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ORIGINAL ARTICLE

Targeted massively parallel sequencing in the management of evaluated as cytogenetically normal lymphoid malignancies

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Summary

The variations in clinical and biological background of lymphoid malignancies trigger researchers to try to find out novel therapeutic targets. A typical treatment includes multiagent chemotherapy and/or targeted therapy in the light of driver mutations. Next generation sequencing (NGS) plays a pivotal role during the identification of genetic alterations in lymphoid malignancies. A total of 52 patients [30 men (58%) and 22 women (42%)] having normal cytogenetic and FISH results were enrolled in this study. Usage of NGS based targeted sequencing can confirm or support a particularly preferred diagnosis (41/52, 78%) or make a differential diagnosis in cases of interference. Notably, in 11 out of these 52 cases (21%), the initial suspect diagnosis was not supported by the NGS result and thereby had to be reconsidered. In this study, we highlight the importance of targeted NGS panel testing for diagnosis, prognosis and treatment decision in highly selected instances of lymphoid malignancies and lymphoproliferative disorders in which histopathology and more conventional molecular analyses remain inconclusive.

Key words: *NGS, lymphoid malignancies, targeted therapy*

Introduction

Advancement of molecular techniques can help the classification of malignancies and also have been rapidly incorporated into daily diagnostic practice of lymphoid malignancies. The prognostic and diagnostic value of mutational analysis play a critical role in targeted therapy.

Lymphoid malignancies show varied biological and clinical behavior and typically based on these characteristics chemotherapy is applied [1]. The treatment strategies of metastatic solid tumors have evolved from chemotherapy toward matching oncogenic driver mutations with targeted therapy [2,4]. The discovery and development of next generation sequencing (NGS) give chance to analyze multiple cancer-related molecular alterations. NGS provides detailed information about risk stratification and finding of potential target gene alterations. Therefore, it plays a key role during the treatment designing and diagnosis of solid cancers [5-8]. This technology has changed the diagnostic significance of pathology and clinical practice. Until now, different commercially available gene panels have been available for advanced solid tumors, but a limited number of gene panels has been described in lymphomas [9-12]. NGS helps identify different genetic alterations in hematologic malignancies, including substitutions, indels, copy number alterations, and gene fusions [13].

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Herein, we summarize our routine practice in 52 consecutive clinical cases of lymphoid proliferations by using NGS. In this report, we identified the frequency of theoretically actionable alterations in lymphoid malignancies. The present study may help shed light to the development of clinical trials in this field.

Methods

Patients

We retrospectively reviewed 52 patients with lymphoid malignancies who had undergone NGS (Table 1) and patients who had normal karyotype results were enrolled.

Only patients with normal karyotype report were selected for further analysis. Patients who were seen at the Trakya University Faculty of Medicine, Department of Medical Genetics from October 2017 until September 2020 were enrolled in this study. This study was performed and consents were obtained in accordance with the guidelines of Trakya University Faculty of Medicine (TUMF) Scientific Research Ethics Committee review board. The study was approved by the Ethics Committee of Trakya University and conducted in accordance with the ethical principles established in the *Declaration of Helsinki.*

NGS

QIAseq Targeted DNA Panel (QIAseq Targeted DNA Panel/Qiagen, Germany) was used for evaluation of molecular alterations in lymphoid malignancies.QIAseq Targeted DNA Panel covers exon/intron boundaries and covered genes has been listed in Table 1.

Amplicons were noted as a dropout and excluded from analysis.If the amplicon coverage (minimum coverage) at any position analyzed in any of the two double-ended sequences was 100x and the allele frequency was> 5%, they were included in the study.

