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BAHÇEŞEHİR UNIVERSITY

DEVELOPMENT OF RAPID AND ACCURATE IDH1 MUTATION DETECTION SYSTEM THAT IS COMPATIBLE FOR INTRAOPERATIVE DIAGNOSIS

Master's Thesis

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NEUROSCIENCEDEPARTMENT

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This is to certify that we have read this thesis and we find it fully adequate in scope, quality and content, as a thesis for the degree of Master of Arts.

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PREFACE

Above all, I would like to thank my supervisors Assist. Prof. Dr. Timuçin AVŞAR and Assoc. Prof. Dr. Melih ACAR for their guidance and contributions. They have professionally guided my first steps in laboratory. I owe my actual knowledge about laboratory discipline and experience to them.

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ABSTRACT

DEVELOPMENT OF RAPID AND ACCURATE IDH1 MUTATION DETECTION SYSTEM THAT IS COMPATIBLE FOR INTRAOPERATIVE DIAGNOSIS

Alihan SÜRSAL

Neuroscience Master Program

Thesis Supervisor: Assist. Prof. Dr. Timuçin AVŞAR

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Isocitrate dehydrogenase I and II (IDH1, IDH2) malformations are marked as cellular energy metabolism altering mutations related with good prognosis upon treatment and are highly frequent in glioma patients. In this study, rapid and accurate intraoperative diagnosis of the IDH1 mutational status was focused in order to inform the neurosurgeon intraoperatively. Steps of analysis were composed of alkaline lysis DNA extraction based novel modified point mutation detection system, which achieved 100 percent accuracy in retrospective analysis of 226 glioma samples, alongside with prospective analysis of 10 glioma samples in maximum 67 minutes. Working principle of utilized techniques were explained alongside with the optimization experiments and the importance of the IDH1/2 mutations on cancer treatment. Besides their approximately 74 percent frequency in gliomas, point mutations of IDH1 and IDH2 genes that reside in glioma patients is directly proportional with good prognosis due to better answer to anti-cancer treatments. Moreover, the frequency of IDH1 R132H mutation is more than 90 percent. As a novel detection tool, 3-mismatch-ARMS technique (3m-ARMS) was optimized in order to achieve our aim. 3m-ARMS is amplification refractory mutational system (ARMS) based point mutation detection system based on three consecutive mismatches at the 3'end between primer and the wild-type allele besides the conventional ARMS that have two consecutive mismatches at the 3'end. In the case of mutant allele, 3m-ARMS primer makes a match at the 3'end followed by two consecutive mismatches used for selective PCR amplification in order to achieve discrimination of mutant alleles. This study focuses the most widespread point mutations of IDH1 gene, which are IDH1 G395A and IDH1 C394G point mutations. These point mutations examined in 236 glial tumor samples via optimized 3m-ARMS technique followed by Sanger sequencing and immunohistochemistry analysis in order to correlate their accuracies. The addition of the appropriate mismatch to the conventional ARMS method, 236 glial tumor sample were analyzed with one hundred percent accuracy when compared with Sanger sequencing methodology. After correlation with immunohistochemistry, 3m-ARMS has been also shown better accuracy when compared to immunohistochemistry analysis. Contrary to conventional Sanger sequencing, 3m-ARMS had successfully analyzed 236 glioma sample without the requirement of mutant specific amplification. This shows the

sensitivity of 3m-ARMS is superior compare to Sanger sequencing. Sensitivity is the most important trait of 3m-ARMS due to the deoxynucleic acid (DNA) source used for detection is heterogeneous in terms of mutant and wild-type allele numbers, as the mutant containing alleles present in the DNA mixture present at much lower frequency compared to wild-type allele. Additionally, we test our specificity and sensitivity by creating our own mutant to wild type ratios and point mutation involving plasmids via cloning and mutagenesis techniques. Quantitatively, up to 14 cycle superiority has been observed in constructs specific to 3m-ARMS primers alongside with all or none detection was achieved at femtogram level. By the help of cloning and mutagenesis methods, IDH2 G515A mutation was also analyzed with 3m-ARMS principle and gave all or none results. With 3m-ARMS, detection of IDH1 mutations can be held during an operation with robust sensitivity and specificity. Thus, it is a novel detection method, which can be used intraoperatively for diagnostic analysis. Lastly, circulatory DNA released by glioma cells also analyzed by using plasma samples with 3m-ARMS method. However, more experiments are required for the future of successful genotyping from blood samples prior to surgical operation. As future prospect, IDH2 will be adapted to alkaline lysis alongside with other point mutations such as TERT promoter mutations in parallel to ctDNA optimizations.

Keywords: ARMS, Glial Tumors, IDH1 Mutation, Intraoperative Diagnosis, Molecular Diagnosis

ÖZET

İNTRAOPERATİF TANI KULLANIMINA YÖNELİK, HIZLI VE HASSAS IDH1 MUTASYONU TEŞHİS SİSTEMİ GELİŞTİRİLMESİ

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İsositrat I ve II (IDH1, IDH2) malformasyonları, hücresel enerji metabolizmasına etki eden mutasyonlardır. IDH1 ve IDH2 mutasyon varlığının uygulanan kanser tedavilerine verilen olumlu cevap ile yakından ilişkileri ve glioma hastalarındaki sıklıkları, bu mutasyonları önemli kılmaktadır. Bu çalışma, ameliyat sırasında IDH1 mutasyonlarını hızlı ve doğru şekilde analiz eden bir tespit sistemi geliştirip cerraha tümör habisliği ile ilgili bilgi vermeyi amaçlamaktadır. Çalışmanın basamakları, alkaline lysis ile DNA çıkarma ve yeni bir nokta mutasyonu tespit sistemi kullanılarak nokta mutasyonlarını analiz etmekten oluşmaktadır. Bu sayede retrospektif biçimde 226 ve prospektif biçimde 10 gliom örneği yüzde 100 doğruluk ile maksimum 67 dakikada analiz edilmiştir. Bu yolda başvurulan optimizasyon deneyleri de tartışılarak ortaya konmuştur. Ayrıca, bulunan tespit sisteminin öneminden ve IDH1/2 mutasyonlarının kanser tedavisini nasıl etkilediğinden de bahsedilmiştir. Gliom hastalarının yaklaşık yüzde 74'ünde görülmelerinin yanı sıra, IDH1 ve IDH2 nokta mutasyonlarını içeren gliomların kanser tedavisine olumlu cevap vermeleri, dolayısıyla iyi prognoz ile olan ilişkileri bilinmektedir. Buna ek olarak, IDH1 R132H nokta mutasyonu sıklığı diğer IDH1/2 mutasyonlarının yüzde 90'ından fazladır. Bu nedenden dolayı, IDH1 mutasyon durumunun ameliyat öncesi veya sırasında beyin cerrahı tarafından bilinmesi, uygulanacak rezeksiyon tipinin belirlenmesi açısından çok önemlidir. Çünkü, IDH1/2 mutasyonları tümör habisliği ile doğrudan ilişkilidir. Çalışmada 3-mismatch-ARMS (3m-ARMS) tekniği optimize edilerek amaçlanan seviyeye geirilmiştir. 3m-ARMS, amplifikasyon refrakter mutasyon sistemi (ARMS) bazlı bir nokta mutasonu tespit sistemidir. Çalışma prensibi, tasarlanan primerin 3' ucuna ardı ardına sıralanmış ve doğal suş alleli ile yapılan üç adet baz uyumsuzluğuna dayanır. Konvansiyonel ARMS tekniğinin farkı ise primer ve doğal suş arasında oluşan iki adet baz uyumsuzluğuna dayanıyor olmasıdır. 3m-ARMS, mutant allel söz konusu olduğunda ise aynı primer 3' ucunda uyumlu bir eşleşme ardından ise iki adet baz uyumsuzluğuna sebep verir. Bu sayede selektif bir PCR ortamı oluşarak mutant allel doğal suş arasından ayırt edilir. Bu çalışmada IDH1 geninde görülen en yaygın nokta mutasyonları ele alınmıştır. Bunlar; IDH1 G395A ve IDH1 C394G nokta mutasyonlarıdır. Bu nokta mutasyonları, toplamda 236 glial tümörde optimize edilmiş, 3m-ARMS yöntemi ile incelenmiştir. İncelemelerin doğruluğunu başka yöntemler ile kıyaslamak amaçlı örnekler Sanger sekanslama ve

immunohistokimya ile analiz edilmiştir. Uygun baz uyumsuzluğunun konvansiyonel ARMS primer tasarımına eklenmesi ile, 236 glial tümör Sanger sekanslama yöntemi baz alınarak yüzde yüz doğrulukla analiz edilmiştir. İmmunohistokimya ile korelasyon sonrası, 3m-ARMS tekniğinin üstün olduğu görülmüştür. Konvansiyonel Sanger sekanslamasının aksine 3m-ARMS, 236 glial hastayı, mutant spesifik amplifikasyona gerek kalmadan başarıyla tespit etmiştir. Bu, 3m-ARMS tekniğinin Sanger sekanslamaya kıyasla daha hassas olduğunu göstermektedir. Hassaslık 3m-ARMS tespit sisteminin en önemli özelliklerden biridir çünkü tespit için kullanılan DNA kaynağı mutant ve doğal suş açısından heterojendir. Bu heterojenite genellikle mutant DNA'nın doğal suşa oranla çok daha az bulunmasıdır. Bundan dolayı, 3m-ARMS'ın sensitivitesini ve spesifitesini test etmek amaçlı klonlama ve mutajenez teknikleri kullanılarak istenilen nokta mutasyonlara ait plazmidler ve oranını kendimiz belirlediğimiz mutant DNA stokları elde edilmiştir. Bu testler sonrası primere spesifik mutasyon içeren reaksiyon, içermeyene oranla 14 döngü önceden başlamıştır. Ayrıca, femtogram seviyesinde DNA içeren reaksiyonlarda bile spesifik sonuçlar alınmıştır. Klonlama ve mutajenez metodları sayesinde IDH2 G515A mutasyonu da 3m-ARMS ile incelenmiştir ve spesifik sonuçlar vermiştir. 3m-ARMS sayesinde, IDH1 mutasyonları operasyon sırasında yüksek sensitivite ve spesifiteyle tespit edilebilir. Bu nedenle, 3m-ARMS, intraoperatif diagnostik analiz için kullanılabilecek yeni bir tespit sistemidir. Son olarak, gliomların saldığı ve kanda serbest dolaşan tümör DNA'ları da plazma örnekleri kullanılarak 3m-ARMS metodu ile incelenmiştr. Ancak, ameliyat öncesi kan örneklerinden genotipleme konusunda başarılı sonuçlara ulaşmak için daha fazla optimizasyon deneyi gereklidir. Gelecekte, IDH2 analizini de alkaline lysis metoduna adapte etmeyi ve TERT promotör mutasyonları gibi başka nokta mutasyonlarını da tekniğimize adapte etmeyi umuyorz. Buna paralel olarak, ctDNA araştırmaları devam edecektir.

Anahtar Kelimeler: ARMS, Glial Tümörler, IDH1 Mutasyonu, İntraoperatif Diagnoz, Moleküler Diagnoz

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ABBREVIATIONS

3m-ARMS	:	3-mismatched amplification refractory mutational system
AFLP	:	Amplified fragment length polymorphism
ALT	:	Alternative lengthening of telomeres
ARMS	:	Amplification refractory mutational system
ASPCR	:	Allele specific polymerase chain reaction
ATRX	:	Alpha-thalassemia/mental retardation syndrome x-linked
C228T	:	Cytosine to thymine change at nucleotide 228
cfDNA	:	Cell-free DNA
CNS	:	Central nervous system
COLD-PCR	:	Co-amplification at lower denaturation temperature-pcr
CSF	:	Cerebrospinal fluid
Ct	:	Cycle treshold
ctDNA	:	Circulating tumor DNA
ddNTPs	:	Dideoxynucleotides
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleotide
E. coli	:	Escherichia coli
EGFR	:	Epidermal growth factor receptor
FFPE	:	Formalin-fixed paraffin-embedded
FN	:	False negative
FP	:	False positive
GB	:	Glioblastoma
GC	:	Guanine-cytosine
GFAP	:	Glial fibrillary acidic protein
H&E	:	Hematoxylin and eosin
HRM	:	High resolution melting
IDH	:	Isocitrate dehydrogenase
IHC	:	Immunohistochemistry

LB	:	Luria-bertani
LR-	:	Negative likelihood ratio
LR+	:	Positive likelihood ratio
MGMT	:	O6-methylguanine-DNA methyltransferase
Mini-Prep	:	Mini preparation
Na_2EDTA	:	Ethylenediaminetetraacetic acid disodium salt dihydrate
NADPH	:	Reduced nicotinamide adenine dinucleotide phosphate
NGS	:	Next-generation sequencing
NPV	:	Negative predictive value
NRAS	:	Neuroblastoma ras viral oncogene homolog
Olig-2	:	Oligodendrocyte transcription factor
OS	:	Overall survival
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PIK3CA	:	Phosphatıdylınosıtol 3-kınase, catalytıc, alpha
PPV	:	Positive predictive value
PTEN	:	Phosphatase and tensin homolog
qPCR	:	Quantitative PCR
R132H	:	Arginine to histidine at amino acid 132
R132H	:	Arginine to histidine at amino acid 132
RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
SNPs	:	Single nucleotide polymorphisms
SOB	:	Super optimal broth
SOC	:	Super optimal broth with catabolite repression
TERT	:	Telomerase reverse transcriptase
TN	:	True negative
TP	:	True positive
WHO	:	World health organization
α-KG	:	A-Ketoglutarate

SYMBOLS

dH_2O	:	Distilled Water
EtBr	:	Ethidium Bromide
KCl	:	Potassium Chloride
$MgCl_2$	:	Magnesium Chloride
$MgSO_4$	:	Magnesium Sulfate
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
TRIS HCl	:	Trizma® Hydrochloride

1. INTRODUCTION

Isocitrate dehydrogenase I and II (IDH1, IDH2) mutations are known as disrupting the cellular energy metabolism by altering the isocitrate to alpha-ketoglutarate transition in Krebs cycle by replacing it with alpha-ketoglutarate to 2-hydroxyglutarate transition. These mutations are strongly related with good prognosis upon standard cancer treatments and highly frequent in glioma patients. In this study, a modified version of the conventional polymerase chain reaction (PCR) based amplification refractory mutational system (ARMS), which is called as 3-mismatched ARMS (3m-ARMS) was used over conventional ARMS as a detection tool for 1 IDH1 mutations on 226 glioma DNA sample of frozen tumor tissue retrospectively and prospectively by 10 prospective samples. 3m-ARMS was selected due to its enhanced specificity and sensitivity during quantitative polymerase chain reaction (qPCR) based mutant specific amplification, and its rapid detection. Quick analysis of the results was maintained owing to compatibility of 3m-ARMS with fluorescence based melting curve analysis. Samples were generated by using alkaline lysis DNA extraction of frozen tumor samples due to its rapidness and efficiency. IDH1 R132H mutations selected as primary focus due to its predominance in glial tumors and its strong value on good prognosis. Sanger sequencing was used as a gold standard method to achieve the accuracy of 3m-ARMS method alongside with immunohistochemistry (IHC). Full co-amplification at lower denaturation temperature–PCR (full-COLD-PCR) method was applied prior to Sanger sequencing in samples with false positivity in 3m-ARMS method to solve the sensitivity problem by increasing the mutant to wild type allele ratio. Thus, mutant to wild type ratio was increased in order to observe which method cause the limitation during detection. More clearly, in order to see if the results are false positive due to low specificity of 3m-ARMS or false negative due to low sensitivity of Sanger sequencing.

As target mutations of IDH1 effecting the amino acid residue 132, G395A and C394G point alterations were focused on. In the case of IDH2, mutation effecting the amino acid residue 172, G515A mutation was focused. Additionally, this study includes the genotyping experiments by the help of circulating tumor DNA (ctDNA), which extracted

from blood samples of glioma patients via 3m-ARMS method aiming to catch glioma-sourced tumor DNA. Main intention of this future prospect is to inform the surgeon about the molecular properties of the tumor prior to surgical operation.

Aim of this study is to produce an accurate intraoperative detection methodology to utilize upon molecular diagnosis of glial tumors within 60 minutes. More specifically, to inform the surgeon about tumor malignancy, prognostic characteristic or treatment response by detecting IDH1 status during a surgical brain operation and guide the operator about the route of neurosurgical resection with ultrasensitive, specific, economic and reproducible manner via 3m-ARMS. In addition to main purpose, generating a novel modification on conventional ARMS methodology in order to overcome probable specificity and sensitivity problems of ARMS itself and conventional immunohistochemistry (IHC) analysis is the second aim of this study. IHC analysis is not always specific nor sensitive upon producing a reliable result since the error rate is significantly high compare to 3m-ARMS analysis maintained in this study. Moreover, intraoperative cytological analysis is not suitable in all applications due to insufficient cellularity in case of diffuse gliomas.(Shankar et al., 2015) Knowledge of IDH1/2 status during or after a surgical operation have a positive effect upon determining the clinical route. Because, glioma patients with IDH1/2 mutation have a better reception to radiotherapy and chemotherapy. Thus, IDH-mutant gliomas have a better prognosis comparing to IDH1/2 wild type. Additionally, information of IDH1/2 status of a patient have a strong importance upon diagnosis of gliomas and differentiating between sub-types of gliomas.(Houillier et al., 2010)

Other studies focusing IDH1 and IDH2 mutations also aims intraoperative diagnosis in order to assist the treatment decisions. Rapidness and high accuracy are the main goal during detection due to the limited time. Similar studies were performed in 2014 and 2015 focusing intraoperative detection of IDH1/2 mutations. According to the study performed in 2014, diagnostic properties of IDH1/2 status helps to assist the discrimination between low-grade gliomas, nonneoplastic lesions and high-grade gliomas. Experiments were performed in order to examine the most suitable procedures for IDH1/2 mutation detection with conventional PCR and COLD-PCR methods prior to targeted sequencing.

Double COLD-PCR was applied before high resolution melting (HRM) analysis in order to reach higher sensitivity of 0.25 percent. Results were analyzed via hybridizing probes, which results a fluorescence based melting curve graph upon mutant specific hybridization.(Kanamori et al., 2014)

Another study published in 2015 discussed the similar significance of IDH1 R132H/C/G/L/S and TERT C228/250T status on 72 WHO grade II gliomas upon guidance of surgical operation. According to the study, in some cases with diffuse gliomas intraoperative IHC analysis is not reliable. Genotyping performed on TERT promoter and IDH1/2 mutational status with using qPCR by the help of wild type specific blockage with enzyme resistant modified nucleic acid called peptide nucleic acid (PNA). Additionally, mutant specific quantitation maintained by incorporation of highly specific modified nucleic acid called locked nucleic acid (LNA) as real-time fluorescence source.(Shankar et al., 2015)

The following parts of the thesis consist of general to specific studies according to publication dates as the literature review in order to form an idea about the foundations of this study. Material and methods section involves utilized tools and methods during experiments followed by results of the experiments. Results were discussed in the discussion part followed by a brief conclusion about the overall significance and result of the study.

2. LITERATURE REVIEW

2.1. CENTRAL NERVOUS SYSTEM TUMORS

All types of tumors can be separated as benign and malign tumors. According to American Brain Tumor Association, malignant central nervous system (CNS) tumors are one of the most dangerous malformations. More than seventy thousand people living with CNS tumors only in 2018, USA. Sixteen thousands of them will lose their lifes despite diagnosis. There are two types of malignant CNS tumors consisting of primary and metastatic, in other words, secondary tumors. Metastatic tumors resulted from another location inside the organism by malfunctioning cell migration capabilities of cancerous cells.(Society, 2018)

2.2. TREATMENTS OF BRAIN TUMORS

Treatment of brain tumors depends on size, type, grade and location of the tumor. Surgical intervention aims to completely remove the risky tissue and prevent any probable recurrence by gross total resection. However, when the tumor resides in a risky anatomical location, gross total resection can be life threatening or may cause reduction of the quality of life after operation. To prevent these results, surgeon may prefer sub-total resection and leave the tumor tissue residing at a risky location. However, in high grade tumors, this decision can be life threatening.

