to elucidate the role of ALCAM in GBM cells, particularly in relation to senescence induction. Despite some promising references, our hypothesis did not come true. Our results were not consistent across different cell lines or within the same cell line. While ALCAM manipulation did cause alterations in GBM cell behavior, the specific connection to senescence remains unclear. Factors such as GBM heterogeneity, the lack of a true tumor microenvironment, and the need for more refined experimental approaches likely contributed to the inconsistent results. Our findings suggest that while ALCAM may play a role in GBM pathology, its exact function, particularly in the context of senescence, requires further investigation.

Future research should address the limitations encountered in this study to better understand the role of ALCAM in GBM. Co-culturing, sorting GBM cell lines according to cell surface markers, using primary cultures, using xenografts, and additional assays to see apoptosis, autophagy and senescence balance would help improve the quality of the research. In addition to that, combinatorial effect of ALCAM with known treatment methods can also be investigated to see if ALCAM manipulation would increase the efficiency of already established treatment methods. These steps will help clarify the potential of ALCAM as a therapeutic target and improve strategies for GBM treatment.

Although it will not explain ALCAM's senescence association in glioblastoma, our literature review suggests that ALCAM may be an effective target gene in breast cancer. Although ALCAM reduction has been examined many times in breast cancer, none of the results have been associated with senescence so far. It will be a new contribution to the literature to see if there is a senescence link behind the role of ALCAM in breast cancer.

Bibliography

- [1] J. Huang, B. Gholami, N. Y. R. Agar, I. Norton, W. M. Haddad, and A. R. Tannenbaum, "Classification of Astrocytomas and Oligodendrogliomas from Mass Spectrometry Data Using Sparse Kernel Machines," *Conf. Proc. IEEE Eng. Med. Biol. Soc.*, vol. 2011, p. 7965, 2011, doi: 10.1109/IEMBS.2011.6091964.
- [2] F. B. Mesfin and M. A. Al-Dhahir, "Gliomas," *StatPearls*, May 2023.
- [3] A. Haque, N. L. Banik, and S. K. Ray, "MOLECULAR ALTERATIONS IN GLIOBLASTOMA: POTENTIAL TARGETS FOR IMMUNOTHERAPY," *Prog. Mol. Biol. Transl. Sci.*, vol. 98, no. C, p. 187, 2011, doi: 10.1016/B978-0-12-385506-0.00005-3.
- [4] A. Liu, C. Hou, H. Chen, X. Zong, and P. Zong, "Genetics and Epigenetics of Glioblastoma: Applications and Overall Incidence of IDH1 Mutation," *Front. Oncol.*, vol. 6, no. JAN, p. 1, 2016, doi: 10.3389/FONC.2016.00016.
- [5] I. Alnahhas *et al.*, "Characterizing benefit from temozolomide in MGMT promoter unmethylated and methylated glioblastoma: a systematic review and meta-analysis," *Neuro-oncology Adv.*, vol. 2, no. 1, pp. 1–7, Jan. 2020, doi: 10.1093/NOAJNL/VDAA082.
- [6] M. A. Azab, "The Potential Role of Histone Modifications in Glioblastoma Therapy: Review Article," *J. Mol. Pathol.* 2023, Vol. 4, Pages 196–212, vol. 4, no. 4, pp. 196–212, Sep. 2023, doi: 10.3390/JMP4040018.
- [7] F. Seker-Polat, N. P. Degirmenci, I. Solaroglu, and T. Bagci-Onder, "Tumor Cell Infiltration into the Brain in Glioblastoma: From Mechanisms to Clinical Perspectives," *Cancers (Basel)*, vol. 14, no. 2, Jan. 2022, doi: 10.3390/CANCERS14020443.
- [8] N. A. Pullen, M. Anand, P. S. Cooper, and H. L. Fillmore, "Matrix metalloproteinase-1 expression enhances tumorigenicity as well as tumor-related angiogenesis and is inversely associated with TIMP-4 expression in a model of glioblastoma," *J. Neurooncol.*, vol. 106, no. 3, pp. 461–471, Feb. 2012, doi: 10.1007/S11060-011-0691-5.
- [9] K. K. Veeravalli and J. S. Rao, "MMP-9 and uPAR regulated glioma cell migration," *Cell Adh. Migr.*, vol. 6, no. 6, p. 509, Nov. 2012, doi: 10.4161/CAM.21673.
- [10] C. F. Tsai, W. L. Yeh, J. H. Chen, C. Lin, S. S. Huang, and D. Y. Lu, "Osthole Suppresses the Migratory Ability of Human Glioblastoma Multiforme Cells via Inhibition of Focal Adhesion Kinase-Mediated Matrix Metalloproteinase-13 Expression," *Int. J. Mol. Sci.* 2014, Vol. 15, Pages 3889–3903, vol. 15, no. 3, pp. 3889–3903, Mar. 2014, doi: 10.3390/IJMS15033889.
- [11] E. Bae *et al.*, "Integrin $\alpha\beta 1$ promotes vessel formation of glioblastoma-associated endothelial cells through calcium-mediated macropinocytosis and lysosomal exocytosis," *Nat. Commun.* 2022 131, vol. 13, no. 1, pp. 1–15, Jul. 2022, doi: 10.1038/s41467-022-31981-2.
- [12] P. Franco *et al.*, " αV -Integrin-Dependent Inhibition of Glioblastoma Cell Migration, Invasion and Vasculogenic Mimicry by the uPAcyclin

Decapeptide,” *Cancers (Basel)*, vol. 15, no. 19, Oct. 2023, doi: 10.3390/CANCERS15194775/S1.

[13] L. Malric *et al.*, “Interest of integrins targeting in glioblastoma according to tumor heterogeneity and cancer stem cell paradigm: an update,” *Oncotarget*, vol. 8, no. 49, p. 86947, Oct. 2017, doi: 10.18632/ONCOTARGET.20372.

[14] R. C. Gimple, S. Bhargava, D. Dixit, and J. N. Rich, “Glioblastoma stem cells: lessons from the tumor hierarchy in a lethal cancer,” *Genes Dev.*, vol. 33, no. 11–12, p. 591, Jun. 2019, doi: 10.1101/GAD.324301.119.

[15] M. Dumba *et al.*, “Imaging in patients with glioblastoma: A national cohort study,” *Neuro-Oncology Pract.*, vol. 9, no. 6, pp. 487–495, Nov. 2022, doi: 10.1093/NOP/NPAC048.

[16] V. Gilard *et al.*, “Diagnosis and management of glioblastoma: A comprehensive perspective,” *J. Pers. Med.*, vol. 11, no. 4, p. 258, 2021, doi: 10.3390/JPM11040258/S1.

[17] W. Szopa, T. A. Burley, G. Kramer-Marek, and W. Kaspera, “Diagnostic and Therapeutic Biomarkers in Glioblastoma: Current Status and Future Perspectives,” *Biomed Res. Int.*, vol. 2017, 2017, doi: 10.1155/2017/8013575.

[18] G. Ceglie *et al.*, “Cancer Predisposition Syndromes Associated With Pediatric High-Grade Gliomas,” *Front. Pediatr.*, vol. 8, p. 561487, Nov. 2020, doi: 10.3389/FPED.2020.561487/FULL.

[19] S. Grochans *et al.*, “Epidemiology of Glioblastoma Multiforme—Literature Review,” *Cancers 2022, Vol. 14, Page 2412*, vol. 14, no. 10, p. 2412, May 2022, doi: 10.3390/CANCERS14102412.

[20] M. Z. Braganza, C. M. Kitahara, A. Berrington De González, P. D. Inskip, K. J. Johnson, and P. Rajaraman, “Ionizing radiation and the risk of brain and central nervous system tumors: a systematic review,” *Neuro. Oncol.*, vol. 14, no. 11, p. 1316, Nov. 2012, doi: 10.1093/NEUONC/NOS208.

[21] Y. K. Shim, S. P. Miynarek, and E. van Wijngaarden, “Parental Exposure to Pesticides and Childhood Brain Cancer: U.S. Atlantic Coast Childhood Brain Cancer Study,” *Environ. Health Perspect.*, vol. 117, no. 6, p. 1002, 2009, doi: 10.1289/EHP.0800209.

