Mature B-Cell Neoplasms

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Frequently Asked Questions

1. **What are commonly used molecular techniques in diagnosis of mature B-cell neoplasms?**
   - Hematopathology has always been the best representative for pathology evolution and on the forefront of integration of diagnostic application of molecular technology.
   - Mature B-cell neoplasms can usually be diagnosed with routine histologic evaluation, combined with flow cytometry and immunohistochemistry (IHC). Molecular techniques, including fluorescence in situ hybridization (FISH), PCR, Sanger sequencing, and next-generation sequencing (NGS), are getting more widely used to determine subclassification of malignancy and provide prognostic or therapeutic information for clinical management (Table 16.1).
2. **How to choose the molecular techniques in lymphoid neoplasms?**
   - The choice of technique should be decided by multiple factors [1], including:
   – The type and amount of available material
   – The desired sensitivity levels
   – The type of target(s) to be analyzed
   – Turnaround time requirement
   – Cost and volumes of the tests
   - Fresh or frozen samples generally show higher quality of nucleic acids and are less prone to artifacts introduced by DNA and RNA degradation, which are

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Table 16.1 Examples of commonly used molecular assays in mature B-cell neoplasms

Molecular assays	Method	Target	Utility	Tissue
Clonality test	PCR, NGS	*IGH, IGK*	Detection of B-cell clonality	FFPE tissue, fresh tissue
Translocation analysis	FISH, PCR, NGS	t(14;18)	Aid in diagnosis of FL	FFPE tissue
	FISH	*BCL2, BCL6, MYC*	Subclassification of DLBCL	FFPE tissue
Mutational analysis	PCR, Sanger sequencing, pyrosequencing NGS	*TP53*	Therapy decisions in CLL	FFPE tissue, fresh tissue
		MYD88 *BRAF*	Differentiate between LPL and MZL	FFPE tissue

FL Follicular lymphoma; *DLBCL* Diffuse large B-cell lymphoma; *CLL* Chronic lymphocytic leukemia; *LPL* Lymphoplasmacytic lymphoma; *MZL* Marginal zone lymphoma; *FFPE* Formalin-fixed paraffin-embedded

generally preferred for RNA-based studies. However, fresh or frozen samples are usually unavailable during the workups of mature B-cell neoplasms due to the traditional workflow setup in the pathology laboratory. Additionally, frozen tissues are not evaluated morphologically. When the lymphoid tissue is only partially involved by a neoplastic process, there might be sampling bias resulting in false-negative results.

- In general, formalin-fixed, paraffin-embedded (FFPE) samples are suitable for most diagnostic purposes. Compared to blood and fresh tissues which are considered biohazards, FFPE tissue is regarded safer for handling.
- Chromosome translocations associated with mature B-cell lymphomas (e.g., *IGH/BCL2*, *IGH/CCND1*) do not usually create fusion transcripts. Therefore, molecular testing to detect these fusion genes will require DNA as a template, which is technically challenging. FISH tests have better diagnostic (not analytic) sensitivity in detecting these chromosome translocations.
- With the development of technologies, there is a move away from single-gene assays toward panel testing in many laboratories because although most diagnostic biopsies are small, the list of requested predictive biomarkers keeps growing. It is more practical to use a panel-based testing approach for economical, time-saving, and material preservation consideration.

3. **What are the principles of B-cell (immunoglobulin gene) clonality tests?**
   - In the development of the lymphoid system, B cells undergo a series of strictly programmed genetic recombination of the surface antigen receptor (immunoglobulin (Ig) in B cells) genes which encode for the various parts of antigen receptor molecules.
   - In bone marrow, the recombination process takes place in committed precursor B cells and follows a sequential order primarily involving different variable (V), diversity (D), and joining (J) gene segments. This process is antigen independent (Fig. 16.1a–b).
   - In periphery, mature B lymphocytes further extend their Ig repertoire upon antigen recognition in germinal centers via somatic hypermutation, a process leading to affinity maturation of the Ig molecules.
   - A normal or reactive immune response typically generates a polyclonal population of lymphocytes with a multitude of different antibodies, whereas a monoclonal proliferation is assumed to be the hallmark of neoplasms.
   - The clonality tests are based on the principle that rearrangement of antigen receptor genes occurs during lymphoid proliferation and each lymphocyte clone has a unique coding sequence for its antigen receptor. The receptor DNA sequences are amplified by multiplex PCR reactions that contain primers binding to the conserved regions. Due to the size difference of the antigen receptor gene in a diversified lymphoid population, the PCR products (amplicons) will have a variety of sizes in Gaussian distribution pattern when running through size differentiating electrophoresis.
   - During a clonal proliferation, such as B-cell neoplasms, the clonal population will be amplified, and its product is also called as "clonal rearrangement positive."
4. **When should I consider a B-cell (immunoglobulin gene) clonality test?**
   - Benign versus malignant lymphoproliferative disorder: Because it can be performed on FFPE tissue, clonality test has been a useful adjunctive method for diagnosis of lymphoproliferative disorder, especially in some cases that morphological and immunophenotypic features can be difficult to interpret between benign versus malignant lesions. It should be particularly considered when biopsy is very small but with predominant B-cell proliferation, without accompanied flow cytometry study due to lack of fresh tissue, appreciable histological architecture, or limited material to complete the immunohistochemistry workup.
   - Lineage verification between T- and B-cell neoplasms, when morphologic and immunophenotypic features are not sufficient to characterize the cell lineage.
   - Clonal relationship determination between two and multiple neoplasms. The test will be valuable in several situations, such as:
     - To determine if a new lymphoproliferative neoplasm in a patient with history of lymphoma is an actual relapse or a de novo malignancy
     - To determine if a higher-grade lymphoma in a patient with a history of low-grade B-cell lymphoma is a disease progression or a separate process

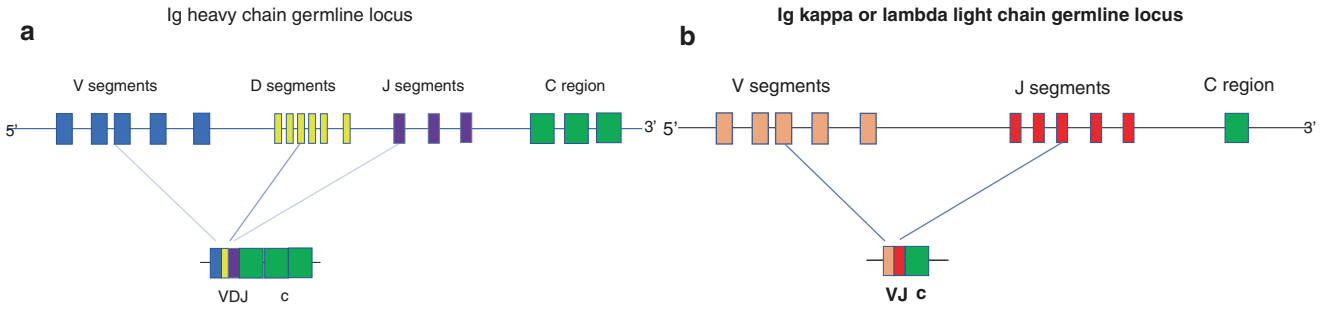


Fig. 16.1 B-cell gene rearrangement (**a**) V-DJ rearrangement in immunoglobulin heavy chain; (**b**) V-J rearrangement in immunoglobulin light chain

Table 16.2 Comparison of different methods of clonality test

	Advantages	Disadvantages
Southern blot	Used to be the gold standard method for clonality testing and now being largely replaced by PCR-based methods Primarily used for TRA tests (too large for PCR or NGS) in research setting	Slow and laborious Large amount of DNA required Relatively insensitive
PCR (Most commonly used)	Fast and inexpensive Much less DNA required Better sensitivity (5–10%) Available and easily instituted into most labs	Separated PCR product by size not by sequence Subjective interpretation Not sensitive enough MRD detection
NGS	Similar DNA input but resulted with sequence data Less subjective More sensitive, suitable for MRD detection	Relative expensive Longer TAT depending on volume

NGS Next-generation sequencing; *MRD* Minimal residual diseases; *TAT* Turnaround time; *TRA* test, T-cell receptor gene rearrangement alpha locus

5. **What methods are commonly used for clonality determination?**
   - Southern blot hybridization-based clonality test for Ig gene rearrangement was introduced to research and later clinical practice in the mid-1990s [2]. It has been replaced by PCR-based assay due to its intrinsic limitations (Table 16.2).
   - Currently multiplex PCR-based clonality assay is commonly used in the clinical practice. The European BIOMED-2 collaborative study has developed and standardized the immunoglobulin gene rearrangement assay, and the BIOMED-2 primer set and kits are commercially available for Ig heavy chain *(IGH),* kappa light chain (*IGK),* and lambda light chain (*IGL)* genes.
   - B lineage cells can express with kappa or lambda Ig light chain; however, the gene rearrangement is programmed to prefer the kappa locus by the recombinase machinery so that *IGK* gene rearrangement begins before *IGL* gene. In addition, *IGK* gene is rearranged in the lambda light chain expressing B cells or plasma cells. Thus, there are more B lineage cells that express kappa than lambda light chain in a healthy individual.
   - B-cell clonality test usually includes both *IGH* and *IGK* but not *IGL* gene rearrangement to keep the balance of higher detection sensitivity yet not too complicated test itself. Studies have shown that testing in both *IGH* and *IGK* will not only ensure the detection of Ig-gene rearrangements in mantle cell lymphomas (MCL) and chronic lymphocytic leukemia/small lymphocytic lymphomas (CLL/SLL) but also increase the detection rate to close to 100% in the heavily somatically mutated follicular lymphomas (FL), marginal zone lymphomas (MZL), and diffuse large B-cell lymphomas (DLBCL) [3].
   - NGS-based clonality tests have been developed in recent years and slowly gain its popularity because of its advantages over traditional PCR-based assays.
6. **What are the limitations of clonality testing we should keep in mind?**
   - During selection of FFPE block or area of slide for testing, we should circle B cell-rich areas or choose B-cell-rich blocks. If only low numbers of B cells are present in the sample tested, there is a possibility of pseudoclonality or false-negative result due to failed amplification or high reactive background.
   - Pseudoclonality, defined as the erroneous detection of a pseduoclonal lymphoid population, is an important pitfall and a major consideration when performing PCR clonality assays on low numbers of lymphocytes. The possibility of pseudoclonality increases when there is less than 20–40 ng of DNA or 800–2000 lymphocytes being analyzed which is more often seen in the interpretation of small biopsies, especially in lymphocytic cutaneous infiltrates [4–8].
   - Duplicate or triplicate tests should be required as the standard procedure, especially for low template samples, to avoid misinterpretation or false diagnosis of a

clonal population. In addition, background knowledge of the sample, including clinical history and histological differential diagnosis, etc., and ample experience are required for accurate interpretation of the clonality tests [9, 10].

