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Cycle XX

Ph.D. in Oncological Genetics

INK4/ARF locus in Burkitt's lymphoma cell lines

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Thesis suitable for the title of "Doctor Europeus"

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The first version of this thesis has been revised according to the following attached reviewers' comments.

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Review of the thesis submitted by Annalisa Roberti
for the title of Ph.D. and "Doctor Europeus"

Supervisor: Prof. Antonio Giordano

"INK/ARF locus in Burkitt`s Lymphoma cell lines"

The thesis submitted by Annalisa Roberti for the title of Ph.D. in Oncological Genetics focuses on a molecular understanding of Burkitt`s lymphoma.

The manuscript is well written. Annalisa wrote a thorough introduction, analyzing in detail the pathology of Burkitt`s lymphoma both at the clinical and molecular levels. I really appreciated the original way in which she presented the INK/ARF locus. She extensively described the alteration affecting the locus in human tumors, cell lines and the animal model, even the evolutionary theory to explain the importance of the whole locus in "safeguarding" cells against tumors.

By using basic methodology she was able to clearly demonstrate a new molecular mechanism accountable for INK/ARF locus inactivation. Given the importance of the tumor suppressor genes encoded by the locus and their common involvement in human tumors, I believe that this work may have relevance in oncological research. I suggest that Annalisa extend her analysis to other categories of lymphoma as well as to other tumors, in order to better understand this mechanism and evaluate whether it is possible to find a link between INK/ARF inactivation modality and tumors.

On the whole, the thesis is clearly presented. Annalisa linearly demonstrates the scientific approach she used to carry out her research. The results are explained in a convincing way and the deduced conclusions are fitting to the experiments performed.

To conclude, in light of the quality of the work and the original results, I believe that Annalisa fully deserves the Ph.D. degree and the title of "Doctor Europeus".

I wish her much success in her future research.

Yours sincerely,

Paul B. Fisher

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January 6, 2009

Dear Antonio,

I read with interest the thesis that Annalisa Roberti has submitted.

My criticisms are minor and are essentially made in hopes that she will further improve her research ability and critical point of view.

My comments:
The Introduction is a little bit long. I think it is well done, but sometimes too much information may distract from the research topic.

Regarding the Material and Methods, I had some difficulty understanding the explanation concerning the method used to normalize the Real Time PCR values. The remaining fields are very clear.

In the Results, Annalisa analyzes a new non-coding RNA. I encourage her to keep studying its involvement in Burkitt`s lymphoma as well in INK4/ARF regulatory mechanisms. Sometimes expression analysis is not enough to rule out other possible mechanisms of action.

My last suggestion is that Annalisa try using different drug concentrations and other time conditions to lengthen the protein stability in GA-10.

The thesis she has submitted is thorough, and is overall well done and original. It could potentially have a considerable impact on oncological and basic research.

In my opinion, Annalisa's work clearly demonstrates that she has a strong ability for academic research that is more than enough to merit the title of Ph.D.

I would strongly support the award of Doctor Europeus for this thesis.

I sincerely hope Annalisa will continue the research she has started and wish her all the best in her career.

Yours sincerely,

Mark A. Feitelson PhD.

Mark Feitelson, Ph.D.

TABLE OF CONTENTS

Introduction

Burkitt`s lymphoma: an overview

Burkitt`s lymphoma was first identified and described by Dennis Burkitt in 1958 while he was working as surgeon for the Colonial Medical Service in Uganda. He noted children with distorted faces, with lesions involving one or both sides of the face and upper and lower jaws, sometimes had huge abdominal masses usually with no lymph node involvement. Burkitt recognized that this was possibly a previously undescribed cancer disease. He undertook a geographical survey of the incidence of the disease and found it to be correlated with the same temperature and rainfall zones as malaria. The geographic and climate association suggested that the occurrence of the disease might be linked with the distribution of certain insect carriers, as with malaria. In 1961, Burkitt made the acquaintance of Epstein, an experimental pathologist, and shared tumor samples with him. In one of these samples Epstain and his assistant Barr were three years later able to isolate the virus now known as Epstein-Barr's virus (EBV). This was the first description of a virus involved in the pathogenesis of a tumor in human. Initially, thought to be a childhood sarcoma of the jaws in African children ([1]), it has been later identified as an aggressive form of non-Hodgkin's B-cell lymphoma, usually diagnosed in children and young adults, but also seen in middle-aged adults in the Western world. Since its initial designation as Burkitt`s lymphoma, this type of lymphoma has received a variety of names in different classifications. Up to the most recent publication of the WHO (World Health Organization) Classification of Hematopoietic and Lymphoid Tumors, it had been recognized as a distinct category of peripheral B-cell lymphoma and it is once again classified as Burkitt`s lymphoma.

Clinical and pathological features of Burkitt`s Lymphoma

Burkitt`s lymphoma (BL) is one of the most rapidly growing malignancies to affect humans. It is a highly aggressive non-Hodgkin's B-cell lymphoma thought to be derived from early germinal center B cells. BL typically produces extranodal tumors rather then lymphadenopathy and often presents as a solid tumor mass in various extranodal sites or as an acute leukemia. Based on geographical distribution and the state of the host immune

system, three clinical variants of BL have been recognized by the WHO classification: endemic, sporadic and immunodeficiency–associated BL. Although these variants are histologically similar, each manifest differences in geographic distribution, clinical presentation, association with Epstein-Barr virus infection and biological future. Endemic BL (eBL), the disease originally described by Burkitt, is most commonly observed in equatorial Africa, the particularly high incidence of BL in this area (50-fold higher than in the United States) and the geographic distribution of this tumor corresponding to the distribution of endemic malaria, have led it to be designated as endemic BL ([2]). The eBL is the most prevalent childhood malignancy in Central Africa, with pick incidence at age 3 to 7, with a male to female ratio of 2:1. The classical presentation of eBL is a destructive tumor deforming the child's face, involving the jaw and other facial bones, as well as the kidneys, gastrointestinal tract, ovarian and other extranodal sites ([3]). Involvement of lymph nodes or bone marrow is uncommon, while involvement of nervous system occurs with greater frequency than in the non-African type.

Virtually all the cases of eBL are 100% EBV genome positive.

The capacity of EBV to stimulate B-cell proliferation inducing malignant phenotype strongly supports an etiological role of EBV in eBL (Orem 166). Although primary infection with EBV appears to be worldwide, the restricted geographic distribution of BL suggests that environmental factors may be important in the reactivation of the EBV genome within latently infected B-lymphocytes ([4])**.**

The endemic variant has long been known to be associated with EBV infection as well as frequent concomitant malaria infection. It is thought that this poly microbial effect leads to a dysregulation of the immune system, thus allowing evasion of the EBV-infected cells ([5]). Sporadic BL occurs worldwide: it includes those cases having no specific geographic or climatic association. In the United States and Western Europe, sBL accounts for up to 40 % of all non-Hodgkin's lymphoma in children and 1%-2% of all lymphomas in adults. As in endemic BL, in sBL males are more often affected than females. The abdomen, especially in the ileocecal areas, is the most common site of involvement and patients with sBL typically present with abdominal masses. Intra-abdominal presentations usually affect the bowel or intra-abdominal lymph nodes; however kidney, pancreas, liver, spleen, breast or ovarian presentations can occur. It has been reported that a bilateral involvement of the breasts may occur in association with the onset of puberty or with lactation. Lymph node involvement is more common with adults than with children. Patients may also have malignant pleural effusion or ascites ([6]). Bone marrow involvement is not usually seen at

presentation, but is more commonly seen in progressive diseases. Bone marrow involvement leads a poorer prognosis and also makes central nervous system involvement more likely. Another way for this type of lymphoma to present is with bone marrow and blood involvement; in this case the lymphoma is called 'acute lymphoblastic leukemia'.

In sporadic BL the frequency of association with EBV infection is lower than in endemic case. Both the degree of incidence and EBV association depends upon the area. Western countries show the lowest incidence rates and the weakest viral association with only 15 % to 20% tumors being EBV positive; by contrast, BL appears to be more common in other locations, for example equatorial areas of Brazil and EBV-association rates are correspondingly higher. Poverty lowers the age of initial EBV infection, and early exposure to EBV could be a trigger for BL development ([7]). It is plausible to assume that chronic immune stimulation from other parasitic infections may also increase the incidence of BL; low socioeconomic factors like poor nutrition and /or hygienic conditions could be associated with a weak immune response.

Immunodeficiency–associated BL occurs in people with depressed immune system, primarily in HIV-infected patients, but may also arise in post transplant patients who are chronically immuno suppressed and in individuals with congenital immunodeficiency. Although BL had been previously considered to be a rare disease in industrialized nations, it has become increasingly common in part due to its association with HIV. Burkitt`s lymphoma was estimated to be 1,000 times more common in HIV^+ individuals than in the immunocompetent populations ([8]).

It is well known that the risk of non-Hodgkin's lymphoma is greatly increased among people who are HIV-positive and 16 % of deaths in AIDS patients have been reported to be due to lymphomas. Although HIV is not directly involved in lymphomagenesis, the increased risk for lymphoma appears to be related to multiple factors, including the transforming properties of the retrovirus itself, the immunosuppression and cytokine dysregulation that results from the disease, and most importantly, opportunistic infections with other lymphotrophic herpes viruses such as the Epstein-Barr virus and human herpesvirus 8 ([9]). Burkitt`s lymphoma makes up the largest group of HIV-associated NHL, comprising up to 30-40% of these neoplasms and the diagnosis of Burkitt's lymphoma in an HIV^+ individual often represents the initial acquired immunodeficiency syndrome (AIDS)-defining illnesses ([10]). In immunodeficiency–associated BL, lymph nodes, bone marrow, and extranodal sites, most often in the abdomen, are commonly involved. As well as for the sporadic variant, the frequency of association with EBV

infection in immunodeficiency–associated BL is relatively low and ranges from 25%-40% of cases, however it shares some pathogenic features with the endemic form.

Burkitt's lymphoma occurring in transplant recipients may be considered a separate entity compared to Post-transplant lymphoproliferative disorders (PTLD) and AIDS- related Burkitt lymphoma. Post-transplant Burkitt lymphoma occurs later after transplantation (mean 4.5 years), whereas the majority of PTLDs occur early, with- in 1–2 years. Posttransplant Burkitt lymphoma is closely related to AIDS-related Burkitt lymphoma; however, the etiopathogenesis of these two conditions is significantly different.

The immunosuppressants given to transplant patients is commonly induced by the regimens used for solid organ transplant, specifically targeting the functional activity of the entire T-cell population. In AIDS patients, immunosuppression is virally induced and selectively depletes helper/inducer T cells ([11]).

Morphology and immunophenotype of Burkitt`s lymphoma

Burkitt's lymphoma is a malignancy of intermediately sized B cells that infiltrate nodal and extranodal tissues in a diffuse pattern. With respect to morphology, the WHO Classification describes in addition to classic Burkitt's lymphoma, two cytological variants: Burkitt's lymphoma with plasmacytoid differentiation and atypical Burkitt's/Burkitt-like lymphoma ([3]).

Morphologically, classic BL is constituted by uniform and medium-sized neoplastic cells with abundant basophilic cytoplasm often containing lipid vacuoles, round nuclei with clumped chromatin and multiple nucleoli, and a diffuse pattern of infiltration.

Histologically, this tumor is characterized by a typical 'starry sky' pattern as the high rate of cell turnover leads to the accumulation of apoptotic debris inside macrophage, giving them their `tingible body` appearance interspersed in a malignant population of round monomorphic B cells ([12]). The rate of cell division in BL is among the highest of any human tumor, as reflected by the presence of numerous mitotic figures and a high fraction of actively growing cells. The latter feature can be demonstrated by staining the cell for cell-cycle specific markers such as Ki-67, that typically shows more than 95% of tumor cells to be progressing through the cell cycle. These characteristic reflects the rapid doubling time of the tumor that is usually between 24 and 48 hours ([13]). Classical BL is found in cases of endemic BL and most cases of sporadic BL affecting children but in only a minority of sporadic and immunodeficiency-associated adult cases. Instead, many

ceases of sporadic BL occurring in adults have a Burkitt-like morphology; whereas the immunodeficiency associated form tends to present a Burkitt's lymphoma with plasmacytoid differentiation. Burkitt-like lymphoma and Burkitt's lymphoma with plasmacytoid differentiation both tend to have greater nuclear pleomorphism than classical Burkitt's lymphoma and both tend to have a smaller number of more prominent nucleoli. The plasmacytoid variant has, in addition, monotypic cytoplasmic immunoglobulin ([3]).

Burkitt-like lymphoma shares several pathologic characteristics with classical BL, such as c-myc translocation and the high proliferation index, but morphologically this variant may more close resemble diffuse large B-cell lymphoma (DLBCL), making pathologic diagnosis difficult and sometimes controversial.

However the high-grade nature of the tumor based on pathologic criteria (Ki-67), the *c-myc* translocation, and a clinical course essentially identical to that of classic BL have led to its classification as a variant of BL by the World Health Organization ([12]). Taken together these data suggest that the diagnosis of Burkitt's lymphoma should ideally be based on a combination of clinical, pathologic, and cytogenetic outcome.

Burkitt's lymphoma, regardless of subtype classification, typically expresses B-cell surface markers such as CD19, CD20, CD22, immunoglobulin (Ig) M and Igκ or λ light chain, as well as germinal center-associated antigens CD10 and BCL6, while they lack expression of CD5 (expressed in mantle cell lymphoma), C23, TdT, and BCL-2 (Jaffe ES 2001).

Their immunophenotype is consistent with their postulated germinal center origin. Moreover, sequence analysis of the Ig variable heavy (V_H)-and light (V_L)-chain in endemic, sporadic, and HIV-associated BL has shown that they have undergone somatic hypermutation, an event that is tipically confined to germinal center B cell and marks B cells as being of germinal center origin.

Genetic features of Burkitt`s lymphoma

BL may be considered a model for the exploration of chromosomal aberrations in cancer.

The molecular hallmark of Burkitt's lymphoma is the inappropriately high activity of the c-Myc oncogene through reciprocal chromosomal translocation, which juxtaposes c-myc to one of the immunoglobulin (Ig) loci. In 1972, it was discovered that endemic BL contained an additional band at the end of the long arm of chromosome 14 ([14]). Several years later, Zech et al. observed

that the telomeric end of the long arm of chromosome 8 was consistently absent, and suggested that the missing part of chromosome 8 was translocated to chromosome 14. The molecular targets of these reciprocal translocations were discovered to be MYC (at 8q24), and genes coding for the immunoglobulin heavy chain (IgH at 14q32); the association between a proto-oncogene with a locus that becomes activated in B-lymphocytes strongly suggested the importance of this translocation in the pathogenesis of BL.

Eighty percent of BL cases harbor t(8;14) translocation that juxtapose the c-Myc gene on chromosome 8 to the immunoglobulin (Ig) heavy chain enhancer elements on chromosome 14, which drive c-Myc mRNA and protein production. In the remaining 20% of BL cases, translocations occurring between chromosomes 2 and 8, t (2;8), or chromosomes 8 and 22, t (8;22), place the c-Myc gene adjacent to either κ or λ light chain loci and enhancer elements, respectively ([2]).

Ig enhancer elements bind to B cell-specific factors capable of activating transcription from genes located up to 500kb away. Because Ig enhancer elements are specifically active in mature B cells, their juxtaposition to c-Myc in BL cells drives a constitutive expression of c-Myc; moreover, negative regulatory sequences residing within c-Myc are often removed as a direct consequence of chromosomal translocation or undergo mutational events, contributing to increase c-myc activity ([13]). In tumors with the t(8;14) translocation, the site of the breakpoints relative to the c-Myc gene on chromosome 8 and the IgH gene on chromosome 14 differs and correlates with the clinical variant of BL. In endemic BL, the break point on chromosome 8 generally occurs more than 100 kilobases (kb) upstream from the c-myc exon 1, whereas the breakpoint on chromosome 14 usually occurs in the joining segments of the IgH gene. This t(8;14) translocation in endemic BL places the c-myc promoter under the control of the IgH Eμ enhancer element ([15], [2]), leading constitutive activation of c-Myc.

In sporadic and HIV-associated BL, the t (8;14) breakpoint tends to fall between c-Myc exon1 and 2 on chromosome 8 and within the IgH Sμ switch region on chromosome14. The Eμ IgH enhancer is eliminated during the translocation, suggesting that others enhancer elements located within the IgH locus such as Eα may be responsible for driving c-Myc transcription ([2]). In addition, in the sporadic BL the t(8;14) breakpoint tends to fall in the c-Myc first intron, thus removing 5` negative regulatory region of the gene. This breakpoint lies downstream of c-Myc normal promoter region, so that the transcription is initiated from a cryptic promoter within the first intron ([13]).

In the two variant translocations, the c-Myc locus is joined to either the Ig κ locus on

chromosome 2 or the Ig locus λ on chromosome 22. The breaks on chromosome 8 occur in noncoding sequences lying 3' of the c-Myc gene, while the breaks on chromosome 2 and 22 occur 5' of the κ and λ gene constant region segments. Ig enhancer elements from either the κ or the λ locus activate the transcription of the c-myc fusion gene from its own promoter. As well as for endemic BL, in the sporadic BL associated with variant translocations, the chromosomal breakpoints of c-Myc preserve its normal genomic organization and transcriptional start sites. However, mutations in the negative regulatory region of c-Myc are commonly found, strengthening the c-Myc activity by revoking its negative regulation ([13]). Taken together, these data suggest that the removal or mutations of *cis*-acting negative regulatory elements cooperates with positive regulatory Ig enhancer elements to drive c-Myc expression in BL. In addition to chromosomal translocations and regulatory sequences mutations that activate c-Myc in BL, the coding sequence also bears hot spot mutations. These alterations may reinforce the oncogenic potential of the protein by impairing c-myc activity and protein stability,

The correlation between BL type and site of chromosomal breakpoint not only suggests a different pathogenesis for these subtypes of BL but also that eBL and sBL may derive from lymphocyte precursors at different stages along the B-cell differentiation pathway.

In sporadic and AIDS-associated BLs with the t(8;14), the positions of the breakpoints in chromosome 14 suggest that they were created during attempted Ig class switching. This event is normally confined to germinal center B cells, providing further support for a germinal center B-cell origin for these forms of BL. In contrast, in endemic BL associated with the t(8;14), the J_H segments immediately flanking the breakpoints usually have deletions and/or additions of base pairs that are characteristic of normal Ig V(D)J segment rearrangement at the pre-B cell stage of differentiation.

Given these observations, it seems to be possible that B cells from sBL are more differentiated than those from eBL. However, several models are reasonable for the timing of t(8; 14) formation in eBL.

One explanation may be that, although c-Myc rearrangement is an early event occurring in a pre-B cell stage of differentiation, the cell, undergoing maturations to a germinal center B cell, acquires additional genetic alterations in c-Myc that collaborate to give BL ([16]). Another possibility is that the V-D-J recombinase, previously believed to be expressed exclusively in pre-B cells, may be re-activated in the germinal center, allowing the t(8; 14) translocation to occur at this latter stage of differentiation ([17]).

Diagnosis and treatment of Burkitt lymphoma

Burkitt lymphoma (BL) has peculiar clinical presentation, morphology, immunophenotype and primary chromosomal aberration, however diagnostic dilemmas may arise due to overlap of BL with subsets of other aggressive, mature B-cell lymphomas such as diffuse large B-cell lymphomas (DLBCL). Burkitt lymphoma and diffuse large-B-cell lymphoma are both mature aggressive B-cell lymphomas that are rapidly fatal if left untreated. This distinction has important clinical implications for treatment and prognosis, as BL responds poorly to standard DLBCL therapy (CHOP-like regimen) but well to high-intensity chemotherapy ([18]).

The diagnostic concern is most pertinent for adults rather than children given the low incidence of BL among the non-Hodgkin's lymphoma in adults and because adults often have the atypical Burkitt`s variant, which has morphologic features closer to those of DLBCL than classic BL. According to WHO classification the parameters that favor BL diagnosis versus DLBCL include the typical morphological feature of a 'starry sky' pattern, an immunophenotype that is CD20+, CD10+, Bcl-6+, Bcl-2−, TdT−, and monotypic sIg+, with virtually all cells Ki67+ (proliferation), and a translocation involving c-myc and IgH or IgL, without rearrangements involving the bcl-2 or bcl-6 genes.

While the differential diagnosis between BL and DLBCL may appear clear-cut in theory, daily practice shows that aggressive B-NHLs are encountered that display some (but not all) morphologic, immunophenotypic and genetic features of classical BL.

On the genetic level, translocations involving MYC are a hallmark feature of classical BL. However, information on the MYC translocation status is insufficient to discriminate between BL and DLBCL, since 5–10% of DLBCLs also carry a MYC translocation. The translocation involving c-Myc can also be found infrequently in follicular lymphoma, mantle-cell lymphoma and plasma-cell lymphoma. In lymphomas other than Burkitt's type, c-Myc is more likely to have variant translocations (whit IgL or other non-Ig partners, rather than IgH), and neoplasmatic cells tend to have more complex karyotypic abnormalities. The c-Myc rearrangement in such cases is considered to be a secondary event ([3]).