[22] Q. T. Ostrom *et al.*, “CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006–2010,” *Neuro. Oncol.*, vol. 15 Suppl 2, no. Suppl 2, 2013, doi: 10.1093/NEUONC/NOT151.

[23] A. F. Tamimi and M. Juweid, “Epidemiology and Outcome of Glioblastoma,” *Glioblastoma*, pp. 143–153, Sep. 2017, doi: 10.15586/CODON.GLIOBLASTOMA.2017.CH8.

[24] A. Colopi *et al.*, “Impact of age and gender on glioblastoma onset, progression, and management,” *Mech. Ageing Dev.*, vol. 211, p. 111801, Apr. 2023, doi: 10.1016/J.MAD.2023.111801.

[25] A. Thomas-Joulié *et al.*, “Prognosis of glioblastoma patients improves significantly over time interrogating historical controls,” *Eur. J. Cancer*, vol. 202, p. 114004, May 2024, doi: 10.1016/j.ejca.2024.114004.

[26] S. S. K. Yalamarty *et al.*, “Mechanisms of Resistance and Current Treatment Options for Glioblastoma Multiforme (GBM),” *Cancers (Basel)*, vol.

15, no. 7, Apr. 2023, doi: 10.3390/CANCERS15072116.

[27] R. S. Angom, N. M. R. Nakka, and S. Bhattacharya, "Advances in Glioblastoma Therapy: An Update on Current Approaches," *Brain Sci.* 2023, Vol. 13, Page 1536, vol. 13, no. 11, p. 1536, Oct. 2023, doi: 10.3390/BRAINS13111536.

[28] D. Petrenko *et al.*, "Temozolomide Efficacy and Metabolism: The Implicit Relevance of Nanoscale Delivery Systems," *Molecules*, vol. 27, no. 11, Jun. 2022, doi: 10.3390/MOLECULES27113507.

[29] I. J. Barani and D. A. Larson, "Radiation therapy of glioblastoma," *Cancer Treat. Res.*, vol. 163, pp. 49–73, 2015, doi: 10.1007/978-3-319-12048-5_4/TABLES/4.

[30] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid cell strains," *Exp. Cell Res.*, vol. 25, no. 3, pp. 585–621, 1961, doi: 10.1016/0014-4827(61)90192-6.

[31] W. Huang, L. T. J. Hickson, A. Eirin, J. L. Kirkland, and L. O. Lerman, "Cellular senescence: the good, the bad and the unknown," *Nat. Rev. Nephrol.* 2022 1810, vol. 18, no. 10, pp. 611–627, Aug. 2022, doi: 10.1038/s41581-022-00601-z.

[32] M. Y. Terzi, M. Izmirlı, and B. Gogebakan, "The cell fate: senescence or quiescence," *Mol. Biol. Rep.*, vol. 43, no. 11, pp. 1213–1220, Nov. 2016, doi: 10.1007/S11033-016-4065-0/TABLES/1.

[33] D. McHugh and J. Gil, "Senescence and aging: Causes, consequences, and therapeutic avenues," *J. Cell Biol.*, vol. 217, no. 1, p. 65, Jan. 2018, doi: 10.1083/JCB.201708092.

[34] R. Kumari and P. Jat, "Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype," *Front. Cell Dev. Biol.*, vol. 9, p. 645593, Mar. 2021, doi: 10.3389/FCCELL.2021.645593/BIBTEX.

[35] S. Miwa, S. Kashyap, E. Chini, and T. von Zglinicki, "Mitochondrial dysfunction in cell senescence and aging," *J. Clin. Invest.*, vol. 132, no. 13, Jul. 2022, doi: 10.1172/JCI158447.

[36] G. P. Dimri *et al.*, "A biomarker that identifies senescent human cells in culture and in aging skin in vivo.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 20, p. 9363, Sep. 1995, doi: 10.1073/PNAS.92.20.9363.

[37] Y. Valieva, E. Ivanova, A. Fayzullin, A. Kurkov, and A. Igrunkova, "Senescence-Associated β -Galactosidase Detection in Pathology," *Diagnostics*, vol. 12, no. 10, Oct. 2022, doi: 10.3390/DIAGNOSTICS12102309.

[38] H. Wong and K. Riabowol, "Differential CDK-inhibitor gene expression in aging human diploid fibroblasts," *Exp. Gerontol.*, vol. 31, no. 1–2, pp. 311–325, Jan. 1996, doi: 10.1016/0531-5565(95)00025-9.

[39] H. Safwan-Zaiter, N. Wagner, and K. D. Wagner, "P16INK4A—More Than a Senescence Marker," *Life*, vol. 12, no. 9, Sep. 2022, doi: 10.3390/LIFE12091332.

[40] M. Shtutman, B. D. Chang, G. P. Schools, and E. V. Broude, "Cellular Model of p21-Induced Senescence," *Methods Mol. Biol.*, vol. 1534, p. 31, 2017, doi: 10.1007/978-1-4939-6670-7_3.

[41] M. G. C. T. Van Oijen, R. H. Medema, P. J. Slootweg, and G. Rijkse, "Positivity of the proliferation marker Ki-67 in noncycling cells," *Am. J. Clin. Pathol.*, vol. 110, no. 1, pp. 24–31, 1998, doi: 10.1093/AJCP/110.1.24.

[42] N. Alessio, D. Aprile, S. Cappabianca, G. Peluso, G. Di Bernardo, and

U. Galderisi, "Different Stages of Quiescence, Senescence, and Cell Stress Identified by Molecular Algorithm Based on the Expression of Ki67, RPS6, and Beta-Galactosidase Activity," *Int. J. Mol. Sci.* 2021, Vol. 22, Page 3102, vol. 22, no. 6, p. 3102, Mar. 2021, doi: 10.3390/IJMS22063102.

[43] A. Freund, R. M. Laberge, M. Demaria, and J. Campisi, "Lamin B1 loss is a senescence-associated biomarker," *Mol. Biol. Cell*, vol. 23, no. 11, p. 2066, Jun. 2012, doi: 10.1091/MBC.E11-10-0884.

[44] J. Il Jun and L. F. Lau, "The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing," *Nat. Cell Biol.*, vol. 12, no. 7, pp. 676–685, 2010, doi: 10.1038/NCB2070.

[45] S. J. Jim Leu *et al.*, "The matricellular protein CCN1 suppresses lung cancer cell growth by inducing senescence via the p53/p21 pathway," *J. Cell. Biochem.*, vol. 114, no. 9, pp. 2082–2093, Sep. 2013, doi: 10.1002/JCB.24557.

[46] S. Xiao *et al.*, "Cellular senescence: a double-edged sword in cancer therapy," *Front. Oncol.*, vol. 13, p. 1189015, 2023, doi: 10.3389/FONC.2023.1189015.

[47] C. A. Schmitt, B. Wang, and M. Demaria, "Senescence and cancer — role and therapeutic opportunities," *Nat. Rev. Clin. Oncol.* 2022 1910, vol. 19, no. 10, pp. 619–636, Aug. 2022, doi: 10.1038/s41571-022-00668-4.

[48] S. S. Park, Y. W. Choi, J. H. Kim, H. S. Kim, and T. J. Park, "Senescent tumor cells: an overlooked adversary in the battle against cancer," *Exp. Mol. Med.* 2021 5312, vol. 53, no. 12, pp. 1834–1841, Dec. 2021, doi: 10.1038/s12276-021-00717-5.

[49] M. K. Saifullah *et al.*, "Expression and Characterization of a Novel CD6 Ligand in Cells Derived from Joint and Epithelial Tissues," *J. Immunol.*, vol. 173, no. 10, pp. 6125–6133, Nov. 2004, doi: 10.4049/JIMMUNOL.173.10.6125.

[50] G. W. M. Swart, "Activated leukocyte cell adhesion molecule (CD166/ALCAM): Developmental and mechanistic aspects of cell clustering and cell migration," *Eur. J. Cell Biol.*, vol. 81, no. 6, pp. 313–321, Jun. 2002, doi: 10.1078/0171-9335-00256.

[51] M. A. Bowen *et al.*, "Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand," *J. Exp. Med.*, vol. 181, no. 6, pp. 2213–2220, Jun. 1995, doi: 10.1084/JEM.181.6.2213.

