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Original Paper

BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas

Joost Kluiver, Sibrand Poppema, Debora de Jong, Tjasso Blokzijl, Geert Harms, Susan Jacobs, Bart-Jan Kroesen and Anke van den Berg*
Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands

**Correspondence to:*
Anke van den Berg, Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.
E-mail: a.van.den.berg@path.umcg.nl

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Abstract

In a previous study we demonstrated high expression of the non-coding *BIC* gene in the vast majority of Hodgkin's lymphomas (HLs). Evidence suggesting that *BIC* is a primary microRNA transcript containing the mature microRNA-155 (miR-155) as part of a RNA hairpin is now accumulating. We therefore analysed HL cell lines and tissue samples to determine whether miR-155 is also expressed in HL. High levels of miR-155 could be demonstrated, indicating that *BIC* is processed into a microRNA in HL. Most non-HL subtypes were negative for *BIC* as determined by RNA-ISH. However, in diffuse large B cell lymphoma (DLBCL) and primary mediastinal B cell lymphoma (PMBL), significant percentages of positive tumour cells were observed in 12/18 and 8/8 cases. A higher proportion of tumour cells were positive for *BIC* in DLBCL with activated B cell-like phenotype than in DLBCL with germinal centre B cell-like phenotype. Differential *BIC* expression was confirmed by qRT-PCR analysis. Northern blot analysis showed expression of miR-155 in all DLBCL and PMBL derived cell lines and tissue samples analysed. In summary, we demonstrate expression of primary microRNA *BIC* and its derivative miR-155 in HL, PMBL and DLBCL.

Keywords: Hodgkin's lymphoma; microRNA; *BIC*; microRNA-155; primary mediastinal B cell lymphoma; diffuse large B cell lymphoma

Introduction

MicroRNAs (miRNAs) are endogenous ~22 nt RNAs that play important regulatory roles by targeting mRNAs for cleavage or translational repression [1]. To date, hundreds of miRNA have been identified, representing approximately 1% of all predicted genes in most species [2]. Chen and colleagues reported a set of miRNAs that can modulate haematopoietic lineage differentiation, and demonstrated that overexpression of miR-181 in haematopoietic stem/progenitor cells resulted in an increase in the number of B lymphoid lineage cells [3]. Low levels of miR-15 and miR-16 were reported in the majority of CLL cases, either by downregulation or by hemizygous and homozygous deletions of a 30 kb region at 13q14 [4]. In addition, miRNA expression profiling revealed different profiles in Zap-70 positive and negative CLL [5], suggesting that miRNA expression patterns contribute to the pathophysiology of this disease. Interestingly, more than half of the miRNAs are mapped within cancer-associated genomic regions or fragile sites [6]. Reduced levels of specific miRNAs were observed in colon and lung tumours, supporting a role for miRNAs in carcinogenesis [7,8]. Taken together, these data provide good evidence that miRNAs play key roles in the regulation of a variety of cellular processes.

We previously reported high expression of *BIC* in more than 90% of Hodgkin's lymphomas (HLs) [9]. The expression was observed in HL/Reed–Sternberg cells (HRS) of classical HL (cHL) and the lymphocytic and histiocytic (L&H) cells of nodular lymphocyte-predominant (NLP) HL. No *BIC* expression was observed in the neoplastic cells of several non-HL (NHL) subtypes [9]. A putative role for *BIC* in lymphomagenesis was supported by frequent over-expression of *BIC* in virally induced lymphomas in chickens [10]. Based on the fact that *BIC* lacks an extensive and conserved open reading frame, it was suggested that *BIC* functions via its RNA transcripts [10]. The high degree of homology observed over a 138 nucleotide (nt) region between human, mouse and chicken, which contains an imperfect stem–loop structure [11], suggests that *BIC* might function as a primary-miRNA (pri-miRNA). Pri-miRNAs are located in the nucleus and processed, based on the presence of a stem–loop structure, into ~70 nt precursor-miRNAs (pre-miRNAs) [12,13]. The nuclear localization of *BIC* transcripts observed in HL and normal tissues is thus consistent with a putative pri-miRNA function. Further support for a pri-miRNA function was obtained by cloning of the *BIC* derived miRNA, miR-155 [14] and by demonstrating that

ectopic *BIC* expression in a *BIC* and miR-155 negative cell line resulted in high levels of miR-155 [15].