Although BLs were characterized by a significantly higher Ki-67 index than DLBCLs and vice versa, DLBCLs were more frequently positive for BCL2, in some studies it has been demonstrated that a significant overlap between these two groups may exist. The morphological feature of a 'starry sky' pattern that is considered a hallmark in BL can

also be present in a subset of highly proliferative DLBCLs raising the question whether these cases may resemble BL molecularly and whether these cases should be subsequently classified as atypical BL. According to a recent study, however, highly proliferative DLBCLs with a 'starry sky' pattern are only rarely positive for CD10 and infrequently carry a MYC translocation. Thus, BL and highly proliferative DLBCL with a 'starry sky' pattern might be reliably distinguished ([19]).

The current diagnostic tools could not be sufficient to discriminate those cases ascribed in the gray zone between BL and BLBCL, making the investigation of new parameters necessary. Two comprehensive gene-expression studies have been published, therefore investigated whether global gene expression profiles might help to discern BL from DLBC on the molecular level. In both studies, BL could be distinguished from other aggressive lymphomas by a distinct gene expression pattern, that is the combination of expression levels of multiple genes being able to predict a diagnosis of BL (predictor or index). Cases with a high index were called 'molecular BL' and cases with a low index 'non-molecular BL.' Although the gene expression signatures of molecular BL are composed of different genes in the two studies, they both showed that 17 ([20]) and 24% ([21]) of cases identified to have the Burkitt gene expression profile had been previously diagnosed as DLBDL or unclassifiable high-grade lymphoma according to current WHO criteria. Both gene expression studies, remarkably, agree in their findings that a subset of aggressive B-NHL with clear-cut morphological features of DLBCL was identified that, nevertheless, showed a gene expression profile of BL ('discrepant BL'). Therefore, both studies help to sharpen the molecular distinction between BL and DLBCL but at the same time, extend the spectrum of molecular BL to some cases that would currently be classified as DLBCL. In addition, besides providing further diagnostic information, these studies also provide some insight into potential therapeutic targets and clinical outcomes. Hummel and colleagues have demonstrated that mBL cases had very few genetic alterations in addition to the MYC translocation (low genetic complexity), whereas intermediate and non-mBL cases carried a higher load of chromosomal imbalances (high genetic complexity). Of importance, non-mBL (DLBCL) cases that harbored a MYC translocation had inferior overall survival as compared with DLBCL cases without MYC translocation in a retrospective analysis of patients receiving mostly a CHOP/CHOP-like therapy. In the gene expression study performed by Denn and coworkers they moreover confirm that CHOP-like regimens are not adequate to treat those groups of tumors carrying both MYC and BCL2 translocations. These dual translocation or 'double hit'

lymphomas have a c-Myc translocation that enhances cell proliferation and a bcl-2 translocation that inhibits apoptosis and thus prolongs cell survival. This devastating combination leads to a tumor with very poor prognosis. A thorough characterization of more such cases will be however needed in order to ascertain whether they represent a variant of Burkitt's lymphoma or have a separate pathogenesis.

The important question that remains is whether the high-grade lymphomas with some but not all features of Burkitt's lymphoma should be treated as BL or as DLBCL.

Burkitt's lymphoma was associated with poor outcome before the advent of high intensity chemotherapy, probably reflecting its high proliferative rate. The high-growth fraction of BL (doubling time of approximately 25 hours) favors re-entry of remaining viable malignant cells into the cell cycle and rapid growth between chemotherapy cycles with subsequent development of resistance. Short-duration, intensive regimens that minimize treatment, delays and maintain serum drug concentrations over at least 48 to 72 hours have the greatest efficacy in BL ([2]). Short-duration combination chemotherapy, first used in pediatric BL, consists of intensive therapy combining non-cross-resistant drugs know to be active agents against BL, including cyclophosphamide, vincristine, methotrexate, doxorubicin and cytarabine. The institution of the Magrath protocol ([22]) represented a major step forward in the treatment of Burkitt's lymphoma. Children and adults treated with this regime had similar outcomes, with an excellent response rate; the event-free survival (EFS) at 2 years was 92% for the group as a whole. However, there were significant associated toxicities, including frequent myelosuppression, mucositis, neuropathy and some treatment-related deaths. Over the past few years, several trials have focused on reducing these associated toxicities, while maintaining the treatment's efficacy. A small recent phase II study, achieved by the Dana-Farber Cancer Institute, utilize a modified Magrath protocol with lower intensity regimes, aimed at decreasing toxicity while maintaining good outcome. In this cohort, there were no treatment-related death, no severe neurotoxicity and a significant decrease in mucositis. The 2 years EFS rate was 64% for all patients, 100% for low-risk patients, and 60% for high-risk patients ([23]). The small study proved that some patients could be effectively treated with lower intensity regimens, with reduced medication-induced toxicities. The inferior survival of high-risk patients may reflect the change in regimen, but may also be explained by other factors distinguishing the two studies. For example, the median age in the latter study was 46 years compared with 24 years for the original study by Magrath *et al.* It has become increasingly clear that age is a significant prognostic factor related to survival, as older

patients do not tolerate the chemotherapy as well, or do not have as significant a response. This again may be the innate difference in the biology of the tumors ([24]). Althought the most important prognostic features have yet to be demonstrated, some features that have been associated with adverse outcome in adults and children include old age, advanced stage, poor performance status, bulky disease and CNS or marrow involvement. A minority of patients with Burkitt`s lymphoma presents with leukemic disease, however, therapy traditionally used to treat lymphoblastic leukemia worked poorly in BL patients. Although, newer chemotherapeutic regimes are associated with a better outcome, they too are associated with a severe toxicity ([3]). Patients positive for HIV infection with BL can be successful treated with intensive chemotherapy, but close observation with transfusion support and antibiotic therapy is necessary. The addition of Highly Active Anti Retroviral Therapy (HAART) to those regimes may improve outcome and minimize the risk of opportunistic infections ([2]). Patients developing BL in a post-transplant setting may be difficult to treat. They may show a good response to therapy that includes a combination of chemotherapy, decreased immunosuppression, and rituximab, although mortality appears to be high ([3]). Despie tremendous progress has been made in recent years, novel treatment regimes are needed, and the high toxicities associated with the current therapeutic regimes needs to be minimalized, especially for patients with poor prognostic futures, patients who fail to achieve a complete response (CR), and patients with relapses.
With the frequent expression of CD20 in BL, the monoclonal anti-CD20 antibody rituximab may be used as adjuvant therapeutic option in BL. It potentially acts through several mechanisms including the activation of cell-dependent cytotoxicity as well as antibody-dependent cytotoxicity ([24]). A recent study demonstrated that for adult patients, the use of rituximab in combination with intensive chemotherapy, might improve survival, especially in older patients, a subclass with known poor outcomes ([25]). Another biological agent the anti-CD22 antibody is being actively studied in the treatment of NHL; it has different mechanism of action to rituximab and may be synergistic with anti-CD20 in inducing cellular apoptosis. Other novel therapies that may have potential benefit, but have not yet been evaluated in BL, include DNA methyltransferase inhibitors, histone deacetylase inhibitors, antisense oligonucleotides targeting c-Myc, proteasome inhibitors, cyclin-dependent kinase inhibitors, selective serotonin re-uptake inhibitors (SSRIs), and a blockade of EBV-related viral proteins. Although the optimal therapeutic strategy for BL is unknown, continued progress in the development of targeted therapies will potentially improve outcomes in this disease.

Molecular biology of Burkitt`s lymphoma.

High-growth fraction lymphomas are characterized by an enhanced proliferative activity as a result of the deregulation of oncogenes with cell cycle regulatory function. The invariable translocation and improper activation of c-Myc is a bottleneck event in BL, sustaining tumors cells in a perpetually proliferative condition. c-Myc rearrangement however, may be considered an early event in lymphomgenesis; subsequent tumor progression involves selection for additional genetic and epigenetic changes, conferring a further growth advantage and apoptosis protection on the cells. A key regulatory pathway determining cell cycle proliferation rate is the cyclin/cyclin-dependent kinase(CDK)/p16Ink4A/retinoblastoma protein (pRb) axis. Alterations to different components of this pathway through overexpression, mutation and epigenetic gene silencing could facilitate c-Myc mediated growth stimulation, providing increased aggressiveness and proliferative advantage to tumor cells. Additional transforming events may overcome the apoptotic signaling induced by c-Myc overexpression by inactivating the $p14^{ARF}$-MDM2-p53 pathway or by perturbing the balance between pro and antiapoptotic members of BCL2 family. Alterations of other genes may occur during tumor progression, selecting cell populations with aggressive phenotype.

Physiologic and oncogenic function of c-Myc.

The c-MYC proto-oncogene encodes the c-MYC transcription factor, and was originally identified as the cellular homologue to the viral oncogene (v-myc) of the avian myelocytomatosis retrovirus ([26]). When reintroduced back into host cells, v-myc promoted cancer in various avian tissues. The discovery of human Myc as a target for activation by chromosomal translocation in Burkitt`s lymphoma first identified it as a prototypical oncogene ([27]).

More recently, elevated or deregulated expression of c-MYC has been detected in a wide range of human cancers, and is often associated with aggressive and poorly differentiated tumors, underlining the key role of this oncogene in tumor progression. Although c-Myc was one of the first cellular oncogenes discovered, it has been difficult to gain a precise understanding of its biological function because it regulates as many as 10% to 15 % of all cellular genes, making it difficult to appreciate how Myc directly or indirectly is involved in physiological as well as neoplastic cell behavior. Through its role as a transcription factor, c-Myc alters the expression of hundreds of target genes, many of

which are themselves oncogenes or tumor suppressors.

c-Myc is a transcription factor implicated in many cellular processes such as cell cycle regulation, apoptosis, cell growth, metabolism, cell adhesion and differentiation, that can contribute positively or negatively to cellular transformation. These effects are mediated through the binding of c-Myc to the promoter elements of a discrete set of downstream genes, thereby either inducing or repressing their transcription.

The c-Myc protein belongs to the large family of helix-loop-helix leucine zipper (HLHzip) transcription factors and is also a member of the Myc family of proteins (B-Myc, L-Myc and N-Myc) that are characterized by two conserve N-terminal regions, termed Myc BoxI and BoxII. The 150-amino-acid amino-terminal region of c-MYC is required for the transactivation of target genes. The carboxy-terminal basic-helix–loop–helix–zipper (bHLHZ) domain of c-MYC binds MAX, another bHLHZ protein, to form MYC–MAX heterodimers that are capable of binding specific DNA sequences, such as the E-box sequence CACGTG7. Mutations in the c-MYC transactivation region or in the bHLHZ domain abolish c-MYC's effects on cell proliferation, transformation and apoptosis ([28]).

The ability of c-Myc to bind to specific DNA sequence and activate transcription requires heterodimerization with Max, which in turn is able to heterodimerize with other Myc family proteins, such as Mad or Mxi1.

The Myc-Max dimers bind to the E-box sequence CACGTG in the promoters of specific target genes and stimulate their transcription. The protein products of these target genes go on to mediate the downstream effects of Myc on cell biology. Myc is then rapidly degraded and the pathway switches to a transcriptionally repressive state when Max dimerizes with the group of related bHLHz proteins, the Mad family, that are thought to operate as Myc antagonists. The Mad-Max dimers bind the same E-boxes that were previously occupied by Myc-Max dimers and repress transcription, at least in some promoter. Myc drives transcription by recruiting co-factors to target gene promoters that through promoting localized modification and remodeling of the chromatin regulate target genes expression. Many of these interactions seem to be mediated by the evolutionary conserved Myc box sequences, MBI and MBII, within the c-Myc N-terminal transactivation domain. C-Myc was found to bind directly to an internal domain of TRAP or Transactivation/transformation Associated Protein ([29]). TRAP is a component of the SAGA (SPT/ADA/GCN5/acetyltransferase) complex; a system consisted of approximately 20 proteins and implicated in transcriptional regulation ([30]). TRAP recruits

GCN5, a known histone acetyltransferase, providing an explanation for transcriptional activation by c-Myc/Max heterodimers. By binding TRAP, C-Myc chaperons in proximity of transcriptional start site the histone acetyltransferase GCN5 that acetylates chromosomal histones specifically at the E-Box and adjacent regions. Nucleosomal acetylation is associated with a more open chromatin structure, allowing accessibility of the MYC–MAX transcriptional-activator complexes to target DNA, resulting in their consequent induction The antagonistic activity between Myc and Mad proteins is supported by the knowledge that Mad proteins contain an amino-terminal repression domain that associates directly with the corepressor Sin3, which, in turn, binds to histone deactylases (HDAC). The recruitment of HDAC leads to histone deacetylation and a more condensed local chromatin structure, shutting down the expression of c-myc target genes ([31]).

In this model a, chromatin-based molecular switch for gene transcription with the ‘‘on’’ or ‘‘off ’’ state seems to be determined by the relative concentration of Myc and Mad proteins. ([32]). Because c-Myc mRNA and protein are short-lived and Max is a relatively stable and abundant protein, the level of Myc/Max heterodimers is largely dependent of c-Myc protein concentration. During tumorigenesis, excess Myc leads to a prevalence of Myc-Max dimers and elevated expression of specific target genes. Along with its ability to activate growth-related genes through E boxes, c-Myc also represses differentiation-associated and growth-suppressing genes through mechanisms that have not been fully understood. Several potential targets of c-Myc–mediated transcriptional repression have been identified, including the genes for adhesion molecules leukocyte function–associated antigen (LFA) 1 and alpha3/beta1 integrin, the growth arrest genes *gadd* 45 and *gas1*, and the cyclin dependent kinase (CDK) inhibitor p27 ([13]).

To understand c-Myc physiologic and tumorigenic activity it is critical to comprehend the gene expression patterns that are induced by c-Myc in both normal and transforming situation. c-Myc expression is closely correlated with growth, and removal of growth factors at any point in the cell cycle results in its prompt downregulation. The protein is normally expressed in all dividing cells, where it enhances cell cycle progression and is downregulated in cells undergoing cell cycle arrest and/or terminal differentiation. C-Myc expression is absent in quiescent cells but is rapidly induced upon the addition of growth factors. Ectopic expression in quiescent cells, under some conditions, may elicit entry in S phase. Overexpression of c-Myc in growing cells leads to reduced growth factor requirement and a shortened G1 phase, while reduced expression causes lengthening of

the cell cycle ([33]).

The global nature of c-Myc's effects on cell cycle progression has been demonstrated in c-*myc* knockout fibroblasts, in which the loss of c-Myc causes a profound growth defect manifested by the lengthening of both G1 and G2 phases of the cell cycle. The largest defect observed in c-Myc$^{-/-}$cells is a 12-fold reduction during G_0-to –S transition in cyclin D1/CDK4 and cyclin D1/CDK6 activity, a pair of protein kinases that promote cell cycle progression. C-Myc promotes proliferation through the induction of genes involved in cell cycle control such as CDK4, CDC25A and cyclins D1, D2 , A and E. The induction of cyclin-E-CDK2 activity early in the G1 phase of the cell cycle is regarded as an essential event in Myc-induced G1-S progression. However the restoration of CDK4 and CDK6 activity does not recuperate the c-Myc defect, which indicates that c-Myc acts through multiple downstream effectors ([33]).

The c-Myc-induced expression of cyclinD1 and CDK4 leads to the sequestration of the CDK inhibitor KIP1 (also known as p27) in the cyclin-D2-CDK4 complex, preventing its binding to the cyclin E-CDK2 complex. The removal of p27 from the cyclin E-CDK2 complex is an essential step for S-phase entry. The cyclin E-CDK2 complex, not longer inhibited, can lead to cell replication.

Subsequent degradation of KIP1 involves two further MYC target genes, *CUL1*and *CKS*. In so doing c-Myc additionally promotes cell growth by enhancing p27 cell cycle inhibitor protein degradation ([34]). c-MYC might also influence the cell cycle by repressing genes, among the c-Myc targets of inactivation there is WAF1, also known as p21, a CDK inhibitor involved in cell-cycle arrest ([31]).

The ability of c-Myc to affect cell proliferation suggests that its ectopic expression may contribute to deregulate DNA synthesis and genomic instability, which in turn may contribute to subsequent transformation. It is proposed that genomic instability stems from accelerated passages through the G1 phase of the cell cycle and perturbation of the G1/S phase checkpoint ([13]). A recent study suggests that inducible transient c-Myc expression is sufficient to increase the transformation of Rat1 fibroblasts at least 50-fold ([35]). This protransforming effect correlates with the appearance of chromosomal abnormality, gene amplification, and hypersensitivity to DNA-damaging agents ([36]: [37]). In most cases the defect appears to be reversible, although in a minority of cells there appears to be a "hit-and-run" effect by c-Myc such that its continuous expression is not necessary to maintain the genomic alterations. C-Myc may act by generating secondary transforming events, which then in turn make c-Myc dysregulation superfluous for the

maintenance of the transformed state. c-Myc actively repress differentiation programs consistently by promoting cell cycle. Because terminal differentiation requires exit from cell cycle, the over activity of c-Myc, supporting persistent cell cycling, inhibits terminal differentiation.

The Eμ-myc transgenic BL mouse model ([38]), a transgenic mouse carrying a human c-myc gene fused to a reconstructed Ig lambda locus mimicking the t(8;22) translocation, has given the first indication that c-Myc partially inhibits the differentiation of primary B cells ([39]). In the prelymphomatous state, these animals show increased numbers of pre-B cells, decreased numbers of mature B cells, and increased numbers of cycling cells in both early and late B-cell populations. In approximately 90% of established lymphoid and myeloid tumors, downregulation of c- Myc elicits cell cycle arrest, terminal differentiation, and tumor regression ([40], [13]).

C-Myc regulates diverse metabolic pathways; among them, the c-Myc induced transcription of LDHA ([41]) may be considered the most relevant in BL. The tendency for tumor cells to increase lactic acid levels under aerobic condition is known as the Warburg effect and may be considered as an adaptive mechanism by which tumors cells keep surviving and proliferating under hypoxic conditions. LDHA overexpression is required for c-Myc-mediated transformation, since antisense LDHA RNA reduces soft agar clonogenicity of c-Myc transformed fibroblasts as well as c-Myc-transformed human lymphoblastoid cells and Burkitt's lymphoma cells ([42]). A number of c-Myc responsive genes appear to be involved in proteins synthesis and translation and hence might contribute to cell growth phenotype. C-Myc also regulates gene involved in iron metabolism; for example it negatively regulates H-ferritine. This downregulation has been shown to be a required event in c-myc induced-cellular transformation ([43]). The connections between c-Myc and cellular metabolism suggest that it is a key integrator of cell proliferation and metabolism.

In some cellular contexts, c-Myc overexpression induces programmed cell death (apoptosis). This association may underline the starry sky appearance that is characteristic of BL due to a high rate of apoptotic cell death. Ectopic expression of c-Myc in fibroblasts deprived of growth factors leads to apoptosis ([44]). It has been well established that oncogenes may activate apoptosis if the proliferative pathway is blocked in some points; cell-proliferative and apoptotic pathway are coupled and the induction of cell cycle sensitizes the cells to apoptosis. However, the apoptotic pathway is suppressed as long as appropriate survival factors deliver anti-apoptotic signals. Given this, the

predominant outcome of these contradictory processes will depend on the availability of survival factors ([31]). C-Myc expression sensitizes cells to a wide range of pro apoptotic stimuli, such as hypoxia, DNA damage and survival factor depletion. Several lines of investigations suggest the existence of p53-dependent and p53-independent c-Myc induced cell death pathways. The *p53*-dependent pathway is triggered by withdrawal of growth factors and is accompanied by the upregulation of the regulatory protein phosphatase cdc25A ([45]).

A second pivotal player in the *p53*-dependent pathway is *ARF*, a c-Myc–inducible gene encoding a protein that, by sequestrating MDM2, prevents the degradation and inactivation of the tumor suppressor p53. Indeed, ectopic expression of c-Myc induces ARF and p53 in wild-type MEFs, triggering replicative crisis and apoptosis. MEFs that survive myc overexpression sustain ARF loss or p53 mutations. The importance of ARF in c-Myc-induced apoptosis in vivo and its antagonistic role in c-Myc–induced lymphomagenesis was underscored in Eμ-Myc transgenic mice that expressed c-Myc only in B-lymphocytes. Tumors arising in Eμ-Myc transgenic mice commonly show a spontaneous inactivation of the ARF-p53 pathway and an ARF-deficient genetic background in the same transgenic models increase lymphomagenesis ([46]).

Conversely, mice that had lost one or both *Bmi*-1 alleles, an oncogene that suppresses the expression of ARF, show decreased c-Myc induced lymphomagenesis by increasing ARF-dependent apoptosis. These observations indicate that ARF participates in a p53-dependent checkpoint that safeguards cells against overexpression of c-Myc ([47]).

The first insight into c-Myc's role in induced apoptosis came from studies showing that c-Myc could induce the release of cytochrome c from mitochondria through the activation of the pro-apoptotic protein BAX during apoptosis ([48]). By contrast, the anti-apoptotic proteins BCL2 and BCL-X_L safeguard cells from c-Myc-induced apoptosis, preventing cytochrome c release. BCL2 protects again both the p53-dependent and independent c-Myc-induced apoptosis. As well as BCL2, N-Ras also protects cell from c-Myc induce apoptosis, an activity that may partially explain the synergism with c-Myc in induction of aggressive murine lymphomas ([49], [50]).

