T.C. BAHÇEŞEHİR UNIVERSITY

EVALUATION OF OPTIMAL KI-67 CUTOFF VALUE DIFFERENTIATING ASTROCYTOMAS, OLIGODENDROGLIOMA AND GLIOBLASTOMA

Master's Thesis

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ISTANBUL ,2020

THE REPUBLIC OF TURKEY
BAHCESEHIR UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCE

NEUROSCIENCE MASTER PROGRAM

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MASTER THESIS APPROVAL FORM

Program Name:	Neuroscience Master Program
Student's Name and Surname:	Tiba Mohammed
Name of The Thesis:	EVALUATION OF OPTIMAL KI-67 CUTOFF VALUE DIFFERENTIATING ASTROCYTOMAS, OLIGODENDROGLIOMA AND GLIOBLASTOMA
Thesis Defense Date:	17.06.2020

This thesis has been approved by the Graduate School which has fulfilled the necessary conditions as Master thesis.

Assoc.Prof.Dr.Burak KÜNTAY
Institute Director

This thesis was read by us, quality and content as a Master's thesis has been seen and accepted as sufficient.

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PREFACE

In the name of Allah, firstly all the praise and thanks to Allah who continuing blessing me to achieve this work by only Allah mercy and grace.

Special thanks for my wonderful parents for their love and encouragement. Words can't express how grateful I am to my father and my mother for all of the sacrifices that you've made on my behalf. your prayers for me was what sustained me thus far. I would also like to thank my beloved husband for the endless amount of support, I can't thank you enough for encouraging me throughout this experience. My thanks for my sisters and my friends who have always been inspired.

I would like to acknowledge the International Academy of Leadership and Development (IALD) for honoring me a partial scholarship to get a Master degree in Bahcesehir University in Turkey. Which has always sought to develop us to be the best example of Muslim and Iraqi youth wherever we are, and made my journey in turkey as remarkable as I imagined.

I would specifically like to express my gratitude to my supervisor, Dr. Ozlem Yapicier for her excellent supervision. My sincere thanks to the Pathology department staff for their assistance. And I would particularly thank Dr. Melike Yavuz for her cooperation and invaluable help for conducting this study.

Finally, great remembrance for all cancer patients. I would like to transfer message for them that I know once you diagnosed with cancer, life becomes quite simple and comes down to just two choices give up or fight back with everything you've got. You are such an inspiration fighting through this battle so bravely. But I want you to remember that Cancer is so limited… It cannot cripple love, it cannot shatter hope, it cannot corrode faith, it cannot destroy peace, it cannot silence courage, it cannot invade the soul, it cannot steal eternal life, it cannot conquer the spirit. Fighting…….

Tiba Idham Mohammed ISTANBUL 2020

ABSTRACT

EVALUATION OF OPTIMAL KI-67 CUTOFF VALUE DIFFERENTIATING ASTROCYTOMAS, OLIGODENDROGLIOMA AND GLIOBLASTOMA

Tiba Mohammed

NEOROSCIENCE MASTER PROGRAM

Thesis Supervisor: Prof. Dr. Ozlem Yapicier

May/2020, 36 pages

Gliomas are a heterogenous group of malignancies, occupying nearly 80% of CNS primary malignant tumors and rank the first among brain tumors. The Ki-67 is a routinely assessed protein in the pathological study of a tumor. It's widely investigated as a proliferation marker throughout the literature for its prognostic, predictive and therapeutic roles. A local Turkish cohort of 102 patient in the Medical Park/ Goztepe, Bahcesehir university, were enrolled in this retrospective study, to identify a precise cut point of ki-67 protein level, separates between glioma WHO assigned grades, and to explore the possible predictive relationship between Ki-67 proliferation index and the preoperatively levels of differential white blood cells in series of archival adult malignant gliomas with grade II, III and IV lesions. The statistical tests revealed that ki-67 expression level of 9.5% is a significant cutoff value between grade IV Glioblastoma and grade II and III astrocytic and oligodendroglia gliomas. And according to our findings, Age and preoperatively measured Monocyte's count showed a statistical difference between patients with low (<10%) and high (≥10%) expression of ki-67. Thereby, our result reinforces the statement that Ki-67 is in proportion to the glioma histologic malignancy degree and is a good tool for assigning grading between GBM and non-GBM gliomas. Hence, encouraging that ki-67 is a potent candidate in development of new classification system combining morphological, molecular and genetic features to better diagnose and treat glial malignant tumors.

Keywords: ki-67, Grading, GBM, Glioma, cutoff

ÖZET

OPTİMAL KI-67 KESME DEĞERİ FARKLILIKLARININ ASTROCYTOMAS, OLIGODENDROGLIOMA VE GLIOBLASTOMA DEĞERLENDİRİLMESİ

Tiba Idham Mohammed

Sinirbilim Yüksek Lisans Programı

Tez Danışmanı: Prof. Dr. Ozlem Yapicier

May/2020, 36 sayfa

Gliomalar, CNS primer malign tümörlerinin yaklaşık %80'ini işgal eden ve beyin tümörleri arasında ilk sırada yer alan heterojen bir malignite grubudur. Ki-67, bir tümörün patolojik çalışmasında rutin olarak değerlendirilen bir proteindir. Prognostik, öngörücü ve terapötik rolleri nedeniyle literatür boyunca bir çoğalma belirteci olarak geniş çapta araştırılmıştır. Bahçeşehir Üniversitesi Medical Park / Göztepe'deki 102 hastanın yerel bir Türk kohortu, bu retrospektif çalışmaya, ki-67 protein seviyesinin kesin bir kesme noktasını belirlemek, glioma WHO tarafından verilen notlar arasında ayrım yapmak ve olası öngörüyü keşfetmek için dahil edildi. II, III ve IV lezyonları olan arşiv erişkin malign glioma serilerinde Ki-67 proliferasyon indeksi ile ameliyat öncesi diferansiyel beyaz kan hücrelerinin ilişkisi. İstatistiksel testler, %9,5 ki-67 ekspresyon seviyesinin derece IV Glioblastoma ile derece II ve III astrositik ve oligodendroglia gliomalar arasında anlamlı bir kesme değeri olduğunu ortaya koydu. Bulgularımıza göre, yaş ve preoperatif ölçülen Monosit sayısı ki-67 düşük (<%10) ve yüksek (%10) ekspresyonu olan hastalar arasında istatistiksel bir fark göstermiştir. Böylece, sonucumuz Ki-67'nin glioma histolojik malignite derecesi ile orantılı olduğunu ve GBM ile GBM olmayan gliomlar arasında derecelendirme atamak için iyi bir araç olduğunu ifade eder. Bu nedenle, ki-67'nin glial malign tümörleri daha iyi teşhis etmek ve tedavi etmek için morfolojik, moleküler ve genetik özellikleri birleştiren yeni sınıflandırma sisteminin geliştirilmesinde güçlü bir aday olması teşvik edilir.

Anahtar kelimeler: ki-67, Grading, GBM, Glioma, cutoff

CONTENTS

TABLES

FIGURES

ABBREVIATIONS

CNS:	Central Nervous System
GBM:	Glioblastoma Multiform
WHO:	World Health Organization
kDA:	kilo Dalton (measurement unite)
DFC:	Dense fibrillar component
RNA:	Ribonucleic acid
PCI:	peri-chromosomal layer
PRE:	proliferating rate estimation
EMT:	epithelial- mesenchymal transition
IDH:	isocitrate dehydrogenase
EGFR:	epidermal growth factor receptor
MGMT:	methylation of 06-methylguanine methyltransferase
LOH:	loss of heterozygosity
GFAP:	glial fibrillary acidic protein
ASO:	antisense oligonucleotide

1. INTRODUCTION

1.1 Summary of The Study

Since its discovery, the expression of Ki-67 shows a strong association with tumor cell proliferation growth and is used in a wide scope among pathologists as a useful immunohistochemical marker of proliferating cells. Intensive researches were conducted to prove the clinically significant value of ki-67 in predicting tumor behavior and providing information about a patient prognosis. It has been shown to be correlated well with histologic grade, mitotic activity, and other proliferation markers.

The fact that the Ki-67 protein is expressed in all active phases of the cell cycle (G1, S, G2, and mitosis), and it is missing in resting cells (G0), this specialty made it an remarkable marker for evaluation the so-called growth fraction of a given cell population, and a clinically important proliferation marker for grading different types of cancers (Dowsett M, 2011).

