T.C.
DOKUZ EYLÜL UNIVERSITY
İZMİR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

INVESTIGATION OF THE POTENTIAL OF THE MUTATIONS IN CELL-FREE CIRCULATING TUMOR DNA AS BIOMARKERS OF DIAGNOSIS AND CHEMO-IMMUNOTHERAPY RESISTANCE IN FOLLICULAR LYMPHOMA PATIENTS

TEVFİK HATİPOĞLU

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

DOCTOR OF PHILOSOPHY THESIS

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Advisor: Assoc. Prof. Dr. Can Küçük

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on the date of 21/06/2023.

Assoc. Prof. Can Küçük
Dokuz Eylül University
CHAIR

Asst. Prof. Yavuz Oktay
Dokuz Eylül University
MEMBER

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Prof.Dr. İnci Alacacıoğlu
Dokuz Eylül University
SUBSTITUTE MEMBER

Prof.Dr. Mahmut Töbü
Ege University
SUBSTITUTE MEMBER

Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı, Moleküler Biyoloji ve Genetik Doktora programı öğrencisi Tevfik Hatipoğlu, "DOLAŞIMDAKI SERBEST TÜMÖR DNA'SINDAKI MUTASYONLARIN FOLİKÜLER LENFOMA HASTALARINDA TANI VE KEMO-IMMÜNOTERAPİ DİRENÇ BİYOMARKERI OLMA POTANSIYELİNİN İNCELENMESİ"

konulu doktora tezini 21/06/2023 tarihinde başarılı olarak tamamlamıştır.

Doç. Dr. Can Küçük
Dokuz Eylül Üniversitesi
BAŞKAN

Dr. Öğr. Üyesi Yavuz Oktay
Dokuz Eylül Üniversitesi
ÜYE

Doç. Dr. Ayşe Caner
Ege Üniversitesi
ÜYE

Dr. Öğr. Üyesi Athanasia Pavlopoulou
Dokuz Eylül Üniversitesi
ÜYE

Doç. Dr. K. Bahriye Payzın
İzmir Katip Çelebi Üniversitesi
ÜYE

Prof.Dr. İnci Alacacıoğlu
Dokuz Eylül Üniversitesi
YEDEK ÜYE

Prof.Dr. Mahmut Töbü
Ege Üniversitesi
YEDEK ÜYE

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LIST OF ABBREVIATIONS

cfDNA: Circulating cell-free DNA ctDNA:

Circulating tumor DNA

DLBCL: Diffuse large B-cell lymphoma

FDG: Fluoro-deoxy-glucose

FFPE: Formalin fixed paraffin embedded

FL: Follicular lymphoma

FLIPI: Follicular Lymphoma International Prognostic Index gDNA:

Genomic DNA

IGV: Integrative Genomics Viewer

IPI: International Prognostic Index

LDH: Lactate dehydrogenase

NGS: Next Generation Sequencing

NHL: Non-Hodgkin lymphoma

OS: Overall survival

PET/CT: Positron emission tomography/computed tomography

PBMC: Peripheral blood mononuclear cells

PFS: Progression-free survival

R-Bendamustine: Rituximab - bendamustine

R-CHOP: Rituximab - cyclophosphamide, hydroxydaunomycin, oncovin, prednisone

R-CVP: Rituximab - cyclophosphamide, vincristine sulfate, prednisone

RT: Room temperature tDNA: Tumor DNA

VAF: Variant allele fraction

WHO: World Health Organization

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INVESTIGATION OF THE POTENTIAL OF THE MUTATIONS IN CELL-FREE CIRCULATING TUMOR DNA AS BIOMARKERS OF DIAGNOSIS AND CHEMO-IMMUNOTHERAPY RESISTANCE IN FOLLICULAR LYMPHOMA PATIENTS

Tevfik Hatipoğlu, Dokuz Eylül University Izmir International Biomedicine and Genome Institute,

ABSTRACT

Follicular lymphoma, a common subtype of non-Hodgkin's lymphoma that originates from abnormal B-cells in the germinal centers of the lymphatic system, has a clinically variable prognosis. Therefore, accurate diagnosis and risk stratification are crucial for determining the most appropriate treatment strategy and improving patient outcomes. In this study, we investigated the potential diagnostic and prognostic value of the concentrations and genotype of circulating cell-free DNA (cfDNA), a non-invasive approach, in patients with follicular lymphoma. We enrolled 20 newly diagnosed cases of follicular lymphoma, including 13 asymptomatic and 7 symptomatic cases. We used ultra-deep targeted next-generation sequencing to identify somatic variants in plasma cfDNA and formalin-fixed paraffin-embedded tumor tissue DNA of FL cases. In treatment-naive follicular lymphoma cases, we identified 91 somatic short variants using a computational bioinformatic pipeline and by applying strict criteria. We observed higher concentrations of plasma cfDNA in symptomatic cases compared to asymptomatic cases, and a higher frequency of somatic variants common in both cfDNA and tumor tissue DNA in symptomatic cases. Consistent with expectations, adverse clinical factors such as high Ki-67 staining, elevated LDH levels, and FDG PET/CT positivity were associated with poorer survival. Furthermore, we observed poorer survival in cases with higher plasma cfDNA concentrations. The presence of *BCL2* mutations in cfDNA was also associated with poorer survival, and the co-occurrence of *BCL2* mutations with adverse clinical variables improved the prognostic utility. In conclusion, our findings suggest that cfDNA, as a non-invasive liquid biopsy method, holds promise for improving the diagnosis and risk stratification of follicular lymphoma patients.

Keywords: Follicular lymphoma, liquid biopsy, cfDNA genotyping, targeted ultra-deep sequencing, non-invasive diagnosis and prognosis, targeted therapy, histological transformation

DOLAŞIMDAKI SERBEST TÜMÖR DNA'SINDAKI MUTASYONLARIN FOLİKÜLER LENFOMA HASTALARINDA TANI VE KEMO-IMMÜNOTERAPİ DİRENÇ BİYOMARKERI OLMA POTANSIYELİNİN İNCELENMESİ

Tevfik Hatipoğlu, Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü,

ÖZET

Foliküler lenfoma, lenfatik sistemin germinal merkezlerindeki anormal B hücrelerinden kaynaklanan Hodgkin dışı lenfomanın yaygın bir alt tipidir. Klinik olarak değişken bir prognoza sahip olması nedeniyle doğru teşhis ve risk stratifikasyonu, en uygun tedavi stratejisini belirlemek ve hasta sonuçlarını iyileştirmek için önemlidir. Bu çalışmada, foliküler lenfoma hastalarında, invaziv olmayan bir yöntem olan dolaşımdaki serbest DNA'nın (cfDNA) konsantrasyon ve genotipinin potansiyel tanısal ve prognostik değerini araştırdık. 13 asemptomatik ve 7 semptomatik olmak üzere 20 yeni teşhis edilmiş foliküler lenfoma vakasını dahil ettik. FL olgularının plazma cfDNA ve formalinle sabitlenmiş parafin gömülü tümör dokusu DNA'sındaki somatik varyantları tespit etmek için ultra-derin hedeflenmiş yeni nesil dizileme kullandık. Tedavi görmemiş foliküler lenfoma vakalarında, sıkı bir kriter uygulayarak ve hesaplamalı biyoinformatik iş akışı kullanarak 91 somatik kısa varyantı belirledik. Semptomatik vakalarda, asemptomatik vakalara kıyasla daha yüksek plazma cfDNA konsantrasyonları ve hem cfDNA hem de tümör doku DNA'sında ortak somatik varyantların daha sık olduğu gözlemlendi. Beklenildiği gibi yüksek Ki-67 boyama, yüksek LDH düzeyleri ve FDG PET/CT pozitifliği gibi olumsuz klinik faktörler, daha kötü sağkalımla ilişkili olduğu gözlemlendi. Ayrıca, daha yüksek plazma cfDNA konsantrasyonları olan vakalarda daha kötü sağkalım gözlendi. *BCL2* mutasyonlarının cfDNA'da varlığı da daha kötü sağkalım ile ilişkilendirildi ve *BCL2* mutasyonlarının olumsuz klinik değişkenlerle birlikte görülmesi, prognostik yararlılığı arttırdı. Sonuç olarak, bulgularımız, cfDNA'nın invaziv olmayan bir sıvı biyopsi yöntemi olarak, foliküler lenfoma hastalarının teşhisini ve risk stratifikasyonunu iyileştirmeye yönelik umut vaat ettiğini düşündürmektedir.

Anahtar Kelimeler: Foliküler lenfoma, sıvı biyopsi, cfDNA genotipleme, hedefe yönelik ultra-derin dizileme, invaziv olmayan tanı ve prognoz, hedefe yönelik tedavi, histolojik dönüşüm.

1. INTRODUCTION AND OBJECTIVES

1.1. Statement and importance of the problem

Follicular lymphoma is the second most common subtype of non-Hodgkin lymphoma, which originates from abnormal B cells in the germinal center of lymphoid tissues. Although it is generally classified as an indolent disease, it exhibits heterogeneity in its clinical presentation, somatic mutation types, molecular features, and response to treatment. Hence, accurate diagnosis and risk stratification are important in managing the treatment of follicular lymphoma. Solid tissue biopsy and its immunohistochemical examination are commonly used in the initial diagnosis of follicular lymphoma. However, this procedure requires invasive surgical intervention, and the limited region of the biopsy taken may not reflect the spatial heterogeneity of the disease, such as metastatic sites. In addition, it is not feasible to monitor response to treatment with repeated sampling, which may cause discomfort to patients and carry risks such as bleeding and infection. In recent years, circulating cell-free DNA has emerged as a complementary liquid biopsy option that overcomes these obstacles and is actively being researched for its clinical utility in the diagnosis and prognostication of certain cancer types. To the best of our knowledge, there was no study that reported the diagnostic and prognostic potential of cfDNA genotyping in patients with follicular lymphoma when this Ph.D. thesis was initiated.

1.2. Objectives of the study

The primary objectives of this study were: **1**) to determine the concordance of mutations detected in tumor tissue of follicular lymphoma patients with those identified in cfDNA extracted from peripheral blood plasma; **2**) to assess the diagnostic and prognostic utility of cfDNA genotyping in follicular lymphoma patients; and **3**) to investigate the presence of mutations associated with resistance to R-chemotherapy in cfDNA.

1.3. Hypothesis of the study

Circulating cell-free DNA in peripheral blood plasma exhibits mutations present in tumor tissues, and its genotyping has the potential to improve the diagnosis and prognosis of follicular lymphoma patients.

2. GENERAL INFORMATION

2.1. B-cell development and germinal centers

The majority of lymphoid cancers arise from abnormalities in B-cells at various stages of their development (Küppers, 2005). Thus, understanding the mechanisms of its normal development help us elucidating the pathogenesis of B-cell lymphomas. B-cells originate from hematopoietic stem cells in the liver during fetal development and then in the bone marrow after birth (O'Byrne et al., 2019). In the bone marrow, hematopoietic stem cells as being multipotent have the capacity to generate all types of blood cells, including red blood cells, platelets, and white blood cells (J. Y. Lee & Hong, 2020). Activation of certain transcription factors such as Ikaros, PU.1, and GATA3 gives rise to common lymphoid progenitor cells from hematopoietic stem cells and loss of their capacity to differentiate into myeloid cells (Mercer et al., 2011). Subsequently, under the influence of specific cytokines in the bone marrow, common lymphoid progenitor cells express specific transcription factors including E2A, EBF1, and Pax5, leading them to the B cell fate, which goes through stages of pro-B cell, pre-B cell, and finally immature B cell (Ramírez et al., 2010).

During differentiation from pro-B cell to immature B cell, with the help of recombination activating genes *RAG1* and *RAG2*, gene rearrangement of antigen receptors occurs in the bone marrow (Papavasiliou et al., 1997). The random recombination of V (variable), D (diversity), and J (joining) segments of the heavy gene locus enables the production of tremendous diversity of antigen receptors in B cells (Roth, 2014). Similarly, light chain gene rearrangement occurs following the heavy chain but carries only V (variable) and J (joining) segments (Collins & Watson, 2018). In the gene rearrangement process, RAG enzymes cleave the DNA from conserved recombination signal sequences (RSS) and introduce double strand breaks which are then joined by the non-homologous end joining repair pathway (G. S. Lee et al., 2004). However, this mechanism is error-prone and may underlie some of the B cell lymphoma pathogenesis, such as the formation of chimeric oncogenes where certain genes are translocated to antigen receptor regulatory sequences (Haines et al., 2006; Vaandrager et al., 2000). Another enzyme involved in V(D)J recombination is terminal deoxynucleotidyl transferase (TdT) which adds nucleotides to the ends of RAG-cleaved DNA without a template and increases the diversity of antigen receptors (Motea & Berdis, 2011). Aberrant expression of TdT is also shown in the pathogenesis

of certain B cell lymphomas (Bhavsar et al., 2022; Bouroumeau et al., 2020; Habeshaw et al., 1979; Kung et al., 1978). After gene rearrangement is completed, B cells undergo certain checkpoints for functional antigen receptors and self-reactivity (Nemazee, 2017). B cells that pass these checkpoints migrate to secondary lymphoid organs such as the spleen, lymph nodes, and mucosal-associated lymphoid tissues (MALT).

In secondary lymphoid organs, naive B cells encounter antigens and are activated through germinal center reactions (Stebegg et al., 2018). One mechanism that enable mature B cells to generate high-affinity antibodies is somatic hypermutation (SHM), where the enzyme activation-induced cytidine deaminase (AID) introduces mutations on the variable region of immunoglobulin genes by converting cytosine to uracil, which are subsequently repaired by error-prone mechanisms (Maul & Gearhart, 2010). Although this mechanism enables improvement in antibody affinity to foreign antigens, it is also associated with tumorigenesis of germinal center B cell lymphomas (Hardianti et al., 2004; Rogozin et al., 2016; Xu-Monette et al., 2019). While B cells with low affinity antibodies are eliminated through apoptosis in the germinal center, B cells with high affinity are selected and undergo class switch recombination (CSR) that changes the isotype of the antibody, allowing for different effector functions without changing the antigen specificity of the antibody it carries (Xu et al., 2012). The selected and differentiated B cells, which have high-affinity antibodies and the appropriate isotype, differentiate into either memory B cells or plasma cells, which secrete large amounts of antibody.

Lymphomas can arise from B cells at various stages of development with different genetic abnormalities, such as changes in chromosomes, alterations in the number of copies of certain genes, or mutations at specific points in the DNA (Esmeray & Küçük, 2020). A summary of lymphomas arising during each stage of development in germinal centers is given in **Figure 1**.

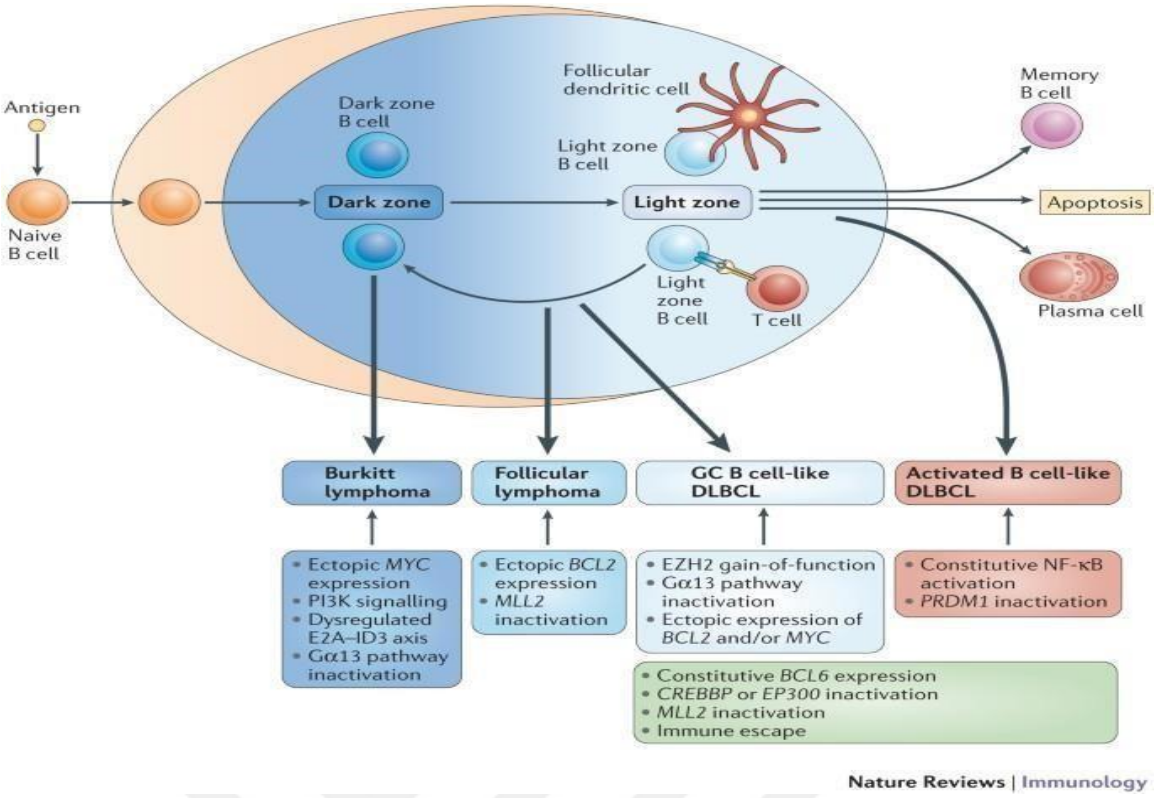


Figure 1: B-cell lymphomas and their origin of developmental stage in the germinal center (adapted from Basso et al. (2015). Nat Rev Immunol).

2.2. Follicular lymphoma

Estimated number of new cases of non-Hodgkin lymphomas (NHL) in the USA in 2023 is 80,550, which account for 4% of all cancer types (Siegel et al., 2023). Follicular lymphoma is the second most common subtype of non-Hodgkin lymphoma after diffuse large B-cell lymphoma (DLBCL) and represents 20-30% of all NHL cases (Teras et al., 2016). Contrary to DLBCL, follicular lymphoma is usually indolent in character; however, it has a risk of transformation to other aggressive B-cell lymphomas such as DLBCL with an estimated rate of 2% per year (Link et al., 2013). FL incidence is slightly higher in men than women with a ratio of 1.2, and at diagnosis, with a median age range of 60-65 years, it is more common in older people (Cerhan, 2020). Etiology of FL is complex and environmental risk factors are weakly associated with disease occurrence (Morton et al., 2014). Genetic susceptibility of FL has been shown with genome-wide association studies and linked family history (Cerhan & Slager, 2015; Skibola et al., 2014).

Symptoms of follicular lymphoma include enlargement of lymph nodes in certain areas such as the neck, armpits, or groin, usually accompanied by fatigue, unexplained weight loss, night sweats, fever, and shortness of breath. Other symptoms, such as anemia, thrombocytopenia, and neutropenia, can be seen in follicular lymphoma with bone marrow involvement, which occurs in 40-70% of cases (Kato et al., 2016). Additional to bone marrow other extra-nodal sites involved in follicular lymphoma include the spleen, liver, bone, lung, and skin (St-Pierre et al., 2019).

Initial diagnosis of FL is made with surgically excised and invasive biopsy specimens from enlarged lymph nodes with histological and immunohistochemical examination. The normal architecture of the lymph nodes in FL is effaced by closely packed follicles (Townsend et al., 2020), and in immunohistochemical staining, FL cells are positive for BCL2, BCL6, and CD10, in addition to pan-B-cell surface antigens CD19, CD20, CD22, and CD79a, and negative for CD5, CD23, CD11c, and CD43 markers (Boyd et al., 2013; Cho, 2022). However, an accurate diagnosis with histological and immunohistochemical staining may be technically challenging due to inadequate samples (Wilkins, 2011) or the requirement for an expert review (Bowen et al., 2014; Lester et al., 2003; Matasar et al., 2012; Proctor et al., 2011), as a recent study showed a 14.5% discrepancy between referral and expert diagnosis of FL (Laurent et al., 2017). According to the 2016 classification of the WHO, the histologic examination of FL is divided into grades based on the centroblast proportion per high-power microscopic field. If there are less than 5 centroblasts, it is classified as grade 1; if there are 6 to 15 centroblasts, it is classified as grade 2; and grade 3 lymphomas have more than 15 centroblasts per high-power field. Grade 3 is further divided into two subgroups: grade 3A, in which centrocytes are present, and grade 3B, when there are solely sheets of centroblasts (Klopčič et al., 2016). Another method in the diagnosis of FL and also monitoring the response to initial treatment is positron emission tomography-computed tomography (PET/CT) imaging. While PET/CT imaging offers advantages such as being non-invasive, it also has limitations, including high costs, limited ability to provide detailed molecular characteristics of tumors, and potential for false-positive results (Barrington et al., 2022; Griffeth, 2005; Wirth et al., 2008).

5-year survival rate of follicular lymphoma is around 90% (Xie et al., 2022), which has improved since the introduction of rituximab, a monoclonal antibody, to the treatment regimens (Dotan et al., 2010; Fisher et al., 2005; Marcus et al., 2008).

Several potentially predictive factors for the prognosis of FL were analyzed in a study involving 4167 patients, leading to the proposal of 5 adverse prognostic factors in the Follicular Lymphoma International Prognostic Index (FLIPI), which include age, Ann Arbor stage, lactate dehydrogenase (LDH) level, hemoglobin level, and the number of nodal areas (Solal-Céligny et al., 2004) (**Table 1**). Further improvements have been made on this index with FLIPI-2, which also includes factors such as age, hemoglobin level, β2 microglobulin level, longest diameter of the largest involved node 6 cm or more, and bone marrow involvement (Federico et al., 2009) (**Table 1**). Risk stratification of patients according to these indexes is based on traditional clinical factors. However, recent advancements in genetic factors related to follicular lymphoma have led to the development of a new clinicogenetic risk model, m7-FLIPI, which integrates mutation status of seven genes (*EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP, and CARD11*) (Pastore et al., 2015).

Table 1: Risk factors evaluated in FLIPI and FLIPI2.

FLIPI risk factors	**FLIPI2 risk factors**
Age >60 years	Age >60 years
Raised LDH	Raised β2 microglobulin
Haemoglobin <12mg/dl	Haemoglobin <12mg/dl
Ann Arbor stage >=3	Maximal diameter of lymph node >6cm
>4 areas of lymph node	Bone marrow involvement

FL is an indolent type of lymphoma, and segregation of FL patients into low- and high-risk groups is important in the management of the disease treatment. FL patients without symptoms, such as enlarged lymph nodes, fever, night sweats, weight loss, etc., are usually monitored with a "watch-and-wait" approach, as treatment does not improve overall survival (OS) in this group, although progression-free survival is improved (Advani et al., 2004; Ardeshna et al., 2014). On the other hand, symptomatic cases are treated with rituximab-containing immunochemotherapy regimens, such as R-CHOP (rituximab - cyclophosphamide, hydroxydaunomycin, oncovin, prednisone), R-CVP (rituximab - cyclophosphamide, vincristine sulfate, prednisone), or R-bendamustine. Understanding the heterogeneous biological background of the disease, such as alterations and interactions on genetic, epigenetic, and tumor

microenvironment factors, will enable more accurate and personalized treatment options and better outcomes (Kumar et al., 2021).

Translocation of t(14;18)(q32;q21), which involves the fusion of the immunoglobulin heavy chain (IGH) enhancer on chromosome 14 with the *BCL2* gene on chromosome 18, resulting in the overexpression of the anti-apoptotic BCL2 protein, occurs in around 85% of FL cases and is considered an early event in the pathogenesis of FL (Green et al., 2013a; Horsman et al., 1995). However, this translocation is not sufficient by itself and is not indicative of FL pathogenesis, as it also occurs in healthy individuals (Schüler et al., 2003). On the other hand, this translocation is predictive of subsequent FL development with a 23-fold higher risk in healthy individuals (Roulland et al., 2014). Other somatic mutations that recurrently occur in FL have been demonstrated with next-generation sequencing studies of whole-genome, whole exome, or targeted sequencing (**Table 2**). Affected pathways and mutated genes leading to FL pathogenesis include the histone-modifying genes (*KMT2D, CREBBP, EP300, and EZH2*), chromatin remodelling genes (*HIST1H1B-E, ARID1A*), BCR/NF-κB signaling pathway (*CARD11, TNFAIP3, CD79A, CD79B, and MYD88*), transcription factors (*BCL6, MEF2B, FOXO1*), JAK-STAT pathway (*SOCS1, STAT6, STAT3*), and NOTCH signaling (*NOTCH1, NOTCH2, DTX1*) (López et al., 2022).

Table 2: Recurrent genetic alterations and their predicted effects in follicular lymphoma (adapted from Carbone et al 2019).

Gene	Alterations (effect)	Frequency inFL (%)	Effect or function
Proliferation			
KMT2D	Mutation (↓)	80–90	Histone modification; tumour suppressor
IgHV, *IgLV*	Mutation (↑)	75–90	N-glycosylation of IgV region of BCR; BCR signalling
RB1	Deletion (↓)	12	Impairment of cell cycle control
CDK4	Copy number gain (↑)	29	Impairment of cell cycle control
BCL6	Translocation (↑) Mutation (↑)	6–15 47	Transcription factor; tumour progression
H1–2, H1–4	Mutation (↓)	44	Chromatin remodelling
MEF2B	Mutation (↓)	13–15	Transcription factor; transcriptional activator

EP300	Mutation (↓)	10–20	Histone modification
SESN1	Epigenetic silencing (↓)	~20	Promotion of mTOR activity
RRAGC ATP6V1B2, ATP6AP1	Mutation (↑)	17	mTORC1 survival signal
EZH2	Mutation (↑)	7–30	Histone modification
ARID1A	Mutation (↓)	15	Chromatin remodelling
GNA13	Mutation (↓)	~10	B cell growth and lymphoma cell dissemination
SGK1	Mutation (↓)	~10	Deregulation of FOXO transcription factors and NF-κB
FOXO1	Mutation (↑)	~10	Transcription factor; survival and proliferation
CARD11	Mutation (↑)	10	Increased BCR signalling
STAT6	Mutation (↑)	10	Activation of JAK–STAT signalling
Survival			
BCL2	Translocation (↑) Mutation (↑)	80–90 50	Suppression of apoptosis
TNFAIP3	Mutation (↓)	2–26	Loss of tumour suppressor
Immune evasion			
EPHA7	Deletion (↓) Epigenetic silencing (↓)	70	Tumour suppressor
TNFRSF14	Mutation (↓)	18–50	Tumour suppressor; increased BCR signalling
CREBBP	Mutation (↓)	33–70	Histone modification; tumour suppressor

2.3. Circulating cell-free DNA

The first description of extracellular DNA found in circulation had been made in 1948 by Mandel and Metais (Mandel & Metais, 1948), far earlier than PCR-based sequencing techniques. After decades, with the improvement of molecular biology and sequencing techniques in sensitivity and specificity, which enabled the detection of mutations with low allele frequencies, cfDNA gained importance in many fields of research such as cancer diagnosis and monitoring, non-invasive prenatal testing, transplantation, and infectious disease diagnosis (Yan et al., 2021).

Cell-free DNA in circulation (cfDNA) is released from apoptotic and necrotic cells, in addition to active secretion with exosomes or NETosis (Neutrophil Extracellular Traps) (Jackson Chornenki et al., 2019; Stroun et al., 2001; Thakur et al., 2014; Ungerer et al., 2021). The size of cfDNA fragments corresponds to the size of DNA wrapping around a nucleosome, which is around 147 bp, and it is thought that histones bound to DNA protect it from degradation by DNAses (Snyder et al., 2016). The concentration of cfDNA has high variability in plasma samples of both healthy individuals, ranging from 0 to 100 ng/ml (Mouliere et al., 2014), and cancer patients, ranging from 5 to 1000 ng/ml (Schwarzenbach et al., 2011), although there is a significant increase in the latter. Other conditions also lead to an increase in cfDNA levels, such as inflammatory diseases, trauma, pregnancy, organ transplantation, and cardiovascular diseases (Bloom et al., 2017; Cuadrat et al., 2021; Del Vecchio et al., 2021; Duvvuri & Lood, 2019; Rodrigues Filho et al., 2014). Clearance of cfDNA from the body can occur via the kidneys and be excreted in urine (Dermody et al., 2022), or in home tissue, blood, other body fluids, or organs such as the liver, spleen, or lymph nodes (Kustanovich et al., 2019). The half-life of cfDNA in circulation ranges from minutes to a few hours (Khier & Lohan, 2018).