[52] P. W. Harrison *et al.*, "Ensembl 2024," *Nucleic Acids Res.*, vol. 52, no. D1, pp. D891–D899, Jan. 2024, doi: 10.1093/NAR/GKAD1049.

[53] G. W. M. Swart, P. C. Lunter, J. W. J. Van Kilsdonk, and L. C. L. T. Van Kempen, "Activated leukocyte cell adhesion molecule (ALCAM/CD166): Signaling at the divide of melanoma cell clustering and cell migration?," *Cancer Metastasis Rev.*, vol. 24, no. 2, pp. 223–236, Jun. 2005, doi: 10.1007/S10555-005-1573-0/METRICS.

[54] J. E. Skonier, M. A. Bowen, J. Emswiler, A. Aruffo, and J. Bajorath, "Recognition of diverse proteins by members of the immunoglobulin superfamily: Delineation of the receptor binding site in the human CD6 ligand ALCAM," *Biochemistry*, vol. 35, no. 38, pp. 12287–12291, 1996, doi: 10.1021/BI961038K/ASSET/IMAGES/LARGE/BI961038KF00004.JPEG.

[55] A. von Lersner, L. Drosen, and A. Zijlstra, "Modulation of cell adhesion and migration through regulation of the immunoglobulin superfamily member ALCAM/CD166," *Clin. Exp. Metastasis*, vol. 36, no. 2, p. 87, Apr. 2019,

doi: 10.1007/S10585-019-09957-2.

[56] A. Bateman *et al.*, “UniProt: the Universal Protein Knowledgebase in 2023,” *Nucleic Acids Res.*, vol. 51, no. D1, pp. D523–D531, Jan. 2023, doi: 10.1093/NAR/GKAC1052.

[57] A. G. Hansen, G. W. Swart, and A. Zijlstra, “ALCAM: Basis Sequence: Mouse,” *AFCS-nature Mol. pages*, vol. 2011, Feb. 2011, doi: 10.1038/MP.A004126.01.

[58] F. Miccichè *et al.*, “Activated Leukocyte Cell Adhesion Molecule Expression and Shedding in Thyroid Tumors,” *PLoS One*, vol. 6, no. 2, p. 17141, 2011, doi: 10.1371/JOURNAL.PONE.0017141.

[59] Y. Yang, A. J. Sanders, Q. P. Dou, D. G. Jiang, A. X. Li, and W. G. Jiang, “The Clinical and Theranostic Values of Activated Leukocyte Cell Adhesion Molecule (ALCAM)/CD166 in Human Solid Cancers,” *Cancers (Basel)*, vol. 13, no. 20, Oct. 2021, doi: 10.3390/CANCERS13205187.

[60] J. M. D. T. Nelissen, I. M. Peters, B. G. De Grooth, Y. Van Kooyk, and C. G. Figdor, “Dynamic Regulation of Activated Leukocyte Cell Adhesion Molecule-mediated Homotypic Cell Adhesion through the Actin Cytoskeleton,” *Mol. Biol. Cell*, vol. 11, no. 6, p. 2057, 2000, doi: 10.1091/MB.C.11.6.2057.

[61] W. Cizelsky, A. Tata, M. Köhl, and S. J. Köhl, “The Wnt/JNK signaling target gene *alcam* is required for embryonic kidney development,” *Development*, vol. 141, no. 10, pp. 2064–2074, May 2014, doi: 10.1242/DEV.107938.

[62] Y. Yang *et al.*, “A novel HDGF-ALCAM axis promotes the metastasis of Ewing sarcoma via regulating the GTPases signaling pathway,” *Oncogene* 2020 404, vol. 40, no. 4, pp. 731–745, Nov. 2020, doi: 10.1038/s41388-020-01485-8.

[63] H. Wang, X. Mao, L. Ye, H. Cheng, and X. Dai, “The Role of the S100 Protein Family in Glioma,” *J. Cancer*, vol. 13, no. 10, pp. 3022–3030, 2022, doi: 10.7150/JCA.73365.

[64] R. von Bauer *et al.*, “CD166/ALCAM Mediates Proinflammatory Effects of S100B in Delayed Type Hypersensitivity,” *J. Immunol.*, vol. 191, no. 1, pp. 369–377, Jul. 2013, doi: 10.4049/JIMMUNOL.1201864.

[65] C. R. Bye, V. Rytova, W. F. Alsanie, C. L. Parish, and L. H. Thompson, “Axonal Growth of Midbrain Dopamine Neurons is Modulated by the Cell Adhesion Molecule ALCAM Through Trans-Heterophilic Interactions with L1cam, Chl1, and Semaphorins,” *J. Neurosci.*, vol. 39, no. 34, pp. 6656–6667, Aug. 2019, doi: 10.1523/JNEUROSCI.0278-19.2019.

[66] Z. Ghazizadeh *et al.*, “Prospective Isolation of ISL1+ Cardiac Progenitors from Human ESCs for Myocardial Infarction Therapy,” *Stem cell reports*, vol. 10, no. 3, pp. 848–859, Mar. 2018, doi: 10.1016/J.STEMCR.2018.01.037.

[67] E. Murayama, C. Vivier, A. Schmidt, and P. Herbomel, “Alcam- α and Pdgfr- α are essential for the development of sclerotome-derived stromal cells that support hematopoiesis,” *Nat. Commun.* 2023 141, vol. 14, no. 1, pp. 1–17, Mar. 2023, doi: 10.1038/s41467-023-36612-y.

[68] M. T. Wiiger, H. B. Gehrken, Ø. Fodstad, G. M. Mælandsmo, and Y. Andersson, “A novel human recombinant single-chain antibody targeting CD166/ALCAM inhibits cancer cell invasion in vitro and in vivo tumour growth,” *Cancer Immunol. Immunother.*, vol. 59, no. 11, pp. 1665–1674, Nov. 2010, doi: 10.1007/S00262-010-0892-3/FIGURES/4.

- [69] A. G. Hansen *et al.*, “ALCAM/CD166 is a TGF β responsive marker and functional regulator of prostate cancer metastasis to bone,” *Cancer Res.*, vol. 74, no. 5, p. 1404, Mar. 2014, doi: 10.1158/0008-5472.CAN-13-1296.
- [70] F. Ferragut *et al.*, “Dual knockdown of Galectin-8 and its glycosylated ligand, the activated leukocyte cell adhesion molecule (ALCAM/CD166), synergistically delays in vivo breast cancer growth,” *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1866, no. 8, pp. 1338–1352, Aug. 2019, doi: 10.1016/J.BBAMCR.2019.03.010.
- [71] L. Devis *et al.*, “Activated leukocyte cell adhesion molecule (ALCAM) is a marker of recurrence and promotes cell migration, invasion, and metastasis in early-stage endometrioid endometrial cancer,” *J. Pathol.*, vol. 241, no. 4, pp. 475–487, Mar. 2017, doi: 10.1002/PATH.4851.
- [72] W. He, L. Huang, M. Li, Y. Yang, Z. Chen, and X. Shen, “MiR-148b, MiR-152/ALCAM Axis Regulates the Proliferation and Invasion of Pituitary Adenomas Cells,” *Cell. Physiol. Biochem.*, vol. 44, no. 2, pp. 792–803, Dec. 2017, doi: 10.1159/000485342.
- [73] K. Fujiwara *et al.*, “CD166/ALCAM Expression Is Characteristic of Tumorigenicity and Invasive and Migratory Activities of Pancreatic Cancer Cells,” *PLoS One*, vol. 9, no. 9, p. e107247, Sep. 2014, doi: 10.1371/JOURNAL.PONE.0107247.
- [74] K. Ikeda and T. Quertermous, “Molecular Isolation and Characterization of a Soluble Isoform of Activated Leukocyte Cell Adhesion Molecule That Modulates Endothelial Cell Function,” *J. Biol. Chem.*, vol. 279, no. 53, pp. 55315–55323, Dec. 2004, doi: 10.1074/JBC.M407776200.
- [75] R. von Bauer *et al.*, “Loss of ALCAM/CD166 partially protects against diabetic nephropathy,” *Diabetol. und Stoffwechsel*, vol. 10, no. S 01, p. FV20, Apr. 2015, doi: 10.1055/S-0035-1549526.
- [76] A. Sulaj *et al.*, “ALCAM a novel biomarker in patients with type 2 diabetes mellitus complicated with diabetic nephropathy,” *J. Diabetes Complications*, vol. 31, no. 6, pp. 1058–1065, Jun. 2017, doi: 10.1016/J.JDIACOMP.2017.01.002.
- [77] I. Witzel *et al.*, “Detection of Activated Leukocyte Cell Adhesion Molecule in the Serum of Breast Cancer Patients and Implications for Prognosis,” *Oncology*, vol. 82, no. 6, pp. 305–312, Jul. 2012, doi: 10.1159/000337222.
- [78] M. Burkhardt *et al.*, “Cytoplasmic overexpression of ALCAM is prognostic of disease progression in breast cancer,” *J. Clin. Pathol.*, vol. 59, no. 4, p. 403, Apr. 2006, doi: 10.1136/JCP.2005.028209.
- [79] J. W. J. Van Kilsdonk *et al.*, “Attenuation of Melanoma Invasion by a Secreted Variant of Activated Leukocyte Cell Adhesion Molecule,” *Cancer Res.*, vol. 68, no. 10, pp. 3671–3679, May 2008, doi: 10.1158/0008-5472.CAN-07-5767.
- [80] J. R. Smith, T. J. Chipps, H. Ilias, Y. Pan, and B. Appukuttan, “Expression and regulation of activated leukocyte cell adhesion molecule in human retinal vascular endothelial cells,” *Exp. Eye Res.*, vol. 104, pp. 89–93, Nov. 2012, doi: 10.1016/J.EXER.2012.08.006.
- [81] Y. M. Yang *et al.*, “Activated Leukocyte Cell Adhesion Molecule (ALCAM), a Potential ‘Seed’ and ‘Soil’ Receptor in the Peritoneal Metastasis of Gastrointestinal Cancers,” *Int. J. Mol. Sci.*, vol. 24, no. 1, Jan. 2023, doi: 10.3390/IJMS24010876.

