Circulating Free RNA as a Therapeutic Evaluation in Diffuse Large B-cell Lymphoma: A Case Series from the Indonesian Cancer Center

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ABSTRACT

Cancer is still the leading cause of death worldwide. Despite advances in diagnosis, management with the rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, and prednisone (R-CHOP) chemotherapy regimen, and careful clinical and radiologic evaluation, diffuse large B-cell lymphoma (DLBCL) still carries high recurrence in clinical practice. This case series aims to assess the potential of circulating free RNA as a biomarker for evaluating therapeutic responses in DLBCL. This case series was conducted at Dharmais National Cancer Center Hospital in Jakarta in 2020. The subjects were 13 DLBCL patients who came for treatment to our hospital in 2020. Sampling was carried out by taking peripheral blood, which was taken 7–14 days after the patient underwent the 3rd and 6th cycles of chemotherapy or before and 7–14 days following the 3rd cycle of chemotherapy. Circulating free RNA (cfRNA) was extracted and assessed. The quantity of cfRNA was subsequently examined twice as matching samples from each patient, with the following results – (1) no mutations detected; (2) mutation detected solely in the second examination; (3) mutation only detected in the first examination; and (4) changes in gene mutations and mutation types. Statistic tabulation neither showed an association between recurrency and clinical variables nor detected cfRNA from the matching samples. This case series underscores the challenges in utilizing cfRNA as a biomarker for therapeutic evaluation in DLBCL due to heterogeneity and increased mutations in post chemotherapy conditions. Further research with larger sample sizes is needed to emphasize the role of cfRNA in DLBCL disease monitoring.

***Keywords**: circulating free RNA, DLBCL, therapeutic evaluation, lymphoma, NGS.*

INTRODUCTION

Cancer is still the leading cause of death worldwide. In 2020 alone, there were 396,914 new cancer cases and 234,511 cancer deaths in Indonesia.[1] Non-Hodgkin's lymphoma (NHL) is a malignant disorder stemming from immune system cells. It primarily manifests as lymphadenopathy or solid tumors.[2] NHL is the seventh most prevalent cancer in Indonesia, accounting for 16,125 new cases reported in 2020, affecting more men than women, with incidence rates of 7.7 and 3.8 per 100,000, respectively. The mortality rate also remains high, with GLOBOCAN reporting that at least 9,024 people died from the malignancy in 2020.[1] Among the various NHL subtypes, DLBCL is the most common histopathologic subtype, accounting for 32.2–32.5% in the United States and higher in Southeast Asia, with a percentage of 58.1% of all NHL cases.[3] DLBCL has a more aggressive molecular nature and can arise in the lymph nodes, spleen, liver, bone marrow, or other organs. Despite advances in diagnosis, management with the R-CHOP chemotherapy regimen, and careful clinical and radiological evaluation, DLBCL still carries short remission and high recurrence rates in clinical practice, with as many as one-third of patients relapsing after achieving a complete response with the R-CHOP regimen and 10% refractory to initial therapy.[4] Radiographic evaluation with computerized tomography scan (CT Scan) and positron emission tomography scan (PET Scan) contribute to these limitations, as these modalities can only macroscopically assess and determine tumor location and size ≥ 5 mm. Furthermore, recurrence is assumed because CT or PET scan modalities cannot trace the dynamic process of the tumor (e.g., response kinetics, clonal evolution, and cellular resistance).[5,6]

Various novel molecular biomarkers, including circulating tumor cells (CTC), have been examined for their potential to assess the dynamic process of the tumor. CTC identification has been shown to provide tumor-specific genomic, transcriptomic, and proteomic information in DLBCL. However, their analysis is less desirable because DLBCL does not typically present with circulating lymphoma cells, unlike follicular lymphoma (FL), mantle cell lymphoma, small lymphocytic lymphoma, marginal zone lymphoma, and a subset of Burkitt lymphoma.[7] CTC analysis, on the other hand, requires a large volume of fresh blood and is time-consuming and costly.[8]