In the scientific medium, proliferation considered to be a key feature for tumor progression, which is now widely estimated by the immunohistochemical assessment of the nuclear antigen ki-67. As long as, ki-67 is found naturally in the normal proliferative cell, but it is continuously and over-expressed in abnormally dividing tumor cell, Immune system attack intensity inside a tumor environment correlates with malignancy activity which can be reflected by overexpression of the Ki-67 which thereby, reflects the growth rate and proliferative activity of a mass of tumor cells.

A high proliferative rate is a hallmark of cancer. It is recorded that aggressive high graded tumors express increased levels of ki-67 expression, concluding that tumors with high expression of Ki-67 are more likely to be malignant. Therefore, we hypothesize that ki-67 can be a reliable marker to categorize glioma malignancies and be a part of a new combined grading system that might help to guide the physicians to better predict tumor behavior and prognosis, more precise diagnosis and hence, best treatment plans.

The aim of this retrospective study was to investigate whether the over-expression of ki-67 (≥10%) in Glioblastoma, Astrocytic, oligodendrocytic differentiated glioma cases is statistically associated with patient and tumor characteristics and other molecular indices and thereby defining the usefulness of ki-67 proliferation index as a predictive marker in glioma assessment protocol.

To help in the early assessment of glioma, we sought it worthy of evaluating the relationship between Ki-67 index and preoperative blood cell count in a population of local adult patients diagnosed with glial tumors from different grades II, III, IV.

1.2 Study background

1.2.1 Glioma overview

Gliomas are highly invasive tumors, considered as the most frequent central nervous system neoplasms, occurs intracranially, occupy the most considerable portion of all primary CNS malignant tumors records with approximately 70-80%. Glioma patients can present a variety of neurological symptoms due to their infiltrative nature, such as epilepsy, motor deficits, or sensory and behavioral changes, for example. Furthermore, because of its location in eloquent areas make it not possible to surgical removal of the tumor completely. The main risk factors are exposure to ionizing radiation and some rare genetic conditions such as neurofibromatosis and tuberous sclerosis and other lifestyle factors and some medical conditions like polyomavirus infections and epilepsy. (Florian, Ungureanu and Berce, 2013)

Gliomas, which called so because it originates in the glial cell, and regarding the cell of origin, it falls into several groups: **oligodendroglioma**, **oliogoastrocytomas**, ependymomas, and **astrocytoma**. The latter (astrocytoma) is the most widespread gliomas, it can be found in many regions in the brain and infrequently in the spinal cord, taking into account the existence of the four major histopathological characteristics (nuclear atypia, mitosis, cell proliferation and presence of necrosis), which are criteria inform us about the malignant potential of a neoplasm and indicate the invasive nature and growth rate of a tumor, **Diffuse Astrocytoma** further graded by world health organization (WHO 2007) into two groups low-grade (benign) which are not aggressive (grade I and II) high-grades (malignant) with much more proliferation rate (Grade III and IV), after then in (2016 WHO classification) we can't find grade I infiltrating astrocytomas and the pathologists started to classify diffuse astrocytomas using a three grade system (II, III and IV).

In details, **diffuse astrocytoma** shows only nuclear atypia (WHO grade II), an **anaplastic astrocytoma i**s a destructive form of astrocytoma, shows nuclear atypia with focal or

dispersed anaplasia, significant proliferative activity and mitoses (WHO grade III) and **glioblastoma** which is rapidly growing tumor, shows nuclear atypia, mitoses, microvascular proliferation or necrosis (WHO grade IV) and it is rare to be fully removed by surgical intervention that may cause patient's death within months.

Although the recent advances in the medical intervention, patients diagnosed with astrocytomas tends to have the worst survival length. The high-grade astrocytomas (grade III and IV) are the most common primary malignant neoplasms of the CNS in adults, which even adequately treated with radiotherapy, surgical resection and chemotherapy, the prognosis is reserved.

Oligodendroglioma is a tumor that could arise from both mature and immature oligodendrocytes, have a high potential to more aggressive type (anaplastic oligodendroglioma), which needs more conventional treatment. The third type of glioma we included in this study is anaplastic mixed glioma of an astrocytic and an oligodendroglioma tumor cells (Grade III).

Glioblastoma is the most frequent primary adult malignant brain tumor, the extremely variable tumor, which conveys 60-70% of all diagnosed gliomas, with a median survival of one year despite different therapies. (according to 2016 World Health Organization) we can find in the classification of central nervous system (CNS) tumors, three glioblastoma morphological variants; histopathologically: giant-cell, epithelioid, and gliosarcoma.

However, the survival improvement of glioma seems to depend on the knowledge and manipulation of the participated molecular markers, and that regulate the growth and aberrant manifestations of these tumors.

1.2.2 Ki-67 Protein Biology

Almost about forty years ago, researchers Scholzen and Gerdes from Kiel University (abbreviated in the name by Ki) in Germany and their group were attempting to produce a cancer-specific monoclonal antibody where they incidentally identified the ki-67 nuclear antigen by using a mouse monoclonal antibody in nuclei of Hodgkin's lymphoma-derived cell line L428. It has been discovered that ki-67 can be found in all vertebrates,

the gene (*MKI67*), located in chromosome 10q25-ter, is coding for ki-67 in a human named with 67 labels referring to the clone number on the 96-well plate.

Ki-67 is relatively large protein exists localized mainly in nucleolus with two isoforms (230 and 359 kDa), has a massive role regarding a very critical biological function, which is cell growth and proliferation, presents only in growing and dividing phases of the cell cycle (GI, S, G2, and M) but it does not exist during the resting phase (G0) (Gerdes J, 1991), since cell growth is crucial to all living organisms to maintain tissue hemostasis, and it is a good indicator of correct cellular function; hence, ki-67 becomes an ideal proliferation marker for the aggressively dividing malignant cells.

Other studies (J. M. Bridger, 1998) had stated that Ki-67 could be also expressed in heterochromatic DNA regions during the early G1 phase of the cell cycle. Ki-67 is predominantly exists within the nucleus, in the dense fibrillar component (DFC) exactly. (I. R. Kill, 1996), which is one of the three principle components in the nucleolus that is free from the ribonucleic acid (RNA) polymerase I transcription apparatus. Nevertheless, (M. Takagi, 2014) mentioned that there were a small amount of Ki-67 which localized within components of RNA polymerase I transcription apparatus and newly synthesized ribosomal RNA in vivo, which contradicts with the Ki-67 role in the early steps of polymerase I-dependent rRNA synthesis (Jonat and Arnold, 2011) (J. Bullwinkel, 2006). Ki-67 gene expression shows a different activity during the cell cycle, begins at the G1 phase and starts to increase during the S phase and reaches the highest expression during the M phase (specifically, metaphase), later it decreases sharply in the anaphase and telophase, as (Dzulkifli, Mashor, and Jaafar, 2018) showed in (Figure 1).

Apart from its role in the cell division, these distribution changes correlate with distinct functions, mainly in the mitotic and interphase in the cell cycle, The precise functional role of Ki-67 is still uncertain, but it has been published that it has multiple functions reviewed in (Booth DG, 2017). (Sobecki M, 2017) stated that for a normal cellular distribution and nucleolar association of heterochromatin antigens in the interphase, Ki-67 is required, it's also primary for the formation of a ribonucleoprotein sheath coating the condensed chromosomes in the M phase, called peri-chromosomal layer (PCL),. (Booth DG, 2014) added that Ki-67 acts to prevent aggregation of mitotic chromosomes and organizes heterochromatin and the mitotic chromosome periphery (Sun *et al.*, 2019),

but it is unnecessary in vivo for the proliferation of a cell thus it be still unclear whether ki-67 confers any benefit to cancer cells or not.

Under the microscope, the proliferating rate estimation (PRE) is represented by the percentage of Ki-67 positive cells using a variety of techniques (Halon, 2017). High proliferative activity is associated with aggressive tumor behavior. In the mouse carcinoma model, (Mrouj *et al.*, 2019) found that the loss of ki-67 leads to widespread deregulation of gene expression, including genes involved in the epithelial-mesenchymal transition (EMT), immune responses and drug metabolism, raises the hope for precise target therapy by seeking to decrease the levels of Ki-67 protein, thereby controlling the proliferation rate.

A better understanding of the proliferation process and interfered molecular markers may help pathologists to deliver the most effective treatment plans to patients with gliomas.