- Many factors could affect the sensitivity of the clonality test, for example, type of lymphoma and length of tissue fixation. It is well known that the sensitivity of clonality test is higher in MCL and CLL/SLL than that of FL [4].
- A positive clonal rearrangement result by itself is supportive but not enough to diagnose a neoplastic lesion, and the clonality test should be used as a valuable tool to clarify morphologic ambiguities. Oligoclonal or monoclonal patterns can be observed in some reactive conditions or B-cell-poor lesions, especially in some small cutaneous biopsy samples [11–13].

7. **Can we use immunoglobulin kappa and lambda light chain stainings by chromogenic in situ hybridization (CISH) to replace clonality tests by molecular methods?**
   - In mature B-cell neoplasms, the majority of immunoglobulin gene rearrangement involves the heavy chain (*IGH*) locus, while a minor part involves the light chain loci, either the kappa light chain (*IGK*) or the lambda light chain (*IGL*).
   - *IGK* and *IGL* by conventional CISH can be used in helping the diagnosis of a mature B-cell neoplasm which expresses abundant kappa or lambda light chains, such as plasma cell neoplasm and marginal B-cell lymphoma with plasma cell differentiation.
   - Majority of B-cell neoplasm, such as follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), nodal and splenic marginal zone lymphoma (MZL), and mantle cell lymphoma (MCL), have lower levels of light chain mRNA expression and do not typically demonstrate an *IGK* or *IGL* restriction by CISH in FFPE tissue.
   - To determine clonality, CISH-based assay is generally less sensitive and specific compared to molecular methods and should not be used as a replacement of the latter.
8. **Are both IGH and IGK clonal gene rearrangement required to call a positive clonality result?**
   - If a clonal gene rearrangement of either *IGH* or *IGK* gene is detected, it is called that the clonality result is positive (aka there is a clonal B-cell population). Although sometimes, if not more common, both *IGH* and *IGK* gene rearrangement can be detected in the same sample, it is not necessary or required for both *IGH* and *IGK* clonal gene rearrangement detected to demonstrate a clonal B-cell population present.
   - However, depending on the detection method used, a standardized interpretation algorithm should be established for result interpretation. For example, in PCR-capillary electrophoresis-based clonality test, it should be considered of peak heights and peak ratio to define "truly clonal" rearrangements.
9. **Is the positive immunoglobulin (Ig) gene rearrangement result required or by Itself enough to diagnose a B-cell neoplasm?**
   - As mentioned above, a positive *IGH* and/or *IGK* gene rearrangement result provides supportive evidence to aid the diagnosis of a B-cell neoplasm. However, it should not be used as the sole evidence to make such diagnosis.
   - In some cases, particularly when biopsy tissues are tiny, determination of the difference between reactive and neoplastic lesions can be challenging. In these cases, the value of immunohistochemistry stains can be limited too due to lack of appropriate architecture. If a B-cell neoplasm is suspected but histological criteria for neoplasia are not met, the clonality analysis would be most useful.
   - The positive clonality test result is not required to establish the diagnosis of a B-cell neoplasm. Histomorphology has a long history and has been essential in the diagnosis of diseases. Additional ancillary tests, such as a clonality test, are not indicated if the histological evidence is clear and enough. In addition, not every B-cell neoplasm has an identifiable Ig gene rearrangement by currently available technology. The cause of the observed difference includes differences in somatic hypermutation rates in the *IGH* locus which result in different recognition and binding efficiency of the PCR primers to their target sequences.
   - Neoplastic B-cell neoplasms could contain cross-lineage T-cell receptor (*TCR*) gene rearrangement. It has been reported that clonal *TCR* gene rearrangement could be detected in up to 90% of children and adults with precursor B-cell lymphoblastic leukemia [14]. This lineage infidelity phenomenon is usually more commonly seen in precursor lymphocytic malignancies than in mature B-cell neoplasms or normal B cells.
10. **What are the advantages of NGS-based clonality tests over length-based analysis, and when should I consider it for clinical samples?**
    - Advantages of NGS-based clonality test include:
      - Allowing identification of the full range of clonal populations

  - Determination of the unique DNA/RNA sequence of clonal rearrangement, in additional to the size of the rearrangement product
  - Detection of clonal events hidden in a polyclonal distribution
- Because of the cost of the test and turnaround time requirement, currently PCR-based clonality test is still the most used method. Detection of clonality using NGS of the immunoglobulin genes has a relatively infrequent but occasionally critical niche in the clinical workup of mature B-cell neoplasms. It should be considered in the following circumstance:
  - By recognizing the unique clonal DNA sequence, it can help determine the clonal relationship between neoplasms of different anatomic sites and time.
  - It can be used for disease monitoring or minimal/measurable residual disease (MRD) detection by comparing to the unique sequence of the original diagnostic sample.

11. **What are the established prognostic biomarkers in chronic lymphocytic leukemia (CLL)?**
    - CLL is a mature B-cell malignancy and the most common lymphocytic leukemia [15].
    - The major diagnostic criteria of CLL include the presence of a circulating clonal B-cell population (>5000/microliter in peripheral blood) with surface co-expression of CD5 and CD23.
    - To assess CLL prognosis, NCCN guideline and iwCLL criteria recommend using several biomarkers, including cytogenetic changes and gene mutation status, which are summarized in Table 16.3 and should be tested in all CLL patients upon diagnosis to facilitate therapy determination [16–20].
    - Because CLL could have different clinical courses and many patients may have an indolent presentation which does not require treatment for many years while others may have more aggressive courses, for patients with newly diagnosed CLL, International Prognostic Index for CLL (CLL-IPI) (Table 16.4) can be used to estimate prognosis and time for start treatment (Table 16.5).
    - In addition, both NCCN and iwCLL also recommend retesting previously treated CLL patients before starting patients on a new treatment.
12. **Besides those established prognostic biomarkers, what are the emerging prognostic biomarkers for CLL?**
    - With advances in DNA sequencing technology and its application in clinical practice, the last decade has seen significant advances in the development of biomarkers in oncology. More predictive biomarkers have been identified and the landscape of CLL therapeutics has changed drastically over the last few years.
    - Among the new markers, *NOTCH1, SF3B1*, and *BIRC3* mutations are present in relatively lower frequency compared to above established biomarkers (*TP53, IGHV*, and cytogenetic abnormalities) in untreated CLL; however, the incidence increases to nearly 25% in patient refractory to chemotherapy, especially fludarabine. Although currently there are limited and conflicting evidence regarding their prognostic significance, it has been demonstrated that *NOTCH1* mutation is independently associated with Richter's transformation of CLL.
    - Recurrent *BTK* and *PLCG2* mutations are usually not detectable at baseline but identified in most patients with CLL progressing on ibrutinib therapy. Activating mutations in *BTK* and *PLCG2* induce increased B-cell receptor signaling and ibrutinib resistance. Because these resistance mutations can usually be detected months prior to clinical disease progression, they could be used for disease/treatment monitoring. Studies have been reported to incorporate these biomarkers into the prognostic and risk stratification model for CLL patients [48].
13. **What are the clinical implications for *MYD88* L265P mutation in B-cell neoplasms?**
    - *MYD88* is a driver gene found in B-cell neoplasms. L265P is a highly recurrent hot spot mutation in *MYD88* which changes leucine at position 265 to proline [49].

Table 16.3 Established CLL prognostic biomarkers and commonly used testing methods [16, 21–47]

Category	Biomarkers	Mean frequency (range) (%)	Prognosis	Methods of detection
Gene	*TP53* mutation	8 (4–12)	Unfavorable	Sequencing
	IGHV mutation[a]	60 (37–78)	Favorable	Sequencing
Cytogenetics	Complex karyotype[b]	–	Unfavorable	Karyotype
	Del(11q)	17 (5–25)	Unfavorable	FISH
	Trisomy 12	14 (12–19)	Intermediate	FISH
	Del(13q)	55 (35–60)	Favorable	FISH
	Del(17p)	6 (3–8)	Unfavorable	FISH

[a]*IGHV* mutated is defined as 98% or less homology with germline gene sequence
[b]Complex karyotype is defined by the presence of at least three unrelated chromosomal abnormalities in more than one cell on karyotype

Table 16.4 International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI)

Variable	Description	Score
Age	≤65 years	0
	>65 years	+1
Clinical stage	Binet A or Rai 0	0
	Binet B-C or Rai I-IV	+1
Serum β2 microglobulin (mg/L)	≤3.5	0
	>3.5	+2
IGHV mutational status	Mutated	0
	Unmutated	+2
TP53 mutational status	Wild type	0
	Del(17p) by FISH and/or *TP53* mutation by sequencing	+4

Table 16.5 Using CLL-IPI to estimate CLL prognosis and time for start treatment

CLL-IPI total score	Risk group	Treatment recommendation[a]	5-yr overall survival (%)
0–1	Low	Do not treat	93.2
2–3	Intermediate	Do not treat unless symptomatic	79.3
4–6	High	Treatment indicated unless asymptomatic	63.3
7–10	Very high	Do not use chemotherapy. Treat on clinical trial or novel targeted inhibitor	23.3

[a]The decision to treat should not be based solely on the risk group. Risk categories should be used to estimate prognosis, and for patients in higher-risk groups, closer monitoring should be considered

- *MYD88* L265P is found in 90–97% of Waldenström macroglobulinemia (WM) or lymphoplasmacytic lymphoma (LPL) and has helped to differentiate these entities from other B-cell neoplasms, particularly marginal zone lymphomas. Besides *MYD88*, other recurrent somatic mutations, including *CXCR4* (30–40%), *ARID1A* (17%), and *CD79B* (8–15%), and deletions in chromosome 6q were also commonly identified in the lymphoplasmacytic cells in WM [50]. In the small number of WM patients who lack *MYD88* L265P mutation, they might have increased risk of disease transformation, poor response to ibrutinib, and shorter overall survival [51, 52].
- *MYD88* L265P also occurs in a small percentage of IgM but not IgG or IgA monoclonal gammopathy of undetermined significance (MGUS), splenic marginal zone lymphoma, 25–30% of DLBCL of activated B cell (ABC) type, and 60–70% of primary large B cell lymphoma of the central nervous system [53, 54]. *MYD88* L265P-positive IgM MGUS patients have shown an increased risk of disease progression to WM [55].
- Because of the above clinical implication and its impact on treatment strategies, it is important to distinguish patients with *MYD88* wild-type WM from those with other IgM-secreting neoplasms, such as plasma cell myeloma.