As a liquid biopsy, cfDNA analysis has attracted attention in translational medicine due to its advantages over tissue biopsy. First, it is non-invasive and does not carry the risks of invasive tissue biopsy, which can cause discomfort and may result in other complications during the procedure (Cigna et al., 2012; Espinosa-Pereiro et al., 2019). Second, cfDNA in circulation is released from any region of the body, providing a global profile of the affected sites, such as metastasis, and better reflecting tumor heterogeneity compared to tissue biopsy, which is restricted to the region it is taken from (Dawson et al., 2013; Parikh et al., 2019). Third, its ease of procedure and rapid turnover enable repeated sampling, providing a more accurate and real-time view of tumor evolution (Lone et al., 2022). Additionally, cfDNA has potential advantages in guiding disease treatment by monitoring response and detecting minimal residual disease (Merker et al., 2018; Oellerich et al., 2017). In fact, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved detection of *EGFR* mutational status in cfDNA in clinical practice for non-small cell lung cancer to guide drug usage (Bardelli & Pantel, 2017), followed by the approval of the first diagnostic test (i.e., Roche cobas® *EGFR* mutation) in 2016 (Malapelle et al., 2017). Similarly, high concordance in *KRAS* mutational status between tumor tissue

and plasma cfDNA has been shown in different studies for colorectal cancer (Demuth et al., 2018; Siravegna et al., 2015).

In recent studies, the potential of cfDNA as a liquid biopsy has been investigated in B-cell lymphomas. In DLBCL, it has been shown that mutations with a variant allele fraction larger than 20% in tissue biopsy can also be detected in plasma cfDNA samples before treatment with a sensitivity of more than 90% and specificity of 100% (Rossi et al., 2017). In the same study, it had been showed that while DLBCL related mutations were cleared in plasma cfDNA samples of patients responding to treatment; in resistant patients these mutations were remained (Rossi et al., 2017). In another study, it had been shown that monitoring the clonal evolution and emerging resistant mutations after treatment is possible with non-invasive ctDNA genotyping in DLBCL patients (Scherer et al., 2016). Furthermore, clinical values such as LDH level, IPI score, Ann Arbor stage and PET features (metabolic tumor volume) were shown significantly to correlate with the ctDNA levels in plasma samples of DLBCL patients (Alig et al., 2021; Bohers et al., 2018). Furthermore, in DLBCL it was shown that analysing cfDNA as liquid biopsy has potential in predicting the patient outcome in different circumstances including the risk of recurrence and prognosis (Kurtz et al., 2018; Rivas-Delgado et al., 2021; Roschewski et al., 2015). Similarly, the diagnostic and prognostic potential of ctDNA genotyping and its applicability in monitoring residual disease were also shown in classical Hodgkin lymphoma (Spina et al., 2018) and other lymphoma subtypes (Kambhampati & Zain, 2022). These recent studies on DLBCL and other lymphoma subtypes reveal the feasibility of ctDNA genotyping in patient management in a clinical setting. However, the potential of cfDNA as a diagnostic and prognostic biomarker is largely unknown in FL. In this study, we aimed to investigate whether cfDNA genotyping in FL has potential as a liquid biopsy in diagnosis, prognosis, and patient management by addressing whether cancer-associated mutations found in tissue biopsy are also present in cfDNA and by investigating their prognostic value.

3. MATERIALS AND METHODS

3.1. Type of the research

The present study employs a prospective cohort design, utilizing observational methods in conjunction with next generation sequencing and bioinformatic analyses in the assessment of follicular lymphoma patients.

3.2. Location and the time frame of the research

All experiments were conducted at the İzmir Biomedicine and Genome Center between 2016 and 2021. Patients enrolled in this study were diagnosed at the Hematology Department of Dokuz Eylül University between 2016 and 2019. The next generation sequencing service was provided by Novogene company (Hong Kong).

3.3. Research population, sampling, and experimental groups

In this study, 20 newly diagnosed FL patients at DEÜ Hematology Department were enrolled and the diagnosis of the disease followed the routine procedures of the clinic according to the WHO 2016 classification. Histological evaluations of biopsy samples from patients showed a follicular pattern of neoplastic lymphoid infiltrate arranged back-to-back, which effaced the normal lymph node architecture in morphology. These infiltrating cells were positive for immunohistochemical staining with CD10, BCL6, BCL2, and CD20 markers, in addition to CD23-positive dendritic network in the center of follicles, which excludes transformation to DLBCL.

Peripheral blood and tumor biopsy FFPE samples were collected from each of the 20 FL cases during diagnosis. Thirteen symptomatic patients received rituximab-based immunochemotherapy regimens as their treatment. The remaining 7 patients were asymptomatic and were monitored with a watch-and-wait strategy. From 12 of the 13 symptomatic cases (as one of them passed away), peripheral blood samples were collected after their treatment for the investigation of potential variants in tumor tissue resistant to immunochemotherapy (**Table 3**).

Table 3: Demographic and clinical characteristics of the FL patients and timing post-treatment sample collection.

FL case number	Gender	Age	Ki-67 index (%)	LDH level (U/L)	Treatment	Sampling time after last chemotherapy cycle
Case-01	Female	50	20	187	R-CHOP	64 weeks
Case-02	Female	78	NA	251	Watch-and-Wait	-
Case-03	Female	40	NA	229	R-CVP	4 weeks
Case-04	Female	71	25	213	Watch-and-Wait	-
Case-05	Male	34	30	110	R-CHOP	19 weeks
Case-06	Female	60	35	193	R-CVP-Radiotherapy	18 weeks
Case-07	Male	38	45	197	R-CHOP	2 weeks
Case-08	Female	41	35	135	R-CHOP	3 weeks
Case-09	Female	35	20	205	Watch-and-Wait	-

Case-10	Female	54	27	336	R-Bendamustine	out of follow-up
Case-11	Male	60	30	262	R-Bendamustine	5 weeks
Case-12	Female	55	23	228	R-CHOP	3 weeks
Case-13	Female	60	23	171	Watch-and-Wait	-
Case-14	Male	62	45	263	R-CHOP	3 weeks
Case-15	Female	47	20	209	Watch-and-Wait	-
Case-16	Female	62	45	518	R-CHOP	4 weeks
Case-17	Male	58	27	170	Watch-and-Wait	-
Case-18	Female	37	NA	224	R-Bendamustine	6 weeks
Case-19	Female	59	55	215	R-CHOP	4 weeks
Case-20	Female	32	23	190	Watch-and-Wait	-

All patients diagnosed with FL without transformation to DLBCL were included in the study, and there were no other exclusion criteria for sampling. All patients involved in the study provided consent to participate, and all samples were collected and processed in accordance with the ethical recommendations approved by the institutional review board of the Medical School at Dokuz Eylül University (protocol no: 2233-GOA).

3.4. Research materials

3.4.1. Solutions

Solutions	**Vendor**
Ficoll-Paque PLUS	GE Healthcare, Uppsala, Sweden
RPMI 1640 medium	Gibco-Invitrogen, CA, USA
TAE buffer (tris-acetate-EDTA) (50X)	Bioshop, Burlington, Canada

3.4.2. Chemicals

Chemicals	**Vendor**
50 bp and 100 bp DNA ladders	New England Biolabs, MA, USA
Agarose	Sigma Aldrich, St Louis, MO, USA
Ethanol (96-100%)	Isolab, Wertheim, Germany
Gel loading dye	New England Biolabs, MA, USA
Isopropanol	Merck, Darmstadt, Germany
Xylen	Sigma Aldrich, St Louis, MO, USA

3.4.3. Kits

Kits	**Vendor**
PAXgene Blood DNA Kit	Qiagen, Hilden, Germany
QIAamp Circulating Nucleic Acid Kit	Qiagen, Hilden, Germany

QIAamp DNA FFPE Tissue Kit	Qiagen, Hilden, Germany

3.4.4. Equipment

Equipments	**Vendor**
Centrifuge	Eppendorf, Hamburg, Germany
Freezer (-80°C)	Eppendorf, Hamburg, Germany
Gel Doc XR+ Gel documentation system	Bio-Rad, Hercules, CA, USA
Heat block	Thermo Fisher Scientific, Waltham, MA, USA
K2 EDTA tubes	BD Vacutainer, Plymouth, UK
Mini centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
NanoDrop 2000	Thermo Fisher Scientific, Waltham, MA, USA
PCR cabinet	Biosan, Riga, Latvia
Pipette set (10, 100, 1000 µl)	Eppendorf, Hamburg, Germany
Power supply unit for electrophoresis	Bio-Rad, Hercules, CA, USA
Refrigerator (+4°C)	Bosch, Stuttgart, Germany
Refrigerator (-20°C)	Bosch, Stuttgart, Germany
Thermal Cycler	Bio-Rad, Hercules, CA, USA
Vacuum manifold	Qiagen, Hilden, Germany
Vacuum Regulator	Megasan, İstanbul, Türkiye
Vortex	Thermo Fisher Scientific, Waltham, MA, USA

3.5. Research variables

Circulating cell-free DNA concentration and somatic variants in cfDNA and tumour tissue DNA are the independent research variables of this study. Dependent variables are the outcomes of patients and their prognosis.

3.6. Data collection tools

3.6.1. Collection of FFPE tumor tissue sections of FL cases

Tumor biopsy samples from lymph nodes of 20 newly diagnosed FL patients were preserved in FFPE blocks in the DEU pathology department. After clinical evaluations of the samples, the remaining archival material was collected for this study. Sections of FFPE tumor tissue samples were sliced to a thickness of 5µm using a microtome device, either at the İBG histopathology department or at the DEÜ pathology department. Five sections from each sample were placed into 1.5 mL microcentrifuge tubes and stored at +4°C to minimize degradation of nucleic acids (Groelz et al., 2018) until isolations were performed.

3.6.2. Collection of peripheral blood samples and separation of plasma

As a part of routine clinical procedure, peripheral blood samples were withdrawn from 20 newly diagnosed FL patients by venipuncture at DEÜ haematology department, and an extra volume was collected into 10 mL K2 EDTA tubes for the study. In order to prevent cellular lysis and contamination of plasma with cellular DNA, peripheral blood samples were transported to the laboratory in less than 30 minutes on ice and the plasma separation process was initiated immediately. Peripheral blood samples were transferred to 15 mL tubes and centrifuged at 1,600g for 10 min at +4°C. After centrifugation, the supernatant part, which is the plasma portion of the peripheral blood, was transferred to 1.5 mL microcentrifuge tubes, and the remaining part was used for granulocyte DNA isolation as explained in section 3.6.5. after adding RPMI 1640 solution up to the original volume. In order to remove cellular debris from the plasma, a second centrifugation was applied at 16,000g for 5 min at +4°C. Then, the supernatant was transferred to new 1.5 mL microcentrifuge tubes without dispersing the pellet, and the clean plasma samples were immediately placed in a -80°C freezer and stored there until cfDNA isolations were performed.

3.6.3. DNA isolation from FFPE tumor tissue sections

In DNA isolations from FFPE tumor tissue sections, the QIAamp DNA FFPE Tissue Kit was used following the manufacturer's instructions. Paraffin wax was first dissolved by adding 1 mL xylene into 1.5 mL microcentrifuge tubes in which sections were stored, and vortexed vigorously for 10 seconds. The dissolved wax and pellet were separated by centrifuging the tubes at 17,000g for 2 min at room temperature (RT). Residual xylene was removed by washing the pellet with 1 mL ethanol (96-

100%), followed by vortexing and centrifuging at 17,000g for 2 min at RT. After centrifugation, the supernatant was removed and discarded with a pipette, keeping the pellet intact. Residual ethanol was removed with a fine pipette tip, and the tube was incubated with the lid open at room temperature for 15 min or until all residual ethanol had evaporated. The dried pellet was then resuspended with 180 µL lysis buffer (ATL buffer), and 20 µL proteinase K was added and mixed by vortexing. The solution was incubated at 56°C for 80 minutes or until the pellet was dissolved, and to reverse the formalin crosslinking, it was then incubated at 90°C for 1 hour. After incubation, the tubes were briefly spun down to lower the drops on the surface of the lid. Next, 200 µL lysis buffer (AL buffer) and 200 µL ethanol (96-100%) were added to the tubes and mixed by vortexing until a homogeneous solution was obtained. The entire lysate was then transferred to the QIAamp MinElute column and centrifuged at 6,000g for 1 min at RT. After centrifugation, the column was placed in a new clean collection tube, and 500 µL of the first washing buffer (AW1 buffer) was added, followed by centrifugation at 6,000g for 1 min at RT. The centrifuged column was then placed into a new collection tube, and 500 µL of the second washing buffer (AW2 buffer) was added and centrifuged again at 6,000g for 1 min at RT. After washing was completed, in order to dry the membrane, the empty column was centrifuged at 17,000g for 3 min at RT. In the elution step, the column was placed into a labelled 1.5 mL microcentrifuge tube in which the eluted DNA would be stored, and 50 µL elution buffer (ATE buffer) was added, followed by incubation at RT for 1 min. Finally, the sample was eluted into the microcentrifuge tube by centrifugation at 17,000g for 1 min at RT. The concentration of the eluted FFPE tumor tissue DNA samples was determined with a Thermo Nanodrop 2000 spectrophotometer and stored at -20°C until shipment for sequencing.

3.6.4. cfDNA isolation from plasma samples

QIAamp Circulating Nucleic Acid Kit was used for the isolation of cfDNA from plasma samples of newly diagnosed FL patients, following the manufacturer's instructions. Plasma samples, which were stored at -80°C, were first equilibrated to room temperature. In a 50 mL centrifuge tube, 500 µL of proteinase K was pipetted, and 5 mL of plasma sample was added to it. Then, 4 mL of lysing buffer (ACL buffer) was added and pulse-vortexed for 30 seconds with a visible vortex until a homogeneous solution was obtained. The solution was immediately placed on a heat block at 60°C and incubated for 30 minutes. After incubation, 9 mL of binding buffer

(ACB buffer) was added to the solution and mixed for 15-30 seconds with pulse-vortexing, then incubated on ice for 5 minutes. Next, the solution was transferred to a vacuum manifold (QIAvac 24 Plus) with a settled QIAamp Mini column with extender, and the vacuum pump was switched on until all lysates were drawn through the columns completely, then the pump was switched off. Six hundred µL of first washing buffer (ACW1 buffer) was added, and the vacuum pump was switched on again until all of the buffer passed through the column. After that, seven hundred fifty µL of second washing buffer (ACW2 buffer) was added to the column, and the vacuum pump was switched on until all the buffer was drawn completely. As the last washing step, 750 µL of ethanol (96-100%) was added, and the vacuum pump was switched on until all ethanol passed through the column, and the vacuum pump was switched off. To remove any residual ethanol, the mini column was placed into a new clean collection tube and centrifuged at 17,000g for 3 minutes at room temperature (RT). The centrifuged column was transferred to a new clean collection tube and incubated on a heat block at 56°C for 10 minutes with an open lid to dry the membrane completely. The dried column was then placed into a labelled 1.5 mL microcentrifuge tube, and 50 µL of elution buffer (AVE buffer) was added, followed by incubation for 3 minutes at RT. The incubated sample was centrifuged at 17,000g for 1 minute at RT, and the column was transferred from the eluted tube to a new labelled 1.5 mL microcentrifuge tube, and again, 50 µL of elution buffer was added as the second elution. After incubation for 3 minutes at RT, the sample was centrifuged at 17,000g for 1 minute at RT. The concentrations of eluted cfDNA samples were measured with a Thermo Nanodrop 2000 spectrophotometer and stored at -20°C until shipment for sequencing.

3.6.5. Granulocyte DNA isolations from peripheral blood samples

Plasma was separated from peripheral blood samples as described in section 3.6.2, and RPMI 1640 medium solution was added to the remaining part, which included granulocyte cells, in the same volume as the plasma. To separate granulocytes from peripheral blood mononuclear cells (PBMC), the ficoll separation method was used. First, 2 mL of blood-RPMI 1640 mixture was transferred to a 15 mL centrifuge tube, and 6 mL of 1X PBS was added and mixed by pipetting. It was then carefully layered onto 6 mL of Ficoll-Paque PLUS in another 15 mL centrifuge tube, taking care not to disturb the layer. The tubes with two layers were placed into the centrifuge rotor with the brake set to zero and centrifuged at 400g for 30 min at room

temperature. After centrifugation, the PBMC part, which settled between the plasma and Ficoll-Paque media, was removed and discarded. DNA isolations from granulocytes were performed following the manufacturer's instructions with volumes of reagents scaled down proportionally. The granulocyte and red blood cell part was transferred to a new 15 mL centrifuge tube and mixed with lysis buffer (BG1 buffer) by inverting the tube 5 times. The mixture was then centrifuged at 2,500g for 5 min at room temperature. The supernatant was removed, and 1.25 mL of wash buffer (BG2 buffer) was added to the pellet and mixed by vortexing vigorously for 5 seconds, followed by centrifugation at 2,500g for 3 min at room temperature. After removal of the supernatant, 12.5 µL of premixed protease and digestion buffer (BG3 buffer) was added, and the solution was vortexed for 20 seconds at high speed. The mixed solution was then incubated on a heat block at 65°C for 10 min. After incubation, the solution was vortexed at high speed for 5 seconds, and 1.25 mL of isopropanol (100%) was added and mixed by inverting the tube at least 20 times until clumps of white-colored DNA strands were visible. Centrifugation at 2,500g for 3 min at room temperature was applied, and the supernatant was removed. The inverted tube was then left on a clean piece of absorbent paper for 1 min to minimize backflow of isopropanol. In order to wash and promote DNA aggregation, 1.25 mL of 70% (v/v) ethanol was added to the pellet, and the solution was vortexed for 1 second at high speed and centrifuged at 2,500g for 3 min at room temperature. After centrifugation, the supernatant was discarded, and the tube was left on a clean piece of absorbent paper for at least 5 min. The tube was then dabbed onto absorbent paper to remove ethanol from the rim and left inverted for an additional 5 min to allow the DNA pellet to dry. For the elution step, 250 µL of elution buffer (BG4 buffer) was added to the pellet and incubated on a heat block at 65°C for 1 hour to dissolve the pellet. Finally, the concentrations of eluted DNA samples were determined with a Thermo Nanodrop 2000 spectrophotometer followed by long-term storage at -20°C until shipment for sequencing.

3.6.6. Ultra-deep targeted next generation sequencing of DNA samples

Investigation of mutations found in isolated DNA samples was performed using ultra-deep next-generation sequencing with a custom-designed targeted NGS panel. This panel covered 110 FL-associated genes (**Table 4**), based on previously published studies (Correia et al., 2015a; Green et al., 2013b, 2015; Karube et al., 2014; Li et al., 2014; Morin et al., 2010; Okosun et al., 2014; Sander et al., 1993), spanning a genomic region of 575,481 bp corresponding to coding and flanking 50 bp intronic sequences

of these genes. All DNA samples were sequenced at Novogene Genome Sequencing Company (Hong Kong, China) using the HiSeq system with 150 bp paired-end reads.

Table 4: The genes included in the custom-designed targeted NGS panel.

ABCC10[b]	*BCL6*[b]	*CCNK*[b]	*DTX1*[b]	*FOXO1*[b]	*HIST1H2BC*[e]	*KMT2D*[b]	*NEB*[f]	*SLC9A6*[f]	*UPF1*[b]
AGTPBP1[b]	*BCL7A*[b]	*CD40*[f]	*EBF1*[e]	*GNA13*[b]	*HIST1H2BD*[e]	*KNDC1*[b]	*NOTCH2*[c]	*SOCS1*[e]	*USP6*[f]
ARHGEF1[b]	*BRWD3*[f]	*CD79A*[e]	*EEF1A1*[b]	*GNAI2*[b]	*HIST1H2BG*[e]	*LAMP1*[b]	*NOTCH1*[c]	*SP140*[b]	*VPS39*[d]
ARID1A[b]	*BTG1*[b]	*CD79B*[e]	*ENPP3*[f]	*GNE*[f]	*IKZF2*[f]	*LPHN1*[b]	*POU2F2*[d]	*STAT3*[e]	*WDR64*[d]
ATP6AP1[b]	*C4orf49*[f]	*CEP112*[f]	*ENTPD4*[f]	*GRM7*[d]	*IKZF3*[e]	*LYST*[d]	*OR2M3*[f]	*STAT6*[e]	*ZCCHC6*[b]
ATP6V1B2[b]	*CALR*[f]	*CHRM3*[d]	*EP300*[b]	*HIST1H1B*[e]	*IL13RA1*[b]	*MATN2*[f]	*PCLO*[d]	*SVIL*[b]	*ZNF141*[b]
AUTS2[f]	*CARD11*[e]	*CPNE1*[b]	*ERBB2*[f]	*HIST1H1C*[e]	*IRF8*[d]	*MCL1*[d]	*PEX14*[f]	*TLR1*[f]	*ZNF236*[b]
B2M[b]	*CASC5*[b]	*CREBBP*[e]	*ETV1*[b]	*HIST1H1D*[e]	*KIR2DL3*[f]	*MEF2B*[b]	*PLCG2*[e]	*TNFAIP3*[e]	*ZNF423*[b]
BAZ2B[f]	*CASZ1*[b]	*CTSS*[f]	*EZH2*[g]	*HIST1H1E*[e]	*KLHDC7B*[d]	*MON2*[b]	*PRKCB*[e]	*TNFRSF14*[e]	*ZNF541*[d]
BCL10[e]	*CCAR1*[f]	*DIRAS3*[f]	*FAT2*[f]	*HIST1H2AC*[e]	*KLHL6*[b]	*MUC4*[f]	*RBM23*[b]	*TP53*[h]	*ZNF595*[b]
BCL2[a]	*CCND3*[b]	*DMRT3*[d]	*FN1*[b]	*HIST1H2AG*[e]	*KMT2C*[b]	*MYD88*[e]	*ROS1*[f]	*TRAFD1*[b]	*ZNF672*[b]

a: Correia et al., 2015; b: Green et al., 2015; c: Karube et al., 2014; d: Li et al., 2014; e: Okosun et al., 2014; f: Green et al., 2013; g: Morin et al., 2010; h: Sander et al., 1993.

The quality of DNA samples was evaluated using the Agilent 2100 automated electrophoresis system, which checks for DNA integrity and quantitation before sequencing. The library preparation was performed using the Agilent SureSelectXT Custom Kit (Agilent Technologies, CA, USA) following the manufacturer's recommendations with a total of 0.5µg DNA. In brief, 180-280 bp fragments were generated using hydrodynamic shearing (Covaris, Massachusetts, USA), and the remaining overhangs were converted to blunt ends by exonuclease/polymerase activities, followed by removal of the enzymes. Then, DNA fragments were adenylated from the 3' ends, followed by ligation of adapter oligonucleotides on both ends and PCR enrichment. Enriched fragments were hybridized to a total of 6385 biotin-labelled probes, and targeted regions were captured with magnetic beads with streptomycin. In order to add index tags for hybridization, captured libraries were enriched in another PCR reaction. Finally, the AMPure XP system (Beckman Coulter, Beverly, USA) was used for purification, and the libraries ready for sequencing were quantified with the Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. Qualified libraries were sequenced, and raw data was produced with a read depth higher than 1,500X, covering the targeted region for the identification of somatic variants with low allele fractions.

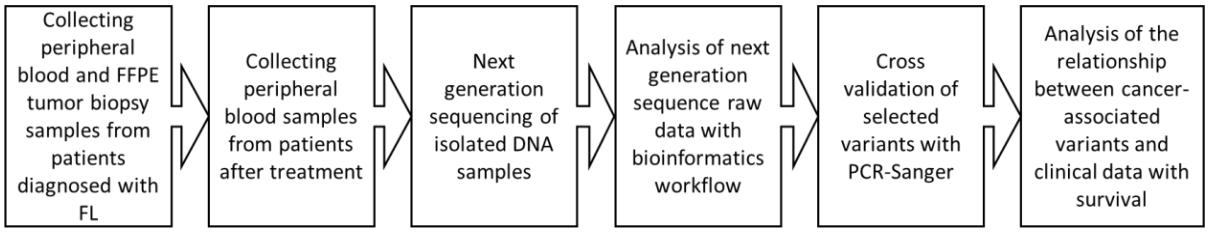
3.6.7. Sanger sequencing for validation and primer design

Variants detected through ultra-deep targeted next generation sequencing were cross-validated using Sanger sequencing. Variants with a variant allele fraction (VAF) higher than 20%, which was chosen as the threshold value based on a previous publication (Tsiatis et al., 2010), were selected for validation. Primers were designed using the PrimerQuest tool (IDT DNA Technologies Inc.) by searching the target region on the UCSC Genome Browser using the COSMIC ID of the variant and obtaining the target sequence with the mutated nucleotide at its center. The amplicon size was limited to a maximum of 160 bp, as cfDNA is typically fragmented with a peak at around 167 bp, which corresponds to the length of DNA wrapped around a nucleosome with 147 bp and an additional 20 bp linker region. The designed primers were synthesized by Sentebiolab Biotech (Ankara, Türkiye), and the optimum annealing temperatures of each primer pair were determined through gradient PCR. Primer pairs that succeeded in the optimization experiments were used in the validation experiments (**Table 5**). Amplified samples were purified using PCR purification and sequenced at Eurofins Genomics (Ebersberg, Germany). The same primer pairs used for PCR amplifications of the mutated regions in cfDNA and/or FFPE tDNA, as well as patient-matched granulocyte gDNA samples as controls, were also used in the Sanger sequencing for forward and reverse reading of the targeted region, with the mutated nucleotide located around the middle of the target to ensure good quality sequencing from both ends.

Table 5: The list of primers used in PCR-Sanger cross-validations.

Primer Name	Forward Sequence	Reverse Sequence	Size (bp)
CREBBP-C1237Y	5'- AAATGACAGGACGGTACTTACG -3'	5'- GGACCAGTTCACCCAAGTATG -3'	139
HIST1H1E-P131S	5'- GGGAAGCCAAGCCTAAG -3'	5'- GGCTTCTTCGCCTTCTTT -3'	142
ARID1A-R1722	5'- AATTCTGTTCTTAGGCCACTTT -3'	5'- CACCCACCTCATACTCCTTTA -3'	147
STAT6-D523V	5'- TATGGCTGCTCAGACTACC -3'	5'- CCCTAGGAGATCCTGCTG -3'	136
CTSS-Y132D	5'- ACTAAGCATTTAAAGAGCTCTACCT -3'	5'- TTGCCTGATTCTGTGGACTG -3'	114
HIST1H1E-A65P	5'- GCTCATTACTAAAGCTGTTGCC -3'	5'- TGTTGTTCTTCTCCACGTCAT -3'	107
ATP6AP1-G363R	5'- CCTCGAAGTCCACAGCAAT -3'	5'- ACGAGGAGACTACCCTTCTT -3'	123
IRF8-Y23H	5'- TCTGTCTTTCCAAGGATGTGTG -3'	5'- CAGCGTGTTTCCAAGGGAT -3'	145
KMT2D-R2417	5'- TGCAGCTGTTTCCTTCTCC -3'	5'- CCCTATATCGCTCCTGTCTCT -3'	158

3.7. Research plan and time-line



3.8. Data analysis

3.8.1. NGS raw data pre-processing

The DNA sequences obtained from the custom designed targeted next-generation sequencing (NGS) gene panel were initially received in FASTQ file format. Prior to further processing, the raw data underwent quality analysis using FastQC program (v0.11.5) to assess read quality. Subsequently, AfterQC program (v0.9.6) was utilized to separate the reads into good quality and bad quality categories. The good quality reads were then subjected to another round of analysis with FastQC (v0.11.5) for comparison. The processing of the NGS data continued with the use of only the good quality reads, which were aligned to the human reference genome hg19 using BWA-MEM (v0.7.12) generating Sequence Alignment Map (SAM) format files. These files were then converted to the Binary Alignment Map (BAM) format using SAMtools (v1.6). Further processing involved the removal of duplicated reads from the aligned reads using Picard tool (v2.15.0), and base recalibration was performed on the unique NGS reads using GATK4 in order to prepare the data for somatic short variant discovery.

