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microRNA expression in lymphoid malignancies: new hope for diagnosis and therapy?

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Abstract

microRNAs are a newly discovered class of short (~22 nt) naturally occurring single-stranded RNA molecules that regulate the expression of target genes post-transcriptionally. Despite only being discovered 7 years ago, microRNAs have been implicated as key regulatory molecules in nearly every biological process examined so far and abnormal expression of microRNAs have been linked to many forms of disease including cancer where they can function as both tumour-suppressors and oncogenes. So why are microRNAs causing so much excitement? And will this excitement translate into new medical breakthroughs? This review attempts to answer these questions in the wider context of cancer, focusing on the role that microRNAs play in normal lymphoid development and malignancy.

Keywords: microRNA • lymphoma • leukaemia • lymphocyte • B cell • T cell

Introduction

The microRNA field has only existed for 7 years, yet it has become one of the hottest topics in biological sciences and there now well over 3000 microRNA publications (PubMed-June 2008). microRNAs have been found to play key roles in nearly every biological process examined so far and abnormal expression of these molecules have been linked with many disease types including infectious diseases, genetic disorders and cancer. So why are microRNAs causing so much excitement? And will this excitement translate into new medical breakthroughs? This review attempts to answer these questions in the wider context of cancer, focusing on the role that microRNAs play in normal lymphoid development and malignancy.

A concise history of small RNA

Despite the fundamental role that microRNAs appear to play in biology, these molecules were unknown to the scientific world until 1993 when *lin-4*, a *C. elegans* developmental regulator was identified [1, 2]. The significance of this finding was not however

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realised until 7 years later when another worm microRNA, *let-7* was discovered [3]. Unlike *lin-4*, the sequence of *let-7* was found to be highly conserved in almost all organisms [4]. It was soon realised that similar sequences were scattered throughout eukaryotic genomes that were first called microRNAs in 2001 [5]. Since then, over 6000 microRNAs have been identified from a huge range of both prokaryotic and eukaryotic organisms [6]. There are currently over 600 human microRNA sequences annotated in the miRBase database (http://microrna.sanger.ac.uk/sequences/), although it is believed that the true figure is closer to one thousand [7, 8]. Despite the relatively small number of microRNAs, because a single microRNA can target several hundred genes, and conversely a single target gene can cooperatively bind multiple microRNAs [9], it is currently believed that between 10% and 30% of all human genes are a target for microRNA regulation [10, 11]. microRNAs are expressed in a tissue-/cell-specific manner, some expressed ubiquitously whilst others appear to be restricted to single cell types [12].

microRNA biosynthesis and function

Mature microRNAs are 19-24 nucleotide non-coding single-stranded RNA molecules which regulate the expression of target genes through perfect (in plants) or imperfect (in animals) binding to the 3'-UTR (un-translated region) and possibly 5'-UTR [13] of mRNA. The majority of human microRNAs are encoded within introns, exgenically, within the exons of non-coding mRNAs, or within the 3'UTR sequence of mRNA [14]. With the exception of microRNAs encoded within Alu repeat sequences, which are transcribed by Pol-III [15], microRNAs are transcribed as 5'-capped polyadenylated transcripts (pri-microRNA) in a Pol II-dependent manner. Approximately 40% of human microRNAs are co-transcribed as clusters encoding up to eight distinct microRNA sequences in a single transcript that can be longer than 1kb [16, 17]. Pri-microRNAs are cleaved by the microprocessor complex consisting of a nulease Drosha, and a co-factor, DGCR8 in human beings, Pasha in *Drosophila* [18]. The resulting 60-70 nucleotide hairpin structure (pre-microRNA) encodes a single microRNA sequence that is exported to the cytoplasm by Exportin5 in a Ran-GTP dependent manner [19]. Cytoplasmic pre-microRNAs are further cleaved, by Dicer associated with TRBP and PACT co-factors (in human beings), to remove the loop sequence forming a short-lived asymmetric duplex intermediate (microRNA: microRNA*) [20]. This intermediate is in turn loaded into the miRISC complex containing Argonaut (Ago) proteins [21]. The strand that becomes the active mature microRNA is dependent upon which has the lowest free energy 5' end, whilst the other strand is degraded by an unknown nuclease [22, 23]. The loaded miRISC is guided by the mature microRNA sequence to its cognate recognition sequence in the UTR of the target mRNA.

Although repression of translation without mRNA degradation appears to be the *modus operandi* of animal microRNAs, the situation appears to be more complex than previously thought, as there is now compelling evidence that microRNAs also effect transcriptional levels through de-adenylation and/or degradation [24] and may even positively affect translation in some instances [25]. How translational repression occurs remains unclear. It has been suggested that mRNA bound to the microRNA-miRISC complex may be sequestered away from the translational machinery in P-bodies that additionally act in concert with enzymes to remove the 5'-cap hence preventing translation [26, 27]. Alternatively it has been suggested that microRNAs may prevent recognition of the 5'cap by translation factors [28].