In this study, we report the presence of miR-155 at high levels in HL cell lines and tissues. In addition, we demonstrate *BIC* and miR-155 expression in some additional NHL subtypes.

Materials and methods

Cell lines and tissues

All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research. The cHL cell lines L591, L428, HDLM-2, KM-H2, L1236 and the NLP HL cell line DEV were used. The HDLM-2 cell line is of T cell origin, while the other HL cell lines are of B cell origin [16–18]. The following NHL cell lines were used: K1106P (primary mediastinal B cell lymphoma, PMBL), SU-DHL-6 [diffuse large B cell lymphoma, DLBCL, t(14;18)], Ver [DLBCL, t(8;14)] and Rose [DLBCL, t(14;18)]. Cell lines were cultured in RPMI-1640 medium (Cambrex Biosciences, Walkersville, MD) supplemented with ultraglutamine 1 (Cambrex Biosciences), 100 U/ml penicillin/streptomycin and 10% FCS (Cambrex Biosciences) or 20% FCS (DEV and K1106P) at 37 °C in an atmosphere containing 5% CO_2. Frozen (northern blotting and qRT-PCR) and paraffin-embedded tissues (*BIC* ISH, IHC and qRT-PCR) of HL and various NHL cases were randomly selected from the tissue bank at the Department of Pathology and Laboratory Medicine, University Medical Centre Groningen. Reactive lymph node and tonsil tissue specimens were included as control tissues.

RNA *in situ* hybridization (ISH)

RNA-ISH was performed as described previously [9], and was used to detect the full-length primary *BIC* transcript. The slides were scored independently by SP and JK, and the average of the two scores was used as the percentage of *BIC*-positive cells. In discrepant cases, slides were scored again simultaneously to reach consensus. All cases were routinely stained with a probe for ß-actin to ensure the RNA quality and fixation of the tissue samples. Cases were scored positive when more than 1% of the tumour cells stained positive.

RNA isolation and northern blotting

Total RNA from frozen tissue sections was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA from the cell lines was isolated using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). After DNAse treatment, the integrity of the RNA was checked on a 1% agarose gel and only good quality RNA samples were used for subsequent analysis. Total RNA (20 μg) was loaded on a 7.5 M urea 12% PAA denaturing gel and after electrophoresis transferred to Hybond N^+ nylon membrane (Amersham, Freiburg, Germany) and cross-linked using UV light. Hybridization with the miR-155 antisense starfire probe 5′-CCCCTATCACGATTAGCATTAA-3′ (to detect miR-155) or miR-155 sense probe 5′-TTAATG CTAATATGTAGGAG-3′ (as a negative control) (IDT, Coralville, IA) was performed according to the manufacturer's instructions. After washing, the membranes were exposed for 20–50 h to Kodak XAR-5 films (Sigma Chemical, St Louis, MO). As a loading control, hybridizations were performed with an antisense U6-snRNA starfire probe, 5′-GCAGGGGCCATGCTA-ATCTTCTCTGTATCG-3′ [19] and the membranes were exposed for 15–30 min. Due to material limitations, we could only analyse a limited number of lymphoma cases by this method.

Quantitative RT-PCR

For the paraffin-embedded DLBCL and PMBL cases, RNA was isolated using a previously described protocol [20]. All RNA samples were treated with DNase, followed by a multiplex PCR with primer sets specific for genomic DNA to monitor the efficiency of the DNAse procedure. Quantitative RT-PCR (qRT-PCR) was performed as described previously and resulted in the specific amplification of *BIC* transcripts [9]. *Glyceraldehyde-3-phophate dehydrogenase* (*GAPDH*) was used for normalization. The *GAPDH* primers and probe were: F, 5′-CCACATCGCTCAGACACCAT-3′; R, 5′-GCGCCCAATACGACCAAAT-3′; and P, 5′-6-FAM-CCCTTCCACTTCCAGCCTCAGTTGC-TAM-RA-3′. The relative amount of *BIC* was calculated by subtracting the average C_T value for *BIC* from the average C_T value of the reference gene (ΔC_T). Relative expression levels were expressed as $2^{-\Delta CT}$.