Libraries were prepared according to the QIAseq Targeted DNA Panel protocol (Qiagen, Hilden, Germany) and after target enrichment process prepared libraries were sequenced on the MiSeq System and NextSeq 550 System (Illumina, San Diego, CA, USA). Quality control and Variant Call Format file generation were completed by using OCI analysis (Qiagen, Hilden, Germany). Ingenuity software (Qiagen, Hilden, Germany) has been used for variant analysis. Identified variants have been interpreted according to the American College of Medical Genetics and Genomic 2015 (ACMG-2015) recommended standards. Then, candidate variants were annotated by ANNOVAR with SIFT, PolyPhen-2, MutationTaster, and the Exome Aggregation Consortium (ExAC) and other databases. Known hotspot or clinically important variants detected below these thresholds were verified by using Sanger sequencing.

Data and statistical analyses

Patient characteristics were obtained through electronic medical record review. Descriptive statistics were used, including medians, ranges and frequencies.

Results

Patient characteristics

Fifty-two patients [30 men (58%) and 22 women (42%)] were enrolled in this study who suffered of different lymphoid malignancies. The median age was ~53 years, ranging from 1 to 79 years and there were 49 adults and 3 children (<18 years) (Table 1).The distribution of malignancies in the cohort were chronic lymphocytic leukemia (CLL; 32%), acute lymphoblastic leukemia (ALL; 21%), non-Hodgkin lymphoma (13%), diffuse large B-cell lymphoma (DLBCL; 4%), Hodgkin lymphoma (HL; 4%), marginal zone lymphoma (MZL; 4%), and atypical lymphoproliferative disease (21%) (Table 2).

The number of patients with at least one or at most 4 variations was determined as 35/52(67%). No variation was detected in 17 out of 52 patients. Using NGS based targeted sequencing could confirm or support a particularly preferred diagnosis (41/52, 78%) or make a differential diagnosis in cases of interference. Notably, in 11 out of these 52 cases (21%), the initial suspect diagnosis was not supported by the NGS result and thereby had to be

Table 1. Patient characteristics

Characteristics	*%*
No. of patients	52
Sex	
Male	30
Female	22
Median age at diagnosis, years (range)	~53 (1-79)
Race	
White	52
Malignancy	
CLL	17
ALL	11
NHL	7
DLBCL	2
HL	2
MZL	2
Atypical lymphoproliferative disease	11
Timing of NGS	
At diagnosis	50
At relapse	2
Median alterations per patient (range)	1.5 (1-4)
Median potentially FDA-actionable alterations per patient (range)	1.9 (0–4)

CLL: chronic lymphocytic leukemia; ALL: acute lymphoblastic leukemia; FDA: US Food and Drug Administration; NGS: next-generation sequencing; NHL: non-Hodgkin lymphoma; DLBCL: diffuse large B-cell lymphoma; MZL: Marginal zone lymphoma; HL: Hodgkin lymphoma.

reconsidered (Figure 1). Therefore, NGS based molecular characterization was useful in patients who had not specific histopathological and molecular (ie, clonality test and / or FISH-related) diagnosis (Table 2).

Sixty-six alterations [39 pathogenic or likely pathogenic/27 variant of uncertain (or unknown) significance (VUS)] were identified by using NGS in the entire cohort of 52 patients. Genetic alterations were distributed on a total of 39 genes. At least 1 genetic aberration was detected in 35 out of the 52 cases (67%) (Table 3).

The mean variant allele frequencies of detected mutations were 37%. The genetic variants included missense mutations (51/52), frame shift insertion or deletion mutations (indels) (13/52), stop gain (3/52), and splice site mutations (1/52) (Table 3).