The cells have evolved mechanisms that protect against ectopic expression of growth-promoting genes; c-Myc mediated apoptosis may be explained as a safeguard mechanism to prevent cell proliferation induced by oncogene activation. The ability of c-Myc to induce apoptosis explains why tumors overexpressing c-Myc recurrently select for

alterations that neutralize its apoptotic program through secondary mutation in the downstream effectors of this tightly regulated pathway.

c-Myc collaborates with activated RAS to transform primary fibroblasts, a simple view of this collaboration may be that c-Myc and RAS induce complementary cell growth pathways that together lead to cellular transformation.

As well as for the c-myc defensive ability to promote apoptosis, constitutive activation of N-Ras induces a form of cellular senescence, which is hypothesized to be a mechanism to eliminate cells with potentially oncogenic N-Ras mutations ([51]). The prosenescent effect of N-Ras is blocked by c-Myc, whereas the proapoptotic effect of c-Myc is antagonized by activated N-Ras ([52]). In a revised view of oncogenic collaboration, c-Myc appears to inactivate cellular responses that are normally required for RAS-mediated growth inhibition, thereby switching RAS into a growth-promoting gene. Reciprocally, RAS is able to inhibit Myc-mediated apoptosis ([42])

C-Myc mediates cellular immortalization by inducing telomerase activity, as well as by the expression of the catalytic subunit of telomerase (TERT) ([53]).

The c-Myc ability to counter the senescent effect of N-Ras could be explained by its regulatory effect on telomerase activity. Telomerase contributes to the immortalization of cells by allowing the indefinite maintenance of the telomeres, which normally shortens as part of the aging process, eventually causing cellular senescence.

C-Myc negatively regulates the expression of genes encoding cell adhesion molecules, as LFA-1 (integrine alphaLbeta2) in lymphoblastoid cell lines and collagen and fibronectine in fibroblasts ([54]).

These associations suggest that down-regulation of adhesion molecules may be necessary for the anchorage-independent growth phenotype associated with c-Myc overexpression and may enable BL cells to escape immune surveillance.

Given the multitude of cellular process mediated by c-Myc, it is arguable that it has to be tightly regulated in a physiological setting and its deregulation has a great relevance in the pathogenesis of cancers. C-Myc activation may occur both directly and indirectly. The direct mechanisms included stabilization of c-Myc both at mRNA transcription and protein translation levels and indirect mechanisms involve the activation of upstream signaling cascades. Novel evidence suggests that c-Myc activation can also occur through stabilization of the c-Myc protein. MYC proteins with mutations at or near Thr58, which abolish phosphorylation at this site, result in inefficient ubiquitylation and stabilization of the protein ([55]). The predominant oncogenic way by which c-Myc deregulation

contributes to the transforming process is through its ability to leads uncontrolled cell proliferation concomitantly with loss of terminal differentiation.

While c-Myc is not required for cell proliferation, evident from the normal proliferation of c-Myc null cells, the pleiotropic transcriptional effect of c-myc suggests that it is a central integrator and accelerator of physiological cell growth, proliferation and cellular metabolism. Ectopic expression of c-Myc most likely contributes to tumorigenesis through temporarily disordered expression of physiological target genes and the abnormal expression of non-physiological target genes that are not regulated by endogenous c-Myc ([28]). Deregulation of c-Myc expression is often associated with aggressive, poorly differentiated tumors, however, given that most human cancers often possess many additional alterations, it is difficult to ascertain at which stage of tumor progression oncogenic Myc is activated.

Alterations in c-myc seem to constitute one of the primary events in the malignant transformation of BL, they could also be a secondary event in tumor progression. Overexpression of c-Myc alone, according to experimental models, may not be enough to induce cellular transformation. Eμ-myc mice develop clonal B-cell malignancies that usually accumulate alterations in the ARF/p53 pathway or BCL2, thereby revoking Myc-induced apoptosis. In BL the unvarying overexpression of c-Myc protein is constantly accompanied by secondary genetic and epigenetic events that enhance c-Myc transforming activities by antagonizing the activity of negative regulatory factors and/or by repressing c-Myc ability to counteract cellular transformation.

Rb pathway deregulation in Burkitt`s lymphoma: assists c-Myc-mediate growth stimulation.

Cell cycle machinery is deregulated at multiple levels in cancer cells. The high incidence of abnormality in the RB pathway underscores how important the maintenance of cell cycle commitment is in the prevention of tumors. Cyclins, CDK inhibitors and pRB are considered putative oncogenes and tumor suppressor genes, and their deregulation often impacts on tumor clinical outcome and therapeutic response. At the restriction point, the cell takes the important decision if grow or not. A wrong choice may lead uncontrolled cell proliferation and subsequent cancer development. c-Myc drives cell cycle progression at least in part by increasing the expression of cyclin D1 and cyclin E, two key components of the regulatory cascade that ensure the cell transition form the G1 to S

phase of the cell cycle. The transition through the restriction point is negatively regulated by the retinoblastoma (Rb) proetein. Rb binds to transcription factors (E2Fs) whose activity is necessary for the expression of S-phase genes, and it assembles a transcriptionally repressing complex that keeps E2F-responsive promoters inactive. Under conditions of mitogenic stimulation, accumulation of D-type cyclins allows for the formation of active CDK4/6-cyclin D complexes, which phosphorylate and inactivate Rb, thus promoting E2F-mediated transcription and subsequent progression through the cell cycle. The pro-proliferative action of CDK-cyclin complexes is counterbalanced by a class of proteins termed CDK inhibitors (CDKIs), which provide an additional lay of control in the ensuring a correctly progression through the cell cycle. Two CDKI gene families have been defined based on their structure and CDK specificities: the INK4 gene family and the Cip/Kip family. Both of them have the ability to block the actions of CDKs at various points in the cell cycle through different regulatory strategies. The INK4 (inhibitors of CDK4) gene family encodes p16INK4a, p15INK4b, p18INK4c, and p19INK4d, all of which bind to CDK4 and CDK6 and inhibit their kinase activities, preventing their association with D-type cyclins. In contrast, the Cip/Kip family members, $p21^{WAF1/Cip1}$, $p27^{Kip1}$and $p57^{Kip2}$ are able to bind both cyclin and CDK subunits and can modulate the activities of cyclin-CDK complexes during the different phases of the cell cycle. Alterations affecting the integrity of this regulatory cascade, termed the Rb pathway (INK4-CDK4,6/cyclin D-Rb-E2F), endow BL cells with a growth advance, by strengthening the c-Myc growth stimulation. This inactivation can be achieved by an increased activity of CDK-cyclin complexes and/or by inactivation of the tumor suppressor genes *INK4a* or *Rb*.

A normal regulation of pRb/p105 in relation to proliferative activity is frequently preserved in different types of lymphomas, in which genetic alterations of the pRb/p105 gene appear to be relatively rare. A positive correlation between p105 expression and proliferative features such as mitotic index and percentage of KI67 has been demonstrated in a large fraction of lymphomas; levels of pRb/p105 are low in low-growth fraction NHLs, whereas they are higher in most high-growth fraction tumors ([56]). Loss of Rb was found to be an adverse prognostic factor in large B-cell lymphomas (LBCL), where an extended overall survival was associated with high Rb proteins level ([57]); whereas in HL the loss of the retinoblastoma protein is associated with an adverse clinical outcome and a failure to achieve complete remission ([58]). In BLs the pRb/p105 expression is often normal and genetic alterations have been found at low frequencies, even if the

inactivation of the Rb pathway is a common feature of this lymphoma. The growth stimulation exerted by oncogenes in high growth fraction lymphomas is often enhanced by the concomitant inactivation of $p16^{INK4}$ tumor suppressor genes. The CDKN2A gene located on chromosome region 9p21 encodes the cyclin-dependent kinase-4 inhibitor, a negative regulator of the cell cycle.

$p16^{INK4}$ binds to and inactivates CDK4/6, preventing them to phosphorylate the Rb protein, thus inhibiting cell cycle progression. In reactive lymphoid tissue (tonsil and lymph node), $p16I^{NK4}$ protein is expressed in all the normal compartments; although it shows a variable concentration in different cellular microenvironments, with a slight increase in the most proliferative one and a decrease in those consist of resting lymphocytes ([59]). This is consistent with the cell cycle dependent expression of $p16^{INK4}$, raised in the S and G2-M phase and explains why alteration in $p16^{INK4}$ are so dramatically different between high-growth and low-grade fraction BCLs. More than 50% of high-growth BCLs exhibit partial or total loss of expression of $p16^{INK4}$protein while in low-grade fraction BCLs $p16^{INK4}$ expression was rarely altered (<5%). The importance of $p16^{INK4}$ alterations in disrupting the cell cycle control in human tumors and thus leading to tumor progression is highlighted by evidence suggesting its role in the transformation of low-growth fraction lymphomas into their aggressive variants. The histological progression of FL to aggressive large cell lymphoma is frequently accompanied by 9p21deletions (absent in the low-grade tumors) that often results in reduction or loss of $p16^{INK4}$ expression, as well as in MCL . In composite tumors, lymphomas consisting of a low-grade and high-grade component, the low-grade fraction component expresses $p16^{INK4}$, but the high-growth fraction component usually exibhit loss of $p16^{INK4}$ protein ([56]).

$p16^{INK4}$ is frequently inactivated by biallelic deletion, although hemizygous deletion associated with mutations or allelic rearrangement has also been described in different types of malignancies. These genetic alterations at the 9p21 region have been found in a high percentage of tumor cell lines (70 to 80 %) whereas in primary human tumors they have been identified with a slightly lower frequency (10 to 70% of case), which differs according to the tumor type ([59]). An additional mechanism for $p16^{INK4}$ gene inactivation arises due to the *de novo* methylation at the 5`-CpG islands in the surrounding regions of its promoter, leading to transcriptional silencing. Both genetic and epigenetic inactivation of $p16^{INK4}$ occurs in high-growth fraction lymphomas, predominantly by 9p21 deletion/LHO or promoter methylation, while point mutations of $p16^{INK4}$ are infrequent. It

has been shown that $p15^{NK4b}$, another CDKI that maps in tandem with $p16^{INK4}$on chromosome 9p21, is either frequently codeleted with $p16^{INK4}$ or hypermethylated at variable frequency, even though epigenetic silencing can occur independently of methylation status of $p16^{INK4}$ promoter region ([56]). As well as for other highly aggressive non-Hodgkin's B-cell lymphoma, $p16^{INK4}$ function results often deregulated in BL. It has been reported ([60]) that both in BL cell lines and biopsies, deletions and mutations in $p16^{INK4}$ and $p15^{NK4b}$ genes occur with a low frequency. However, $p16^{INK4}$ exon 1 methylation arises with a high incidence in BL cell lines as well as in BL biopsies, respectively 89.5% and 42% of the analyzed samples but not in non-neoplastic lymphoid cell lines, suggesting that $p16^{INK4}$ inactivation through promoter methylation could be a common mechanism involved in the development of BL. Since, the methylation status of $p16^{INK4}$ was found regardless of EBV status, the virus presence does not reduce the selection for $p16^{INK4}$gene inactivation during BL progression.

Exon 1 of $p15^{NK4b}$ is also methylated in the BL lines and primary tumors, although at a lower frequency than p16/INK4a. Consistently with the observation that $p15^{NK4b}$ gene methylation does not necessarily lead to complete silencing of the gene, many BL lines with methylated $p15^{NK4b}$ normally express $p15^{NK4b}$ mRNA ([61]). Loss of p16/ INK4a results in functional RB inactivation through CDK4/ 6-mediated phosphorylation and could collaborate with c-Myc medietd-induction of cyclin D1 in BL aberrant growth proliferation.

It is understandable how Rb pathway inactivation, predominantly by $p16^{INK4}$ silencing and more rarely by Rb alteration or CDK4/6-cyclin D hyperactivation, is a key event in BL genesis and progression.

ARF-MDM2-p53 pathway inactivation: protects the BL cells from c-Myc-induced apoptosis.

Given that c-Myc overexpression can induce p53-dependent and independent apoptosis, it could be concluded that secondary lesions that abrogate programmed cell death pathways may have a key role in driving BL transformation. The p53 protein is a transcription factor that induces the expression of genes involved in cell cycle arrest or apoptosis in response to a variety of toxic or oncogenic stimuli; the final cellular response depends on cellular context. p53 protein is short-lived and expressed at very low levels in normal cells but it is stabilized and rapidly accumulates in response to a multiplicity of pro

apoptotic signals. Negative regulation of the cell cycle progression by p53 is partially mediated by the induction of p21, a CKI of the CIP/KIP family that neutralizes the kinase activity of CDK2-cyclin E complex ([62]). Another key target is MDM2, which acts in a feedback loop to limit the action of p53, both by inhibiting its transactivating activity and by catalyzing its destruction ([63]). In response to aberrant growth or oncogenic stress such as c-Myc activation, the p53-MDM2 interaction is disrupted through the activation of the ARF protein ([64]). ARF binds and inactivates MDM2 that is no longer able to induce p53 degradation, allowing the execution of programmed cell death in response to genotoxic stress. P53–dependent apoptosis in response to activated oncogenes represents a putative mechanism for the elimination of incipient tumor cells. The p53 pathway is frequently altered in most human cancers, either by the inactivation of the tumor suppressor genes p53 or $p14^{ARF}$ or by the hyperactivation of MDM2 oncogene. p53 is the most commonly mutated gene in all human tumors (>50% of all cases) and the majority of these mutations tend to cluster in conserved regions that are part of the sequence encoding the DNA binding domain. Mutations of *p53* are detected in 17% to 25% of cases of LBCL and BL ([65]). This frequency is relatively low compared with other types of cancer but is still significantly higher than that observed in most low-growth fraction BCLs, supporting the hypothesis that *p53* inactivation is one of the events leading to high-grade progression ([56]). A majority of BL lines and at least 30% of BL biopsies carry the p53 mutation and like in other tumor types, they cluster in the core domain and include so-called hot spot residues such as Arg-175, Arg-248, and Arg-273. Many of these mutations were shown to functionally affect p53 and no correlation with EBV infection has been found. It has been suggested that acquisition of p53 mutations is a late event occurring during BL progression. Inactivation of normal p53 increases the tumorigenicity of BL cell lines, whether restoration of normal p53 function in p53 mutant BL cell lines reduces tumorigenicity, demonstrating that functional defects of wt p53 protein are associated with an enhanced tumorigenic potential of BL cells ([66], [13]).

MDM2 overexpression may represent an alternative mechanism of p53 inactivation that is not dependent on p53 mutations. High expression of MDM2 has been detected in some BL carrying wt p53, overexpression of MDM2 protein seems to be due to impaired posttranscriptional regulatory mechanisms and it is able to induce an escape from p53-controlled cell growth. The fact that MDM2 may contribute to lymphomagenesis is supported by the high frequency of its overexpression in both HL and NHL and by the correlation between high level of MDM2 and shorter overall survival in LBCL and low-

growth fraction NHL ([56]).

$p14^{ARF}$, the other INK4a-encoded tumor suppressor, is specifically induced by inappropriate hyperproliferative signals and mediates apoptosis in response to oncogenic stimuli. In this way the ARF-induced p53 apoptosis may represent an `oncogene checkpoint that guards the cell against hyperfroliferative stimuli.

Unlike what has been found in other neoplasias, molecular alterations resulting in loss of p14ARF expression are rare in NHLs. Hypermethylation of 14^{ARF} promoter and deletions within the 9p21 region, the two main mechanisms for $p14^{ARF}$ silencing have been detected only exceptionally in BLs. In a screen of 47 BL lines, only 3 had lost p14ARF by homozygous deletion of the INK4a locus. Interestingly, these 3 lines BL carried wt p53. Further analysis of wt p53-carrying BL revealed elevated levels of MDM2 in 3 of 4 BL. Thus, 6 out of 7 BL lines that carried wt p53 had loss of ARF or MDM2 overexpression, indicating that the ARF–p53 pathway is inactivated in a majority of BL and that mutations in the different genes are mutually exclusive ([67]). $p14^{ARF}$ may be additionally inactivated through the up regulation of BIM-1 and Twist transcription factors that negatively regulate its activity on tumors. It has been demonstrated that BIM-1 prevents c-Myc induced expression of p14ARF, thus overexpression of BIM-1 may collaborate with c-Myc through antagonizing apoptosis: the MIB-1 mediated prevention of p14ARF induction by c-Myc forms the basis for the efficient cooperation in oncogenic transformation between oncogenes ([68]).

A study indicated that anomalies in BIM-1 expression are common and may play a role in human B-cell NHL ([69]). An apparently contradictory finding has demonstrated that a $p14^{ARF}$ atypical overexpression defines a group of lymphomas characterized by higher aggresivity. A similar correlation existed at the prognostic level since cases with abnormal p14ARF overexpression were also characterized by a shorter overall survival. An explanation for these findings can be found considering the central role of p14ARF in cell cycle control, as a nexus between the major tumor suppressor pathways. Therefore p14ARF should integrate all these stimuli, its level of expression being a measure of the accumulation of alterations in different points of the cell cycle, and consequently a marker of tumor aggressivity ([70]). The importance of ARF-MDM2-p53 pathway in BL is finally supported by the Eμ-Myc transgenic model, in which mice develop clonal-pre B and B cell lymphomas. The emerging tumors carry p53 mutations (24%), or an overexpression of INK4/ARF deletion (24%), or overexpresses MDM2 in some cases.

Lack of INK4/ARF abrogates B-cell apoptosis in Eμ-Myc transgenic mice, and greatly accelerates lymphomgenesis ([67]).

Inactivation of the Rb and p53 pathways allows c-Myc to efficiently drive the cell growth and alleviate c-Myc induced apoptosis respectively. BL cells often undergo accumulated disruption in both these cascades, bestowing an advantage on the tumor cells and a more and more aggressive behavior.

INK4a/ARF a cancer-safeguarding locus

In a small 35 kb stretch of the human genome the INK4a/ARF locus encodes three distinct tumor suppressor genes that, controlling the two critical cell cycle regulatory pathways, the p53 pathway and the Rb retinoblastoma pathway, safeguards cells against uncontrolled proliferation and represents a powerful protection against cancer. For this reason it is inferable as the INK4a/ARF locus, located on chromosome 9p21 is one of the most frequently altered genomic regions in human cancers. Two distinct lines of research converged on the identification of a tumor suppressor gene located on human chromosome 9p21.

The first report was of the existence of a 16-kDa protein that became associated with cyclin dependent kinases (CDKs) particularly in cells, as SV40 transformed human fibroblast, lacking a functional retinoblastoma protein ([71]).

The interaction with CDK4 was exploited to isolate the corresponding cDNA; using CDK4 as bait in a yeast 2-hybrid screen, Serrano et al. (1993) cloned human p16 (INK4) cDNA and it quickly became clear that the 16-kDa protein, which they designated $p16^{INK4}$, through the binding to CDK4 inhibited the catalytic activity of the CDK4/cyclin D enzymes ([72]; [73]).

A great achievement in the chromosomal location of the gene was reached by genetic linkage studies showing that inherited predisposition to melanoma could be, in some families, traced to a putative tumor suppressor gene on chromosome 9p21([74]). Subsequent positional cloning efforts recognized the INK4a as the main suspect, the melanoma susceptibly gene found in this region, originally called MTS1 (for multiple tumor suppressor-1), has an identical sequence to that of the p16 gene previously described by Serrano et al. Additionally, these works revealed the presence of the closely related INK4b gene; $p16^{INK4a}$ and 15^{INK4b} are within 30 kb of one another on chromosome 9p21 and in the same transcriptional orientation. 15^{INK4b} has its own and physically distinct open reading frame, with the two exon composing the gene upstream to the first exon of p16. $p16^{INK4a}$ and 15^{INK4b} are 85% similar at the amino acid level this homology is reflected upon a analogous biologic function. They both have appropriate biochemical proprieties for tumor suppression by acting as inhibitors of the CDKs that regulate progression through the G1 phase of the cell cycle. Although it is assumed that INK4a and INK4b arose from gene duplication, the simple tandem arrangement is complicated by the presence of an additional exon (called exon 1β) between them ([75]). Stone et al.,

identified a novel transcription with 3'sequence identical to INK4a but with a distinctive 5`end. This transcript, called ARF (for alternative reading frame), is derived from a distinct first exon (exon 1β) that is 13-20 kb centromeric to the first exon of INK4a (exon 1α) and is spliced into the common second and third exons shared with INK4a. However, the protein are encode in an alternative reading frame, as a consequence $p16^{INK4a}$ and $p14^{ARF}$ are not isoforms and have no amino acid homology. Their expression begin controlled by individual promoters and are independently regulate by different signaling patterns. ([76]). (Fig Locus). Importantly, ARF product also has the potential to function as tumor suppressor by modulating the p53 activity as part of a checkpoint response to oncogenic ([64]), hyperproliferative signals. Since, both the proteins encoded by INK4A/ARF locus have anti-cancer activity and frequently tumors harbor homozygous deletions that abrogate the expression of all three proteins, it is difficult to establish which members of the locus represents the principal tumor suppressor activity located at human chromosome 9p21.While the encoding of opening reading frames in overlapping stretches of the genome is common in viruses and bacterial, such genomic structure is practically unique in mammalian genome. The unusual genomic arrangement of the INK4a/ARF locus appears illogical in terms of cancer genetic, as it rends three critical regulators of the Rb and p53 tumor suppressor pathway vulnerable to a single, relatively small deletion ([77]).

