UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI

PATOLOGIA E DIAGNOSTICA

DOTTORATO DI RICERCA IN

PATOLOGIA ONCOLOGICA E CELLULE STAMINALI

CICLO **XXIII**

Integrated approach to the study of mantle cell lymphoma

S.S.D. MED/08

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INTRODUCTION

Mantle cell lymphoma (MCL) is a unique type of B cell malignancy that includes most cases which were formerly recognized in the European literature as centrocytic lymphoma according to the Kiel classification and which were identified in the American literature under the terms lymphocytic lymphoma of intermediate differentiation, intermediate lymphocytic lymphoma, and finally, mantle zone lymphoma (Weisenburger 1996). The revised European–American classification of lymphoid neoplasms (REAL) and subsequently, the world health organization (WHO) classification of neoplastic disease of the haematopoietic and lymphoid tissues accepted the term MCL as the name for this group of lymphomas (Harris 1994 and 1999). It was described for the first time as a distinct entity in 1982 in the same period by Weisenburger and Rappaport on Cancer and by Palutke et al. on Blood.

MCL constitutes 6% of all non-Hodgkin lymphomas (NHLs) (Jares 2007), it is typically disseminated at presentation, with a leukemic component in 20%-30% of patients. Classic and blastoid variants are recognized, the latter associated with inferior clinical outcome. The genetic hallmark of MCL is the translocation t(11;14)(q13;q32) leading to aberrant expression of cyclin D1, which is not typically expressed in normal lymphocytes. However, cyclin D1–negative cases having typical morphology and gene expression profile have been described and often show over-expression of cyclin D2 or D3 (Rosenwald 2003).

Recently, SOX11 has been described as a diagnostic maker that seems to be equally expressed in D1-positive and D1-negative MCL (Ek 2008). MCL is one of the most difficult to treat B-cell lymphomas. Although conventional chemotherapy induces high-remission rates in previously untreated patients, relapse within a few years is common, contributing to a rather short median survival of 5-7 years. (Martin 2008). One of the best predictor of survival seems to be tumour proliferation (Rosenwald 2003). Intensification of first-line treatment has improved progression-free survival, but no curative regimen has been defined so far (Ghelmini 2009).

CLINICAL MANIFESTATIONS

Most MCL patients have disseminated disease, including generalized lymphadenopathies and bone marrow involvement. Bulky disease and B symptoms are less common (Argatoff 1997, Bosch 1998). Extranodal involvement is almost constant, occurring in more than two extranodal sites in 30–50% of the patients. An extranodal presentation without apparent nodal involvement is observed in only 4–15% of cases. Asymptomatic involvement of the gastrointestinal tract (figure 1) with or without macroscopic lesions is very common, but the detection of this microscopic infiltration rarely modifies the clinical management of the patients (Romaguera 2003, Salar 2006). Central

nervous system involvement occurs in 10–20% of the patients; it usually appears as a late event and is part of a resistant disease or generalized relapse with ominous significance (Montserrat 1996, Ferrer 2008). Other extranodal sites are also commonly involved (Argatoff 1997). Peripheral blood involvement at diagnosis varies among studies, depending partly on the disease definition.

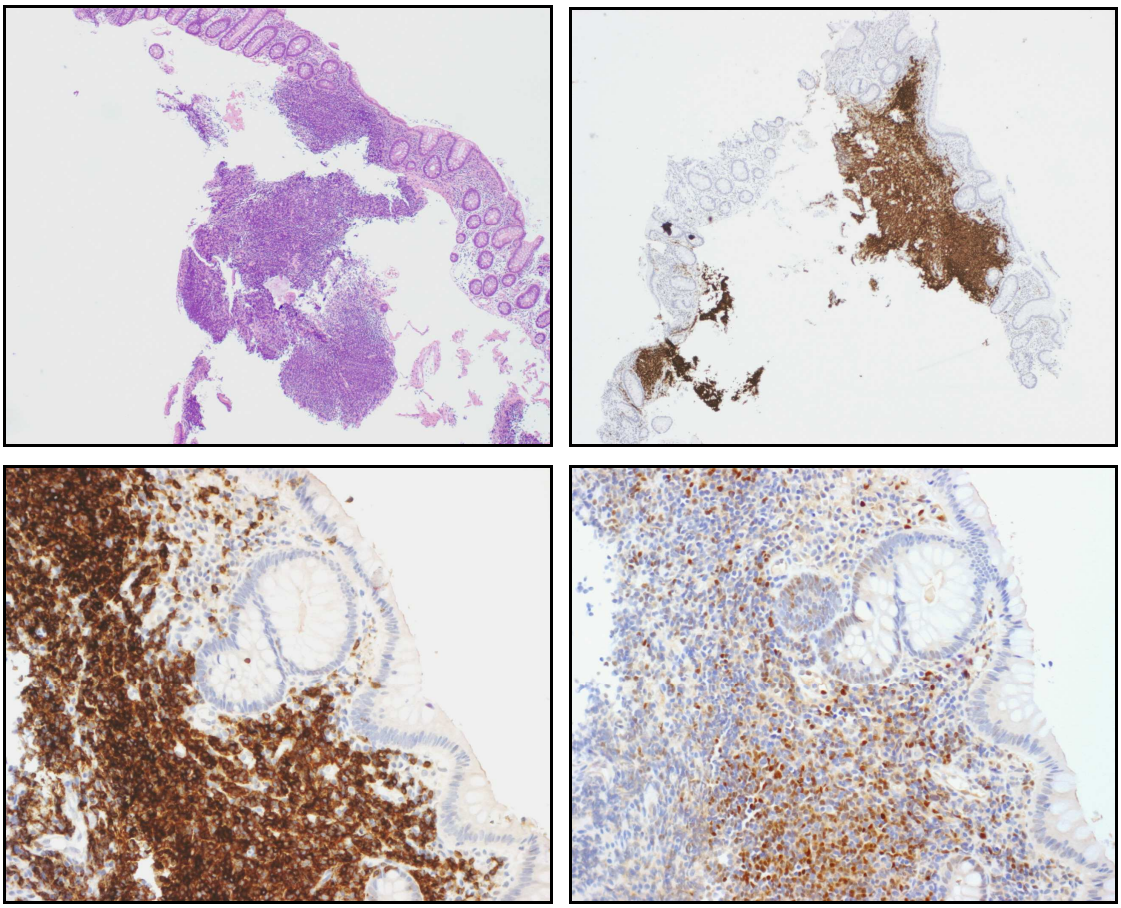


Figure 1. Upper left: Haematoxylin&Eosin (H&E) slide of a big bowel biopsy with MCL localization; upper right: CD20 positive staining; in the lower part of the figure ICH for CD5 on the left and for cyclin D1 on the right.

Atypical lymphoid cells may be observed in the peripheral blood in the absence of lymphocytosis (Pittaluga 1996) and they may be detected by flow cytometry in virtually all the patients (Ferrer 2007). Leukemic involvement may also appear during the evolution of the disease and could represent a manifestation of disease progression. A very aggressive leukemic form mimicking acute leukaemia has been described in a few patients. These cases have blastoid morphology, complex karyotypes, occasionally with 8q24 anomalies, and very rapid evolution with a median survival of only 3 months (Viswanatha 2000). The clinical behaviour of MCL patients is aggressive with a median overall survival (OS) of around 3–4 years. However, recent studies have identified a subset of patients with an indolent lymphoid proliferation and longer survival (5–12 years) even without the need of any treatment, suggesting that the biological behaviour of MCL may be more

heterogeneous that initially thought (Nodit 2003, Orchard 2003). These patients present with a leukemic form usually with splenomegaly, in absence of lymphadenopathies. The tumour cells tend to show small cell morphology and carry somatic hypermutations of the IGHV genes. Unfortunately, although these characteristics are commonly seen in patients with indolent evolution, similar manifestations may occur in patients with a more aggressive clinical behaviour, compromising its diagnostic usefulness.

PATHOLOGY

Morphological and phenotypic characteristics

A better definition of the disease has expanded our knowledge of the morphological spectrum and the phenotypic variations of MCL (Swerdlow 2001). MCL cells in the lymph nodes adopt a mantle zone, nodular (figure 2), or diffuse growth pattern, which might represent different stages of tumour infiltration. The mantle zone growth pattern may be difficult to distinguish from follicular or mantle cell hyperplasia and is characterized by an expansion of the follicle mantle area by neoplastic cells surrounding reactive germinal centres. This pattern is usually seen in areas of partially involved lymph nodes that otherwise show the most common nodular or diffuse involvement by the tumour.

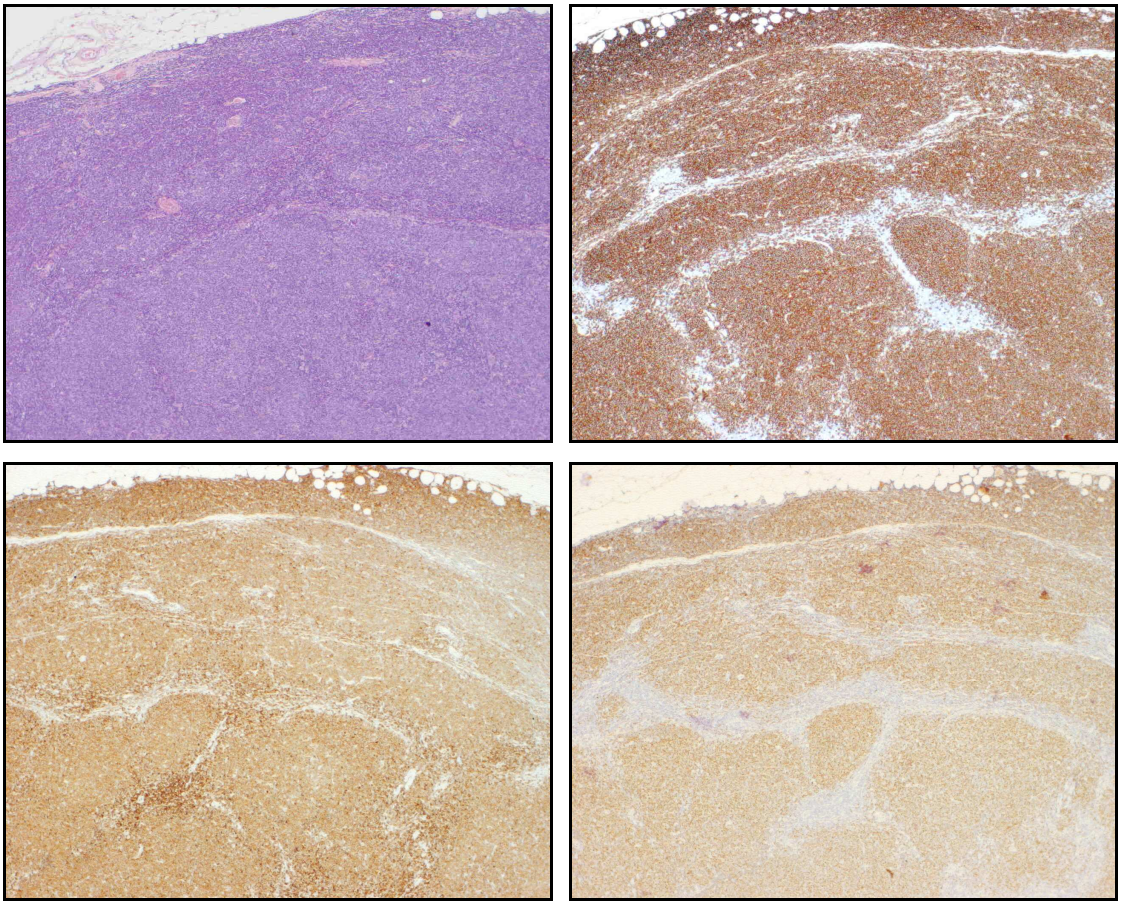


Figure 2: nodular growth of MCL in a lymph node (H&E upper left, strong staining for CD20 in the upper right, CD5 lower left and cyclin D1 lower right.

The observation of this pattern in isolated lymph nodes of occasional patients with an indolent clinical course (Nodit 2003; Espinet 2005), suggest that it represents the initial infiltration of the follicle by the tumour cells. Cyclin D1 staining has revealed that not uncommonly the tumour cells infiltrate the germinal centre of the follicles. These cells may totally replace the lymphoid follicle leading to the nodular pattern that may retain a meshwork of follicular dendritic cells. Later in the course of the disease, invasion and obliteration of internodular areas by neoplastic cells results in a diffuse pattern of growth that it is the more commonly observed in MCL. The MCL has two major cytological variants (figure 3) that are associated with different biological and clinical characteristics, the classic variant occurs in 80–90% of cases and the blastoid variant is present in 10–20% of patients. The classical cytological appearance of MCL is a monotonous proliferation of small/medium cells with irregular nuclei and inconspicuous nucleoli. Some tumours may show small cells with round nuclei mimicking CLL but in these cases the cells do not have the characteristic central nucleoli of the prolymphocytes and paraimmunoblast present in the latter entity. Interestingly, this cytological variant has been frequently recognized in patients with a leukemic and splenomegaly presentation without lymph nodes and a more indolent clinical course (Angelopoulou 2002; Orchard 2003). Although MCL proliferation activity may vary from case to case, it is generally low, with Ki-67 positive cells around 15–30%. The two cytological variants identified as classic blastoid and pleomorphic MCL in the current World Health Organization (WHO) classification are associated with more aggressive clinical evolution. Classic blastoid MCL show an extremely high proliferative activity with numerous mitotic figures, high percentage of Ki-67 positive cells (>40%), and sometimes a ‘starry sky pattern’ similar to Burkitt’s lymphoma. Pleomorphic MCLs are composed of a more heterogeneous population of larger cells; although the proliferation activity is high it is usually lower than in blastoid cases. These tumours are frequently tetraploid and it is not uncommon to observe mitotic figures highly hyperchromatic with an apparent high number of chromosomes (Ott 1997). These cytological variants probably represent the ends of a morphological spectrum and intermediate forms between them may be observed in some tumours in which it may be difficult to decide whether the cytology is classical, blastoid, or pleomorphic (Tiemann 2005). Blastoid variants occur usually de novo and less frequently in patients with previous diagnosis of classical MCL (Norton 1995; Argatoff 1997). Recent data supports the view that blastoid MCL arising in patients with previous diagnosed classical MCL represents histological transformation of the initial neoplastic clone rather than a de novo tumour (Yin 2007).

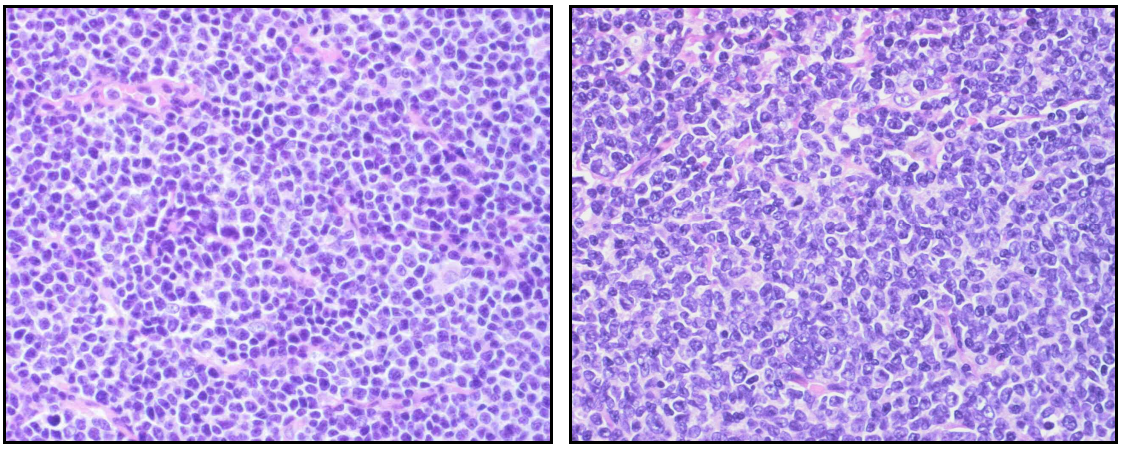


Figure 3: H&E of a classic MCL on the left and blastoid variant on the right.

The phenotype of MCL is relatively characteristic with expression of mature B-cell antigens and co-expression of the T-cell associated antigens CD5 and CD43. The IgM/IgD surface immunoglobulins are usually intense and frequently associated with the lambda light chain, and CD23 is generally negative. Some MCL may show phenotypic variants that may make the diagnosis difficult. Thus, some tumours may be CD5 negative, particularly among blastoid variants, and CD23 may be detected by flow cytometry in a number of cases (Gong 2001; Schlette 2003). Detection of CD8 and CD7 by flow cytometry has been reported in isolated cases of MCL (Hoffman 1998). MCL is usually negative for the follicular germinal centre markers BCL6, CD10, and the plasma cell differentiation antigen MUM1, even if the expression of these antigens has been detected in occasional cases (Camacho, 2004). The clinical and biological significance of these phenotypic variants is currently unclear.