Aberrant expression of microRNA is a common feature of cancer

There is now compelling evidence that dysfunctional expression of microRNAs is a common feature of malignancy [29]. Aberrant expression of specific microRNAs has now been associated with all cancer types including solid and haematopoietic tumours (Table 1). Currently there are $>$650 publications relating to microRNA involvement in cancer (source PubMed database (www.pubmed.gov)). Moreover, it has been suggested that microRNA expression profiling can distinguish cancers according to diagnosis and developmental stage of the tumour to a greater degree of accuracy than traditional gene expression analysis [30]. microRNAs are proposed to play a direct role in oncogenesis as they can function as both oncogenes (*e.g.*, *miR-155* and members of *miR-17-92* cluster) and tumour suppressor molecules (*e.g.*, *miR-15a* and *miR-16*) (see below).

What causes microRNAs to be aberrantly expressed in cancer is fundamental to understanding their mode of action and hence identifying clinically useful properties. There are currently understood to be at least three mechanisms whereby microRNAs are de-regulated in cancer; (*i*) chromosomal lesions at regions encoding microRNAs, (*ii*) defects in the microRNA biosynthetic pathway machinery and (*iii*) epigenetic regulation.

The majority of human and mouse microRNAs have been found to be encoded at cancer-associated genomic regions such as fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification and common break point regions [31, 32]. Using array comparative genomic hybridization of 283 microRNA loci in solid tumours, a large proportion were found to be associated with DNA copy number alterations [33]. These data suggest that dysregulation of microRNA expression by genomic alterations is probably a widespread phenomenon in cancer.

Global effects on microRNA expression can be exerted through aberrant expression/activity of components of the microRNA biosynthetic machinery. For example, reduced levels of Dicer but not Drosha in lung cancer have been associated with poor prognostic outcome [34]. Dicer was also found to be down-regulated in B-cell lymphomas (Lawrie- unpublished data). Conversely up-regulation of Dicer has been reported in lung adenocarcinoma [35] and prostate adenocarcinoma [36] and Drosha levels in cervical squamous cell carcinoma are up-regulated [37]. When endogenous microRNA processing was silenced in both cell lines and

Table 1 Common aberrantly expressed microRNAs in lymphoid malignancies

microRNA	Expression	Target	Regulation	Reference
miR-15a/16-1	↓CLL; ↑APL	*BCL2*	13q14 deletion (CLL)	[55–62, 64–67, 125]
miR-17-92 cluster	↑DLBCL, BL, MCL, lymphoma	*E2F1, CDKN1A, Bim*	E2F3, c-myc, 13q31 (*c13orf25*) amplification	[49, 99–102, 104, 107–109]
miR-155	↑DLBCL (ABC), FL CLL, HL, PMBL, PTLD, pediatric BL; ↓adult BL	*AGTR1, FADD, RIPK1, BACH1, PU.1*	FOXP3, AP-1	[49, 64, 71, 76, 93, 94, 96]
miR-29	↑CLL; ↓SMZL	*TCL1*		[68, 69]
miR-181a	↑CLL, AML(M1/M2); ↓APL, AML(M3/M4)	*TCL1, HOXA11, BCL2*		[64, 65, 68, 116, 126]
miR-143/145	↓CLL, DLBCL, MALT, BL	*ERK5*		[83]
miR-21	↑CLL, FL, DLBCL (ABC), HL, myeloma	*PTEN, BCL2*		[64, 70, 76, 82]
miR-221	↑DLBCL (ABC), FL	*c-KIT, p27*		[76]
miR-196a	↑HL		ERK5	[49,72]

Abbreviations: CLL, chronic lymphocytic leukaemia; APL, acute promyelocytic leukaemia; AML, acute myeloid leukaemia; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; PMBL, primary mediastinal B-cell lymphoma; FL, follicular lymphoma; PTLD, post-trasplantation lymphoproliferative disorder; BL, Burkitt lymphoma; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; SMZL, splenic marginal zone lymphoma.

mice, they displayed enhanced cellular transformation and tumourgenesis, giving a pathological significance to the down-regulation of tumour suppressor microRNAs in cancer [38].

More recently, it has been found that microRNA expression can be influenced by treatment with either de-methylating agents or histone modifying agents (reviewed by [39]), suggesting that epigenetic control of microRNA expression may also be involved in malignancy.

