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DOTTORATO DI RICERCA IN MEDICINA SPERIMENTALE
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miR-*221/222*: New Insights in Burkitt Lymphoma

DOTTORANDA
Jessica Consiglio

DOCENTE GUIDA
Prof. Andrea Vecchione

COORDINATORE DEL DOTTORATO
Prof.ssa Maria Rosaria Torrisi

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Dedication

In memory of Denise Saridakis. You left fingerprints of grace on my life; thanks to you I found hope and faith again.

You shan't be forgotten.

To all my family. The warmth of your love and support even ocean apart gave me strength to overcome all the difficulties I found in this beautiful but crazy journey.

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Abstract

Burkitt Lymphoma (BL) is a highly aggressive B cell non Hodgkin lymphoma. It's considered the fastest growing human tumor and it is commonly associated with EBV infection. It was the first type of cancer shown to have a chromosomal translocation that activates *c-Myc* to become an oncogene. This genetic rearrangement places *myc* that usually is on chromosome 8 under the immunoglobulin gene regulatory element on chromosome 14 resulting in tumor promoting effect. BL is a highly malignant B-cell neoplasm that occurs endemically in equatorial Africa and sporadically throughout the world. The endemic BL (eBL) is the pediatric form positive for EBV in most cases. The variant of BL affecting the rest of the world is the sporadic BL (sBL) which is found in older patients and it is considered EBV negative because only a minority is EBV infected. The World Health Organization recognizes also a third form, HIV-BL which develops in HIV positive patients.

Although, until now the translocation 8-14 and its variants are considered the major mechanism for the pathogenesis of BL, other molecular mechanism such as microRNA expression profile have been used to characterize and classify different types of BL from other lymphoma malignancies. However, the differential expression of microRNAs between BL patients and healthy control has not been studied before. For this reason our goal is to investigate the functional role of microRNAs that are disregulated in BL patients compared to healthy (cancer free) individuals. MicroRNAs are noncoding RNA, 18-24 nucleotides long. They are transcribed in the nucleus as long primary transcripts, and then cut by Drosha and

DGCR8 into 70 nucleotides long precursors (pre-miRNA). This Pre-Mir is exported to the cytoplasm by Exportin-5 and then cleaved into a mature dsRNA by Dicer. Only one strand of the duplex miRNA-miRNA* binds the target mRNA to modulate the gene expression through two principle mechanisms which are the degradation of mRNA or the inhibition of the protein translation.

To gain further insight into the molecular pathology of BL, we performed miRNA expression profile using a set of 5 sporadic, and 2 endemic BL patients, compared to B cells from reactive lymph nodes of 9 healthy patients and 11 patients affected by mononucleosis. MiRNAs expression signature shows, among the group of downregulated miRNAs, miR-*221* that usually is upregulated in solid tumors. This is the first microRNA profiling that has been done in BL using as negative control lymph nodes taken from reactive patients or patients affected by the EBV virus, whereas the literature shows microRNA profiles in BL using as negative control T cells or different type of B cell lymphomas like for example DLBL (diffuse large B cell lymphoma). To confirm the remarkable down-regulation of miR-*221* a nanoString analysis in 2 different cohorts of BL cell lines was also performed. We observed a common trend of altered expression of microRNAs, highlighting once again the down-regulation of miR-*221/222*, suggesting a different role of these miRNA in liquid tumours compared to their well-known pro-tumorigenic function in epithelial tumors The down-modulation of miR-*221/222* was also confirmed by the qRT-PCR method in a bigger cohort of BL cell lines compared to 4 normal B lymphoblast EBV transformed cell lines. The four cell lines representing the controls express high levels of miR-*221* compared to the group that represents the BL cell lines where the miR-*221* is lost. The same trend is shown for miR-*222*.

We found that interesting considering the up-regulation of miR-*221* and miR-*222* previously confirmed in a lot of solid tumors by multiple studies, such as breast, liver and lung cancer. Here, we are investigating a different role of the cluster *221/222* in lymphomas that have a different process in carcinogenesis than solid tumors. To better understand the potential role of miR-*221/222* in BL, we also analyzed their expression levels in EμMyc transgenic mouse model which has been considered for a decade a good in vivo model of BL. We investigated the expression of miR-*221* and *222* in B cells extracted from both transgenic and wild type mice. The miRNA levels detected by qRT-PCR show a down-regulation in 80% of the transgenic samples when compared to normal B cells derived from the spleen of wild type mice littermates.

Once we determined that miR-*221* and *222* were down regulated in both human and mouse models, we wanted to understand what pathways both of the models had in common and how miR-*221* and *222* play a role in these pathways. Therefore, in order to establish the effect of miR-*221* and *222* in a human model, we transfected BL cell line Bjab, which lacks of miR-*221* and *222* expression with mature miRNA. A gene expression analysis was then conducted on extracted RNA from treated Bjab cells collected 48 hours after transfection compared to its negative control collected at the same time point. We then performed a parallel gene expression profile on the mouse model using the RNA extracted from CD-19+ of the wild type spleen and the transgenic spleen of littermates. Then we picked the down-regulated gene of the human gene expression profiling and compared it to the gene expression profile of the mouse model. From this comparison, we found some genes that were up-regulated in the mouse model that were also down-regulated by miR-*221* and *222*

in the human model. One of these genes is *DUSP6/MKP-3*, a MAP kinase phosphatase that dephosphorylate phosphothreonine and phosphotyrosine within *ERK* pathway, playing a role in the induction of apoptosis. This dual specificity phosphatase has been found also acting as an oncogene but no further studies have been conducted, leaving its function in a contradictory background. The level of expression in BL cell lines of *DUSP6* has been evaluated by qRT-PCR, compared to negative lymphoblastoid cell lines and results show an up-regulation of the mRNA in 80% of BL cell lines whereas it's lost in the controls, suggesting an oncogenic role of this protein in BL but additional studies need to be performed to confirm this hypothesis.

Since these new findings may highlight a different role of these miRNA in BL compared to their well-known pro-tumorigenic function in epithelial tumors we cross a miR-*221/222* KO mouse with the well-known EμMyc transgenic mouse model. The miR-*221/222* KO doesn't show any particular phenotype but when we breed the KO with the transgenic EμMyc we observe an early development of the BL pathogenesis in 5 out of 8 miR-*221/222* KO/ EμMyc tg positive and death at 3-4 months of age while the wild type miR-*221/222*/ EμMyc tg are still alive at 6 months of age without showing any enlarged lymph nodes. Unfortunately, even these preliminary results indicate that the loss of miR-*221/222* can play an important role in the pathogenesis of BL, the number of wild type miR-*221/222* are not enough for the statistical analysis; for this reason we are increasing the numbers of litters and we need further investigations.

Our findings indicate that miR-*221/222* can be critical mediators for BL pathogenesis and together with other important genetics alteration such as translocation of *MYC* can lead to the aggressive phenotype that this B cell malignancy usually shows. These results highlight the potential role of this cluster of microRNAs to be a good tool of diagnosis and prognosis for BL.

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Abbreviations

BL	Burkitt Lymphoma
EBV	Epstein-Barr Virus
eBL	Endemic Burkitt Lymphoma
sBL	Sporadic Burkitt Lymphoma
HIV-BL	Immunodeficency-related Burkitt Lymphoma
IGH	Immunoglobulin Heavy Chain
B-NHL	B cell Non-Hodgkin Lymphoma
B-CLL	B cell Chronic Lymphatic Leukemia
DLBL	Diffuse Large B cell Lymphoma
siRNA	Small Interfering RNA
C. elegans	*Caernorhabditis elegans*
miRNAs	MicroRNAs
pri-miRNA	Primary transcript miRNA
pre-miRNA	Precursor miRNA

DGCR8	DiGeorge critical region 8
D. melanogaster	*Drosphila melanogaster*
Exp5	Exportin-5
miRISC	MiRNA-induced silencing complex
miRNA*	Star microRNA
mRNP	MiRNA-containing ribonucleoprotein complex
miR-RISC	MiRNA-containing RNA-induced silencing complex
AGO1	Argonaute Protein 1
AGO2	Argonaute Protein 2
hnRNPA1	Ribonucleoprotein A1
dsRBD	Double strand RNA-binding domain
TRBP	Transactivating response RNA-binding protein
PACT	Protein activator of PKR
PKR	Protein Kinase RNA-activated
3'UTR	3' untranslated region
KO	Knock-out

Chapter 1-Introduction

1.1 Burkitt Lymphoma

1.1a History

Burkitt lymphoma is a non-Hodgikin lymphoma that has had an important role in the understanding of tumorigenesis in recent years because of its genetic and molecular features[1]. Indeed, it was one of the first tumour shown to have a chromosomal translocation that activates a regular transcription factor to become an oncogene[2]. The disease is also associated with Epstein-Barr virus (EBV) and in fact it was the first tumor found to be correlated with a virus[3], and the first lymphoma reported to be associated with HIV infection[4]. Burkitt lymphoma is a highly aggressive B-cell lymphoma and is considered the fastest growing human tumor, with a cell doubling time of 24-48 hrs, and was the first childhood tumor to respond to chemiotherapy alone[5].

Furthermore, it is the most common childhood cancer in areas where malaria is holoendemic and where there is an early acquisition of EBV—eg, equatorial Africa, Brazil, and Papua New Guinea[6]. Interestingly the epidemiological maps of these two diseases overlap[7] and nowadays a Burkitt lymphoma belt has been defined which stretches across central Africa between latitude 15° on either side of the equator, where the climate is typically humid (more than 50 cm annual rainfall) and hot. Early in the 20th century, Sir Albert Cook, a missionary doctor in Uganda, and other medical staff working in west, east, and central Africa reported the high frequency of jaw tumors and childhood lymphomas[8]. Later on in 1958, Denis Burkitt, an Irish surgeon working in Uganda, described cases of children

presenting with rapidly growing jaw or abdominal tumors[9]. At first Burkitt suggested that these tumors were round-cell sarcoma. However, in 1960 George O'Connor, a pathologist, concluded that the cancer was of lymphoma lineage[10]. In 1964, three virologists, Michael Anthony Epstein, Yvonne Barr, and Bert Achong identified viral particles in the tumor tissue; this virus became known as Epstein-Barr virus (EBV)[3]. Meanwhile, Burkitt travelled through eastern and central Africa to map the tumor spread and found records of affected children in all the malarial areas of the region[11].

1.1b Classification

Burkitt Lymphoma is listed in the World Health Organization (WHO) classification of lymphoid tumors as a single genetic and morphologic entity with variable clinical presentation. In particular, the WHO classification describes three different variants: endemic (eBL), sporadic (sBL) and immunodeficiency-related (HIV-BL). Each clinical subset affects different population and can present different features but they are similar in morphology, immunophenotype and genetic features.

The endemic form is overall the commonest type; it is EBV infection associated in most cases and is consider a pediatric variant, being the most frequent childhood cancer in equatorial Africa. This variant is also correlated with malaria endemicity as well as with local environmental pathogens (ie, *Euphorbia tirucalli*) and coinfection with arbovirus also appear to be important for its pathogenesis.

The sporadic variant is the predominant type found in non-malarial areas, whereas it occurs mainly in the rest of the world, especially in Europe and United States. This form doesn't

have a special climatic or geographical link and is rarely associated with EBV infection; only 20% of cases occurs concomitantly with Epstein-Barr virus infection. Sporadic BL also predominantly affects adults.

The immunodeficiency-related BL subset is seen most often in patients affected by HIV and rarely in patients who have undergone organ transplantation[12]. In the latter case the risk increases 4/5 years after the transplantation, but still remains less than the risk associated with HIV. HIV-BL is more common in patients with relatively low CD4 counts which is a typical condition of the early stage of HIV infection. The immunosuppression itself however is not sufficient to explain the high prevalence of BL in this setting[13].

1.1c Epidemiology

Nowadays in the United States, lymphoma is the most common form of blood cancer and it comprises over 5% of all cancers all over the world (Figure 1). Many forms continue to have 5-year survival rates as low as 50% and require patients to undergo aggressive chemotherapies.

The exact worldwide incidence of BL is not known due to the limited collection of epidemiologic data, which is in turn due to a lack of resources needed for case ascertainment and accurate diagnosis in the developing countries that have the highest apparent incidence (eg equatorial belt of Africa and New Guinea)[14].

As previously mentioned Burkitt lymphoma has been subdivided into three epidemiologically distinct forms, differing with respect to geographical distribution and EBV association[15].

High-risk areas for Burkitt Lymphoma correspond to areas of equatorial Africa and New Guinea where malaria is holoendemic and there is an early acquisition of EBV. Here endemic BL accounts about half of all childhood cancers diagnosed with an estimated incidence at 40-50 per million children per year[16]. The peak incidence occurs in children age 4 to 7 years and the disease is twice as common in boys as in girls.

SBL is seen in low-risk areas such as North America, Europe and East Asia at an annual incidence of 2 per million children. Though this form is found also in adults, it occurs most commonly in children of 6-12 years of age, affecting from 3 to 5 times more males than females[17].

Parts of South America, North Africa, southern Europe and Middle East are considered areas of intermediate risk.

1.1d *c-Myc* translocation: the hallmark of Burkitt Lymphoma

BL was the first type of cancer to be shown to have a chromosomal translocation that activates the transcription factor *c-Myc* to become an oncogene. This genetic rearrangement places *Myc* locus, which is usually on chromosome 8, under gene regulatory elements that lead to the overexpression of the protein c-*Myc*. All BL variants show high level of this transcription factor as a consequence of genomic aberration involving the chromosomes 14, 2 and 22 where the DNA coding sequence of *c-Myc* is transposed under the control of immunoglobulin gene enhancers that are constitutively active in mature B cells (Figure 2). Translocation t(8;14) is the most common representing 80% of BL cases and it causes the overproduction of *c-Myc* under the control of the immunoglobulin heavy chain (IGH)

regulatory element on chromosome 14, resulting in a tumor promoting effect. In all other cases, *c-Myc* is translocated close to one of the immunoglobulin light chain genes on chromosome 2 (IGK- kappa light chain), resulting in the t(2;8) found in 15% of BL, or 22 (IGL- lambda light chain), resulting in the t(8;22) found in 5% of BL cases[18] . The common effect of these translocations is that the juxtaposed *c-Myc* allele is expressed constitutively in tumor cells, as opposed to the strict regulation of *c-Myc* levels in normal B cells[19] where it affects different pathways regulating cell cycle, growth, adhesion, differentiation and apoptosis.

c-Myc rearrangements have been demonstrated for the first time in 1982[20] but in the last decade it has been shown that up to 5% of tumors with BL clinical, morphologic and immunophenotipic features lack of *c-Myc* translocation and its overexpression is due to other molecular mechanisms[21]. Other mechanisms leading tit h increased expression of *c-Myc* include interference with the 5' regulatory regions and/or stabilization of the protein product. 5' regulatory regions normally present within the *c-Myc* DNA sequence can be mutated or moved as a direct result of the translocation. Liberation of *c-Myc* from normal regulation can result in increased expression of the protein. The *c-Myc* first exon/first intron border, where *c-Myc* regulatory sequences are located, can be either decapitated by the translocation described previously or undergo mutation in translocated alleles[22].

1.1e Other molecular mechanisms

The overexpression of c-Myc via its juxtaposition with immunoglobulin gene enhancer induces genes like cyclin D2, TRAP1 and HLA-DRB, whereas others like p21 and platelet

derived growth factor receptor-alpha (*PDGFRα*) are consistently repressed, possibly playing a role in the pathogenesis of BL[23].

In addition to the presence of c-*Myc* translocations in the vast majority of BL cases, most tumors contain additional chromosomal abnormalities, the molecular implication of which are poorly understood[24].

Several studies have reported a high frequency of somatic mutations that appear to involve the transcription factor *TCF3*, its inhibitor ID3, or downstream targets (eg. *CCND3*)[25-27]. In one study authors report that approximately 70% of sBL cases had mutations in *TCF3* or *ID3*. Mutations in *CCND3* (a gene encoding cyclin D3, a key regulator of cell cycle progression) were present in 38 %[25].

Interestingly, in recent years, *E2F1* was found to be overexpressed in most sBL. *E2F1* is a member of E2F family of transcription factors that is involved in regulation of cell growth. Furthermore, reduction of *E2F1* expression led to decreased growth capacity in sBL cells in vitro[28].

1.1f Epstein-Barr virus infection

Epstein-Barr virus (EBV) is a member of the herpesvirus family, which includes herpes simplex I and II and varicella zoster virus, cytomegalovirus and human herpesvirus (HHV)-6-7 and -8[29]. EBV is one of the best understood of the human herpesvirus, for which there is an established biological model of infection and persistence. An icosahedral nucleocapsid and a tegument surround a DNA core that is contained in the enveloped virus. The EBV genome encodes for a series of products interacting with or exhibiting homology to a wide

variety of anti-apoptotic molecules, cytokines, and signal transducers, hence promoting EBV infection, immortalization, and transformation[30,31].

EBV has been strongly implicated in the endemic form of BL. Several observations suggest a direct causative role for EBV in endemic Burkitt's lymphoma; monoclonal EBV infection is present in virtually all cases of eBL, approximately 30 percent of sporadic BL, and 40 percent of immunodeficiency-associated BL[32]. The pathobiologic role of EBV is poorly understood and the link in EBV- associated BL is less clear than with endemic BL. Most US cases of HIV-associated BL are EBV-negative whereas the vast majority of HIV-associated BL in Africa are EBV-positive[33,34]. For example, EBV is consistently present in these tumours; infection of malignant B cells precedes tumorigenesis[35]; EBV induces immortalization of B cells in culture; and very high EBV antibody titers are recorded in children before development of the disease[36]. However, the underlying mechanism linking EBV infection of B cells to the emergence of malignancy remains undiscovered.

One hypothesis is that EBV tends to cause a latent infection of B lymphocytes, some of which evade the T cell mediated immune response and enter the germinal center. This subsequently results in excessive B cell proliferation[37] during which process translocations may occur, leading to overexpression of c-Myc.

1.1g Clinical presentation

The abdomen is the most common site of presentation in sporadic BL (60–80%)[38]. Presenting symptoms include abdominal pain (25% of patients have ileocaecal disease—either a right lower quadrant mass or pain from intussusception), distension, nausea and vomiting, and

gastrointestinal bleeding[39,40]. The next most common site is the head and neck, including lymphadenopathy and involvement of the nasal or oropharynx, tonsils, or sinuses. Rarely the jaw is implicated. Bone marrow is infiltrated only in 20% of patients. We observe also some cases classified as Burkitt's leukaemia that are characterized by extensive marrow infiltration (more than 25% blasts)**,** with possible bone pain as a common manifestation at the time of diagnosis. We can also find rare presenting sites include the mediastinum, CNS, skin, testes, breasts, and thyroid gland. Patients with endemic BL most frequently show with jaw or periorbital swellings, or abdominal involvement (of retroperitoneal tissue, gut, ovary, or kidney)[41]. 15% present with sudden paraplegia and incontinence. Infiltration of bone marrow is rare. Jaw involvement is common in young children (peak ages of incidence 3–7 years)[42]. It has been observed in low income regions, such as in sub-Saharan Africa, an advanced disease in many children. In a study of 84 Malawian children with BL, 26 (31%) presented with facial disease only and 52 (62%) with abdominal disease; 58 (69%) had St Jude stage III or IV disease[41].

1.1h Histopathology and immunohistochemistry

BL is a highly aggressive B-cell non-Hodgkin lymphoma characterized by monomorphic medium-sized cells with a very high proliferation rate. The cells are intermediate in size and contain coarse chromatin and prominent basophilic nucleoli. Some plasmacytoid and atypical variants show more nuclear pleiomorphism. In tissue sections, typically the cells seem to be moulded and the cytoplasm is deeply basophilic with squared-off cytoplasmic margins. The proliferation index is almost 100%, with a high turnover shown by increased apoptosis. A "starry sky" appearance is due to scattered

tangible-body-laden macrophages that contain apoptotic tumour cells[43]. The cells are always of B-cell lineage (CD20 positive and CD79a positive). CD10 and Bcl-6 are commonly coexpressed, but the cells are generally negative for Bcl-2. There is also a low number of T cells in the background[44]. Epstein-Barr-encoded RNA can be identified by fluorescence in-situ hybridization. Classification is difficult when the cells have the morphology of diffuse large B-cell lymphoma but the genetic and immune-phenotypic features of BL. Some of these cases are now classified as “B-cell lymphoma, unclassifiable, with features between diffuse large B-cell lymphoma and BL”. However, distinct molecular changes in BL could provide a more reliable diagnosis.