The most frequently altered genes in the entire cohort were TP53 (11, 53%, and 6/52), NOTCH1 (9, 61%, and 5/52), and ATM (7, 69%, and 4/52). As demonstrated in Table 3, 18 patients (35%) had no reportable alterations, 13 (25%) had one alteration, and 19 (37%) had two or more alterations. The maximum number of alterations identified was 5, which

Table 2. The distribution of potentially actionable alterations in 52 patients with various lymphoid malignancies

Diagnosis	*No reportable alterations*	*Patients with alterations but none potentially actionable by FDA-Approved Drug and/or Clinical Trial.*	*Patients with one or more alterations potentially actionable by approved and/or experimental drug*
	n	*n*	*n*
CLL (17)	2	2	13
ALL(11)	2	2	7
NHL(7)	4	1	3
DLBCL(2)	1	-	1
HL(2)	-	-	2
MZL(2)	1	-	1
Atypical lymphoproliferative disease (11)	8	3	-

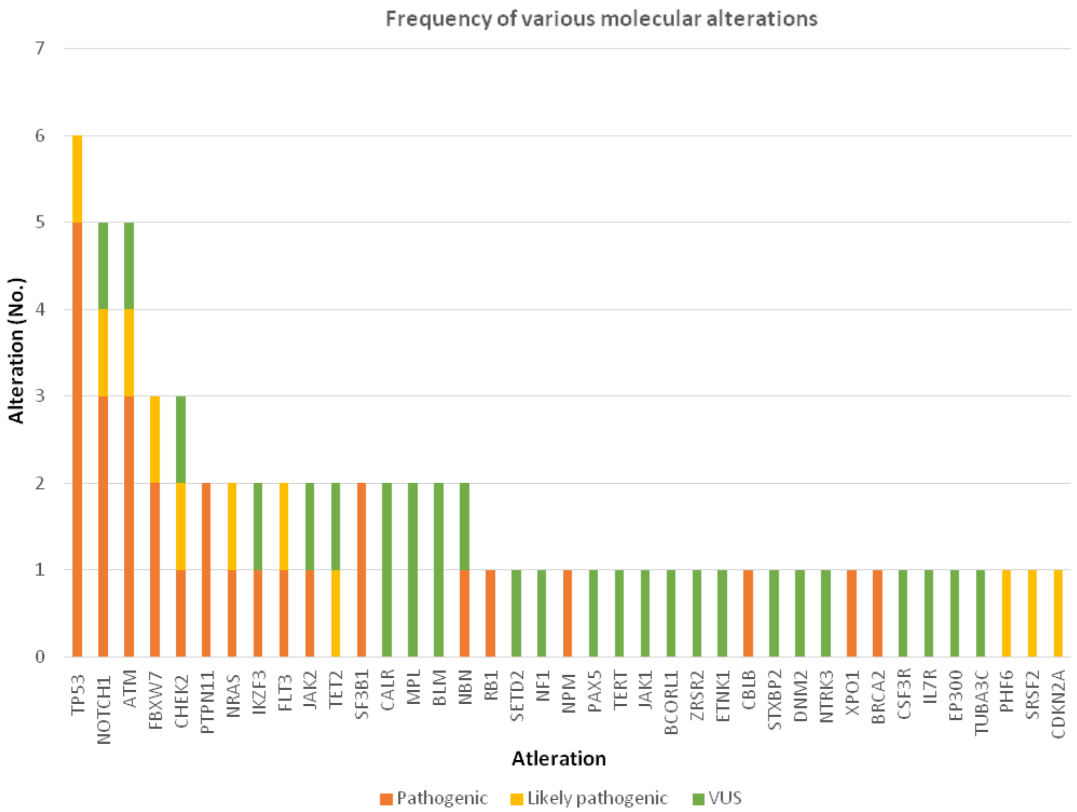

Figure 1. Frequency of molecular alterations which was found among 52 individuals with lymphoid malignancies.