14. **What methods are commonly used for *MYD88* mutation detection?**
    - Like detection of other gene mutations, *MYD88* L265P can be detected using different molecular methods such as relatively simple, straightforward techniques like allele-specific PCR (AS-PCR), pyrosequencing, and Sanger sequencing or more complicated but comprehensive analysis techniques such as NGS.
    - The selection of the specific method depends on many factors, which usually include the following:
      1. Desired test sensitivity or lower limit of detection.
      2. Volume of the test.
      3. Cost of the test, including instrument, reagent, technologists, quality control, proficiency test, and maintenance of the equipment.
      4. Is it usually ordered as a single biomarker or combined with other biomarkers?
      5. Requirement of turnaround time.
15. **What genetic changes are largely seen in extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) but not in other types of marginal zone lymphomas?**
    - There are three main groups of marginal zone lymphoma (MZL), including MALT lymphoma, nodal marginal zone lymphoma (NMZL), and splenic marginal zone lymphoma (SMZL).
    - Chromosomal translocations are commonly associated with certain types of MALT lymphomas but not in SMZL or NMZL. For example, t(11;18)(q21;q21) is associated with pulmonary and gastric MALT, whereas t(3;14)(p14.1;q32) is more commonly associated with MALT lymphoma arising in the thyroid, ocular adnexa, orbit, and skin. A summary of commonly seen chromosomal translocation in MALT is listed in Table 16.6.
16. **What are the clinical implications for *EZH2* mutation in B-cell neoplasms?**
    - *EZH2* (enhancer of zeste homolog 2) gene plays an essential role in the development of lymphocytes and is required for germinal center formation. When deregulated, mutant *EZH2* can induce germinal center hyperplasia and B cell neoplasm, which mainly includes FL and germinal center type DLBCL [56, 57].
    - Activating *EZH2* mutation resulting in aberrant methylation of histone H3 lysine 27 (H3K27) can be identified in 20–25% of FL. The *EZH2* mutations can also be detected by sequencing methods. Copy num-

Table 16.6 Recurrent chromosomal translocation in MALT lymphoma

Chromosome translocation	Gene partners	Site of disease	Detection methods
t(11;18) (q22;q21)	*BIRC3-MALT1*	Lung and stomach	FISH, RT-PCR, NGS
t(3;14) (p14.1;q32)	*FOXP1-IGH*	Thyroid, ocular adnexa, orbit, and skin	FISH, RT-PCR, NGS
t(1;14)(p22;q32)	*BCL10-IGH*	GI tract and lung	FISH, RT-PCR, NGS
t(14;18) (q32;q21)	*IGH-MALT1*	Ocular, salivary gland, and skin	FISH, RT-PCR, NGS

GI tract gastrointestinal tract

ber changes of *EZH2*, which is also clinically relevant, can be detected by SNP arrays when indicated. Immunohistochemical stain for EZH2 expression or H3K27 methylation may be a useful surrogate for *EZH2* mutation analysis [58].

- There are emerging interests in recent years to investigate the role of *EZH2* in lymphomagenesis with dozens of therapeutic agents that have been developed to target the EZH2 enzymatic domain. For example, tazemetostat (TAZVERIK™) is a protein known as a methyltransferase, which is an *EZH2* gene inhibitor and works by targeting *EZH2* and can be used to treat *EZH2* mutation bearing follicular lymphoma, after other treatments have been tried. However, further studies and clinical trials are still required to advance our knowledge in the tumorigenesis of *EZH2* mutants.

17. **When should the mutational analysis be considered in mature B-cell neoplasm diagnosis, prognosis, and treatment?**
    - Besides the molecular biomarkers discussed above, high-throughput technology has identified many emerging markers with prognostic potentials in other mature B-cell neoplasms, such as MCL and DLBCL.
      - Besides traditional *CCND1* gene rearrangement in MCL, molecular aberrations such as *ATM*, *TP53*, *CDKN2A,* and *MYC* are also frequently seen in at least 20% of patients [59].
      - In DLBCL, somatic mutations in *MYD88*, *CD79B*, *EZH2, NOTCH1,* and *NOTCH2* and gene rearrangement in *BCL2* and *BCL6* genes could further distinguish DLBCL into genetic subtypes with different clinical courses [60, 61].
    - Although the understanding of the genetic landscape of mature B-cell neoplasm has grown rapidly in recent years and had a significant impact on our understanding of lymphoma pathobiology and by extension on the current WHO classification, mutational analysis has not been a standard clinical practice in mature B-cell neoplasm diagnosis and currently being used only in a few entities and specific clinical situations, such as *BRAF* V600E in hairy cell leukemia and *MYD88* L265P in lymphoplasmacytic lymphoma. This is mainly because:
      - Lymphoma diagnosis can usually be achieved with routine histologic evaluation, combined with immunohistochemistry (IHC), flow cytometry (FCM), and sometimes with addition of fluorescence in situ hybridization (FISH) assay.
      - Mutational analysis has limited direct impact on lymphoma prognostic purpose and therapy decisions.
      - As the newer member of ancillary tests, the current cost of mutational analysis is similar or more expensive compared to IHC, FISH, or FCM.
    - It is likely that we will experience a significant increase in molecular testing, especially sequencing-based mutational analysis, in B-cell neoplasm for subclassification of lymphoma, as well as prognostic and treatment stratification. It will bring many benefits in practical applications such as:
      - Mutational analysis, especially panel-based NGS testing, is a more efficient and timely technology for genomic profiling of mature B-cell neoplasm. As we see more cytology and core biopsy samples in lymphoma workup, NGS test minimizes the amount of tissue consumed and avoids the need for iterative reflex testing.
      - Greater use of panel-based mutational analysis will allow the discovery of more driver mutations.
      - Utilization of molecular tumor boards allows molecular pathologists and oncologists to integrate genomic reports in the context of a broader knowledge base and the patient to support better treatment decisions.

Cases Presentation

Case 1

Learning Objectives

1. Indication of clonality test in mature B-cell neoplasm
2. The utility and limitation of PCR-based clonality assay
3. Use of NGS-based clonality assay in determination of clonal relationship

Case History

A 70-year-old male presented to his primary care physician complaining of several days of black tarry stools, occasional bright red blood per rectum, fatigue, and abdominal pain. Patient's past medical history includes basal cell carcinoma diagnosed 10 years ago which was treated with resection.

Initial Workup

His complete blood count (CBC) was significant for mild anemia with a hemoglobin of 11.7 g/dL (reference 13.7–17.5), hematocrit of 30% (reference 40–51%), and leukocytosis of 14.5 thousand/uL (reference 4.2–9.1). An esophagogastroduodenoscopy revealed a 5–6 cm mass with central ulceration in the lesser curvature of the stomach which was biopsied.

Histologic Findings

The biopsy of the gastric lesion showed intact gastric mucosa with a diffuse atypical infiltrate of small lymphoid cells with scattered larger forms in the submucosa (Fig. 16.2a–b). The atypical infiltrates were positive for B-cell markers CD20 (Fig. 16.2c) and CD79a while negative for CD5 (Fig. 16.2d), cyclin D1, and CD10. Flow cytometry was not performed.

Question 1: After reviewing this preliminary information, which hematologic diseases are in the differential diagnosis?

Question 2: Which molecular studies could be ordered to help the diagnosis?

Based on the described findings, there are two primary considerations: reactive lymphoid proliferation and a mature B-cell neoplasm. Testing for *IGH* and *IGK* gene rearrangement was ordered, and the result was positive for clonal proliferation of both *IGH* and *IGK* genes. Taken together with its immunophenotype, a diagnosis of low-grade extranodal marginal zone B cell lymphoma/mucosa-associated lymphoid tissue (MALT) lymphoma was made. Fluorescence in situ hybridization (FISH) for t(11;18) *BIRC/MALT1* fusion was performed on the gastric biopsy with no translocation detected. However, approximately 90% of the nuclei had trisomy 18 at 18q21 resulting in three copies of the *MALT1* gene.

Next, positron emission tomography (PET) scan and bone marrow biopsy were performed for staging purposes. PET identified increased fluorodeoxyglucose (FDG) uptake and gastric thickening in the region of the gastric cardia with max SUV of 8.6 consistent with known diagnosis of MALT lymphoma. No other abnormal regional FDG uptake is identified.

The bone marrow biopsy was mildly hypocellular for age with progressive trilineage hematopoiesis (Fig. 16.3a). Flow cytometry identified a population of clonal B-cell population which expresses CD19, CD20 (dim), CD5, and CD23 with dim kappa light chain restriction (Fig. 16.3b). The monoclonal B-cell population has an immunophenotype most typical of chronic lymphocytic leukemia (CLL). However, the absolute count of clonal B cells is too low to reach formal criteria for a diagnosis of CLL in the absence of symptoms or adenopathy. Given the disparity between this phenotype and the impression based on the gastric biopsy, additional molecular studies were ordered.

Question 3: Is the monoclonal B-cell proliferation identified in marrow related to the patient's gastric MALT lymphoma? Which molecular studies could be ordered now?

Because the flow cytometry was not performed on the gastric biopsy, the immunophenotype cannot be compared with that of the patient's bone marrow. The gastric B-cell lymphoma was CD5 negative by IHC, whereas the clonal B cells in bone marrow were expressing CD5 and CD23.

Molecular Genetic Study

First, the PCR-based gel detection of *IGH* and *IGK* gene rearrangements was also performed on the bone marrow aspirate, and the result was also positive for clonal proliferation. Although clonal amplification products were detected in both gastric lymphoma and bone marrow B-cell proliferation, the sizes of the products are not identical, and it is inconclusive that the marrow B cell lymphocytosis was originated from the same clone of the gastric lymphoma (Fig. 16.4a–b).

Question 4: What else could we do to clarify this inconclusive result?

NGS-based IGH clonality assay was next performed on the gastric lymphoma and marrow B-cell lymphocytosis samples. In bone marrow, the only clone identified is a 302 bp sequence using *IGHV3* and *IGHJ6* segments with cumulative of 51.7% of total reads. In the gastric MALT lymphoma, the dominant clone identified is a 303 bp sequence using *IGHV4* and *IGHJ5* segments with cumulative of 58.3% of total reads. Interestingly, another small clone (3.1% of total reads) is also identified in this sample which is using the same V and J segments as in the clone identified in the marrow (Fig. 16.5a-b) with 100% match of their sequence (Fig. 16.5c).

Final Diagnosis

1. Gastric MALT lymphoma without bone marrow involvement
2. Monoclonal B-cell lymphocytosis (MBL) with CLL type

Follow-Up

The patient completed radiation therapy for gastric MALT lymphoma and is doing well. A recent PET scan indicated complete remission. His monoclonal B-cell lymphocytosis, CLL type, was monitored clinically on blood-based flow cytometry test every 6 months without any treatment.