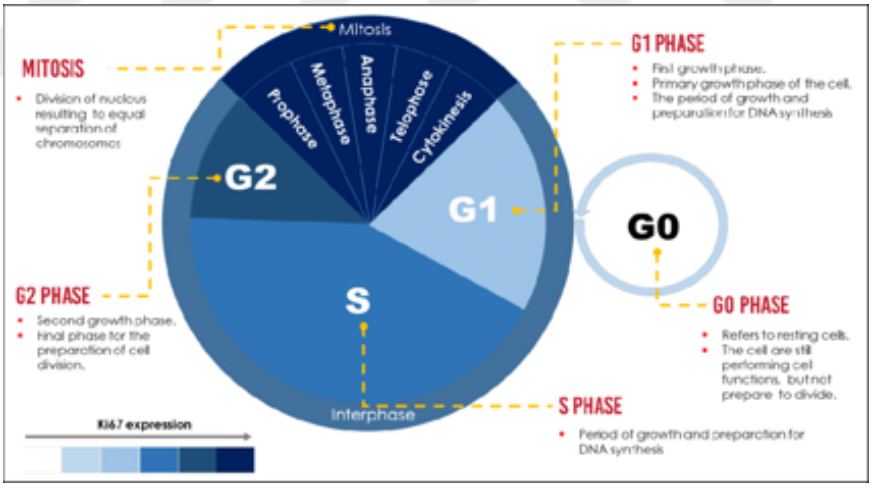


Figure 1 shows the level of expression of Ki67 during the different phases of the cell cycle.

1.3 Statement of the Hypothesis

The variation of ki-67 protein expression levels in glioma malignances, qualify it to be a diagnostic marker between WHO assigned glioma grades, and this emphasizes the substantial need to take these molecular markers into account to develop better diagnosis protocols and treatment modalities.

Main queries of this research were:

1. Does ki-67 could be used at its own to predict tumor behavior?
2. What is the relation between patient's and different tumor characteristics and the overexpression of ki-67?
3. Which type of white blood cells has the potential to predict ki-67 overexpression preoperatively?
4. How much it's vital to integrate the molecular features of the WHO glioma grading system?

2. LITERATURE REVIEW

Cell proliferation rate indicates the status of the overall population of cells going through the cell division process. ki-67 is a nuclear protein which generally expressed in the nucleus of dividing cells during (G1, S, G2) and mitotic phases, but it is missing during the resting phase (G0) and the beginning of (G1). Taking into account this critical fact, ki-67 became an excellent immunohistochemical marker of cell proliferation. (Sales Gil and Vagnarelli, 2018)

The highly proliferative cell is one of the hallmarks of cancerous tumors, the score of ki-67 expression has been routinely used and widely documented among pathologists as a proliferation marker for various human tumors, including the brain malignancies. (Wakimoto *et al.*, 1996). The Ki-67 protein expression has been established as an indicator for prognosis and prediction in the evaluation of cancer patients' biopsies.

In this chapter we are going to highlight the correlation between ki-67 overexpression and patient survival time; also, this chapter focuses on the importance of the index of cell proliferation, measured by Ki-67 index, in the diagnosis and classification of various cancer types in general, and the clinical role of ki-67 in glioma, specifically. However, the role of Ki-67 in glial cancers remains ambiguous and needs to be more investigated. Lastly, we are going to enlighten the increased pieces of evidence about the effectiveness of ki-67 as a therapeutic target.

2.1 Ki-67 protein measurements

In 1983 was the discovery of (ki-67 antibody) monoclonal IgG antibody against a protein (ki-67), of an uncertain specific function, which cannot be found in cells at rest (quiescent) while it is easily recognizable in the active cell (proliferative). Samples that can be treated with this antibody was restricted to the fresh tissue, not formaldehyde-fixed tissue, due to low immunoreactivity between the antibody and its antigen. Since the description of Ki-67 in the literature, and the success of extracting the antigen, several antibodies such as; MM-1, Ki-S5, and SP6 have been used on paraffin sections. The prospective and retrospective studies were applicable by virtue of the discovery of (MIB1 antibody), which could be used to detect the antigen in formalin-fixed tissues and

paraffin-embedded. Now, Ki-67 is mainly calculated from paraffin sections by an immunohistochemical method, using the MIB-1 antibody. (Jonat and Arnold, 2011)

There is a variety of ki-67 counting and scoring methods: automated counting device or image analysis software, manual counting of camera captured or digital images, visual counting using a microscope or viewer software (Barricelli *et al.*, 2019), the most commonly used protocol in the laboratories is 'eye-balling' estimation method, which is accomplished by looking at the slide at low power magnification of 10x under a microscope. In general, the overall number of nuclear stained tumor cells corresponded to the percentage of Ki-67 index, while other investigators conclude a total average index by counting hundreds of consecutive nuclei to.

Since we do not have standardization of IHC staining procedures, and there is a notable variation in technical issues such as fixation, dilution, staining, incubation, and antigen retrieval protocols between the laboratories and among the observers themselves and the counting method they use. We noticed that the reported studies about prognostication of glioma patients showed very varied cutoff values of Ki-67 expression in survival curves. These notable differences may be responsible for the problems in determining a standard limit in daily practice. Some used the average value of the ki-67 index (Fisher, 2002) and (Kanyilmaz *et al.*, 2018), some authors have used median as the arbitrary value, whereas others proposed a range of cutoff values in their articles from 1.5% (Hsu DW, 1997), 5 % (Jaros *et al.*, 1992) to 15.3% (Sallinen PK, 1994). While (Schiffer D, 1997) and (Di X, 1997) employed 8% as the cutoff in their work (Eneström S, 1998) suggested value of 10% level of expression as the arbitrary value.

Therefore, ki-67 standard measurement methods and clinically precise cutoff values are highly critical in predicting the biological behavior of CNS neoplasms. In our study, we are trying to explore the significant prognostic cutoff point for isolation of high-grade gliomas in Bahcesehir Medical Park- Goztepe.

2.2 Clinicopathological significance of Ki-67 in other tumors

Recently, there were an increased interest on the attractive prognostic ability of Ki-67 as well as, predictive and therapeutic potentiality in malignant tumors associated with many malignant tumors. Its classically known that tumor size, grade, and unrevealed symptoms are the main basis for the prognosis for a cancer. Ki-67 acts as an excellent molecular representative of the aggressive behavior revealed by tumors and therapy response for survival outcome assessment in multiple types of cancers, including gliomas.

A systematic correlation between cell proliferation with the postoperative follow-up of patients is necessary to determine the value of the ki-67 proliferative index in predicting the biological behavior of CNS neoplasms survival time and hence, identify the subgroups of patients who are more likely to respond to a given therapy.

Over the years, articles by many authors have been concluded that scoring of ki-67 proliferative index yields very significant prognostic information in prostate needle biopsies (Berlin *et al.*, 2017) and cervical cancer patients (Pan *et al.*, 2015). Also, the ki-67 expression is correlated to the metastatic potential and stage of renal cancer (Menon, 2019). Similarly, a higher rate of metastasis and increased progression founded to be associated with higher ki-67 expressing chordoma patients (cancer of the notochord) (von et al., 2016)

Ki-67 is officially considered a prognostic factor of breast cancer also as (Thakur SS, 2018) shows that the automated calculated score of ki-67 is significantly contributing to the empirical models that tell in advance the risk of recurrence in breast cancer.

In addition to that, higher grade and recurrence in patients diagnosed with superficial noninvasive papillary urothelial neoplasms of the bladder were interrelated with high expression of the ki-67 protein (Ogata *et al.*, 2012).

The same relationship has been recorded in the work of (Ko *et al.*, 2017), who found that a high expression of ki-67 is linked to higher progression and more likelihood of recurrence in non-muscle invasive bladder cancer and vice versa. Similarly, (Luo *et al.*, 2016) indicated in his meta-analysis that poor clinical outcome and high risk of recurrence in patients with superficial bladder tumors is correlated high ki-67 reactivity.

However, the survival improvement seems to depend on our accumulative knowledge and manipulation of the genetic pathways that regulate the growth of these aberrant tumors

2.3 Clinical significance of Ki-67 proliferative index in glioma

Ki-67 proliferation index was presented more than three decades ago as a prime molecular marker of tumor proliferation, and the application of immunohistochemical Ki-67 staining is very common in clinical pathology department. It has shown to be a potent independent prognostic marker for survival time in high-grade glioma patients.