1.2 MicroRNA

1.2a History

In the traditional central dogma of molecular biology, the RNA molecule was considered just an intermediate of gene expression from DNA to protein. However, the finding of RNA interference involved RNA molecules in the regulation of gene expression.

Small RNA molecules can bind to other specific RNAs and either decrease or increase the activity of the targeted RNAs. For example, small interfering RNA (siRNA) binds to specific target mRNAs, resulting in decrease of translation of the mRNAs[45].

The discovery in 1993 of a small endogenous regulatory RNA molecule in the nematode *Caernorhabditis elegans* (*C. elegans*) set the scenes for the description of a large family of short (≈22 nt) single-stranded ribonucleic acids termed microRNAs (miRNAs)[46]. Since the discovery, many microRNAs have been identified in most eukaryotic cells and have been considered critical posttranscriptional regulators of gene expression in complex life. It is not surprising, therefore, that miRNAs are themselves tightly regulated to allow the shaping of gene expression in a temporally restrained and tissue-specific manner instrumental for properly structured organismal development and growth[47].

To date it has been demonstrated in several studies that miRNAs participate in many cellular pathways and have provided many clues for missing part of cellular pathways[48-51].

1.2b Canonical miRNA biogenesis

The generation of miRNAs is a multistage process and takes place first in the nucleus and it continues in the cytoplasm (Figure 4). Long primary transcripts, called pri-miRNAs, are transcribed from genomic DNA by RNA polymerase II or III[52]. The majority of miRNAs are found in intergenic regions and for this reason the transcription of intergenic miRNAs is usually dependent on their own promoters. On the other hand, other miRNAs genes are located in protein-coding genes resulting in the name of intragenic miRNAs: this group is divided into exonic and intronic miRNAs based on their localizations. The expression of intragenic miRNAs depends on the promoter of their host genes[52]. The miRNA must therefore be excised during its biogenesis to cause gene silencing; two endoribonucleolytic enzymes are responsible for this excision. The first endonucleolytic reaction takes place in the nucleus and is driven by two proteins associated with each other; the transcribed pri-miRs are processed to precursor miRNAs (pre-miRNAs) by the endoribonuclease Drosha in complex with the dsRNA binding protein DGCR8 (DiGeorge critical region 8). Drosha is a highly conserved 160 kDa protein containing two RNAse III domains and one double-strand RNA-binding domain. Drosha forms a huge complex, 500 kDa in *Drosphila melanogaster* (*D. melanogaster*) and 650 kDa in *Homo sapiens*, called Microprocessor and containing the co-factor DGCR8. DGCR8, known also as Pasha in *C. elegans* and *D. melanogaster*, recognizes and binds to the double strand region of the pri-miRNA, functioning as a molecular ruler: DGCR8 arranges the Drosha cut site 11bp from the base of the hairpin stem. This progression releases a pre-miRNAs that possesses a 3' extension. After being processed to pre-miRNAs, the precursors are exported to the cytoplasm via Exportin-5 (Exp5) in complex with Ran-GTP.

Once exported, the pre-miRNA is processed by a second endoribonucleolytic reaction, catalyzed by RNAse III Dicer[53], which digests an ≈22nt RNA duplex with protruding 3' projections at both ends. At this stage of the processing an Argonaute protein binds the duplex miRNA/miRNA* and one strand, complementary to the target mRNA (guide strand), is selected and subsequently forms the miRNA effector (mature miRNA) as part of the miRISC (miRNA-induced silencing complex). Whereas one of the two strands is selected as guide strand according to thermodynamic properties, the remaining strand, termed also as passenger strand or miRNA* (star miRNA; many publications refer to the two strand pair as miR-3p/miR-5p, referring to the direction of the functional miRNA) is released and degraded. The so-called miRNA* was initially thought to be the strand subjected to degradation, while more recent evidences suggest that it does not simply represent a non-functional bioproduct of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles[54]. Once the processing steps are completed, the mature single stranded miRNA product is incorporated in the complex known as miRNA-containing ribonucleoprotein complex (miRNP), or miRNA-containing RNA-induced silencing complex (miRgonaute or miR-RISC, a ribonucleoproteic complex containing Argonaute proteins, of which AGO1 and 2 have been the most widely studied. As a part of this complex, the mature miRNA is able to regulate gene expression at the post-trascriptional level, binding for the most part through partial complementarity to target mRNAs in mammals, and mainly leading to mRNA degradation or translation inhibition.

1.2c Alternative miRNA biogenesis pathways

The two endoribonucleolytic cleavage steps represent the most obvious points at which functional miRNA production can be regulated. The first endoribonucleolytic digestion takes place in the nucleus as previously described and presents some unconventional processing steps of miRNAs.

An alternative miRNA biogenesis pathway, called miRtron pathway, has been discovered among diverse mammals, drosophila and nematodes[55-58]: miRtrons are regulatory RNAs which get processed to form pre-miRs using the splicing machinery without Drosha-mediated cleavage. Moreover Drosha-DGCR8-mediated processing of let-7 pri-miRNA can be inhibited by Lin28B[59], although the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) binds specifically to pri-miR-18a to promote its processing[60]. In the cytoplasm, Dicer catalyzes the second endonucleolytic reaction and is essential for miRNA maturation; it's been also demonstrated that its knockdown in human cell lines marks the production of aberrant miRNAs[61] and moreover the accumulation of pre-miRNAs.

A number of recent reports have described regulatory mechanisms that affect on Dicer processing, labeling Dicer as a regulatory node in the cytoplasm. One such controlling pathway modifies via 3'end uridylylation of the pre-miRNA which alternatively promotes or inhibits dicing and the pre-miRNA maturation[62,63] (Fig.4B). Dicer is a very large enzyme (approximately 200 kDa) which is conserved among the species and presents several different domains: a double strand RNAbinding domain (dsRBD); two RNAse III catalytic domains, one which binds the 30-end small RNAs (PAZ domain) and a second which binds

other domains with ATPasic and RNA-helicasic activity. Since Dicer does not function alone but in association with additional proteins (Fig.4C), such as TRBP (transactivating response RNA-binding protein) and PACT (protein activator of PKR), we find even more regulatory mechanisms at this level. TRBP and PACT interacting with Dicer may alter its activity to give different miRNA isoforms with new target specificities[47].

Furthermore, recent discoveries showed that members of the Argonaut family participate in the Dicer-mediated processing of pre-miRs, playing an important role in stabilizing the complex Dicer-miRNA[64]. In mammalians Ago2 protein complex, characterized by RNAse H activity, cooperates in the Dicer-mediated processing of some pre-miRNAs, yielding to another intermediate processing product, called AGO2-cleaved precursor miRNA (ac-pre-miRNA)[52].

1.2d Mechanism of action and function of human microRNAs

Once the microRNA processing steps are completed, the mature single stranded miRNA is integrated in the miRNP or miR-RISC complex to inhibit their target genes by pairing the 3'UTR messenger RNA (mRNA). The mature form of miRNA is about 22 nucleotides long but only a small region of the non-coding RNA binds to its complementary sequence in the 3'UTR of the target gene: the 7-9 nucleotides from the 2^{nd} to 8^{th} (9^{th} or 10^{th}) nucleotide are identified as "seed region"[65]. The seed sequence has a major role in microRNA function and its interaction with 3'UTR target leads to repression of gene expression through three different mechanisms: (a) site-specific cleavage; (b) enhanced mRNA degradation; and (c)

translation inhibition. The first one is restricted to miRNAs with a prefect or near-perfect match to the target mRNA and it's considered a very rare event in mammals, and it's exclusively Ago2 dependent. The second and the third mechanisms just listed represent the common scenario in mammals and are frequently associated with mismatched miRNA/target sequences. Nonetheless the exact pathway through which miRNAs can impair translation is still debated, this mechanism together with the enhanced mRNA degradation are commonly defined as a non-cleavage repression, and can be carried out by any of the four components of the Ago family[66]. In addition, at this step we can find further pathways that can control the microRNA-mediated regulation of gene expression, like for example the formation of mRNA secondary structure capable of inhibiting the access of the target by the small non-coding RNA[67]. Likewise RNA binding protein can cover the complementary region of target mRNA by binding it and avoiding the microRNA seed sequence to pair with its target gene. Besides this canonical targeting regulation, more recent studies report that microRNA can also bind the 5'-untraslated region (5' UTR) or the ORF[68,69] and, even more remarkably, they can up-regulate translation upon growth arrest condition[70]. It is not surprising that each miRNA has the potential to target a large number of genes, since they are controlled by highly developed regulatory pathway and fine-tuning mechanisms in both miRNA processing and recognizing the target[71-73]. For this reason miRNAs are considered versatile regulators of gene expression and to date we can count roughly 60% of the mRNAs as sharing one or more sequences that are evolutionarily conserved and predicted to interact with miRNAs. Bioinformatics analysis predicts that 3' UTRs of single genes are often targeted by several different miRNAs[74].

1.2e microRNAs in cancer: oncogenes or tumor suppressors' functional evidence

Since their discovery in 1993 the role of microRNAs has been elucidated in various cellular pathways and diseases. For instance, members from the family of let-7 have been studied and found involved in several diseases such as asthma, hypertrophy, inflammation, Parkinson's disease and various cancers[75-77].

Over the time, cancers develop refined networks of biological events allowing them to grow and, in some cases, escape treatment. In the pathogenesis of cancer, dysregulated miRNAs may function as either tumour suppressor or oncogenes (Figure 5) by targeting one of the six essential features of cancer progression described by Hanahan and Weinberg: self-sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis evasion, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis[78]. Experiments of gain- and loss-of-function have provided new insights into the role of miRNAs in carcinogenesis. For example, tumour suppressor miRNAs have been studied through gain-of-function approaches showing crucial roles in various cancer pathways by targeting oncoproteins such as *BCL2*, found regulated by miR-*15a* and miR-*16-1*[79], *MYC* or *RAS*, both targeted by let-*7*[80,81] and *MCL1* (myeloid cell leukemia sequence 1) modulated by miR-*29b*[82]. Vice versa, to delineate the biological effects of oncogenic miRNAs, often overexpressed in cancer cells, in vitro silencing was performed using antisense oligonucleotides. For instance, a cohort of miRNAs such as miR-*221*, miR-*222*, miR-*21*, miR-*24*, miR-*133*, miR-*17-5p*, miR-*19*, miR-*25* and miR-*128* targets pro-apoptotic genes, resulting in inhibition of apoptosis.

The first examples of miRNAs with oncogenic activity validated in engineered animal models were miR-*17-92* cluster and miR-*155*, both discovered to be over-expressed in lymph proliferative disorders, including lymphomas and leukemia[83,84]. Infection of murine hematopoietic stem cells with a retrovirus carrying the mir-*17-92* cluster accelerated the development of lymphomas in Myc transgenic mice[83]. Two different studies describe the development of lymphoproliferative disease and autoimmunity in transgenic mice overexpressing miR-*17-92* cluster in B cells[85,86]. Lymphocytes higher rate of proliferation and lower rate of activation-induced cell death in these mice were partially accredited to the direct targeting of the anti-apoptotic genes *BIM* and *PTEN* by miR-*17-92* cluster. Moreover, Ventura and colleagues showed that mice deficient for miR-*17-92* cluster died shortly after birth with lung hypoplasia and a ventricular septal defect[85].

Lastly, a subsequent study showed that deletion of the whole cluster miR-*17-92* reduces Myc-induced oncogenesis[87]. In contrast, miR-*155* overexpression in the lymphoid compartment was sufficient to cause cancer without any other cooperative mutation or Myc expression. Costinean and colleagues developed the miR-*155* transgenic mice that showed polyclonal lymphoid proliferation followed by acute lymphocytic lymphoma or leukaemia[88]. These data were the first to report that the dysregulation of a single miRNA can lead to malignancy. Further, it was one of the first, and still few, miRNA engineered animal models, which, through knock out or transgene introduction, can provide the genetic demonstration of the causative involvement of a specific microRNA in a biological phenomenon. Regardless of notable recent studies in cancer-related microRNA, it is unlikely that miRNAs will be found responsible for a specific phenotype by aiming at a specific target. Though many critical

questions about microRNAs remain to be addressed, it is largely accepted that miRNAs engage in a complex interactions with the machinery that controls the transcriptome and simultaneously target multiple mRNAs. This is probably the most captivating foundation supporting the idea of using microRNAs as anticancer drugs.

1.3 Thesis significance

The revolution in cancer research carried to a conclusion that cancer is, in essence, a genetic disease. Alterations in three types of genes contribute to tumorigenesis: oncogenes, tumor-suppressor genes and stability genes are considered responsible for initiation and development of cancer. Recent studies outlined miRNAs as an important stride in maintaining the balance among genes and regulating cell fate through critical mechanisms such as cell growth, cellular differentiation and programmed cell death. Imbalance in miRNAs expression levels has been shown to be a frequent hallmark in different human malignancies that can destabilize this equilibrium, thus contributing to cancer development and/or progression at different levels from initiation to metastatic disease. Data available to date clearly support the involvement of miRNAs in cancer etiology, and strongly suggest a possible use of these molecules as markers of diagnosis and prognosis, and eventually as new targets or tools for a specific therapy. Over the past few years miRNA signatures have been described to characterize different types of BL or to investigate the expression of miRNAs possibly regulated by c-*Myc* in BL cases positive or negative for Myc translocation. Furthermore microRNA expression profiles have been used to classify different types of BL from other lymphoma malignancies, such as different type of B cell Non-Hodgkin lymphoma (B-NHL), like B cell Chronic Lymphatic leukemia (B-CLL) or Diffuse Large B cell Lymphoma (DLBL). However, it remains unclear what the functional role of differentially expressed miRNAs is and no further studies have been conducted.

In the present thesis the reported researches focused on identifying the differentially expressed miRNAs in Burkitt Lymphoma using as control tissues extracted from lymph nodes of healthy patients. Previously, only T-cell have been used as negative control to compare RNA extracted from B cells obtained by BL patients. In this study we present a microRNA profile acquired by analyzing lymph nodes from BL patients and reactive lymph nodes from healthy patients or mononucleosis affected patients where by definition we can find a large cell population of B cells.

In addition, the present thesis focused on miR-*221/222*, an important family cluster of miRNA previously described as oncogenes in solid tumours. We found this cluster to be interestingly down-regulated in BL and we investigated the role of miR-*221* and miR-*222* in both human and mouse models of BL. Many studies to date have been conducted on the role of miR-*221/222* in cancer development, outlining the cluster as an oncomiR in solid tumors. Our hypothesis deviates from the theory developed in the last few years about the role of miR-*221/222* in carcinogenesis, indicating that miRNAs' function is extremely connected to cellular context. In fact, tumours can be mostly classified as liquid or solid and besides having diverse clinical and pathological features, they can have also different types of oncogenes, tumor suppressor genes and stability genes to be altered[89]. Liquid tumours include leukemias and lymphomas, are composed of neoplastic cells whose precursors are normally mobile. Solid tumours are composed of epithelial (epithelial tumors) or mesenchymal cells that normally are immobile. Liquid and solid tumours show numerous other differences, such as the number of mutations required to develop liquid or solid tumours. On the genetic level at last three mutations seem to be necessary to advance a

malignant solid tumour in adults[90]; in contrast, only one or two mutations may be required to develop a malignant liquid tumour[91]. For this reason also miRNAS can have multiple and conflicting roles in solid tumours than in liquid tumours. To date only one evidence show a down-regulation of miR-*221/222*, whereas the overexpression of miR-*221/222* has been observed in a number of advanced malignancies, indicating that miR-*221/222* could be potential therapeutic targets for epithelial cancer such as glioblastomas[92], thyroid papillary carcinomas[93], breast cancer[94,95], hepatocellular carcinoma[96] and, lung cancer[97]. These studies demonstrated that miR-*221/222* overexpression has important consequences on the proliferation rate and the cell cycle distribution. Nonetheless lately also a downregulation of this family of miRNA encoded in tandem on the X chromosome in human, mouse and rat has been observed; in erythropoietic (E) culture of cord blood CD34+ progenitors cells, miR-*221/222* levels are markedly down-modulated, indicating that microRNA function depends on the cellular setting. In erythropoietic culture undergoing exponential cell growth, miR down-modulation is inversely related to increasing Kit protein expression. Treatment of CD34+ progenitors with miR-*221/222* is able to induce impaired proliferation and increased differentiation of E cells. Likewise our study evidences a tumor suppressive role of the cluster *221/222*, indicating once more that microRNA function is exclusively dependent on the cellular context and tumour type.

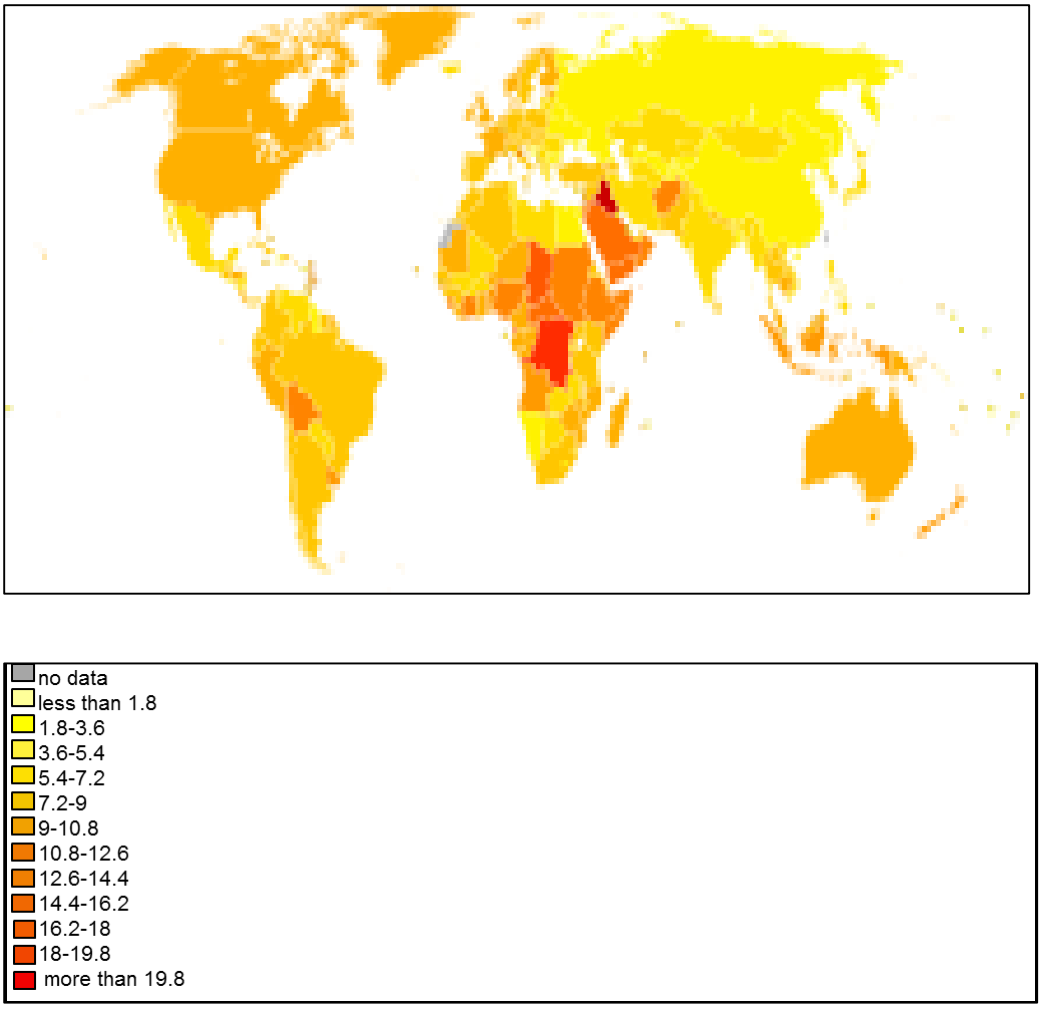


Figure 1. Worldwide lymphoma-related mortality rates as reported by the World Health Organization according to data retrieved in 2009. This displays the estimated average number of deaths by country for every 100,000 inhabitants. Notes: the data/color given for the following former countries were assigned as follows: "*Serbia and Montenegro*": Serbia, Montenegro. The following groupings/assumptions were made: France includes the overseas departments as well as overseas collectivities. The United Kingdom includes the Crown dependencies as well as the overseas territories. The United States of America includes the insular areas. The Netherlands includes Aruba and the Netherlands Antilles. Denmark includes Greenland and the Faroe islands. China includes the SARs of Hong Kong and Macao.