The t(11;14)(q13;q32) translocation (figure 4) is the genetic hallmark of MCL. This translocation is detected by conventional cytogenetics in up to 65% of MCLs. However, using different fluorescent in situ hybridization (FISH) techniques, it can be found in virtually all cases of MCL (Vaandrager 1996). This translocation involves the immunoglobulin heavy chain gene (IGH) at 14q32 locus and a region at 11q13 designated BCL-1. The majority of breakpoints sites at 11q13 occur in a region named the major translocation cluster (MTC). CCND1, encoding cyclin D1, is the closest gene located 120 kb downstream of the MTC locus, and its expression is deregulated by the translocation. Although normal B lymphocytes might express cyclin D2 and D3 (Teramoto 1999), cyclin D1 is not normally expressed in these cells, but it is over-expressed at both mRNA and protein levels in MCL (Bosch 1994; de Boer 1995). Cyclin D1 cloning identified two transcripts of approximately 4.5 and 1.5 kb. Both transcripts contain the whole coding region that codify for a 36 kDa polypeptide (isoform a), but differ in the length of the 3' untranslated region (UTR) (Xiong 1991), that contains an AU-rich element involved in transcript instability (Seto 1992). Some MCL

lack the long mRNA transcript but over-express shorter cyclin D1 transcripts missing the AU destabilizing elements. These shorter transcripts are generated by secondary 3' rearrangement in the CCND1 locus (Bosch 1994; de Boer 1997), or by genomic deletions and point mutations at the 3'UTR (Wiestner 2007). Although the role that these shorter transcripts could have in MCL is not clear, its expression correlates with high levels of CCND1 mRNA, increased proliferation, and poor survival of the patients (Rosenwald 2003; Sander 2005; Wiestner 2007), suggesting that these secondary events in the 3' region of the gene may be important in the progression of the disease. CCND1 also encodes for a less abundant isoform protein generated by an alternative splicing (isoform b) (Betticher 1995). Interestingly, this isoform has been related to a common SNP (G/A 870) that seems to modulate the splicing process of cyclin D1 (Howe & Lynas, 2001). Although this SNP has been associated with increased cancer risk or poor outcome in different tumours (Knudsen 2006), it does not seem to have an impact in MCL (Carrere 2005). In addition, despite the evidences suggesting the tumorigenic role of this isoform (Solomon 2003), its implication in MCL pathogenesis is not clear since human MCL cells mostly express the canonical cyclin D1a isoform and only low levels of the cyclin D1b mRNA (Marzec 2006).

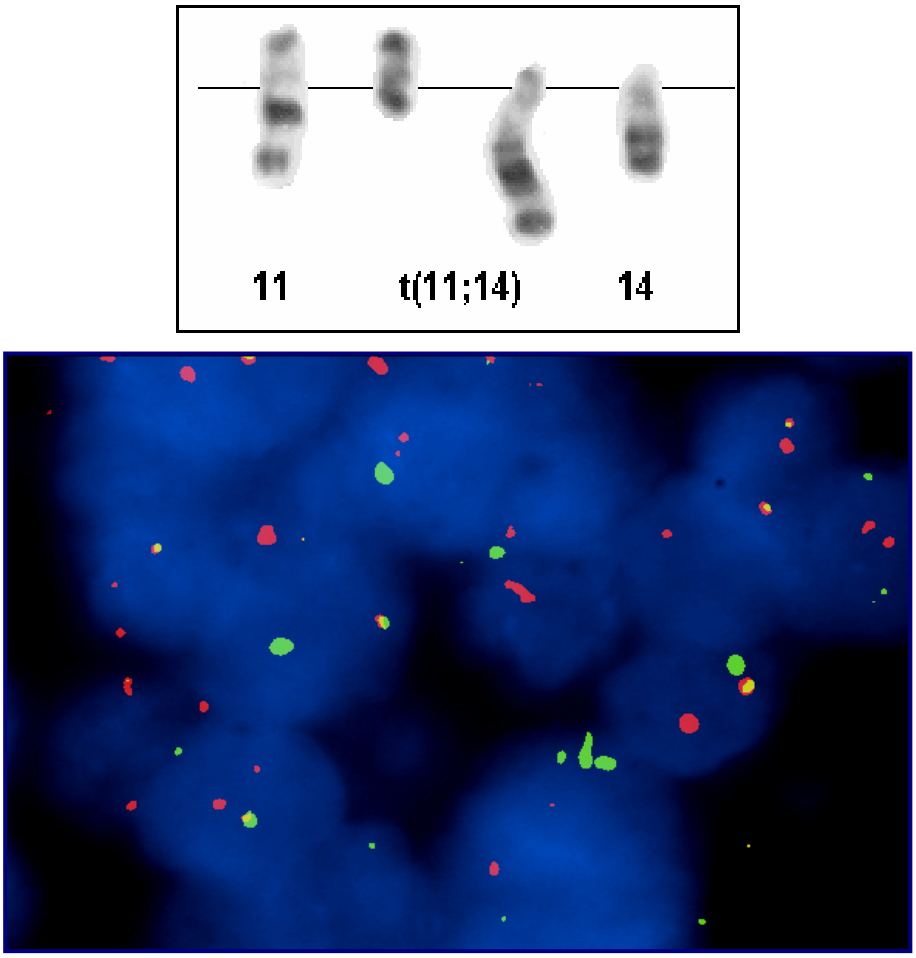


Figure 4: t(11;14) translocation evidenced by classic cytogenetic and FISH analysis.

Cyclin D1 negative MCL

The MCL expression profile analysis has identified rare tumours that, despite being negative for cyclin D1 and the t(11,14), showed a morphology, phenotype, and global expression profile undistinguishable from conventional MCL. These cases seem to have a clinical behaviour and a secondary genetic alteration profile similar to conventional MCL suggesting that they correspond to the same disease. However, only a few of these cases have been reported (Fu 2005; Salaverria 2007). Interestingly, these cases have high expression of cyclin D2 or D3. Although the mechanism deregulating these cyclins is not well understood, a recent report has identified cases carrying a t(2;12)(p12;p13) translocation fusing CCND2 to the kappa light Ig chain gene locus (IGK@) (Gesk 2006). This data suggest that deregulation of other cyclins may be an alternative mechanism to cyclin D1 overexpression in MCL tumorigenesis. The right diagnosis of this MCL cyclin D1-negative variant in routine practice is challenging. Some small B-cell lymphomas, such as marginal zone lymphomas, follicular lymphomas, or small lymphocytic lymphomas, may mimic MCL both morphologically and phenotypically. The differential diagnosis between these tumours and a cyclin D1-negative MCL may be relevant for patient management. Unfortunately, the only reliable criteria to establish the diagnosis of cyclin D1 negative MCL seems to be the microarray profile that it is limited to research environments. The immunohistochemistry detection of cyclin D2 or D3 may not be helpful because these cyclins are also expressed by other types of lymphomas. However, the array study has shown significant higher levels of these cyclins in cyclin D1 negative MCL than in other lymphomas. Therefore, the development of quantitative assays (e.g. qPCR) for the detection of these cyclins may be useful in this differential diagnosis. In the next future, as SOX11 seems to be highly sensible expressed in MCL, we'll have a new chance to make a proper diagnosis in those unusual forms.

MOLECULAR PATHOGENESIS

Several studies have investigated chromosomal alterations in MCL, for example, by conventional comparative genomic hybridization (CGH) (Salaverria 2007, Bea 1999), by array CGH (Tagawa 2005, de Lee 2004, Kohlhammer 2004, Sebradero 2008), and very recently, by single nucleotide polymorphism (SNP) arrays (Vater 2009, Bea 2009, Kawamata 2009). Taken together, these studies identified the most frequently altered chromosomal regions in MCL, including gains of chromosomal material in 3q, 7q, and 8q and losses in 1p, 6q, 8p, 9p, 9q, 11q, 13q, and 17p. In addition, these studies have narrowed down some of the minimal regions affected by genomic copy number alterations (CNAs) in MCL and have led to the suggestion of putative target genes of these chromosomal abnormalities, with particular emphasis on genes involved in cell cycle regulation and DNA damage response pathways. Furthermore, recent SNP array studies of MCL cell lines and

small series of primary MCL samples revealed recurrent regions of copy number neutral loss of heterozygosity (CNN-LOH; also referred to as acquired/partial uniparental disomy [UPD]) that may represent an alternative mechanism to an allelic deletion in the process of a tumor suppressor gene inactivation because the CNN-LOH regions appear to cluster in chromosomal locations that are also frequently affected by deletions (Vater 2009, Bea 2009, Kawamata 2009, Nielander 2006).

Previous copy number profiling studies in MCL were performed on relatively small numbers of primary tumour specimens, used low resolution techniques, or did not include accompanying gene expression profiling experiments or survival data. Recently Hartman et al (Blood 2010) present high-resolution gene expression and 500K SNP array data (mean intermarker spacing, 5.8 kb) from 77 primary MCL samples, including 72 cyclin D1–positive and 5 cyclin D1–negative MCLs with available survival data representing the largest MCL series studied to date. Using an integrated analysis approach, gene expression and dosage integrator (GEDI), which was recently developed (Lentz 2008) they refine the minimal regions of recurrent CNAs and CNN-LOH in MCL and identify novel putative target genes and pathways that might be pathogenetically relevant and show an association with the clinical outcome. Several genes of the Hippo signaling pathway exhibit altered expression in MCL and may therefore deserve more detailed future studies.

How does cyclin D1 contribute to MCL lymphomagenesis?

Cyclin D1 plays an important role in the cell cycle regulation of G1-S transition following mitotic growth factor signalling (figure 5). Cyclin D1 binds to CDK4 and CDK6 to form a CDK/cyclin complex able to phosphorylate the tumour suppressor gene retinoblastoma (RB1) facilitating cell cycle progression. RB1 plays a master role in the G1-S transition by sequestering and inactivating E2F transcription factors involved in the transactivation of essential genes required for S phase entry and DNA replication, including cyclin E (Harbour & Dean, 2000). The initial phosphorylation of RB1 by cyclin D1/CDK4-6 will initiate the release of E2F transcription factors promoting the accumulation of Cyclin E/CDK2 complexes that will produce the irreversible inactivation of RB1 and the subsequent progression into S phase. Thus, cyclin D1 over-expression would contribute to the lymphomagenesis in MCL by overcoming the suppressor effect that retinoblastoma performs in the G1/S transition. In that sense RB1 seems to be normally expressed in the majority of MCL cases and the protein appears to be hyperphosphorylated (Zukerberg 1996), particularly in highly proliferative blastic variants (Jares 1996). The different CDK/cyclin complexes are tightly regulated by the action of two families of CDK inhibitors. The INK4 family is specific for CDK4/6. However, the Cip/Kip family, which includes p27 among others, shows a broad CDK inhibition activity targeting all the different CDK/cyclin complexes (Sherr & Roberts, 1999). Moreover, the Cip/Kip proteins appear to have an important role in the formation of active CDK4/cyclin D

complexes (LaBaer 1997). This property seems to be responsible for the p27 titration into CDK4/cyclin D1 complexes by deregulated cyclin D1, preventing the p27-dependent inactivation of CDK2/cyclin E and G1 cell cycle arrest. Moreover, the presence of active CDK2/cyclin E complexes will phosphorylate p27, targeting it for ubiquitination and proteosome degradation allowing cell cycle progression (Montagnoli 1999). In NHL other than MCL, p27 protein expression is inversely related to the proliferation activity of the tumours. However, in MCL p27 is detected immunohistochemically mainly in blastic variants (Quintanilla-Martinez 1998). The mechanism responsible for this p27 pattern in MCL is not clear but may imply both an increased p27 protein degradation by the proteosome pathway (Chiarle 2000) that in a subset of cases may be related to the accumulation of SKP2 (Lim 2002), and the sequestration of p27 protein by the over-expressed cyclin D1, rendering it inaccessible to antibody detection (Quintanilla- Martinez 2003). Cyclin D1 may also have an oncogenic potential independently of its CDK cell cycle regulatory function. For example, Cyclin D1 has been shown to regulate a number of transcription factors and transcriptional co-regulators, including STAT3, C/EBPb, and B-MYB among others, which in some cases appears to occur independent of CDK4-binding activity (Coqueret 2002). However, it is not known if this transcriptional regulation function is present in MCL cells.

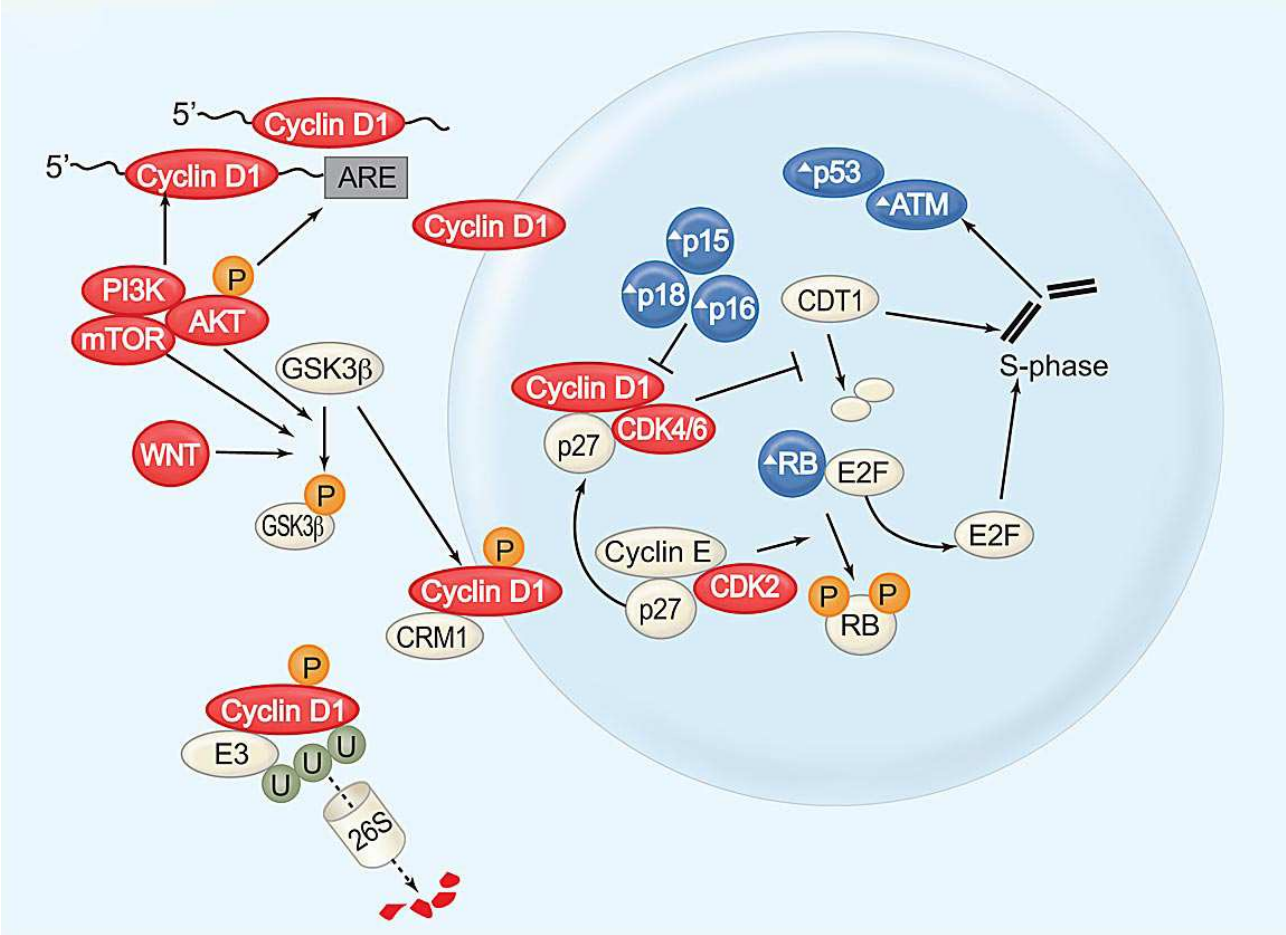


Figure 5: Cyclin D1 pathway and interactions (Perez-Galan 2011).

Secondary molecular events contributing to the pathogenesis of MCL

Several experimental observations suggest that cyclin D1 deregulation, although important for MCL initiation, may not be responsible for the complete cell transformation. Transgenic mice that over express cyclin D1 did not develop a spontaneous lymphoma, and cooperation with other oncogenes like MYC were required for lymphomagenesis (Lovec 1994). Further, a mouse model expressing a constitutively nuclear cyclin D1 in murine lymphocytes developed mature B-cell lymphomas carrying alterations similar to blastoid variants of MCL, including deregulation of the ARF/ MDM2/p53 pathway and BCL2 over-expression (Gladden 2006). These results suggest that the development of tumours requires additional oncogenic events, despite the highly lymphomagenic effect of nuclear restricted cyclin D1 expression. In addition, the identification of the t(11;14) translocation in blood cells of 1–2% of healthy individuals without evidence of disease (Hirt 2004) supports the need for additional oncogenic events in the progression of MCL. Genetic studies have revealed that MCL is one of the malignant lymphoid neoplasms with the highest level of genomic instability (Salaverria 2006), with blastoid variants having more complex karyotypes. In addition to frequent chromosomal imbalances, tetraploidy occurs frequently in pleomorphic (80%) and blastic variants (36%) (Ott 1997). Although the pathogenesis of this phenomenon is not well understood, tetraploid cases contain more centrosome anomalies (Kramer 2003) and over-express centrosome-associated genes (Neben 2007). Preliminary genomic studies of MCL have identified uniparental disomies (UPD) in regions similar to the ones commonly deleted (Nielaender 2006; Rinaldi 2006). These results would support the view of UPD as an alternative mechanism to inactivate tumour suppressor genes (Fitzgibbon 2005). The identification of the genes targeted by the described chromosome abnormalities has disclosed that most of them are involved in two common pathogenetic pathways, the cell cycle machinery and the cellular response to DNA damage (Fernandez 2005). However, genes implicated in cell survival might also be involved in MCL lymphomagenesis (see below).

