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DRUG RESISTANCE EFFECT ON METASTASIS BY ACTIVATING EMT PATHWAY IN MENINGIOMA

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

İDİL GÜL KARAKAYA

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FOR
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DRUG RESISTANCE EFFECT ON METASTASIS BY ACTIVATING EMT PATHWAY IN MENINGIOMA

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ABSTRACT

DRUG RESISTANCE EFFECT ON METASTASIS BY ACTIVATING EMT PATHWAY IN MENINGIOMA

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Cancer is one of the leading and often fatal diseases in humans. Cancerous cells can arise within any tissue or organ in the body. Treatment of cancer includes but is not limited to radiotherapy, chemotherapy, immunotherapy, or targeted therapy options. Meningioma, one of the brain malignancies, is the subject of this research. As it is known, cancer cells can spread to other organs and this is called metastasis. Different biological pathways contribute to the migration of cells. Epithelial to mesenchymal transition (EMT) is the pathway that primarily affects cancer metastasis. Certain EMT indicators and transcription factors are differentially regulated as the EMT pathway is activated. E-cadherin, N-cadherin, Vimentin, and β–Catenin are important EMT indicators. In our study, *CDH1* expression is lower in a benign meningioma cell line, AC599, compared to a malignant meningioma cell line, IOMM Lee. It is well known that *CDH2* and *VIM* expressions increase, while *CDH1* expressions decrease during EMT. This increment of *VIM* and *CDH2* has been seen in the AC599 meningioma cell line upon gaining JQ1 drug resistance (AC599 JR). We observed that the expression of *SNAIL*, *SLUG*, and *TWIST* is increased in the

JQ1 Resistant AC599 cell line (AC599 JR) more than in the JQ1 Resistant IOMM Lee (IOMM Lee JR) cell line. Chemoresistance is a major obstacle in cancer therapy and could be caused by various factors and pathways, among which the EMT mechanism has recently received attention for its role in drug resistance in addition to metastasis. EMT contributes to drug resistance in many cancer types, especially pancreatic, bladder, and breast cancer. Overall, our study indicates that JQ1 drug resistance increases migration and mesenchymal properties of AC599, benign meningioma cell line more than IOMM Lee, malignant meningioma cell line. RNAseq data indicates that STAT family transcription factors play a major role in JQ1 chemoresistant meningioma cell lines. *STAT3* inhibition by using Stattic reduces meningioma cell proliferation and migration, and in some cases stops cell migration. This observation is specific to the NF2 mutation status of meningioma. NF2 protein is known to play a role in EMT activation and NF2 loss is very common in meningioma which is associated with the downregulation of *CDH1* in meningioma. In conclusion, JQ1 and Stattic drug treatments are viable therapy options for meningioma chemotherapy. Furthermore, our results suggest the importance of cell and pathway-specific approaches for effective cancer treatment.

Keywords: Meningioma, EMT, Drug Resistance, JQ1, Bromodomain Inhibitor

ÖZ

MENİNJİOMDA EMT YOLUNU AKTİVE EDEREK İLAÇ DİRENCİNİN METASTAZ ÜZERİNDE ETKİSİNİN ARAŞTIRILMASI

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Kanser, insanlarda en önde gelen ve genellikle ölümcül hastalıklardan birisidir. Kanserli hücreler vücuttaki herhangi bir doku veya organda ortaya çıkabilir. Kanser tedavisi radyoterapi, kemoterapi, immünoterapi veya hedefe yönelik tedavi seçeneklerini içerir fakat bu tedavi seçenekleri bunlarla sınırlı değildir. Beyin malignitelerinden biri olan menenjiyom bu araştırmanın konusunu oluşturmaktadır. Bilindiği gibi kanser hücreleri diğer organlara yayılabilir ve buna metastaz denir. Farklı biyolojik yolaklar, hücrelerin göçüne katkıda bulunmaktadır. Epitelden mezenkimal geçiş yolağı (EMT), birincil olarak kanser metastazını etkileyen yolaktır. Belirli EMT göstergeleri ve transkripsiyon faktörleri, EMT yolu etkinleştirildiğinde farklı şekilde düzenlenir. E-cadherin, N-cadherin, Vimentin ve β–Catenin önemli EMT göstergeleridir. Çalışmamızda *CDH1* ekspresyonu, malign meninjiyoma hücresi, IOMM Lee, ile karşılaştırıldığında iyi huylu meninjiyoma hücreleri AC599'da azalmıştır. EMT sırasında özellikle *CDH2* ve *VIM* ifadeleri artarken *CDH1* ifadeleri azalır. *VIM* ve *CDH2*'deki bu artış JQ1 ilaç direnci kazanması üzerine AC599 meningioma hücre hattında (AC599 JR) görülmüştür. *SNAILl, SLUG* ve *TWIST* ifadesinin JQ1 dirençli AC599 (AC599 JR) hücre hattında,

JQ1 dirençli IOMM Lee (IOMM Lee JR) hücre hattına göre daha fazla arttığını tespit ettik. İlaç direnci, kanser tedavisinde önemli bir engeldir. Son zamanlarda EMT mekanizması metastazın yanı sıra ilaç direncindeki rolü nedeniyle dikkat çekmektedir. EMT mekanizması bunu çeşitli faktörler ve yolakları etkileyerek yapmaktadır. EMT, başta pankreas, mesane ve meme kanseri olmak üzere birçok kanser türünde ilaç direncinde rol oynamaktadır. Genel olarak, çalışmamız JQ1 ilaç direncinin iyi huylu meningioma hücre dizisi AC599'un migrasyon ve mezenkimal hücre özelliklerini IOMM Lee, malign meningioma hücre dizisine göre daha fazla artırdığını göstermektedir. RNAseq verileri, STAT ailesi transkripsiyon faktörlerinin JQ1 dirençli meningioma hücre hatlarında önemli bir rol oynadığını göstermektedir. Stattic kullanarak *STAT3* susturulduğunda, meningioma hücre bölünmesi ve hücre göçü azaldığını gözlemledik hatta bazı durumlarda hücre göçünü tamamen durdu. Elde edilen bu sonuç, meningioma hücresinin NF2 mutasyon durumuna özgüdür. NF2 proteininin EMT aktivasyonunda rol oynadığı bilinmektedir ve meninjiyomda NF2 kaybı en yaygın mutasyon olup *CDH1* ekpresyonunun azalmasına da neden olduğu bilinmektedir. Sonuç olarak, JQ1 ve Static ilaç tedavileri, meningioma kemoterapisi için uygun tedavi seçenekleridir. Ayrıca, çalışmalarımız etkili kanser tedavisi için hücreye ve yola özgü yaklaşımların önemini de göstermektedir.

Anahtar Kelimeler: Meninjiyoma, EMT, İlaç Direnci, JQ1, Bromodomain İnhibitör

To my family and my biggest supporters

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LIST OF ABBREVIATIONS

ABBREVIATIONS

CDH1 E- Cadherin

CDH2 N-Cadherin

CTNNB1 β-Catenin

EMT Epithelial-Mesenchymal transition

JR JQ1 Resistance

NR Non-Resistant

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate-buffered saline

PBST Phosphate-buffered saline and Triton X-100

PI Propidium iodide

SDS Sodium dodecyl sulfate

TBS-T Tris-buffered saline and Tween-20

VIM Vimentin

CHAPTER 1

INTRODUCTION

Cancer which results from the uncontrolled growth of cells in our body (Gouzerh et al., 2022) is one of the most common diseases and it is the second leading cause of death worldwide. Cancer can occur in any part of our body, and there are more than 100 types of diseases based on the cell of origin and the underlying mutations. Lung cancer, female breast cancer, bowel cancer, and prostate cancer are the four types of cancer that are most frequently diagnosed worldwide which, together make up more than 40 percent of all cancer malignancies diagnosed worldwide (Figure 1.1). By 2040, there will be 27.5 million new instances of cancer annually throughout the world based on the UK Cancer Research Institute. With an incidence of 100.000, the first five most common cancers in Turkey are lung (30.13), prostate (24.33), skin (18.91), breast (17.96), and stomach (9.92) cancers. Cancer incidence growth rates are higher in males compared to female cancer incidence growth rates (Yilmaz et al., 2010).

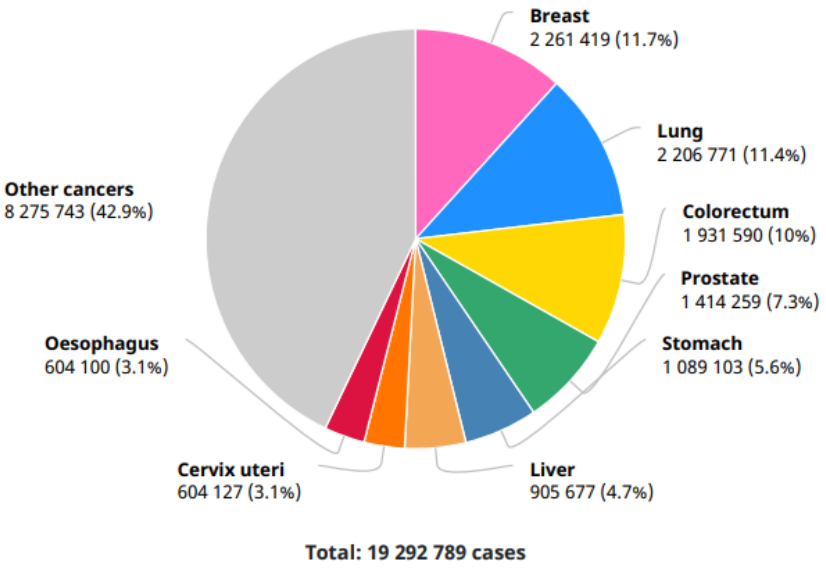


Figure 1.1. Number of new cases in 2020, both genders, all ages

Number and type of new cancer cases in the world (2020) in both gender and all ages. The figure is taken from the World Health Organization (WHO) cancer fact sheet.

In general, cancer can be subdivided into two main categories based on its prognosis; while some types of tumors are malignant, others are benign. Malignant cancer (or cancerous) cells are the group posing the real danger. This group of cancer cells begins to divide irregularly and uncontrollably and is abnormal (Figure 1.2). They also affect the cells within their surrounding tissue by compressing them, and infiltrating into or destroying them. Furthermore, they migrate to distant parts of the body via blood and lymph circulation. In contrast to malignant cancer cells, benign tumors are considered non-malignant and can grow larger in size but they do not invade another part of the body as malignant ones and often stays as a non-life threatening mass in the body.

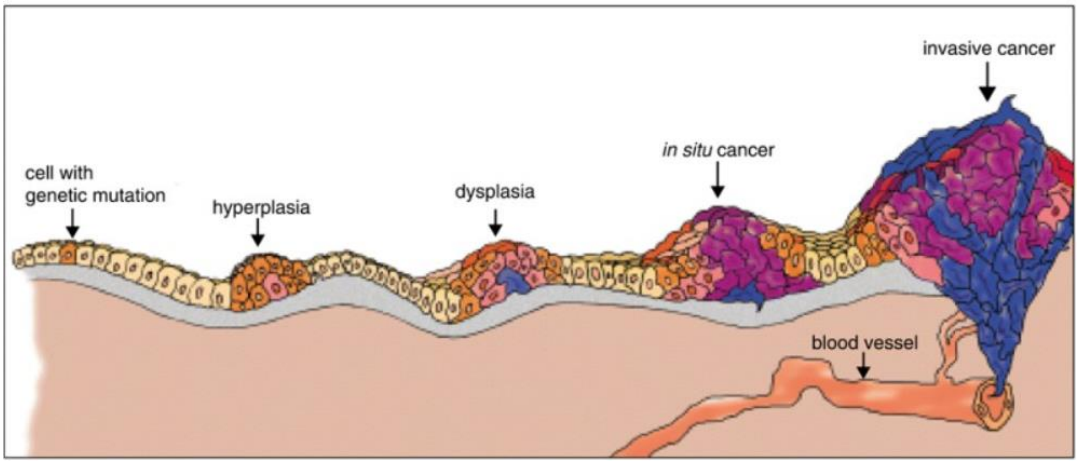


Figure 1.2. Cells change due to genetic mutation, proliferation, and metastases throughout tumor development.

The figure is taken from "National Institutes of Health (US), Biological Sciences Curriculum Study. NIH Curriculum Supplement Series [Internet]. Bethesda (MD): National Institutes of Health (US); 2007. Understanding Cancer".

1.1 One of The Brain Cancers: Meningioma

Cancer cells can be seen in every organ and tissue. They can have different types, properties, and genetic changes; such as mutations, copy number variations, epigenetic modifications, and various other malignancies. Those differences are all based on the cell of origin and the location where cancer cells begin to grow. An intracranial tumor also referred to as a brain tumor, is an abnormal mass of tissue where brain cells start to divide and grow out of control, appearing to be unaffected by the systems that regulate normal cells' proliferation as well as apoptosis. Although there are over 100 known types of brain tumors; primary and metastatic are the two basic classifications. Gliomas make up about 78 percent of malignant brain tumors in adults, making them the most common type which arises from the glia, or the brain's supporting cells. Astrocytes, ependymal cells, and

oligodendroglial cells are the different types of these cells (or oligos), and cancer that arises from these cells are called astrocytomas, ependymomas, and glioblastomas respectively. (Herholz et al., 2012).

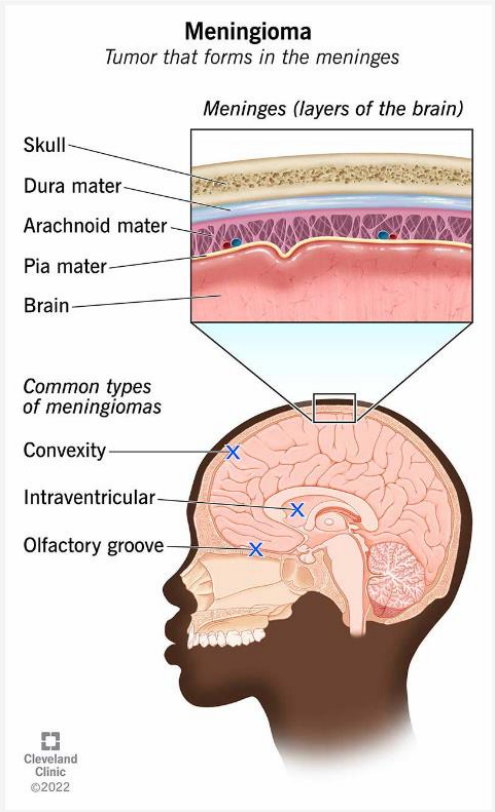


Figure 1.3. Cross-section of a human skull. The figure is focused on the brain-skull interface where the meninges covers the outermost layer of the brain and spinal cord.

The marks in the brain figure indicate the most common types of meningiomas and their corresponding locations. The figure was taken from Cleveland Clinic.

Meningiomas, make up 10 to 15 percent of all brain neoplasms out of which only a small fraction is malignant; however, they are still the most frequent among all intracranial tumors. The meninges, the membrane-like structures that cover and protect the brain and spinal cord, are the cell of origin where these malignancies

arise. The brain and the spinal cord are surrounded by three protective layers: Dura mater, Arachnoid, and Pia mater. All these three are called 'Meninx' (Figure 1.3). The origin of the term meningioma is the spider web-shaped ‘arachnoid’ membrane; however, it is now used to refer to ‘Meninx’, which is the tumor of the cerebral membrane (Marosi et al., 2008). Nearly 30% of the central nervous system (CNS) tumors in adults are due to meningiomas, which originate from arachnoidal cap cells of the leptomeninges (Kamamoto et al., 2019). Most meningiomas are benign tumors and grow slowly; however, some meningiomas pose risks due to their location, pathology, and size. Its incidence is 4.5 per 100,000 for healthy people; however, most meningiomas can develop unnoticed therefore the number of incidences is estimated to be much higher (Rodríguez-Hernández et al., 2022). The incidence in the female population is two times higher than in males. Its incidence increases with age and is most commonly observed between the ages of 30 to 70. Moreover, people with a genetic disorder called 'Neurofibromatosis Type 2' (*NF2* gene deficiency) are more likely to develop meningiomas (Bachir et al., 2021).

The *NF2* mutation is the common molecular change found in meningiomas, a mutation in the Neurofibromatosis Type 2 (*NF2*) gene located on chromosome 22 (Ruttledge et al., 1994). Studies show that *NF2* mutation is more common in fibroblasts than in meningothelial meningiomas (Kros et al., 2001). The gene product of the *NF2* gene is called Merlin (moesin-ezrin-radixin like) or Schwannomin. Actin acts as a linker between the cytoskeleton and transmembrane proteins. Thus, it plays a role in cell-cell or cell-matrix bonding. It is argued that merlin functions as a tumor suppressor protein and inhibits cell proliferation (McClatchey, 2003).

Using animal models, the role of *NF2* changes in meningioma development has been demonstrated (Kalamarides et al., 2002, Kalamarides et al., 2011). Although human meningiomas generally show *NF2* changes, some meningiomas are the *NF2* wild type. In addition to *NF2* mutation, mutations in other genes such as *SMO, AKT1*, and *KLF4/TRAF7* are observed in meningiomas (Clark et al., 2013, Abedalthagafi et al., 2016).

World Health Organization (WHO) stated that primary CNS malignancies are graded according to the type, size, and location of the tumor, as well as the extent of tumor metastasis, genetic evidence, patient age, and tumor remnants following surgery if that is an option. Meningiomas can be categorized into three histological grades, according to the World Health Organization (WHO): benign (Grade I), atypical (Grade II), and anaplastic meningiomas (Grade III) (Combs et al.,2011).

Grade I meningiomas, are the most common ones, are low-grade tumors. They are benign tumor cells that grow slowly.

Grade II atypical meningiomas are intermediate-grade tumors, which hold a higher chance of coming back after removal.

Grade III anaplastic meningiomas are malignant. In other words, they are fast-growing tumors.

Nowadays, *NF2* mutations are used in meningioma classifications besides WHO classification. Cytogenetic alterations of chromosome 22 and the *NF2* gene are characteristic genetic alterations in early meningioma tumorigenesis and are frequently noted in higher-grade tumors (Alahmadi & Croul, 2011). Grade II and III tumors, as opposed to benign meningiomas, exhibit a more complex cytogenetic and molecular background with oncogene activation, tumor suppressor gene inactivation, and changes in other genes implicated in various cellular processes (Rodríguez-Hernández et al., 2022).

Radiotherapy, chemotherapy, and surgical methods are generally used in the treatment of cancer patients. In addition to these, hormonal treatments, targeted therapies, and biochemical drug treatment methods are applied. These treatment methods can be applied alone or together. For example, targeted therapies can be employed along with chemotherapy. Targeted therapies are a special type of treatment in which biomolecule binds to the specific cancer biomarkers in tumor cells inhibiting their function. In recent years, researchers try to figure out personalized therapies for more efficient cancer treatments. Personalized therapies

target specific mechanisms that are affected by certain genes and signaling pathways. Cancer cells have unique gene expressions, mutations, and properties, therefore personalized therapies offer the best treatment for cancer patients. A better understanding of the genetic and epigenetic makeup of cancer allows us to design more efficient personal therapies in near future.

1.2 Metastasis and Meningioma Interaction

Cancer treatments, such as chemotherapy, immunotherapy, or radiation therapy are therapy procedures that stop cancer cell proliferation, metastasis, and growth. Most cancer types expand onto other organs or tissues by metastasis. Cancer therapies fight all cancer cells in the body which are primer or metastatic cells. Metastasis is a combination of the Greek words "meta" (next) and "stasis" (displacement) (Babaei et al., 2021). It is the case when tumor cells migrate from the tissues directly or via blood lymph to other regions; and the resultant secondary tumors, metastases, cause the death of 90% of cancer patients. The formation of new capillaries (a process known as angiogenesis) provides tumor cells with higher demand for oxygen and nutrients and provides a gateway for a subset of tumor cells that are capable of transforming and activating migration (Figure 1.4). Malignant tumors not only harm themselves but also damage other tissues and cells. Metastasized cells carry the cellular characteristics of the original tumor into the tissues they migrate (Eble & Niland, 2019).

Multiple causes trigger metastasis in a tumor cell. Factors such as changes in the genetic code, epigenetic factors, inhibited or activated biochemical pathways, and resistance of cells to drugs affect the metastasis of cancer cells.

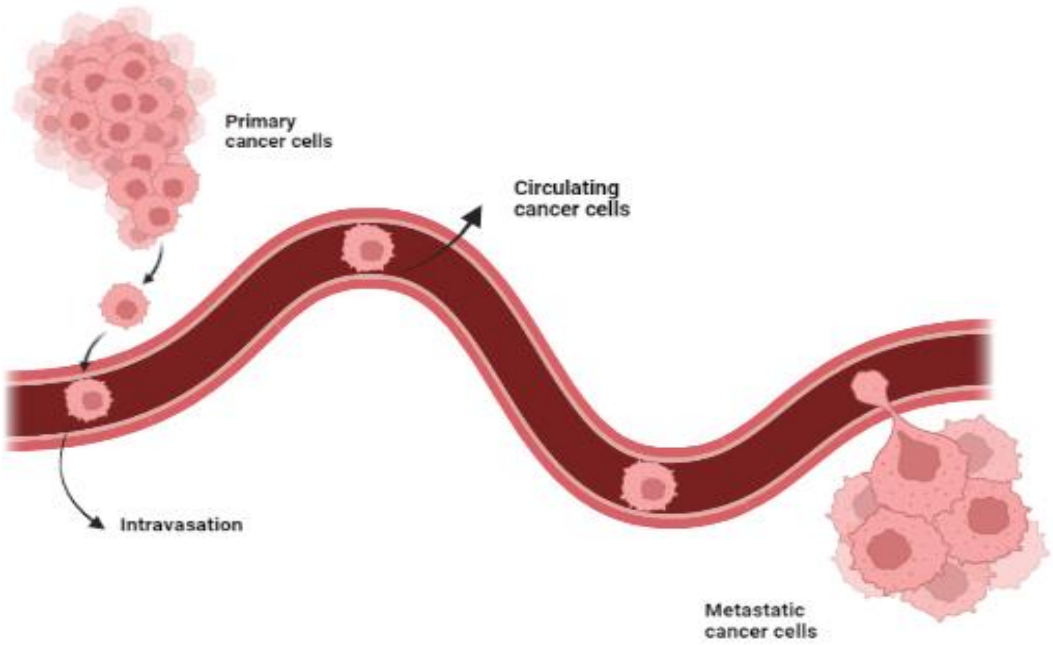


Figure 1.4. Healthy cells gain cancer cell properties.

After they become cancer cells primary cancer cells start to grow and proliferate. After a while cancer cells acquire metastatic properties. If there are any blood vessels near the primary cancer cells intravasation occurs. Then cancer cells circulate in blood vessels until they locate in another organ or tissue.

1.3 Epigenetics Mechanism and Meningioma

A study has highlighted the six biological abilities that are acquired throughout the evolution of human malignancies, namely, sustaining proliferative signals, evading growth suppressors, resisting cell death, permitting replicative immortality, generating angiogenesis, and activating invasion and metastasis collectively known as Cancer Hallmarks (Hanahan, and Weinberg et al. 2011). In 2022, two more

hallmarks are added to the list which are reprogramming cellular metabolism and avoiding immune destruction (Nedelcu, 2022).

There are an increasing number of studies in recent years indicating the importance of epigenetics in meningioma pathology. Increasing amounts of data point to the etiology of meningiomas being significantly influenced by aberrant epigenetic regulation (Murnyák et al., 2015). In meningiomas with prognostic and therapeutic significance, altered DNA methylation, microRNA expression, histone, and chromatin alterations are frequently observed.

Epigenetics refers to changes that are not caused by DNA alterations such as mutations or misalignment but consist of changes in gene expression. These changes directly affect the events taking place in the cell. Epigenetic changes also affect the formation or progression of diseases and play a crucial role in many human diseases. For instance, epigenetic changes play a significant role in the formation of the development of cancerous cells. These changes affect the biochemical pathways in cells, protein and RNA synthesis, cell death, and even the morphological shapes of cells. Meningiomas have an incomplete epigenetic profile. Mutant epigenetic modifiers are thought to be involved in abnormal microRNA synthesis, DNA methylation, and histone and chromatin modifications are among the biomarkers of progression and relapse. (Murnyák et al., 2015).

The primary events that cause epigenetic changes are methylation, acetylation, histone modifications, non-coding RNAs, and polycomb mechanisms (Talebian et al., 2020). Although several of these events are sometimes seen together, different epigenetic regulations are observed in different tumors. The epigenetic regulations can be changed based on the genetic and biochemical characteristics of tumors, their location, and the environmental factors to which they are exposed. Gene expression linked to EMT is seen to be significantly influenced by epigenetic regulatory mechanisms (Jia et al., 2019). Alterations in the frequency of DNA methylation within the CpG islands and post-translational covalent changes of histone proteins are major epigenetic modifications that go along with aberrant genetic

reprogramming in cancer cells (Nowak & Bednarek, 2021). Overall, these are stable but reversible changes in our genome which ultimately regulate gene expression that contributes to the onset and progress of cancer (Braicu et al., 2022). Therefore, aberrant epigenetic status offers a viable target for personalized cancer therapy approaches.

1.3.1 DNA Methylation

One of the epigenetic features that occur with DNA methylation is hypermethylation. DNA methylation occurs when a methyl group is added to the position of the 50-cytosine ring of the CpG dinucleotides. When methyl binds to CpG islands, it usually leads to the arrest of gene activation. CpG islands include regions where RNA transcription or DNA transcription occurs. When there is methylation in CpG regions, the gene region to be read is closed by methyl, thus preventing the transcription of the gene region. Nevertheless, inhibition of transcription is not permanent. DNA methylation can be recycled, and this phenomenon is called demethylation.

One of the epigenetic features that occur with DNA methylation is hypermethylation (i.e., an increase in methylation). Hypermethylation occurs by methylation transfer to cytosine in CpG dinucleotides of histone methyltransferases (HDAC) (Kulis & Esteller, 2010). Conversely, numerous other processes link extensive hypomethylation (i.e., decrease in methylation) to the occurrence of cancer and malignancy. Hypomethylation affects cancer and malignancy by playing a role in the promoter region of the tumor suppressor gene and transcription (Kato, 2022).

In cancers, global hypomethylation occurs in the DNA, which is accompanied by hypermethylation at other sites. Oncogenes and other genes are expressed when there is abnormally low methylation, whereas tumor suppressor genes are inhibited when there is excessive methylation (Fardi et al., 2018). Dysregulation of DNA methylation can result in incorrect repression of tumor suppressors or overexpression

of oncogenes, influencing the formation of a variety of illnesses, including cancer. Abnormal DNA methylation happens at certain genes in practically all neoplasms, suggesting that such a change could be used as a diagnostic biomarker in cancer prevention and treatment. Histone methylation is a process that can be reversed. When histone methylation-induced epigenetic modifications are reversed, the expression of oncogenes, the expression of other genes, and the activation of pathways are altered, and cancer formation can be avoided.

