bjh short report

Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma

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Summary

Circulating nucleic acids have been shown to have potential as non-invasive diagnostic markers in cancer. We therefore investigated whether microRNAs also have diagnostic utility by comparing levels of tumour-associated *MIRN155* (*miR-155*), *MIRN210* (*miR-210*) and *MIRN21* (*miR-21*) in serum from diffuse large B-cell lymphoma (DLBCL) patients ($n = 60$) with healthy controls ($n = 43$). Levels were higher in patient than control sera ($P = 0{\cdot}009$, $0{\cdot}02$ and $0{\cdot}04$ respectively). Moreover, high *MIRN21* expression was associated with relapse-free survival ($P = 0{\cdot}05$). This is the first description of circulating microRNAs and suggests that microRNAs have potential as non-invasive diagnostic markers for DLBCL and possibly other cancers.

Keywords: microRNA, diffuse large B-cell lymphoma, lymphoma, sera, plasma.

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MicroRNAs are a recently discovered class of naturally occurring short non-coding RNA molecules that negatively regulate eukaryotic gene expression through binding to complementary sequences in the 3′UTR of target mRNA. There are more than 400 known human microRNAs and more than 1000 predicted microRNA sequences await scientific confirmation (Griffiths-Jones *et al*, 2006). It is currently believed that between 10–30% of all human genes are targets for microRNA regulation. MicroRNAs play key regulatory roles in a diverse range of pathways, including haematopoiesis, and there is rapidly accumulating evidence to suggest that dysfunctional expression of microRNAs is a common feature in haematological malignancy (Lawrie, 2007).

The search for non-invasive tools for the diagnosis and management of cancer has long been a goal of cancer research that has led to great interest in the field of circulating nucleic acids in plasma and serum (Tsang & Lo, 2007). Many studies have shown that specific cancer characteristics, both genetic and epigenetic, are detectable in the plasma and serum of cancer patients and may be useful as a tool for early detection, diagnosis and follow-up of cancer patients (Tsang & Lo, 2007).

The current study investigated whether circulating microRNAs can be detected in serum and whether expression levels of specific microRNAs differ between cancer patients [diffuse large B-cell lymphoma (DLBCL)] and healthy individuals. As yet there is no detailed study of microRNA expression in DLBCL, therefore we investigated expression of *MIRN155* (*miR-155*) and *MIRN21* (*miR-21*), both found to be highly expressed in DLBCL (Lawrie *et al*, 2007) as well as *MIRN210* (*miR-210*), which is associated with a more general malignant phenotype (Kulshreshtha *et al*, 2007).

Methods

We performed a retrospective study that analyzed serum samples from 60 patients diagnosed with *de novo* DLBCL,

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Table I. Summary of clinical details of DLBCL patients used for sera analysis ($n = 60$).

	n (%)
Sex	
Men	39 (65)
Women	21 (35)
Age range, years	
Mean (SD)	63 (11)
Stage	
1	20 (33)
2	14 (23)
3	18 (30)
4	8 (13)
Extranodal involvement	
Yes	33 (55)
No	27 (45)
International Prognostic Index score	
0	6 (10)
1	11 (18)
2	25 (42)
3	12 (20)
4	6 (10)
Follow-up, months ($n = 52$)*	
Mean [relapse-free ($n = 38$)] (SD)	27 (16)

*No survival data available for eight patients.

taken at the time of diagnosis (summarized in Table I). Further patient details, including treatment regimes and *MIRN21* expression, can be found in Table SI. Forty-three serum samples from healthy individuals (27 males, 16 females, median age 57) were used as controls. Ethical permission and informed consent was obtained for the use of all samples.