Whilst these three mechanisms may, in part, help explain aberrant microRNA expression in cancer, a paucity of understanding of how microRNAs are regulated means that in the majority of cases, the causes behind dysfunctional microRNA expression remains unknown.

microRNAs in lymphocyte development

The first study to suggest a role for microRNAs in normal lymphocyte development was by Chen *et al.* who cloned ~150 microRNAs from murine bone marrow and found that *miR*-181a, *miR*-223 and *miR*-142 were preferentially expressed in haematopoietic tissue [40]. *miR*-181a, more highly expressed in B-cell lineage samples, when expressed ectopically, resulted in a dramatic increase in the proportion of B-lineage cells produced. Expression of *miR-181a* was also found to be up-regulated in $CD4^+/CD8^+$ double positive (DP) thymocytes where it was shown to target *BCL2*, *CD69* and *TCRα in vitro*, although levels of *BCL2* were unexpectedly decreased in *Dicer*-deficient mice [41]. Furthermore, the inhibition of *miR-181a* in DP cells by antagomirs was found to impair sensitivity to antigens and to inhibit positive and negative selection in T-cell development [42] pointing to a fundamental role for this microRNA in normal T-cell function.

Another microRNA found to be lymphocyte associated, *miR-150* was shown to be down-regulated in response to T-cell stimulation in both T_h1 or T_h2 subsets [43]. The role of *miR-150* in lymphocyte maturation was explored further by Zhou *et al.* who observed that ectopic expression of this microRNA in haematopoietic stem cells transplanted into mice had no effect on T cells, macrophages or granulocytes but greatly impaired formation of mature B cells [44]. More detailed investigation revealed that over-expression of *miR-150* reduced numbers through increased apoptotic rates in different developmental stages of B cells with the exception of pro-B cells suggesting that this microRNA inhibits the transition from pro- to pre-B cell. *miR-150* was recently shown to control B-cell differentiation *in vivo* though specific targeting of *c-Myb* levels [45].

Mir-155, originally identified as being abnormally expressed in lymphoma (see below), has more recently been found to play a pivotal role in normal lymphocyte development using knockout

mice. Rodriguez *et al.* found that *miR-155*-deficient mice were also immunodeficient and displayed increased lung airway remodelling [46]. These mice when challenged with the pathogen *Salmonella typhimurium* were not protected by immunization unlike wild-type mice. Furthermore, B cells from these mice produced significantly reduced levels of immunoglobulins in response to antigen treatment, whilst T cells produced reduced levels of IL-2 or IFN-γ and dendritic cells failed to efficiently activate T cells. Gene expression analysis of $CD4^+$ cells from *miR-155*-deficient mice showed that many of the genes aberrantly expressed in these cells were computationally predicted as targets for this microRNA. Another study by Thai *et al.* used both *BIC/miR-155*-deficient and mice overexpressing *miR*-155 to investigate germinal centre (GC) B cell responses [47]. They found a reduction in total numbers of GC B cells in deficient mice and an increase in numbers in mice overexpressing *miR-155*. *In vitro* activation of wild type B cells resulted in strong up-regulation of *miR-155* expression as did activation of $CD4^+$ T cells. Similar to the study of Rodriguez *et al*., immunization resulted in decreased antibody titres in *miR-155*-deficient mice but the authors observed no difference in B-cell proliferation levels or somatic hypermutation when stimulated *in vitro*. It was also observed that activated *miR-155*-deficient B cells expressed about a third of normal levels of TNF leading to the suggestion that *miR-155* controls B-cell activation through control of cytokine production. Further research on *miR*-155-deficient revealed that *miR*-155 is required for B-cell responsiveness to both thymus-dependent and independent antigens and that B cells lacking this microRNA generated reduced extrafollicular and GC responses and failed to generate high affinity IgG antibodies [48]. This phenotype was reproduced by overexpressing a target gene of *miR*-155, the transcription factor *PU.1*.

Microarray analysis of *in vitro* B-cell activation by IL-2 or IgM resulted in changes to the expression of eleven microRNAs (including *miR-155*), the majority (9/11) of which were up-regulated. In contrast, plasma cell development was associated with the down-regulation of (10/11) of microRNAs [49]. Three microRNAs implicated in both B-cell developmental stages (*miR*-17, *miR*-20b and *miR*-106a) form part of the *miR*-17-92 cluster leading to the proposition that this cluster played a role in normal B-cell development. Subsequently, this has been found that the *miR-17-92* cluster is indeed essential for normal B-cell development as its deletion in a murine model resulted in increased levels of the pro-apoptotic protein Bim and inhibited B-cell development at the stage of pro- to pre-B-cell transition [50].

In addition, to the role of specific microRNAs, the general necessity of microRNAs in lymphocyte development was investigated by selective deletion of Dicer in the thymus at both an early stage of T-cell development and at a much later stage [51, 52]. Surprisingly, this deficiency, in both experiments, did not inhibitT-cell differentiation but did cause a severe block in peripheral $CD8^+$ development and reduced numbers of $CD4^+$ cells which when stimulated underwent increased apoptosis and proliferated poorly.

microRNAs in lymphoid malignancy

Lymphoma is the fifth most common cancer type in the UK with approximately 12,000 cases per annum. The age-adjusted incidence of non-Hodgkin's lymphoma (NHL) in the United States has increased 74% between 1976 and 2001 [53]. The WHO classification divides NHL into either B-cell (85%) or T- and NK-cell (15%) neoplasms. Compared with solid tumours the identity and role of microRNAs in lymphomas remains little studied with the exception perhaps of chronic lymphocytic leukaemia (CLL) (Fig. 1).

