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INVESTIGATING THE RELATIONSHIP OF BRAF AND PSUDO BRAF GENETIC POLYMORPHISM IN LEUKEMIA

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INVESTIGATING THE RELATIONSHIP OF BRAF AND PSUDO BRAF GENETIC POLYMORPHISM IN LEUKEMIA

By Mustafa Haytham Jameel AL-KHSHALI

September 2023

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

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ABSTRACT

INVESTIGATING THE RELATIONSHIP OF BRAF AND PSUDO BRAF GENETIC POLYMORPHISM IN LEUKEMIA

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Master of Science in Biology

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September 2023

Leukemia is the cancer of the body's blood-forming processes, including the bone marrow and lymphatic systems. There are many types of leukemia. Some forms of leukemia are more common among children, while other forms of leukemia often affect adults. This study aims to detect the prevalence of V600E pathogenic mutations and other mutations in the BRAF gene and PBRAF that cause leukemia. In this study, BRAFP1 was looked at in 14 people with leukemia. This was done to find and record new polymorphisms that cause leukemia, as well as to figure out the genetic change and how it affects the normal function of the gene. 19 samples were collected at the Medical City Hospital in Baghdad between June 2022 and September 2022. The samples were divided into two groups. The first group was made up of 14 leukemia patients. And the second group had five samples as a control group (normal individuals). Each participant provided a single blood sample in a container with a mouth-tight lid and a capacity of 5 mL for the study. Standard phlebomy procedures were followed to collect blood samples from volunteers. These samples were placed in 5 mL ethylenediamine-tetraacetic acid tubes. There were significant differences in the DNA sequence of the BRAF gene that could be seen among patients; second, no difference could be observed among normal or control patients. Nine patients showed the highest divergence in the BRAF gene sequence among patients. Upon examining the difference in colors of squares in the matrix, patients showed almost the same percentage of difference in the BRAF DNA sequence of 73%–79%, indicating a common change at a specific location that will be investigated. While BRAFP1 compares results between control and patients,

the following conclusion can be drawn: first, there is a specific difference in similarity among patients since the score generated did not exceed 60%. Comparing patients with normal, the score generated is about 99%. This can be explained on the basis of the position of the sequence aligned in patients; second, when patients sequences were aligned with control, only a few changes in the sequence were observed, which means there are specific changes at certain positions in BRAFP1, the gene in patients with leukemia.

2023, 60 pages

Keywords: BRAF, Oncogenes, Leukemia, Genetic polymorphism, Epigenetics

ÖZET

LÖSEMİDE BRAF VE PSUDO BRAF GENETİK POLİMORFİZMİ İLİŞKİSİNİN İNCELENMESİ

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Lösemi, kemik iliği ve lenfatik sistemler de dâhil olmak üzere vücudun kan oluşturan süreçlerinin kanseridir. Birçok lösemi türü vardır. Bazı lösemi türleri çocuklar arasında daha yaygınken, diğer lösemi türleri genellikle yetişkinleri etkiler. Bu çalışma, BRAF geninde ve PBRAF'ta lösemiye neden olan V600E patojenik mutasyonlarının ve diğer mutasyonların yaygınlığını tespit etmeyi amaçlamaktadır. Bu çalışmada lösemili 14 kişide BRAFP1'e bakılmıştır. Bu, lösemiye neden olan yeni polimorfizmleri bulmak ve kaydetmek, ayrıca genetik değişikliği ve genin normal işlevini nasıl etkilediğini anlamak için gerçekleştirilmiştir. 19 örnek Haziran 2022 ve Eylül 2022 tarihleri arasında Bağdat'taki Medical City Hastanesi'nde toplanmıştır. Örnekler iki gruba ayrılmıştır. İlk grup 14 lösemi hastasından oluşmuştur. İkinci grupta ise kontrol grubu (normal bireyler) olarak beş örnek alınmıştır. Her katılımcı, çalışma için ağzı sıkı kapaklı ve 5 mL kapasiteli bir kapta tek bir kan örneği vermiştir. Gönüllülerden kan örneklerini almak için standart flebomi prosedürleri izlenmiştir. Bu örnekler 5 mL'lik etilendiamin-tetraasetik asit tüplerine yerleştirilmiştir. BRAF geninin DNA diziliminde hastalar arasında görülebilen önemli farklılıklar söz konusu olmuştur; ikinci olarak, normal veya kontrol hastaları arasında herhangi bir farklılık gözlenmemiştir. Hastalar arasında BRAF gen diziliminde en yüksek farklılığı dokuz hasta göstermiştir. Matristeki karelerin renklerindeki farklılık incelendiğinde, hastalar BRAF DNA dizisinde %73-%79 arasında neredeyse aynı oranda farklılık göstermiştir, bu da araştırılacak belirli bir konumda ortak bir değişiklik olduğunu göstermektedir. BRAFP1 kontrol ve hastalar arasındaki sonuçları karşılaştırırken, şu sonuç çıkarılabilir: ilk olarak, oluşturulan skor

%60'ı geçmediği için hastalar arasında benzerlik açısından belirli bir fark söz konusudur. Hastalar normallerle karşılaştırıldığında, elde edilen skor yaklaşık %99'dur. Bu, hastalarda hizalanan dizinin pozisyonuna dayanarak açıklanabilir; ikincisi, hasta dizileri kontrol ile hizalandığında, dizide sadece birkaç değişiklik gözlenmiştir, bu da lösemili hastalarda gen olan BRAFP1'de belirli pozisyonlarda spesifik değişiklikler olduğu anlamına gelmektedir.

2023, 60 sayfa

Anahtar Kelimeler: BRAF, Onkogenler, Lösemi, Genetik polimorfizm, Epigenetik

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Mustafa Haytham Jameel AL-KHSHALI

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LIST OF SYMBOLS

^{o}C	Degree celsius
hr	Hour
µL	Microliter
mg	Milligram
min	Minute
mL	Milliliter
%	Percentage
rpm	Revolutions per minute

LIST OF ABBREVIATIONS

Kinase/ERK	Extracellular-signal-regulated kinase
KRAS	Ki-ras2 kirsten rat sarcoma viral oncogene homolog
RAS/MAPK	Mitogen-activated protein kinase
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
BRAF	Serine/threonine-protein kinase B-raf
SOS1	Son of sevenless homolog 1
PTPN11	Tyrosine-protein phosphatase non-receptor type 11-gallus

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1. INTRODUCTION

Leukemia is the cancer of the body's blood-formingassue, including bone marrow and lymphatic system. There are many types of leukemia. Some forms of leukemia are more common among children. While other forms of leukemia often affect adults (Aken *et al.* 2017).

Leukemia typically includes white blood cells. White blood cells are the first line for defense in your body for infection control, and they grow and divide in an orderly way, depending on the body's needs. But in the case of patients with leukemia, the bone marrow produces excess amounts of abnormal white blood cells, which do not function properly (Straniero *et al.* 2017).

Treatment for leukemia may be complicated, depending on type of leukemia and some factors. But there are strategies and resources that some strategies and resources canatment. The symptoms of leukemia are often vague and indeterminate. Early leukemia symptoms may be ignored because they may resemble flu symptoms and other common diseases (Liu *et al.* 2018).

Leukemia is often detected while undergoing blood tests for some other conditions.

Leukemia is hypothesized to arise when certain blood cells experience DNA abnormalities. DNA in cells includes instructions for carrying out each activity, and cell DNA usually directs two things: growth at a sure rate and death at a specified time. In case of leukemia, mutations direct blood cells to continue dividing and expanding throughout the process of creating new blood cells (Hong *et al.* 2019). The amount of healthy white blood cells, red blood cells, and platelets decreases as a result of these aberrant blood cells' ability to overtake healthy blood cells in the bone marrow over time ,showing indications of leukemia and its symptoms (Chowdhry *et al.* 2019).

V600E is a mutation of the BRAF gene in which valine (V) at amino acid 600 is replaced by glutamic acid (E). It is a driver mutation in several diagnoses, such as hairy cell leukemia, melanoma the non-Langerhans-cell histiocytosis Erdheim-Chester sickness, papillary thyroid carcinoma, colorectal cancer, non-small-cell lung cancer, Langerhans cell histiocytosis, and ameloblastoma (Adeniran *et al.* 2011).

Due to its acidic glutamic acid residue's negative charge, which mimics phosphorus, which is the mechanism of mutation. The neighboring T599 threonine and S602 serine residues in activation section of BRAF, which are phosphorylated to activate the protein's wild-type version, are mimicked by this action (Al-Rahawan *et al.* 2007). As a result, the mutant's wild-type residue serves to activate BRAF, making interaction between glycine-rich loop of BRAF and the activation seglycine-rich normally inhibitory. BRAF's baseline activity increases when its inhibition is removed, which makes it carcinogenic (Ascierto *et al.* 2012).

Aim of the study

This study aim is to detect the prevalence of V600E pathogenic mutation and othe once in BRAF gene and PBRAF causing Leukemia by detect and registering new polymorphisms causing leukemias and it allelic distribution.

2. LITERATURE REVIEW

Leukemia is a malignant blood illness, according to a research taken by the Middle East Cancer Research Group, 515 Egyptians and 1,354 Jordanians were diagnosed with leukemia between 1999 and 2001. Additionally known as leukemia or lymphoma. This illness starts in the bone marrow and then spreads throughout the body (Schutte *et al.* 2017).

Leukemia is a category of malignant tumors that often develop in bone marrow and are propertized by an abnormally large number of white blood cells. These fully undeveloped cells are referred to be tumors. Symptoms include bleeding, complications, exhaustion, fever, and an elevated risk of infection (Brandl *et al.* 2018). These symptoms are caused by a deficiency of normal blood cells and are detected by blood testing or a bone tissue biopsy. Blood cancer has an unknown etiology, which varies depending on the kind of leukemia (Farinello *et al.* 2018). Leukemia may be caused by both genetic and environmental causes. Smoking, drinking, and being overweight are risk factors for leukemia, ionizing radiation and some chemicals like the gasoline ring or in case of prior chemotherapy and Down's syndrome (Blagitko *et al.* 2019).

People with a family history of leukemia also have an increased incidence of leukaemia. There are four main types of leukemia: chleukemiaphoblastic leukemia, acute myeloid leukemia, acute lymphoblastic leukemia and chronic myeloid leukemia. Leukemia and lymphoma, fall under the type of cancer that affects the blood, bone marrow, lymph node system and is called tumors of blood compand onents and lymph tissue. Leukemia treatment includes a combination of chemotherapy, radiotherapy, targeted therapy, bone marrow transplantation, and supportive and painkiller care (Miller *et al.* 2018). The effectiveness of therapy relies on the kind of leukemia and the patient's age. In wealthy nations, treatment outcomes are highly refined. Infected individuals have a 57% five-year survival rate in the USA. Depending on the kind of cancer, the 5-year survival rate for children under 15 is between 60 and 85 percent (Wang *et al.* 2019).

Children who developed acute leukemia and then were treated and remained healthy for 5-years will often not suffer from the cancer in 5 years. In 2012 a 352 thousand people were diagnosed with leukemia around the world, resulting the death of 265 thousand cases. Leukemia is most common among children, with three quarters infected children with acute leukemia three-quarters of leukemia is diagnosed in adults and the type is often chronic leukemia or acute lymph (Rothe *et al.* 2019).

Table 2.1 Leukemia Types

Cell Type	Severe	Chronic
Blood Bleaching for Mvaoui "Lymphoblastic Bleaching"	Acute Lymphoblastic Leukemia (ALL)	Chronic Lymphoblastic Leukemia (CLL)
Acute Myeloid Leukemia (AML or aromas)	Chronic Myeloid Leukemia (CML)	Bleaching Blood ("Marrow" or "non-MFU")

Acute cancer is characterized by an increase in the number of immature blood cells, which crowds the bone marrow and decreases its capacity to create normal cells (Moosavi *et al.* 2019). Due to its fast progression, acute leukemia need early treatment and gathers cancer cells that have moved from the blood to other organs. Acute leukemia is the most prevalent form of cancer among children (Shen *et al.* 2018).

Chronic cancer is characterized by a huge number of relatively mature white cells but possesses an abnormal form. White blood cells often need months or years to grow; however, in this scenario, white blood cells mature rapidly, resulting in a significant number of aberrant white cells (Wright *et al.* 2020). Acute leukemia need prompt treatment, but chronic leukemia necessitates monitoring for a period of time prior to therapy in order to maximize therapeutic efficacy. Chronic leukemia mainly affects elderly folks but may afflict individuals of any age (Shetty *et al.* 2021).