3.8.2. Identification of the somatic short variants (SNVs and indels)

Somatic short variants were identified from pre-processed next-generation sequencing (NGS) reads using a bioinformatic pipeline that involved the use of Mutect2 (v4.0.1.2) tool. In this pipeline, a panel of normals was created using pre-processed NGS reads from healthy granulocyte DNA samples, which were expected to carry only germline variants. This panel of normals file was then used to filter out germline variants as well as technical sequencing artifacts from the NGS reads of tumor tissue DNA or cfDNA samples during the process of somatic variant discovery with Mutect2 (v4.0.1.2). The list of variants obtained using this bioinformatic pipeline was annotated with wANNOVAR, and the presence and somatic nature of these mutations were visually verified using Integrative Genomics Viewer (IGV).

To reduce the likelihood of false positives, the obtained variant list was further filtered. Variants that were present in the COSMIC database (release v89) and previously identified in hematopoietic and lymphoid tissue were retained. Variants detected in circulating cell-free DNA (cfDNA) or formalin-fixed paraffin-embedded (FFPE) tissue DNA were filtered if the same variant was also detected in granulocyte DNA of the same patient and had a variant allele fraction (VAF) less than 4-fold of the VAF of granulocyte DNA. Additionally, variants with less than 0.5% VAF in general and variants with less than 6 forward or less than 6 reverse NGS reads were filtered out to obtain a confident list of variants **(Figure 2)**.

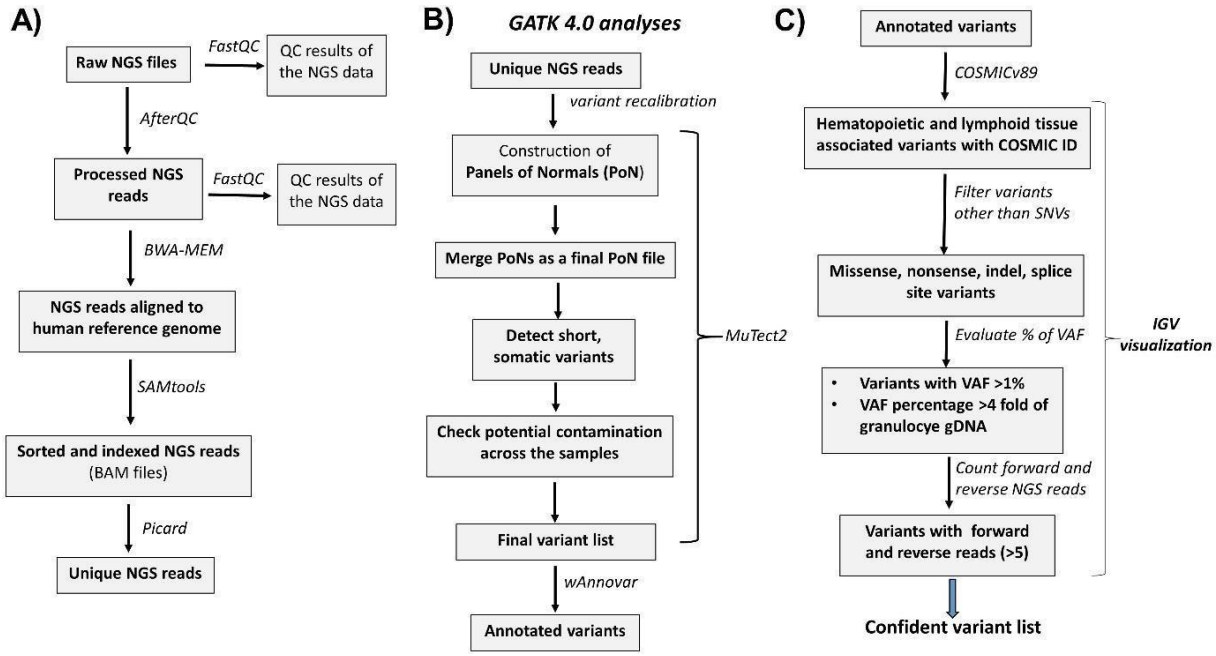


Figure 2: Computational bioinformatics pipeline used for identification of a confident list of somatic variants in FL cases.

3.8.3. Statistical analyses

Statistical analyses were conducted using RStudio (Build 351) with different packages for specific types of analyses. Survival analyses, including the generation of Kaplan-Meier graphics and comparison of grouped samples with the log-rank test, were performed using the R package Survival (version 3.4.0). The cut-off levels for continuous variables were determined using the R package Survminer (version 0.4.9), which utilizes maximally selected rank statistics. Pathway enrichment analysis was performed by applying the “ReactomePA” R package. For comparing cfDNA

concentrations, a t-test was employed, and Spearman correlation model was used for correlations. In all statistical analyses, a significance threshold of $p<0.05$ was considered statistically significant.

3.9. Limitations of the research

This study is considered exploratory in nature due to the limited number of cases included, and the statistical findings obtained need to be validated in independent studies with a larger sample size. The variant detection method utilized in this study has the capability to identify low allele frequency variants, but other techniques, such as the ones incorporating unique molecular identifiers (UMIs) in sequencing may further enhance sensitivity. Additionally, the targeted next generation sequencing approach employed in this study only covers a number of genes known to be associated with FL, and there is a possibility of missing rare variants in other genes, which have not yet been reported in the literature. Moreover, certain cancer-associated variants located outside of the coding sequences including but not limited to intergenic regions or enhancers can not be identified with the ultra-deep targeted NGS platform used in this study.

3.10. Ethical committee approval

The patient samples used in this study were obtained with the approval of the institutional review board of the Medical School at Dokuz Eylül University (protocol no: 2233-GOA, decision no: 2015/19-11, date: 21-08-2015). Informed consent was obtained from all patients who participated in the study.

4. RESULTS

4.1. Clinicopathological characteristics of the FL cases

The patient cohort of this study comprised of 20 cases diagnosed with follicular lymphoma (FL) as per the World Health Organization (WHO) 2016 classification. Among these cases, 13 were symptomatic and underwent immunochemotherapy treatment with rituximab-based regimens, including R-CHOP (8 cases), R-bendamustine (3 cases), R-CVP (1 case), and R-CVP-radiotherapy (1 case). The remaining 7 cases were asymptomatic and followed a watch-and-wait approach. The male-to-female ratio for the entire cohort was 1:3, and for symptomatic cases, it was 1:2.2. The mean age of the FL cohort in the study was 50.2 years for symptomatic cases and 54.4 years for asymptomatic cases, respectively, based on data obtained

from the database of DEU hospital. Other clinical parameters, such as Ki-67 index of biopsy specimens, LDH level before treatment, and PET/CT images (when available), were also collected. As expected, the mean Ki-67 index was higher in symptomatic cases (35.5%) compared to asymptomatic cases (23.0%). Similarly, the mean LDH level before treatment was higher in symptomatic cases with 238 U/L compared to asymptomatic cases with 201 U/L. **Table 6** provides a summary of the clinicopathological characteristics of the FL cohort in the study.

Table 6: Clinicopathological and demographic characteristics of the FL cases.

	Asymptomatic	**Symptomatic**
	(N=7)	**(N=13)**
Gender		
Female	6 (85.7%)	9 (69.2%)
Male	1 (14.3%)	4 (30.8%)
Age (years)		
Mean (SD)	54.4 (17.4)	50.2 (10.6)
Median [Min, Max]	58.0 [32.0, 78.0]	54.0 [34.0, 62.0]
Ki-67 Index (%)		
Mean (SD)	23.0 (2.76)	35.5 (10.9)
Median [Min, Max]	23.0 [20.0, 27.0]	35.0 [20.0, 55.0]
Missing	1 (14.3%)	2 (15.4%)
LDH Level (U/L)		
Mean (SD)	201 (28.0)	238 (101)
Median [Min, Max]	205 [170, 251]	224 [110, 518]
Treatment		
Watch-and-Wait	7 (100%)	0 (0%)
R-Bendamustine	0 (0%)	3 (23.1%)
R-CHOP	0 (0%)	8 (61.5%)
R-CVP	0 (0%)	1 (7.7%)
R-CVP-Radiotherapy	0 (0%)	1 (7.7%)

4.2. Concentrations of cfDNA, tumor tissue DNA and granulocyte DNA in FL patients

DNA concentrations of isolated samples were measured using a Thermo Nanodrop 2000 spectrophotometer prior to shipment to Novogene for targeted ultra-deep next-generation sequencing, as outlined in **Table 7**. It is important to note that the spectrophotometer measurements may not accurately reflect the actual cfDNA concentrations, as carrier RNA was used during cfDNA isolation to enhance yield according to the kit protocol. Therefore, for further analyses pertaining to plasma cfDNA concentrations, we utilized the concentrations determined by Novogene using the Qubit® DNA Assay Kit and Qubit® 2.0 Fluorometer.

Table 7: Concentrations of FL DNA samples based on spectrophotometer measurements.

FL case number	Granulocyte gDNA concentration (ng/µl)	FFPE tDNA concentration (ng/µl)	cfDNA concentration before therapy (ng/µl)	cfDNA concentration after therapy (ng/µl)
Case-01	539.2	1850.6	23	42.6
Case-02	61.8	50	25.2	N/A
Case-03	138.4	14.21	28.4	35.3
Case-04	83.4	637.6	29.8	N/A
Case-05	135.7	123.4	32	32.7
Case-06	260	136.8	21.4	37.3
Case-07	112.2	680	28.4	30.9
Case-08	220.9	172	21.2	24.1
Case-09	161.7	104.6	40.4	N/A
Case-10	125.1	253.2	48.6	N/A
Case-11	43.6	490.5	38.2	37.1
Case-12	96.2	378.8	43.8	40.4
Case-13	116.9	184.1	42.1	N/A
Case-14	101.1	138.9	54.7	22.5
Case-15	55.8	413.2	31.5	N/A
Case-16	62.9	115.8	24.1	28.9
Case-17	260.3	296.3	17.1	N/A

Case-18	69.4	221.5	44.2	26.5
Case-19	100.7	79.8	19.4	21.5
Case-20	40.9	27.8	21.5	N/A

N/A: not available

The plasma cell-free DNA (cfDNA) concentrations for each case were determined by multiplying the cfDNA concentrations with the elution volume and then dividing the resulting value by the plasma volume used during the isolation process, as detailed in **Table 8**.

Table 8: Qubit-based cfDNA concentrations in the plasma of treatment-naive FL patients.

Case Number	**Symptomaticity**	**cfDNA concentration (ng/µl) measured with Qubit**	**Used plasma volume in cfDNA isolation (ml)**	**Used elution buffer volume (µl)**	**cfDNA concentration in plasma (ng/ml)**
Case-01	Symptomatic	0.304	5	50	3.04
Case-02	Asymptomatic	0.468	5	50	4.68
Case-03	Symptomatic	1.600	5	50	16.00
Case-04	Asymptomatic	0.640	5	50	6.40
Case-05	Symptomatic	1.700	5	50	17.00
Case-06	Symptomatic	1.850	4	50	23.13
Case-07	Symptomatic	0.100	5	50	1.00
Case-08	Symptomatic	0.216	5	50	2.16
Case-09	Asymptomatic	0.692	5.5	50	6.29
Case-10	Symptomatic	2.460	5.5	50	22.36
Case-11	Symptomatic	0.668	5.5	50	6.07
Case-12	Symptomatic	4.840	5.5	50	44.00
Case-13	Asymptomatic	0.928	5.5	50	8.44
Case-14	Symptomatic	1.860	5	50	18.60
Case-15	Asymptomatic	1.310	5	50	13.10
Case-16	Symptomatic	3.990	5	50	39.90
Case-17	Asymptomatic	0.490	2.5	50	9.80

Case-18	Symptomatic	1.930	5	50	19.30
Case-19	Symptomatic	0.340	5	50	3.40
Case-20	Asymptomatic	0.360	5	50	3.60

4.3. Basic statistics of targeted next generation sequencing raw data

The DNA samples were subjected to quality checks and were sequenced using a panel specifically designed for 110 genes associated with follicular lymphoma (FL). A total of 684.29 gigabytes of raw data were obtained, which corresponded to 2.2 x 10^9 raw reads.

Granulocyte DNA samples exhibited an average of 26.8 x 10^6 ± 5.4 x 10^6 raw reads per sample, with an average percentage of clean reads to raw reads of 98.9% ± 0.5 (**Table 9**). When compared to plasma cfDNA and FFPE tDNA samples, this average effective read percentage of granulocyte DNA samples was higher than the others. FFPE tDNA samples had an average of 35.5 x 10^6 ± 19.2 x 10^6 raw reads per sample on average, with the lowest percentage of clean reads to raw reads at 89.1% ± 12.7 (**Table 10**). This could be attributed to DNA degradation during the fixation procedure (Guyard et al., 2017). For cfDNA samples, the average percentage of effective reads was 93.8% ± 3.27, with an average of 32.3 x 10^6 ± 8.7 x 10^6 raw reads per sample. These values were obtained from both pre- and post-treatment samples (**Table 11-12**).

Table 9: Basic statistics of raw NGS data of granulocyte DNA samples.

Case number	**Sample type**	**Raw reads**	**Raw data (G)**	**Effective (%)**	**Error (%)**	**Q20 (%)**	**Q30 (%)**	**GC (%)**
Case-01	gDNA	21882109	6.56	98.58	0.01	97.12	93	47.99
Case-02	gDNA	31057521	9.3	98.36	0.01	96.6	92.05	48.51
Case-03	gDNA	21557107	6.47	98.77	0.01	96.97	92.69	47.71
Case-04	gDNA	21347311	6.4	98.99	0.01	97.03	92.76	47.77
Case-05	gDNA	17999553	5.4	98.35	0.01	97.06	92.84	48.14
Case-06	gDNA	22065195	6.62	98.84	0.01	97.01	92.78	47.94
Case-07	gDNA	21827691	6.5	99.52	0.01	96.96	92.59	47.73
Case-08	gDNA	24348793	7.3	99.42	0.01	97.18	93.09	47.36

Case-09	gDNA	28307102	8.5	99.3	0.01	97.13	93.07	47.45
Case-10	gDNA	28796310	8.6	99.4	0.01	97.13	93.09	48.34
Case-11	gDNA	27052238	8.1	99.55	0.01	96.5	91.59	47.34
Case-12	gDNA	28036495	8.4	99.18	0.01	97.27	93.33	47.94
Case-13	gDNA	28620332	8.6	99.36	0.01	97.03	92.88	47.94
Case-14	gDNA	23764241	7.1	99.5	0.01	97.11	92.97	47.58
Case-15	gDNA	32222469	9.7	98.72	0.01	96	90.68	52.48
Case-16	gDNA	37571829	11.3	98.58	0.01	96.06	90.8	52.72
Case-17	gDNA	38301358	11.5	98.58	0.01	95.89	90.43	53.73
Case-18	gDNA	31245081	9.4	98.22	0.01	96.72	92.31	47.33
Case-19	gDNA	25116904	7.5	98.07	0.01	96.56	91.97	47.31
Case-20	gDNA	25337582	7.6	98.65	0.01	96.45	91.72	48.38

Table 10: Basic statistics of raw NGS data of tumor tissue DNA samples.

Case number	**Sample type**	**Raw reads**	**Raw data (G)**	**Effective (%)**	**Error (%)**	**Q20 (%)**	**Q30 (%)**	**GC (%)**
Case-01	FFPE tDNA	33009228	9.9	93.5	0.01	96.99	92.78	51.27
Case-02	FFPE tDNA	21727482	6.52	86.79	0.01	95.44	90.22	48.91
Case-03	FFPE tDNA	32671752	9.8	95.98	0.01	96.66	92.32	46.73
Case-04	FFPE tDNA	64090823	19.23	95.76	0.01	96.97	92.73	52.3
Case-05	FFPE tDNA	34065445	10.22	95.71	0.01	96.95	92.77	48.19
Case-06	FFPE tDNA	34735008	10.42	90.87	0.01	96.16	91.52	48.25
Case-07	FFPE tDNA	22383054	6.7	95.55	0.01	97.12	93.21	48.65
Case-08	FFPE tDNA	22554851	6.8	97.46	0.01	96.05	90.9	47.12
Case-09	FFPE tDNA	34789809	10.4	96.85	0.01	97.16	93.32	46.34
Case-10	FFPE tDNA	33694729	10.1	98.39	0.01	97.01	93.06	46.09
Case-11	FFPE tDNA	25135696	7.5	95.61	0.01	97.2	93.41	46.84
Case-12	FFPE tDNA	25587131	7.7	96.79	0.01	96.28	91.32	49.36
Case-13	FFPE tDNA	25262116	7.6	95.51	0.01	97.35	93.73	47.23
Case-14	FFPE tDNA	29828087	8.9	98.85	0.01	96.17	91.19	44.95
Case-15	FFPE tDNA	58048036	17.4	88.74	0.01	96.16	91.14	49.21
Case-16	FFPE tDNA	102120822	30.6	83.4	0.01	96.17	91.16	47.59

Case-17	FFPE tDNA	25348580	7.6	48.93	0.01	96.03	91.12	53.75
Case-18	FFPE tDNA	37352964	11.2	64.16	0.02	95.42	90.09	49.52
Case-19	FFPE tDNA	25639445	7.7	79.21	0.02	95.24	89.64	43.89
Case-20	FFPE tDNA	22813618	6.8	82.98	0.02	94.94	89.1	45.67

Table 11: Basic statistics of raw NGS data of pre-treatment plasma cfDNA samples.

Case number	Sample type	Raw reads	Raw data (G)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)
Case-01	cfDNA	26542159	7.96	92.33	0.01	97.06	93.09	47.97
Case-02	cfDNA	29995498	9	93.71	0.01	97.23	93.38	47.5
Case-03	cfDNA	32619874	9.79	96.05	0.01	97.41	93.69	46.63
Case-04	cfDNA	28189581	8.46	95.28	0.01	97.23	93.38	47.84
Case-05	cfDNA	33659244	10.1	92.9	0.01	97.16	93.25	47.48
Case-06	cfDNA	42135475	12.64	88.61	0.01	97.42	93.72	48.16
Case-07	cfDNA	62746738	18.8	86.69	0.01	96.86	92.67	52.93
Case-08	cfDNA	58496258	17.5	91.55	0.01	97.14	93.25	53.14
Case-09	cfDNA	21410620	6.4	92.24	0.01	96.88	92.6	47.23
Case-10	cfDNA	31747997	9.5	96.09	0.01	97.42	93.75	47.09
Case-11	cfDNA	37119371	11.1	89.09	0.01	97	93	51.03
Case-12	cfDNA	31856623	9.6	95.09	0.01	97.5	93.89	46.84
Case-13	cfDNA	24846277	7.5	94.59	0.01	96.06	91.01	46.3
Case-14	cfDNA	24546828	7.4	96.56	0.01	97.56	94.17	46.68
Case-15	cfDNA	30472773	9.1	96.41	0.01	96.53	91.83	50.8
Case-16	cfDNA	37679176	11.3	87.73	0.01	97.49	93.93	50.47
Case-17	cfDNA	34193278	10.3	97.21	0.01	96.31	91.38	51.72
Case-18	cfDNA	26756794	8	89.97	0.02	96.33	91.18	46.87
Case-19	cfDNA	27017054	8.1	93.6	0.02	96.16	90.84	46.6
Case-20	cfDNA	27980837	8.4	92.94	0.02	96.21	90.97	46.78

Table 12: Basic statistics of raw NGS data of post-treatment plasma cfDNA samples.

Case number	Sample type	Raw reads	Raw data (G)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)

Case-01	cfDNA-AT	32620105	9.8	87.29	0.01	97.38	93.77	47.5
Case-03	cfDNA-AT	34349554	10.3	94.42	0.01	97.44	93.76	46.8
Case-05	cfDNA-AT	29994313	9	97.5	0.01	97.66	94.34	46.47
Case-06	cfDNA-AT	23600055	7.1	93.95	0.01	96.52	92.01	47.19
Case-07	cfDNA-AT	24112384	7.2	98.05	0.01	97.46	93.94	47.17
Case-08	cfDNA-AT	32133893	9.6	97.92	0.01	97.12	93.17	46.97
Case-11	cfDNA-AT	32141097	9.6	96.2	0.01	96.15	91.05	53.13
Case-12	cfDNA-AT	35346954	10.6	96.5	0.01	97.07	93.07	51.6
Case-14	cfDNA-AT	31354302	9.4	95.56	0.01	96.89	92.72	50.46
Case-16	cfDNA-AT	28475159	8.5	98.32	0.01	96.91	92.68	48.2
Case-18	cfDNA-AT	27387129	8.2	93.53	0.01	98.28	95.53	50.58
Case-19	cfDNA-AT	33653084	10.1	92.77	0.01	98.37	95.97	49.86

4.4. Variants observed in cfDNA and/or tumor tissue DNA of FL cases

Following the application of the final criteria, as described in section 3.8.2, to the variants identified using Mutect2, a total of 91 somatic variants associated with hematological cancers, as defined in COSMICv89, were identified in either cfDNA or FFPE tDNA, or both. Notably, no variants were detected in Case-13, an asymptomatic case, and Case-19, a symptomatic case, after applying the filtration process. **Table 13** presents the list of variants identified in our cohort of follicular lymphoma (FL) cases, along with the sample type in which the mutation was detected, and their respective variant allele fractions. Only one variant was observed in cfDNA samples obtained after treatment, specifically in Case-06.

Table 13: Somatic variants identified in cfDNA and/or tumor tissue DNA of 20 FL cases.

Case	Gene	Variant Class	COSMIC_ID	AA mutation	Sample Type	VAF (%)
Case-01	*CARD11*	Missense	COSM133703	p.Q249P	FFPE tDNA	34.6
Case-01	*CARD11*	Missense	COSM133703	p.Q249P	Plasma cfDNA	7.1
Case-01	*CARD11*	Missense	COSM43452	p.F115I	Plasma cfDNA	1.0
Case-02	*TNFRSF14*	Missense	COSM4170622	p.S112F	FFPE tDNA	5.7
Case-02	*CREBBP*	Indel	COSM291735	p.S1680del	FFPE tDNA	29.5
Case-02	*SOCS1*	Nonsense	COSM4170737	p.Q6*	FFPE tDNA	15.7

Case-02	*STAT6*	Missense	COSM221036	p.G375R	FFPE tDNA	12.1
Case-03	*CARD11*	Missense	COSM133703	p.Q249P	FFPE tDNA	3.2
Case-03	*CREBBP*	Missense	COSM88744	p.Y1503H	FFPE tDNA	18.5

Case-03	*KMT2D*	Missense	COSM3753295	p.P813L	FFPE tDNA	4.2
Case-03	*STAT6*	Missense	COSM1988332	p.D419N	FFPE tDNA	17.9
Case-03	*BCL7A*	Splice site	COSM5715227		FFPE tDNA	2.1
Case-03	*EZH2*	Missense	COSM37028	p.Y646F	FFPE tDNA	18.7
Case-03	*EZH2*	Missense	COSM3762470	p.D185H	FFPE tDNA	2.6
Case-03	*BAZ2B*	Missense	COSM3757702	p.S2024N	FFPE tDNA	2.2
Case-03	*CREBBP*	Missense	COSM88744	p.Y1503H	Plasma cfDNA	0.7
Case-03	*STAT6*	Missense	COSM1988332	p.D419N	Plasma cfDNA	1.2
Case-04	*CARD11*	Missense	COSM41660	p.D357V	FFPE tDNA	28.5
Case-04	*CREBBP*	Indel	COSM291735	p.S1680del	FFPE tDNA	28.4
Case-05	*KMT2D*	Missense	COSM5019244	p.M3398V	FFPE tDNA	0.7
Case-05	*KMT2D*	Nonsense	COSM220686	p.R2687*	FFPE tDNA	32.6
Case-05	*KMT2D*	Nonsense	COSM4170426	p.R2645*	FFPE tDNA	6.3
Case-05	*KMT2C*	Missense	COSM4006667	p.C1114R	FFPE tDNA	0.7
Case-05	*KMT2D*	Nonsense	COSM220686	p.R2687*	Plasma cfDNA	6.9
Case-06	*TNFRSF14*	Nonsense	COSM220659	p.Q148*	FFPE tDNA	5.5
Case-06	*CARD11*	Indel	COSM1724481	p.S622del	FFPE tDNA	1.1
Case-06	*CREBBP*	Missense	COSM703032	p.C1408Y	FFPE tDNA	41.8
Case-06	*USP6*	Missense	COSM4997892	p.R912Q	FFPE tDNA	0.7
Case-06	*IKZF3*	Missense	COSM145512	p.L162R	FFPE tDNA	20.9
Case-06	*STAT6*	Missense	COSM220668	p.D419G	FFPE tDNA	17.4
Case-06	*EZH2*	Missense	COSM37028	p.Y646F	FFPE tDNA	9.4
Case-06	*EZH2*	Missense	COSM37031	p.Y646N	FFPE tDNA	2.7
Case-06	*CARD11*	Indel	COSM1724481	p.S622del	Plasma cfDNA	0.6
Case-06	*CREBBP*	Missense	COSM703032	p.C1408Y	Plasma cfDNA	2.1
Case-06	*IKZF3*	Missense	COSM145512	p.L162R	Plasma cfDNA	0.8
Case-06	*STAT6*	Missense	COSM220668	p.D419G	Plasma cfDNA	1.4
Case-06	*EZH2*	Missense	COSM37028	p.Y646F	Plasma cfDNA	1.2
Case-06	*CARD11*	Indel	COSM1724481	p.S622del	Plasma cfDNA-AT	0.8
Case-07	*BCL2*	Missense	COSM220811	p.D31N	FFPE tDNA	13.5
Case-07	*BCL2*	Missense	COSM220811	p.D31N	Plasma cfDNA	8.3

Case-08	*TNFRSF14*	Splice site	COSM3356560		FFPE tDNA	24.7
Case-09	*HIST1H1E*	Missense	COSM145482	p.A65P	FFPE tDNA	53.3
Case-09	*CTSS*	Missense	COSM220485	p.Y132D	FFPE tDNA	39.1

Case-09	*ATP6AP1*	Missense	COSM1316108	p.G363R	FFPE tDNA	44.6
Case-09	*HIST1H1E*	Missense	COSM145482	p.A65P	Plasma cfDNA	5.2
Case-09	*CTSS*	Missense	COSM220485	p.Y132D	Plasma cfDNA	11.1
Case-09	*ATP6AP1*	Missense	COSM1316108	p.G363R	Plasma cfDNA	7.5
Case-10	*CCND3*	Missense	COSM144647	p.P284S	FFPE tDNA	17.8
Case-10	*BCL2*	Missense	COSM1580569	p.E179D	FFPE tDNA	2.8
Case-10	*BCL2*	Missense	COSM220915	p.P53S	FFPE tDNA	0.5
Case-10	*BCL2*	Missense	COSM1580569	p.E179D	Plasma cfDNA	1.1
Case-11	*CREBBP*	Missense	COSM96466	p.C1237Y	FFPE tDNA	27.2
Case-11	*FOXO1*	Missense	COSM4805886	p.S22W	FFPE tDNA	28.9
Case-11	*STAT6*	Missense	COSM5946952	p.D523V	FFPE tDNA	44.9
Case-11	*BCL2*	Missense	COSM5947452	p.G197S	FFPE tDNA	31.4
Case-11	*BCL2*	Missense	COSM220800	p.A131V	FFPE tDNA	1.1
Case-11	*BCL2*	Missense	COSM5654988	p.L119M	FFPE tDNA	28.8
Case-11	*BCL2*	Missense	COSM3357021	p.A113G	FFPE tDNA	14.9
Case-11	*IRF8*	Missense	COSM974537	p.Y23H	FFPE tDNA	31.2
Case-11	*DTX1*	Missense	COSM4609972	p.V61L	FFPE tDNA	19.7
Case-11	*CREBBP*	Missense	COSM96466	p.C1237Y	Plasma cfDNA	1.1
Case-11	*STAT6*	Missense	COSM5946952	p.D523V	Plasma cfDNA	1.6
Case-11	*BCL2*	Missense	COSM5947452	p.G197S	Plasma cfDNA	0.8
Case-11	*IRF8*	Missense	COSM974537	p.Y23H	Plasma cfDNA	0.9
Case-11	*DTX1*	Missense	COSM4609972	p.V61L	Plasma cfDNA	1.0
Case-12	*BCL2*	Missense	COSM5654982	p.R129H	FFPE tDNA	4.2
Case-12	*BCL2*	Missense	COSM220796	p.P57S	FFPE tDNA	1.5
Case-12	*BCL2*	Missense	COSM220825	p.P57A	FFPE tDNA	1.0
Case-12	*BCL2*	Missense	COSM4170947	p.G36D	FFPE tDNA	0.7
Case-14	*HIST1H1E*	Missense	COSM5715116	p.P131S	FFPE tDNA	67.4
Case-14	*ARID1A*	Nonsense	COSM51418	p.R1722*	FFPE tDNA	33.5
Case-14	*BCL2*	Missense	COSM5656306	p.A198T	FFPE tDNA	40.2
Case-14	*BCL2*	Missense	COSM220800	p.A131V	FFPE tDNA	0.7
Case-14	*BCL2*	Missense	COSM220816	p.L119V	FFPE tDNA	37.1