Table 3. The distribution of detected variants in lymphomas

P. N	*Gene*	*WHO class.*	*After NGS*	*Variant (VAF)*	*Alterations type*	*Pathogenicity*	*Patient age*
P2	SETD2	ALL	Resistance to chemotherapy	p.Val2229Met (53%)	Missense	VUS	37
P3	NF1	Lyphoma	JMML	p.Ser641Gly (41%)	Missense	VUS	58
P5	FBXW7NOTCH1	ALL	T-ALL	p.Arg465Cys (65%)	Missense	L.Pathogenic	29
				p.Leu1574Pro (54%)	Missense	Pathogenic	
P7	PTPN11	ALL	B- ALL	p.Tyr62Asp (47%)	Missense	Pathogenic	42
	NPM			p.Leu366Phe (63%)	Frameshift	Pathogenic	
	PAX5			p.Trp288Cysf (75%)	Missense	VUS	
P8	CHEK2	HL	HL	p.Ser356Leu (56%)	Missense	VUS	20
	MPL			p.Lys355Glu (34%)	Missense	VUS	
P9	TP53	DLBCL	Precursor B-ALL	p.Pro190Thr (67%)	Missense	Pathogenic	48
	NRAS			p.Gly12Asp (38%)	Missense	Pathogenic	
	TERT			p.Thr644Met (45%)	Missense	VUS	
P10	PTPN11NOTCH1	CLL	SLL	p.Glu69Lys (76%)	Missense	Pathogenic	63
				p.Pro2514Argfs (38%)	Frameshift	Pathogenic	
P11	NBN	CLL	CLL	p.Gln291Ter (23%)	Stop gain	Pathogenic	53
	JAK1			p.Gly741Ser (59%)	Missense	VUS	
P12	BCORL1	CLL	CMML	p.R816W (20%)	Missense	VUS	79
	ZRSR2			p.R366fs*18 (23%)	Frameshift	VUS	
	BLM			p.D565N (47%)	Missense	VUS	
	ETNK1			p.S250F (65%)	Missense	VUS	
P13	IKZF3	CLL	CLL	p.Leu162Arg (21%)	Missense	Pathogenic	56
P16	FLT3	CLL	CLL	p.Gly583Asp (35%)	Missense	Pathogenic	76
	CBLB			p.Arg670Trp (39%)	Missense	Pathogenic	
	NOTCH1			p.Gln2444Ter (48%)	Missense	Pathogenic	
P17	JAK2	NHL	Subacute Lymphoid Leukemia	p.Val617Phe (50%)	Missense	Pathogenic	70
	TET2			p.Glu796Gln (34%)	Missense	VUS	
	STXBP2			p.Arg529Pro (23%)	Missense	VUS	
P18	DNM2	Lyphoma	ALL	p.Arg271Cys (78%)	Missense	VUS	79
P19	ATM	CLL	CLL	p.Ser2489Phe (53%)	Missense	Pathogenic	37
	SF3B1			p.Glu622Asp (25%)	Missense	Pathogenic	
	MPL			pPhe41Leu (13%)	Missense	VUS	
	CALR			p.Pro228Ser (23%)	Missense	VUS	