Deregulation of cell cycle

Highly proliferative and clinically aggressive MCL carry oncogenic alterations in two major regulatory pathways, INK4a/CDK4/RB1 and ARF/MDM2/p53, which are involved in cell cycle control and senescence. Homozygous deletions of the CDKN2A locus on 9p21 have been detected in 20–30% of blastoid variants but in less than 5% of typical cases (Dreyling 1997, Pinyol 1997). Inactivation of this locus by hypermethylation occurs in other lymphomas but this phenomena seems uncommon and of uncertain significance in MCL (Hutter 2006). This locus encodes for two key regulatory elements, the CDK4 inhibitor INK4a and the p53 regulator ARF. The presence of INK4a deletion may cooperate with cyclin D1 deregulation, promoting G1/S-phase transition in

MCL cells by increasing the amount of active cyclin D1/CDK4 complexes. A pathogenetic mechanism alternative to INK4a deletion might be the amplification and overexpression of BMI1, a transcriptional repressor of the CDKN2A locus (Jacobs 1999, Bea 2001). Inactivation of other members of the INK4 family, such as CDKN2B and CDKN2C, occur by homozygous deletion in cell lines and in occasional MCL cases (Williams 1997, Mestre-Escorihuela 2007). The identification of CDK4 amplification in some aggressive blastoid MCL strengthens the significance of the G1/S transition deregulation during MCL progression (Hernandez 2005). These gene amplifications occurred almost exclusively in MCL with a wild-type CDKN2A locus, suggesting that CDK4 amplifications are another alternative mechanism of disrupting the RB1-dependent G1/S phase control. Interestingly, early studies of MCL did not identify alterations of this tumour suppressor gene. However, inactivating microdeletions of RB1 have recently been described in some highly proliferative MCL cases (Pinyol 2007). Similarly to the amplification of CDK4, these alterations occurred in cases with a wild-type CDKN2A locus supporting the idea that oncogenic alterations of more than one member of the INK4a/CDK4/RB1 pathway do not seem to provide additional biological advantage for the tumour. The homozygous deletion of the CDKN2A locus in MCL usually also involves ARF, the main function of which is to stabilize the p53 protein by preventing its MDM2-mediated degradation. The homozygous deletion of this locus determines the simultaneous deregulation of the cell cycle and the p53 pathway. TP53 itself is frequently targeted by genetic alteration in MCL patients. Although TP53 mutations are rarely observed in classical low proliferative MCL they are identified in approximately 30% of highly proliferative blastoid MCL, usually associated with 17p deletion (Greiner 1996, Hernandez 1996). An alternative mechanism to p53 inactivation may be the overexpression of MDM2 that it is detected in a small subset of MCL cases (Hartmann 2007). However, the mechanism driving this MDM2 upregulation is not known. Although, inactivation of TP53 occurs in tumours with wild-type CDKN2A locus, it is associated with CDK4 amplification or RB1 deletions suggesting that the tumours cells may obtain a selective advantage inactivating both ARF/MDM2/p53 and INK4a/CDK4/RB1 pathways. The simultaneous inactivation of these pathways may occur by homozygous deletion of the CDKN2A locus, BMI1 amplification, or by TP53 mutation with concomitant CDK4 amplification or RB1 deletion (Hernandez 2005, Pinyol 2007).

DNA damage response pathway dysfunction

The identification of a high number of chromosome aberrations suggest that alterations of the mechanisms involved in genome stability, such as DNA damage response pathways, may be important for MCL pathogenesis. In this sense, the ataxia telangiectasia mutated gene (ATM) that plays an essential role in the cellular response to DNA damage is located in 11q22-23, a frequently

deleted locus in MCL (Stilgenbauer 1999).ATM mutations have been described in 40–75% of MCL usually associated with the deletion of the wild type allele (Camacho 2002, Fang 2003). ATM is required for the activation of p53 in response to DNA damage and participates during normal immunoglobulin V-D-J recombination in B cells promoting the repair of double strand break lesions (DSBs) (Perkins 2002). A deficient response to DSBs in lymphoid cells might produce genomic instability facilitating the development of lymphomagenic alterations (Kuppers & Dalla-Favera 2001). In fact, ATM inactivation in MCL is associated with a high number of chromosomal alterations suggesting that its deregulation is important for the accumulation of chromosomal aberrations (Camacho 2002). ATM alterations occur in classical and blastoid MCL variants independently of the proliferation activity and do not appear to be associated with any clinical behaviour or prognosis of the patients, suggesting that ATM inactivation may occur early during MCL lymphomagenesis. The finding of heterozygous germ line ATM mutations in MCL patients in which tumour cells subsequently lost the wild-type allele would support the idea that these alterations might represent a predisposing event in these neoplasms. Downstream ATM targets, such as CHEK2 and CHEK1, are also occasionally deregulated in MCL. Decreased protein levels and mutations of CHEK2 have been described in a subset of MCL with a high number of chromosomal imbalances. Similarly to what happen with ATM, CHEK2 mutations have been observed in the germline of some MCL patients suggesting that these mutations might predispose to the development of the tumours (Hangaishi 2002, Tort 2002). CHEK1 protein is downregulated in occasional cases of MCL, but no mutations of the gene have been detected (Tort 2005). The identification of an altered expression of DNA replication licensing factors in MCL with high number of chromosome abnormalities and checkpoint defects suggest that the failure to appropriately regulate DNA replication could facilitate the accumulation of chromosome aberrations in MCL with a compromised DNA damage response (Pinyol 2006).

Cell survival pathways in MCL

Additional molecular events (figure 6) that mainly deregulate survival and apoptosis mechanisms seem to contribute to the MCL oncogenesis. Amplification of the antiapoptotic BCL2 and homozygous deletions of BCL2L11, a member of the BH-3 only family with pro-apoptotic activity, have been described in several MCL cell lines, but the importance of these alterations in primary MCL is not clear (Tagawa 2005). Overexpression of MCL1, an antiapoptotic protein of the BCL2 gene family, has been associated with blastoid variants (Khoury 2003). Deletions of 8p21·3, targeting two members of the tumour necrosis factor receptor superfamily (DR4 and DR5), also have been reported in a subset of MCL (Rubio-Moscardo 2005), but they do not seem to correlate with protein down regulation (Roue 2007). The expression of two tumour necrosis family receptors,

CD40 and FAS, which may transduce survival or death signals respectively in B cells, are deregulated in some MCL patients (Clodi 1998, Rummel 2004). Moreover, constitutive activation of the nuclear factor-jB (NFjB), which regulates expression of various genes involved in both survival and apoptotic signalling pathways, has been detected in MCL cell lines and primary tumours with over-expression of downstream targets, such as FADD-like apoptosis regulator or B-lymphocyte stimulator (Martinez 2003, Pham 2003, Fu 2006, Roue 2007). The activation of the AKT survival pathway in MCL (Rizzatti 2005), particularly in blastoid cases, associated with the loss of PTEN expression (Rudelius 2006), and the activation of the downstream mTOR pathway might also confer to MCL cells higher proliferation and survival capacity (Peponi 2006). The tyrosine kinase SYK involved in the B-cell receptor signalling pathway and activation of AKT and NFkB in B cells is over-expressed in a subset of MCL due to genomic amplification (Rinaldi 2006).

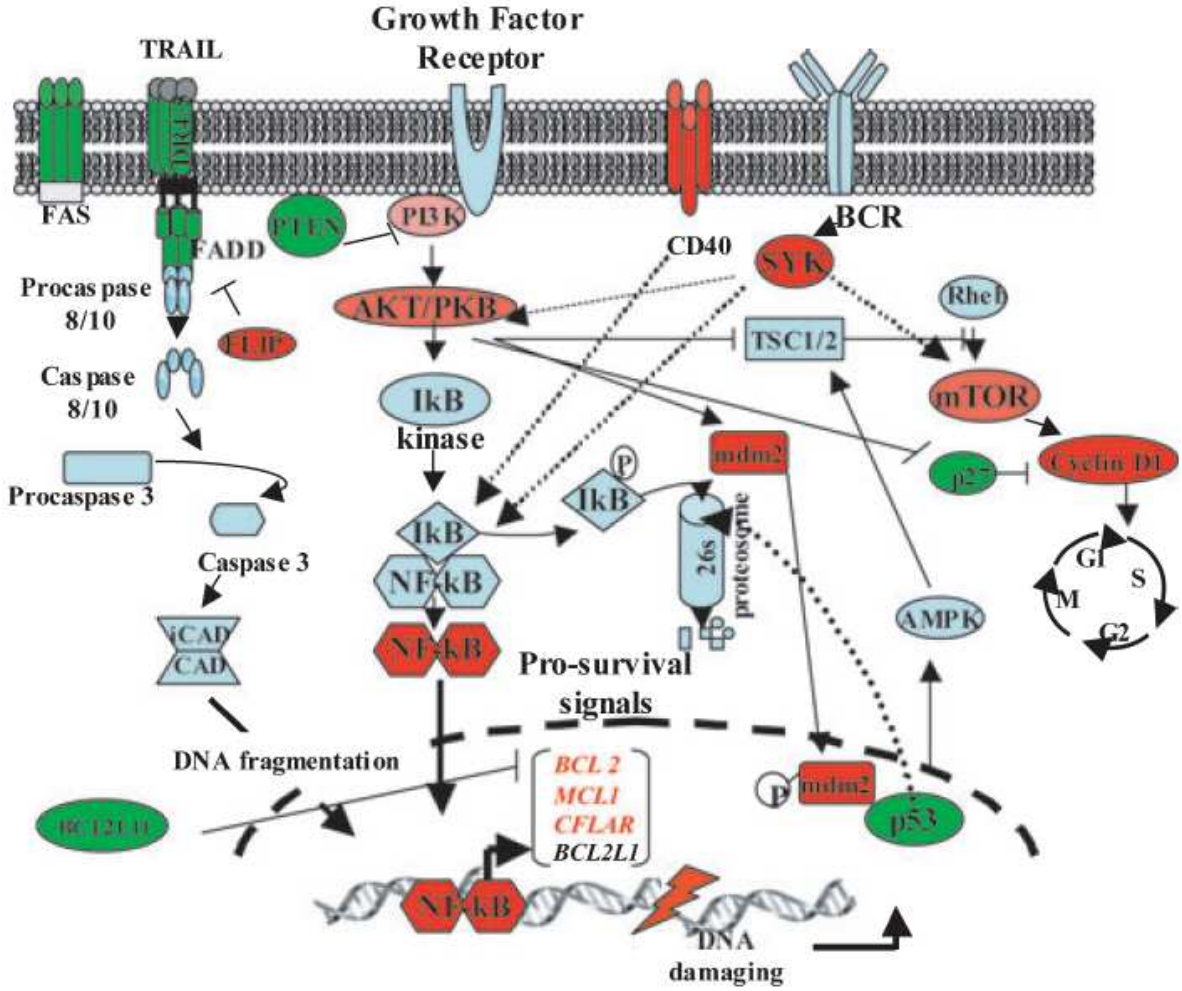


Figure 6 (Campo 2008). Genetic alterations, such as amplifications and over-expression of genes, that will promote cell survival are shown in red, while deregulation (such as deletion or downregulation) of genes raising pro-apoptic signals are represented in green. MCL cells show activation of AKT, NFjB, and mTOR pathways resulting in cell cycle progression and resistance to apoptosis. The upregulation of pro-survival signals like BCL2, MCL1, or FLIP, together with the downregulation of proapoptotic genes, such as BCL2L11, TNFRSF10A (DR4) and TNFRSF10B (DR5), FAS, and FADD among others will facilitate the survival of MCL tumour cells.

NOVEL TREATMENT APPROACHES IN MCL IN CLINICAL DEVELOPMENT

As long as many different pathways turned out to be involved in the pathogenesis of MCL, many new target treatment are recently being tested either with in vitro than in vivo studies (table 1).

Drug class	Drugs	Mechanism of action and effects on MCL biology	Clinical development and results
Proteasome inhibitors	Bortezomib (CT-L),[48-50] NPI-0052 (CT-L, T-L, C-L),[59] PR-171 (CT-L),[60] MLN9708 (CT-L)[61]	Reversible or irreversible inhibition of ≥ 1 proteasome activities; cell-cycle arrest and induction of apoptosis through oxidative and ER stress–mediated up-regulation of NOX	Bortezomib (phase 2): ORR 33%-58% in relapsed/refractory MCL; NPI-0052 and PR-171 (phase 1); MLN9708 (phase 1)
CDK inhibitors	Flavopiridol (pan-CDK),[63] PD0332991 (CDK4/6)[62]	Flavopiridol: pan-CDK inhibitor, decreased RNA stability; PD0332991: blocks cyclin D1/CDK4, leading to cell-cycle arrest	Flavopiridol (phase 1, 2): minimal responses as a single agent, modified dosing schedules may be more effective; PD0332991(phase 1)
Serine/threonine and tyrosine kinase inhibitors	Enzastaurin (PKC-β II),[66] fostamatinib (SYK),[65] PCI-32765 (BTK)[67]	Inhibition of BCR signal transduction cascade	Mostly stable disease for fostamatinib and enzastaurin; objective responses in phase 1 study of PCI-32765
PI3K/AKT inhibitors	CAL-101 (PI3Kδ),[73,131] ON1910.Na (multikinase /PI3Kα),[72] perifosine (AKT)[75]	Inactivation of AKT and mTOR; cell-cycle arrest, cyclin D1 down-regulation; activation of p53 and BAD-mediated apoptosis	CAL-101 (oral agent, phase 1): PRs in MCL and CLL; ON-01910.Na (phase 1); Perifosine (phase 1)
mTOR inhibitors (rapalogs)	Rapamycin, temsirolimus (CCI-779),[79-81] everolimus (RAD001),[83,132] deforolimus (AP23573)[78]	Partial allosteric TORC1 inhibition; cell-cycle arrest; inconsistent effects on expression of cell-cycle regulators (cyclin D1, p21, p27); may induce autophagy but not apoptosis	Temsirolimus (phase 2 and 3): ORR 30%-40% in relapsed patients, mostly PRs of rapid onset (median, 1 mo), median duration of response of 6.9 mos; everolimus (phase 1 and 2): some objective responses; deferolimus (phase 2): 30% PR and 40% SD
BH3 mimetics	*ABT-263,[103] AT-101,[104] obatoclax (GX15-070)[105]	Inhibition of antiapoptotic members of BCL-2 family, BCL-2, BCL-X_L; GX 15-070 inhibits also MCL-1; mitochondrial depolarization through release of BH-3–only proteins and BAX/BAK activation	Obatoclax (phase 1 and 2) with or without bortezomib; AT-101 (phase 2); ABT-737 (phase 1 and 2)
IMIDs	Lenalidomide,[113,114] thalidomide[115]	Modulation of immune response; microenvironment or direct effect on tumor cells	Lenalidomide (phase 2): ORR 42%-53%, CR 20%, PFS 5.6 mos in relapsed MCL
HDAC inhibitor	Vorinostat (SAHA)[119,120]	Cyclin D1 down-regulation, p21 and p27 up-regulation, and cell-cycle arrest; up-regulation of the BH3-only proteins BIM and BMF	SAHA (phase 1 and 2) with or without bortezomib
HSP90 inhibitors[122]	Ansamycins: 17-AAG, 17-DMAG: IPI-504; synthetic: SNX-5422, KW-2478	Degradation of client proteins, including cyclin D1, CDK4, IKKβ, AKT, c-MYC, and c-RAF[133]; cell-cycle arrest; activation of the mitochondrial apoptotic pathway	17-AAG (phase 2): mostly disease stabilization, with or without bortezomib; 17-DMAG clinical development stopped; IPI-504: water-soluble 17-AAG derivative; synthetic inhibitors: entering early stage testing

Information on ongoing clinical trials at is available at http://www.clinicaltrials.gov.

CT-L indicates chymotrypsin-like; T-L, trypsin-like; C-L, caspase-like; ORR, overall response rate; ER, endoplasmic reticulum; PR, partial response; SD, stable disease; CR, complete response; and PFS, progression-free survival.

*ABT-263 is the oral formulation of the compound used in clinical trials; ABT-737 is the equivalent compound typically used in preclinical studies.

Table 1. Novel treatment approaches in MCL in clinical development (Perez-Galan 2011).

Proteasome inhibitors

The ubiquitin-proteasome system, the main nonlysosomal pathway through which intracellular proteins are degraded, exhibits 3 proteolytic activities: chymotrypsin-like (CT-L), trypsin-like, and caspase-like, each localized to a distinct 20S proteasome β subunit. Bortezomib, a peptide boronic acid that reversibly inhibits primarily the CT-L activity, is the first proteasome inhibitor approved by the Food and Drug Administration (FDA). Phase 2 clinical trials of single-agent bortezomib showed durable responses in 33%-58% of patients with relapsed or refractory MCL (Goy 2009 and

2005, O'Connor 2009). Bortezomib is now increasingly combined with other agents. Interestingly, some studies suggest a sequence-dependent synergism with chemotherapy (Weigert 2007).

A second generation of proteasome inhibitors aiming for continued activity against bortezomib-resistant cells, simplified dosing schemes, and reduced toxicity is now entering the clinic. NPI-0052 (salinosporamide A), a natural product related to lactacystin, targets all 3 catalytic sites (CT-L, trypsin-like, and caspase-like), provides more potent and more durable proteasome inhibition and is active against bortezomib-resistant MM cells (Chauhan 2005). PR-171 (carfilzomib), a modified peptide related to the natural product epoxomicin, selectively and irreversibly disables the CT-L activity, which may explain its greater cytotoxicity (O'connor 2009). MLN9708, a second-generation peptide boronic acid derivative, hydrolyzes to MLN2238, the biologically active form that reversibly blocks CT-L activity, and has shown preclinical efficacy in hematologic malignancies (Kupperman 2010) .

Cyclin D1, cell-cycle control, and cell-cycle inhibitors

Cyclin D1 and cell-cycle control appear as natural targets in MCL. Two basic strategies have been pursued: down-regulation of cyclin D1 expression and inhibition of CDK function. PI3K and mTOR inhibitors can inhibit cyclin D1 translation and promote its degradation, whereas PD0332991 blocks cell-cycle progression through potent inhibition of CDK4/6 (Marzec 2006, Leonard 2008). The pan-CDK inhibitor flavopiridol, whose mechanism of action involves inhibition of RNA synthesis, has minor clinical activity as a single agent, but a modified dosing regimen has shown considerable activity in CLL (Lin 2009) and is currently under investigation in MCL.

BCR pathway

Recent studies reported constitutive activation of the BCR signal transduction components SYK and PKCβ II (Boyd 2009, Rinaldi 2006) SYK was amplified in both Jeko-1 cells and some primary MCL samples, but constitutive activity of SYK was only shown in the cell line (Rinaldi 2006). Jeko-1 cells were more sensitive to an SYK inhibitor than MCL cell lines without constitutive SYK activation, indicating some dependence on the pathway. In a screen for phosphoproteins, PKCβ II was found to be phosphorylated in primary MCL samples in contrast to normal B cells (Boyd 2009). However, it was not localized to lipid rafts as would be expected after classic BCR activation. Inhibitors targeting the signaling cascade downstream of the BCR have entered clinical testing. Fostamatinib, an inhibitor of SYK, achieved a 55% response rate in CLL, but only 1 in 9 patients with MCL responded (Friedberg 2010). Enzastaurin, a PKCβ inhibitor, induced no objective responses in MCL (Morshhouser 2008). More recently, a phase 1 study of the BTK inhibitor PCI-327 (Friedberg 2010) reported objective responses in a few patients with MCL

(Advani 2010). Thus, a role for BCR signaling in MCL pathogenesis and the possible clinical benefit of agents targeting this pathway deserve further investigation.