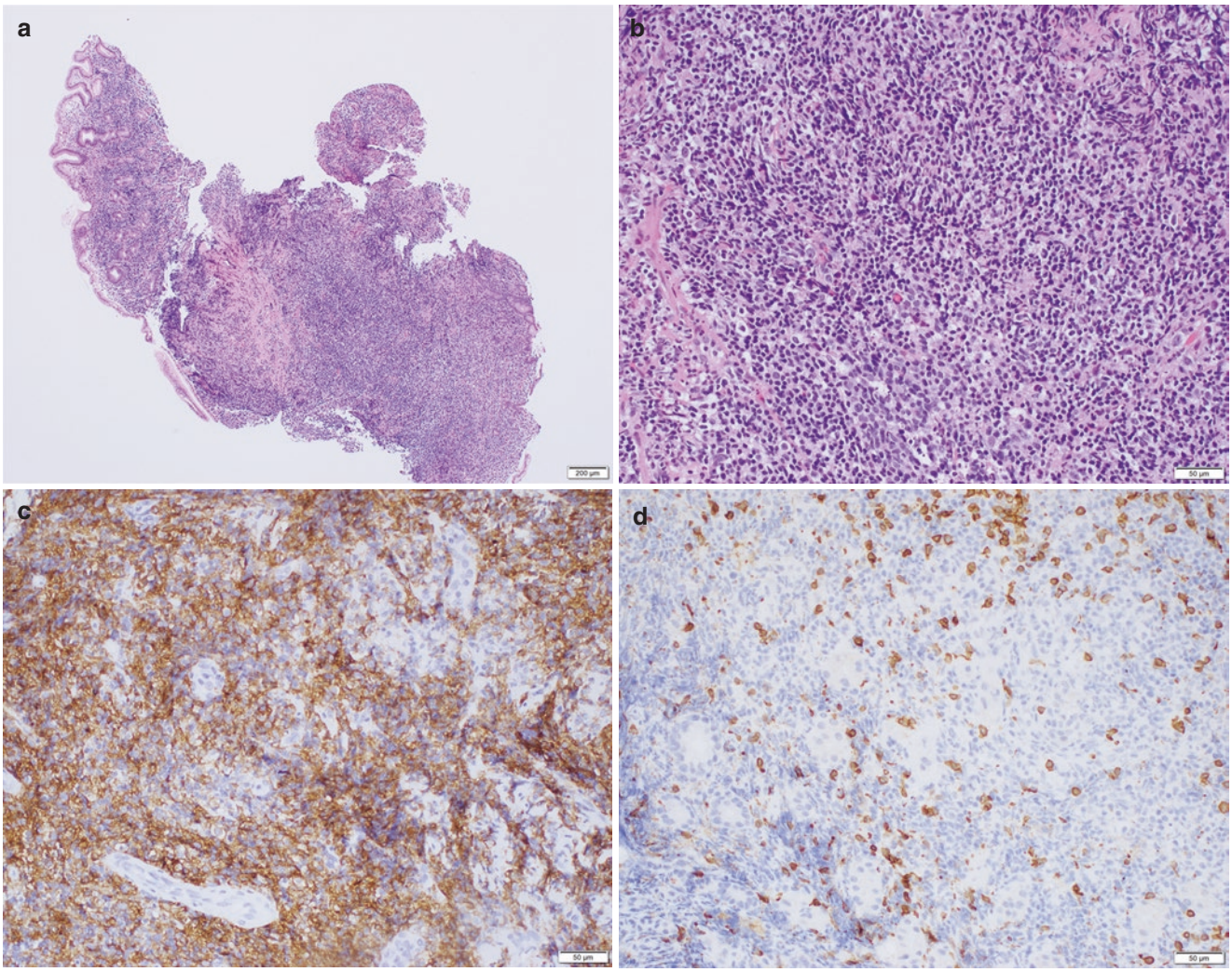


Fig. 16.2 Gastric biopsy showing sheets of atypical lymphoid cells in the gastric submucosa. (**a**) Hematoxylin and eosin, 40×; (**b**) hematoxylin and eosin, 200×; (**c**) CD20 was positive in atypical cells, 200×; (**d**) CD5 highlighted scattered small T lymphocytes and was negative in atypical cells, 200×

Discussion

This interesting but not uncommon case illustrates the utility and limitation of PCR-based clonality assay. It also highlights the advantage of NGS-based tests in determination of clonal relationship of mature B-cell neoplasms. In summary, there were two clonally unrelated neoplastic diseases identified in this patient: a MALT lymphoma which is localized at the stomach only and another MBL of CLL type which involves the patient's blood and bone marrow. The B-cell clone identified in the bone marrow which was also identified in the patient's gastric sample could be explained by blood circulating neoplastic cells in the stomach. Clonality test should not be performed on clear-cut lymphomas; however, because NGS-based clonality assay can identify the unique DNA sequence, clonal prevalence, and V-J family identity for each gene rearrangement, it is a power tool in the determination of clonal relationship. In this case, it has helped the clinical treatment stratification and patients did not need to receive systemic treatment.

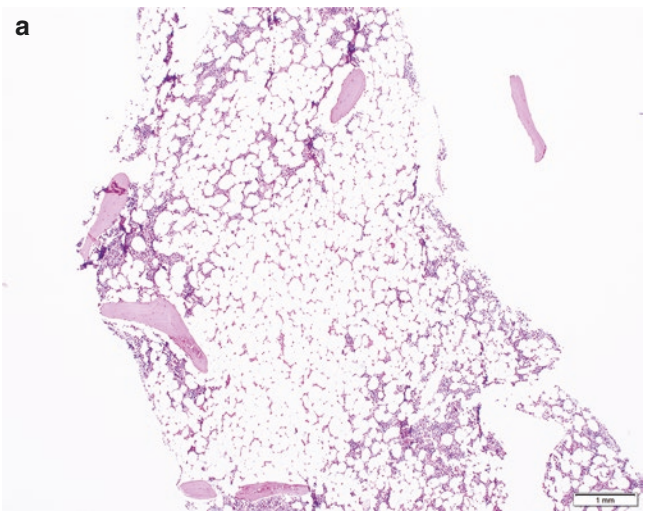


Fig. 16.3 Bone marrow biopsy. (**a**) Mildly hypocellular bone marrow for age with trilineage hematopoiesis; (**b**) flow cytometry results showing a population of lymphocytes expressing CD19, CD20 (dim), CD5, and dim kappa light chain restriction

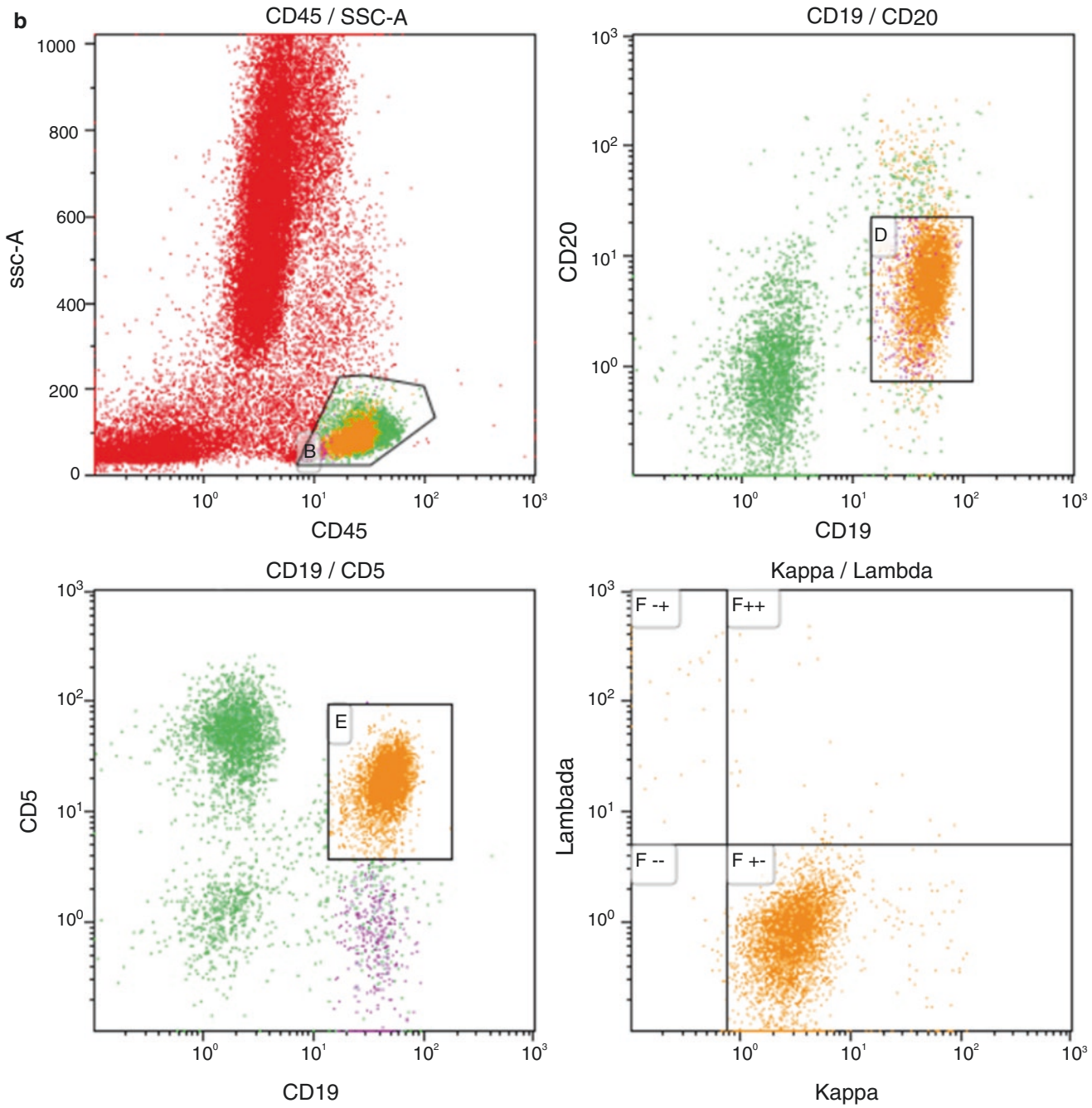


Fig. 16.3 (continued)

Case 2

Learning Objectives

1. Use of NGS-based clonality assay in determination of clonal relationship and help on clinical management
2. The utility of clonality assay in Hodgkin lymphoma

Case History

A 26-year-old female, with no past medical history, presented to the emergency department complaining of acute chest pain radiating to her shoulder accompanied by shortness of breath. Deep inspiration provoked pain radiating to the middle of her back. In addition, she was experiencing general malaise, recent drenching night sweats, and weight loss.