In order to discuss the progression of a disease from an oncology point of view, we should categorize the markers into two main domains: Prognostic marker which gives information about the disease behavior without taking in consideration the treatment used and Predictive marker which informs about what implications patient may face if a particular intervention is performed. Because of the need for a prognostic marker that might help clinicians to direct therapy and predict tumor behavior and prognosis, ki-67 has been heavily investigated to examine the relationship between the overall survival of the patients with gliomas and ki-67 protein expression. In the systematic review with meta-analysis done by (Chen WJ, 2015), he showed that an increased level of ki-67 expression is a statistically significant biomarker for the worst prognosis of patients with glioma (Yoshida Y, 2010) (Preusser M, 2012). Later in his prospective study (Saha R, 2014) underscored, using the Kaplan-Meier curve, that astrocytomas patients with the level of ki-67 expression equal and greater than 14.3% showed lower survival time. Similarly, (Montine TJ, 1994) concluded that patients with ki-67 < 3% showed higher survival time than patients with a ki-67 index ≥3.

On the contrary, other studies in the literature mentioned that the higher expression of ki-67, the longer the survival (Bredel *et al.*, 2002). This raised the hypothesis of (Wong *et al.*, 2018) in glioblastoma and (Fasching *et al.*, 2011) in breast cancer previously that the higher susceptibility to adjuvant therapy agents could be achieved by patients with higher proliferative index. Otherwise, (Dirven CM, 1998) showed that there is no prognostic relationship with the ki-67 index, and nowadays (Alkhaibary *et al.*, 2019) used the median of Ki-67 expression as a cutoff, to concluded that there is no established correlation between the Ki-67 expression and overall survival. Yet now, findings regard the association between time of survival and proliferative activity of glioma tumors is conflicting.

The Ki-67 level of expression has the potential to be a postoperative biomarker of poor prognosis in astrocytomas recurrences cases also. (Uehara K, 2012) concluded that ki-67 has a predictive ability beside its previously mentioned prognostic role, he found that GBM patients with a higher rate of recurrence had a higher proliferative rate. Furthermore, in his study, (Gzell C, 2016) categorized the patients in the IHC examination of recurrence into two groups, above and under 10% level of ki-67 expression, to conclude that there is an inverse correlation between ki-67 proliferative index and survival time of the patient.

Theoretically, tumors with high proliferative index reflected by ki-67 percentage, expected to be associated with higher rates of radiodensity (Fisher BJ, 2002), (Willett CG, 1994), these opinions conflicts with the claims of (JB., 1969) who said that the proliferative cell has relative radio-sensitivity compared with non-proliferating cell.

In the majority of studies, the rate of cellular proliferation, measured by the antibody against the ki-67 nuclear protein, is proven to be a prognostic marker. Most of these studies point to a significant increase in the levels of Ki-67 proliferative index between astrocytomas of high grade when compared to those of low grade. (Hsu DW, 1997) (Saha R, 2014). However, there are articles that mention the importance of anti-Ki67 as a method of gliomas classification, especially (Khalid H, 1997), who set the ki-67 values above 10% seem to be a cutoff point for differentiating the astrocytomas in benign and malignant tumors. (Zhu L, 2015) underscored the significant positive correlation between histological different glioma grades and ki-67 proliferative index ($P<0.01$). (Johannessen AL, 2006) mentioned that increased expression of Ki-67 has been shown to be positively correlated with the higher degree of malignancy and worst prognosis in gliomas patients, and it is not enough to use it alone as a diagnostic indicator and must be combined with other prognostic parameters. In his work, (Skjulsvik AJ, 2014) highlighted the statistically significant difference in the values of a ki-67 index between grade II and IV glioma patients. (Hilton DA, 1998)

2.4 Ki-67 as a target in cancer therapy

There is a few of studies sought that ki-67 could be a favorable molecular factor for targeted therapy of a variety of tumors. Clinically, Ki-67 show promises to be related with metastasis and the clinical level of a tumor. It has been shown that gene silencing technologies that work on blocking of Ki-67 by means of microinjection of antibodies or using of antisense oligonucleotides lead to the stoppage of the proliferation process. (Figure 2) illustrates the currently used different treatment approaches aim to decrease the ki-67 expression level as a target.

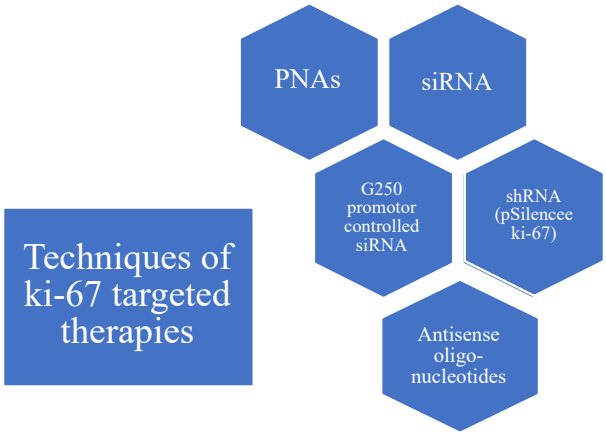


Figure2: illustration of techniques of ki-67 targeted therapies

(Peng Zhang, 2007) proved that the cell division rate could be decreased using antibodies directed against the Ki-67 in a process called microinjection. The major obstacle to accomplish this technique is the requirement to deliver targeting antibodies intracellularly and, due to ki-67 nuclear localization, intranuclearly. (Zhang P, 2007) have designed a photo immunoconjugate-encapsulating liposomes (PICELs) by the aid of liposomal encapsulated construct. In the case of subcellular delivery of antibodies, non-cationic PICELs, showed a great usefulness also provide multi-functional constructs for imaging and therapy.

(Sioud, 2010) had constructed a novel oncolytic adenovirus-based shRNA expression system named ZD55, which has the ability to deliver Ki-67- shRNA along with oncolytic adenoviruses with lytic ability.

ZD55-Ki67 have been used in vitro and in nude mice to cause tumor cell apoptosis by influencing the silencing of Mki-67 gene, allowing for efficient tumor-specific viral replication (Zhu *et al.*, 2005).

Antisense oligonucleotides (ASOs) is a powerful tool in treating malignancies, the author (Zheng JN, 2006) showed that antibodies against Ki-67 protein (Ki-67-ASOs) found to participating in cell cycle progression inhibition in animal models, and he observed in his previous work (Zheng JN, 2005) that anti-Ki-67 PNAs (peptide nucleic acids), which are RNA and DNA very specific DNA mimic with high affinity, had markedly increased in-vitro inhibition of ki-67 expression than ASOs, and had greater impact on the human renal carcinoma cells proliferation and apoptosis processes.

Yet there are no substantial pieces of evidence about the importance of the Ki-67 proliferation marker as a promising tool for treatment and prognosis in glioma malignances. However, we can see that ki-67 heralding the potential for better treatment options. Consequently, a deeper comprehension of the molecular basis linked with the very specific role of Ki-67 and therapies targeting Ki-67 are required to be more explored.

2.5 Molecular Classification of Gliomas

All the time, authors tried to correlate postsurgical evaluated molecular markers (e.g., ki-67, IDH, EGFR, BCL-2 and p53, telomerase...etc.) to better understand the parameters contributing to the long-term survival complex phenomenon. Given that brain tumors have multiple independent prognostic markers, the ki-67 proliferative index has been mentioned as a contributing prognostic indicator for glioma cases, increasing the clinicians' ability to accurately predict the patient course. (DM1, 1999) in his review had summed up the efforts done in the past century to accumulate proved molecular information. He mentioned that GFAP sounds to be a more trustworthy biomarker in high-grade gliomas tissues than the commercially available S-100B. The existence of RNA telomerase or telomerase activity be revealed to be linked to the extent of malignancy in different types of CNS tumors, including gliomas, and that the molecular markers, p53 gene and EGFR, are promising prognostic indicators of recurrence and overall survival in GBM patients.

A systematic review of molecular and genetic predictors of survival of gliomas in 14678 patients (Thuy *et al.*, 2015) concluded that, beside IDH1/2 (mutation of isocitrate dehydrogenase 1 and 2), 06- MGMT (methylation of 06-methylguanine methyltransferase) and LOH 10/10q (loss of heterozygosity of chromosome 10/10q), Ki-67 (which represent the proliferative cell rate) is a main informative biomarker in active highly proliferative gliomas.