RNA was extracted from 2 ml of serum using Trizol LS reagent (Invitrogen, Paisley, UK) as described by the manufacturer. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using Taqman microRNA probes as described by the manufacturer (Applied Biosystems, Warrington, UK), using 10 ng of total RNA per reaction in a Roche LightCycler 480 machine (Roche, Sussex, UK). Triplicate samples were used throughout. As there is no current consensus on the use of house-keeping microRNAs for qRT-PCR analysis, and because we could not detect U6 or 5s in sera samples, based on previously published results (Mattie *et al*, 2006; Zhang *et al*, 2006) and as recommended by the manufacturer (Applied Biosystems), we used *MIRN16* (*miR-16*) levels for normalization. In order to test variability of *MIRN16* expression with other microRNAs that have previously been used for qRT-PCR normalization (Bandres *et al*, 2006; Lawrie *et al*, 2007), we compared sample *MIRN16* C_t values with those of *MIRNLET7A* (*let-7a*) and *MIRN24* (*miR-24*) and found a very good level of concordance ($P < 0{\cdot}0001$; $r^2 = 0{\cdot}85$ and 0·87 respectively; data not shown). There was no significant difference in *MIRN16* C_t values, or any other microRNAs measured between control and lymphoma samples, suggesting there was no general over-representation of microRNA in lymphoma samples (data not shown). The ΔC_t method was used for analysis [ΔC_t = mean C_t (*MIRN16*) − mean C_t (microRNA of interest)].

MicroRNA expression levels were compared using Mann–Whitney independent *t*-test. Kaplan–Meier survival analysis was carried out on relapse-free survival times of DLBCL patients for whom follow-up details were available ($n = 52$) as a function of microRNA expression level, using the median value as a high/low cut-off. Relapse-free and overall survival times were calculated from the time of diagnosis to the date of relapse, death or last contact. Curves were compared by univariate (logrank) analysis. Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad, San Diego, CA, USA). *P*-values ≤ 0·05 were considered significant.

Results and discussion

The expression levels of three tumour-associated microRNAs (*MIRN155*, *MIRN210* and *MIRN21*) were compared in serum samples from DLBCL patients ($n = 60$) with those of healthy controls ($n = 43$). We found levels of these microRNAs to be upregulated in patient sera ($P = 0{\cdot}009$, 0·02 and 0·04 respectively) (Fig 1A–C), corresponding to an average fold-change of 5·24, 4·15 and 2·56 respectively [fold change = $2^{(\text{mean control } \Delta C_t - \text{mean patient } \Delta C_t)}$].

Moreover, high expression levels of *MIRN21* in DLBCL patient sera were found to be associated with improved relapse-free survival times ($P = 0{\cdot}05$) (Fig 1D) but not overall survival ($P = 0{\cdot}07$; data not shown). These data are consistent with our previous findings in biopsy material from a different cohort of DLBCL patients, where high tumoural *MIRN21* expression was also associated with a better prognostic outcome (Lawrie *et al*, 2007). We found no significant correlation between expression of either *MIRN155* or *MIRN210* and prognosis in this series of cases ($P = 0{\cdot}22$ and 0·86 respectively). We also investigated associations between microRNA levels and clinicopathological features of the DLBCL patients (i.e. sex, International Prognostic Index, stage and presence of extranodal disease) but found no significant correlations (data not shown).

Why high levels of *MIRN21* should be associated with a more favourable clinical outcome for DLBCL patients remains to be determined. Although *MIRN21* has been found to have an anti-proliferative effect in some cancers (Si *et al*, 2006), the opposite appears to be true for other cancers (Cheng *et al*, 2005). Presumably it is the targeting of oncogenes and/or anti-apoptotic molecules by *MIRN21* that is important in determining clinical outcome in DLBCL patients. Amongst the 72 putative *MIRN21* targets listed by two or more predictive algorithms (Table SII) there are several interesting candidate genes including *PLAG1*, *E2F3*, *JAG1*, *SKI* and *STAT3* that should be explored further.