1.3.2 Histone Modification

The DNA consists of two long helix structures. Due to their length, they do not fit into the cell as they are, forming a more condensed structure by being wrapped in essential proteins called histones. A nucleosome is made up of DNA that spins one and two-thirds around the protein core containing 2 copies of each H2A, H2B, H3, and H4 histone (Ors Kumoglu et al., 2022). The binding of nucleosomes to each other forms histone proteins. The binding of the DNA to the histones forms chromosome structures that fit into the cell nucleus more easily. The stability or instability of chromatin for transcription, replication, and repair is influenced by the precise arrangement of changed amino acids in the histone tail (Fardi et al., 2018). Histones are modified by environmental conditions, the location of the cell, or biochemical events. The continuous and interconnected structure of the DNA and the histones creates coordination between DNA methylation and histone modifications (Cao & Yan, 2020). Besides DNA condensation, histone proteins are also involved in the regulation of gene expression by posttranslational modifications which mainly occur along their N-terminal tail (Miller & Grant, 2012). The processes that produce these modifications are methylation, acetylation, phosphorylation, ubiquitination, and sumoylation (Nathan et al., 2003; Calabrese et al., 2012). The structure of chromatin changes and regulates gene transcription as a result of histone modification. Among the histone modifications, acetylation and methylation are the most common. Histone lysine acetylation is a well-studied histone alteration that is

often associated with activated genes (Figure 1.5). Histone acetylation of histone deacetylases (HATs) is responsible for the acetylation of histones and histone deacetylases (HDACs) lead to their deacetylation (Marks et al., 2004).

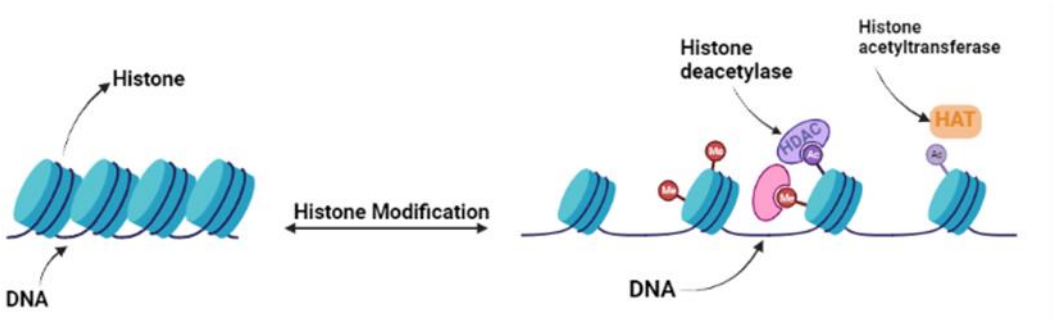


Figure 1.5. Histone modification (acetylation) was shown in the figure.

HATs (histone acetyltransferase) play role in histone acetylation while HDAC plays role in histone deacetylase. Acetylation of histone changes gene expression.

Despite the importance of altered DNA methylation, aberrant microRNA expression, and mutant epigenetic modifiers (EMGs) during the modification processes of histone and chromatin in cancer biology; understanding of the epigenetic configuration appears to be incomplete in meningioma (Murnyák et al., 2015). Therefore, the role of epigenetics in regulating critical cellular pathways in meningioma development and progress needs to be extensively studied.

1.4 Role of Bromodomain and Extra Terminal Domain (BET) Proteins BRD4 in Epigenetic Regulations

Bromodomain and Extra Terminal Domain (BET) Proteins carry out a variety of functions, including histone modifications, chromatin remodeling, transcription factor binding, and enhancer or mediator complex formation. Furthermore, they are responsible for both transcription's start and elongation. Most BET protein family proteins also contain other structurally conserved modular domains that work independently or in tandem with the BET protein family to influence protein-protein or protein-nucleic acid interactions (Donati et al., 2018).

The modified histones sometimes bind more tightly to DNA, silencing certain parts of the DNA and preventing transcription. Sometimes they are separated from the DNA parts to which they are attached, causing the regions that should not be transcripted usually to be read (Shi & Vakoc, 2014). As a result of bromodomain protein - DNA interactions, some of the genes or transcription factors (TF) are read while others are not. These changes in the transcription of DNA are effective in realizing pathways or biochemical events in the cell.

Whether a cell will be metastatic or not may be based on earlier stages before the tumor develops. A variety of differentiation pathways to which cells are exposed can affect the fate of cells. Epigenetic factors cause genes in certain regions to be activated or inhibited by the histones they bind, affecting gene expression and downstream biochemical pathways.

BRD4 is a crucial transcriptional and epigenetic regulator that affects both tumor progression and embryogenesis (Donati et al., 2018). Therefore, inhibiting the activity of any of these BrDs and/or the BET protein provides an effective treatment for cancer that relies on these modifications (Muddassir et al., 2021). Changes in the control of BET protein activities, particularly BRD4, have been strongly linked to inflammatory and cancerous disorders. Due to this, BET protein is a desirable therapeutic target (Taniguchi, 2016). In this study, the JQ1 drug, a small molecule

BRD4 inhibitor, is investigated in meningioma cell lines both with their after-treatment effects but also in JQ1 chemoresistant states particularly focusing on their role in EMT regulatory pathways.

1.5 Epithelial-Mesenchymal Transition in Meningioma

One of the pathways affected by epigenetic regulations is Epithelial-Mesenchymal Transition (EMT). EMT markers' or EMT TFs' gene expression can be affected by enhancing or inhibiting gene expression. In addition to cell migratory properties, cancer cells' resistance to drugs was shown to change during the EMT process.

The Epithelial-Mesenchymal Transition (EMT) pathway plays a role in the mesenchymal acquisition of epithelial cells (Gloushankova et al., 2018). Epithelial cells are round, fixed cells with complete intercellular bonds and thus, cannot move much (Figure 1.6). Suppose that these cells become active in the EMT pathway. In that case, they acquire the characteristics of more extensive mesenchymal cells, in which the intercellular bond is weakened. Therefore, can move quickly and cause metastasis in cancer cells (Figure 1.6). Dividing EMTs into three separate biological subtypes according to the biological context they occur in was proposed in two subsequent EMT meetings in Poland (2007) and at Cold Spring Harbour Laboratories (2008) (Kalluri & Weinberg, 2009). This pathway is divided into three groups: EMT I, II, and III which play role in development, wound healing, and metastasis respectively. EMT I is mainly seen in embryonic development, neural crest formation, and the gastrulation phase (Figure 1.6). During embryonic development, cells need to migrate to different places to form different structures and tissues, and EMT I is active at this stage (Debnath et al., 2021). EMT II participates in healing tissues and wounds and the formation of myofibrils and the closing of the wounds by communicating with each other and extending the cells (Marconi et al., 2021). EMT III is seen during the metastasis of tumor cells. Tumor cells metastasize to move from one tissue to another (Lee et al., 2006). Briefly, cell-cell connections between cells are weakened, allowing cells to move from one place

to another upon gaining metastatic properties (Micalizzi et al., 2017). In addition to its major role in metastasis, type III EMT also plays role in other biological mechanisms such as cell death, resistance to cellular aging, resistance to different types of therapy, immune regulation, acquisition of stem cell-like properties that are important for carcinogenesis (Kalluri & Weinberg, 2009).

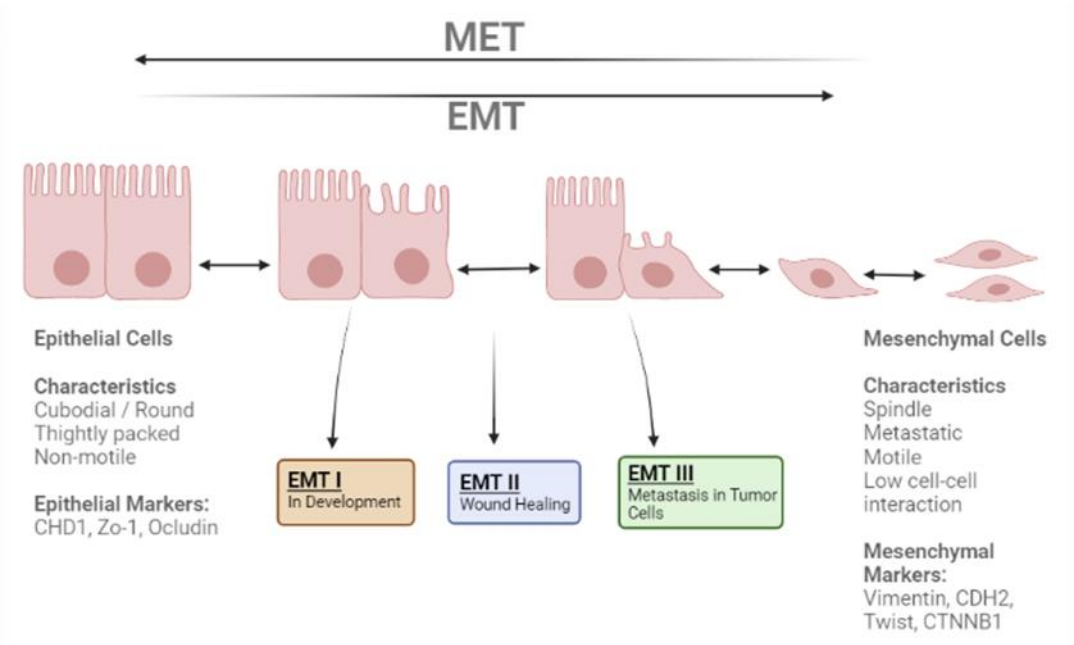


Figure 1.6. EMT is a reversible pathway.

Epithelial cells are cuboidal, round, tightly packed, and non-motile. They gain spindle, metastatic, motile, and low cell-cell interaction properties with their transition to mesenchymal cells. This figure was adapted from Debnath et al., 2021.

When the EMT pathway is active, there is either an increase or decrease in specific EMT markers and transcription factors as these phenomena can be bi-directional (Figure 6). Significant EMT markers are *E-cadherin (CDH1), β-catenin, Claudin*, and *ZO-1* are markers of epithelial cells; while *N-cadherin (CDH2), Vimentin*, and

β–Catenin are markers of mesenchymal cells (Riemann et al., 2019) (Figure 1.6). For cells to develop metastatic properties, they must lose their epithelial properties first (Y. Wang & Zhou, 2013). This event changes cells' stability, morphology, and gene expression. While CDH1 decreases in the cell, the epithelial properties are lost; mesenchymal markers such as CDH2 and Vimentin increase as they gain mesenchymal properties (Y. Wang & Zhou, 2013). *CDH2* is a marker of mesenchymal cells such as fibroblasts. CDH1 is an essential protein for cells to stick together and resist mechanical stress (Bruner & Derksen, 2017). The suppression of *CDH1* causes the cells to become active.

Besides specific EMT biomarkers, it is well-known that EMT is controlled by many transcription factors (TFs). The TFs of the primary EMT tokens are *SNAIL, SLUG, ZEB1, ZEB2,* and *TWIST* (Snatamaria et al., 2017). Transcription Factors: *SNAIL* and *SLUG* suppress the *CDH1* gene expression. Cancer cell proliferation, invasiveness, and metastasis have been demonstrated to increase when *CDH1* is down-regulated (Cordani et al., 2019). Numerous mesenchymal markers have been demonstrated to be upregulated by EMT TFs (J. M. Lee et al., 2006). Another signaling molecule that affects EMT is transforming growth factor beta (TGF-β). The increase in *TGF-β* expression causes a decrease in *CDH1* expression and epithelial properties in cells with epithelial characteristics. In contrast to *TGF-β*, *CDH1* is activated by *KLF4*, another critical TF especially known for its role in stem cell maintenance. *KLF4* functions as a MET inhibitor or a negative regulator of EMT (Debnath et al., 2021). *KLF4* expression; however, was discovered to be downregulated in cells going through EMT when *TGF-β* was present (Garg, 2013). EMT is also affected by the activation of different pathways. Studies have shown there are crucial signaling pathways for cells to maintain their mesenchymal character; canonical and non-canonical *WNT* signaling pathways. Although epithelial cells also have these signaling pathways and secrete their ligands, a high number of inhibitory proteins are also secreted simultaneously to prevent the activation of the EMT program to retain epithelial cell identity.

EMT is a reversible process, and cells can regain their epithelial character after completing the invasion and metastasis processes; the reverse process of EMT is named Mesenchymal to Epithelial transition (MET) (Chen et al., 2017) (Figure 1.6). It should be noted here that not every cell in a tumor exhibits EMT characteristics and sometimes only partial EMT is observed (Nieto et al. 2016). Activation of the EMT pathway may be affected by epigenetic changes which is a reversible process. When epigenetic changes trigger EMT pathway activation, the resulting EMT pathway must also be reversed at a certain level after these epigenetic changes are modified back to their normal state. In fact, whenever the EMT pathway is reversed upon which malignant cells start to lose mesenchymal properties and regain epithelial properties, this can also reduce or even reverse the drug resistance of malignant cancer cells. These reversible epigenetic changes affect many other critical pathways which involve in cancer pathology; therefore, targeting epigenetic change provides new approaches to cancer therapy.

1.6 WNT- β–Catenin Pathway and Meningioma Interaction

Cells produced by Type III EMT have the potential to penetrate and spread across the circulatory system, leading to systemic symptoms of cancerous tumor development (Acloque et al., 2009). When EMT III-generated migratory cancer cells have reached distant tissue beds, cells become secondary tumors with an epithelial character (Trimboli et al., 2008). Several signaling pathways are involved in EMT III (metastasis of a tumor). These include transforming growth factor beta (TGFβ), WNT, Notch, and Hedgehog, which activate EMT transcription factors (EMT TFs). Some transcription factors such as *SNAIL*, *TWIST*, and *ZEB* have been identified as master regulators of EMT. In addition to these, there are some EMT TFs, including *SOX* and *FOX* transcription factors, which bind to certain DNA sequences to control the EMT-target genes. In addition to *CDH1*, a transcription target for repression by SNAIL and several other EMT TFs, there are some key proteins engaged in epithelial cell–cell junction formation.

WNT-β–Catenin signaling is a growth control pathway that plays a role in EMT regulation in cancer cells. WNT signaling branches out into canonical (*β–Catenin* dependent activity) and noncanonical (*β–Catenin* independent activity) pathways (Komiya & Habas, 2008). A crucial structural element of the adhesion complex is β–Catenin (Shapiro et al., 1997). It also acts as a transcriptional co-activator in the WNT signaling pathway; a pathway used reiteratively during development to control cell fate decisions and one implicated in cancer in many tissues.

The WNT signaling pathway is one of the main cellular pathways involved in the embryonic development of cells (Sanz-Ezquerro et al., 2017). Studies carried out so far indicate that the WNT pathway also plays a role in the formation of meningioma tumors. The main element of the canonical WNT signaling pathway is *β–Catenin* (MacDonald et al., 2009). When the WNT signaling pathway is inactive, β–Catenin deposited in the cytoplasm is targeted, phosphorylated, and cleaved by a degradation complex composed of AXIN, APC, GSK3β, and CK1 (Lecarpentier et al., 2019). When the WNT pathway is activated, the degradation complex is attracted to the cell membrane, and β–Catenin is not phosphorylated. Non-phosphorylated β–Catenin enters the nucleus and increases the expression of TFs, which play a role in cell metastasis and development. When TFs with increased expression increase in an uncontrolled manner, the metastatic and tumoral properties of the cells also increase (Rutkowski et al., 2018).

1.7 Drug Resistance in Meningioma Interaction with EMT

Cancer treatment drugs are first put on the market by going through the stages of laboratory and then clinical research. Currently, there are approximately seven hundred drugs on the market. Furthermore, approximately 1300 new cancer drugs are in the final phase of clinical trials (Kunnumakkara et al., 2019). As the number of drugs increases, the resistance of patients to drugs also changes and increases. Therefore, determining drug resistance and understanding its causes will play a major role in increasing the treatment efficiency of cancer chemotherapy (Housman

et al., 2014). Since the genetic codes and profiles of cancer cells are different from normal cells, we can divide chemoresistance into two groups. The first is intrinsic (or de novo) drug resistance, which is caused by the genetic code that cancer cells have before treatment (Mansoori et al., 2017). The second is acquired drug resistance, which is caused by the fact that drugs change the metabolism of cancer cells during chemotherapy and cause mutations in the genes they target (Emran et al., 2022). Although drug resistance is due to different causes and different pathways, the EMT mechanism has recently attracted more attention in its role in tumor chemoresistance.

According to the literature, cancer chemoresistance is becoming more widespread, and more research are focused on the subject recently. EMT contributes to drug resistance in different cancer types, especially pancreatic, bladder, and breast cancer (Du & Shim, 2016). Instead of taking a role alone in cancer cells, EMT causes drug resistance by affecting other pathways (Georgakopoulos-Soares et al., 2020). Pathways such as WNT and Hedgehog play a role in drug resistance by activating the EMT pathway. The WNT pathway is more effective in EMT activation and most research pathways with EMT. According to the literature, overexpression of *WNT3* promoted EMT by activating the WNT/β–Catenin signaling pathway (Qi et al., 2014). Thereupon, overexpression of *WNT3* rendered human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer cells under trastuzumab treatment (Wu et al., 2012). Besides different pathways, EMT TFs promote drug resistance in cancer cells. *TWIST* upregulation was shown to increase the expression of multi-drug resistant protein 1 (MDR1) in colorectal cancer cells by activating EMT, which in turn causes oxaliplatin resistance (Deng J. et al. 2016). Various EMT TFs, including *SNAIL, SLUG,* and *ZEB,* have also been linked to drug resistance (Du & Shim, 2016). When EMT is active, the cells exhibit similar properties to cancer stem cells, such as increased drug efflux pumps and anti-apoptotic effects, together contributing to chemoresistance (Hermawan et al., 2015).

In conclusion, EMT is established as an important cancer cell phenotype that leads to chemoresistance. Inhibitors of this cellular process will be effective “partners” for

chemotherapy or other targeted therapy drugs, through which the clinical outcomes of current cancer therapeutics can significantly improve (Laham-Karam et al., 2020).

1.8 Aim of the Study

This study aimed to understand the effect of JQ1 (a small molecule BRD4 inhibitor) chemoresistance on the malignancy of meningioma cells. Moreover, we focus on discovering and targeting significant pathways responsible for acquired JQ1 chemoresistance. Accordingly, two different meningioma cell lines, IOMM Lee and AC599 are used in this study. Both IOMM Lee and AC599 cell lines are exposed to increasing doses of JQ1 drug to make them acquire resistance to that drug. RNA-seq data obtained from these cell lines before and after JQ1 treatments revealed that the EMT pathway and STAT family transcription factors plays role in JQ1 chemoresistance in meningioma. To further investigate the role of the EMT pathway, we studied changes in EMT markers in both resistant and non-resistant IOMM Lee and AC599 meningioma cell lines. We identified the STAT family as a critical player in JQ1 chemoresistance therefore we tested whether STAT inhibitor, Stattic is a viable supplementary chemotherapy option for JQ1 chemoresistant meningioma treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Line Characteristics

IOMM Lee, CH157 MN, and AC599 (Ben-Men1 599) cell lines are three different meningioma cell lines. While IOMM Lee and CH157 MN cells are malignant meningioma cell lines, AC599 cell line is benign meningioma cell line. IOMM Lee is non-*NF2* deficient, whereas CH157 MN and AC 599 carry *NF2* mutation. IOMM Lee JQ1 Resistant (JR), CH157 MN JQ1 Resistant (JR), and AC599 JQ1 Resistant (JR) were obtained after a long time of JQ1 drug exposure where JQ1 was gradually added to the cell lines to make them resistant to JQ1 drug. This procedure occurred in the School of Medicine, Yale University. In our study, we used IOMM Lee and AC599 cell lines for comparison of malignancy and *NF2* mutation.

2.2 Cell Culture

IOMM Lee, CH157 MN, and AC599 (Ben-Men1 599) are immortalized primary meningioma cell lines. Non-resistant and Resistant IOMM Lee, CH157 MN, and AC599 were ensured from Yale University. IOMM Lee, CH157 MN, and AC599 were grown in High Glucose Dulbecco's Modified Eagle Medium (DMEM) (BI- 01-052-1A) with 10% FBS, and 1% Penicilline-Streotomycin solution (growth medium). Cells were grown in a humidified incubator with 5% CO2 at 37 °C. For maintenance of JQ1 Resistant IOMM Lee, CH157 MN and AC599, (+)- JQ1 (Cayman 11187) which is a bromodomain inhibitor, was dissolved in DMSO and 10µM stock solution was prepared for cells. For IOMM Lee 3:1000, CH157 MN

2,5:1000, and AC 599 1,2:1000 JQ1 stock solution was added to the growth medium for each cell to maintain them in a chemoresistant state.

2.3 Wound Healing Assay

0.2 mg Mitomycin C was dissolved in 1 ml PBS. 500 µL Mitomycin C ((Serva) was added into a 9.5 ml medium, the final concentration should be 10 µg/ml. 0.05x106 cells were seeded into the 24-well plate containing 500 µL of growth medium. When cells reached 80% confluency, the medium was removed and washed with the DMEM (Basal medium). Cells were treated with (0.2 mg/ml) Mitomycin C for 2 hours. Cells were then washed with basal medium, thoroughly as it might reduce viability afterward. Cells were incubated with normal DMEM for 1 hour. 200 µL pipette tip was used for making a scratch. After wounding, the cells were washed with PBS or DMEM and incubated with a growth medium. Cells were treated with JQ1, Stattic, and DMSO (control).

2.4 Cell Cycle Assay

Cells were cultured in 100 mm cell culture dishes, followed by Mitomycin C (Serva) (600 µL) for 2h, JQ1, and Stattic treatment (IC50 values) for 24h. After the incubation period, 1x10^6 cells were harvested and washed with basal DMEM and 500 µL cold Dulbecco's PBS (DPBS), complete the volume to 4 mL with 70% ethanol, and incubated at − 20 °C for 30 min. Next, the cells were washed with cold DPBS and suspended in propidium iodide (PI) staining solution (40 µg/mL PI and 40 µg/mL RNase A in PBS). This suspension was transferred to Flow cytometer tubes, and flow cytometry was used to analyze the cell cycle.

2.5 Immunofluorescence Assay

AC599 and JQ1 Resistant AC599 JR cells were grown on coverslips. Then, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in 1% BSA. The expression of N-cadherin, E-cadherin, Beta-catenin, and Vimentin was detected using primary antibodies against N-cadherin (#ab18203, Abcam), E- cadherin (#sc-8426, Santa Cruz), Beta-catenin (#ab32572, Abcam) and Vimentin (#sc6260, Santa-Cruz) respectively at 4°C overnight. After the incubation, the coverslips were washed and further incubated with secondary Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (#Ab150113, Abcam) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) (#Ab150080, Abcam) respectively for 1 hour at room temperature in the dark. Nuclei were stained using Antifade Mounting Media with DAPI (Vectashield). Immunofluorescent images were obtained using a fluorescent microscope in 40x and 60x magnified objectives.

2.6 RNA Isolation

Cells were briefly washed with PBS to get rid of dead cells. 500 µL TRIzol per 6 well plates was added and the standard procedure was followed according to the provider's description (Ambion, 15596-026). RNA was quantified via a spectrometer. (BioDrop) RNA integrity was checked by inspecting two intact and distinct RNA bands; 18S and 28S on 1% agarose gel.

2.7 cDNA Synthesis

cDNA was synthesized from 0.7- 1 µg RNA template using BioRad iScript cDNA Synthesis Kit per manufacturer’s protocol (1708890). 1000 ng RNA was used as a template. 4 µl 5x iScript Reaction Mix and 1 µl Reverse Transcriptase was added to 1000 ng RNA. The total volume was completed to 20 µl with nuclease-free water. Samples were incubated with the complete reaction mix in a thermal cycler using the

following PCR protocol: Priming: 5 min at 25ºC, Reverse transcription: 20 min at 46ºC, RT inactivation: 1 min at 95ºC, Optional step: Hold at 4ºC.

2.8 Gene Expression Analysis with RT-PCR

Primers for candidate genes were designed using NCBI Primer Blast and UCSC In-Silico PCR tool, validated by UCSC InSilico PCR (Appendices Table A.1.). To determine the optimal conditions for primers, a gradient PCR from 62C-55C was run for each primer pair.

Quantitative RT-PCR (qRT-PCR) was carried out in a total volume of 10 µl containing 0.5 µl of cDNA (1:10 dilution), 5 µl of Advanced Universal I SYBR Green Supermix (1725271), 0.6 µM forward and reverse primers (Appendices Table A.1.) and molecular biology grade water. β-actin was used as an internal control for all reactions. The reactions were performed in the Rotor-Gene Q 6000 System (Qiagen, Germany). Data collected was analyzed using a Rotor-Gene Q Series Software and Microsoft Excel. The delta-delta Ct method was conducted by normalizing data to β-Actin expression and calculating fold change relative to DMSO (control) samples.

2.9 Statistical Analysis

All experiments were carried out with at least two or three different biological replicates; each had at least three technical replicates. GraphPad Prism 8 (GraphPad Software Inc., USA) was used for data analysis. Two-way ANOVA was carried out to determine significance. p-value < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Morphological Changes Based on JQ1 Drug Resistance

Cancer cells are exposed to chemotherapy and radiotherapy during treatment. It has been observed that most cancer cells develop resistance to drugs following chemotherapy. Drug resistance is affected by altered biochemical pathways, morphology, or the behavior of cells. JQ1, one of the novel cancer drugs examined for use in cancer treatment, is a small-molecule bromodomain inhibitor. Research has been carried out to see the effects of JQ1 in the treatment of meningioma.

In this study, IOMM Lee and CH157 cell lines were used as malignant meningioma cells, and the AC599 cell line was used as benign meningioma cells. These cells were obtained from Yale University School of Medicine, where they were made JQ1 resistant. To find out the cells' resistance levels to JQ1, the IC50s of the cell lines were determined by dose response curve, and non-resistant IOMM Lee, CH157, and AC599 cell lines were made resistant to JQ1 by exposure to increasing doses of the drug over the course of three months (Appendices A.7). The IC50 values of JQ1 for each cell lines were found by our collaborators at Yale University School of Medicine Laboratory and confirmed in our lab as well.

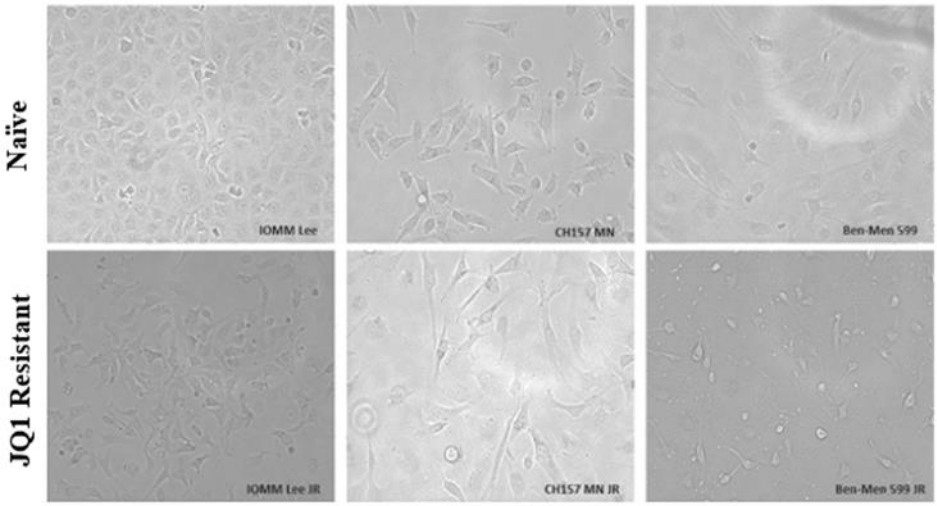


Figure 3.1. IOMM Lee- IOMM Lee JR, CH157- CH157JR, AC599- AC599 JR bright field image for a morphological change. JR stands for JQ1 Resistant.