Chronic lymphocytic leukaemia (CLL)

CLL is the most common leukaemia among adults in the western world and despite its name is classified by WHO as a B-cell lymphoma being characterized by clonal expansion of $CD5^+$ mature B lymphocytes. Although generally an indolent disease some patients develop a more aggressive form with consequent poor prognostic outcome. Several factors have emerged as having prognostic power in CLL including certain genetic aberrations, somatic IgV_H mutation status and levels of ZAP-70 protein (reviewed by Chiorazzi *et al.* [54]).

The first report of aberrant microRNA expression in CLL or indeed in any cancer was by Calin *et al.* in 2002, who found that that two microRNAs, *miR-15a* and *miR-16-1* encoded at the 13q14 locus, a region deleted in the majority of CLL cases, were down-regulated in 68% of cases that harboured this deletion [55]. These microRNAs were subsequently shown to target *BCL2* and to induce apoptosis *in vitro*, suggesting they have a tumour-suppressor role in CLL [56]. Consistent with this role, it has recently been demonstrated that *miR-16* negatively regulates cellular growth and cell cycle progression [57] and exogenous delivery of *miR-16* to a mouse model of CLL with low endogenous levels of *miR-16* resulted in altered cell-cycle and increased apoptosis [58]. In addition, the potential effect of *miR-15a/miR-16-1* expression in a leukaemic model was investigated *in vitro* by restoring endogenous expression levels in an acute megakaryocytic leukaemia cell line (MEG-01) containing the 13q14 deletion [59]. Gene expression and proteomic profiling identified a signature enriched for AU-rich elements (ARE) as well as cancer genes as *ETS1, JUN, BCL2* and *MCL1*. A previous involvement of *miR-16* in ARE-mediated mRNA decay has also been reported [60]. Ectopic expression of *miR-15a* and *miR-16* in Dicer-deficient cell lines led to a decrease in cell growth and down-regulation of genes that caused an accumulation at G0/G1 [57].

However, a number of factors call into question the importance of *miR-15a/16-1* in the pathogenesis of CLL. Firstly, the link between the 13q14 deletion and *miR-15a/16-1* levels is unclear. Recently, it was found that ~85% of 171 CLL cases displayed a wide expression range of these microRNAs that was independent of 13q14 status [61] and in a study of 56 patients although all cases with 13q14 homozygous deletions were significantly down-regulated, those with a heterozygous deletion were not significantly