- [82] P. J. Killela *et al.*, “TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 15, pp. 6021–6026, Apr. 2013, doi: 10.1073/PNAS.1303607110.
- [83] J. A. Ewald, J. A. Desotelle, G. Wilding, and D. F. Jarrard, “Therapy-Induced Senescence in Cancer,” *JNCI J. Natl. Cancer Inst.*, vol. 102, no. 20, pp. 1536–1546, Oct. 2010, doi: 10.1093/JNCI/DJQ364.
- [84] L. Chuaire-Noack, M. Carolina Sánchez-Corredor, and S. Rocío Ramírez-Clavijo, “The Dual Role of Senescence in Tumorigenesis Papel Dual de la Senescencia en la Tumorigénesis,” *Int. J. Morphol.*, vol. 28, no. 1, pp. 37–50, 2010.
- [85] S. Qin, B. A. Schulte, and G. Y. Wang, “Role of senescence induction in cancer treatment,” *World J. Clin. Oncol.*, vol. 9, no. 8, p. 180, Dec. 2018, doi: 10.5306/WJCO.V9.I8.180.
- [86] L. Beltzig *et al.*, “Senescence Is the Main Trait Induced by Temozolomide in Glioblastoma Cells,” *Cancers (Basel)*, vol. 14, no. 9, p. 2233, May 2022, doi: 10.3390/CANCERS14092233/S1.
- [87] D. Aasland *et al.*, “Temozolomide induces senescence and repression of DNA repair pathways in glioblastoma cells via activation of ATR–Chk1, p21, and NF-κB,” *Cancer Res.*, vol. 79, no. 1, pp. 99–113, Jan. 2019, doi: 10.1158/0008-5472.CAN-18-1733/653444/AM/TEMOZOLOMIDE-INDUCES-SENESCENCE-AND-REPRESSION-OF.
- [88] L. Beltzig, M. Christmann, and B. Kaina, “Abrogation of Cellular Senescence Induced by Temozolomide in Glioblastoma Cells: Search for Senolytics,” *Cells*, vol. 11, no. 16, p. 2588, Aug. 2022, doi: 10.3390/CELLS11162588/S1.
- [89] E. A. Pérès *et al.*, “Silencing erythropoietin receptor on glioma cells reinforces efficacy of temozolomide and X-rays through senescence and mitotic catastrophe,” *Oncotarget*, vol. 6, no. 4, pp. 2101–2119, 2015, doi: 10.18632/ONCOTARGET.2937.
- [90] H. Y. Jeon *et al.*, “Irradiation induces glioblastoma cell senescence and senescence-associated secretory phenotype,” *Tumour Biol.*, vol. 37, no. 5, pp. 5857–5867, May 2016, doi: 10.1007/S13277-015-4439-2.
- [91] E. H. Kim, “Tumor treating fields induced senescence on glioblastoma,” *Am. J. Cancer Res.*, vol. 13, no. 11, p. 5626, 2023.
- [92] R. Kumar, A. Gont, T. J. Perkins, J. E. L. Hanson, and I. A. J. Lorimer, “Induction of senescence in primary glioblastoma cells by serum and TGFβ,” *Sci. Rep.*, vol. 7, no. 1, Dec. 2017, doi: 10.1038/S41598-017-02380-1.
- [93] Y. Li *et al.*, “Copper induces cellular senescence in human glioblastoma multiforme cells through downregulation of Bmi-1,” *Oncol. Rep.*, vol. 29, no. 5, pp. 1811–1818, 2013, doi: 10.3892/OR.2013.2333.
- [94] I. J. Restall *et al.*, “PKC α depletion initiates mitotic slippage-induced senescence in glioblastoma,” *Cell Cycle*, vol. 14, no. 18, pp. 2938–2948, 2015, doi: 10.1080/15384101.2015.1071744.
- [95] R. Salam *et al.*, “Cellular senescence in malignant cells promotes tumor progression in mouse and patient Glioblastoma,” *Nat. Commun.*, vol. 14, no. 1, Dec. 2023, doi: 10.1038/S41467-023-36124-9.
- [96] N. Kijima *et al.*, “CD166/activated leukocyte cell adhesion molecule is

expressed on glioblastoma progenitor cells and involved in the regulation of tumor cell invasion,” *Neuro. Oncol.*, vol. 14, no. 10, pp. 1254–1264, Oct. 2012, doi: 10.1093/NEUONC/NOR202.

[97] W. Wang, W. Liu, J. Xu, and H. Jin, “MiR-33a targets FOSL1 and EN2 as a clinical prognostic marker for sarcopenia by glioma,” *Front. Genet.*, vol. 13, p. 953580, Aug. 2022, doi: 10.3389/FGENE.2022.953580.

[98] O. Allmendinger *et al.*, “Activated leukocyte cell adhesion molecule is expressed in neuroepithelial neoplasms and decreases with tumor malignancy, matrix metalloproteinase 2 expression, and absence of IDH1R132H mutation,” *Hum. Pathol.*, vol. 43, no. 8, pp. 1289–1299, Aug. 2012, doi: 10.1016/J.HUMPATH.2011.10.007.

[99] C. Hartmann *et al.*, “Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age: Implications for classification of gliomas,” *Acta Neuropathol.*, vol. 120, no. 6, pp. 707–718, Dec. 2010, doi: 10.1007/S00401-010-0781-Z/FIGURES/6.

[100] A. Virtuoso *et al.*, “Tumor Microenvironment and Immune Escape in the Time Course of Glioblastoma,” *Mol. Neurobiol.*, vol. 59, no. 11, p. 6857, Nov. 2022, doi: 10.1007/S12035-022-02996-Z.

[101] N. Kijima *et al.*, “ANGI-03 Functional roles of CD166/activated leukocyte cell adhesion molecule (CD166/ALCAM) for glioblastoma invasion,” *Neuro-Oncology Adv.*, vol. 2, no. Supplement_3, pp. ii3–ii3, Nov. 2020, doi: 10.1093/NOAJNL/VDAA143.012.

[102] G. C. Sajina *et al.*, “ α 2,6 Sialylation mediated by ST6GAL1 promotes glioblastoma growth,” *JCI Insight*, vol. 7, no. 21, Nov. 2022, doi: 10.1172/JCI.INSIGHT.158799.