BenMen1 AC599 is a benign low-grade *NF2* meningioma, CH157MN and IOMM Lee are higher-grade malignant, and *NF2* and non*NF2* meningioma respectively. When we acquired meningioma cell lines, first we aimed to identify their morphological differences if there are any. Non-resistant IOMM Lee, CH157, and AC599 cell lines were compared with JQ1 Resistant IOMM Lee JR, CH157 JR, and AC599 JR cells under a bright field. During this process, it was observed that there were morphological differences between non-resistant and JQ1 Resistant cells (Figure 3.1). Whereas non-resistant cell lines were observed to be small, roundish, and short, JQ1 Resistant cells appeared to be larger, filamentous, and elongated (Figure 3.1).

When the morphology of the cells was compared with each other primary changes were seen between non-resistant and resistant IOMM Lee and AC599 meningioma cell lines morphology. Besides morphology, IOMM Lee is a malignant meningioma, and AC599 is a benign meningioma cell line. Moreover, while AC599 is *NF2* mutant cell, IOMM Lee meningioma cell lines are non-*NF2* mutant cells. We used IOMM Lee and AC599 meningioma cell lines during our research to understand how malignancy and *NF2* mutant differences affect cell morphology. Based on this

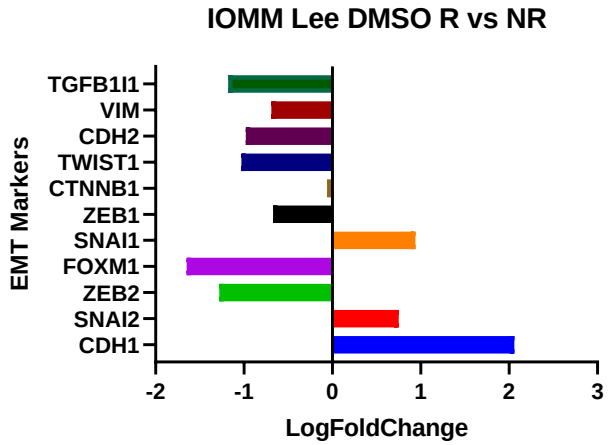
observation, it was concluded that JQ1 Resistant cells might have activated the EMT pathway to gain more mesenchymal properties. To check whether our hypothesis has any biological relevance, the gene expression levels of EMT markers and genes affecting EMT were examined by analyzing RNA-seq data provided by our colleagues at Yale University (Figures 3.2 and 3.3) and we performed Ingenuity Pathway Analysis using the sequence data to understand upstream and downstream regulatory pathways in our data set. Our analysis showed that the STAT family proteins were among the genes significantly regulated (Appendices A.6). It is well established in the scientific literature that the STAT family affects the EMT pathway, apoptosis, and cell proliferation (Verhoeven et al., 2020). We then further investigate the RNAseq data to monitor the changes in EMT genes during the acquisition of JQ1 resistance.

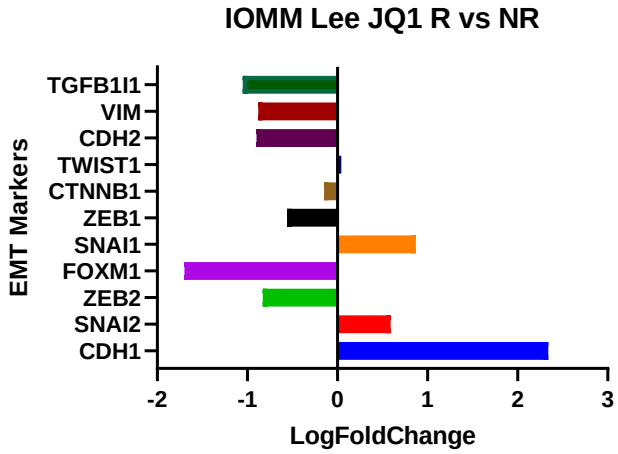
3.2 JQ1 Treatment Effect on Different Genes and Transcription Factors

Meningioma cell lines that were made resistant to the JQ1 drug. JQ1 Resistant meningioma cells and non-resistant meningioma cells were exposed to DMSO as a control group and JQ1 as a treatment group for 3 hours. Those cells were later harvested for RNA isolation and these RNA samples were sent to the Yale Genome Center for RNA sequencing and ultimately sequence analysis is performed. IOMM Lee and AC599 meningioma cell lines were compared in terms of resistant and non-resistant cells under DMSO treatment, which is the control group of gene expressions, and JQ1 treatment, which is the drug treatment group to see the JQ1 drug effect on resistant and non-resistant cells gene expressions, according to the data obtained from the Yale Bioinformatics team. (Figures 3.2 and 3.3).

Then we analyze these RNA-Seq data specifically for EMT markers to better understand the morphological changes observed in vitro upon chemoresistance. When the IOMM Lee and AC599 cell lines' gene expression changes in DMSO control group comparisons were analyzed, it was understood that when AC599 meningioma cell lines become resistant to JQ1, their mesenchymal markers were

upregulated whereas the mesenchymal markers of the cells in IOMM Lee meningioma cell lines were downregulated. *CDH1*, one of the epithelial markers, enables the cells to bind to each other by cell-cell adhesion bonds. When there is a decrease in the expression of this gene or if there is a loss of function, the proliferation, invasion, or metastasis properties of genes increase (Debnath et al., 2021). During this process, *CDH1* genes together with other epithelial markers are downregulated, and mesenchymal markers such as *CDH2*, *VIM*, *CTNNB1*, and *TWIST* are upregulated (Debnath et al., 2021). When mesenchymal markers are upregulated, the cell-cell interaction between the cells decreases while the metastatic properties of the cells increase. In our study, when the RNA-seq comparisons of DMSO control groups of AC599 and IOMM Lee meningioma cell lines were analyzed. It was observed that *CDH1* was downregulated whereas *VIM* and *CDH2* were upregulated in AC599 compared to IOMM Lee (Figures 3.2 and 3.3). When resistant and non-resistant AC599 cell lines following JQ1 treatment were compared, it was particularly observed that *CDH2* expression tripled in the resistant cell line compared to the non-resistant counterpart (Figure 3.3). When the DMSO control group and JQ1 treated group of IOMM Lee meningioma cell lines were compared the expression of *TWIST* upregulated in resistant IOMM Lee meningioma cell lines after JQ1 exposure.



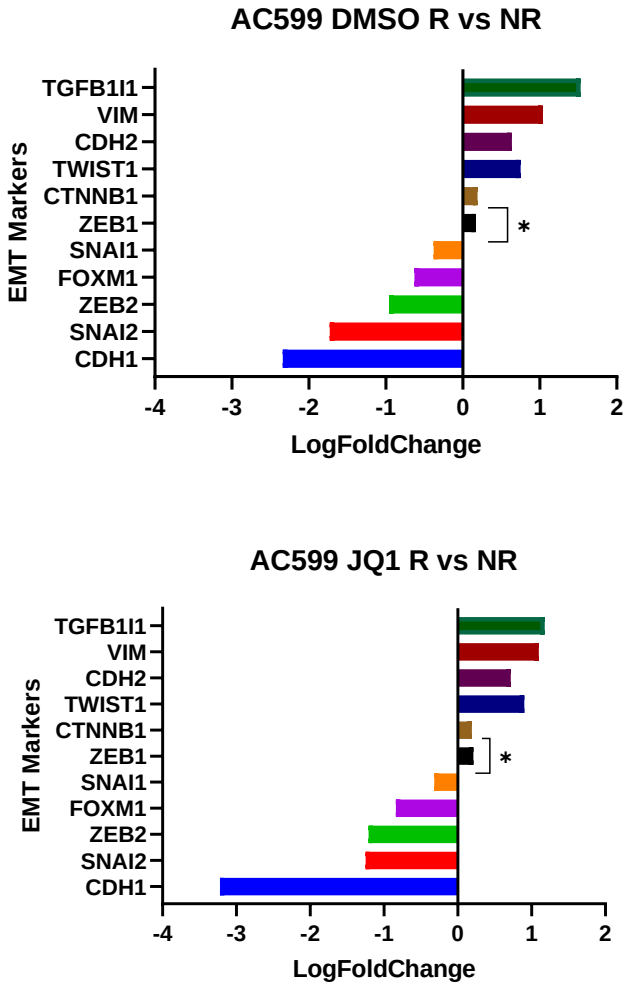


IOMM Lee R vs NR After JQ1 Treatment			
Upregulated	Downregulated	No change	
ZEB2		CDH1	ZEB1
TWIST1		SNAI2	CTNNB1
TGFB1		FOXM1	CDH2
		SNAI1	VIM

Figure 3.2. Resistant and Non-resistant IOMM Lee were treated with DMSO and JQ1.

RNA sequencing data were analyzed for JQ Resistant (R) and Non-resistant (NR) cell lines and upregulated and downregulated genes were shown in the graph and table. RNA sequencing data were analyzed for JQ Resistant (R) and Non-resistant (NR) cell lines Statistical values of RNA-Seq were shown (Appendices A1).

*LogFoldChange for EMT markers in this comparison is shown. ≤ 0 downregulated; ≥0 upregulated . * shows significant p-values.*



AC599 R vs NR After JQ1 Treatment			
Upregulated	**Downregulated**	**No change**	
CDH1	TGFB1	SNAI2	ZEB1
		ZEB2	CTNNB1
		FOXM1	TWIST1
		SNAI1	CDH2
			VIM

Figure 3.3. Resistant and Non-resistant AC599 were treated with DMSO and JQ1.

RNA sequencing data were analyzed for JQ Resistant (R) and Non-resistant (NR) cell lines and upregulated and downregulated genes were shown in the graph and table. RNA sequencing data were analyzed for JQ Resistant (R) and Non-resistant (NR) cell lines Statistical values of RNA-Seq were shown (Appendices A1). LogFoldChange for EMT markers in this comparison is shown. ≤ 0 downregulated; ≥0 upregulated . * shows significant p-values.

3.3 EMT Markers Expression and Pathway Analysis Based on RNA-Seq Data

RNA-Seq data provides the whole transcriptome of the cell that shows every gene expression change. After initial bioinformatics data construction, RNA-Seq data can be examined in different ways. We applied Ingenuity Pathway Analysis (IPA) (QIAGEN©) which allow us to predict upstream and downstream changes for the given transcriptomic profile gene regulations based on JQ1 treatment and resistance (Appendices A.2, A.3, A.4, and A.5).

Based on the Ingenuity Pathway prediction of our RNAseq data, *CTNNB1* is predicted to be increased in non-resistant and JQ1 Resistant AC599 cell lines. Ingenuity prediction shows that *CDH1* expression, on the other hand, decreases at non-resistant and JQ1 Resistant AC599 cell lines. The interaction between *CDH1* and *CTNNB1* was well known as they have an association at adherens junctions. This decrease affects the *CTNNB1* amount in the cytoplasm. When *CDH1* is expressed in a cell, they connect with *CTNNB1* in adherens junctions. Following EMT pathway activation, *CDH1* expression starts to decrease in the cell which then transitions to mesenchymal cell fate. After *CTNNB1* becomes free in cytoplasm WNT ligands bind to CTNNB1 and translocate it to the nucleus. Within the nucleus, *CTNNB1* regulates *TWIST* gene expression. In Appendices A2, an increase in *TWIST* was seen when *CTNNB1* translocate to the nucleus. *TWIST* is a transcription factor that plays role in the mesenchymal differentiation of cells. Based on this data, the mesenchymal markers increase in JQ1 treated resistant AC599 cell line (Appendices A.5). In IPA

the system shows some pathway predictions based on bioinformatics data, such as RNA-Seq and next-generation sequencing. IPA results, including both resistant and non-resistant IOMM Lee and AC599 meningioma cell lines, showed gene expression predictions such as a decrease in *TWIST* expression (Appendices A.3, A.4 and A.5). In IOMM Lee cell lines, with JQ1 and DMSO treated ones, IPA prediction shows an increase in *CDH1* expression. Based on the *CDH1* increase in IOMM Lee meningioma cell lines the expression of *CTNNB1* decreased. By implication of *CTNNB1* decrease, the expression of mesenchymal differentiation marker *TWIST* expression was decreased. Decreasement of these two genes inhibits mesenchymal differentiation depending on Ingenuity Pathway Analysis (Appendices A.4, A.5). We are predicting to see EMT pathway activation and an increase in EMT properties in non-resistant and resistant AC599 meningioma cell lines.

RNAseq data analysis for each cell line in comparison to their resistant counterparts (ie. AC599 vs AC599 JR, CH157 vs. CH157 JR, and IOMM Lee vs. IOMM Lee JR) by using Ingenuity Pathway Analysis Program (IPA) were investigated (Appendices A.2, A.3, A.4, A.5 and A.6). This bioinformatic research also highlights STAT family transcription factors as being the primary regulators of these specific signaling pathways, in particular *STAT1* and *STAT3* (Appendices A.6). The JAK/STAT system is a key pathway that regulates cellular functions like immunity, apoptosis, cell cycle, and division that are crucial for both healthy and cancerous cells. In contrast to their parental, healthy cells, resistant meningioma cells have a distinct regulation of STAT members, according to our RNAseq data. Particularly, the *STAT* RNA levels of the BenMen1 AC599 cell line appear to be significantly impacted. We show that *STAT1* downregulation seems to play a significant role in benign AC599 meningioma cell line as it acquires JQ1 chemoresistance. RNAseq data showed that STAT family members *STAT1* and *STAT2* are significantly downregulated as benign meningioma cell line AC599 acquires JQ1 resistance (AC599 JR) (Appendices A.9).

Next, we hypothesized that *STAT3* overexpression would be an indirect critical component to target in this situation because Ingenuity Pathway Analysis (IPA)

revealed that upon *STAT1* downregulation, its downstream target *STAT3* was more likely to be upregulated (Appendices A.6). Numerous research groups have examined the functions of *STAT1* and *STAT3*, which are typically regarded as tumor suppressor and oncogene, respectively, in cancer. The relative abundance of *STAT1:STAT3* appears to be the key, according to these studies; however, our knowledge of STAT's role in cancer is still limited (Appendices A.6). Based on our data and current literature, we decided to use Stattic as a STAT3 inhibitor to understand STAT family impact on our meningioma cells with particular focus on EMT pathway.

In addition to focusing on top EMT markers and TFs, other pathways interaction that also play role in EMT were investigated with Ingenuity Pathway Analysis. After checking EMT interactions with other pathways, one of the top predictions shows that *STAT3* expression affects *TWIST* expression. In the AC599 cell line upon JQ1 treatment, *STAT3* causes an increase in *TWIST* expression. In IOMM Lee cell lines; however, in both DMSO control and JQ1 treatment, a decrease in *STAT3* expression results in a decrease in *TWIST* expression as well. *TWIST* plays a big role in mesenchymal differentiation. The expression changes of *TWIST* affect EMT pathway activation and cells epithelial to mesenchymal transition. Upon these IPA data analysis results, we decided to further investigate the role of *STAT3* on the EMT pathway in the meningioma cell lines, especially its regulatory role on *TWIST* expression. We used a specific STAT3 inhibitor, Stattic, for this experiment. IC50 value of Stattic dose was determined by dose-response assay for each meningioma cell line (Appendices A.8).

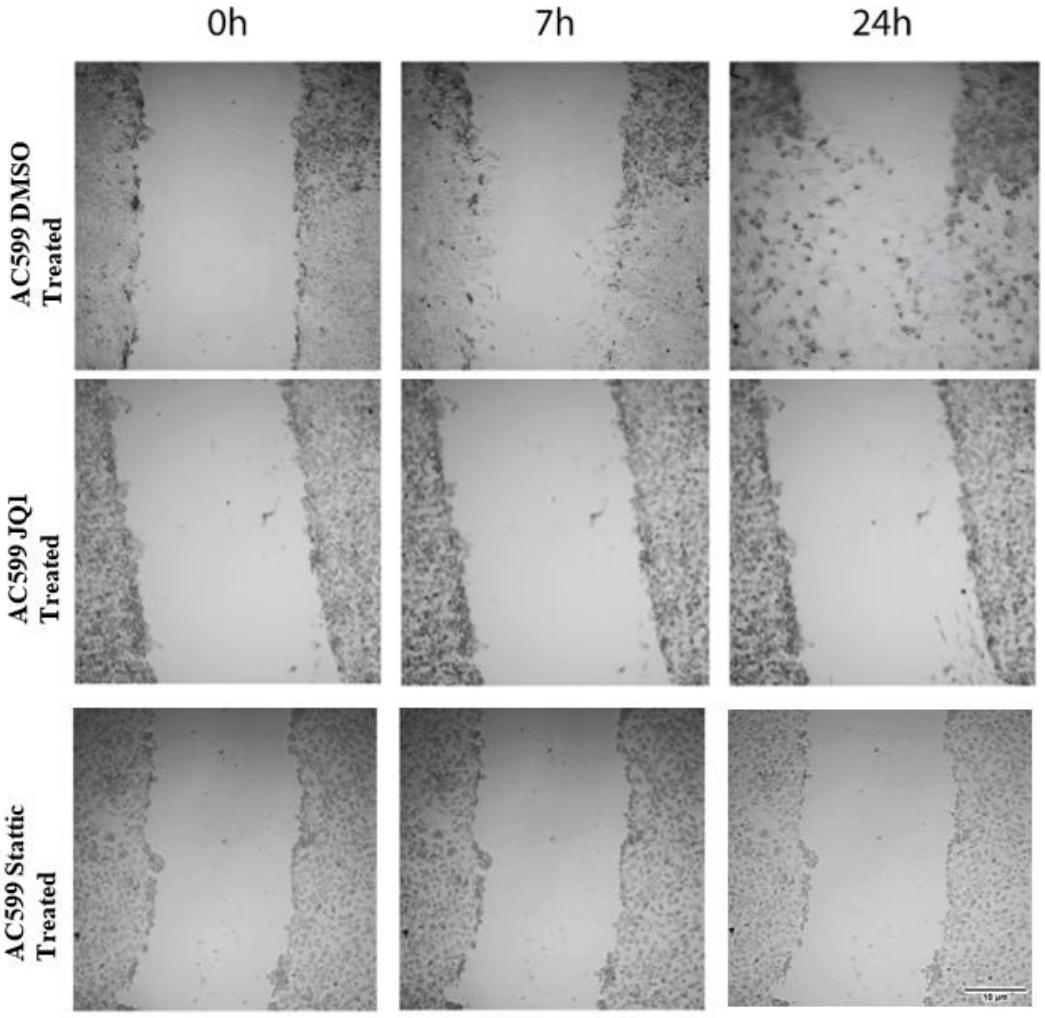
3.4 Drug Treatment Affects on Migration

When malignant and benign meningioma cell lines were compared in terms of morphological changes, the morphological changes seen after activation of the EMT

pathway are predominantly seen in cell lines with JQ1 resistance (AC559 JR) of the benign cell line AC599 (Figure 3.4). It is well known in the literature that there is a relationship between drug resistance and EMT. In cancer cells gaining drug resistance, EMT causes drug resistance by affecting other pathways instead of playing a role alone. It is known that EMT has an effect on metastasis along with its effect on drug resistance. After the cells acquire drug resistance, they also undergo morphological changes (Figure 3.1). On top of that, after proving that there was no change in proliferation in the cells, the increase and change in the metastatic properties of the cells were examined by in vitro wound healing assay. The scratch wound area was monitored by microscopy daily for 24 hours (Figures 3.4, 3.5, 3.6, and 3.7). Images were taken at 0h,7h, and 24h and analyzed with ImageJ. After 24 hours, the proportion of migrated cells increased in the AC599 DMSO-only controls. Wound healing assay images showed that JQ1 and Static treatment in AC599 inhibit cell proliferation and migration compared to the AC599 DMSO-only control group. These data are consistent with the literature (Bid et al., 2016, Guo et al., 2022).

It is well known that acquired chemoresistance can affect DNA, biochemical pathways, morphology, or behavior of the cells. One such well-known mechanism is epithelial to mesenchymal transition (EMT) which typically results in the transition of epithelial (E) cells to cells with a mesenchymal (M) phenotype, defined by prototypical markers, such as E-cadherin and Vimentin. When cells gained mesenchymal properties, they are more likely to migrate because they lose cell-cell contact which migratory potential of the cells. To better understand the role of EMT on meningioma cell migration we set up a wound healing assay to monitor migration in real-time. Furthermore, our RNAseq data already showed the importance of the *STAT* regulatory network in chemoresistance and it is well-known in the literature STAT family affects the EMT pathway, apoptosis, and cell proliferation. To test whether Stattic (*STAT* inhibitor) treatment affects the cell migration ability compared to the untreated control, we set up three groups (no treatment, JQ1 treatment, and Stattic treatment) and monitor the migration behavior in real time.

A.


0h
7h
24h
AC599 DMSO Treated
AC599 JQ1 Treated
AC599 Stattic Treated

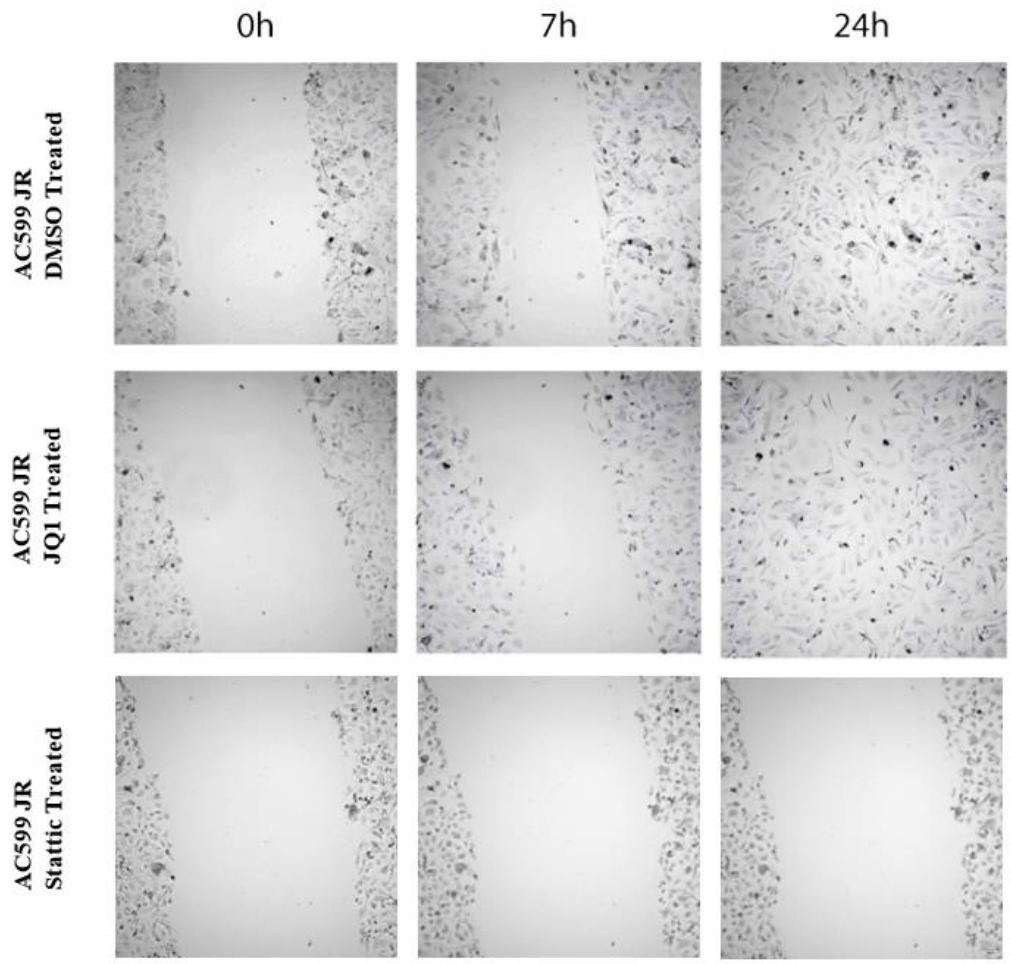
B.

	Scratch Area Change Based on Treatment Time (Scratched Area %)		
	AC599		Percent Change (Area JQ1 Treatment- DMSO Control)
	DMSO Control	JQ1 Treatment	
0 Hour	45.8	49.2	3.4
7 Hour	35.0	50.3	15.2
24 Hour	22.1	48.3	26.3
	AC499		Percent Change (Area Stattic Treatment- DMSO Control)
	DMSO Control	Stattic Treatment	
0 Hour	45.8	40.0	-5.8
7 Hour	35.0	40.4	5.4
24 Hour	22.1	41.1	19.0

Figure 3.4. Migration trace of AC599 after 24 hours DMSO control, JQ1, and Stattic treatment was analyzed with a fluorescent microscope and microscope chamber.

Scratch was done with a 200 µL pipet tip, then cells were washed with basal DMEM. IC50 value JQ1 and Static were added to cells. Images were taken at 0 hours, 7 hours, and 24 hours after scratch (A). The percentage change of the scratched area was shown in table (B).

A.


0h
7h
24h
AC599 JR
DMSO Treated
AC599 JR
JQ1 Treated
AC599 JR
Stattic Treated

B.

	Scratch Area Change Based on Treatment Time (Scratched Area %)		
	AC599		
	DMSO Control	JQ1 Treatment	Percent Change
0 Hour	48.8	58.5	9.7
7 Hour	33.5	44.4	10.9
24 Hour	5.9	8.9	3.0
	AC599		
	DMSO Control	Stattic Treatment	Percent Change
0 Hour	48.8	58.6	9.8
7 Hour	33.5	44.2	10.7
24 Hour	5.9	44.1	38.2

Figure 3.5. Migration trace of AC599 JR after 24 hours DMSO control, JQ1, and Stattic treatment was analyzed with a fluorescent microscope and microscope chamber.