On the other hand, radiotherapy, chemotherapy and targeted drug therapy are other approaches aiming to stabilize or diminish the tumor tissue. These methods can be applied separately or together. Thus, any information about the tumor malignancy is extremely important before the surgical operation ends, which makes intraoperative molecular diagnosis crucial.

2.2.1. Intraoperative Diagnosis

Intraoperative diagnosis aims to inform the surgeon about the detailed location or histopathological diagnosis of the tumor during surgery. Intraoperative magnetic resonance imaging is conventionally used to maintain real-time guidance of operation. Additionally, cytological methods such as crush smear and touch imprint commonly used to evaluate tumor specimens. However, due to limited duration of operation, intraoperative diagnosis should not exceed one hour. Thus, applying molecular diagnosis on specimens is not possible with traditional IHC methods.

2.3. TYPES AND CATEGORIZATION OF GLIOMAS

Glial tumors, gliomas, forms more than seventy percent of all brain tumors in adults making them the most common brain tumor type.(Ricard et al., 2012, Goodenberger and Jenkins, 2012) Glioma is the generalized name that given to numerous tumor types including ependymal tumors, astrocytomas, oligodendrogliomas, optic nerve gliomas and brain stem gliomas. Further classification of glial tumors is maintained by World Health Organization (WHO) according to pathological assessment of tumors by IHC procedures.

WHO classification is composed of four grades, which specify the malignity of tumor. WHO Grade I and II gliomas classified as low-grade gliomas. Low-grade gliomas indicate benign properties with a better prognostic value. They are not anaplastic as they fully differentiated. High-grade gliomas include WHO grade III and IV gliomas. High-grade gliomas show malign properties, as they are anaplastic.(Louis et al., 2016) Glioblastoma (GB) is the worst scenario upon all glial tumors and classified as WHO grade IV glial tumor. GB originates from astrocytes and have the lowest prognosis. Oligoastrocytomas on the other hand, belongs to WHO in 2007 is the mixed tumor tissue composed of both oligodendrogliomas and astrocytomas. However, newly formed WHO grading system in 2016 eliminates this term and takes into account the genetic testing to determine the class and grade of the tumor. In addition to terminological modifications, sole histological evaluation based 2007 WHO grading system include novel epigenetic

and genetic properties of gliomas, which enables new diagnostic and prognostic hallmarks to consider upon WHO grading of tumors.

In 2016, WHO grading system upgrade its conventional profile by opening the doors for molecular markers.(Masui et al., 2016) Histology based old WHO grading system separates cancer cells according to their morphological properties in order to suppose the level of differentiation. Thus, immunohistochemistry techniques under light microscope was the sole procedure during the estimation of WHO grades until 2016. Updated WHO grading system utilizes different staining methods using thin-sectioned tissues in order to observe the structural properties of cells as well as genotyping methodologies. (Masui et al., 2016, Louis et al., 2016)

2.3.1. Types Of Glial Tumors

2.3.1.1. Astrocytoma

Astocytomas originate from astrocytes. Astrocytes are star-shaped glial cells excessively found in central nervous system (CNS). They are the most prominent glial cell type found in CNS. There are four distinct group of WHO grade in terms of astrocytomas. Grade I astrocytic tumors can be defined morphologically as having nuclear atypia. Grade II astrocytomas are diffuse astrocytomas and cellular infiltration via increased rate of mitotic activity observed morphologically. Anaplastic astrocytomas belongs to grade III and have a greater proliferative activity compare to grade II astrocytomas. Lastly, GBs are the grade IV astrocytomas, which defined phenotypically with necrosis and vascular proliferation as well as high proliferative activity.(Louis et al., 2016)

Astrocytes have numerous functions such as forming the blood-brain barrier and transmit required nutrients to nerve cells and regulation of ion concentration in extracellular matrix via their sodium and potassium channels. Astrocytes also play a role in scarring process in order to form glial scar tissue following a traumatic injury by the event called reactive astrogliosis.(Sofroniew and Vinters, 2010) Reactive astrogliosis known to result following a disease or injury in CNS by differentiating into morphologically distinct

phenotype that have an altered molecular profile.(Sofroniew, 2009) Reactive astrogliosis is an important signal of pathological event in the CNS. Besides their indicative features of long-term reaction of pathogenesis, cancerous formations of swollen reactive astrocytes are present as high grade gliomas with worse prognostic value compare to its non-gemistocytic phenotype.(Tonn et al., 2006) Tumor incorporated reactive astrogliosis can also be seen in GB, which is the worst-case scenario of astrocytoma with WHO grade IV. Because reactive astrogliosis enhance the progression of tumors by increasing the overall progression and aggression by the help of cytokine release.(Guan et al., 2018)

2.3.1.2. Oligodendroglioma

Oligodendrogliomas are another type of glial tumors that originate from oligodendrocytes. Oligodendrocytes are glial cells found in CNS and have primary function of axonal insulation. Thus, preventing the ion leakage during dispersion of action potential. Oligodendroglial insulation maintained by myelin sheath wrapped around axonal projections in CNS. Besides the insulator effect, oligodendrocytes nourish nerve projections with nutrient support.(Bradl and Lassmann, 2010)

Cancerous form of oligodendrocytes, oligodendrogliomas, could not maintain their functions, which is why the most encountered symptom of oligodendroglioma is seizure activity. Even though there is no treatment of oligodendrogliomas, their growth is not fast as astrocytomas, makes them to cause longer OS rate.(Ohgaki and Kleihues, 2005) There are two grades of oligodendrogliomas according to WHO. Grade II oligodendrogliomas have very slow growth rate compared to malignant and fast growing anaplastic oligodendrogliomas that belongs to grade III.(Louis et al., 2016)

2.3.1.3. Ependymoma

Ependymal cells are another type of glial cells, which present in the neuroepithelial foundation in CNS. Ependymal cells cover the perimeter of the ventricles and have important functions as absorption, production and circulation of cerebrospinal fluid (CSF). Ventricles of the brain create an interconnected network of CSF.(Sadler, 2010)

CSF inside the cavities of ventricles acts as a protective layer, and maintain both nourishment and cleanse of the brain as well as the spinal cord. Additionally, it supports the brain against gravity and shocks.(Center and Campus, 2011)

Ependymomas are the cancerous formation of ependymal cells appears from the neuroepithelial lining. Fourth ventricle is the most common location which known to arise ependymomas. Other locations such as spinal cord also seen to emerge ependymomas. Ependymomas are less encountered glial tumors compare to astrocytomas and oligodnedrogliomas. They generally form benign tumors with non-anaplastic properties. However, some ependymomas are malign and have anaplastic features that have low prognosis.(Poppleton and Gilbertson, 2007)

2.3.2. Categorization Of Gliomas

2.3.2.1. Immunohistochemistry

IHC is an imaging technique, which utilizes specific antibodies to bind targeted antigens in cells contained by a specific thin tissue section. Antibody staining of molecular markers conventionally used to diagnose tumors, including gliomas. Different molecular markers used via IHC analysis have distinctive roles upon expression on cellular events such as proliferation and apoptosis as well as utilized in cell discrimination via observing the expression and distribution of indicative markers in tissue.(Duraiyan et al., 2012)

Categorization of gliomas depend on both molecular markers identified via IHC analysis and the genetic profiling via genotyping methods according to WHO-2016. Important hallmarks of glial cells are glial fibrillary acidic protein (GFAP) and oligodendrocyte transcription factor (Olig-2). Some important tumor markers are TP53, MIB-1, Alpha-thalassemia/mental retardation syndrome X-linked (ATRX) and IDH1 arginine to histidine at amino acid 132. (R132H). These markers utilized to categorize most of the astrocytomas and oligodendrogliomas.

2.3.2.1.1. Immunohistochemical markers of glial cells

GFAP is the structural protein of intermediate filaments, which is abundant in many cells of the CNS as well as some cells outside of the CNS. It maintains mechanical stamina as well as shape of the cells. GFAP have another important role in cell-to-cell interactions and mitosis. Astrocytes strongly express GFAP as it maintains neuron-astrocyte communications, which also makes it an important marker of astrocytomas. (Weinstein et al., 1991)

Olig-2 is another diagnostic marker used to discriminate astrocytomas, oligodendrogliomas and GBs. Olig-2 is an important transcription regulatory protein, which have role during oligodendrocyte development. However, other glial cells also express Olig-2 protein making it insufficient to make a certain separation between tumors of astrocytic and oligodendroglial origin. (Ligon et al., 2004)

2.3.2.1.2. Immunohistochemical markers of tumors

Gene of TP53 expresses a tumor protein, which also known as tumor suppressor protein, which have a crucial role on cancer prevention via genome protection. Several pathways act on tumor suppression involves TP53 protein. Such as, regulation of angiogenesis, apoptosis, DNA repair and stability, cell cycle arrest via DNA damage sensor property and senescence. Thus, loss of TP53 expression is a conventional indicator of tumor presence.(Mendrysa et al., 2011, Surget et al., 2013)

MIB-1 is an antibody against Ki-67 protein encoded from Ki-67 gene. Ki-67 expression conventionally correlated with the proliferative index of a cell population. Thus, it gives an approximate value about the rate of mitosis. Ki-67 protein can observed in all stages of the cell cycle process. Ki-67 labelling gives an approximate clue about the percentage of cell population entered cell cycle. It is crucial to determine the aggressiveness of cancerous tissue so Ki-67 expression is directly proportional with the low survival rate in cancer. (Vasudev et al., 2012, Scholzen and Gerdes, 2000)

ATRX is a transcriptional regulatory protein and have important roles upon chromatin remodeling and stability via histone interactions. (Nandakumar et al., 2017) According to a study, mutations in the ATRX gene caused disturbed methylation profile.(Gibbons et al., 2000) ATRX loss is a characteristic profile for anaplastic gliomas especially tumors with astrocytic origin. However, low percentage of GB cases appear to have ATRX loss, which indicates the presence of different pathways during GB formation. It accommodates with other tumor characteristics such as alternative lengthening of telomeres (ALT) phenotype, IDH1/2 mutation, 1p/19q co-deletion and O6-methylguanine-DNA methyltransferase (MGMT) methylation status.(Wiestler et al., 2013) The rare concurrence of 1p/19q co-deletion and ATRX expression loss provides facility upon differentiating astrocytomas and oligodendrogliomas, since presence of 1p/19q co-deletion is a characteristic of oligodendrogliomas.(Nandakumar et al., 2017)

Anti-IDH1 R132H is another antibody, which is specific for the mutant protein expressed from the mutated IDH1 gene.(Dimitrov et al., 2015) IDH1 have two additional isozymes named IDH2, IDH3 that expressed from five different gene called IDH1, IDH2, IDH3A, IDH3B and IDH3G. IDH1 is a cytosolic NADP+-dependent dehydrogenase enzyme that acts as a catalyzer of the reversible oxidative decarboxylation reaction of the citric acid cycle resulting α-ketoglutarate (α-KG) from isocitrate. (Guo et al., 2011) Nowadays, IDH1 and IDH2 mutations gained crucial importance upon diagnosis and prognosis of glial tumors. Moreover, IDH1 gene have a greater percentage of mutational status comparing to IDH2 mutations and it has been previously shown that majority of astrocytic and oligodendrocytic tumors of WHO grades II and III have mutations on the IDH1 gene.(Hartmann et al., 2009)

2.3.2.2. Genotyping

Protocols of human genetics are flourishing every day as novel methods creating the basis of modifications on these methods in the direction of efficiency. History of genetics based on Mendelian genetics in 1865. In the 20th century, new discoveries forged the foundations of the following improvements and made the molecular basis of genetics

more clear. These improvements lead the completion of Human Genome Project in 2003.(Durmaz et al., 2015)

Genotyping techniques includes numerous conventional methods, which uncovers the differences of genetic profile between species or specifically chosen reference sequences by comparing their gene sequences. There are plenty of commercially available DNA extraction kits in order to extract pure DNA prior to genotyping. However, fast and reliable technique called alkaline lysis is another option resulting impure but PCR friendly DNA stock via alkaline degradation of the cell components at high temperature.(Ehrt and Schnappinger, 2003)

At present time, genotyping methods used extensively in order to attribute the relationship between specific mutations and diseases. Thus, the opportunity of creating specific cancer models are emerged. This opportunity in modelling technology is directly proportional to prediction of the route of tumor development and to find a therapeutic response has become possible.(Ragoussis, 2009) There are several conventional genotyping methods such as sequencing, DNA microarray, technologies based on DNA-probe relationship and other genotyping technologies based on restriction enzyme cleavage based methodologies and PCR.(Kim and Misra, 2007)

2.3.2.2.1. DNA sequencing techniques

DNA sequencing is a powerful tool that aims to define the targeted DNA sequence of a specific organism according to its nucleotide order. Information about the nucleotide order of an organism is crucial upon biotechnology, forensic biology, diagnosis and virology. Before the fluorescence based sequencing methods, two-dimensional chromatography was used during DNA sequencing in early 1970s.(Pettersson et al., 2009)

Sanger sequencing is a popular method of DNA sequencing, which utilizes dideoxynucleotides (ddNTPs) as a terminator base in order to stop additional elongation by the polymerase enzyme. Additionally, each ddNTP have a tagged fluorophore, which results emissions with different wavelengths according to each base followed by

fluorescent reading that gives the information about the nucleotide order. After 1977, Sanger sequencing settled as the most widespread DNA sequencing technique. However after few decades, due to the slowness of Sanger sequencing, next-generation sequencing (NGS) techniques will replace the conventional Sanger sequencing in the case of large DNA sequences. For short sequence analysis, Sanger sequencing is still a preferred DNA sequencing technique.(Sanger and Coulson, 1975)

NGS is the group of modern sequencing techniques widely used during genomic researches. NGS is like Sanger sequencing, as it based on capillary electrophoresis technology. Despite the similarity, NGS is very fast due to its parallel sequencing ability of numerous small DNA fragment at the same time by using an array-based technology. These small scale readings then rendered together by bioinformatics in order to achieve the information about the nucleotide order as complete and continuous sequence. Thus, whole genome analysis become much faster and easier to achieve.(Behjati and Tarpey, 2013)

2.3.2.2.2. DNA microarray and restriction enzyme based techniques

DNA microarray is an artificial biochip formed of nanoscale fabric that have specific probes. Probes consist of specific DNA sequences at picomole range in order to cross with fluorophore-labelled target DNA. Thus, a quantitative detection achieved directly proportional to the hybridized probes, which further used to characterize the expression profile of a selected organism.(Pollack et al., 1999)

On the other hand, enzyme based genotyping techniques rely on specific restriction sites. These sites may disappear or emerge due to an alteration on the sequence, which effects the sizes of the fragments during the presence of an alteration. Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) are common restriction enzyme based genotyping techniques. Both technique turn out with unique template of the selected genes to discriminate specific alterations on the gene. There are various appliactions based on RFLP and AFLP such as, determining the genetic

variation between species, creation of a genetic maps, paternity testing and elucidation of the genetic background for genetic diseases.(Paun and Schonswetter, 2012)

2.3.2.2.3. Polymerase chain reaction

PCR was first revelaed by Kary Mullis in 1983, which make him won the Nobel Prize in chemistry. Afterwards, it was first explained as enzymatic amplification of DNA in 1988.(Saiki et al., 1988) PCR is valuable multidisciplinary tool, which generally used to enhance the copy number of a selected region of a specific gene. By the help of PCR technique, single copy of a DNA sequence can exponentially increase to millions of copies. In addition to its amplification-based utility, PCR used to quantitatively analyze the expression rates of specific genes or selectively eliminate unwanted amplification to achieve the knowledge of the produced sequence. Numerous applications utilizes various PCR methods and most of them based on thermal cycler tool.(Chakrabarti and Schutt, 2001)

Thermal cycler increase or decrease the temperature according to the settled conditions depending on the intended size of the product, melting temperature of the primer and working conditions of the selected polymerase enzyme. Every cycle of PCR, exponentially increase the DNA number by doubling the present DNA amount with three thermally different step. Each cycle consist of denaturation step, which melts the double stranded DNA sequence to single stranded form. Annealing step, which maintains the primer binding its specific target sequence. Primers are specifically designed oligonucleotides that have affinity to its target sequence and present a suitable domain for polymerase binding. Lastly, extension step, which the polymerase enzyme binds to 3' ends of annealed primers and creates the anti-sense strand of template DNA by adding dNTPs present inside the reaction buffer.(Saiki et al., 1988, Lorenz, 2012)

Different additives, such as dimethyl sulfoxide (DMSO), formamide and betaine, can used to destroy the secondary structures on the template in the case of high GC content product requirement. Additionally, besides the ionic content used in enzyme buffers, supplementary ions, such as $MgCl_2$ and KCl utilized to increase the efficiency of

polymerase enzyme by neutralizing the negative charge present in the backbone of the DNA. As a result, Mg^{2+} and K^{+} ions removes the unwanted repulsion between the primer molecule and the DNA strand thus, facilitating primer-DNA binding during the annealing step of each PCR cycle.(Lorenz, 2012, Cheng et al., 1995)

Generally, during analysis of the PCR results, agarose gel electrophoresis used in order to observe the product size, non-specific amplifications, DNA contamination and primer secondary structures. Agarose forms a porous matrix, which can penetrated by charged macromolecules during presence of driving force. Electric field used as the driving force, which pushes negatively charged macromolecules towards the positive pole. An electrophoresis buffer used as ion source that allows current flow inside the electrophoresis tank. According to size, macromolecules move fast or slow at the same current. As a result, small molecules form a separate band from larger molecules. Approximate product size can observed via molecular-weight size markers.(Viovy, 2000)

2.3.2.2.3.1. Quantitative PCR

QPCR (qPCR) or real-time PCR is a type of PCR, which the constant observation of the amplification can made during the reaction. The real-time monitoring of the reaction maintained by DNA specific fluorescent dyes that have affinities to DNA molecule. Thus, the increase of the DNA copy number is directly proportional to emitted fluorescent light heading to the detector. Two different intercalating options are available. Non-specific intercalating dyes that have affinity to every double stranded DNA present inside the reaction tube. On the other hand, probes can preferred in order to detect specific amplifications during PCR reaction. Probes are small oligonucleotides like primers however, a specific fluorophore previously labelled to it. In the case of hybridizing probes, when the crossing between the template strand and the probe molecule provided, fluorophore starts to emit light that gives the real-time knowledge about the copy number status. TaqMan probes on the other hand depends on polymerase cleavage to emit light because of the emission inhibitory effect due to quencher-fluorophore relationship.(Heid et al., 1996, Peirson and Butler, 2007) There are several commercial quantitative dye, which can used to maintain a real-time analysis. Most common non-saturating dyes are

SYBR Green I and EvaGreen. According to a study, SYTO-13 and SYTO-82 are other intercalating dyes, which have reduced reaction inhibitory effects with no melting curve alteration and no superior affinity to GC rich sequences. Thus, offers more sensitive qPCR reaction.(Gudnason et al., 2007)

QPCR is a strong tool when it comes to produce a map of expression rates of specific genes by applying a cycle threshold (Ct) discrimination between them. Additionally, separating of different sized products by melting curve analysis is another utility of qPCR. Ct value is the fluorescence threshold, which the detected emission passes the background emission inside the reaction tube. Thus, it informs the observer about which amplification reach the threshold copy number first. Low Ct value indicates high concentration of starting DNA and visa-versa. As a result, one may compare the starting number of templates according to specific Ct values in order to generate a profile of gene expression. Melting curve analysis also based on intercalation of the DNA strand by specific dyes. When double stranded products formed after PCR reaction, fluorescent dyes hybridize with the DNA content inside the tube and emits light. Melting analysis performed afterwards by increasing the temperature to denature double stranded DNA, which removes the crossing between fluorescent dye and the template. Longer products denatures at higher temperatures and vice versa. Thus by analyzing the melting curve peaks, primer secondary structures, short products and long products can distinguished. Melting curve peaks emerges when the derivative of fluorescence loss over time applied. High-resolution melting (HRM) curves are more sensitive tool for differentiating products with lengths close to each other. However, non-saturating dyes like SYBR green I is not suitable since it leaves non-hybridized parts on the double stranded DNA. There are saturating dyes suitable for HRM analysis such as LCGreen dyes**.** By analyzing melting peaks and Ct values in optimized qPCR reactions, further imaging techniques like gel electrophoresis can evaded.(Rutledge and Côté, 2003)