According to (Von Deimling *et al.*, 2018) and after the era of IDH testing, we need a new grading system combining histological and genetic aspects to grade diffuse astrocytoma cases. The molecular classification in glial tumors may aid in the selection of individualized therapy strategy, design of therapeutic trial, and evaluation of clinical prognosis.

It is not surprising to found the ki-67 proliferative index involved in glioma classification suggested models, independent of WHO grades. Many studies in the recent literature tried to reclassify gliomas into distinct subgroups combing ki-67 with other prognostic factors in glioma. In his work, (Zeng *et al.*, 2015) found that IDH-1/2 mutated cases survived longer, as well as cases with low expression of ki-67, then he linked the prognostic value

of using IDH-1/2 mutation status combined with ki-67 level of expression, to classify astrocytomas into five biologically different prognostic groups, the worst prognosis groups were defined with IDH-1/2 wildtype and moderate to the high level of ki-67 (≥10%). They were suggesting a potentially clinically applicable molecular approach to better treatment options.

(Yan *et al.*, 2012) showed that low level of ki-67 expression is linked to IDH-1 mutation in GBM patients, which further confirmed by (Cai *et al.*, 2014) findings, who set a molecular classification of astrocytic tumors into three groups regardless of WHO grades, combing ki-67 expression and IDH-1/2 with ATRX mRNA expression. He concluded that patients who had the shortest survival time were those who carry IDH-1/2 wildtype and high ki-67 proliferative index percentage (>10%). (Yan *et al.*, 2012) discovered that IDH-1 mutated patients had a significantly low level of ki-67 and EGFR. This is powerfully suggesting that the best patient outcome patients' group in the combined stratification system for glioma should include a low rate of proliferation accompanying with IDH-1 mutation. This raises questions regarding the efficiency of these biomarkers as an informative candidates for new glioma classification models. These documented data must be justified by further prospective studies to reach greater knowledge for the physicians about the disease course. Given the recent breakthrough discoveries have highlighted the intermolecular associations and to further support the molecular categorization of glioma tumors, we encourage the incorporation of Ki-67 protein expression with the other molecular prognostic markers into the classification model.

In a nutshell, the cell proliferation rate in glioma tissues, as evaluated by ki-67 immuno-reactivity, has been marked as an informative indicator and independent marker for predicting the length of survival, tumor grade, and clinical outcome for glioma patients.

3. DATA AND METHOD

3.1 study setting and cohort population

A non-clinical, retrospective simple random approach have been chosen for patient selection fits with the purpose of this study. and to reduce possible bias caused by the retrospective nature of the study. A total number of 102 patients were considered in this study cohort were diagnosed for brain malignancies with astrocytic and oligodendroglioma differentiation in the hospital of Bahcesehir University (Goztepe Medical Park), Istanbul, Turkey, in the period from July 2012 to January 2019.

All eligible patients were required to meet the following criteria to be enrolled in this single-institution retrospective study:

1. Eighteen years old or older.
2. Confirmed histopathological diagnosis grade II, III astrocytoma and oligodendroglioma and GBM grade IV
3. Availability of postoperative tested ki-67 expression value

3.2 Data collecting

Information was obtained included: demographic, patient history, biopsy report of the routinely stained sections after the surgery and blood test right before entering the operating room, for 102 local Turkish patients' cohort, diagnosed with oligodendroglioma and astrocytoma glial tumors grade II, III and IV, were included in this study.

Pathology records pertaining to these patients have been retrieved from archives and through departmental medical records of anatomic pathology division. Of them, 44 patients were grade IV glioblastoma multiforme (GBM), grade I, and ependyma glioma cases have been excluded from the study. Of the remaining 58 patients, 18 patients with grade II diagnosis, and 40 were patients in Grade III. Furthermore, we have retrieved the numerical data for preoperative blood cell count (total and differential white blood cell count) to find the most promising peripheral blood index for predicting ki-67 expression.

3.3 Data management and analysis plan

Data was organized in an EXCEL datasheet (Microsoft Corporation, Redmond, WA, USA), and all analyses were performed with a statistical software SPSS version 22.0. Patient baseline characteristics were summarized using descriptive statistics. Data shown to have skewed distribution as the statistical analysis made known. So, non-parametric tests have been selected. Receiver operating characteristics (ROC) test was taken to define the optimal cutoff value of ki-67 expression, which could be used to separate Grade II, III from grade IV glioma cases.

Then cohort cases have been regrouped, two-tailed hypothesis tests were the basis for the analysis of categorical variables such as; chi-square, Mann–Whitney U tests, and Kruskal–Wallis tests. Variables were expressed by the median, to evaluate the relationship between ki-67 overexpression and other patients' or tumor variables. A P-value of lower than 0.05 was defined to point to a statistically significant divergence in the two-tailed hypothesis tests.

4. FINDINGS

4.1 Correlation of ki-67 proliferative index and Sex

Of the total 102 patients (median age 48, range between 22-81), 57 were male (56%), while 45 were female (44%). The median of Ki67 expression in males is %15, in female %12, But the difference in ki-67 expression rate is not significant between sexes (p=0.769). (Table 1).

Table 1 **Ki-67% variation in relation to Sex**

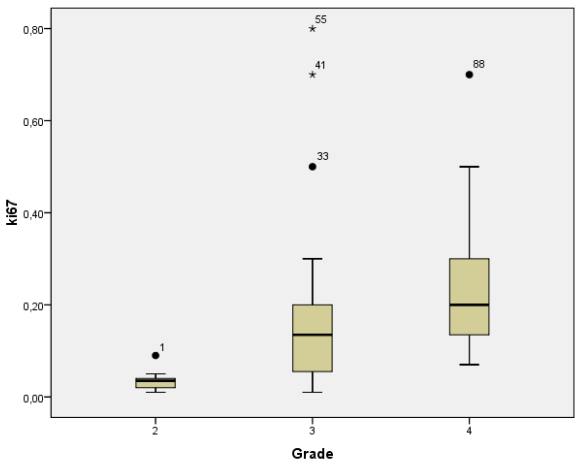
Variables	Number	%	Ki-67 mean + SD	Ki-67 range	Ki-67 Median
Sex					
Male	57	55.9%	17.18 ± 14.27 %	1-70 %	15 %
Female	45	44.1%	16.84 ± 16.27%	2-80 %	12 %
Statistics (Grouping Variable: SEX)				Ki-67	
Mann-Whitney U				1239,000	
Wilcoxon W				2274,000	
Z				-,294	
Asymp. Sig. (2-tailed)				**,769**	

4.2 Correlation of ki-67 proliferative index with glioma histological grades

The WHO histopathological groups have been included in this analysis, involved no WHO grade I, eighteen, forty and forty-four WHO grade II, III and IV with an average expression of ki-67 protein 3.56 ± 1.75, 16.9 ± 16.71, 22.66 ± 13.17, respectively.
With the lowest index being 1% and the highest one being 80% amongst the whole cohort. (Table 2) shows information about the distribution of ki-67 between histological grades, the median of the Ki-67 proliferative index was 3.5% in grade II, 13,5%, in grade III, and 20% in grade IV. As (Figure 3) illustrates and by using of Kruskal Wallis Test (chi-square), we can conclude that the difference of ki-67 value between grades was statistically significant **(p=0,000).**

Table 2 **Ki-67% correlation with histopathological glial grades**

Glial Grade	Number (percent)	Ki-67 mean (with SD)	Median	Range
Grade II	18 (17.65%)	3.56 ± 1.75	3.5	(1-9 %)
Grade III	40 (39.21%)	16.9 ± 16.71	13.5	(1-80 %)
Grade IV	44 (43.14%)	22.66 ± 13.17	20.0	(7-70 %)



Kruskal Wallis Test	
Grouping Variable: Grade	
Test Statistics	
	Ki-67
Chi-Square	44,167
df	2
Asymp. Sig.	**,000**

Figure 3: Ki-67 quartiles according to the grade

Table 3 Ki-67% correlation between grade II, III and grade IV

Variables	Number	Ki-67 mean + SD	(Range)	Median
Grade				
Grade II -III	58	12.76 + 15.19	(1-80 %)	6.5
Grade IV	44	22.66 +13.17	(7-70 %)	20
Statistics (Grouping Variable: grade)			Ki-67%	
Mann-Whitney U			556,500	
Wilcoxon W			2267,500	
Z			-4,872	
Asymp. Sig. (2-tailed)			**,000**	

According to the illustration shown in (Figure 4), Ki-67 was not normally distributed in grade II, III, and grade IV. So, non-parametric tests were performed (Mann-Whitney u), which illustrated the significant positive difference between the group of grades II, III and grade IV glioma group set patients (p=0.000) (Table 3) supporting the potential of using the ki-67 proliferative index to differentiate between GBM and lower glioma histological grades.