Continued on the next page

P. N	*Gene*	*WHO class.*	*After NGS*	*Variant (VAF)*	*Alterations type*	*Pathogenicity*	*Patient age*
P20	ATM	CLL	CLL	p.Ser2283LeufsTer5 (56%)	Frameshift	Pathogenic	65
	RB1			p.Ser811ValfsTer15 (43%)	Frameshift	Pathogenic	
	NTRK3			NM_002530.3(NTRK3):c.2293-3C>T (18%)	Missense	VUS	
	BIRC3			p.Phe602Leu (47%)	Missense	VUS	
P21	XPO1	CLL	CLL	p.Glu571Gln (48%)	Missense	Pathogenic	68
	SF3B1			p.Gly742Asp (76%)	Missense	Pathogenic	
	TP53			p.Lys132Arg (65%)	Missense	Pathogenic	
P22	BRCA2	CLL	CLL	p.Thr3033Asnfs (34%)	Frameshift	Pathogenic	57
P23	CHEK2	ALL	ALL	p.Asp438Tyr (76%)	Frameshift	Pathogenic	21
	ATM			p.Thr1011LysfsTer11 (33%)	Missense	L.pathogenic	
	CSF3R			p.Pro767Ala (55%)	Missense	VUS	
P24	BLM	R. ALL	R. ALL	p.Asn92Asp (86%)	Missense	VUS	40
P25	NBN	CLL	CLL	p.Thr55_Leu57 (45%)	Frameshift	VUS	61
	IL7R			p.Tyr201Cys (67%)	Missense	VUS	
P26	JAK2	ACLD	LALL	p.Met1Ile (55%)	Missense	VUS	37
P27	TP53	CLL	CLL	p.Arg213Leu (85%)	Missense	Pathogenic	72
	IKZF3			p.Glu333Gly (54%)	Missense	VUS	
P29	CALR	CLL	CLL	- (56%)	Missense	VUS	63
	EP300			p.Pro2323Ser (55%)	Missense	VUS	
	TUBA3C			p.Val182Met (56%)	Missense	VUS	
	NOTCH1			p.V2453fs*24 (46%)	Missense	VUS	
P32	PHF6	ALL	T-ALL	p.Arg342Ter (43%)	Stop gain	L.pathogenic	68
	TET2			p.Asn275Ilefs (65%)	Frameshift	L.pathogenic	
	SRSF2			p.Pro95Leu (45%)	Missense	L.pathogenic	
	FLT3			p.Trp603_Glu604insGlyGluTyrGluTyrAspLeuLysTrp (78%)	Frameshift	L.pathogenic	
	STAG2			p.Arg1082IlefsTer6 (56%)	Frameshift	L.pathogenic	
P34	NRAS	CLL	CLL	p.Gln61His (20%)	Missense	L.pathogenic	58
P35	TP53	CLL	CLL	p.S166L (23%)	Missense	L.pathogenic	42
	NOTCH1			p.Pro2514Argfs (56%)	Frameshift		
P37	CHEK2	HL	HL	NM_001349956.1:c.444+1G>A (67%)	Splicing	L.pathogenic	52
P39	TP53	CLL	CLL	p.Arg273Pro (15%)	Missense	Pathogenic	50
P42	CDKN2A	NHL	NHL	p.Lys85Argfs (34%)	Frameshift	L.pathogenic	57
P43	FBXW7	NHL	ALL	p.Arg465His (48%)	Missense	Pathogenic	37
	FBXW7			p.Arg465Cys (62%)	Missense	Pathogenic	
P47	ATM	ALL	ALL	p.Gln2108Ter (32%)	Stop gain	Pathogenic	63
P51	TP53	MZL	CLL	p.Arg282Gly (44%)	Missense	Pathogenic	77

was observed in one patient (1, 9%), with ALL (Table 3). Variations of 4 genes were detected in 4 patients (7, 69%) who were clinically diagnosed as CLL.

NGS was performed on clinical demand to assess potentially druggable alterations that might have therapeutic implication in 27 out of 52 (51%) cases. Harbored actionable or potentially actionable mutation were identified in 27 cases (51%) with an average 1,9 alterations/patients (range 0-4).

Mutation of *NOTCH1* is a potential targeted therapy with Gamma-secretase inhibitors and was detected in a significant body of cases in our cohort (n=5). The second predictive group included cases who had *ATM*/Mutations (n=4) with a certain sensitivity for Olaparib. NRAS mutations were identified in 2 cases which precision medicine might be available using NRAS-targeted therapy in affected cases. TET2 (n=2) mutations potentially targetable with agents against dysfunctional TET2 (azacytidine, decitabine) and JAK1, 2 (n=3) were determined in our cohort which JAK-inhibitors might have been useful based on mutational status (Table 4) [15-20].