Chest x-ray showed a widened mediastinum, most prevalent on the right. A subsequent CT scan revealed a 7 cm mass in the anterior mediastinum compressing the medial aspect of the lung right upper lobe. A video-assisted thoracoscopic (VATS) biopsy of the mass was performed and sent to pathology for evaluation.

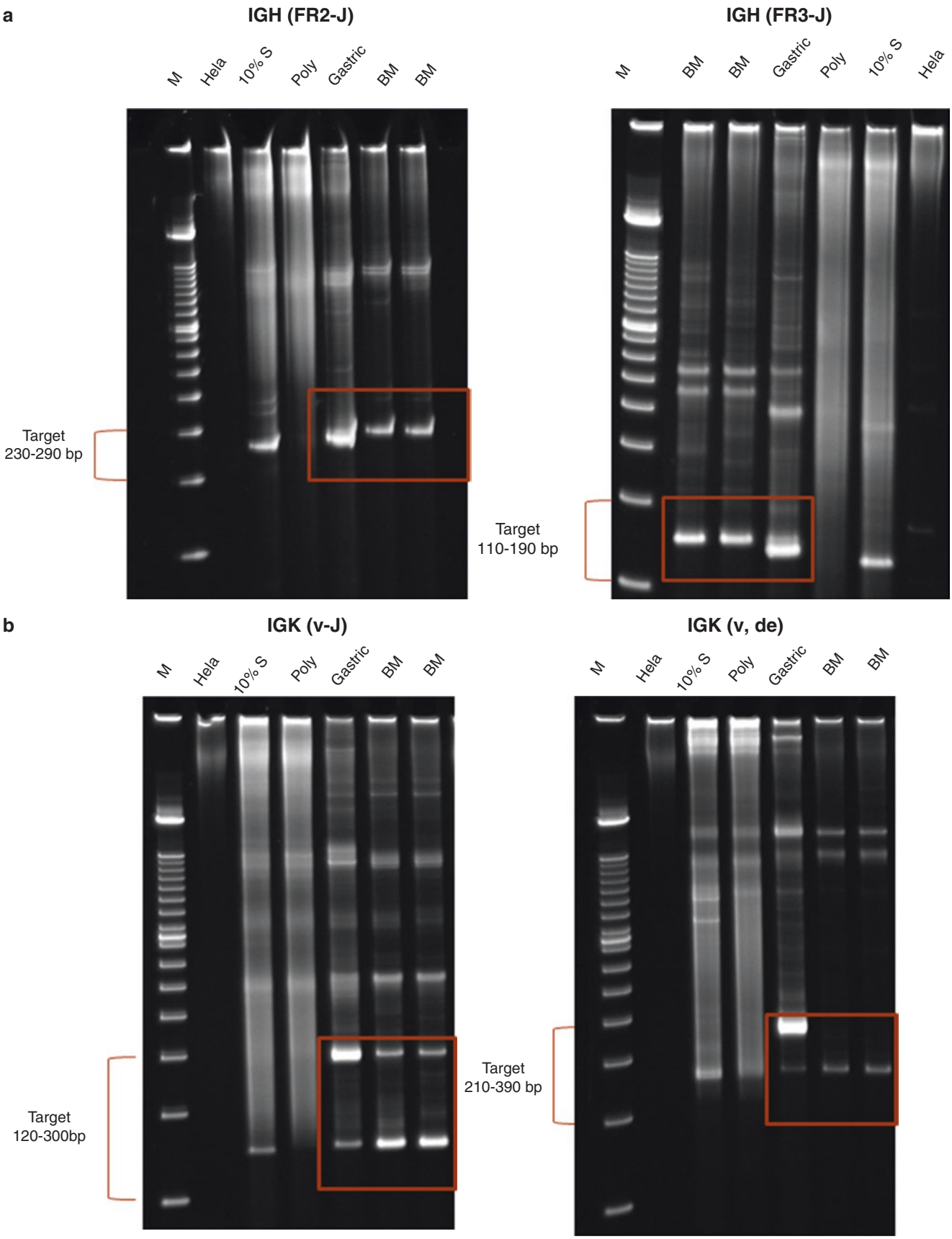
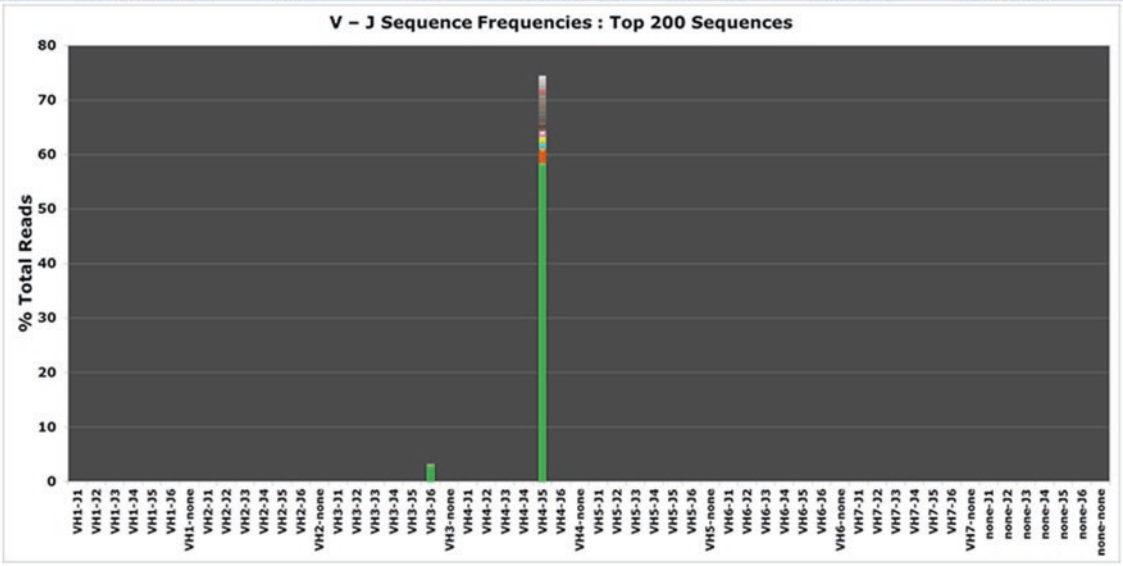


Fig. 16.4 PCR-based clonality assay with gel detection. (**a**) *IGH* gene rearrangement, FR2 primers, and FR3 primers. (**b**) *IGK* gene rearrangement, two sets of primers. (M, DNA size marker; BM, bone marrow aspirate; Gastric, gastric biopsy; Poly, polyclonal control; 10% S, 10% sensitivity and positive control; Hela, negative control)

a

Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %	V-coverage
1	CGCTGTCTATGGT	303	1161654	IGHV4-34_02	IGHJ5_02	58.3908871	58.3908871	97.3700000
2	GCCTCTGGATTCA	302	61085	IGHV3-21_02	IGHJ6_03	3.0704559	61.4613430	98.6800000
3	CGCTGTCTATGGT	303	52868	IGHV4-34_02	IGHJ5_02	2.6574259	64.1187689	97.3700000
4	CGCTGTCTATGGT	303	22331	IGHV4-34_02	IGHJ5_02	1.1224744	65.2412433	97.3700000
5	CGCTGTCTATGGT	303	20798	IGHV4-34_02	IGHJ5_02	1.0454177	66.2866610	97.3700000
6	CGCTGTCTATGGT	303	20636	IGHV4-34_02	IGHJ5_02	1.0372747	67.3239357	97.3700000
7	CGCTGTCTATGGT	303	8019	IGHV4-34_02	IGHJ5_02	0.4030774	67.7270132	97.3700000
8	CGCTGTCTATGGT	303	7441	IGHV4-34_02	IGHJ5_02	0.3740241	68.1010373	97.3700000
9	CGCTGTCTATGGT	303	7345	IGHV4-34_02	IGHJ5_02	0.3691986	68.4702359	97.3700000
10	CGCTGTCTATGGT	303	6598	IGHV4-34_02	IGHJ5_02	0.3316505	68.8018864	97.3700000



b

Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %	V-coverage
1	GCCTCTGGATTCA	302	981752	IGHV3-21_02	IGHJ6_03	51.6529706	51.6529706	98.6800000
2	GCCTCTGGATTCA	302	25127	IGHV3-21_02	IGHJ6_03	1.3220082	52.9749788	98.6800000
3	GCCTCTGGATTCA	302	16418	IGHV3-21_02	IGHJ6_03	0.8638011	53.8387799	98.6800000
4	GCCTCTGGATTCA	302	13290	IGHV3-21_02	IGHJ6_03	0.6992275	54.5380074	98.6800000
5	GCCTCTGGATTCA	269	9991	IGHV3-7_01	IGHJ4_02	0.5256570	55.0636644	99.5600000
6	GCCTCTGGATTCA	256	9137	IGHV3-20_01	IGHJ4_02	0.4807255	55.5443899	95.5900000
7	GCCTCTGGATTCA	299	8735	IGHV3-21_02	IGHJ6_03	0.4595750	56.0039649	97.3600000
8	GCCTCTGGATTCA	281	8407	IGHV3-74_02	IGHJ4_02	0.4423179	56.4462829	99.5600000
9	GCCTCTGGATTCA	302	8028	IGHV3-21_02	IGHJ6_03	0.4223776	56.8686605	98.6800000
10	GCCTCTGGATTCA	269	7351	IGHV3-74_02	IGHJ4_02	0.3867586	57.2554190	99.5600000

