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To the editor:

Vitreous microRNA levels as diagnostic biomarkers for vitreoretinal lymphoma

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Primary vitreoretinal lymphoma (PVRL) is often associated with primary central nervous system lymphoma (PCNSL) and is a rare high-grade diffuse large-cell B-cell lymphoma occurring within the vitreous and/or retina.[1-3]

Overlapping clinical signs observed in PVRL and chronic vitritis often mislead ophthalmologists to diagnose the latter rather than PVRL. The pathological diagnosis of PVRL is usually established by morphological examination of vitreous biopsies and/or subretinal aspirates, followed by immunoprofiling and genetic analyses.[1,4] Despite this workup, the false negative diagnostic rate is still estimated to be up to 30% in PVRL,[2] particularly in paucicellular specimens containing only necrotic tumor cells. Therefore, novel noncellular biomarkers within the sample supernatant are required in the diagnostic repertoire of PVRL.

Previously, we have demonstrated an upregulation of certain microRNAs (miRNA; miR-92, mi-19b, mi-21), in the cerebrospinal fluids of PCNSL patients, compared with cerebrospinal fluid of patients with inflammatory and nonneoplastic central nervous system diseases.[5,6] Because there are distinct similarities between PVRL and PCNSL,[7] we hypothesized that the same miRNAs could be upregulated in PVRL vitreous specimens compared with those with vitritis.

Ruhr University ethic committee approval (register no. 4704-13; Bochum, Germany) was obtained for the vitreous sample collection.

All vitreous samples were collected prospectively by pars-plana vitrectomy before initiation of any chemotherapy, and following discontinuation of cortisone therapy. Vitreous samples from 10 PVRL patients were included. In all PVRL samples, the diagnosis of diffuse large-cell B-cell lymphoma was established following standard practice in the ocular oncology reference centers. Immunoglobulin H–polymerase chain reaction (IgH-PCR) was also performed in 3 cases. The PVRL was newly diagnosed in all patients. We used 40 vitreous samples from histopathologically confirmed vitritis patients and 7 vitreous samples from noninflammatory macular pucker patients as controls. The clinical data were recorded for all patients and are summarized in Table 1. Vitreous samples were centrifuged within 30 minutes (at 500*g*, 10 minutes, room temperature). The supernatants were stored at −80°C for further processing and miRNA analysis.

Total RNA was extracted using the mirVana RNA isolation kit (Ambion; ThermoFisher Scientific, Waltham, MA), according to the manufacturer's instructions. Briefly, 0.4 mL of vitreous was diluted with mirVana PARIS 2× denaturing solution. Equal volumes of acid/phenol/chloroform were added to each aliquot and centrifuged. Following this, glycogen was added to the aqueous phases, which were subsequently mixed with 1.25 volumes of 100% ethanol. After passage through a mirVana PARIS column, several washing steps were carried out.

TaqMan miRNA assays (Applied Biosystems) to quantify miRNA levels were applied as has previously been published.[5,6] The synthetic miRNAs, cel-54 and miR-769, not present in human vitreous, were chosen as reference molecules for normalization. The amount of miR-92, miR-19b, and miR-21 was normalized relative to the mean amount of cel-miR-54 and miR-769 (Ct = (Ct(cel-miR-54) + Ct(miR-769))/2 – Ct(miR-92 or miR-19b or miR-21)).

Statistical analyses were performed using SPSS (v.20; SPSS) and GraphPad Prism (v.5.0). Group-wise comparisons of distributions of clinical and biologic data were performed, applying 2-tailed Mann-Whitney *U* tests and Kruskal-Wallis tests with Dunn multiple comparisons.

Distinguishing PVRL and vitritis remains a major clinical challenge, even in some cases following extensive pathological evaluation of vitreous biopsies using a variety of diagnostic techniques. In this study, we assessed whether miR-92, miR-19b, and miR-21 levels in the noncellular component (ie, the supernatant) of centrifuged vitreous biopsies could be used to differentiate patients with PVRL (n = 10) from macular pucker patients without any inflammation (n = 7). All 3 miRNA candidates were significantly upregulated in vitreous of PVRL patients (Table 2; Figure 1A-C). Furthermore, when comparing miR-92, miR-19b, and miR-21 levels in the vitreous of PVRL patients with those from vitritis patients (n = 40), we found miR-92, miR-19b, and miR-21 levels to be significantly upregulated in PVRL patients (Table 2; Figure 1A-C).