Data retrieved: http://www.who.int/healthinfo/global_burden_disease/estimates_country/en/index.html

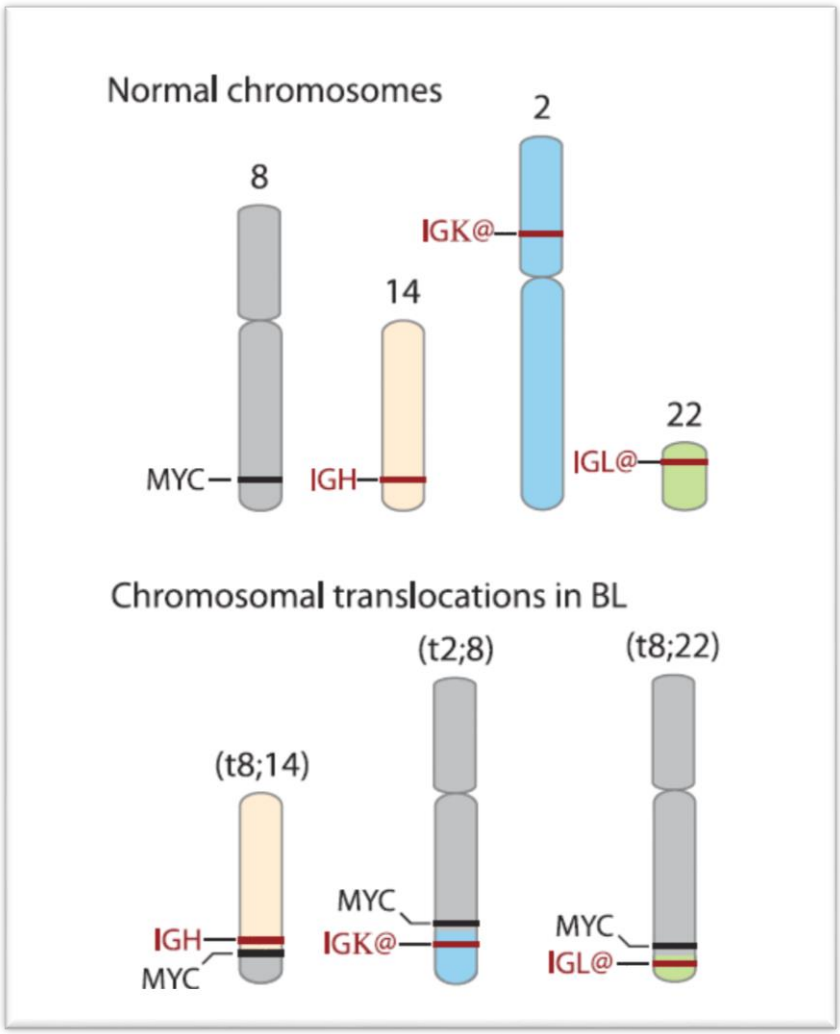


Figure 2. Chromosomal translocation occurring in Burkitt Lymphoma.

In Burkitt Lymphoma overexpression of c-*Myc* is invariably connected to a chromosomal translocation of the Myc proto-oncogene to either chromosome 14, 2 and 22, harbouring the genes for the immunoglobulin μ (IGH) heavy chain and κ (IGK@) or λ (IGL@) light chains.

Figure by (Klapproth and Wirth, 2010)

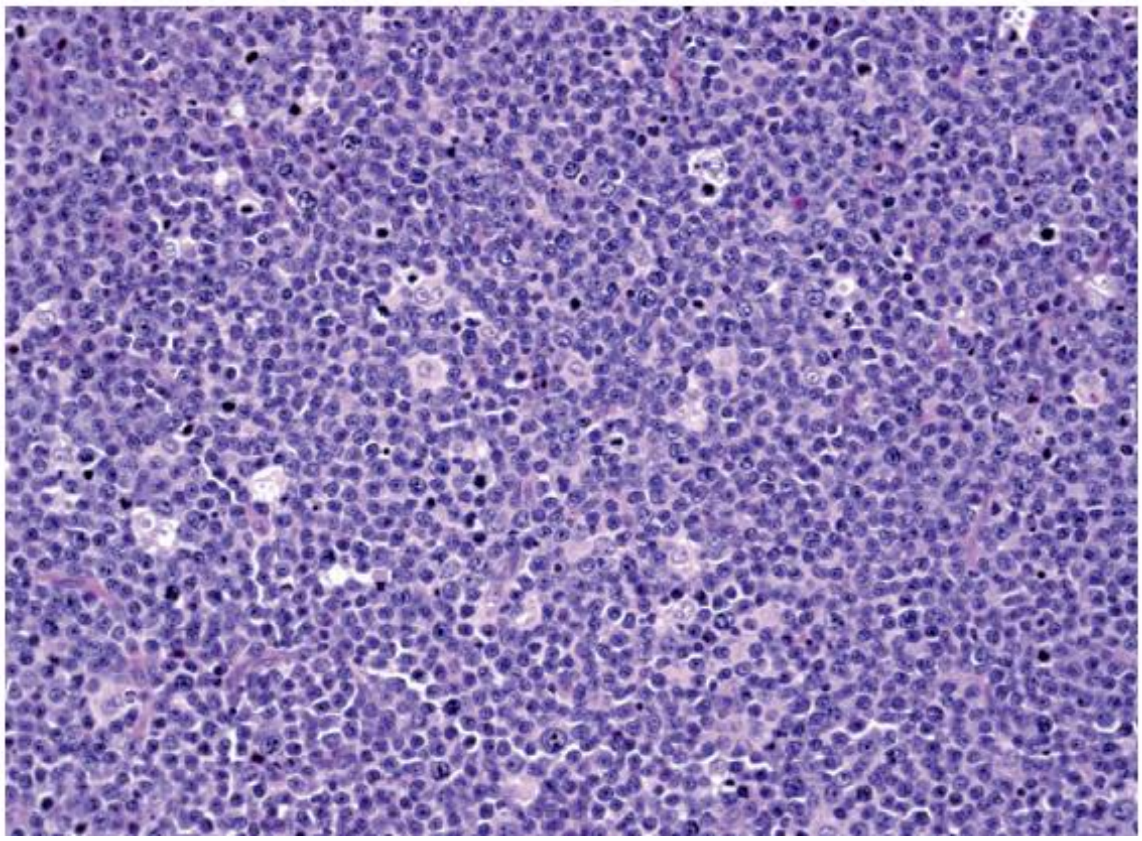


Figure 3. Burkitt Lymphoma "starry-sky" pattern.

Low-power magnification showing sheets of uniform neoplastic cells with consistently scattered reactive tangible-body macrophages resulting in characteristic "starry-sky" appearance. Hematoxylin-eosin stain.

Figure adapted from Molyneux EM, 2012 [98]

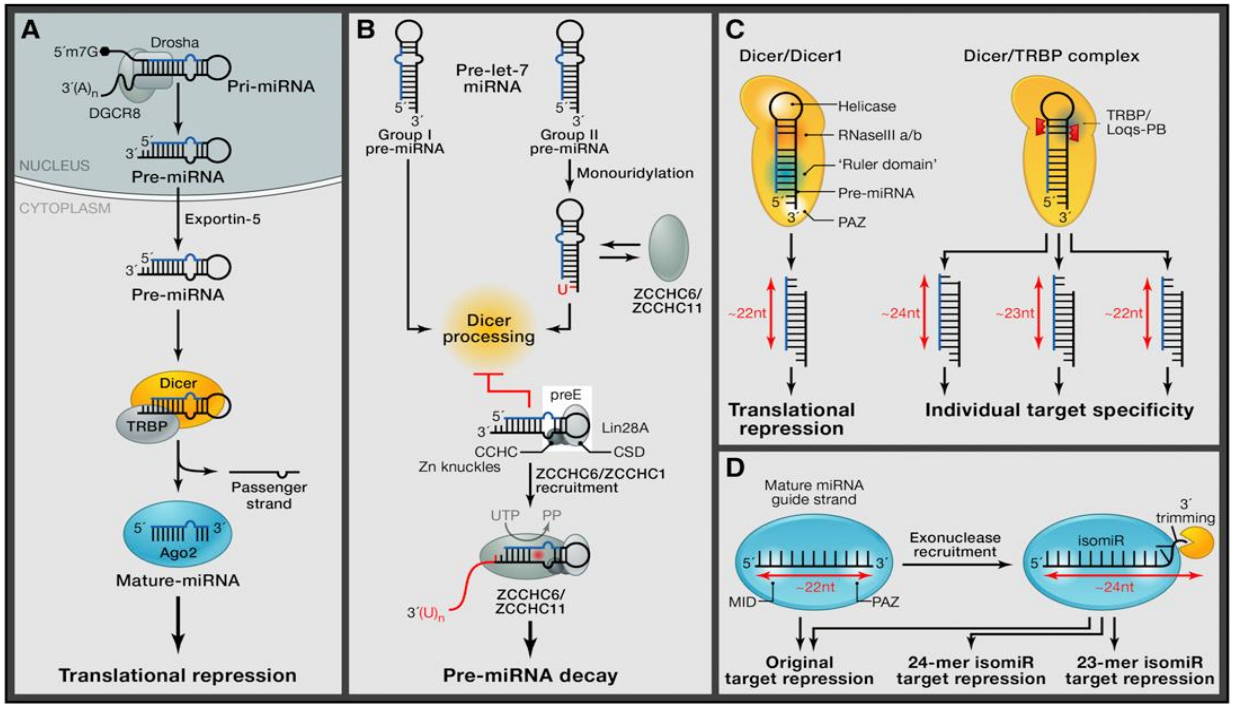


Figure 4. The Regulation of miRNA Function by Means of RNA Length.

- **A.** Canonical maturation pathway of miRNA
- **B.** Modification of the 3' end of pre-miRNA by the untemplated addition of uridines catalized by cytoplasmatic TUTs. Mono-uridylylation facilitates Dicer processing, whereas Lin28 binding and oligo-uridylylation are inhibitory.
- **C.** Tuning of Dicer cut-site selection (shifted by 1-2 nt) by its binding partners allows the generation of specific isomiRs with altered target specificities.
- **D.** Exonucleolytic 3' trimming to the Ago-loaded mature miRNA alters the length and thus target specificity.

Figure by (Yates et *al.*, 2013)

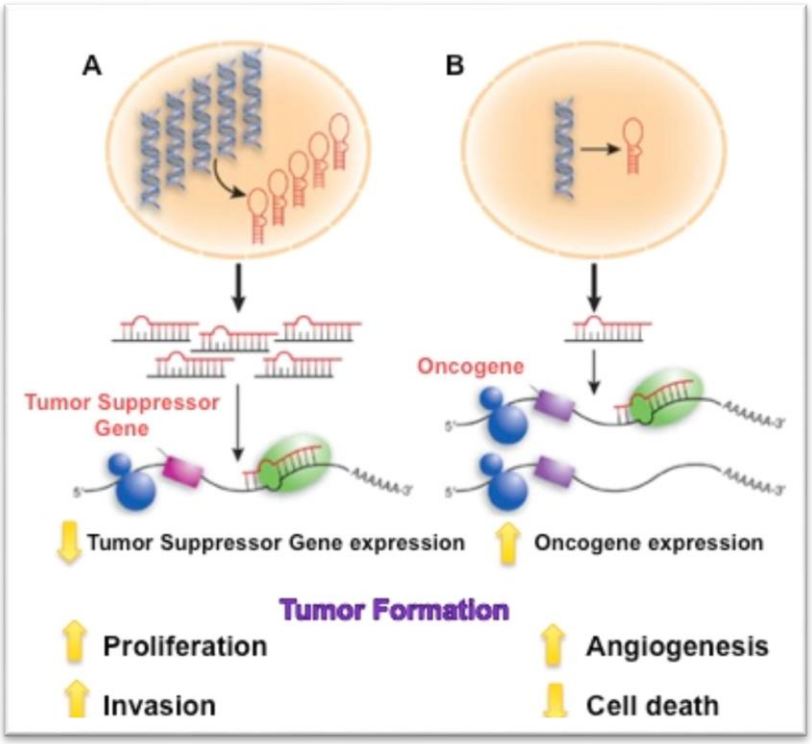


Figure 5. MicroRNAs as oncogenes and tumour suppressor genes.

MiRNAs can have oncogenic effects (oncomiRNA) when they target tumour suppressor genes. When an oncomiRNA is overexpressed, for example because the encoding gene is located in an amplified region of the genome, this will lead to down-regulation of the targets and to tumour formation (A). Conversely, a miRNA can be characterized by tumour suppressor properties if the main target in that specific cellular context is an oncogene; in this case, if the miRNA expression is lost, for example because the encoding gene is located in a deleted region of the genome, the resulting effect will be tumorigenic (B). In summary, what usually happens in a tumour is the over-expression of an oncogenic miRNA, and/or the loss of a miRNA with oncosuppressive properties.

Figure adapted from Caldas C, 2005[99].

Chapter 2- Materials and Methods

2.1 Samples and reagents

2.1a Patients

Tissue specimens were obtained from the University of Bologna, Sant'Orsola-Malpighi Hospital. We examined 5 sporadic, and 2 endemic BL patients, compared to B cells from reactive lymph nodes of 9 healthy patients and 11 patients affected by mononucleosis.

2.1b Cell lines

All the cell lines used in this work were either purchased from the American Type Culture Collection (ATCC, Manassas, VA) or previously generated in Prof. Croce CM's laboratory and kindly provided to me. Burkitt Lymphoma cell lines EBV positive (Daudi, Namalwa, Raji, EB3 and P3HRI) and EBV negative (Loukes, BL41 and LY91) were obtained from ATCC. The cell line BJAB were obtained by Life Tecnologies. The cell line EW36 was derived from an American case diagnosed as undifferentiated lymphoma and EBV is not present in the tumour of origin, nor is found in the cell line but in certain respects displays the molecular features of eBL cases and it was formerly studied and well characterized[100] by Prof. Croce's group. RS11896, SK-DHL, REH and WNM of unknown EBV status were also obtained from ATCC. Lymphoblastoid cell lines (ADO cell lines) were made in Prof. Croce's lab and obtained from peripheral blood lymphocytes of patients with Alzheimer's disease by transformation with EBV following the protocol reported previously[101]. Pekarsky *et al* described and characterized ADO2069, ADO1476, ADO1471 and ADO1701 in their manuscript[102].

Burkitt lymphoma cell lines, ADO cell lines and IM9 were grown in Roswell Park Memorial Institute 1640 (RPMI) medium, containing 10% heat-inactivated fetal bovine serum (FBS), 2mM l-glutamine and 100 U/mL penicillin–streptomycin.

2.1c Animals

All animals were housed under specific pathogen free conditions and all experiments were performed according to animal use protocols and guidelines approved by the Ohio State University and approved by the Institutional Animal Care and Use Committee of the OSU Wexner Medical Center Comprehensive Cancer Center.

The EμMyc tg mouse used for our in-vivo studies was purchased from The Jackson Laboratory company. The former name was C57BL/6J-Tg (IghMyc) 22Bri/J. First, the TgN (IghMyc) 22Bri transgenic strain was made in the laboratory of Dr. Ralph Brinster, University of Pennsylvania in collaboration with Dr. Alan Harris, currently of the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The transgene construct consists of the mouse Myc oncogene (*c-myc*) in association with Emu immunoglobulin heavy chain enhancer and Myc promoter. Expression of the mouse *Myc* is restricted to the B cells lineage. The strain was maintained by mating hemizygous male to a wild-type C57BL/6J female.

For the validation of downregulation of miR-*221* and miR-*222* EμMyc tg mice and wild-type C57BL/6 littermates were sacrificed by cervical dislocation. Selected tissues (listed in the results section) were collected to purify B cells for subsequent RNA extraction.

Constitutive miR-*221/222* null mice were generated by a standard gene-targeting approach using the R1 embryonic stem cell line as previously described[103,104] by Gianpiero Di Leva (data

unpublished) and kindly given to me. The miR-*221/222*-specific targeting vector was obtained by recombineering technology[105]. All the mice used in the experimental procedures were backcrossed at least four generations.

2.1d RNA extraction protocols

Recover all kit from Ambion was used to extract RNA from tissue paraffin embedded samples obtained from lymph nodes of cancer and healthy patients, following the manufacturer's instructions.

Total RNA from cell lines and B cells (CD19+) isolated from enlarged lymph nodes or spleen tissues collected from Eμ-Myc transgenic mice was isolated by TRIzol (Invitrogen) according to manufacturer's instructions, starting from at least $1x10^6$ cells.

2.2 Detection of microRNAs expression

2.2a MicroRNA Expression arrays

Microarray analysis was performed as previously described[106]. Briefly, 5 micrograms of total RNA were used for hybridization of miRNA microarray chips. These chips contain gene-specific oligonucleotide probes, spotted by contacting technologies and covalently attached to a polymeric matrix. The microarrays were hybridized in 6X SSPE (0.9 M NaCl/60 mM NaH2PO4 ·H2O/8 mM EDTA, pH 7.4)/30% formamide at 25°C for 18 hrs, washed in 0.75X TNT (Tris·HCl/ NaCl/ Tween 20) at 37°C for 40 minutes, and processed by using a method of detection of the biotin-containing transcripts by streptavidin-Alexa647 conjugate. Processed slides were scanned using a microarray scanner (Axon), with the laser set to 635 nm, at fixed PMT setting, and a scan resolution of 10 mm. Microarray images were analyzed by using GenePix Pro and post-processing was performed essentially as described earlier[107]. Concisely, average values of the replicate spots of each miRNA were background-subtracted and subject to further analysis. miRNAs were retained when present in at least 20% of samples and when at least 20% of the miRNA had fold change of more than 1.5 from the gene median. Absent calls were thresholded prior to normalization and statistical analysis. Normalization was performed by using the quantiles method. MicroRNA nomenclature was according to the microRNA database at Sanger Center. Data analysis. F635-background values were used. Bad spots were removed. Non-Expressed spots were averaged for each gpr files (chip). For each mature miRNA, we computed the geometric mean of its multiple

reporters in the chip. A NaN value was assigned to miRNAs with more than 50% of corrupted spots, as reported by the GenePix image analysis software. All the results were log2-transformed. The Normalization was performed by using the quantiles normalization, as implemented in Bioconductor "affy"" package[108]. We performed class comparisons algorithms in BRB-ArrayTools using the paired t-test ($p < 0.05$, FDR <0.05). Genes were considered statistically significant if their p-value was less than the chosen value of each analysis. A significance threshold was used to limit the number of false positive findings. Class comparison algorithms in BRB-ArrayTools were used to determine whether gene expression patterns could differentiate between lymphoma samples and controls[109].

2.2b Nanostring analysis

The NanoString nCounter Human miRNA Expression Assay Kit was used to profile more than 700 human micro-RNAs (miRNAs) in normal mammary gland or tumour tissues from MMTV-HER2/neu mice (http://www.nanostring.com); 100 ng total RNA were used as input for nCounter miRNA sample preparation reactions. All sample preparation was performed according to the manufacturer's instructions (Nano-String Technologies, Seattle, WA). Preparation of small RNA samples involves the ligation of a specific DNA tag onto the 3' end of each mature miRNA. These tags are designed to normalize the Tms (melting temperatures) of the miRNAs as well as provide a unique identification for each miRNA species in the samples. The tagging is accomplished in a multiplexed ligation reaction using reverse complementary bridge oligonucleotides to direct the ligation of each miRNA to its

designated tag. After the ligation reaction, excess tags and bridges are removed, and the resulting material is hybridized with a panel of miRNA tag specific nCounter capture and barcoded reporter probes. Hybridization reactions were performed according to the manufacturer's instructions with 5 μL fivefold diluted sample preparation reaction. All hybridization reactions were incubated at 64 °C for a minimum of 18 h.