PI3K/AKT/mTOR pathway

The PI3K/AKT pathway is involved in the transduction of a variety of extracellular signals and plays a prominent role in many cancers (Engelman 2009). In normal B cells, PI3K functions as a transducer of BCR signaling that regulates proliferation, differentiation, apoptosis, and survival. Gene expression profiling implicated the PI3K/AKT pathway in the pathogenesis of MCL (Rizzatti 2005), and several key components of the PI3K/AKT/mTOR pathway are activated in MCL (Peponi 2006) indicating a possible contribution of this pathway to MCL pathogenesis. Constitutive activation of AKT was found in most blastoid and many classic MCL tumours and was associated with the phosphorylation of downstream targets, including MDM2, Bad, and p27 (Dal Col 2008, Rudelius 2006). Furthermore, AKT mediated activation of mTOR, and its downstream targets S6K and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) can increase translation of key proteins.

Several mechanisms may cause constitutive activation of AKT, including activation of upstream kinases such as SYK, and amplification of PI3KCA, the gene encoding the catalytic subunit p110 (Psyrri 2009). In contrast to solid tumours, no activating somatic mutations of PI3KCA have been identified (Rudelius 2006, Psyrri 2009). Loss of PTEN, a phosphatase that turns the PI3K pathway off, is another recurrent feature in MCL and may be the result of mutations, deletions, or promoter methylation. PTEN can also be inactivated by phosphorylation at serine 380 and threonine 382/383, which has been found in MCL cases with constitutively active AKT (Dal Col 2008). The mechanism of PI3K/AKT/mTOR activation can determine the therapeutic potential of small molecule inhibitors. In tumours addicted to an upstream kinase such as SYK, PI3K/AKT signaling can effectively be turned off by inhibition of the upstream kinase. In contrast, in tumours with constitutive activation of PI3K or AKT, inhibitors directed at these kinases may be required.

NF-kB pathway

The NF-kB family of transcription factors, (p50/p105, p65/RELA, c-REL, RELB, and p52/p100) binds DNA as heterodimers and homodimers that activate the transcription of genes involved in survival, proliferation, and apoptosis (Karin 2002). The canonical pathway is activated through phosphorylation and consequent degradation of IB, a cytosolic inhibitor that sequesters p50 and RELA. Constitutive activation of the canonical NF-kB pathway has been reported in MCL cell lines evidenced by the presence of pIB and nuclear p65, p50, and c-REL. Gene-expression profiling of MCL patient samples showed frequent high expression of NF-kB target genes that correlated with strong pIB expression on tissue microarrays (Martinez 2003, Tracey 2005). NF-kB target genes

highly expressed in MCL include the antiapoptotic proteins BCL-2, BCL-XL, XIAP, and cFLIP (Pham 2003, Roue 2007). Activation of the noncanonical pathway (p52, RELB) has been reported in some MCL cell lines (Granta-519, JVM-2, and NCEB), but this could be due to Epstein-Barr virus transformation. BCR engagement and TNF signaling in the lymphoma microenvironment may contribute inducible NF-kB activation. NF-kB signaling can also drive transcription of the TNF family member BAFF/BlyS, a potent B-cell survival factor that binds BAFF-R, BCMA, and TACI. BAFF in turn stimulates both canonical and alternative NF-kB pathways, activating a positive feedback loop that could contribute to tumour cell survival (Fu 2006). Recently, BAFF-R has been detected in the nucleus of MCL cells, where it colocalized with inhibitor of nuclear factor B kinase β (IKKβ) and cooperated in histone H3 phosphorylation. BAFF-R associated with c-REL, leading to an increase transcription of genes that promote cell survival and proliferation (Fu 2009). Constitutive activation of NF-kB signaling may also be caused by the inactivation of TNFAIP3/A20, a ubiquitin-editing enzyme that acts as a negative regulator. TNFAIP3/A20 is often inactivated in MCL through genomic deletions, mutations, and increased promoter methylation Honma 2009). Moreover, MCL cases showed mono- and bi-allelic deletions of FAF1, which inhibits p65 and IKKβ (Bea 2009).

IKK inhibitors

In keeping with the role of NF-kB in MCL survival, inhibitors of this pathway, such as BAY-117082, curcumin, or the IKKβ inhibitor BMS-345541, have shown in vitro activity in MCL (Pham 2003, Roue 2007, Shishodia 2005). However, despite its preclinical promise it has apparently proven difficult to translate this class of compounds into the clinic, and except for curcumin no clinical trials of these compounds are currently ongoing.

Proteasome inhibition to block NF-kB signaling

Inhibition of the NF-kB pathway has initially been hypothesized as mechanisms of bortezomib-induced apoptosis. However, in MM it has recently been shown that bortezomib efficiently inhibits inducible but not constitutive NF-kB activity and can even cause canonical NF-kB activation (Hideshima 2009). Proteasome inhibitor–resistant NF-kB activity has also been described in MCL (Yang 2008). However, in activated B cell–like diffuse large B-cell lymphoma, an aggressive lymphoma having constitutive activation of NF-kB, bortezomib appeared to sensitize cells to combination chemotherapy (Dunleavy 2009), suggesting that NF-kB inhibition may play a role in some tumours but not others.

WNT pathway

WNT signaling can occur through two main mechanisms, a canonical pathway through β-catenin and an alternative pathway leading to c-Jun N-terminal kinase activation. The canonical pathway is

considered to be more relevant in cancer (Angers 2009). Microarray analysis provided a first indication that the WNT pathway may be activated in MCL (Rizzatti 2005). Recently, nuclear expression of transcriptionally active β-catenin has been detected in MCL cell lines and tumour biopsies and correlated with GSK3β inactivation (Chung 2010). WNT3 and WNT10a are consistently expressed in MCL cell lines and patient biopsies, and amplification of WNT11 on 11q13.4-q13.5 has been found in MCL cell lines. High expression of several Frizzled receptors and low density lipoprotein receptor-related protein 5 has been documented, indicating a possible autocrine loop of WNT signals. Furthermore, the serine-threonine kinase CK2, which is constitutively phosphorylated in MCL (Cecconi 2008), phosphorylates β-catenin at Thr393, thereby impeding its proteasomal degradation. The resultant stabilization of β-catenin increases its transcriptional activity. Inhibition of the canonical pathway by small interfering RNA mediated knockdown of DVL2 and Frizzled 2 or by the pharmacologic agent quercetin reduced proliferation and induced apoptosis (Ortega 2008, Gelebart 2008).

Hedgehog pathway

Recent studies have suggested that stromally produced hedgehog proteins Indian hedgehog, sonic hedgehog (SHH), and desert hedgehog have a role in the proliferation of hematopoietic stem cells, lymphoid cells, and in B-cell malignancies (Dierks 2007). The molecules associated with SHH signaling and its target transcripts PTCH, GLI1, and GLI2 are over-expressed in MCL primary cells compared with normal B cells. Interestingly, GLI is located at 12q13 and may be coamplified with CDK4 and MDM2 in some tumours (Sander 2008). Activation of PTCH by SHH may increase proliferation of MCL cells, an effect that could be inhibited by cyclopamine, a specific inhibitor of SHH-GLI signaling. Furthermore, down-regulation of GLI transcription factors with the use of antisense oligonucleotides significantly reduced cyclin D1 and BCL-2 transcript levels, decreased proliferation of MCL cells, and increased their susceptibility to chemotherapy (Hedge 2008). These results indicate a role for SHH-GLI signaling in MCL proliferation and identify the hedgehog pathway as a potential therapeutic target in MCL.

Antiapoptotic BCL-2 family proteins

BCL-2 family proteins are key regulators of apoptosis, determining cellular fate in response to numerous insults (Adams 2007). The BCL-2 pathway is deregulated in MCL; high-level copy number gains of the BCL2 locus at 18q21.3 are frequent (Bea 2009), high MCL1 expression is common in more aggressive tumours (Khoury 2003) and can be increased by AKT/mTOR signaling, and BCL-XL over-expression has been linked to constitutive activation of the NF-kB pathway (Shishodia 2005). Furthermore, the proapoptotic BH3-only protein BIM is frequently inactivated through genomic deletions of the BCL2L11 gene. Homozygous deletions of the

BCL2L11 locus are found in MCL cell lines (Jeko, Z138, SP53, UPN1, SP-49), and immunohistochemistry on tissue arrays showed the absence of BIM expression in 32% of primary tumours (Jares 2007, Mestre-Escorihuela 2007).

Targeting BCL-2 family members to restore apoptosis.

The recognition of the importance of the BCL-2 family proteins in cancer has sparked efforts to target these critical survival molecules. One approach has been to reduce BCL-2 expression with compounds affecting gene or protein expression (Kang 2009). A BCL-2 antisense oligonucleotide, oblimersen (Banerjee 2001), has undergone extensive clinical testing in various malignancies, including MCL (Leonard 2003). However, despite encouraging preclinical data and some activity in clinical trials, the compound has repeatedly failed to gain FDA approval and clinical trial activity has virtually stopped. In addition, oblimersen contains CpG motifs and acts as a Toll-like receptor 9 ligand, giving rise to many effects beyond BCL-2 inhibition (Kang 2009). The disappointing activity of oblimersen may be due to pharmacokinetic issues or redundancy within the BCL-2 system that makes inhibition of BCL-2 alone not very effective. A different strategy that may overcome this later limitation is based on small molecules directly inhibiting the function of BCL-2 antiapoptotic proteins. Several compounds have been isolated or chemically synthesized that mimic the action of BH3-only proteins and thereby promote apoptosis. Several such BH3-mimetics (ABT-737 [oral compound in clinical trials is ABT-263], AT-101, GX15-070) have shown in vitro and in vivo activity, both as single agents and in combination with proteasome inhibitors or conventional chemotherapy (Paoluzzi 2008, Perez 2007). BH3-mimetic compounds differ in their specificity and affinity for various BCL-2 family members (Zhai 2006). GX15-070 and AT-101 are considered pan–BCL-2 inhibitors and can antagonize MCL-1, which is up-regulated by proteasome inhibition. Thus, combination of these compounds with bortezomib yields synergistic antitumor activity.105 In contrast, ABT-737 does not neutralize MCL-1 or A1/BFL-1, high expression of which may cause resistance to this BH3 mimetic (Yecies 2010).

Microenvironment in MCL pathogenesis and immunomodulatory drugs

The microenvironment has been found to play an important role in the biology of several B-cell malignancies. There are some indications that tumour host interactions might be more important in MCL than is currently appreciated. First, MCL almost invariably involves the gastrointestinal tract, often only microscopically, but large tumours growing in the intestinal wall may cause presenting symptoms. This suggests that microenvironment factors determine disease localization or promote tumour cell expansion or both at these sites. Second, in vitro studies show that MCL cells interact with bone marrow stromal cells, and these interactions can contribute to chemoresistance (Kurtova 2009). Third, gene expression profiling showed increased expression of factors involved in cell-cell

crosstalk such as CCL4 and TNFSF9 (4-1BB-L) in MCL cells compared with normal B cells (Ek 2006). An intriguing observation is that cell adhesion to bone marrow stromal cells can decrease the proteasomal destruction of the cell-cycle inhibitor p27, leading to reversible growth arrest (Lwin 2007). Such a "dormant" state could protect cells from cytotoxic therapy and may provide an explanation how rapidly proliferating MCL tumours can remain in remission for years before relapsing again as a highly proliferative cancer.

The immunomodulatory drugs (ImiDs), thalidomide and lenalidomide, are active in MM. Although the precise mechanism of action is not understood, effects on the microenvironment either by disruption of tumour-stroma interactions or through activation of immune effector mechanisms are hypothesized to contributed to the therapeutic effect (Chanan-Khan 2008). Recently, lenalidomide has been shown to improve formation of so-called "immune synapses" between CLL cells and autologous T cells, which could enhance immune-mediated antitumor effects (Ramsay 2008). Phase 2 studies of single-agent lenalidomide in lymphoma have yielded promising results in MCL (Habermann 2009). In the NHL-002 study lenalidomide induced responses in 53% of 15 heavily pretreated patients. The progression-free survival (PFS) of 5.6 months and the median diagnostic odds ratio of 13.7 months compares favorably with other treatment options for these patients. In the international NHL-003 study the response rate in 57 patients with MCL was 42% with a median PFS of 5.7 months (Witzig 2009). A study of thalidomide in combination with rituximab found a response rate of 81% and a PFS of 20 months in 16 patients with relapsed MCL (Kaufmann 2004). Despite these encouraging results no follow-up study with the use of this combination has been reported.

Epigenetic regulation of tumor biology

In contrast to the detailed profiling of genetic lesions in MCL, epigenetic changes have only recently begun to be explored. Acetylation of histones leads to an open chromatin conformation that facilitates the access of transcription factors to DNA. In contrast, methylation of gene promoters can silence gene expression. Both acetylation and methylation can be altered in tumours and can contribute to disease pathogenesis. A recent study of DNA methylation in primary MCL identified several genes that were hypermethylated and thereby silenced in tumour cells compared with normal B cells (Leshchenko 2010, Hutter 2006). The DNA methyltransferase inhibitor decitabine not only reversed the aberrant hypermethylation but also synergized with a histone deacetylase inhibitor (HDACi) and induced cytotoxicity. Several hypomethylated and thereby up-regulated genes could play a pathogenic role, including NOTCH1, CDK5, and HDAC1. Surprisingly, the frequently observed methylation of CDKN2B did not correlate with increased tumour proliferation.

HDAC inhibitors

Hypoacetylation of histones is found in lymphomas compared with normal lymphoid tissue, and data suggest that transformed cells are more sensitive to HDAC inhibition than normal cells (Fraga 2005). Studies with MCL cell lines have shown that suberoylanilide hydroxamic acid (SAHA), the only HDACi currently approved by the FDA, induces histone acetylation, up-regulates p21 and p27, causes cell-cycle arrest, and induces apoptosis. Interestingly, SAHA also reduced cyclin D1 expression at the protein but not mRNA level, through an unexpected inhibition of the PI3K/AKT/mTOR pathway (Kawamata 2009). There is compelling evidence that HDACis also affect the handling of misfolded proteins. HDAC6 is a key factor in the aggresome pathway, a proteasome-independent pathway of protein degradation. The aggresome pathway is up-regulated in response to proteasome inhibition, suggesting that simultaneous inhibition of both degradation pathways may result in an improved therapeutic effect. In this regard, preclinical data indicated a synergistic interaction between SAHA and bortezomib, resulting in increased reactive oxygen species generation and apoptosis (Heider 2008).

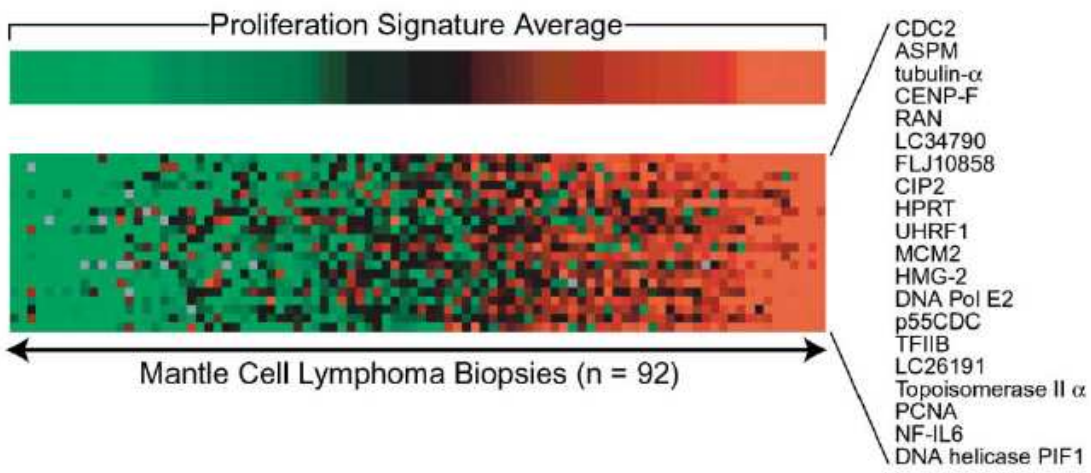
Heat shock proteins, chaperones of tumor biology, and HSP90 inhibitors

Heat shock proteins (HSPs) are ubiquitously expressed chaperones that facilitate and guard the proteome from misfolding and aberrant aggregation. HSP90 is of particular interest because many of its clients are oncogenic signaling proteins, including cyclin D1, c-MYC, p53, CDKs, AKT, IB kinases, and survivin. HSP90 inhibitors displace the client proteins and target them for destruction by the proteasome system (Taldone 2008). Resultant depletion of essential oncoproteins can induce cell-cycle arrest and apoptosis. HSP90 in tumour cells is present entirely in active, complexed form, rendering cancer cells more sensitive to HSP90 inhibitors than normal cells. HSP90 inhibitors have been a focus of drug development for more than 10 years, but no compound of this class has gained clinical approval (Kim 2009). The first generation of HSP90 inhibitors 17-AAG and 17-DMAG are derivatives of the natural product geldanamycin. Most clinical trials used 17-AAG, showing moderate antitumour activity, which in part may be due to poor water solubility. Additional 17-AAG derivatives and several synthetic HSP90 inhibitors have been developed and could overcome some of the limitations of the older compounds. HSP90 inhibitors may be of particular value in combination with proteasome inhibitors. For example, IPI-504 synergized with bortezomib in a MM xenograft model (Sydor 2006) and is active against bortezomib-resistant MCL cells possibly because of the down-regulation of the endoplasmic reticulum chaperone BIP/GRP78 (Roue 2009). An intriguing feature of this combination is that HSP90 inhibitors may reduce the neurotoxicity observed with bortezomib.