Studies have proposed the role of cell-free RNA (cfRNA) as a biomarker of therapy response. The use of cfRNA, however, has shown promise as a precision medicine biomarker for early detection, diagnosis, prognostication, and therapy evaluation in a wide range of cancers[9-11], including nasopharyngeal, liver, multiple myeloma, bladder, breast, colorectal[11], lung[11-12], pancreas[13], and endometrial cancers.[14] Due to its more challenging analysis technique, the analysis of cfRNA also harbors some advantages as a biomarker compared to circulating free DNA (cfDNA). Moreover, cfRNA molecules are thought to be capable of providing a more comprehensive understanding of the crucial pathways involved in normal differentiation, as well as lymphoma initiation and transformation, owing to their ability to mediate or influence intercellular communication. Variations in cfRNA patterns during the course of the disease or treatment may also represent functional and longitudinal changes in the tumor and the non-malignant compartment. Additionally, due to their distinctive and dynamic characterization, including tumor–environment crosstalk, cfRNA analysis is thought to be able to guide the development of individualized diagnostic and therapeutic options, particularly in genetically heterogeneous malignancies, such as DLBCL.[8] RNA expression changes are dynamic processes that may reveal tissue damage or disease.[15] Furthermore, the research of cfRNA is based not only on the differential abundance of a set of specified genes but also on supplementary variables, such as pathogenic alternative splicing[16] or A-to-I RNA editing[17], changes that are only observable in the transcriptome and not the genome. A study by Zaporozhchenko et al. found that the scope of RNA biomarkers extends beyond expression analyses.[18] Transcripts specific to certain tissues, RNA molecules resulting from alternative or defective splicing, fusion events, unconventional processing,

or post-transcriptional alteration of RNAs are examples of qualitative RNA biomarker choices.[10,20] The detection of isoforms and changed transcripts, in addition to expression data, increases RNA's capacity to convey valuable clinical information, and regulatory RNA levels can be directly associated with valuable clinical characteristics.[18] Furthermore, RNA biomarker detection and analysis are carried out primarily using the same or similar DNA analysis techniques, including "omics" approaches[21,22], which are sensitive to even minor changes in RNA expression and can be adjusted to be specific to RNA structure, allowing them to capture the molecular landscapes of normal and tumor cells and utilize them to determine molecular cancer portraits.[23] Transcriptional signatures have been proven to distinguish cancer subtypes and predict disease progression and prognosis.[24-26] These characteristics of RNA expression in normal and cancerous tissues can be utilized to identify novel potential biomarkers as well as aid in the deconvolution of circulating RNA.[27,28] Another benefit of high amounts of RNA analysis is the capacity to create pan-cancer signatures that can be used to detect cancer and identify its source.[29] This can be extremely useful in locating the primary sites of metastatic tumors and cancers of unknown primary sites and monitoring recurrence and metastases.[18]

The utilization of circulating free RNA is still lacking worldwide, particularly in DLBCL patients. Thus, given the limitations as well as the benefits that cfRNA analysis may provide in clinical evaluation, the authors are interested in conducting a case series of cfRNA's profile and role in therapeutic evaluation of DLBCL, particularly at Dharmais National Cancer Center, Indonesia.

CASE ILLUSTRATION

This case series described cases at Dharmais Cancer Hospital (Rumah Sakit Kanker Dharmais; RSKD), Jakarta, in 2020. We included 13 NHL patients with DLBCL subtype who came to RSKD for treatment in 2020 and met the following inclusion criteria: (1) DLBCL patients as confirmed by histopathological reports from RSKD or other hospitals and (2) over 18 years of age. The exclusion criteria were patients (1) afflicted with primary cancers elsewhere and (2) refusing to participate in the research. The sampling technique was carried out using *total sampling.*