This is the first study, to our knowledge, that demonstrates the presence of microRNAs in biological fluids. Whilst it would be tempting to hypothesize that the detected

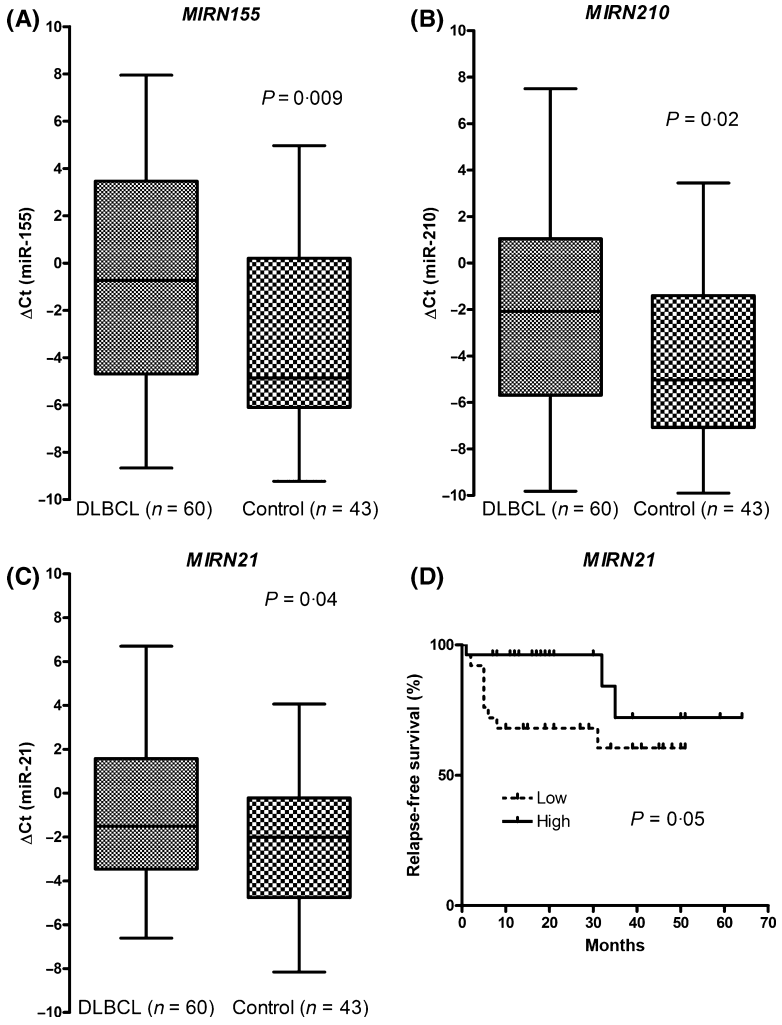

Fig 1. Sera levels of (A) *MIRN155*, (B) *MIRN210* and (C) *MIRN21* are higher in DLBCL patient sera ($n = 60$) than healthy controls ($n = 43$) and (D) sera levels of *MIRN21* are associated with relapse-free survival in DLBCL patients. Values shown (ΔC_t) are relative to levels of *MIRN16*. *P*-values were calculated by Mann–Whitney independent *t*-test. Kaplan–Meier survival curves were compared by univariate (logrank) analysis and *MIRN21* expression levels in patients defined as high or low relative to the median.

extra-cellular microRNAs are derived from tumour cells, it is equally possible that these microRNAs arose from cells infiltrating the lymphomas or local organ or node involvement and may be part of an indirect response to cancer. For example *MIRN155*, in addition to being upregulated in certain lymphomas but not others (Kluiver *et al*, 2005), is necessary for normal immune function (Rodriguez *et al*, 2007). This is an area that clearly needs further research.

For this study we analysed serum samples but we have also successfully amplified microRNAs from plasma samples (data not shown). It would also be interesting to investigate microRNA expression in other body fluids, such as urine. Circulating microRNAs may have clinical utility for diagnosis, monitoring and follow-up of patients and this should be explored by conducting further studies with larger sample numbers and by recruiting patients prospectively within clinical trials so that serial measurements of the microRNA expression could be achieved before, during and after therapy.