Case-14	*BCL2*	Missense	COSM4170918	p.A77V	FFPE tDNA	6.2
Case-14	*BCL2*	Missense	COSM220803	p.A60V	FFPE tDNA	21.6
Case-14	*BCL2*	Missense	COSM220907	p.P59A	FFPE tDNA	0.9

Case-14	*BCL2*	Missense	COSM2881912	p.G8E	FFPE tDNA	5.1
Case-14	*DTX1*	Nonsense	COSM145349	p.W30*	FFPE tDNA	0.6
Case-14	*BCL7A*	Missense	COSM1580594	p.R11G	FFPE tDNA	48.0
Case-14	*HIST1H1E*	Missense	COSM5715116	p.P131S	Plasma cfDNA	33.8
Case-14	*ARID1A*	Nonsense	COSM51418	p.R1722*	Plasma cfDNA	20.2
Case-14	*BCL2*	Missense	COSM5656306	p.A198T	Plasma cfDNA	13.0
Case-14	*BCL2*	Missense	COSM220816	p.L119V	Plasma cfDNA	16.7
Case-14	*BCL2*	Missense	COSM220803	p.A60V	Plasma cfDNA	8.6
Case-14	*BCL2*	Missense	COSM220809	p.G47D	Plasma cfDNA	1.4
Case-14	*BCL7A*	Missense	COSM1580594	p.R11G	Plasma cfDNA	21.3
Case-15	*CREBBP*	Missense	COSM166406	p.G1411R	FFPE tDNA	31.1
Case-15	*FOXO1*	Missense	COSM5948479	p.R21H	FFPE tDNA	16.6
Case-15	*STAT6*	Missense	COSM1988332	p.D419N	FFPE tDNA	12.1
Case-15	*NOTCH1*	Missense	COSM33748	p.G1656S	Plasma cfDNA	0.5
Case-16	*CD79B*	Missense	COSM1737939	p.Y196D	FFPE tDNA	9.3
Case-16	*B2M*	Nonsense	COSM1580548	p.Q22*	Plasma cfDNA	1.8
Case-16	*CD79B*	Missense	COSM1737939	p.Y196D	Plasma cfDNA	13.5
Case-16	*BTG1*	Missense	COSM220618	p.E46D	Plasma cfDNA	8.2
Case-17	*CREBBP*	Nonsense	COSM88763	p.R1360*	FFPE tDNA	0.7
Case-17	*CREBBP*	Nonsense	COSM88760	p.R1173*	FFPE tDNA	1.6
Case-17	*TP53*	Splice site	COSM85958		FFPE tDNA	0.9
Case-17	*SOCS1*	Missense	COSM1161151	p.R160H	FFPE tDNA	1.7
Case-17	*PRKCB*	Missense	COSM5706062	p.R142C	FFPE tDNA	0.6
Case-17	*HIST1H1E*	Missense	COSM3357987	p.L107F	FFPE tDNA	2.2
Case-17	*MYD88*	Missense	COSM2986019	p.A146T	FFPE tDNA	0.9
Case-17	*BCL10*	Nonsense	COSM220637	p.S136*	FFPE tDNA	2.1
Case-17	*ENPP3*	Missense	COSM5650796	p.V13I	FFPE tDNA	6.2
Case-17	*TNFAIP3*	Nonsense	COSM4766046	p.E388*	FFPE tDNA	0.8
Case-17	*EZH2*	Missense	COSM37028	p.Y646F	FFPE tDNA	31.3
Case-17	*KMT2C*	Nonsense	COSM1166702	p.R4478*	FFPE tDNA	1.2

Case-18	*ARID1A*	Indel	COSM1582021	p.Q2176Rfs*48	FFPE tDNA	27.3
Case-18	*BCL2*	Missense	COSM1316823	p.S203N	FFPE tDNA	29.4
Case-18	*BCL2*	Missense	COSM5947452	p.G197S	FFPE tDNA	29.5
Case-18	*BCL2*	Missense	COSM220880	p.A60D	FFPE tDNA	27.7
Case-18	*BCL2*	Missense	COSM220897	p.A60T	FFPE tDNA	27.3
Case-18	*BCL2*	Missense	COSM220907	p.P59A	FFPE tDNA	28.0
Case-18	*BCL2*	Missense	COSM1649430	p.A4P	FFPE tDNA	2.6
Case-18	*CD79B*	Missense	COSM5045028	p.E198D	FFPE tDNA	1.6
Case-18	*CD79B*	Splice site	COSM5045045		FFPE tDNA	32.2
Case-18	*EZH2*	Missense	COSM220529	p.A692V	FFPE tDNA	4.9
Case-18	*ARID1A*	Indel	COSM1582021	p.Q2176Rfs*48	Plasma cfDNA	22.1
Case-18	*BCL2*	Missense	COSM1316823	p.S203N	Plasma cfDNA	16.0
Case-18	*BCL2*	Missense	COSM5947452	p.G197S	Plasma cfDNA	15.5
Case-18	*EZH2*	Missense	COSM220529	p.A692V	Plasma cfDNA	4.8
Case-20	*CREBBP*	Missense	COSM88743	p.Y1503D	FFPE tDNA	55.9

It is noteworthy that a statistically significant and positive moderate correlation, with a correlation coefficient of R=0.53 and a p-value of 0.002, was observed between the variant allele fractions of variants identified in both cfDNA and FFPE tDNA samples (**Figure 3**).

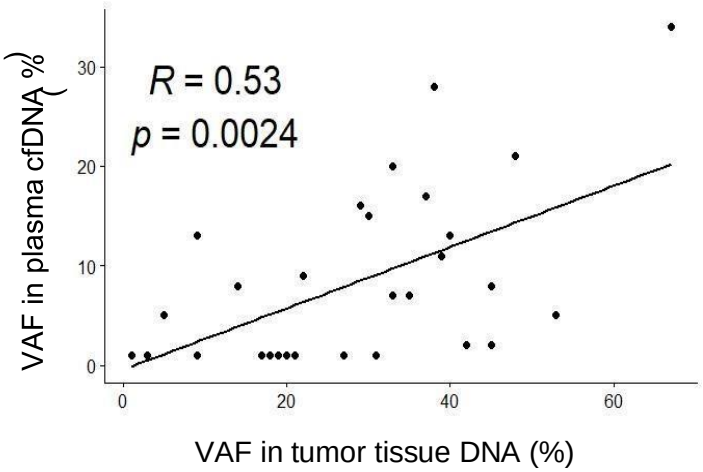


Figure 3: Spearman correlation of variant allele fractions for mutations detected in both cfDNA and tumor tissue DNA.

Among the 110 genes included in our custom targeted next-generation sequencing (NGS) panel, mutations were detected in 31 of them. *CREBBP* was the most frequently mutated gene in our cohort of follicular lymphoma (FL) cases that was observed in 40% of cases, followed by *BCL2* (30%), *STAT6* (25%), *CARD11* (20%), and *EZH2* (20%) in descending order of frequency. The remaining genes had mutation frequencies of less than 20% in our cohort. Among the 91 variants identified, 71 were missense mutations, 12 were nonsense mutations, 4 were indel mutations, and 4 were splice site mutations. Notably, all *BCL2* mutations were observed exclusively in symptomatic cases (**Figure 4**).

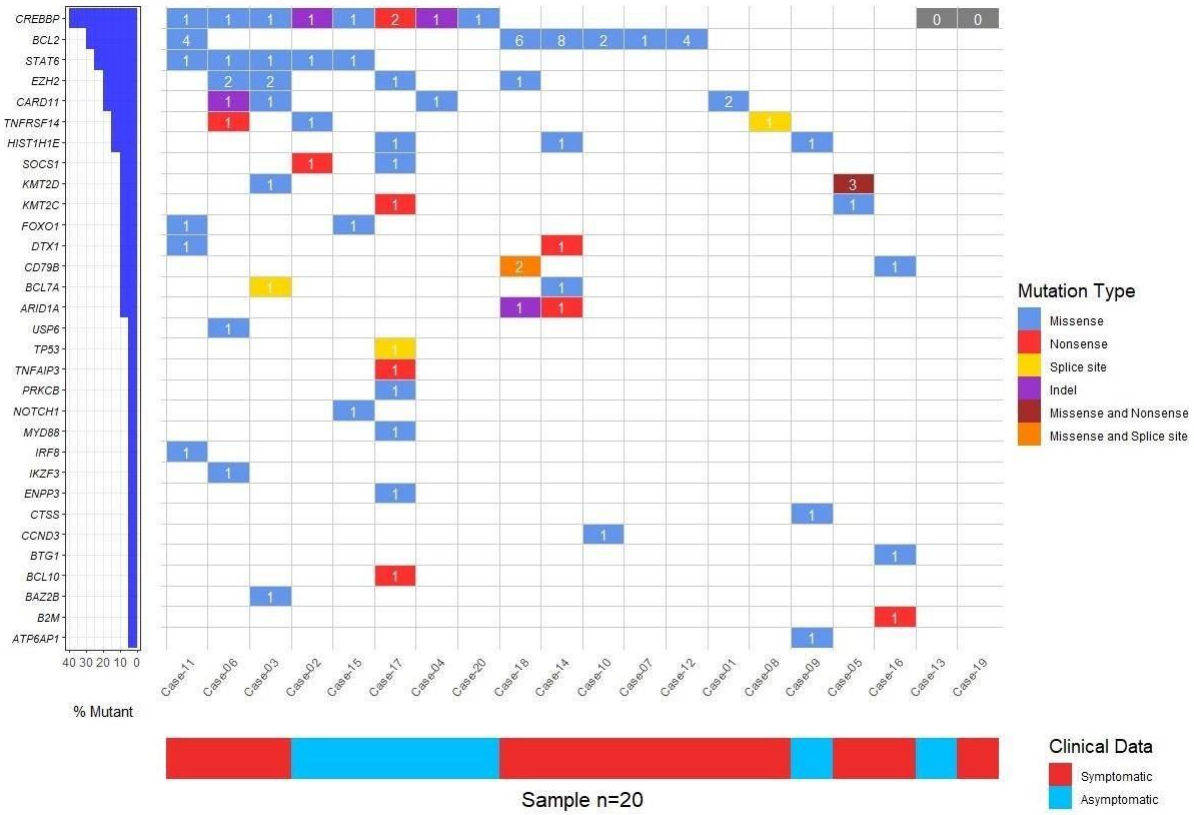


Figure 4: Waterfall plot showing mutated genes and the type of mutations detected in cfDNA and/or tumor tissue DNA in FL cases.

4.5. Comparison of variants detected in cfDNA and/or FFPE tDNA in symptomatic and asymptomatic follicular lymphoma cases: distribution and mutation types

When analyzing the distribution of observed variants based on the DNA type in a cohort of 20 cases of follicular lymphoma (FL), we observed that 30 variants (33%) were detected in both circulating free DNA (cfDNA) and tumor tissue DNA, while 56 variants (61.5%) were detected only in tumor tissue DNA, and 5 variants (5.5%) were

detected only in plasma cfDNA (**Figure 5A**). This distribution showed slight changes when we analyzed the variants separately in symptomatic and asymptomatic cases. In symptomatic cases, 27 variants (41.5%) were found to overlap in both tumor tissue DNA and plasma cfDNA, which was higher compared to the overall cohort.

Additionally, 34 variants (52.3%) were found only in tumor tissue DNA, and 4 variants (6.2%) were found only in plasma cfDNA (**Figure 5B**). In asymptomatic cases, the number of variants detected in both tumor tissue DNA and plasma cfDNA, as well as the number of variants detected only in plasma cfDNA, was quite low, with three variants (11.6%) and one variant (3.8%), respectively. The majority of variants in asymptomatic cases were detected only in tumor tissue DNA, with a total of 22 variants and a percentage of 84.6% (**Figure 5C**). A more detailed visualization of the distribution of variants based on DNA type and a comparison between symptomatic and asymptomatic groups is shown in **Figure 6A**.

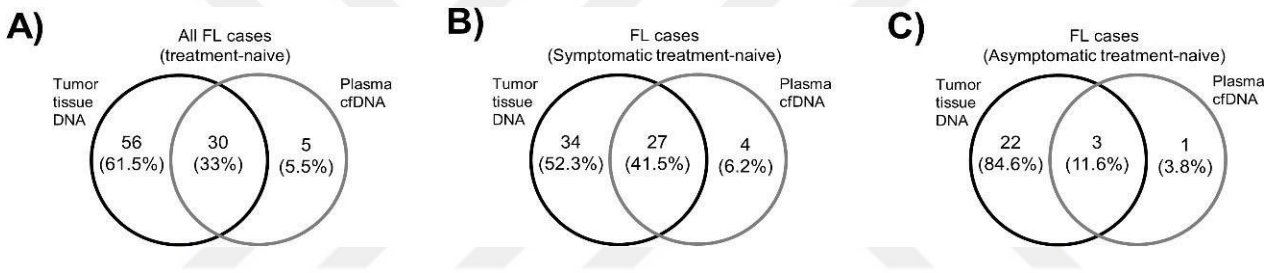


Figure 5: The venn diagrams showing the numbers and percentages of somatic mutations detected in tumor tissue DNA and/or cfDNA of symptomatic, asymptomatic or all FL cases.

When comparing the number of variants per case between symptomatic and asymptomatic groups in the cohort, we observed a slightly higher average of 5.0 variants per case (median: 4.0) in the symptomatic group, compared to an average of 3.7 variants per case (median: 3.0) in the asymptomatic group (**Figure 6B**). The majority of mutations identified in both symptomatic and asymptomatic groups were missense mutations, accounting for 54 mutations (83.1%) in the symptomatic group and 17 mutations (65.4%) in the asymptomatic group. Nonsense mutations were identified in 6 cases in both groups, with a higher percentage in the asymptomatic group (9.2% vs. 23.1%). Additionally, we found two indel mutations (3.1%) and three splice site mutations (4.6%) among the 65 mutations identified in the symptomatic

group. In the asymptomatic group, there were two indel mutations (7.7%) and one splice site mutation (3.8%) among the 26 mutations identified (**Figure 6C**).

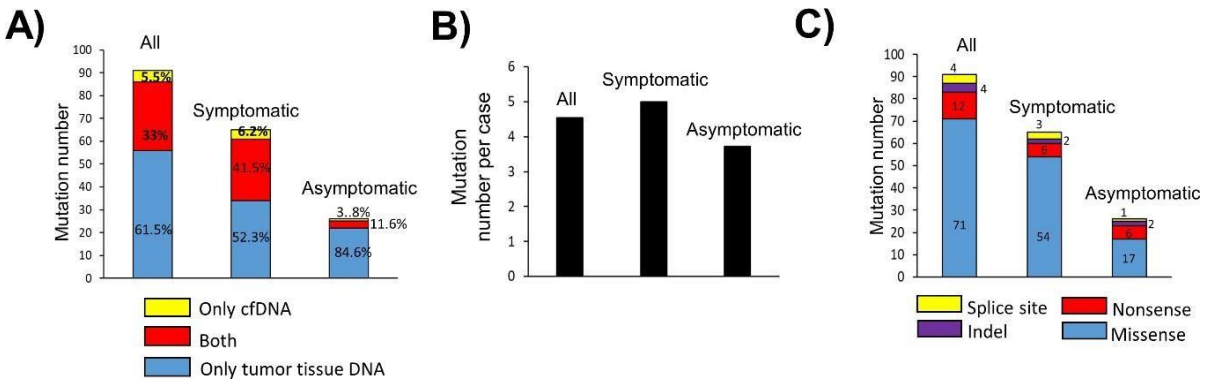


Figure 6: Comparison of symptomatic and asymptomatic FL cases in terms of the number of mutations identified in each DNA type and the types of mutations detected.

4.6. The degree of overlap of mutations in cfDNA and tumor tissue DNA for each case

In the 13 symptomatic FL cases, we detected at least one variant in both tumor tissue DNA and plasma cfDNA in 10 cases (77%). On the other hand, in all 7 asymptomatic cases, only one case (14.3%) showed the same mutation in both tumor tissue DNA and plasma cfDNA. The overlap of mutations found exclusively in either tumor tissue DNA, plasma cfDNA or in both types is depicted in Venn diagrams (**Figure 7A-M** for symptomatic cases and **Figure 8A-G** for asymptomatic cases).

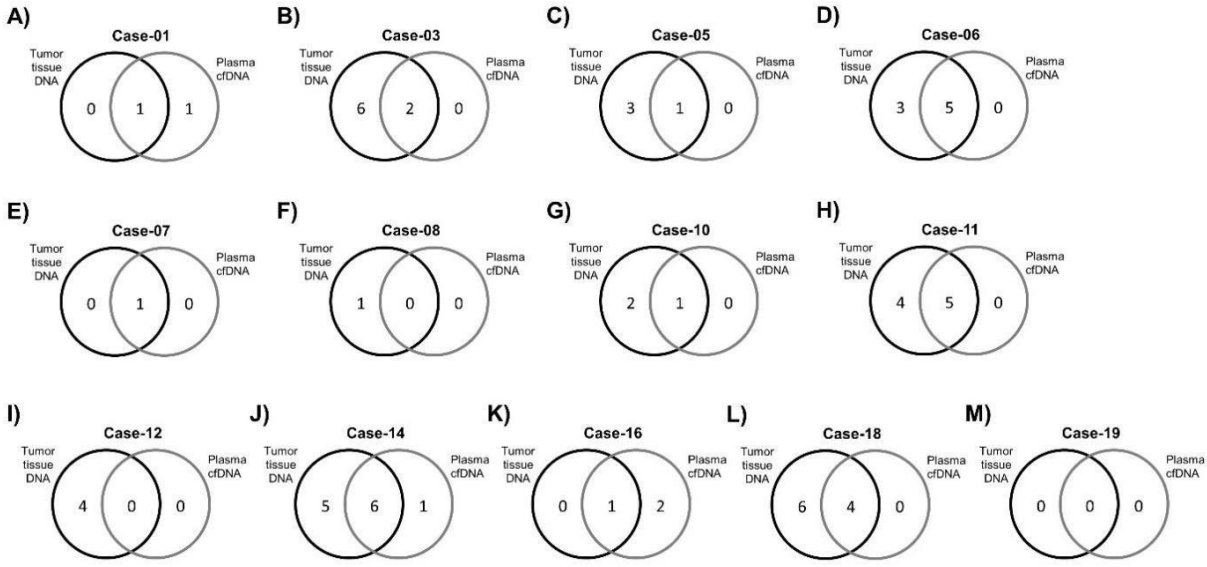


Figure 7: Venn diagrams showing the number of mutations detected in cfDNA and/or tumor tissue DNA of symptomatic FL cases.

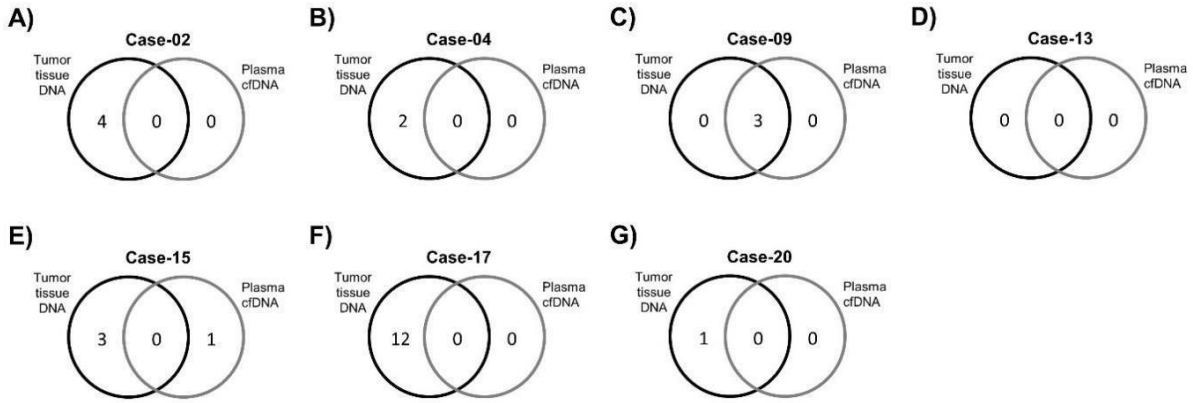


Figure 8: Venn diagrams showing the number of mutations in cfDNA and/or tumor tissue DNA of asymptomatic FL cases.

The frequency and distribution of mutated genes for each case, categorized by the observed DNA type, is depicted using a waterfall graph in **Figure 9**.

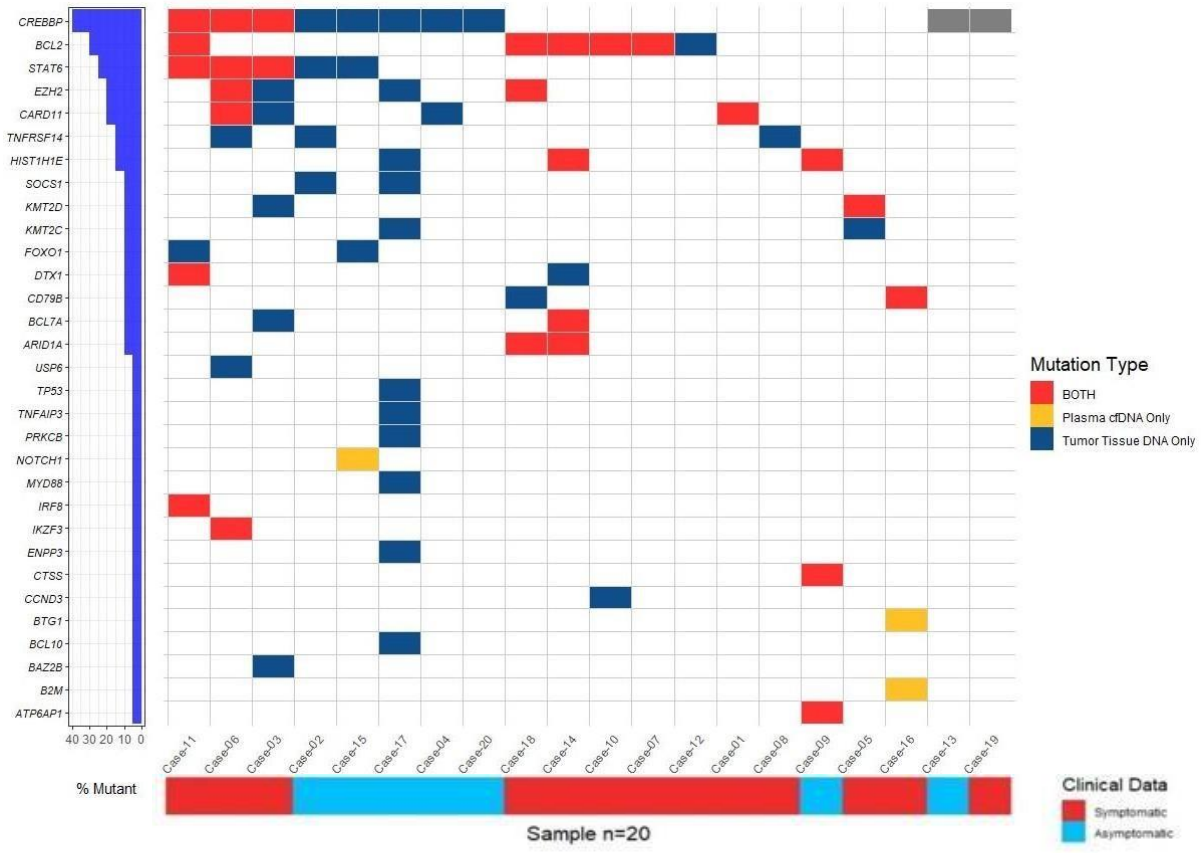


Figure 9: Waterfall plot illustrating the mutated genes in FL cases based on the DNA type in which they were detected.

4.7. The domains and affected residues of recurrently mutated genes

We conducted an analysis of the domains impacted by mutations in genes that exhibited a recurrence rate of 20% or higher in cases of follicular lymphoma (FL), as depicted in **Figure 10**. Our findings revealed that mutations in the *CREBBP* gene tended to accumulate around the histone acetyltransferase domain. Conversely, mutations in the *BCL2* gene were distributed more widely. Mutations in the *STAT6* gene were located within the STAT binding domain, while mutations in the *EZH2* gene were enriched in the SET domain, which governs the enzymatic activity of histone lysine methyltransferases. Finally, mutations in the *CARD11* gene were also widely distributed.

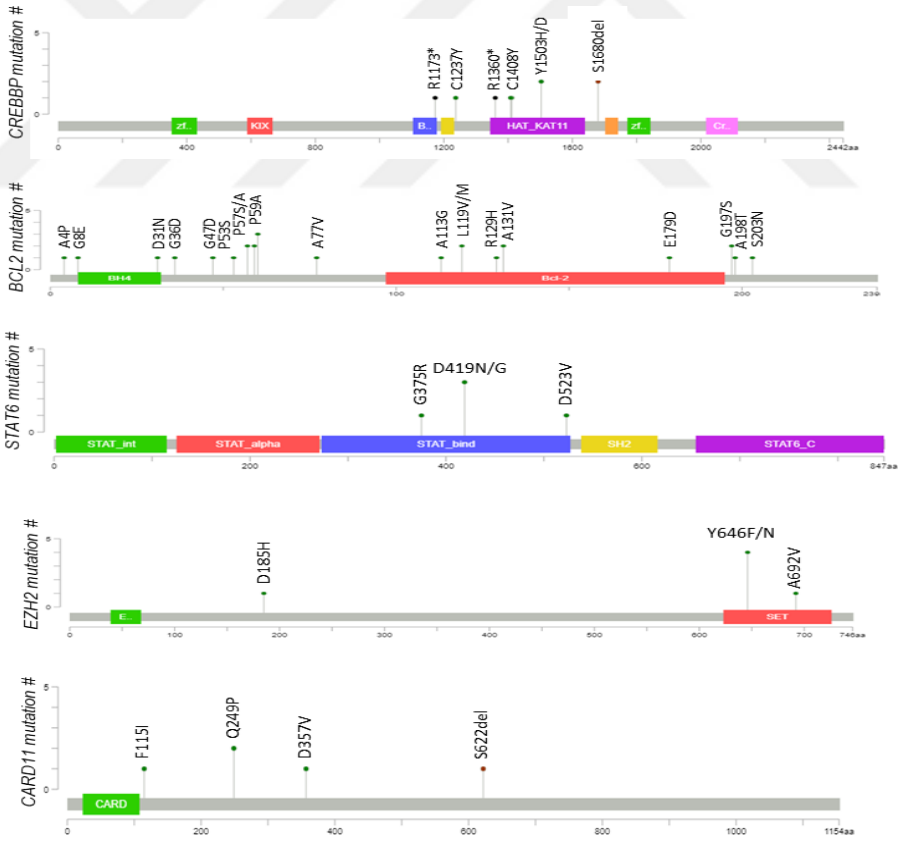


Figure 10: The domains and affected residues of recurrently mutated genes in FL cases.