Sampling was carried out by taking as much as 15 ml of peripheral blood from the patient. Blood samples were taken at 7–14 days after the patient underwent the 3rd and 6th cycles of chemotherapy. However, due to poor compliance during the COVID-19 pandemic, samples were collected prior to chemotherapy and 7–14 days following the 3rd cycle of chemotherapy. The blood sample was subsequently transferred to an EDTA-coated tube, immediately used for RNA extraction, and analyzed using *Next-Generation Sequencing (NGS)*. RNA was prepared and processed multiple times using Archer® FusionPlex® for Illumina from Qubit quantification, random priming, first- and second-strand synthesis, and ligation. Multiple indexing adapters were tied to the ends of the fragments and then quantified using the Kapa Library Quantification Kit for Illumina sequencing platforms. The RNA samples were then effectively enriched and fed to the Illumina MiSeq™ Sequencing System (Illumina, Inc., San Diego, California, United States of America). The sequencing data was mapped and analyzed using Archer® Analysis, which allows the analysis and interpretation of the data of genes, as shown in **Table 1**.

The collected data was processed using SPSS Statistics Version 25 for descriptive statistics. In the univariate or descriptive analysis, nominal and ordinal data were expressed in frequency and percentage. The research ethics were assessed by the Ethics Commission of Dharmais Cancer Hospital (number 179/KEPK/IX.2019). All data was kept confidential by researchers.

Of the 13 non-Hodgkin lymphoma participants, seven (53.8%) were females while six (46.2%) were males. The median age was 44, ranging from 22 to 74 years old, with two subjects over 60 years old. Ten patients (76.9%) were diagnosed at stages III and IV based on the Ann Arbor staging system. The median overall survival (OS) was 19 months, ranging from 6–21 months, and the progression-free

Table 1. List of gene targets covered by the analysis of Archer® FusionPlex® Lymphoma Kit[30]

SNV or indel	Expression		Fusion, splicing, or exon skipping
AKT3	AICDA	IRF4	AKT3
BAX	ASB13	IRF8	ALK
BCL2	BATF3	ITPKB	BCL2
BIRC3	BCL2	KIAA0101	BCL6
BRAF	BCL2A1	LIMD1	BCR
BTK	BCL3	LMO2	BIRC3
CARD11	BCL6	LRMP	CBFB
CCND1	BLNK	LZTS1	CCND1
CD79B	BMF	MAL	CCND3
CREBBP	BMP7	MAML3	CDK6
DNMT3A	CCDC50	MME	CHIC2
ETV6	CCND1	MUC1	CIITA
EZH2	CCND2	MYBL1	DEK
FBXW7	CCND3	MYC	DUSP22
IDH1	CD274	NEK6	EIF4A1
IDH2	CD44	NFKB1	ETV6
JAK1	CDC25A	NME1	FGFR1
JAK3	CDKN2A	PAICS	JAK2
KRAS	CDKN2B	PDCD1	KMT2A
MYD88	CEBPD	PDCD1LG2	MALT1
NOTCH1	CEBPE	PIM1	MKL1
NOTCH2	CEBPG	PIM2	MLF1
NRAS	CREB3L2	PPAT	MLLT10
PLCG1	CTLA4	PRKAR2B	MYC
PLCG2	CYB5R2	PTPN1	NFKB2
RHOA	DENND3	PYCR1	NOTCH1
SF3B1	DLEU1	RAB29	P2RY8
STAT3	DNMT3B	RAG1	PDCD1LG2
STAT5B	DNTT	RAG2	PDGFRA
STAT6	E2F2	RANBP1	PRDM16
WT1	EIF4A1	S1PR2	STIL
XPO1	ENTPD1	SERPINA9	TCF3
	EXOC2	SH3BP5	TP63
	FAM216A	STRBP	
	FOXP1	TNFRSF13B	
	FUT8	TNFSF4	
	IL16		

survival (PFS) was 13 months, ranging from 3–20 months. Seven patients had extranodal cancer involvement, with two of them having multiple (> 1) extranodal disease sites and four having bone marrow involvement. Of the 13 subjects involved in this study, only 5 patients (38.5%) were found to have increased lactate dehydrogenase (LDH) prior to therapy. Eight patients had restrained activities, as shown by the Eastern Cooperative Oncology Group (ECOG) score of ≥ 1. The remaining patients had normal daily functioning. Based solely on CT scan examination, four patients had complete response after chemotherapy with the existing regiment guidelines. The same number of patients had partial response, two had stable disease, and three experienced progressive disease. Five patients with progressive disease and partial response following complete cycles of chemotherapy were declared relapsed, and four of them subsequently died from malignancy—the demographic data of the patients are presented in **Table 2**. Statistically, there was no association between recurrency throughout the follow-up period and the observed