PROGNOSTIC PARAMETERS

The different therapeutic approaches do not consider the high heterogeneity in the evolution of the disease in MCL patients. The increasing number of therapeutic options is opening new perspectives for patients but the evaluation of these approaches will require a correct stratification of the patients according to the specific biological risk of their disease. Recently, a specific MCL prognostic index (MIPI, Mantle-cell lymphoma International Prognostic Index) based on four independent prognostic factors [age, Eastern Cooperative Oncology Group (ECOG) performance score, lactate dehydrogenase (LDH) and leucocyte count] has shown the capacity to clearly separate MCL patients into three groups with significantly different prognoses (Hoster 2008).

Early histopathological studies recognized the proliferation of the tumour, evaluated either as the mitotic index or cells expressing the proliferation-associated antigen Ki67, as the best predictor of survival in MCL patients (Argatoff 1997, Tiemann 2005). A tight correlation between Ki67 levels and survival is observed, suggesting that this marker may be useful in the stratification of patients for clinical trials (Katzenberger 2006). New proliferation markers recognized by immunohistochemistry, such as topoisomerase IIalpha or MCM6, may be useful in routinely processed samples and may improve the predictive value of the Ki-67 detection (Schrader 2004). However, these methods may be hampered by the difficulties in the standardization of the techniques and the reproducibility of the evaluation among observers (De Jong 2007). As already tested Pich et all (Am J Clin Pathol 1994) S-phase fraction analized by flow cytometry could be a more objective way to check proliferative index. The analysis of gene expression profiling in MCL has identified a proliferation signature based on the expression of 20 genes able to clearly discriminate patients with different median survival, confirming that increased proliferation was the best predictor of poor survival (Rosenwald 2003, picture below).



Previous studies had identified other morphological, genetic, and molecular parameters with prognostic value in MCL patients. Thus, the blastoid cytological variants are associated with shorter

survival. Tumours with complex karyotypes and specific chromosomal alterations, such as gains in 3q, 12q, Xq and losses in 9p, 9q, and 17p have a more aggressive clinical evolution. Molecular investigations have identified the inactivation of p53, CDKN2A locus, and the presence of high levels of cyclin D1 or MDM2 as predictors of shortened survival. The prognostic impact of most of these parameters merely reflects the proliferation of the tumours; they lose their predictive value in multivariate analysis, suggesting that the proliferation activity may represent an integrator of different oncogenic events (Rosenwald 2003). Interestingly, some molecular and genetic alterations maintain their prognostic prediction independently of the proliferation of the tumours, suggesting that other factors may also influence the behaviour of the lymphoma or the response to the current treatments. Thus, the quantitative gene expression of MYC, MDM2 and CCND1 seem to have prognostic value independently of the tumour proliferation (Kienle 2007). Similarly, the concomitant inactivation of the two regulatory pathways INK4a/CDK4 and ARF/p53 in MCL was associated with a poor survival that was independent of Ki-67 proliferation index (Hernandez 2005). Interestingly, the impact of the chromosome 3q gains and 9q losses on survival is independent of the microarray proliferation signature, suggesting that a predictive model combining the quantification of the proliferation activity and the genetic profile may improve the estimation of the risk of the patient (Salaverria 2007). These robust molecular and genetic prognostic predictors may become an essential tool in the clinical practice to tailor the best therapy for each patient.

IN VITRO AND IN VIVO MODEL

Cell lines

The advent of continuous human leukemia-lymphoma (LL) cell lines as a rich resource of manipulable living cells has contributed significantly to a better understanding of the pathophysiology of hematopoietic tumors. The major advantages of cell lines were the unlimited supply and worldwide availablility of identical cell material and the infinite viable storability in liquid nitrogen. LL cell lines are characterized generally by monoclonal origin and differentiation arrest, sustained proliferation in vitro under preservation of most cellular features, and notably specific genetic alterations (Drexler 1999, Drexler 2000). Over the last several years, a number of cell lines have been established from patients with MCL or a lymphoma which would nowadays be classified as MCL (table 2) (Drexler 2002, Drexler 2006).

Various groups in Canada, Germany, Japan, Sweden and Spain have characterized several bona fide and presumptively MCL cell lines in astonishing detail, using classical G-banding cytogenetics, fluorescence-in situ hybridization (FISH), spectral karyotyping (SKY), comparative genomic hybridization (CGH) and cDNA microarray analysis (Sanchez-Izquierdo 2003, Rudolph 2004, Ek 2005, de Leuuw 2004, Rubio-Moscardo 2005, Tagawa 2005).

Mantle cell lymphoma-derived cell lines

Cell line	Patient	Sample	Year	Diagnosis	Ref.
Granta 519	58 F	PB	1991	MCL, leukemic transformation	[20]
HBL-2	84 M	LN	1985	B-NHL, diffuse large cell	[21]
JeKo-1	78 F	PB		MCL, leukemic conversion	[22]
JVM-2	63 F	PB	1984	B-PLL	[9]
M-1	58 M	PB	2001	MCL, leukemic phase	[23]
MAVER-1	82 M	PB	2003	MCL, leukemic phase	[24]
Mino	68 F	PB	1997	MCL, leukemic phase	[25]
NCEB-1	57 M	PB	1987	B-NHL, diffuse centroblastic-centrocytic	[18]
Rec-1	61 M	LN or PB	1987	MCL, transformed from DLCL	[26]
SP-53	58 F	PB	1986	B-NHL, intermediate lymphocytic, leukemic conversion	[27]
UPN1	52 M	PE		MCL, leukemic phase	[28]
UPN2	57 M	PE		B-CLL → MCL	[28]
Z-138	70 M	BM	1990	B-CLL → B-ALL – reclassified as MCL	[11]

Table 2. MCL cell lines (Drexler et al 2006).

Animal models

Progress in MCL therapy has been slow in part because of its relative rarity, but also because no suitable animal models have existed. Preclinical evaluation of investigational agents for MCL has been limited by lack of suitable animal models that mimic the natural history of human MCL and provide the microenvironment in which MCL cells thrive. The currently available in vivo model of MCL is s.c. xenograft of human MCL cell lines in severe combined immunodeficient (SCID) or nonobese diabetes– SCID mice (Bodring 1994, Lavec 1994).

It is well known however that primary MCL cells from patients are very difficult to grow in culture medium in vitro and in the mouse model (Wiestner 2007).

M'kacher et al. (Cancer Genetics and Cytogenetics 2003) have established two cell lines from the pleural effusions of two patients with MCL that they have used for further cytogenetic characterization to better define the incidence and nature of secondary chromosome abnormalities using multicolor fluorescence in situ hybridization, whole chromosome paint, and specific probes. Both cell lines grew independently without growth factors. Using CCND1/IGH-specific probes, patient UPN1 was found to have a masked t(11;14). Numerous and complex chromosomal abnormalities were found in both cell lines affecting chromosomes 2, 8, 13, 18, 22, X, and Y. These abnormalities included 8p losses, suggesting the presence of an anti-oncogene in this region, rearrangements of 8q24, MYC gene, and translocations involving 8, X, and Y chromosomes, which might be significant in the pathogenesis of MCL progression. The use of the cell lines (UPN1) allowed them to generate a mouse model of human MCL, mimicking a disseminated lymphoma and leading to the death of the animals in 4 weeks. That blastoid MCL model could be of major interest to determine molecular events involved in MCL progression, allowing isolation of involved genes and their functional characterization, and to study the effects of new chemotherapy regimens in mouse models. Xenografted UPN1 recapitulates faithfully some features of blastoid MCL in NOD/SCID mice with evidence of migration of leukemic cells to the spleen, lymph nodes, and

CNS. A similar model expressing CCND1 on a xenografted tumour cells, however, was not validated for the presence of the specific chromosomal abnormalities (Bryant 2000).

Tucker et al (2006) wanted to study growth rate of four MCL cell lines in mice. Female and male Rag2-M mice with transgenic knockout of the Rag-2 gene were obtained from the breeding colony at the BC Cancer Agency at age 6–9 weeks. The mice, which lack B and T lymphocytes, were maintained in a pathogen-free environment. The 5×10^6 exponentially growing cultured cells were injected subcutaneously with Matrigel (volume of 100 µl) or without Matrigel (volume of 50 µl) (Collaborative Biomedical Products Inc., Chicago,USA), intraperitoneally, or intravenously via tail vein. Animals were assessed for the time course of illness and for signs of tumour growth, and were sacrificed for progressing subcutaneous tumour mass >1 cm^3, paralysis, ascites, scruffy coat and lethargy or for survival beyond 90 days. Harvested tumour specimens were snap frozen at −70 ◦C and also placed in buffered formalin and subsequently prepared for histopathological analysis and immunohistochemistry to confirm that tumour specimens exhibited MCL traits. All animal studies were completed in accordance with the current guidelines of the Canadian Council of Animal Care and with the approval of the University of British Columbia Animal Care Committee. All cell lines showed subcutaneous growth in Rag2-M mice. Growth was enhanced by injection with Matrigel, with the exception of HBL-2 which showed equally rapid growth with or without Matrigel as most injected sites showed growth by days 25–30, although in the cell lines other than HBL-2 growth in the absence of Matrigel was delayed beyond day 50 or not observed. JVM-2 and NCEB-1 had a slower growth pattern of about 40–50 days as compared to Z-138 and HBL-2. A shorter survival time was observed for animals injected subcutaneously with HBL-2 (27 days) and Z-138 (31 days) while longer survival times were observed for both JVM-2 (50 days) and NCEB-1 (47 days). IP and IV injection of HBL-2 led to tumour growth and dissemination with a somewhat slower time course of about 45–50 days. Parenteral injection of Z-138 did not generate any systemic disease. The i.v. injection of JVM-2 and NCEB- 1 led to establishment of cohesive deposition of systemic MCL after 60–90 days. Parenteral injection of the HBL-2, NCEB-1 and JVM-2 cell lines showed tropism for extranodal sites, although the number of animals tested was small.

Ford et al. (Blood 2007) developed another murine model of MCL-Blastoid variant by crossing interleukin 14α (IL-14α) transgenic mice with c-Myc transgenic mice (double transgenic [DTG]). 14α is a B-cell growth factor that is expressed in a number of high-grade lymphomas, including MCL-BV. Ninety-five percent of 14α transgenic mice develop CD5positive large B-cell lymphomas by 18 months of age. Sixty percent of c-Myc transgenic mice develop pre-B-cell lymphomas by 12 months of age. Close to 100% of DTG mice develop an aggressive, rapidly fatal lymphoma at 3 to 4 months of age that is CD5+, CD19+, CD21-, CD23-, sIgM+. The tumour was

found in the blood, bone marrow, liver, spleen, lymph nodes, gastrointestinal tract, and lungs and rarely in the brain, similar to the involvement seen in human MCL-blastoid variant. Immunoglobulin gene rearrangements document the monoclonality of the tumour. Cyclin D1 was highly expressed in these tumours, as it is in MCL-blastoid variant. However, MCL cells in these transgenic mouse models are murine, not human.

As MCL usually involves the bone marrow, Wang et al (Clin Canc Res 2008) developed an in vivo SCID model for human primary MCL cells. They adopted the SCID-hu mouse model developed for myeloma (Yaccoby 1998) and injected primary human MCL cells directly into the microenvironment of human fetal bone, which had been s.c. implanted into SCID mice. The engrafted MCL cells in human fetal bone have been shown to produce measurable levels of human h2-microglobulin (h2M) in mouse serum. In this model, the engraftment and growth of patient MCL cells were dependent on human bone marrow microenvironment and these cells metastasized to mouse lymph node, spleen, bone marrow, and gastrointestinal tract. Primary tumour cells such as myeloma and lymphoma usually do not survive and grow in SCID mice because they require the supporting human microenvironment. To establish a mouse model for primary myeloma cells, investigators developed SCID-hu mice, in which a fetal human bone chip was implanted s.c. into SCID mice (Yaccoby 1998). After a 4- to 6-week postprocedure recovery phase, the bone implants are found to be vascularized and histologically similar to normal human bone marrow and could provide bone marrow microenvironment for the growth of primary tumour cells (Kyozumi 1992). Injected myeloma cells grow within and around the bone chip and seldom migrate and infiltrate murine tissues (Yaccoby 1998). Through interactions with the human bone marrow microenvironment, myeloma cells induce typical myeloma manifestations, such as the appearance of M-protein in the serum, and changes in the density of implanted human bone. The SCID-hu mouse model has been used not only in studies of human hematopoiesis, immune function, and biological development (Kaneshima 1990, McCune 1996, Carbadillo 2000) but also in studies of microenvironment and growth and metastasis of human primary myeloma cells in vivo (Yaccoby 2004). MCL shares the same feature of common bone marrow involvement as myeloma. Previous studies indicated that MCL was identified in 83% to 92% of bone marrow aspirate specimens and in 91% of bone marrow biopsy specimens (Salar 2006, Cohen 1998, Romaguera 2005). Based on the characteristics of the high frequency of bone marrow involvement with MCL cells, Wang et al. reasoned that by implanting a human fetal bone in SCID mice, primary MCL cells might survive and grow in the SCID mice. They successfully established a reproducible in vivo model closely resembling the clinical situation of patients with MCL. This approach tourned out to be especially practical and useful because they can establish a mouse host by injecting as few as $0.5x10^6$ of

freshly isolated primary MCL cells from patients. Unlike myeloma, MCL cells do not produce M-proteins. To monitor tumor growth, they examined various factors secreted by MCL cells such as IL-6, h2M, and lactate dehydrogenase (Argatoff 1997, Williams 2005). Based on preliminary studies monitoring and comparing serum levels of these factors and the sizes of tumour masses in MCL-bearing SCID-hu mice, they found a positive correlation between tumour burdens and the level of circulating human h2M. IL-6 and lactate dehydrogenase were less useful for this purpose. Therefore, they chose human h2M as the surrogate marker for tumour burden in MCL–SCID-hu mice. They recently showed that levels of circulating human h2M correlated well with the sizes of s.c. MCL tumours in the xenograft SCID mouse model. Therefore, circulating human h2M seems indeed a good indicator for MCL tumour burdens in SCID-hu mice. This study provides strong evidence for the crucial requirement of implanted human bone for the engraftment of primary MCL cells in the host. They injected primary MCL cells i.v. or directly into the bone chips of SCID-hu mice and found that intrabone, but not i.v. injection, led to the establishment of MCL in the majority of SCID-hu mice. Further support came from SCID mouse experiments in which i.v. injection of large numbers of primary MCL cells failed to establish MCL in the mice. Furthermore, in one of five SCID-hu mice that successfully engrafted MCL after i.v. injection of the tumour cells, a tumour mass formed within and around the implanted human fetal bone. These results indicate that the implanted bone chips are absolutely required, particularly during early stages of tumour engraftment, for injected MCL cells to survive and grow because at 2 months after tumor inoculation, primary MCL cells were also found in murine tissues such as bone marrow, lymph nodes, spleen, and gastrointestinal tract. This is a very interesting finding because in patients with MCL, tumour metastasis is often observed in these tissues or organs. Romaguera and coworkers (Romaguera 2005) published results from their phase II study with 97 patients with MCL using Hyper CVAD regimen and found that 90% of patients were confirmed to have gastrointestinal tract involvement, gastrointestinal tract involvement by MCL at diagnosis has become very frequent (Salar 2006). Therefore, Wang et al believe that their MCL–SCID-hu model will also be useful to elucidate the mechanisms underlying tumour metastasis in MCL. High engraftment rates with a short time for tumour establishment are required to make the in vivo model feasible for research purposes. Wang et al treated MCL-bearing SCID-hu mice i.p. with atiprimod for 6 consecutive days. The data showed that atiprimod decreased serum h2M to low levels, comparable with serum h2M levels of mice treated with PBS. The results indicate that this SCID-hu model not only allows for the study of MCL biology but also is rapid and useful for the preclinical evaluation of novel agents against MCL.

Testing Olaparib effect on MCL, Weston et al (Blood 2010) use a nonobese diabetic/severe combined immunodeficient (NOD/SCID) murine xenograft model of an ATM mutant MCL cell line and demonstrated significantly reduced tumour load and an increased survival of animals after olaparib treatment in vivo. For all experiments, tumour cell engraftment in the bone marrow and spleen before initiation of olaparib treatment was confirmed both by FACS analysis of human anti-CD45 (eBioscience)– and murine anti-CD45 (BD Pharmingen)–stained cells and by immunohistochemistry using anti–human CD5 (Leica Microsystems), anti–human Pax5 (Thermo Scientific), and anti–human ki-67 (Dako) antibodies. For assessment of MCL tumour load in murine primary lymphoid organs, sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (aged 5 weeks) were intravenously injected with $3x10^6$ Granta-519 cells. Fourteen days after injection, animals received either 50 mg/kg/d olaparib or vehicle, 10% (wt/vol) 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich), via intraperitoneal injection, daily for 14 days. Mice were culled at day 30-36, and tumour load in the bone marrow and spleen was assessed by FACS analysis of human CD45+ cells. To compare tumour size and survival between treated and untreated animals, subcutaneous tumours were initiated by injection of $3x10^6$ Granta-519 cells. Tumours were allowed to grow for 5 days before initiation of treatment with 100 mg/kg/d olaparib (tumor size, survival) or vehicle alone (tumour size, survival) via oral gavage for no more than 28 days. Tumour volume was measured manually using a calliper 3 per week. Mice were killed upon signs of illness or when tumours reached 1250 mm^3 Any mice that died early in the experiments due to graft-unrelated causes were excluded from the experiments.

To investigate the in vivo impact of olaparib, they generated murine xenograft models of the ATM mutant MCLcell line, Granta-519. To determine whether infiltration and engraftment of the tumour cell line had already taken place before initiation of olaparib treatment in the different animal cohorts, 3 representative mice from each cohort were analyzed on the day that treatment was to begin (14 days after intravenous or 5 days after subcutaneous injection of cells). The presence of tumour cells at the level of at least 1% of all cells, which is considered to be engraftment, was observed by FACS analysis in the bone marrow and spleen both at 5 days (subcutaneous) and 14 days (intravenous) after injection. Furthermore, using immunocytochemistry and anti–human CD5, Pax-5, and Ki-67 antibodies, they confirmed significant infiltration of proliferating human B-lymphoid tumour cells in both the spleen and bone marrow at both time points before treatment initiation. Subsequently, the degree of tumour load was compared in the lymphoid organs of 23 NOD/SCID Granta-519 cell–injected mice 5 weeks after intravenous injection of cells and 14 days after treatment with olaparib. However, early in the experiment, 7 mice died of graft-unrelated causes, leaving 16 mice, which were treated with either olaparib (8 mice) or vehicle alone (8 mice).