4.8. Pathways associated with the mutated genes in follicular lymphoma cases

We investigated the pathways associated with the 31 mutated genes in our follicular lymphoma cohort using the "ReactomePA" package of R, which is based on the REACTOME pathway database (**Figure 11**). Our analysis revealed enrichment of several pathways, including "Transcriptional regulation by RUNX1," "IL-4 and IL-13 signaling," "Chromatin modifying genes," and "Signaling by the B-cell receptor," with the mutated genes in our FL cohort. However, it is important to note that the low number of genes may lead to an overestimation of these pathways, and caution should be taken when interpreting these results.

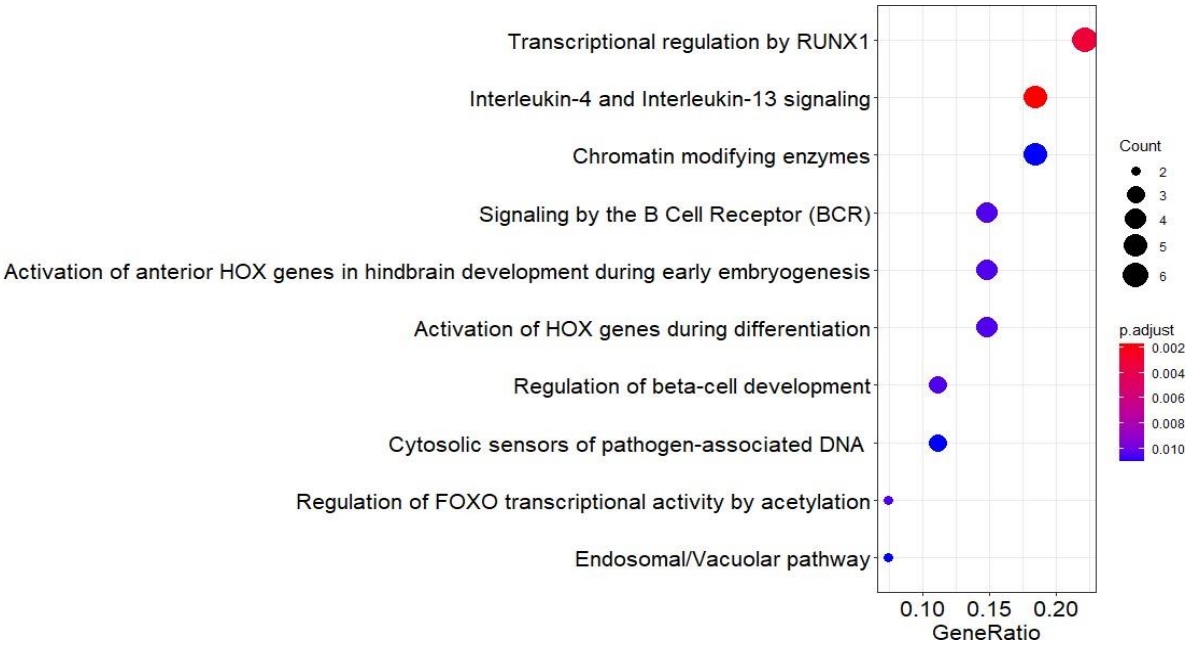


Figure 11: Pathway enrichment analysis result of 31 mutated genes in FL cases.

4.9. Post-therapy plasma cfDNA concentrations of symptomatic FL cases and persistence of variants

Peripheral blood samples were collected from 12 out of 13 patients diagnosed with symptomatic follicular lymphoma (FL) after immunochemotherapy treatments, in order to investigate whether the somatic variants identified at the time of diagnosis persisted after treatment. As Case-10 passed away during the course of the project, we were not able to obtain post-therapy peripheral blood sample. Out of the 91 variants identified in the entire FL cohort, 65 variants were identified in all symptomatic cases.

Among these variants 62 of them were detected in the 12 patients whose peripheral blood samples were also collected after therapy. In the circulating cell-free DNA (cfDNA) samples obtained before treatment from these 12 symptomatic cases, 30 variants were identified, but after therapy, only one indel variant (i.e. CARD11 p.S622delS with a variant allele frequency (VAF) of 0.8%) was observed in Case-06. When comparing the plasma cfDNA concentrations between the samples obtained before treatment and after treatment, there was no significant difference (**Figure 12**).

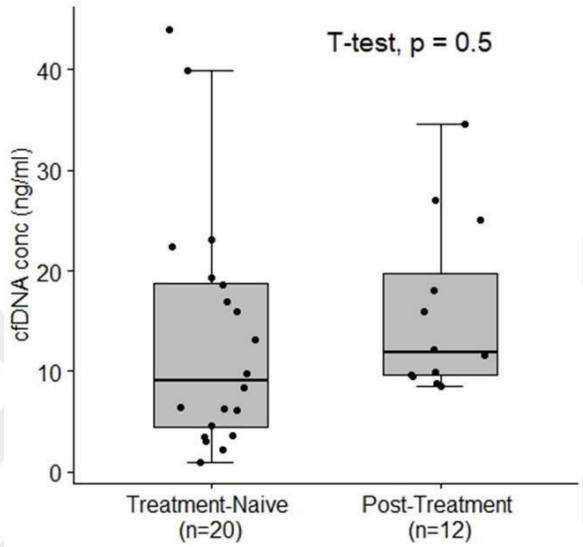


Figure 12: Comparison of plasma cfDNA concentrations of symptomatic FL cases before and after therapy.

4.10. Correlation analysis of plasma cfDNA concentrations with clinical parameters, and comparison of symptomatic and asymptomatic cases

We conducted a correlation analysis using Spearman correlation to examine the association between the concentration of circulating cell-free DNA (cfDNA) in plasma from all cases of follicular lymphoma (FL), which was calculated as described in section 4.2, with their demographic, clinical, or pathological characteristics. Our analysis did not reveal any significant correlations between plasma cfDNA concentrations and variables such as age, Ki-67 index, FDG PET/CT positive regions, highest SUVmax value, or LDH levels prior to treatment (**Figure 13**). However, it should be noted that we observed a tendency for positive correlation between plasma cfDNA concentrations pre-treatment LDH levels, which did not reach statistical signficance due to the limited number of samples analyzed (**Figure 13E**).

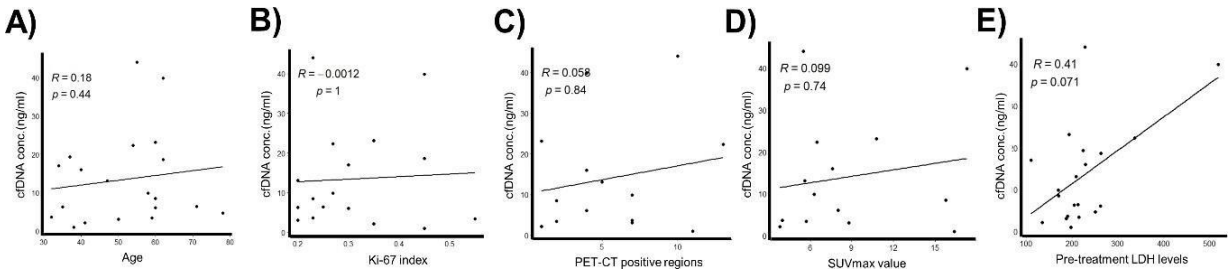


Figure 13: Spearman correlations between plasma cfDNA concentrations and demographic or clinical parameters.

On the other hand, when we compared the concentrations of circulating cell-free DNA (cfDNA) between symptomatic and asymptomatic cases, we observed a statistically significant difference between the two groups with a p-value of 0.039 (**Figure 14**).

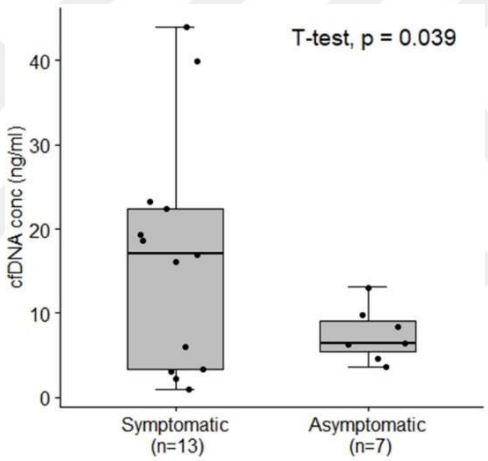


Figure 14: Comparison of plasma cfDNA concentrations between treatment-naive symptomatic and asymptomatic FL cases.

4.11. Prognostic predictors of survival in follicular lymphoma: clinical variables, cfDNA concentrations, and gene mutations

We conducted overall survival and progression-free survival analyses with our cohort of follicular lymphoma (FL) patients, utilizing clinical information collected from the DEU hospital database, including variables such as Ki-67 index of biopsy specimens, LDH level before treatment, PET/CT results, age, and plasma cfDNA concentrations before treatment. Additionally, we analyzed the impact of mutated genes on the survival of FL patients, examining each gene separately. Furthermore,

we investigated the improvement in prognostic predictive value of the co-occurrence of adverse clinical variables and mutations in the *BCL2* gene, which is not typically expressed in germinal centers under normal conditions but is activated in FL.

4.11.1. The prognostic value of age, symptomaticity, SUVmax, Ki-67, LDH, and FDG-PET/CT in follicular lymphoma

In our study of follicular lymphoma (FL), we employed the "surv_cutpoint" function from the "survminer" R package to group patients into old and young cohorts based on an age cut-off of 38 years. However, this age-based grouping did not yield significant predictive value for overall survival (p=0.33) or progression-free survival (p=0.2) in FL patients, as demonstrated in **Figure 15A** and **Figure16A**, respectively. Similarly, the symptomaticity of FL cases did not predict overall survival (p=0.15) (**Figure 15B**) or progression-free survival (p=0.11) (**Figure 16B**).

Subsequently, we evaluated the predictive value of the highest SUVmax value obtained from FDG-PET/CT images of FL cases. Using a cut-off value of 15.8, we divided the cohort into high and low SUVmax groups. Patients with SUVmax values exceeding this cut-off exhibited inferior progression-free survival (p=0.017) compared to the other group (**Figure 15C**). However, no significant difference was observed in overall survival (p=0.062) between the two groups based on SUVmax (**Figure 16C**).

On the other hand, we observed that FL patients with high Ki-67 percentage (p=0.014), high LDH level before treatment (p=0.00049), and a high number of involved anatomical regions based on FDG-PET/CT images (p=0.00014) exhibited inferior overall survival, as demonstrated in **Figure 15D-F**. The cut-off levels for Ki-67 percentage, LDH level, and FDG-PET/CT positive regions were determined using the survminer R package as 35%, 262.0 units per litre, and 10 regions, respectively. Similarly, based on these variables, the groups with high values for each variable also showed inferior progression-free survival, as depicted in **Figure 16D-F**.

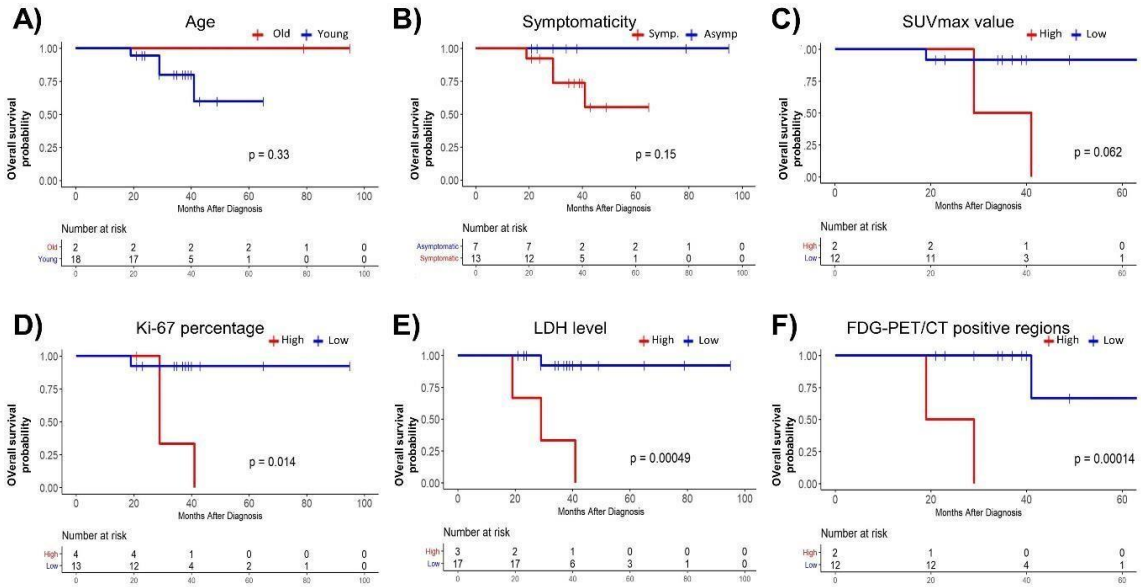


Figure 15: Kaplan-Meier plots showing overall survival probabilities based on values of demographic or clinical variables of FL patients.

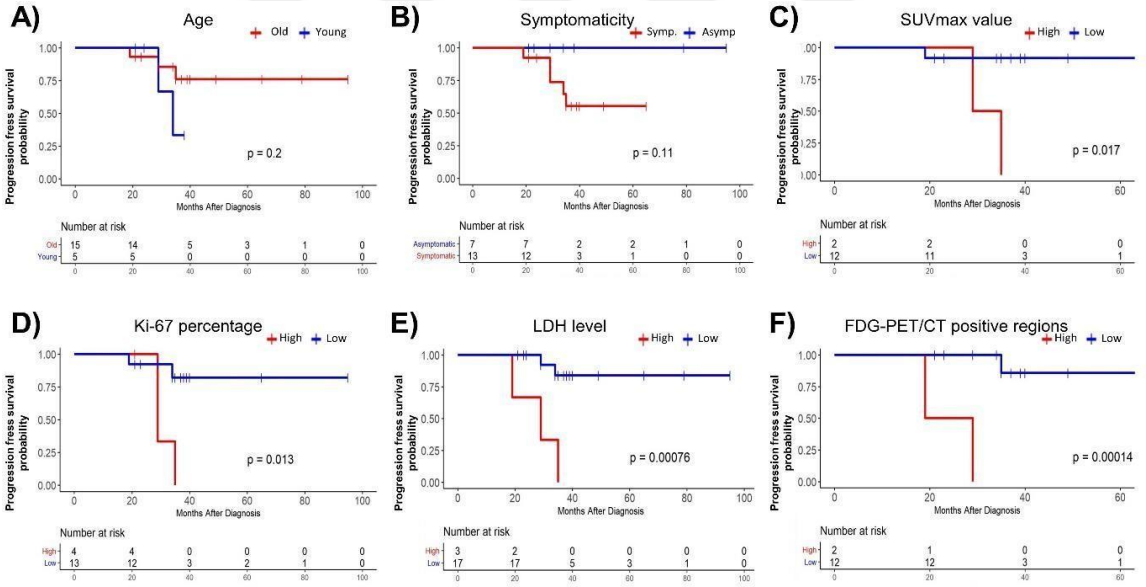


Figure 16: Kaplan-Meier plots showing progression-free survival probabilities based on values of demographic or clinical variables of FL patients.

4.11.2. The prognostic value of plasma cfDNA concentrations in FL

We assessed the survival outcomes of our follicular lymphoma (FL) cohort based on plasma cfDNA concentrations, categorized into high and low concentration groups. Notably, the high cfDNA concentration group exhibited a significant difference compared to the low cfDNA concentration group in both overall survival (p=0.018) and progression-free survival (p=0.023) analyses (**Figure 17**). Plasma cfDNA concentrations were determined as described in section 4.2, and cut-off value was 17 ng/µl for overall survival and 16 ng/µl for progression-free survival analyses.

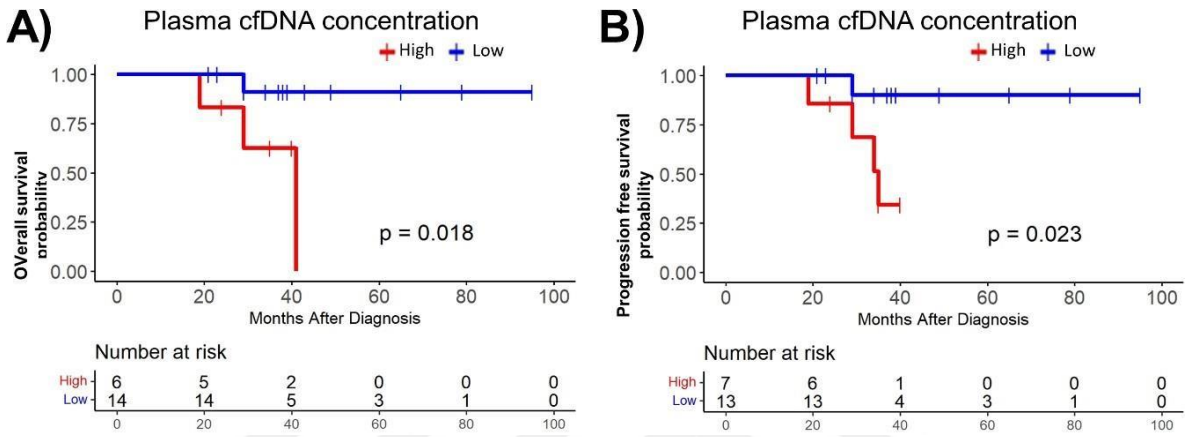


Figure 17: Kaplan-Meier plots showing survival probabilities based on plasma cfDNA concentration levels in treatment-naive FL patients.

4.11.3. The prognostic value of mutation status of genes in FL

This study included generation and application of a targeted next-generation sequencing (NGS) panel that comprehensively covered the coding regions of 110 genes, including their 50 bp flanking intron regions on FL DNA samples. With this targeted platform, mutations were identified in 31 out of these 110 genes using stringent criteria for variant identification. The influence of the mutation status of each of these 31 genes on the survival outcomes of the follicular lymphoma cohort was thoroughly analyzed. Analyses were conducted based on the DNA type in which the mutations were detected, that is, dichotomizing FL cases based on the presence of mutations only in formalin-fixed paraffin-embedded tissue DNA (FFPE tDNA), mutations only in circulating cell-free DNA (cfDNA), or considering all mutations found in FFPE tDNA and/or cfDNA. *BCL2*, *BCL7A*, and *CCND3* were identified as genes that

exhibited prognostication potential based on their mutation status in any of the analyses conducted as described above.

The presence of *BCL2* mutation, when considering only formalin-fixed paraffin-embedded tissue DNA (FFPE tDNA), was observed to be predictive of inferior overall survival (p=0.0042) but not associated with progression-free survival (p=0.054). On the other hand, when considering only circulating cell-free DNA (cfDNA), the mutation status of *BCL2* was associated with both overall survival (p=0.00074) and progression-free survival (p=0.0072). When considering the mutation status of *BCL2* in both FFPE tDNA and/or cfDNA, it was observed to be predictive of overall survival (p=0.0042) but not progression-free survival (p=0.054) (**Figure 18**).

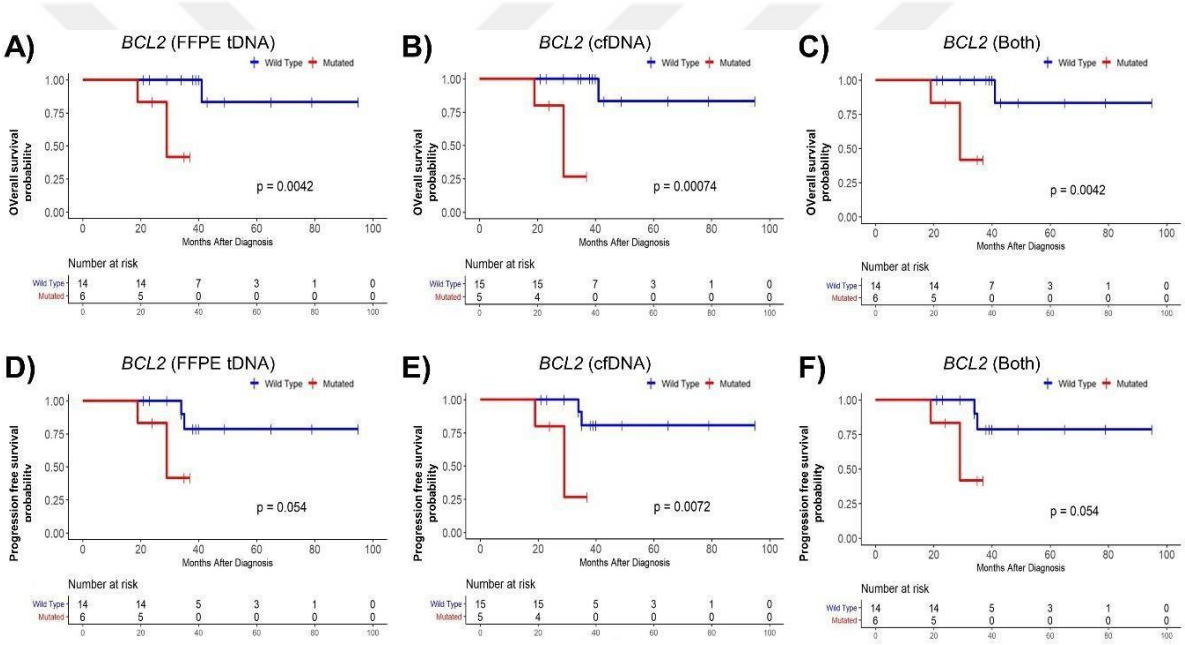


Figure 18: Kaplan-Meier plots showing survival probabilities based on *BCL2* mutation status in FFPE tDNA and/or cfDNA of FL patients.

To further evaluate the statistical significance of the findings, Fisher's exact test was employed to analyze the mutation status of the *BCL2* gene in the cases of follicular lymphoma using plasma circulating cell-free DNA (cfDNA) alone, as well as considering both DNA types (FFPE tDNA and cfDNA). The presence of *BCL2* mutation in cfDNA was still found to be associated with inferior overall survival, although not consistently when considering both FFPE tDNA and/or cfDNA (**Table 14**).

Table 14: Contingency tables showing the relationship between *BCL2* mutations and follicular lymphoma survival.

	2	**14**	16	Survived	**3**	**13**
	3	**1**	4	Not Survived	**3**	**1**

***BCL2* (cfDNA)**

	Mutated	Wild Type	Total
Survived	16		
Not Survived	4		
Total	5	15	20
p-value:	**0.031992**		

***BCL2* (Both)**

	Mutated	Wild Type	Total
Survived	16		
Not Survived	4		
Total	6	14	20
p-value:	**0.060888**		

Furthermore, our analysis revealed that the mutation status of *CCND3*, when considering only formalin-fixed paraffin-embedded tissue DNA (FFPE tDNA) or when considering both DNA types (FFPE tDNA and/or cfDNA), was associated with significantly inferior overall survival ($p < 0.0001$) and progression-free survival ($p < 0.0001$). However, it should be noted that *CCND3* mutation was observed in only one case of FL, and it was detected exclusively in FFPE tDNA (**Figure 19**).

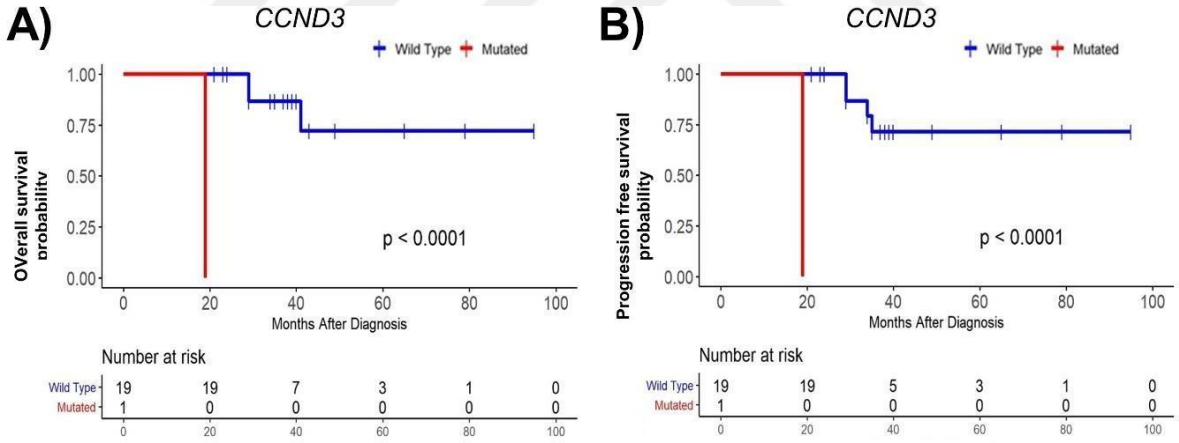


Figure 19: Kaplan-Meier plots showing survival probabilities based on *CCND3* mutation status present in any DNA type of FL patients.

The mutation status of the remaining genes, other than *BCL2*, *BCL7A* and *CCND3,* analyzed in this study did not exhibit predictive value in terms of survival outcomes for follicular lymphoma patients. The corresponding p-values obtained from the analyses of these genes are presented in **Table 15**.

Table 15: The p-values obtained from the log-rank test in survival analyses based on mutation status of all mutated genes identified in FL cases.

	FFPE tDNA		cfDNA		Both FFPE tDNA and/or cfDNA	
Gene	**OS**	**PFS**	**OS**	**PFS**	**OS**	**PFS**
ARID1A	p = 0.091	p = 0.091	p = 0.091	p = 0.091	p = 0.091	p = 0.091
ATP6AP1	p = 0.65	p = 0.52	p = 0.65	p = 0.52	p = 0.65	p = 0.52
B2M			p = 0.24	p = 0.27	p = 0.24	p = 0.27
BAZ2B	p = 0.52	p = 0.52			p = 0.52	p = 0.52
BCL10	p = 0.82	p = 0.82			p = 0.82	p = 0.82
BCL2	**p = 0.0042**	p = 0.054	**p = 0.00074**	**p = 0.0072**	**p = 0.0042**	p = 0.054
BCL7A	p = 0.48	p = 0.51	**p = 0.043**	**p = 0.043**	p = 0.48	p = 0.51
BTG1			p = 0.24	p = 0.27	p = 0.24	p = 0.27
CARD11	p = 0.16	p = 0.14	p = 0.42	p = 0.35	p = 0.16	p = 0.14
CCND3	**p < 0.0001**	**p < 0.0001**			**p < 0.0001**	**p < 0.0001**
CD79B	p = 0.31	p = 0.34	p = 0.24	p = 0.27	p = 0.31	p = 0.34
CREBBP	p = 0.079	p = 0.051	p = 0.34	p = 0.23	p = 0.079	p = 0.051
CTSS	p = 0.65	p = 0.52	p = 0.65	p = 0.52	p = 0.65	p = 0.52
DTX1	p = 0.25	p = 0.51	p = 0.65	p = 0.52	p = 0.25	p = 0.51
ENPP3	p = 0.82	p = 0.82			p = 0.82	p = 0.82
EZH2	p = 0.38	p = 0.31	p = 0.61	p = 0.49	p = 0.38	p = 0.31
FOXO1	p = 0.51	p = 0.42			p = 0.51	p = 0.42
HIST1H1E	p = 0.32	p = 0.57	p = 0.25	p = 0.51	p = 0.32	p = 0.57
IKZF3	p = 0.65	p = 0.52	p = 0.65	p = 0.52	p = 0.65	p = 0.52
IRF8	p = 0.65	p = 0.52	p = 0.65	p = 0.52	p = 0.65	p = 0.52
KMT2C	p = 0.5	p = 0.2			p = 0.5	p = 0.2
KMT2D	p = 0.33	p = 0.62	p = 0.52	p = 0.13	p = 0.33	p = 0.62
MYD88	p = 0.82	p = 0.82			p = 0.82	p = 0.82
NOTCH1			p = 0.65	p = 0.65	p = 0.65	p = 0.65
PRKCB	p = 0.82	p = 0.82			p = 0.82	p = 0.82
SOCS1	p = 0.5	p = 0.49			p = 0.5	p = 0.49
STAT6	p = 0.17	p = 0.11	p = 0.34	p = 0.23	p = 0.17	p = 0.11

TNFAIP3	p = 0.82	p = 0.82			p = 0.82	p = 0.82
TNFRSF14	p = 0.34	p = 0.23			p = 0.34	p = 0.23
TP53	p = 0.82	p = 0.82			p = 0.82	p = 0.82
USP6	p = 0.65	p = 0.52			p = 0.65	p = 0.52

4.11.4. The prognostic value of the co-presence of BCL2 mutation status and adverse clinical variables in follicular lymphoma

In order to understand whether the presence of *BCL2* mutation(s) in plasma cfDNA together with adverse clinical variables can potentially improve the prognostication of FL patients, we integrated the mutation status of *BCL2* in cfDNA with clinical variables such as Ki-67 index, LDH level, and positive FDG-PET/CT regions, and performed survival analyses by categorizing the FL patients into three groups: **1**) those with *BCL2* mutation in cfDNA and high values of adverse clinical variables; **2**) those with *BCL2* mutation in cfDNA and low values of adverse clinical variables or wild-type *BCL2* in cfDNA with high values of adverse clinical variables; and **3**) those with wild-type *BCL2* in cfDNA and low values of adverse clinical variables. The co-presence of mutated *BCL2* in cfDNA with each of these clinical variables was associated with the worst overall and progression-free survival outcomes (**Figure 20**).