Table 2. Demographic data

No	Name	Age	Ann Arbor Stage	Extranodal Involvement	Baseline LDH (U/L)	B2M (mg/L)	ECOG	Response***	Progression-Free Survival (Months)	Relapse	Overall Survival (Months)	Outcome
1	Mrs.*	36	IV	Lung	793.52	1.30	1	PR	3	Yes	16	Dead
2	Mr.*	48	IV	Bone marrow	323.73	1.26	1	SD	20	No	20	Live
3	Mr.*	44	IV	Lung	604.41	2.04	1	SD	20	No	20	Live
4	Mrs.*	59	II	x	316.00	3.34	1	PR	14	No	21	Live
5	Mr.*	33	III	x	2525.00	1.72	2	PR	14	No	21	Live
6	Mrs.**	66	II	x	552.00	4.83	0	PD	7	Yes	9	Dead
7	Mr.**	74	IV	Bone marrow	318.00	8.9	0	PD	5	Yes	19	Dead
8	Mr.**	39	III	x	1148.00	x	0	PR	3	Yes	17	Live
9	Mrs.**	53	IV	Adrenal gland, skin, mesentery, colon, ileocecal	413.00	5.99	1	CR	13	No	15	Live
10	Mrs.**	47	IV	Lung, subpleural, bone marrow	407.00	3.35	0	CR	12	No	19	Live
11	Mrs.**	34	I	x	**201**	1.63	0	CR	13	No	14	Live
12	Mrs.**	41	III	x	**385**	1.39	2	PD	3	Yes	6	Dead
13	Mr.**	22	IV	Bone marrow	**201**	1.36	1	CR	17	No	19	Live

*Subjects who were assessed two weeks following the 3rd and 6th cycles of chemotherapy.
** Subjects who were assessed prior to chemotherapy and two weeks following the 3rd cycle of chemotherapy.
***CR: Complete Response, PR: Partial Response, SD: Stable Disease, PD: Progressive Disease.

clinical variables or with the cfRNA detected from the examinations on paired samples. This statistic tabulation is presented in **Table 3**.

The quantity of cfRNA was examined and measured; six subjects (46.15%) were found to have no circulating tumor RNA during the first molecular evaluation. Among the subjects whose circulating tumor RNA was detected, one subject (7.69%) had FOXP1 mutation on chromosome 3 with fusion type, four subjects (30.77%) had

Table 3. Association between recurrence throughout the follow-up period and clinical variables