2.3.2.2.3.2. Co-amplification at lower denaturation temperature

COLD-PCR is another type of conventional PCR based on a critical denaturation temperature, which is the first step of each cycle in PCR reaction in order to separate the

double stranded structure to single stranded form in order to provide conditions for primer annealing step. COLD-PCR is a strong tool aiming to enhance the content of mutation versus wild type DNA amount in the reaction tube. Critical denaturation temperature of the COLD-PCR technique tuned according to the melting temperature of the mismatched sequence or the melting temperature caused by single base change due to a specific point mutation. Two different sub-naming of COLD-PCR is present depending on these properties.(Li and Makrigiorgos, 2009, Milbury et al., 2011)

Fast-COLD-PCR is one type of COLD-PCR methodology, which based on critical denaturation temperature depending on single base change. However, single base change should cause a loss of hydrogen bonding between two strands in order to be suitable for fast-COLD-PCR. Because the denaturation temperature of the mutant allele should lower than the wild type allele for proper COLD-PCR reaction. Thus, the limitation of fast COLD-PCR is the requirement of specific base changes like, guanine or cytosine changes to adenine or thymine.(Milbury et al., 2011)

On the other hand, full-COLD-PCR covers all types of point mutations but have a longer reaction time. Full-COLD-PCR relies on intentional mismatch generation between two strands by integrating another step to the reaction, which separates mutant and wild type alleles by high denaturation temperature and rejoining of all strands to each other. During rejoining phase, the hybridization chance of mutant strands with wild type strands is higher than the chance of homogeneous hybridization because of high wild type containing ratio at the beginning. Thus, single mismatch appears between heterogeneously hybridized DNA content, which causes the emergence of less stable double strands with lower denaturation temperatures. As a result, the critical temperature chosen according to denaturation temperature of mismatch containing alleles. This will cause a mutant-specific denaturation, making them appropriate for the following annealing step while retaining the homogeneous alleles from denaturation, thus inhibiting primer binding.(Ghalamkari et al., 2018)

2.3.2.2.3.3. Allele specific polymerase chain reaction

Allele specific PCR (ASPCR) is another method in order to detect single nucleotide polymorphisms (SNPs) by the help of one intentional mismatch at the terminal base of 3' end location on primer. Enzymes without 3' to 5' exonuclease activity should selected in order to prevent the removal of the intentional mismatch. In a study, primer pairs with 3' end terminal mismatch have a reduced extension speed comparing to matched version based on Drosophila DNA polymerase, which lacks 3' to 5' exonuclease activity.(Petruska et al., 1988) As a result, intentional mismatch against the wild type allele cause lower amplification kinetics or completely inhibits the PCR reaction, which then observed via agarose gel electrophoresis. In opposite to wild type inhibition, mutant specific 3' end base allows proper amplification.(Ugozzoli and Wallace, 1991)

2.3.2.2.3.4. Amplification refractory mutational system

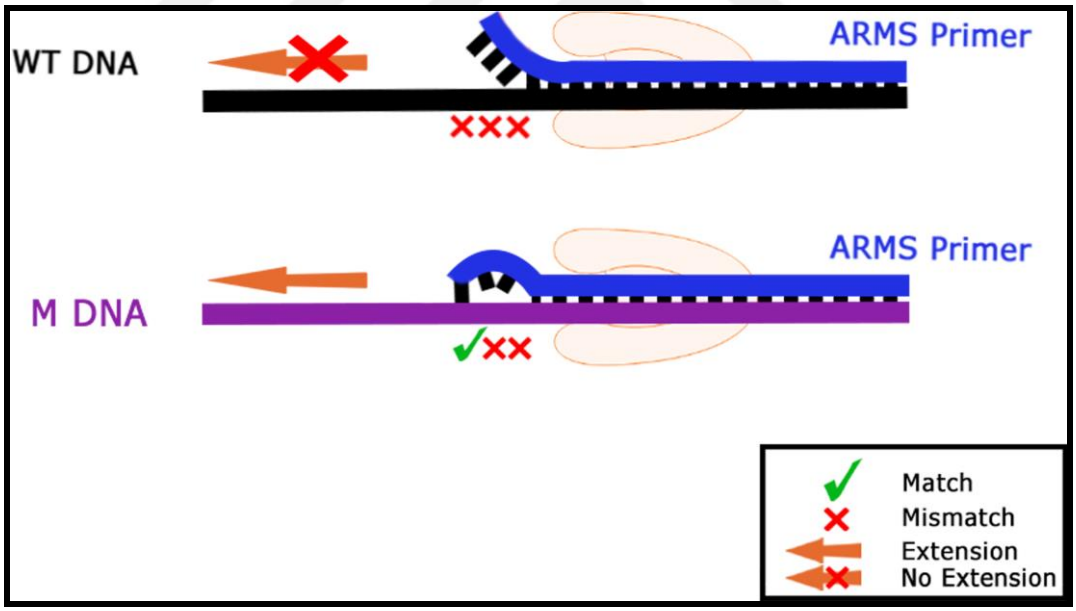
ARMS is another type of PCR similar to ASPCR, which conventionally used as a selective amplification reaction. It also requires enzymes lacking 3' to 5' exonuclease activity due to its 3' end based separation. ARMS is also based on intentional mismatch containing 3' ends in primer design, which will selectively binds to mutant allele and enables mutant specific amplification. Specific selection of 3' ends for mismatch formation is due to 3' to 5' activity of commonly used polymerase enzymes. That is why 3' end priming is very important for proper extension.(Ferrie et al., 1992)

Conventional ARMS primers involve one intentional mismatch with wild type but match with mutant alleles at the last base in 3' end. This modification allows the primer structure to have higher affinity against mutation containing alleles. However, one mismatch with the wild type allele is not enough to inhibit primer-DNA hybridization. Thus, in addition to the first base, second intentional mismatch is present at the penultimate base in 3'end of the primer. Second mismatch selected according to terminal mismatch thermodynamics. Strong terminal mismatch requires weak mismatch at the penultimate base and vice versa. Moreover, penultimate mismatch is not specific to both wild type

and mutant alleles. Thus, when the target is wild type, combined disparity of two mismatch between primer and DNA inhibits annealing of the 3' ends.(Little, 2001)

3-m ARMS is a modified version of the conventional ARMS. 3-m ARMS resulted by the incorporation of the third intentional mismatch between the primer and the template DNA in order to enhance the accuracy of mutant specific amplification via PCR reaction. Third mismatch also chosen in order to generate a mismatch with both mutant and wild type alleles like the second mismatch. Thus, creating less stable 3' end matching during wild type-primer annealing with lower affinity for polymerase. Two serial mismatch formation during the presence of mutant alleles can tolerated by the help of the terminal match but in the case of wild type allele, three serial mismatch is strongly inhibits the amplification reaction.(Avsar, In submission)

Figure 2.1: 3m-ARMS working principle



WT wild type
M mutant
Reference: This figure was prepared by Kutay CANTAŞIR and Alihan SÜRSAL.

2.3.2.3. Site-directed mutagenesis, Gibson assembly and transfection

Producing artificial mutant alleles via genome editing is very important during optimizations of genotyping techniques as well as studying the oncogeneous activities of

specific gene mutations. Site-directed mutagenesis is an efficient and strong tool of intentional introduction of mutation on a specific site by using site-directed primers. Site-directed primers have a specific mutational change on its middle base, which produces point mutation containing products and a hybridization site at the both sites of the mismatched site, which allows proper binding of the primer to template DNA. After a low cycled PCR reaction with site-directed primers, mutation-containing products generated inside the reaction tube. Sequencing techniques can used to observe if the introduction of point mutation is present. Efficient and correct amplification of the mutant containing DNA can maintained via bacteria colony formation following proper transfection.(Shortle et al., 1981)

Proper transfection requires a suitable plasmid DNA including the target sequence in order to let the target bacteria recognize the genetic element. The involvement of the target sequence inside the plasmid requires cloning. Gibson assembly is widely used cloning methodology that enables up to fifteen fragment joining without the requirement of restriction digestion. Gibson assembly was discovered by Daniel G. Gibson in 2009 and rapidly gains interest afterwards as a fast and economical cloning technique. Gibson assembly based on overlapping target sequence and a suitable vector. The reaction maintained by three different enzyme containing cocktail including polymerase, ligase and exonuclease. Exonuclease digest the 5’ ends of the overlapping fragment and the vector. Due to the overlapping parts, the target sequence and the vector joins together, enabling the polymerase to fill the digested sequence according to the undigested template. Lastly, ligase enzyme repairs the nicks on the hybridized DNA.(Gibson et al., 2009)

Transfection is the intentional presentation of specific non-viral nucleic acids in eukaryotic cells. There are numerous ways to introduce the wanted nucleic acid sequence inside the eukaryotic cell. Generally, transfection method relies on pore generation on the lipid membrane in order to let the modified nucleic acids inside the bacteria, called as electroporation. Pore generation maintained by a strong electric field applied on electrocompetent bacteria. Other applications such as DNA endocytosis via calcium

phosphate directed DNA binding on the cell surface and liposome generation via introduction of cationic lipids to nucleic acid are other tools for transfection.

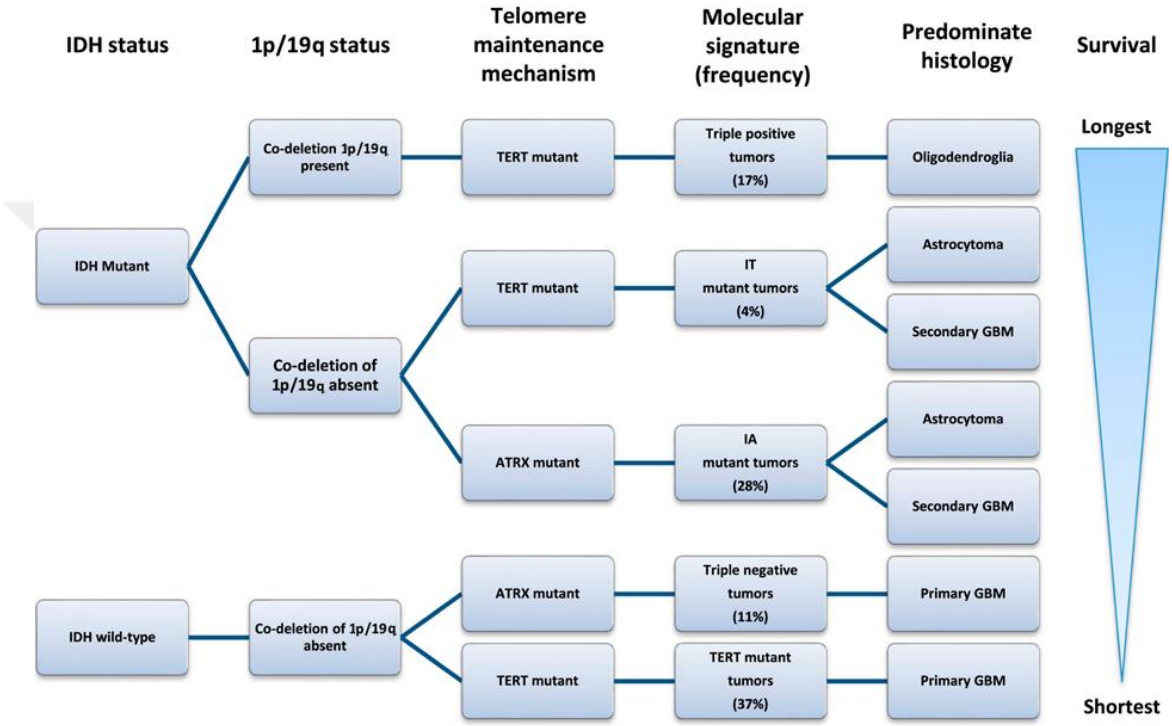
2.4. MOLECULAR MARKERS IN PRIMARY GLIOMAS

The term oncogene defines potentially cancerous genes that generally have different alterations in tumor cells. A tumor cell formed due to a combined alteration rather than a single mutation in its genome. High expression levels also another property of oncogenes in tumor cells other than the presence of mutations. Most important properties of oncogenes are their inhibitory effects on apoptotic pathways or excitatory effects on cell cycling processes. Apoptosis normally cleans malfunctioning cells from the tissue in order to protect the organism. Excitation of cell cycle process involves up-regulation of related genes commonly caused by gain of function mutations. Thus, hyper-proliferation of malfunctioning cells occurs, which causes a rapid increase in tumor mass over time. Newly formed tumor masses have distinctive properties according to the secondary or higher genetic mutations. Thus, specific clinical management may followed according to the cytogenetic difference and heterogeneity in tumor tissue based on indicative markers. Additionally, hypermethylation or missense mutations of tumor suppressor genes is another type of oncogene alteration, which causes the removal of inhibition on tumor formation. Oncogenes generally produce typical proteins such as transcriptional factors, growth factors, growth factor receptors, chromatin remodelers, signal transducers, regulators of cell cycle and apoptosis.(Croce, 2008)

Some important gene alterations in gliomas involves mutations in IDH1/2 and (TERT) gene promoter. There are numerous other gene alterations seen in glioma patients such as mutations in TP53, Phosphatase and tensin homolog (PTEN), neuroblastoma RAS viral oncogene homolog (NRAS), epidermal growth factor receptor (EGFR), phosphatıdylınosıtol 3-kınase catalytıc alpha (PIK3CA), CDKN2A, H3F3A, BRAF and CIC. (Bleeker et al., 2014, Taylor et al., 2012, McNeill et al., 2018, Reis et al., 2015, López et al., 2017, Vuong et al., 2018, Chittaranjan et al., 2014) It is previously known that the mutations in IDH1/2 gene plays role in glioma proliferation. Presence of combined mutations indicates glioma sub-types such as in astrocytoma with co-presence

of TP53, ATRX and IDH1/2.(Wakimoto et al., 2014) Additionally, co-presence of 1p/19q co-deletion alongside with TERT and CIC mutations correlated with oligodendroglioma histology.(Ceccarelli et al., 2016)

Figure 2.2. Survival based distribution of important molecular markers according to predominate glioma histology



Reference: Foote, M. B., Papadopoulos, N. & Diaz, L. A., Jr. 2015. Genetic Classification of Gliomas: Refining Histopathology. Cancer Cell, 28**,** 9-11.

2.4.1. Isocitrate Dehydrogenase 1 And 2 Mutations In Gliomas

IDH1 and IDH2 mutations commonly present in low-grade gliomas as well as non-primary high-grade gliomas, which makes them crucial for glioma biology. IDH1/2 mutations present on 2q34 and 15q26.1 locations respectively. IDH1/2 mutations ascertained in 2009, and enhances the knowledge about gliomagenesis and drastically effects the clinical results because of its effects on molecular mechanisms in the tumor tissue. Mutations of the IDH1/2 genes results an altered state of the enzyme isocitrate dehydrogenase. The altered enzyme disrupts the isocitrate to alpha-ketoglutarate transition in the original mechanism of the citric acid cycle and form an oncometabolite

called 2-hydroxyglutarate by nicotinamide adenine dinucleotide phosphate (NADPH) dependent fashion.(Cohen et al., 2013) As a result, it decreases intracellular NADPH levels, which is required during the regulation of reactive oxygen species (ROS) in order to prevent oxidative stress. That is why IDH1/2 mutations closely related with increased oxidative stress and sensitization of tissue to anticancer treatments. (Rinaldi et al., 2016)

Mutations in the IDH1/2 genes emerges at early stages of glioma development and related with good prognosis according to OS rates compare to IDH1/2 wild type patients with gliomas, which makes the information of the mutational status is important in terms of diagnosis and prognosis. In grade III gliomas, the median survival duration of patients with IDH1 mutation is 81.1 months vs 19.4 months, which is approximately four fold increment of lifetime.(Labussiere et al., 2010) Decrement of NADPH, reduced glutathione, PDGFRA degradation and α-ketoglutarate causes the increased rate of chemo/radio sensitivity due to increased ROS. Together, these traits support the better prognosis of IDH1/2 mutant glioma patients.(Houillier et al., 2010, Flavahan et al., 2016, Labussiere et al., 2010) Vast majority of IDH1 mutations present as R132H mutations, which is resulted by the G395A. Some other scarce IDH1 mutations with descending frequency are also present at the amino acid 132 such as; R132C by the nucleotide change C394T, R132S by the nucleotide change C394A, R132G by the nucleotide change C394G and R132L by the nucleotide change G395T respectively. Similarly, mutations of IDH2 generally resides at the amino acid 172 and composed of R172K by the nucleotide change G515A, R172M by the nucleotide change G515T, R172W by the nucleotide change A514T.(Hartmann et al., 2009)

Table 2.1: Distribution and types of 716 IDH1 and 31 IDH2 point mutations among 1,010 WHO grade II and III oligodendrogliomas and astrocytomas

Gene	Nucleotide change	Amino acid change	N (%)
IDH1	G395A	R132H	664 (92.7%)
	C394T	R132C	29 (4.2%)
	C394A	R132S	11 (1.5%)
	C394G	R132G	10 (14%)
	G395T	R132L	2 (0.2%)
IDH2	G515A	R172K	20 (64.5%)
	G515T	R172M	6 (19.3%)
	A514T	R172W	5 (16.2%)

N (%) number of cases having specific mutation and percentage among all cases

Reference: Hartmann, C., Meyer, J., Balss, J., Capper, D., Mueller, W., Christians, A., Felsberg, J., Wolter, M., Mawrin, C., Wick, W., Weller, M., Herold-Mende, C., Unterberg, A., Jeuken, J. W., Wesseling, P., Reifenberger, G. & Von Deimling, A. 2009. Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta Neuropathol,* **118,** pp.469-74.

2.4.2. 1p/19q Co-deletion And 1q/19p Polysomy In Gliomas

Detection of 1p/19q co-deletion is a unique indicative of patients diagnosed with oligodendroglioma. Most commonly encountered chromosome polysomy is 1q and 19p in the case of 1p/19q co-deletion. According to a study, from a cohort of 148 1p/19q co-deleted oligodendrogliomas, 1q and 19p polysomy indicates with bad prognosis with lower OS. Alongside the importance of 1p/19q co-deletion as the indicative of oligodendrogliomas, 1q and 19p polysomy correlates with worse answer to standard care of treatment.(Ren et al., 2013) Additionally, in the case of astrocytic tumors, 1q and 19p polysomy is related with longer OS according to a study, which accomplished an experiment on 421 astrocytic glioma case.(Zeng et al., 2017)

2.4.3. Telomerase Reverse Transcriptase Promoter Mutations In Gliomas

Telomere is a repetitive sequence resides at the ends of chromosomes in order to prevent degradation and inter-chromosome coactions. Additionally, another crucial role of telomeres is maintaining a complementary sequence for ribonucleic acid (RNA) primer, aiming Okazaki fragment production during chromosome replication on lagging strand. As a result, it prevents the end-replication problem to prune the genes instead of telomere sequences.(Witzany, 2008)

TERT gene expresses a reverse transcriptase enzyme, which maintains the addition of telomere caps consist of TTAGGG nucleotide sequence to the ends of telomeres by the help of telomerase ribonucleic acid (RNA) component as a template so, replaces the chromosome replication-based telomere loss. Telomerase formed of two sub-units. One is the catalytic part with reverse transcriptase property, which translated from TERT mRNA and the second part is the RNA template. That is why telomerase belongs to the RNA-dependent polymerase family.(Weinrich et al., 1997) Malignant mutations of the TERT promoter in cancerous cells results increased functioning of the enzyme, which provides unlimited proliferation for the tumor tissue. In opposite, other malignant mutations resides in telomerase and its fragments causes shortening of the telomeres over time due to chromosome replication, which is called telomere shortening syndromes.(Kirkpatrick and Mokbel, 2001)

TERT promoter mutations are indicative markers of GB's and oligodendrogliomas. However, telomerase malfunctioning is an important alteration that causes tumorigenesis of relevant glioma types as well as astrocytomas.(Lee et al., 2017) Through several point mutation, mostly occurring point mutations in the TERT promoter region are cytosine to thymine change at nucleotide 228 (C228T) and C250T mutations, which abolish the regulation of telomerase activity and causes telomerase hyperactivity alongside with other probable mechanisms.(Masui et al., 2018) Thus, supports immortality of tumor cells. TERT promoter polymorphisms generally correlated with bad prognosis and higher risk of glioma, which causes it to be a valuable glioma marker.(Killela et al., 2013)

2.5. CIRCULATING TUMOR DNA

CtDNA is tumor-sourced alone DNA fragments, which have not surrounded by cell structure. CtDNA should not mixed with cell-free DNA (cfDNA), which is not essentially released by tumor cell. CtDNA release have numerous probable mechanisms such as apoptosis, necrosis or actively released from viable cells, which are still completely unclear. CtDNA generally originated from circulating tumor cells. Circulating tumor cells are metastatic tumor cells, which separated from their origin, primary tumor location.(Akca et al., 2013)

CtDNA gained its popularity due to its informative genomic content, which can utilized in tumor genotyping by liquid biopsy. Thus, in place of taking a tissue sample from the tumor area by a surgical operation, sole blood draws can be utilized. This makes ctDNA-based genotyping very valuable as it presents preoperative diagnosis. According to two separate studies based on humans and xenografted mice models, specific ctDNA fragmentations of 166 base pair (bp) has been encountered predominantly, which is an indicative of apoptosis.(Thierry et al., 2010) (Heitzer et al., 2013) Normally, necrotic and apoptotic cells are eliminated by immune system. However, ctDNA amount is may enhanced in cancer patients compare to healthy ones due to disrupted immune cell infiltration to tumor areas, which makes ctDNA-based genotyping easier in these patients.(Pisetsky and Fairhurst, 2007)

Despite the encouraging property of blood-based liquid biopsies due to its preoperative diagnosis potential and invasiveness, very confined development has been made in the case of central nervous system tumors. Because, blood-brain-barrier (BBB) prevents the release brain tumor-sourced biomarkers to bloodstream. Thus, extreme sensitivities should be reached in order to catch brain tumor-sourced ctDNA or disrupting the BBB with technologies like focused ultra-sound.(Zhu et al., 2018)

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Kits And Chemicals

All kits used in this research are listed in Table 3.1. All chemicals used in this research are listed in Table 3.2.