In addition, Leukemia may be subdivided based on the kind of blood cell that is afflicted, resulting in lymph tissue cancer and bone marrow cancer.

In lymph tissue cancer, a kind of bone marrow cell that typically transforms or creates lymphocytes, which are immune cells that combat illness, undergoes malignant transformation. The majority of lymph tissue tumors comprise beta lymph cells (Trager *et al.* 2022).

Blood marrow cancer is characterized by the malignant transformation of bone marrow cells that ordinarily generate red blood cells, some types of white blood cells, and platelets (Cives *et al.* 2020).

2.1 Types of Leukemia

Acute lymphoblastic Leukemia (ALL) is the most frequent kind in children and mostly affects adults over the age of 65. chemotherapy is included in treatment and radiotherapy, the rate varies depending on the patient's age like 85% in children and 50% in adults with this ratio its shown that its more effective in children more than adults (Coccaro *et al.* 2020). Branches of the type are acute lymphatic tissue cancer of B cells, acute lymphatic tissue cancer of B cells, burkat cancer, acute leukemia which has two formative representations (Ilie *et al.* 2019).

Chronic Lymphoblastic Leukemia (CLL), which often affects adults above the age of 55. Chronic cancer is incurable, however several effective therapies are available. For chronic leukemia, there is one subcategory, the bleaching of precursor lymphocytes B, which is a more aggressive disease (Corné *et al.* 2021).

Acute myeloid leukemia (AML) effects adults more often than children and men more frequently than woman, treatable with chemotherapy. The 5-year survival rate is 40%, with exception of acute proplymphocene leukemia, for which it is 90%. Acute myeloid leukemia has three subtypes: acute bromphocytic leukemia, acute myoplasty leukemia

characterized by alymphocytic muscle cells, and megakaryoblastic leukemia (Arance *et al.* 2022).

Chronic myeloid leukemia (CML) often occurs affecting adults and extremely few children. It is treated with Lmatinib or other medications. 90% of patients survive after five years (Vanni *et al.* 2020).

Hairy cell leukem (HCL) is myelomonocyticonic lymphocyte carcinoma but does not fit exactly into this category. 80% of those infected are male adults. It was not reported that any cases had been recorded in the children. There are treatments for (HCL), but the (HCL) cannot be cured. Survival rate from 96 to 100% in ten years (Chen *et al.* 2021).

The survivalolymphocytic leukemia (T-PLL) is a very rare and dangerous disease that affects adults and often more male than female. Although scarce, it is most common than blood cancers that affect T cells, most types of blood cancers affect B cell. Curing it is challenging, and the survival rate is only measured in months (Ghanadan *et al.* 2021).

Lymphoblastic leukemia that affects large pellets and either contains Tcell or NKcells, is a rare disease such as hyperleukemia, which occurs only in B cells and is not aggressive (Ottaviano *et al.* 2021).

Leukemia to adult T cells. It is caused by a virus called the human virus that infects Tcell (HTLV), a virus similar to even HIV; Where both infect and multiply CD4 + Tcell cells. Anyway, it's different from HIV in that it doesn't destroy these cells. Instead, HTLV "immortalizes" infected T cells, allowing them to reproduce abnormally. Human T Cell Virus Type I and II (HTLV-I/II) is endemic in specific areas of the world (Nikolouzakis *et al.* 2021).

2.2 Symptoms and Signs

2.2.1 Common signs of acute or chronic leukemia

In leukemia, immature white blood cells replace normal bone marrow cells in vast numbers resulting in broken bone marrow. As a result, there are fewer platelets that help normal in the process of corrosion. This means that people with bruises are easily bruised, suffer from excess bleeding, and have bleeding on the surface of the skin or so called freckles (Li *et al.* 2012). White blood cells (responsible for fighting nursing organisms) number will decrease, or they will lose their normal function. This weakens the immune system and becomes unable to cope with minor infections or starts by fighting other body cells (Stonesifer *et al.* 2021).

Leukemia suffers from repeated infections to weakened immune systems and these infections range from tonsillitis, oral ulcers, diarrhoea to pneumonia or opportunistic infections. The number of red blood cells decreases, leading to anemia, which in turn causes pale and shortness of breath (Davidsson *et al.* 2018). Some sufferers experience other symptoms such as feeling sick, high temperature, sweating during the night, feeling exhausted and other symptoms similar to those associated with infonza. It is also possible to feel nauseous and full due to enlarged liver and spleen resulting in unintended underweight. Immature cells may accumulate and form liver bloating or lymph nodes, resulting in pain and nausea (Gouda *et al.* 2022).

If cancer cells over run the central nervous system, symptoms and neuromarkers will show the most important headaches. Other symptoms include migraines and neurological convulsions, and high pressure on the brainstem leads to fainting. All these symptoms may be associated with other diseases, so leukemia is diagnosed by medical examinations. Leukemia is derived from the presence of large numbers of white blood cells (Pardo *et al.* 2020). These numbers appear when a sample of blood is examined

using a microscope. These excess cells have abnormal shapes or abnormal function. The presence of a large number of white blood cells affects the numbers of other blood cells resulting in an imbalance in the amount of blood (Chen *et al.* 2018).

Some leukemia patients may not have an increase in white blood cells when conducting a blood count test. This condition is called aleukemia. In this case, the bone marrow contains cancerous white blood cells that destroy the normal cells responsible for producing blood cells. The cancer cells themselves remain in the bone marrow instead of going out into the blood (Lopez *et al.* 2019). The scarcity of leukemia can occur in the previous four types of leukemia but the most common is accompanied by hyperbolic cancer. Bone marrow filling with cancer cells inhibits the process of building normal blood cells resulting in a lack of red blood pellets (anemia) and platelet stalks, which play an essential role in the process of blood clotting. Hence, leukemia patients may suffer from bleeding that often occurs in the gums, or the surface of the skin and appears as small subcutaneous bleeding spots the size of the needle head known as inks (Haugh *et al.* 2019).

2.3 The Cause of Leukemia

There is no one cause for this harm disease, although there are many reasons for its various subtypes. The reason behind most of its species is unknown, the causes may be due to the family history of the disease, ionizing radiation and chemotherapy, and the cause varies according to the type of cancer (Masetti *et al.* 2018). Leukemia results from a mutation of genetic material by activating carcinogenic genes or disrupting tumor suppressor genes and thus disrupting the regulation of cell death. Differentiation or division of these steps can occur automatically (i.e. without any external effect) or can occur due to exposure to ion radiation or carcinogens (Picharski *et al.* 2019). Natural and industrial ion radiation is the most common cause in adults and some viruses such as human virus of lymphatic tissue and some chemicals, especially benzene and basal chemotherapy, may lead to this cancer. Smoking increases the risk of acute myeloid leukemia in adults by a small proportion (Kim *et al.* 2014).

During some studies such as Cohort and Case-Control, exposure to certain petrochemicals and hair dyes was linked to leukemia. Diet has no role in infection, but eating vegetables may protect. Viruses that have been traced to this cancer, including Tlymphatic tissue human virus, which causes adult T lymphocytes' leukemia. Some individuals have a genetic factor for the development of leukemia, which is determined by family history and twin studies. Infected people have one or several common genes (Shiba *et al.* 2019). In some families, individuals develop the same type of leukemia and in others they develop different types or different types of blood-related cancers. Individuals with chromosomal anomalies have a higher incidence of this cancer. For example, those with Down's syndrome have a higher risk of leukemia (especially acute leukemia). A mutation in Jenspred1 also plays a role in developing leukemia during childhood (Bager *et al.* 2018).

Decades of research have been devoted to determining whether non-ionizing radiation may cause leukemia. The World Cancer Research Organization's working group of specialists has undertaken detailed research into all data on static and low-frequency electromagnetic fields that appear naturally when generating, transporting and using electrical energy (Duployez *et al.* 2018). They found that there is limited evidence that exposure to large amounts of low-frequency magnetic waves may cause certain types of leukemia in children and that no relationship with other blood cancers and tumors in adults has been established. Exposure to such quantities (if proven as a causative agent) is uncommon (Niktoreh *et al.* 2019). The World Health Organization found that such radiation caused 100-2400 cases of leukemia in children annually, 0.2 to 4.9% of cases of paediatric leukemia in that year and 0.03-0.9% of all cases of leukemia were recorded in children as a result of transmission from mother to fetus in the case of leukemia during pregnancy. Children born to mothers who use fertility medications to stimulate ovulation are times as likely as other children to acquire childhood leukemia (Aplenc *et al.* 2020).

2.3.1 Factors that can lead to Leukemia

Radiation exposure. It was noted that radiologists from doctors, technicians and patients treated for Ankylosis Spondylitis using radiation compared to patients with non-radiotherapy treatments and atomic bomb survivors in Nakazaki and Hiroshima all showed a higher incidence of leukemia (Conneely and Stevens 2021).

Exposure to chemical chemicals such as chloramphenicol, benzene, and some pesticides. Several medications are used to treat malignancies, such as vinyl alanine vinegar, which is used to treat multiple myeloma, as well as some immunosuppressant pharmaceuticals and basal chemicals used to treat Hodgkin's disease (Roberts and Izraeli 2014).

2.3.2 Genetic disorders which lead to Leukemia

Viruses, like Human T-Lymphocyte virus, have the first HTLV-1 and the second HTLV-2 by introducing a gene and a throw (Oncogene) of human cells which makes them cancerous (Niemeyer and Mecucci 2017).

2.4 Diagnosis

Diagnosis of the disease is heald with several tests:

1. Complete Blood Count CBC. Through this examination, initial signals of possible leukemia can be given.
2. Clinical examination to detect inflation of the liver, spleen or lymphatic nodes
3. A biopsy of the bone marrow that is examined under a microscope after the addition of specific pigments that reveal the presence of blood washing.

Sometimes a patient's leukemia is not detected by blood testing, especially during the onset of the disease or during the latency of the disease, i.e. without symptoms. In some

cancers a biopsy of lymphatic nodes can be used. After diagnosis, Chemical blood tests may be used to assess the patient's response to chemotherapy as well as the patient's kidney and liver damage. It is possible to use x-ray, which is X-ray, ultrasound ultrasound or MRI (Zhang *et al.* 2016, Arber *et al.* 2016). These tests are used to detect damage to bones, brain (MRI), kidney, spleen or liver (ultrasound). CT can used to examine the lymphatic nodes of the chest but this method is rarely used. Although these methods are used to diagnose the patient if he is infected or recovered, people haven't been diagnosed due to misdiagnosis of the similarity of symptoms with other diseases. For this reason, the American Cancer Society estimates that at least one fifth of people with leukemia have not been diagnosed with the disease processing (Laing *et al.* 2017).

2.5 Treatments

There are several treatments for leukemia. Appropriate therapy is administered based on the patient's state, severity of patients and kind of disease (Sanz *et al.* 2019). One of the most commonly used treatments is:

1. Chemotherapy: in which the patient is administered drugs such as (hydroxyurea, busulfan, etoside, glivec, tasigna, daunorubicin) and others to eliminate cancer cells.
2. Radiotherapy: exposing the patient to radiation to eliminate cancer cells.
3. Bone marrow transplantation

2.5.1 Auxiliary treatments

- Blood transfusion: To treat anemia and to treat blood losing.
- Antibiotics and Interferon: To be immunologically supported to increase the patient resistance against diseases.

2.6 BRAF Gene

The BRAF gene on chromosome 7 (7q34) encodes the BRAF protein, which participates in the MAP kinase/ERK signalling pathway. This pathway regulates important cell functions including cellular growth, differentiation, proliferation, senescence and apoptosis. Different variant products of the BRAF gene have been reported as both activating and silencing the RAS/MAPK pathway. Additionally, an increase in protein expression or activity can disturb the Ras–MAPK signalling pathway, which in turn can result in different developmental disorders such as Noonan syndrome (NS), cardio-facio-cutaneous (CFC) syndrome, Costello syndrome, and different types of human cancers (Badalian *et al.* 2010).

Similar to BRAF, germline mutations in the PTPN11, SOS1, RAF, and KRAS genes also influence the RAS/MAPK signalling pathway and contribute to the development of NS in approximately 70% of patients (Badalian *et al.* 2012).