Immunohistochemistry

Immunohistochemical staining was performed with mouse anti-human monoclonal antibodies against CD10 (1 : 20, 56C6, Novacastra, Newcastle, UK), MUM1 (1 : 25, MUM1p, DAKO, Copenhagen, Denmark) and Bcl-6 (1 : 20, PG-B6P, DAKO) on paraffin-embedded tissue sections after antigen retrieval. Positive staining was visualized using a peroxidase-labelled second step and diaminobenzidine (DAB). DLBCL cases were classified as germinal centre B cell-like (GCB-like) or activated B cell-like (ABC-like) according to Hans *et al* [21].

Results

BIC RNA-ISH

We previously reported positive staining for *BIC* in a few cases of DLBCL, typically in a minority of tumour cells [9]. We have now extended the

Table 1. Overview of *BIC* RNA-ISH results in various NHL subtypes and HL

Tissue	BIC$^-$	BIC$^+$
FL*	15	0
BL*	8	1
MCL	6	0
MZL	0	2
DLBCL	6	12
PMBL	0	8
HL*	5	53
TCRBCL†	16	1
ALCL*	7	0
TCL	0	3

FL, follicular lymphoma; BL, Burkitt's lymphoma; MCL, mantle cell lymphoma; TCRBCL, T cell-rich B cell lymphoma; MZL, marginal zone lymphoma; DLBCL, diffuse large B cell lymphoma; PMBL, primary mediastinal B cell lymphoma; HL, Hodgkin's lymphoma; ALCL, anaplastic large cell lymphoma; TCL, T cell lymphoma. * Cases described previously [9]. † Cases partly described previously [9].

DLBCL group and screened additional NHL subtypes (Table 1). *BIC* RNA-ISH revealed characteristic nuclear staining for *BIC* in a variable percentage of tumour cells in the majority of DLBCL cases (12/18, ~67%) (Table 2). DLBCL cases were stained for CD10, Bcl-6 and Mum1 to classify them into GCB-like or ABC-like [21]. Eleven cases (61%) were assigned to the GCB-like group and seven cases (39%) to the ABC-like group (Table 2). Interestingly, ABC-like DLBCL showed more than twice as many *BIC*-positive cells as GCB-like DLBCL. In addition, screening of eight PMBL cases by RNA-ISH revealed *BIC*-positive staining in 12% on average (range 2–30%) of the tumour cells. All PMBL cases were negative for CD10 and positive for MUM1 and in 7/8 cases positive for Bcl-6, indicating an ABC-like phenotype (Table 2).

RNA-ISH in other NHL subtypes revealed no *BIC* expression in 11 TCRBCL and six MCL cases (Table 1). In some cases a few weakly positive small cells were observed that, based on the morphology, most likely do not represent the tumour cell population. One TCRBCL case was positive in a low percentage of tumour cells. The three TCL cases showed *BIC* staining in 1–2% of the tumour cells (Figure 1). Two MZL cases were positive; in one case the majority of cells in the marginal zone were positive, while in the other case the positive cells were mainly present in the mantle zone.

Quantification of *BIC* expression levels in DLBCL and PMBL

To confirm differences in *BIC* expression levels observed by RNA-ISH in DLBCL and PMBL we applied qRT-PCR (Table 2) on paraffin-embedded or frozen tissue samples. To determine the accuracy of the qRT-PCR on paraffin-embedded tissues, we tested several tissues both on paraffin-embedded tissues and frozen material, which revealed similar *BIC* expression levels (bottom of Table 2). The relative *BIC* levels in ABC- and GCB-like DLBCL and PMBL, eg highest in PMBL and lowest in GCB-like DLBCL, were in agreement with the results of the RNA-ISH data.

MiR-155 analysis

Using northern blot analysis with an antisense miR-155 probe, a strong 22 nt hybridization signal was observed in HL cell lines DEV, L1236, L591 and KM-H2, in the PMBL cell line K1106P and in the DLBCL cell line Rose. HL cell line L428 and DLBCL lines VER and SU-DHL-6 were weakly positive. The T cell HL cell line HDLM-2 was negative for miR-155 (Figure 2A). Using a miR-155 sense probe no signal was observed, providing experimental support that only one of the two stem sequences is retained as a miRNA (data not shown).