Table 3.1: List of kits

Kit	**Trademark**	**Catalog No.**
E.Z.N.A Blood DNA Mini Kit	Omega Bio-tek®	R6814-01
NEBuilder® HiFi DNA Assembly Cloning Kit	New England BioLabs	E5520S
NucleoSnap® DNA plasma	MACHEREY-NAGEL	740300,10
NucleoSpin® gel and PCR clean-up	MACHEREY-NAGEL	740609,50
NucleoSpin® plasmid	MACHEREY-NAGEL	740588,50

Reference: This table was prepared by Alihan SÜRSAL.

Table 3.2: List of chemicals

Chemical Product	**Trademark**	**Catalog No.**
1 Kb plus DNA ladder	ThermoFisher	LSG-SM1331
10% Buffered formalin	bright-slide	BS01-112-5000
6X Loading dye	ThermoFisher	LSG-SM1331
Absolute ethanol	Sigma-Aldrich	32221-2.5L
Agarose	Biomax	HS-800
Alcoholic eosin	DDK Italia	09-209
Ampicllin sodium salt	Panreac Applichem	A0839-10G
Anti-ATRX antibody [C3], C-term	GeneTex	GTX101310
Anti-IDH1 R132H	DIANOVA	DIAH09
Boric acid	VWR	33601.261-500G
CutSmart	New England BioLabs	B7204S
Dimethyl sulfoxide	Amresco	WN182-10ML
DpnI	New England BioLabs	R0176S
Ethidium bromide	VWR	0492-5G
Ethylenediaminetetraacetic acid disodium salt dihydrate	VWR	33600.267-500G
EvaGreen 20X	BIOTIUM	31000
Hematoxylin H	BIOGNOST	HEMH-OT-1L
Liquid nitrogen	HABAŞ	

Chemical Product	**Trademark**	**Catalog No.**
Magnesium chloride 25mM	New England BioLabs	M0270L
Magnesium sulfate	Sigma-Aldrich	M7506-1KG
pBluescript SK(+)	Kindly provided from Dr. Melih Acar	
Phosphate buffered saline	Gibco	70011-036
Potassium chloride	Sigma-Aldrich	12636-1KG
Q5® high fidelity DNA polymerase	New England BioLabs	M0491S
Sodium chloride	Sigma-Aldrich	31434-1KG-R
Sodium hydroxide	DAEJUNG	7570-4100
Sterile water (molecular grade)	MULTICELL	809-115-CL
SYBR green 2X master mix	ThermoFisher	4309155
Taq 2X master mix	New England BioLabs	M0270L
TOP10 electrocompetent Escherichia coli	Kindly provided from Dr. Melih Acar	
Trizma® base	Sigma-Aldrich	T6066-1KG
Trizma® hydrochloride	SIGMA ALDRICH	T5941-500G
Tryptone	VWR	84610.05
Yeast extract	VWR	84601.05

Reference: This table was prepared by Alihan SÜRSAL.

3.1.2. Equipment

All equipment used in this research are listed in Table 3.3.

Table 3.3: List of equipment

Equipment	**Trademark**
Armadillo PCR Plate, 96-well, clear, semi-skirted, low profile, white wells	Thermo Fisher, USA
ARPEGE 170	AIR LIQUIDE, France
ChemiDoc MP Imaging system	BIO-RAD, USA
Digital heat block	Benchmark, USA
Digital scale	ISOLAB, Germany
DM2000 LED light microscope	Leica, Germany
HI-2211 pH/ORP meter	HANNA instruments, USA
Lightcycler 96	Roche, Switzerland
Microcentrifuge® 16	BECKMAN COULTER, USA
MicroPulser electroporator	BIO-RAD, USA
MX-S vortex	SCILOGEX, UK
nanoPAC-300	Cleaver Scientific, UK
Optically clear adhesive seal sheets	Thermo Fisher, USA
Orbital shaker incubator	mrc, Germany
OT 90L pressure vessel	NÜVE, Turkey
PURELAB Option-Q	ELGA, UK

Equipment	Trademark
SL 40-FR centrifuge	ThermoFisher, USA
Sub-Cell® GT	BIO-RAD, USA
T100 Thermal cycler	BIO-RAD, USA
T80+ UV/VIS spectrometer	pg instruments, UK
Ultra-low temperature freezer	DAIHAN scientific, South Korea
UN55 sketch universal oven	memmert, Germany

Reference: This table was prepared by Alihan SÜRSAL.

3.2. METHODS

3.2.1. Tumor Samples

Sample source of this study is based on retrospective and prospective patient samples with the total number of 236 samples. 228 sample that were retrospectively analyzed were provided from "Bahcesehir University Brain Tumor Tissue Bank" as frozen specimens inside the nitrogen tank. All patients were previously updated about the experiment and written approvals were taken. After taking the required confirmation from Research Ethics Committee (2017-14/01), diagnostic information about glioma samples were taken from pathology reports. Sample selection was based on gliomas with different grades according to WHO-2016 histopathological classification. Diagnostic knowledge has shaped the sample selection as pilocytic astrocytomas from the WHO grade I content. As for grade II and III samples; diffuse astrocytomas, oligodendrogliomas, anaplastic astrocytomas and anaplastic oligodendrogliomas were taken. For grade IV content, GBs were chosen as an only tumor source. The remaining 8 sample were receipt in prospective fashion while retrospective analyses were continuing.

3.2.2. Preparation Of Tumor Samples And Alkaline Lysis DNA Extraction

Optimizations of the alkaline lysis DNA extraction method were based on minimum duration with maximum efficiency. Two separate tests were applied in order to understand if the dissected tumor size effects the product yield of the PCR reaction and to set the minimum amount of incubation time at 99°C in heat block for efficient lysis to minimize the diagnosis duration. For Both PCR reactions were tested with CFTR-21 primers, which were mentioned in the Supplementary 1: Table 1. Big and small sized tumor samples were dissected from two different glioma patients with IDH1 G395A mutation. Wild type blood-extracted DNA, which was taken from healthy volunteer was also incorporated to the experiment. To test the minimum required incubation time for proper lysis process, different heat block removal times were applied to one tumor sample with IDH1 G395A mutation as 5, 6, 7, 8, 9 and 10 minutes. Moreover, in order to compare the column extraction method with alkaline lysis and to involve an wild type control to following experiments, blood samples were taken from healthy volunteers. DNA extraction from blood were handled with E.Z.N.A Blood DNA Mini Kit by considering the manufacturer's protocol.

For alkaline lysis procedure, frozen tumor samples that were taken from "Bahcesehir Universtiy Brain Tumor Tissue Bank" than weighted as 5 to 25 mg with sensitive scale and shredded in small pieces in order to increase the surface area during lysis process. After dissection, sample were washed with 1X PBS (Phosphate Buffered Saline) in order to remove blood. Alkaline lysis DNA extraction method were applied. As an alkaline solution 200 µl 1mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), 25mM NaOH, pH: 12) was used and the sample was incubated at 99°C for 8 minutes inside the alkaline solution. When the incubation time was over, neutralization solution was added inside the alkaline solution (40mM Trizma® HCl, pH: 7.5) containing the sample in order to stop the lysis reaction.

3.2.3. Sequencing

In order to verify 3m-ARMS detection method and analyse the mutational status, Sanger sequencing was taken as golden standard. IDH1 and IDH2 regions were amplified for Sanger sequencing by using IDH1 and IDH2 region primers, which were mentioned in Supplementary 1: Table 1. Region specific primers covers all mutational hot spots G395 and C394 of IDH1 gene together with the A514, G515, G516 hot spots for IDH2 gene. Region specific amplification reaction was maintained with T100 thermal cycler as the reaction master mix, Q5® High-Fidelity DNA Polymerase was used in order to minimize probable error formation. Reaction conditions for IDH1 region specific amplification were as follow; 98°C for 30 seconds, 35 cycles of PCR as denaturation at 98°C for 7 seconds, annealing at 68°C for 20 seconds and extension at 72°C for 30 seconds and with a final extension of 72°C for 2 minutes. Reaction conditions for IDH2 region specific amplification were same as IDH1 except, annealing at 67°C for 20 seconds.

3.2.4. ASPCR Analysis

ASPCR was applied to test if single mismatch can prevent the primer to form heteroduplex so the polymerase enzyme only amplifies the target sequence. IDH1 G395A mutation was the primary focus during ASPCR analysis. Four different primer sets were used in ASPCR in order to select the most efficient primer size and direction with IDH1 G395A mutant tissue-extracted glioma patient DNA and blood-extracted DNA from heatlhy volunteer. Allele specific primers were mentioned in the Supplementary 1: Table 1. ASPCR was accomplished with T100 thermal cycler and Taq 2X Master was used as polymerase enzyme master mix. ASPCR conditiones were as follow; 95°C for 30 seconds, 36 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 12 seconds with the final extension of 68°C for 5 minutes. Final concentration of the primers were set to 0.5 uM. DMSO was used at a 5 percent final concentration as a fidelity enhancer. Additionally, harder reaction conditions were tested with same primers by increasing the annealing temperature to 63°C and decreasing the extension duration to 8 seconds.

All PCR and qPCR samples were migrated via agarose gel electrophoresis inside tris/borate/EDTA (TBE). TBE was prepared by mixing 10.8 percent trizma® base, 5.5 percent boric acid and 25 percent 0.5M Na_2EDTA pH:8.0 in dH_2O. Agarose gel concentrations were calculated according to product sizes such as; 2 percent agarose concentration was used for products between 50-500bp, 1 percent for products longer than 500bp. In order to sink the DNA content, final concentration of 1X loading dye and to estimate the product sizes, GeneRuler 1 kb Plus DNA Ladder was used in every experiment in this study. All visualization procedures were maintained by using UV imaging system. For UV based visualization, 5 percent final concentration of ethidium bromide (EtBr) was added during preparation of the agarose gel. If not specifically mentioned, all PCR and qPCR master mix ingredients were settled according to the manufacturer's protocol of the polymerase enzyme or its master mix.

3.2.5. ARMS Analysis

Due to the toleration of single mismatch during ASPCR analysis and the requirement of all or none results, second mismatch was decided to add as a penultimate mismatch next to terminal base during IDH1 G395A point mutation analysis. Penultimate mismatch selection was made according to penultimate mismatch selection table mentioned in the study.(Little, 2001) Six different mismatch containing ARMS primer were designed including four reverse primers and two forward primers. However, mismatch containing reverse primers were selected as ARMS primers due to penultimate point mutation resides at C394, which may interlope forward primer binding. ARMS primers were mentioned in Supplementary 1: Table 1. ARMS based PCR was accomplished with T100 thermal cycler and Taq 2X Master Mix was used as enzyme master mix. ARMS conditions were as follow; 95°C for 30 seconds, 37 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 7 seconds with the final extension of 72°C for 5 minutes. Final concentration of the primers were set to 0.5 uM. DMSO was used at a 5 percent final concentration as a fidelity enhancer.

3.2.5.1. QPCR based ARMS

SYBR Green 2X Master Mix was selected as polymerase enzyme and qPCR fluorescence dye during quantitative analysis. DMSO was eliminated due to its specificity enhancement property, which overlaps the working principle of ARMS. IDH1 G395A specific C2MP-R + C2-F primer pair was selected for qPCR based ARMS reaction due to their superior results and mutant specific amplification was accomplished via LightCycler-96. QPCR working conditions were as follow; 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 10 seconds, annealing at 63°C for 5 seconds and extension at 72°C for 10 seconds. Melting curve was obtained by following conditions: 95°C for 10 seconds, 60°C for 60 seconds and 97°C for 1 second. Final concentration of the primers was set to 0.5 uM.

3.2.6. 3m-ARMS Analysis

Conventional ARMS method was not enough to discriminate between wild type and IDH1 G395A alleles as all or none results. Thus, additional mismatch was decided to merge with C2MP-R primer. Our 3-mismatch containing design involves the conventional ARMS based primer selection, which the terminal mismatch formation between the wild type DNA was prepared according to the point mutation at the primer terminal site and the penultimate mismatch was chosen according to table mentioned in the study.(Little, 2001) However, in the case of third mismatch selection, every option was taken into consideration. So, three different primer type for each point mutation was designed. 3m-ARMS primers was mentioned in Supplementary 1: Table 1. 3-mismatched primer design involving the most suitable third intentional mismatch was chosen according to two optimization experiments. PCR reaction in order to test if 3m-ARMS primers were compatible to allele specific amplification. The reaction conditions were as follow; 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 63°C for 20 seconds and extension at 72°C for 8 seconds and final extension at 72°C for 5 minutes. Reactions was held with T100 thermal cycler with the Taq 2X Master Mix as reaction solution. Final concentration of the primers were set to 0.5 uM. Due to high concentration of primer secondary structure, same conditions and

materials was used with increased annealing temperature, 65°C from 63°C and the annealing duration was raised to twenty seconds from five. Most accurate primer selection was made after these results.

3.2.6.1. QPCR based 3m-ARMS optimizations

In order to adapt the 3m-ARMS method to quantitative analysis, SYBR Green 2X Master Mix based qPCR was accomplished by using LightCycler-96 on three 3m-ARMS primer sets. Reaction conditions were as follow, 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 10 seconds, annealing at 65°C for 20 seconds and extension at 72°C for 8 seconds. Melting curve was obtained by following conditions: 95°C for 10 seconds, 63°C for 60 seconds and 97°C for 1 second. Same primer final concentration of 0.5 uM was applied.

3.2.6.2. Re-selection of quantitative fluorescence dye

Due to probable exonuclease activity of SYBR Green 2X Master Mix, EvaGreen 20X was selected as quantitative dye for qPCR. In order to test the probable inhibitory effects of EvaGreen 20X, PCR reaction was prepared with T100 thermal cycler with the Taq 2X Master Mix by using 1X, 0.5X and 0.35X concentrations with 0.5 uM final primer concentration. PCR conditions were 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 10 seconds, annealing at 65°C for 20 seconds, extension at 72°C for 8 seconds and final extension at 72°C for 5 minutes.

3.2.6.3. Optimized 3m-ARMS detection of IDH1 G395A mutation

As shown previously in figure 2.1, suitable 3-mismatch containing primer inhibits annealing of 3’ ends of the primer and prevent polymerase binding while allowing mutant amplification by the help of 3’ end terminal match. C2MP_R-ATT-long primer was chosen for all or none detection of G395A mutation of IDH1. 3m-ARMS reaction was accomplished via LightCycler-96 using Taq 2X Master Mix. EvaGreen 20X was selected as fluorescent quantitation dye according to previous experiments and utilized as 0.35X

concentration in reaction tube. Optimized qPCR working conditions were as follow; 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 62°C for 20 seconds, extension at 72°C for 10 seconds and final extension at 72°C for 5 minutes. Melting curve was obtained by following conditions: 95°C for 10 seconds, 60°C for 60 seconds and 97°C for 1 second. Same primer final concentration of 0.5 uM was applied during all analyses. Moreover, extra magnesium was added from 25mM $MgCl_2$ solution in order to reach 0.5Mm final concentration excluding the magnesium content inside the master mix solution. DMSO was reviewed due to rarely occurring non-specific results with 5 percent final concentration as a fidelity enhancer.

3.2.6.4. Optimized 3m-ARMS detection of IDH1 C394G mutation

Same primer design method was applied on C394G point mutation of IDH1 gene. By using same the equipment during IDH1 G395A genotyping, 3m-ARMS conditions were applied as follow; 95°C for 30 seconds, 40 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 64°C for 20 seconds, extension at 72°C for 10 seconds and final extension at 72°C for 5 minutes. Melting curve was obtained by following conditions: 95°C for 10 seconds, 60°C for 60 seconds and 97°C for 1 second. As additives, same DMSO and $MgCl_2$ concentrations were used as in IDH1 G395A analysis.

3.2.7. Mutant DNA Construction

3.2.7.1. Getting IDH1 wild type gene region and addition of overlapping sequence for cloning

Gibson assembly compatible cloning primers were used to amplify the IDH1 and 2 regions while adding the overlapping sequences suitable for the vector, pBluescript SK(+), which involves ampicillin resistant gene. Cloning primers were mentioned in the Supplementary 1: Table 1. Region specific amplification reactions were accomplished with T100 thermal cycler by using Q5® High-Fidelity DNA Polymerase. Reaction conditions were as follow; 98°C for 30 seconds, 35 cycles of PCR including; denaturation at 98°C for 7 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30

seconds with the final extension of 72°C for 2 minutes. PCR purification was applied to all region specific products in order to increase the efficiency of Gibson assembly by using NucleoSpin® gel and PCR clean-up.

3.2.7.2. Gibson assembly, electroporation and plasmid isolation

Gibson cloning reaction solution was prepared as follow; 1 ul pBluescript SK(+) vector, 1 ul purified insert, 2.5 ul NEBuilder® HiFi DNA Assembly Cloning Kit master mix and 1 ul dH_2O in order to form a total 5 ul reaction solution. Reaction was incubated at 50°C for 15 minutes. Electrocompetent escherichia coli (E. coli) was prepared in order to make bacteria suitable for the high current flow. 1 ul Gibson assembly product was applied to 40 ul electrocompetent E. coli solution then the whole mixture was transferred to electroporation cuvette while setting the micropulser® electroporator to bacteria-E1 at 2500V. After proper electroporation, super optimal broth with catabolite repression (SOC) medium was added immediately. SOC media was prepared by addition of 2 percent glucose to super optimal broth (SOB) medium. SOB medium is a rich medium composed of 0.5 percent yeast extract, 2 percent tryptone, 2.5mM final concentration of KCl, 10mM final concentration of NaCl and 20mM $MgSO_4$. After the addition of SOC media in order to prevent further stress on bacteria, mixture was incubated in shaker incubator at 250 rpm in 37°C for one hour in order to let the bacteria maintain gene expression and become ampicillin resistant. After gene expression, 100 ul E. coli media was planted on ampicillin containing agar plates and incubated overnight at 37°C in order produce transfected colonies. Ampicillin containing agar plates were prepared manually by mixing 1 percent tryptone, 1 percent NaCl, 0.5 percent yeast extract and 1.7 percent agarose inside dH_2O. 1 percent ampicillin was added after autoclave sterilization in order to prevent denaturation of the antibiotic. Stock ampicillin solution was prepared by adding 10 mg ampicillin sodium salt to every 1mL of dH_2O.