In addition to germline mutations, somatic mutations of BRAF have been identified in Langerhans cell histiocytosis, Erdheim–Chester disease, lung, colon, thyroid, and melanoma malignancies, as well as non-Hodgkin lymphoma (Benlloch et al. 2006). More than 80% mutations in the BRAF gene have been reported with varied frequencies. For instance, ~67% of melanomas exhibit BRAF mutations, whereas 30% of other tumours, such as thyroid cancer (15%), lung cancer (3%) and colorectal cancer (12%), present with BRAF mutations (Bertola *et al.* 2001). Among all cancers, V600E, has been examined in 66% of malignant melanomas, and specifically localised to the serine/threonine kinase domain. Additionally, somatic mutations resulting in BRAF heterozygous variants in kinase domain, such as G1382T (R461I), T1385G (I462S), G1388A (G463E), and A1798G (K600E), have been identified in colorectal cancer patients. According to some recent reports of the past 2–3 years, somatically acquired T1799A (V600E) mutation has been characterised in hairy cell leukaemia and related lymphoproliferative disorders (Bissler *et al.* 2013). However, the somatic mutations with G465V (exon 11) and L596R (exon 15) amino acid substitutions have been characterised in human lung adenocarcinoma. An in-frame fusion of the AKAP9 gene (exons 1–8) to the BRAF gene (exons 9–18), which occurs through a paracentric inversion of chromosome 7, has been preferentially recognised in radiation-induced

papillary carcinomas, compared to BRAF point mutations. Moreover, two novel BRAF gene fusions-KIAA1549-BRAF and FAM131B-BRAF-have been identified in pilocytic astrocytomas and one (PAPSS1-BRAF) in melanoma (Blombery *et al.* 2012).

The essence of the new method discovered by scientists from the British University of Lester, in the use of medical preparations for treatment of skin cancer such as vemurafenib (BRAF inhibitors). Tests have shown that these tablets clean blood from cancer cells and lead to rapid treatment within a few days (Bollag *et al.* 2012).

The tests included the study and analysis of several blood samples of patients with leukemia, where they were observed to have mutations in the gene Braf, which is observed in melanoma. Based on these findings scientists decided to use these preparations in the treatment of leukemia (Bosmuller *et al.* 2013).

The results were surprising, with significant improvements observed in patients even those in difficult condition. Despite these good results, scientists are afraid that this will be the hunger of the disease (Brose *et al.* 2002).

The BRAF genetic test checks for a change in the BRAF gene, often known as a mutation. It should be noted that genes are basic units of mother and father's inheritance (Cardarella *et al.* 2013).

The BRAF gene encodes a protein that helps regulate cell development. It is known as the tumor gene. The tumor gene works as a gasoline pedal in the car where it develops (Chappell *et al.* 2011).

However, if you have a BRAF mutation, you are susceptible to cancer. It means that the throttle breaks down, and the gene cannot then prevent cell growth. Unregulated cell growth may result in cancer (Cho *et al.* 2006).

The BRAF mutation is either inherited from one's parents or acquired later in life. Mutations acquired later occur due to the environment or an error in the body during cell division. Although genetic BRAF mutations are very uncommon, they may have major health consequences (Cin *et al.* 2011).

Acquired BRAF mutations (also known as physical mutations) are more common and widespread. The most dangerous kind of skin cancer, melanoma, has these alterations in nearly half of cases (Clancy *et al.* 2013). BRAF mutations have been linked to a number of diseases and cancers, including ovarian, thyroid, and colon cancer. Tumors that have the BRAF mutation are often more deadly than cancers without it (Davies *et al.* 2002).

This test will be used to identify BRAF mutations in patients with skin cancer or other BRAF-related malignancies. Certain anti-cancer medications are especially effective in patients with a BRAF mutation. The medications themselves are ineffective and occasionally harmful for those who lack a mutation (Denayer *et al.* 2010).

Also, The BRAF test may be used to determine cancer risk based on family history or personal medical history (Deng *et al.* 2004).

Most BRAF tests are done by biopsy of the tumor. During biopsy, the specialist takes out a small piece of tissue by cutting or cavity the surface of the tumor. If the specialist needs to test the tumor tissue from inside the body, he may use a special needle to pull the sample (Elisei *et al.* 2008).

2.7 BRAF-Genetic Mutation's Test

If the patient has skin cancer or any other type of cancer, the findings reveal the existence of a BRAF mutation, the doctor may prescribe drugs meant to target the mutation. These medications can be more successful than other therapies (El-Osta *et al.* 2011).

If the patient suffers from skin cancer or any other type of cancer, and results show that he does not have a BRAF mutation, the doctor will prescribe different types of drugs for cancer treatment (Fargnoli *et al.* 2008).

If cancer is not diagnosed and results show that a person has a BRAF genetic mutation does not indicate cancer, but it does increase the chance of cancer. But periodic and frequent cancer tests, such as skin screening, can reduce the risk of infection (Feng *et al.* 2013). During the skin examination, the doctor carefully examines the skin to check for moles or any other growth that raises suspicions (Forschner *et al.* 2013). There are several BRAF mutation kinds, the most prevalent kind of BRAF mutation is the V600E variant (Gear *et al.* 2004).

BRAF Pseudogene are similar to coding BRAF Gene but contain defects that render them unable to encode fully functional proteins (Haroche *et al.* 2012). Mistakenly considered non-functunal, pseudogenes have long been dismissed in the understanding of the functions. Pseudogenes have been established as versatile regulators of gene expression and key players in the pathogenesis of human cancer (Huang *et al.* 2013).

3. MATERIALS AND METHODS

3.1 Material

3.1.1 Instruments and Equipments

Different kinds of instruments and many equipment have been used to process the collected samples and they are listed in Table 3.1.

Table 3.1 Instruments and equipment have been used in this study

Instrument / Equipment	Company	Origin
Autoclave	Hirayama	Japan
Cooling Centrifuge	Eppendorf	Germany
Deep Freeze -80	Binder	Germany
Electrophoresis System	Cleaver	United Kingdom
Gel Documentation	Cleaver	United Kingdom
Gel Tube	HiLab	India
Gradient Thermocycler	Eppendorf	Germany
Hot plate	VELP	Germany
Micropipettes	Eppendorf	Germany
Qubit 4	Invitrogen	USA
Refrigerator	Kelon	Korea
Sensitive balance	Denver	European Union
Thermomixer	Eppendorf	Germany
Vortex	VELP	Germany
Water Distillater	GFL	Germany
Water-bath	GFL	Germany

3.1.2 Chemicals

The chemicals and their origins have been used in this study are been mentioned in Table 3.2.

Table 3.2 Chemicals used in this study with their origins

Chemical Compound	Company (Origin)
Absolute ethyl alcohol (99.9%)	Diamound® (France)
Agarose	Cleaver® (England)
ddH2O	Bariq (Iraq)
DNA Marker (100-1000) bp	NEB® (England)
Free Nuclease Water	NEB® (England)
Normal Saline	Bioneer® (Iraq)
RedSafe dye	Intron®(South Korea)
TAE Buffer (50x)	Carl ROTH® (Germany)

3.1.3 Kits

The kits used in this study with their sources are given in Table 3.3.

Table 3.3 Kits used in this study with description and origin

Product Name	Description and Components	Company (Origin)
Monarch® Genomic DNA Purification Kit	The Monarch Genomic DNA Purification Kit is a comprehensive solution for cell lysis, RNA removal, and purification of intact genomic DNA (gDNA) from a wide variety of biological samples, including cultured cells, blood, and mammalian tissues. Additionally, bacteria and yeast can be processed with extra steps to enhance lysis in these tough-to-lyse samples.	NEB® (USA)
OneTaq® 2X Master Mix	One*Taq*® DNA Polymerase is an optimized blend of *Taq* and Deep Vent™ DNA polymerases for use with routine and difficult PCR experiments.	NEB® (England)
Qubit™ dsDNA HS Assay Kit	The Qubit dsDNA HS (High Sensitivity) Assay Kit is designed specifically for use with the Qubit Fluorometer. The assay is highly selective for double-stranded DNA (dsDNA) over RNA	ThermoFisher® (USA)

3.1.4 Working group

19 samples where colected in medical city hospital-baghdad between June 2022 and Septemper 2022. The samples were devided into two groups, first group was a 14 leukemia patients. And the secound group was a 5 samples as a control group (Normal Individuals). Each participant provided a single blood sample in a container with a mouth-tight lid and a capacity of 5 mL for the study. Standard phlebomy procedures were followed to collect blood samples from volunteers. These samples were placed in 5 mL ethylenediaminetetraacetic acid tubes. We centrifuged the tubes at room temperature for 10 minutes at 1000 Rpm. Cryovial tubes were stuffed with plasma that had been collected. Aliquots were separated into different tubes, with one being used right away for RNA extraction, while other ones was tagged and stored at a temperature of -80 ^{o}C. In the first three hours after sampling, we kept everything at -80 ^{o}C.

3.2 Scales and Methods Used in the Study

3.2.1 RNA isolation from blood

Trizol Reagent was used to perform RNA isolation from total blood. For the lysis of blood cells according to the kit protocol, 1 mL of trizol and 300 µL of blood were taken into an ependropod and vortexed. It was incubated on ice for 15 min. At the end of the incubation, 200 µL of chloroform was added and vortexed for 10 seconds. It was centrifuged at 4 ^{o}Celsius and 12,000 times its original weight for 15 minutes. A fresh Eppendorf tube was used to store the remaining 80% of the supernatant that had been created by centrifugation. After adding 500 µL of isopropanol, the mixture was placed in an incubator on ice for ten minutes. Incubation was centrifuged at 12.000 Rpm for 10 min at 4°C. 1000 µL of 70% ethanol was added for the washing step. Afterwards, The samples were centrifuged at x7500g for ten minutes at a temperature of 4 ^{o}C. The RNAs acquired in Ependrof were processed to eliminate 70% of the alcohol. dry at room temperature for ten minutes after being left alone. On top of the RNA pellet, an additional 40 L of nuclease-free water was added.

Before miRNA expression analysis, RNA samples were stored at -80 ^{o}C and their optical densities and concentrations were determined using a UV-Vis Spectrophotometer/ Nano Drop (Beckman Coulter, USA) at a wavelength of 260/280 nm.

3.2.2 Quantity and quality determination of obtained RNAs

The wavelength at which heterocyclic nucleotide rings are most absorbent is 260 nm. For this reason, the absorption values measured at 260 nm wavelength are used to determine the amount of nucleic acids isolated in ng/µL or µL/mL level. The spectrophotometric methods used to determine concentration of RNA are exactly same as the spectral analysis of DNA. Only the formula used to quantify single-stranded

RNA is different (Total RNA (ng/ µL) = absorbance at 260 nm x 50 x Dilution factor) (Zheng *et al.* 2019).

It is known that 1 optical density corresponds to 40 µL/mL for RNA molecules. However, The purity of nucleic acids may be determined by comparing the results obtained at 260 and 280 nm wavelengths. They exhibit absorbance at 280 nm, as is typical for proteins. The A260/A280 ratio therefore decreases when a value measured at 280 nm is increased.This ratio should be 2.00 to talk about the purity of the isolated total RNA samples.

The quantity and quality of the RNA samples isolated in our study were measured in a nanodrop device and their suitability for expression analysis was evaluated.

3.2.3 cDNA Synthesis from blood

The MicroRNA Universal cDNA Synthesis Kit II was used in order to accomplish the synthesis of microRNA cDNA (Exiqon, Denmark).The procedure for the kit, after the RT reaction was prepared on the cold block under the conditions specified in Table 3.1, cDNAs were obtained after incubation at 42ºC for 60 minutes, at 95ºC for 5 minutes and at 4ºC. The cDNAs were diluted 1:50 -1:500 with nuclease water and stored at -20ºC.

Table 3.4 Preparation of RT-PCR mix for miRNA expression

RT-PCR mix	
Multiple by a factor of five buffer	2.0 µL
Zero-nuclease water	4.5 µL
Enzym	1.0 µL
Elevated RNA	0.5 µL
RNA	2.0 µL
Total volume	10 µL

3.2.4 Evaluation of RT-PCR based microRNA expression profiles

A comprehensive RT-PCR experiment was conducted, with negative controls. In order to determine whether or not there were differences in the levels of miRNA expression, the Ct technique was used. The endogenous controls, RNU6, were used for the normalization of the data as reference levels. For miRNA expressions, the initial copy number and Ct values of the samples were determined in the software program of the RT-PCR device. The mean Ct values obtained from the raw data were normalized with the house keeping gene RNU6.