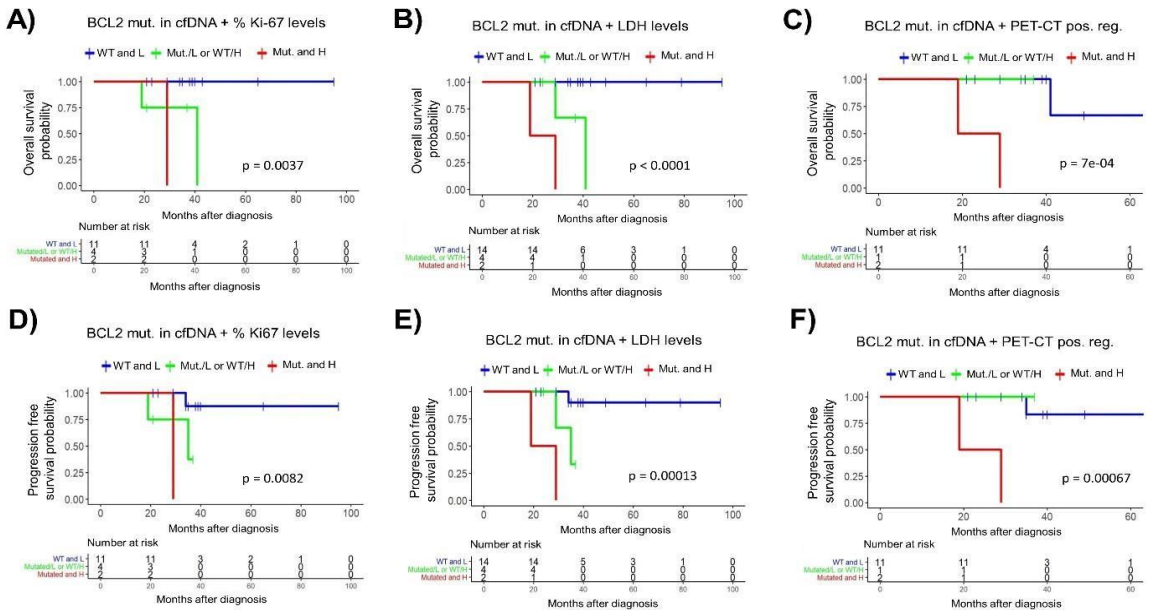


Figure 20: Kaplan-Meier plots showing survival estimates based on co-presence of *BCL2* mutation in cfDNA and different levels of adverse clinical variables of FL patients.

4.12. The presence of histological transformation associated *BTG1* and *B2M* mutations exclusively in cfDNA of a FL case

In one of the FL cases, we detected BTG1 p.E46D and B2M p.Q22* mutations, which have been previously reported to be associated with early progression in FL (Kridel et al., 2016), exclusively in the cfDNA samples of the patient. This patient received R-CHOP immunochemotherapy and showed complete remission on PET/CT imaging at 6 months post-therapy. However, disease progression was observed at 12 months post-therapy (**Figure 21**).

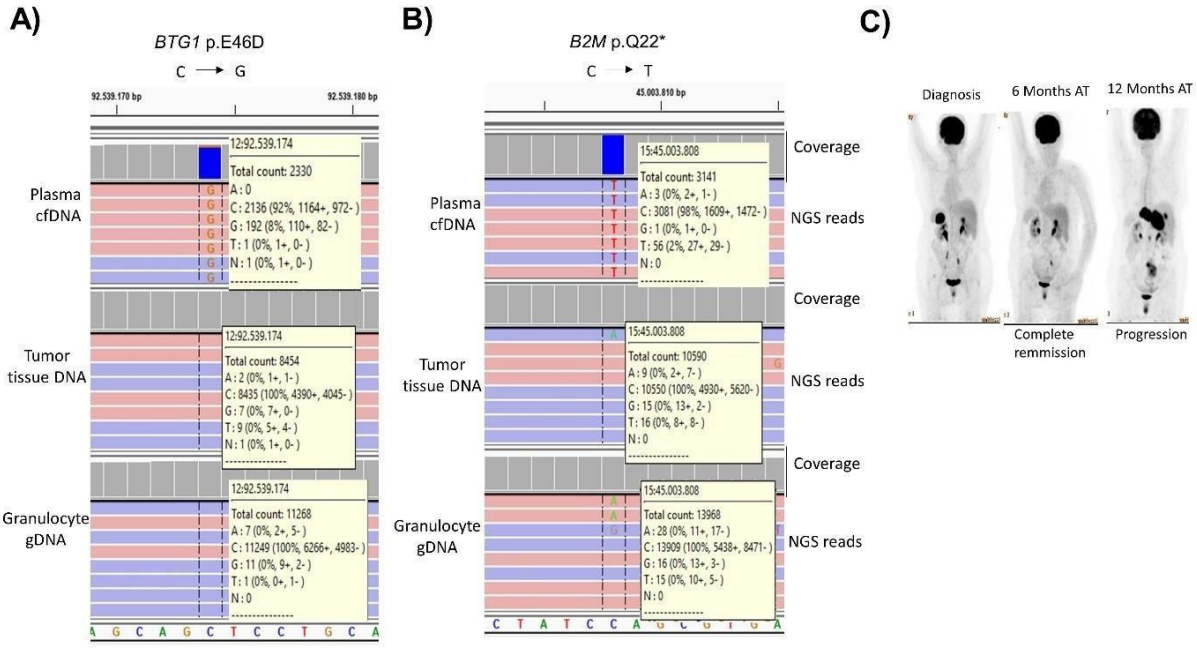


Figure 21: IGV snapshots showing BTG1 p.E46D and B2M p.Q22* mutations in the plasma cfDNA sample and images of FDG PET/CT of Case-16.

4.13. Cross-validation of selected variants with PCR-Sanger sequencing

Sanger sequencing was employed as a cross-validation method to confirm the variants identified by ultra-deep next generation sequencing. The results are presented in **Table 16**. As an example, a snapshot view of the Integrative Genomics Viewer (IGV) and Sanger sequencing chromatogram sections for the ARID1A p.R1722* variant are shown side by side for each type of DNA in **Figure 22**. Notably, in the Sanger chromatograms, nucleotide change from cytosine to thymine was observed in plasma cfDNA and tumor tissue DNA samples but was absent in the granulocyte DNA sample.

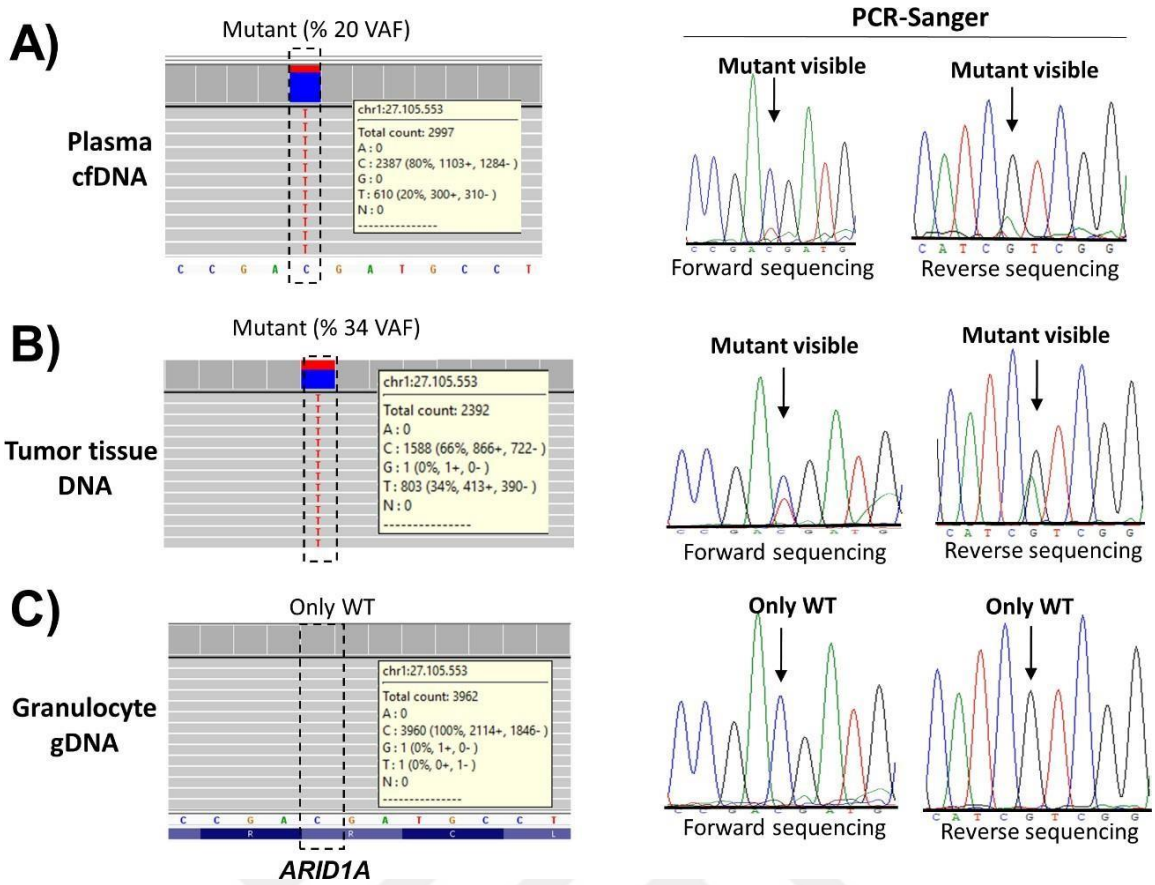


Figure 22: Represantative IGV snapshots of ultra-deep NGS results and cross-validation of the ARID1A R1722* mutation with PCR Sanger in DNA samples of a FL case.

Table 16: The PCR-Sanger cross-validation status of the selected variants in FL cases.

Case number	Gene	Sample type	Nucleotide change	AA change	Mutation detection	NGS VAF percentage (%)
Case-11	*CREBBP*	FFPE tDNA	C > T	C1237Y	+	27
Case-14	*HIST1H1E*	Granulocyte	C > T	P131S	-	0
Case-14	*HIST1H1E*	FFPE tDNA	C > T	P131S	+	67
Case-14	*HIST1H1E*	cfDNA	C > T	P131S	+	34
Case-14	*ARID1A*	Granulocyte	C > T	R1722*	-	0
Case-14	*ARID1A*	FFPE tDNA	C > T	R1722*	+	33
Case-14	*ARID1A*	cfDNA	C > T	R1722*	+	20
Case-11	*STAT6*	Granulocyte	T > A	D523V	-	0

Case-11	*STAT6*	FFPE tDNA	T > A	D523V	+	45
Case-09	*CTSS*	Granulocyte	A > C	Y132D	-	0
Case-09	*CTSS*	FFPE tDNA	A > C	Y132D	+	39
Case-09	*HIST1H1E*	Granulocyte	G > C	A65P	-	0
Case-09	*HIST1H1E*	FFPE tDNA	G > C	A65P	+	53
Case-09	*ATP6AP1*	Granulocyte	G > A	G363R	-	0
Case-09	*ATP6AP1*	FFPE tDNA	G > A	G363R	+	45
Case-11	*IRF8*	Granulocyte	T > C	Y23H	-	0
Case-11	*IRF8*	FFPE tDNA	T > C	Y23H	+	31
Case-05	*KMT2D*	Granulocyte	G > A	R2417*	-	0
Case-05	*KMT2D*	FFPE tDNA	G > A	R2417*	+	33

5. DISCUSSION

Follicular lymphoma (FL) is a frequent subtype of non-Hodgkin lymphoma, typically characterized by a relatively indolent disease course. However, in some cases, FL may transform into more aggressive types of lymphoma, with diffuse large B-cell lymphoma (DLBCL) being the most common (Link, 2018). The standard treatment for FL can involve a watch-and-wait approach for asymptomatic patients, whereas patients with symptomatic or advanced-stage FL may require chemotherapy and/or immunochemotherapy (Cahill & Smith, 2022). Despite the availability of several treatment options, the heterogeneous nature of FL poses significant challenges in identifying the optimal treatment approach for individual patients (Matasar et al., 2019). Hence, there is an ongoing need to develop better methodologies that can improve diagnosis or prognostication for better patient management.

In this study, we investigated the potential of cfDNA as a liquid biopsy, which could be used as a complementary diagnostic and prognostic tool due to its advantages. Firstly, it is non-invasive and feasible for repeated sampling from patients, enabling monitoring of disease progression or response to treatment. Secondly, circulating cfDNA represents the genotype of all tumor regions in the body that release cfDNA into circulation, providing information on metastatic sites or in cases where tissue biopsy is not available.

Consistent with previously reported recurrent mutations of genes in FL (Okosun et al., 2014), we observed mutations in *CREBBP, BCL2, STAT6, EZH2, CARD11, TNFRSF14, HIST1H1E, SOCS1*, and *KMT2D* genes in our FL cases with next-generation sequencing. Among all mutations found in our FL cohort, 38.5% of them were found in cfDNA, and 33% of them were common both in tumor tissue DNA and cfDNA. A higher concordance of mutations observed in tumor tissue DNA and cfDNA samples of DLBCL, observed previously, may be due to the more aggressive character of DLBCL compared to the relatively indolent character of FL (Rossi et al., 2017). The potential of ctDNA genotyping in non-Hodgkin lymphoma in the detection of minimal residual disease or recurrence had been discussed previously (Roschewski et al., 2016). We identified only one mutation in cfDNA samples of 12 FL cases after treatment; however, this may be due to the short time of sampling with a median of 4 weeks after the last chemotherapy cycle, in addition to the limit of detection of the method.

We analyzed the prognostication value of plasma cfDNA concentration in the progression of the disease. High levels of cfDNA concentration in plasma were associated with poor prognosis in both progression-free survival and overall survival. Indeed, in symptomatic FL cases, the level of cfDNA concentrations were significantly higher than in asymptomatic FL cases. Given the larger overlap of detected mutations in tumor tissue DNA samples and cfDNA samples in symptomatic FL cases compared to asymptomatic cases, with 41.5% and 11.6%, respectively, cfDNA genotyping may be more informative in patients showing disease-associated symptoms. On the other hand, we did not observe a difference in cfDNA concentration levels between pre-treatment and post-treatment samples.

In our FL cohort, LDH levels and FDG PET/CT positivity, which are also parts of FLIPI, showed poor survival, consistent with the previous studies (Le Dortz et al., 2010; Montoto et al., 2004). Although there are some controversial results in the literature, a high Ki-67 proliferation index was also predictive of poor survival in our study (Kawaguchi et al., 2018; Xue et al., 2020). On the other hand, when we analyzed the correlation of plasma cfDNA concentration, which has prognostic value in our study, with other clinicopathological variables, we did not observe any significant correlation. However, it is worth noting that LDH levels showed a positive correlation with cfDNA concentrations, although this correlation did not reach statistical significance, possibly

due to the relatively low number of FL cases. This finding is consistent with the observation reported in a recent publication (Fernández-Miranda et al., 2023).

In 10 out of 13 symptomatic FL cases, after applying the criteria for variants, we observed at least one common variant detected in both tumor tissue DNA and plasma cfDNA. In asymptomatic FL cases, we observed common variants in both DNA types only in one case, and in one case while there were no overlapping variants, we detected a variant exclusively in the cfDNA sample. In five of seven asymptomatic FL cases, all variants were only found in FFPE tumor tissue samples. Considering the low tumor burden in asymptomatic FL cases (Chen et al., 2012), the cfDNA released into circulation may not be reaching the detection limit of the method we used.

Importantly, we detected variants which have been reported to be associated with FL pathogenesis in cfDNA, including the KMT2D variant p.R2417* (Pasqualucci et al., 2014); activating STAT6 variants p.D419G, p.D419N, and p.D523V (Yildiz et al., 2015); and a known mutational hotspot of EZH2 variant Y646 (Bödör et al., 2013). Being a heterogeneous disease, with the improvement of novel agents, FL patients may benefit from more personalized and targeted treatments with the help of cfDNA genotyping, which is more feasible than tissue biopsy in monitoring disease progression with repeated sampling (Lauer et al., 2022). Indeed, there are already inhibitors under development for STAT6 and its signaling pathways (Miklossy et al., 2013); and EZH2 inhibitors that have shown promising results in clinical trials (Knutson et al., 2014). Notably, *STAT6* was co-mutated with *CREBBP* in all patients carrying *STAT6* gene mutations, which is consistent with a previous study (Xian et al., 2020).

We applied survival analyses for each of the 31 mutated genes in all FL cases by stratifying based on the presence of mutation in cfDNA and/or tumor tissue DNA. *BCL2* mutations were shown to be associated with poor overall survival either in tumor tissue DNA, in cfDNA, or both. Although a previous study showed that *BCL2* mutations are associated with shortened survival and an increased risk of transformation in follicular lymphoma (Correia et al., 2015b), to the best of our knowledge, this is the first time such an association has been shown for *BCL2* mutations in cfDNA. This finding may have potential clinical implications for the use of cfDNA as a liquid biopsy and as a guide for therapy. We further evaluated the prognostic value of *BCL2* mutations in cfDNA in the presence of adverse clinical variables. FL cases with a high Ki-67 index,

high LDH level, or a high number of positive FDG PET/CT regions showed the worst overall survival in the presence of *BCL2* mutation in their cfDNA samples.

Of note, in Case-16, we identified two mutations in genes *B2M* and *BTG1* exclusively in cfDNA. These two genes were shown to be associated with early progression and histological transformation in a recent clonal evolution study (Kridel et al., 2016). Additionally, variants of BTG1 p.E46D and B2M p.Q22* had been reported in two DLBCL cases, one of which was transformed FL (Morin et al., 2011; Pasqualucci et al., 2014). Consistent with these findings, the disease of Case-16 showed early progression in 12 months post-therapy after complete remission. The lack of these mutations in the tumor biopsy sample of this FL patient suggests that cfDNA fragments carrying these mutations may have originated from metastatic site(s) other than where the biopsy was taken. Although this observation in a single case requires further confirmation in larger cohorts, this reveals the importance of liquid biopsy, which has the ability to represent the spatial heterogeneity of tumor burden in FL.

When we cross-validated the selected variants of the NGS results with a VAF higher than 20% using Sanger sequencing, we obtained 100% concordance between the two methods. However, it is worth noting that variants with lower VAF values may require more sensitive techniques for validation, such as digital droplet PCR (ddPCR) or allele-specific PCR (AS-PCR) (Oxnard et al., 2014; Pinzani et al., 2010). Additionally, we observed a positive correlation between the VAF values of variants in tumor tissue samples and cfDNA samples (**Figure 3**), which implies that the variants in cfDNA originated from tumor tissue.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Follicular lymphoma typically has a favourable prognosis, with a median overall survival of more than 15 years. However, the ability to accurately identify low-risk and high-risk FL patients would enable a more personalized approach to treatment management, minimizing unnecessary toxicity for those with an indolent disease course. Therefore, it is important to develop advanced risk stratification methods that can help identify patients who are likely to benefit from more aggressive treatment strategies, as well as those who may be managed with less intensive approaches. Such personalized risk stratification would optimize clinical outcomes and improve the overall quality of life for patients with FL. In conclusion, we showed that, in principle,

with cfDNA genotyping and concentration measurement, a better, non-invasive diagnostic and/or prognostic evaluation of FL patients is possible. Further studies are required to validate the findings of this study by employing other sensitive methods with a larger sample size.

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8. APPENDIX

8.1. Ethics committee report

DOKUZ EYLÜL ÜNİVERSİTESİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU

Konu: Karar hk.- 646 21.08.2015

Sayın Yard.Doç.Dr.Can KÜÇÜK,

Kurulumuz tarafından 21.08.2015 tarih ve 2233-GOA protokol numaralı 2015/19-11 karar numarası ile görüşülen **"Dolaşımdaki Serbest Tümör DNA'sındaki Mutasyonların Foliküler Lenfoma Hastalarında Tanı ve Kemo-İmmünoterapi Direnç Biyomarkeri Olma Potansiyelinin İncelenmesi "** konulu araştırmanıza ilişkin Kurulumuz kararı ekte sunulmuştur.

Bilgilerinizi ve gereğini rica ederim.

Prof.Dr.Banu ÖNVURAL
Başkan

Dokuz Eylül Üniversitesi Sağlık Yerleşkesi İnciraltı 35340 İZMİR-TÜRKİYE
Tel:0 232 4122254 - 0 232 4122258 Faks: 0232 4122243 Elektronik posta:etikkurul@deu.edu.tr

DOKUZ EYLÜL ÜNİVERSİTESİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURUL KARARI

ETİK KOMİSYONUN ADI	**DOKUZ EYLÜL ÜNİVERSİTESİ GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU**
AÇIK ADRES	**Dokuz Eylül Üniversitesi Tıp Fakültesi Dekanlığı 2. Kat İnciraltı-İZMİR**
TELEFON	**0 232 412 22 54-0 232 412 22 58**
FAKS	**0 232 412 22 43**
E-POSTA	**etikkurul@deu.edu.tr**

BAŞVURU BİLGİLERİ	DOSYA NO:	2233-GOA	
	ARAŞTIRMA	UZMANLIK TEZİ ☐	AKADEMİK AMAÇLI ☐
	ARAŞTIRMANIN AÇIK ADI	**Dolaşımdaki Serbest Tümör DNA'sındaki Mutasyonların Foliküler Lenfoma Hastalarında Tanı ve Kemo-İmmünoterapi Direnç Biyomarkeri Olma Potansiyelinin İncelenmesi**	
	ARAŞTIRMA PROTOKOL KODU		
	SORUMLU ARAŞTIRMACI ÜNVANI/ADI/SOYADI ve UZMANLIK ALANI	Yard.Doç.Dr.Can KÜÇÜK	
	DESTEKLEYİCİ VE AÇIK ADRESİ	-	
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ VE ADRESİ	-	
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ☐

	Belge Adı	**Tarihi**	**Versiyon Numarası**	**Dili**
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ	Mevcut		Türkçe ☒ İngilizce ☐ Diğer ☐
	ARAŞTIRMA İLE İLGİLİ LİTERATÜR	Mevcut		Türkçe ☐ İngilizce ☒ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU	Mevcut		Türkçe ☒ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU	Mevcut		Türkçe ☒ İngilizce ☐ Diğer ☐

KARAR BİLGİLERİ	Karar No:2015/19-11	Tarih: 20.08.2015
	Yard.Doç.Dr.Can KÜÇÜK'ün sorumlusu olduğu **"Dolaşımdaki Serbest Tümör DNA'sındaki Mutasyonların Foliküler Lenfoma Hastalarında Tanı ve Kemo-İmmünoterapi Direnç Biyomarkeri Olma Potansiyelinin İncelenmesi"** isimli klinik araştırmaya ait başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, etik açıdan çalışmanın gerçekleştirilmesinin uygun olduğuna oy birliği ile karar verilmiştir.	
ETİK KURUL BİLGİLERİ		
ÇALIŞMA ESASI	Dokuz Eylül Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu İşleyiş Yönergesi İyi Klinik Uygulamaları Kılavuzu	
ETİK KURUL ÜYELERİ		

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet	Araştırma ile İlişkili mi?		İmza
Prof.Dr.Banu ÖNVURAL (Başkan)	Tıbbi Biyokimya	DEU Tıp Fakültesi Tıbbi Biyokimya Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Ş.Reyhan UÇKU (Başkan Yardımcısı)	Halk Sağlığı	DEU Tıp Fakültesi Halk Sağlığı A.D.	Kadın	E ☐	H ☒	
Prof.Dr.Nejat SARIOSMANOĞLU	Kalp Damar Cerrahisi	DEU Tıp Fakültesi Kalp Damar Cerrahisi Anabilim Dalı	Erkek	E ☐	H ☒	
Prof.Dr.Ece BÖBER	Pediatrik Endokrinoloji	DEU Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Vesile ÖZTÜRK	Nöroloji	DEU Tıp Fakültesi Nöroloji Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Sevinç ERASLAN	Endokrinoloji	DEU Tıp Fakültesi İç Hastalıkları Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Mukaddes GÜMÜŞTEKİN	Tıbbi Farmakoloji	DEU Tıp Fakültesi Tıbbi Farmakoloji Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Ayşe Aydan ÖZKÜTÜK	Tıbbi Mikrobiyoloji	DEU Tıp Fakültesi Tıbbi Mikrobiyoloji Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Nihal GELECEK	Fizik Tedavi ve Rehabilitasyon	DEU Fizik Tedavi ve Rehabilitasyon Yüksek Okulu	Kadın	E ☐	H ☒	
Doç.Dr.Müge KİRAY	Fizyoloji	DEU Tıp Fakültesi Fizyoloji Anabilim Dalı	Kadın	E ☐	H ☒	
Doç.Dr.Şeyda Seren İNTEPELER	Hemşirelik Yönetimi	DEU Hemşirelik Fakültesi Hemşirelik Yönetimi A.D	Kadın	E ☐	H ☒	
Doç.Dr.Seta KIZILDAĞ	Tıbbi Biyoloji ve Genetik	DEU Tıp Fakültesi Tıbbi Biyoloji ve Genetik A.D	Erkek	E ☐	H ☒	
Prof.Dr.Sevda ÖZKARDEŞLER	Anesteziyoloji	DEÜ Tıp Fakültesi Anesteziyoloji ve Reanimasyon A.D.	Kadın	E ☐	H ☒	
Uzm.Dr.Ahmet Can BİLGİN	Hukuk	DEU Tıp Tarihi ve Etik A.D	Erkek	E ☐	H ☒	
Mehmet Erhan ÖZKUL	Sağlık mensubu olmayan üye	D.E.U Tıp Fakültesi İdari Mali İşler	Erkek	E ☐	H ☒	

8.2. Curriculum Vitae

TEVFİK HATİPOĞLU

Personal Information

Contact Information

Contact Address	Eğitim mah., Adalet sok., Ceylan apt., No 16/4
Telephone	(5
Email	te
Web Page	

Education Information

01 February 2018 - Now (5 Year 4 Month)
Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, T
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENS
GENETİK (DR) (İNGİLİZCE)
Diploma Number: -
Cumulative Grade Point Average: 3.79 / 4

13 September 2012 - 02 January 2
Yüksek Lisans, Tezli Program, AN
BİYOTEKNOLOJİ ENSTİTÜSÜ, T
Diploma Number: 2017-41-
Cumulative Grade Point Av