Hybridized probes were purified using the nCounter Prep Station (NanoString Technologies) following the manufacturer's instructions to remove excess capture and reporter probes and immobilize transcript-specific ternary complexes on a sterptavidincoated cartridge. Date collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high density (600 fields of view) was performed. The samples analyzed using the Nanostring technology are subject to lane-to-lane variations that can arise during processing. To minimize the impact of these sample preparation and detection anomalies, the data were normalized to the positive control count values. The positive controls are 63 synthetic miRNA sequences included in the assay to confirm successful ligation of the miRNA to the tags. This type of normalization is called "Technical Normalization". Next,a biological normalization method "Quantile Normalization" has been applied[108]. To identify differentially expressed miRNAs between samples from twoclasses we used the non-parametric statistical test called Significance Analysis of Microarrays (SAM)[110]. SAM identifies miRNAs with statistically significant changes in expression by assimilating a set of miRNA-specific t tests. Once the set of significant (Positive and Negative) miRNAs have been computed, a cluster analysis has

been conducted to identify subgroups of significant miRNAs sharing a similar expression level. This has been done through the Average Linkage hierarchical clustering algorithm. These results have been obtained using the SAM and cluster analysis module of the Tmev (TM4) system[111].

2.2c Quantitative Real Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed with the TaqMan PCR Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, followed by detection with the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. Simultaneous quantification of 54 small endogenous nucleolar RNA U44/U48/U6 (for human samples) or snoRNA-135/snoRNA-234 (for mouse tissues) was used as a reference for TaqMan assay data normalization. The comparative cycle threshold (Ct) method for relative quantization of gene expression (User Bulletin #2; Applied Biosystems) was used to determine miRNA or mRNA expression level. For all the qRT-PCR experiments, values on the *y*-axis equal to $2^{-(\Delta Ct)}$, where ΔCt is the difference between gene Ct and normalizer gene Ct. For quantification of DUSP6 mRNAs, the appropriate TaqMan probes were purchased from Applied Biosystems (Hs04329643_s1). Data were normalized using GAPDH or OAZ1.

2.3 Target identification and validation

2.3a Transfection

Bjab cell line was transfectd by electroporation using AMAXA 3D machine and supplies according to manufacturer's instructions. Both the pre-miRNA molecules (Ambion) and the corresponding scrambled oligonucleotides, were transfected at a final concentration of 100 nM, unless otherwise indicated and the transfection efficiency was greater than 95% for all the cell lines used.

2.3b Mouse B cells purification

Enlarged spleens, enlarged cervical, brachial or inguinal lymph nodes were isolated from EuMyc tg mice. Magnetic activated negative selection B-cell isolation kit was used to purify B cells from tissues (Miltenyi Biotec) after lysing red blood cells with 155 mM ammonium chloride.

2.3c Gene expression profiles

Total RNA was extracted with the use of the TRIzol reagent (Invitrogen and Life Technologies), purified with the use of the RNeasy Kit (QIAGEN), and processed according to the Affymetrix Expression Analysis Technical Manual. Fragmented cRNA (15 g) was

hybridized to HG-U133 2.0 Plus microarrays (Affymetrix). The gene expression values were determined by MAS 5 algorithm in Expression Console (Affymetrix), and normalization was performed by scaling to a target intensity of 500. The normalized data were managed and analyzed by BRBArrayTools Version 4.3.2. Affymetrix CEL files were imported and RMA normalized. Genes whose expression differed by at least 1.5-fold from the median in at least 20% of the arrays were used. We performed class comparisons algorithms in BRB-ArrayTools using the paired t-test ($p < 0.05$).

2.4 Phenotyping

At autopsy, selected tissues were immediately fixed in 10% (v/v) Gurr neutral buffered formalin (pH 7.4) for 48 hours before dehydration and embedding in paraffin wax. 5 µm sections from paraffin blocks were examined for histopathologic features: 2 sections per tissues were stained with Mayer's haematoxylin and eosin. Tissue sections were examined by a clinical pathologist from Ohio State University Veterinary School experienced in the histological analysis of mouse tissues. For antibody staining, high-temperature antigen retrieval was performed overnight by incubating sections with 0.01 M citrate buffer. Endogenous peroxidase was quenched by 1% v/v H_2O_2 in PBS for 30 minutes. Sections were blocked with either goat or rabbit serum (10% v/v in PBS) depending on the host species of the secondary antibody. Primary antibodies were incubated for 1 hour at room temperature and were detected by HRP polymer anti-mouse or anti-rabbit Envision antibodies (DAKO). Sections were developed using DAB substrate (DAKO) then counterstained with Mayer's haematoxylin.

2.5 Bioinformatics tools and statistical analysis

miRNA nomenclature, sequences and related information:

- Genome Browser: http://genome.ucsc.edu

- miR Registry: http://www.sanger.ac.uk/Software/Rfam/mirna/

miRNA target prediction*:*

- Targetscan: www.targetscan.org

- miRGen Database: http://www.diana.pcbi.upenn.edu/miRGen.html

- PITA: http://genie.weizmann.ac.il/pubs/mir07/index.html

Statistical analysis:

Two-tails Student's *t*-test was used to determine statistical significance and the *P* value was calculated for each experiment ($p<0.05$, $p<0.01$).

Chapter 3-Results

3.1 Downregulation of miR-221 and miR-222 in BL patients and human cell lines

3.1a Introduction

BL is a B-cell non-Hodgkin lymphoma (NHL) which has been characterized throughout the years based on a combination of clinical data and morphological, phenotypic and cytogenetic features. New, reliable markers continue to be required to improve the accuracy of lymphoma characterization, diagnosis and therapy selection. In the recent years, the discovery of a new type of non-coding small RNA, miRNAs, has given innovative impulse to cell differentiation and cancer pathogenesis studies[49]. MiRNAs post-trascriptionally regulate the expression of thousands of genes, including key genes in cell differentiation and cancer pathogenesis[80]. First evidence of the relationship between miRNAs and cancer arose with the description of the loss of miR-15/16 in CLL cases by researchers from Prof. Croce's group[112]; since then, an increasing number of specific miRNA changes have been identified in many tumour types. The potential of using miRNAs for differential diagnosis of tumours and hematopoietic malignancies has been recognized in a numbered of disorders and it has been applied also to distinguish two different tumour types. For instance, in acute lymphoblastic and myeloid leukemias, the expression signatures of at least two of four microRNAs (miR-128a, miR-128b, miR-223 and let-7b) discriminated the two tumour types with about 95% of accuracy[113]. The importance of miRNAs as diagnostic markers in B-cell

lymphomas is linked to their role in cellular differentiation, as demonstrated in hematopoietic cells and for example, by miR-150, in B-cell differentiation[114]. Furthermore, altered miRNA expression profiles have been used to test whether different B-cell lymphoma types have specific miRNAs signatures, and to apply this knowledge to identify potential markers for distinguishing different types of B-cell non-Hodgkin lymphomas (NHLs)[115]. In BL, a prior study has demonstrated the value of c-Myc translocations throughout the expression of miRNAs possibly regulated by this gene in BL cases positive or negative for the translocation[116]. Mainly, Onnis *et al* identify miR-9* as potentially relevant for malignant transformation in BL cases with no detectable Myc translocation. Moreover microRNAs signatures in BL have been used to delineate the boundaries between this B-cell malignancy and other NHLs such as DLBCL[115]; these two different lymphoma types show different up-regulated miRs, improving their classification among B-cell NHLs. Although in recent years there have been many significant developments in BL classification, additional molecular markers enabling a better distinction between BL and healthy patients is still needed.

Here we show for the first time a microRNA signature acquired by analyzing and studying a group of BL patients compared to a group of healthy (non-cancer) controls. This is the first profile that has been prepared to described differences in miRNAs expression between disease and healthy in BL. Moreover we identify the down-regulation of a family/cluster of miRNAs previously defined as oncogenes. MiR-221/222 are strongly down-modulated in BL patients when we compared them to reactive lymph nodes from healthy patients. The same pattern of miR-221/222 expression is shown in BL cell lines, when related to their control (non-cancer), analyzed by nanostring and then confirmed by qRT-PCR.

3.1b Results and discussion

To identify miRNAs that are deregulated in BL patients versus non-cancer patients, we used miRNA microarray analysis[117] on a set of 2 eBL, 5 sBL, 9 reactive lymph nodes from non-cancer patients and 11 lymph nodes from mononucleosis affected patients (non-cancer). RNA was extracted by paraffin-embedded lymph nodal tissues as described in chapter 2 (Material and Methods). Interestingly all BLs were grouped within one cluster, presenting with relatively homogeneus profiles for both upregulated and downregulated miRNAs (Figure 6). Furthermore, the two heat maps in figure 6 also show all non-cancer samples clustering together, presenting a distinguished miRNA expression signature differing from BL cluster. Apparently, such intriguing result is not related to EBV status since in both cancer and non-cancer groups we observed the same trend of miRNA expression whether the sample is negative or positive for EBV infection. Indeed in BL cluster we found that each downregulated or upregulated miRNA has the same pattern in both EBV negative (sBL) or EBV positive (eBL) patient samples. Likewise reactive lymph nodal tissues (EBV negative) clustered together with mononucleosis affected lymph nodes (EBV positive). The link between EBV infection and BL is strong, but the mechanism underlying that association is still unclear because conflicting hypotheses have been described throughout the years. A recent study compared gene expression profiles among BL subtypes, including endemic, sporadic, and HIV-associated BL samples. Although the BL samples were clearly distinct from other B-cell malignancies, distinguishing among BL subtypes was less precise and the samples did not cluster according to EBV status[118]. In our research we show a microRNA profile that is not related to EBV infection indicating that the virus is not contributing to the

microRNA pattern and the role of EBV in BL pathogenesis needs further investigations. Indeed, we observed copious miRNAs down-modulated like for example miR-*23a*, let-*7a*, miR-*96*, miR-*24*, miR-*29a*, miR-*182*, miR-*146a*, miR-*21*, miR-*101* and miR-*22* in all cancer patients compared to healthy controls where all previously listed miRs are up-modulated. We also found many miRNAs upregulated in the "tumour" cluster related to their expression in "non-tumour" cluster, as miR-*9*, miR-*122*, miR-*17* and miR-*579*. Among the downregulation of several miRNAs we identified an important miRNA which has been described already involved in several solid tumours, miR-221. MiR-*221/222* are encoded in tandem on the X chromosome in human, mouse and rat and are highly conserved in vertebrates[93]. Moreover, they have the same seed sequence[97]. In accordance with current literature, many studies have been reported on the role of miR-*221/222* in cancer development as oncomiR and only in few cases as oncosuppressor-miR[119,120]. Overexpression of miR-*221/222* has been observed in a number of advanced malignancies indicating that miR-*221/222* could be potential therapeutic targets for epithelial cancers[121]. In our research we observe the downregulation of miR-*221*, suggesting a different role than the one formerly defined in multiple studies on solid tumours, like for example lung, liver and breast cancer. We found this new finding innovative considering that to date only two studies support the hypothesis of the cluster *221/222* to play an oncosuppressive role[119,120]. Our study could be added to these previous indications, leading to the theory that microRNA function depends on the cellular context. After identifying a downregulation of miR-*221* by microarray analysis (Figure 7) in human samples we investigate the pattern of miRNAs expression also by nanostring analysis in two different sets of BL cell lines compared to their

controls. We divided human BL cell lines in two different groups according or not to EBV presence. MiRNAs expression of five EBV positive BL cell lines (Daudi, Namalwa, Raji, EB3 and PH3RI) was compared to five ADO cell lines, B lymphoblastoid cells EBV transformed (IM9, ADO2069, ADO1471, ADO1476, ADO1017), whereas miRNAs level of five EBV negative BL cell lines (BJAB, Loukes, BL41, LY91 and EW36) was related to their negative control, CD19+ B cells (EBV negative). A similar pattern of expression was identified between the two nanostring analyses (Figure 8) and moreover we found some miRNAs downregulated in common with the microRNA signature. For instance miR-*101* and miR-*29a* are shown to be downregulated in both custom microarray platform analysis and EBV negative set of BL cell lines comparison; whereas we detected low expression of miR-*22*, miR-*146a*, miR-*23a*, miR-*21* and miR-*24* in EBV positive BL cell lines set of nanostring investigation in common with our microarray signature. MiR-*221* and miR-*222* are identified downregulated in EBV positive BL cell lines compared to EBV positive lymphoblastoid cell lines and miR-*222* is found downregulated in nanostring analysis of EBV negative BL cell lines cluster. The down-regulation of miR-*221/222* was further confirmed by the qRT-PCR method in a bigger cohort of human BL cell lines (Namalwa, Raji, Bjab, RS11896, SK-DHL, EW36, REH, WNM, EB3, Louches, BL41, PH3RI, Daudi) compared to 4 normal B lymphoblast EBV transformed cell lines (Figure 9). Moreover the statistical analysis established that miR-*221/222* are down-regulated in Burkitt Lymphoma's cell lines compared to normal control with Fold Change miR-221=-33.85, miR-222=-134.80 and p-value for both: $P<0.01$.

3.2 BL in-vivo model shows down-modulation of miR-*221/222*

3.2a Introduction

MicroRNAs as a new class of regulatory molecules have been investigated in many specific cells and organs in healthy and diseased conditions in several types of tumours. These small non coding RNAs are known to play significant roles in animals and humans at the post-trascriptional level and miRNAs signatures have been described in in-vivo model of severe diseases such as pancreatic cancer[122] and gastric cancer[123]. Several mouse models have been created to mimic human malignancies and most of them have been proven useful for studying tumour progression, prevention and therapy. Recent reports from Ali *et al* and Shah *et al* showed that expression profiles of miRNAs in mouse models of respectively pancreatic and gastric cancer can be highly informative in discriminating the malignant from the normal because of the unique patterns of expression of miRNAs of their in-vivo models. These findings indicate the significance and untapped potentiality of comparing miRNAs level in both human and engineered mouse models which emulate genetic alterations found in the corresponding human anomalies. We assessed miRNAs signatures in patients' paraffin specimens from lymph nodes and in BL human cell lines, finding a down-regulation of miR-*221/222*. For a better understanding of our miRNAs' contribution to BL we screened the level of miR-*221* and miR-*222* in a well-known mouse model of BL. Previously in Chapter 1

(introduction), we discussed how chromosomal translocation juxtaposing the MYC protooncogene with regulatory sequences of immunoglobulin (Ig) H chain, on chromosome 14, or kappa (Igκ), on chromosome 2, or lambda (Igλ), on chromosome 22, chain genes and effecting deregulated expression of *Myc* are considering the hallmarks of human BL. The renowned mouse model described for the first time in 2000 and considered the most suitable system to study the pathogenesis of BL used the foundation of the genetic hallmark for BL[124]. A BL with striking similarities with the human BL develops in mice bearing a mutated *Myc* gene controlled by a reconstructed Igλ locus including all the elements required for establishment of locus control in vitro. Since studying miRNA expression levels in mouse models of human malignancies has been previously described to be fundamental in characterizing the molecular basis of the human disease, we investigated expression level of miR-*221/222* in transgenic mice EμMyc. In summary here we propose a new role for the cluster *221/222* after examining a miRNAs signature in BL for the first time compared to "cancer-free" controls in a human model which is also confirmed in a recognized engineered in-vivo model of BL.

3.2b Results and discussion

We used a mouse model of BL purchased from the Jackson Labs (description in Chapter 2) where expression of the mouse *Myc* transgene is restricted to the B cell lineage. The literature reporting this in-vivo model of BL showed that hemizygotes have increased pre-B cells in the bone marrow throughout life and a transient increase in large pre-B cells in the

blood at 3-4 weeks of age. Spontaneous pre-B and B cell lymphomas reach an incidence of 50% at 15-20 weeks in hemizygous progeny of a wildtype female mated with a hemizygous male. Before checking the expression of the cluster *221/222* in the mouse model we characterize and analyze the phenotype of our in-vivo model of BL. We analyze two different transgenic mice of 6 months old, one female and one male. We observed that the massive enlargement of lymph nodes and spleen (Figure 10) coupled with widespread involvement of kidney, liver, and lung is consistent with widespread BL with leukemia. The primary cellular traits of the neoplastic lymphocytes appeared to represent a middle ground between images of BL and Burkitt-like lymphoma as reported in a recent proposal on the nomenclature for lymphoma in mice[125]. However, the distribution of tumor cells at various sites and the extensive apoptosis ("starry sky" phenotype) closely resembled images of BL in Myc-transgenic mice[124]. The tumor cells expressed the B-cell marker B220 but were not producing the T-cell marker CD3. We used B cells negatively selected as described in Chapter 2 (Materials and Methods) collected from enlarged lymph nodes and spleens from six EμMyc transgenic mice to validate miR-*221* and miR-*222* expression patterns. The expression data were compared to B cells isolated from the spleen of three wild-type littermate mice; the relative expression of miR-*221/222* is strongly down-regulated in 13 out of 17 samples obtained from transgenic mice when we evaluate the data sets with the expression of B cells from wild-type mice (Figure 11). To note that we didn't compare the transgenic group of samples to lymph nodes from wild type mice because these small lymphatic organs are often difficult to identify in mice using standard dissection techniques. In facts while enlarged lymph nodes can be readily identified in mice with acute inflammation or advanced cancer,

the lymph nodes of normal mice are small and difficult to distinguish from surrounding adipose and connective tissue[126].

3.3 Gene expression profiles in human and mouse models of BL identify *DUSP6* as common target

3.3a Introduction

Over the past years gene expression profiling has been shown to be a powerful tool that can be used to classify, clarify the origins, and potentially even identify the underlying causes of malignancies. In the field of molecular biology, this technology is defined the measurement of the activity (expression) of thousands of genes at once, to create a global picture of cellular function. The first profiles were shown in early 2000 and if reliable, microarrays could be used for anything from testing a drug's effect to identify differential pathways involved in two or more diverse experimental conditions[127]. In the understanding of BL pathogenesis we identify two pioneering studies published in 2006 which used gene expression profiling to define a molecular BL phenotype that would aid accurate diagnosis[128,129]. They both investigated the gene expression patterns in classical BL, atypical BL (DLBCL with BL-like characteristics, in terms of either c-myc/Ig translocation or immunophenotype) and typical DLBCL. Since BL and DLBCL require different treatment regimes, they assessed through gene expression profiles a molecular BL phenotype that could clarify their diagnosis. Using 95% similarity to consensus molecular BL gene signature as the standard, all classical BLs and most all atypical BLs displayed a molecular BL phenotype; correspondingly, the molecular BL

signature differed from that of typical DLBCLs by mainly relative up-regulation of c-myc target genes. Nevertheless, these revolutionary studies focused on only European and North American tumours, and the EBV status was neither specified nor taken into consideration for the analysis. Concerning the importance of EBV infection in understanding the molecular phenotype of BL, more recent work suggests that endemic BL tumours are broadly similar to sporadic tumours in gene expression profiles indicating that all BL subtypes converge on gene expression profiles that are highly connected and lie within the restricted molecular BL boundary. The last gene expression signature in BL has been conducted to gain further understanding in two types of endemic BL which display different patterns of EBV antigen expression and, by implication, arise by different pathogenic routes[130]. Kelly *et al* concluded that the two types of endemic BL show only slightly distinct phenotypes and both display gene expressions profiles that fit within the molecular BL defined boundary. Furthermore, we can find in gene expression profiles' history of BL a study which demonstrates a shared gene expression signature in mouse models of EBV-associated and non-EBV-associated BL to elucidate the mechanism underlying the link between EBV and BL pathogenesis[131].

Though a few gene expression profile's discoveries have been made throughout the last decade, whether there are patterns within the molecular BL signature that distinguish precise pathways of BL pathogenesis remains to be determined.