[103] O. Rosso *et al.*, “The ALCAM shedding by the metalloprotease ADAM17/TACE is involved in motility of ovarian carcinoma cells,” *Mol. Cancer Res.*, vol. 5, no. 12, pp. 1246–1253, Dec. 2007, doi: 10.1158/1541-7786.MCR-07-0060.

[104] J. Behnan *et al.*, “Recruited brain tumor-derived mesenchymal stem cells contribute to brain tumor progression,” *Stem Cells*, vol. 32, no. 5, pp. 1110–1123, 2014, doi: 10.1002/STEM.1614.

[105] B. Er Baba *et al.*, “Zebrafish brain RNA sequencing reveals that cell adhesion molecules are critical in brain aging,” *Neurobiol. Aging*, vol. 94, pp. 164–175, Oct. 2020, doi: 10.1016/J.NEUROBIOLAGING.2020.04.017.

[106] R. Jeannet, Q. Cai, H. Liu, H. Vu, and Y. H. Kuo, “Alcam regulates long-term hematopoietic stem cell engraftment and self-renewal,” *Stem Cells*, vol. 31, no. 3, pp. 560–571, Mar. 2013, doi: 10.1002/STEM.1309.

[107] J. Nautiyal, J. Du, Y. Yu, S. S. Kanwar, E. Levi, and A. P. N. Majumdar, “EGFR regulation of colon cancer stem-like cells during aging and in response to the colonic carcinogen dimethylhydrazine,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 302, no. 7, p. G655, Apr. 2012, doi: 10.1152/AJPGI.00323.2011.

[108] G. Ou *et al.*, “Fibroblast Growth Factor-2 Stimulates the Proliferation of Mesenchyme-Derived Progenitor Cells From Aging Mouse and Human Bone,” *Journals Gerontol. Ser. A Biol. Sci. Med. Sci.*, vol. 65A, no. 10, p. 1051, Oct. 2010, doi: 10.1093/GERONA/GLQ114.

[109] B. W. Kunkle *et al.*, “Novel Alzheimer Disease Risk Loci and Pathways

in African American Individuals Using the African Genome Resources Panel: A Meta-analysis,” *JAMA Neurol.*, vol. 78, no. 1, p. 1, Jan. 2021, doi: 10.1001/JAMANEUROL.2020.3536.

[110] C. D. Whelan *et al.*, “Multiplex proteomics identifies novel CSF and plasma biomarkers of early Alzheimer’s disease,” *Acta Neuropathol. Commun.*, vol. 7, no. 1, pp. 1–14, Nov. 2019, doi: 10.1186/S40478-019-0795-2/FIGURES/3.

[111] A. R. V. R. Horimoto, D. Xue, T. A. Thornton, and E. E. Blue, “Admixture mapping reveals the association between Native American ancestry at 3q13.11 and reduced risk of Alzheimer’s disease in Caribbean Hispanics,” *Alzheimer’s Res. Ther.*, vol. 13, no. 1, pp. 1–14, Dec. 2021, doi: 10.1186/S13195-021-00866-9/TABLES/4.

[112] M. Ghanbari *et al.*, “A functional variant in the miR-142 promoter modulating its expression and conferring risk of Alzheimer disease,” *Hum. Mutat.*, vol. 40, no. 11, pp. 2131–2145, Nov. 2019, doi: 10.1002/HUMU.23872.

[113] J. Chen *et al.*, “Increase of ALCAM and VCAM-1 in the plasma predicts the Alzheimer’s disease,” *Front. Immunol.*, vol. 13, Jan. 2023, doi: 10.3389/FIMMU.2022.1097409/FULL.

[114] K. Tominaga and H. I. Suzuki, “TGF- β Signaling in Cellular Senescence and Aging-Related Pathology,” *Int. J. Mol. Sci.*, vol. 20, no. 20, Oct. 2019, doi: 10.3390/IJMS20205002.

[115] A. P. Patel *et al.*, “Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma,” *Science (80-.)*, vol. 344, no. 6190, pp. 1396–1401, Jun. 2014, doi: 10.1126/SCIENCE.1254257/SUPPL_FILE/TABLE_S3.XLSX.

[116] Q. Zhu *et al.*, “Single cell multi-omics reveal intra-cell-line heterogeneity across human cancer cell lines,” *Nat. Commun.* 2023 141, vol. 14, no. 1, pp. 1–21, Dec. 2023, doi: 10.1038/s41467-023-43991-9.

[117] D. Eisenbarth and Y. A. Wang, “Glioblastoma heterogeneity at single cell resolution,” *Oncogene* 2023 4227, vol. 42, no. 27, pp. 2155–2165, Jun. 2023, doi: 10.1038/s41388-023-02738-y.

Appendix A

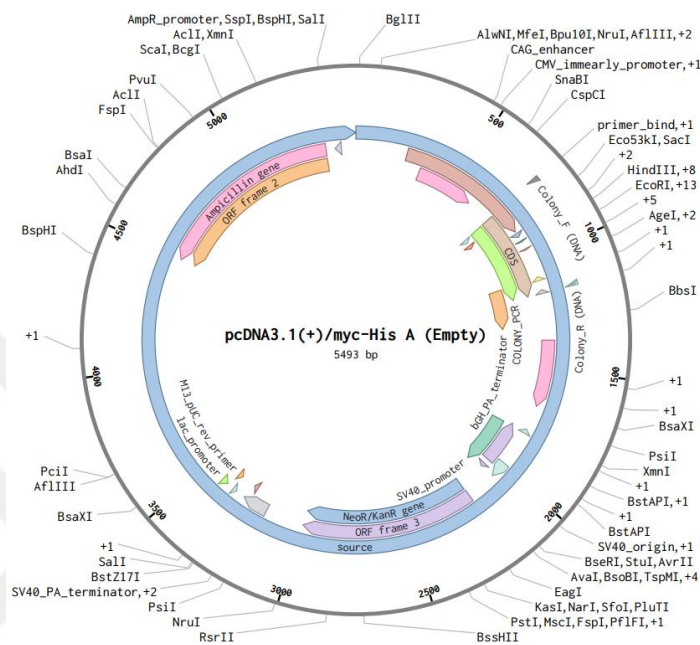


Figure A.1 *pcDNA3.1 (+)/myc-HisA Empty plasmid*. Green annotation shows the colony PCR product

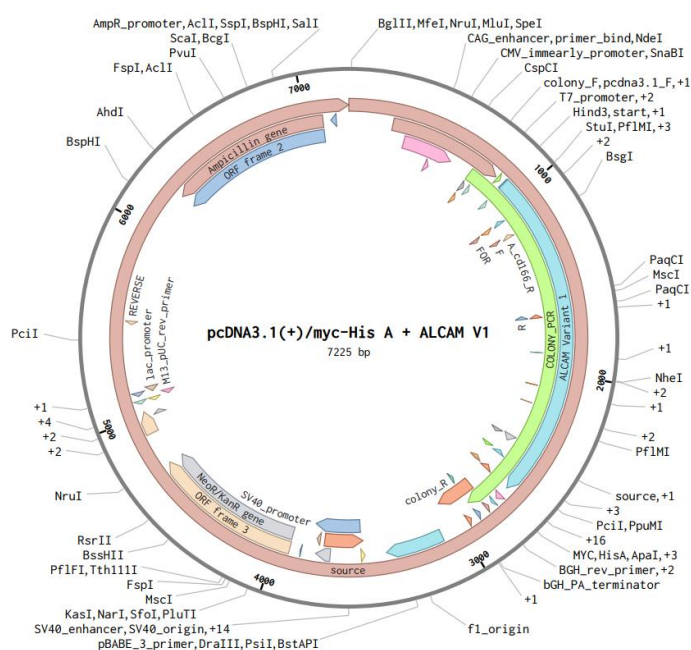


Figure A.2 *pcDNA3.1 (+)/myc-HisA plasmid inserted with ALCAM V1 sequence.*
Green annotation shows the colony PCR product