06 October 2003 - 0
Lisans, Anadal/Nor
FEN-EDEBİYAT F
Diploma Numb
Cumulative

Experience Information

01 Ma
DİĞF

Foreign Language Inf

R&D Competency

Articles

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8.3. Publications from the thesis

1. Hatipoğlu, T., Esmeray, E., Hu, X., Yuan, H., Danyeli, A. E., Şeyhanlı, A., Süzek, T.

Ö., Zhang, W., Olgun, A., Akman, B., Özkal, S., You, H., Alacacıoğlu, İnci, Özcan, M. A., & Kucuk, C. (2020). Ultra-Deep Targeted Sequencing Reveals Cancer-Associated, Prognostically Significant Mutations in Circulating Cell-Free DNA in Treatment-Naive Patients with Follicular Lymphoma. *Blood*, *136*(Supplement 1), 20–21. https://doi.org/10.1182/blood-2020-136281

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ORIGINAL RESEARCH
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Plasma Concentrations and Cancer-Associated Mutations in Cell-Free Circulating DNA of Treatment-Naive Follicular Lymphoma for Improved Non-Invasive Diagnosis and Prognosis

Tevfik Hatipoğlu[1,2†], Esra Esmeray Sönmez[1,2†], Xiaozhou Hu[1,2], Hongling Yuan[2], Ayça Erşen Danyeli[3], Ahmet Şeyhanlı[4], Tuğba Önal-Süzek[5], Weiwei Zhang[6], Burcu Akman[1,2], Aybüke Olgun[4], Sermin Özkal[3], İnci Alacacıoğlu[4], Mehmet Ali Özcan[4], Hua You[7] and Can Küçük[1,2,8*]*

[1] İzmir Biomedicine and Genome Center, İzmir, Turkey, [2] İzmir International Biomedicine and Genome Institute, Dokuz Eylül University, İzmir, Turkey, [3] Department of Pathology, Faculty of Medicine, Dokuz Eylül University, İzmir, Turkey, [4] Department of Hematology, Faculty of Medicine, Dokuz Eylül University, İzmir, Turkey, [5] Department of Bioinformatics, Graduate School of Natural and Applied Sciences, Muğla Sıtkı Koçman University, Muğla, Turkey, [6] Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, United States, [7] Department of Oncology, Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou, China, [8] Department of Medical Biology, Faculty of Medicine, Dokuz Eylül University, İzmir, Turkey

Follicular lymphoma (FL) is the second most frequent non-Hodgkin lymphoma accounting for 10-20% of all lymphomas in western countries. As a clinically heterogeneous cancer, FL occasionally undergoes histological transformation to more aggressive B cell lymphoma types that are associated with poor prognosis. Here we evaluated the potential of circulating cell-free DNA (cfDNA) to improve the diagnosis and prognosis of follicular lymphoma patients. Twenty well-characterized FL cases (13 symptomatic and 7 asymptomatic) were prospectively included in this study. Plasma cfDNA, formalin-fixed paraffin-embedded (FFPE) tumor tissue DNA, and patient-matched granulocyte genomic DNA samples were obtained from 20 treatment-naive FL cases. Ultra-deep targeted next-generation sequencing was performed with these DNA samples by using a custom-designed platform including exons and exon-intron boundaries of 110 FL related genes. Using a strict computational bioinformatics pipeline, we identified 91 somatic variants in 31 genes in treatment-naive FL cases. Selected variants were cross-validated by using PCR-Sanger sequencing. We observed higher concentrations of cfDNA and a higher overlap of somatic variants present both in cfDNA and tumor tissue DNA in symptomatic FL cases compared to asymptomatic ones. Variants known to be associated with FL pathogenesis such as STAT6 p.D419 or EZH2 p.Y646 were observed in patient-matched cfDNA and tumor tissue samples. Consistent with previous observations, high Ki-67 staining, elevated LDH levels, FDG PET/CT positivity were associated with poor survival. High

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Edited by:
Stefano Luminari,
University of Modena and Reggio Emilia, Italy

Reviewed by:
Benedetta Donati,
AUSL Reggio Emilia, Italy
Simone Ferrero,
University of Turin, Italy

***Correspondence:**
Can Küçük
canlucukibgizmir@gmail.com
Hua You
youhua307@163.com

†These authors have contributed equally to this work

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plasma cfDNA concentrations or the presence of *BCL2* mutations in cfDNA showed significant association with poor survival in treatment-naive patients. *BCL2* mutation evaluations in cfDNA improved the prognostic utility of previously established variables. In addition, we observed that a FL patient who had progressive disease contained histological transformation-associated gene (i.e. *B2M* and *BTG1*) mutations only in cfDNA. Pre-treatment concentrations and genotype of plasma cfDNA may be used as a liquid biopsy to improve diagnosis, risk stratification, and prediction of histological transformation. Targeted therapies related to oncogenic mutations may be applied based on cfDNA genotyping results. However, the results of this study need to be validated in a larger cohort of FL patients as the analyses conducted in this study have an exploratory nature.

Keywords: follicular lymphoma, liquid biopsy, cfDNA genotyping, targeted ultra-deep sequencing, non-invasive diagnosis, prognosis, targeted therapy, histological transformation

INTRODUCTION

Follicular lymphoma (FL) is the second most common type of non-Hodgkin lymphoma, which accounts for 20-30% of all non-Hodgkin lymphomas (1). FL originates from germinal center (GC) B cells (2), and displays substantial clinic and genetic heterogeneity (3). FL cases occasionally undergo histological transformation to more aggressive B cell lymphoma subtypes with poor prognosis such as diffuse large B cell lymphoma (DLBCL) or Burkitt lymphoma, which are usually associated with poor survival and chemotherapy resistance (4). Asymptomatic FL patients are usually observed without any chemotherapeutic intervention whereas symptomatic FL cases are treated with rituximab-containing immunochemotherapy regimens (R-CHOP, R-CVP, etc.) (5). In 85-90% of FL cases, the anti-apoptotic *BCL2* gene is overexpressed as a result of t(14, 18)(q32;q21) translocation (2).

Genetic aberrations other than *BCL2* translocations are required for FL development including somatic mutations of epigenetic regulatory genes (i.e. *CREBBP*, *EZH2*, and *KMT2D*) or the genes of the JAK-STAT pathway (e.g. *STAT6*, *SOCS1*) (6). *CREBBP* mutations were detected in *in situ* follicular neoplasia (7), a neoplasm recently reported to preceed follicular lymphoma (8). Hotspot mutations of *EZH2* (i.e. Y646, A682, and A692) were detected to be frequent and early event in FL pathogenesis (9). Among these *EZH2* mutations, the role of Y646 (previously referred as Y641) was reported to inhibit EZH2 activity *in vitro* in FL (10), thereby potentially reducing H3K27me3 histone marks, which is associated with transcriptional silencing. Importantly, genetic depletion of the histone lysine transferase KMT2D led to B cell lymphoma development *in vivo*, suggesting that it may act as a tumor suppressor of FL development (11).

Diagnostic and prognostic evaluations offollicular lymphoma require histological, immunohistochemical (e.g. CD19, CD20 staining), and molecular genetic analyses in tumor tissue sections of biopsies obtained through invasive procedures such as incisional biopsy (12). However, biopsies obtained through invasive methodologies have several disadvantages: 1) patients may feel some pain and have other complications related to the procedure; 2) tumor biopsy may be difficult to obtain, and repeated sampling is not feasible; 3) genetic landscape of the entire tumor tissue or tumors at metastatic sites cannot be fully identified; 4) monitoring response to immunochemotherapy treatment is challenging. Currently available non-invasive methods (e.g. PET, PET/CT) used for cancer diagnosis and staging are expensive (13). Moreover, they may generate false-positive results (14, 15); and the results of these imaging tests do not exclude the need for tumor tissue evaluation. To overcome these challenges associated with diagnosis and cancer patient management, circulating cell-free DNA (cfDNA) genotyping has been proposed as an alternative approach for diagnosis, prognostication, management, and monitoring of different cancers (16) including B cell lymphoma subtypes (6).

Plasma cfDNA derives from the genomic DNA of apoptotic or necrotic cells released into the blood circulation (17). In cancer patients, cfDNA includes circulating cell-free tumor DNA (ctDNA) fragments reflecting cancer genomes such that cancer-associated genetic alterations including point mutations can be detected in plasma cfDNA samples when appropriate methodologies are used. Indeed, this possibility was already shown for a variety of carcinoma types such as lung (18) or prostate cancer (19). Recent studies showed the feasibility of ctDNA genotyping for diagnosis, prognosis, and monitoring response to therapy of classical Hodgkin lymphoma (20) and diffuse large B-cell lymphoma (21). However, the clinical potential of cfDNA genotyping in follicular lymphoma has been largely unknown. In this study, our main goal was to address whether it is possible to identify cancer-associated or prognostically-related mutations in plasma cfDNA samples of FL patients which may provide basis for future investigations focusing

Abbreviations: cfDNA, Circulating cell-free DNA; ctDNA, Cell-free tumor DNA; DLBCL, Diffuse large B cell lymphoma; FDG PET/CT, 18F-fluorodeoxyglucose positron emission tomography/computed tomography; FFPE, Formalin-fixed paraffin-embedded; FL, Follicular lymphoma; gDNA, Genomic DNA; IGV, Integrative Genomics Viewer; LDH, Lactate dehydrogenase; NGS, Next-generation sequencing; R-CHOP, Rituximab-cyclophosphamide hydroxydaunorubicin-oncovin-prednisone; SUVmax, Maximum standard uptake value; VAF, Variant allele fraction.

on non-invasive diagnosis and risk stratification of patients for improved patient management.

MATERIALS AND METHODS

FL Patients and Clinicopathological Data

Twenty FL cases diagnosed and clinically followed up in Dokuz Eylül University hospital were included in this study. The patient samples used in this study were approved by the institutional review board of the Medical School at Dokuz Eylül University (protocol no: 2233-GOA). All patients consent to participate in the study. All FL cases were evaluated according to the WHO 2016 classification, using morphological criteria and an appropriate immunohistochemical panel. All of the samples had a follicular pattern of neoplastic lymphoid infiltrate and the follicular dendritic cell meshworks were present in follicles. Based on these findings transformation to DLBCL was excluded. All available clinical and pathological data of the FL patients were obtained from the online clinical database of DEU hospital. **Table 1** shows the clinicopathological characteristics (e.g. type of therapy, cancer stage, the percentage of Ki-67$^+$ cells) of the FL cases. In FDG PET/CT imaging, malignant tissues can be distinguished with their higher uptake of FDG than normal. Briefly, FDG PET/CT was performed as follows: Six hours after starvation, F-18 FDG was applied intravenously to FL patients. With the purpose of attenuation correction, low dose CT and then PET images were obtained 60 mins post-injection. The number of anatomical regions potentially having tumor involvement was identified from the FDG PET/CT reports based on their SUVmax measurements.

TABLE 1 | Clinicopathological characteristics of the FL cases.

	Asymptomatic (N = 7)	Symptomatic (N = 13)
Gender		
Female	6 (85.7%)	9 (69.2%)
Male	1 (14.3%)	4 (30.8%)
Age (years)		
Mean (SD)	54.4 (17.4)	50.2 (10.6)
Median [Min, Max]	58.0 [32.0, 78.0]	54.0 [34.0, 62.0]
Stage		
I	1 (14.3%)	1 (7.7%)
II	2 (28.6%)	3 (23.1%)
III	3 (42.9%)	3 (23.1%)
IV	1 (14.3%)	6 (46.2%)
Ki67 Index (%)		
Mean (SD)	23.0 (2.76)	35.5 (10.9)
Median [Min, Max]	23.0 [20.0, 27.0]	35.0 [20.0, 55.0]
Missing	1 (14.3%)	2 (15.4%)
LDH Level (U/l)		
Mean (SD)	201 (28.0)	238 (101)
Median [Min, Max]	205 [170, 251]	224 [110, 518]
Treatment		
Watch-and-Wait	7 (100%)	0 (0%)
R-Bendamustine	0 (0%)	3 (23.1%)
R-CHOP	0 (0%)	8 (61.5%)
R-CVP	0 (0%)	1 (7.7%)
R-CVP-Radiotherapy	0 (0%)	1 (7.7%)

LDH, Lactate dehydrogenase.
R-Bendamustine, Rituximab-Bendamustine.
R-CHOP, Rituximab- Cyclophosphamide, Hydroxydaunomycin, Oncovin, Prednisone.
R-CVP, Rituximab- Cyclophosphamide, Vincristine Sulfate, Prednisone.

Plasma cfDNA and Granulocyte DNA Isolation From Peripheral Blood of FL Patients

Peripheral blood was collected from newly diagnosed 13 symptomatic and 7 asymptomatic FL cases before treatment. From 12 of 13 symptomatic FL cases, peripheral blood was collected second time after treatment as one FL patient passed away. The treatment and timeframe for peripheral blood samplings are shown in **Supplementary Table S1**. For each sample, 12 ml of peripheral blood was withdrawn into K2 EDTA tubes (BD Vacutainer, USA) in the department of hematology at DEU, and placed immediately on ice. Blood samples were transferred into 15 ml tubes, and centrifuged at 1600g for 10 min at 4°C. Supernatants were transferred to 1.5 ml tubes with each containing 1 ml plasma. The tubes were spun down to remove cellular debris at 16.000g for 5 min at 4°C. The supernatants were then transferred into new tubes, and stored in the -80°C freezer until cfDNA isolations. Circulating cell-free DNA (cfDNA) isolation was performed using QIAamp Circulating Nucleic Acid Kit (Qiagen Inc., Germany) according to the manufacturer's recommendations. The size distribution of plasma cfDNA samples were evaluated with Agilent Bioanalyzer 2100 system to ensure fragment size distribution at around 150 bp (**Supplementary Figure S1**), which is the typical size for circulating cell-free DNA (22). Granulocytes was obtained from the pellet part of the blood sample by using Ficoll® Paque Plus (MilliporeSigma, Burlington, MA) per the manufacturer's instructions. After that, genomic DNA was isolated from granulocytes using PAXgene Blood DNA Kit (Qiagen Inc., Germany).

Determination of cfDNA Concentrations in the Plasma of Treatment-Naive Follicular Lymphoma Patients

Concentrations of cfDNA isolated with QIAamp Circulating Nucleic Acid Kit were determined by using Qubit® DNA Assay Kit and Qubit® 2.0 Fluorometer. Plasma cfDNA concentrations per 1 ml of plasma were calculated using the following formula: (Qubit concentration measurement as ng/µl * elution volume as µl)/ (plasma volume as ml used during cfDNA isolations).

DNA Isolation From Formalin-Fixed Paraffin-Embedded Tumor Tissue Samples

Five µm-thick sections were prepared from tumor tissue blocks that were fixed with formalin and embedded in paraffin for each FL patient. The sections were then placed into 1.5 ml tubes, and stored at 4°C until isolations to prevent nucleic acid degradation (23). Tumor tissue genomic DNA samples were isolated using QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions.

Custom-Designed Targeted Sequencing Gene Panel

The custom-designed NGS panel including FL and transformed FL-associated 110 genes with somatic mutations was constructed

based on previously published studies (24–31). In this targeted NGS panel 575.481 bp genomic region, including the coding sequences and 50 bp flanking intronic sequences of 110 FL-related genes was targeted. FL-associated genes included in the targeted NGS panel are shown in **Table 2**.

Library Preparations and Ultra-Deep Next-Generation Sequencing

The targeted sequencing experiments on plasma cfDNA, tumor tissue DNA, and granulocyte gDNA samples from 20 diagnostic FL cases, and 12 plasma cfDNA samples of immunochemotherapy treated FL cases were performed in Novogene Genome Sequencing Company (Hong Kong, China). These target regions were captured with 6385 probes; and Agilent SureselectXT Custom Kit was used for library preparations. Captured regions were paired-end sequenced using a HiSeq system, and 150 bp NGS reads were generated. Targeted genomic regions were covered with >1500X in-depth for the identification of somatic variants with low variant allele fractions (VAF).

Computational Bioinformatics Analyses for Somatic Variant Identification

Somatic variants were identified by using a computational bioinformatics pipeline that includes the MuTect2 variant caller (**Supplementary Figure S2**). The workflow of this pipeline is briefly as follows: First, the quality of raw NGS reads were evaluated with the FASTQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Secondly, the quality of raw NGS reads were improved by applying the AfterQC program (32). Processed NGS reads were then aligned to the human reference genome (hg19) with the BWA-MEM tool (33). Picard tool (https://broadinstitute.github.io/picard/) was used to mark and filter off the PCR duplicates in NGS reads processed with SAMtools (34). MuTect2 variant caller, which can identify somatic variants with a low percentage of VAF, was used to identify somatic variants in plasma cfDNA and tumor tissue DNA samples (35). Patient-matched granulocyte gDNA samples were used to filter off the germline SNPs. Identified variants were annotated to genomic regions with Annovar (36). In the final stage, the aligned NGS reads corresponding to the genomic locations of the identified variants were visually investigated with Integrative Genomics Viewer (IGV) to verify the presence and somatic nature of the mutations (37). During IGV visualizations, the following inclusion criteria were applied for the identification of a confident list of somatic variants: 1) Variants present in the COSMIC database (release v89), and associated with hematopoietic and lymphoid tissues; 2) Missense, nonsense, indel, or splice site mutations; 3) Variants with a VAF > 0.5%; 4) Variants in cfDNA or tumor tissue DNA with a VAF > 4 fold compared to that in the granulocyte gDNA; and 5) Variants with >5 forward and >5 reverse NGS reads.

PCR-Sanger Sequencing

PCR-Sanger was applied on FL cfDNA and/or tumor tissue DNA to cross-validate selected somatic variants with a percentage of VAF greater than 20%. The DNA nucleotide sequences nearby the selected variants were obtained using the "Get DNA" bioinformatics tool available in UCSC Genome Browser (http://genome.ucsc.edu/). The primer pairs to be used for amplification of the genomic regions including the evaluated variants were designed with the PrimerQuest software (IDT DNA technologies, Coralville, IA). Designed primers were produced by Sentebiolab Biotech (Ankara, Turkey). Optimum annealing temperatures of the primer pairs were determined by gradient PCR. FL cfDNA and tumor tissue DNA samples were amplified with PCR and then send to Eurofins Genomics (Ebersberg, Germany), where PCR purifications and Sanger sequencing reactions were performed with forward and reverse primers. Vector NTI 10.3.0 (Invitrogen, Carlsbad, CA) was used to detect mutations in sequenced FL DNA samples by comparing to the human reference gene sequences. The primers used in PCR-Sanger experiments are shown in **Supplementary Table S2**.

Statistical Analysis

RStudio software (https://www.rstudio.com/) was used for linear regression, survival, and signaling pathway analyses. Survival (38) and Survminer (39) R packages were used during survival analyses and generation of Kaplan-Meier graphics. The statistical significance of the differences in survival of FL patients were evaluated with the log-rank test. Cutoff level for high and low values for each evaluated quantitative variable (i.e. cfDNA concentrations, serum LDH levels, Ki-67 index, and FDG PET/CT positive regions) were determined with maximally

TABLE 2 | The list of 110 genes included in ultra-deep targeted sequencing of follicular lymphoma DNA samples.

ABCC10[b]	BCL6[b]	CCNK[b]	DTX1[b]	FOXO1[b]	HIST1H2BC[a]	KMT2D[b]	NEB[f]	SLC9A6[f]	UPF1[b]
AGTPBP1[b]	BCL7A[b]	CD40[f]	EBF1[a]	GNA13[b]	HIST1H2BD[a]	KNDC1[b]	NOTCH2[c]	SOCS1[a]	USP6[f]
ARHGEF1[b]	BRWD3[f]	CD79A[a]	EEF1A1[b]	GNAI2[b]	HIST1H2BG[a]	LAMP1[b]	NOTCH1[c]	SP140[b]	VPS39[a]
ARID1A[b]	BTG1[b]	CD79B[a]	ENPP5[f]	GNE[f]	IKZF3[f]	LPHN1[b]	POU2F2[d]	STAT3[a]	WDR84[a]
ATP6AP1[b]	C4orf49[f]	CEP112[f]	ENTPD4[f]	GRM7[d]	IKZF3[a]	LYST[d]	OR2M3[f]	STAT6[a]	ZC3HC6[b]
ATP6V1B2[b]	CALR[f]	CHRM3[d]	EP300[b]	HIST1H1B[a]	IL13RA1[b]	MATN2[f]	PCLO[d]	SVIL[b]	ZNF141[b]
AUTS2[f]	CARD11[a]	CRNE1[b]	ERBB2[f]	HIST1H1C[a]	IRF8[d]	MCL1[d]	PEX14[f]	TLR1[f]	ZNF236[b]
B2M[b]	CASC5[b]	CREBBP[a]	ETV1[b]	HIST1H1D[a]	KIR2DL3[f]	MEF2B[b]	PLCG2[a]	TNFAIP3[a]	ZNF423[b]
BAZ2B[f]	CASZ1[b]	CTSS[f]	EZH2[a]	HIST1H1E[a]	KLHDC7B[a]	MON2[b]	PRKCB[a]	TNFRSF14[a]	ZNF541[d]
BCL10[a]	CCAR1[f]	DIRAS3[f]	FAT2[f]	HIST1H2AC[a]	KLHL6[b]	MUC4[f]	RBM23[b]	TP53[b]	ZNF595[b]
BCL2[a]	CCND3[b]	DMRT3[d]	FN1[b]	HIST1H2AG[a]	KMT2C[b]	MYD88[a]	ROS1[f]	TRAF3[b]	ZNF672[b]

[a], (24); [b], (25); [c], (26); [d], (27); [e], (28); [f], (29); [g], (30); [h], (31).

The genes reported to be mutated in FL tumor tissues in 8 previous research articles are shown in alphabetical order.

selected rank statistics using the "surv_cutpoint" function of the Survminer R package. The cutoff levels classifying low and high cfDNA groups were 17 ng/µl for overall survival and 16 ng/µl for progression-free survival analyses. These cutoff levels were 35% for Ki-67 index, 262.0 units per liter for LDH level, and 10 regions for FDG PET/CT positive regions. The R-value of linear regression analyses for the percentage of VAFs was calculated on the Spearman correlation model; and the p-value was generated with t-test. Hypergeometric distribution test was used to determine the statistical significance of enriched pathways by using the ReactomePA package (40). $p<0.05$ was considered statistically significant for the analyses performed with the RStudio software.

RESULTS

Overall Plan of the Study

The overall plan of the study involves the following steps: 1) Construction of a custom-designed targeted NGS panel based on previously mutated genes in FL and tFL; 2) Collection of peripheral blood and tumor tissue samples from treatment-naive and immunochemotherapy-treated FL patients; 3) DNA isolations from peripheral blood plasma, granulocyte, and FFPE tumor tissue samples; 4) Identification of cancer-associated mutations through computational bioinformatics analyses; 5) Investigation of the mutual presence of somatic variants in plasma cfDNA and tumor tissue DNA; 6) Survival analyses with identified variants; 7) PCR-Sanger cross-validation of selected variants. The workflow of this study is shown in **Supplementary Figure S3**.

Basic Statistics for Ultra-Deep Targeted Next-Generation Sequencing

Full set of plasma cfDNA, tumor tissue gDNA, granulocyte gDNA from 20 treatment-naive FL cases (n=60) as well as 12 post-treatment plasma cfDNA samples passed the quality parameters. The average basic NGS parameters per FL DNA samples are as follows: Total raw reads: 31.7*10^6 ± 12.3*10^6, raw data: 9.5 ± 3.7 gigabyte, the percentage of clean reads to raw reads: 93.9% ± 7.8, the average error rate in all bases: 0.011% ± 0.003, Q20: 96.8% ± 0.6, Q30: 92.5% ± 1.3, and GC content: 48.5% ± 2.2. Basic ultra-deep targeted sequencing statistics are shown in **Supplementary Table S3**.

The Impact of cfDNA Concentrations on Survival of Follicular Lymphoma Patients

To address whether plasma cfDNA concentrations have any relationship with FL patient prognosis or not, we compared survival of treatment-naive FL patients stratified based on plasma cfDNA concentrations. We observed significantly inferior overall survival (**Figure 1A**) and progression-free survival (**Figure 1B**) of FL patients with high plasma cfDNA concentrations compared to those having low cfDNA concentrations. Importantly, plasma cfDNA concentrations of symptomatic FL patients were significantly higher compared to those of asymptomatic ones (**Figure 1C**). Next, by performing linear regression analyses we evaluated whether plasma cfDNA concentrations correlate with demographic, clinical, or pathological parameters (i.e. age, Ki-67 index, pre-treatment LDH levels, FDG PET/CT positive regions, or SUVmax value) or not. We did not observe any correlation between plasma cfDNA levels and age, Ki-67 index, FDG PET/CT positive regions, SUVmax value, or LDH levels (**Figures 1D–H**).

The Numbers and the Extend of Overlap of Mutations in cfDNA and Tumor Tissue DNA

In 20 treatment-naive FL patients, in total 91 hematological cancer-associated somatic variants were detected in cfDNA and/or tumor tissue DNA samples for 31 of 110 FL-related genes targeted. Among these variants, 30 (33%) of them were common in both plasma cfDNA and tumor tissue DNA, whereas 56 (61.5%) and 5 (5.5%) of them were observed only in tumor tissue DNA or plasma cfDNA, respectively (**Figure 2A**). Of significance, we observed a significant positive correlation ($R=0.53$, $p=0.002$) between variant allele fractions of variants in tumor tissues and plasma cfDNA samples (**Figure 2B**). Next, we comparatively analyzed the number and percentage of mutations in cfDNA and/or tumor tissue DNA of symptomatic and asymptomatic FL cases. Among 91 cancer-associated somatic variants, 65 of them were identified in symptomatic, and 26 of them were identified in asymptomatic cases. Twenty seven (41.5%) of these 65 mutations were observed in both cfDNA and tumor tissue DNA, 34 (52.3%) of them were observed only in tumor tissue DNA, and four (6.2%) of them were present only in cfDNA (**Figure 2C**). In asymptomatic FL patients, 3 (11.6%) of 26 somatic mutations were observed in both cfDNA and tumor tissue DNA, 22 (84.6%) of them only in tumor tissue DNA, and just one (3.8%) of them were present only in cfDNA (**Figure 2D**). The proportion of somatic mutations present in cfDNA samples was markedly higher in symptomatic cases and most of the variants detected in asymptomatic cases were only present in tumor tissue DNA (**Figure 2E**). Importantly, we observed at least one variant present in both DNA samples in 10 of 13 (77%) of symptomatic FL cases (**Supplementary Figure S4**) and in 1 of 7 (14.3%) of asymptomatic cases (**Supplementary Figure S5**). When we compared the number of mutations per FL case in these two FL groups, we observed that the number of mutations per FL patient was marginally higher in symptomatic cases (average: 5.0; median: 4.0) compared to asymptomatic cases (average: 3.7; median: 3.0) (**Figure 2F**). In 65 mutations observed in symptomatic cases there were 54 missense, 6 nonsense, 2 indel, and 3 splice site mutations. In 26 mutations observed in asymptomatic cases there were 17 missense, 6 nonsense, 2 indel, and only one splice site mutations (**Figure 2G**). The details of 91 cancer-associated somatic mutations identified through ultra-deep targeted NGS are shown in **Supplementary Table S4**.