	Recurrency during Follow-Up Period		Univariate analysis HR (95% CI)	P
	Yes	No		
Age				
≤ 60 years	3 (60)	8 (100)	Ref	ref
> 60	2 (40)	0 (0)	0.271 (0.045–1.638)	0.155
Ann Arbor Stage				
I–II	1 (20)	2 (25)	Ref	ref
III–IV	4 (80)	6 (75)	0.691 (0.077–6.234)	0.742
Sex				
Male	2 (40)	4 (50)	Ref	ref
Female	3 (60)	4 (50)	1.308 (0.218–7.840)	0.769
Extranodal Involvement				
0–1	5 (100)	6 (75)	Ref	ref
>1	0 (0)	2 (25)	0.036 (0.000–762.914)	0.514
Bone Marrow Involvement				
No	4 (80)	5 (62.5)	ref	ref
Yes	1 (20)	3 (37.5)	0.503 (0.056–4.521)	0.54
Raised LDH				
No	2 (40)	6 (75)	ref	ref
Yes	3 (60)	2 (25)	2.712 (0.451–16.317)	0.276
Raised β2-microglobulin				
No	2 (50)	2 (25)	ref	ref
Yes	2 (50)	6 (75)	2.735 (0.376–19.900)	0.32
ECOG Score				
Score 0–1	4 (80)	7 (87.5)	ref	ref
Score ≥ 2	1 (20)	1 (12.5)	0.620 (0.069–5.589)	0.67
Chemo Response				
Complete Response (CR)	0 (0)	4 (50)	ref	ref
Stable Disease (SD)	0 (0)	2 (25)		
Partial Response (PR)	2 (40)	2 (25)	80.921 (0.056–117193.700)	0.237
Progressive Disease (PD)	3 (60)	0		
RNA Detection on 1st evaluation				
No	3 (60)	3 (37.5)	ref	ref
Yes	2 (40)	5 (62.5)	1.867 (0.311–11.199)	0.494
RNA Detection on 2nd evaluation				
No	3 (60)	4 (50)	ref	ref
Yes	2 (40)	4 (50)	0.828 (0.138–4.967)	0.837
Δ cfRNA				
Not Applicable	1 (20)	2 (25)	Ref	ref
Decreased/Stable	2 (40)	4 (50)	1.144 (0.103–12.674)	0.913
Increased	2 (40)	2 (25)	1.994 (0.177–22.405)	0.576

Hazard ratios (HR), 95% confidence intervals (95% CI), and P values were calculated using Cox proportional hazard. No assumptions of Cox proportional hazard were violated.

KMT2A mutation on chromosome 11 with oncogenic isoform type, one subject had JAK2 mutation on chromosome 9 with fusion intergenic type, and, interestingly, another had two types of mutations simultaneously, namely intergenic in JAK2 gene on chromosome 9 and oncogenic isoform in KMT2A gene on chromosome 11. All mutation findings in the first examinations harbored potential clinical significance (Tier II). In contrast, seven participants (53.85%) had no peripheral circulating RNA in the second evaluation. Of the 6 subjects whose mutations were detected in the second evaluation, three had JAK2 mutations, two of which were fusion type and the other intergenic. KMT2A mutations were also discovered in the same number of subjects (3 subjects, 23.08%), all oncogenic isoforms on chromosome 11. The data for these mutation findings is demonstrated in **Table 4**.

The examination of cfRNA has been utilized more in precision molecular medicine as it is considered to be less invasive[31-33]; since it only involves sampling from peripheral blood, and the results obtained are considered to be able to demonstrate the dynamic processes of cancer, such as response kinetics, clonal evolution, and cellular resistance.[5,6] However, as a molecular biomarker, cfRNA has begun to be developed as a supplementary, and even complementary, evaluation of existing limited modalities. Tissue biopsy is difficult to employ to detect recurrence or evaluate minimal residual disease (MRD) because biopsies are intrusive, and repeated examination may cause patient's discomfort. Imaging has low sensitivity and cannot detect changes at the molecular level. The advantages of evaluating circulating RNA have been discussed in depth in several studies, and its molecular dynamics have been shown to be employed as an evaluation parameter in post-therapy DLBCL patients.

The utilization of cfRNA is also more promising than ctDNA[31], which still has significant drawbacks, including a short half-life[34] and wild-type DNA contamination from leukocyte lysis[35]. Thus, detection still requires numerous techniques with high analytical sensitivity and specificity to identify the low-concentration and highly variable ctDNA fractions.[36] Furthermore, ctDNA analysis is less appropriate for detecting gene rearrangements, regardless of the fact that Russo et al. successfully detected gene fusions in ctDNA[37], where the process still requires extensive deep sequencing or break-point involving targeted assays to detect rearrangements. In establishing the molecular profile, cfRNA analysis could supplement ctDNA.[31] Thus, the discovery of cancer-associated abnormalities using cfRNA analysis in circulation or embedded in vesicles or tumor-educated platelets (TEPs) has shown immense clinical potential.[38-40]