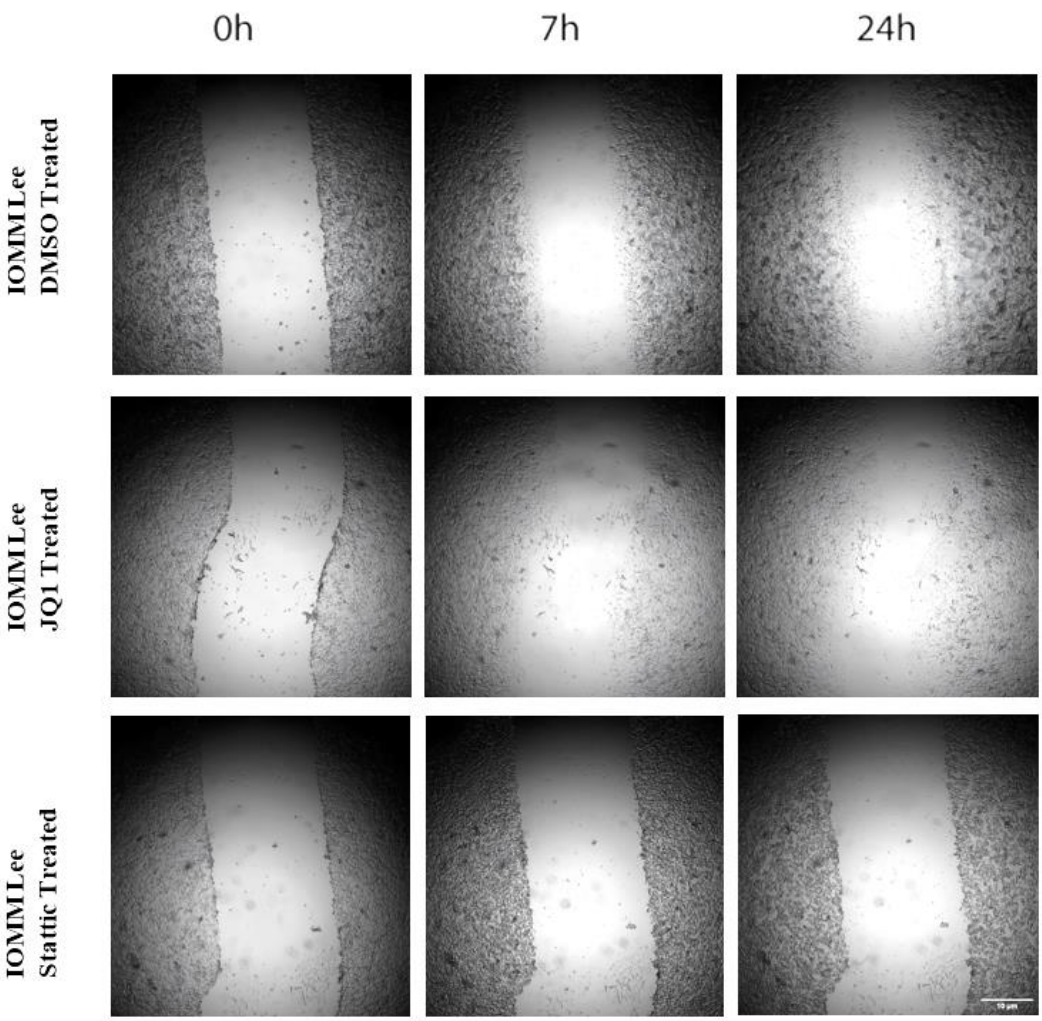
Scratch was done with a 200 µL pipet tip, then cells were washed with basal DMEM. IC50 value JQ1 and Stattic were added to cells. Images were taken at 0 hours, 7 hours, and 24 hours after scratch (A). The percentage change of the scratched area was shown in table (B).

STAT3 interacts with some EMT biomarkers and TFs, leading to the activation of the EMT pathway. Stattic is a drug that inhibits the interaction of STAT3 with other genes by inhibiting STAT3 phosphorylation. On top of that, with the inhibition of STAT3 by Stattic treatment, the migration of cells will also decrease.

The Non-resistant AC599 cell line does not appear to close the scratch wound and does not move. This immobility is seen not only in Stattic treatment but also in JQ1 treatment. In conclusion, Stattic and JQ1 flow did not affect the migration of cells in AC599 meningioma cell line that was JQ1 resistant (Figures 3.4 and 3.5). As it is well known in the literature, EMT is active in units with drug resistance and this activation is the right regulator with drug resistance (Singh & Settleman, 2010).

As malignancy increases and cells gain resistance to drugs in meningioma, the metastatic properties of the cells increase. However, there is a difference in migration rates between non-resistant and resistant cells. No matter how malignant or benign the cells are, they seem more prone to metastasis once they have become resistant to the drug. As seen in the wound healing assay, cells after 24 hours of DMSO control IOMM Lee JR and AC599 JR close the wound completely (Figures 3.5 and 3.7) In addition, when non-resistant IOMM Lee and AC599 meningioma cell lines were compared, non-resistant AC599 meningioma cell line closed part of the wound at the end of 24 hours. The non-resistant IOMM Lee barely moved and closed the wound. However, when JQ1 treatment was applied to non-resistant IOMM Lee meningioma cell line, the cells closed the wound almost completely. In non-resistant IOMM Lee meningioma cell line, it seems likely that JQ1 treatment induced the metastatic properties of the cells (Figure 3.6).

A.


0h
7h
24h
IOMM Lee DMSO Treated
IOMM Lee JQ1 Treated
IOMM Lee Stattic Treated

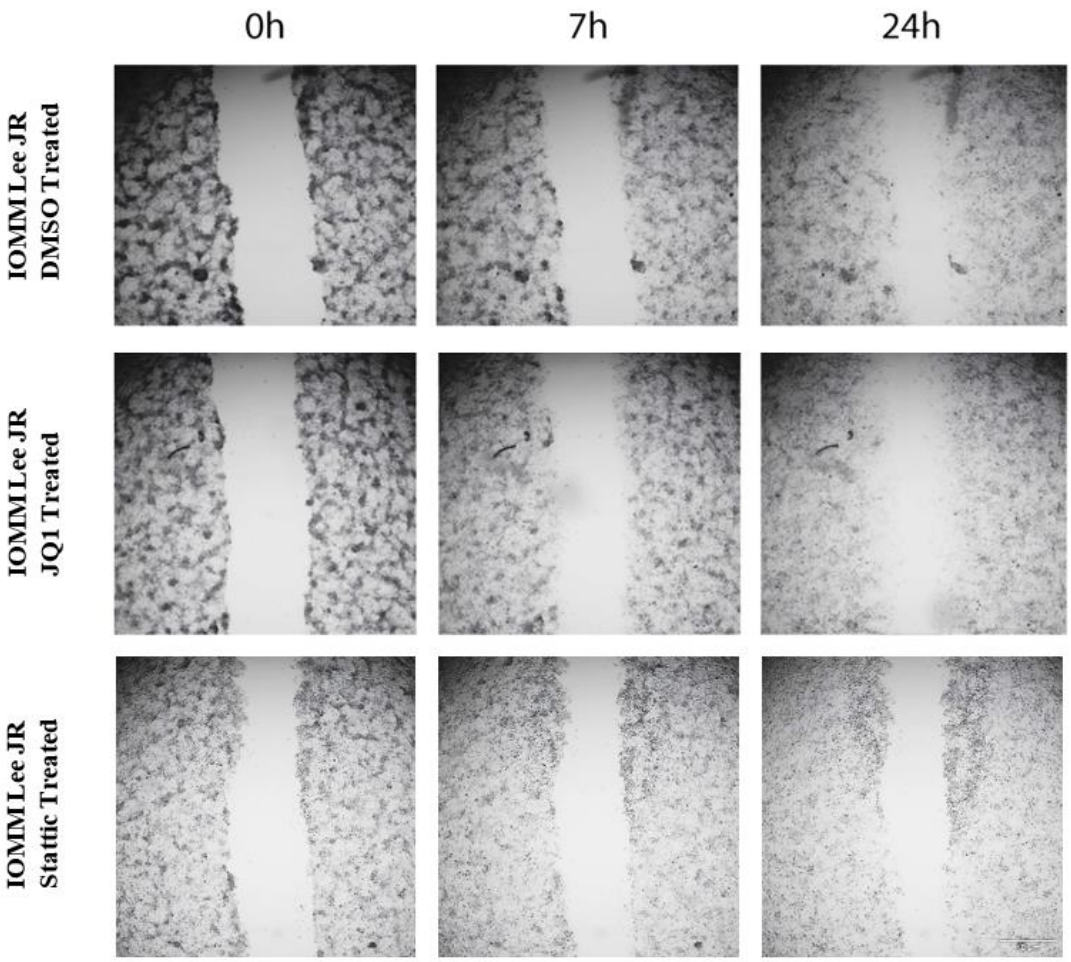
B.

	Scratch Area Change Based on Treatment Time (Scratched Area %)		
	IOMM Lee		
	DMSO Control	JQ1 Treatment	Percent Change
0 Hour	42.7	41.4	-1.3
7 Hour	37.3	38.3	1.0
24 Hour	24.0	35.5	11.4
	IOMM Lee		
	DMSO Control	Stattic Treatment	Percent Change
0 Hour	42.7	31.8	-10.9
7 Hour	37.3	31.8	-5.5
24 Hour	24.0	31.9	7.9

Figure 3.6. Migration trace of IOMM Lee after 24 hours DMSO control, JQ1, and Stattic treatment was analyzed with a fluorescent microscope and microscope chamber.

Scratch was done with a 200 µL pipet tip, then cells were washed with basal DMEM. IC50 value JQ1 and Stattic were added to cells. Images were taken at 0 hours, 7 hours, and 24 hours after scratch (A). The percentage change of the scratched area was shown in table (B).

A.


0h
7h
24h
IOMM Lee JR DMSO Treated
IOMM Lee JR JQ1 Treated
IOMM Lee JR Stattic Treated

B.

	Scratch Area Change Based on Treatment Time (Scratched Area %)		
	IOMM Lee JR		Percent Change (Area JQ1 Treatment- DMSO Control)
	DMSO Control	JQ1 Treatment	
0 Hour	24.2	22.9	-1.2
7 Hour	22.3	20.2	-2.1
24 Hour	24.0	16.9	-7.2
	IOMM Lee JR		Percent Change (Area Stattic Treatment- DMSO Control)
	DMSO Control	Stattic Treatment	
0 Hour	24.2	17.0	-7.2
7 Hour	22.3	15.4	-6.9
24 Hour	24.0	13.3	-10.8

Figure 3.7. Migration trace of IOMM Lee JR after 24 hours DMSO control, JQ1, and Stattic treatment was analyzed with a fluorescent microscope and microscope chamber.

Scratch was done with a 200 µL pipet tip, then cells were washed with basal DMEM. IC50 value JQ1 and Stattic were added to cells. Images were taken at 0 hours, 7 hours, and 24 hours after scratch (A). The percentage change of the scratched area was shown in table (B).

The tumoral characteristics of malignant cells are also increasing with drug resistance. Resistance to higher doses of drugs, more persistent enlargement, and metastatic features are more prone to more extensive metastasis. After 24 hours of DMSO, JQ1, and Stattic treatments were placed on IOMM Lee and IOMM Lee JR meningioma cell lines. In IOMM Lee JR meningioma cell line, the wounds approached nearly complete enlargement. After Stattic treatment in non-resistant IOMM Lee meningioma cell line, the wound is not closed by cells. IOMM Lee JR meningioma cell line has closed the wound partially after Static treatment. Based on this data JQ1 and Stattic treatments have effects on cell migration and proliferation.

Stattic and JQ1 treatments have been shown to affect cell cycle progression in different cancer cell lines. As mentioned previously, we aimed to see the impact of Stattic and JQ1 treatment on cell cycle progression, apoptosis, and EMT. Our EMT and migration experiments were mainly based on the motion movement of the cells. Therefore, we monitored the cell cycle progression upon treatment of the JQ1 and Stattic on parental and resistant cell lines (IOMM Lee, AC599 and IOMM Lee JR, and AC599 JR).

3.5 Effects of Stattic and JQ1 Treatment on Cell Cycle Progression

Whether the expression changes in EMT markers observed in the analysis of RNA-seq had any effect on the cells' migration characteristics had to be analyzed. Therefore, a Wound Healing assay was performed to examine the cellular migration characteristics of meningioma cell lines. Whether this wound healing data solely resulted from cellular migration we then checked the cell cycle status of these cells before and after drug treatments.

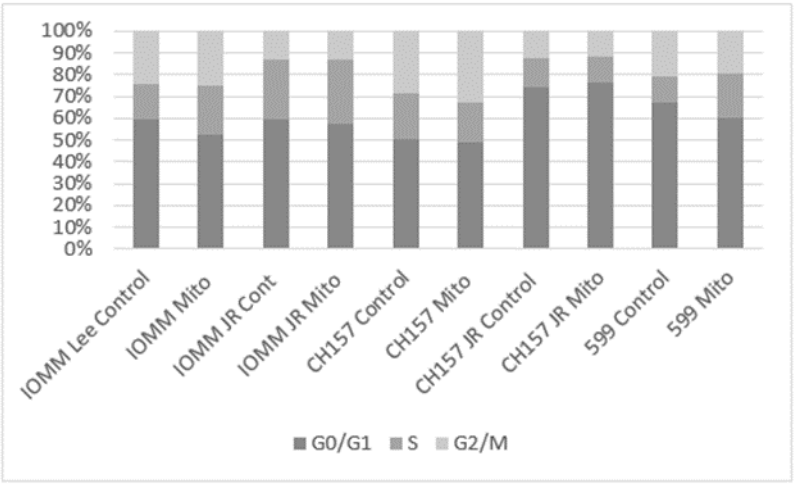


Figure 3.8. Cells were treated with Mitomycin for 2 hours to achieve cell cycle synchronization before the wound healing assay.

Then washed with basal medium (DMEM). Cells were treated with DMSO, JQ1, and Stattic for 24 hours. Cells were collected and stained with PI after fixation. BD Accuri C6 Software (BD Biosciences, USA) was used to generate cell cycle distribution histograms. Cell cycle phase ratios are analyzed with BD Accuri C6 Software.

It cannot be stated that movement is just due to cellular migration movement if JQ1 and Stattic treatments increase cell proliferation. To synchronize cells in the cell cycle for wound healing assay, we first used Mitomycin C, which is commonly used in cell cycle synchronization and cancer treatment (Figure 3.8). The reason why Mitomycin treatment was applied only before JQ1 and Stattic treatment is that the long-term treatment with Mitomycin has a toxic effect on cells. Cells were treated with Mitomycin C for two hours for cell cycle synchronization so that their migration could be observed. Afterward, either Stattic or JQ1 drug treatments were applied to cells at their corresponding IC50 value for 24 hours. To understand the effects of these drugs on the cell cycles of IOMM Lee, IOMM Lee JR, AC599, and AC599 JR meningioma cell lines, the cell cycle distributions were examined using flow cytometry after they were stained with propidium iodide (PI). BD Accuri C6 Software (BD Biosciences, USA) was used to generate cell cycle distribution

histograms, and the appropriate gate was applied to identify cell types and exclude duplicate cells (Figure 3.9).

While the results of Mitomycin C treatment were expected to be synchronized, the ratios of G1/M/G2 stages in Mitomycin C treated non-resistant and resistant IOMM Lee and AC599 meningioma cell line turned out to be the same as the G1/M/G2 ratios of control non-resistant and resistant IOMM Lee and AC599 versions (Figure 3.10 B and C). In other words, Mitomycin C treatment did not show the expected synchronization effect on meningioma cells used in the experiments. Consequently, the cells were prepared once more without receiving Mitomycin treatment and were subjected to only JQ1 and Stattic treatments.

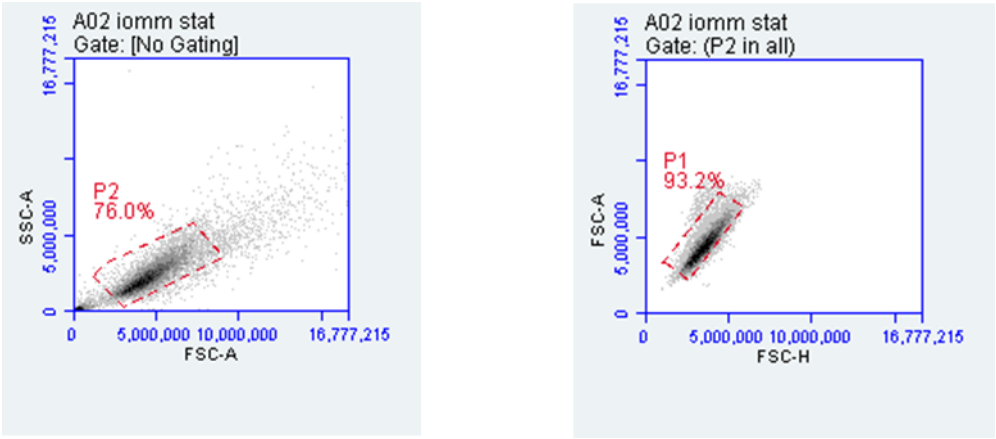


Figure 3.9. The effects of Stattic and JQ1 treatment on cell cycle in non-resistant and JQ1 Resistant meningioma cells were detected by flow cytometry assay.

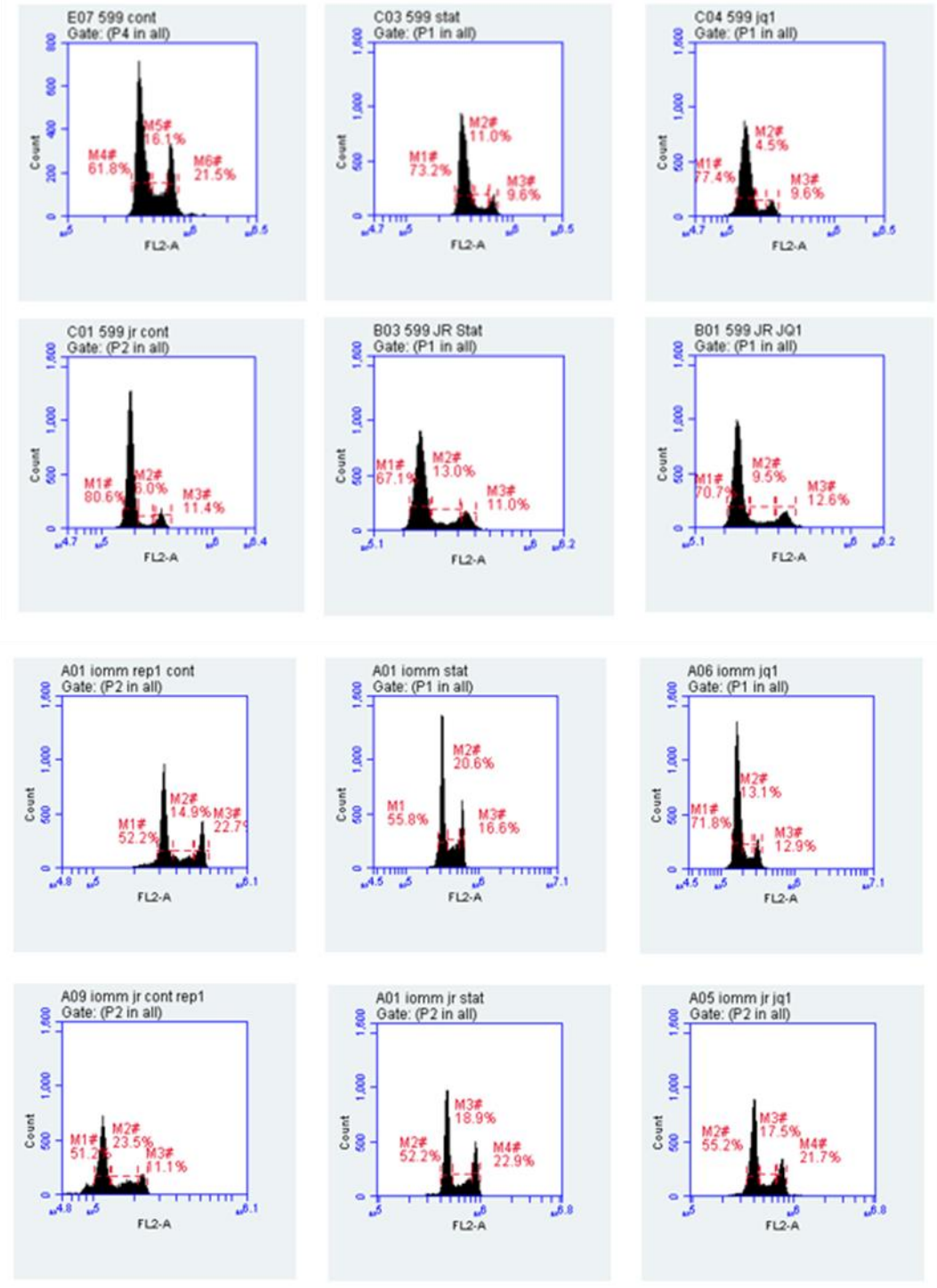
In the scientific literature, it is asserted that Stattic and JQ1 treatments affect cell cycle progression in different cancer cell lines.

This experiment aimed to see/analyze the effect of Stattic and JQ1 treatment on cell cycle progression, migration, and EMT. EMT and migration assay are mainly based on the metastasis movement of cells. Therefore, the cell cycle progression effect of drugs on non-resistant and resistant IOMM Lee and AC599 cell lines was observed (Figure 3.10). Our cell cycle data showed that JQ1 and Stattic drug treatments did not increase proliferation in the meningioma cell lines tested.

However, according to the data obtained after a 24 hours treatment with Stattic and JQ1, no significant changes were observed in cell cycle progression at all cell cycle phases in non-resistant and resistant IOMM Lee cell lines. Their G0/G1, S, and G2/M phase ratios remained the same. However, an increase was observed in the G1 phase of the resistant and non-resistant cells that received JQ1 treatment (Figure 3.10 B), which indicates that cells generally change position by movement, not by division, during migration assay. Compared to malignant cells (IOMM Lee), benign AC599 meningioma cell lines were arrested at the end of G0/G1 and the initial phases of S (Figure 3.10 C).

However, the G2/M phase ratio decreased significantly. In AC599 JR meningioma cell line treated with Stattic and JQ1 treatment, when the control AC599 JR graph was examined, the G1 ratio was around 80%, while this ratio decreased to 70% after the treatment. However, since the G1 ratio of AC599 meningioma cell line was higher than that of IOMM Lee meningioma cell line, it is thought that most of the 80% of cells can move forward without dividing. Briefly, these treatments did not show a significant effect on the cell cycle progression of malignant cell line. However, they greatly influence the cell cycle progression of the benign cell line. Next, we aim to immunostain and perform western blotting with cell cycle and EMT markers before and after treatment to better understand how STAT inhibition affects the signaling pathways.

A.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M4# 61.8%
M5# 16.1%
M6# 21.5%
C03 599 stat
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M2# 11.0%
M3# 9.6%
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M1# 77.4%
M2# 4.5%
M3# 9.6%
C01 599 jr cont
Gate: (P2 in all)
M1# 80.6%
M2# 6.0%
M3# 11.4%
B03 599 JR Stat
Gate: (P1 in all)
M1# 67.1%
M2# 13.0%
M3# 11.0%
B01 599 JR JQ1
Gate: (P1 in all)
M1# 70.7%
M2# 9.5%
M3# 12.6%
A01 iomm rep1 cont
Gate: (P2 in all)
M1# 52.2%
M2# 14.9%
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Gate: (P1 in all)
M1 55.8%
M2# 20.6%
M3# 16.6%
A06 iomm jq1
Gate: (P1 in all)
M1# 71.8%
M2# 13.1%
M3# 12.9%
A09 iomm jr cont rep1
Gate: (P2 in all)
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M2# 23.5%
M3# 11.1%
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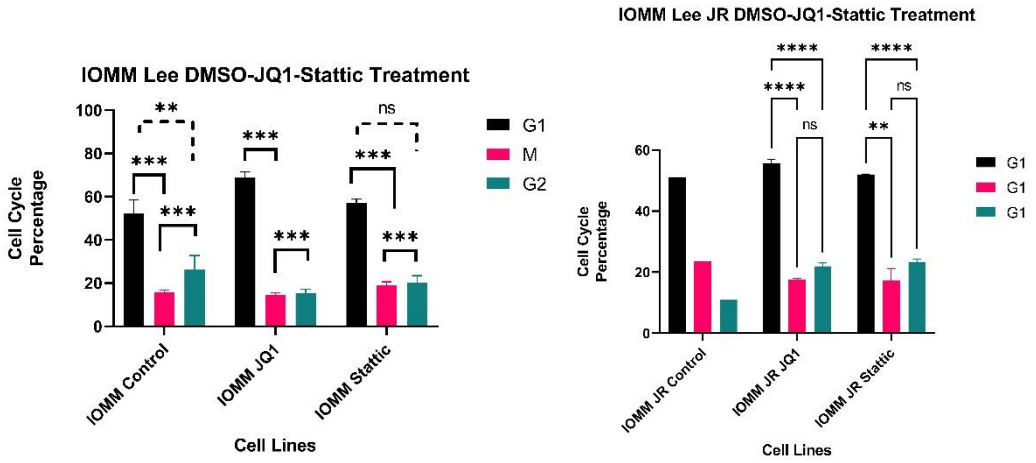
Gate: (P2 in all)
M2# 52.2%
M3# 18.9%
M4# 22.9%
A05 iomm jr jq1
Gate: (P2 in all)
M2# 55.2%
M3# 17.5%
M4# 21.7%
Count
FL2-A

B.



C.

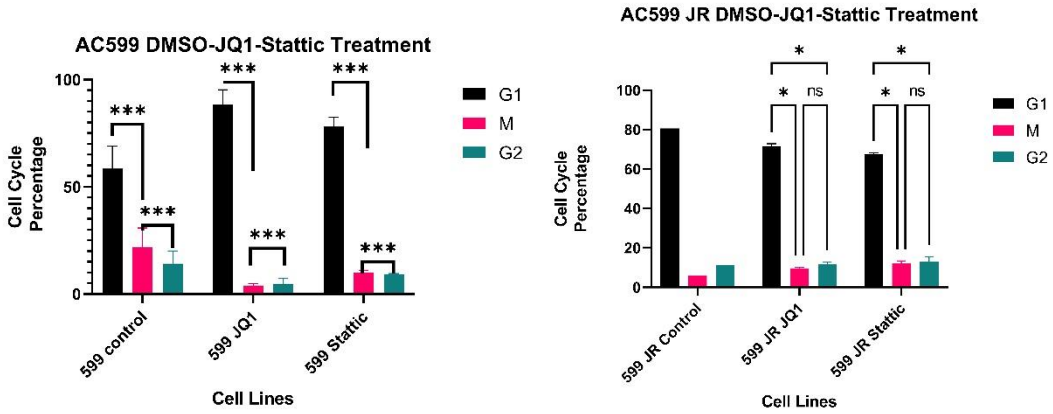


Figure 3.10. Cell cycle progression of IOMM Lee, AC599, IOMM Lee JR, and AC599 JR after 24 hours Stattic and JQ1 treatment.

BD Accuri C6 Software was used for cell cycle progression (A). Graphs show the comparison of drug treatment (JQ1 or Stattic) and DMSO control cells cell cycle phase ratio (%) of IOMM Lee and IOMM Lee JR (B). Graphs show the comparison of drug treatment (JQ1 and Stattic) and DMSO control cells cell cycle phase ratio (%) of AC599 and AC599 JR (C). Cell cycle data are presented as bar graphs. Black column G1; Red column M; Green column G2.

3.6 EMT Marker Expression

CDH1, *N-cadherin*, and *Vimentin* are the markers that show the most changes and the activation of EMT. EMT, activated by decreased *CDH1* expression and increased *N-cadherin* expression, plays an important role in tumor development, growth, and metastasis. There is an important link between the classical WNT signaling pathway and EMT. It has been shown in the literature that the subcellular localization of β–Catenin is critical for the EMT mechanism (Rutkowski et. al. 2018).