Northern blot analysis on various tissue samples revealed a strong miR-155 hybridization signal in seven HL cases, two randomly chosen DLBCL cases and one PMBL case. In reactive lymph node and tonsil, which show a low percentage of *BIC* RNA-ISH positive cells, miR-155 was also detected at high levels (Figure 2B).

To compare expression of miR-155 with *BIC*, we quantified *BIC* levels using qRT-PCR in all samples analysed for miR-155 (Table 3). The four HL cell lines and the PMBL cell line with strong miR-155 signals also showed high *BIC* levels (DEV, KM-H2, L1236, L591 and K1106P). Cell lines without (HDLM-2) or low miR-155 levels (L428, VER and SU-DHL-6) all showed lower amounts of *BIC*. The only cell line with an inconsistent result was Rose, which demonstrated a strong signal for miR-155 and only a low level of *BIC*. Whole tissue sections from seven HL cases, two DLBCL and one PMBL revealed a uniform *BIC* expression level consistent with the miR-155 levels.

Discussion

In this study we demonstrated high *BIC* and miR-155 levels in four of the six HL cell lines (L1236, L591, KM-H2 and DEV). The other two HL cell lines, L428 and HDLM-2, expressed low levels of *BIC* and miR-155 analysis revealed low or no expression, respectively. The lack of miR-155 in HDLM-2 may relate to its T cell derivation or to reduced processing potential of *BIC* transcripts. These observations demonstrate a good relation between high *BIC* and miR-155 levels and support the hypothesis that *BIC* is a pri-miRNA that can be processed to miR-155. This was recently confirmed by induction of ectopic *BIC* expression in a *BIC*- and miR-155-negative cell line, resulting in a high level of miR-155 [15].

In HL tissues, the absolute levels of both the primary *BIC* transcripts and miR-155 are on average similar to those observed in the HL cell lines (Figure 2, Table 3).

Table 2. Quantification of *BIC* expression levels in DLBCL, PMBL, HL and controls

Tissue	Case No	CD10	MUM1	Bcl-6	Site	*BIC* ISH (%)	*BIC* ($\times 10^{-3}$) (paraffin tissue)	*BIC* ($\times 10^{-3}$) (frozen tissue)
GCB-like	1	+	+	+	N	–	11	
DLBCL	2	+	–	+	N	1–2	5	
	3	+	–	+	N	–	2	
	4	–	–	+	N	–	3	
	5	+	+	+	E	20	47	23
	6	+	+	+	E	–	9	
	7	+	+	+	E	5	19	
	8	+	+	+	E	–	3	
	9	–	–	+	E	5	4	
	10	–	–	+	E	5	11	
	11	–	–	+	E	10	24	
	Average					4.2	12.4	
ABC-like	12	–	+	+	N	1–2	23	19
DLBCL	13	–	+	+	N	10	nd	
	14	–	+	+	N	–	9	
	15	–	+	–	E	50	32	
	16	–	+	–	E	5	30	
	17	–	+	–	E	5	28	
	18	–	+	–	E	10	11	
	Average					11.6	21.9	
DLBCL	Average					7.1	15.8	
PMBL	1	–	+	+		10	38	
	2	–	+	+		3–10	nd	
	3	–	+	+		2	16	
	4	–	+	+		2	77	
	5	–	+	+		10	34	
	6	–	+	+		30	nd	
	7	–	+	+		25	26	5
	8	–	+	–		10	21	
	Average					11.9	35	
HL	1						7	5
	2						4	7
	3						5	12
	4						7	4
	5						12	7
	Average						7.0	6.8
RL	1						4	4
	2						5	6
	3						6	4
	4						4	4
	5						3	3
	Average						4.6	4.0

GCB, germinal centre B cell; ABC, activated B cell; N, nodal presentation; E, extranodal presentation; nd, not determined; RL, reactive lymph node.

This finding is unexpected, since it is known that HRS and L&H cells in HL tissues make up only 1% or less of the total cell population. A possible explanation for this discrepancy might be that HRS and L&H cells in HL tissues are triggered for enhanced *BIC* expression by the T cells directly surrounding the tumour cells, while this interaction is lacking in the cell lines. Another possible explanation might be that in the cell lines only a low percentage of the cells are *BIC*-positive. Due to lack of a reliable RNA-ISH protocol for cell lines, we could not investigate this aspect further.