In this study NGS testing was applied in cases whose clinical follow-up information was available because NGS panel testing plays a crucial role during the treatment decision and clinical management of cases. In our cohort, we also confirmed the importance of NGS testing during the patient management. Two patients with CLL and harboring a highly unfavorable SFB3B1 mutation were treated with a more intense cyclophosphamide + mitoxantrone (RFCM) chemotherapy rather than

Table 4. Potentially actionable targets and relevant FDA-approved drugs

Actionable Gene	*Examples of FDA Approved Drugs*	*Comment*
ATM	Olaparib	Olaparib is a PARP inhibitor.
TP53	Bevacizumab Pazopanib	Bevacizumab (anti-VEGF-A antibody) has been associated with longer median PFS in patients with tp53 than tp53 wild type (11 v 5 months; retrospective study), and TP53 mutation is associated with increased VEGF-A. In patients with sarcoma, pazopanib (a VEGFR inhibitor) response is associated with the presence of TP53 mutations.
NOTCH1	Gamma-secretase inhibitors (GSI)/ OMP-52M51	Targeting Notch signaling
CHEK2	neoadjuvant chemotherapy	Targeting DNA repair, cell cycle regulation and apoptosis
FLT3	Sorafenib, Sunitinib	Both sorafenib and sunitinib are multitargeted kinase inhibitors
JAK1	Tofacitinib	Tofacitinib is FDA approved for the treatment of rheumatoid arthritis
JAK2	Ruxolitinib, Tofacitinib	Ruxolitinib is approved for use in polycythemia vera and primary myelofibrosis.
NF1	Trametinib Temsirolimus Everolimus	Temsirolimus and everolimus are mTOR inhibitors.
NRAS	Trametinib	Trametinib is an MEK inhibitor.
TET2	Azacitidine Decitabine	Azacitidine and decitabine are hypomethylating agents.
CDKN2A/B	Palbociclib	Palbociclib is a CDK4 and CDK6 inhibitor.
RB1	Glucocorticoids	Hypophosphorylation
SF3B1	Spliceostatin A, Pladienolide, Meayamycin, Sudemycins	Spliceosome modulators
MPL	Ruxolitinib	JAK 1/2 inhibitor
PTPN11	Dasatinib	Multikinase inhibitor
IKZF3	Lenalidomide	IMiD
BCORL1	CHOP	TCR signaling
CALR	PD-L1, IFN-α	immunomodulatory treatments
XPO1	Selinexor (KPT-330)	Small-molecule inhibitors of XPO1-mediated nuclear protein export
BRCA2	Lynparza	PARP inhibitor
CSF3R	Dasatinib	Tyrosine kinase inhibitor
EP300	YF2	HDAC inhibitors
PHF6	Prednisolone	Glucocorticoid

less intense rituximab monotherapy. In a case of MZL, NGS helped resolve the clinical dilemma of whether to give R-CHOP or R-bendamustin. NGS testing helped identify TP53 mutated in 6 patient's clinical decision and treatment protocol. NGS panel analysis was clinically requested in 26 (50%) of 52 cases to clarify the prognosis. Remarkably, NGS rendered prognostic information in all these instances.

Discussion

In this study, we highlighted the importance of targeted NGS panel testing for diagnosis, prognosis and treatment decision in highly selected instances of lymphoid malignancies and lymphoproliferative disorders. Moreover, NGS analysis was a useful tool able to detect potentially therapeutically relevant mutations in lymphoid malignancies. We identified actionable mutations in 27 patients in our cohort and investigated 25 of these mutated cases for use as biomarkers upon clinical request. NGS analysis, nevertheless was meaningful for the clinicians in the case of actionable mutations were not identified. In this study group, clinical follow-up information was available approximately to 50 % of our patients. Thus, the information which was gained from NGS had treatment implications in our cohort.

Molecular alterations of numerous lymphomas have been well characterized by whole-exome sequencing studies. In DLBCL, *MLL2, CREBBP* and *TP53* alterations described by Pasqualucci et al [21] mutations were detected in patients with DLBCL in the current study.

NOTCH1 mutations identified in 5 out of 19 patients suffered with CLL (26%). Also, Puente et al [22] identified *NOTCH1* mutation in 12% of patients with CLL [22]. The frequency of variations in the current cohort were included; TP53 (12%), *NOTCH1* (11%) and ATM (11%). In theoretical usage, these alterations can be used for decision of targeted treatment by bevacizumab/pazopanib, NOTCH pathway inhibitors and olaparib respectively [21,22].