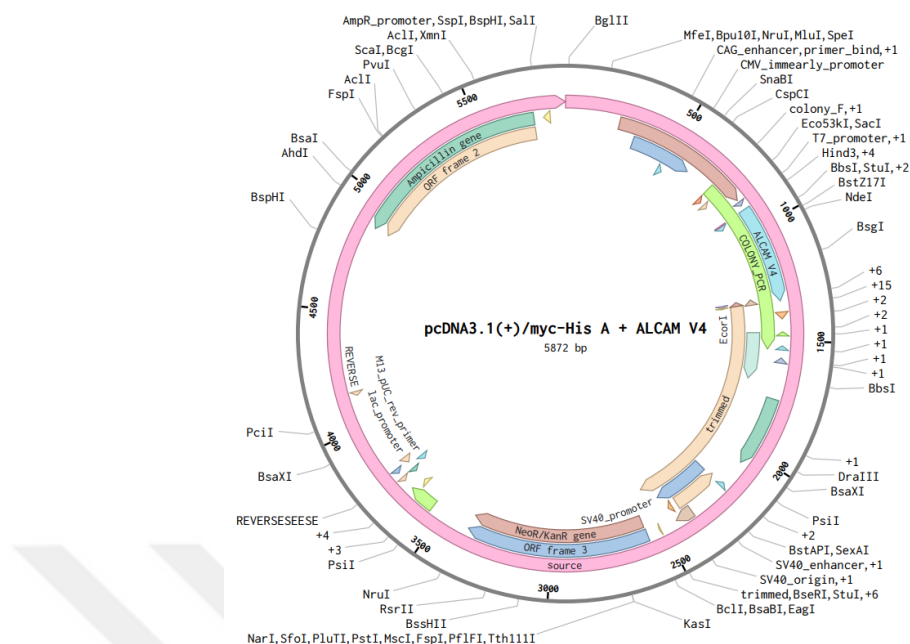


Figure A.3 *pcDNA3.1 (+)/myc-HisA plasmid inserted with ALCAM V4 sequence.*
Green annotation shows the colony PCR product.

Appendix B



Figure B.1 *Colony PCR results of ALCAM V1 and V4 constructs.* ~2300 bp was expected for V1 while ~810 bp for V4.

Appendix C

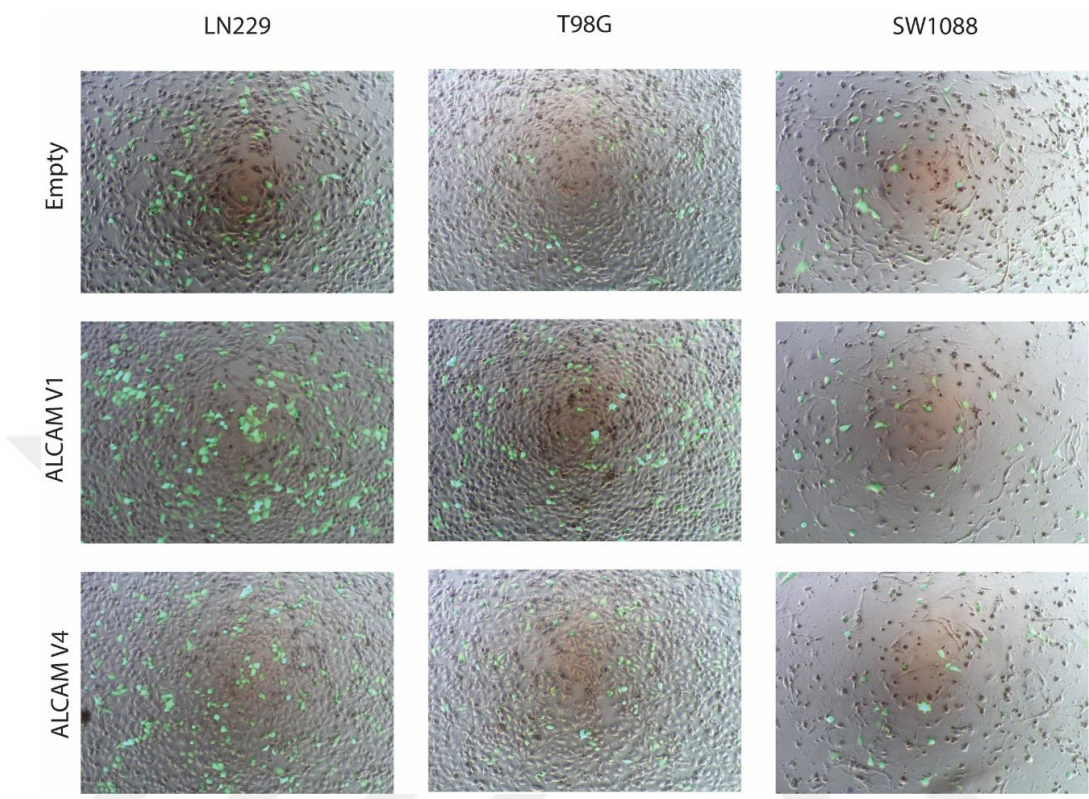


Figure C.1 Transfection efficiency for plasmid transfection. pEGFPC1 plasmid was used to make sure there is efficient transfection to proceed.

Appendix D

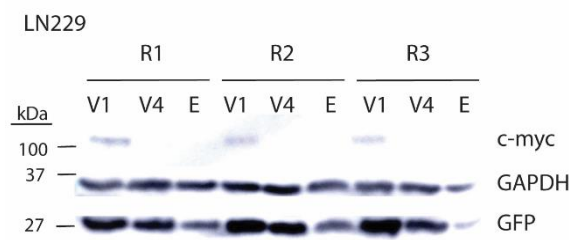


Figure D.1 LN229 western blot image showing the myc tag belonging to plasmid and GFP protein. Antibodies targeting GFP, c-myc and GAPDH were used.

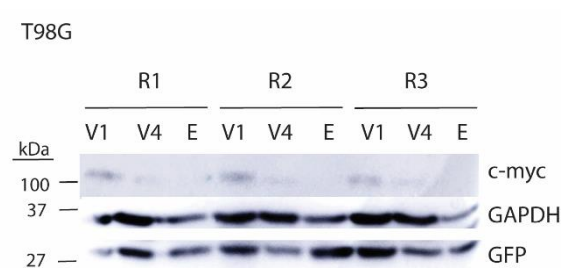


Figure D.2 T98G western blot image showing the myc tag belonging to plasmid and GFP protein. Antibodies targeting GFP, c-myc and GAPDH were used.

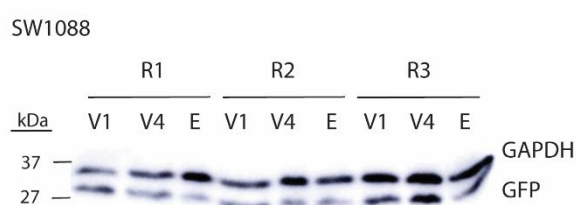


Figure D.3 SW1088 western blot image showing only GFP protein. Antibodies targeting GFP, c-myc and GAPDH were used. However, c-myc could not be detected.

Appendix E

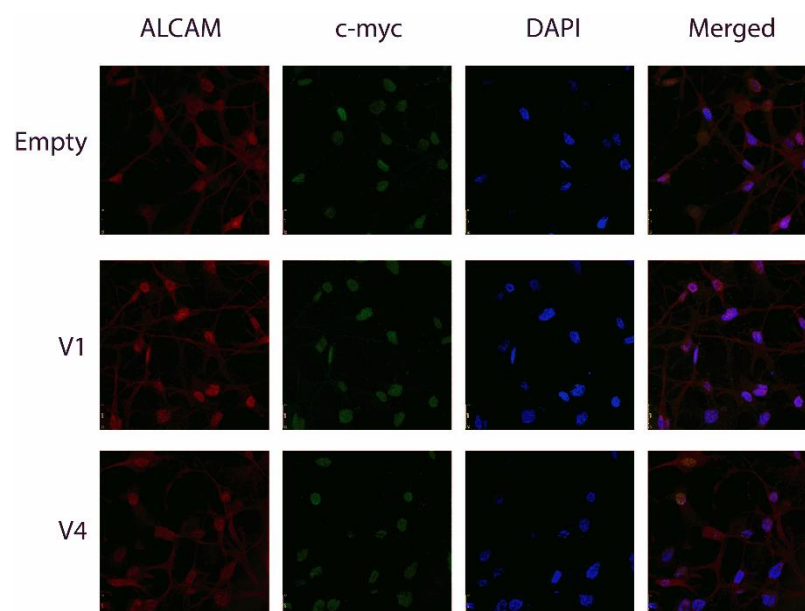


Figure E.1 Immunofluorescence assay of plasmid transfection in A172 cell line: replicate 2.