Mutated Genes and the Mutation Types Observed in cfDNA and Tumor Tissue DNA of Follicular Lymphoma Cases

To address whether genes reported to be recurrently mutated in previous studies are also frequently mutated in our cohort of FL

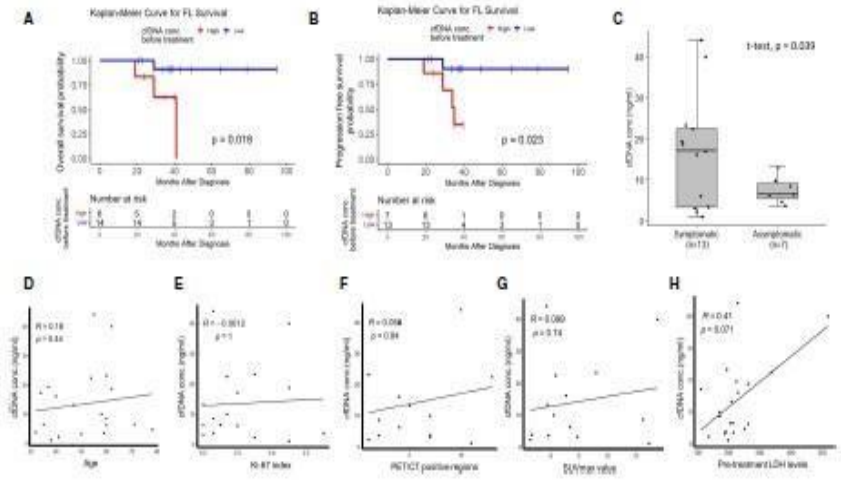

FIGURE 1 | The relationship between high plasma cfDNA concentrations and clinical variables in treatment-naive FL patients. Kaplan-Meier plots showing the relationship between high plasma cfDNA concentrations and overall survival **(A)** or progression-free survival **(B)** in treatment-naive follicular lymphoma patients. **(C)** Box-whisker plot comparing plasma cfDNA concentrations in symptomatic and asymptomatic FL patients. Linear regression graphics showing the relationship between plasma cfDNA concentrations and age **(D)**, Ki-67 index **(E)**, PET-CT positive region number **(F)**, SUVmax value **(G)**, and LDH levels **(H)**.

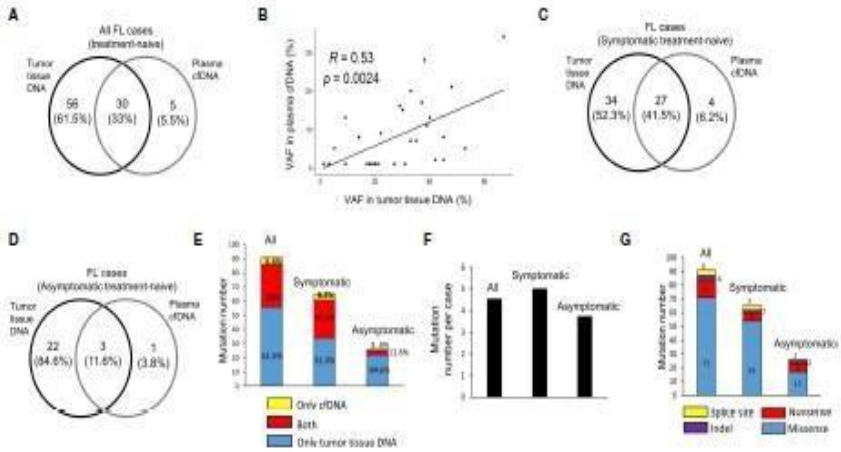

FIGURE 2 | Cancer-associated somatic variant numbers in DNA samples of FL patients. **(A)** The numbers of somatic mutations detected in plasma cfDNA and tumor tissue DNA for all FL patients shown as intersecting Venn diagrams. **(B)** Spearman correlation analysis between variant allele fractions (VAF) of mutations detected in both plasma cfDNA and tumor tissue DNA samples. The numbers and percentages of somatic mutations detected in plasma cfDNA and tumor tissue DNA for symptomatic **(C)** and asymptomatic **(D)** FL patients shown as intersecting Venn diagrams. **(E)** Bar graph of cancer-associated somatic mutation number per patient for all, symptomatic, or asymptomatic FL patients. **(F)** Bar graph of total numbers of somatic mutations detected in cfDNA and/or tumor tissue DNA for symptomatic and asymptomatic FL patients. **(G)** Bar graph of total number of somatic missense, nonsense, indel and splice site mutations detected in 13 symptomatic and 7 asymptomatic FL cases.

patients, we visually observed the frequency of the mutated genes as well as the presence of these genes in different DNA types by generating waterfall graphics (**Figures 3A, B**). The genes mutated in at least 20% of 20 FL cases are as follows: *CREBBP* (40%), *BCL2* (30%), *STAT6* (25%), *CARD11* (20%), and *EZH2* (20% of cases) (**Figure 3A**). Of note, more than one mutation per gene was observed in some FL cases. *CREBBP* mutations were generally located on the HAT/KAT domain (**Figure 3C**).

Mutations of *BCL2* were distributed in different parts of the protein whereas *STAT6* mutations were enriched in the STAT binding domain, and the *EZH2* mutations were mainly on the SET domain (**Figure 3C**). Among five cancer-associated STAT6 missense variants, three of them (i.e. p.D419G, p.D419N, and p.D523V) were present in both cfDNA and tumor tissue DNA in FL patients. Moreover, all patients with *STAT6* mutations were also carrying *CREBBP* mutations consistent with the observations in a previously reported study (41). In total 6 *EZH2* variants were identified in four FL patients, of which two were present in both cfDNA and tumor tissue DNA. In addition, we identified the enriched signaling pathways and biological processes associated with the 31 genes identified to have somatic variations in cfDNA and/or tumor tissue DNA samples. This analysis showed that mutated genes are mainly enriched in "Transcriptional regulation by RUNX1", "IL-4 and IL-13 signaling", "Chromatin modifying genes", and "Signaling by the B-cell receptor" (**Supplementary Figure S6**). However, it should be noted that the number of genes used as input for the gene ontology analysis is low, which may result in overestimation of the signaling pathways or biological processes identified.

We then asked whether any of the somatic variations identified in diagnostic FL cases persisted after immunochemotherapy treatment in symptomatic FL patients. Sixty-five somatic mutations were identified in cfDNA and/or FFPE tumor tissue DNA in 13 symptomatic FL cases. We could not obtain plasma cfDNA from one of the FL cases (i.e. Case-10) as this patient passed away. Based on targeted ultra-deep NGS analyses, 9 of these 12 symptomatic FL cases had at least one somatic mutation in their plasma cfDNA before treatment started. The total number of somatic mutations detectable in pre-treatment plasma cfDNA of these 9 symptomatic FL cases was 30. We specifically evaluated these 30 mutations in cfDNA samples after immunochemotherapy. In plasma cfDNA samples obtained post-therapy, only one indel (i.e. CARD11 p.S622delS, %VAF: 0.8) among 30 missense, nonsense, and indel variant was detectable based on our stringent variant identification criteria. Of note, there was no significant difference in plasma cfDNA concentrations between treatment-naive and post-therapy FL samples (**Supplementary Figure S7**).

The Influence of *BCL2* Mutations on Survival of Follicular Lymphoma Patients

To address whether the identified mutations predict overall and progression-free survival of FL patients, we applied survival analyses for each of 31 mutated genes with or without mutations in cfDNA and/or tumor tissue DNA samples. When we evaluated mutations detected in cfDNA and/or tumor tissue DNA, mutations in the *BCL2* gene predicted a poor overall survival ($p = 0.0042$) (**Figure 4A**). Similarly, when FL patients are dichotomized based on the presence or absence of *BCL2* mutations only in plasma cfDNA, FL patients with *BCL2* mutations were significantly ($p = 0.0007$) associated with inferior overall survival (**Figure 4B**). We also observed poor overall survival ($p < 0.0001$) of a CCND3-mutated FL patient (**Figure 4C**); however, CCND3 was mutated only in the tumor tissue of this FL case. When FL cases were dichotomized based on the presence of *BCL2* mutations in cfDNA and/or tumor tissue DNA, *BCL2*-mutated FL cases were associated with poor progression-free survival albeit not statistically significant ($p = 0.05$) (**Figure 4D**). Importantly, FL patients with *BCL2* mutations were significantly associated with progression-free survival when only cfDNA mutation status was taken into account (**Figure 4E**). Finally, a *CCND3*-mutated FL case showed significantly poor progression-free survival (**Figure 4F**). When Fisher's exact test was used to evaluate the statistical significance of these survival differences, presence of *BCL2* mutation in cfDNA was still associated with poor FL survival; whereas, no statistical significance was observed in terms of survival for the CCND3-mutated FL case (**Supplementary Table S5**). No association with FL patient survival was detected for other mutated genes evaluated. Kaplan-Meier curves for the recurrently (15% or more of FL cases) mutated genes with no association to progression-free survival are shown in **Supplementary Figure S8**.

The Effect of Co-Presence of *BCL2* Mutations and Adverse Clinical Variables on Follicular Lymphoma Survival

Next we evaluated the relationship between previously established clinicopathological variables (i.e. Ki-67 index, serum LDH levels, PET/CT positivity) routinely used in prognostic evaluations during FL diagnosis on patient survival. As expected, high expression of Ki-67 ($p = 0.013$), high serum LDH levels ($p = 0.00076$), and high number of FDG PET/CT positive regions ($p = 0.00014$) were significantly associated with inferior prognosis (**Supplementary Figure S9**). To address whether *BCL2* genotyping can improve current prognostic evaluations in a non-invasive fashion, we evaluated the co-presence of *BCL2* mutations in plasma cfDNA and adverse clinical variables on predicting overall and progression-free survival. Of significance, FL patients carrying *BCL2* mutation with high Ki-67 index (**Supplementary Figure S10A**), high LDH level (**Supplementary Figure S10B**), or high number of positive FDG PET/CT regions (**Supplementary Figure S10C**) showed the worst overall survival. Similarly, FL patients with *BCL2* mutations and high value for one of these parameters had the most inferior progression-free survival (**Supplementary Figures S10D–F**). The relationship between FL patient survival and different variables (*BCL2* mutation status, clinical variables, different treatment types) are altogether shown in **Table 3**.

Histological Transformation-Associated *BTG1* and *B2M* Mutations Were Exclusively Present in the cfDNA of a FL Patient

It has been recently shown that mutations in certain genes including those of *BTG1* and *B2M* could identify FL cases with a tendency for early progression and histological transformation (42). In our study, one FL case (Case-16) had the BTG1 p.E46D (**Figure 5A**) and B2M p.Q22* (**Figure 5B**) mutations specifically in plasma cfDNA but not in the tumor tissue DNA of the same FL patient. As expected, patient-matched granulocyte gDNA did not have variant reads, which shows the somatic origin of these

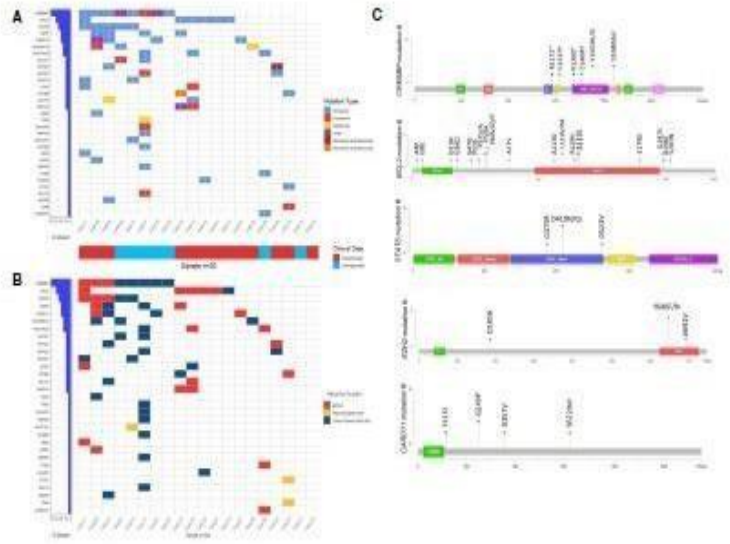

FIGURE 3 | The somatic variants identified by ultra-deep sequencing in ctDNA and/or tumor tissue DNA FL patients. **(A)** Waterfall plot showing the genes with missense, nonsense, splice site, and indel mutations detected in FL cases. Number labels indicate the number of mutations found on that gene for that FL case. Horizontal blue bars on the left show the percentage of FL cases mutated for the genes. If a gene displayed as having two mutation types includes three or more mutations in a FL case, it means at least one of these mutations belong to a mutation type different than others. **(B)** Waterfall plot showing the DNA type mutations found in plasma ctDNA, FFPE tDNA or both. Horizontal blue bars on the left show the percentage of FL cases mutated for the genes. If a gene in a FL case includes more than one mutation that is displayed as present in both DNA types, it means at least one of these mutations is present in both plasma ctDNA and FFPE tumor tissue DNA samples. **(C)** The domains and affected residues of recurrently mutated genes in FL cases determined with Cbioportal mutation mapper.

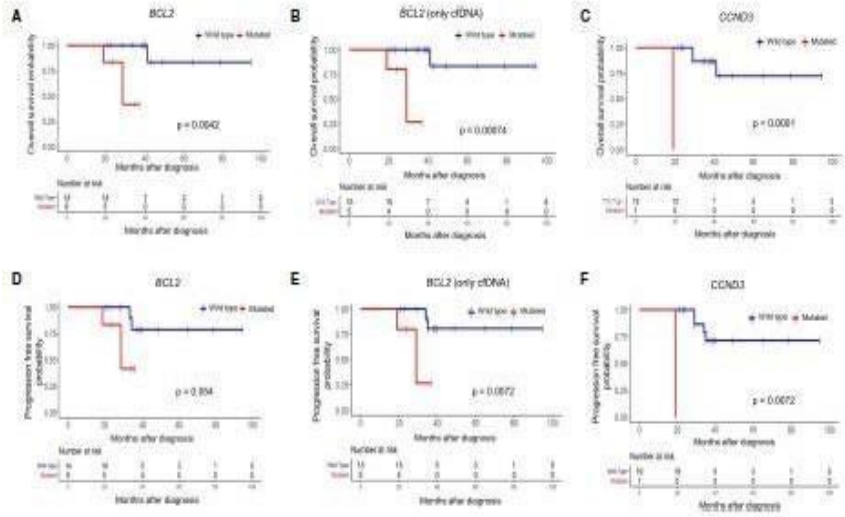

FIGURE 4 | *BCL2* mutations in plasma ctDNA predict poor prognosis in follicular lymphoma. Kaplan-Meier plots showing overall survival for FL patients based on mutation status of *BCL2* gene in ctDNA and/or tumor tissue DNA **(A)**, *BCL2* gene in only ctDNA **(B)**, or *CCND3* gene in ctDNA and/or tumor tissue DNA **(C)**. Kaplan-Meier plots showing progression-free survival for FL patients based on mutation status of *BCL2* gene in ctDNA and/or tumor tissue DNA **(D)**, *BCL2* gene in only ctDNA **(E)**, or *CCND3* gene in ctDNA and/or tumor tissue DNA **(F)**. p-values represent the statistical significance based on the log-rank test.

mutations. PET/CT imaging showed complete remission 6 months after R-CHOP immunochemotherapy; however, Case-16 showed progressive disease 12 months post-therapy (**Figure 5C**). The observations with this FL case suggest the possibility of identification of high-risk FL patients non-invasively during diagnosis through cfDNA genotyping.

Cross-Validation of Selected Variants With PCR-Sanger Sequencing

Next, we performed cross-validation of selected variants detected with ultra-deep targeted sequencing using PCR-Sanger. As the threshold for the percentage of VAF for Sanger sequencing is 20%, we chose the variants with a VAF percentage higher than 20%. Cross-validation of selected somatic variants using PCR-Sanger showed 100% consistency with NGS results (**Table 4**). PCR-Sanger cross-validation of ARID1A p.R1722* variant identified in plasma cfDNA and tumor tissue DNA is shown in **Supplementary Figure S11** as a representative.

DISCUSSION

Recurrent mutations of genes involved in epigenetic regulation (e.g. *KMT2D*, *CREBBP*, and *EZH2*), JAK-STAT pathway (e.g. *SOCS1*, *STAT6*), immune signaling (i.e. *TNFRSF14*), and BCR/NFKB signaling (e.g. *CARD11*, *CD79B*) were previously reported in follicular lymphoma cases (28). Consistent with these results, we observed recurrent mutations in *CREBBP*, *BCL2*, *STAT6*, *EZH2*, *CARD11*, *TNFRSF14*, *SOCS1*, and *KMT2D* in our FL cases with targeted next-generation sequencing (**Figure 3A**). Importantly, KMT2D variations reported to be associated with FL pathogenesis such as p.R2417* were observed in plasma cfDNA and tumor tissue DNA of our FL cases (**Supplementary Figure S12**) (43).

Previous studies revealed activating mutations of *STAT6* in the DNA binding domain, which is associated with FL pathogenesis (44). Given that in three FL patients activating STAT6 (i.e. p.D419G, p.D419N, and p.D523V) mutations present in tumor tissue DNA were detectable in plasma cfDNA, it is possible to apply therapies targeting the JAK-STAT6 pathway for the FL patients with these mutations in cfDNA. Of note, specific inhibitors of STAT6 or its signaling pathway are under development (45).

Importantly, four of the EZH2 variants were located on Y646, which is a known mutational hotspot (**Figure 3C**). H3K27 methyltransferase EZH2 gain-of-function mutations including Y646 were reported to be frequently observed as an early alteration in FL pathogenesis (9). A report showed anti-tumor efficacy for EPZ-6438, a selective inhibitor of EZH2, in Y646F-mutant xenograft tumors of non-Hodgkin lymphoma (46). Given that tazemetostat as an EZH2 inhibitor has shown activity against mutant EZH2 patients in a recent clinical trial (47), plasma cfDNA genotyping of pre-treatment FL patients may be used to decide on EZH2 targeting therapy.

Consistent with the previous studies revealing the prognostic utility of certain clinicopathological variables such as FDG PET/CT positivity (48) and serum LDH positivity (49), we observed poor survival of FL patients with high values for these variables. We also observed poor survival of FL patients with a high Ki-67 proliferation index despite the controversy on its prognostic value (50). Interestingly, in a previous study the prevalence of *BCL2* mutations detected in FL tumor tissue samples was 12% at diagnosis and 53% at the transformation; and FL patients with *BCL2* mutations had shortened survival and increased risk of histological transformation to aggressive B cell lymphomas (24). Observation of the prognosis-associated *BCL2* mutations for the

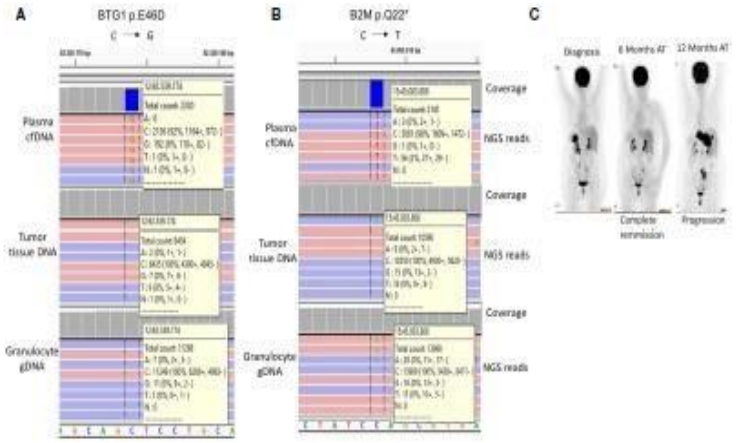

FIGURE 5 | Histological transformation-associated *BTG1* and *B2M* mutations detected exclusively in cfDNA of a FL patient. IGV snapshots showing BTG1 p.E46D **(A)** and B2M p.Q22* **(B)** mutations in the plasma cfDNA sample of a FL patient (Case-16). The mutation status of the patient-matched granulocyte gDNA is also shown as a negative control sample. **(C)** FDG PET/CT images of Case-16 are shown before and after immunochemotherapy. AT, After therapy.

TABLE 3 | The relationship between clinical variables as well as *BCL2* or *CCND3* mutation status and follicular lymphoma survival.

Variable		Number	Event	OS (%)	p	Event	PFS (%)	p
Ki-67 percentage (%)	> 35%	4	3	25.0	0.014	3	25.0	0.013
	≤ 35%	13	1	92.3		2	84.6	
LDH level before therapy (units/L)	> 262 units/L	3	3	0.0	0.00049	3	0.0	0.00076
	≤ 262 units/L	17	1	94.1		2	88.2	
Number of positive FDG PET/CT regions	> 10	2	2	0.0	0.00014	2	0.0	0.00014
	≤ 10	12	1	91.7		1	91.7	
ctDNA concentration before therapy (ng/ml)	> 16 ng/ul	6	3	50.0	0.018	4	33.3	0.023
	≤ 16 ng/ul	14	1	92.9		1	92.9	
BCL2 gene	Mutated	6	3	50.0	0.0042	3	50.0	0.054
	Wild Type	14	1	92.9		2	85.7	
CCND3 gene	Mutated	1	1	0.0	<0.0001	1	0.0	<0.0001
	Wild Type	19	3	84.2		4	78.9	
BCL2 gene and Ki-67 percentage (%)	Mutated and High (> 35)	2	2	0	0.0037	2	0	0.0082
	Mutated and Low (≤ 35) or Wild Type and High (> 35)	5	2	60		2	60	
	Wild Type and Low (≤ 35)	10	0	100		1	90	
BCL2 gene and LDH level before therapy (units/L)	Mutated and High (> 262)	2	2	0	<0.0001	2	0	0.00013
	Mutated and Low (≤ 262) or Wild Type and High (> 262)	5	2	60		2	60	
	Wild Type and Low (≤ 262)	13	0	100		1	92.3	
BCL2 gene and Number of positive FDG PET/CT regions	Mutated and High (> 10)	2	2	0	0.0007	2	0	0.00067
	Mutated and Low (≤ 10) or Wild Type and High (> 10)	2	0	100		0	100	
	Wild Type and Low (≤ 10)	10	1	90		1	90	
Treatment	R-Bendamustine	3	1	66.7	0.42	1	66.7	0.47
	R-CHOP	8	3	62.5		4	50.0	
	R-CVP	2	0	100.0		0	100.0	

OS, Overall Survival; PFS, Progression-Free Survival.

TABLE 4 | List of genes and variants cross-validated with PCR-Sanger sequencing and their confirmation status.

Case number	Gene	Sample type	Nucleotide change	AA change	Mutation detection	NGS VAF percentage (%)
Case-11	CREBBP	FFPE tDNA	C > T	C1237Y	+	27
Case-14	HIST1H1E	Granulocyte	C > T	P131S	–	0
Case-14	HIST1H1E	FFPE tDNA	C > T	P131S	+	67
Case-14	HIST1H1E	ctDNA	C > T	P131S	+	34
Case-14	ARID1A	Granulocyte	C > T	R1722*	–	0
Case-14	ARID1A	FFPE tDNA	C > T	R1722*	+	33
Case-14	ARID1A	ctDNA	C > T	R1722*	+	20
Case-11	STAT6	Granulocyte	T > A	D523V	–	0
Case-11	STAT6	FFPE tDNA	T > A	D523V	+	45
Case-09	CTSS	Granulocyte	A > C	Y132D	–	0
Case-09	CTSS	FFPE tDNA	A > C	Y132D	+	39
Case-09	HIST1H1E	Granulocyte	G > C	A65P	–	0
Case-09	HIST1H1E	FFPE tDNA	G > C	A65P	+	53
Case-09	ATP6AP1	Granulocyte	G > A	G363R	–	0
Case-09	ATP6AP1	FFPE tDNA	G > A	G363R	+	45
Case-11	IRF8	Granulocyte	T > C	Y23H	–	0
Case-11	IRF8	FFPE tDNA	T > C	Y23H	+	31
Case-05	KMT2D	Granulocyte	G > A	R2417*	–	0
Case-05	KMT2D	FFPE tDNA	G > A	R2417*	+	33

AA, Amino acid.
NGS, Next generation sequencing.
VAF, Variant allele fraction.
FFPE tDNA, Formalin-fixed paraffin-embedded tumor tissue DNA.
Granulocyte, Granulocyte gDNA.

first time in plasma cfDNA samples of FL cases have important implications for risk assessment as well as the guidance of therapy. Indeed, it may be more effective to choose aggressive therapies for the subset of FL patients having both *BCL2* mutations in cfDNA with any of these other poor prognostic indicators. However, validation in independent, larger cohorts of FL cases is needed to address these observations in our study with a relatively low number of cases. Furthermore, cross-validation of targeted NGS results of *BCL2* mutations having low VAFs in FL cfDNA with approaches more sensitive than Sanger sequencing (e.g. ddPCR (18)) will be one of the priorities of future research. The association of highly poor survival and the presence of *CCND3* mutations requires further investigation with larger FL patient cohorts as well to address whether prognostic *CCND3* mutations are observed in plasma cfDNA or not as we observed *CCND3* mutation in tumor tissue of only one FL case. Importantly, all seven genes of the m7-FLIPI (51) was included in our targeted NGS panel (**Table 2**). Given that none of the 5 mutated genes of m7-FLIPI (i.e. *ARID1A*, *CREBBP*, *CARD11*, *EZH2*, and *FOXO1*) showed association with survival in our FL patient cohort is consistent with a recent report indicating that its prognostic value in FL patients may be context dependent (52).

The marked difference observed between symptomatic and asymptomatic FL cases with respect to the percentage of mutations in cfDNA suggests that cfDNA genotyping may be more feasible for FL patients showing disease-associated symptoms. It should also be noted that 61.5% of the identified somatic mutations were specific to tumor tissues. This observation may be related to the relatively indolent character of follicular lymphomas compared to more aggressive lymphomas such as DLBCL where a higher proportion of tumor tissue-derived mutations was observed in patient-matched plasma cfDNA (21). However, we do not exclude the possibility that certain cancer-associated mutations in cfDNA of asymptomatic FL patients may have been missed due to the limitations of the targeted ultra-deep NGS approach applied in this study or the limited amount of cfDNA available from asymptomatic patients.

Identification of mutations in *BTG1* and *B2M* genes associated with early disease progression and/or histological transformation (42) exclusively in the plasma cfDNA sample of a FL case suggests that plasma cfDNA genotyping may provide additional prognostic information that may not be obtained from tumor tissues under certain circumstances. Of note, BTG1 p.E46D and B2M p.Q22* mutations were previously reported in two DLBCL cases, one of which was a transformed FL (43, 53). Our observation that the FL case with these mutations in cfDNA had a progressive disease is consistent with these previous reports. The lack of *BTG1* and *B2M* mutations in tumor tissue DNA suggests that ctDNA fragments with these mutations may have originated from distant metastatic tumor tissues with different genotypes in this FL patient. Nevertheless, it should also be noted that these mutations were observed in only one FL case; and plasma cfDNA genotyping of independent patient cohorts is needed to address whether these observations can be generalized for follicular lymphoma.

Given that many FL pathogenesis-associated mutations present in tumor tissues are also observed in patient-matched plasma cfDNA samples, it is possible -in principle- to detect these somatic mutations non-invasively with targeted next-generation sequencing during clinical applications. The positive correlation between the variant-allele fractions of mutations in patient-matched tumor tissue and plasma cfDNA implies that mutations in cfDNA originate from tumor tissues. As somatic mutations with VAF percentages >20% were cross-validated with Sanger sequencing in this study (**Supplementary Figure S11**), more sensitive methodologies such as ddPCR (18) or BEAMing (54) will be needed to specifically cross-validate these NGS-identified mutations with low percentages of VAFs in FL cases in the future.

In conclusion, we showed in this study the possibility that concentration measurement and/or genotyping of FL-related genes in plasma cfDNA of treatment-naive FL patients may be used -in principle- to improve current diagnostic and prognostic evaluations, thereby leading to more appropriate selection and timing for therapeutic interventions including targeted therapies.

DATA AVAILABILITY STATEMENT

All targeted NGS data files were submitted to the NCBI SRA database (accession no: PRJNA832096), and can be directly accessed through the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA832096.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Medical School at Dokuz Eylül University (protocol no: 2233-GOA). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TH, HLY, XH, and BA: Performed research and analyzed data; EES and WZ: Computationally analyzed the targeted sequencing data; TÖS: Contributed to supervision of the computational bioinformatics analyses; AE and SÖ: Pathologically diagnosed the FL cases and provided tumor sections; AŞ, IA, AO, and MÖ: Clinically followed FL patients, provided the peripheral blood samples, compiled, and organized clinical data of the FL patients; TH and CK: Wrote the manuscript; HY. and CK: Financially supported the project; CK: Conceived the study, and supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.870487/full#supplementary-material

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