We detected TP53 variation in 12% of our patients and this provided information about the progression of disease. Despite the development of new therapeutic choices, those patients who had TP53 aberrations are defined as a high-risk group. Monotherapy or in combinational therapies have become the first-line treatment in patients with CLL harboring TP53 mutations or del (17p), as well as in relapsed or refractory CLL and have led to recent updates in treatment guidelines.

Staging of CLL is based on *NOTCH1* variations and recent studies showed the interaction between *NOTCH1* mutation and aggressive clinical behavior of CLL [23]. Also, *NOTCH1* mutations plays a key role for decision of the therapeutic approach in hematological B cell malignancies [24].

ATM is another common variation in lymphoid malignancies. *ATM* variation was detected in 11% of the cases in our cohort. Ten-twenty % of CLL patients carried del (11q22–23) before the treatment and this will increase to over than 40% upon progression following chemoimmunotherapy.

Discovery of ATM deficient individuals provides prognostic importance for these subgroups to therapies that target redundant, cooperative DNA damage response pathways such as ATR, DNA-PK and PARP1 inhibition. In a recent study, researchers demonstrated ATR inhibition which led to synthetic lethality in ATM-defective CLL cells [25].

A limitation of our study is that germline DNA was not investigated. Targeted NGS was enriched for known cancer-relevant genes and hotspots, and this may be linked to difficulties to differentiate between rare germline variants and somatic alterations. The other important point of NGS is the detection of subclonal, low frequency variants in samples with low tumor cell numbers. This will be solved by using a VAF cutoff of 5% for non-hotspot mutations which inevitably filters out some somatic variants. Besides, other types of relevant genetic alterations (like CNVs, insertion, translocation) are not detected by NGS technology. Therefore, additional techniques such as FISH and aCGH were needed to address copy number alterations and translocations. Targeted RNA sequencing may offer possibilities for the simultaneous NGS-based detection of fusion genes and gene dosage so this will be the use in future approaches during biomarker evaluation [26-29].

In this study, the patients who had a normal FISH and karyotype were enrolled. Our aim was to show the molecular changes that are likely to be found in cases where no alterations were detected by FISH and karyotyping. Despite these above-mentioned limitations, NGS panel testing is a feasible and may be clinically useful in lymphomas. Additionally, our cohort reflects the reality of clinical practice and our study demonstrates the robustness and applicability of dedicatedly designed NGS panels. In general, molecular data should be interpreted with the histopathological and clinical background. NGS results should be evaluated context-dependent and carefully. During the evaluation of NGS results should avoid overcalling of pathogenic mutations and the clinical diagnosis or indication should be done cautiously. In clinical practice, analysis is not generally useful for patients as first-line investigation but should be applicable to further define diagnosis and/or to

identification of mutations will guide treatment decisions in cases in which standard treatment options are limited or exhausted.

After all current molecular genetics advances, personalized medicine provides an accurate and personalized diagnosis that contributes to precise and targeted therapy to reduce adverse outcomes or side effects [30].

Conclusion

The advancement of personalized oncology/personalized medicine identification of copy number alterations and fusion genes play a crucial role during the identification of novel treatment targets. In cases, which standard treatment options are limited or resistant NGS platforms will help re-design novel scientific information with respect to diagnosis, prognosis and therapy and will likely become part of standard investigations in the near future.

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Authors' contribution

HG, AMD: conceived and planned the experiments. EA: carried out the statistical analysis. DE: carried out the experiment. EIA and RK wrote the manuscript. Both SD and SY contributed to the final version of the manuscript. EIA and RK provided critical feedback and helped shape the research, analysis and manuscript.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (TUMF Scientific Research Ethics Committee Directive) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interests

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this article.

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