Analysis of the percentage of human CD45 staining by FACS analysis revealed a significant reduction in the percentage of Granta- 519 cells in the bone marrow and a trend toward reduced tumour cell load in the spleen of mice treated with olaparib compared with those receiving vehicle alone. They next assessed the effect of olaparib on the growth of subcutaneous tumours generated by the localized injection of ATM mutant Granta-519 cells into mice and found a significant positive correlation between olaparib treatment and reduced tumour size. Finally, the overall survival of mice engrafted with Granta-519 cells was significantly increased by olaparib treatment compared with vehicle alone.

Liu et al (Clin Cancer Res 2010) tested in vivo therapeutic activity of FTY720 in severe immunodeficient mice engrafted with the Jeko MCL cell line. Six- to eight-week-old female severe combined immunodeficient (SCID) mice (cb17 scid/scid; Taconic Farms) were depleted of murine natural killer cells with intraperitoneal injections of 0.2 mg of rat anti-mouse interleukin-2 receptor β monoclonal antibody (TMβ1), 1 day before engraftment with human tumour cell lines and then every week, as previously described (Tananka 1993). Cell-dose titration trials with three separate MCL cell lines (SP53, Jeko, and Mino) were done to determine the optimal dose of cells that would lead to consistent engraftment and development of tumour burden in all mice. This dose was found to be 4×10^7 cells (injected i.v. through tail vein) for Mino and Jeko cell lines. SCID mice treated with the monoclonal antibody TMβ1 to deplete murine natural killer cells were engrafted with 4×10^7 MCL cells (Jeko, Mino, and SP53) i.v. and observed daily for signs of tumour burden. Animals engrafted with Jeko cells developed cachexia/wasting syndrome and respiratory distress between days 18 and 20 following engraftment. All mice engrafted with Jeko cells showed central nervous system and diffuse organ tumour burden and splenomegaly. Evaluation of brain tissue showed an impressive meningeal infiltrate and perivascular invasion. Mice engrafted with Mino cells developed cachexia/wasting syndrome and respiratory distress from 30 to 50 days. Mice engrafted with Mino cells developed large bilateral neck masses, mediastinal tumour, and splenomegaly. Histopathologic examination of neck masses showed tumour effacement of parotid glandular tissue. Mice engrafted with SP53 cells survived longest, developing respiratory distress from 40 to 60 days. All mice engrafted with SP53 cells developed retroperitoneal and abdominal tumour burden. Histopathologic examination of retroperitoneal lymph nodes showed marked effacement of normal lymph node architecture. Tumour burden was verified by immunohistochemistry staining for the human CD19 and CD45 antigen. Engraftment of each cell line resulted in a characteristic pattern of tumour burden and highly reproducible time to develop advanced disease. To determine the in vivo therapeutic activity of FTY720 against MCL, they engrafted 20 SCID mice with 4×10^7 Jeko cells and randomized mice to two treatment groups. The dose used in this study (5 mg/kg/d) was

determined based on in vivo PK studies of FTY720 in a rat model. This regimen, in prior studies using a murine Raji xenograph model (Liu 2008), did not lead to significant toxicity, which further justified its implementation for our in vivo studies using the MCL preclinical model. Mice treated with 5 mg/kg FTY720 (n = 10) have a median survival of 38 days (95% confidence interval, 30-39), which is significantly longer than the median survival of 26.5 days (95% confidence interval, 26-27) for PBS-treated mice (n = 10; P = 0.001). The histopathologic analysis of the spleens from both FTY720- and PBS treated mice showed diffuse infiltration of human CD19+/CD45+ cells, with neoplastic lymphocytes completely effacing the normal splenic parenchyma, consistent with our observation that FTY720 increases the median survival of MCL-engrafted mice but, as a single agent, does not result in complete elimination of disease.

PROJECT AND GOAL

The project based its scientific philosophy into two main phases.

First of all a large collection phase that we decided to do looking inside protein expression of MCL cell lines, either by using immobilized metal affinity chromatography (IMAC) pre-fractionation (a column-based technique that selectively enriches all phospho-proteins by a reversible binding to metal oxydes), followed by 2D-PAGE/MS (Cecconi 2008), than with PhosphoScan analysis. We chose protein and phosphoprotein because they are the final effectors of cell… and good candidate drive targets.

Second we needed to validate data obtained by such a collection phase, finding antibodies against some of the most expressed phosphoprotein and testing them on tissue (figure 7).

We did this on human fixed tissue, trying to find cases with clinical follow-up and building tissue micro arrays to perform immunohistochemistry at low cost and hopefully find new prognostic parameter or confirm and validate old ones. The proliferative index scored by Ki-67, a time-proven immunohistochemical antibody, has been shown to be a powerful prognostic marker in MCL and a 30% cut-off has recently been proposed as clinically meaningful. However, its determination is subject to inter-laboratory and inter-observer variation, limiting study-to-study comparison. S-phase index, determined by flow cytometry, might be a potentially more objective method for the determination of proliferation, and might retain the prognostic value of Ki-67 labelling.

In order to validate our experimental findings in vivo we also developed a xenograft mouse model useful for further experimental work.

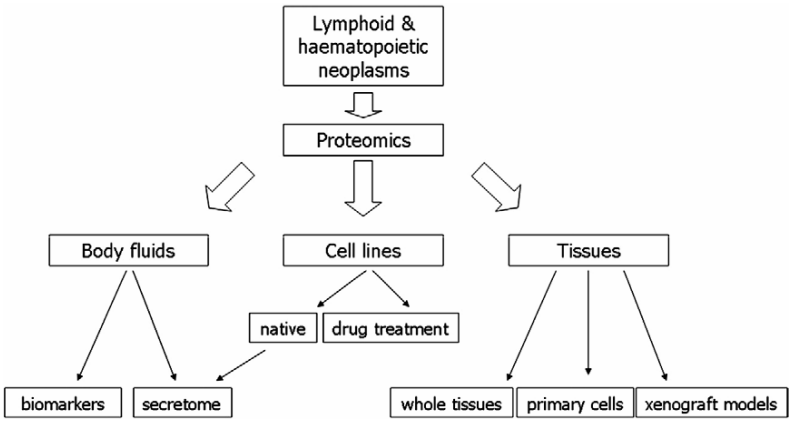


Figure 7. Options for performing proteome analysis of lymphoid and haematopoietic neoplasms focusing on body fluids, cell lines, and/or tissues.

MATERIAL AND METHODS

PROTEIN

Cell culture

The MAVER-1 cell line was established in our laboratories and previously described (Zamò 2006). Cell lines Granta-519 (Jadayel 1997), Rec-1 (Raynoud 1993), and Jeko-1 (Jeon 1998) were obtained from DSMZ (Braunschweig, Germany). UPN-1 (M'kacher 2003), UPN-2 (M'kacher 2003), and NCEB-1 (Saltman 1988 were provided by Professor Elias Campo (Hospital Clinic, University of Barcelona, Spain). Cell lines were cultured in RPMI 1640 (figure 8) medium containing 10% FCS (Bio- Whittaker, Walersville, MD, USA), and gathered at plateau phase (1.5–2 x 10^6 cells/mL) by centrifugation. Cells were washed twice in sterile 0.9% NaCl followed by freezing in liquid nitrogen.

Figure 8. Cell lines coltures in RPMI 1640 medium.

IMAC (figure 9)

A phosphoprotein purification kit (Qiagen, Valencia, CA, USA) was utilized for purifying phosphorylated proteins. Cells ($1x10^7$) were harvested and processed following the manufacturer's instructions. Briefly, cells were gently lysed in a buffer containing 0.25% w/v CHAPS, Benzonase (a DNase/RNase), and a mixture of protease inhibitors to prevent proteolytic degradation. Subsequently, lysates were added to the ready-to-use columns and the flow-through fractions (containing nonphosphorylated proteins) were collected. After a wash step, proteins carrying phosphate groups were eluted in PBS. Free phosphate in the elution buffer inhibits phosphatase activity, and therefore stabilizes the phosphorylation status of the eluted fractions during downstream processing and storage.

High-resolution 2-DE (figure 10)

Phosphorylated protein fractions were concentrated and desalted before 2-DE by ultrafiltration (Nanosep® Ultrafiltration columns 10 kDa cut-off) and addition of 450 µL 10mM Tris-Cl, pH 7.0.

Protein fractions were then precipitated by acetone/methanol (8:1 v/v) and the pellets resuspended in 2-D solubilizing solution: 7 M urea (Sigma–Aldrich, St. Louis, MO, USA), 2 M thiourea (Sigma), 3% CHAPS (Sigma), 20 mM Tris (Sigma), 1% pH 3–10 Ampholyte (Fluka, Buchs, SG, Switzerland), and protease inhibitor cocktail tablet (Complete Mini; Roche, Basel, Switzerland). After 1 h samples were centrifuged for 10 min at 10000 x g at 4°C to remove nucleic acids complexed with ampholytes. Supernatants were incubated with 5 mM ributylphosphine and 20 mM acrylamide for 60 min at room temperature to reduce protein disulfide bonds and alkylate sulfhydryl groups. The reaction was blocked by the addition of 10 mM DTT and protein samples were collected and stored at -80°C. Protein concentration was evaluated with the DC Protein assay (BioRad, Hercules, CA, USA). Seventeen centimetres long, pH 3–10 IPG strips (BioRad) were rehydrated for 8 h with 450 µL 2-D solubilizing solution (7 M urea, 2 M thiourea, 3% CHAPS, and 20 mM Tris) containing 2.5 mg/ mL total phosphoprotein. IEF was performed using a Multiphor II apparatus (GE Healthcare BioSciences, Little Chalfont, UK). The total product time x voltage applied was 70 000 V·h for each strip at 20°C. For the second dimension, IPG strips were equilibrated for 26 min in a solution of 6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl, pH 8.8. The IPG strips were then laid on an 8–18%Tgradient SDS-PAGE with 0.8% agarose in Tris/glycine/SDS running buffer (192 mM glycine, 0.1% SDS and Tris to pH 8.3). The second dimension was run in a Protean Plus Dodeca cell (BioRad) with Tris/glycine/SDS running buffer.

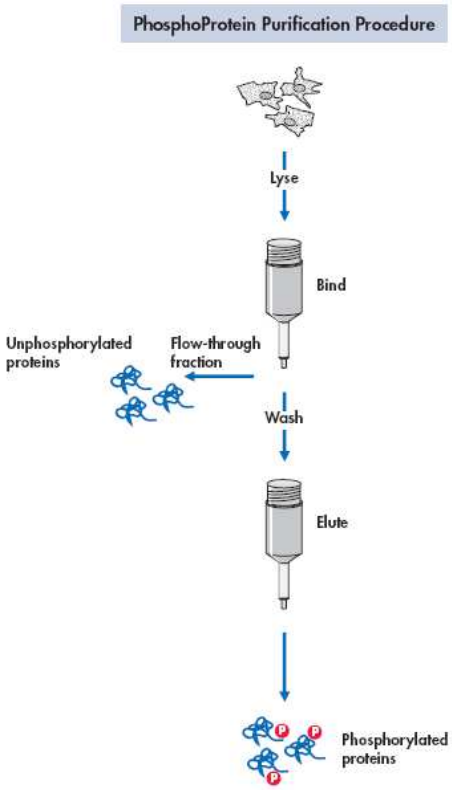


Figure 9. Phosphorylated proteins by immobilized metal affinity chromatography (IMAC) (PhosphoProtein Purification Kit, Qiagen).

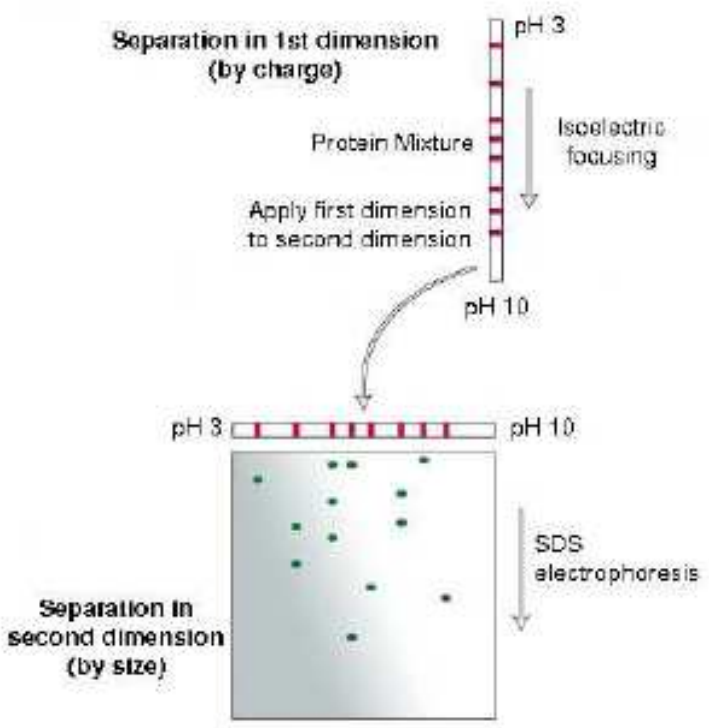


Figure 10. 2D-page analysis.

The electrophoresis was conducted with continuous cooling and mixing by setting a current of 40 mA for each gel for 3 min, then 2 mA/gel for 1 h, and 20 mA/gel until the track dye, bromophenol blue, reached the anodic end of the gels. Protein spots were revealed with Sypro Ruby stain. Gels were incubated in a fixing solution containing 40% ethanol and 10% acetic acid for 30 min followed by overnight staining in a ready-to-use Sypro Ruby solution. Destaining was performed in 10% methanol and 7% acetic acid for 1 h, followed by rinsing at least 3 h in distilled water. Detection of phosphoproteins by Pro-Q Diamond staining (Molecular Probes) after separation on 2-DE gels was performed following the manufacturer's instructions. In brief, 2-DE gels were fixed in solution containing 50% methanol and 10% acetic acid, washed with several changes of water to remove SDS, and stained with the Pro-Q Diamond dye. After destaining, gel images were recorded using a Typhoon 9600 fluorescence scanner. The spots consistently stained with Pro-Q Diamond dye were considered as putative phosphoproteins.

Protein pattern differential analysis

Gels were scanned using a BioRad VersaDoc 1000 imaging system. 2-D gel analysis was performed by PDQuest software version 7.3 (BioRad). Each gel was analyzed for spot detection, background subtraction, and protein spot OD intensity quantification (spot quantity definition). The gel image showing the highest number of spots and the best protein pattern was chosen as a reference template, and spots in a standard gel were then matched across all gels. Spot quantity values were normalized in each gel dividing the raw quantity of each spot by the total quantity of all

the spots included in the standard gel. For each protein spot, the average spot quantity value and its variance coefficient in each group were determined. The average spot intensities of each cell line were ranked in decreasing order, and the 100 spots with the highest average spot intensity value were chosen for the identification.

In-Gel digestion

Spots were selectively excised from the gel using the Proteome Works robotic Spot cutter (BioRad), which allows for reproducible processing of gels. Excised spots were then subjected to in-gel trypsin digestion. Spots were destained (15 min wash in 300 mL 100 mMNH4HCO3; 15 min wash in 300 μL 50% 100 mM NH4HCO3 v/v, 50% ACN; 5 min wash in 100% ACN) and dried at 37°C. The gel pieces were swollen in 10 mL of digestion buffer containing 100 mM NH4HCO3 and 40 ng/mL trypsin (Promega, Madison, WI, USA). After 10 min, 40 μL of 100 mM NH4HCO3 was added to the gel pieces and digestion was performed at 37°C overnight. The supernatants were collected, peptides were extracted in an ultrasonic bath for 10 min (twice in 50 mL 50% ACN, 50% H2O with 1% formic acid v/v; once in 25 mL ACN), and the supernatants were pooled. Tryptic peptides were vacuum-dried with centrifugation, resuspended in 20 μL 0.1% formic acid and purified using a ZIP-TIP C18 column (Millipore, Bedford, MA, USA).

Peptide sequencing by nano-RP-HPLC-ESI-MS/MS

Peptide mixtures were analyzed by nanoflow HPLC: a 10 μL sample was loaded onto a homemade 2 cm fused-silica precolumn (75 mm id, 375 mm od, packed with Reprosil C18-AQ, 3 m/m, at a flow rate of 2 μL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from solution A (2%ACN, 0.1%formic acid) to 50% of solution B (98% ACN, 0.1% formic acid) over 40 min. Desorbed peptides were injected directly into a Q-TOF microhybrid mass spectrometer (model microTOF-Q, Bruker-Daltonik) equipped with a modified ESI ion source (the spray capillary was a fused-silica capillary, 0.090 mm od, 0.020 mm id). The mass spectrometer was operated in the positive ion MS mode, and data-dependent analysis was employed for survey scans (m/z 350–1500) to choose up to three most intense precursor ions. For CID mass spectrometric (MS/MS) analysis, collision energies were chosen automatically as a function of m/z and charge. The collision gas was argon. The temperature of the heated sample source was 180°C, and the electrospray voltage was 4000 V. External mass calibration in quadratic regression mode using sodiumformiate resulted in mass errors of typically 5 ppm in the m/z range 50–2000. Data searching was accomplished with MASCOT software (Matrix Sciences, London, UK) using the following constraints: only tryptic peptides up to two missed cleavage sites were allowed; ±0.25 Da tolerance for both MS and MS/MS fragment ions; complete propionamide formation on cysteines and partial oxidation of methionines were specified.

Protein phosphorylation status validation by Western blot analysis

Protein electroblotting and chemiluminescent signal detection were performed as previously described (Cecconi 2007). Briefly, 1-D SDS-PAGE gels were transferred to a PVDF membrane and treated with the respective antiphosphoprotein antibodies at the appropriate dilutions. Bound antibody was detected with the enhanced chemiluminescent (ECL) detection kit (Amersham Biosciences Europe) and recorded on X-Omat AR film (Kodak, Rochester, NY, USA). Membranes were immunoblotted again with a monoclonal anti-b actin antibody (Sigma–Adrich, 1:4.000) for normalization.