We have previously observed *BIC* expression in some of the cells within germinal centres of reactive lymphoid tissues, suggesting that *BIC* positivity may be a reflection of the activation status of the lymphoid cells [9]. This is indeed confirmed by the strong induction of *BIC* expression in both activated B and T cells [9,22]. Nevertheless, we did not detect *BIC*-positive reactive cells in HL tissues. This difference might be due to the different cellular composition of HL tissues and reactive lymphoid tissues. In addition, it might also be explained by the intrinsic differences of the reactive cells of HL, such as the anergic/immunoregulatory phenotype of the T cells [23] or the different microenvironment due to abnormal cytokine production in HL tissues [24].

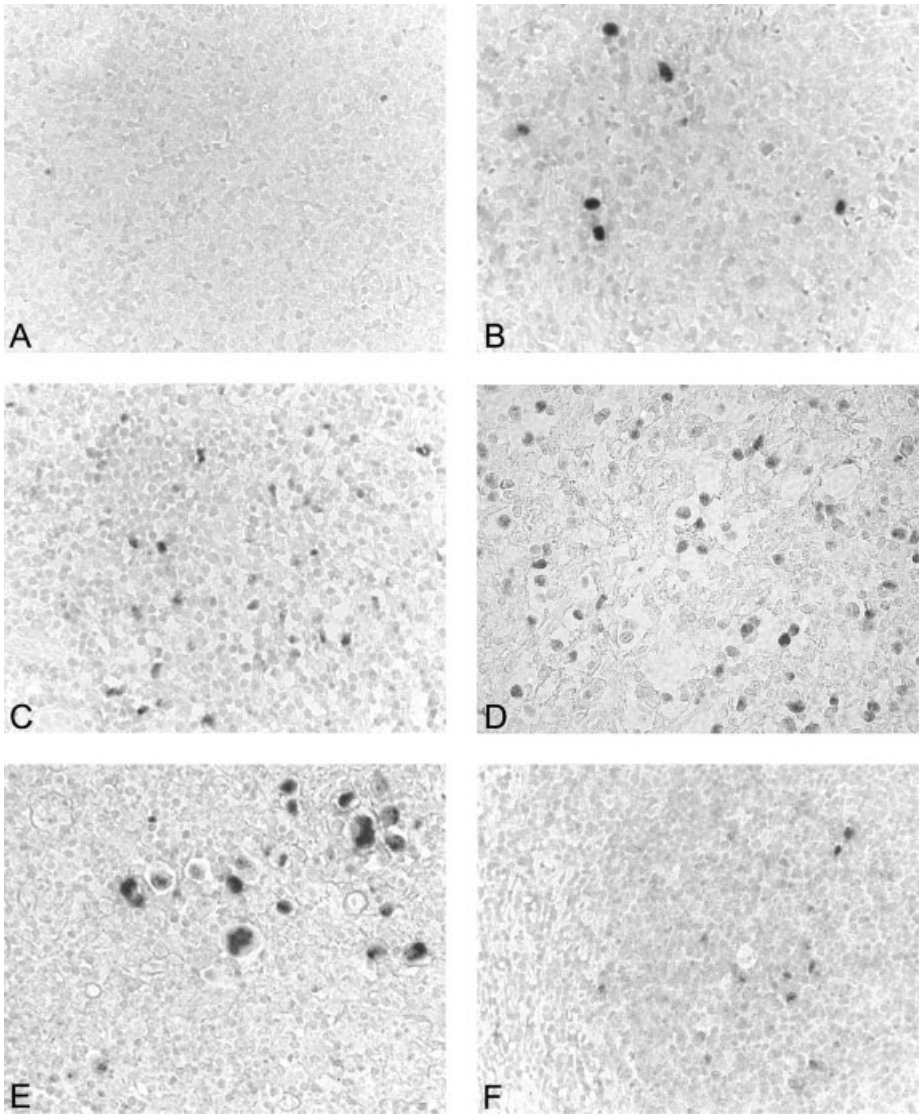

Figure 1. *BIC* RNA-ISH in various NHL subtypes. (A) *BIC*-negative mantle cell lymphoma; (B) T cell lymphoma showing approximately 2% *BIC*-positive cells; (C) diffuse large B cell lymphoma with 5% positive cells; (D) primary mediastinal B cell lymphoma with 10% of the cells positive for *BIC*, as controls; (E) classical Hodgkin's lymphoma case with a group of strongly positive Reed–Sternberg cells; and (F) a tonsil with a few positive cells within the germinal centre (original magnifications ×400)