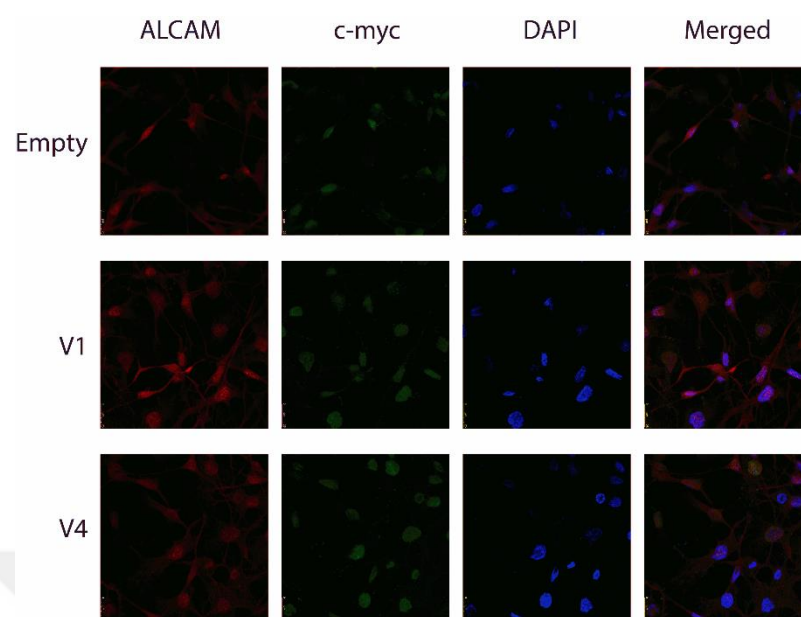


Figure E.2 Immunofluorescence assay of plasmid transfection in A172 cell line: replicate 3.

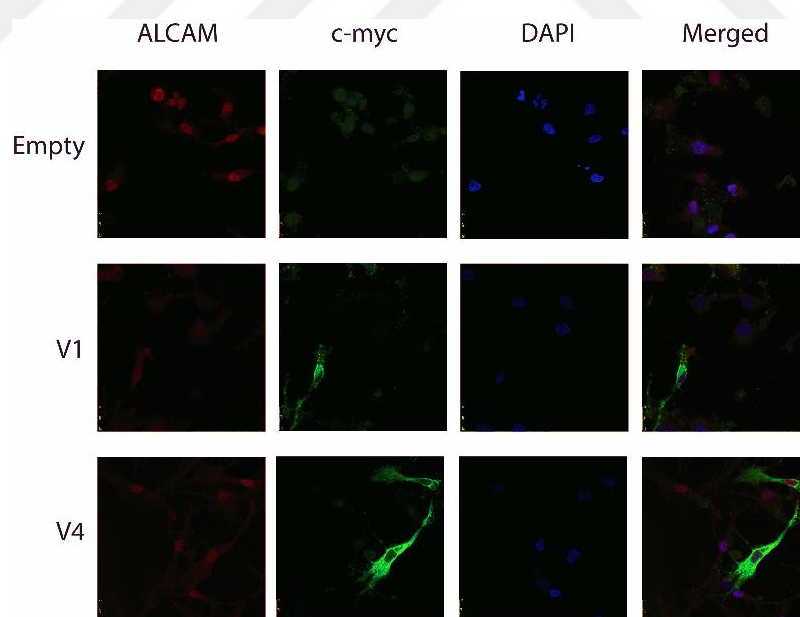


Figure E.3 Immunofluorescence assay of plasmid transfection in LN229 cell line: replicate 2.

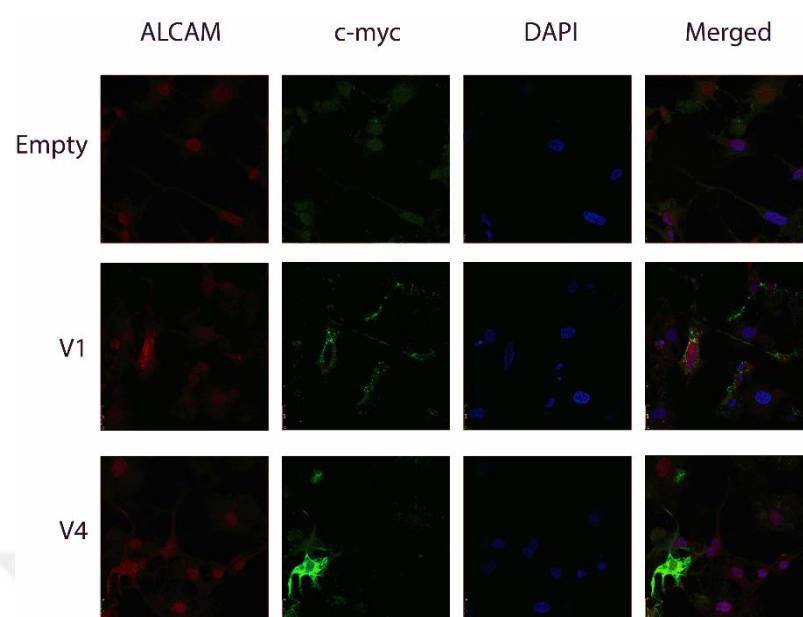


Figure E.4 Immunofluorescence assay of plasmid transfection in LN229 cell line: replicate 3.

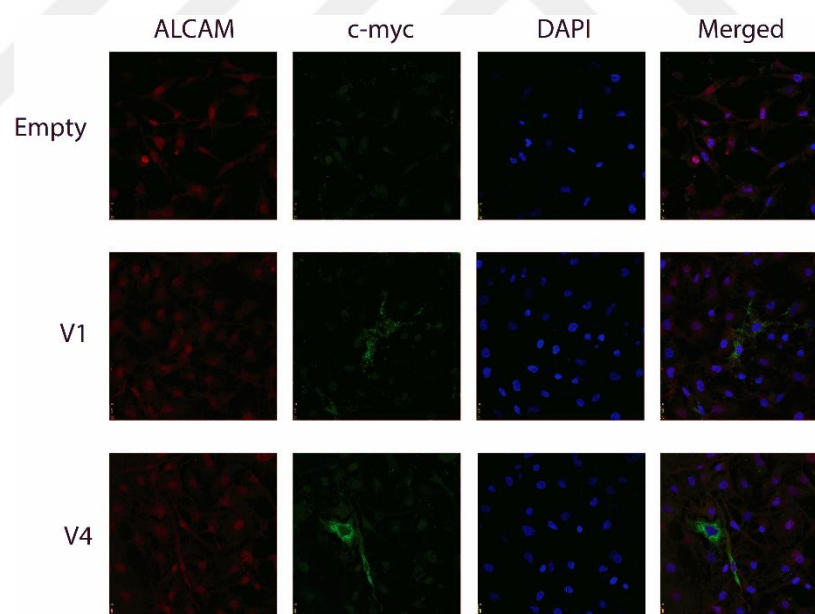


Figure E.5 Immunofluorescence assay of plasmid transfection in T98G cell line: replicate 2.