Classification of identified phosphoproteins and phosphorylation sites

GO lists were downloaded using FatiGO (http://fatigo. bioinfo.cipf.es/) from Babelomics (Al-Shahour 2006). Each protein was classified with respect to its cellular component, biologicalprocess, and molecular function using GO annotation. When no GO annotation was available, proteins were annotated manually based on literature searches and closely related homologs. The NetPhos 2.0 server (http:// www.cbs.dtu.dk/services/NetPhos/) (Blom 1999) was used to predict the number of potential protein phosphorylation sites. Further database searches within PhosphoBase 6.0 (http:// phospho.elm.eu.org/) (Blom 1998) were also carried out to confirm the phosphorylation of identified proteins and gain information about the exact position of known phosphorylated instances. Scansite 2.0 (http://scansite.mit.edu) (Obenoure 2003) was used to search for potential Ser/Thr- or Tyr-kinases responsible for the phosphorylation by searching for specific recognized motifs within the phosphorylated protein.

XplorMed (http://www.ogic.ca/projects/xplormed/) (Perz-Iratxzeta 2001) was used to search the literature for signal transduction pathway reconstruction.

SNP-array analysis and correlation

DNA was extracted by column-purification methods (AllPrep columns, Quiagen). The Affymetrix GeneChip® Mapping Assay was used according to the manufacturer's instructions. Briefly, 250 ng of genomic DNAper array were used for restriction enzyme (Xba I) digestion and ligation of sequences onto the DNA adaptor fragments. PCR products were then fragmented, end-labeled, and hybridized to a Gene- Chip array following the manufacturer's instructions. Raw data analysis was performed using GTYPE 4.1. Copy number and LOH were analyzed using the Affymetrix Chromosome Copy Number Analysis Tool (CNAT) 4.0. For all samples, we performed unpaired analysis using the reference datasets from Affymetrix. To correlate the expression of identified phosphoproteins with putative underlying alterations in DNA copy number, we selected the data relative to probes mapping within 500 kb from each protein locus from the SNP-Chip and averaged

their values. Next, we evaluated the presence of common alterations among cell lines, performing a series of one-sample t-tests on the assumption that the expected mean was 0 (no change).

PhosphoScan analysis (figure 11) was performed in collaboration with Cell Signalling Technologies (Danvers, MA, USA) on MCL cell lines MAVER-1, Granta-519, Jeko-1, and Rec-1 as previously described (Rikova 2007). The technique requires a total protein lysate digestion followed by immunoprecipitation of tyrosine-phosphorylated peptides, and then identified by LC-MS/MS. The corresponding proteins were then ranked semi-quantitatively on the basis of the number of identified peptides for each protein. Kinases were identified by categorizing proteins into KEGG terms using the DAVID Ease web framework.

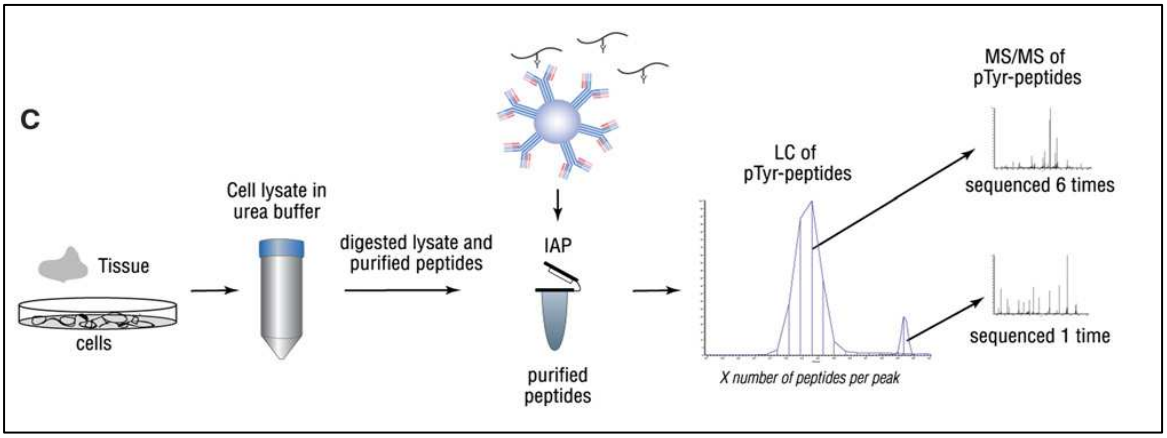


Figure 11. Phosphoscan analysis

CASE COLLECTION

TMA

For validation studies, cases of MCL diagnosed on lymph node or spleen and in one case on testis, between 1993 and 2008, were collected from the files of the Department of Pathology of the University of Verona. Cases were reviewed in order to verify the diagnosis and to select those with enough material to perform a tissue microarray (TMA). Representative areas were identified and properly marked on slides.

For TMA construction a Beecher industry instruments tissue microarrayer was used, which allows to take a core of 1 mm diameter from donor blocks and put it in a host block. Each case was sampled three times from microscopically identified areas. Two TMAs were constructed, containing triplicate cores from 40 cases of MCL (figure 12-16).

Figure 12. Cases from archives with slides and blocks.

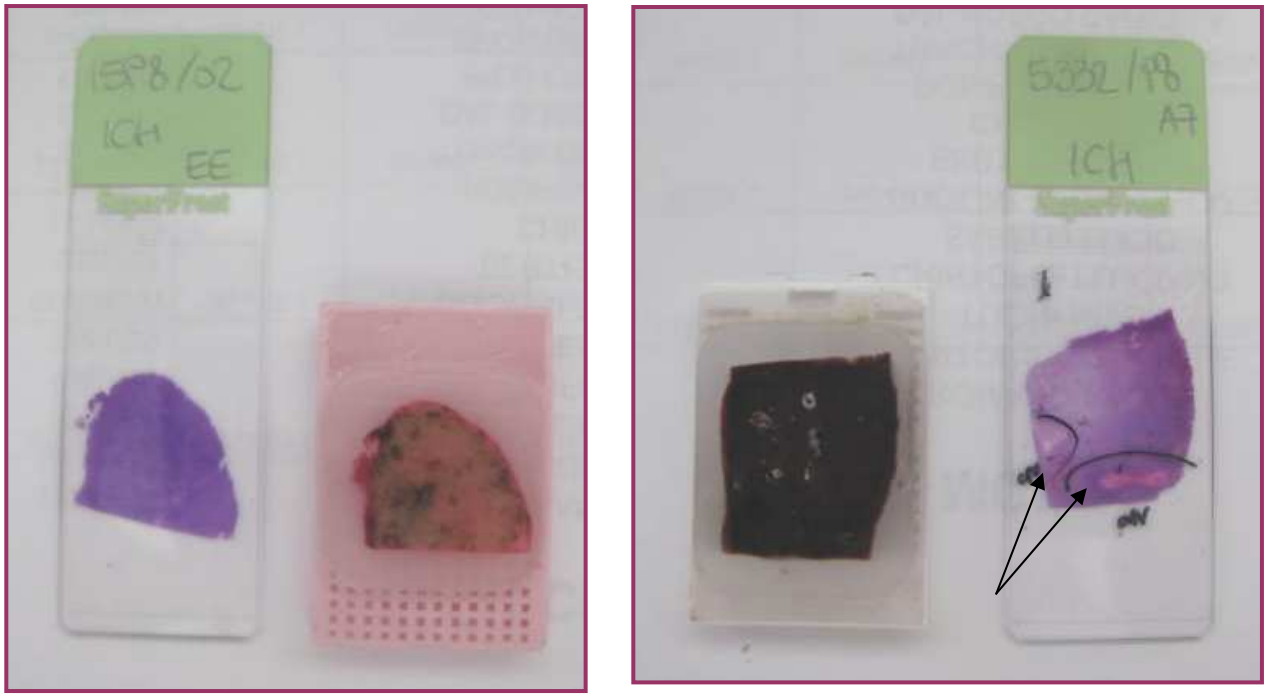


Figure 13. Selection of good areas to make the TMA.

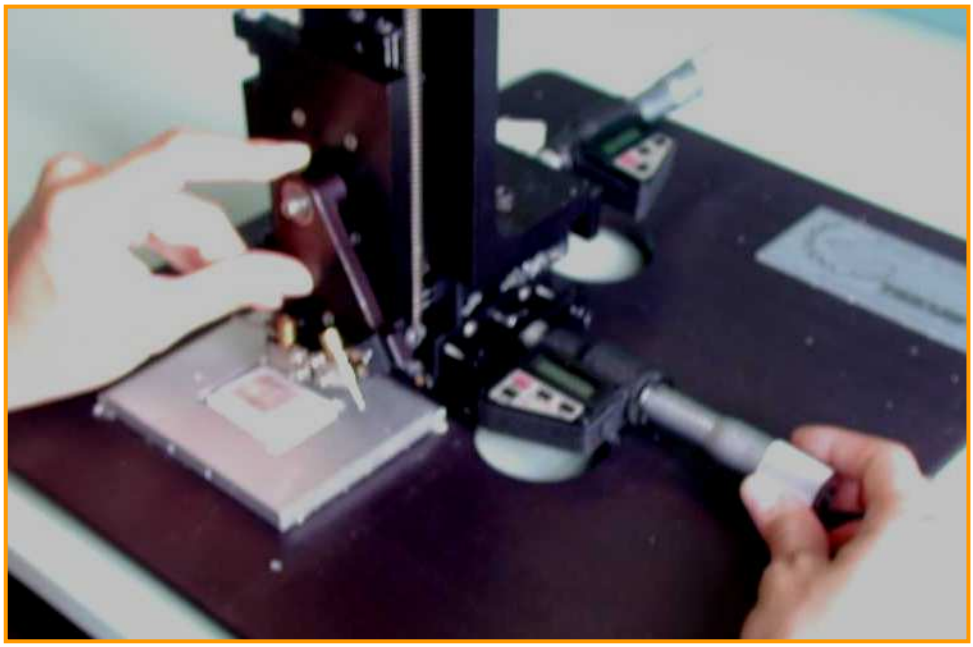


Figure 14. TMA building with tissue microarrayer, which allows to take a core of 1 mm diameter from donor blocks and put it in a host block

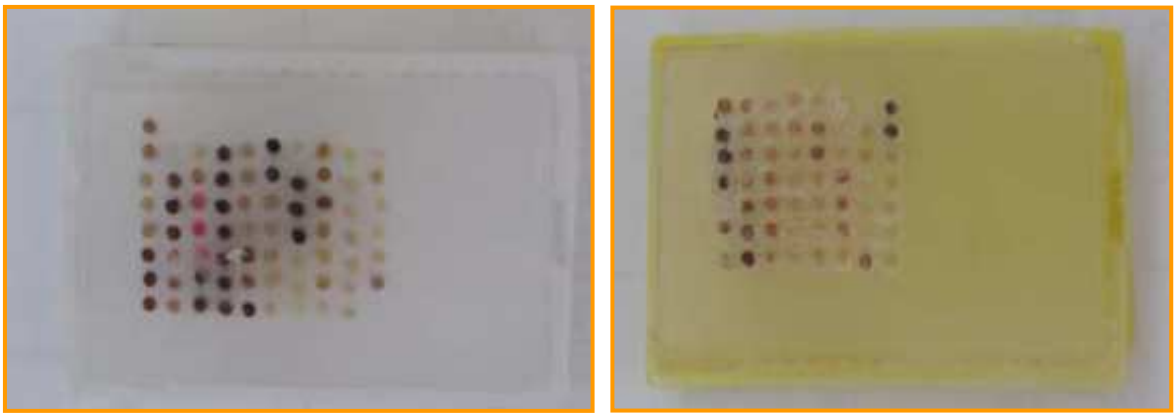


Figure 15. Two TMA of MCL lymphoma cases.

CLINICAL PROGNOSTICATOR

S-phase

We have performed S-phase analysis by flow cytometry (figure 16) on cases from 1994 to 2009, using paraffin embedded tissue before 2002 and fresh tissue from 2002 till 2009 on 38 patients with MCL, clinically followed up by the haematology department of the University of Verona. We collect the Ki67 index from our diagnostic reports and we analyzed the data, in order to verify the prognostic significance of Ki67 index and S-phase fraction in mantle cell lymphoma, analysing their correlation with overall survival.

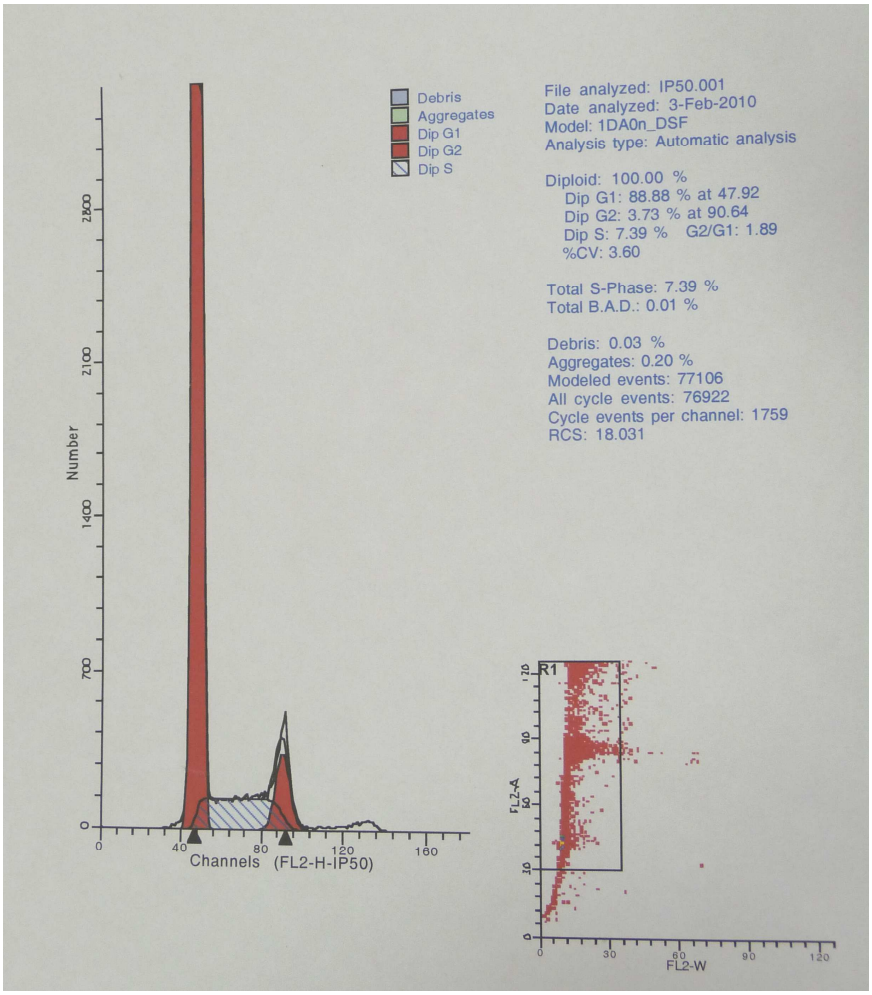


Figure 16. Example of S-phase analysis performed by flow cytometry.

MODELS

Cell lines

We keep growing and freezing many different cell lines as already explained in order to make all different studies. All cells were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in a humidified atmosphere (see “cell culture” page 34).

Mouse model

Attempts to grow primary MCL cells in NOD/SCID mice (in collaboration with the Pathology department of the University of Torino) were unsuccessful because after 3-4 weeks mice developed GHVD and died. So we've decided to change kind of model and we implanted MCL cell lines in rag -/- γ -/- grey mice (figure 17).

We injected $5x10^6$ cells in eight mice per MCL cell lines (JEKO-1, UPN-1 and MAVER-1), 2 subcutis, 2 intraperitoneum and 4 intravenous way with $0.5x10^6$ cells (table 3).

We performed autopsy of 11 mice that already developed a mass and we processed tissue samples to obtain H&E and ICH analysis too.

Figure 17. Mouse before perform autopsy.

n. RES	mouse model	MCL cell line	way of inoculation	n° cells injected
C	Rag2(-/-) γ chain (-/-)	Jeko-1	subcutaneous (s.c)	$5x10^6$ cells/mouse
D	Rag2(-/-) γ chain (-/-)	Jeko-1	intraperitoneum (i.p.)	$5x10^6$ cells/mouse
E	Rag2(-/-) γ chain (-/-)	Jeko-1	intraperitoneum (i.p.)	$5x10^6$ cells/mouse
I	Rag2(-/-) γ chain (-/-)	Jeko-1	intravenous (i.v)	$0,5x10^5$ cells/mouse
J	Rag2(-/-) γ chain (-/-)	Jeko-1	intravenous (i.v)	$0,5x10^5$ cells/mouse
A	Rag2(-/-) γ chain (-/-)	UPN-1	subcutaneous (s.c)	$5x10^6$ cells/mouse
B	Rag2(-/-) γ chain (-/-)	UPN-1	subcutaneous (s.c)	$5x10^6$ cells/mouse
K	Rag2(-/-) γ chain (-/-)	UPN-1	intraperitoneum (i.p.)	$5x10^6$ cells/mouse
G	Rag2(-/-) γ chain (-/-)	UPN-1	Intravenous (i.v)	$0,5x10^5$ cells/mouse
H	Rag2(-/-) γ chain (-/-)	UPN-1	Intravenous (i.v)	$0,5x10^5$ cells/mouse
F	Rag2(-/-) γ chain (-/-)	Maver-1	intraperitoneum (i.p.)	$5x10^6$ cells/mouse

Table 3. Mouse injected with different MCL cell lines, named as "RES" followed by a letter.

ICH CLASSIC AND NEW ANTIBODIES PROTEOMIC BASED

We looked for commercially available antibodies against at least some of the protein that turned out to be highly expressed from our studies (table 4). We chose those antibodies possibly useful either to perform western blot analysis than for immunohistochemical assay (PHOSPO-STAT3, Pragmin, PRKCD, GCET2, CdK2, CDC2, PRPF4 beta).

We perform immunohistochemistry for ciyclinD1, TCL1A, SOX11 ki67, cd20 cd5, tubulin3beta on 5 micron slides obtained from our 2 TMA.