The cutoff value for the ki-67 index that could significantly separate patients in grade II and III from patients in grade IV was determined by receiver operating characteristics (ROC) curve analysis. After proving that the ki-67 proliferative index could be statistically used to distinguish between low- and high-grade glioma cases.

According to the ROC curve analysis, when **ki-67 is equal to 9,5%,** the sensitivity is 93,2%, and specificity is 62,1% for separating glioma grade II, III from grade IV (Figure 5) (Table 4).

Based on the reported ki-67 cutoff value previously (9.5%), we considered 10% to be a threshold to describe a glioma tumor cell to be overexpressing ki-67 or not.

Among the involved primary human central nervous system (CNS) tumors, 17 anaplastic oligodendrogliomas, 11 astrocytomas, 13 anaplastic astrocytomas, and 17 oligodendrogliomas, 44 Glioblastoma multiform were included. The average value of the ki-67 expression in this study amongst the entire population was 17% (range 1-80%).

Based on this result, 10% and above values of ki-67 were defined as over-expression. The statistical analysis of the cases regarding the Ki-67 index was divided dichotomously into

two categories regarding patient and tumor parameters analysis, <10% ki-67 and overexpressed (≥10%) categories, representing by 57% and 43%, among the whole population sample, respectively. (Table 5)

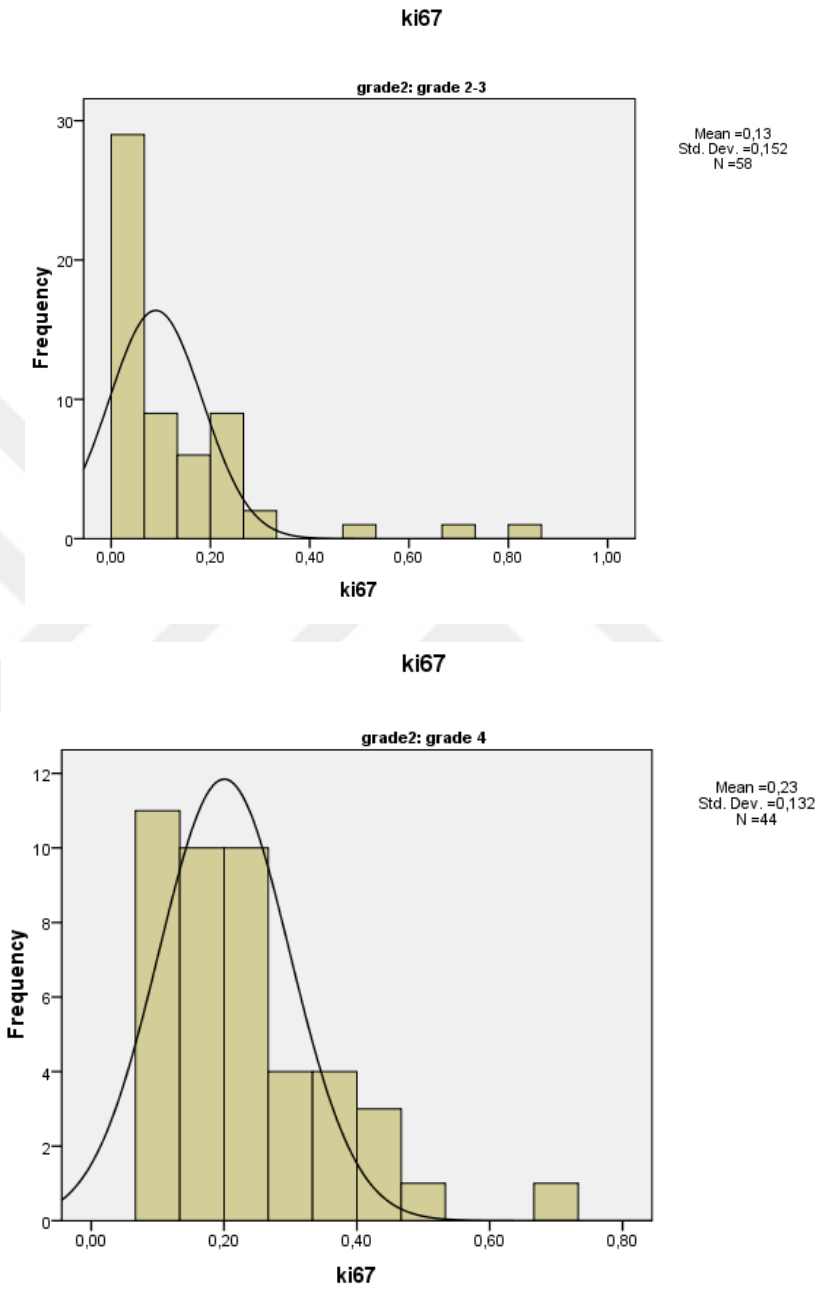
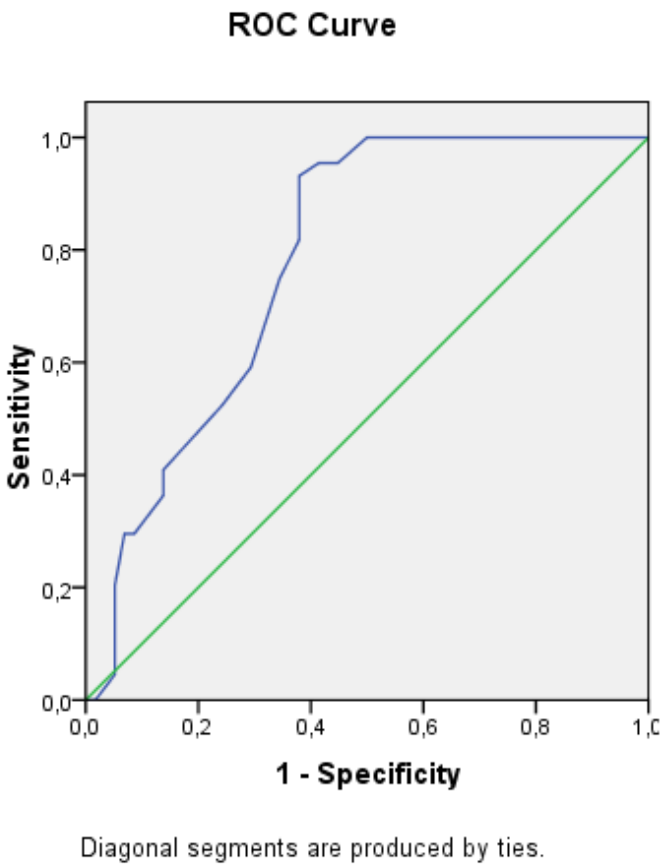


Figure 4: distribution of ki-67 percentage values between grade II, III and grade IV



Area Under the Curve				
Test Result Variable(s): **ki-67**				
Area	Std. Error	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
,782	,045	,000	,693	,871
The test result variable(s): ki-67 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.				
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Figure 5: representative image of receiver operating characteristics (ROC) curve to determine the cutoff value of ki-67 for separating grade II, III and group IV patient subset

Table 4: Coordinates of the ROC Curve

Test Result Variable(s):ki-67		
Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
,0000	1,000	1,000
,0150	1,000	,966
,0250	1,000	,879
,0350	1,000	,810
,0450	1,000	,690
,0550	1,000	,534
,0650	1,000	,500
,0750	,955	,448
,0850	,955	,414
,0950	,932	,379
,1100	,818	,379
,1350	,750	,345
,1650	,591	,293
,1900	,523	,241
,2100	,409	,138
,2350	,364	,138
,2650	,295	,086
,2900	,295	,069
,3250	,205	,052
,3750	,114	,052
,4250	,091	,052
,4750	,045	,052
,6000	,023	,034
,7500	,000	,017
1,0000	,000	,000

The test result variable(s): ki67 has at least one tie between the positive actual state group and the negative actual state group.

a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

Table 5 Tumor characteristics for glioma patients regarding ki-67 overexpression

Variables	Number (cohort)	%
Histopathology		
Grade II Astrocytoma	6 (102)	5.9 %
Grade II Oligodendroglioma	12 (102)	11.7 %
Grade III Astrocytoma	5 (102)	4.9 %
Grade III Oligodendroglioma	5 (102)	4.9 %
Grade III anaplastic astrocytoma	13 (102)	12.7 %
Grade III anaplastic oligodendroglioma	17 (102)	16.7 %
Grade IV Glioblastoma	44 (102)	43.2 %
Ki-67 labeling index		
≥10%	44 (102)	43.2 %
<10%	58 (102)	56.8 %

4.3 Correlation of patients' and tumor characteristics regarding ki-67 overexpression

In our archive-based retrospective study, we were limited by the available data of other tumor characteristics. We have analyzed other parameters such as; tumor laterality and intracranial location (Table 6), and routinely assessed molecular markers for glioma biopsy examination (GFAP, P53 and IDH-1 immunohistological status) (Table 7), all **did not** show a statistically significant difference between ki-67 proliferation index groups (<10% and ≥10%), we attribute this to the limited available information of tested markers, and the low sensitive immunohistochemical approach used to define these molecular antigens.