Expressions of EMT markers were examined on the change in migration of cells in the 24 hours wound healing assay. After scratch and 24 hours drug treatment, cells were fixed with immunofluorescence assay and stained with EMT marker antibodies. In parallel with the wound healing assay, CDH1, N-cadherin, Vimentin, and β–Catenin protein translations were examined in the cells to understand what kind of expression change occurred in the migrating cells. DMSO, JQ1, and Stattic-treated cells were harvested every 0 hours, 7 hours, and 24 hours. A small amount of β–Catenin and E-cadherin protein translations was seen in non-resistant IOMM Lee meningioma cell line (Figure 3.11). β-catenin was also seen to be concentrated around the cell nucleus. After 7 and 24 hours, an increase in Vimentin and N-cadherin protein translations are seen in both JQ1 and Stattic treatments (Appendices A.10). CDH1 protein translations, on the other hand, do not appear to be significant (Figure 3.11 and Appendices A.10). Since Vimentin and N-cadherin are the main EMT markers, it was seen that the EMT pathway was activated at the end of 24 hours and the cells gain mesenchymal characteristics. However, an increase in

mesenchymal markers is observed in cells close to the scratch part, not in every cell, and this is called partial EMT.

A.

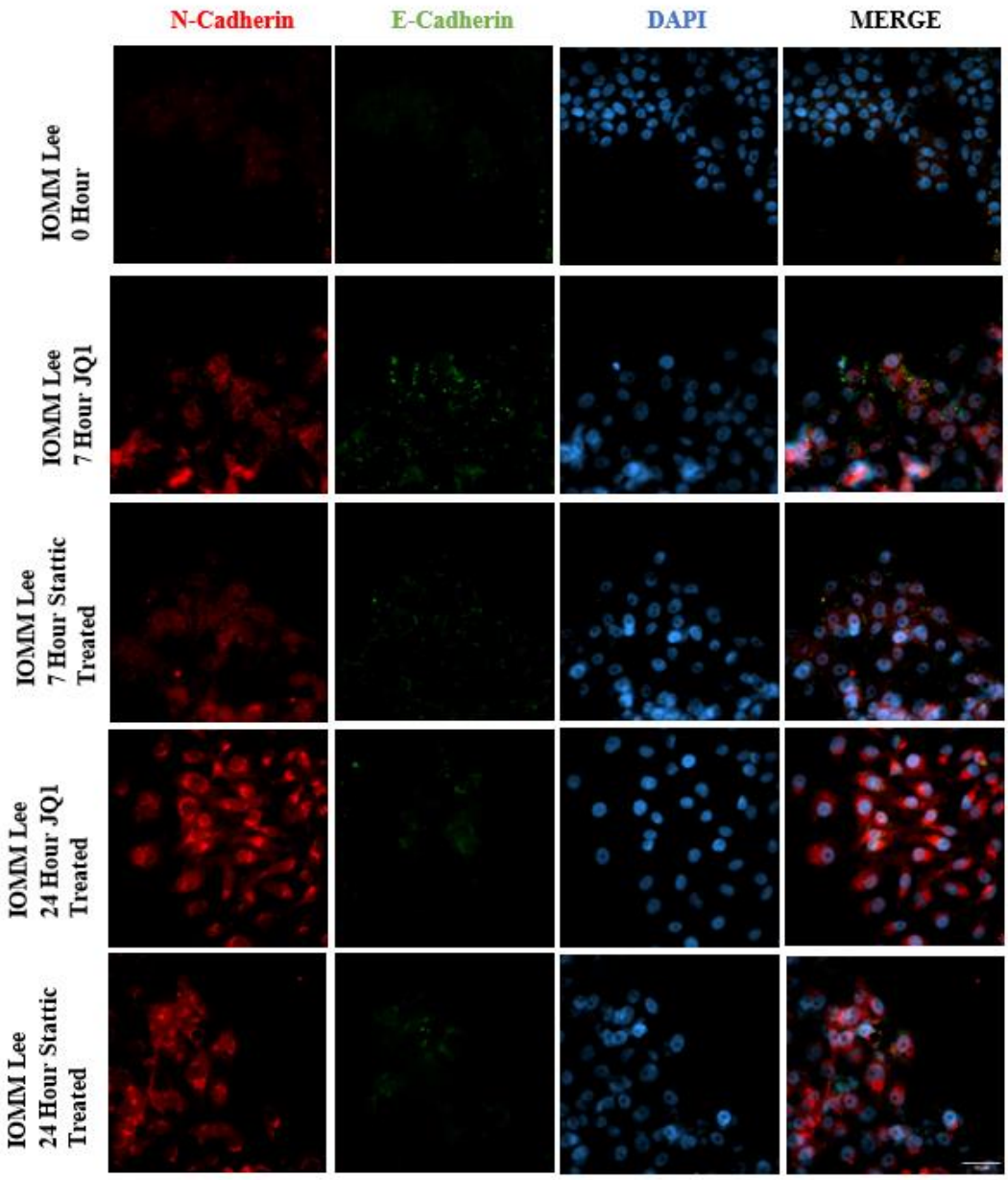


Figure 3.11. IOMM Lee meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

B.

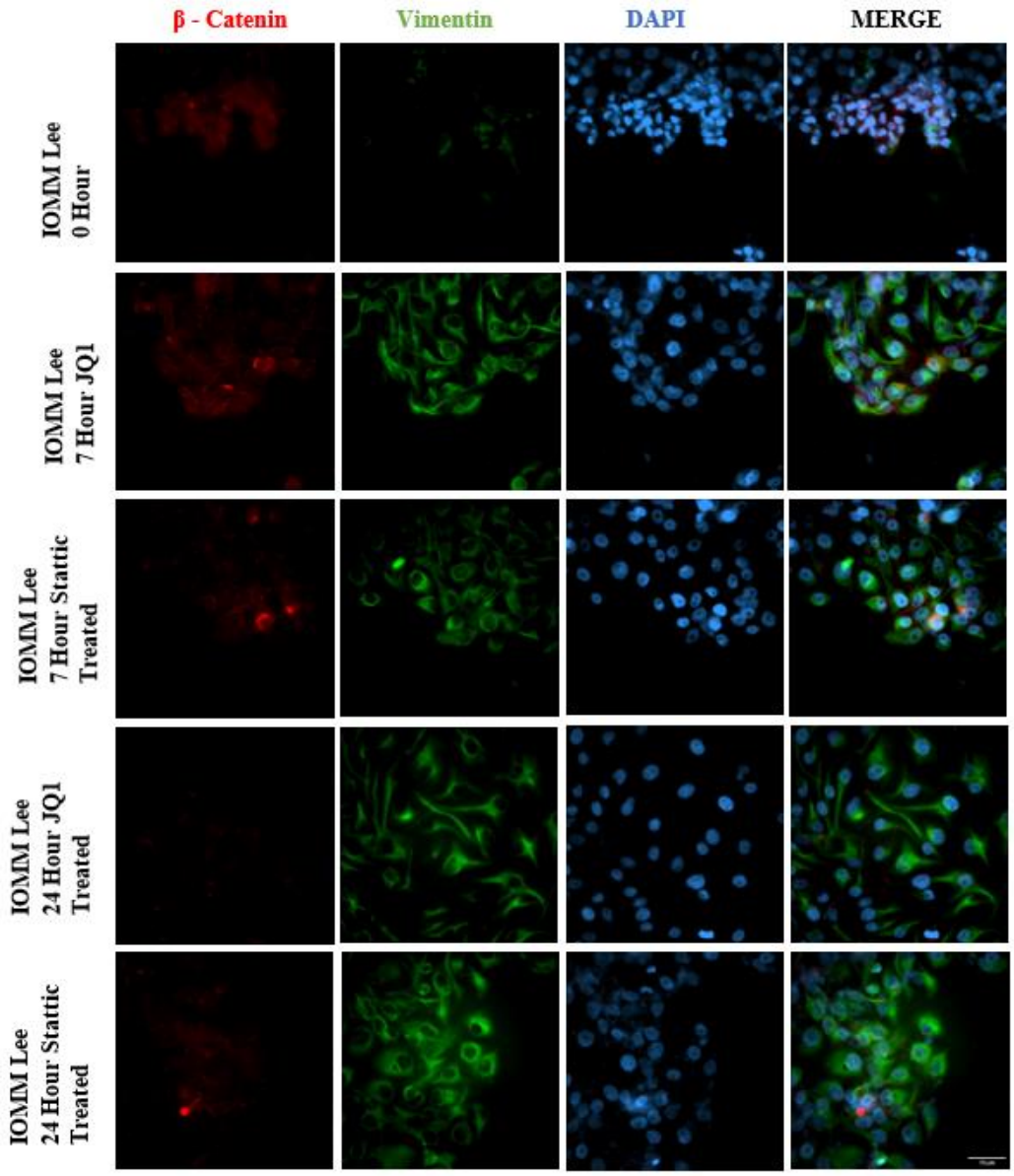


Figure 3.11. IOMM Lee meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

Cells were collected after 0 hours, 7 hours, and 24 hours. After collection cells were fixed with paraformaldehyde. When the fixation period was finished, cells were stained with antibodies. A. Cells stained with N-cadherin and CDH1. Vectashield with DAPI was used to stain the nucleus. B. Cells stained with β–Catenin and Vimentin Vectashield with DAPI were used to stain the nucleus. Immunofluorescent images were obtained using a fluorescent microscope in 40x and 60x magnified objectives.

A.

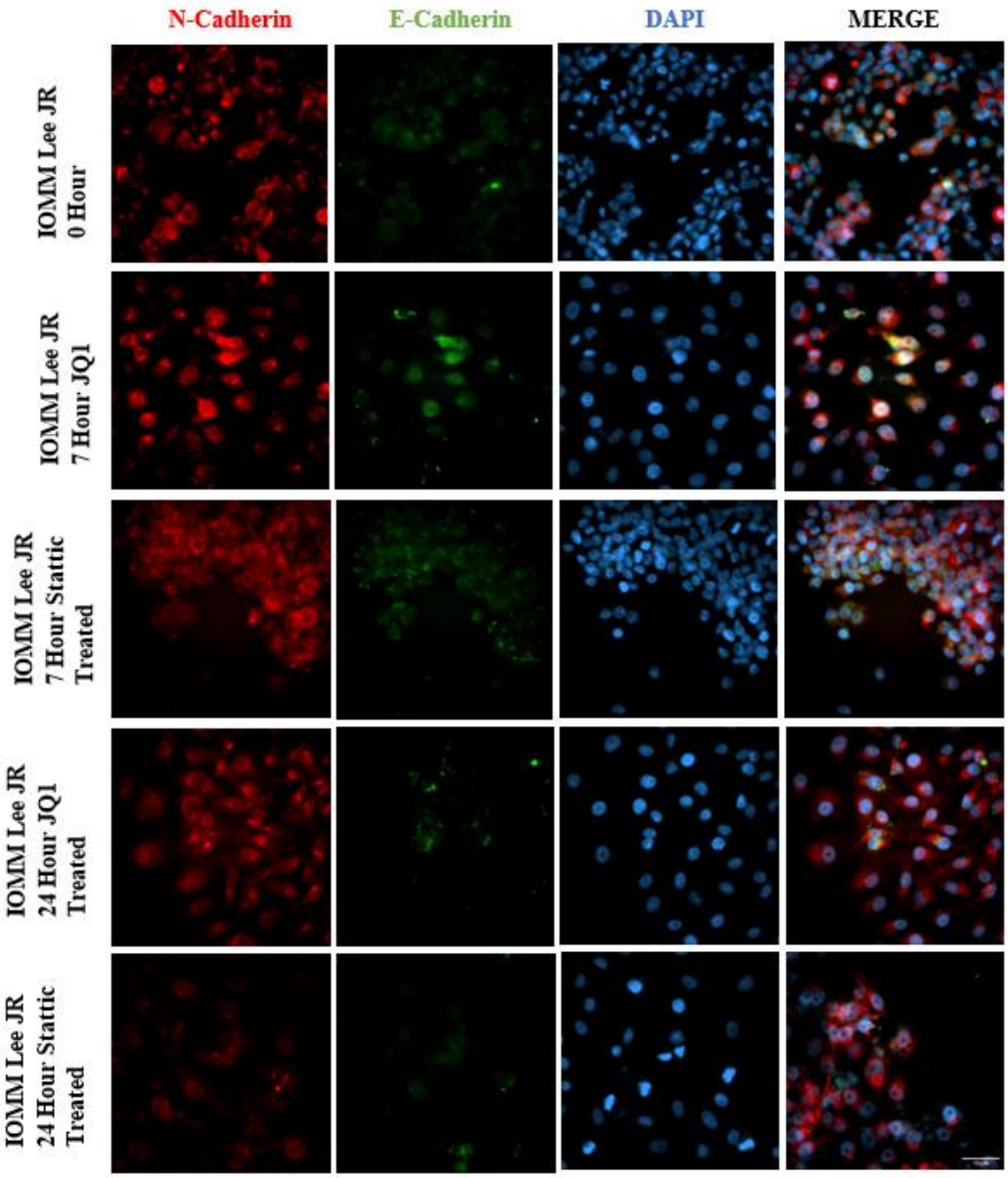


Figure 3.12. IOMM Lee JR meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

B.

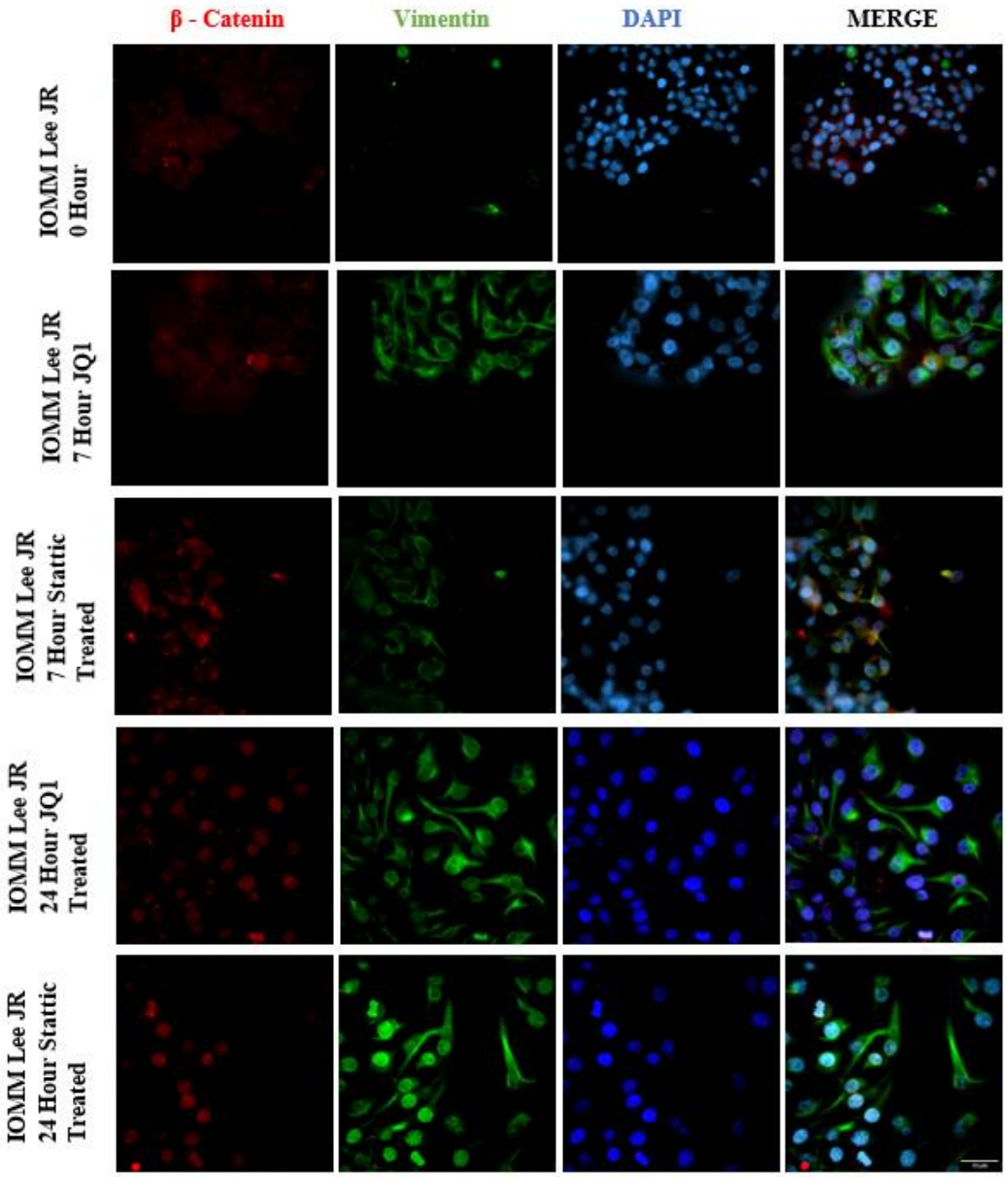


Figure 3.12. IOMM Lee JR meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

Cells were collected after 0 hours, 7 hours, and 24 hours. After collection cells were fixed with paraformaldehyde. When the fixation period was finished, cells were stained with antibodies. A. Cells stained with N-cadherin and CDH1. Vectashield with DAPI was used to stain the nucleus. B. Cells stained with β–Catenin and Vimentin Vectashield with DAPI were used to stain the nucleus. Immunofluorescent images were obtained using a fluorescent microscope in 40x and 60x magnified objectives.

When we looked at JQ1 Resistant IOMM Lee meningioma cell line, it is seen that the cells express N-cadherin and CDH1 at 0 hours (Appendices A.11). However, another mesenchymal marker, Vimentin protein translation, is not seen at 0 hours in the immunofluorescence assay. After 7 hours, CDH1 protein translation is seen in both JQ1 and Stattic-treated IOMM Lee JR meningioma cell lines (Figure 3.12). However, after 24 hours, CDH1 expression decrease by 12.8% after JQ1 treatment. For 24 hours, N-cadherin is protein translations in both JQ1 and Stattic-treated cells but the expression decreases in Stattic treatment (Figure 3.12 and Appendices A.11). Vimentin, another mesenchymal marker, has an increased expression in this process (Appendices A.11). In IOMM Lee JR meningioma cell line, β–Catenin protein translations at 0 hours is seen intensely around the cell nucleus. After 7 hours of JQ1 and Stattic treatment, expression of β–Catenin is seen both around and inside the cell nucleus (Figure 3.12). At the end of 24 hours, after both drug treatments, β-catenin expression is predominantly seen in the cell nucleus and increases by around 15%. According to the literature, non-phosphorylated β–Catenin enters the nucleus and increases the expression of TFs, which plays a role in cell metastasis and development (Rutkowski et al, 2018). As we know from the study, the cellular location of β–Catenin activates the EMT pathway. Thereupon, after drug resistance in IOMM Lee meningioma cell line, the WNT pathway may have been activated and may have induced the EMT mechanism.

A.

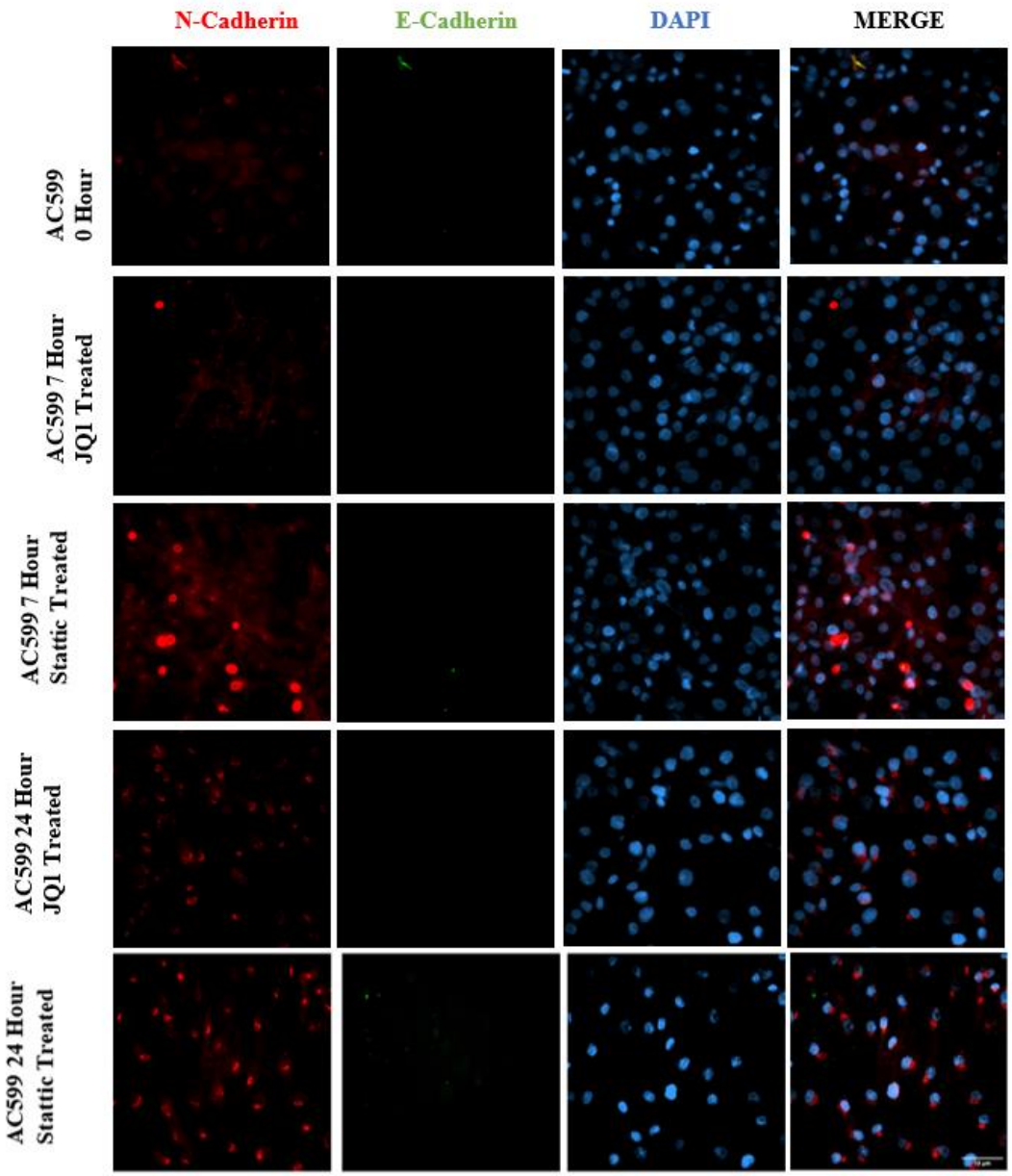


Figure 3.13. AC599 meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

B.

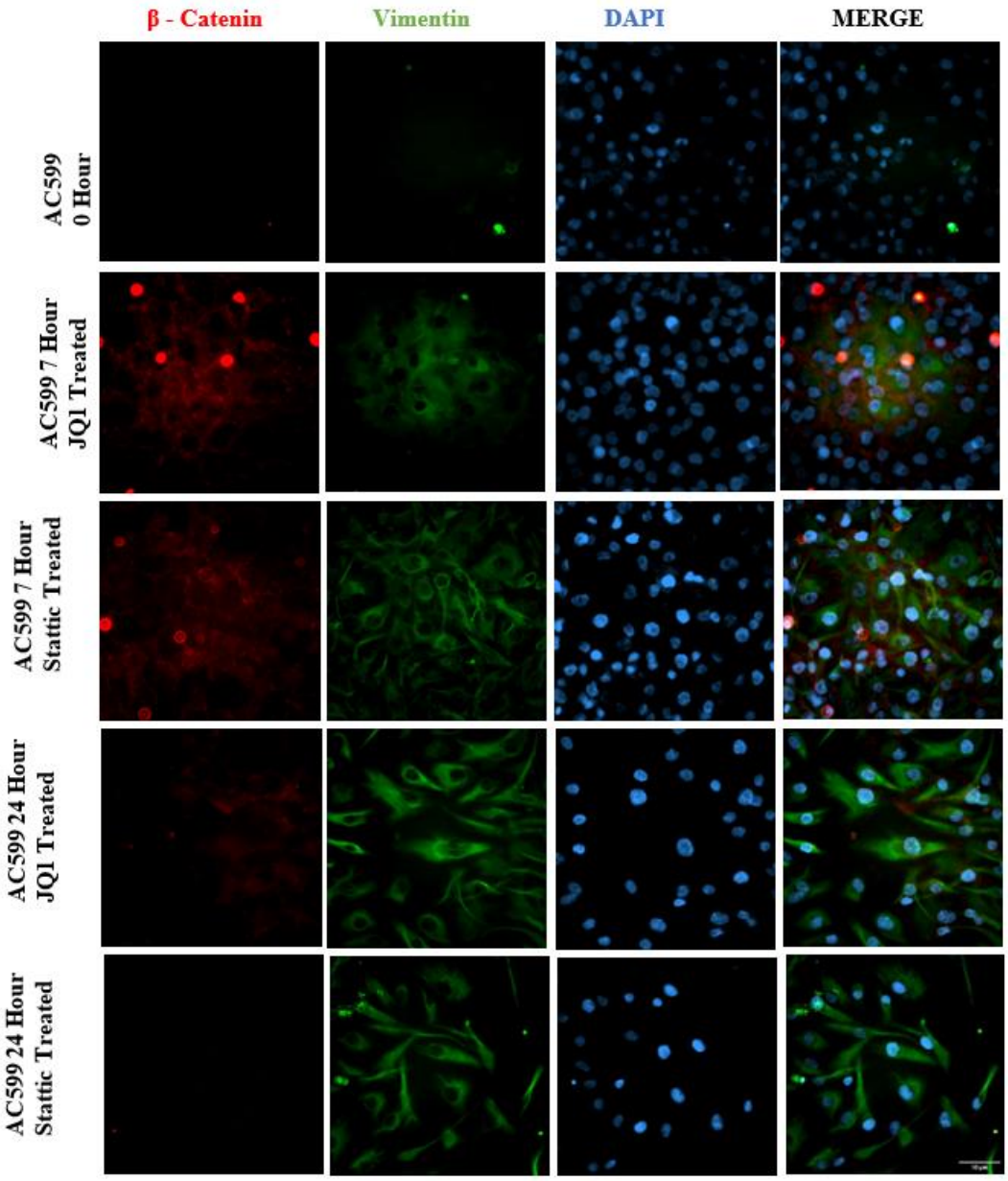


Figure 3.13. AC599 meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

Cells were collected after 0 hours, 7 hours, and 24 hours. After collection cells were fixed with paraformaldehyde. When the fixation period was finished, cells were stained with antibodies. A. Cells stained with N-cadherin and CDH1. Vectashield with DAPI was used to stain the nucleus. B. Cells stained with β–Catenin and Vimentin Vectashield with DAPI were used to stain the nucleus. Immunofluorescent images were obtained using a fluorescent microscope in 40x and 60x magnified objectives.