To test all 3 miRNA candidates for their ability to discriminate between PVRL and vitritis, area under the curve (AUC) calculations were performed. MiR-92 revealed an AUC of 0.9725 (Figure 1D). Corresponding to this analysis, cutoff vitreous relative quantification value (REL) with the highest accuracy for miR-92 was determined as follows: REL −10.82 with 100% sensitivity and 90% specificity. MiR-19b and miR-21 demonstrated AUC of 0.94 and 0.8125 by comparison of PVRL with vitritis, respectively (Figure 1E-F).

Various methods are used in PVRL diagnostics: cytological and immunocytochemical analyses are standard procedures.[1] Diagnostic rates of PVRL using cytological analysis only are estimated to be between 45% and 60%.[8-10] Additional immunophenotyping for B-cell markers (eg, CD20, PAX5, and CD79a) has improved this rate, with a sensitivity of 80% and a specificity of 100%.[1,8,11] Some centers also use evaluation of interleukin-6 (IL-6) and IL-10 ratios in the aqueous and vitreous, as an adjunctive diagnostic tool in PVRL.[12-14] Other centers prefer clonality analysis (ie, IgH-PCR or IgL-PCR) when there is sufficient residual sample. The sensitivity for PCR-based clonality does vary between 65% and 95%, as PVRL

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Table 1. Clinicopathological characteristics of included PVRL patients

Vitreous no.	Age (y)	Sex	PCNSL	Vitreous collection procedure	Prev. intraocular surgery	Syst. prev. GCs	Interval since syst. GCs (d)	Top. GCs	Interval since top. GCs (d)	AC cells	Vitreous haze	CME by FLA	CME by OCT	PVRL confirmation
1	63	F	Yes	23G VTX	None	No	—	No	—	1+	3+	None	None	IH
2	67	M	Yes	23G VTX	None	No	—	No	—	0	2+	None	None	IH
3	51	M	Yes	23G VTX	None	Yes	7	No	—	0	3+	None	None	IH + IgH-PCR
4	78	M	Yes	23G VTX	None	No	—	No	—	0	3+	Not done	None	IH
5	74	M	Yes	23G VTX	None	No	—	No	—	0	2+	Not done	Yes	IH + IgH-PCR
6	53	F	Yes	23G VTX	None	No	—	No	—	0	3+	Not done	Not done	IH + IgH-PCR
7	69	F	Yes	23G VTX	None	No		No		0	Hemorrhage	Not possible	Not possible	IH
8	42	F	No	20G VTX, chorioretinal biopsy	VTX	Yes	7	Yes	7	0.5+	2+	None	None	IH
9	60	F	Yes	23G VTX	None	Yes	19	Yes	19	0	2+	None	Not done	IH
10	67	F	Yes	23G VTX	Cataract surgery 3 months ago	No	—	No	—	1+	2+	None	Not done	IH

Biodata of all PVRL patients (Bochum/Germany: 5 PVRL specimens; Münster/Germany: 4 PVRL specimens; Mulhouse/France: 1 PVRL specimen) include age, sex, presence/absence of PCNSL, HIV status, biopsy method, previous cortisone therapy, and relevant clinical signs. All PVRL cases were diagnosed on the basis of morphology and immunoprofiling in the ocular oncology reference centers in Liverpool/United Kingdom, Lübeck/Germany, and Mulhouse/France. For 3 specimens, additional IgH-PCR was performed.

AC, anterior chamber; CME, cystoid macular edema; FLA, fluorescein angiography; GCs, glucocorticoids; IH, immunohistology; OCT, optical coherence topography; prev., previous; syst., systemic; top., topical; VTX, vitrectomy.

cells are highly mutated in the IgH-variable region, and they can be masked by a dense reactive inflammatory infiltrate, leading to false negatives.[1,4,10,12,15-18]

More recent tests have developed targeting genetic alterations in vitreoretinal lymphoma, requiring smaller DNA concentrations. Bonzheim et al demonstrated the presence of *MYD88* mutation L265P in a retrospective analysis in 71% of PVRLs[18] and demonstrated an increase in PVRL diagnosis from 62% to 90% when MYD88 mutation analysis was included in the sample workup.[18] Furthermore, next generation analysis identified MYD88 mutation S243N and copy number losses in CDK2NA as potentially actionable target in PVRL.[19]