The potential was further explained by a German study by Metzenmacher et al. that the analysis of cfRNA has become an intriguing non-invasive approach for biomarker identification in cancer's early detection. The study addressed how NGS-based cfRNA profiling and real-time digital droplet PCR (RT-ddPCR) could be used to analyze cfRNA for the early detection of solid malignancies (lung, pancreas, bladder, and skin cancers).[32] In a similar fashion to metastatic prostate cancer, Ladurner et al. found that PSA was significantly increased in the plasma-derived cfRNAs of metastatic prostate cancer patients compared to healthy controls. High prostate specific antigen (PSA) expression was similarly correlated to poor overall survival, implying that cfRNA from plasma could be a useful tool for molecular expression analysis.[41]

Among the 13 patients involved in our study, it was found that females were more prevalent than males, with the gender ratio between males and females being 0.86, contradicting the findings of a general DLBCL population study that revealed men to be likelier than women to be affected by DLBCL. This was due, in particular, to the fact that COVID-19 infection was more prevalent in male patients in our hospital during the pandemic, requiring male patients to undergo recovery therapy before receiving chemotherapy regimens. Thus, a higher number of females were eligible for inclusion as study subjects in this study. Statistical analysis also showed that clinical variables, such as being over 60 years old and being at a higher stage (Ann Arbor stages III and IV) and having more than one extranodal involvement, elevated LDH and B2M levels,

Table 4. Cell-free RNA results

No.	Name	1st Evaluation							2nd evaluation							
		Gene	Fusion & Isoform Onc	Breakpoint, type	Clinical Significance	Qbit	%reads	RNA Qty	Gene	Fusion & Isoform Onc	Breakpoint, type	Clinical Significance	Qbit	%reads	RNA Qty	Δ cfRNA (%)
1	Mrs.*	x	x	x	x	0.02	0	**0**	NOTCH1	Fusion	chr9_139390700_21_-_A1_GS P2	Tier III	13.2	28.42	**3.75**	NA
		x	x	x	x	0.02	0	**0**	KMT2A	Onc isoform	chr11_118359430_27_+_A1_GS P2	Tier II	13.2	0.75	**0.09**	+100
2	Mr.*	DEK	Fusion	chr6_18264130_20_-_A1_GSP2	Tier III	4.38	27.66	**1.21**	KMT2A	Onc isoform	chr11:118359475,chr11:118348682, exons out of order	Tier II	72	0.52	**0.37**	NA
		JAK2	Fusion: intergenic	chr9_5089695_22_-_A1_GSP2	Tier II	4.38	12.63	**0.55**	KMT2A	Onc isoform	chr11:118352807,chr11:118339490, exons out of order	Tier II	72	0.54	**0.39**	NA
3	Mr.*	KMT2A	Onc isoform	chr11_118352754_27_+_A1_GS P2	Tier II	47.6	0.51	**0.24**	KMT2A	Onc isoform	chr11:118352807,chr11:118339490, exons out of order	Tier II	45.7	0.62	**0.28**	+16.67
		KMT2A	Onc isoform	chr11_118348873_22_+_A1_GS P2	Tier II	47.6	0.55	**0.26**	KMT2A	Onc isoform	chr11:118348916,chr11:118339490, exons out of order	Tier II	45.7	0.88	**0.4**	+53.85
4	Mrs.*	x	x	x	x	17.4	0	**0**	JAK2	Intergenic	chr9_5089695_22_-_A1_GSP2	Tier II	8.59	40.91	**3.51**	+100
5	Mr.*	x	x	x	x	16	0	**0**	x	x	x	x	20.4	0	**0**	0
6	Mrs.**	x	x	x	x	12.3	0	**0**	x	x	x	x	12.2	0	**0**	0
7	Mr.**	EIF4E3 → FOXP1	Fusion	chr3:71739161,chr3:71542706, N/A	NA	21	13.46	**2.83**	x	x	x	x	29.1	0	**0**	-100
8	Mr.**	x	x	x	x	15.6	0	**0**	JAK2	Fusion	chr9:5126446,chr9:5140632|chr9:5140689,chr9:5089674	Tier II	40.3	32.5	**13.09**	+100
9	Mrs.**	KMT2A	Onc isoform	chr11_118359430_27_+_A1_GS P2	Tier II	24.7	1.18	**0.29**	x	x	x	x	12.8	0	**0**	-100
		KMT2A	Onc isoform	chr11_118352754_27_+_A1_GS P2	Tier II	24.7	0.73	**0.18**	x	x	x	x	12.8	0	**0**	-100
		KMT2A	Onc isoform	chr11_118350924_24_+_A1_GS P2	Tier II	24.7	0.68	**0.17**	x	x	x	x	12.8	0	**0**	-100
10	Mrs.**	JAK2	Intergenic	chr9_5089695_22_-_A1_GSP2	Tier II	14.3	21.82	**3.12**	JAK2 → INTERGENIC → JAK2	Fusion	chr9:5126446,chr9:5140632|chr9:5140689,chr9:5089674	Tier II	16.2	14.29	**2.31**	-25.96
		KMT2A	Onc isoform	chr11_118352754_27_+_A1_GS P2	Tier II	14.3	0.69	**0.09**	NOTCH1 → HBA1	Fusion	chr9:139390696,chr16:227068	Tier III	16.2	10.26	**1.66**	NA
11	Mrs.**	x	x	x	x	35.2	0	**0**	x	x	x	x	7.6	0	**0**	0
12	Mrs.**	KMT2A	Onc isoform	chr11:118355690,chr11:118339490 (Exons out of order)	Tier II	13.4	0.57	**0.08**	x	x	x	x	11.8	0	**0**	-100
13	Mr.**	KMT2A	Onc isoform	chr11_118352754_27_+_A1_GS P2	Tier II	8.49	0.58	**0.05**	x	x	x	x	37.8	0	**0**	-100