The metastasis characteristics of malignant and benign cancer cells are completely different. Because malignant cells are more aggressive, they are more prone to metastasis. Benign cells, on the other hand, have fewer metastatic properties. However, in meningioma, there is a group of benign meningioma cells that show metastatic properties. Our data showed that AC599 meningioma cell line migrated more in DMSO control during the wound healing assay, and after treatment with drugs, the migration rate decreased and there was almost no migration (Figure 3.4). Thereupon, EMT markers were examined with the immunofluorescence assay (Figure 3.13). When we compared the expressions of CDH1 and N-cadherin, we did not detect any CDH1 staining however some cells exhibit positive N-cadherin staining in JQ1 treated AC599 meningioma cell line (Figure 3.13 and Appendices A.12). This reveals the possibility of cells exhibiting partial EMT characteristics. Vimentin expression was not seen in the very first hours, in DMSO control (Figure 3.13). When the cells were treated with either JQ1 or Stattic drug, after 24 hours, there is almost a 30% increase in the protein translations of Vimentin (Figure 3.13). However, we did not see Vimentin protein translations in every cell suggesting that cellular heterogeneity exists within the cell lines tested. With the increase in Vimentin protein translations, there was an increase in β–Catenin expression which was mostly located in the cell cytoplasm in JQ1-treated AC599 meningioma cell line (Appendices A.12.B). At the end of 24 hours of Stattic treatment, the protein translations of β–Catenin in the cells decreased and it was almost undetectable (Figure 3.13). It was known that β–Catenin accumulated in the cytoplasm was targeted by the degradation complex upon which was phosphorylated and destroyed.

As seen after 7 hours of treatment, β–Catenin accumulated in the cell cytoplasm (Figure 3.13). As a result of this accumulation, it may have been destroyed because it could not pass into the cell nucleus (Figure 3.13). In conclusion, the WNT pathway might not be the only major pathway that controls the EMT pathway in AC599 meningioma cells.

A.

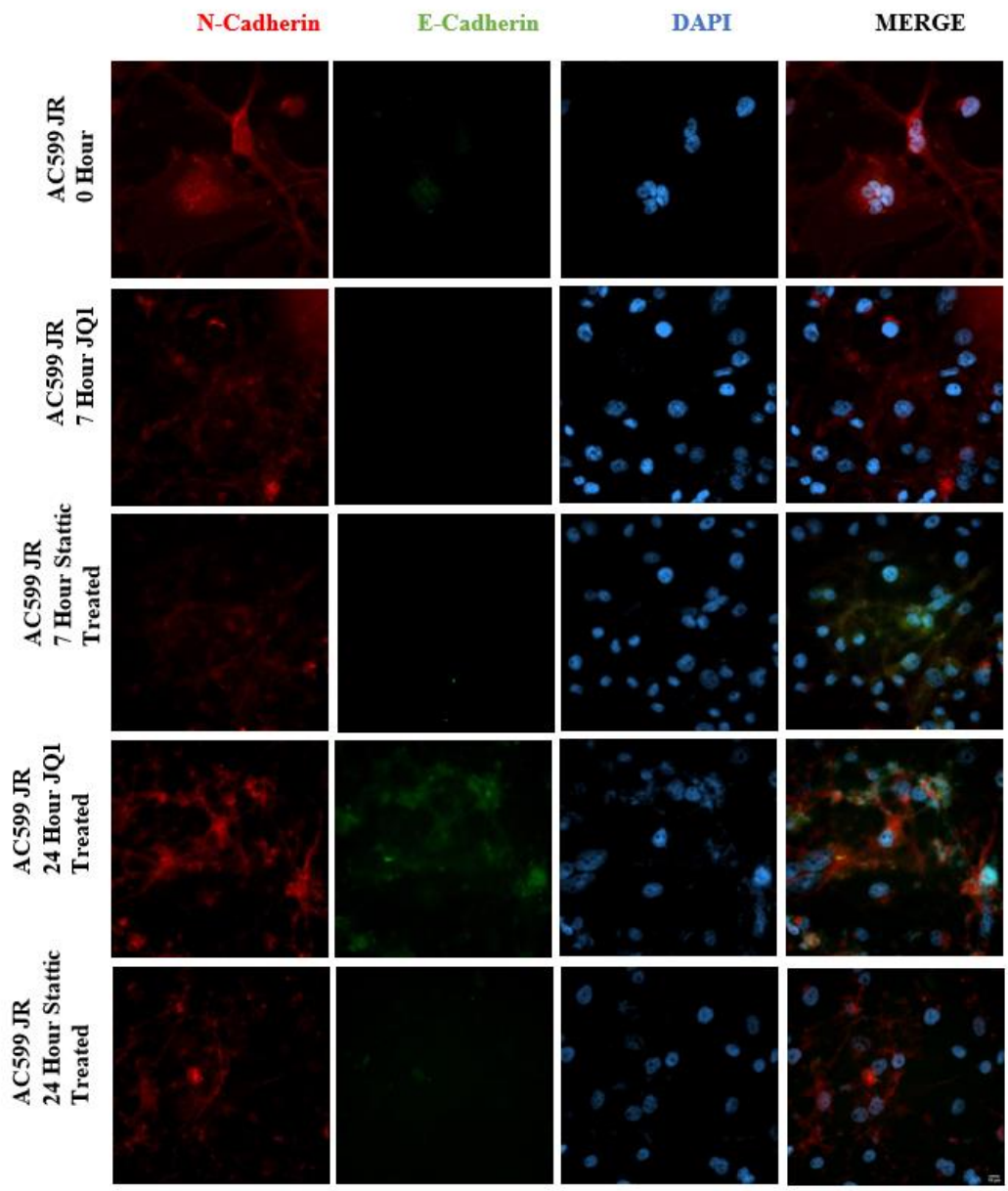


Figure 3.14. AC599 JR meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

B.

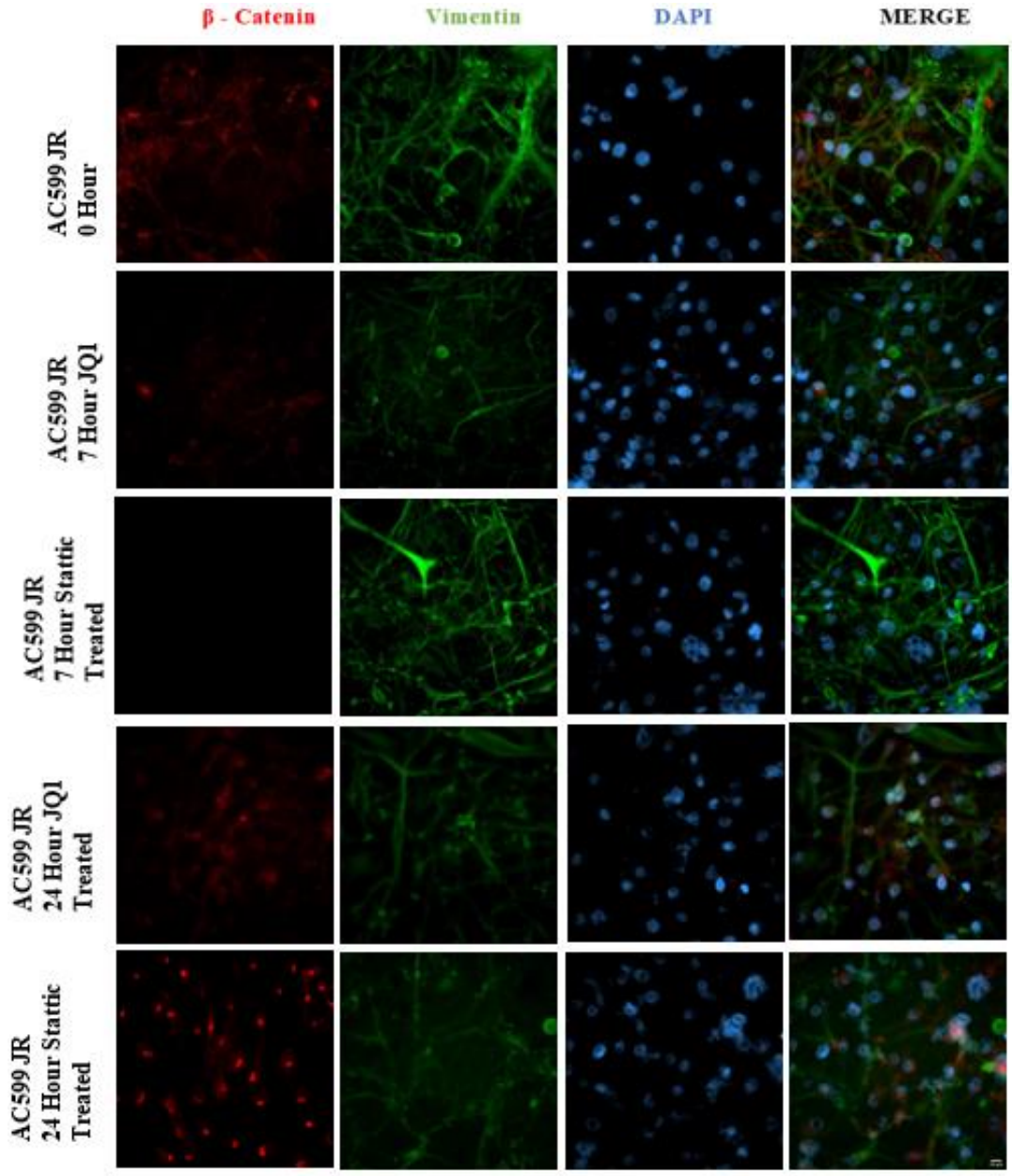


Figure 3.14. AC599 JR meningioma cell line was scratched with 200 µl pipet tip, then JQ1 and Stattic with IC50 value of drugs.

Cells were collected after 0 hours, 7 hours, and 24 hours. After collection cells were fixed with paraformaldehyde. When the fixation period was finished, cells were stained with antibodies. A. Cells stained with N-cadherin and CDH1. Vectashield with DAPI was used to stain the nucleus. B. Cells stained with β–Catenin and Vimentin Vectashield with DAPI were used to stain the nucleus. Immunofluorescent images were obtained using a fluorescent microscope in 40x and 60x magnified objectives.

The AC599 JR meningioma cell line has longer, and thinner morphology than non-resistant AC599 meningioma cell line. An increase in mesenchymal biomarkers expression could be the reason for these changes in the morphology of the cell. While N-cadherin is a mesenchymal marker, its expression increases 43.5 times in AC599 JR meningioma cell line (Figure 3.14.A and Appendices A.13). The AC599 meningioma cell line gain mesenchymal properties the E-cadherin expression decreases. The protein translation of the E-cadherin (9.8%) is almost none in AC599 JR meningioma cell line. However, after 24 hours of JQ1 treatment in AC599 JR meningioma cell line decrease was seen in CDH1 protein level (Figure 3.14.A and Appendices A.13.B). When compares with the Stattic treatment the protein translation of CDH1 was not seen in the cells. This showed that Stattic treatment affects epithelial markers protein translation or even gene expression. The N-cadherin proteins were located in the cytoplasm of AC599 JR meningioma cell line (Figure 3.14.A). After JQ1 treatment the N-cadherin protein translation increased in AC599 JR meningioma cell line compared to Stattic treatment AC599 JR meningioma cell line (Appendices A.13). The adherent protein in the cytoplasm was changed to mesenchymal proteins in AC599 JR meningioma cell line. Vimentin expression is mostly seen in mesenchymal cells' cytoplasm. Similarly, we observed that in non-resistant AC599 meningioma cell line, Vimentin expression was higher compared to AC599 JR meningioma cell line based on our immunofluorescence assay (Figure 3.14.B and Appendices A.12 - A.13). Our RNA-Seq data analysis also confirms that the Vimentin expression increase 2 fold in AC599 JR cell line compared to non-resistant AC599 meningioma cell line (Figure 3.3). Also, there is

an increase in β–Catenin expression in AC599 JR meningioma cell line compared to the non-resistant AC599 meningioma cell line (Figure 3.3). Our imaging data showed that β–Catenin localized mostly within and/or around the nucleus (Figure 3.14). The subcellular localization of β–Catenin is crucial for its role as a transcription factor. When β–Catenin is localized in the nucleus it enhances EMT TFs transcription. This data shows that AC599 JR meningioma cell line have a higher potential to activate the EMT pathway regulated by β–Catenin (Figure 3.14). To sum up, JQ1 resistance in AC599 meningioma cell line induces mesenchymal properties and EMT markers expression. The benign AC599 meningioma cell line are capable of metastasis after gaining drug resistance as it induces activation of the EMT pathway.

3.7 EMT Transcription Factors Relative Gene Expressions

As known in the literature *CDH1, N-cadherin* and *Vimentin* are not the sole markers of the EMT pathway. Other biomarkers, pathways, or TFs contribute to the activation of the EMT pathway. After showing CDH1, N-cadherin and Vimentin protein expression changes with the immunofluorescence assay, we then decided to look at some EMT TFs expression differences with 7 hour JQ1 and Stattic treatment after wound healing assay.

Numerous cellular pathways are regulated as a result of changes in the expression of key transcription factors. Various such transcription factors were identified for their role in EMT activation. The most significant among these TFs are *SNAIL, SLUG, TWIST*, and *β-catenin*. In addition to being the most significant EMT inducer, *SNAIL* is also crucial for cell survival, immunological control, and stem cell biology (Wu & Zhou, 2010). The earliest and most significant transcriptional repressor of *CDH1* was revealed to be *SNAIL*. Additionally, *SNAIL*'s most fundamental function in the EMT process is to inhibit the *CDH1* gene expression (Martin et al., 2005).

To understand the EMT pathway mechanism in our cell assay groıups, EMT TFs expressions were checked with qRT-PCR assay. *SNAIL* gene expression was significantly increased in AC599 JR cell line after 7 hours of JQ1 and Stattic drug treatment (Figure 3.15). When JQ1 and Stattic treatment of AC599 JR meningioma cell line is compared, there is a significant increase in *SNAIL* expression after JQ1 treatment. Although there is a significant difference between non-resistant AC599 JQ1 treated cells and AC599 JR JQ1 treated cells (Figure 3.15). While there is an increase in *SNAIL* expression in AC599 JR after JQ1 treatment, there is a significant decrease in non-resistant AC599 meningioma cell line after JQ1 treatment (Figure 3.15). In addition to this, there is not any significant difference in *SNAIL* expression in non-resistant and resistant IOMM Lee cell line after JQ1 and Stattic treatment for 7 hours (Figure 3.15). If malignant (IOMM Lee) and benign (AC599) meningioma cell lines are compared, there is a significant increase in *SNAIL* expression after JQ1 treatment in AC599 JR cell line compared to IOMM Lee JR cell line. In the immunofluorescence assay, the expression of *CDH1* was just seen in IOMM Lee JR cell lines after 7 hours of JQ1 and Stattic treatment. As known in the literature there is an interaction between *CDH1* and *SNAIL* expression. It is known that *SNAIL* enlists chromatin modifying complexes targeted to the *CDH1* promoter to inhibit CDH1 production during tumor development and EMT (Wang et al., 2013). Our immunofluorescence assay and RNA-Seq data showed that *CDH1* expression decreases in non-resistant and resistant AC599 cell line (Figure 3.3 and Figure 3.13).

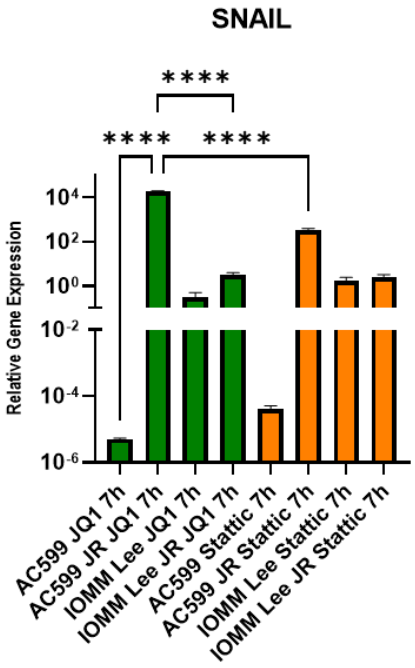


Figure 3.15. *SNAIL* which is an EMT TFs relative gene expression among meningioma cell line with different treatments was shown.

Non-resistant and JQ1 resistant AC599 and IOMM Lee cell lines were treated with DMSO (control), JQ1, and Stattic for 7 hours after wound healing assay. Qiagen Rotor-Gene Q Series Software was used for analysis. DMSO-treated cell line were used as a control. JQ1 and Stattic treated cell lines normalized based on DMSO treatment. Graphs were drawn with GraphPad Prism 9.4.1 Software. (Relative gene expression level showed in Log10).

Another important EMT TF is SLUG which plays an important role in regulating the EMT process, such that it decreases *CDH1* expression in the cells. This causes the adhesion bond between cells to decrease and the cells to gain mobility (Grzegrzolka et al., 2015). As a result, the EMT pathway becomes active. When *SLUG* expression is compared after 7 hours of JQ1 and Stattic treatment, there is a significant increase in AC599 JR JQ1 treated cell line. 7 hour JQ1 treated AC599 JR cell line compared to the AC599 cell line there is a significant increase in *SLUG* expression after JQ1 treatment (Figure 3.16). JQ1 affects EMT pathway activation because when AC599

JR meningioma cell line both JQ1 and Stattic drug treatment were compared to *SLUG* expression significantly increase after JQ1 treatment in a resistant cell line (Figure 3.16). Based on previous experiments, AC599 JR meningioma cell lines' metastatic properties were higher than IOMM Lee JR cell lines. We could therefore assume that JQ1 resistant benign meningioma cell lines have a higher potential of activating the EMT pathway. In qRT-PCR analysis, it shows that the expression of *SLUG* increased in AC599 JR cell line compared to IOMM Lee JR cell line after JQ1 treatment. Significant morphological changes were seen in AC599 JR cell line (Figure 3.1). EMT pathway activation is higher in the AC599 JR cell line in comparison to non-resistant AC599, as well as resistant and non-resistant IOMM Lee meningioma cell line.

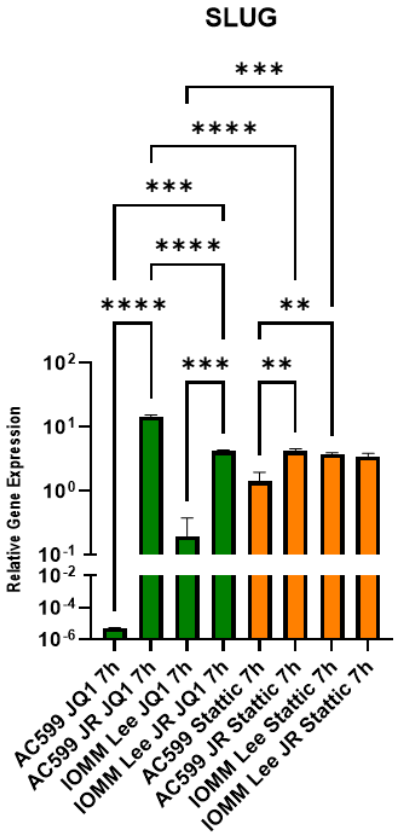


Figure 3.16. *SLUG* which is an EMT TFs relative gene expression among meningioma cell line with different treatments was shown.

Non-resistant and JQ1 resistant AC599 and IOMM Lee cell lines were treated with DMSO (control), JQ1, and Stattic for 7 hours after wound healing assay. Qiagen Rotor-Gene Q Series Software was used for analysis. DMSO treated cell line were used as a control. JQ1 and Stattic treated cell lines normalized based on DMSO treatment. Graphs were drawn with GraphPad Prism 9.4.1 Software. (Relative gene expression level showed in Log10).

As known in the literature *SNAIL*, *SLUG*, and *TWIST* are EMT regulators, as evidenced by their reduction of *CDH1* and other epithelial indicators and cell adhesion (H. Wang et al., 2019). *SNAIL* and *SLUG* expressions were significantly increased in 7 hours JQ1 treated AC599 JR (Figures 3.15 and 3.16). Instead of *SNAIL*

and *SLUG*, there is a significant increase in TWIST expression in 7 hours JQ1 treated AC599 cell line (Figure 3.17). When non-resistant AC599 treated with JQ1 is compared to resistant AC599 JQ1 treated cell line, there is a significant decrease in TWIST expression. When JQ1 treated benign (AC599) and malignant (IOMM Lee) meningioma cell lines were compared, there is an increase in *TWIST* expression in JQ1 resistant benign meningioma cell line, AC599 JR (Figure 3.17). There is a significant increase in *TWIST* in JQ1 treated AC599 JR cell line compared to Stattic-treated AC599 JR cell line. Furthermore, our Ingenuity Pathway Analysis suggests that STAT3 and *TWIST* interact in mesenchymal transition. While *STAT3* expression decreases, *TWIST* expression should also decrease based on Ingenuity predictions. When *STAT3* expression is inhibited with Stattic treatment, *TWIST* expression decreases significantly in 7 hour Stattic treated AC599 JR cell line (Figure 3.17). Also, there is a non-significant decrease in Stattic treated AC599 cell line compared to JQ1 treated AC599 cell lines. On the other hand, there are not any significant changes in *TWIST* expression in non-resistant and resistant IOMM Lee cell lines after both JQ1 and Stattic treatment (Figure 3.17).

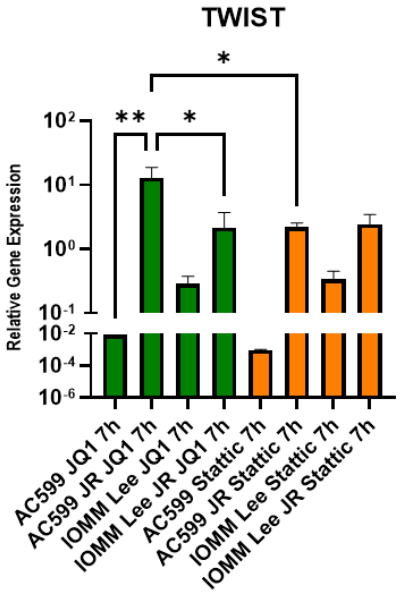


Figure 3.17. *TWIST* which is an EMT TFs relative gene expression among meningioma cell line with different treatments was shown.

Non-resistant and JQ1 resistant AC599 and IOMM Lee cell lines were treated with DMSO (control), JQ1, and Stattic for 7 hours after wound healing assay. Qiagen Rotor-Gene Q Series Software was used for analysis. DMSO treated cell line were used as a control. JQ1 and Stattic treated cell lines normalized based on DMSO treatment. Graphs were drawn with GraphPad Prism 9.4.1 Software. (Relative gene expression level showed in Log10).

In addition to the *SNAIL, SLUG, and TWIST* genes, *CTNNB1*, which is also called *β-catenin*, is another EMT related biomarker. The WNT/ β–Catenin signaling pathway influences EMT by modulating the expression of EMT transcription factors (Xiao et al., 2021). β–Catenin is important for *CDH1* expression in the EMT pathway. CDH1 and β–Catenin plays role in cell-cell adhesion and connections. β–Catenin is an intracellular protein that is attached to the actin cytoskeleton of a cell. CDH1 is attached to β–Catenin to form a complex that can associate with nearby cells to form bonds with the cell's cytoskeleton (Ramis-Conde et al., 2008). Phosphorylated β–Catenin is released when it is disconnected from CDH1. It is ready

to interact with other molecules, and if it does not interact with other molecules, phosphorylated β–Catenin is detected and destroyed by cellular proteasome systems for recycling. The intracellular location of β–Catenin is crucial for the preservation of tissue architecture. Associated with cell migration and the epithelial-mesenchymal transition is the high regulation of free β–Catenin (Wong & Gumbiner, 2003). Free β–Catenin upregulation in the nucleus, is thought to communicate with EMT TFs in the nucleus and cause cell migration (Jankowski et al., 1997). Based on this literature knowledge, *CTNNB1* (*β-catenin*) expression was examined by quantitative qRT-PCR analysis. The previous data shows us that *CDH1* expression is higher in AC599 cell line. From this point of view, the *β–Catenin* expression could be higher in IOMM Lee cell lines in direct proportion to *CDH1*. Cells were collected after 7 hours of JQ1 and Stattic treatment and wound healing assay. 7 hour JQ1 treated IOMM Lee meningioma cell line *β–Catenin* expression is significantly upregulated compared to 7 hour JQ1 treated AC599 meningioma cell line (Figure 3.18).

As known in previous data both non-resistant and resistant AC599 meningioma cell line has decreased *CDH1* expression rate while they have increased *Vimentin* and *N-cadherin*. When *CDH1* expression is downregulated, *β–Catenin* expression is also decreased. Even in immunofluorescence assay β–Catenin signals were only detected in 7 hour JQ1 and Stattic treatment nevertheless β–Catenin expression was only present in the cytoplasm. After 7 hours, the β–Catenin signal disappeared, which suggests that these free β–Catenin in the cytoplasm could be degraded (Figure 3.13). When static treatments of AC599 and IOMM Lee meningioma cell line was compared, there is a significant 4-fold increase in *β–Catenin* expression in 7 hour Stattic treated IOMM Lee meningioma cell line (Figure 3.18). Non-resistant and resistant cells compared after benign and malignant cells comparison. In AC599 meningioma cell line, AC599 JR meningioma cell line have higher expression levels of *β–Catenin* compared to non-resistant AC599 meningioma cell line (Figure 3.18). But still have no protein translation of β-catenin. This means that β–Catenin is not functional in EMT pathway activation in AC599 cell line. But β–Catenin seems functional in IOMM Lee cell line. The β–Catenin protein expression seems higher

in the immunofluorescence assay (Figure 3.12). Also, the expression rate of *CDH1* in IOMM Lee JR meningioma cell line is 4 times higher than in non-resistant IOMM Lee meningioma cell line based on RNA-Seq data (Figure 3.2). These protein translations of β–Catenin were in the nucleus in IOMM Lee JR meningioma cell line (Figure 3.12). The β–Catenin plays a role in EMT TFs transcript based on its localization even if it seems IOMM Lee meningioma cell line have an increase in *CTNNB1* expression (Figure 3.18).

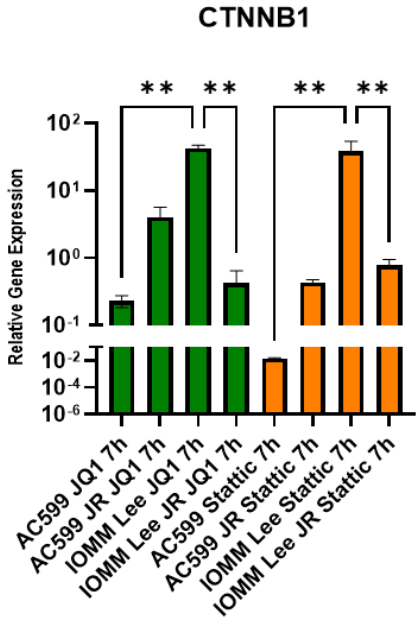


Figure 3.18. *CTNNB1* which is an EMT TFs relative gene expression among meningioma cell line with different treatments was shown.