3.2.5 Clinical evaluation

Statistical analysis was performed by comparing the obtained miRNA expression levels of the study participants with the EDSS (Expanded Disability Status Scale) parameters, which included age range which was between 27 to 60 years old, gender which was a 55% male and the fwmale was 45%, immunomodulatory treatment, the overall number of attacks up to the time the research was conducted, the types of leukemia found (AML leukemia and other types) also included, the yearly attack rates, and an extended handicap status scale indicating the severity of condition.

3.3 Statistical Analysis

The Mann-whitney was used to compare groups' demographic information. The duration of the follow-up was established by counting backwards in time from the initial diagnosis to the time when samples were taken. To evaluate if there are The Independent Sample T Test, the Mann-Whitney Test, and the Kruskal Wallis Test were used in order to explore the association between shifts in miRNA expression levels between the RRMS, the Mann-Whitney Test, and the Kruskal Wallis Test. CIS, and RIS groups and the clinical features of individuals diagnosed with RRMS. Reciever Operator Characteristics Curve (ROC) analysis was used compare miRNA expression levels across patient groups and determine diagnostic test's applicability. SPSS 17.00, a

statistical analysis software package, was used to analyze the study's results. The significance level used in analyzing the data was $p<0.05$, and the 95% confidence interval was used.

4. RESULTS AND DISCUSSION

4.1 DNA Alignment and Identity Matrix

BRAF is a gene found on chromosome seven that encodes a protein also called BRAF. This protein plays a role in cell growth by sending signals inside the cell promoting, among other functions, cell division. When any pathogenic change occurs it reflects its effect on normal cell function that eventually influences DNA replication that in some cases leads to malignant tumors or cancer. In this study, 14 patients were subjected to molecular analysis of BRAF against 5 normal and healthy persons. The results of PCR products were subjected to thorough analysis to determine the pattern of DNA change in this gene (Sandahl *et al.* 2015). According to the BRAF gene sequence among the patient group, there were complete and higher genetic similarities among all BRAF gene sequences. In contrast, lower genetic similarity was found between BRAF gene number 9 and other BRAF gene sequences, as shown in Table 4.1.

Percent Identity Matrix

BRAFPatien9	100.00%	68.65%	67.78%	69.61%	68.31%	68.33%	68.33%	68.85%	68.13%	69.19%	68.89%	67.78%	70.45%	70.39%
BRAFPatien7	68.65%	100.00%	90.86%	79.62%	91.49%	92.47%	92.47%	91.49%	91.49%	73.06%	91.94%	94.62%	96.70%	95.68%
BRAFPatien5	67.78%	90.86%	100.00%	98.91%	98.39%	98.92%	98.92%	98.92%	98.92%	98.92%	98.92%	94.62%	94.51%	94.57%
BRAFPatien3	69.61%	79.62%	98.91%	100.00%	99.46%	100.00%	100.00%	100.00%	99.46%	74.18%	100.00%	93.48%	95.60%	95.68%
BRAFPatien2	68.31%	91.49%	98.39%	99.46%	100.00%	100.00%	100.00%	99.47%	98.94%	99.47%	99.46%	93.55%	95.60%	95.14%
BRAFPatien13	68.33%	92.47%	98.92%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	94.05%	95.60%	95.63%
BRAFPatien14	68.33%	92.47%	98.92%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	94.05%	95.60%	95.63%
BRAFPatien11	68.85%	91.49%	98.92%	100.00%	99.47%	100.00%	100.00%	100.00%	99.47%	100.00%	100.00%	93.55%	95.60%	95.68%
BRAFPatien4	68.13%	91.49%	98.92%	99.46%	98.94%	100.00%	100.00%	99.47%	100.00%	99.47%	100.00%	93.55%	95.60%	95.14%
BRAFPatien1	69.19%	73.06%	98.92%	74.18%	99.47%	100.00%	100.00%	100.00%	99.47%	100.00%	100.00%	93.55%	95.60%	95.68%
BRAFPatien6	68.89%	91.94%	98.92%	100.00%	99.46%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	93.55%	95.60%	95.65%
BRAFPatien8	67.78%	94.62%	94.62%	93.48%	93.55%	94.05%	94.05%	93.55%	93.55%	93.55%	93.55%	100.00%	98.35%	97.83%
BRAFPatien10	70.45%	96.70%	94.51%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	98.35%	100.00%	100.00%
BRAFPatien12	70.39%	95.68%	94.57%	95.68%	95.14%	95.63%	95.63%	95.68%	95.14%	95.68%	95.65%	97.83%	100.00%	100.00%

Figure 4.1 Identity matrix of DNA alignment resulted from sequencing PCR product in patients with leukemia

Furthermore, DNA alignment among healthy people was performed to generate an identity matrix for them that can be used as a baseline for comparison, as shown in Figure 4.1. The current study found there were complete and greater genetic similarities among BRAF gene sequences in the control group.

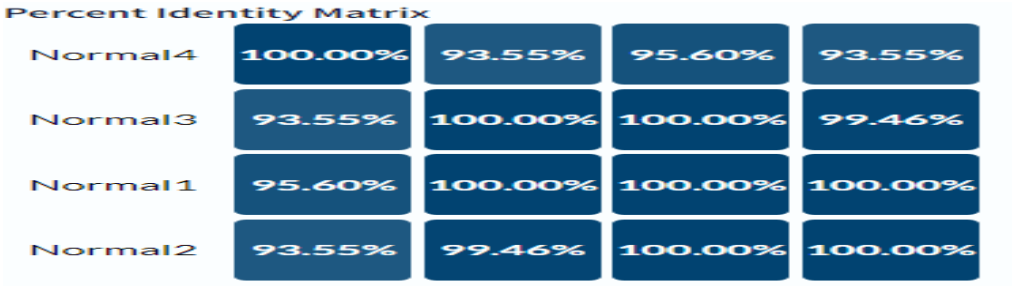
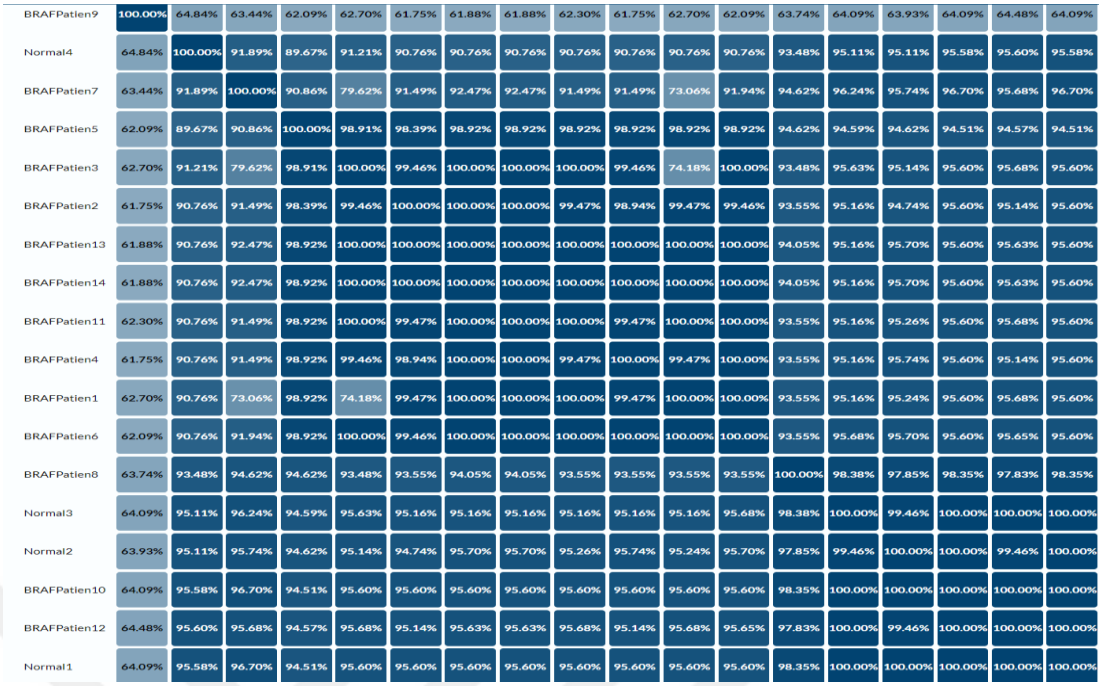


Figure 4.2 Identity matrix of healthy persons used as control generated from DNA alignment of BRAF gene

According to the comparison between the alignment of the BRAF gene DNA sequence in patients and control groups, the current study showed there were complete and greater percentage similarities between all BRAF gene sequences among patients and controls, with the exception of a lower similarity found in BRAF gene sequence number 9 of the patient with leukemia when compared with other BRAF gene sequences in both patients and control subjects, as shown in Figure 4.3.



BRAFPatien9	100.00%	64.84%	63.44%	62.09%	62.70%	61.75%	61.88%	61.88%	62.30%	61.75%	62.70%	62.09%	63.74%	64.09%	63.93%	64.09%	64.48%	64.09%
Normal4	64.84%	100.00%	91.89%	89.67%	91.21%	90.76%	90.76%	90.76%	90.76%	90.76%	90.76%	90.76%	93.48%	95.11%	95.11%	95.58%	95.60%	95.58%
BRAFPatien7	63.44%	91.89%	100.00%	90.86%	79.62%	91.49%	92.47%	92.47%	91.49%	91.49%	73.06%	91.94%	94.62%	96.24%	95.74%	96.70%	95.68%	96.70%
BRAFPatien5	62.09%	89.67%	90.86%	100.00%	98.91%	98.39%	98.92%	98.92%	98.92%	98.92%	98.92%	98.92%	94.62%	94.59%	94.62%	94.51%	94.57%	94.51%
BRAFPatien3	62.70%	91.21%	79.62%	98.91%	100.00%	99.46%	100.00%	100.00%	100.00%	99.46%	74.18%	100.00%	93.48%	95.63%	95.14%	95.60%	95.68%	95.60%
BRAFPatien2	61.75%	90.76%	91.49%	98.39%	99.46%	100.00%	100.00%	100.00%	99.47%	98.94%	99.47%	99.46%	93.55%	95.16%	94.74%	95.60%	95.14%	95.60%
BRAFPatien13	61.88%	90.76%	92.47%	98.92%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	94.05%	95.16%	95.70%	95.60%	95.63%	95.60%
BRAFPatien14	61.88%	90.76%	92.47%	98.92%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	94.05%	95.16%	95.70%	95.60%	95.63%	95.60%
BRAFPatien11	62.30%	90.76%	91.49%	98.92%	100.00%	99.47%	100.00%	100.00%	100.00%	99.47%	100.00%	100.00%	93.55%	95.16%	95.26%	95.60%	95.68%	95.60%
BRAFPatien4	61.75%	90.76%	91.49%	98.92%	99.46%	98.94%	100.00%	100.00%	99.47%	100.00%	99.47%	100.00%	93.55%	95.16%	95.74%	95.60%	95.14%	95.60%
BRAFPatien1	62.70%	90.76%	73.06%	98.92%	74.18%	99.47%	100.00%	100.00%	100.00%	99.47%	100.00%	100.00%	93.55%	95.16%	95.24%	95.60%	95.68%	95.60%
BRAFPatien6	62.09%	90.76%	91.94%	98.92%	100.00%	99.46%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	93.55%	95.68%	95.70%	95.60%	95.65%	95.60%
BRAFPatien8	63.74%	93.48%	94.62%	94.62%	93.48%	93.55%	94.05%	94.05%	93.55%	93.55%	93.55%	93.55%	100.00%	98.38%	97.85%	98.35%	97.83%	98.35%
Normal3	64.09%	95.11%	96.24%	94.59%	95.63%	95.16%	95.16%	95.16%	95.16%	95.16%	95.16%	95.68%	98.38%	100.00%	99.46%	100.00%	100.00%	100.00%
Normal2	63.93%	95.11%	95.74%	94.62%	95.14%	94.74%	95.70%	95.70%	95.26%	95.74%	95.24%	95.70%	97.85%	99.46%	100.00%	100.00%	99.46%	100.00%
BRAFPatien10	64.09%	95.58%	96.70%	94.51%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	98.35%	100.00%	100.00%	100.00%	100.00%	100.00%
BRAFPatien12	64.48%	95.60%	95.68%	94.57%	95.68%	95.14%	95.63%	95.63%	95.68%	95.14%	95.68%	95.65%	97.83%	100.00%	99.46%	100.00%	100.00%	100.00%
Normal1	64.09%	95.58%	96.70%	94.51%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	95.60%	98.35%	100.00%	100.00%	100.00%	100.00%	100.00%

Figure 4.3 The complete identity matrix of control subjects and patients with leukemia was generated from the DNA sequence alignment of the BRAF gene obtained from PCR amplification

4.3 Phylogeny of BRAF gene

Phylogeny is an important tool to analyze the proximity among living organisms depending on specific or multiple criteria. In this work, phylogenesis was performed depending on sequence data obtained from BRAF gene amplified by PCR. Each group were subjected to phylogenesis to confirm convergence and divergence among each group and together for final analysis.