The three TCLs showed a low percentage of *BIC*-positive tumour cells. Induction of *BIC* expression was demonstrated in $CD4^+$ T cells upon activation with anti-CD3 and anti-CD28 antibodies, demonstrating that *BIC* can be induced in T cells [22]. Whether *BIC*-positive cells in TCL can be attributed to an activated phenotype remains to be investigated.

In this study we show that, in addition to HL, PMBL and a large proportion of DLBCL cases are also *BIC*-positive, albeit in a smaller proportion of the tumour cells. Currently, the biological implication of the variation of *BIC* expression within these lymphomas is not known. It can be speculated that, in contrast to HL, in which consistent *BIC* expression is demonstrated in the vast majority of tumour cells, the induction of *BIC* expression in some of the tumour cells in DLBCL and PMBL cases is dependent on specific external or internal stimulatory signals. This suggests that, in DLBCL and PMBL, the high *BIC* expression in some of the cells displays a specific (activation) state rather than an oncogenic marker.

Comparison of ABC-like with GCB-like DLBCL revealed more pronounced expression of *BIC* in ABC-like cases by RNA-ISH and qRT-PCR (Table 2). These findings are in agreement with a recent publication of Eis *et al* (2005), who also demonstrated low *BIC* and miR-155 level in two GCB-like DLBCL cases and an enhanced *BIC* and miR-155 expression level in nine ABC-like DLBCL cases, using specific invader mRNA assays [15]. In addition, they showed accumulation of *BIC* and miR-155 in five HL cell lines, three CLL cases and one case of marginal zone lymphoma.

Based on high *BIC* levels observed by both RNA-ISH and qRT-PCR, we speculate that the ABC-like phenotype is related to higher expression of *BIC*. Interestingly, it was shown that nuclear factor (NF)-κB transcription factor activity correlates with

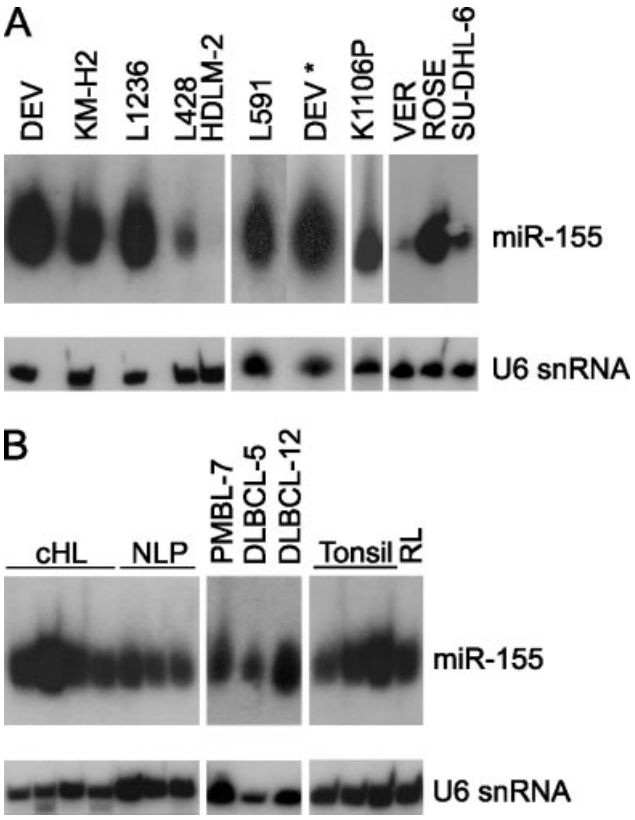

Figure 2. Northern blot analysis for miR-155. (A) The majority of the Hodgkin's, primary mediastinal and diffuse large B cell lymphoma cell lines express miR-155 strongly, as do (B) primary cases of HL, PMBL and DLBCL. As a control, tonsils and a reactive lymph node were analysed. U6 snRNA hybridizations results are shown as a quality and loading control. *Note that L591 was analysed on a separate blot, to compare it with the other HL cell lines DEV was also analysed on this blot