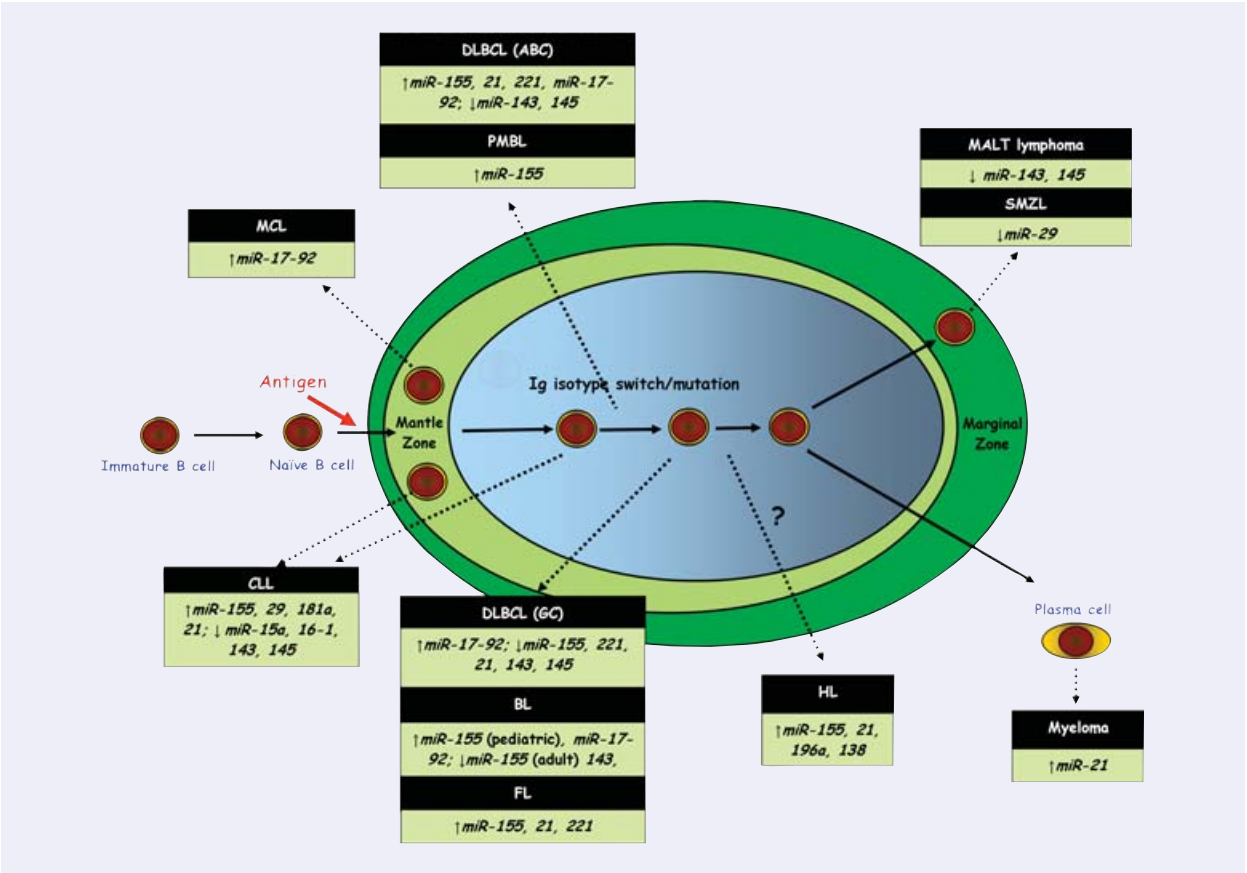

Fig. 1 microRNA involvement in lymphoid malignancy shown relative to proposed counterpart B-cell developmental stage in the context of the lymph node germinal centre. Abbreviations used: CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; PMBL, primary mediastinal B-cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; SMZL, splenic marginal zone lymphoma.

different from controls [62]. Additionally, although an inverse relationship between *miR-15a/miR-16-1* and BCL2 was reported in 26 CLL cases [56], other reports using much larger patients cohorts did not find a correlation [61, 62]. Secondly, a homologous cluster to *miR-15a/16-1* is encoded at 3q25 (*miR-15b/16-2*), a region not commonly deleted in CLL. It is possible that consequent residual expression of these microRNAs might explain the more favourable prognosis observed in CLL patients with 13q14 deletions compared to other common CLL chromosomal abnormalities such as 17p13 or 11q23. Consistent with this idea it has recently been reported that CLL patients with a monoallelic 13q14 deletion had slower lymphocyte growth kinetics than those with a biallelic deletions [63].

Asides from *miR-15a/miR-16-1* involvement several groups have investigated the microRNA expression profile of CLL using a variety of techniques. Microarrays were used by Calin *et al.* to identify microRNA signatures that distinguished between karyotype, the presence of ZAP-70 and IgV_H mutation status in a series of 38 CLL patients [64]. A follow-up study by the same authors extended the profiling studies to 94 CLL cases and defined a prognostically significant 13-microRNA signature [65]. Fulci *et al.* used a combination of cloning and qRT-PCR technologies to profile 56 CLL patients [62]. They found that *miR-21* and *miR-155* were highly overexpressed in CLL compared to controls whilst *miR-92* was down-regulated and *miR-223, miR-29b* and *miR-29c* were up-regulated in cases with IgV_H mutations. Consistent with these findings high expression of *miR-16*, *miR-21* and *miR-150* were reported in 37 CLL cases by qRT-PCR [66]. Furthermore, *in situ* hybridization (ISH) was used to show that CLL proliferation centres were characterized by low *miR-150* but high *miR-155* expression. A cloning strategy was used by Marton *et al.* to clone pools of patients ($n = 3$) with either mutated (M) or non-mutated (NM) IgV_H from which they obtained 287 and 237 clones respectively of which 32 and 24 clones represented 13 previously annotated microRNAs as well as the identification of 5 novel microRNAs [67].

A further indication of the role that microRNAs could play in the pathogenesis of CLL was provided by Pekarsky *et al.* who found that the *TCL1* oncogene, previously shown to be linked with an aggressive CLL phenotype, was regulated by *miR-29* and *miR-181b in vitro* and that levels of these microRNAs inversely correlated with levels of *TCL1* expression in clinical samples of CLL [68]. Interestingly a similar finding was reported in cases of splenic marginal zone lymphoma (SMZL) [69].