The current study found greater convergence between the DNA of BRAF gene sequences in patient numbers 14, 2, 13, and 3. There was high convergence between the DNA of BRAF gene sequences in patient's numbers 4, 6, 11, and 1, with moderate genetic similarity with the DNA of BRAF gene sequence number 5. In addition, the DNA of BRAF gene sequences in patient numbers 8, 10, 12, and 7 had greater convergence. In contrast, lower similarity was found between the DNA of BRAF gene sequence number 9 and other DNA of BRAF gene sequences, as shown in Figure 4.4.

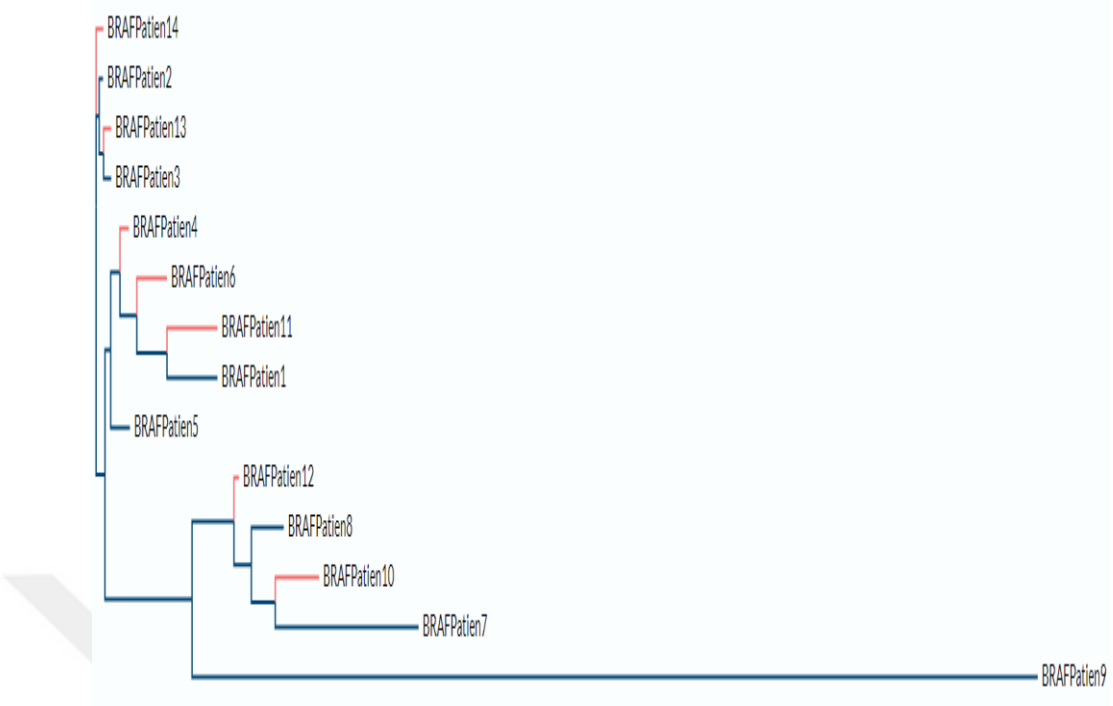


Figure 4.4 Phylogenetic Tree for patients generated depending on BRAF DNA sequence

Furthermore, the phylogenetic tree among control group subjects found greater convergence between all DNA sequences of the BRAF gene among the control group, as illustrated in Figure 4.5.

Figure 4.5 Phylogenetic tree of control patients depends on BRAF DNA sequence

In combination between patients and control subjects, the phylogenetic tree of DNA sequences of the BRAF gene showed convergence between the BRAF gene sequence of patient number 10 and control number 2, with moderate convergence with the BRAF gene sequence of patient number 8. Also, there was greater convergence between the

BRAF gene sequences of patient numbers 2, 13, 11, and 1. Furthermore, there was greater convergence between the BRAF gene sequences of patient numbers 4, 14, 6, 3, and 5. In addition, higher convergence between BRAF gene sequences between patient number 12 and control numbers 3 and 2 was found. Genetic similarity was found between BRAF gene sequences in patient number 7 and control number 4, with lower similarity in patient number 9, as shown in Figure 4.6.

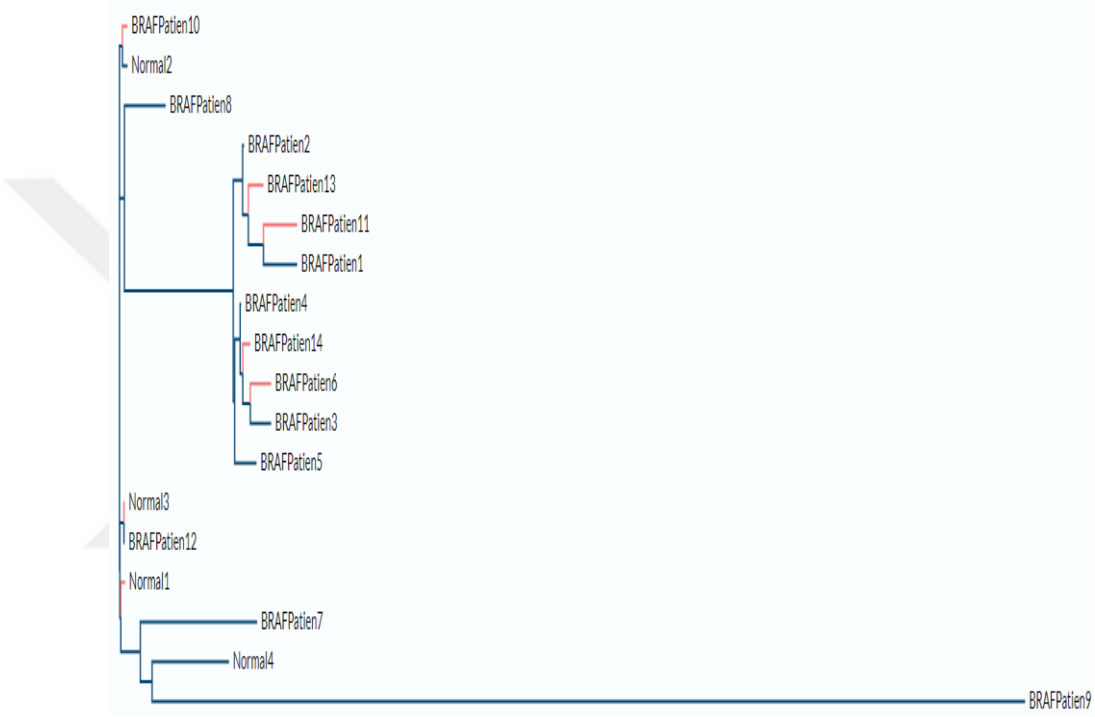


Figure 4.6 Phylogenetic tree of patients and control combined generated depending on BRAF DNA sequence

4.4 BLAST, genomic location, and alignment of BRAF PCR sequence

It is a crucial step to blast PCR sequence data against gnome to determine the location and, position, and record the critical data that results from this process. All participants PCR sequences where subjected to this process and data of some participants listed in Table 4.1. all BRAF gene sequences fall in chromosome 7 in which BRAF gene is found, second, they fall in exon 15, and region been studied is 7:140753198-140753406 that covers a critical location in which alter BRAF function if a pathogenic DNA change occur.

Table 4.1 Genomic location, chromosome number and position of BRAF gene blast results

Patients no.	Genomic location	Overlapping gene	Query start	Query end	Length	Score	E – value	ID percentage
1	7:140753208-140753397	BRAF	39	229	191	359	2.1e-99	99.8
2	7:140753208-140753407	BRAF	28	225	198	377	1.1e=104	98.99
3	7:140753208-140753405	BRAF	23	226	204	384	5.5e-107	99.51
4	7:140753208-140753410	BRAF	30	230	201	374	6e-104	99
5	7:140753208-140753407	BRAF	28	225	198	373	1.5e-103	99.49
6	7:140753209-140753413	BRAF	22	227	206	388	3.7e-108	99.51
7	7:140753222-140753404	BRAF	30	212	183	355	4.5e-98	100
8	7:140753209-140753413	BRAF	21	226	206	388	3.7e-108	99.51
9	7:140753209-140753350	BRAF	86	227	142	272	5.3-e73	99.3
10	7:140753209-140753413	BRAF	20	225	206	379	2.8=e105	98.06
11	7:140753209-140753413	BRAF	21	225	205	388	6.2e-108	98.54
12	7:140753219-140753340	BRAF	92	214	123	192	4.1e-49	91.87
13	7:140753209-140753411	BRAF	20	222	203	393	1.2e-109	100
14	7:140753208-140753405	BRAF	33	231	199	375	4e-104	99.5

4.5 Determination of DNA change effect on BRAF gene

DNA change in some cases can be lethal and alter or diminish gene function. In case of this study, multiple DNA change was found that may affected gene function in patients with leukemia. These changes are shown in Figure 4.7.

Figure 4.7 Changes in nucleotide sequence in some places that might lead to alteration of BRAF function

With further analysis to nucleotide sequence change, it was possible to identify multiple RSs listed in Table 4.2.

Table 4.2 Nucleotide sequence change details and effect of the change on BRAF gene

Variant	Class	Location	Alleles	Ambiguity code	Global MAF	Consequence
Rs573408357	SNP	7:140753213	G/C	S	0.000599 (C)	intron variant
rs759260016	SNP	7:140753227	T/C	Y	CliVar	intron variant
rs1198315740	SNP	7:140753230	C/T	Y	CliVar	intron variant
rs1255031025	SNP	7:140753233	G/A	R	CliVar	intron variant
rs373560312	SNP	7:140753235	C/A /T	H	CliVar	intron variant
rs1195545325	SNP	7:140753237-140753241	A/A	-	CliVar	intron variant
rs375646647	SNP	7:140753249	A/G	R	CliVar	intron variant
rs1302550107	SNP	7:140753252	G/A	R	CliVar	intron variant
rs368859030	SNP	7:140753259	T/A /C	H	CliVar	intron variant
rs1412035678	SNP	7:140753267	A/G	R	CliVar	splice region variant
rs886041842	SNP	7:140753272	T/C	Y	CliVar	splice donor region variant
rs1255101216	SNP	7:140753315	G/C	S	CliVar	missense variant
rs104886015	SNP	7:140753328	G/A	R	CliVar	stop gained
CM122892	SNP	7:140753340	HGMD_MUTATION		CliVar	coding sequence variant

Distribution of detected DNA mutations may inflect a dramatic change in BRAF function. All detected mutation were of pathogenic or likely pathogenic with no benign type as was obtained when these mutation were compared to clinvar interpretation shown in Figure 4.8.