3.3b Results and discussion

Once we established that miR-*221* and *222* were down-regulated in both human and mouse models, we wanted to understand what pathways both of the models had in common and how miR-*221* and *222* played a role in these pathways. Therefore, in order to establish the effect of miR-*221* and *222* in a human model, we transfected BL cell line Bjab, which lacks of miR-*221* and *222* expression, with the mature form of both miRNAs (as described in Chapter 2, Materials and Methods). A gene expression analysis was then conducted on extracted RNA from treated Bjab cells collected 48 hours after transfection compared to its negative control collected at the same time point. As shown in figure 13, we analyzed comparative expression levels of both miR-*221* and miR-*222* at 24 hours, 48 hours and 72 hours' time points after transfection and the time point showing the highest expression levels of both miRs is at 48 hours; this time point was selected to establish the gene expression profile. Subsequently we performed a parallel gene expression profile using the mouse model; we compared the RNA extracted from CD-19+ of the wild type spleen with the RNA extracted from CD19+ of the transgenic spleen of littermates. Then we picked the down-regulated gene of the human gene expression profiling and compared it to the gene expression profiling of the mouse model. From this comparison (Figure 12), we found some genes that were up-regulated in the mouse model that were also down-regulated by miR-*221* and *222* in the human model, to indicate their candidacy to be targets of miR-*221/222*. One of these genes is *DUSP6/MKP-3*, a *MAP* kinase phosphatase that dephosphorylates phosphothreonine and phosphotyrosine within ERK pathway, playing a role in the induction of apoptosis. This dual specificity phosphatase has been found also acting as an oncogene

but no further studies have been conducted, leaving its function in a contradictory background. Before performing the experiment for the validation of Dusp6 as target of miR-*221/222* we first investigated if this protein was listed as expected target by bioinformatics prediction analysis. The binding site for miR-*221-3p* in *DUSP6* 3'UTR was predicted by the computational tool PITA (figure 14). Since miR-*222* has the same seed of miR-*221* because are from the same family, the target prediction is also effective for miR-*222*. The level of expression in BL cell lines of DUSP6 has been evaluated by qRT-PCR, compared to negative lymphoblastoid cell lines and results show an up-regulation of the mRNA in 80% of BL cell lines whereas it's lost in the controls (Figure 15). To better understand the role of *DUSP6* in BL we are planning to conduct further studies and using different methods such as WB in BL cell lines, qRT-PCR and WB in B cells extracted from lymph nodes of transgenic mice compared to their wild type littermates, immunohistochemistry in both human and mouse lymph nodes to check the expression of the protein in the specific tissue and the luciferase assay, to confirm Dusp6 as a target gene of miR-*221* and miR-*222*.

3.4 Constitutive knock-out miR-*221/222 in vivo* model can anticipate BL pathogenesis in EuMyc tg mouse model: preliminary data

3.4a Introduction

The discovery of microRNAs (miRNAs) has revealed a new level of gene expression regulation that affects many normal and unusual biological pathways. MiRNAs are thought to have tumor suppressive or tumor promoting properties, depending on the nature of their specific targets and countless malignancies have been found affected by the dysregulation of miRNAs. In the last 6-7 years, the experimental field that provided the deepest insights into the *in vivo* biology of miRNAs is that of mouse modeling in which transgenic and knockout animals mimic, respectively, over-expression or down-regulation of specific miRNAs involved in human malignancies. The first in vivo evidence that microRNAs are essential in the development process came from the generation of the miRNA-processing factors Dicer, DGCR8, Drosha and AGO2 knockout mice. These mouse models, lacking these key miRNA processing genes, died indeed during early gestation with severe developmental defects[132-134]. Individual miRNA knockouts were first reported in 2007 by four independent groups[135-138]. Following these initial discoveries, the number of miRNA knockouts generated has critically increased. Observations from published knock-out mouse models suggest that

miRNAs can control expression of numerous functionally related proteins to apply their biological functions and these mechanisms of action may vary from one miRNA to another, over the course of development, or in a tissue-dependent manner[139]. Although these and others knock-out studies clearly demonstrated the importance of miRNAs on specific developmental stages and tissues, it is not clear which miRNAs are responsible for the observed phenotypes.

3.4b Results and discussion

To further explore miR-*221/222* function in the biology of Burkitt Lymphoma development and its involvement in the tumourigenesis process, mice with constitutive loss-of-function alleles for miR-*221/222* were crossed with EμMyc mice in collaboration with Gianpiero Di Leva from Prof. CM Croce's laboratory. The miR-*221/222* KO doesn't show any particular phenotype (data unpublished) but when we breed the KO with the transgenic EμMyc (Figure 17) we observe an early development of the BL pathogenesis in 5 out of 8 miR-*221/222* KO/ EμMyc tg positive and death at 3-4 months of age while the wild type miR-*221/222*/ EμMyc tg are still alive at 6 months of age without showing any enlarged lymph nodes (Figure 18). Unfortunately, even these preliminary results indicate that the loss of miR-*221/222* can play an important role in the pathogenesis of BL, the number of wild type miR-*221/222* is not enough for the statistical analysis; for this reason we are increasing the numbers of litters and we need further investigations.

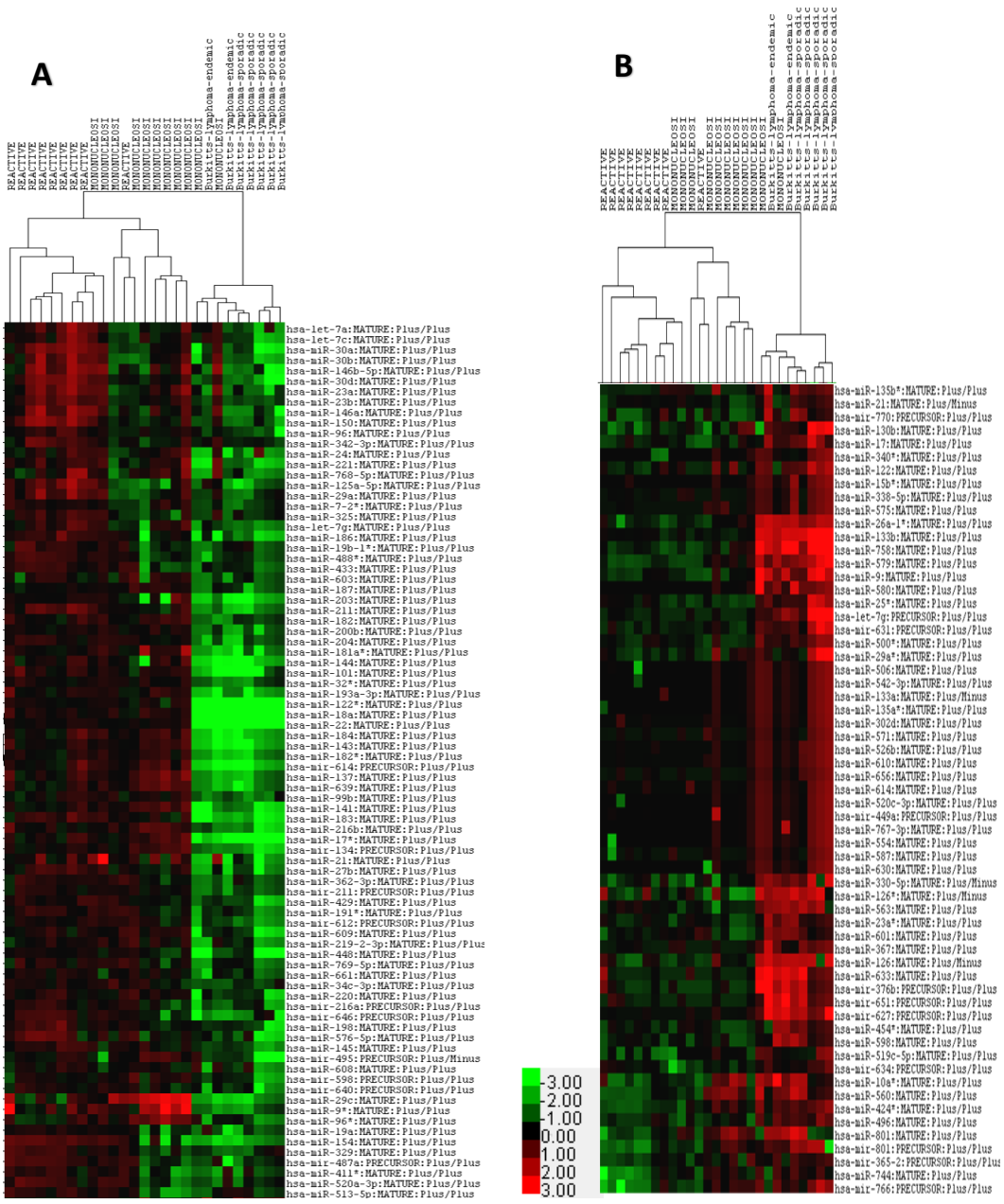


Figure 6. BL has a unique miRNAs signature distinct from healthy (non-cancer) patients. The unsupervised hierarchical clustering, based on the expression of almost 900 miRNAs, clearly separated BL patients from the "healthy" (cancer free) patients, in particular grouping eBL cases with sBL cases and reactive lymph nodes from cancer free cases with lymph nodes from cancer free mononucleosis cases. The dendograms were generated with the use of a hierarchical clustering algoritm that was based on the average-linkage method. In the matrix, each column represents a sample and each row represents a miRNA. The color scale bar shows the relative gene expression changes normalized by the standard deviation (0 is the mean expression level of a given

miRNA). Dendogram **A** shows the miRNAs downregulated (green) in BL versus non-cancer patients, whereas dendogram **B** shows the miRNAs upregulated (red) in BL versus non-cancer patients. Observing both heat-maps, at the cut-off of 0.05, there is a homogeneous expression pattern of disregulated miRNAs between the tumor samples and the negative controls.

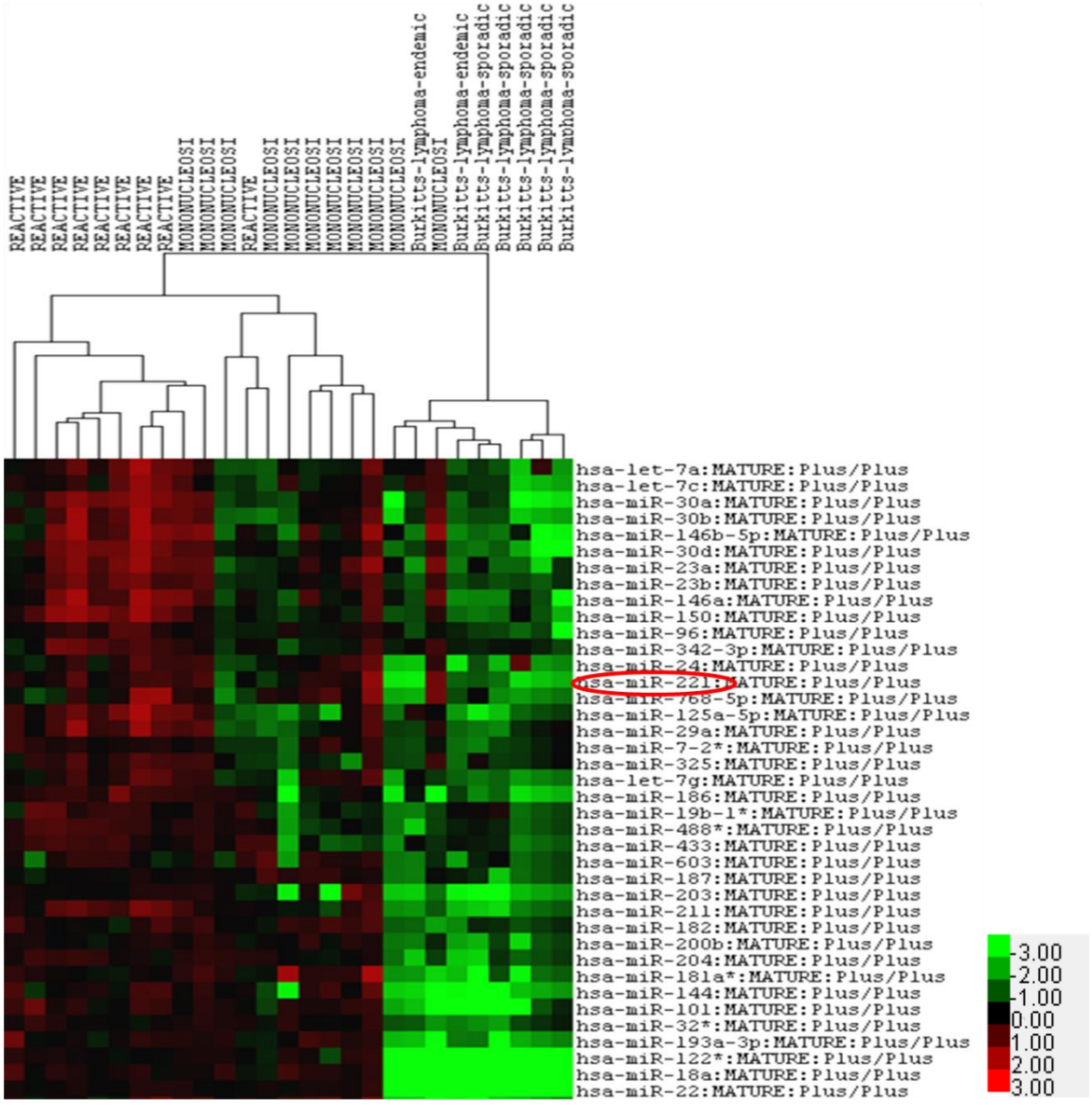
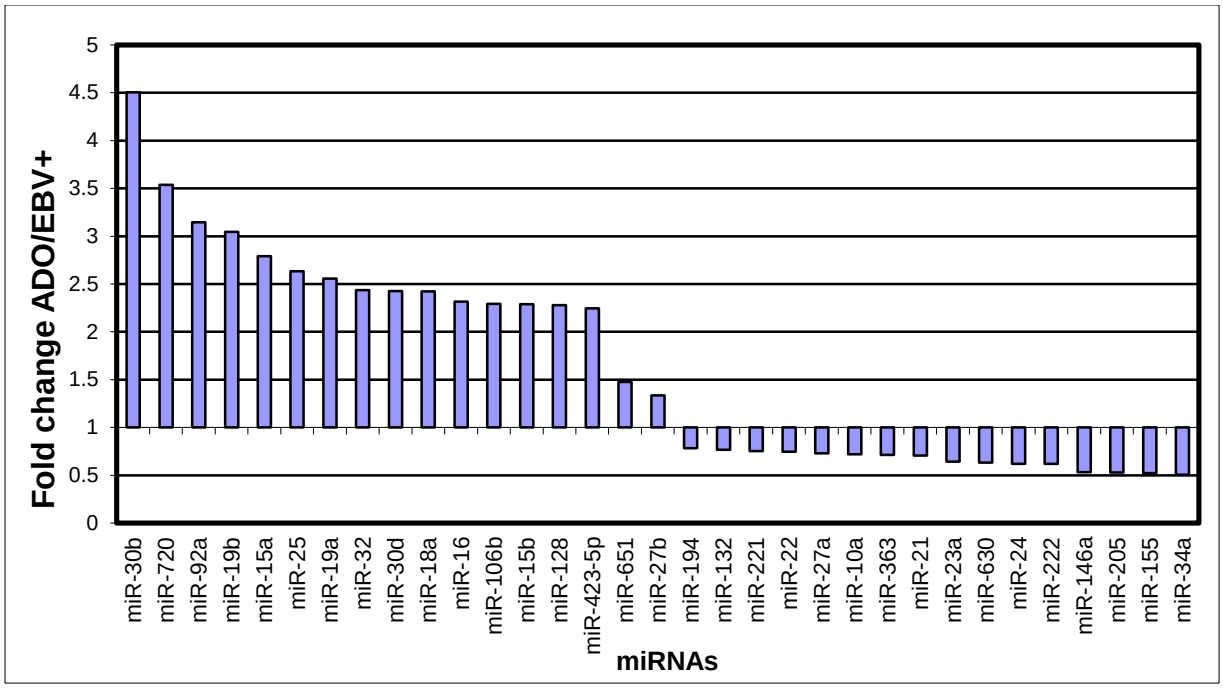


Figure 7. Down-regulation of miR-*221* found in the microarray profile. Magnification of the dendogram in figure 6A to illustrate that miR-*221* is among the most down-regulated miRNAs in BL patients cluster compared to non-cancer cluster. The color scale bar shows the relative gene expression changes normalized by the standard deviation (0 is the mean expression level of a given miRNA).



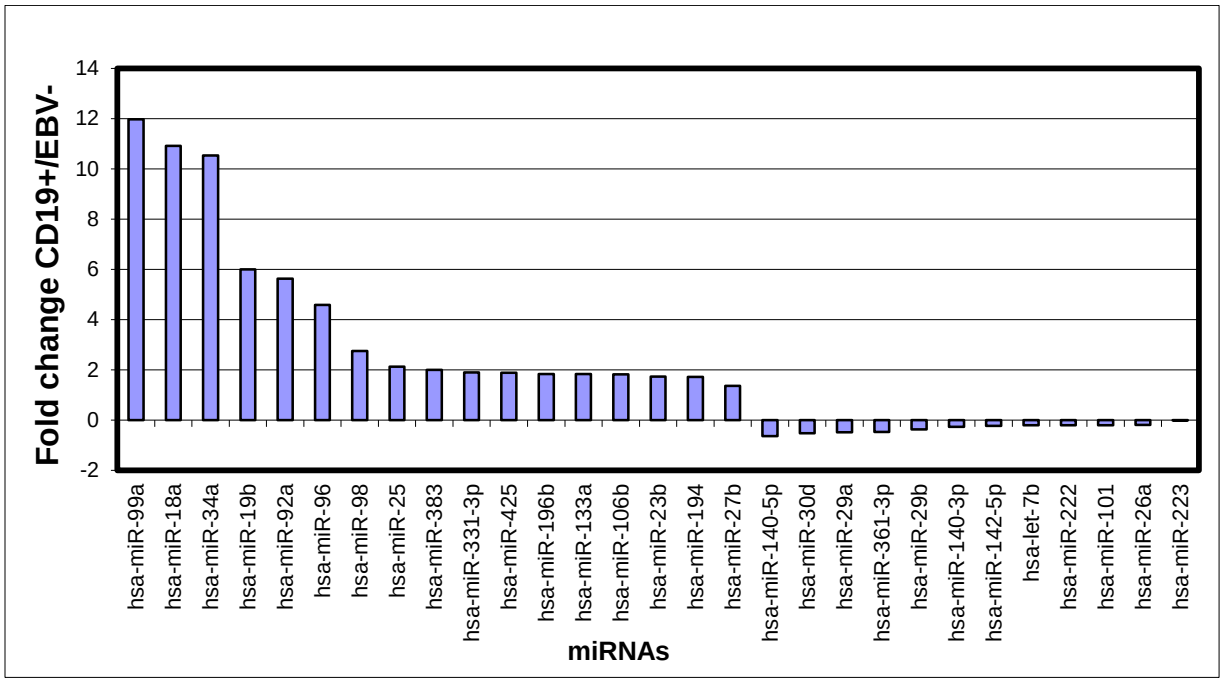
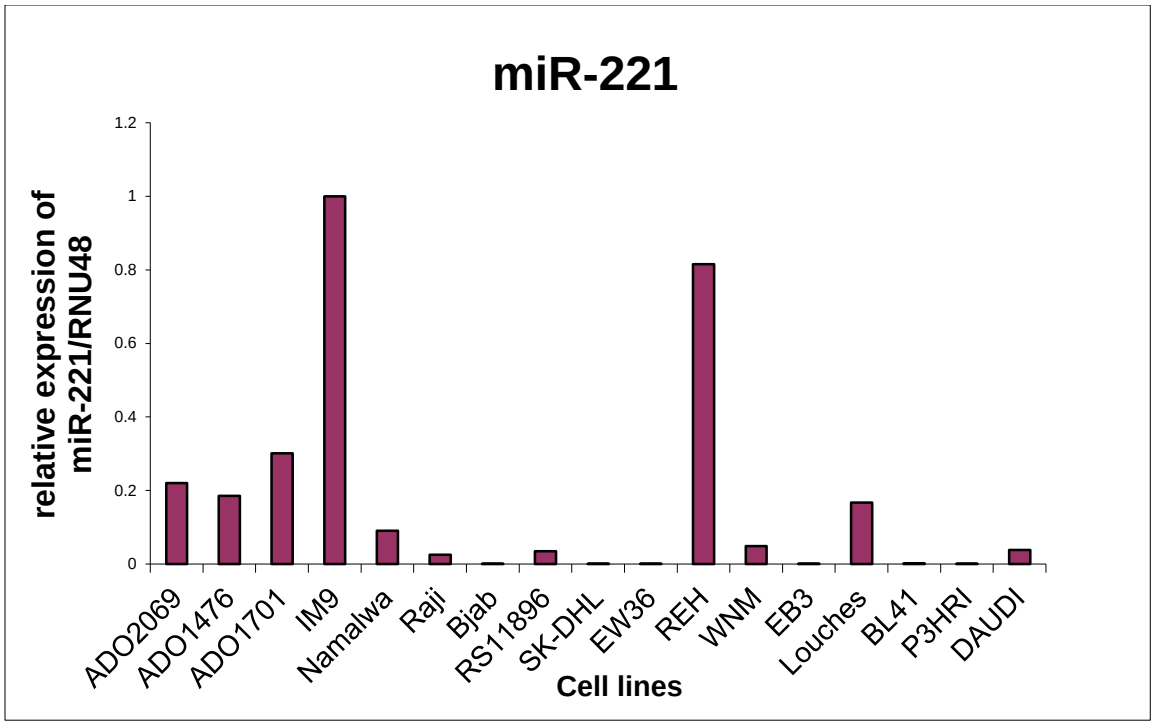


Figure 8. Nanostring analysis on two different sets of BL cell lines compared to their controls, respectively CD19+ B cells (EBV negative) or ADO cell lines (EBV positive). miRNA expression patterns analyzed by

nanostring analysis in a cohort of 5 EBV negative BL cell lines (BJAB, Loukes, BL41, LY91 and EW36) compared to 5 CD19+ B cells (top chart) and in a second cohort of 5 EBV positive BL cell lines (Daudi, Namalwa, Raji, EB3 and P3HRI) compared to EBV transformed B lymphoblastoid cell lines (IM9, ADO2069, ADO1471, ADO1476, ADO1701).