*Subjects who were assessed two weeks following the 3rd and 6th cycles of chemotherapy.
** Subjects who were assessed prior to chemotherapy and two weeks following the 3rd cycle of chemotherapy.

and an ECOG score ≥2, were not associated with recurrence during the follow-up period. This contradicts prior findings that showed patients with the aforementioned characteristics have lower progression-free survival (PFS), as determined by the Revised International Prognostic Index (R-IPI) score.[42-45]

Our study also successfully extracted circulating RNA from patients on the first and second examinations as evaluation parameters. Almost all of the mutation findings in our study carried potential clinical significance, as expressed in the "Tier II" group, but the findings from matching samples could not be concluded as a continuous therapeutic evaluation, which we believe is due to the high heterogeneity of mutations in DLBCL that has been extensively studied.

Heterogeneity in DLBCL has been suggested in a study conducted by Nijland et al. (2018), where they suggested that there were non-synonymous mutations in 30 to 100 genes per case (median 3.3 to 6.6 mutations per megabase) from more than 2,000 DLBCL cases studied in the last decade. Overall, 1,000 gene mutations have been described in DLBCL. To support these previous studies, Nijland performed whole genome sequencing on formalin-fixed paraffin-embedded (FFPE) DLBCL biopsies to assess the global evolution of mutations and to identify specific gene mutations in relapsed patients treated with the R-CHOP regimen. A small proportion of mutations found in primary samples (median 7.6%, range 4.8–66.2%) could not be detected in matched relapse samples, while relapsed DLBCL samples showed a mild increase in mutations (median 12.5%, range 9.4–87.6%) when compared to primary tumor biopsies.[46]

The landscape of heterogeneity in DLBCL has also been discussed in several studies.[47-50] DLBCL is a malignancy that yields extreme genetic heterogeneity, phenotypes, and clinical entities. It differs from other hematologic malignancies and shows a higher number of mutations per patient and a larger proportion of recurrently mutated genes, with long mutated gene tails observed in a limited number of cases. Overall, the field has now converged on roughly 150 protein-coding driver genes in DLBCL that are recurrently mutated or functional targets of somatic copy number alterations.[47]

In conjunction with the findings of the studies that were conducted by Nijland et al.[46] and Lee et al.[49], the following four groups may be drawn from our findings – (1) no detection of mutations in matching samples; (2) mutation detected in the second examination, after not being detected on the first examination in a matching sample; (3) mutation not detected after being initially detected on the first examination; and (4) changes in gene mutations and mutation types in a matching sample. All of these findings support a molecular mechanism of mutation heterogeneity in DLBCL. Henceforth, the four events described in our findings are explained by the heterogeneity in DLBCL as well as the evolution of mutations following chemotherapy.