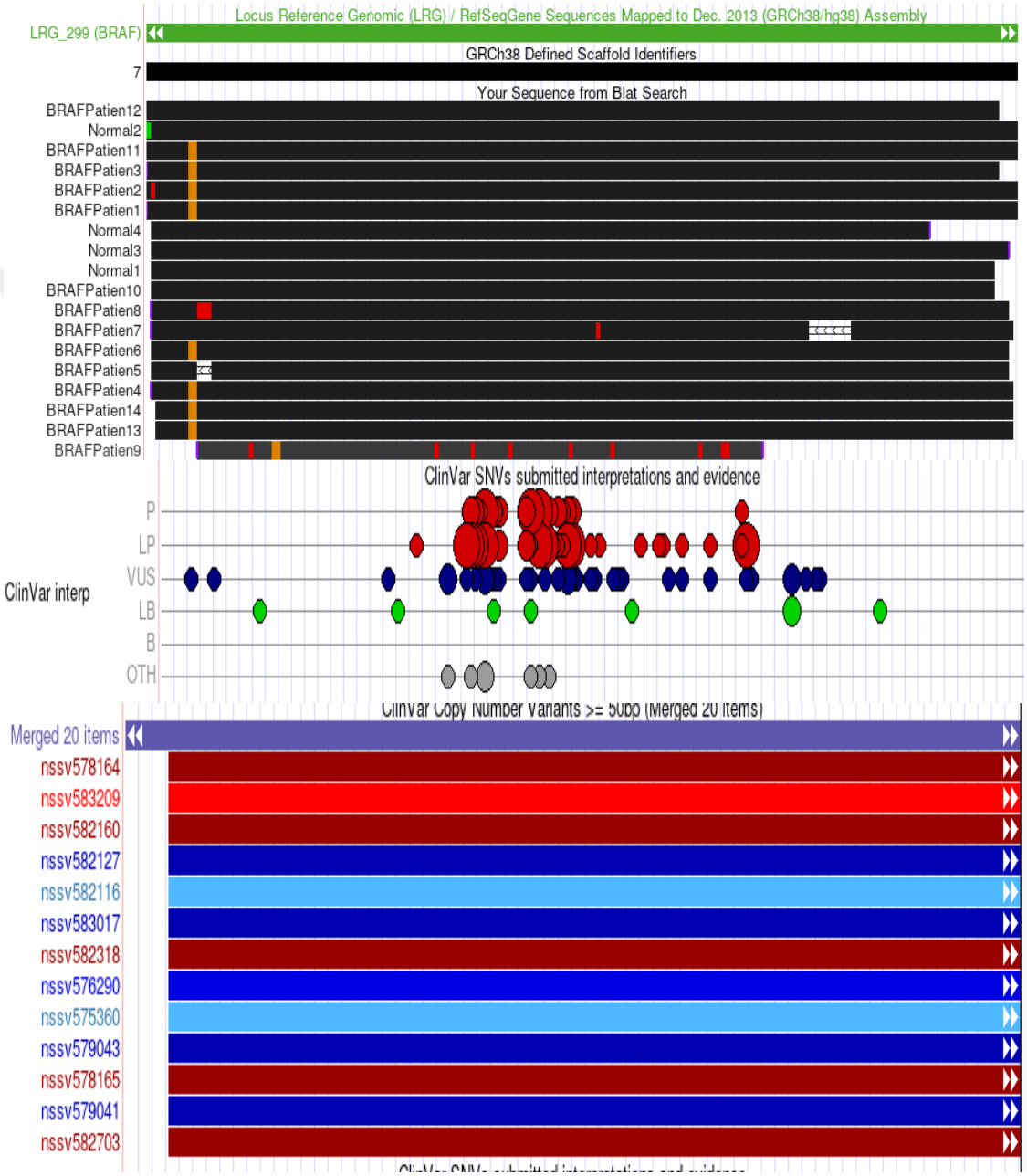


Figure 4.8 Type of DNA mutation determined in patients with leukemia as determined by clinvar database. P: pathogenic, LP: likely pathogenic, VUS: Variant of Unknown Significance, LB: Likely Benign, and OTH: Others

The BRAF gene encodes a protein known as RAF kinase, which functions as a signaling molecule downstream of RAS and activates the mitogen-activated protein kinase (MAPK) pathway have identified it as a significant catalyst for cancer development and a potential therapeutic focus in several types of solid tumors and hematological malignancies (Vultur *et al.* 2011). The signaling pathway, including BRAF, plays a crucial role in the processes of cell division and differentiation. When BRAF mutations are activated, it leads to unregulated cellular proliferation and the development of tumors. The majority of activating BRAF mutations in cancer cells, specifically more than 90 percent, are located inside the kinase domain at amino acid V600. Among these changes, the most prevalent is the V600E. This particular mutation has been identified as a target for authorized inhibitors such as dabrafenib and vemurafenib, which are used for the management of metastatic malignant melanoma (Cantwell-Dorris *et al.* 2011). According to Dadu et al., several non-melanoma cancers characterized by activating BRAF changes, namely the V600E mutation, have shown positive responses to therapeutic interventions targeting the BRAF protein (Dadu *et al.* 2015). The activation of the BRAF gene via fusions is a distinct mechanism that has been seen in several kinds of solid tumors (Pettirossi *et al.* 2015).

The whole BRAF gene sequencing among leukemic patients was the first investigation in our country; however, the mutations in the BRAF gene play an important role in the development of other types of tumors. Another previous study focused on the analysis of the exome of the BRAF gene only (encoding region) among patients with hairy-cell leukemia and identified five missense clonal mutations among all patients (Tiacci *et al.* 2011). In our study, fourteen mutations were identified within the BRAF gene sequence. All mutations were single-nucleotide polymorphisms located on chromosome 7. The majority of these mutations were in the intron region; two of them were in the splicing donor region, and one was in the coding variant region. A separate research conducted in Iraq examined the mutations occurring in the BRAF gene sequence among individuals diagnosed with brain tumors. The study detected a total of thirty mutations in the BRAF area, consisting of twenty-eight single nucleotide polymorphisms resulting in missense translation, as well as two deletions classified as inframe type. Furthermore, it is worth noting that all of these mutations have been shown to have a pathogenic

impact on the molecular outcome, specifically situated on chromosome 7 (Hade *et al.* 2020).

In The occurrence of various forms of Gangliogliomas, dysembryoblastic neuroepithelial tumors, and astrocytomas may be attributed to the presence of the BRAF V600E mutation. In a significant proportion of investigated cases, around twenty-sixty percent, thirty percent, and five percent respectively, these tumors are shown to be benign tumors and exhibit the BRAF V600E mutation. (Fang *et al.* 2018). The clinical significance of BRAF mutations was seen in epithelioid glioblastomas, where they were shown to occur at a frequency of 50%. Similarly, in pleomorphic xanthoastrocytomas, including anaplastic forms, the frequency of BRAF mutations ranged from fifty percent to seventy-eight percent (Pakneshan *et al.* 2014). In the research conducted by Louis *et al.* (2016) a comprehensive examination was performed on a specific amino acid change occurring at position 600. This alteration was identified in 14 out of the 31 brain tumors, specifically in 3 gangliogliomas, as a consequence of a BRAF mutation. Additionally, a total of 31 tumors were tested for activating mutations in exons eleven and fifteen (Louis *et al.* 2016). About 66 percent of malignant gliomas have been shown to have activating mutations in the BRAF gene. These mutations result in the continuous production of BRAF protein and are seen in several forms of brain tumours, including pilocytic astrocytomas and a limited number of grade II astrocytomas (Sievert *et al.* 2009). In contrast, the findings of direct sequencing from many studies revealed a lack of detection of BRAF gene mutations in colorectal cancer patients (Baldus *et al.* 2010, Lurkin *et al.* 2010).

Given that all BRAF pathogenic mutations, irrespective of their classifications, induce ERK phosphorylation, it is postulated that transcription factors (TFs) regulated by the ERK signalling pathway may serve as potential downstream targets of BRAF mutations. It has been claimed that a significant proportion, around 30%, of cancer tissues exhibit constitutively active RAS-RAF-MEK-ERK signalling pathway (Scholl *et al.* 2005). Hence, it is crucial to clarify the regulatory mechanisms governing downstream consequences of ERK phosphorylation within the framework of cancer development, with the aim of impeding tumour formation and advancement. However,

there is a scarcity of research that establish a connection between abnormal phosphorylation of ERK resulting from BRAF mutation and the subsequent activation of transcription factors in malignant cells. However, several studies have successfully identified proteins targeted by ERK, including various transcription factors. cMyc is a transcription factor that is well recognized for its regulation by ERK phosphorylation in cancer cells (Maik-Rachline *et al*. 2019). The continuous activation of cMyc was seen with phosphorylation at Thr58 as well as Ser62 (Sears *et al.* 2000). Phosphorylation serves as a mechanism to inhibit protein breakdown. Furthermore, the occurrence of a mutation at Thr58 in the cMyc protein, resulting in the prevention of its degradation, has been seen to provide resistance to FGFR inhibition in many cancer cell lines that are dependent on FGFR signaling (Liu *et al.* 2017).

The findings of this study indicate that the phosphorylation of ERK, mediated by BRAF mutations, leads to increased stability of cMyc and perhaps facilitates the transmission of tumorigenic signals associated with BRAF mutations. One other prominent carcinogenic transcription factor, which is activated by ERK phosphorylation, is cFos. The transcription factor cFos functions as a heterodimer with c-Jun, serving as an activator for the complex known as protein-1 (AP1). The AP1 dimer protein exhibits binding affinity towards the AP1-specific DNA sequence found inside the promoter and enhancer regions of target genes. In addition to c-Jun, cFos has been seen to engage in interactions with many nuclear transcription factors, including NCOA1 and SMAD3. These interactions are subject to regulation by ERK phosphorylation. In aggregate, it is postulated that the phosphorylation of ERK induced by BRAF mutation has the capacity to dynamically alter the activity of transcription factors that regulate gene expression in cancer cells, hence manifesting tumor characteristics. In addition to the extensively investigated transcription factors, an extensive investigation has identified over 100 transcription factors that are recognized as targets of ERK phosphorylation (Maik-Rachline *et al*. 2019).

There are several components inside cells that are typically localised outside of the nucleus and then transferred into the nucleus upon phosphorylation of ERK. It is worth noting that SMAD 1-4 have been identified as ERK substrates that exhibit localization

inside the nucleus and other organelles. The TGF-β signalling pathway is facilitated by SMAD proteins, which are recognized as influential regulators of the epithelial to mesenchymal transition (EMT). Transforming growth factor beta 1 (TGF-β1) exhibits binding affinity towards its receptor II (TβRII), hence initiating the activation of the TGF-β receptor type I (TβRI)-kinase. Consequently, the cytoplasmic environment triggers the phosphorylation process of SMAD2 and SMAD3 (Lan 2011). Following this, the SMAD2/3 complex interacts with SMAD4 and performs its transactivation function to facilitate the expression of target genes. The process of epithelial-to-mesenchymal transition (EMT) is well recognized as a critical mechanism in the metastatic progression of several forms of cancer (Dongre and Weinbreg 2019). Indeed, the suppression of the transforming growth factor-beta (TGF-β) signalling pathway via the use of pharmacological inhibitors effectively impedes the process of epithelial-mesenchymal transition (EMT) in several forms of malignancies (Guo *et al.* 2019). The results of this study indicate that the presence of BRAF mutations might lead to increased phosphorylation of ERK, which in turn can boost the phosphorylation of SMAD2/3 and activate the epithelial-mesenchymal transition (EMT) potential of cancer cells. The activation of NRF2, a protein encoded by the NFE2L2 oncogene, is facilitated by ERK (Zipper and Mulcahy 2003).

Furthermore, it is responsible for the regulation of other genes associated with oxidative stress, such as heme-oxygenase and NQO1, which are considered important target genes of NRF2 (Cho *et al.* 2019). Multiple natural substances, such as Sesamin and Curcumin, have shown the ability to modulate genes associated with oxidative stress via the ERK-NRF2 pathway (Bai *et al.* 2019). The primary function of BRAF is to govern the phosphorylation of ERK. It is probable that NRF2 is controlled by BRAF, as well as its mutant V600E variant, through the ERK signalling pathway in several types of cancer. Indeed, previous studies have provided evidence that mutations in BRAF and RAS genes elicit the transcription of Nrf2 in primary mouse cells (DeNicola *et al.* 2011). Aberrant activation of the nuclear factor erythroid 2-related factor 2 (NRF2) has been seen in several forms of cancer (Zimta *et al.* 2019).

During the process of organogenesis, a number of transcription factors play a crucial role as regulatory molecules in coordinating the determination of cell destiny. Recent investigations have shown a strong correlation between the development of tissue-specific cancers and the abnormal functioning of these components. The GATA transcription factors are a class of DNA binding proteins that include zinc finger motifs and have the ability to recognise a common consensus sequence for binding. Transcription factors are known to activate several target genes and are crucial in the first stages of development. Additionally, their involvement in the progression of cancer has also been shown (Huilgol *et al.* 2019). Hepatocyte nuclear factor 3 β (HNF3β) as well as GATA6 transcription factors are well recognised as important components in the process of lung formation (Bonner *et al.* 2003). The abnormal expression of GATA6 plays a significant role in several types of malignancies, including lung cancer (Ma *et al.* 2019).