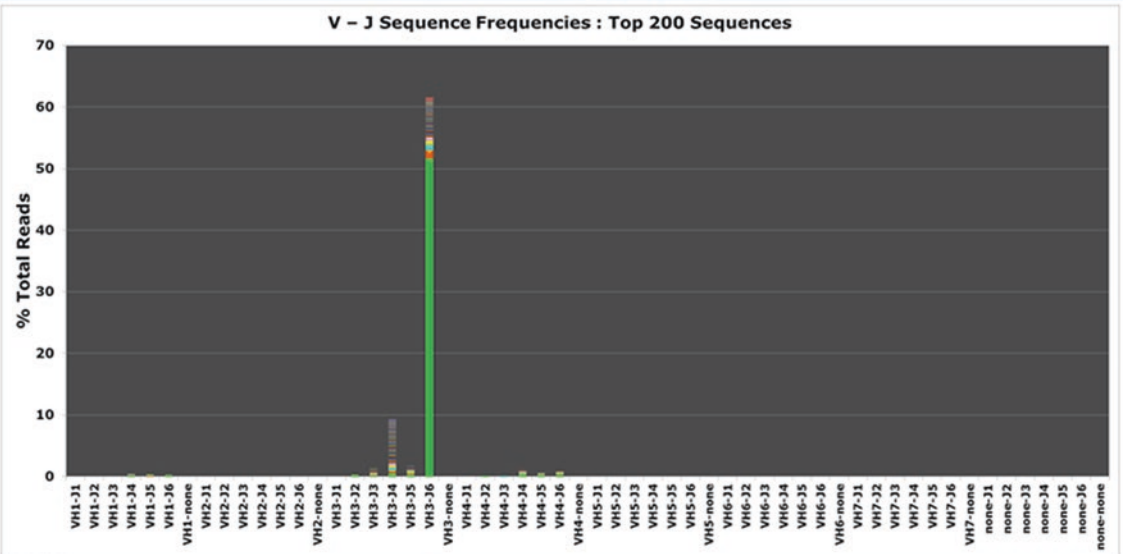


Fig. 16.5 NGS-based clonality assay. (**a**) Gastric MALT lymphoma. (**b**) Bone marrow. (**c**) 100% identical sequence match between rank 2 clone from gastric lymphoma to that of rank 1 clone from bone marrow

c

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gastric          1 GCCTCTGGATTCACCTTCAGTAGTTATAACATGAACTGGGTCCGTCAGGC     50
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM               1 GCCTCTGGATTCACCTTCAGTAGTTATAACATGAACTGGGTCCGTCAGGC     50

gastric         51 TCCAGGGAAGGGGCTGGAGTGGGTCTCATCCATTAGTAGTAGTAGTACTT    100
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM              51 TCCAGGGAAGGGGCTGGAGTGGGTCTCATCCATTAGTAGTAGTAGTACTT    100

gastric        101 ACATATACTACGCAGACTCACTGAAGGGCCGATTCACCATCTCCAGAGAC    150
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM             101 ACATATACTACGCAGACTCACTGAAGGGCCGATTCACCATCTCCAGAGAC    150

gastric        151 AACGCCAACAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGA    200
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM             151 AACGCCAACAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGA    200

gastric        201 CACGGCTGTGTATTACTGTGCGAGGTCCCCGTCTGACTATTTTTGGACTG    250
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM             201 CACGGCTGTGTATTACTGTGCGAGGTCCCCGTCTGACTATTTTTGGACTG    250

gastric        251 GTTATTCTTCGACTTACTATTACTACATGGACGTCTGGGGCAAAGGGACC    300
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
BM             251 GTTATTCTTCGACTTACTATTACTACATGGACGTCTGGGGCAAAGGGACC    300