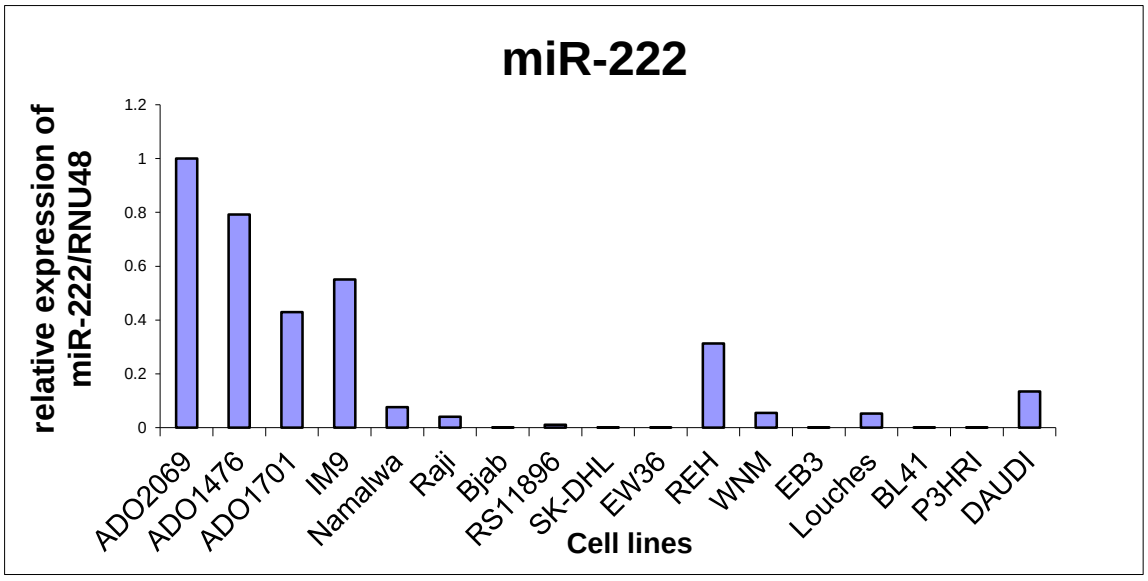


Figure 9. qRT-PCR on a cohort of human BL cell lines compared to ADO cell lines (EBV positive). Comparative level of expression of miR-*221* (top chart) and miR-*222* (bottom chart) in the same cohort of human BL cell lines (EBV positive, EBV negative and unknown EBV status) compared to 4 B lymphoblastoid EBV transformed cell

lines. Data are mean of three replicates for each sample and each sample is compared to the highest expression level found in the controls. For miR-*221*, each comparative level of expression for each sample is compared to the level of expression of IM9, whereas for mir-*222* expression, we compared to the level of expression of ADO2069.

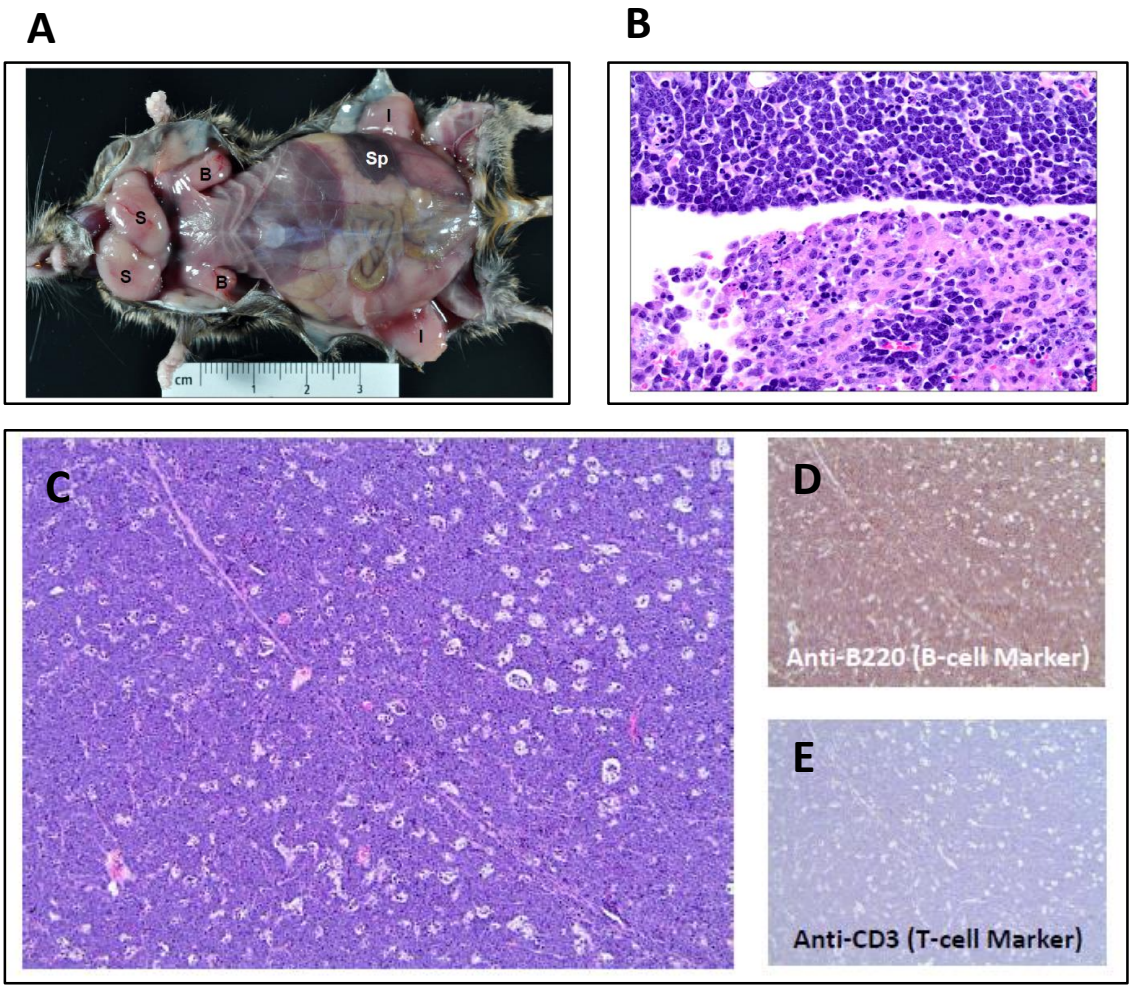


Figure 10. Characterization and phenotypic analysis of one of the EμMyc transgenic mouse from the case selection used in our qRT-PCR analysis to assess level of miR-*221/222*. The primary gross findings (A) were massive enlargements of all lymph nodes (lymphadenopathy) and the spleen (splenomegaly [sp]). Visible lymph nodes are identifying by letters B= brachial, I=inguinal, S= superficial cervical. The pleural plaque of neoplastic lymphocytes (B) stained with H&E illustrates the closely packed, dark basophilic traits of most neoplastic lymphocytes (top) as well as the unusual appearance of a small population of large, loosely attached, pale eosinophilic lymphocytes (bottom). Enlarged lymph node were filled with continuous sheets of neoplastic lymphocytes (H&E, panel C). Apoptosis of the tumour was widespread, typically occurring in clusters (scattered

clear spaces, termed a “starry sky” appearance). Cancer cells expressed B-lymphocytes marker B220 (IHC, panel D) but did not produce the T-lymphocytes marker CD3 (IHC, panel E). B magnification: 400X. C-E magnification: 100X

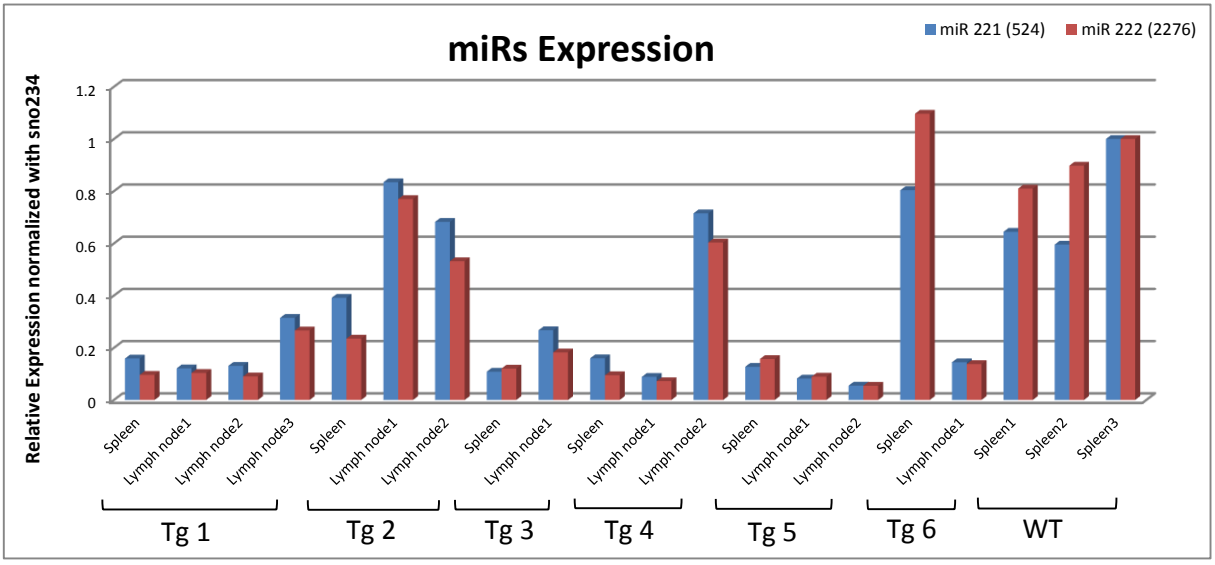


Figure 11. Down-modulation of miR-*221/222* in EμMyc transgenic mice, in vivo model of BL. qRT-PCR analysis showing level of expression of miR-*221-222* in a panel of 17 samples taken from 6 different EμMyc tg mice and compared to their controls: three spleens obtained from wild type littermates. Data are mean of three replicates per sample and each sample is compared to the expression level of spleen number 3 from the negative controls cluster.

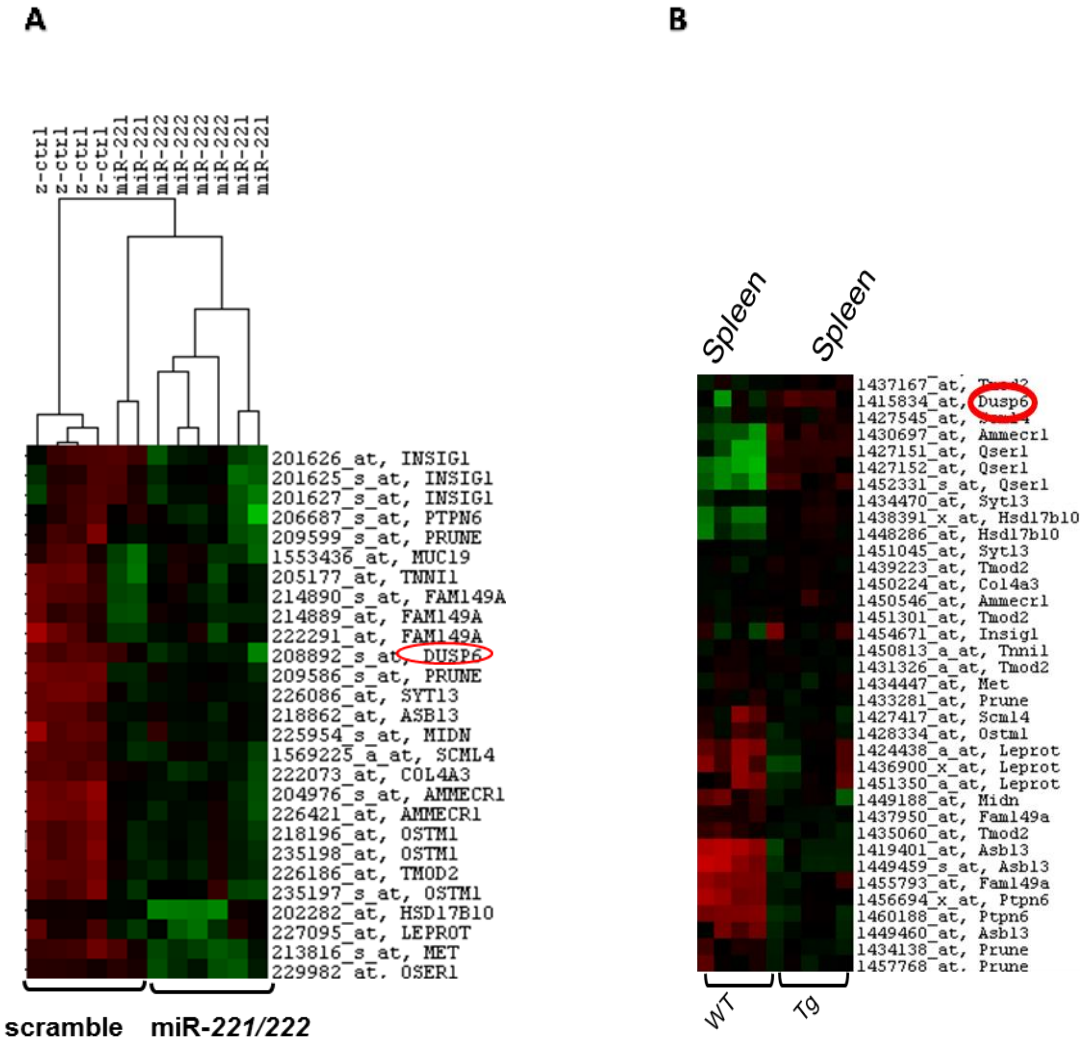


Figure 12. Comparison of gene expression profile in a human BL cell line upon 48 hrs transfection of miR-*221/222* and gene expression signature in EuMyc tg mice. Gene expression patterns in two different systems: the cluster dendogram A represents the microarray analysis performed on RNA extracted from a BL cell line BJAB (which lacks of miR-*221/222* expression) after reestablishing the level of the two miRNAs, whereas the cluster dendogram B represents the gene expression profile of B cells samples purified from spleen of EuMyc tg mice. For both analyses we used 4 replicates of each condition: in dendogram A we evaluated the differential expression of genes after 48 hrs of mature miRNAs transfection and we used 4 different replicates for each transfection (scramble (control oligo), mature miR-*221* and mature miR-*222*); in dendogram B instead we

analyzed spleens from 4 EuMyc tg mice and 4 WT littermates. If we compare downregulated genes upon re-expression of miRs in human BL cell line with upregulated genes in BL mouse model, where we previously showed having a down-regulation of miR-*221/222*, we find *DUSP6* as common target.

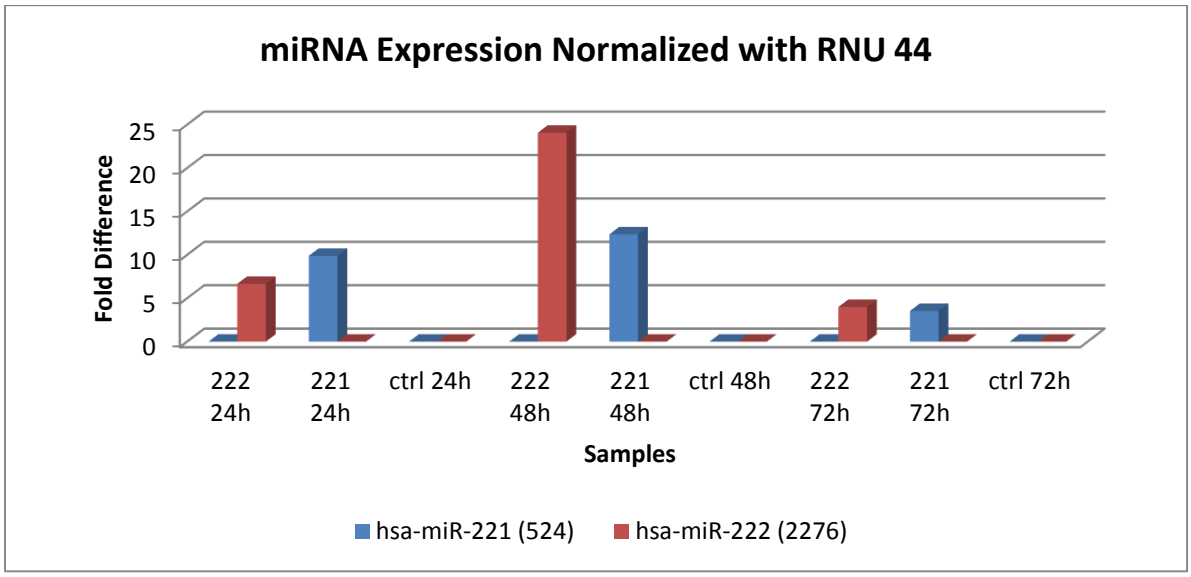


Figure 13. q-RT PCR showing expression levels of miR-*221/222* upon transfection. Comparative level of expression of miR-*221* and miR-*222* in BJAB cell line (which lacks of miR-*221/222* expression) after restoring their level through transfection of the mature miRs. At 48 hrs after reestablishing their levels, we see the highest level of expression for both miRs. This time point was used to perform the gene expression profile shown in figure 12 (dendogram A).