The evolutionary issue is why the selection pressure has not selected against a so weakness genomic organization. Probably tumorigenesis provide such a strong pressure that an entire set of tumor suppressor genes has been selected for at the INK4a/ARF locus to prevent cancer. The evolutionary history of the locus demonstrates that initially, a duplication of the single *INK4* gene occurred to place p16INK4a and p15INK4b approximately 30 kbp spaced out in cis on the same chromosome. At some time subsequent to that, ARF was introduced as a single exon gene (exon 1β) between exon 2 of p15INK4b and exon 1□ of p16INK4a, ARF appears to have used exons 2 and 3 of p16INK4a for a 3′ UTR and poly-A signal. The insertion of ARF gene into an existing regulatory framework allows for message stability and translation. ([75],[78])

The evolutionary history is in accord with the possibility that *INK4a*/*ARF* locus structure has been preserved in mammals as it provides additional resistance to tumorigenesis over the expression of $p16^{ARF4a}$ alone. The obvious advantage to grouping genes within a single chromosomal domain is that they can provide a common function and could be

activated in concert under similar conditions. In this prospective the INK4a/ARF locus seems to be no longer a flaw of human genome, but rater a multi-functional tumor suppressor locus efficiently in prevent cancer. Consistently with this evolutionary point of view seems that not a single gene, but collectively all the genes belonging to the locus could be considered the candidate for the tumor suppression activity ascribed to the human chromosome 9p21.

INK4a/ARF locus in human cancer.

Inactivation of the Rb and p53 pathways is observed in almost all human cancers; given its direct commitment in both pathways, $p14^{ARF}$-$p15^{INK4b}$-$p16^{INK4a}$ gene cluster at chromosome 9p21 is a frequent target of inactivation in a variety of tumors.

The aim of recognizing if a lone protein may be considered the tumor suppressor candidate of the locus requires to find a link between a specific tumor and a target definite lesion in one member of the locus in the absence of mutation of the other.

There are extensive evidences supposing the ubiquitously tumor suppression function of $p16^{INK4a}$ in human cancers. This link was established following its localization to the chromosome 9p21, a key cancer hot spot that is the target of deletion in many types of human tumors. $p16^{INK4a}$ Germline point mutations were found to cosegregate with tumor susceptibility in kindreds with familiar melanomas ([79], [80]); in rare case alterations that target $p16^{INK4a}$ biochemical partners have also been found, supporting the relevance of this functional pathway in conferring genetic predisposition to melanoma. The fact that mutually exclusive events in any given tumors result in the loss of either $p16^{INK4a}$, or Rb, or in the overexpression of cyclin D or CDK4, suggests that they constitute equivalent step in a single critical tumor surveillance pathway. Significantly, the ability of $p16^{INK4a}$ to induce cell cycle arrest and in turn to block tumorigenesis is lost in cells lacking functional retinoblastoma protein, demonstrating that RB is the major target for the $p16^{INK4a}$ tumor suppressor activity ([81]). Its role as eligible candidate tumor suppressor gene of the locus is supplied by the high frequency of inactivation in several human tumors. Multiple types of genetic alterations have been reported to affect protein functionality with modalities that vary among different tumor types and some times within the same oncological variant. Somatic inactivation of $p16^{INK4a}$, through point mutation or small deletion, is remarked primarily in pancreatic cancer, esophageal cancer, glioblastoma and acute T-cell lymphoblastic leukemia. More than 56 distinct germline

mutations, that individually target p16INK4 and unconcern ARF and p15INK4b, have been described in unrelated kindreds that are cancer prone ([82]). The *de novo* methylation of the CpG islands spanning the promoter and first exon of the gene is a further inactivation mechanism by which $p16^{INK4a}$ expression may be turned off or significantly reduced in cancer cells. The epigenetic gene silencing is described at high frequencies in a growing number of tumors and it is now a well established mechanism of tumor suppressor gene inactivation ([83]). Methylation of the CpG islands within the p16/INK4a has been detected in approximately 20% of different primary neoplasms, remarkably in breast and colon cancer ([84]), highlighting the unquestionable $p16^{INK4a}$ tumor suppressor function in human cancer. The $p16^{INK4a}$ expression is induced in response to several cancer-relevant stimuli including passage in culture, growth at high density, DNA damage, oncogene activation and advancing age, the protein exerts its tumor suppression activity by contrasting uncontrolled cellular proliferation. Mice lacking $p16^{INK4a}$ are phenotypically normal except prone to tumors and sensitive to carcinogens. These data suggest that, although, $p16^{INK4a}$ has marginal or not role in normal development it provide to limit inappropriate or aberrant cellular proliferation ([85]).

Adjacent to $p16^{INK4a}$ lies the $p15^{INK4b}$ gene encoding a homologue protein, which demonstrates analogous binding to and inhibitory activity on CDK4 and CDK6. Genetic lesions exclusive for $p15^{INK4b}$ and unaffecting $p16^{INK4a}$ or ARF are not well described. Since $p15^{INK4b}$ is frequently deleted along with the other members of the locus, it cannot be considered alone as a specific target for deletion or distinct point mutations. Given their overlapping biochemical function, co-deletion of p15INK4b and p16INK4a, may be more oncogenic in certain tissues than loss of either alone. Instead, remarkable is the methylation-induced $p15^{INK4b}$ silencing observed specifically in cases hematologic neoplasms including leukemia and myelodysplasia ([86]). In the report cases the p15INK4b promoter hypermethylation does not involve the adjacent p16 promoter, rising the possibility of an independent contribute in some human cancer. The $p15^{INK4b}$ value as tumor suppressor gene in human cancer fits well with the information obtained in animal models.

In agreement with data indicating that INK4a mutations are much more common than those affecting INK4b in human cancers, Ink4b-null mice neither show evident developmental anomalies nor spontaneously develop many tumors later in life. But, disruption of INK4b in the mouse does predispose to extramedullary haematopoiesis and lymphoid hyperplasia ([87]), consistent with observations that INK4b expression is silenced

in certain human myeloid and lymphoid tumors ([88]; [89]). $p15^{INK4b}$ probably is an important tumor suppressor in human cancers, particularly in the hematopoietic lineages.

Splicing of alternative first exons (1-alpha vs 1-beta) to a common second exon within the INK4A gene generates $p14^{ARF}$ mRNAs in which exon 2 sequence is translated in different reading frames. The difference in amino acid sequences is in turn a sign of a completely unrelated biological function between $p14^{ARF}$ and $p16^{INK4a}$. $p14^{ARF}$ does not bind CDKs or inhibited the activity of to cyclin-CDK complexes, conversely its exerts its tumor suppression function by regulating the MDM2-mediated degradation of p53. ARF binds to and promotes the rapid degradation of MDM2, allowing the p53-imposed G1 cell cycle arrest that otherwise is abrogated by MDM2. The E1-ß-encoded N-terminal domain of $p14^{AR}$ mediates this interaction ([90]), enforcing the biochemical and genetic evidences that the in vivo function of $p14^{AR}$ is entirely encodes by the exon 1ß. The establishment of a univocal tumor suppressor activity for $p14^{AR}$ in human tumor seems to be complicated since in most cases the loss of the protein occurs in the setting of genetic deletion that is concomitant with loss of $p15^{INK4b}$ and/or $p16^{INK4a}$. In addition, inactivating missense mutations in the unique exon1 ß of $p14^{AR}$ were found rarely, whereas more common are those in the shared exon 2 that may simultaneously disrupt both $p14^{AR}$ and $p16^{INK4a}$, making difficult to attribute a individual tumor suppressor activity for each protein. Unlike the neighbor $P16^{INK4a}$ promoter, epigenetically silencing of $p14^{AR}$ was observed just occasionally, being the two genes under control of different regulatory sequences. Finally, the interaction between ARF and MDM2 appears biochemically stable and requires only a few (~25 N-terminal) amino acids, and therefore inactivating missense mutations may be far more unlikely than those in $p16^{INK4a}$. Although infrequent, selective inactivation of $p14^{ARF}$ with intact $p15^{INK4b}$ and $p16^{INK4a}$ function occurs in some human tumors. In kindreds with familiar melanoma and astrocitoma exon 1-ß mutations that do not alter the $p16^{INK4a}$ function have been found and in colon cancer $p14^{ARF}$ is exclusively silenced by promoter methylation without affecting p16 levels and activity. These collective observations in human cancer, as well as the potent anti-transforming activities of ARF in vitro and the compelling murine genetic data indicate that $p14^{ARF}$ has a significant tumor suppressor activity ([77]). This makes good sense, as a matter of fact p53 is directly targeted in >50% of tumors, then p-53 positive malignancies have likely sustained epistatic mutations such as MDM2 amplification or ARF loss.

Therefore, the answer to the question of which protein is being targeted by inactivation on 9p21 appears to be $p16^{INK4a}$ in some tumors, $p14^{ARF}$ in others and both in the majority,

leading to the possibility of a non-redundant, cooperative function of INK4/ARF proteins in tumor transformation. Numerous mouse models have underscored the collaboration of INK4a-ARF locus with other tumor suppressor genes and oncogenes in tumorigenesis and have aided to establish the individual role of the INK4-ARF locus genes in cancer formation and progression. Knockout studies of mice specifically deficient for ARF (or $p19^{ARF}$ the corresponding mouse gene), $p15^{INK4b}$ or $p16^{INK4a}$ revealed that all three strains are more prone to spontaneous cancer than wild-type littermates, but each of these single knockouts appears significantly less prone than animals lacking both the genes (INK4/ARF-/-)([91]). As a matter of fact, it has been shown that the median tumor latency of $p16^{INK4a}$-null mice was 76 weeks, $p19^{ARF}$-null 62 weeks and Ink4a/Arf-/- null 38 weeks. The cancer-prone conditions of mice singly deficient for either $p16^{INK4a}$ or $p19^{ARF}$ agree with data derived from human cancer genetics, and reinforce the view that both gene products play significant and non-redundant roles in suppressing malignant transformation *in vivo* ([91]). Overexpression of the Ink4a/Arf locus in mice also supports its role in tumor suppression. Indeed, the "super Ink4a/Arf" mouse, a mouse model with increased activity for the Ink4a and Arf tumor suppressors, demonstrates a 3-fold reduction in the incidence of spontaneous cancers, indicating that modest increases in the activity of the Ink4a/Arf tumor suppressor result in a beneficial cancer-resistant phenotype without affecting normal viability or aging ([92]). Similar cooperation was seen in murine melanoma study, in which alleles of only $p16^{INK4a}$ or $p19^{ARF}$ were targeted to validate the individual role of the proteins.

Animals specifically deficient of $p16^{INK4a}$ ($p16^{INK4a}$ -/-) demonstrate low frequencies of melanoma spontaneously or after the exposure to carcinogens, such as DMBA, which is significantly increased in the setting of $p19^{ARF}$ hapolinsufficiency. On the other hand, mice with exclusive targeting of $p19^{ARF}$ do develop melanoma neither spontaneously nor after DMBA exposure. Additionally, Ink4a/Arf-null and Arf-null strains show little evidence of melanoma development unless crossed with animals that express a Ras transgene under the control of a tyrosinase promoter. Since Rb lesions are commonly found in melanomas from $p19^{ARF}$-/- mice, while p53 pathway lesions can be detected in $p16^{INK4a}$ -/- tumors, it is inferable that both tumor suppressors of INK4a/ARF locus play prominent role in melanoma formation ([93]).

The tumor spectrum of the single knockdown strains shows however as the inactivation of a distinct gene may be specific for a given tumor phenotype. Generally, $p19^{ARF}$-null animals develop a tumor spectrum more similar to p53-null than $p16^{INK4a}$-null or INK4a-

ARF-null mice. The most common tumor in *p19ARF* -null mice is a lymphocytic lymphoma of the thymus and/or lymph nodes. Animals lacking p19ARF also developed an increased incidence of soft tissue sarcomas, and carcinomas, particularly of the lung. In contrast, mice lacking p16INK4a spontaneously develop sarcoma, melanoma and a widely disseminated histiocytic tumor involving the spleen, peripancreatic and other lymph nodes or other sites ('histiocytic lymphoma'). This latter tumor is by far the most common histology seen in *Ink4a*/*Arf*-null animals, but does not occur in *p19ARF* −/− mice. Therefore, this tumor in particular seems to be most enhanced by the combined inactivation of p16INK4a and p19ARF, although there appears to be cooperation between p16INK4a and p19ARF loss for most tumor histologist (Review Sharples [77]). Treatment with DMBA produces similar tumor spectra, predominantly lymphomas, sarcomas and lung tumor, in wild type, $p16^{INK4a}$ -/- and $p19^{ARF}$ −/−, although the latency is considerably shortened by $p16^{INK4}$ or $p19^{ARF}$ inactivation, consistently with the observation that both gene inhibit DBMA-induced tumor, although by different mechanism of action at different stages of tumorigenesis ([91]).

INK4a/ARF deletions can impact tumor development and anticancer therapy by disabling p53-induced apoptosis. The Eu-Myc transgenic mouse has been a useful model to study not only the biology of Burkitt lymphoma but also how the INK4/ARF locus may collaborate whit c-Myc oncogene to drive tumor progression and evolution along a worst phenotype. These mice constitutively express c-Myc in the B-cell lineage and develop B-cell lymphoma ([94]). Early in the course of the disease, an increased number of B cells in lymph nodes are stimulated to enter the cell cycle but their expansion is counterbalance by c-Myc induced apoptosis. However, when the apoptotic response fails, lymphoma cell emerge and about half of them bring mutation in either p53 or ARF. Moreover, when the Eu-Myc transgene is expressed in an INK4a/$ARF^{-/-}$ or ARF heterozygous background, the tumors arise much more rapidly and exhibit loss of the second ARF allele ([95]). Like $p53^{-/-}$ tumors, INK4a/$ARF^{-/-}$ lymphomas form rapidly, are highly invasive and displayed apoptotic defects, furthermore they display an attenuated p53 activity despite the presence of a wild-type p53 gene. The profound impact of INK4a / ARF and p53 mutations on Myc-induced lymphomagenesis indicates that the ARF–p53 pathway contributes to oncogene-induced cell death in developing tumors and underscores the importance of this fail-safe mechanism in tumor suppression ([46]). In complete INK4a/ARF-null or pure ARF-null background, Eu-Myc transgenic mice die of aggressive disseminated lympholeukemias by only seven weeks of age. By contrast, Eu-Myc lymphomagenesis is

not accelerated by loss of Ink4a or Rb alone. Therefore, ARF or p53 loss (with or without maintenance of $p16^{Ink4a}$ expression) accelerates lymphoma onset.

Tumors with p53 mutations responded poorly to cyclophosphamide therapy in vivo. Surprisingly, transplanted Eu-Myc lymphomas lacking ARF alone responded to cyclophosphamide therapy and were cured, whereas Ink4a/Arf-null tumors were resistant. This implies that, whereas Arf loss accelerates disease onset in Eu-Myc transgenic mice, Ink4a loss instead results in a poor response to drug treatment ([95]) and find that a senescence program controlled by p53 and p16INK4a is an important determinant of treatment outcome in vivo.

Schmitt et al. found that murine lymphomas responded to chemotherapy by engaging a senescence program controlled by p53 and p16(Ink4a). Hence, tumors with p53 or Ink4a/Arf mutations, but not those lacking Arf alone, responded poorly to cyclophosphamide therapy in vivo. Block of p-53-dependent and –independent apoptotic pathway by Bcl2 uncovers senescence as a drug-induced response program. Senescence is disrupted in the context of p53 or INK4a/ARF loss, whereas ARF deficiency alone is not sufficient to disable senescence. Mice bearing tumors capable of drug-induced senescence had a much better prognosis following chemotherapy than those harboring tumors with senescence defects. Therefore, cellular senescence contributes to treatment outcome in vivo. The way in which the INK4a/ARF locus is mutated during tumor development produces heterogeneity in treatment responses. ARF mutations promote lymphomagenesis, whereas INK4a mutations (alone or in combination with ARF) have no additional impact. However, mice harboring tumors that have disrupted both genes have a worse prognosis than those with tumors lacking ARF alone, responding poorly to cancer treatments. Given theyr physical proximity, almost 25% of Eu-Myc lymphomas, acquired mutation that disable both genes; this closeness also allows mutations targeting one gene (ARF) during tumor development to facilitate loss of the other gene (INK4a) under therapy ([96]).

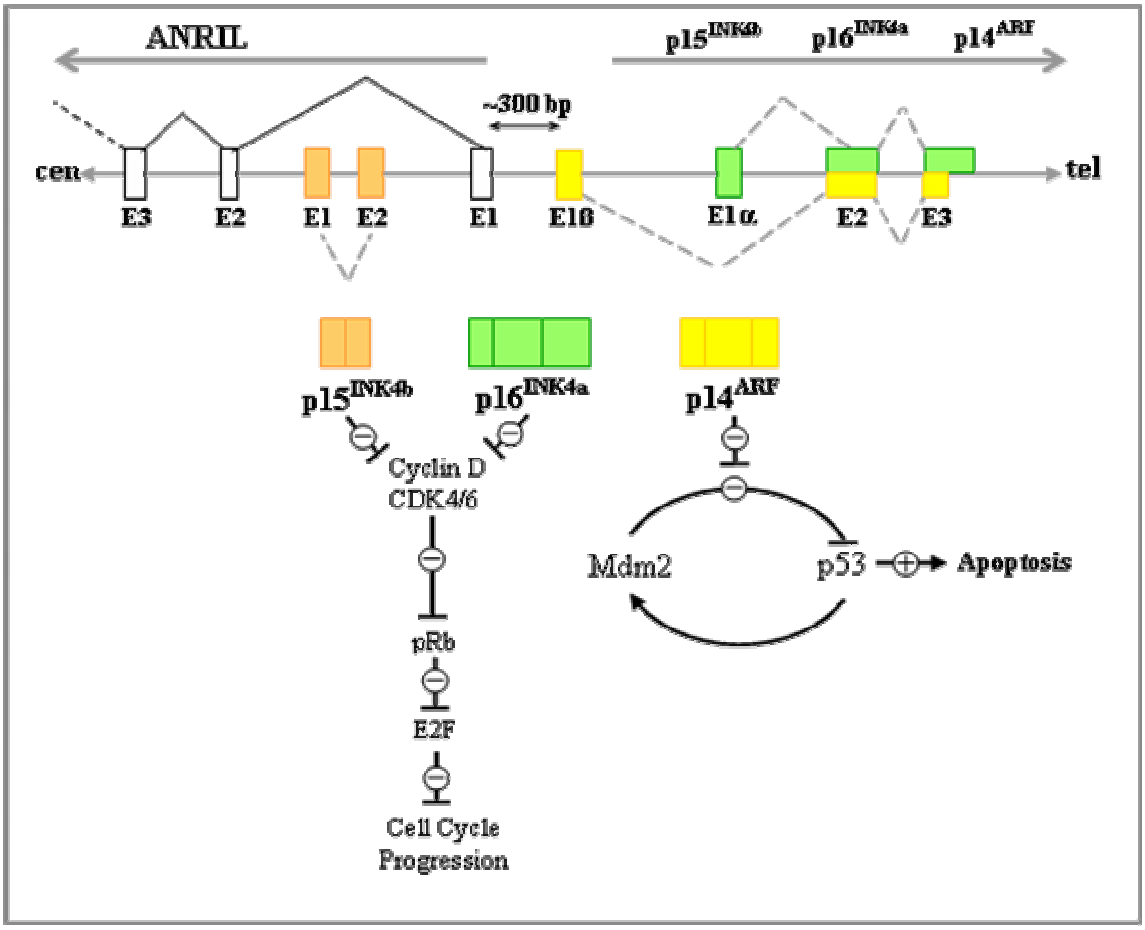


Fig1. Genomic organization of INK4/ARF Locus

The INK4a/ARF/INK4b locus encodes three genes within 35 kilobases: ARF, $p15^{INK4b}$, and $p16^{INK4a}$.
Alternative first exons (1α and 1β) that are transcribed from different promoters specify the 5' ends of the p^{Ink4a} and $p14^{Arf}$ transcripts, respectively. E1α (Exons 1α), E2, and E3 encode $p16^{INK4a}$ protein, whereas E1β, spliced to E2 and E3 of $p16^{INK4a}$ in a different reading frame encodes $p14^{ARF}$ protein. 15^{INK4b} has distinct open reading frame, with the two exon (E1 and E2) composing the gene upstream to the first exon of $p16^{INK4a}$. Members of the INK4 family of cyclin-dependent kinase inhibitors bind to and inactivate CDK4/6, thus inhibiting cell cycle progression. ARF inhibits MDM2, resulting in p53 stabilization, and apoptosis. The ANRIL gene overlaps the two exons of $p15^{INK4b}$ and is transcribed in the orientation opposite to the INK4a/ARF/INK4b gene cluster. E1 (Exon 1) of ANRIL is located about 300 bp upstream of the transcription start site of $p14^{ARF}$ (exon 1β).