Proper colony was selected via colony PCR reaction by using 1 uM final concentration of T3 and T7 primers that were mentioned in table 1.1 with the manufacturer's protocol of Taq 2X Master Mix. Reaction conditions were as follow; 95°C for 3 minutes, 35 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds

and extension at 72°C for 75 seconds with the final extension of 72°C for 5 minutes. In place of DNA, tipped colony was spreaded inside the reaction tube and let the longer denaturation duration expose the genetic content outside of the reaction mixture. Sanger sequencing was used to validate if the insertion was properly completed. Liquid culture was prepared in order to increase the bacteria number of the selected colonies by mixing 5 mL Luria-Bertani (LB) media with 5 ul ampicillin. LB media was prepared manually by mixing by mixing 1 percent tryptone, 1 percent NaCl, 0.5 percent yeast extract and inside dH_2O. 5 uL ampicillin was added after autoclave sterilization. Inoculation was performed via overnight incubaton at shaker incubator at 250 rpm followed by mini preparation (mini-prep) of plasmid DNA via NucleoSpin® plasmid according to manufacturer's protocol from incubated transfected bacteria. DNA concenration was measured with T80+ UV/VIS spectrometer.

3.2.7.3. Site-directed mutagenesis

Site-directed mutagenesis PCR reactions were accomplished with T100 thermal cycler by using Q5® High-Fidelity DNA Polymerase. Final DNA concentration in reaction tube was set to 800pg via spectrometer. Reaction conditions were as follow; 95°C for 30 seconds, 16 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute and extension at 68°C for 4 minutes.

Restriction enzyme, DpnI was used to maintain methylation specific digestion of the wild type DNA template by preparing the reaction buffer as; 25 ul site-directed mutagenesis PCR product, 5 ul CutSmart® buffer, 1 ul DpnI and 19 ul dH_2O. Reaction buffer was incubated at 37°C overnight. Same transfection procedures were applied as before. Respectively; purification of the sample via NucleoSpin® gel and PCR clean-up, electroporation via micropulser® electroporator with bacteria-E1 at 2500V, transfected E.coli planting with overnight incubation at 37°C, colony PCR with Sanger sequencing validation of the mutation containing insert, inoculation of selected colonies and mini-prep for bacterial DNA extraction with NucleoSpin® plasmid. Construct concentrations were again measured with T80+ UV/VIS spectrometer. Total of fifteen mutagenesis construct were prepared with site-directed mutagenesis composed of G395A, G395T,

G395C, C394T, C394A and C394G mutations of IDH1 gene. Alongside with; G515A, G515T, G515C, A514T, A514G, A514C, G516A, G516T and G516C mutations of IDH2 gene.

3.2.8. Sensitivity And Specificity Tests With IDH1 And IDH2 Mutations

In order to see the upper and the lower limit of DNA concentration inside the reaction tube, point containing DNA constructs of G395A, G395T, G395C together with wild type DNA were tested with C2MP_R-ATT-long/C2_F-long primer pair. Respectively; 20ng/reaction, 10pg/reaction and 100fg/reaction DNA concentrations were tested with qPCR method by using LightCycler-96 with Taq 2X Master Mix. As quantitative dye, previously optimized 0.35X EvaGreen 20X was used. Reaction conditions were as follow; 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 62°C for 20 seconds, extension at 72°C for 10 seconds and final extension at 72°C for 5 minutes. Melting curve was obtained by following conditions: 95°C for 10 seconds, 60°C for 60 seconds and 97°C for 1 second. As additives, same DMSO and $MgCl_2$ concentrations were used as in IDH1 G395A analysis.

From IDH2 constructs with point mutations, G515A was selected again due to its higher occurrence comparing to other point mutations of IDH2 gene. Point mutation containing DNA constructs of G515A together with the wild type DNA were tested with I2G5AR-AAT/I2F primer pair. Respectively; 100pg/reaction, 10pg/reaction, 1pg/reaction and 1fg/reaction DNA concentrations were tested with qPCR method by using LightCycler-96 with Taq 2X Master Mix. As additives, same DMSO and $MgCl_2$ concentrations were used as in IDH1 G395A analysis. Reaction conditions were as follow; 95°C for 30 seconds, 45 cycles of PCR including; denaturation at 95°C for 30 seconds, annealing at 68°C for 20 seconds, extension at 72°C for 10 seconds and final extension at 72°C for 5 minutes. Melting curve was obtained by following conditions: 95°C for 10 seconds, 60°C for 60 seconds and 97°C for 1 second.

3.2.9. COLD-PCR Enhanced Sequencing

Four sample, which resulted IDH1 G395A mutant via 3m-ARMS shown wild type property by Sanger sequencing results. Non-coherent samples re-analyzed with COLD-PCR in order to enhance the mutant to wild type ratio prior to sequencing after purification of the samples via NucleoSpin® gel and PCR clean-up. Previously tested COLD-PCR primers were mentioned in Supplementary 1: Table 1. Reaction conditions were as follow; 95°C for 30 seconds, 40 cycles of PCR at lower denaturation temperature between 75.7 to 75.9°C for 30 seconds, annealing at 60°C for 20 seconds and extension at 72°C for 10 seconds, and with a final extension of 72°C for 5 minutes. Final concentration of the primers was set to 0.5 uM.

3.2.10. IHC Analysis

106 formalin-fixed paraffin embedded (FFPE) sections were analyzed by histopathologic assessment. As fixative, 10 percent buffered formalin were used on tissue components. 5-µm sections were prepared by using microtome and stained with hematoxylin and eosin (H&E), IDH1 R132H and ATRX antibodies. As IHC staining procedure, conventional avidin-biotin method was considered. Stained samples were observed under light microscope.

3.2.11. Statistical Analysis

Triple correlation between IHC, Sanger and 3m-ARMS methods were done in order to test the accuracies of both techniques comparing them with the Sanger sequencing, which is selected as the golden standard. Both tests were statistically analyzed by sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. Additionally, positive likelihood ratio (LR+) and negative likelihood ratio (LR-) were taken into account in order to rate diagnostic utilities of both techniques. Values of sensitivity and specificity were calculated via 2x2-probability table based on

variables called true positive (TP), true negative (TN), false positive (FP) and false negative (FN). All graphs were prepared with Graphpad Prism 6®.

3.2.12. Circulatory DNA Extraction And 3m-ARMS Analysis

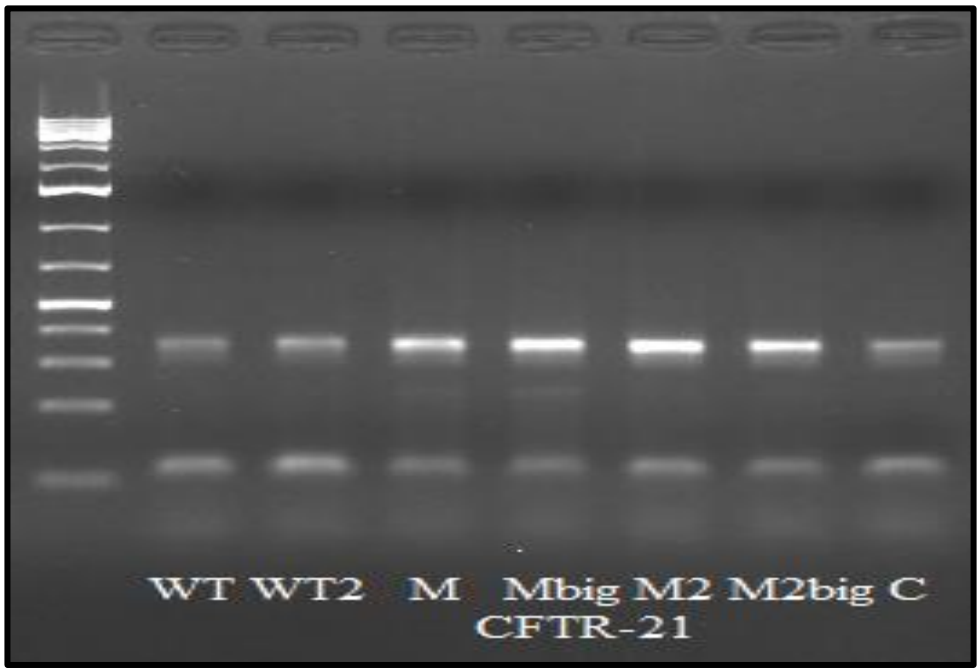
Plasma samples from IDH1 R132H positive glioma patients were obtained from three blood sample, which were taken from same glioma patients as the tissue samples in our tumor bank by centrifugation at 400g for 15 minutes followed by 10000g for 5 minutes more centrifugation. In both process, the supernatant was isolated and transferred to another tube. CtDNA extraction was achieved via NucleoSnap® DNA Plasma kit according to manufacturer's protocol. Then, by using primers that are mentioned in Supplementary 1: Table 1, which targeting IDH1 genomic region, ctDNA extracts were tested in order to both analyze IDH1 R132H positivity via Sanger sequencing and to see if there is enough DNA for an ordinary PCR reaction. All samples were purified prior to Sanger sequencing. Region specific amplification reaction was maintained with T100 thermal cycler alongside with the reaction master mix, Q5® High-Fidelity DNA Polymerase. Reaction conditions for IDH1 region specific amplification were as follow; 98°C for 30 seconds, 35 cycles of PCR as denaturation at 98°C for 7 seconds, annealing at 65°C for 20 seconds and extension at 72°C for 30 seconds and with a final extension of 72°C for 2 minutes. Then, by the aid of 3m-ARMS, tissue-sourced DNA samples as positive control, together with plasma-sourced ctDNA samples belongs to same patients were quantitatively analyzed respective to IDH1 R132H mutation by using C2MP_R-ATT-long primer, which is mentioned in Supplementary 1: Table 1. The same PCR settings and additives as optimized 3m-ARMS analysis were used during ctDNA genotyping.

4. RESULTS AND DISCUSSION

4.1. EFFECT OF TUMOR SAMPLE SIZE AND HEAT BLOCK REMOVAL TIME ON ALKALINE LYSIS DNA EXTRACTION

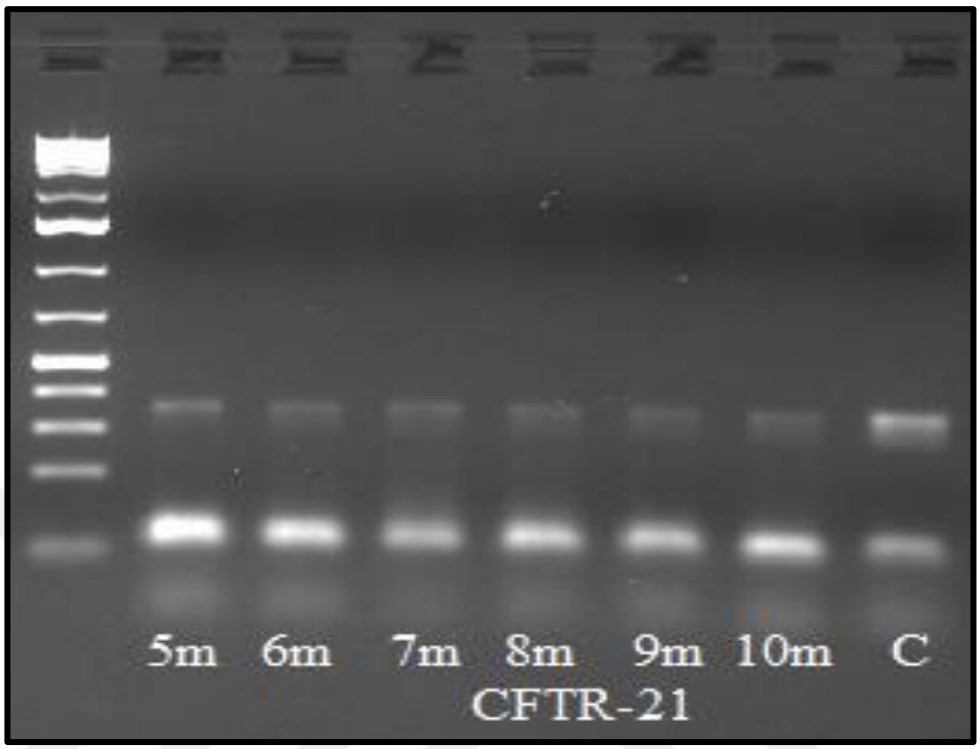
As the primal aim of our study is to perform an intraoperative detection of IDH1 point mutations, rapidness is one the most important thing. That is why, the approximate weight of tumor sample should optimized to prevent insufficient or excessive amount of template DNA, which in both cases it may cause FN or FP results. Moreover, 10 minutes of alkaline lysis at 99°C may not be required for extraction of sufficient amount of DNA template. To test the minimum time and tumor size required for efficient lysis process, two separate experiments were performed.

Figure 4.1: Gel electrophoresis results of PCR based amplification on alkaline lysed tumor samples with different sizes by using CFTR-21 primers



WT Blood-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
C Control
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.2: Gel electrophoresis results of PCR based amplification on different durations of alkaline lysis with CFTR-21 primers



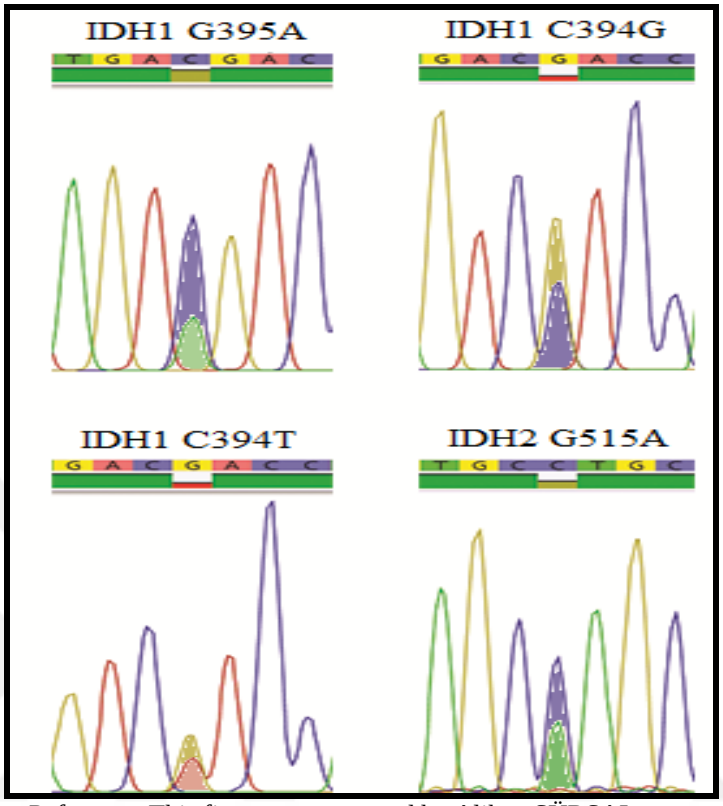
m Minutes at 99°C
C Control
Reference: This figure was prepared by Alihan SÜRSAL

Band formations of both samples in figure 4.1 and were similar to the control DNA. There is no significant difference between the smaller and bigger sized tumor samples, which means there is no FP or FN probability caused from initial tumor size. Different heat block removal times also did not cause difference in amplification in figure 4.2. This result indicates that, minimum amount of time, five minutes, can used to efficiently lyse tumor samples.

4.2. SEQUENCING

We tested our 236 sample with Sanger sequencing in order to trust our positive control samples, which have specific point mutation. In this way, we compare our results and interpret our experiments to increase the efficiency and accuracy of the primer design and reaction mixture ingredients.

Figure 4.3: Sanger sequencing results of wild type and IDH1/IDH2 mutant samples



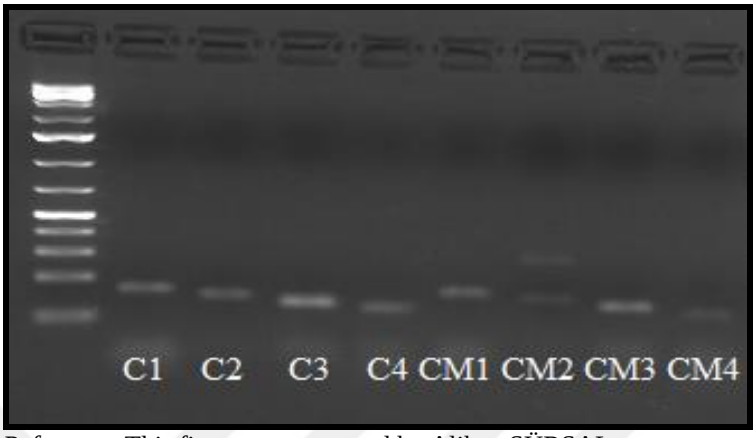
Reference: This figure was prepared by Alihan SÜRSAL

By the aid of Sanger sequencing, which was taken as a golden standard, we provide our positive and negative controls. These controls are composed of alkaline lysed DNA samples with specific point mutations or without them to optimize further experiments of point mutation detection.

4.3. ASPCR ANALYSIS

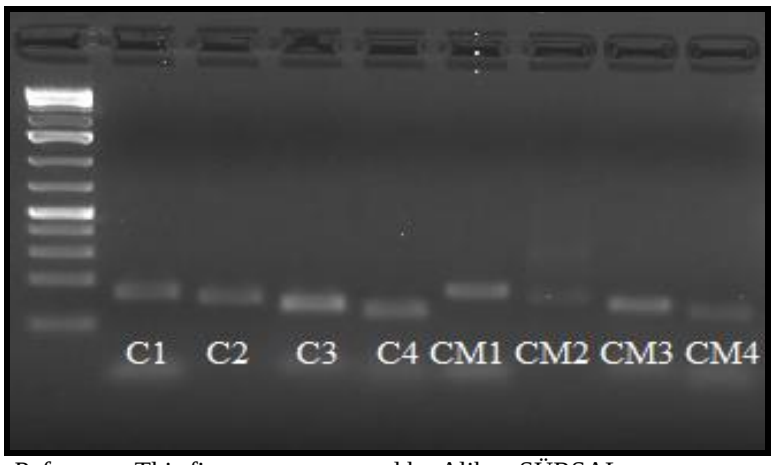
ASPCR analysis was performed with four different primers sets in order to select the most efficient primer set to work with. ASPCR wild type primers C1, C2, C3, C4 and mutant primers C1M, C2M, C3M and C4M were labelled in the following figures. In the first following figure 4.3, DNA extracted from the blood of healthy volunteer was used as template DNA. In the second figure 4.4, DNA extracted from tumor tissue of a glioma patient, which contains IDH1 G395A point mutation, was selected as template DNA.

Figure 4.4: Gel electrophoresis results of ASPCR based amplification with four ASPCR primer set on wild type DNA extracted from blood



Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.5: Gel electrophoresis results of ASPCR based amplification with four ASPCR primer set on IDH1 G395A mutant DNA extracted from tumor tissue



Reference: This figure was prepared by Alihan SÜRSAL

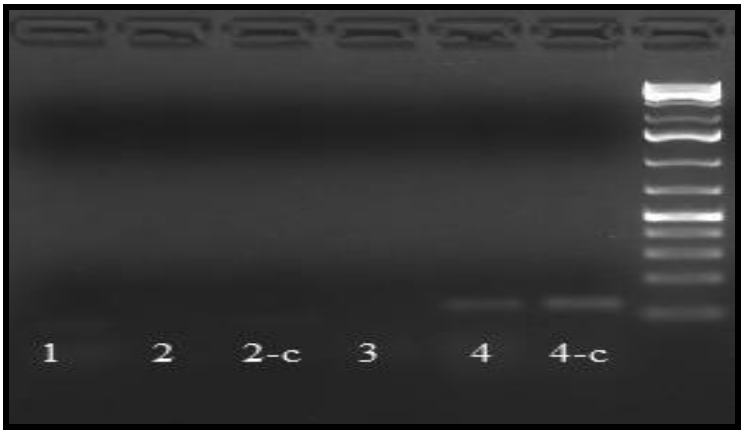
When figure 4.4 was observed, it is clear that mutant specific primers also amplified the wild type DNA template. In figure 4.5, it is also clear that wild type specific primers amplified mutant DNA template. Thus, ASPCR results are not specific and allele specific primers working with single mismatch elimination do not produce all or none results due to single mismatch toleration during amplification reaction. Our main aim of

intraoperative detection requires very accurate results under one hour duration. Because of that, addition of penultimate mismatch to allele specific primers according to conventional ARMS methodology may stop mismatch toleration during wild type amplification.