Organism	RefSeq	me	microRNA	Position	Seed	dGduplex	dGopen	G	Conservation
Human	NM_001946; NM_022652	SP6	hsa-miR-221-	797	6mer	-9.4	-4.76	-4.63	0.46

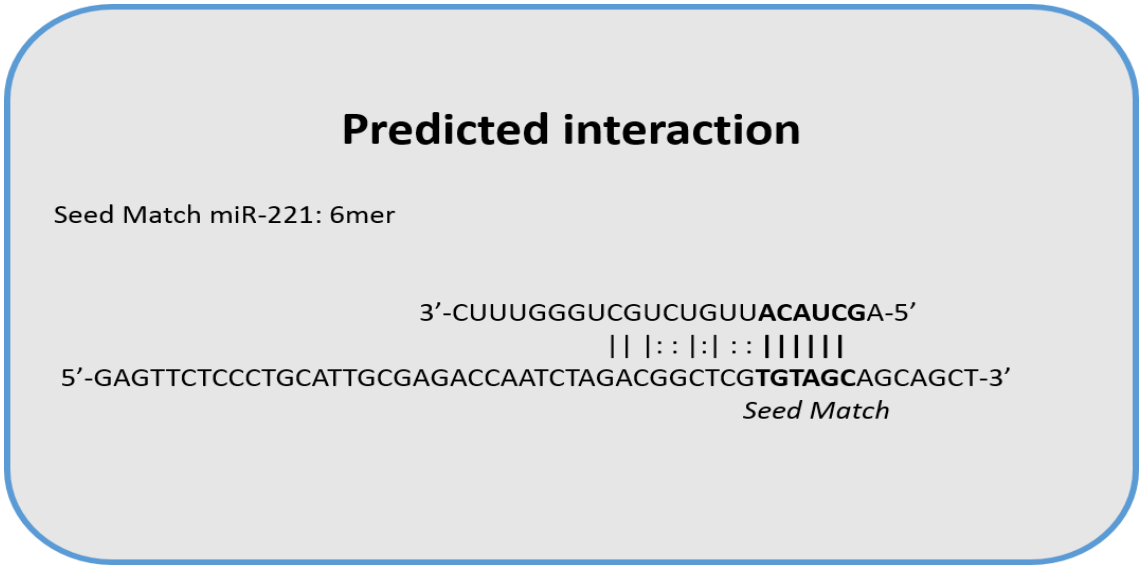
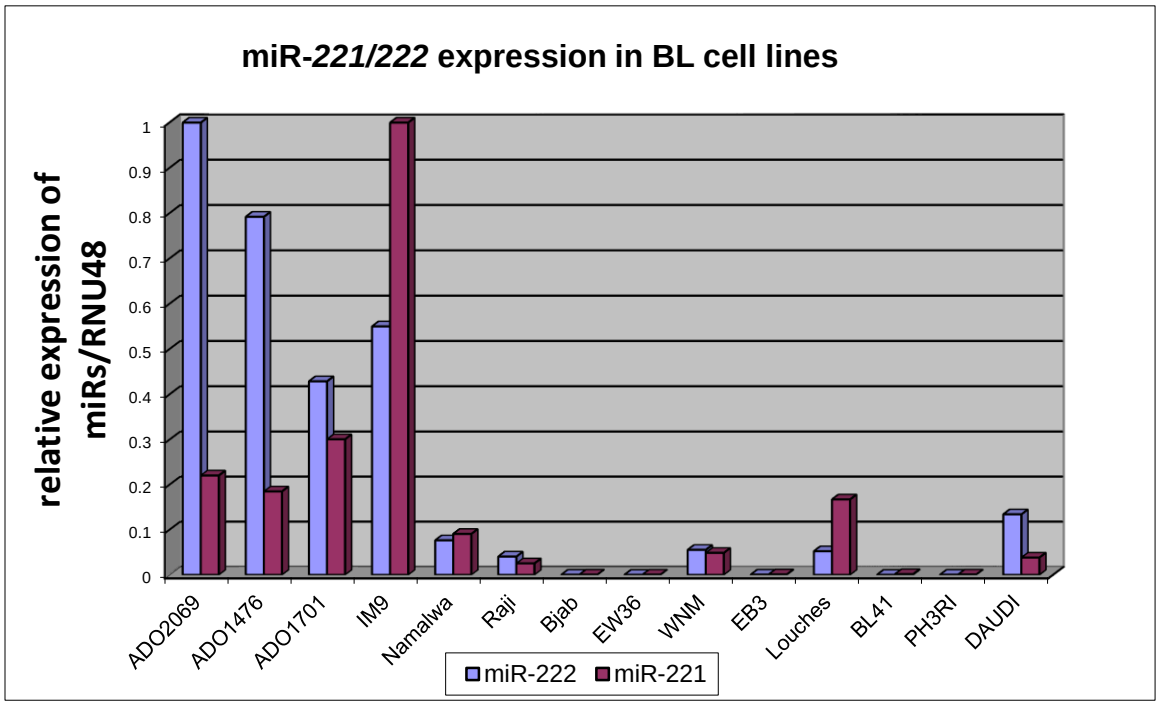


Figure 14. Seed sequence of miR-*221* and its binding to 3'UTR of target mRNA *Dusp6*. Schematic representation of the interaction between miR-*221* and the binding site on the wild type DUSP-6-3'UTRThe binding site for miR-*221* in *DUSP6* was predicted by the computational tool PITA. This software is based on the structural accessibility of the target mRNA. It computes the difference between the free energy gained from the formation of the microRNA-target duplex and the energetic cost of unpairing the target to make it accessible to the microRNA. PITA was able to find a 6mer site on both isoforms of *DUSP6* (NM_001946, NM_022652). The predicted interaction is shown in figure.



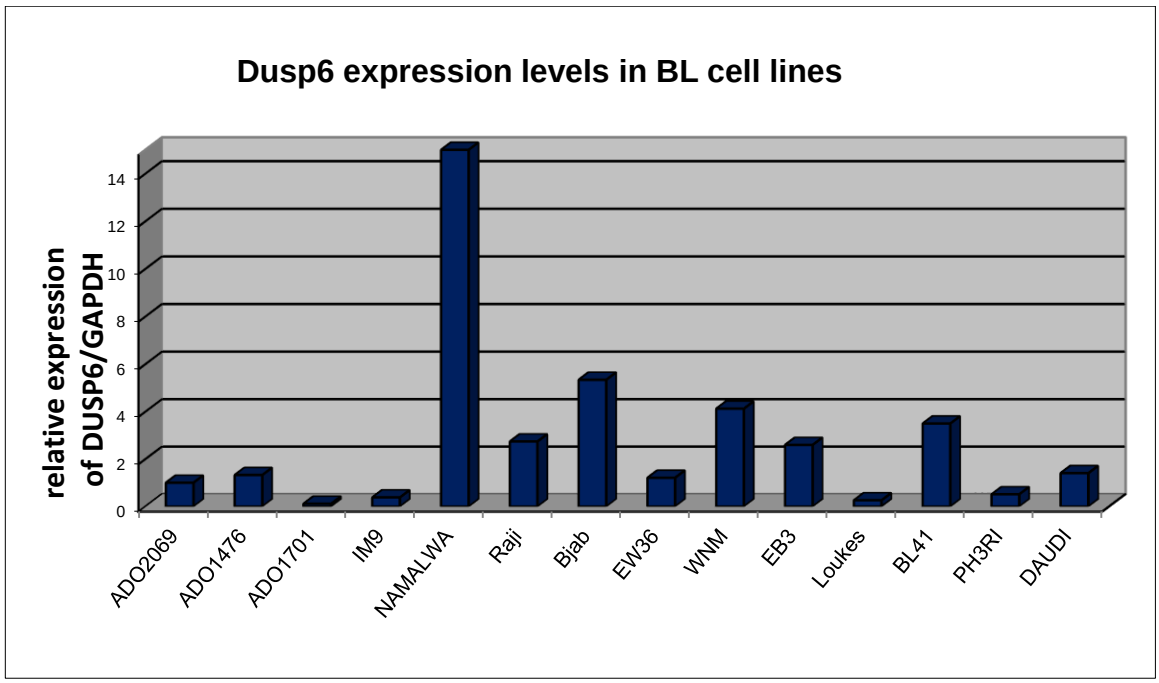


Figure 15. Comparison between comparative expression of miR-*221* and miR-*222* detected by qRT-PCR and expression level of *DUSP6* detected by qRT-PCR. Comparative level of expression of miR-*221* and miR-*222*

shown in the same chart (top graph) and previously described in figure 9, to be related to comparative level of expression of *DUSP6* in the same cohort of BL cell lines. Statistical analysis: miR-*221/222* are down-regulated in Burkitt Lymphoma's cell lines compared to normal control (Fold Change miR-221=-33.85, miR-222=-134.80, $P<0.01$); *DUSP6* is up-regulated in Burkitt Lymphoma's cell lines compared to normal control (Fold Change DUSP6=+5, $P<0.05$).

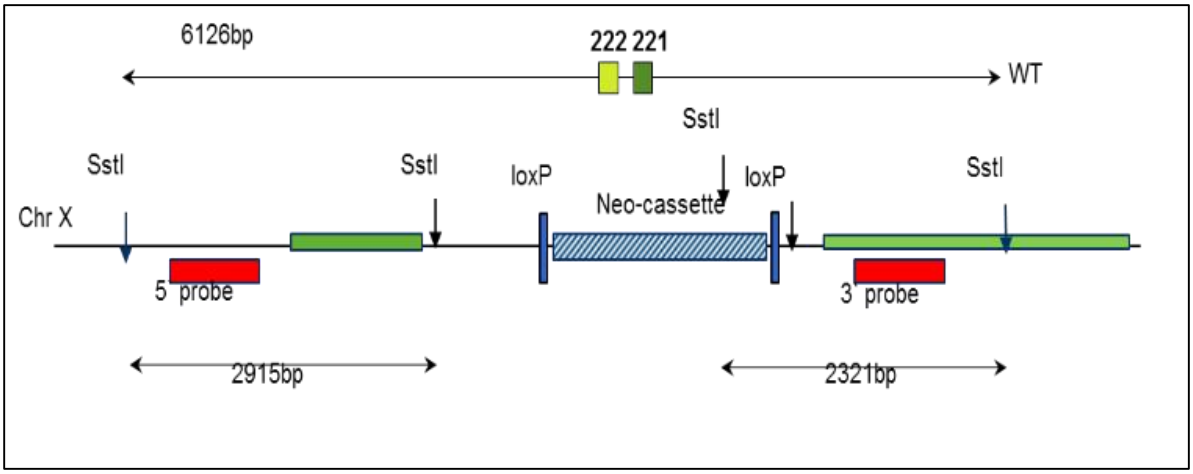


Figure 16. Schematic view of miR-*221/222* knockout generation. Schematic representation of miR-*222/221* null allele. MiR-*222/221* have been replaced by the Neo cassette. Strategy of cloning: right and left arm were amplified from mouse genomic DNA and cloned in the TOPO-TA vector where the loxP-Neo-loxP cassette was previously cloned in EcoRI restriction site. Both probes for southern blot analysis at the 5` and 3` arms are reported and are represented by red boxes.

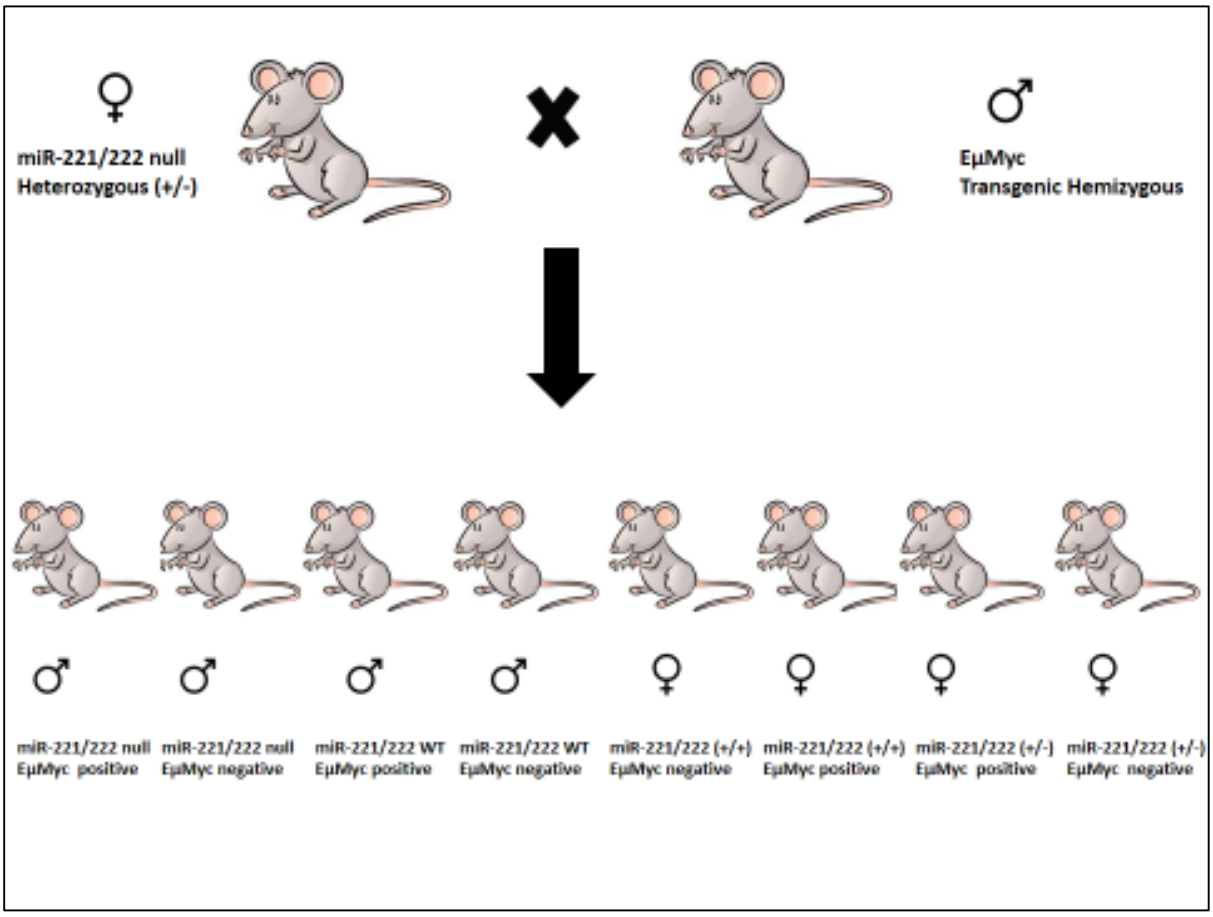


Figure 17. Breeding strategy. The picture shows the breeding approach applied to study the role of miR-*221/222* downregulation in vivo. A female miR-*221/222* null mouse was bred with a male which is a well-known model of BL in vivo, the transgenic mouse for Myc. In our study we used only males of the first generation: since miR-*221/222* cluster is on X chromosome the males obtained were either homozygous for miR-*221/222* WT or miR-*221/222* null whether instead at the first generation of females we had only heterozygous miR-*221/222* null.

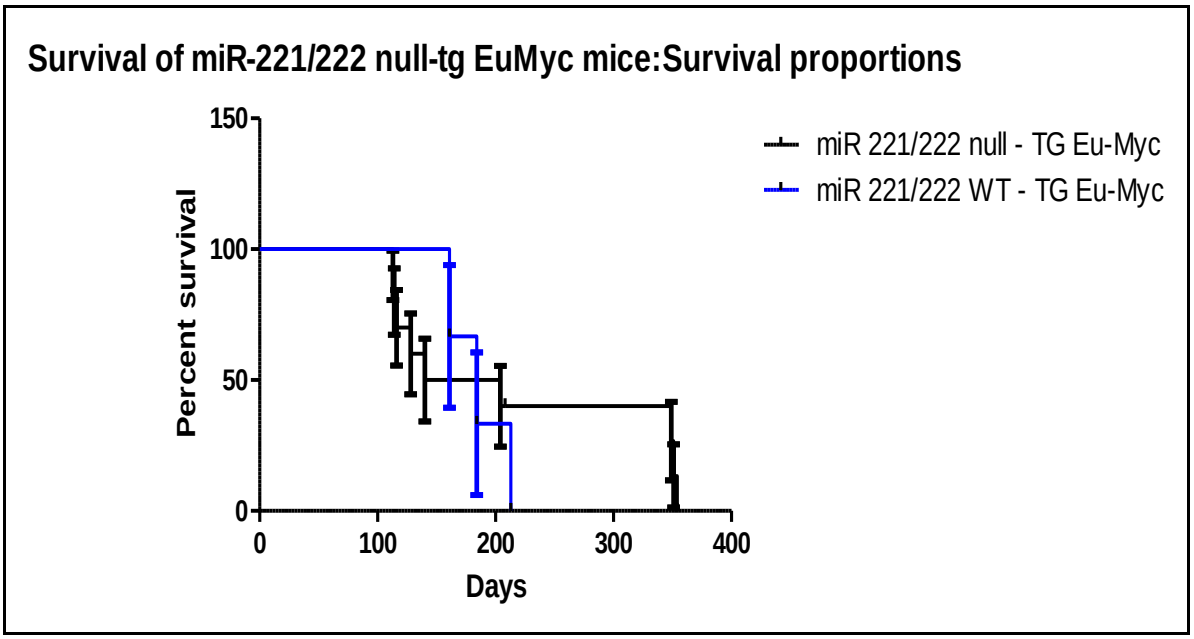


Figure 18. Survival rates of miR-*221/222* null/EμMyc tg mice compared to their WT littermates. Graft showing the Kaplan-Meier survivorship curve, with conversion to death as the endpoint. The survival rate of the control group (miR-*221/222* WT- tg Eμ-Myc) is represented by only two cases (blue bars) and it's not enough to have a statistical significance but indicates the importance of the ablation of miR-*221/222* as an important event that contributes to the aggressiveness of the disease and its progression. "Steps" in the survival curve indicate the occurrence of the permanent failure (death).

Chapter 4-Conclusions and future directions

Here, we studied for the first time the miRNAs expression signature of BL subtypes compared with normal B lymphocytes obtained from reactive lymph nodes of cancer free patients. First, we showed that BL is a distinct entity, supporting the current WHO classification. In fact, we showed that the miRNAs profile of all BL subtypes is quite homogeneous and distinct from those of cancer free lymph nodes. In addition, significantly, we showed that the described BL microRNAs signature is not related to EBV infection. Moreover, we are reporting that miR-*221* and miR-*222* are significantly down-regulated in BL when compared to healthy (cancer free) controls. These new findings may highlight a different role of these miRNA in lymphoma cells compared to their well-known pro-tumorigenic function in epithelial tumors. To better investigate the potential role of miR-*221* and miR-*222* in Burkitt pathogenesis, we also analyzed their expression levels in Eμ-*Myc* transgenic mouse model that resembles an aggressive Burkitt pathogenesis at 6/8 months of life. By analyzing CD-19+ B cells from the spleen of Eμ-*Myc* transgenic mice and litter mate WT we found that miR-*221* and miR-*222* levels show a severe down-regulation. This common pattern between human and mice suggests that miR-*221* and miR-*222* are involved in BL pathogenesis. In agree with these results, mir-*221* and miR-*222* ectopic re-expression in BL cell line (Bjab) reveals interesting new cellular pathways that could be regulated by this class of miRNAs in lymphoma cells. Also, comparing this gene expression profile with a gene expression profile obtained from the mouse model we were able to find a common target that could be considered to play a role in the BL pathogenesis. Thus, further studies, such as western blot analysis in BL cell lines, qRT-PCR and western blot analyses in mouse samples, need to be assessed to validate this protein as a target of the cluster *221/222*.

Our preliminary data on the in vivo mouse model obtained by mating miR-*221/222* KO with EμMyc tg mice support our results previously reported: the knockout mouse for miR-*221/222* doesn't show any particular phenotype but when we cross the miR-*221/222* KO with the well-known in vivo model for BL we observe an anticipation of the disease appearance of at least 60 days compared to the wt *221/222*-EμMyc tg, indicating that the translocation of Myc needs also the dysregulation of the miR-*221* and miR-*222* to develop the malignancy. Unfortunately the number of mice is not enough for statistical analysis so we are increasing the litters and furthers studies need to be carry out to establish this KO/tg mouse model as a good in vivo BL model.

Our findings indicate that miR-*221/222* can be critical mediators for BL pathogenesis and together with other important genetics alteration such as translocation of Myc can lead to the aggressive phenotype that this B cell malignancy usually shows. These results highlight the potential role of this cluster of microRNAs to be a good tool of diagnosis and prognosis for BL.

Chapter 5: Publications

Oncogenic role of miR-483-3p at the IGF2/483 locus.

Veronese A, Lupini L, **Consiglio J**, Visone R, Ferracin M, Fornari F, Zanesi N, Alder H, D'Elia G, Gramantieri L, Bolondi L, Lanza G, Querzoli P, Angioni A, Croce CM, Negrini M.

Cancer Res. 2010 Apr 15;70(8):3140-9. doi: 10.1158/0008-5472.CAN-09-4456. Epub 2010 Apr 13.

Mutated beta-catenin evades a microRNA-dependent regulatory loop.

Veronese A, Visone R, **Consiglio J**, Acunzo M, Lupini L, Kim T, Ferracin M, Lovat F, Miotto E, Balatti V, D'Abundo L, Gramantieri L, Bolondi L, Pekarsky Y, Perrotti D, Negrini M, Croce CM.