As it shows, there is a notable statistically significant difference between grades before (Table 2) and after sub-grouping of data set (Table 8). by performing Chi-Square tests, there was a statistical difference in grades of normal and over-expressed ki-67 groups **(p=0,000)**. We saw that 100% of grade II cases showed <10% ki-67 proliferative index, increasing with grade; to reach 93% of the total grade IV (GBM) cases were overexpressing ki-67 protein. This result makes sense regarding that ki-67 increased expression is proportional to the malignancy degree of the tumor.

As expected, the mean and median age in patients with ki-67 over 10% found to be 52, 54 years, much older than 40, 38 years for patients expressing ki-67 under 10%, respectively. Two-tailed Mann-Whitney U statistical tests revealed a significant difference between ki-67 categorial groups regarding age (**p=0,000**) (Table 9).

Table 6 Correlation of Tumor characteristics regarding ki-67 overexpression

Variables	Number	10% > Ki-67	10% ≤ Ki-67	*P value*
Laterality				
left	40 (77)	37.5 %	62.5 %	P= 0.374
Right	36 (77)	50 %	50%	
Tumor intracranial site				
Temporal	24 (95)	41.7 %	58.3 %	
Frontal	31 (95)	51.6 %	48.4 %	
Parietal	6 (95)	33.3 %	66.7 %	
Occipital	6 (95)	16.6 %	83.3 %	
Multiple	18 (95)	27.8 %	72.2 %	P= 0.397
Corpus callosum	4 (95)	0 %	100%	
Insular cortex	4 (95)	75 %	25 %	
Spinal	1 (95)	100 %	0%	
Cingulated gyrus	1 (95)	0 %	100%	

Table 7 Correlation of other molecular markers regarding ki-67 overexpression

Variables	Number	Ki-67 <10%	Ki-67 ≥10%	*P-value*
IDH-1 wildtype	13 (42)	15.4 %	84.6 %	P= 0.084
IDH-1 Mutant	29 (42)	48.3 %	51.7 %	
GFAP +ve	95 (102)	34.7 %	65.3%	P= 0.252
GFAP -ve	7 (102)	75.1 %	42.9 %	
P53 +ve	51 (74)	27.5 %	72.5 %	P= 0.252
P53 -ve	23 (74)	43.5 %	56.5 %	

Table 8 Correlation of Tumor malignancy grade regarding ki-67 overexpression

Variables	Number	Ki-67 <10%	Ki-67 ≥10%	*P-value*
Grade II	18 (102)	100 %	0%	
Grade III	40 (102)	45 %	55 %	P= 0.000
Grade IV	44 (102)	6.8 %	93.2%	

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	48,242[a]	2	,000
Likelihood Ratio	58,746	2	,000
Linear-by-Linear Association	47,104	1	,000
N of Valid Cases	102		

a. 0 cells (0%) have expected count less than 5. The minimum expected count is 6,88.

Table 9 **Distribution of age between ki-67 categories**

	Ki-67 <10%	Ki-67 ≥10%
Number	39	63
Mean	40,67	51,89
Median	38,00	54,00
Std. Deviation	10,628	13,931
Minimum	22	26
Maximum	69	81
Correlation between Age and ki-67 categories		
Mann-Whitney U	652,500	
Wilcoxon W	1432,500	
Z	-3,969	
Asymp. Sig. (2-tailed)	,000	

4.4 Peripheral blood markers predicting ki-67 overexpression

The second endpoint of the study was to examine the potential of using preoperative differential white blood cell count for the patient to predict Ki-67 index score, some trend in the values was notable, the percentage of patients with high abnormal were 82%, 92% 62.7% for WBC, Neutrophil and Monocyte, respectively.

On the other hand, 50%, 19.6%, was the population of patients with a low abnormal reading of Lymphocyte and Monocyte, respectively. The mean of WBC, Neutrophil, between the two ki-67 categories of <10% and ≥10%, were in the range of high abnormal, whereas Lymphocyte mean value was in the low abnormal range for both ki-67 categories. As we can see from the reported result below (Table 10), there is a statistically significant difference in **Monocytes'** value between ki-67 expression <10% and over-expressed ki-67 group (≥10%) (**p= 0.040). High** levels of **monocytes** may indicate the presence of cancer or other medical conditions. In our study population, Monocyte average count was in the normal range for both ki-67 categories, but higher Monocyte in the group of higher expression of ki-67 indicates the active immune response inside the body is proportional with the level of proliferative and malignancy of the tumor.

Table 10			**Distribution of white blood cell count differential regarding ki-67 categories**					
ki67_group			WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophile
Ki-67 <10%	N	Valid	39	39	39	39	39	39
		Missing	0	0	0	0	0	0
	Mean		14,1669	12,0454	1,2826	,5477	,0077	,0228
	Median		14,2500	12,4200	1,1700	,3600	,0000	,0200
	Std. Deviation		4,71783	4,62339	,66003	,53241	,01739	,02127
	Minimum		4,80	1,40	,18	,09	,00	,00
	Maximum		30,52	24,28	3,32	2,81	,09	,11
Ki-67 ≥10%	N	Valid	63	63	63	63	63	63
		Missing	0	0	0	0	0	0
	Mean		14,2483	12,1700	1,3365	,7100	,0127	,0214
	Median		14,0300	11,5500	1,1800	,5700	,0000	,0200
	Std. Deviation		5,23304	4,69166	,76619	,59346	,02548	,01777
	Minimum		2,70	1,37	,49	,09	,00	,00
	Maximum		29,37	23,97	4,32	3,05	,13	,08

Table 11	Correlation of white blood cell count differential regarding ki-67 overexpression					
Grouping Variable: ki-67 groups						
	WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Mann-Whitney U	1221,000	1221,000	1209,000	930,500	1099,000	1215,000
Wilcoxon W	2001,000	3237,000	1989,000	1710,500	1879,000	3231,000
Z	-,052	-,052	-,134	-2,052	-1,055	-,096
Asymp. Sig. (2-tailed)	,959	,959	,893	**,040**	,291	,923

5. DISCUSSION

Ki-67 is majorly expressed in active dividing cells but sharply down-regulated in resting cells (Gerdes, 1984). This distinctive feature has made Ki-67 a clinically significant proliferation marker for scoring numerous types of cancers. Ki-67 proliferative index represented by percentages is usually used to evaluate the growth fraction of various human malignancies, including the intracranial tumors (Jonat and Arnold, 2011).

Different studies have linked the location of the tumor to better and worse patient outcome, and some found no significant correlation (Krishnan *et al.*, 2019), (Gehan EA, 1977), (Stark AM, 2005) and (Pigott TJ, 1991). We noted that the frontal lobe (39%) was most frequently involved in this cohort (31 cases being only frontal lobe and 9 cases within the temporal lobe region and 4 being a combination of frontal with parietal lobe). Still, statistics showed no correlation between ki-67 overexpression and neither lobe laterality nor the lobar location of the tumor.

In the study at your hands, the median age for the patients' group expressing ki-67 <10% and ≥10% were 38 and 54 years, respectively. This statistically significant variation (p= 0,000), suggest that older age could be a predictor of ki-67 over-expression, and vice versa. This result is consistent with the study of (McKeever *et al.*, 2001), who concluded that a low level of ki-67 proliferative index expression in younger glioblastoma patients linked with longer survival time and thereby better prognosis.