gastric        301 AC    302
                   ||
BM             301 AC    302
```

Fig. 16.5 (continued)

Histologic Findings

Biopsy sections showed sheets of large atypical cells, with abundant pale cytoplasm and relative round or ovoid nuclei. The mass had a vaguely nodular growth pattern with coarse fibrotic band (Fig. 16.6a-b). The neoplastic cells were positive for CD45, CD20, and CD79a while negative for pancytokeratin, CD3, CD30, and TdT (Fig. 16.6c–d). The Ki67 proliferation index was approximately 60% (Fig. 16.6e).

Question 1: After reviewing this histology and its immunophenotype, what is the diagnosis?

A diagnosis of primary mediastinal (thymic) large B-cell lymphoma was rendered. The patient received six cycles of R-CHOP treatment and achieved complete remission. Five years after the completion of her treatment, routine surveillance imaging revealed a new development of anterior mediastinal soft tissue adjacent to the left brachiocephalic vein, suspicious for a recurrence of lymphoma. PET scan showed increased uptake in the mediastinum and multiple lymph nodes. Excisional biopsy of a supraclavicular lymph node was performed. Biopsy sections consisted of enlarged lymph node with effaced architecture. Numerous large cells with amphophilic cytoplasm, two to multiple nuclei or one large lobated nucleus with clear karyoplasm, and huge viral inclusion-like nucleoli were present in a background of mixed inflammatory cells which consist mainly of small lymphocytes, histiocytes, eosinophils, and plasma cells (Fig. 16.7a). These large neoplastic cells were positive for CD30, CD15, MUM-1, and weakly positive for PAX5 while negative for CD20 and CD3 (Fig. 16.7b–f).

Question 2: In a patient with a known history of primary mediastinal large B-cell lymphoma, chemotherapy, and current findings in the enlarged lymph node, which hematologic diseases are in the differential diagnosis?

Although the relapse of a patient's previously diagnosed primary mediastinal large B-cell lymphoma was suspected at

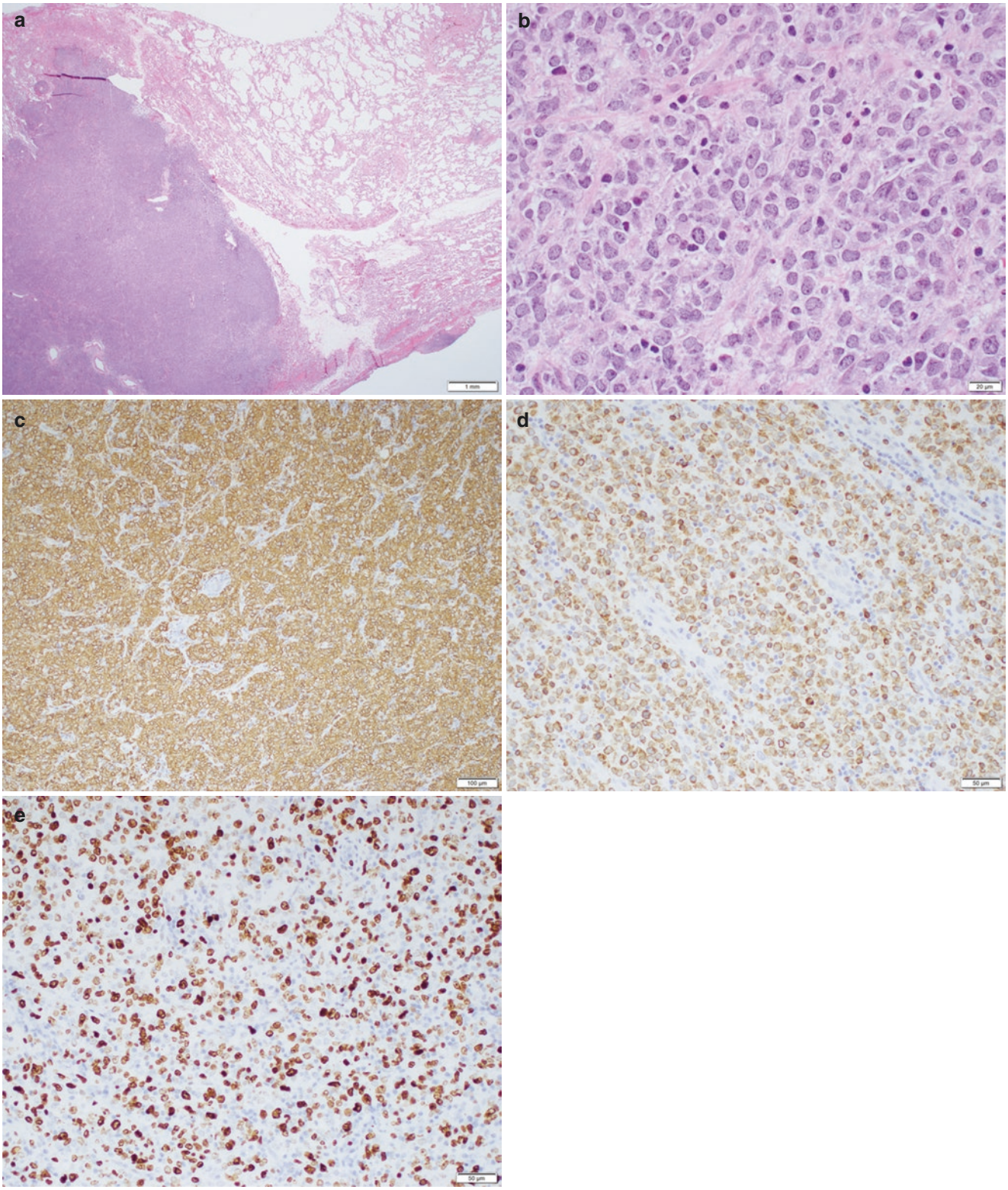

Fig. 16.6 Biopsy of mediastinal mass showed sheets of large atypical cells, (**a**) Hematoxylin and eosin, 20×; (**b**) hematoxylin and eosin, 400×; (**c**) CD20, strongly positive, 100×; (**d**) CD79a, positive, 200×; (**e**) proliferation index Ki67, approximately 60%, 200×

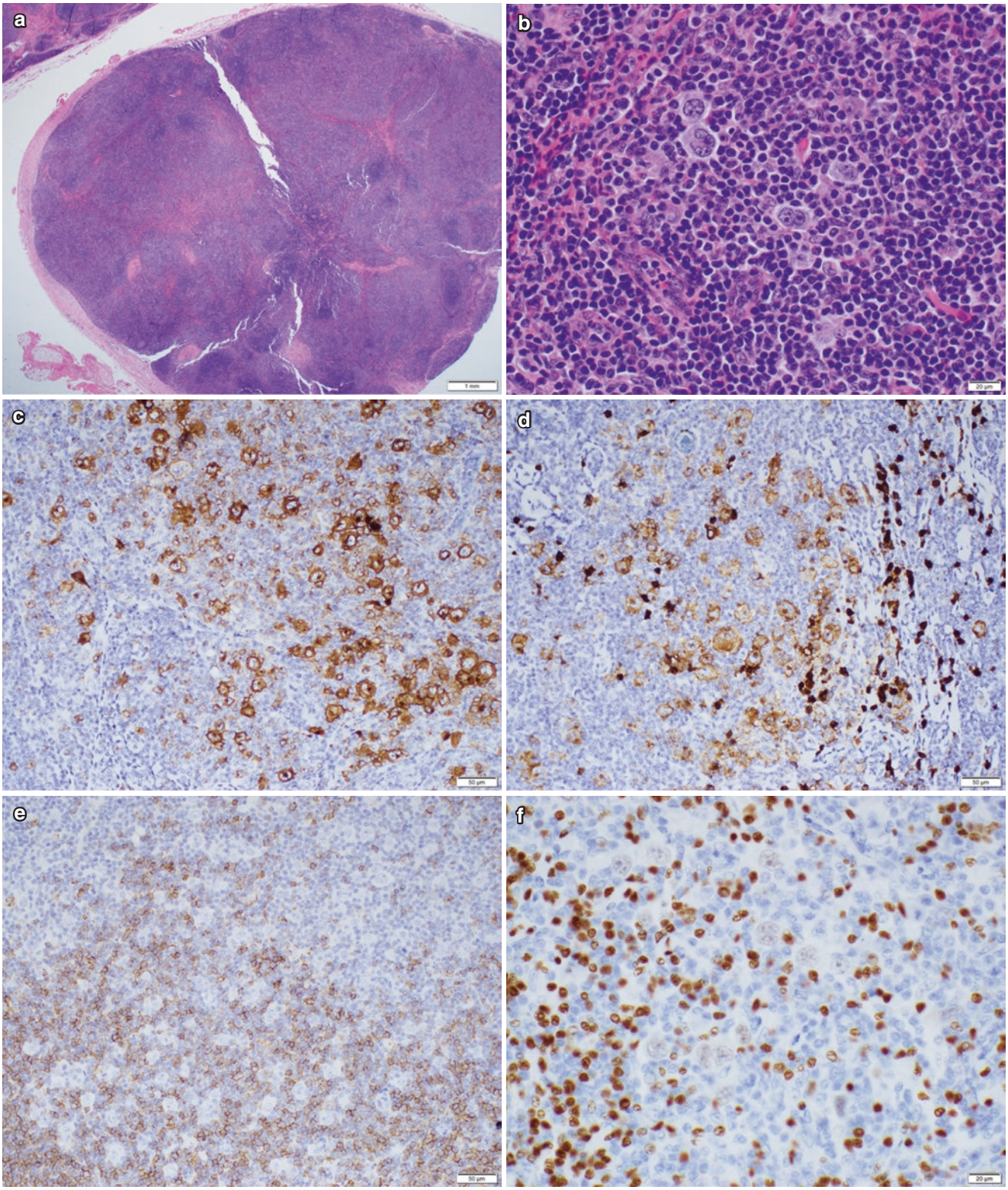

Fig. 16.7 Biopsy of supraclavicular lymph node with Reed-Sternberg cells in a background of mixed inflammation, (**a**) enlarged lymph node with effaced architecture, hematoxylin and eosin, 20×; (**b**) Reed-Sternberg cells, hematoxylin and eosin, 400×; (**c**) CD30 positive in neoplastic cells, 200×; (**d**) CD15, positive in neoplastic cells, 200×; (**e**) CD20 negative in neoplastic cells, 200×; (**f**) PAX5, weakly positive in neoplastic cells, 200×

first, the histologic finding and immunophenotype of this subsequent lesion did not support that diagnosis. Instead, a diagnosis of nodular sclerosis classical Hodgkin lymphoma (cHL) was rendered for this subsequent mass after careful review of the previous lymphoma and comparison of the overall presentation of two tumors.

Question 3: For treatment purposes, the patient's oncologist would like to know if these two lymphomata were clonally related. Which molecular studies could be ordered to help answer this question?

Molecular Genetic Study

NGS-based clonality assay was used to determine the clonal relationship of these two tumors in this patient. A clone was identified in each sample; however, the clones were clearly distinct (Fig. 16.8a–b) with 84.2% of nucleotide difference. Thus, the molecular study supported the presence of two separate metachronous lymphomas in this patient.

Final Diagnosis

1. Primary mediastinal large B- cell lymphoma and several years later
2. Classical Hodgkin Lymphoma, Nodular Sclerosis Type

Follow-Up

The patient completed ABVD chemotherapy and radiation therapy for Hodgkin lymphoma and is currently in complete remission for both lymphomas.

Discussion

The patient was diagnosed with primary mediastinal large B-cell lymphoma first, received chemotherapy, and was in remission for several years before another new mass, classical Hodgkin lymphoma, was diagnosed. Although histologically these two lymphomata could share some overlapping features, their immunophenotypes are distinctly different. Because of the young age of the patient, the oncologist was considering using more aggressive treatment if it is proved that these two tumors were clonally related.

Due to the scarcity of tumor cells (Reed-Sternberg (RS) cells and Hodgkin cells) in cHL and lack of immunoglobulin expression [62], although being classified as a B-cell lymphoma, it has been challenging to detect the clonal rearrangement in cHL by standard CISH, flow cytometry, or molecular techniques. Therefore, clonality assay has generally been considered as a supportive ancillary test for only B-cell non-Hodgkin lymphomas (NHL). With the advancement of research in defining the immunophenotype of the HRS cells as well as the background cells found in Hodgkin lymphoma (HL), Fromm et al. have demonstrated a six-color flow panel to diagnose HL in clinical practice [63, 64]. However, standard PCR-based clonality assay has not been useful in the past in HL diagnosis. The NGS-based clonality assay successfully detected clonal proliferation in this case could be resulted from its relatively high tumor cell load. Based on the completely different clonal origins, standard chemotherapy for Hodgkin lymphoma was selected for treatment.

Case 3

Learning Objectives

1. Established prognostic biomarkers in chronic lymphocytic leukemia (CLL)
2. Role of genetics and risk-stratified approach in CLL treatment

Case History

A 75-year-old female presented to her primary care physician for routine annual checkup. Her past medical history included hypertension, hypothyroidism, remote history of colon cancer, and uterine cancer treated with surgery and chemotherapy. Patient had no current complaint. No swollen lymph nodes or spleen was detected during physical examination.