Furthermore, it has been shown that GATA6 has a role in regulating the chromatin architecture of lung cancer cells, hence influencing their proliferation and diverse lineage dependencies during the course of tumor advancement. HNF3β has been identified as a tumor suppressor in the context of lung cancer, and its upregulation has been seen to impede the proliferation of lung cancer cells (Halmos *et al.* 2004). The precise impact of ERK-mediated phosphorylation on HNF3β activity remains uncertain; nevertheless, extensive evidence exists about the augmentation of GATA6 function in target gene expression via ERK phosphorylation in the context of colon cancer. CaCo-2 cells are a commonly used cell line in biomedical research (Adachi *et al.* 2008). In contrast, the co-expression of HNF4A and HNF3α, β, γ, or HNF1A/HNF3γ/GATA4 with the inactivation of p19Arf has been seen to induce the generation of hepatocyte-like cells. This observation implies that these factors are significant contributors to the process of liver formation (Huang *et al.* 2011). The induction of hepatocellular carcinoma (HCC) in mice may be seen by the targeted removal of the Gata4 allele, which is unique to the liver and hampers the functionality of GATA4. (Enane *et al.* 2017). The hepatic transcription factor HNF4A has been identified as a regulator that suppresses the ERK pathway in the liver. This suppression is achieved by reducing the levels of phosphorylated ERK and JunD. Consequently, this dysregulation of the ERK

pathway has been seen to contribute to the development of liver cirrhosis in rats (Fan *et al.* 2013). HNF4A, a hepatic tumour suppressor, has the potential to function as a suppressor of tumour formation by inhibiting ERK-phosphorylation. In its whole, the regulation of transcription factor activity via ERK phosphorylation is controlled by a complex regulatory network. Additional research is necessary to explore the role of ERK phosphorylation produced by BRAF mutations in the development of cancer (Ning *et al.* 2010).

4.6 BRAFP1 Data Analysis

BRAFP1 belongs to a subclass of long noncoding RNAs (lncRNAs) that developed from protein-coding genes but are now considered pseudogenes due to loss of their protein-coding abilities. BRAFP1 has the potential to participate in posttranscriptional regulation of its parental transcript, BRAF. It is reported that human BRAFP1 pseudogene, is over 3 kb long. The authors failed to detect peptide translation for mouse or human BRAF pseudogenes. Quantitative PCR analysis detected BRAFP1 expression in 30% of primary human diffuse large B-cell lymphomas (DLBCLs) and in 20% of human DLBCL cell lines, but not in normal human B cells. BRAFP1 was also expressed in melanoma, prostate cancer, and lung cancer cell lines. In human DLBCL cell lines, BRAFP1 was several-fold less abundant than BRAF and had a much shorter half-life (Karreth *et al.* 2015).

BRAFP1 was studied in 14 leukemia patients and 5 healthy control to determine the genetic change and effect of such change on normal function of the gene. PCR products of BRAFP1from patients and control were sequenced to and all sequences were aligned to generate the identity score among participants including patients separately, control, and both patients and control together results. According to the Figure 4.9, there was divergenic among all leukemia patients with respect to BRAF1 gene sequence. In contrast there was high convergence among all control group with respect to BRAF1 gene sequence, as shown in Figure 4.10. Comparing results between control and patients, the following conclusion can be drawn: first, there is a specific difference in similarity among patients since the score generated did not exceed 60%, second when

patients are compared with normal, the score generated is reached about 99%. This can be explained on the basis of the position of the sequence aligned in patients, second, when patients sequence aligned with control, only few changes in the sequence was observed that means there are specific change at certain positions in BRAFP1 gene in patients with leukemia, as shown in Figure 4.11.

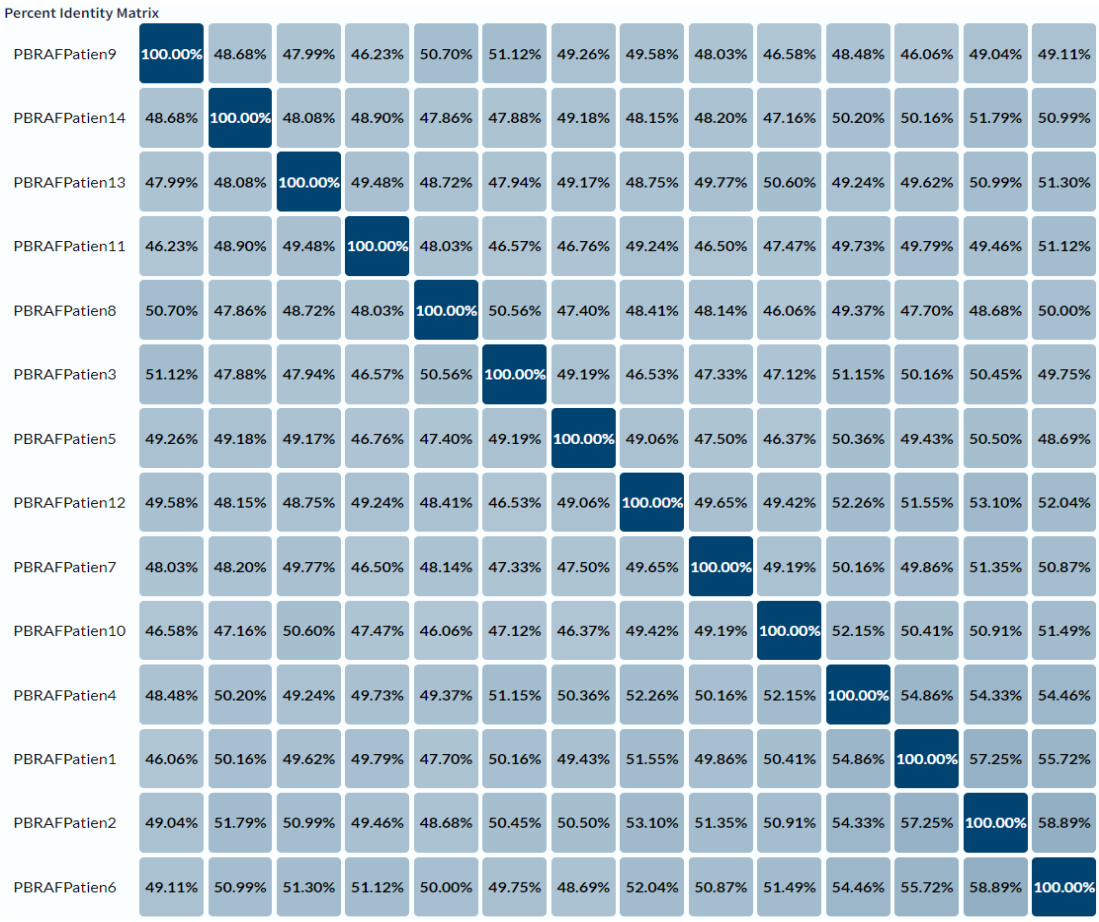


Figure 4.9 Identity matrix of BRAFP1 gene in patients with leukemia

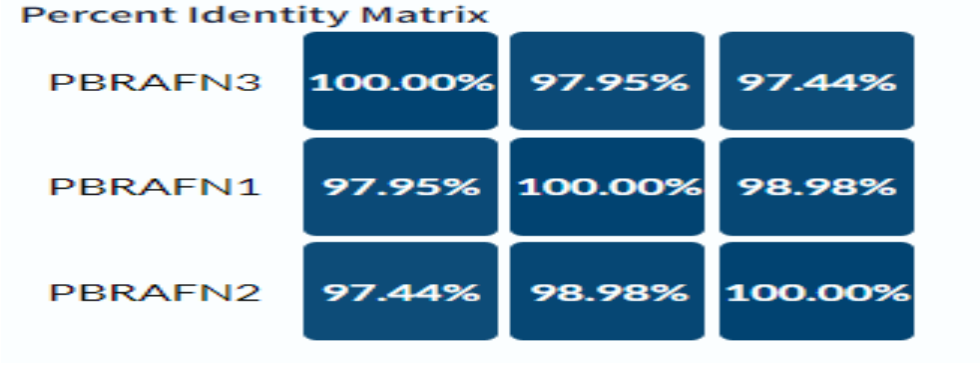


Figure 4.10 Identity matrix of BRAFP1 gene in control group

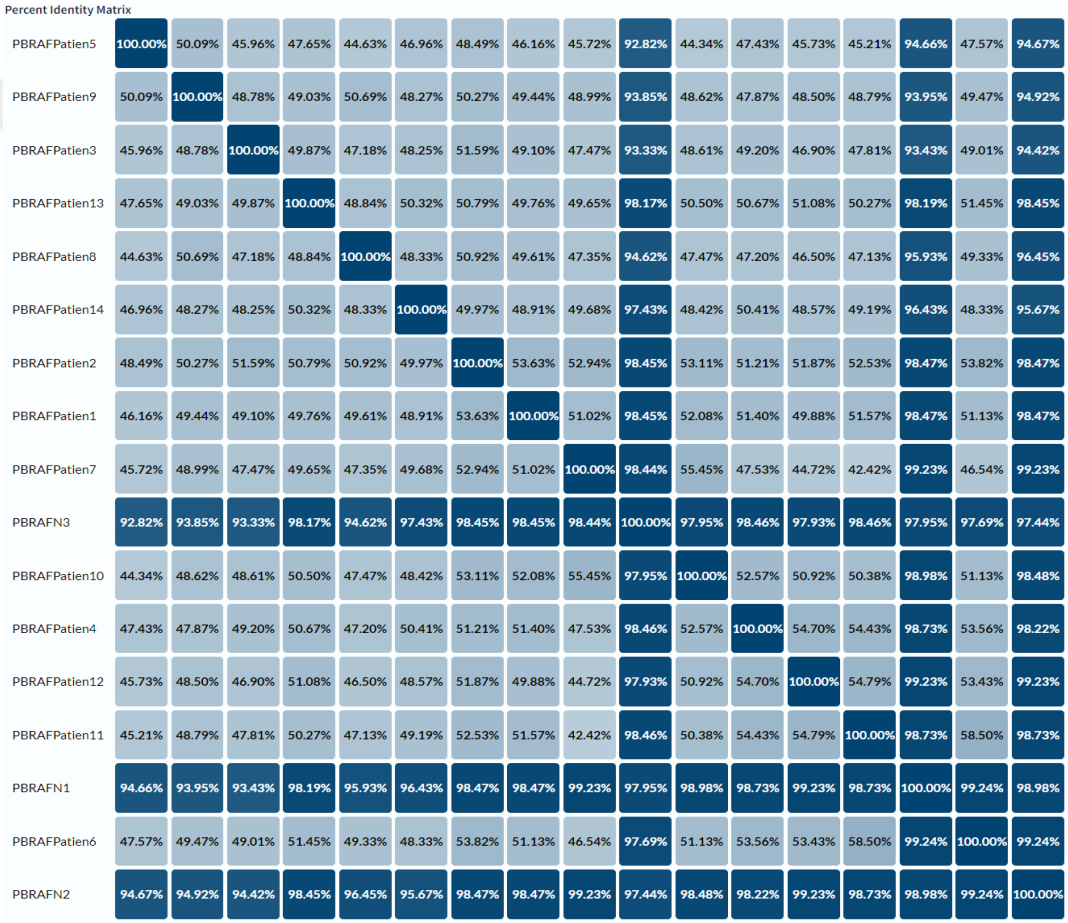


Figure 4.11 Identity matrix of both patients and control regarding BRAFP1 gene

4.7 Phylogenesis Analysis of BRAFP1 Gene

Using sequences from the BRAFP1 gene to make a phylogenetic tree for each participant is an important step in figuring out how close or far apart people are

genetically. Both groups were subjected to phylogenetic analysis independently and together to generate the phylogenetic trees. Figure 4.12 shows that the BRAF1 gene sequences of all the leukemia patients in this study were very different from each other. On the other hand, Figure 4-13 shows that the BRAF1 gene sequences of all the control groups were very similar. Furthermore, when patients were compared to the control, the genetic divergence was low and grouped as follows: patients 5, 9, 3, 13, 8, 14, 2, 1, and 7 can be grouped in one cluster with high similarity to control subject 3, while patients 10, 4, 12, 11, and 6 are close to controls 1 and 2, as demonstrated in Figure 4.14.