In parallel to the above, evaluations of vitreous samples for miRNA levels have been undertaken. Tuo et al investigated miRNA levels in 3 PVRL samples and compared these to 3 vitreous specimens with uveitis, using a real-time PCR-based miRNA panel.[20] Of the 168 miRNAs analyzed in the panel, 3 miRNAs, miR-484, -197, and -132, were upregulated in PVRL, and 3 miRNAs, miRNA-155, -200c, -22*, were upregulated in the ocular fluid.[20] After individual miRNA real-time PCR in all specimens (PVRL, n = 17; uveitis, n = 12), only miRNA-155 was approximately twofold upregulated in uveitis specimens.[20] Interestingly, in Tuo's report, miR-21, miR-19b, and miR-92 were not described to be differentially expressed.[20] The data of Tuo et al and of our study are not comparable, due to the differences in PVRL diagnosis analysis, sample preparation, and the usage of different assays.

To our knowledge, this is the first observation of deregulated miR-21, miR-19b, and miR-92 expression in vitreous samples from PVRL patients. The relatively small number in the examined cohort is due to the rarity of this disease, and a larger validation study is required. However, the results of this study are of clear importance, due to the high level of discrimination, when miRNA expression in PVRL is compared with that of vitritis and macular pucker. We propose that miRNA analysis could represent a new adjunctive test in the PVRL diagnostic repertoire, providing supportive evidence to the morphological, immunocytological, and genetic evaluations. An advantage is that the miRNA would use the supernatant of the centrifuged vitreous sample, leaving the cellular component for profiling and DNA-based tests. We suggest that time delays to definitive diagnosis of this high-grade malignancy could be reduced. Nevertheless, a larger prospective study is required to validate our results.

The miRNA candidates analyzed here have also been established for disease course monitoring in PCNSL patients.[5,6] To date, disease course monitoring is difficult in PVRL, although IL-6:IL-10 aqueous levels are used in some centers. Because of the small fluid volumes required, targeted miRNA analysis should be considered in PVRL diagnostics and monitoring.

Table 2. Relative miR-92, miR-19b, and miR-21 expression in vitreous of patients with PVRL vs patients with vitritis and patients with macular pucker

	PVRL (n = 10)		Vitritis (n = 40)			Macular pucker (n = 7)		
	REL*	SD†	REL*	SD†	*P* value	REL*	SD†	*P* value
miR-92	−7.29	0.65	−11.72	0.81	.0020‡	−13.60	0.44	<.0001§
miR-19b	−9.32	0.84	−12.92	0.97	.0250‡	−15.45	0.50	<.0001§
miR-21	−6.78	0.77	−9.45	1.12	.0431‡	−10.59	0.51	.0025§

*Data are means of RELs (group-wise).

†Standard deviation.

‡The *P* value is for comparison of expression levels of miR-92, miR-19b, and miR-21 among PVRL patients and vitritis patients and was calculated using the Mann-Whitney *U* test.

§The *P* value is for comparison of expression levels of miR-92, miR-19b, and miR-21 among PVRL patients and macular pucker patients and was calculated using the Mann-Whitney *U* test.