4.4. ARMS ANALYSIS

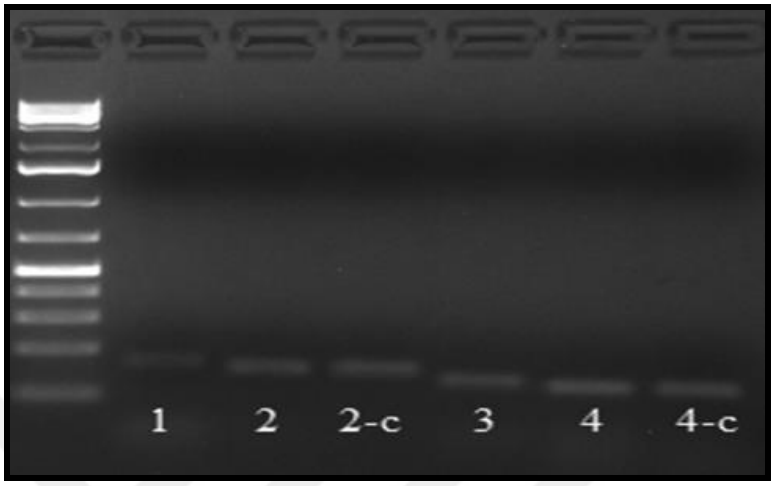
Introduction of penultimate mismatch according to traditional ARMS protocol was applied only to mutant specific primers because the primal aim of this study is to selectively amplify mutant alleles. C1MP-F, C2MP-R, C2MP-R-c, C3MP-F, C4MP-R, C4MP-R-c were designed ARMS primers. Reverse primers were chosen due to probable presence of point mutation in the forward primer binding site as previously mentioned in the materials and methods section. However, in order to test all penultimate mismatch containing ARMS primers, all sets were introduced in optimization experiment. Some reverse primers have a second variant due to the ARMS penultimate mismatch table.(Little, 2001)

Figure 4.6: Gel electrophoresis results of ARMS based amplification of wild type DNA extracted from blood



1 C1MP-F
2 C2MP-R
2-c C2MP-R-c
3 C3MP-F
4 C4MP-R
4-c C4MP-R-c
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.7: Gel electrophoresis results of ARMS based amplification of IDH1 G395A mutant DNA extracted from tumor tissue



1 C1MP-F
2 C2MP-R
2-c C2MP-R-c
3 C3MP-F
4 C4MP-R
4-c C4MP-R-c
Reference: This figure was prepared by Alihan SÜRSAL

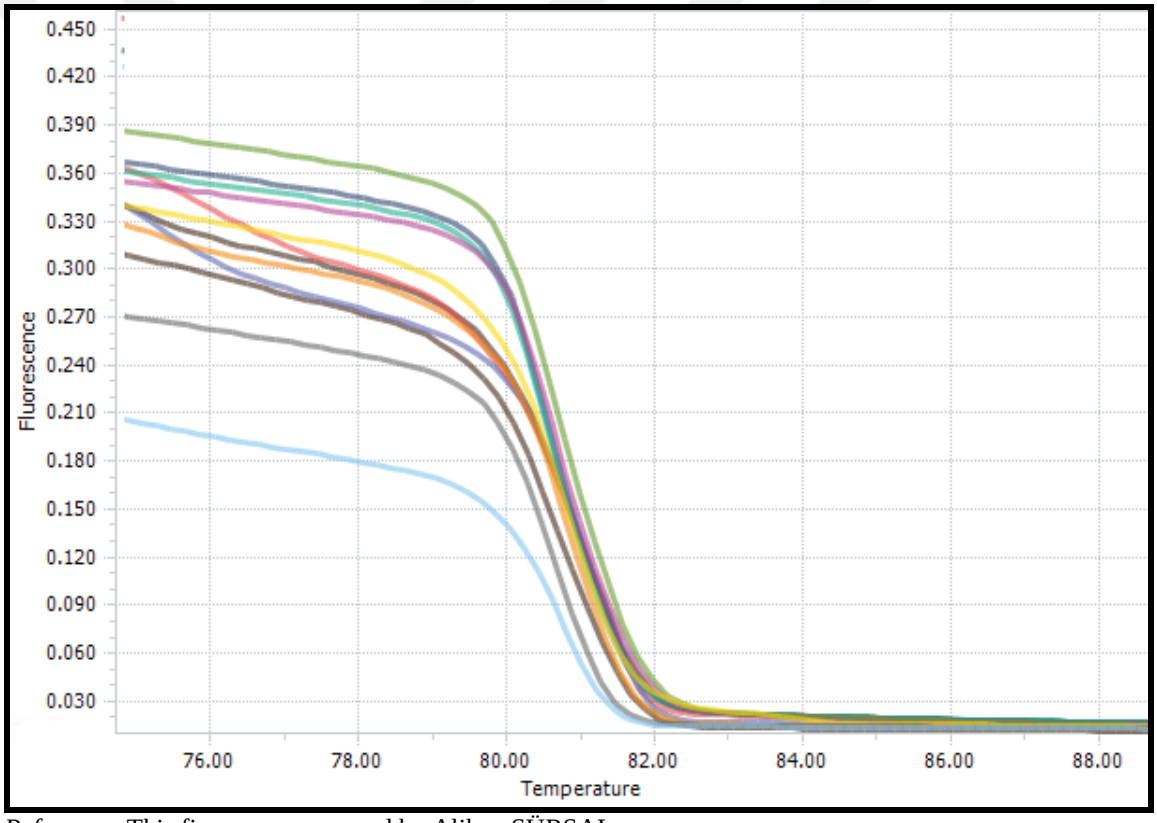
ARMS analysis resulted in specific amplification in case of blood-extracted wild type DNA template except C4MP-R, C4MP-R-c primers, which can be observed from figure 4.6 since the amplification is not inhibited by penultimate mismatch in the presence of these primers. Additionally, C1MP-F primer resulted very faint amplification. When figure 4.7 was observed, it can be seen that ARMS primers that were designed as mutant specific terminal mismatch resulted amplification in all cases so ARMS reaction contains wild type DNA template should be the limiting reaction during selection of the most effective ARMS primer. Despite C2MP-R, C2MP-R-c and C3MP-F primers are the most promising primers, C2MP-R was chosen and C3MP-F was eliminated due to probable IDH1 C394 point mutation on C3MP-F binding site.

4.4.1. QPCR Based ARMS

Even if the normal PCR concludes with specific results with two ARMS primer, the main purpose of this study is to produce a real-time separation of the mutant alleles from wild

type samples. This aim requires an addition of a quantitative dye to the reaction. Because there are commercially available quantitative dye containing master mixes, first quantitative PCR based experiment was performed with SYBR Green 2X Master Mix. It is known that DMSO is an additive which removes secondary structures of the template and enhance specificity of primer binding.(Lorenz, 2012) Thus, DMSO may improve the effect of the mismatch so it may inhibit the reaction even there is only single mismatch. Therefore, DMSO was removed from following experiments in order to specifically observe the sole effect of the quantitative dye on the reaction.

Figure 4.8: Melting curve of QPCR based ARMS with C2MP-R primer



Reference: This figure was prepared by Alihan SÜRSAL

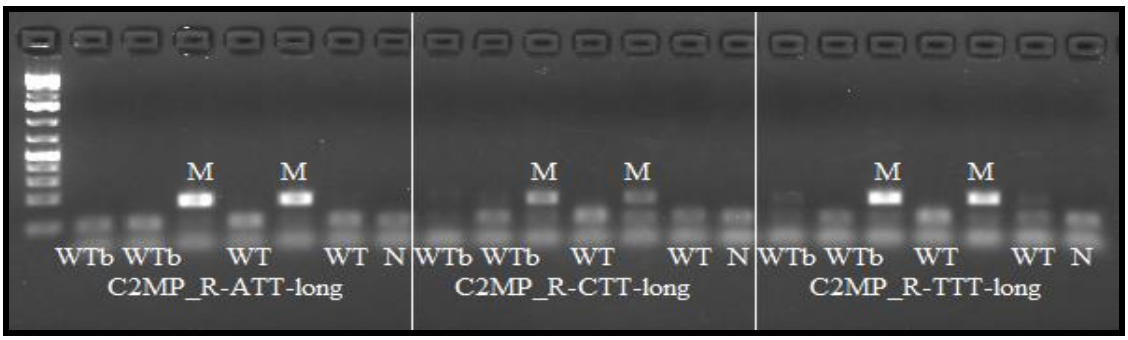
In figure 4.8, there are two blood-extracted DNA from healthy volunteers, two tumor-extracted wild type DNA and two tumor-extracted IDH1 G395A mutant DNA. Each sample was tested twice in the same experiment in order to increase the significance of results. C2MP-R ARMS primer was selected from previous experiments. When looking the melting curve generated by SYBR green dye, there is no any difference in case of

critical temperature of denaturation of the products. This means, ARMS primers did not worked as they were in normal PCR reaction. Normally, melting point of an inhibited reaction should contain the melting point of primer secondary structures. That is why we assume to see a lower point of melting temperature in in wild type DNA containing reaction compare to mutant DNA containing samples in the case of desired ARMS reaction. We decided to increase the mismatch number by introducing additional mismatch to the base next to the penultimate mismatch. Thus, there will be three mismatches in the case of wild type allele and two mismatches in the case of mutant allele. We proposed that, this novel modification to conventional ARMS may enhance the specificity of the reaction

4.5. 3M-ARMS ANALYSIS

As previously mentioned in the materials and methods section, incorporation of third mismatch was selected considering all probable mismatch formations since there is no any reference providing the third mismatch thermodynamics. C2MP-R primer was selected based on previous experiments. We changed the antepenultimate guanine base of the 3' end to adenine, thymine and cytosine and designed 3m-ARMS primers called C2MP_R-ATT-long, C2MP_R-TTT-long and C2MP_R-CTT-long. Firstly, 3m-ARMS primers were tested with PCR reaction prior to quantitative analysis.

Figure 4.9: Gel electrophoresis results of 3m-ARMS based amplification with wild type and IDH1 G395A mutant DNA samples



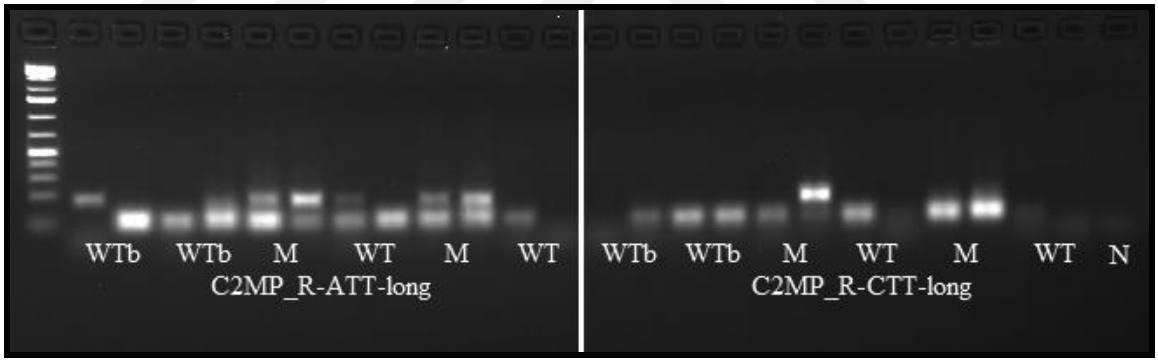
WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
N Negative control without DNA template
Reference: This figure was prepared by Alihan SÜRSAL

From figure 4.9, C2MP_R-ATT-long primer has the most accurate and strong results comparing to other two 3m-ARMS primers. C2MP_R-CTT-long primer however, is more specific comparing to C2MP_R-TTT-long but have weaker bands. Specificity is the most important property of our study. Thus, we choose C2MP_R-ATT-long as our primary and C2MP_R-CTT-long as our secondary primer for further optimizations.

4.5.1. QPCR Based 3m-ARMS Optimizations

After we observed accurate results via normal PCR with 3m-ARMS method. In order to produce a real-time analysis based on 3m-ARMS, we start the quantitative optimization experiments with SYBR Green dye. QPCR reaction was handled as twice from each sample to increase the significance of the experiment.

Figure 4.10: Gel electrophoresis results of qPCR based 3m-ARMS based amplification with wild type and IDH1 G395A mutant DNA samples



WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
N Negative control without DNA template
Reference: This figure was prepared by Alihan SÜRSAL

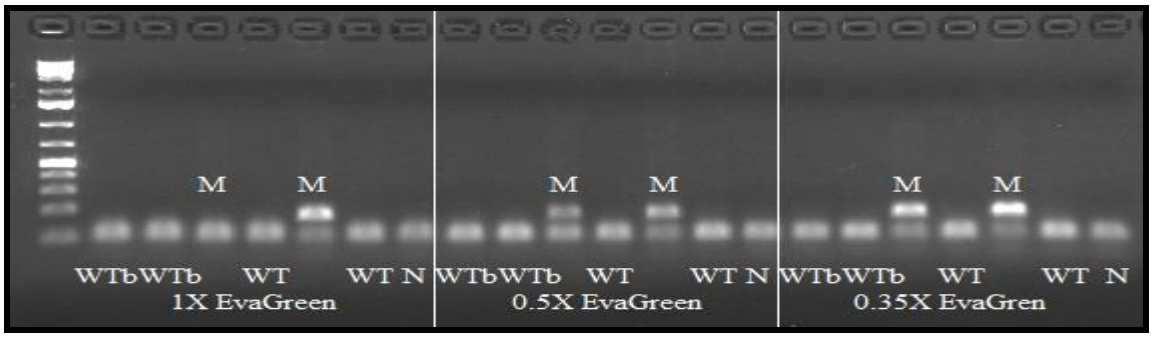
When figure 4.10 is observed, it is clear that there is still problems in specificity when it comes to qPCR analysis of IDH1 G395A point mutation. Gel electrophoresis image was preferred in order to see non-specific amplifications more accurately. These results are far away from all or none. Thus, after some research, we found that there is a problem in our master mix, which contains polymerase enzyme. From the official site of ThermoFisher, we confirmed that SYBR Green 2X Master Mix contains the same enzyme as AmpliTaq GOLD^{TM} DNA Polymerase, which have 3' to 5' exonuclease activity.

Exonuclease activity is not compatible with ARMS reaction due to mismatch removal of the required site, which enables wild type amplification without any detention. That may the reason of random non-specific amplification during qPCR analysis and specific amplification during PCR analysis. Moreover, that may the reason of complete toleration of mismatches in the case of qPCR based conventional ARMS. To remind, during our PCR reactions, Taq 2X Master Mix was selected as polymerase enzyme containing media. It is known that Taq 2X Master Mix have no 3' to 5' exonuclease activity. This information can be re-examined from the commercial datasheet of the product.

4.5.2. Re-selection Of Quantitative Fluorescence Dye

In order to use reliable Taq 2X Master Mix in quantitative analysis, we need to find another quantitative dye and add it manually to our master mix. By considering excitation/emission levels of SYBR Green dye, we found EvaGreen dye as usable in our detection system. We performed an experiment according to EvaGreen 20X protocol which should have 1X final concentration inside the reaction tube. However, based on experience of my supervisors, we performed a reaction to test the most efficient EvaGreen final concentration in the reaction tube. C2MP_R-ATT-long was selected as the most efficient primer for further optimizations.

Figure 4.11: Gel electrophoresis results of PCR based amplification via C2MP_R-ATT-long primer with different EvaGreen concentrations



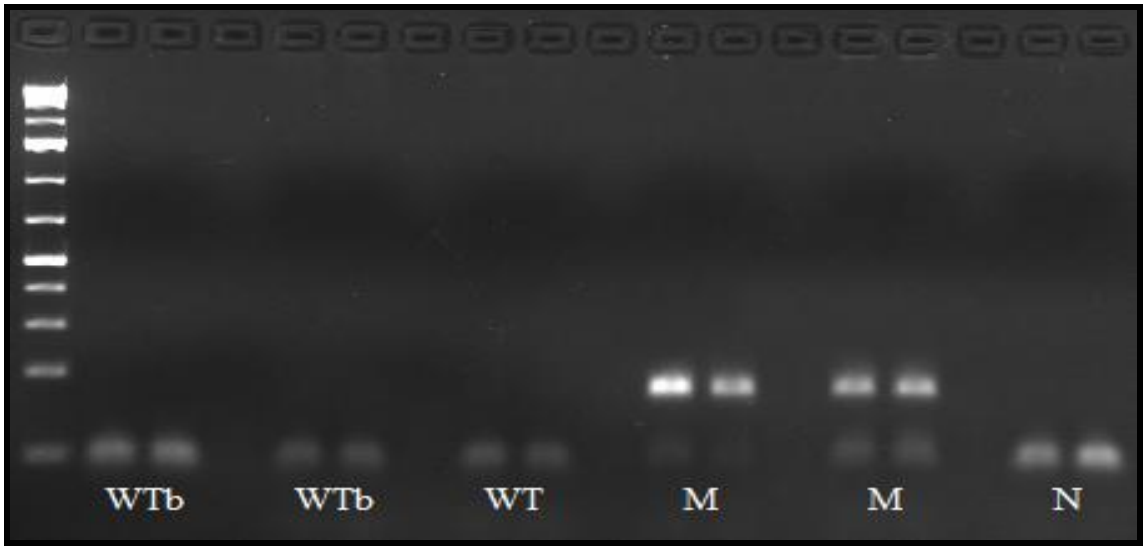
WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
N Negative control without DNA template
Reference: This figure was prepared by Alihan SÜRSAL

Based on figure 4.11, it is clear that 1X EvaGreen concentration is not optimal for 3m-ARMS analysis. 0.35X concentration of EvaGreen resulted strong and specific bands in PCR reaction. So, it can be used as quantitative dye in qPCR analysis to test if 0.35X concentration of EvaGreen is enough for sufficient emission in order to produce an accurate melting curve. We also consult further PCR additives in order to strengthen the results. Extra $MgCl_2$ was selected in order to increase the efficiency of amplification reaction.

4.5.3. Optimized 3m-ARMS Detection Of IDH1 G395A Mutation

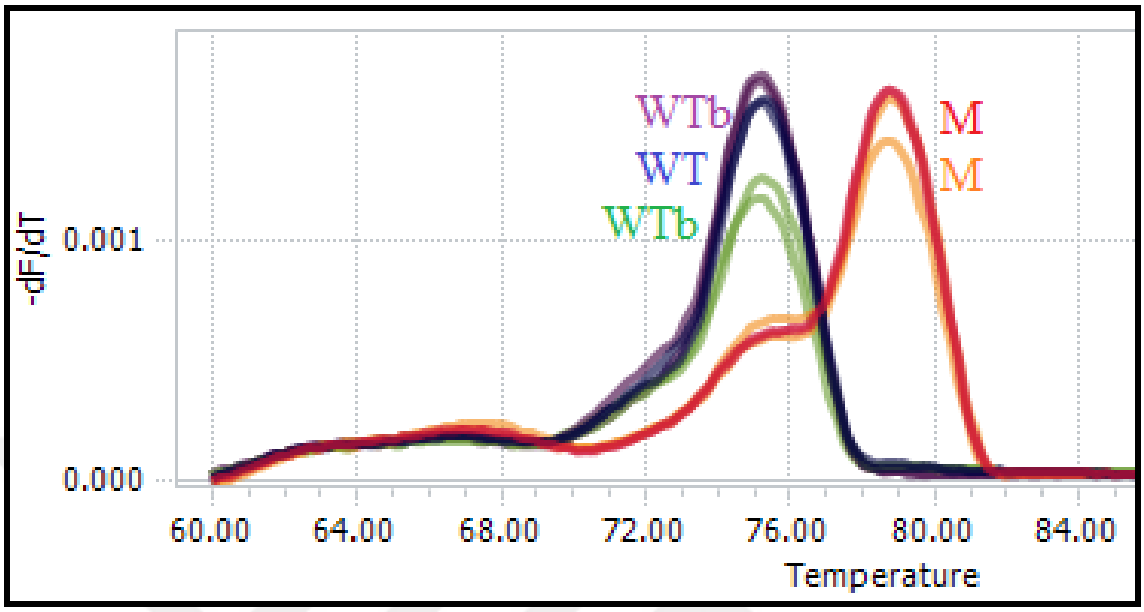
3m-ARMS master mix was optimized as the proper quantitative dye and 3' to 5' exonuclease free polymerase enzyme were previously tested. Best 3m-ARMS primer was selected in order to use in retrospective analysis. Therefore, we performed the quantitative analyze with same samples and observe the relationship between the melting curve and gel electrophoresis results.