Material and Methods

Burkitt`s lymphoma cell culture

The human Burkitt B-cell lines Ramos (EBV-), Ga-10 (chemo refractory EBV-), DG-74 (metastatic EBV-) and Daudi (EBV+) were purchased from American Type Culture Collection (ATCC) and healthy B-lymphocyte purchased from Corriel Institute for Medical Research. Both the cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% L-glutamine. Gal-01 and Gal-02 (gently provided by Laurence de Leval laboratory) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1 mM sodium pyruvate, 1%minimum essential medium non-essential amino acids and 0.5 uM 2-Mercaptoethanol. All cell cultures were were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. The final cells concentration was adjusted to 5 · 106 cells/mL in 25 cm2 flasks.

MSP-PCR

Genomic DNA from cell lines was purified using DNeasy Tissue kit (QIAGEN) according to the manufacturer's instruction.

The methylation-specific polymerase chain reaction (MSP) for gene promoter methylation was performed as described in detail previously ([97]). Briefly, bisulfite modification of DNA (1ug) was performed for conversion of unmethylated, but not methylated, cytosine to uracil, with a commercially available kit (CpGenome^{TM} DNA modification kit, CHEMICON international) according to the manufacturer's instructions. PCR of modified DNA from was performed using specific set of primers for the methylated and unmethylated forms of each gene. The primers for the methylated (M-MSP) and unmethylated (U-MSP) gene promoter regions for *p16, p14 and p15* and the annealing temperature applied are shown in Table 1. The PCR mixture contained 50 ng of bisulfite treated DNA, 0.2 mM dNTPs, 2 mM MgCl2, 10 pmol of each primer, 1 X PCR Buffer II and 2.5 units of AmpliTaq Gold (Applied Biosystem) in a final volume of 50 µl. Thermal cycling was initiated at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at the specific temperature for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 10 min. A methylated DNA (CpGenome Universal Methylated DNA, CHEMICON international) was used as positive control in all the

experiments.

The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

Reverse Transcriptase-PCR and Real time

Total RNA from Burkitt`s lymphoma cell lines was extracted using RNeasy Mini kit (QIAGEN), following the manufacturer's procedure. 2 ug of total RNA in a 20ul reaction was reverse transcribed with MultiScribe Reverse Transcriptase (Appled Biosystem), using RT Buffer, dNTP (100mM), random primer and RNase Inhibitor provided by the supplier. A RT reaction without added Reverse Transcriptase (RT-) was used as control for DNA contamination of the RNA. 2 ul of the RT+ and RT- was used for the PCR. cDNA was amplified using specific intragenic primers for p16, p15 and p14 and for β-actin gene (described in Tab.1) used as internal control. The PCR conditions were: 200uM of each dNTPs, 0,5uM of each primer, 1,5mM Mg^{++} and 1,5U of Taq Polimerase (Promega) in 50ul. Reactions were performed in a Perkin Elmer-Gene Amp 2400 thermal cycler (Perkin Elmer Ltd, UK), at 95^0C for 5min, then 35 cycles at 95^0C for 30 sec, 60^0C for 30 sec, and 72^0C for 30 sec, followed by an extension step at 72^0C for 10min. The PCR products were separated on a 2% agarose gel and visualized with ethidium bromide stain.

All cell lines were subject to semi-quantitative Real-Time RT-PCR in order to accurately quantify the expression levels of p16, p14 and p15. In addition we analyzed the expression level of a new large antisense noncoding RNA (named ANRIL) with a first exon located in the promoter of the p14/ARF gene and overlapping the two exons of p15/CDKN2B. Equal amount of cDNA were subjected to real time PCR using 7300 Real Time PCR System (Applied Biosystem) and the SYBR Green (Roche), following the manufacturer's instructions. Forty cycles of PCR were performed using an annealing temperature of 60^0 C for each analyzed genes, mRNA levels for each gene were established using the ΔΔCt comparative method utilizing β-actin as the normalization control gene. Each sample was analyzed in triplicate and as positive control we used Hela cell line which normally expressed all the INK4/ARF locus gene.

Western Blotting Analysis

Actively growing cells were harvested from culture, washed in PBS and then lysed in lysis buffer (50 mM

Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0,1% triton-X-100, 0,1mM Na3Vo4) containing Protease Inhibitor Cocktail (Roche, Complete, Mini, EDTA-Free). The lysates were kept on ice for 30 min, vortexed twice, and centrifuged at 4°C for 20 min at 14,000 rpm in an Eppendorf microcentrifuge; and clear supernatant recovered. Protein concentration was determined by the Bradford assay. Equal amount of cell extracts (50 µg) were electrophoresed through 10%-12% (depending on protein analyzed) acrylamide SDS-denaturing gel, electroblotted onto nitrocellulose membrane and probed with the appropriate primary and secondary antibodies. Blocking and incubation with primary and secondary antibodies were performed respectively in 5% and 3% nonfat dry milk and 0.1% Tween20 in TBS.

The membranes were probed using antibody against p16INK4a diluted 1:200 (Santa Cruz, C-20), p14 ARF (Santa Cruz, DCS-241)diluted 1:200, p15 (Santa Cruz, 15PO6)diluted 1:500, p53 (Santa Cruz, FL393) diluted 1:500, p27 (Santa Cruz, C-19) diluted 1:200, p21 (BD, Pharmigen), c-Myc (Santa Cruz, C-33) diluted 1:500 and ß-tubulin 1:10000 (Santa Cruz) as equal loading control. Immunodetection using the ECL system was carried out according to manufacturer's instructions (Amersham).

Cell cultures and treatments

Exponentially growing cells, harvested 24 h after feeding, were seeded 1 x 10^5cells/ml in medium containing 5-Aza-Cdr (Sigma Aldrich) at final concentration of 1 uM, 2,5 uM and 5 uM respectivelly and cultured for 96h. For each time point (24h, 48h, 72h and 96h) treated and untreated cells were collected for DNA, RNA and protein assays and cell viability was estimated by Trypan Blu quantitative colorimetric assay. MG132, purchased from Sigma-Aldrich was initially dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mM. The aliquoted MG132 was then stored at 20^0C. At the time of the experiment, MG132 was thawed and diluted with tissue culture media to a final concentration of 10 mM.

Cells were incubated with 10 uM MG-132 alone or in combination with 5-Aza-Cdr. Both healthy B-lymphocyte and Burkitt`s lymphoma cell lines were incubated in the presence of 2,5 uM of 5-AZA for 72h and treated with MG132 (10 uM) and harvested at 4h, 8h

and 10h after the addition of MG132. The timing and drug concentration have been chosen based on our preliminary data demonstrating that these conditions are those better tolerated in terms of cell viability and drugs efficiency. At these concentration the 5-azaC has a cytostatic effect, while at higher (5 uM) the drug results toxic for the cell lines analyzed in our analyses (Fig.4), and no additional effect is obtained with both higher drug concentration and time exposure (96h). The final concentration and exposure time of MG-132 used throughout this study was based on Han at al. study ([98]). MG132, purchased from Sigma-Aldrich was initially dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mM. The aliquoted MG132 was then stored at 20^0C. At the time of the experiment, MG132 was thawed and diluted with tissue culture media to a final concentration of 10 mM.

Coding sequence direct sequencing

By using the total RNA as a template we performed a RT-PCR using specific primers designed to amplified the p16, p14 and p15 full length coding sequence respectively (tab 1: CDS primes). The PCR reaction was carried out using The Fast Start Hight Fidelity PCR system (Roche), an high fidelity Taq with the additional ability to amplify fragments up to 5kb and GC-rich template fragments, features common to the cDNA sequences in examination. PCR was conducted foe each samples using the following setting: 95^0C 5 min, 35 cycles (30s at 95^0C, 30s 56^0C, 30s 72^0C) and 72^0C 4min. The PCR conditions were in a final reaction volume of 50ul: 100pgcDNA, 200uM of each dNTPs, 0,4uM of each primer, 1,8mM Mg^{++}, 2,5U of Taq FastStart High Fidelity Enzyme (roche) and 5ul GC-rich solution. The provided GC-RICH solution, a PCR additive that facilitates amplification of difficult templates by modifying the melting behavior, will improve PCR performance on templates rich in secondary structures or GC content. PCR products were electrophoresed on a 1% low melting point agarose gel and visualized using ethidium bromide. The desired bands were excised and gel purified using Qiagen purification columns (Qiagen). The purified PCr products were sequenced using Big Dye Terminator v.3.1 Cycle Sequencing Kit from ABI for sequencing reactions. Products are analyzed on the 3730 DNA Analyzer from Applied Biosystems.

Tab.1

Primers used for DNA sequence, mutation analysis, MSP and RT-PCR

	Primer sequence (5'→3')		
Analysis	Sense	Antisense	Annealing temp. (°C)
RT-PCR and Real Time			
$P16^{INK4a}$	GCT GCC CAA CGC ACC GAA TA	ACC ACC AGC GTG TCC AGG AA	60
$P15^{INK4b}$	CTA GCG GAG AAG GTG CGA CA	CGC TGC CCA TCA TCA TGA C	60
$P14^{ARF}$	CCC TCG TGC TGA TGC TAC TGA	ACC ACC AGC GTG TCC AGG AA	60
ANRIL	TGA CGC GAC ATC TGG ACA CGG	AGG AGC TGA GGA ATC ATC ACA GC	60
β-actin	ACC ATG GAT GAT GAT ATC GC	ACA TGG CTG GGG TGT TGA AG	60
Mutational analysis			
p16 CDS	ACC GGA GGA AGA AAG AGG AG	TGT AGG ACC TTC GGT GACT G	56
p15 CDS	TTA AGT TTA CGG CCA ACG GT	AAA TAA AGT CGT TGT GGG CG	56
p14 CDS	CTC AGG GAA GGC GGG T	CGC TCA GGG AAG GCG	56
MSP-PCR			
M-p16	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA	60
U-p16	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A	60
M-p15	GCG TTC GTA TTT TGC GGT T	CGT ACA ATA ACC GAA CGA CCG A	60
U-p15	TGT GAT GTG TTT GTA TTT TGT GGT T	CCA TAC AAT AAC CAA ACA ACC AA	60
M-p14	GTG TTA AAG GGC GGC GTA GC	AAA ACC CTC ACT CGC GAC GA	62
U-p14	TTT TTG GTG TTA AAG GGT GGT GTA GT	CAC AAA AAC CCT CAC TCA CAA CAA	62

Results

Characterization of INK4/ARF locus in a novel human EBV-negative Burkitt lymphoma cell line, the GAL-01.

In 2003 Thielen C. et. al.([99]) have established and characterized two novel Burkitt lymphoma cell lines, designated GAL-01 and GAL-02, from the ascitic fluid of one patient with sporadic EBV-negative BL presenting as a primary lymphomatous effusion. The two cell lines were derived from samples obtained at diagnosis and shortly after one cycle of chemotherapy, respectively. Both cell lines were composed of medium-sized lymphoid cells with clumped chromatin, multiple medium-sized nucleoli and moderate amounts of vacuolated cytoplasm. GAL cells display the phenotype and genotype of a B-cell lineage (positive for CD20, CD79a and clonal rearrangement of Ig heavy chain), carry the c-MYC rearrangement by t(8;22)(q24;q11) translocation and are characterized by the expression of the germinal centre-associated antigens CD10, BCL6, CD38 and absent to low BCL2 expression. EBV and HHV8 were not identified within parental tumor or in cultured cells. Subcutaneous injection of both cell lines to NOD/ SCID mice induced tumor formation.

The in vivo effusion occurred in a very peculiar clinical setting; the patient had a previous history of intestinal diffuse large B- cell lymphoma. However morphologic, immunophenotypic and cytogenetic features demonstrated that Burkitt effusion did not represent disease progression of the intestinal tumor, but represented a second primary hematological malignancy. In addition, although secondary lymphomatous effusions are a common finding in patients with BL, presentation of BL as a primary effusion lymphoma is exceptional. Moreover, most cases of primary effusion lymphoma are classified as a clinical-pathologic subtype of DLBCL, tend to occur in HIV-infected or immunocompromised individuals and are co-infected with the Kaposi's sarcoma associated herpesvirus and the EB. Rare cases of primary effusion lymphomas manifest as BLs and usually are HHV8-negative and EBV-positive.

In the case reported by Thielen et. al. the parental ascites as well as the derived cell lines were negative for both HHV8 and EBV. Gal-01 and Gal-02 are the first cell lines derived from and HIV, HHV8 and EBV-negative primary effusion BL. These cell lines represent a suitable model to study the biology of BL possibly including some aspects of primary lymphomatous effusion and resistance to chemotherapy.

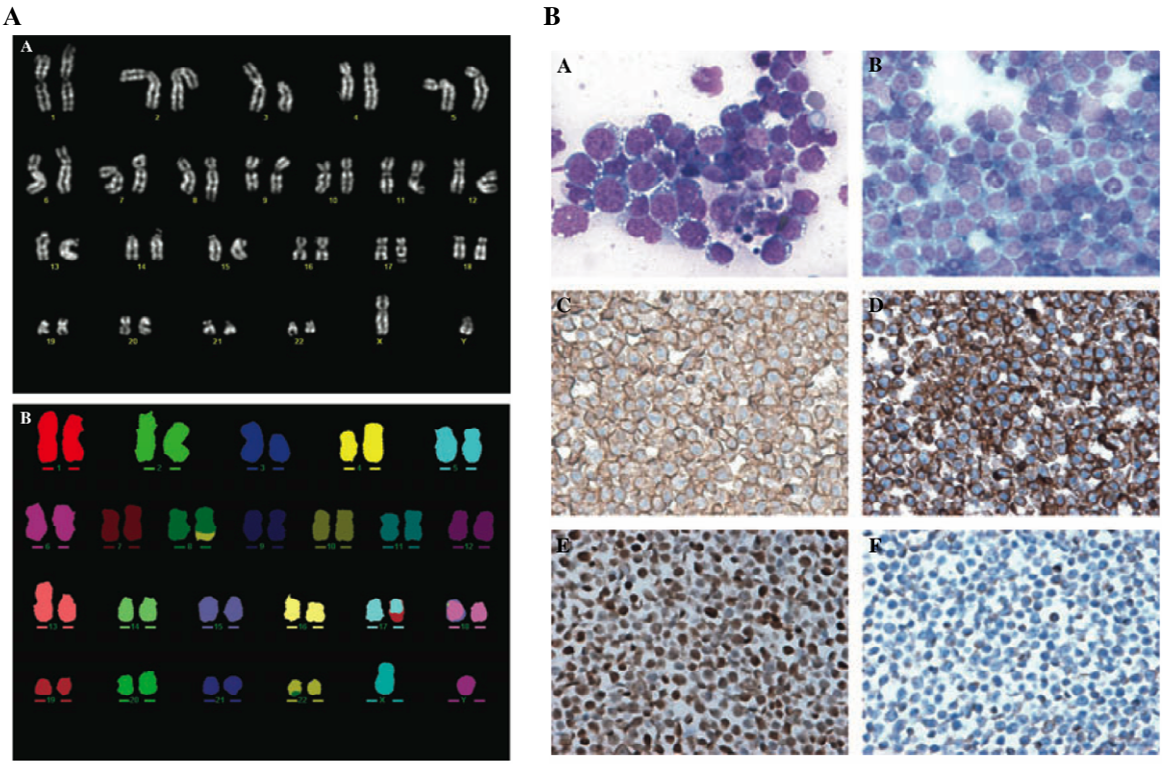


Fig.1 (A) A representative Q-banded karyotype of the GAL-02 cell line. Note the presence of the t(8;22)(q24;q11). (B) M-FISH karyotype from GAL-02. (B) Cytomorphology of primary lymhomatous effusion Burkitt's type (A) and derived GAL-02 (B) cell line (May- Grunwald-Giemsa coloration, original magnification ·400). Immunohistochemical staining show CD20 (C), CD10 (D), BCL-6 (E), BCL-2 (F) immunoreactivities in GAL-01 cells. (Thielen C. et al. Eur J Haematol 2006: 77: 318–326)

Inactivation of INK4/ARF locus in GAL-01 and GAL-02 Burkitt`s lymphoma cell lines.

In view of the fact that INK4/ARF locus is frequently inactivated in human malignancies and that INK4/ARF loss in Eu-Myc transgene abrogate B-cell apoptosis and significantly accelerate lymphomagenesis ([46]) we analyzed the locus expression pattern in Gal-01 and Gal-02 cell line to evaluate the contribute provided from each single gene in this particular Burkitt lymphoma setting.

$P16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ mRNA and protein expression level were investigated in both GAL-01 and GAL-02 cell lines.

Western blotting analysis (Fig.2A) revealed no detectable signal for 16^{INK4a} protein in both the cell lines in contrast with the high levels observed in the Hela cell lines, used as a positive control as well as in the healthy B-lymphocytes. The fact that normal B-lymphocytes express high level of 16^{INK4a} license us to infer that 16^{INK4a} inactivation is a critical trait of the cell lines analyzed and that its loss may drive B-cell lymphocytes through the malignant transformation. The same result was obtained for $p15^{INK4b}$;

although its expression is moderately high in normal B-lymphocytes the protein is completely loss in the GAL cell lines, confirming that the simultaneous inactivation of INK4a/b has a critical role in Burkitt lymphoma progression, strengthening the c-Myc-mediated growth stimulation. As expected quite low levels was detectable for $p14^{Arf}$ in healthy B-lymphocytes, since it is not usually activated in normal tissues but it is induced by sustained and elevated mitogenic signal ([64]), while the loss of the protein found in both the Gal cell lines may be considered as an additional tumorigenic future acquired by the BL cells from escape c-Myc-induced apoptosis (Fig.2A). In order to investigate the possible mechanism liable to INK4/ARF proteins absence, we analyzed $p16^{INK4a}$,$p15^{INK4b}$ and $p14^{Arf}$ mRNA level in the GAL-01 and GAL-02 cell lines, comparing their gene expression pattern with both those of the Hela cell line (a positive control) and the normal B-lymphocytes. For each examined gene we analyzed the mRNA status by RT-PCR (Fig.2 B) and semi-quantitative Real-Time RT-PCR (Fig. 2C) in order to have a more accurate quantification of the transcript abundance. Consistently with protein lack, no $p16^{INK4a}$ mRNA level was detected in both GAL cell lines, suggesting that $p16^{INK4a}$ inactivation in BL is due to transcriptional silencing. Unexpectedly the situation is not so linear for the other two locus genes, in which there is not correlation between gene and protein expression. In GAL-01 cell line p15 mRNA level is comparable to the level detected in normal B-lymphocytes that nevertheless express the protein. It is possible that posttranscriptional mechanisms may affect protein stability and be responsible for $p15^{INK4b}$ loss. Instead, in the GAL-02 both protein and mRNA are undetectable, leading us to consider the $p15^{INK4b}$ gene silencing as a secondary event that cells acquire progressing through a drug resistant phenotype. Healthy B-lymphocyte showed barely, almost undetectable, $p14^{Arf}$ mRNA as expected in a physiological background, while in BL cell lines the gene expression is induced by C-myc mitogenic signal. In both GAL-01 and GAL-02 cell lines $p14^{Arf}$ was upregulated as regards to the normal, but gene induction did not correspond with protein expression, supporting that $p14^{Arf}$ is downregulated through a posttranscriptional regulatory mechanism.

Taken together our results showed that in GAL cell lines the genes belonging to the INK4/ARF locus are simultaneously deregulated, although at different regulatory level.