Hodgkin's lymphoma (HL)

Hodgkin's lymphoma (HL) accounts for approximately 1% of cancers worldwide and is characterized by the presence of relatively few Hodgkin/Reed-Sternberg (HRS) tumour cells surrounded by a non-neoplastic cellular infiltrate. Navarro *et al.* investigated the microRNA profile of 49 classical HL (cHL) cases along with 10 reactive lymph nodes (RLN) by qRT-PCR [70]. They identified a 25-microRNA signature associated with disease and 36 microRNAs differentially expressed between two histological subtypes of cHL (nodular sclerosing and mixed cellularity). However, only a subset of these microRNAs were also expressed in cHL cell lines suggesting the remainder of microRNAs were associated with non-neoplastic cells. Of note *miR*-138 expression was associated with clinical stage in both training and validation sets and *miR-21, miR-134* and *miR-138* expression was visualized in HRS cells by ISH. Although this study did not identify *miR-155* expression as being highly expressed in cHL cases, at odds with other publications [71], they did visualize *miR-155* expression in HRS cells by ISH. Microarray analysis of four cHL cell lines identified seven differentially expressed microRNAs [49]. As well as *miR-155* this study found that *miR*-196a was up-regulated, a microRNA that was shown in another study to be up-regulated along with neighbouring homeobox gene *HOXB9* in cHL cell lines as a result of constitutively active ERK5 pathway [72].

Non-Hodgkin's lymphoma

Diffuse large B-cell lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) is the most common form of adult lymphoma accounting for nearly 40% of all lymphoid tumours [73]. Gene expression and immunohistochemical studies of this clinically heterogeneous disease have revealed the presence of at least two prognostically distinct subtypes of DLBCL; those with a good prognostic outcome that are germinal centre B cell-like (GCB) and those with a worse prognosis that are activated B cell-like (ABC) [74, 75]. microRNA profiling of five cell lines representing these molecular subtypes identified differentially expressed microRNAs [76]. Three of the microRNAs associated with ABC-type cell lines (*miR-155*, *miR-221* and *miR-21*) were found to be up-regulated in clinical cases of follicular lymphoma (FL), transformed DLBCL and were more highly expressed in cases of *de novo* DLBCL that had an ABC-type immunophenotype than those with a GCB-immunophenotype. Furthermore, *miR-21* expression level was shown to be an independent prognostic indicator in these patients.

Recently, *miR-155* levels measured in 22 DLBCL cell lines revealed that although high expression was seen in the ABC-type cell lines OCI-Ly3 and OCI-Ly10, expression was also high in RC-K8 and Farage cell lines that are GC-type. The authors observed a positive correlation between *miR-155* and NFκB levels in eight of these cell lines [77]. Using data mining of existing microarray data from 176 DLBCL cases that contained pri-*miR-155* probe data it was found that *miR-155* levels were indeed higher in ABC-type cases than GC-type (or type III) cases but not in other schemes that sub-classify DLBCL. Forty-two genes were found to be correlated inversely with *miR-155* expression, nine of which were targets of *miR-155* by multiple predictive algorithms.

Primary effusion lymphoma (PEL) is a rare form of post-germinal centre DLBCL caused by Kaposi sarcoma-associated herpesvirus (KSHV) that is also associated with EBV infection in about 80% of cases. Nine cases of PEL were subject to DNA copy-number, pre-microRNA and mature microRNA profiling by qRT-PCR [78]. A 68-microRNA specific signature was identified as well as microRNAs associated with KSHV and EBV infection. KSHV itself encodes 12 microRNAs, including KSHV-*miR*-K12-11, which shares 100% seed sequence homology with *miR-155*. The viral microRNA was shown to function as an orthologue of *miR-155* [79, 80] and in PEL, KSHV-*miR*-K12-11 but not *miR-155* is expressed. Both microRNAs were expressed in 293T cells, leading to the identification of 66 genes that were commonly down-regulated, 20 of which had 3'-UTR seed matches that were involved in cell signalling, cell division and T-cell activation. This suggests that the overexpression of KSHV-*miR*-K12-11 caused by KSHV infection in B cells mirrors the malignant phenotype of *miR-155* overexpression (see below) in non-virally induced DLBCL.

Other non-Hodgkin's lymphoma

Mantle cell lymphoma (MCL) accounts for about 6% of NHLs and is an aggressive disease of poor prognosis with a median survival of only 3 years. The majority of patients harbour a t(11;14) translocation that leads to truncation and overexpression of cyclin D1 (CCND1). Recently it has been found that the truncation of the CCDN1 gene in the t(11;14) translocation alters *miR-16-1* binding sites and that *miR-16-1* alters CCDN1 mRNA expression [81], suggesting a profound role for microRNAs in the pathogenesis of this disease.

Myeloma is a plasma cell neoplasm that is essentially incurable with a median survival of 3 years accounting for nearly 2% of all deaths from cancer and about 20% of deaths from haematological cancers. Using myeloma cell lines Löffler *et al.* found that *miR-21* expression is controlled by an up-stream enhancer that binds Stat3 and that *miR-21* expression by IL-6 induction was dependent on Stat3 and that ectopically expressing *miR-21* without IL-6 reduced apoptosis [82].