Figure 4.12: Gel electrophoresis results of qPCR based amplification via C2MP_R-ATT-long primer with 0.35X EvaGreen concentration



WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
N Negative control without DNA template
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.13: Melting curves of qPCR based amplification via C2MP_R-ATT-long primer with 0.35X EvaGreen concentration



WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
Reference: This figure was prepared by Alihan SÜRSAL

From figure 4.12, we clearly understand that 3' to 5' exonuclease activity of the previous enzyme is the problem for our quantitative analysis. We accomplished all or none results via qPCR. By the help of real-time quantification, we observed that the Ct of mutant samples are range from 24 to 35 cycles, which sets our maximum analyze time as 67 minutes. Moreover, when we observe figure 4.13, melting point differences between the mutant and wild type samples are approximately 3°C. This means, the product size of the mutant DNA template containing reaction tube has the wanted products which melts approximately at 79°C while in the wild type containing tube, products melts at approximately 76°C. The melting peaks of the wild type DNA template containing reaction tube is the result of primer dimer formations. Primer dimer formations are also present in the mutant samples, which can also observed from the gel electrophoresis result in figure 4.12. Labelling colors of figure 4.13 was selected according to melting peak colors aiming to provide the reader a simple observation.

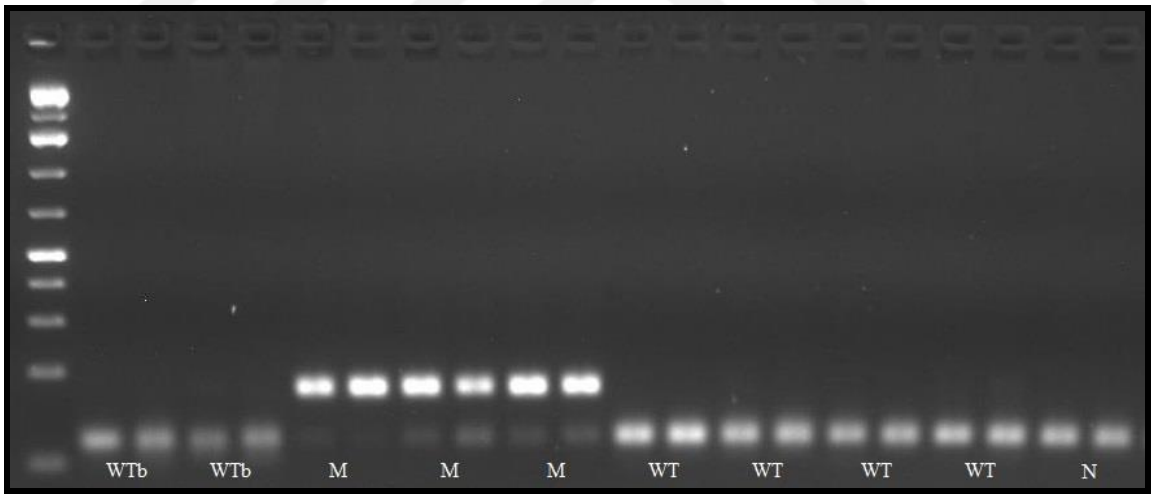
Retrospective scanning and recording of 228 glioma patient for IDH1 G395 mutation is the next station of our study. Moreover, DMSO was selected again as fidelity enhancer

in order to prevent rarely occurring non-specific results. Alkaline lysis causes random distribution of degraded materials inside the solution which may cause a chaotic environment. This random cellular material distribution between different tumor samples may causes rarely occurring non-specific results, which were eliminated after the addition of DMSO and extra $MgCl_2$.

4.5.4. Optimized 3m-ARMS Detection Of IDH1 C394G Mutation

Based on previous knowledge about quantitative dye concentration and type, we tested our designed primers for IDH1 C394G mutation for qPCR analysis by using similar optimizations. Double sample was studied again in order to prevent the risk of non-significance.

Figure 4.14: Gel electrophoresis results of qPCR based amplification via I1C4GF-GCG primer



WTb Blood-extracted wild type DNA
WT Tissue-extracted wild type DNA
M Tissue-extracted IDH1 G395A mutant DNA
N Negative control without DNA template
Reference: This figure was prepared by Alihan SÜRSAL

We achieved all or none results for IDH1 C394G mutation with I1C4GF-GCG primer as it can be seen from figure 4.14, which can temporize for further retrospective detection. Due to low frequency of IDH1 C394G mutations, sample number is limited for this point

mutation for retrospective analysis. However, we tested our primer with many wild type samples in order to concretize the accuracy of our primers and 3m-ARMS method.

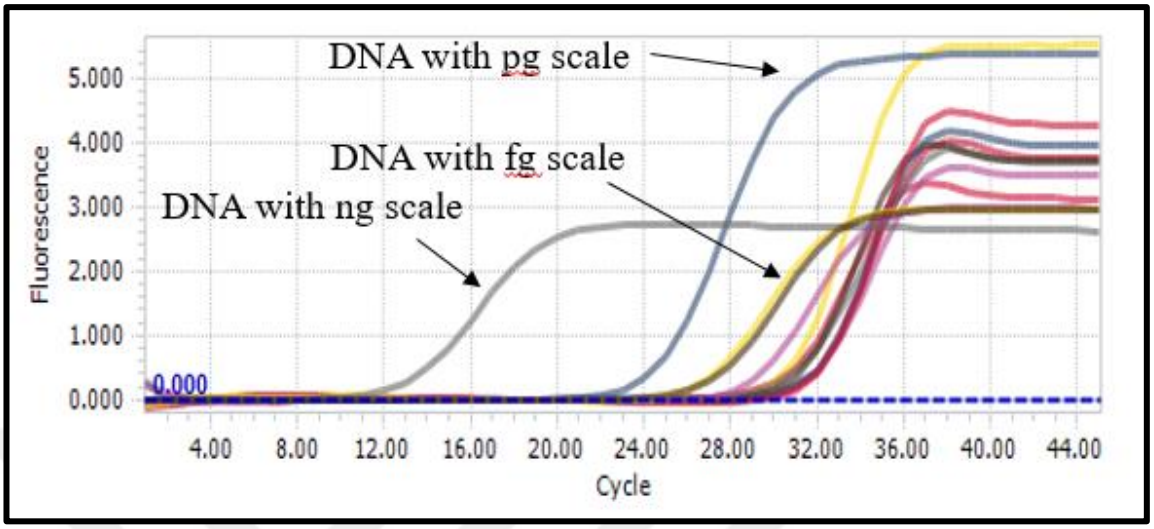
4.6. SENSITIVITY AND SPECIFICITY TESTS

We generated mutant containing DNA constructs by site-directed mutagenesis technique on previously designed plasmids containing IDH1 or IDH2 genes via Gibson assembly. 3m-ARMS analysis of IDH2 G515A mutation was also tested for the first time, which will set our optimizations for further optimizations in the case of IDH2 analysis on alkaline lysed samples. Wild type IDH1 and IDH2 constructs were already prepared prior to mutagenesis, was used as negative controls without the need of applying site-directed mutagenesis. These constructs are pure and specific mutant or wild type DNA containing solutions. Thus, we can examine our 3m-ARMS method on purified positive and negative controls. Additionally, we used these constructs as internal controls during retrospective analysis. Furthermore, by the aid of these constructs, low mutant to wild type ratio samples was mimicked. Thus, the minimum amount of mutant DNA resides in wild type-concentrated solution required for proper analysis was found. The upper limit of DNA content for specific amplification reaction was observed.

Figure 4.15: Gel electrophoresis result of sensitivity and specificity test for IDH1 G395A mutation detection with C2MP_R-ATT-long primer via 3m-ARMS

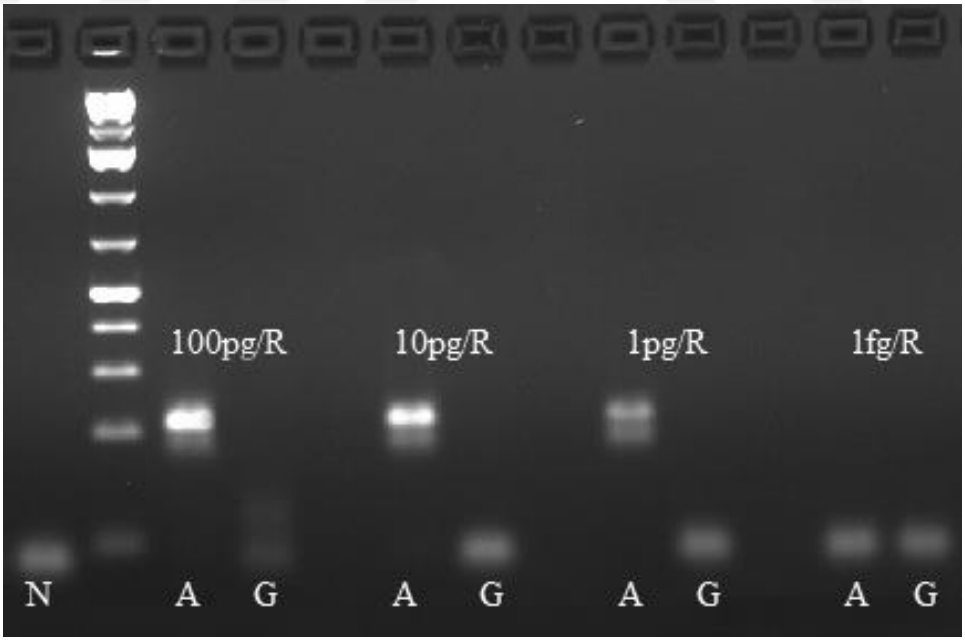
R Reaction
G IDH1 G395
A IDH1 G395A
T IDH1 G395T
C IDH1 G395C
N Negative control
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.16: Melting curve analysis of sensitivity and specificity test for IDH1G395A mutation detection with C2MP_R-ATT-long primer via 3m-ARMS



Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.17: Gel electrophoresis result of sensitivity and specificity test for IDH2 G515A mutation detection with I2G5AR-AAT primer via 3m-ARMS



R Reaction
G IDH2 G515
A IDH2 G515A
N Negative control
Reference: This figure was prepared by Alihan SÜRSAL

When figure 4.15, 4.16 and 4.17 were analyzed, 10pg DNA concentration in reaction is efficient and specific in terms of 3m-ARMS detection. Additionally, the lower limit of 3m-ARMS detection limit is between 1pg and 1fg DNA template in reaction tube, which is also supported by quantitative analysis that shows up to 14 cycle superiority in the case of primer specific mutant containing construct compare to wild type. Even in fg-level, up to 4 cycle superiority was achieved. At 20ng/R concentration, slight non-specificity was observed which is expected. Based on our findings and coherency with Sanger sequencing, our dissected tissue samples between 5 to 25mg always have sufficient DNA template to reach the mutant to wild type ratio compatible for point mutation detection with 3m-ARMS. These figures supports the robust sensitivity of 3m-ARMS as even in fg-level, all or none results were obtained. Despite the all or none results of 3m-ARMS analysis of IDH2 in mutagenesis samples, which have pure population of constructs with no other contaminants, alkaline lysis adaption of IDH2 analysis requires more optimization experiments due to high guanine cytosine content of IDH2 mutation region alongside with chaotic environment produced via alkaline lysis.

Table 4.1: QPCR based Ct values of diminishing DNA concentrations wild type and mutagenesis constructs of IDH1 G395 with C2MP_R-ATT-long and IDH2 G515 with I2G5AR-AAT primers

Base change of gene	DNA amount in reaction tube			
IDH1 G395	20 ng/R	10 pg/R	100fg/R	
G	27,99	33,76	35,42	
A*	13,46*	23,26*	30,18*	
T	30,22	33,24	33,05	
C	27,30	33,65	33,78	
IDH2 G515	100pg/R	10pg/R	1pg/R	1fg/R
G	37,35	37,29	38,17	N/A
A*	27,77*	30,57*	34,97*	39,05*

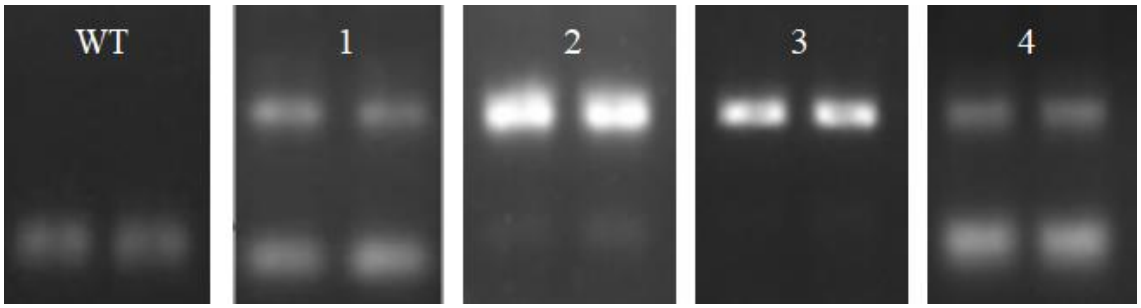
N/A No result
* Base change targeted by selected primer and their Ct results
Reference: This table was prepared by Alihan SÜRSAL

As it can clearly observed, the reaction tube containing the construct targeted by the selected primers, IDH1 G395A construct was targeted by C2MP_R-ATT-long and IDH2 G515A construct was targeted by I2G5AR-AAT, have lower cycle threshold. As expected, non-specific constructs have harder reaction conditions compare to specific ones due to three-mismatch formation with the template, which provides superiority to two mismatch containing mutant allele. By limiting the PCR cycles to the point of true target amplification, we can concretize our all or none results.

4.7. COLD-PCR ENHANCED SEQUENCING

Among 236 specimen, 4 samples resulted false positively with 3m-ARMS when compared with Sanger sequencing, which is worrying since we aimed clinical diagnosis under one hour of duration with all or none results. Because of these inaccurate results, reliability of our method was damaged.

Figure 4.18: Gel electrophoresis of 4 samples resulted IDH1 R132H positive via 3m-ARMS but wild type via Sanger sequencing

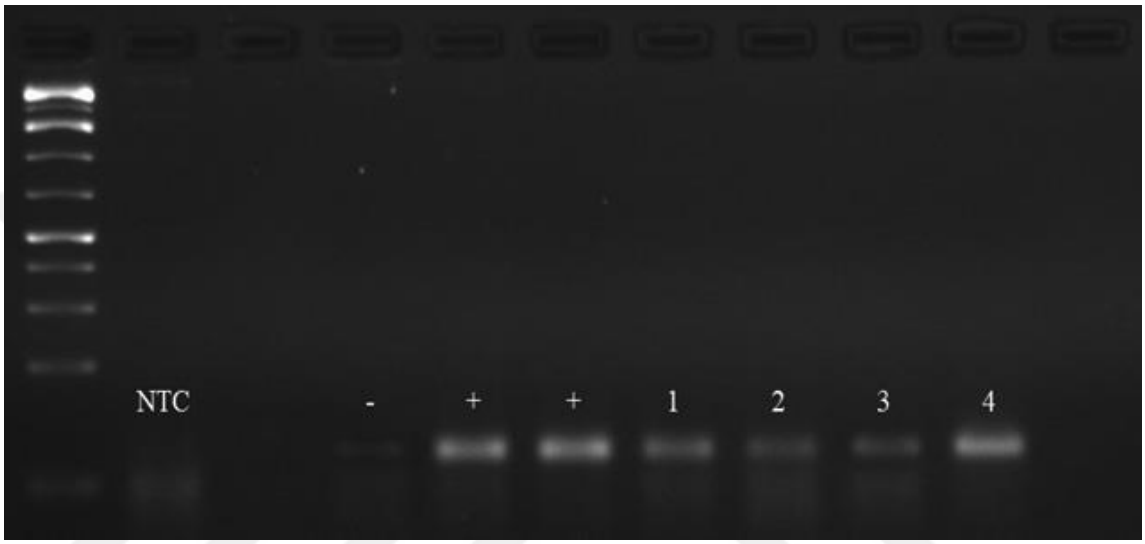


WT wild type
Reference: This figure was prepared by Alihan SÜRSAL

In order to find out the reason behind this false positivity. We choose a skeptical pathway, which may relocates the false positivity or insufficient specificity of 3m-ARMS with false negativity or insufficient sensitivity of Sanger sequencing. To test the probable sensitivity limitation of Sanger sequencing, full-COLD-PCR was used to increase the mutant to wild type ratio since according to our hypothesis, in these 4 problematic specimens, wild type alleles forms the very large proportion of resected tumor tissues. COLD-PCR was

increased the mutant to wild type ratio prior to Sanger sequencing, which may overcome the probable sensitivity problem of our golden standard.

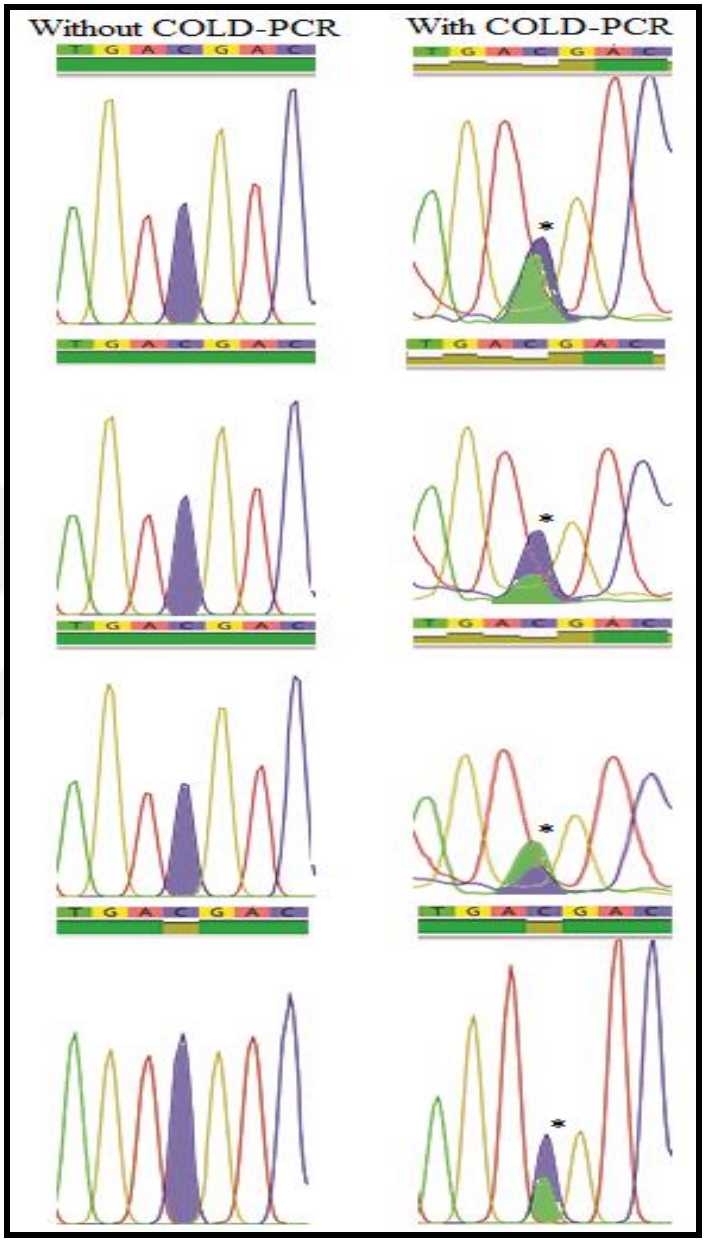
Figure 4.19: Gel electrophoresis result of COLD-PCR with 4 problematic sample alongside with positive and negative controls by using Cold-BAU primers



NTC Non-template control
- Negative control
+ Positive control
Reference: This figure was prepared by Alihan SÜRSAL

Four problematic sample were successfully enhanced in the case of mutant allele number prior to Sanger sequencing after purification. Slight non-specific amplification in the negative sample is not important in this case because our aim is not all or none detection via COLD-PCR but only enhance the mutant to wild type ratio. It is obvious that, 4 problematic sample alongside with the positive controls were amplified specifically.