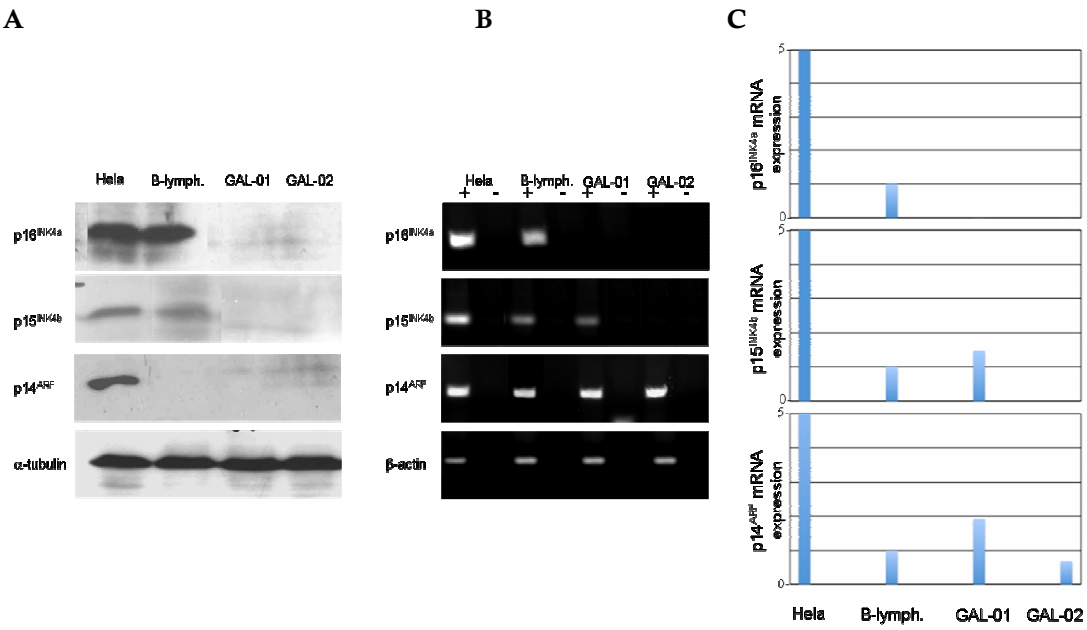


Fig.2 Molecular characterization of GAL-01 and GAL-02 cell lines for INK4/ARF locus genes expression. Evaluation of $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ mRNA and protein expression levels in GAL-01 and GAL-02 cell lines. (A) Western blot analysis using respective antibodies for $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ were performed on whole-cell lysate from GAL cell lines and Normal B-lymphocytes. Hela cell line was used as strong positive control for any reaction. Blots were stripped and re-probed for α-tubulin as a loading control. Expression of $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ mRNA levels were determined by Reverse Transcription-PCR (B) and Real Time PCR (C). The total RNA from GAL-01, GAL-02 and Normal B-lymphocytes was reverse transcribed with (+) and without (−) reverse transcriptase, and amplified with intragenic primer specific for $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$. RNA extracted from Hela cell line was used as positive control for the reaction. Amplification of β-actin was used to demonstrate RNA integrity (B). $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ expression levels were independently evaluated by real-time quantitative RT-PCR for any cell line (indicated on the X asses). The plot diagrams show the mRNA expression levels, calculated according to the ΔΔCt method (as described in Materials and methods) (**C**).

DNA methylation status of $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ gene promoter in GAL cell lines.

De novo methylation of CpG islands within gene promoter region is a well-established mechanism of tumor suppressor gene silencing in human tumors ([83]). We performed MSP-PCR to determine whether the complete or partially lack of $p16^{INK4a}$ and $p15^{INK4b}$ respectively in GAL cell lines was due to methylation of the CpG island in their promoter regions**.** Although gene transcription levels were found to be regular in the GAL cell lines, we as well analyzed the methylation status of $p14^{Arf}$ to reinforce the previously made observation and to completely exclude any possible correlation between $p14^{Arf}$ gene expression-promoter methylation and protein loss.

The *de novo* methylation of the CpG islands spanning the promoter and first exon of the gene is a common inactivation mechanism by which $p16^{INK4a}$ expression may be turned off or significantly reduced in cancer cells. It has been previously reported that $p16^{INK4a}$ is frequently methylated in BL cell lines ([60]) and that $p16^{INK4a}$ promoter methylation status correlates with gene silencing. To establish if also in GAL cell lines $p16^{INK4a}$ expression is

suppressed by promoter methylation we performed an MSP-PCR using two sets of primers that distinctively recognize the methyled (M) and unmethyled (U) CpG sequences. As internal control for both the successful bisulfite modification of DNA and proper primers conditions we used a methylated control DNA that was chemically modified in order to be completely methylated within all CpG islands. As show in Fig.3 with exception of the normal B-lymphocytes in which only the unmethyled U primers generated a PCR detectable product, both the GAL cell lines displayed methylation of $p16^{INK4a}$ promoter. As well as for the methylation control, no bands were detected using the U set of primers, while we obtained genomic amplification product exclusively with M set of primers. The methylation status of promoter regions fits with mRNA expression pattern found earlier. As a matter of fact only the healthy B-lymphocytes, in which the p16 expression is activated, had an unmethyled promoter, in contrast to GAL cell lines where the lack of mRNA happened concurrently with promoter methylation. This data confirmed that in GAL cell lines the loss of $p16^{INK4a}$ is due to improper methylation of its promoter region and, together with published knowledge, that in BL $p16^{INK4a}$ is commonly inactivated as a result of epigenetic silencing. We also performed MSP-PCR to analyze the $p15^{INK4b}$ methylation status in Gal-01 and GL-02 cell lines, using specific set of primers specifically designed to discriminate between unmethyled and methyled CpG islands within $p15^{INK4b}$ promoter region. The GAL-01 were found to be heterozygous for $p15^{INK4b}$ promoter methylation, indeed both M-and U-primers-generated bands have been identified as amplification products. The hemimethylated condition is not sufficient to lead a complete silencing of the gene as demonstrated by $p15^{INK4b}$ mRNA expression in GAL-01 cell line. Probably, the unmethyled allele may account for the $p15^{INK4b}$ mRNA presence previously detected. An overall methylation of $p15^{INK4b}$ gene promoter was found in the drug resistant GAL-02 clone, in which only the M primers generated a detectable PCR product. The $p15^{INK4b}$ mRNA loss is associated with the completely methylation within the gene promoter, that may be acquired during the phenotypic evolution through a drug resistant tumor. $p14^{Arf}$ gene promoter was found to be unmethyled in all the cell lines analyzed regardless of their phenotype, this condition correlate with the normal mRNA level previously found for the GAL cell lines and confirm that in physiological condition the $p14^{Arf}$ expression is not normally submitted to epigenetic control.

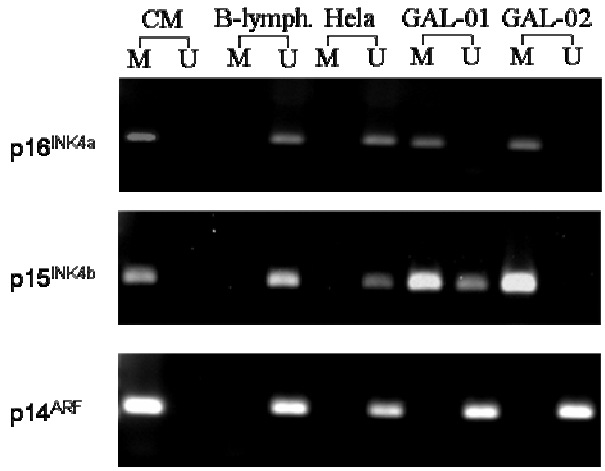


Fig.3 MSP analysis of the INKA/ARF locus in GAL-01 and GAL-01 cell lines.
Bisulfite-modified DNA derived from GAL-01, GAL-02, Normal B-lymphocytes and Hela cell line, which normally express all INK4/ARF locus gene, was used for Methylation-Specific Polymerase Chain Reaction (MSP-PCR). MSP-PCR for $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ was performed with Unmethylation-specific (U) and methylation–specific (M) primer set. Products of MSP were resolved on a 2% agarose gel. A visible PCR product in lanes marked *U* and *M* indicates the presence of unmethylated and methylated promoters, respectively. A methylated DNA (CM) was used as positive control in all the reactions for each set of primers.

5-Aza-dC induced $p16^{INK4a}$ mRNA, but not protein expression in GAL-01 cell line

We next examined the effect of the DNA methyltransferase inhibitor 5Aza-dC on $p16^{INK4a}$ expression both at mRNA and protein level in GAL-01 to assay if it is possible to revert the gene silencing due to the improper promoter methylation. Since the INK4/ARF gene expression in GAL-02 traced those of GAL-01 we decide to border the analysis to this latter cell line. GAL-01 was exposed to different drug concentrations (1μM, 2,5 μM and 5 μM) for 24, 48, 72 and 96 h, to examine the effect of each experimental condition on cell growth kinetic. Cell viability for treated cell was estimated by Trypan Blu quantitative colorimetric assay and compared to those of control cells cultured in the absence of 5-Aza-dC (Fig.4). High drug concentration, 5μM 5-Aza-dC, had an early toxic effect on GAL-01 cell line, just following 24h exposure the fraction of death cells is much higher than both untreated controls and samples treated with lower drug concentration. 1μM 5-Aza-dC treatment did not induce a sensible cell growth arrest, while 2,5μM 5-Aza-dC had a cytostatic effect, blocking cell growth without killing the cells. $p16^{INK4a}$ exerts its tumor suppressive function by limiting cell cycle progression. We chose 2,5μM 5-Aza-dC concentration in order to evaluate if the drug induced- $p16^{INK4a}$ re expression may also be accountable for drug-induced cell cycle arrest. Based on these data we have treated GAL-01 cell line for 96 hours with 2,5 μM Aza-5dC, to assay if it is

possible to restore $p16^{INK4a}$ expression by inhibiting DNA methylation cellular machinery. MSP-PCR performed on DNA extracted respectively from treated and untreated cells demonstrated that 5-Aza-dC is able to revert the methylated status of $p16^{INK4a}$ promoter region; after the treatment with DNA methyltransferase inhibitor both methyled and unmethyled bands were observed, whereas in the untreated control only the methyled form appeared (Fig.5). The transcriptional silencing imposed by improper methylation was reverted by inhibiting the DNA methyltransferase, an up regulation of $p16^{INK4a}$ transcription was observed after 72h of treatment and it was maintained in next experimental time point. As shown in Fig.6 $p16^{INK4a}$ mRNA became detectable in GAL-01 cell line following 5-Aza-dC treatment, while in the untreated control no amplification products were generated in each experimental time. These data strictly confirmed that in GAL cell lines $p16^{INK4a}$ methylation correlates with $p16^{INK4a}$ downregulation and suggests that the protein loss may be due to methylation-induced gene silencing. As expected in light of our previously results, $p15^{INK4b}$ mRNA level was not influenced by 5-Aza-dC treatments, remaining stable before and after demethylation treatment. The hemimethylated condition of its promoter is not enough to silence the gene; indeed the mRNA level observed in the untreated control was comparable to that of treated cell lines. This is consistent with the observation that $p15^{INK4b}$ gene methylation does not necessarily lead to complete silencing of the gene and that in some BL cell lines methylated $p15^{INK4b}$ could express $p15^{INK4b}$ mRNA ([60]). We performed western blotting analysis on 5-Aza-dC treated and untreated cell lines in order to verify $p16^{INK4a}$ protein expression following the transcriptional silencing reversion. As show in Fig.7 after 72h of treatment a barely detectable signal was obtained. This result has been found to be analogous also increasing drug concentration and drug exposure time (data not show), suggesting that even if 5-Aza-dC is able to revert gene silencing due to promoter methylation, it is not enough to restore protein expression. Additional mechanisms might cooperate with the epigenetic gene silencing by affecting mRNA or protein stability.

In order to evaluate the presence of mutations that could generate an aberrant mRNA, we examined $p16^{INK4a}$ coding sequence after 5-Aza-induced $p16^{INK4a}$ mRNA re-expression. Total RNA, extracted from treated samples, was retro transcribed and used as template for PCR reaction using specific set of primers designed in order to amplify the $p16^{INK4a}$ full length coding sequence (471bp). Analysis of PCR products by agarose gel electrophoresis revealed a correct size $p16^{INK4a}$ segment, excluding the presence of a truncated, atypical transcript product.

No mutations were found following sequencing analysis, identifying the PCR amplification product as the *wildtype* $p16^{INK4a}$ coding sequence. This result allowed us to except the possibility that the lack of 16^{INK4a} protein reexpression after 5Aza-dC treatment may be due to genetic alterations affecting mRNA fidelity and stability.

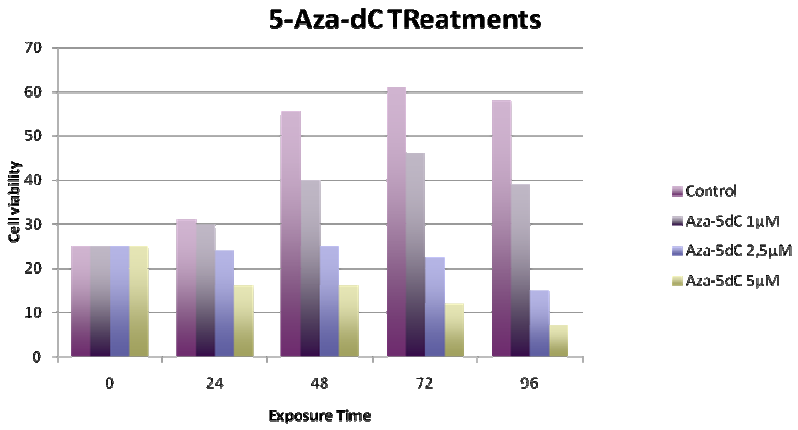


Fig.4 Time- and dose-dependent effect of 5-Aza-dC on GAL-01 growth kinetic
GAL-01 cells were either untreated or treated with increasing concentrations (1µM, 2,5 µM and 5 µM) of 5-Aza-dC for 96h. Every 24h the cells were harvested and the growth kinetic were evaluated by Trypan blu viability assay. Concentration of drug *versus* percent survival was plotted.

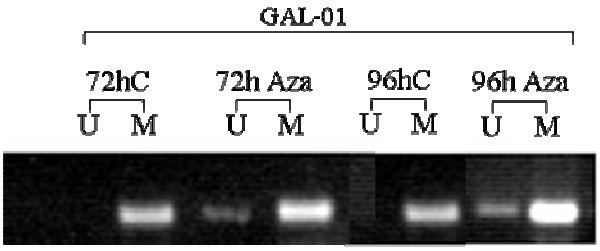


Fig.5 MSP-PCR analysis of p16INK4a promoter region after 5-Aza-dC treatment. MSP-PCR performed using bisulfite modified DNA form GAL-01 cell line following 72h and 96h of 2,5µM 5-Aza-dC treatment (72h Aza and 96h Aza). Untreated samples were harvested at 72h and 96h and used as control. After 72h of 2,5µM 5-Aza-dC exposure both the methyled and unmethyled bands were observed, whereas in the 72hC only the methyled band appeared. The same result was achieved for cell lines 96h treated cells. 5-Aza-dC is able to revert partially p16INK4a promoter methylation.

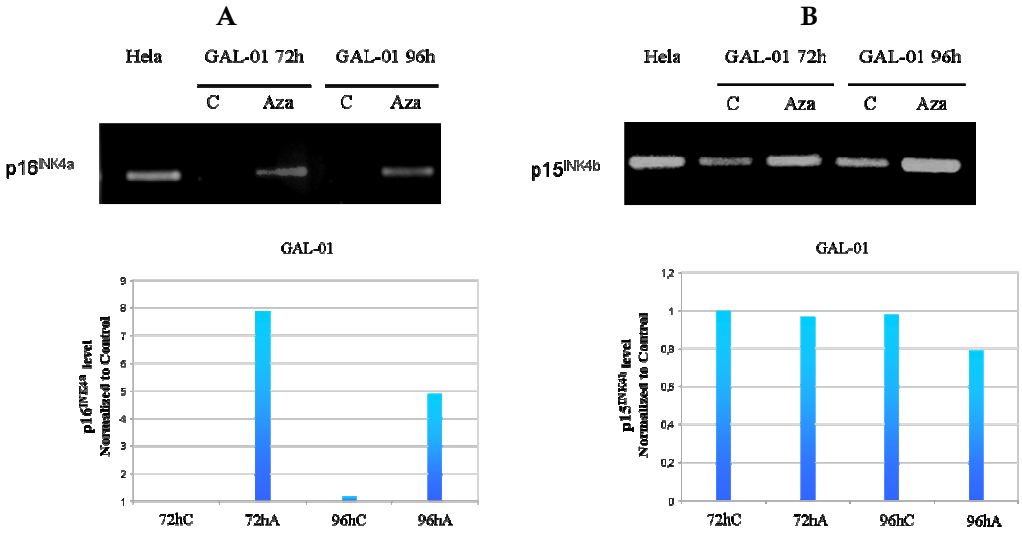


Fig.6 5-Aza-dC induced p16^{INK4a} expression in GAL cell lines.
2,5µM 5-Aza-dC treated and untreated (C) GAL-01 samples were harvested at different times and total RNA was retro transcribed and amplified using specific sets of primer for p16^{INK4a} and p15^{INK4b}. mRNA levels were evaluated by RT-PCR and Real time PCR. p16^{INK4a} and p15^{INK4b} expression values were normalized to that obtained in the untreated cells (control). (A) After 72h of 5-Aza-dC exposure the p16^{INK4a} mRNA became detectable in treated samples and it remained stable in next experimental time point, whereas no amplification products were generated at any time in the untreated control. p16^{INK4a} up regulation following 5-Aza-dC treatment was further confirmed by real-time quantitative RT-PCR. (B) Aza-dC treatments did not influence p15^{INK4b} mRNA level. Both RT-PCR and real-time quantitative RT-PCR analysis, shown that 15^{INK4b} expression was comparable between treated and untreated samples, as well as to the positive control (Hela cell line).

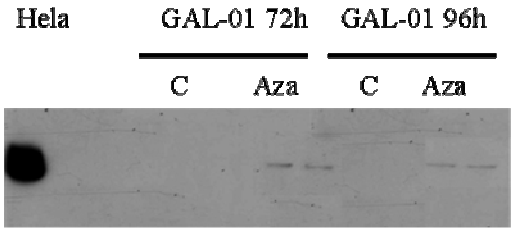


Fig.7 p16^{INK4a} protein expression was not induced following 5-Aza-dC treatment in GAL cell lines.
Western blot analysis of p16 expression in GAL-01 cell line incubated with (+) or without (-) 2,5µM 5-Aza-dC for 72 h and 96h. A barely detectable signal was obtained in both 72h(+) and 96(+) samples, almost equivalent to the undetectable p16 stain in the untreated (-) controls. Hela cells were used as positive control and α-tubulin was use to confirm loading integrity.

Concomitant inactivation of INK4/ARF locus genes is a common feature in BL cell lines.

The concurrent inactivation of INK4/ARF locus genes seems to be a distinctive marker in

GAL cell lines, underscoring the well known tumor suppressor functions of these genes and their critical involvement in tumor progression. To overdraw our finding we evaluated a board of BL cell lines, differing each other for tumor stage, drug sensitivity and EBV infection. As show in Fig.8 for any cell lines under consideration we determined respectively $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ status at both transcriptional and posttranscriptional level, correlating for each gene the expression level with the promoter methylation status (Fig.9). The results previously obtained for the GAL-01 cell lines roughly overlapped the data achieved for the other BL cell lines. Indeed, $p16^{INK4a}$ has been found to be silenced in all the cell lines at both mRNA and protein level. As well as for the GAL cell lines, no staining was detected by western blotting analysis for the other cell lines under consideration with the exception of the normal B-lymphocytes and the Hela cell line, used as positive control. Similarly, no amplification products have been obtained through both RT-and Real Time- PCR in any BL cell lines, contrary to the high level found for Hela cell line as well as for the normal one. We used the previously described MSP-PCR strategy to determine whether the lack of $p16^{INK4a}$ expression in the BL cell lines was due to the improper methylation of $p16^{INK4a}$ promoter region, similarly to the GAL cell line. For all the BL cell lines we obtained a genomic amplification product in the reaction carried out with M primers, moreover in the Ramos and Ga-10 cell lines the U band appeared too, indicating that these cell lines are heterozygous for promoter methylation. The complete lack of $p16^{INK4a}$ mRNA in these cell lines demonstrated that the hemimethylated condition is enough to lead a complete gene silencing. These data demonstrated that in BL cell lines p16/INK4a promoter methylation and gene expression silencing are reciprocally related and further suggest that $p16^{INK4a}$ methylation might be considered a common feature in BL. The same experimental design has been followed to investigate $p15^{INK4b}$ expression pattern. As well as for $p16^{INK4a}$ protein, $p15^{INK4b}$ loss was found to be a common signature among all the cell lines under consideration. Otherwise, $p15^{INK4b}$ mRNA levels differed according to the cell specific phenotype. As well as GAL-01 and Normal B-lymphocytes, Ramos, DG-75 and Daudi cell lines expressed regular $p15^{INK4b}$ mRNA level, while it was down regulated in Ga-10 cell line, closely resembling the $p15^{INK4b}$ expression level observed in the drug resistant GAL-02 clone. The fact that both the cell lines lacking $p15^{INK4b}$ derived from patients with clinically aggressive, chemo-refractory Burkitt`s lymphoma, might suggest that $p15^{INK4b}$ gene silencing may confer upon the cell a more aggressive phenotype and contribute to the development of a drug-resistant one. However, the MSP-PCR analysis

revealed that $p15^{INK4b}$ gene methylation cannot be considered a common mechanism for gene inactivation, since the methylation status of the promoter did not correlate with gene expression pattern. Although both GAL-02 and GA-10 have not shown $p15^{INK4b}$ expression, the promoter region in the latter cell line was unmethyled, therefore in a transcriptional permissive chromatin state. In all the remaining BL cell lines, we have found that $p15^{INK4b}$ promoter was unmethyled according to the previously obtained mRNA level. The lack of $p15^{INK4b}$ protein in a positive mRNA setting seems to be a common state in BL, suggesting that in tumor cells posttranscriptional mechanisms may be responsible for mRNA and/or protein instability. An analogous system may be accountable for $p14^{Arf}$ protein loss, as we have observed in some cell lines. Despite the high mRNA level (Fig.8 A) estimated in each cell lines, protein expression was restricted to the DG-75 and GA-10 that respectively showed the same or quite less expression level than the positive control. In the remaining cell lines, as well as for the normal B-lymphocytes, no detectable signal was found. Given the constitutive activation of c-Myc proto-oncogene in BL, it is expected that $p14^{Arf}$ will be upregulated in response to mitogenic signal. As expected we observed higher $p14^{Arf}$ mRNA levels in tumor cell lines than in the normal one, however this correlation was lost at posttranscriptional level in some of the cell lines we have analyze. Alike the previously examined GAL cell lines, no detectable protein staining was found in both Ramos and Daudi cell lines. Given that in Burkitt`s lymphoma cell lines the $p14^{Arf}$ inactivation can impact tumor development and anticancer therapy by disabling c-Myc-induced apoptosis, the variability observed in protein expression among the tumor cell lines may reflect difference in tumor progression stages. $p14^{Arf}$ protein inactivation could be considered a prognostic marker for Burkitt`s lymphoma. The latter observation seems to be contradictory in regard with the high protein expression level found in DG-75 and Ga-10 cell line, representing respectively a metastatic and drug resistant BL clones. However, our results were in line with recent observations ([70]), showing that p14ARF abnormal overexpression is a marker of high tumor aggressivity by acting as a sensor of malfunction of the major cell cycle regulatory pathways. In this case however an impaired cellular localizations is accountable for $p14^{ARF}$ inactivation and the sustained gene induction is not correlate with protein over activity. Methylation-specific PCR assays were performed to determine the methylation status of the CpG islands of the $p14^{ARF}$ promoter in each cell lines under investigation. Consistently with the general mRNA expression, only the unmethyled band resulted as amplification product in all the samples evaluated, with the alone exception for the

methyled DNA control in which $p14^{ARF}$ promoter methylation was appropriately found. $p14^{ARF}$ promoter methylation as well as its transcriptional silencing do not represent a specific marker of Burkitt lymphomagenesis, nevertheless protein inactivation may have a significant diagnostic and prognostic value.