Initial Workup

Her CBC was significant for mild anemia with a hemoglobin of 8.9 g/dL (reference 13.7–17.5), hematocrit of 29% (reference 40–51%), and leukocytosis of 118.6K/μL (reference 4.2–9.1) with lymphocytosis of 100.81K/μL (reference 1.0–4.8). Peripheral blood smear review is referred. Her serum β2 microglobulin was 9.3 mg/L (reference ≤2.51) and elevated.

Histologic Findings

Peripheral blood smear slides showed significant increase in small lymphocytes with round nuclei, scant cytoplasm, and clumped chromatin. Also noted were numerous "smudge" or "basket" cells where the cellular remnants form a lattice-like pattern. An albumin preparation slide removed these smudge cells which showed the majority of WBCs were small lymphocytes mixed with a few unremarkable neutrophils and monocytes (Fig. 16.9). Multicolor flow cytometry study was performed and on CD45 versus dot plot histogram, the lymphoid population comprises approximately 87% of the total events, which contained a predominant population of monoclonal B-lymphoid cells of small to medium cells. The clonal B-lymphoid population was positive for CD45, CD19, CD20 (low), CD5, CD23, CD38, and CD43, and reveals surface kappa immunoglobulin light chain restriction, and the population of B cells is negative for FMC7, CD10, Zap70, and

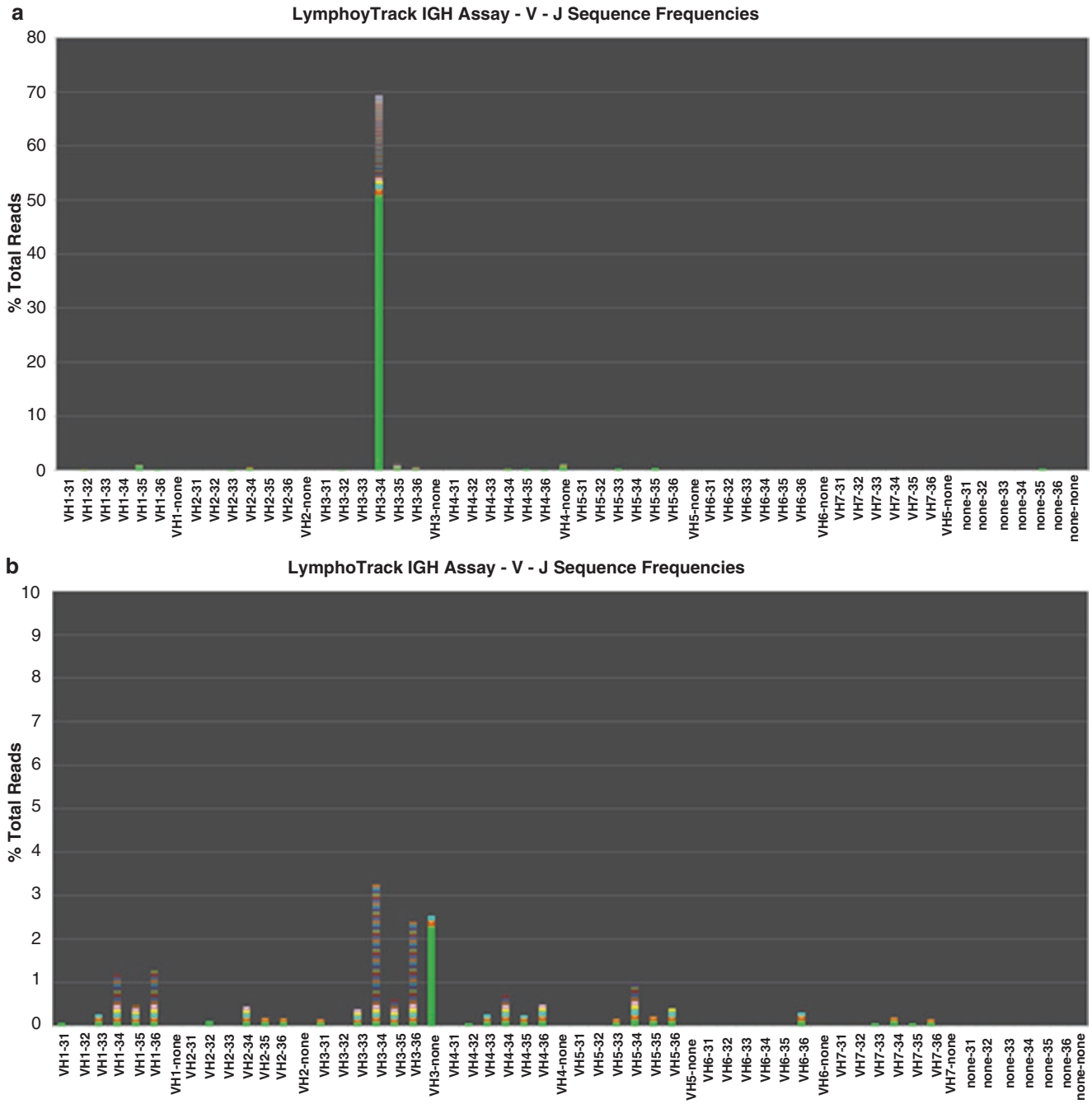

Fig. 16.8 NGS-based clonality assay showing two distinct clonal populations in the (**a**) mediastinal mass and (**b**) supraclavicular lymph node

other T-cell markers tested from this study. There was no increase in the blast population.

Question 1: After reviewing this preliminary information, what neoplastic hematologic disease is most likely?

Question 2: What additional laboratory studies might be helpful?

Data from CBC, peripheral blood smear, and flow cytometry demonstrated a phenotypically distinct population of chronic lymphocytic leukemia (CLL). A typical workup for a patient with CLL includes cytogenetic analysis on the peripheral blood and molecular testing.

Molecular Genetic Study

Cytogenetic analysis on the patient's blood showed a complex karyotype, and FISH studies, performed using a panel of DNA probes for chromosomes 6q, 11,12,13, and 17 to detect abnormalities frequently involved in CLL, were positive for deletion of chromosome 13q (Fig. 16.10). Next-generation

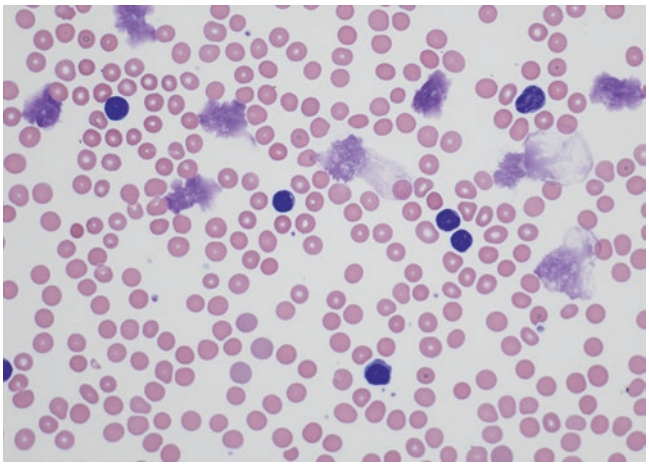

Fig. 16.9 Peripheral blood showed predominant small lymphocytes with round nuclei and clumped chromatin. Numerous "smudge" cells are present. Wright-Giemsa stain, 400×

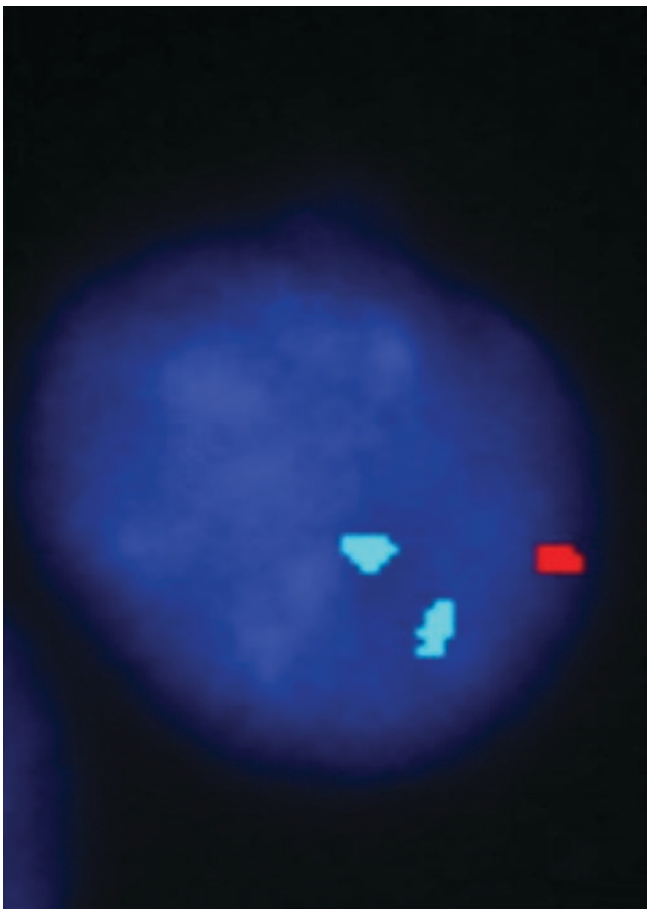

Fig. 16.10 FISH for CLL prognostic panel detected deletion of chromosome 13q with signal pattern of 1 red and 2 aqua. Probe color red for *DLEU1&2* gene at 13q14.3 and probe color aqua for *LAMP1* gene at 13q34

sequencing for *TP53* was positive for p.R342* (c.1024C > T) mutation with allele frequency of 81.2% and unmutated IgVH with expressed VH5–10 family (Fig. 16.11).

Final Diagnosis

Chronic lymphocytic leukemia with complex karyotype, *TP53* mutation, and unmutated IgVH

Follow-Up

According to CLL-IPI (Table 16.7), this patient got a total score of 9, which was in the very-high-risk group. The current treatment recommendation for the very-high-risk group is to treat on clinical trial or novel targeted inhibitor instead of regular chemotherapy. Patient was enrolled in the clinical trial A041702 (a randomized phase III study of ibrutinib plus obinutuzumab versus ibrutinib plus venetoclax and obinutuzumab in untreated older patients (>70 years of age) with CLL).

Discussion

As one of the most common leukemias in adults, chronic lymphocytic leukemia is incurable in most cases, and the standard approach used to be a "watch and wait" strategy based on the majority of cases being diagnosed in early stage and irrespective of risk factors [65]. Although the pathogenesis of CLL is not fully understood, it is known associated with constitutive activation of the B-cell receptor (BCR) signaling pathway but also with substantial heterogeneity in the disease course. For active disease, combined chemoimmunotherapy of CD20 antibody (rituximab, ofatumumab or obinutuzumab) and chemotherapy (fludarabine, cyclophosphamide and rituximab (FCR)) have been the main choice and widely used [66]. However, this approach does not have satisfactory efficacy in CLL patients with older age, comorbidity, or del [17] or *TP53* mutations [67]. The CLL-IPI was developed using patient data from before and categorizes CLL patients into different risk groups. This scoring system has not been incorporated into CLL management guidelines and should be used with caution to provide prognosis information and at least may warrant closer initial monitoring for patients with higher risk.

Bruton's tyrosine kinase (BTK), a kinase in the downstream of BCR signaling, plays a crucial role in the survival of neoplastic cells, and more recently, small-molecular inhibitors of BTK, such as ibrutinib, have shown excellent antitumor activity and induce prolongation of progression free with CLL and MCL, including patients with high-risk genetic changes [68, 69].

In summary, a standard CLL workup is strongly recommended for every CLL patient at the time of diagnosis to facilitate risk evaluation and treatment stratification [70].

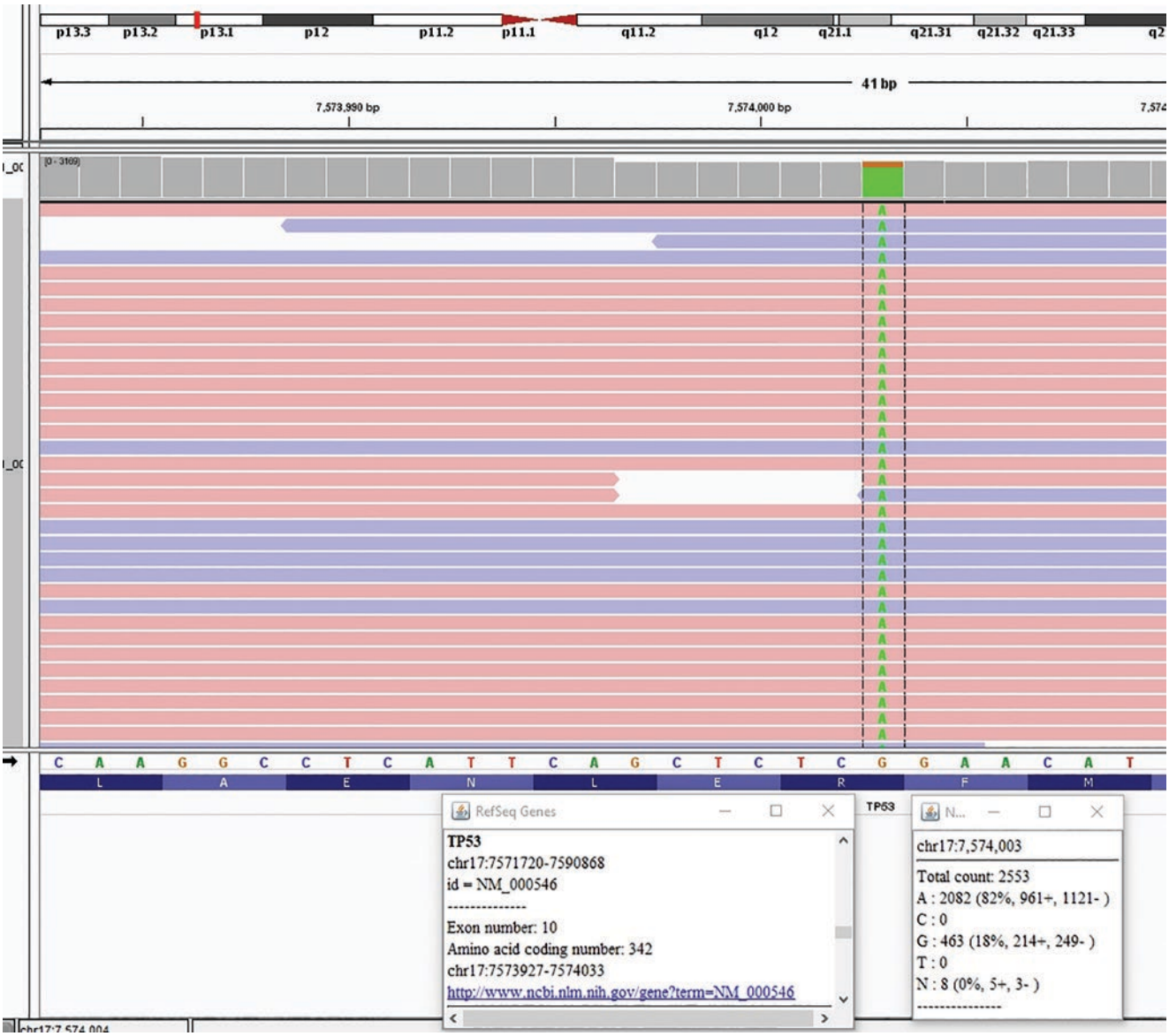

Fig. 16.11 Aligned next-generation sequencing data revealed *TP53* p.R342* mutation with allele frequency of 81.2%

Table 16.7 CLL-IPI risk score calculation for Case 3

Variable	Description	Patient	Score
Age	≤65 years	75 years	+1
	>65 years		
Clinical stage	Binet A or Rai 0	Rai 0	0
	Binet B-C or Rai I-IV		
Serum β2 microglobulin (mg/L)	≤3.5	9.3	+2
	>3.5		
IGHV mutational status	Mutated	Unmutated	+2
	Unmutated		
TP53 mutational status	Wild type	*TP53* R342* detected	+4
	Del(17p) by FISH and/or *TP53* mutation by sequencing		
International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) Total score			9

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