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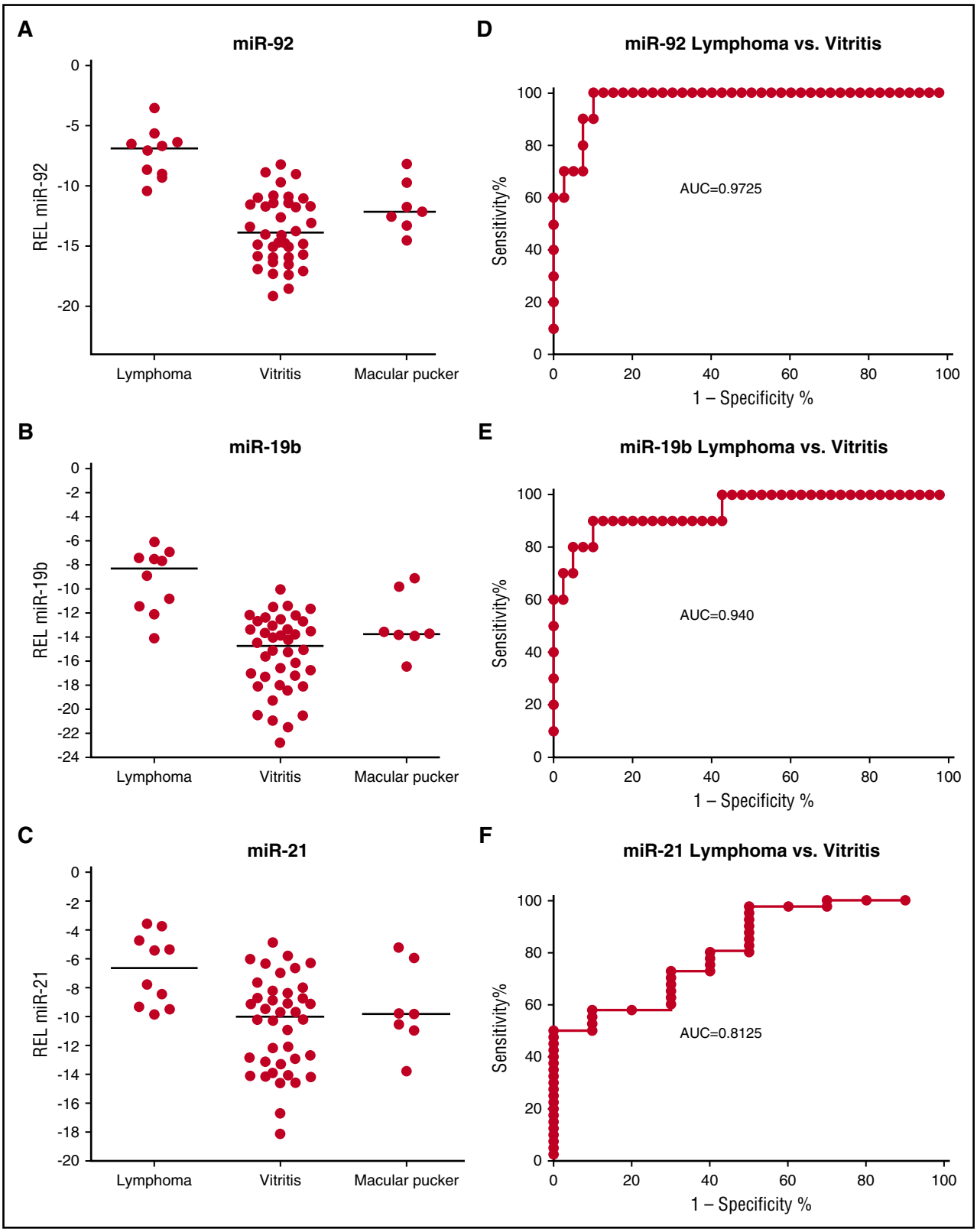

Figure 1. Scatterplots of expression levels: receiver-operating characteristics curve analyses. (A-C) Scatter plots of expression levels. Scatter plots of expression levels of miR-92 (A), miR-19b (B), and miR-21 (C) in vitreous samples from patients with PVRL (n = 10) compared with subgroups of patients with vitritis (n = 40), and noninflammatory macular pucker patients (n = 10). The black horizontal lines represent median REL values. Group-wise, *P* values are indicated as determined in Kruskal-Wallis tests with Dunn multiple comparisons (*$P < .05$). (D-E) Receiver-operating-characteristics curve analyses. Receiver-operating-characteristics curve analyses with RELs of vitreous miRNAs for discrimination of patients with PVRL (n = 10) and vitritis patients (n = 40). Vitreous relative expression of miR-92 yielded an AUC of 0.9725 (95% confidence interval [CI], 0.9340-1.011) (D), for miR-19b an AUC of 0.9400 (95% CI, 0.8555-1.024) (E), and for miR-21 an AUC of 0.8125 (95% CI, 0.6723-0.9527) (F), respectively.

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Contribution: V.K., R.S., and A.B. designed the study; R.S. and A.B. performed the miRNA experiments; V.K., M.-I.W., M.S., C.H., S.W., U.S., P.L., G.A., and S.E.C. contributed samples, evaluated data, and performed clinic-pathological correlation; V.K., R.S., C.H., A.H., U.S., H.B.D., W.S., S.E.C., and A.B. interpreted the results; and V.K., R.S., S.E.C., and A.B. wrote the manuscript. All authors critically reviewed the manuscript.

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