Taken together these data demonstrated that in BL cell lines the INK4/ARF locus functions are concurrently inactivated, even though by distinctive mechanisms. Although aberrant promoter methylation seems to be accountable for $p16^{INK4a}$ inactivation and as consequence for protein loss, the same mechanism is not appropriate to explain the lack of both p15 and $p14^{ARF}$ in BL cell lines. Alterations affecting mRNA and/or protein stability may justify the overlooked protein expression in cells that normally express the corresponding mRNA.

B

A

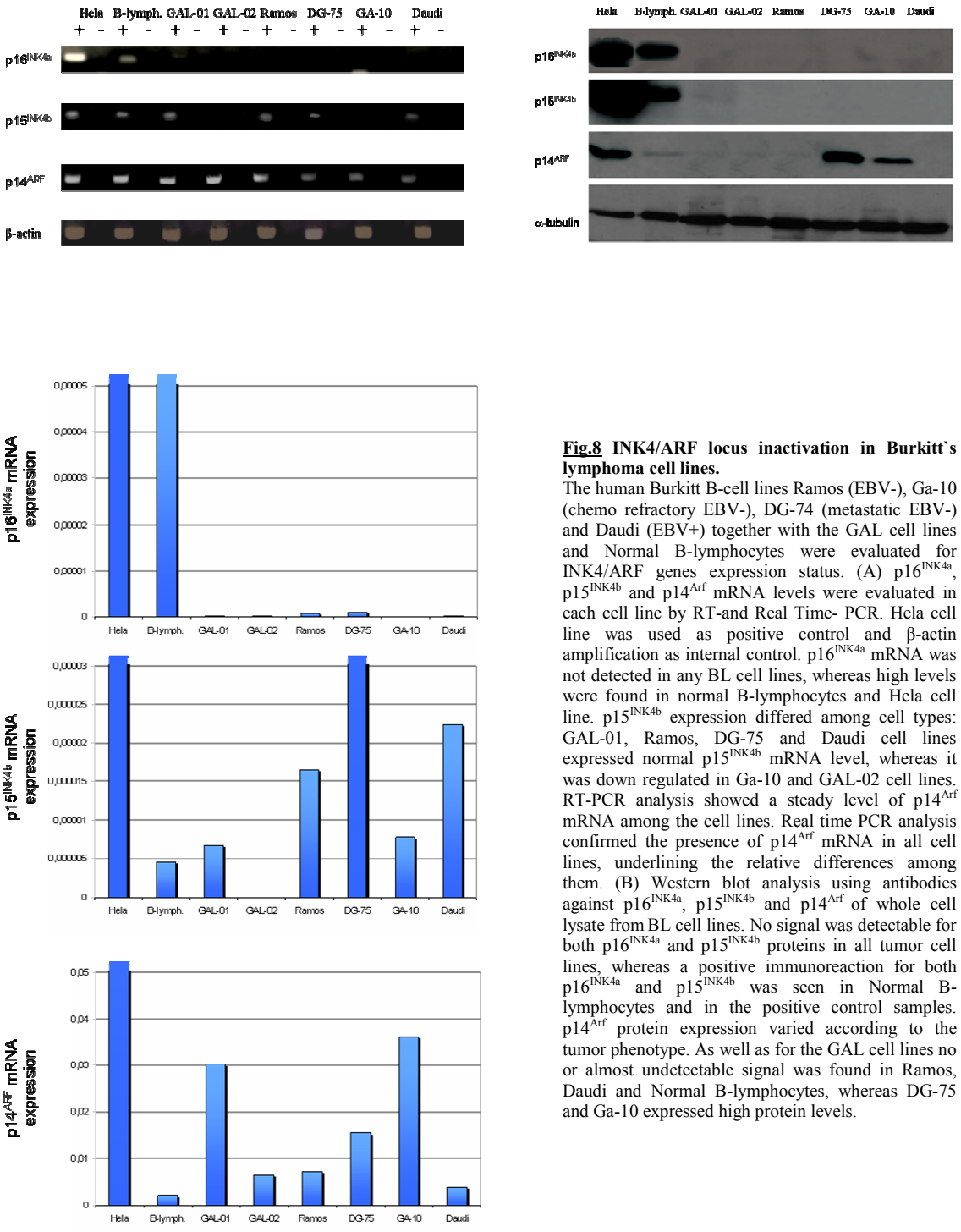


Fig.8 INK4/ARF locus inactivation in Burkitt`s lymphoma cell lines.
The human Burkitt B-cell lines Ramos (EBV-), Ga-10 (chemo refractory EBV-), DG-74 (metastatic EBV-) and Daudi (EBV+) together with the GAL cell lines and Normal B-lymphocytes were evaluated for INK4/ARF genes expression status. (A) $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ mRNA levels were evaluated in each cell line by RT-and Real Time- PCR. Hela cell line was used as positive control and β-actin amplification as internal control. $p16^{INK4a}$ mRNA was not detected in any BL cell lines, whereas high levels were found in normal B-lymphocytes and Hela cell line. $p15^{INK4b}$ expression differed among cell types: GAL-01, Ramos, DG-75 and Daudi cell lines expressed normal $p15^{INK4b}$ mRNA level, whereas it was down regulated in Ga-10 and GAL-02 cell lines. RT-PCR analysis showed a steady level of $p14^{Arf}$ mRNA among the cell lines. Real time PCR analysis confirmed the presence of $p14^{Arf}$ mRNA in all cell lines, underlining the relative differences among them. (B) Western blot analysis using antibodies against $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ of whole cell lysate from BL cell lines. No signal was detectable for both $p16^{INK4a}$ and $p15^{INK4b}$ proteins in all tumor cell lines, whereas a positive immunoreaction for both $p16^{INK4a}$ and $p15^{INK4b}$ was seen in Normal B-lymphocytes and in the positive control samples. $p14^{Arf}$ protein expression varied according to the tumor phenotype. As well as for the GAL cell lines no or almost undetectable signal was found in Ramos, Daudi and Normal B-lymphocytes, whereas DG-75 and Ga-10 expressed high protein levels.

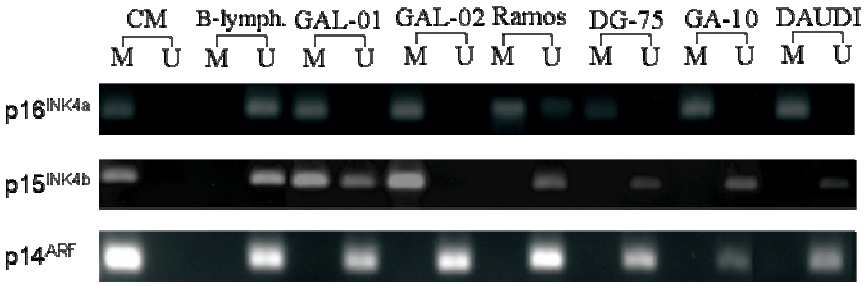


Fig.9 $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ promoters methylation status in Burkitt`s lymphoma cell lines.
MSP-PCR was used to evaluate the methylation condition of INK4/ARF locus, using definite set of primers (M and U) for each gene. $p16^{INK4a}$ promoter resulted methylated in all the BL cell lines examined. p15INK4b promoter region was exclusively unmethylated in normal B-lymphocytes, DG-75, GA-10 and Daudi cell lines whereas was emy- or totally-methylated respectively in GAL-01 and GAL-02 cell lines. As well as for GAL cell line and the normal B-lymphocytes the $p14^{Arf}$ promoter region was unmethylated in each the samples examined. For each reaction a totally methylated DNA was used as internal control, showing as amplification products only the M band for each specific set of primers.

	$p16^{INK4a}$				**$p15^{INK4b}$**				**$p14^{ARF}$**			
	Protein	mRNA	MSP	CDS	Protein	mRNA	MSP	CDS	Protein	mRNA	MSP	CDS
B-lymp.	+	+	U	wt	+	+	U	wt	+	+	U	wt
GAL-01	-	-	M	wt	-	+	U/M	wt	-	+	U	wt
GAL-02	-	-	M	wt	-	-	M	wt	-	+	U	wt
Ramos	-	-	M	wt	-	+	U	wt	-	+	U	wt
DG-75	-	-	M	wt	-	+	U	wt	+	+	U	wt
GA-10	-	-	M	wt	-	-	U	wt	Low	+	U	wt
Daudi	-	-	M	wt	-	+	U	wt	-	+	U	wt

Tab.1 INK4/ARF locus in BL cell lines.
$p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ were analyzed for protein expression, mRNA level, promoter methylation and mutation analysis of gene coding sequences.

No abnormalities were found in both INK4/ARF coding-and coding-RNA.

We analyzed $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{AR}$ coding sequences in order to identify possible mutations that may impair the mRNA ability to be traduced in a functional protein. For

each cell line we used the total RNA as the template for a RT-PCR reaction carried out with specific sets of primers specifically designed for amplify the full-length coding sequence of the genes belonging to the locus. Since $p16^{INK4a}$ expression was silenced in all the cell line by aberrant promoter methylation, we had treated the cell lines with 5-Aza-dC in order to revert the transcriptional inactivation and afterward we carried out the RT-PCR using the re expressed mRNA as template for the reaction.

As well as we had previously observed for the GAL cell lines, all the samples under evaluation have revealed an amplification product having the same length (471 bp) and nucleotide sequence of the wildtype $p16^{INK4a}$ coding sequence. The lack of mutations in the mRNA sequence exclude the possibility that incomplete $p16^{INK4a}$ protein reexpression after demethylating treatment may be due to anomalies affecting mRNA structure and stability. These data confirmed that in BL cell lines additional mechanisms cooperate with methylation-induced gene silencing to achieve a complete $p16^{INK4a}$ functional inactivation by affecting protein stability.

Both $p15^{INK4b}$ and $p14^{ARF}$ mRNA were normally expressed by the BL cell lines under examinations; this allow us to amplify directly the respectively coding sequences, using as template for reaction the total RNA extracted from any cell lines. The amplification products obtained from the RT-PCR were equivalent in length and identical in base sequence to the reciprocal wild type. $p15^{INK4b}$ and $p14^{ARF}$ full-length sequencing did not reveal any mutations within the opening reading frame. These data shown that no atypical transcript product for both genes and for each cell was generated and moreover excluded the possibility that the previously observed proteins lack was due to genetic alterations affecting mRNA fidelity and stability. The lack of mutation in the coding sequences and their fidelity to a normally translated CDS, assured that the sequences under examination have both the potential to be translated in a wildtype protein. Given the growing recognized importance of the non-protein coding RNAs in regulating mRNA stability and translation, we have moreover investigated for the presences of a known non-coding RNAs within the INK4/ARF locus. Pasmant et al. has ([100]) have recently identified a new large antisense noncoding RNA (named ANRIL) with a first exon located in the promoter of the $p14^{ARF}$ gene and overlapping the two exons of $p15^{INK4b}$. In this study they demonstrated that ANRILL expression coclustered with, $p14^{ARF}$, $p16^{INK4a}$ and $p15^{INK4b}$ both in physiologic and in pathologic conditions (human breast tumors) with a stronger positive correlation between ANRIL and $p14^{AR}$than between ANRIL and $p16^{INK4a}$ and $p15^{INK4b}$. We analyzed ANRILL expression in BL cell lines in order to investigate if there

were some correlations between ANRILL expression and INK4/ARF proteins lack previously observed. To assay the relationship linking ANRILL and INK4/ARF locus gene expression we quantified ANRIL, $p14^{ARF}$, $p16^{INK4a}$ and $p15^{INK4b}$ mRNA in BL cell lines by Real Time PCR. As show in Fig. 10 our data demonstrated a coordinated transcriptional regulation of ANRIL and INK4/ARF locus genes, with a stronger positive correlation between ANRIL and $p14^{ARF}$ than between ANRIL and $p16^{INK4a}$ and $p15^{INK4b}$, as well as for the previously published data.

These correlations were found in all the cell lines under examination regardless of protein expression status. Since the expression of ANRILL and $p14^{ARF}$ mRNA are maintain in both protein expressing- (Hela, DG-75and GA-10) and non expressing- (GAL-01, GAL-02, Ramos and Daudi) cell lines as well as in normal B-lymphocytes, we can deduce that this non coding RNA did not interferer with $p14^{ARF}$ mRNA stability and translation and that it was co expressed with $p14^{ARF}$ in physiologic and pathological conditions.

Although we have found a correlation between ANRILL and both $p16^{INK4a}$ and $p15^{INK4b}$ expression this is less significant than between $p14^{ARF}$. However through gene expression analysis we have not found any possible link between ANRILL expression and $p16^{INK4a}$ and $p15^{INK4b}$ lack. Taken together these data demonstrate that despite a normal coding and non-coding gene expression, other posttranscriptional mechanisms could be accountable for the concomitant loss of INK4/ARF proteins in BL cell lines. We can furthermore assume that in BL cell lines, although wild type proteins were normally traduced, mechanisms affecting INK/ARF proteins stability lead an improper protein turnover.

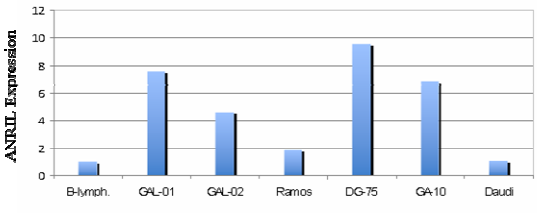
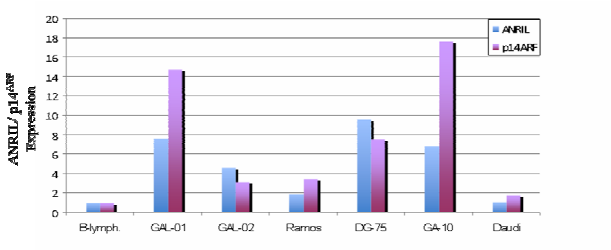


Fig.10 ANRIL expression mainly coclustered with $p14^{Arf}$ in Burkitt`s lymphoma cell lines.
Real Time PCR showing ANRILL expression levels in BL cell lines (A). For each cell lines under investigation $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{Arf}$ transcription levels were



respectively compared with ANRIL expression. (B) A Strong correlation was found between ANRIL and $p14^{Arf}$ in all tumor cell lines as well as in normal B-lymphocytes, regardless of protein expression levels.

Proteasome inhibitor induces INK4/ARF proteins re expression in BL cell lines

Ubiquitin-proteasome pathway (UPP) is the major system for the selective degradation of cellular proteins and its overactivation plays a key role in the pathogenesis of common human cancers. Proteasome inhibitors are a new class of antitumor agents that, by inhibiting the ubiquitin-proteasome pathway, prevent the selective degradation of intracellular proteins. To test the possibility that in BL cell lines the weakened INK4/ARF proteins expression was owing to improper protein degradation, we analyzed whether the proteasome inhibitors MG-132 could increase $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ levels in the cell lines under consideration. Since we had previously established that p16 gene was silenced by aberrant promoter methylation, we evaluated if MG-132 could cooperate with 5-Aza-dC-induced gene transcription to achieve a steady protein expression. We therefore treated BL cell line with MG-132 alone or in combination with 5-Aza-dC and compared the protein expression levels to untreated and 5-Aza-dC treated controls. Moreover, normal B-lymphocytes were examined in order to evaluate the effects of these treatments in a physiological setting and to validate if the proteasome-mediated INK4/ARF proteins degradation is a tumor specific feature. Since $p14^{ARF}$ was the only INK4/ARF protein that shown a tumor specific expression, while the both p16 and 15 were commonly found down regulated among the cell lines under our investigation, we selected for MG-132 treatment the cell lines characterized by dissimilar $p14^{ARF}$ expression status. Both GAL-01, DG-75 and GA-10 as well as Normal B-lymphocytes were incubated for 72h respectively in the presence or absence of 2,5 µM of 5-Aza-dC, treated with 10 µM MG-132 and harvested at 4, 8 and 10 h after the addition of MG-132. $p16^{INK4a}$, $p15^{INK4b}$ and $p14^{ARF}$ protein expression levels were evaluated by western blotting analysis and, comparing the effects of each treatment alone and in combination we have hypothesized a plausible mechanism for explaining INK4/ARF locus inactivation of in BL cell lines. MG-132 treatment alone did not induced significant change in $p16^{INK4a}$ protein in all cell lines and for each considered time points. This data was consistent with the observation

that in BL cell lines $p16^{INK4a}$ is epigenetically silenced by promoter methylation and gene expression is induced following demethylathing treatments. However, 5-Aza-dC treatment alone is not able to ensure a steady expression of p16 protein that, as previously shown and confirmed again by analyzing 5-Aza-dC control in both GAL-01 and GA-10, was barely detectable compared to the positive control (Fig 10 A). Following 8h exposure to MG-132 p16 protein level was found to increase compared to the same samples treated with 5-Aza-dC alone. MG-132 induced $p16^{INK4a}$ up regulation in a time dependent manner; in the early 4h its action was indiscernible from the 5-Aza-dC effect alone and after 10h exposure it went to the bottom. The normal distribution of the MG-132 action time on $p16^{INK4a}$ protein stability represented an additional evidence for the $p16^{INK4a}$ proteasome-mediated downregulation in BL cell lines. These data demonstrated for the first time that, together with the well-established epigenetic silencing, $p16^{INK4a}$ expression might be furthermore inhibited in BLs at posttranscriptional level by overactivation of proteasome-mediated $p16^{INK4a}$ degradation. Differently, we have found that in DG-75 cell line the protein expression was comparable between samples treated with 5-Aza-dC alone as well as in combination with MG-132, suggesting that in this cell line $p16^{INK4a}$ protein reexpression was a exclusively consequence of 5-aza-2'-deoxycytidine hypomethylating action that, by reverting the transcriptional silencing, allowed the transcription of a stable protein. Taken together these data demonstrate that $p16^{INK4a}$ down regulation may be also governed by improper activation of the proteasome pathway in a tumor specific manner. Tumor cells, evolving through a more aggressive phenotype, select supplementary mechanisms for tumor suppressor genes inactivation that allowing them to escape the thinly regulated progression thought the cell cycle. In normal B-lymphocytes neither the single treatment nor the combination exerted any effect on $p16^{INK4a}$ expression protein, suggesting that both methylation induced-p16 silencing and ubiquitin-dependent proteasomal degradation are selectively activated in a tumor setting and not normally involved in the physiological turnover of the protein. The same samples have been examined for $p15^{INK4b}$ expression status in order to investigate the mechanisms accountable for protein downregulation in BL cell lines. $p15^{INK4b}$ promoter methylation could not be considered a general signature among the BL cell lines under our consideration. As well as for the untreated control no protein stain was detected in 5-Aza-dC treated samples in any cell lines, ruling out the involvement of $p15^{INK4b}$ epigenetic silencing in BL cell lines. Indeed, MG-132 treatment alone as well as in combination with5-Aza-dC induced an early, (following 2h exposure) $p15^{INK4b}$ up regulation in both