(Karamitopoulou E1, 1994) one of the first literature documents highlighted the significant difference of the Ki-67 proliferative index between low-grade and high-grade gliomas (p= 0.004). Although it was known then, that higher expression of ki-67 is linked with higher malignant CNS tumors, he mentioned an increase in the expression of the ki-67 index in many benign glioma tumors.

We went through studying the capability of ki-67 to tell a piece of information about the malignancy of the tumor. This study showed a strong divergence in ki-67 expression between grade IV GBM and grade II and III (non-GBM glioma), concluding that **9.5 % ki-67 index** to be the cut-point to differentiate between WHO grade II, III and grade IV.

Our study proved the findings of the latest published work by (Krishnan *et al.*, 2019), who concluded that there is a marked statistical divergence in the expression of ki-67 between grade III and grade IV with (P = 0.00025) and (0.04) respectively. There was a significantly prominent existence of grade IV patients, with a ki-67 index value above a cutoff of 10% compared to grade III tumors (P < 0.0001). without consideration of the histological grade, he concluded that a low ki-67 index (≤10) to be correlated with longer survival among high-grade gliomas.

Our result is similar to work done by (Rathi KR, 2007) and (Wakimoto *et al.*, 1996), who used the previous and current antibody, respectively, to show a strong relationship between the ki-67 index and WHO tumor grading. And after a while, (Hu *et al.*, 2014) defined the correlation of different molecular markers in 152 glioma patients to conclude that the increase in the expression of EGFR and Ki-67 significantly correlate with higher graded glioma tumors. Nevertheless, there is no novel identified molecular marker with precise cut point value to work out better diagnosis and thereby better therapy plans.

It is hard to determine a standard threshold for ki-67 to be used in routine clinical practice, and this variation in ki-67 cutoff values is obvious in the meta-analysis conducted by (Chen *et al.*, 2015). Our result concluded that 9.5 -10% to be the most accurate threshold range to describe overexpression of ki-67, consensus the claim of (Yuan *et al.*, 2013) who considered (ki-67 ≥10%) as the overexpression cut point in his study with grade II WHO glioma patient set. And generally, there is a proportional relationship that ki-67 increases definitely with increased heterogenicity and malignancy of glial tumor mass. This further support the notion that most malignant tumor (GBM) with high ability to infiltrate and a higher probability for metastasis and thereby shortest survival time amongst glioma malignancies, is probably a reflection of higher ki-67 expression which means more capable cells to continue to proliferate after resection of the tumor.

Holding in mind that ki-67 overexpression is associated with worse prognosis and lower overall survival time (Chen *et al.*, 2015) which also a special characteristic for highly malignant glioma (GBM), and based on our result we can confirm that high expression of ki-67 is not the only but a potent predictor for grade IV glioblastoma multiform, demonstrating a potential tool to assign WHO grading of glioma.

The World Health Organization (WHO) stratification and diagnosis system of glioma considered to be not optimal, because it depends on subjective and personal perspective the evaluation of the histopathological criteria. Because the routine histological assessment of tumor mass is limited in predicting tumor behavior. (Kim BH, 2013) introduced Ki-67 as an excellent representative for growth rate and tumor behavior, empowering it its potential to improve the information the grading system provides and thereby, ki-67 might be used in routine grading of cancer (Nabi U, 2008) (Klöppel G, 2004) (Inwald EC, 2013). Nevertheless, ki-67 cannot on its own be used as a cornerstone for the classification of glial tumors, we join the voices of many authors, who raised the attention toward the need for supplementary molecular and immunohistochemical diagnostic parameters to be employed in the assessment and diagnosis of astrocytomas (Habberstad AH, 2011) (Jansen M, 2010). The value of Ki-67 for molecular staging of glioma also needs to be confirmed in controlled trials involving a larger number of patients before any definitive conclusions can be made.

The ki-67 percentage is one of the crucial parameters obtained postoperatively by biopsies in the pathology laboratories. Therefore, exploring the area of noninvasive ki-67 preoperatively prediction parameters could be clinically useful. Whereas (Peng M, 2015) found an equation to predict ki-67 preoperatively in GGO nodules pathological assessment using 3D CT images parameter analysis, we sought to identify peripheral blood cell indices associated with ki-67 proliferative index amongst patients diagnosed with different grades of glial malignancies. (Wang *et al.*, 2019) proved in his work that the Neutrophil-to-Lymphocyte ratio (NLR) <3.2 statistically corresponds with poor prognosis glioma patients, and vice versa. In general, he concluded that the count of preoperative Neutrophil, Eosinophil, and Lymphocyte was strongly associated with glioma grades. However, on account of parameters such as white blood cells count, to a certain extent, manifesting the body's immune attack against the highly proliferative dividing cancerous cells. In the same manner, as the same with Ki-67 in lung cancer, we considered that the correlation between them is biological and pathophysiological more than just statistical and mathematical.

Based on the reported results, a preoperatively differential white blood count test cannot be used to estimate the expression of ki-67 in glioma patients. Except for Monocyte

because the difference in median of Monocyte count between the two groups of the low and high ki-67 proliferative index was statistically significant ($P < 0.004$).

Nowadays, checkpoint inhibitors considered the most promising target in glioma treatment options. However, there are limited reported studies regarding the association between PD-L1 (programmed cell death protein ligand-1) and Ki-67 expression in glioma. However, they share similar implications, PD-L1 expression also, as Ki-67 higher expression, is much higher in GBM compared to grade II and III gliomas (Caccese *et al.*, 2019), and to be negatively correlated with the survival time of patients in glioma and especially GBM patients (Chen *et al.*, 2019). The point of interference was that (Xue *et al.*, 2017) concluded that PD-L1 also is important for glioma proliferation process, raised the attention in his first of kind study to the relation between these two promising markers. He found a positive correlation of PD-L1 with the ki-67 level of expression ($p = 0.002$), and as expected with tumor grade ($p = 0.013$), suggesting that PD-L1 can be a potential target for additional clinical research in the future for Ki-67 over-expressing GBM patient to predict the best patient receiver. These results indicate that further research about the association of the process of angiogenesis and proliferation in the progression of malignant glioma should be conducted to include ki-67 in the protocol of stratification, diagnosis, and treatment plans, including immunotherapy.

Taking into account that our study has some limitations regarding it is a single-institution cohort, our heterogenous population number of patients. Besides, 50% of the cases included in this study have been evaluated according to WHO classification of central nervous system tumors 2007 which needed to be reclassified after the updating in 2016, the retrospective feature of the study was the major limitations of the study. We should mention here that ki-67 evaluated values varied amongst the literature studies due to using different protocols and techniques, ki-67 level heterogenicity, and the personnel subjectivity. (Raghavan R, 1991)

Although we were keen to consider the very preoperative blood test to ensure the patient was in the best health condition qualify him to enter surgery; we could not make sure about the exact patient manifestations may affect the white blood cell counts, as well as, for defining such a multivariant correlation relationship between different blood

parameters and two categories of ki-67 expression, our sample considered to be small, this makes this result vulnerable to bias. However, Ki-67 remains a valuable IHC pathological tool, and further efforts to explore markers that allow us to predict ki-67 preoperatively are warranted.

Irrespective of the potential ki-67 shows, there is no enough solid shreds of evidence yet to definitely consider ki-67 proliferative index as a diagnostic or predictor of prognosis in gliomas due to the lack of published prospective studies validating the preliminary findings of multiple authors who have conducted single-center retrospective studies.

Acknowledgment

The study was done in collaboration with the department of pathology at the Medical Park / Goztepe at Bahcesehir University. And much thanks for my supervisor Prof. Dr. Ozlem Yapicier. And I can't forget Dr. Melike Yavuz for her valuable support and help in the statistical analysis.

6. CONCLUSION

In a nutshell, the primary finding of the present retrospective study was to determine the value of ki-67 is a priority in predicting the biological behavior of grade II, III (astrocytoma and oligodendroglioma) and IV (glioblastoma) patients. Most notably, we identified that ki-67, besides its prognostic and therapeutic attribution in glioma assessments, it has the validity to be used in molecular grading of glioma. Furthermore, this study confirms that the ki-67 cutoff point of (9.5%) index level separates between high-grade GBM and non-GBM glioma cases. Our overall impression that Age and Monocyte are a reliable predictor of higher expression of ki-67 in glioma tumor, which already known to be linked to higher malignances and poorer prognosis.

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