Heterogeneity in DLBCL, aided by an increased incidence of mutations in the post-chemotherapy condition (compared to de novo DLBCL),[47,49] may lead to the limitation of circulating RNA detection and identification. The limitation of the mutation profile we obtained from the study also cannot determine the diagnostic and prognostic value of DLBCL patients treated with R-CHOP. Still, our study can serve as a highlight to demonstrate that cfRNA detection in DBLCL patients can be further expanded into a diagnostic method and even a therapeutic evaluation before relapse through paired identification with FFPE samples at the pre-chemotherapy examination.

In addition to the fact that DLBCL heterogeneity may affect cfRNA identification, an American study conducted by Wagner et al. also discovered that patient-specific baselines should be addressed when employing this clinical assay for patient risk stratification, diagnosis, or disease progression monitoring since the interpersonal variability is remarkably high, with several individuals showing a perpetually higher expression of particular genes than others. Hence, the biomarker threshold should be tailored for each patient.[51]

Owing to its stability in body fluids as extracellular RNA, cfRNA certainly yields good potential as a biomarker for early detection,

diagnosis, prognosis, and therapeutic evaluation. Biological and technical variations in circulating RNA expression have also been studied, and the results reveal that many of the RNA molecules identified in body fluids of cancer patients, such as plasma, serum, and urine,[52] contain signs of biological variation, making them unlikely to be random breakdown by-products.[9] Increased levels of cfRNA have also been discovered circulating in cancer patients, as indicated by the detection of 68% of gene mutations detected solely in cancer patients compared to healthy individuals, making cfRNA implementable as a non-invasive biomarker for cancer diagnosis. The ability of cfRNA as a non-invasive biomarker for cancer detection has also been shown to be able to identify 10^5 to 10^6 copies per cell in studies conducted on nasopharyngeal cancer. The detection of cfRNA is not confined to a single type of biological sample or malignancy. Furthermore, Hieter et al. performed plasma cfRNA sequencing to use cfRNA as a biomarker for hepatocellular carcinoma and multiple myeloma, which showed that cfRNA has different expressions across cancer development stages; thus, cfRNA-based analysis can be used to identify precancerous and cancerous conditions in patients. Cancer diagnosis through cfRNA can also be performed using urine analysis. Kim et al. reported that the urine of bladder cancer patients preserved higher levels of cfRNA compared to healthy individuals. Several studies have demonstrated that cfRNA has adequate diagnostic capacities in various cancers, including breast, colorectal, lung, and several others.[11]

To the best of our knowledge, this study is the first case series to report the measurement of cfRNA in DLBCL patients in Indonesia. This study can be a cornerstone for future studies on the role of cfRNA as a biomarker in various stages of DLBCL. However, due to the limited samples in the COVID-19 pandemic surge, we are unable to provide clear and compelling evidence of this. Therefore, further research on this topic in Indonesia is recommended to more deeply analyze the utility of cfRNA toward various clinical stages of DLBCL, be it at the pre-diagnostic, diagnostic, prognostic, and therapy evaluation stages. Further research with better protocols is required to provide better evidence. More research with larger sample sizes and follow-up samples is also required.

CONCLUSION

This case series underscores the challenges in utilizing cfRNA as a biomarker for therapeutic evaluation in DLBCL due to heterogeneity and increased mutations in post chemotherapy conditions. Further research with larger sample sizes is needed to emphasize the role of cfRNA in DLBCL disease monitoring.

DISCLOSURE STATEMENT

The authors report that there are no competing interests to declare. Disclosure forms provided by the authors are available with the full text of this article online.

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