Non-resistant and JQ1 resistant AC599 and IOMM Lee cell lines were treated with DMSO (control), JQ1, and Stattic for 7 hours after wound healing assay. Qiagen Rotor-Gene Q Series Software was used for analysis. DMSO treated cell line were used as a control. JQ1 and Stattic treated cell lines normalized based on DMSO treatment. Graphs were drawn with GraphPad Prism 9.4.1 Software. (Relative gene expression level showed in Log10).

CHAPTER 4

DISCUSSION

Meningioma is one of the most challenging brain cancers. It can be relatively easily removed via surgery in most cases if diagnosed at early stages; however, often it is diagnosed at later stages and its location might exclude surgery options. Although it is difficult for tumors formed in the brain to cross the blood-brain barrier, they can still reach the blood vessels and or lymphatic system through which they can migrate to other parts of the body. Primary benign cancer cells cannot migrate to another organ or tissue unless they are close to blood vessels or lymph nodes. Some gene groups or pathways are involved in the migration of cancer cells. One of these pathways is the epithelial-to-mesenchymal transition (EMT) pathway. After the activation of the EMT pathway, epithelial cells lose their properties and acquire mesenchymal cells' properties. In this process, original cells with tight cell-cell junctions lose these properties, and cell-cell and ECM interactions get weaker. This increases the possibility of cells migrating from one place to another. Few critical genes or transcription factors are known to play a role in the activation of the EMT pathway. The most important of them are *CDH1*, *CDH2* (*N-cadherin*), *and VIM*. These are the fundamental EMT markers. If *CDH1* expression is high in the cell and there is almost no expression of *N-cadherin* and *Vimentin*, that cell has epithelial properties. However, if *Vimentin* and *N-cadherin* expression become high, those cells are now considered to have acquired mesenchymal characteristics. After all gene transitions occur, the EMT pathway gets activated. Migration features of cells are shown to increase with the increment of mesenchymal features.

Meningioma cells can become metastatic depending on their location and EMT gene program activation. In addition, changes are observed in the metastatic properties of tumor cells depending on whether they are malignant or benign. In our study, we use malignant and benign meningioma cell lines as a model which we compare using

IOMM Lee (Grade III), non-*NF2* mutant, and AC599 (Grade I), *NF2* mutant, cell models. Meningioma cells with *NF2* losses generally showed higher levels of protein translation of various EMT markers. E-Cadherin binds actin which is directly or indirectly attached to cortical actin fibers in meningiomas and is necessary for the formation of epithelial bonds. NF2/Merlin, which is accepted as a meningeal tumor suppressor, enables the fibers to transform into membrane proteins and ensures the protection of meningeal cell-cell contacts. Thereupon, it was stated that there is a correlation between NF2 and E-Cadherin (James et al., 2008). But current literature supporting this statement is limited and it should be investigated in the future.

In addition to non-resistant (parental) IOMM Lee and AC599 meningioma cell lines, we also use their JQ1 drug-resistant counterparts namely IOMM Lee JR and AC599 JR meningioma cell lines. Different research groups have shown that BRD4 inhibition changes epithelial cell morphology to mesenchymal cell morphology. Other groups also reported similar morphological changes in embryonic stem cells after JQ1 treatment (Horne et al., 2015). To understand how JQ1 resistance affects the morphology of meningioma cells, we decided to examine first if meningioma cell lines show any morphological changes upon JQ1 drug resistance. We have found that JQ1 Resistant cell lines have acquired distinctive morphological changes during this process. The non-resistant parental meningioma cell lines were smaller, rounder, and shorter, while the JQ1 Resistant counterparts appeared larger, broad, and slender (Figure 3.1). It was thought that the EMT pathway might be active in the cells whose features we evaluated further in our study. These morphological changes showed features of epithelial and mesenchymal cell characteristics. To understand these differences at the molecular level non-resistant (parental) and their JQ1 Resistant counterpart meningioma cell lines were subjected to RNA sequencing. Following RNA sequence analysis, we have found that there is an increase in the expression of *E-cadherin (CDH1),* which plays a role as an epithelial marker, *SNAI1 (SNAIL)* and *SNAI2 (SLUG)* in the IOMM Lee cell line, which represents more malignant properties in our study (Figure 3.2). In the non-resistant (parental) group of the AC599 meningioma cell line, there was a significant increase in the expression of

TGFB1, *VIM*, *CDH2* (*N-cadherin*), and *TWIST1* genes (Figure 3.3). Based on the differences in these gene expressions, we can say that upon JQ1 resistance acquisition, the AC599 meningioma cell line gained more mesenchymal cell characteristics compared to its non-resistant (parental) AC599 counterpart as well as the IOMM Lee meningioma cell line in our study. Therefore, in resistant IOMM Lee JR, *CDH1* gene expression increases 4 times compared to non-resistant parental IOMM Lee (Figure 3.2). While there is an increase in *CDH1* in the IOMM Lee JR meningioma cell line, there is a decrease in *CDH1* in the AC599 JR meningioma cell line. This difference shows that the EMT pathway should be activated in the AC599 JR meningioma cell line. *CDH1* is downregulated (with fold change -2) in DMSO-only control treatment as well as the JQ1 treatment group of AC599 meningioma cell lines (with fold change -3) (Figure 3.3). When we compared IOMM Lee JR with AC599 JR, we found that *CDH1* gene expression is lower in the AC599 JR meningioma cell line. This comparison showed that AC599 JR cells have the potential for EMT pathway activation and migration. Besides the decrease of *CDH1*, *VIM*, and *CDH2* gene expressions were upregulated in the AC599 JR meningioma cell line (fold change of 2) compared to the non-resistant (parental) AC599 meningioma cell line (Figure 3.3). Another critical EMT regulator *TWIST* gene was also found upregulated in the AC599 JR meningioma cell line group compared to the non-resistant (parental) AC599 meningioma cell line (Figure 3.3). In summary, our RNA-seq data suggest that upon gaining JQ1 resistance, AC599 JR shows more mesenchymal properties based on their corresponding relative gene expression. Moreover, both *CDH2* and *TWIST* genes were downregulated in the IOMM Lee JR meningioma cell line compared to non-resistant (parental) IOMM Lee (Figure 3.2). We also investigated changes in gene expression patterns after JQ1 treatment in these meningioma cell lines. As a result, it was observed that the AC599 meningioma cell line showed mesenchymal characteristics compared to other cells after JQ1 treatment. EMT is observed upon JQ1 resistance (Calder et al., 2021). Thus, we further analyze our RNA-seq data using Ingenuity Pathway Analysis to understand

the possible upstream and downstream regulatory mechanisms that may be involved in our observed EMT gene expression pattern (Appendices A.2, A.3, A.4, and A.5).

We found that one of the most significant such regulators is the *STAT3* transcription factor. In healthy cells, *STAT3* is typically momentarily activated, but it is highly upregulated in a diverse range of tumor types and blood cancers, such as breast cancer, prostate cancer, head and neck cancer, melanoma, brain cancer, and gastrointestinal cancers (Li & Huang, 2017). Moreover, *STAT3* involves nearly entire biological processes like metastasis, apoptosis, stemness, and cell cycle. Our IPA data suggested that the STAT family could be the master regulator of EMT markers in meningioma. In cholangiocarcinoma, metastasis regulates via the activation of *STAT3* (Sun et al., 2019). As we know from the literature, the JAK/STAT pathway is well established for its role in EMT pathway regulation. Recent research indicates that *STAT3* functions as a stimulator and regulator of cells' epithelial to mesenchymal transition, both in healthy and pathological circumstances (Wendt et al., 2014). To test this hypothesis in our meningioma model cell system, we use Stattic treatment, which is a STAT3 inhibitor. We then use wound healing assay to evaluate how inhibition of STAT3 can affect cellular migration in vitro. To exclude the possibility of wound closure occurring due to cell proliferation we also evaluated the effect of STAT3 inhibitor in a cell proliferation assay. Targeting cell cycle-related regulatory factors, STAT3 changes the progression of the cell cycle (Qin et al., 2019). Stattic treatment induced G1 arrestment in pancreatic cancer cells (Guo et al., 2022). Our main reason of doing this is to see that cells move only with their metastatic properties during the wound healing assay. After 24 hours of drug treatment, the cells were fixed and stained with PI to look at cell cycles by flow cytometry (Figure 3.9). Our cell cycle studies indicated that there appears to be a decrease in the M and G2 phases and an increase in the G1 phases of the cells after both JQ1 and Stattic treatment. We see that with the increase of the G1 phase, the drugs significantly reduce the division properties of the cells (Figure 3.10). In conclusion, JQ1 and Stattic treatment does not affect cell cycle distribution. JQ1 and

Stattic treatment both induce G1 arrest in non-resistant and resistant IOMM Lee and AC599 meningioma cells.

This data suggested that the cells would close the wound not by dividing but by their metastatic properties during the wound healing assay. After creating the gap by scratching the cells in the plate, JQ1 and Stattic were added to the culture, and cell migration was monitored for up to 24 hours. When AC599 meningioma cell line is exposed to JQ1 and Stattic treatment, cells did not progress and; therefore ,did not close the wound (Figure 3.4). This was consistent with the literature while BRD4 inhibition with JQ1 treatment induces cell cycle arrest, it reduces migration and wound healing of breast cancer cell MDA-MB-231 (Choi et al., 2015). Cancer cells often gain resistance to drugs after a while upon treatment. If BRD4 inhibitor, JQ1 treatment affects cell cycle arrest and wound healing in different cancer cells, gaining resistance to JQ1 drug should have the opposite effect in these cells. However, when we looked at AC599 JR meningioma cell line, we observed that they closed the wound completely after 24 hours in both control and JQ1 and Stattic treatments (Figure 3.5). Even though JQ1 treatment reduces wound healing in non-resistant cancer cells, in JQ1 resistant cells we did not detect any effect on wound healing in the IOMM Lee meningioma cell line (Figure 3.6). When we compare the results of the wound healing assay in the light of RNA-Seq data, the increase in the mesenchymal markers of the cells correlates with the increase in their metastatic properties in JQ1 resistant AC599 meningioma cell line (Figure 3.4). JQ1 resistance in AC599 meningioma cell line affects EMT pathway activation. When we look at the IOMM Lee meningioma cell line, there is no significant wound closure at the end of 24 hours after drug treatments in both non-resistant and resistant cells (Figures 3.6 and 3.7). When the RNA-Seq data of the IOMM Lee meningioma cell line are compared with the wound healing data, it is seen that the epithelial feature is more dominant in the cells, and; therefore, they behave more like epithelial cells in culture as well (Figures 3.2, 3.6, and 3.7). STAT3 inhibition with Stattic also affects cell proliferation. Stattic treatment works with the inhibition of STAT3 phosphorylation on Tyr705. The inhibition of phosphorylation affects the activation of STAT3 and

interaction with other molecules in signaling pathways. The Stattic treatment should decrease cell proliferation. STAT3 alters the cell cycle's development by focusing on regulatory elements that are connected to the cell cycle. (Qin et al., 2019). STAT3 inhibition also inhibits cell migration. Stattic treatment in AC599, IOMM Lee, and IOMM Lee JR meningioma cell lines was shown to inhibit the migration of cells (Figures 3.4, 3.6, and 3.7). However, in AC599 JR meningioma cell line Stattic treatment did not affect the migration of the cells (Figure 3.5). They close the wound after 24 hours of Stattic treatment which suggests that *STAT3* might not play any role in EMT activation in the AC599 JR meningioma cell line. Based on our data *STAT3* plays a role in cell migration in AC599, IOMM Lee, and IOMM Lee JR meningioma cell lines.

There is a change in EMT biomarkers and TFs expression based on RNA-Seq data. But when a gene is expressed in a cell, it does not mean there is a protein translation based on this gene expression. Therefore, gene expression must be translated at the protein level for its biological role to take place in the cell. To understand EMT activation at the protein level we decided to look at the antibody staining of the main EMT markers in cell culture, whose expression changes were also observed in the RNA-Seq data (Figure 3.2 and Figure 3.3). To achieve this, cells were stained with CDH1, N-cadherin, Vimentin, and β–Catenin antibodies, which are the main EMT markers. As a result of staining, cells close to the part of the wound were observed while imaging. Vimentin and N-cadherin protein translation were seen in the IOMM Lee meningioma cell line upon treatment with both JQ1 and Stattic within 24 hours (Figure 3.11). On the contrary, CDH1 protein translation was not seen. This may be due to the translation of the CDH1 protein was not seen in the IOMM Lee cell. Expression of N-cadherin and Vimentin was not seen in all the cells but cells that are close to the gap area have strong signals (Figure 3.11). When we look at the IOMM Lee JR meningioma cell line, CDH1 protein translation increases at 7 hours and then decreases, while an increase in N-cadherin protein translation was observed for 24 hours (Figure 3.12). We can say that cells get used to drugs and reveal their metastatic properties. When the RNA-Seq and immunofluorescence assay are

compared, there is a 2 times increase in CDH1 expression but there was not a significant protein translation of CDH1 in the immunofluorescence study (Figures 3.2 and 3.12). This shows that the IOMM Lee JR meningioma cell line expresses epithelial markers compared to mesenchymal markers. But the cells that are near the wound do not translate epithelial marker proteins. Even though those cells express higher amounts of Vimentin and N-cadherin protein. This can be shown that IOMM Lee and IOMM Lee JR meningioma cell lines could have partial mesenchymal properties. The particular reason for the circumstance for 24 hours, N-cadherin and Vimentin protein translation was increased in cells close to the wound.

When we look at the benign, AC599, meningioma cell line as well as the malignant, IOMM Lee, meningioma cell lines, we did not see CDH1 expression in the immunofluorescence assay (Figure 3.13). Expression of epithelial markers is also decreased in RNA-Seq data (Figure 3.3). It is possible not to detect protein translation when gene expression is significantly downregulated. The decrease of *CDH1* expression was also affected by upstream EMT TFs' expression. *SNAIL* and *SLUG* transcription factors are known to regulate *CDH1* expression. However, there is an increase in N-cadherin and Vimentin expression after both JQ1 and Stattic treatments within 24 hours (Figure 3.13). When we encounter other cells, N-cadherin appears to be concentrated around the cell nucleus. In the AC599 JR meningioma cell line, there was an increase in Vimentin protein translation after 24 hours of JQ1 and Stattic treatment (Figure 3.14). But there is a difference between the AC599 JR meningioma cell line and the non-resistant and resistant IOMM Lee meningioma cell line. AC599 JR meningioma cell line expressed mesenchymal markers in every cell, in the IOMM Lee meningioma cell line some of the cells express mesenchymal markers. However, in the AC599 JR cell line, there was not any protein expression of *β–Catenin* within 24 hours of treatment. β–Catenin protein translation was not only low in protein level but also its gene expression was lower than the non-resistant and resistant IOMM Lee meningioma cell line. Whenever CDH1 and β–Catenin join, a complex is created that can connect with the cell's cytoskeleton and nearby cells to form connections. If CDH1 is downregulated, its connection with β–Catenin breaks

down and β–Catenin is released into the cytoplasm (Ramis-Conde et al., 2008). After this process, β–Catenin becomes a target for degradation, and its expression was downregulated. In figure 9, *CDH1* expression was decreased 8 times more than AC599 NR (non-resistant) meningioma cell line. There was a decrease in gene expression of epithelial markers based on RNA-Seq data (Figure 3.3). However, there is an increase in mesenchymal markers in both the non-resistant AC599 meningioma cell line and the AC599 JR meningioma cell line.

After seeing translation and expression differences in EMT markers, we decided to examine the differential expression of EMT TFs' genes. The proteasome system quickly breaks down the main EMT TFs like *SNAIL, TWIST*, and *ZEB* that modulate EMT (Díaz et al., 2014). Besides *CTNNB1, SNAIL, TWIST*, and *SLUG* play role in *CDH1* expression. While their expression increased, *CDH1* expression decreased.

The regrowth of tumors was aided by *SNAIL*-driven EMT in a mouse model of breast cancer recurrence (Moody et al., 2005). Patients' remaining breast cancer cells after conventional therapy frequently display EMT and stem cell characteristics (Creighton et al., 2009). Overviews of EMT, which is a factor in the metastatic characteristic of epithelial cancer cells, is characterized by the stimulation of *SNAIL* and consequent decrease of *CDH1* (Tian et al., 2020). The *CDH1* expression also decreases in AC599 and AC599 JR meningioma cell lines after JQ1 and Stattic treatments. When non-resistant and resistant AC599 meningioma cell lines are compared to the non-resistant and resistant IOMM Lee cell lines the epithelial markers' expressions are decreased. The decrease of epithelial markers especially *CDH1* affects *β–Catenin* expression at the same time. The *β–Catenin* expression is positively correlated with *CDH1* because of the complex they constitute together. Non-resistant and resistant AC599 meningioma cell lines have a decrease in *β–Catenin* expression and *CDH1* expression after both JQ1 and Stattic treatment (Figures 3.13 and 3.18). Contrary to this *SNAIL*, *SLUG*, and *TWIST* expressions are increased in both non-resistant and resistant AC599 meningioma cell lines after JQ1 and Stattic treatment (Figures 3.15, 3.16, and 3.17). However, their expressions significantly increase, especially in the AC599 JR meningioma cell line. The

increase of *SNAIL, SLUG,* and *TWIST* affected *CDH1* negatively and its expression decreases based on EMT TFs. Decreasement of CDH1 induces N-cadherin and Vimentin protein translation (Figures 3.13 and 3.14). Drug resistance affects EMT TFs' and markers' expressions. Further, it triggers epithelial to mesenchymal transition.

IOMM Lee cell lines are malignant meningioma cell lines. The hypothesis was EMT activation and malignancy have a positive correlation. But previous data does not show any positive correlation between IOMM Lee and EMT activation. *SNAIL*, *SLUG*, and *TWIST* are the basic TFs of EMT. However, they are not expressed enough to affect EMT activation. After JQ1 and Stattic treatment of IOMM Lee and IOMM Lee JR meningioma cell lines, basic EMT TFs' expressions were examined. The examination showed that *SNAIL, SLUG,* and *TWIST* expressions were decreased (Figures 3.15, 3.16, and 3.17). Based on these expressions *CDH1* expression should be increased in the IOMM Lee meningioma cell line. However, in the immunofluorescence assay, CDH1 protein translation was not detected in the non-resistant IOMM Lee meningioma cell line while it is expressed in the IOMM Lee JR meningioma cell line (Figure 3.12). *CDH1* gene might be expressed in the IOMM Lee meningioma cell line but not translated at the protein level. The downregulation of EMT TFs, such as *SNAIL*, *SLUG*, and *TWIST* leads to the expression of *CDH1*. Overall, we observed differences in EMT marker expression among meningioma cell lines which highlight the fact that EMT pathway activations depend on cell identity and their exposure to chemotherapy.

In addition, according to the literature, it is known that the expression of epithelial markers, especially *E-cadherin,* is also dependent on *NF2* mutation. When we look at all the experimental results, the AC599 meningioma cell line that carries *NF2* mutation has low *E-cadherin* expression even before they acquire JQ1 resistance. Accordingly, we can say that *NF2* loss decreases the expression of *E-cadherin* and other epithelial markers in the AC599 meningioma cell line. According to the literature, *NF2* has a negative correlation with *SNAIL*. Accordingly, the effect of *NF2* loss also plays a role in the *SNAIL* expression increase in the AC599 meningioma

cell line. Changes in the transcription of these genes increase the tendency of the AC599 meningioma cell line to migrate. After acquiring JQ1 resistance, the AC599 meningioma cell line gains more aggressive mesenchymal cell properties and; therefore, the AC599 JR meningioma cell line is willing to migrate. We found that JQ1 resistance increased the expression of mesenchymal markers in AC599 meningioma cell lines (ie. AC599 JR). Furthermore, the positive correlation of *NF2* loss with epithelial markers caused a significant increase in mesenchymal markers after JQ1 resistance.

When we look at the malignant IOMM Lee meningioma cell line, we see that the expression of *E-cadherin* is high even before gaining JQ1 resistance. We expect the IOMM Lee cell, which is more aggressive than AC599, to have fewer epithelial features and to be more willing to migrate. However, when the *NF2* mutation is involved, we see that the AC599 meningioma cell line with *NF2* loss shows more mesenchymal properties than the IOMM Lee meningioma cell line and they gain more migratory features. The IOMM Lee meningioma cell line does not have the *NF2* loss (ie. Non-*NF2* meningioma) and has high *E-cadherin* expression. The IOMM Lee meningioma cell line without *NF2* loss exhibits more epithelial features as compared to *NF2* mutant AC599 meningioma cell line. Therefore, the genetic makeup of the tumor is critical to design personalized therapies to target the underlying molecular mechanism in cancer treatment.

CHAPTER 5

CONCLUSION

EMT and chemoresistance are closely related subjects. Tumor cells that acquire drug resistance may develop EMT pathway characteristics (Shah et al., 2007). On the other hand, tumor cells that went through EMT also develop resistance to treatment (Yin et al., 2007). In addition, decreasing EMT can improve drug sensitivity (Fischer et al., 2015; Zheng et al., 2015). EMT TFs establish a resistance to chemotherapy and radiotherapy through different mechanisms including resistance to apoptosis, enhanced DNA damage repair, and altered drug metabolism (van Staalduinen et al., 2018). Our study results show that metastatic properties increase in meningioma cell lines with chemoresistance. EMT and chemoresistance are two pathways that affect each other and work with positive feedback. Activation of the EMT pathway increases in cells that develop drug resistance (Du & Shim, 2016). When we compared malignant and benign meningioma cell lines, we observed that benign meningioma cells gain more metastatic properties after they acquired drug resistance. In light of these results, we see that there is no relation between the metastatic properties of malignant cells gaining drug resistance or not. This could be explained by the fact that chemoresistance might not change malignant cell biology as much as it changes benign cell biology during the process. While the EMT pathway has the potential to be seen in a colony of tumor cells, it can be seen in some cells and not in others contributing to the tumor heterogeneity which makes tumor therapy challenging in often cases. This is also called partial EMT. As a result of our experiments, we can say that EMT markers increase in the cells that have the opportunity to migrate. To understand the fact of EMT markers in wound healing and migration the Wound Healing Assay was done. Based on this assay while the malignancy decreases the migration property increases. However, when a cell gains drug resistance, the migration property and EMT marker expression increase. Even

though EMT markers are expressed, if they are not translated at the protein level, the gene cannot be affected by EMT pathway activation. However, the expression of EMT markers is affected by different pathways and TFs.

The EMT-related cellular changes were only partially inhibited by *STAT3* suppression (Rocha et al., 2018). *STAT3* does not always play a role in every pathway activation. Based on our results, only some of the cells receiving Stattic treatment showed that genes related to the EMT pathway and TFs were affected. *STAT3* inhibition reduces cell proliferation and metastasis, and in some conditions, it stops migration. When the effects of *STAT3* inhibition on EMT genes are not observed, we can say that the *STAT3* gene does not play a role in the activation of the EMT pathway. According to our results, Stattic treatment has no impact on mesenchymal markers in a malignant meningioma cell, IOMM Lee. We can say that Stattic treatment will not be our first choice as a treatment specific to EMT in malignant meningioma. Although some of the epithelial markers influence gene expression in malignant cells, there is no effect on the expression of mesenchymal markers. Stattic treatment inhibits metastasis and cell proliferation in other cancer types and causes cells to enter apoptosis. For this reason, we can say that *STAT3* inhibition is more likely specific to tumors and could be potentially used as a treatment for tumors with a specific gene profile.

JQ1 therapy is known to cause morphological alterations in embryonic stem cells (Horne et al., 2015). Numerous studies have demonstrated that BRD4 inhibition converts epithelial cells to mesenchymal cells. Activation of the EMT pathway has been observed in the literature and after morphological changes in our meningioma cells. In addition to the effect of JQ1 treatment, the activation of the EMT pathway is also seen in our cells that have become resistant to JQ1. When malignant and benign meningioma cell lines were compared, more morphological changes and metastatic features are observed in the benign AC599 meningioma cell line after is made resistant to the JQ1 drug. According to our data, upon gaining JQ1 resistance, the benign meningioma cell, AC599, gains more aggressive properties and increases its metastatic properties. JQ1 treatment suppresses the migratory properties of the

non-resistant benign AC599 meningioma cell line. However, after the AC599 meningioma cell line gains resistance to JQ1 (ie. AC599 JR), JQ1 and Stattic treatments work partially in these cells because they show more aggressive properties upon acquiring chemoresistance. In non-resistant IOMM Lee, a malignant meningioma cell line, JQ1 treatment does not show a significant response. It partially inhibits EMT activation and metastasis. However, when the IOMM Lee meningioma cell line gains JQ1 resistance, it becomes even more aggressive and the effects of JQ1 and Stattic treatments were not effective as expected. To sum up, JQ1 and Stattic treatments are viable therapy options for cancer treatment. However, they should be used in cell and pathway-specific manner for effective cancer treatment.

EMT pathways' most important TFs are *SNAIL, SLUG,* and *TWIST*. In this study traces of the EMT pathway in the meningioma cell lines are seen in the AC599 meningioma cell line, which is benign and JQ1 resistant. After it was understood that AC599 JR growth differed morphologically, EMT markers were examined. One of the most notable suppression targets during the EMT procedure is the traditional epithelial biomarker *CDH1* (Aiello & Kang, 2019), which is a crucial element of adherent connectivity. Transcriptional regulation of *CDH1* and several other epithelial genes is specifically targeted by several EMT TFs, such as *SNAIL*, *SLUG*, and *Zinc-finger E-box-binding homeoboxes 1* and *2 (ZEB1/2)* (Comijn et al., 2001; Bolós et al., 2003). EMT TFs support the expression of crucial mesenchymal genes such as *Vimentin (VIM),* and *N-cadherin (CDH2)* (Aiello & Kang, 2019). On top of that, *N-cadherin* and *Vimentin,* which are mesenchymal markers, are also seen in the sequence, while *CDH1* is the decreased scene. EMT TFs *TWIST* is an important promoter of the mesenchymal transcription program (Yang et al., 2004; Ocaña et al., 2012). A series of works made in the supplement edition of *TWIST*. Also, other notable TFs are *SNAIL* and *SLUG*. Research shows that *SNAIL*-derived EMT promotes metastasis in melanoma (Kudo-Saito et al., 2009).

In our study *SNAIL* and *SLUG* expressions play a role in *CDH1* expression level. Our malignant meningioma cell line, IOMM Lee, has an expression of *CDH1* and correlating to this expression *SNAIL* and *SLUG* have low expression rates. In

conclusion, the benign meningioma cell line, AC599, gains EMT activation after JQ1 resistance while the IOMM Lee JR meningioma cell line has a low amount of activation in the EMT pathway. EMT pathway activation is mostly based on *CDH2, VIM,* and *TWIST* which are EMT TFs. These three genes are the most important markers of mesenchymal properties. *CDH1* and *β–Catenin* are important epithelial markers. Identification of these genes and expression rates show EMT pathway activation. However, our malignant cell lines, even if they are non-resistant or JQ1 resistant IOMM Lee meningioma cell lines have partial EMT. The cells which are near the wound or the circulation mechanism have a higher possibility to activate the EMT pathway. Because they are more able to migrate to another place, compared to the IOMM Lee meningioma cell line, benign AC599, and AC599 JR cell lines express mesenchymal markers, and all the cells of AC599 and AC599 JR can migrate. To sum up, the benign meningioma cell line, AC599, gains migrational and mesenchymal properties after gaining JQ1 resistance. The malignancy of cancer cells does not always affect their migrational properties.