Several B-cell malignancies including CLL, DLBCL, BL and EBV-transformed lymphoblastoid cell lines were associated with

the down-regulation of *miR-143* and *miR-145* [83]. Expression of these microRNAs in the BL cell line Raji inhibited growth, which was associated with ERK5 protein levels.

microRNA expression in T-cell lymphoma

T cell and natural killer cell lymphoma account for about 15% of total lymphomas and represent a heterogeneous group of diseases that compared with their B-cell counterparts are little understood. The direct role of microRNAs in these lymphomas has not yet been studied; rather the little research that has been carried out suggests a role for microRNAs in these diseases. Recently the T lymphoma causing murine leukaemia virus (MLV), was used in a screen to identify common proviral integration sites and found a high density of integrations upstream of the *miR-106a* cistron [84]. In tumours containing this integration, the *miR-106a* cistron, as well as component mature microRNA sequences, was found to be overexpressed. Using the same technique, the same group reported that the *miR-17-92* cluster was also a common integration site [85]. Additionally, microRNAs have been found to be encoded in the avian T-cell lymphoma-causing virus, Marek's disease virus (MDV) [86–88]. EBV-associated microRNAs were recently profiled in a peripheral T-cell lymphoma case, which were consistent with a type II latency infection [89].

Lymphoma-associated oncomirs (*miR-155* and the *miR-17-92* cluster)

The non-coding *BIC* locus was originally identified as a common retrovirus integration site for avian leukosis virus (ALV) that despite being poorly conserved between avian, mouse and human genomes, enhanced lymphogenesis in a mouse *myc* model [90, 91]. Commenting on the observation by van den Berg *et al.* that *BIC* was highly expressed in over 90% of Hodgkin's lymphoma (HL) cases [92], Metzler and colleagues proposed that a phylogenically conserved region of 138 nucleotides in the *BIC* gene encoded a functional precursor sequence of *miR-155* [93]. Subsequently, both *BIC* and *miR-155* transcript levels were found to be up-regulated 10-30 fold in DLBCL cases with higher levels of expression observed in cases with an ABC-immunophenotype than those with a GCB- immunophenotype [94]. Similar findings were reported by Kluiver *et al.* who also found *BIC* overexpression in HL, primary mediastinal B-cell lymphoma (PMBL), but not other non-Hodgkin's lymphomas [71]. Overexpression of *miR-155*, in BL and post-transplantation lymphoproliferative disorder (PTLD) at least, appears to be associated with EBV latency type-III infections [95, 96]. Consistent with this hypothesis high *miR-155* expression was reported in latency type III infected lymphoblastoid, Raji and Granta-519 cell lines but not EBV-negative BL cell line Ramos or latency type II infected cell YT [49].

Transgenic mice that overexpressed *myc* carrying the *miR-155* precursor sequence under control of a V_H promoter-Ig heavy chain E_μ enhancer, which becomes active at the late pro-B cell stage of B-cell development, were found to develop initially pre-B-cell proliferation in spleen and bone marrow followed by a frank B-cell malignancy resembling high-grade lymphoma after 6 months [97]. It should be noted however that these mice developed a polyclonal lymphoproliferation, suggesting additional factors are necessary for oncogenesis in this model. Intriguingly, overexpression of *miR-155* in mouse haematopoietic stem cells resulted in a myeloproliferative disorder that resembled acute myeloid leukaemia [98].

A commonly found amplification in B-cell lymphomas, the 13q31 locus encodes a functional precursor microRNA sequence, the *miR-17-92* cluster, that itself encodes seven mature microRNA sequences [99]. Levels of the *miR-17-92* cluster were found to be elevated in cell lines harbouring this amplification compared to cell lines, which did not. Additionally, amplification of 13q31 has been linked to overexpression of *miR-17-92* in BL, mantle cell lymphoma (MCL) and lung cancer [100–102]. However, the link between overexpression of the *miR-17-92* cluster and 13q31 amplification is not clear. Recently, it has been suggested that that overexpression of these microRNAs is a more common occurrence in haematological malignancies than previously thought as high expression levels were observed ubiquitously in 40 haematological cell lines examined including cell lines lacking the 13q31 amplification [49]. Although He *et al.* reported that 65% of 46 lymphoma cases, including 13 DLBCL, overexpressed *miR-17-92*, comparative genomic hybridisation suggests that just over quarter of DLBCL cases have the 13q31 amplification [103]. In addition, overexpression of *miR-17-92* has been described as a common feature of solid tumours irrespective of 13q31 amplification status [104, 105], and indeed loss-of-heterozygosity covering the 13q31 locus is frequently observed in these tumours [33].

Similar to *BIC* [90, 91], He *et al.* found that expression of components of the *miR-17-92* cluster in mice also overexpressing *myc* greatly accelerated lymphogenesis [99]. Compared to the Eμ-Myc mice, mice also overexpressing the *miR-17-92* cluster showed reduced levels of apoptosis, suggesting that the main effect of these microRNAs was to suppress cell death. Increased lymphogenesis was only observed however when these microRNAs were expressed together, but not as individual microRNAs, suggesting a cooperative effect. In contrast, a tumour suppressor role has been proposed for this cluster as expression of *miR-17* in breast cancer cell lines resulted in reduced proliferation [106].