the analyzed cell lines. However, as show in Fig.10 B the time interval in which MG-132 was effective varied in strength and among the different cell lines. This could be explained assuming that different cell lines, having diverse growth kinetic, have singular timing for proteins turnover. These result underscored as the lack of $p15^{INK4b}$ protein, commonly found in BL cell lines, was dependent on the proteasome degradation pathway, as a proteasome inhibitor, MG-132, efficiently blocked the $p15^{INK4b}$ degradation and restored its normal expression level. No supplementary effects were conferred by the concurrent hypomethylating action of 5-Aza-dC, demonstrating that $p15^{INK4b}$ silencing in BL cell lines was exclusively executed at posttranscriptional level, consistently with the normal mRNA levels we had previously found among the cell lines. Additionally the noncurrent 5-Aza-dC action on the MG-132 ability to revert back $p15^{INK4b}$ expression licensed us to exclude an indirect involvement of demethylating agent in any protein regulation mechanisms. As well as for $p16^{INK4a}$ protein expression, no variations in $p15^{INK4b}$ levels have been observed following the treatments in normal B-lymphocytes, this suggested that neither promoter methylation nor ubiquitin-dependent proteasomal degradation overseen protein activity in physiological conditions. The levels of $p14^{ARF}$ expression in normal tissues are quite low, since gene transcription is induced by sustained oncogenic signals. Indeed, as expected in Normal B-lymphocytes no protein staining was detected and its expression was not influenced by treatments. This result underlined as in physiological condition the lack of mitogenic stimulations retains $p14^{ARF}$ in a transcriptional repressive condition and its inactivation is achieved neither by promoter methylation nor by protein degradation. As previously shown (Fig.8 A), $p14^{ARF}$ was transcriptionally regulated in normal B-lymphocytes as well as in BL cell lines, with mRNA induction in the c-Myc overexpressing Burkitt`s lymphoma cell lines. DG-75 cell line expressed both high levels of $p14^{ARF}$ mRNA and protein, and treatment with 5-AZA-dC alone or in combination with MG-132 did not influence its expression (Fig.10 C). Rather, western blotting analysis seemed to reveal decree in protein expression levels following the treatments. We have found an explanation for this atypical effect supposing an overall toxicity rather than a direct treatment involvement in gene regulation. In GAL-01 and GA-10 cell lines respectively the total loss or barely detectable presence of $p14^{ARF}$ protein in a c-Myc-induced $p14^{ARF}$ expression setting may be explained by a posttranscriptional regulatory mechanisms. The totally unmethylated $p14^{ARF}$ promoter together with mRNA presence found in any cell lines may explain the ineffective 5-Aza-dC action on $p14^{ARF}$ protein expression in both GAL-01 and GA-10 cell lines. 2h

following MG-132 exposure $p14^{ARF}$ protein expression was induced regardless of 5-Aza-dC concurrent presence. These data demonstrate that in BL cell lines $p14^{ARF}$ activity was impaired by ubiquitin-proteasome pathway overactivation, this allows the cell to escape c-Myc induced apoptosis and to acquire a more aggressive phenotype. Both $p16^{INK4a}$ and $p15^{NK4b}$ are very stable proteins with half-live over 8h while $p14^{ARF}$ is more rapidly degraded (half-life ~30 minutes) ([101]), this may explain the shorter effect of MG-132 treatment on $p14^{ARF}$ protein stability than on $p15^{INK4b}$ and $p16^{INK4a}$ one (spanning for 8-10h following MG-132 exposure). In GA-10 the $p14^{ARF}$ expression could be considered exclusively governed by proteasome-mediated degradation and its loss as direct result of ubiquitin-proteasome pathway overactivation. In GAL-01, in which there was a complete lack of the protein, the MG-132 treatment was as well able alone to inhibit protein degradation in the early 2h (GAL-01 treated 2h with MG-132 alone), but its effect kept working longer where used in combination with 5-Aza-dC. By an indirect action the 5-Aza-dC could influence protein stability and/or expression by repressing methylation-induced gene silencing of unknown regulatory genes. Finally, to be definitely confident that what we had demonstrated before was due to inhibition of proteasome activity, we have analyzed p27 levels, a protein known to be a target for ubiquitin-proteasome pathway degradation. In normal B-lymphocytes, in which the protein turnover takes place in physiological fashion, p27 expression was not much influenced by MG-132 treatment alone or in combination with Aza-5-dC. As underlined in this work, INK4/ARF tumor suppressor genes are additional inactivated in BL cell lines by an enhanced activity of ubiquitin-proteasome pathway and their activity may be recovered by MG-132. The overactivation of proteasome machinery could be confirmed by the re expression of p27 protein following proteasome inhibitor treatment both in the GAL-01 and GA-10 in which we had previously demonstrated an improper activity of proteins degradation cellular machinery. In both the untreated control the p27 levels were much lesser than in the samples expose to MG-132. Of note, in GAL-01 the same synergistic effect between 5-AZA-dC and MG-132 has been observed, confirming that the demethylating drug may indirectly regulate the expression of some actually unknown regulator of protein stability.

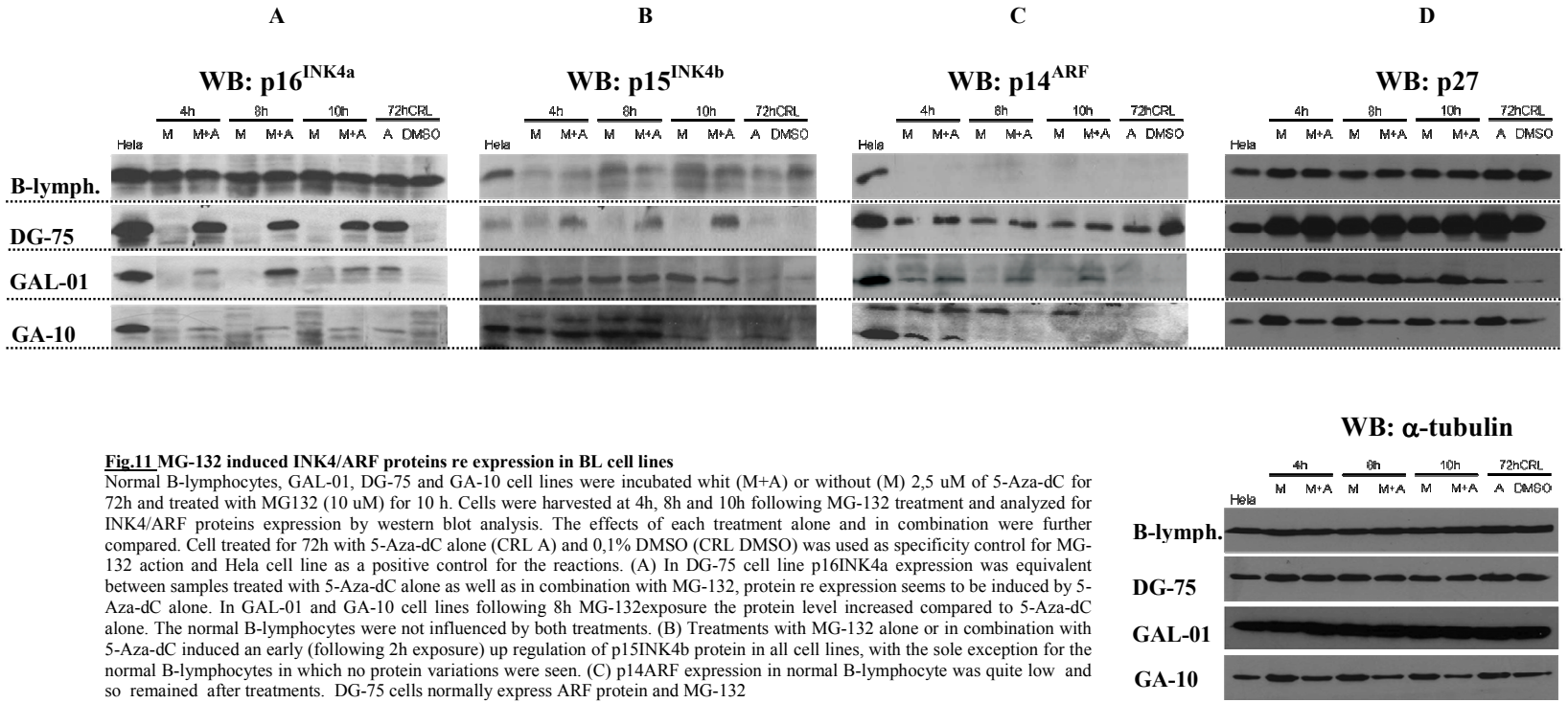


Fig.11 MG-132 induced INK4/ARF proteins re expression in BL cell lines

Normal B-lymphocytes, GAL-01, DG-75 and GA-10 cell lines were incubated whit (M+A) or without (M) 2,5 uM of 5-Aza-dC for 72h and treated with MG132 (10 uM) for 10 h. Cells were harvested at 4h, 8h and 10h following MG-132 treatment and analyzed for INK4/ARF proteins expression by western blot analysis. The effects of each treatment alone and in combination were further compared. Cell treated for 72h with 5-Aza-dC alone (CRL A) and 0,1% DMSO (CRL DMSO) was used as specificity control for MG-132 action and Hela cell line as a positive control for the reactions. (A) In DG-75 cell line p16INK4a expression was equivalent between samples treated with 5-Aza-dC alone as well as in combination with MG-132, protein re expression seems to be induced by 5-Aza-dC alone. In GAL-01 and GA-10 cell lines following 8h MG-132exposure the protein level increased compared to 5-Aza-dC alone. The normal B-lymphocytes were not influenced by both treatments. (B) Treatments with MG-132 alone or in combination with 5-Aza-dC induced an early (following 2h exposure) up regulation of p15INK4b protein in all cell lines, with the sole exception for the normal B-lymphocytes in which no protein variations were seen. (C) p14ARF expression in normal B-lymphocyte was quite low and so remained after treatments. DG-75 cells normally express ARF protein and MG-132
alone or combined with 5-Aza-dC did not influence protein expression. p14 protein expression was induced 2h following MG-132 exposure regardless of 5-Aza-dC presence in both GAL-01 and GA-10 cell lines. The effect of MG-132 treatment on p14ARF expression was prolonged by an indirect action of 5-Aza-dC in GAL-01 cell line. (D) Western blot analysis using antibodies against p27 (a known target for ubiquitin-mediated proteasome degradation) were performed in order to evaluate the MG-132 efficiency in the settled conditions. (E) After the immuno blotting the membranes were stripped and reprobed with β-actin antibody to assess the loading.

Discussions

The genetic signature of Burkitt`s lymphoma is the reciprocal chromosomal translocations that activate c-myc oncogene through juxtaposition to one of the immunoglobulin (Ig) loci, driving tumor cell growth. Several lines of investigation, however, indicate that c-Myc overactivation is necessary but not sufficient requirement and tumor progression involves selection for additional genetic and epigenetic changes. INK4/ARF locus at chromosome 9p21 is a frequent target of inactivation in a variety of tumors, since it encodes for three tumor suppressors genes that distinctively regulate p53- and Rb-pathways. The aim of this work, for the first time, was to identify the mechanisms accountable for the simultaneously inactivation of INK4/ARF genes in Burkitt`s lymphoma and demonstrate the functional importance of proteasome-mediated INK4/ARF proteins downregulation not only in BL genesis and progression, but also as a new general mechanism of INK4/ARF inactivation.

The loss of INK4/ARF in Eu-Myc transgene significantly accelerate lymphomagenesis and abrogate c-Myc induced apoptosis ([46]). Knockout studies of mice specifically deficient for ARF (or $p19^{ARF}$ the corresponding mouse gene), $p15^{INK4b}$ or $p16^{INK4a}$ have revealed that all three strains are more prone to spontaneous cancer than wild-type littermates, but each of these single knockouts appears significantly less prone than animals lacking both the genes (INK4/ARF-/-)([91]). These observation prompted us to undertake a comprehensive molecular analysis of 9p21 chromosome region, where the TSGs $p15^{INK4}b$, $p14^{ARF}$, and $p16^{INK4a}$ map in tandem, in a series of Burkitt`s lymphoma cell lines, to test the possibility of a simultaneously INK4/ARF genes inactivation. Multiple genetic and epigenetic mechanisms affecting INK4/Arf locus expression have been described, differing among tumor types and sometimes within the same oncological variant. Frequently tumors harbor homozygous deletions or point mutation, in the sharing exons, that abrogate the expression of all three proteins. Both the genes were analyzed for protein expression, promoter methylation, gene mutation, and also investigated for the expression of their relative transcripts. By the analysis of a Burkitt`s lymphoma cell lines panel we observed a broad deregulation of INK4/ARF proteins level in almost all the cells. In particular lack of both $p16^{INK4a}$ and $p15^{INK4b}$ resulted to be a common feature in BL,

whereas the $p14^{ARF}$ varied in a tumor specific manner. RT-PCR and real time studied were performed to evaluate the relative abundance of INK/ARF mRNA expression levels for each gene. Consistently with the general $p16^{INK4a}$ protein loss no relative transcript was found in all the BL cell lines under investigation, with the sole exception for the normal B-lymphocytes that we used as untransformed control. By contrast, the homologue gene $p15^{INK4b}$, was normally expressed among the cell lines and became transcriptionally silenced during the evolution through a drug resistant phenotype. No mRNA was instead detected in GAL-01 and GA-10 cell lines derived both from chemorefractary patients. Our study, for the first time, underscored a correlation between $p15^{INK4b}$ inactivation and BL evolution through a drug resistant phenotype. The loss of $p15^{INK4b}$ expression may be considered as a secondary event acquired by the cells, following drug exposure, in order to keep proliferating under normally non-allowed conditions. According to its oncogenic inducible expression, $p14^{ARF}$ was found upregulated in c-Myc overexpressing BL cell lines compared with the almost undetectable level in normal B-lymphocites. $p16^{INK4a}$ was one of the first genes discovered to be silenced epigenetically in human cancers ([83]), and silencing through promoter methylation is well described in numerous types of cancers as well as in BL lines and in a primary tumors ([60]). We performed MSP-PCR analysis in order to verify the methylation status of $p16^{INK4a}$ promoter region and we found that $p16^{INK4a}$ promoter methylation correlated with gene silencing in all the cell lines analyzed. Our results confirmed the previously reported data, corroborating the epigenetical silencing as the general mechanism for $p16^{INK4a}$ inactivation in BL cell lines. As umpteenth proof, we demonstrated that 5-Aza-dC treatment resulted in demethylation of $p16^{INK4a}$ promoter CpG islands and in reactivation of the geneexpression. Since we did not find $p16^{INK4a}$ methylation in both normal B-lymphocytes as well as in positive expressing control, we can conclude that $p16^{INK4a}$ methylation is a BL-associate phenomenon. Consistently with the normal $p15^{INK4b}$ and $p14^{ARF}$ mRNA levels, respective promoter methylation analysis demonstrated that in BL cell lines these genes are not inactivated at transcriptional level neither by genetic nor by epigenetic mechanisms. Furthermore, the results that we obtained concerning the $p15^{INK4b}$ promoter methylation status were in accordance with data demonstrating as $p15^{INK4b}$ gene methylation does not necessarily lead to complete silencing of the gene ([61]). In GAL-01 the emy-methylated status of $p15^{INK4b}$ promoter is not sufficient for the silencing of the gene whereas in GA-10 the complete loss of

transcription is not corresponded by a methylated promoter. Although many genetic alterations and stimuli regulate the mRNA expression of the *INK4a*/*ARF*/*INK4b* locus, less is known about the posttranslational regulation of the proteins. Our data demonstrated that, although transcriptional inactivation was found only for $p16^{INK4a}$, INK/ARF protein expression is broadly impaired in BL cell lines. In addition, the achieved $p16^{INK4a}$ mRNA expression after treatments with demethylating agent was not followed by an equivalent protein re expression. Together these data licit us to infer that post transcriptional mechanisms affecting mRNA and/or protein stability, rather than the recognized gene silencing, might be liable for the simultaneously inactivation of INK4/ARF locus in BL cell lines. In order to test the possibility that the lack of INK/ARF proteins was due to the transcription of an untranslatable mRNA, we respectively analyzed the normally expressed $p15^{INK4b}$ and $p14^{ARF}$ and the 5-aza-dC induced $p16^{INK4a}$ coding sequences. A Normal mRNA, equivalent in length and bases sequence to the corresponding wild types, was found in any cell lines under investigation. The absence of mutation in the coding sequences and their fidelity to a normally translated CDS, assured that the sequences under examination have the potential to be translated in a wildtype protein.

In recent years a large number of non-coding mRNA has been discovered, even though they are not translate in any corresponded proteins, they may have regulatory functioning as regulators of other mRNAs, at transcriptional and post-transcriptional level, and controlling protein ubiquitination and degradation ([102]). Pasmant et al. has ([100]) have recently identified a new large antisense non-coding RNA (named ANRIL) with a first exon located in the promoter of the $p14^{ARF}$ gene and overlapping the two exons of $p15^{INK4b}$, although its rule is still not completely elucidated. In our study we investigated the ANRILL expression pattern in order to clarify its role in BL and to search a potential link between this non-coding RNA and the INK4/ARF proteins deficiency in cell lines that normally express the corresponding mRNAs. We demonstrated that in BL cell lines, as well as in the reported breast and NF1-associated tumors, the transcriptional regulation of ANRIL and INK4/ARF locus genes was correlated, with a stronger positive correspondence between ANRIL and $p14^{ARF}$ than between ANRIL and $p16^{INK4a}$ and $p15^{INK4b}$, in both physiological and pathological conditions. The fact that this correlation was maintained in all the cell lines regardless of protein expression status, allow us to rule out the involvement of this non-coding RNA in regulating INK4/ARF mRNAs stability and translation.

Together these data demonstrate that, although wild type proteins were normally traduced, mechanisms affecting proteins stability may be accountable for the down regulation of INK4/ARF locus products in BL cell lines. The coordinated expression and degradation of intracellular proteins are essential for normal cellular functions. In eukaryotic cells the key proteolytic pathway responsible for proteins degradation is the ubiquitin–proteasome system (UPS) that works in a highly regulated and tightly controlled manner in order to maintain the cellular homeostasis ([103]). Proteasome is a multicatalytic enzyme complex that degrades numerous types of proteins, many of which are regulatory proteins that control the cell cycle or play a role in survival pathways. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including malignant transformation. In this context proteasome represents an attractive therapeutic target in cancer. Since we found that the loss of INK4/ARF proteins in BL cell lines was due to an impaired stability of protein, we analyzed the effect of MG-132 ([104]), a proteasome inhibitor, on $p16^{INK4a}$, $p15^{INK4b}$ and 14^{ARF} proteins in order to assay if their impaired expression in Burkitt`s lymphomas was mediated by deregulation in UPS pathway. Furthermore, in view of the fact that $p16^{INK4a}$ is silenced by aberrant promoter methylation, we assayed the effect of 5-Aza-dC and MG-132 alone and in combination in order to clarify as both UPS and promoter methylation pathways contribute and collaborate in INK4/ARF inactivation. MG-132 stabilized the weakly $p16^{INK4a}$ protein expression achieved following 5-Aza-dC treatment, but it was not able alone to allow protein re expression. These data demonstrated as in BL cell lines $p16^{INK4a}$ was concurrently inactivated through two epigenetic mechanisms, although remain to proven if the aberrant degradation of the protein was the primary inactivation mechanism or a secondary one, in a specific context in which the whole locus was deregulated. Our study demonstrated that in BL cell lines also $p15^{INK4b}$ and $p14^{ARF}$ inactivation was mediated by ubiquitin-proteasome pathway overactivation. Given their chromosomal proximity it is possible that mechanisms still unknown may make the whole locus a target for Ubiquitin-Dependent proteasomal degradation. $p16^{INK4a}$ promoter methylation and proteasome-mediated INK4/ARF proteins degradation were mechanisms selectively activated in a tumor setting and not normally involved in the physiological regulation of the locus, as demonstrated by the unaffected expression of both $p16,^{INK4a}$ $p15^{INK4b}$ and $p14^{ARF}$ in normal B-lymphocytes following treatments. Although promoter hypermethylation and UPS overactivation were tumor-associated

mechanisms, both 5-Aza-dC and MG-132 are wide action ranging drugs and may control the expression of other cellular proteins that in turn might influence INK4/ARF expression pattern. As a matter of fact, this work not only demonstrated the existence of double hit mechanism on $p16^{INK4a}$ inactivation, but also an indirect effect of 5-Aza-dC on UPS pathway, as demonstrated in GAL-01cell line comparing $p14^{ARF}$ expression changes between samples treated with 5-Aza-dC and MG-132 alone and in combination. The synergism between treatments suggested that the demethylating drug might indirectly regulate the expression of some, still unknown, regulator of protein stability. This study provides information regarding the in vitro effects of proteasome inhibitors on BL cell lines, and the molecular mechanisms involved in INK4/ARF locus deregulation. It also gives support to the future use of such inhibitors in the treatment of patients with BL, either administered alone or in combination with 5-AZA-dC.

Considering the highly aggressive nature of Burkitt`s lymphoma we can infer that multiple an concurrent inactivation mechanisms may cooperate in bestowing on the cells supplementary protection against both inhibition of cell cycle progression and c-Myc induced apoptosis, conferring upon the cells a more aggressive phenotype.

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