We know that the AC599 meningioma cell line has *NF2* mutation which plays a role in cell-cell adherence. NF2/Merlin is bound to actin in the cytoskeleton, and its loss triggers the loss of tight junctions. In addition, E-cadherin plays a role in adhesion/tight junctions in cells and is associated with actin filaments. In our study, we see that E-cadherin decreases in the AC599 meningioma cell line more than the IOMM Lee meningioma cell line. The *NF2* loss in the AC599 meningioma cell line affects *E-cadherin* expression in the AC599 meningioma cell line. Despite being an aggressive meningioma cell, the IOMM Lee meningioma cell line expresses *E-cadherin*. This expression must be associated with the non-*NF2* mutation. In conclusion, *NF2* mutation plays a role in EMT activation. *NF2* loss correlates with a decrease in *E-cadherin* expression in meningioma. Even if the meningioma cells are benign the *NF2* mutation increases the aggressiveness and migration properties of the cells. However, in the malignant IOMM Lee meningioma cell line, non-*NF2* mutations decrease the aggressiveness of the cells and play an important role in epithelial markers' expression. To understand this, aggressive CH157MN cells with

NF2 mutation should be compared with the IOMM Lee meningioma cell line in future studies. Thus, the effect of *NF2* mutation on EMT markers and activation in malignant meningioma cells should be examined. Further, the molecular interplay between JQ1 chemoresistance and *NF2*, which is a main mutation in almost half of the meningioma tumors, should be investigated and aberrant molecular targets must be identified for potential targeted therapies.

In future studies, the relations between EMT and chemoresistance should be further investigated. In particular, we must address how the migrational properties of cell lines with different degrees of malignancy change. The tumor microenvironment and epigenetic landscape need to be integrated for their contribution to the tumor state. For instance, since there is a partial EMT observed in non-resistant and resistant IOMM Lee and AC599 meningioma cell lines, 3D organoid models could be established to address these questions.

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APPENDICES

A. RNA- Seq Data Table

IOMM DMSO RvsNR		
symbol	log2FoldC	p-value
CDH1	2.06	0.04
SNAI2	0.75	7.10E-08
ZEB2	-1.28	4.53E-43
FOXM1	-1.65	2.87E-51
SNAI1	0.94	3.40E-153
ZEB1	-0.67	4.03E-37
CTNNB1	-0.06	3.02E-08
TWIST1	-1.03	1.04E-48
CDH2	-0.98	5.11E-08
VIM	-0.69	9.88E-20
TGFB1	-1.18	0.17

IOMM JQ1 RvsNR		
symbol	log2FoldC	p-value
CDH1	2.34	0.04
SNAI2	0.59	8.06E-06
ZEB2	-0.83	1.58E-21
FOXM1	-1.7	1.6E-162
SNAI1	0.87	1.38E-07
ZEB1	-0.56	2.47E-14
CTNNB1	-0.15	0
TWIST1	-0.0063	0.97
CDH2	-0.9	2.35E-32
VIM	-0.88	2.29E-77
TGFB1I1	-1.05	6.5E-34

599 DMSO RvsNR		
symbol	log2FoldC	p-value
CDH1	-2.34	4.24E-13
SNAI2	-1.73	9.24E-18
ZEB2	-0.96	4.15E-32
FOXM1	-0.63	1.62E-13
SNAI1	-0.38	0.62
ZEB1 *	0.17	0.07
CTNNB1	0.19	3.57E-05
TWIST1	0.75	2.18E-10
CDH2	0.64	1.53E-21
VIM	1.04	5.76E-60
TGFB1	1.53	1.83E-05

599 JQ1 RvsNR		
symbol	log2FoldC	p-value
CDH1	-3.22	3.11E-11
SNAI2	-1.25	3.34E-08
ZEB2	-1.21	9.90E-38
FOXM1	-0.84	9.45E-18
SNAI1	-0.32	0.7
ZEB1 *	0.21	0.06
CTNNB1	0.19	0
TWIST1	0.9	3.25E-11
CDH2	0.72	2.90E-21
VIM	1.1	1.00E-53
TGFB1	1.18	3.48E-12

Figure A.1. Resistant and Non-resistant IOMM Lee and AC599 were treated with DMSO and JQ1.

*RNA sequencing data were analyzed for JQ1 Resistant (R) and Non-resistant (NR) cell lines Statistical values of RNA-Seq were shown (B). LogFoldChange for EMT markers' in this comparison is shown. ≤ 0 downregulated; ≥0 upregulated . * shows significant p-values.*

B. Ingenuity Pathway Analysis

A **B**

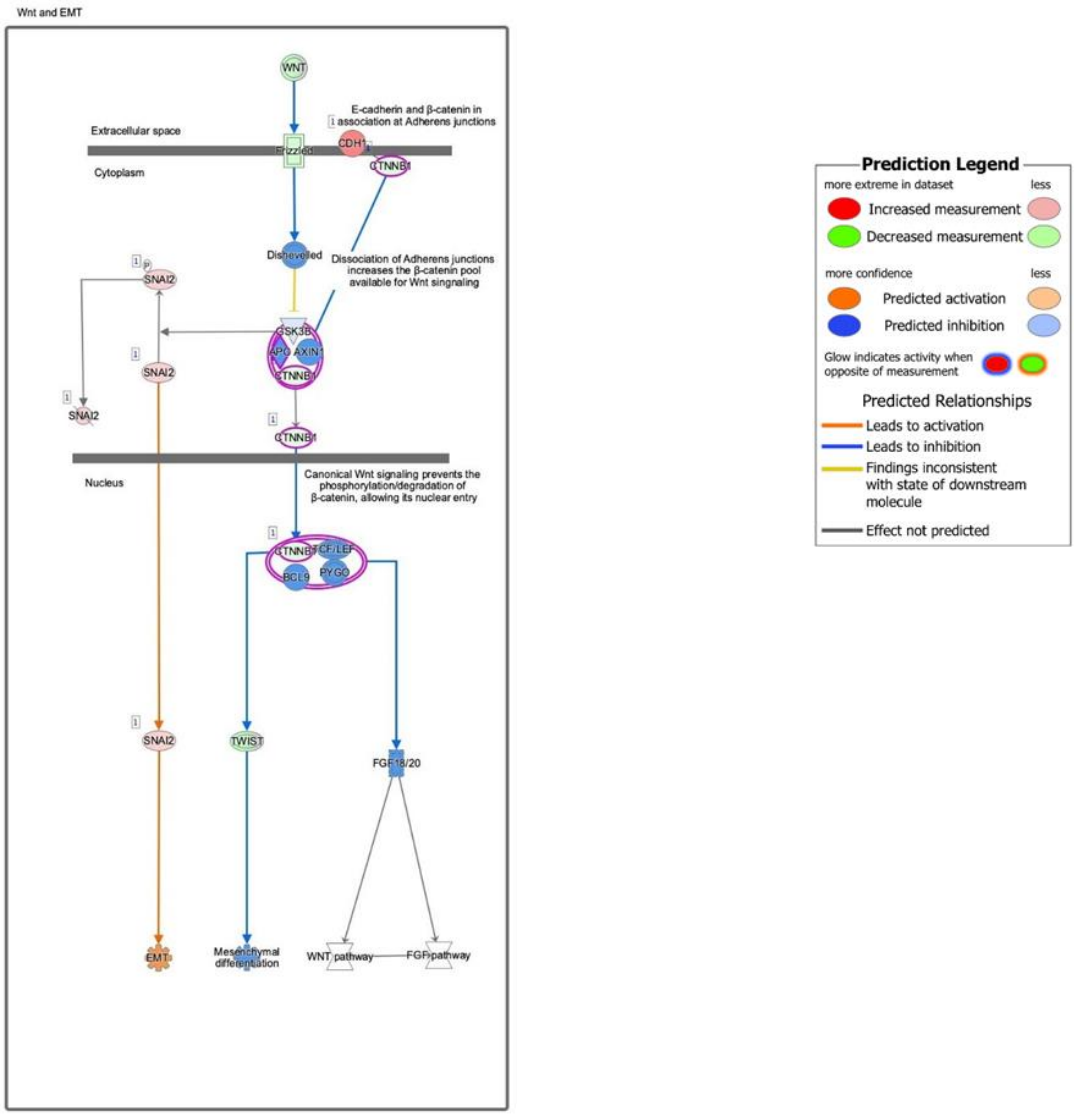


Figure A.2. IOMM Lee DMSO R vs NR Canonical EMT pathway, Ingenuity Pathway Analysis. RNA-Seq data were analyzed by Ingenuity and the predictable expression of genes is shown in figure (A). Prediction legend of figure (B).

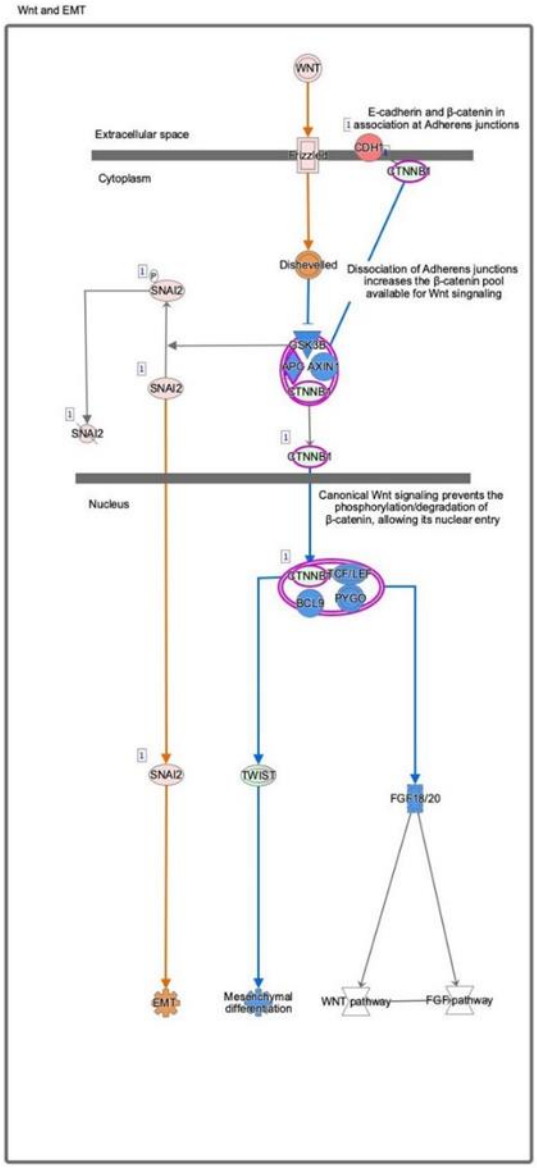


Figure A.3. IOMM Lee JQ1 R vs NR Canonical EMT pathway, Ingenuity Pathway Analysis. RNA-Seq data were analyzed by Ingenuity and the predictable expression of genes is shown in the figure.

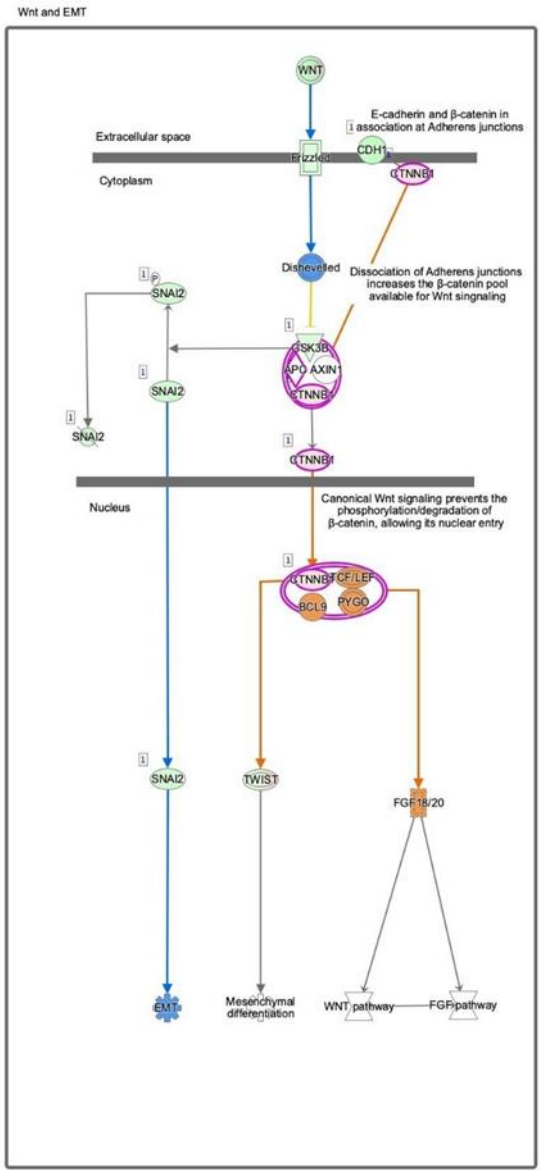


Figure A.4. AC599 DMSO R vs NR Canonical EMT pathway, Ingenuity Pathway Analysis. RNA-Seq data were analyzed by Ingenuity and the predictable expression of genes is shown in the figure.

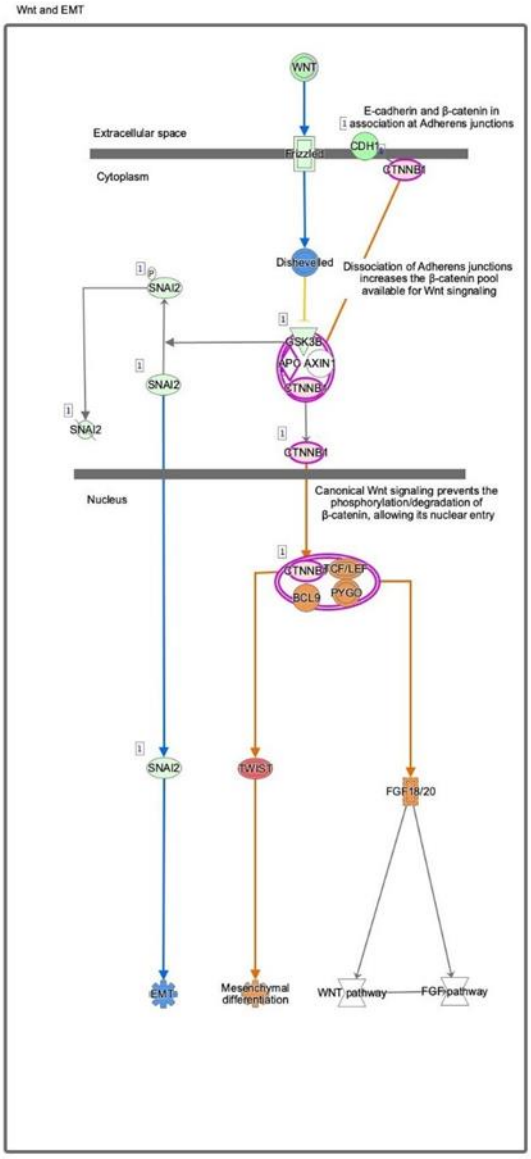


Figure A.5. AC599 JQ1 R vs NR Canonical EMT pathway, Ingenuity Pathway Analysis. RNA-Seq data were analyzed by Ingenuity and the predictable expression of genes is shown in the figure.

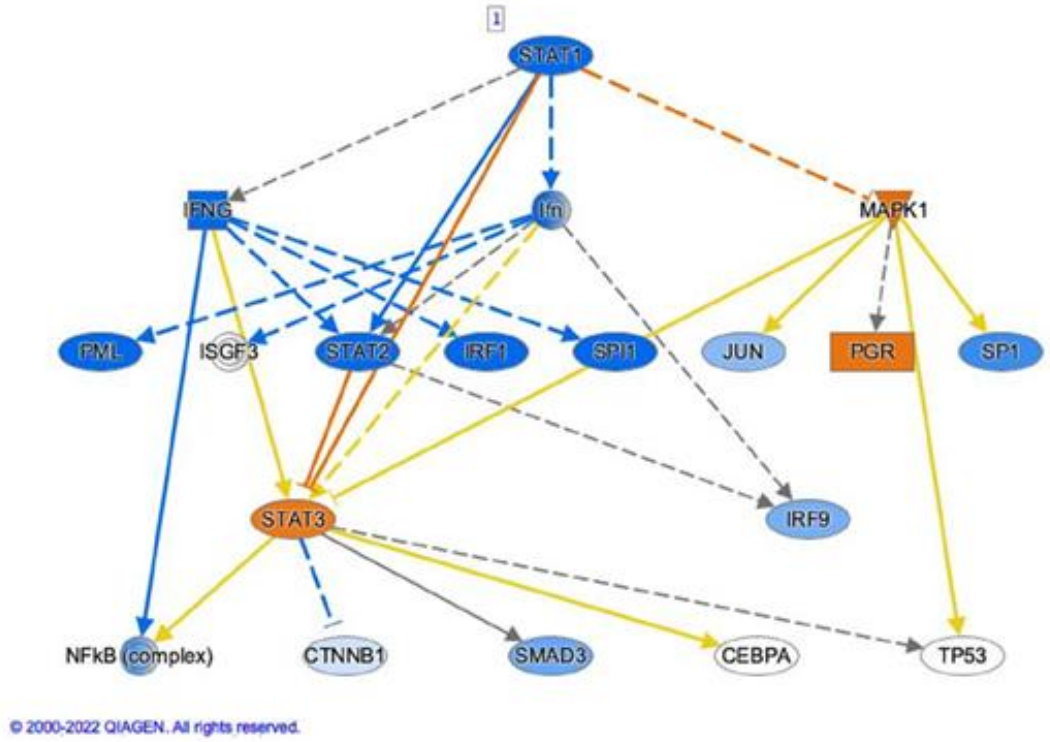
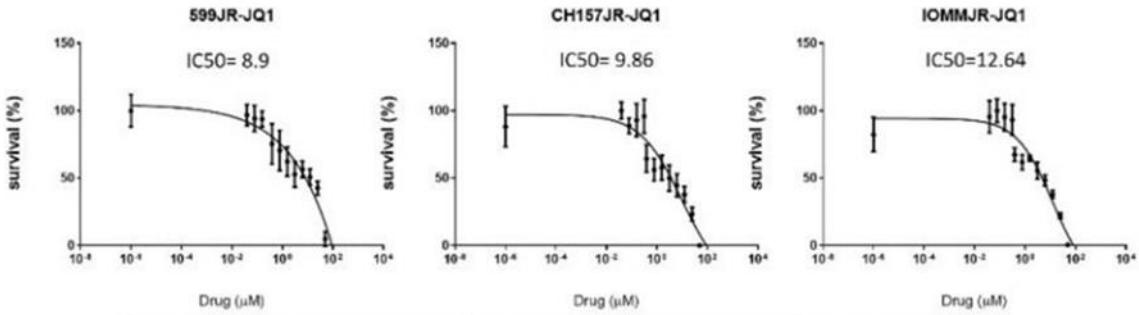


Figure A.6. Upon chemoresistance there is a significant *STAT1* downregulation in the AC599 JR cell line compared to the parental AC599 cell line. Ingenuity Pathway Analysis combines our RNA-Seq data with the database to predict the downstream effect where *STAT3* (shown in orange) is predicted to be upregulated.

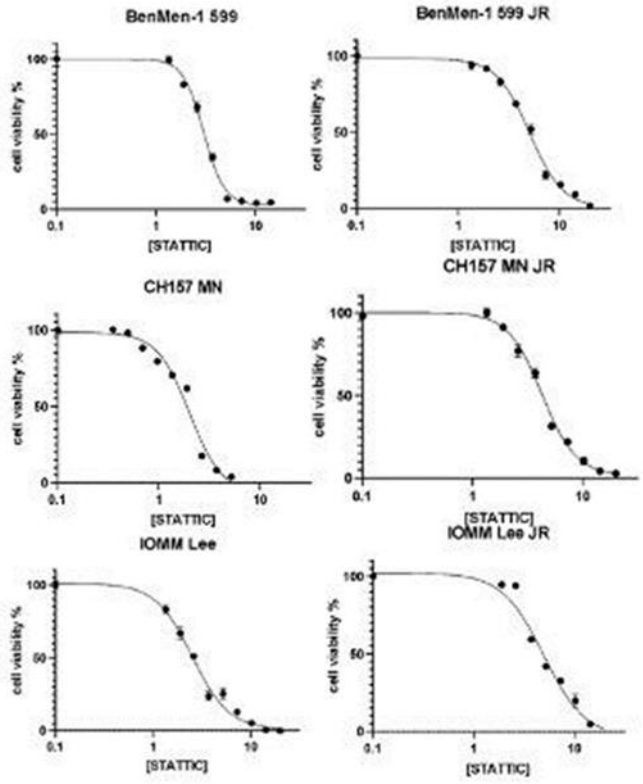
C. JQ1 IC50 Values of IOMM Lee, CH157 -MN and AC599



Naive	IC50 (μM)	JQ1-resistant	IC50 (μM)	FOLD CHANGE
AC599	0.1-0.2	599-JR	8.9	60
CH157-MN	0.3	CH157-JR	9.86	30
IOMM-Lee	2-3	IOMM-JR	12.64	5

Figure A.7. JQ1 was added to cells gradually in 96 well plates. After 72 hours of adding JQ1, Cell Titter Glo Assay was observed. Cell Titter Glo Assay is a viability assay that is used for finding the IC50 value in this experiment.

D. Stattic IC50 Values of Non-Resistant and Resistant IOMM Lee, CH157 - MN and AC599



Cell Line	Stattic IC_{50} (μM)
BenMen1	3,021
BenMen1-JR	5.076
CH157	2.014
CH157-JR	4.260
IOMM	2,516
IOMM-JR	4,951

Figure A.8. Stattic was added to cells gradually in 96 well plates. After 72 hours of adding Stattic, MTT Assay was observed. MTT Assay is a viability assay that is used for finding the IC50 value in this experiment.

E. *STAT1* Log Fold Change in JQ1 Resistant AC599 Cell Line Compared to Parental

Molecule	Experimetal Log Fol Change	Predicted Log Fold Change	Experimental pvalue	Experimetnal padj	Predicted p value
STAT1	-2,06	-4,007	3.37E-44	3.37E-44	4,64E-13
STAT2	-0,804	-2,607	9.93E-21	9.93E-21	0,000000206
STAT4	2,162	-2,303	4.19E-14	4.19E-14	0,0373

Figure A.9. *STAT1* is downregulated in JQ1 resistant AC599 cell line compared to parental.

F. Immunofloerecense Staining Intensity Comparision

	Positive Antibody Staining (Intensity Per Area)		
	Stattic Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	22.1	49.5	62.7
β-catenin (red)	0.0	0.0	16.2
E-cadherin (green)	0.0	12.0	10.6
N-cadherin (red)	35.5	30.6	20.1
	JQ1 Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	22.1	59.5	40.8
β-catenin (red)	0.0	19.3	5.5
E-cadherin (green)	0.0	0.0	16.0
N-cadherin (red)	35.5	35.4	66.2

Figure A.10. The IOMM Lee meningioma cell line was stained with Vimentin, β-catenin, E-cadherin, and N-cadherin antibodies. The table shows antibody stainings intensity in 24 hours of Stattic and JQ1 treatment. Intensity per area changes in percentage.

	Positive Antibody Staining (Intensity Per Area)		
	Stattic Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	12.2	63.4	51.2
β-catenin (red)	26.1	43.4	17.3
E-cadherin (green)	28.4	15.7	-12.8
N-cadherin (red)	73.9	26.2	-47.7
	JQ1 Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	12.2	44.1	31.9
β-catenin (red)	26.1	36.9	10.7
E-cadherin (green)	28.4	27.3	-1.1
N-cadherin (red)	73.9	64.3	-9.6

Figure A.11. The IOMM Lee JR meningioma cell line was stained with Vimentin, β-catenin, E-cadherin, and N-cadherin antibodies. The table shows antibody stainings intensity in 24 hours of Stattic and JQ1 treatment. Intensity per area changes in percentage.

	Positive Antibody Staining (Intensity Per Area)		
	Stattic Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	22.1	49.5	27.4
β-catenin (red)	0.0	0.0	0.0
E-cadherin (green)	0.0	12.0	12.0
N-cadherin (red)	35.5	30.6	-4.9
	JQ1 Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	22.1	59.5	37.4
β-catenin (red)	0.0	19.3	19.3
E-cadherin (green)	0.0	0.0	0.0
N-cadherin (red)	35.5	35.4	-0.1

Figure A.12. The AC599 meningioma cell line was stained with Vimentin, β-catenin, E-cadherin, and N-cadherin antibodies. The table shows antibody stainings intensity in 24 hours of Stattic and JQ1 treatment. Intensity per area changes in percentage.

	Positive Antibody Staining (Intensity Per Area)		
	Stattic Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	45.3	34.0	-11.3
β-catenin (red)	27.3	36.0	8.7
E-cadherin (green	17.4	9.8	-7.5
N-cadherin (red)	29.2	19.9	-9.3
	JQ1 Treatment		
	0 Hour	24 Hour	Percent Change
Vimentin (green)	45.3	27.9	-17.4
β-catenin (red)	27.3	32.4	5.1
E-cadherin (green	17.4	13.3	-4.1
N-cadherin (red)	29.2	72.6	43.5

Figure A.13. The AC599 JR meningioma cell line was stained with Vimentin, β-catenin, E-cadherin, and N-cadherin antibodies. The table shows antibody stainings intensity in 24 hours of Stattic and JQ1 treatment. Intensity per area changes in percentage.

G. Compositions of the Buffers Used in This Study

Propidium Iodide* (*PI) Staining

50 µl 0.4x Triton X

4 µl RNase A (10 mg/ml)

4 µl Propidium Iodide (PI) (1 mg/ml)

142 µl 1x PBS (Cold)

Immunofluorescence TBST

50 ml 1x PBS

50 µl 0.1% Triton X-100

Immunofluorescence Blocking Buffer

50 ml 1x PBS

50 µl 0.1% Triton X-100

0.50 gr BSA

H. EMT Biomarker qPCR Primers

Table A.1 qPCR Primers of EMT TFs

Genes	*Primer Sequence*	*TM*
ACTIN Forward	5'TTGCCCTGAGGCTCTTTTCCA'3	61.95
ACTIN Reverse	5'GGTCTTTGCGGATGTCCACG'3	61.64
SNAIL Forward	5'AGCCCAGGCAGCTATTTCAG'3	60.11
SNAIL Reverse	5'CTGGGAGACACATCGGTCAG'3	59.83
SLUG Forward	5'CAACGCCTCCAAAAAGCCAA'3	59.90
SLUG Reverse	5'ACTCACTCGCCCCAAAGATG'3	60.04
TWIST Forward	5'CAAAGAAACAGGGCGTGGGG'3	61.81
TWIST Reverse	5'GCAGAGGTGTGAGGATGGTG'3	60.39
CTNNB1 Forward	5’TCTTACACCCACCATCCCAC’3	58.70
CTNNB1 Reverse	5’GCACGAACAAGCAACTGAAC’3	58.97