O'Donnell and colleagues found that the *miR-17-92* cluster itself was up-regulated through direct c-myc binding [107] and it has been suggested that the two factors act synergistically in carcinogenesis [101]. They also reported that components of the cluster, *miR-17-5-p* and *miR-20*, negatively regulate expression of *E2F1*, a pro-apoptotic transcription factor. Conversely it was recently found that the promoter sequence of the *miR-17-92* cluster contains two functional E2F binding elements and chromatin immunoprecipitation analysis demonstrated that E2F3 binds this region, forming a negative regulatory loop between the

pro-apoptotic E2F1 and the proliferative E2F3 [108]. Recently, the cluster has been shown to control cell cycle progression through targeting of CDKN1A (p21) [109]. In some cell lines, overexpression or inhibition of members of the cluster promoted or delayed entry into S-phase respectively and overexpression inhibited DNA-damage mediated cell cycle arrest. Overexpression of these microRNAs in gastric cancer cells made them insensitive to TGFβ-mediated cell cycle arrest, attributable in part at least to their effect on p21 [104].

MicroRNA: a new class of diagnostic and therapeutic agents for lymphoma?

MicroRNAs as diagnostic/prognostic bio-markers

Many studies have shown that microRNA expression levels have potential diagnostic and/or prognostic significance in cancer [30, 34, 65, 76, 110–114]. Specifically in haematological malignancies, a 13-microRNA prognostic signature was identified from a study of 94 CLL patients, which was associated with other prognostic markers including IgV_H status and ZAP70 expression [65]. More recently, a 27-microRNA signature was identified that could distinguish ALL from AML with 97–99% accuracy [115], whilst *miR-181a* expression was found to discriminate between AML M1/M2 and M3/M3 stages [116]. Additionally, the expression of *miR-21* was found to be an independent indicator of overall survival in DLBCL patients [76]. With the advent of more standardised and widely available high throughput technologies to identify microRNAs, this list can only increase in the coming years.

MicroRNAs as non-invasive diagnostic tools

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 [117]. 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 [117]. microRNAs due to their small size are relatively resistant to RNase degradation and unlike mRNA can be successfully recovered intact from archival formalin-fixed paraffin-embedded (FFPE) material [76, 118, 119], suggesting they would be eminently suitable candidates for detection in biological fluids. We were able to show the presence of circulating microRNAs for the first time and found that tumour-associated microRNAs (*miR-21, 155* and *210*) were present at significantly higher levels in DLBCL patient sera ($n = 60$) than in healthy controls ($n = 43$) [120]. Furthermore, we found that expression levels of serum *miR-21*, like that of tumoural material [76], were associated with relapse-free survival times for these patients. More recently, placental microRNAs have also been detected in maternal plasma [121]. These findings open a new diagnostic potential for microRNAs that will undoubtedly grow in the future.

microRNAs as therapeutic agents

The association between aberrant expression of microRNAs and malignancy is now so compelling that research is rapidly focusing on the therapeutic use of these molecules. As microRNAs can function as both tumour-suppressor molecules and oncogenes, the delivery of exogenous microRNAs and silencing of microRNAs can be envisaged as possible therapeutic scenarios. The power of the first scenario is perhaps best illustrated by Lim and colleagues who expressed *miR-124* and *miR-1* in HeLa cells and saw that the gene expression profiles of transfected cells shifted towards that typically seen in brain and skeletal muscle respectively, the organs where these microRNAs are preferentially expressed [122].

Several researchers have explored the potential of microRNA-based therapeutics using an antisense approach to inhibit specific microRNAs. Both *in vitro* and more recently *in vivo* studies have successfully used antisense technologies to inhibit specific microRNAs (reviewed by Hammond [123]). Most dramatically Krutzfeldt *et al.* injected modified 2-*O*-methyl antisense molecules (antagomirs) into mice and found remarkably efficient and long-lasting inhibition of specific microRNAs in all tissues except the brain [124]. Furthermore, inhibition of liver-specific *miR-122* resulted in a 40% decrease in plasma cholesterol levels.

Perhaps one of the biggest problems facing the use of microRNAs and/or associated agents as therapeutics is their pleiotropic mode of action. As a single microRNA can target several hundred genes their perturbation may be expected to give rise to a complex phenotype that may not be readily predictable. It is therefore imperative that such therapeutics is precisely delivered in order to mitigate effects on non-targeted cells.

What is clear however is that microRNAs promise a great deal in furthering understanding of malignancy and of biology in general and can be expected to deliver further therapeutic tools in the not too distant future, although equally, a great deal is yet to be discovered before this vision becomes reality.

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