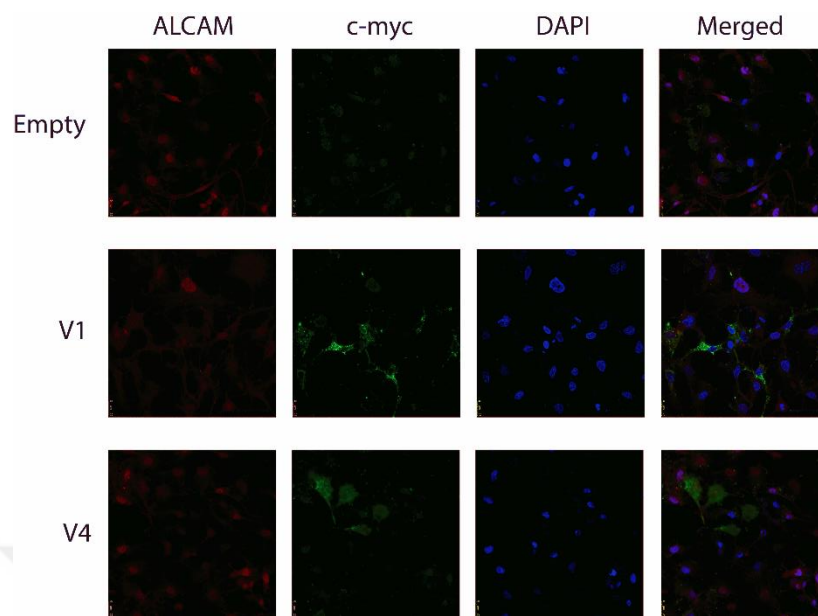


Figure E.6 Immunofluorescence assay of plasmid transfection in T98G cell line: replicate 3.

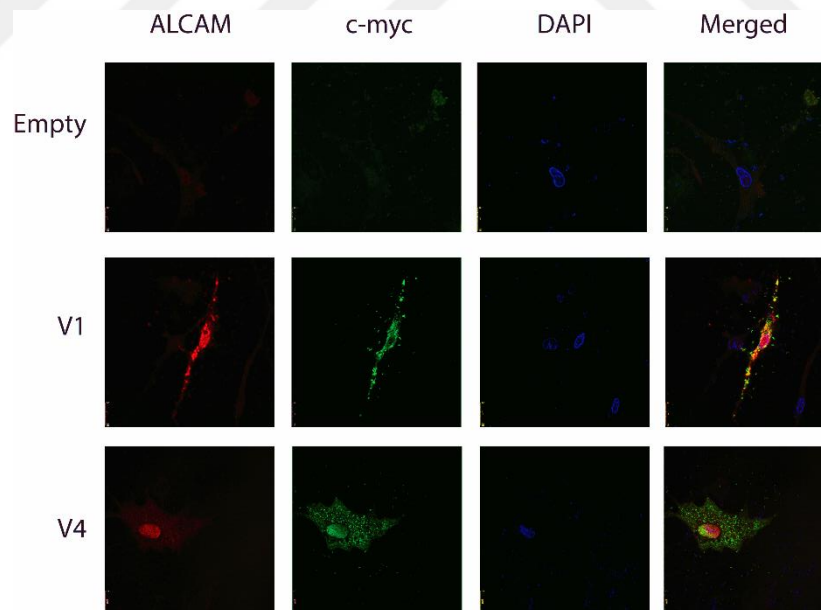


Figure E.7 Immunofluorescence assay of plasmid transfection in SW1088 cell line: replicate 2.

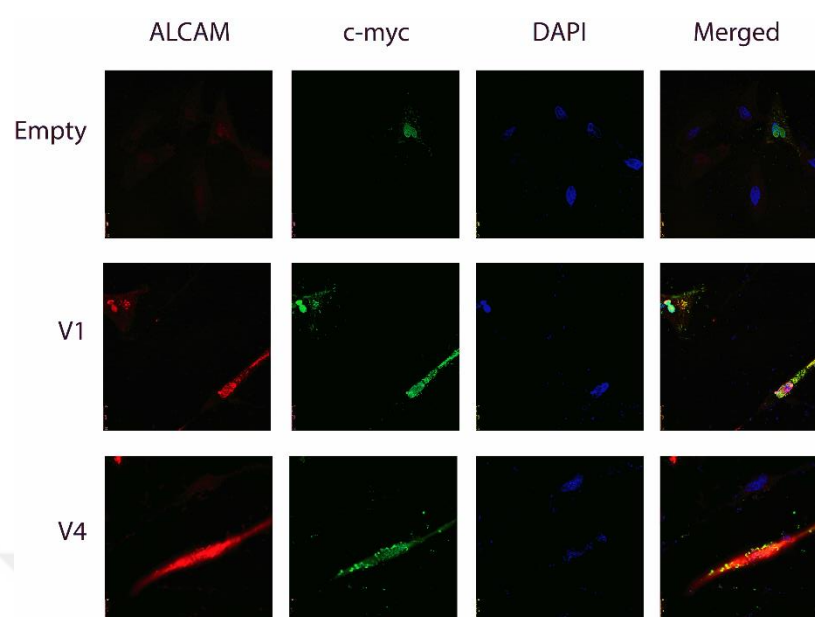


Figure E.8 Immunofluorescence assay of plasmid transfection in SW1088 cell line: replicate 3.

Appendix F

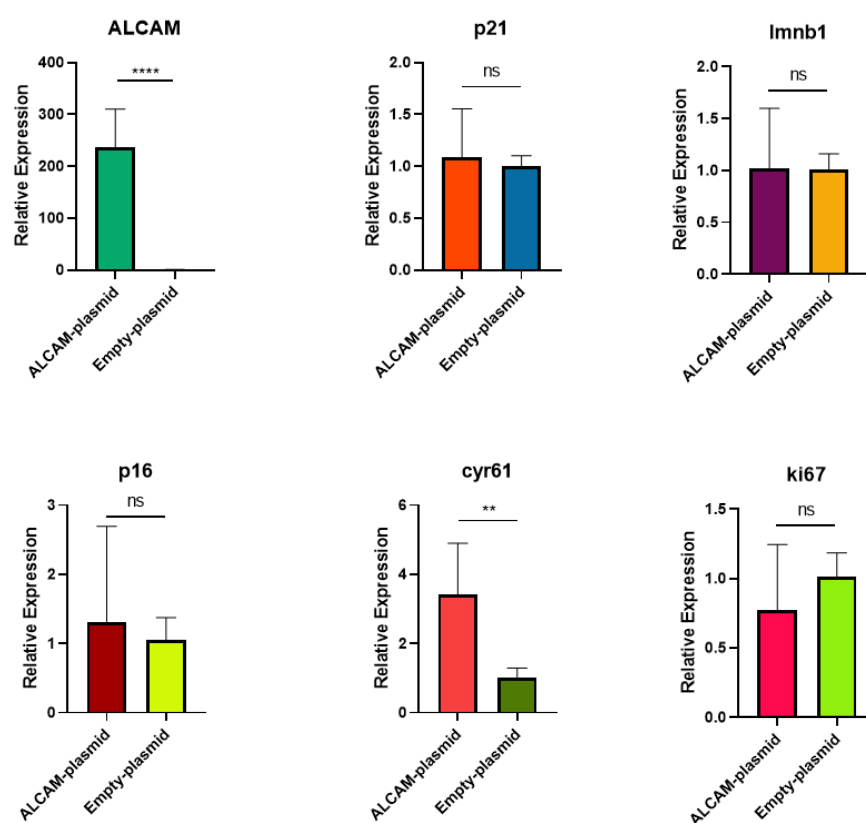


Figure F.1 HEK293 plasmid transfection results

Appendix G



Figure G.1 Western blot optimization of ALCAM antibody. The other 2 variant were also observed. 72 kDa band can be variant 3 while the 17 kDa band can be variant 4.

Appendix H

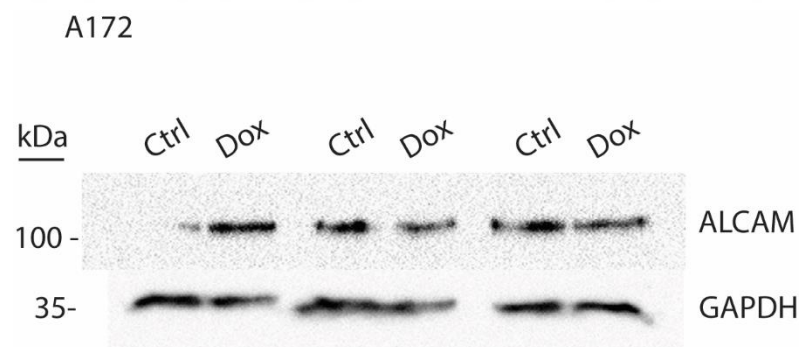


Figure H.1 Immunoblotting of ALCAM on dox treated and control A172 cells. GAPDH was used as housekeeping gene.