Table 3. Overview of qRT-PCR for *BIC* in HL, PMBL, DLBCL cell lines and tissues and normal tissues that were also analysed for miR-155 by Northern blot

	Cell line	Rel. *BIC* ($\times 10^{-3}$)	Tissue	Rel. *BIC* ($\times 10^{-3}$)
HL	KM-H2	6	cHL-1	5
	L1236	4	cHL-2	7
	L428	0.03	cHL-3	3
	HDML-2	0.08	cHL-4	4
	L591	10	NLP HL-1	3
	DEV	8	NLP HL-2	4
			NLP HL-3	3
PMBL	K1106P	3	PMBL-7	3
DLBCL	VER	0.08	DLBCL-5	11
	ROSE	0.09	DLBCL-12	9
	SU-DHL-6	0.4		
Normal tissues			T-1	2
			T-2	3
			T-3	3
			RL	5

cHL-1, classical HL 1; NLP HL-1, nodular lymphocyte predominant HL-1; T-1, Tonsil-1.

the ABC-like DLBCL phenotype [25]. Constitutively activated NF-κB is also one of the hallmarks of HRS cells [26] and a putative NF-κB binding site is present in the promoter region of the *BIC* gene [9]. Despite our original findings that triggering of the B cell receptor in a RAMOS cell line, transfected with a non-degradable form of IκB, did not completely block the induction of *BIC*, a strongly reduced *BIC* expression level was observed at 24 h compared to wild-type RAMOS [9]. This reduced *BIC* induction has now been confirmed in several additional experiments (unpublished data). Therefore, it can be speculated that NF-κB activity plays a role in the induction of a high *BIC* expression.

Several studies have reported a close relationship between HL and PMBL cases, based on similar gene expression profiles and common genomic aberrations [27,28]. It will be of interest to analyse the miRNA expression profiles of HL and PMBL cases to determine whether these two entities are also related with respect to miRNA expression profile. Our finding that *BIC* and miR-155 are expressed in both lymphoma subtypes indicates a new common pathophysiological feature of HL and PMBL and warrants further studies.

Our results show that, with the exception of PMBL and DLBCL, the tumour cells of most NHL subtypes are *BIC*-negative. Nonetheless, we could detect miR-155 in whole tissue of some NHL cases with a percentage of *BIC*-positive cells below the 1% cutoff used for RNA-ISH (data not shown). Since similar low percentages of *BIC*-positive cells in normal tissues give rise to detectable miR-155 levels, it can be anticipated that miR-155 is also present at similar levels in these NHL cases.

The relevance of high miR-155 expression in HL is not yet known. Given the high expression of *BIC* and miR-155 in HL, PMBL and DLBCL, it may be speculated that downregulation of one or more of the miR-155 target genes is involved in the pathophysiology of these diseases. So far, a number of potential miR-155 target genes have been reported based on computational algorithms [19,29–32]. None of these target genes were verified *in vitro* or *in vivo* and different targets have been reported in each study (see Supplementary data table). One potentially interesting putative target of miR-155 is ICOSL [19]. ICOS-ICOSL signalling is important in T cell activation, proliferation and cytokine production [33,34]. HRS are surrounded by ICOS expressing T cells (unpublished results) and the lack of ICOSL expression may thus influence the immune response. Another miR-155 target is the transcription factor PU.1 [29], a protein required for early B cell differentiation [35]. Absence of PU.1 protein expression is thought to be associated with defective immunoglobulin transcription in HRS cells of cHL [36,37].

In summary, we demonstrate high levels of *BIC* in the majority of tumour cells in HL and in a variable percentage of tumour cells in DLBCL and PMBL. Consistent with these findings, Northern blot

analysis of various cell lines and tissues demonstrated high miR-155 levels in HL, PMBL and DLBCL. In DLBCL, *BIC* expression appears to be associated with an ABC-like phenotype, possibly as a result of NF-κB activation.

Supplementary material

A supplementary table showing putative target genes for miR-155 can be found at the website: http://www3.interscience.wiley.com/cgi-bin/jabout/1130/suppmatt.htm

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