In summary, we have shown that circulating microRNAs are clearly detectable in serum samples and that higher levels of specific microRNAs are associated with diagnosis and prognostic outcome in DLBCL patients. This data suggests that microRNAs have potential as clinically useful non-invasive biomarkers in cancer.

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References

Bandres, E., Cubedo, E., Agirre, X., Malumbres, R., Zarate, R., Ramirez, N., Abajo, A., Navarro, A., Moreno, I., Monzo, M. & Garcia-Foncillas, J. (2006) Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Molecular Cancer*, **5,** 29–39.

Cheng, A.M., Byrom, M.W., Shelton, J. & Ford, L.P. (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Research*, **33,** 1290–1297.

Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A. & Enright, A.J. (2006) miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Research*, **34,** D140–D144.

Kluiver, J., Poppema, S., de Jong, D., Blokzijl, T., Harms, G., Jacobs, S., Kroesen, B.J. & van den Berg, A. (2005) BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *Journal of Pathology*, **207,** 243–249.

Kulshreshtha, R., Ferracin, M., Wojcik, S.E., Garzon, R., Alder, H., Agosto-Perez, F.J., Davuluri, R., Liu, C.G., Croce, C.M., Negrini, M., Calin, G.A. & Ivan, M. (2007) A microRNA signature of hypoxia. *Molecular and Cellular Biology*, **27,** 1859–1867.

Lawrie, C.H. (2007) microRNAs and haematology: small molecules, big function. *British Journal of Haematology*, **136,** 503–512.

Lawrie, C.H., Soneji, S., Marafioti, T., Cooper, C.D.O., Palazzo, S., Paterson, J.C., Cattan, H., Enver, T., Mager, R., Boultwood, J., Wainscoat, J.S. & Hatton, C.S.R. (2007) MicroRNA expression distinguishes between germinal center B cell-like (GCB) and activated B cell-like (ABC) subtypes of diffuse large B cell lymphoma. *International Journal of Cancer*, **121,** 1156–1161.

Mattie, M.D., Benz, C.C., Bowers, J., Sensinger, K., Wong, L., Scott, G.K., Fedele, V., Ginzinger, D., Getts, R. & Haqq, C. (2006) Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. *Molecular Cancer*, **5,** 24.

Rodriguez, A., Vigorito, E., Clare, S., Warren, M.V., Couttet, P., Soond, D.R., van Dongen, S., Grocock, R.J., Das, P.P., Miska, E.A., Vetrie, D., Okkenhaug, K., Enright, A.J., Dougan, G., Turner, M. & Bradley, A. (2007) Requirement of bic/microRNA-155 for normal immune function. *Science*, **316,** 608–611.

Si, M.L., Zhu, S., Wu, H., Lu, Z., Wu, F. & Mo, Y.Y. (2006) miR-21-mediated tumor growth. *Oncogene*, **26,** 2799–2803.

Tsang, J.C. & Lo, Y.M. (2007) Circulating nucleic acids in plasma/serum. *Pathology*, **39,** 197–207.

Zhang, L., Huang, J., Yang, N., Greshock, J., Megraw, M.S., Giannakakis, A., Liang, S., Naylor, T.L., Barchetti, A., Ward, M.R., Yao, G., Medina, A., O'Brien-Jenkins, A., Katsaros, D., Hatzigeorgiou, A., Gimotty, P.A., Weber, B.L. & Coukos, G. (2006) MicroRNAs exhibit high frequency genomic alterations in human cancer. *Proceedings of the National Academy of Sciences of the United States of America*, **103,** 9136–9141.

Supplementary material

The following supplementary material is available for this article online:

Table SI. Clinical details of DLBCL patients. *IPI group calculated according to *N Engl J Med* (1993) 329: 987–994. NA, not applicable because patient had no available follow-up data.

Table SII. Target genes predicted by two or more algorithms for miR-21 indicating cancer associations.

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