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In vivo NCL targeting affects breast cancer aggressiveness through miRNA regulation.

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Chapter 6: Bibliography

1. Molyneux, E.M.*, et al.* Burkitt's lymphoma. *Lancet* **379**, 1234-1244.
2. Manolov, G. & Manolova, Y. Marker band in one chromosome 14 from Burkitt lymphomas. *Nature* **237**, 33-34 (1972).
3. Epstein, M.A., Achong, B.G. & Barr, Y.M. Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. *Lancet* **1**, 702-703 (1964).
4. Schulz, T.F., Boshoff, C.H. & Weiss, R.A. HIV infection and neoplasia. *Lancet* **348**, 587-591 (1996).
5. Burkitt, D.P. Etiology of Burkitt's lymphoma--an alternative hypothesis to a vectored virus. *Journal of the National Cancer Institute* **42**, 19-28 (1969).
6. DM, P. Cancer in Africa: epidemiology and prevalence, Burkitt Lymphoma. *IARC Scientific Publications* **153**, 324-328 (2003).
7. Kafuko, G.W. & Burkitt, D.P. Burkitt's lymphoma and malaria. *International journal of cancer. Journal international du cancer* **6**, 1-9 (1970).
8. Davies, J.N. Pathology of Central African Natives; Mulago Hospital post mortem studies. *East African medical journal* **25**, 454-467 (1948).
9. Burkitt, D. A sarcoma involving the jaws in African children. *The British journal of surgery* **46**, 218-223 (1958).
10. O'Conor, G.T. & Davies, J.N. Malignant tumors in African children. With special reference to malignant lymphoma. *The Journal of pediatrics* **56**, 526-535 (1960).
11. Burkitt, D. A "tumour safari" in East and Central Africa. *British journal of cancer* **16**, 379-386 (1962).
12. Gong, J.Z*., et al.* Burkitt lymphoma arising in organ transplant recipients: a clinicopathologic study of five cases. *The American journal of surgical pathology* **27**, 818-827 (2003).
13. De Falco, G*., et al.* Interaction between HIV-1 Tat and pRb2/p130: a possible mechanism in the pathogenesis of AIDS-related neoplasms. *Oncogene* **22**, 6214-6219 (2003).
14. Magrath, I. Epidemiology: clues to the pathogenesis of Burkitt lymphoma. *British journal of haematology* **156**, 744-756 (2012).
15. Thorley-Lawson, D.A. & Allday, M.J. The curious case of the tumour virus: 50 years of Burkitt's lymphoma. *Nature reviews. Microbiology* **6**, 913-924 (2008).
16. Ogwang, M.D., Bhatia, K., Biggar, R.J. & Mbulaiteye, S.M. Incidence and geographic distribution of endemic Burkitt lymphoma in northern Uganda revisited. *International journal of cancer. Journal international du cancer* **123**, 2658-2663 (2008).
17. Sant, M*., et al.* Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project. *Blood* **116**, 3724-3734 (2010).
18. Dalla-Favera, R*., et al.* Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 7824-7827 (1982).
19. ar-Rushdi, A*., et al.* Differential expression of the translocated and the untranslocated c-myc oncogene in Burkitt lymphoma. *Science* **222**, 390-393 (1983).
20. Bornkamm, G.W. Epstein-Barr virus and the pathogenesis of Burkitt's lymphoma: more questions than answers. *International journal of cancer. Journal international du cancer* **124**, 1745-1755 (2009).
21. Hecht, J.L. & Aster, J.C. Molecular biology of Burkitt's lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **18**, 3707-3721 (2000).
22. Pelicci, P.G., Knowles, D.M., 2nd, Magrath, I. & Dalla-Favera, R. Chromosomal breakpoints and structural alterations of the c-myc locus differ in endemic and sporadic forms of Burkitt lymphoma. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 2984-2988 (1986).
23. Blum, K.A., Lozanski, G. & Byrd, J.C. Adult Burkitt leukemia and lymphoma. *Blood* **104**, 3009-3020 (2004).

24. Toujani, S*., et al.* High resolution genome-wide analysis of chromosomal alterations in Burkitt's lymphoma. *PloS one* **4**, e7089 (2009).
25. Schmitz, R*., et al.* Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature* **490**, 116-120 (2012).
26. Richter, J*., et al.* Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. *Nature genetics* **44**, 1316-1320 (2012).
27. Love, C*., et al.* The genetic landscape of mutations in Burkitt lymphoma. *Nature genetics* **44**, 1321-1325 (2012).
28. Molina-Privado, I*., et al.* E2F1 expression is deregulated and plays an oncogenic role in sporadic Burkitt's lymphoma. *Cancer research* **69**, 4052-4058 (2009).
29. Young, L.S. & Rickinson, A.B. Epstein-Barr virus: 40 years on. *Nature reviews. Cancer* **4**, 757-768 (2004).
30. Thompson, M.P. & Kurzrock, R. Epstein-Barr virus and cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**, 803-821 (2004).
31. Rezk, S.A. & Weiss, L.M. Epstein-Barr virus-associated lymphoproliferative disorders. *Human pathology* **38**, 1293-1304 (2007).
32. Niedobitek, G*., et al.* Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma. *Blood* **86**, 659-665 (1995).
33. Subar, M., Neri, A., Inghirami, G., Knowles, D.M. & Dalla-Favera, R. Frequent c-myc oncogene activation and infrequent presence of Epstein-Barr virus genome in AIDS-associated lymphoma. *Blood* **72**, 667-671 (1988).
34. Lazzi, S*., et al.* HIV-associated malignant lymphomas in Kenya (Equatorial Africa). *Human pathology* **29**, 1285-1289 (1998).
35. Neri, A*., et al.* Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma. *Blood* **77**, 1092-1095 (1991).
36. Geser, A., de The, G., Lenoir, G., Day, N.E. & Williams, E.H. Final case reporting from the Ugandan prospective study of the relationship between EBV and Burkitt's lymphoma. *International journal of cancer. Journal international du cancer* **29**, 397-400 (1982).
37. Babcock, G.J. & Thorley-Lawson, D.A. Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 12250-12255 (2000).
38. Mbulaiteye, S.M., Biggar, R.J., Bhatia, K., Linet, M.S. & Devesa, S.S. Sporadic childhood Burkitt lymphoma incidence in the United States during 1992-2005. *Pediatric blood & cancer* **53**, 366-370 (2009).
39. Patte, C*., et al.* The Societe Francaise d'Oncologie Pediatrique LMB89 protocol: highly effective multiagent chemotherapy tailored to the tumor burden and initial response in 561 unselected children with B-cell lymphomas and L3 leukemia. *Blood* **97**, 3370-3379 (2001).
40. Patte, C*., et al.* High survival rate in advanced-stage B-cell lymphomas and leukemias without CNS involvement with a short intensive polychemotherapy: results from the French Pediatric Oncology Society of a randomized trial of 216 children. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **9**, 123-132 (1991).
41. Hesseling, P., Molyneux, E., Kamiza, S., Israels, T. & Broadhead, R. Endemic Burkitt lymphoma: a 28-day treatment schedule with cyclophosphamide and intrathecal methotrexate. *Annals of tropical paediatrics* **29**, 29-34 (2009).
42. Magrath, I.T. African Burkitt's lymphoma. History, biology, clinical features, and treatment. *The American journal of pediatric hematology/oncology* **13**, 222-246 (1991).

43. Fujita, S*., et al.* Early stage of Epstein-Barr virus lytic infection leading to the "starry sky" pattern formation in endemic Burkitt lymphoma. *Archives of pathology & laboratory medicine* **128**, 549-552 (2004).
44. Jaffe, E.S., Harris, N.L., Diebold, J. & Muller-Hermelink, H.K. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. A progress report. *American journal of clinical pathology* **111**, S8-12 (1999).
45. Tang, G. siRNA and miRNA: an insight into RISCs. *Trends in biochemical sciences* **30**, 106-114 (2005).
46. Lee, R.C., Feinbaum, R.L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854 (1993).
47. Fukunaga, R*., et al.* Dicer partner proteins tune the length of mature miRNAs in flies and mammals. *Cell* **151**, 533-546 (2012).
48. He, L. & Hannon, G.J. MicroRNAs: small RNAs with a big role in gene regulation. *Nature reviews. Genetics* **5**, 522-531 (2004).
49. Lu, J*., et al.* MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-838 (2005).
50. Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A. & Enright, A.J. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic acids research* **34**, D140-144 (2006).
51. O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. & Mendell, J.T. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839-843 (2005).
52. Krol, J., Loedige, I. & Filipowicz, W. The widespread regulation of microRNA biogenesis, function and decay. *Nature reviews. Genetics* **11**, 597-610 (2010).
53. Kim, V.N. MicroRNA biogenesis: coordinated cropping and dicing. *Nature reviews. Molecular cell biology* **6**, 376-385 (2005).
54. Bhayani, M.K., Calin, G.A. & Lai, S.Y. Functional relevance of miRNA sequences in human disease. *Mutation research* **731**, 14-19 (2012).
55. Berezikov, E., Chung, W.J., Willis, J., Cuppen, E. & Lai, E.C. Mammalian mirtron genes. *Molecular cell* **28**, 328-336 (2007).
56. Okamura, K., Hagen, J.W., Duan, H., Tyler, D.M. & Lai, E.C. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell* **130**, 89-100 (2007).
57. Ruby, J.G., Jan, C.H. & Bartel, D.P. Intronic microRNA precursors that bypass Drosha processing. *Nature* **448**, 83-86 (2007).
58. Yi, R., Qin, Y., Macara, I.G. & Cullen, B.R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development* **17**, 3011-3016 (2003).
59. Viswanathan, S.R., Daley, G.Q. & Gregory, R.I. Selective blockade of microRNA processing by Lin28. *Science* **320**, 97-100 (2008).
60. Guil, S. & Caceres, J.F. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nature structural & molecular biology* **14**, 591-596 (2007).
61. Hutvagner, G*., et al.* A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**, 834-838 (2001).
62. Heo, I*., et al.* Mono-uridylation of pre-microRNA as a key step in the biogenesis of group II let-7 microRNAs. *Cell* **151**, 521-532 (2012).
63. Heo, I*., et al.* TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**, 696-708 (2009).
64. Chendrimada, T.P*., et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740-744 (2005).
65. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297 (2004).
66. Su, H., Trombly, M.I., Chen, J. & Wang, X. Essential and overlapping functions for mammalian Argonautes in microRNA silencing. *Genes & development* **23**, 304-317 (2009).

67. Kedde, M.*, et al.* RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* **131**, 1273-1286 (2007).
68. Lytle, J.R., Yario, T.A. & Steitz, J.A. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 9667-9672 (2007).
69. Moretti, F., Thermann, R. & Hentze, M.W. Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *Rna* **16**, 2493-2502 (2010).
70. Vasudevan, S., Tong, Y. & Steitz, J.A. Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318**, 1931-1934 (2007).
71. Krek, A.*, et al.* Combinatorial microRNA target predictions. *Nature genetics* **37**, 495-500 (2005).
72. Betel, D., Wilson, M., Gabow, A., Marks, D.S. & Sander, C. The microRNA.org resource: targets and expression. *Nucleic acids research* **36**, D149-153 (2008).
73. Lewis, B.P., Burge, C.B. & Bartel, D.P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15-20 (2005).
74. Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P. & Burge, C.B. Prediction of mammalian microRNA targets. *Cell* **115**, 787-798 (2003).
75. Gehrke, S., Imai, Y., Sokol, N. & Lu, B. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature* **466**, 637-641 (2010).
76. Yang, Y., Ago, T., Zhai, P., Abdellatif, M. & Sadoshima, J. Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7. *Circulation research* **108**, 305-313 (2011).
77. Barh, D., Malhotra, R., Ravi, B. & Sindhurani, P. MicroRNA let-7: an emerging next-generation cancer therapeutic. *Current oncology* **17**, 70-80 (2010).
78. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
79. Cimmino, A.*, et al.* miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13944-13949 (2005).
80. Johnson, S.M.*, et al.* RAS is regulated by the let-7 microRNA family. *Cell* **120**, 635-647 (2005).
81. Sampson, V.B.*, et al.* MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer research* **67**, 9762-9770 (2007).
82. Garzon, R.*, et al.* MicroRNA 29b functions in acute myeloid leukemia. *Blood* **114**, 5331-5341 (2009).
83. He, L.*, et al.* A microRNA polycistron as a potential human oncogene. *Nature* **435**, 828-833 (2005).
84. Garzon, R.*, et al.* Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3945-3950 (2008).
85. Ventura, A.*, et al.* Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132**, 875-886 (2008).
86. Xiao, C.*, et al.* Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nature immunology* **9**, 405-414 (2008).
87. Mu, P.*, et al.* Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes & development* **23**, 2806-2811 (2009).
88. Costinean, S.*, et al.* Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7024-7029 (2006).
89. Vogelstein, B. & Kinzler, K.W. Cancer genes and the pathways they control. *Nature medicine* **10**, 789-799 (2004).

90. Komarova, N.L., Sengupta, A. & Nowak, M.A. Mutation-selection networks of cancer initiation: tumor suppressor genes and chromosomal instability. *Journal of theoretical biology* **223**, 433-450 (2003).
91. Nowell, P.C. Tumor progression: a brief historical perspective. *Seminars in cancer biology* **12**, 261-266 (2002).
92. le Sage, C.*, et al.* Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *The EMBO journal* **26**, 3699-3708 (2007).
93. Visone, R.*, et al.* MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. *Endocrine-related cancer* **14**, 791-798 (2007).
94. Di Leva, G.*, et al.* MicroRNA cluster 221-222 and estrogen receptor alpha interactions in breast cancer. *Journal of the National Cancer Institute* **102**, 706-721 (2010).
95. Miller, T.E.*, et al.* MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. *The Journal of biological chemistry* **283**, 29897-29903 (2008).
96. Fu, X.*, et al.* Clinical significance of miR-221 and its inverse correlation with p27Kip(1) in hepatocellular carcinoma. *Molecular biology reports* **38**, 3029-3035 (2011).
97. Garofalo, M.*, et al.* MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene* **27**, 3845-3855 (2008).
98. Molyneux, E.M.*, et al.* Burkitt's lymphoma. *Lancet* **379**, 1234-1244 (2012).
99. Caldas, C. & Brenton, J.D. Sizing up miRNAs as cancer genes. *Nature medicine* **11**, 712-714 (2005).
100. Haluska, F.G., Tsujimoto, Y. & Croce, C.M. The t(8;14) breakpoint of the EW 36 undifferentiated lymphoma cell line lies 5' of MYC in a region prone to involvement in endemic Burkitt's lymphomas. *Nucleic acids research* **16**, 2077-2085 (1988).
101. Ounanian, A., Guilbert, B. & Seigneurin, J.M. Characteristics of Epstein-Barr virus transformed B cell lines from patients with Alzheimer's disease and age-matched controls. *Mechanisms of ageing and development* **63**, 105-116 (1992).
102. Pekarsky, Y., Hallas, C., Isobe, M., Russo, G. & Croce, C.M. Abnormalities at 14q32.1 in T cell malignancies involve two oncogenes. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2949-2951 (1999).
103. Bonin, A., Reid, S.W. & Tessarollo, L. Isolation, microinjection, and transfer of mouse blastocysts. *Methods in molecular biology* **158**, 121-134 (2001).
104. Tessarollo, L. Manipulating mouse embryonic stem cells. *Methods in molecular biology* **158**, 47-63 (2001).
105. Copeland, N.G., Jenkins, N.A. & Court, D.L. Recombineering: a powerful new tool for mouse functional genomics. *Nature reviews. Genetics* **2**, 769-779 (2001).
106. Liu, C.G., Calin, G.A., Volinia, S. & Croce, C.M. MicroRNA expression profiling using microarrays. *Nature protocols* **3**, 563-578 (2008).
107. Volinia, S.*, et al.* A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2257-2261 (2006).
108. Bolstad, B.M., Irizarry, R.A., Astrand, M. & Speed, T.P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193 (2003).
109. Zhao, Y. & Simon, R. BRB-ArrayTools Data Archive for human cancer gene expression: a unique and efficient data sharing resource. *Cancer informatics* **6**, 9-15 (2008).
110. Tusher, V.G., Tibshirani, R. & Chu, G. Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5116-5121 (2001).

111. Saeed, A.I*., et al.* TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* **34**, 374-378 (2003).
112. Calin, G.A*., et al.* Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15524-15529 (2002).
113. Mi, S*., et al.* MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19971-19976 (2007).
114. Xiao, C*., et al.* MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* **131**, 146-159 (2007).
115. Di Lisio, L*., et al.* The role of miRNAs in the pathogenesis and diagnosis of B-cell lymphomas. *Blood* **120**, 1782-1790 (2012).
116. Onnis, A*., et al.* Alteration of microRNAs regulated by c-Myc in Burkitt lymphoma. *PloS one* **5**(2010).
117. Liu, C.G*., et al.* An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 9740-9744 (2004).
118. Piccaluga, P.P*., et al.* Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes. *Blood* **117**, 3596-3608 (2011).
119. Felli, N*., et al.* MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18081-18086 (2005).
120. Liu, X*., et al.* MicroRNA-222 regulates cell invasion by targeting matrix metalloproteinase 1 (MMP1) and manganese superoxide dismutase 2 (SOD2) in tongue squamous cell carcinoma cell lines. *Cancer genomics & proteomics* **6**, 131-139 (2009).
121. Zhang, C*., et al.* PUMA is a novel target of miR-221/222 in human epithelial cancers. *International journal of oncology* **37**, 1621-1626 (2010).
122. Ali, S*., et al.* Inactivation of Ink4a/Arf leads to deregulated expression of miRNAs in K-Ras transgenic mouse model of pancreatic cancer. *Journal of cellular physiology* **227**, 3373-3380 (2012).
123. Shah, A.A*., et al.* A set of specific miRNAs is connected with murine and human gastric cancer. *Genes, chromosomes & cancer* **52**, 237-249 (2013).
124. Kovalchuk, A.L*., et al.* Burkitt lymphoma in the mouse. *The Journal of experimental medicine* **192**, 1183-1190 (2000).
125. Morse, H.C., 3rd*, et al.* Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood* **100**, 246-258 (2002).
126. Harrell, M.I., Iritani, B.M. & Ruddell, A. Lymph node mapping in the mouse. *Journal of immunological methods* **332**, 170-174 (2008).
127. Couzin, J. Genomics. Microarray data reproduced, but some concerns remain. *Science* **313**, 1559 (2006).
128. Hummel, M*., et al.* A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *The New England journal of medicine* **354**, 2419-2430 (2006).
129. Dave, S.S*., et al.* Molecular diagnosis of Burkitt's lymphoma. *The New England journal of medicine* **354**, 2431-2442 (2006).
130. Kelly, G.L*., et al.* Different patterns of Epstein-Barr virus latency in endemic Burkitt lymphoma (BL) lead to distinct variants within the BL-associated gene expression signature. *Journal of virology* **87**, 2882-2894 (2013).

131. Bieging, K.T., Fish, K., Bondada, S. & Longnecker, R. A shared gene expression signature in mouse models of EBV-associated and non-EBV-associated Burkitt lymphoma. *Blood* **118**, 6849-6859 (2011).
132. Bernstein, E.*, et al.* Dicer is essential for mouse development. *Nature genetics* **35**, 215-217 (2003).
133. Wang, Y., Medvid, R., Melton, C., Jaenisch, R. & Blelloch, R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nature genetics* **39**, 380-385 (2007).
134. Fukuda, T.*, et al.* DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nature cell biology* **9**, 604-611 (2007).
135. Rodriguez, A.*, et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **316**, 608-611 (2007).
136. Thai, T.H.*, et al.* Regulation of the germinal center response by microRNA-155. *Science* **316**, 604-608 (2007).
137. van Rooij, E.*, et al.* Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**, 575-579 (2007).
138. Zhao, Y.*, et al.* Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* **129**, 303-317 (2007).
139. Park, C.Y., Choi, Y.S. & McManus, M.T. Analysis of microRNA knockouts in mice. *Human molecular genetics* **19**, R169-175 (2010).

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"If we have no peace it is because we have forgotten that we belong to each other.

You should never lose heart. God is merciful and kind. He has endowed you with the best gift-SMILE- which can make millions happy"

Mother Teresa