Figure 4.12 Phylogenetic tree of patients with leukemia depending on BRAFP1 sequence

Figure 4.13 Phylogenetic tree of control subject depending on BRAFP1 sequence

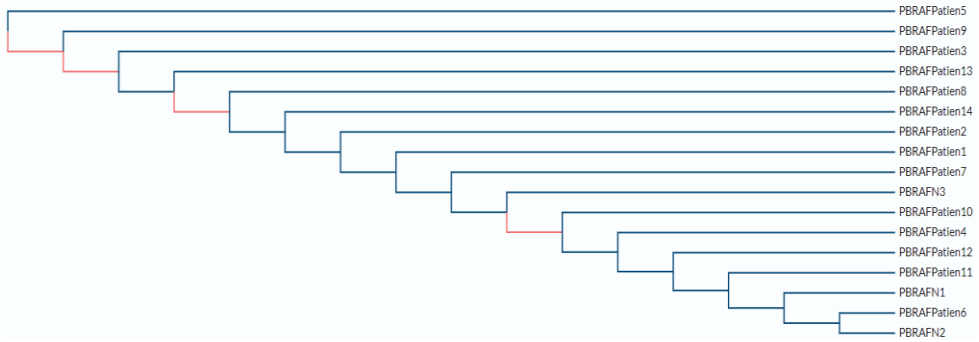


Figure 4.14 Phylogenetic tree of patients and control generated depending on BRAFP1 DNA sequence obtained from PCR amplification

4.8 BLAST, genomic location, and alignment of BRAF PCR sequence

To identify the genomic location, chromosome, and sequence position, all data obtained from BRAFP1 PCR amplification were blasted to achieve this goal. Table 4.3 shows the following: All sequences subjected to blast are for the BRAFP1 gene and are positioned on the X chromosome; second, they fall within an exon region of the gene; and third, they extend in the distance of X:75582814 –75583226.

Table 4.3 Genomic location, chromosome, and position of PCR product sequence of BRAF gene blast results

Patients no.	Genomic location	Overlapping gene	Query start	Query end	Length	Score	E – value	ID percentage
1	X:75582833-75583208	BRAFP1	40	419	380	699	1.2e-201	98.95
2	X:75582827-75583217	BRAFP1	32	423	392	739	1.4e-213	99.23
3	X:75582831-75583209	BRAFP1	39	417	379	732	1.8e-211	100
4	X:75582826-75583209	BRAFP1	30	413	384	741	2.5e-214	100
5	X:75582827-75583209	BRAFP1	30	413	384	730	4.6e-211	99.74
6	X:75582831-75583207	BRAFP1	38	417	380	701	2.2e-202	99.21
7	X:75582833-75583209	BRAFP1	41	417	377	728	2.4e-210	100
8	X:75582807-75583207	BRAFP1	10	414	406	715	2.1e-206	98.28
9	X:75582833-75583221	BRAFP1	42	431	390	740	6.7e-214	99.49
10	X:75582827-75583217	BRAFP1	34	425	392	743	6.5e-215	99.49

4.9 Determination of DNA Change and Effect on BRAFP1 Gene

When reviewing previous results regarding the identity matrix and phylogenetic trees, it can be concluded the presence of DNA change when analyzing the BRAFP1 sequences. Position, and effect of such change is illustrated in Figure 4.15 and Table 4.4.

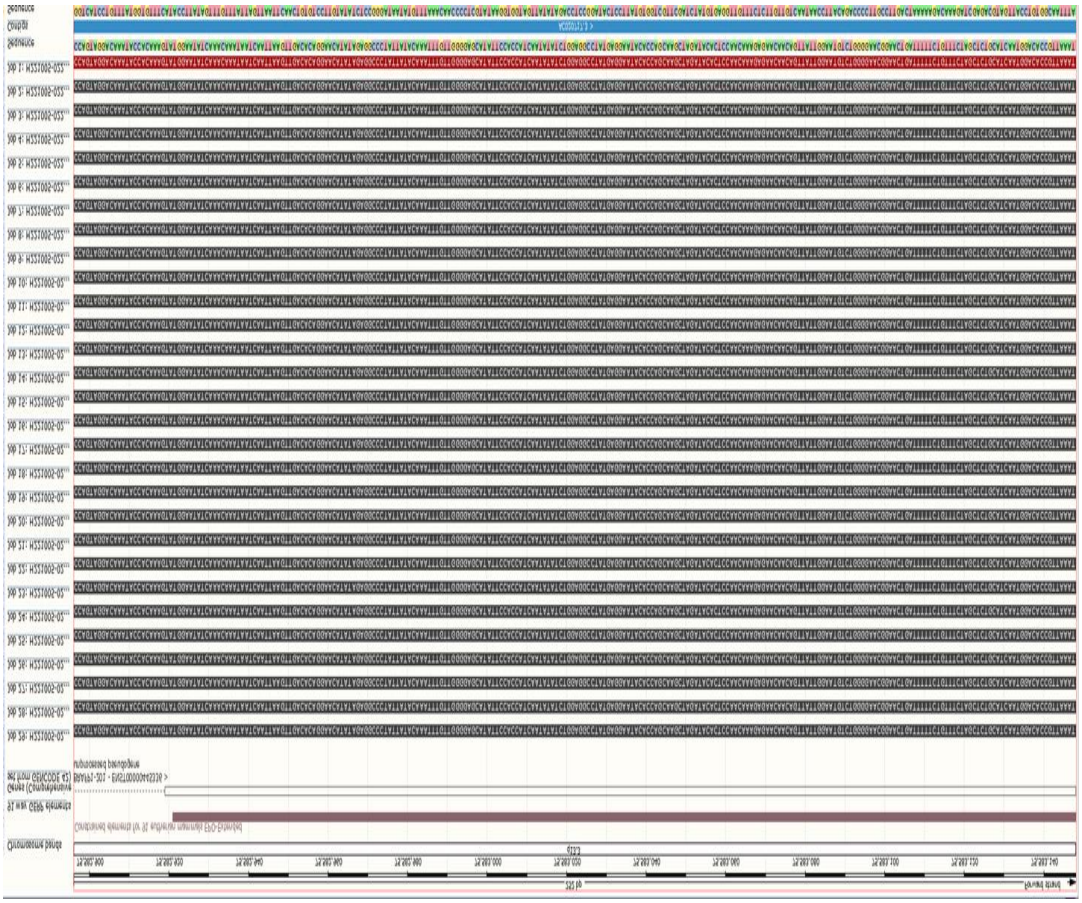


Figure 4.15 Detailed DNA alignment within the genomic sequence of BRAFP1 depending on DNA sequence obtained from PCR amplification

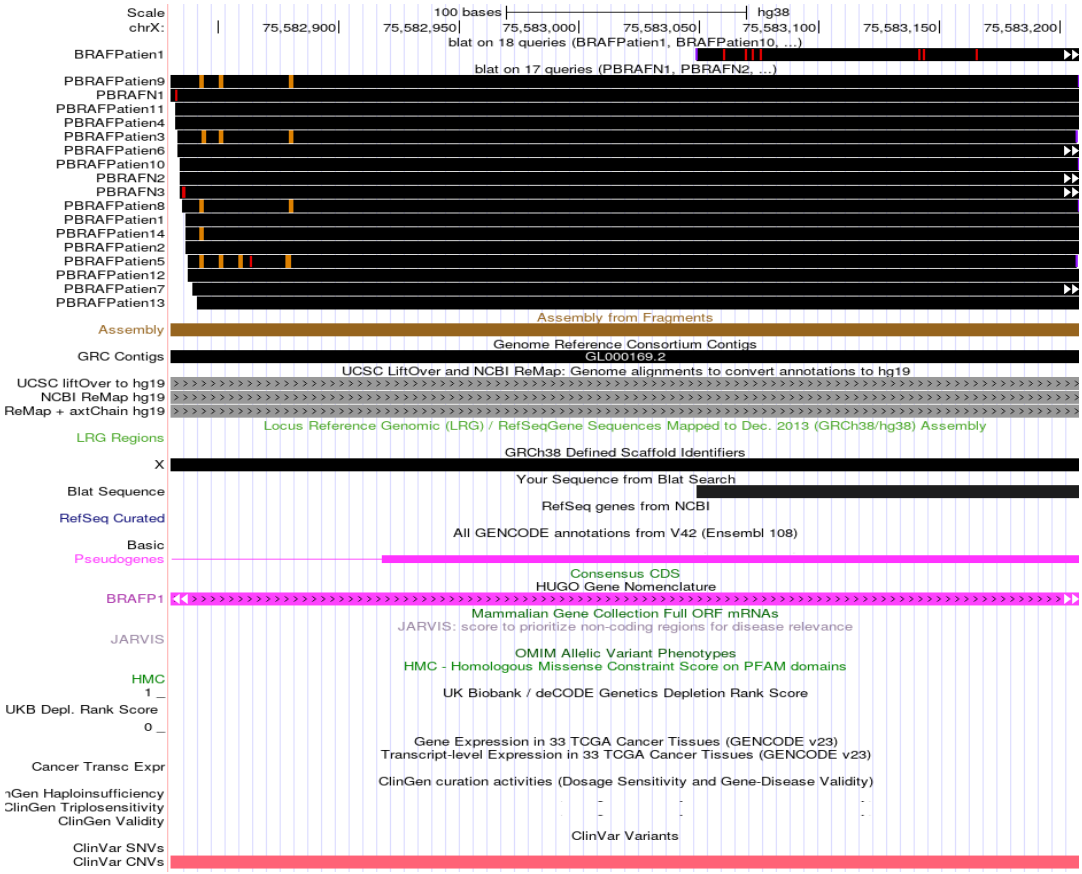


Figure 4.15 Detailed DNA alignment within the genomic sequence of BRAFP1 depending on DNA sequence obtained from PCR amplification (Continue)

Table 4.4 Nucleotide sequence change details and effect of the change on BRAFP1 gene

Variant	Class	Location	Alleles	Ambiguity code	Global MAF	Consequence
rs1313663496	SNP	X:75582915	A/G	R	dbSNP	splice polypyrimidine tract variant
rs1340099037	SNP	X:75582932A/G	A/G	R	dbSNP	non coding transcript exon variant
rs185657234	SNP	X:75582937	T/A	W	dbSNP	non coding transcript exon variant
rs1277118792	SNP	X:75582989	T/A	W	dbSNP	non coding transcript exon variant
rs1350448260	SNP	X:75582993	G/T	K	dbSNP	non coding transcript exon variant
rs12851473	SNP	X:75583016	A/T	W	dbSNP	non coding transcript exon variant
rs1212069356	Indel	X:75583029	T/TT		dbSNP	non coding transcript exon variant
rs1262977879	SNP	X:75583081	T/C	Y	dbSNP	non coding transcript exon variant

Since BRAFP1 is a regulatory gene and doesn't involve in coding protein, all DNA alterations detected in patients with leukemia fall with regulatory region in the gene

produces an alternative long non – coding RNA (LncRNA) with a variant sequence. With such variation affinity to annealing site of BRAF promoter, and RNA decay mechanism differ in these patients that eventually affected DNA methylation, and rate of gene expression that bear itself DNA mutations vary in their effect on coded protein. In our study, eight mutations were identified within the BRAFP1 gene sequence. All mutations were single-nucleotide polymorphisms located on chromosome X. The majority of these mutations were in the non- coding transcript exon variant; one of them was in the splice polypyrimidine tract variant. There have been no previous studies investigating the mutations in the BRAFP1 gene; the current study is considered the first study focused on this issue.

Hematopoietic malignancies in humans have been shown to be linked to an excessive dosage of the X chromosome, specifically in relation to the presence of the BRAF pseudogene locus. The phenomenon of XIST deletion and X chromosome duplication has been seen in women diagnosed with myeloid tumors. Additionally, the presence of additional X chromosomes has been documented in many hematological cancers affecting individuals of both genders. A prior research has shown that a range of human malignancies exhibit copy-number increases and amplifications of the genomic region including BRAFP1 (Karreth *et al.* 2015). The suggestion that an increased number of X chromosome copies and potential overexpression of BRAFP1 may play a role in the proliferation and metastasis of malignancies exhibiting multiple copies of the active X chromosome is an enticing notion. Furthermore, there has been documented evidence of increased expression of BRAFP1 in malignancies other than diffuse large B-cell lymphoma (Kalyana-Sundaram *et al.* 2012). Further studies are needed to assess the role of BRAFP1 gene polymorphisms in the development of leukemia and other types of cancer.

5. CONCLUSIONS AND RECOMMENDATION

Conclussions

- There is a specific difference in similarity among patients since the score generated did nor exceed 60%.
- Comparing patients with normal, the score generated is reached about 99%.
- There is a genetic distance divergence among patients. And when patients are compared to control, the genetic distance is low.
- BRAFP1 is a regulatory gene and doesn't involve in coding protein, all DNA alterations detected in patients with leukemia fall with regulatory region in the gene produces an alternative long non – coding RNA (LncRNA) with a variant sequence.

Recomendations

- Condect More research about Leukemia in Children.
- Studying relations between BRAF and Pseudo BRAF genes in other cancers.

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