Figure 4.20: False negative Sanger results versus COLD-PCR enhanced Sanger results of four problematic tumor sample



* IDH1 G395A point mutation
Reference: This figure was prepared by Alihan SÜRSAL

As it can be seen from figure 4.17, COLD-PCR enhancement is successful in terms of increasing mutant to wild type ratio, which enables Sanger sequencing to distinguish mutant alleles through wild type alleles. By the help of COLD-PCR enhanced Sanger

sequencing, 3m-ARMS method regained its reliability as an intraoperative diagnostic tool.

4.8. STATISTICAL ANALYSIS

As previously mentioned, molecular marker based classification of glial tumors have positive effects upon prognosis with standard treatments. As numerous studies indicates IDH1/2 mutations as one the most important markers of glial tumors.(Pouratian and Schiff, 2010, Soffietti et al., 2010) Due to the required resection is not always possible due to risky locations of glial tumors, correctly produced prognostic profile of the tumor begins to play an important role. Thus, to correlate our data with two conventional techniques, we performed a detailed statistical analysis to our results. Alongside with the aim to test our accuracy, we also test the accuracy of IHC in order to uncover the probable FN and FP results traditionally revealed by IHC analysis.

The frequency of the mutational status according to grades is another step to validate our tumor bank information and results. Moreover, the frequency of mutational status depends on the type of glial tumor is another tool to validate our results. Since, it is known that WHO grade II and III oligodendrogliomas should contain IDH1 mutation and 1p/19q co-deletion.(Louis et al., 2016) Thus, having a non-coherent result with these types of information is an indication of an error.

Table 4.2: Distribution of 90 IDH1 and 3 IDH2 mutation among 91 WHO grade II, III, IV glioma case

Gene	Nucleotide change	Amino acid change	N (%)
IDH1	G395A	R132H	86 (95.6%)
	C394T	R132C	1 (1.1%)
	C394G	R132G	3 (3.3%)
IDH2	G515A	R172K	3 (100%)

N (%) number of cases having specific mutation and percentage among all cases
Reference: This table was prepared by Alihan SÜRSAL

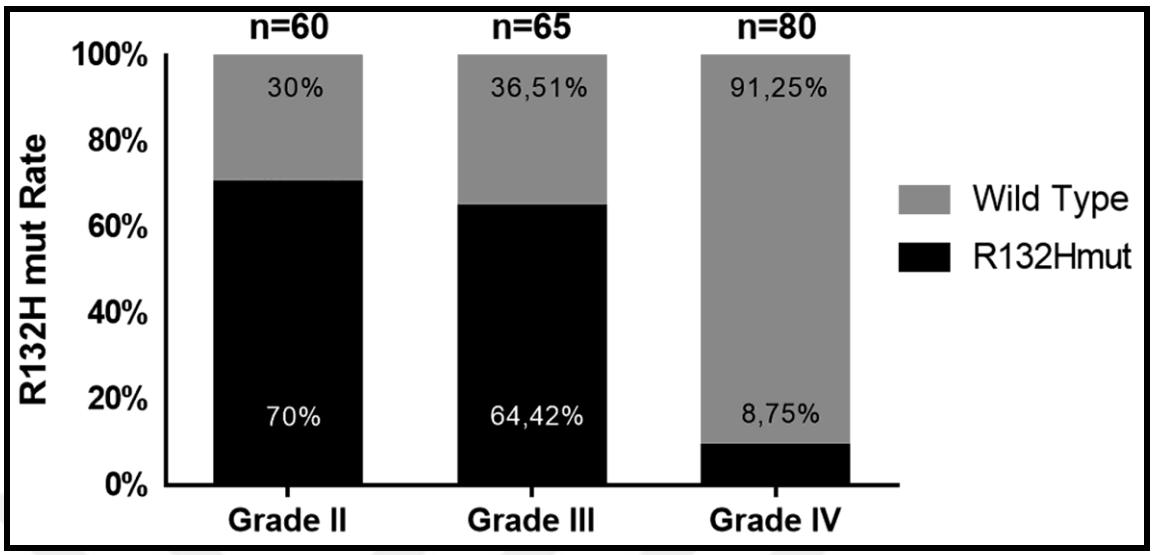
Table 4.3: Distribution of IDH1 and IDH2 mutation presence among 186 glioma patient including GBs, diffuse and anaplastic astrocytomas, oligodendrogliomas and anaplastic oligodendrogliomas

IDH1 status	Total	DA	AA	O	AO	GB
	(n=186)	(n=14)	(n=35)	(n=35)	(n=23)	(n=79)
R132H (+)	85 (45.7%)	6 (42.9%)	15 (42.9%)	35 (100%)	23 (100%)	6 (7.6%)
Wild-type	101 (54.3%)	8 (57.1%)	20 (57.1%)	0 (0%)	0 (0%)	73 (92.4%)

N (%) number of cases having specific mutation and percentage among all cases
DA = Diffuse astrocytoma
AA = Anaplastic astrocytoma
O = Oligodendroglioma
AO = Anaplastic oligodendroglioma
GB = Glioblastoma
Reference: This table was prepared by Alihan SÜRSAL

When the table 4.2 observed, the results achieved via our experiments are compatible with the data covering 1,010 cases of glioma, which positively indicates the accuracy of our results. Additionally, table 4.3 indicates the precision of diagnostic information both after and before 3m-ARMS detection. Since the percentages are also compatible with the information of the related study based on one of the largest glioma population. (Hartmann et al., 2009) Additionally, all of our oligodendrogliomas and only the lower population composed of secondary GBs have IDH1/2 mutation which is also compatible with the WHO grading system 2016.

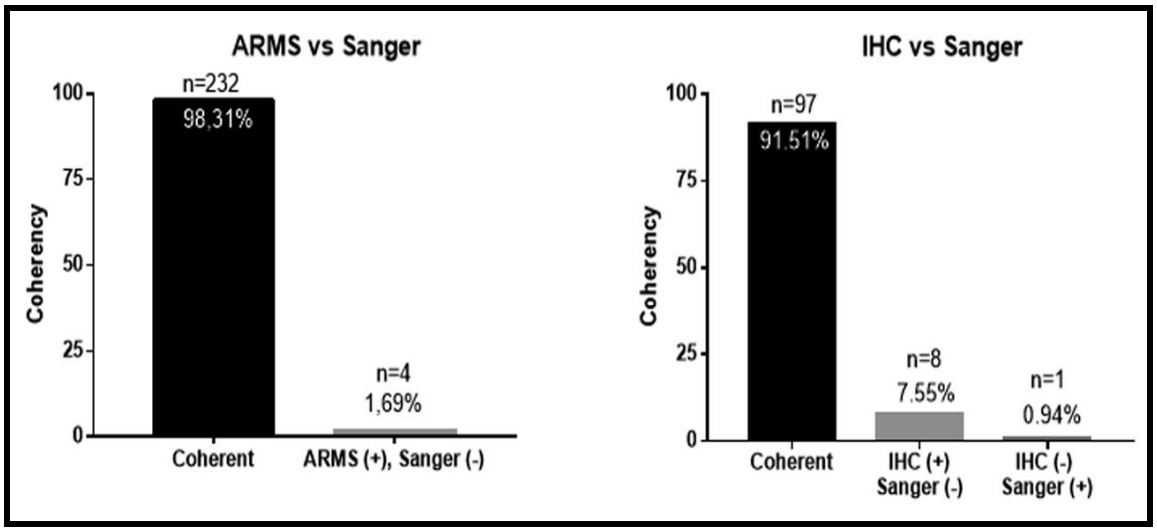
Figure 4.21: Distribution of IDH1 R132H mutations in terms of WHO grades



n Number of cases
mut Mutant
Reference: This figure was prepared by Alihan SÜRSAL

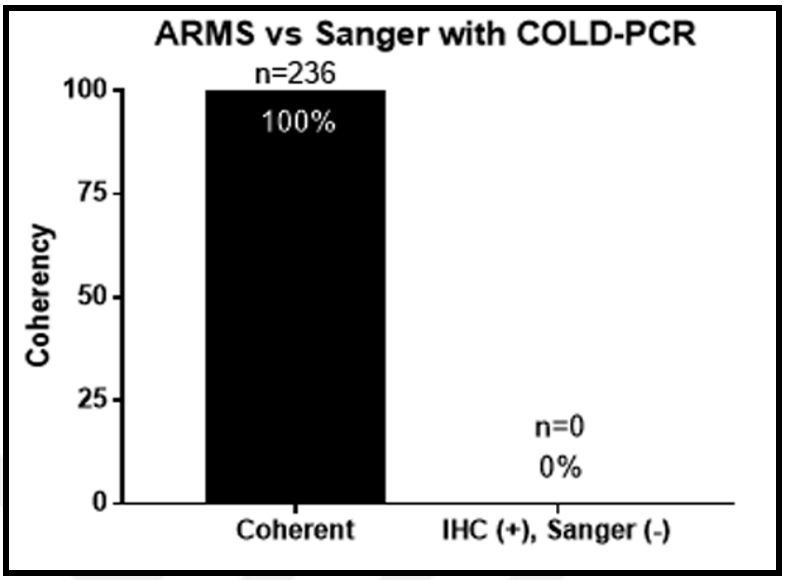
As previously monitored by the table 4.3, WHO grade IV cases have lower IDH1 R132H mutation rate comparing to WHO grades II and III. IDH1 R132H mutations present in GBs represent the percentage of secondary GB presence in our tumor bank. According to WHO 2016, frequency of secondary GBs are lower compare to primary ones.(Louis et al., 2016) These results shows compatibility with these knowledge.

Figure 4.22 Correlation of 3m-ARMS and IHC analysis with Sanger sequencing



n Number of cases
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.23: Correlation of 3m-ARMS with COLD-PCR enhanced Sanger sequencing



n Number of cases
(+) IDH1 R132H positive
(-) IDH1 R132H negative
Reference: This figure was prepared by Alihan SÜRSAL

Figures 4.19 and 4.20 shows that, 3m-ARMS accuracy was reached to 100 percent after COLD-PCR correction of results, which though to be FP. IHC analysis however, have some problematic samples with non-specific staining. Despite the sample size is lower comparing to the 3m-ARMS analysis due to the lack of old FFPE samples in the pathology laboratory, it is significant since 106 sample was tested with IHC alongside with Sanger sequencing and 3m-ARMS analysis. In addition to graphical presentation, overall performance results were calculated by 2x2 contingency table in order to produce an apparent correlation between these detection methods. Unfortunately, IHC analysis results did not affected from COLD-PCR enhanced sequencing results because these 4 problematic sample are not belongs to the IHC analysis cohort.

Table 4.4: Performance results of 3m-ARMS versus Sanger sequencing, IHC versus Sanger sequencing and 3m-ARMS versus COLD-PCR enhanced Sanger sequencing respectively

Performance Factors	3m-ARMS vs Sanger Sequencing	IHC vs Sanger Sequencing	3m-ARMS vs COLD-PCR Enhanced Sanger Sequencing
Sensitivity	100%	97,50%	100%
Specificity	97,40%	87,88%	100%
PPV	95,35%	84,79%	100%
NPV	100%	98,31%	100%
Accuracy	98,30%	91,51%	100%
LR+	38,5	8,04	max.
LR-	0	0,028	0%

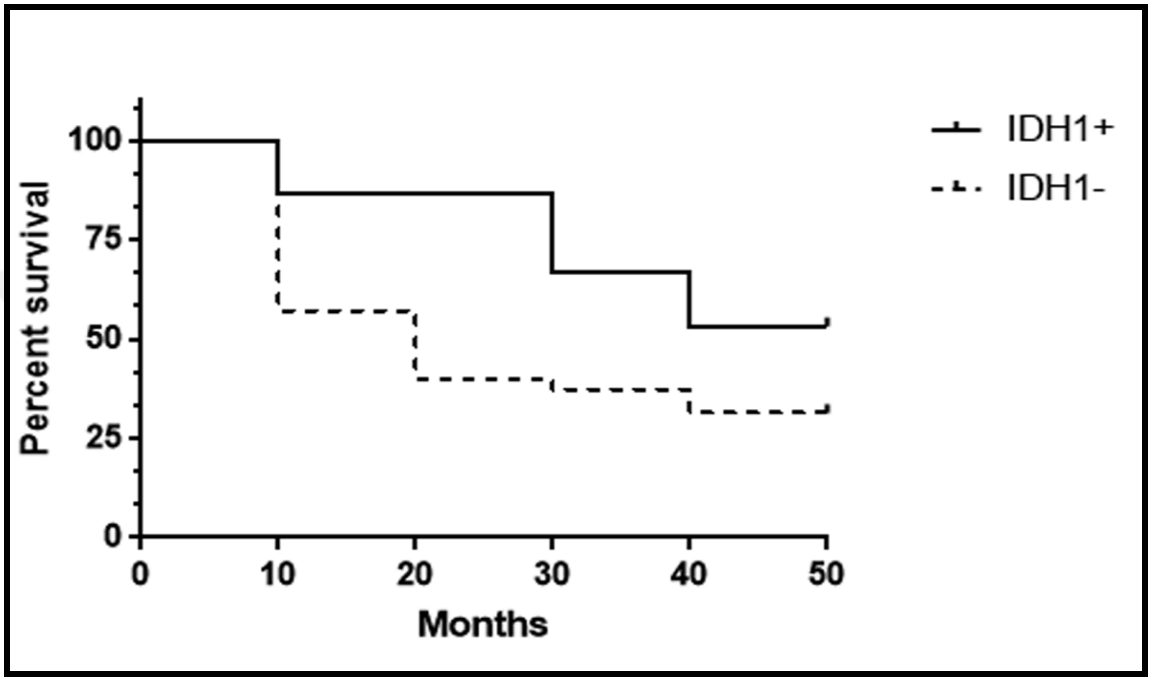
vs Versus
max. Maximum value
Reference: This table was prepared by Alihan SÜRSAL

By the help of traditional formulations of performance values, diagnostic potential and screening potential of 3m-ARMS is compared to IHC analysis by taking Sanger sequencing and COLD-PCR enhanced Sanger sequencing as golden standard. High specificity and PPV indicates the diagnostic potential. High sensitivity and NPV indicates the screening potential. As it can be seen from table 4.4, 3m-ARMS have higher potential comparing to IHC, which makes it more effective tool for diagnosis. Diagnostic utility can be uncovered by high LR+ and low LR- values. 3m-ARMS reached the maximum value in case of utility and accuracy after COLD-PCR enhanced Sanger detection of samples having low concentration of mutant allele.

The effects of IDH1 mutations on good prognosis was previously mentioned.(Houillier et al., 2010) Therefore, we designed a Kaplan-Meier graph for 50 months in order to observe the part of subjects survived for specific proportion of time by contacting our patients or their family. 48 IDH1 wild type and 27 IDH1 mutant subject were contacted

after reluctantly limiting our subjects due to insufficient antiquity of tumor samples for 50 months of surveillance and problems during contact.

Figure 4.24: Kaplan-Meier survival graph for 50 months of surveillance of 48 IDH1 wild type and 27 IDH1 mutant subjects

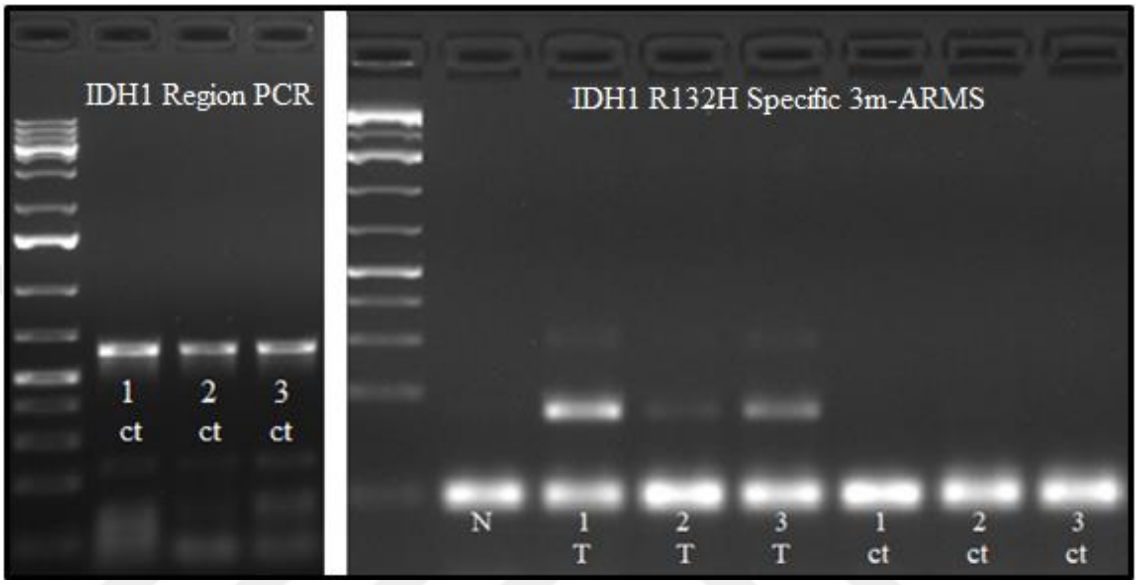


\+ IDH1 mutant
\- IDH1 wild type
Reference: This figure was prepared by Alihan SÜRSAL

Figure 4.21 indicates that, the presence of IDH1 mutation in glioma patients is a determiner of higher OS value. These results are also compatible with literature since it is known that IDH1 mutation is an indicator of longer OS and hallmark of good prognosis, which was seeded the main idea behind this study.(Wang et al., 2016)

4.9. CTDNA EXTRACTION AND 3M-ARMS ANALYSIS

Figure 4.25: Gel electrophoresis result of IDH1 region specific amplification of DNA extracted from plasma samples together with gel electrophoresis result of IDH1 R132H specific 3m-ARMS analysis



N Negative control
T IDH1 R132H positive DNA sample from tissue
ct IDH1 R132H positive DNA sample from plasma
Reference: This figure was prepared by Alihan SÜRSAL

CtDNA was extracted successfully with the commercial kit however after analysis with Sanger sequencing and 3m-ARMS, neither methods could not find IDH1 R132H mutations despite the IDH1 R132H positivity of tissue-sourced samples of the same patient. Other probable mutations were also analyzed via Sanger sequencing but ctDNA samples seem to be wild type again. As previously mentioned, BBB prevents the major proportion of ctDNA release into bloodstream. We may require parallel detection of the same patient to increase the chance of getting a glioma-sourced ctDNA among numerous cfDNA. During the region specific amplification of ctDNA IDH1 region, lower annealing temperature of 65°C was used in order to eliminate the risk of low numbered DNA amount in reaction tube. That is why, some non-specific results occurred but eliminated by purification prior to Sanger sequencing.

5. CONCLUSION

In conclusion, by addition of the third mismatch to conventional ARMS, 3m-ARMS detection method was produced with higher accuracy in the case of IDH1 point mutation detection comparing to both conventional ARMS and IHC as well as higher sensitivity comparing to Sanger sequencing. 3m-ARMS introduces rapid methodology to analyze 236 glioma sample with maximum accuracy in maximum 67 minimum 40 minutes of duration. In the case of sensitivity and specificity, all or none analysis at femtogram levels and up to 14 cycle superiority was achieved respectively. In the case of IDH2 analysis via 3m-ARMS method on mutagenesis samples, all or none results with robust sensitivity were also achieved, which seems promising upon intraoperative detection of IDH2 mutations. When 3m-ARMS is compared with IHC, it is clear that 3m-ARMS showed higher potential to be a diagnostic tool with maximum specificity and sensitivity based on Sanger sequencing. However, both techniques can be used concomitantly in order to overcome problematic samples and to correlate the results. In the future, alongside with the adaption of IDH2 to alkaline lysed samples, additional 3m-ARMS primers can be designed specific for other point mutations such as TERT promoter mutations, which are compatible with the working principle of 3m-ARMS in order to accelerate and strengthen the diagnostic response. Additionally, ctDNA analysis via 3m-ARMS experiments will be continued to catch a circulating IDH1 mutant DNA as a preoperative detection method among cfDNA.

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