Antybodies	source	diluition	retrieval	control tissue
CD20	Novocastra	1:100	ER2 BOND pH 8	
CyclinD1 (bcl-1 clone Sp4)	NeoMarkers	1:10	ER2 BOND pH 8	
SOX11	Atlas Sigma	1:50	ER2 BOND pH 8	
ki67 (MM1)	Novocastra	1:50	ER1 BOND pH 6	
CD5 (4C7)	Novocastra	1:150	ER2 BOND pH 8	
TCL1A	Santa Cruz	1:100	ER2 BOND pH 8	
ph STAT3	Cell signaling	1:50	ER2 BOND pH 8	tonsil
Pragmin	Abnova	1:400	ER2 BOND pH 8	adrenal gland
PRKCD	Abnova	1:500	ER1 BOND pH 6	tonsil
GCET2	Abnova	1:1000	ER2 BOND pH 8	tonsil
CdK2	Cell signaling	1:50	ER2 BOND pH 8	tonsil
CDC2	Cell signaling	1:50	ER2 BOND pH 8	breast
PRPF4 beta	Sigma	1:500	ER2 BOND pH 8	breast

Table 4. ICH: classic and new antibodies, with annotation of the tissue tested for the new ones.

RESULTS

IMAC and phosphatase-treated separated fractions by a phospho-stain such as Pro-Q Diamond, demonstrate enrichment in phosphoproteins in the IMAC separated fractions. The 100 spots (figure 18) with the highest average intensity were subjected to RP-HPLC coupled with MS/MS analysis for protein identification. The typical high resolution 2-DE phosphoprotein pattern obtained from MAVER-1 cell line is reported in fig. 19a, while representative 2-D maps of the un-phosphorylated fraction and phosphorylated fraction treated with alkaline phosphatase (from MAVER-1) are shown in figs. 19b and 19c, respectively. Figure 20 shows the staining of IMAC and phosphatase-treated separated fractions by a phospho-stain such as Pro-Q Diamond, demonstrating enrichment of phosphoproteins in the IMAC separated fractions.

A total of 76 unique, highly expressed phosphoproteins were identified (figure 21). Functional annotation of the phosphoproteins identified was carried out by categorizing the proteins into different groups based on GO terms (figure 22). Out of the 76 proteins identified, 23% were involved in RNA splicing and translation, 18% in cell proliferation, cell cycle, and apoptosis, 13% in chromatin/cytoskeleton organization and protein folding and 9% in signal transduction. We characterized the putative phosphorylation sites of each protein by using different phosphorylation prediction programs. Potential binding sites for the Erk D-domain and casein kinase 2 were most commonly predicted; casein kinase 1, PKC epsilon, and zeta sites were also found frequently. To correlate the expression of phosphoproteins with possible changes in DNA copy number, we extracted the information relative to the identified proteins from the SNP-Chip profiles of MCL lines. Out of 76 unique proteins, 62 had a corresponding set of probes on the SNP-Chip. The presence of significant DNA copy number alterations, among the seven cell lines investigated, was evaluated with a t-test, and proteins were subsequently ranked based on significance. There was a trend for most highly correlated proteins to have copy-number gains, although some proteins (e.g., MSN, RPSA, and RLP0) showed an inverse correlation.

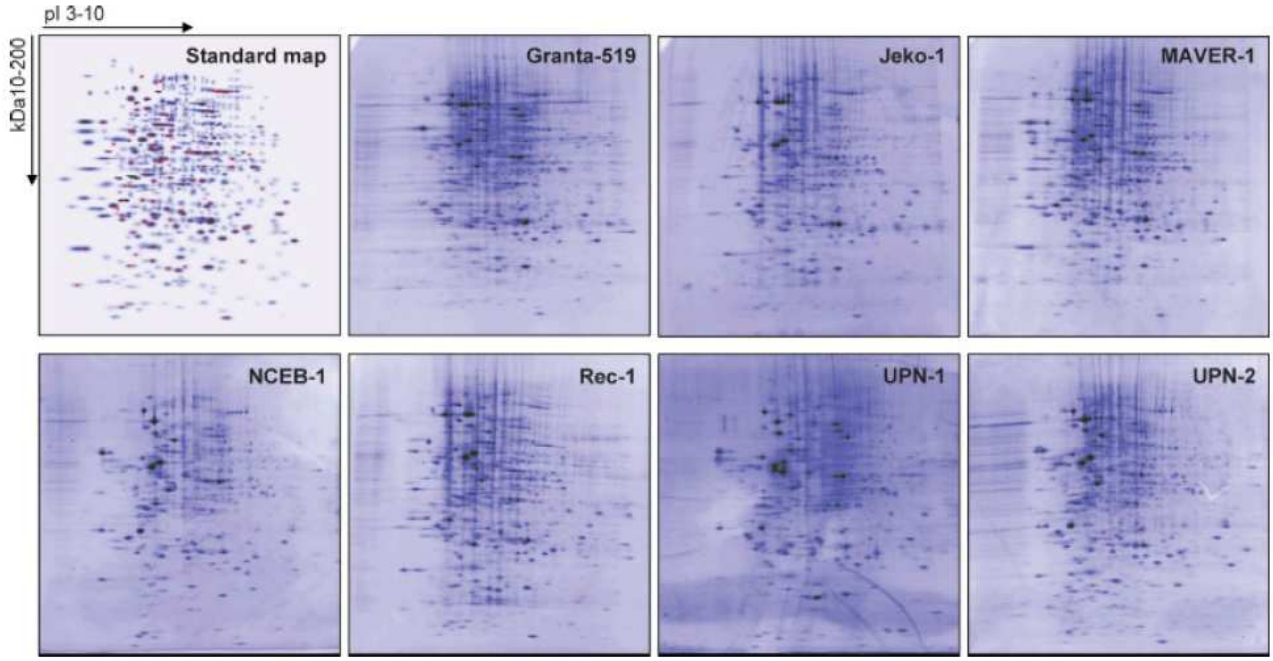


Figure 18. 2-DEphosphoproteins maps: the first 100 most abundant phosphoproteins are marked by red squares in the upper left.

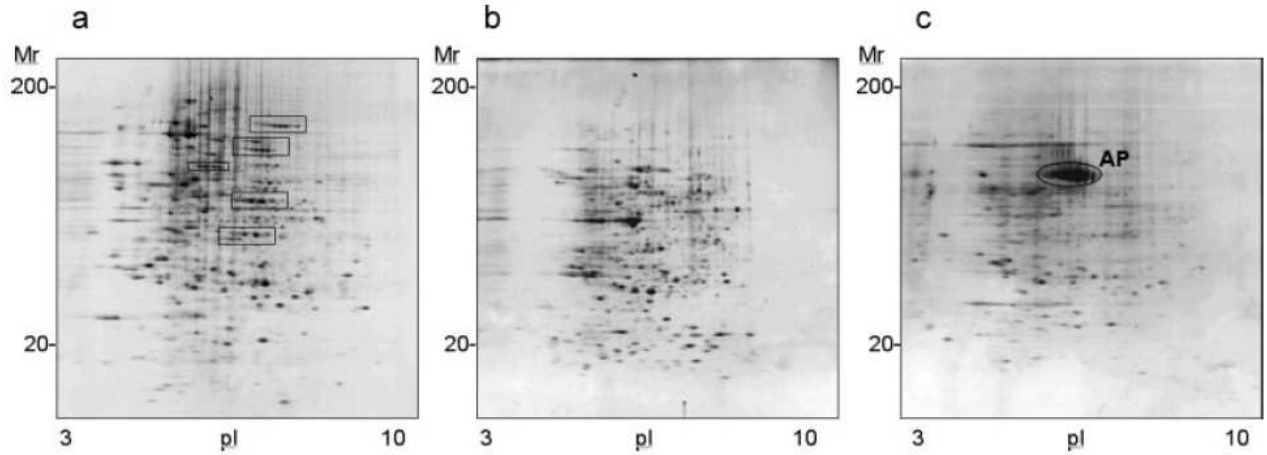


Figure 19. (a) Example of Sypro Ruby stained 2-D gel of phosphoproteins ("phosphorylation trains" are marked by rectangle); (b) unphosphorylated fraction; (c) the phosphorylated fraction treated with alkaline phosphatase (marked as AP) extracted from the MAVER-1 cell line.

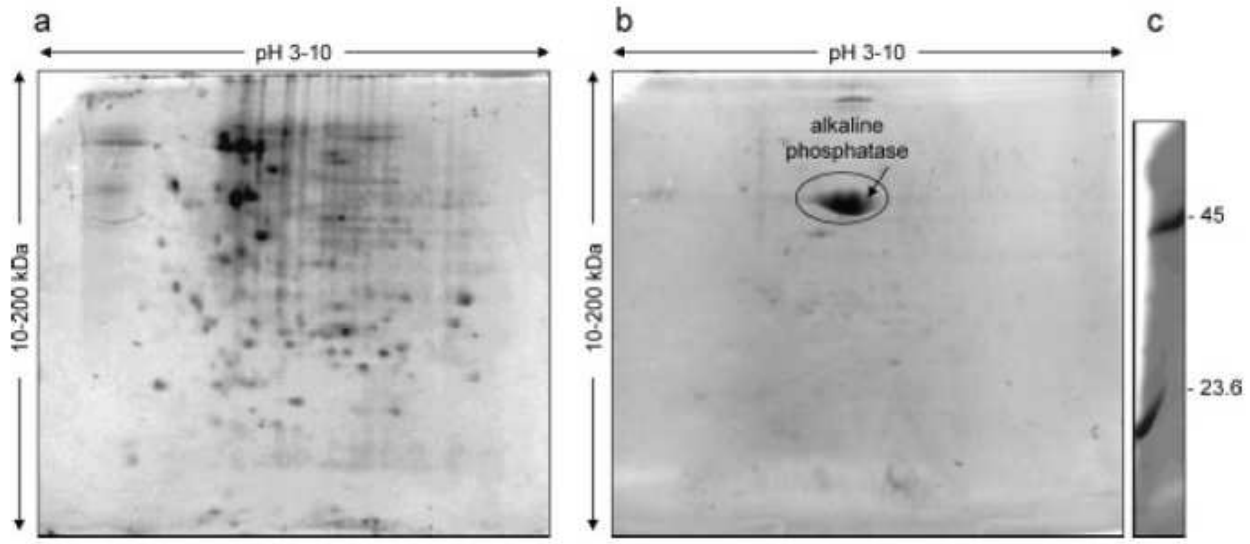


Figure 20. Phosphoprotein staining by Pro-Q Diamond. Total proteins from both (a) phosphorylated IMAC fraction and (b) phosphorylated IMAC fraction treated with protein phosphatase were separated by 2-DE gels and stained with fluorescent Pro-Q Diamond for phosphoproteins. Stained gels were scanned with a Typhoon 9600 fluorescence scanner. (c) PeppermintStickTM phosphoprotein molecular weight standards.

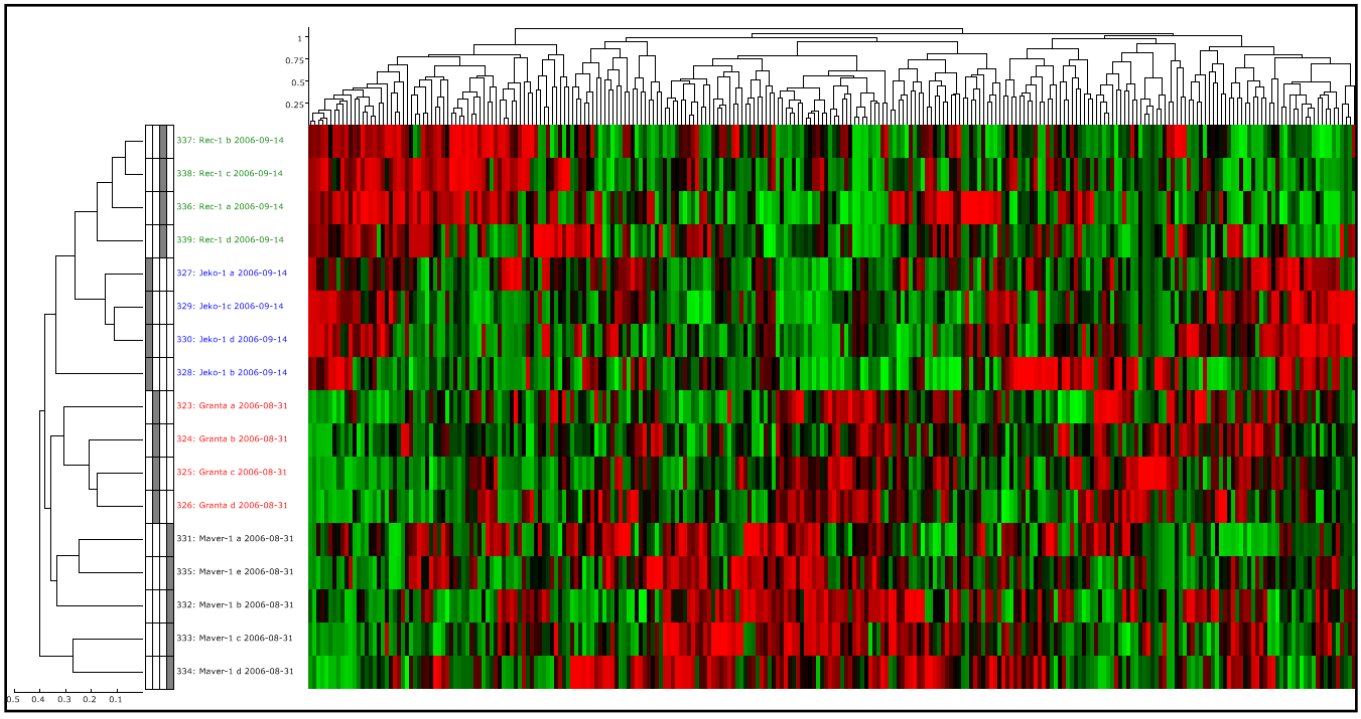


Figure 21. Bioinformatical analysis: a bioinformatical approach is necessary to analyze all data. Clustering cell lines results we can see they have a profile close to each other (meaning that the experiment is reproducible), on the other hand there are some differences, which make necessary to replicate each experiment to avoid false findings.

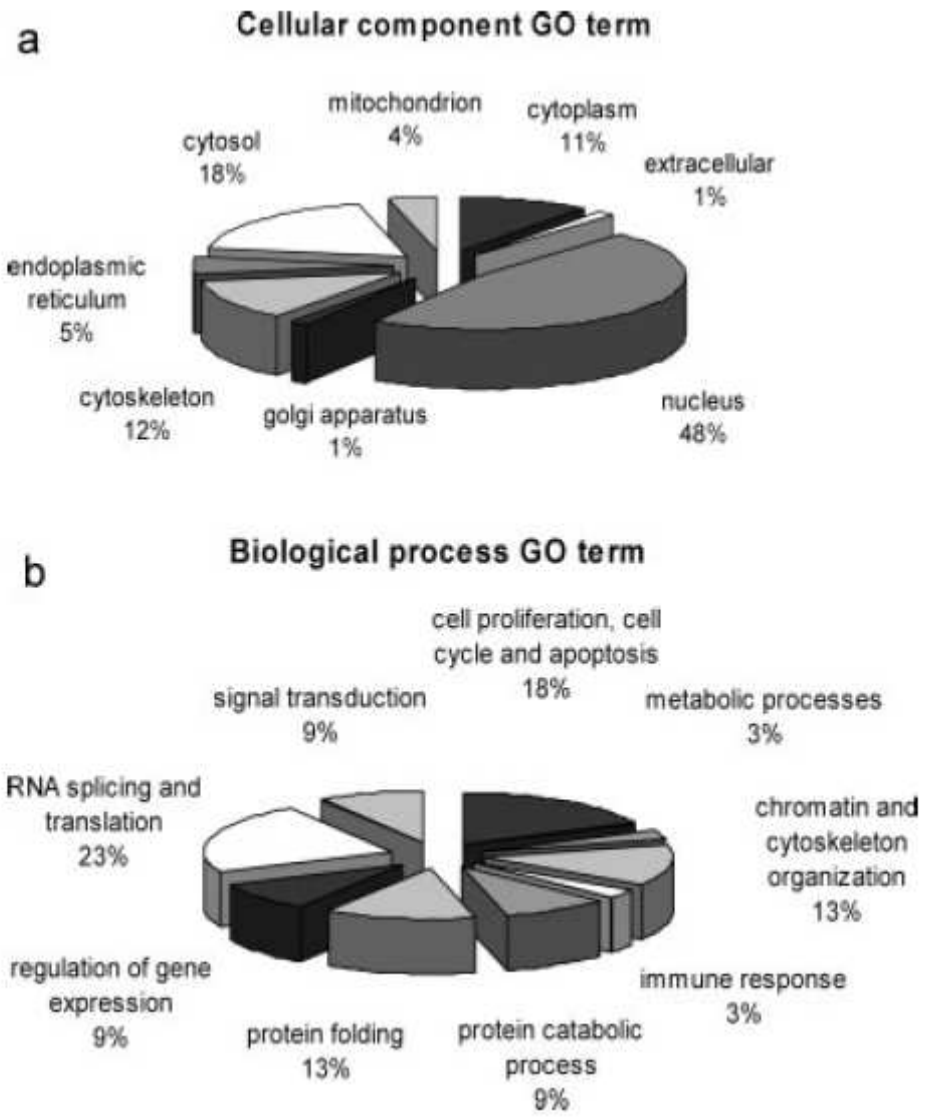


Figure 22. Distribution of identified proteins according to the (a) cellular component or the (b) biological process in which they are involved. Assignments were made on the basis of information provided by GO lists downloaded from FatiGO (http://fatigo. bioinfo.cipf.es/).

Phosphoscan analysis identified 421 unique peptides, corresponding to 341 proteins (table 5). Among these, several activated protein kinases were found. Interestingly, several identified proteins mapped to cytogenetic loci that have been reported to be altered in MCL by previous literature. Our data support a pro-survival role of BCR signaling in MCL and suggest that this pathway might be a candidate for therapy. Our findings also suggest that Syk activation patterns might be different in MCL compared to other lymphoma subtypes.

Protein name	Granta-519	Jeko-1	MAVER-1	Rec-1	BCR pathway	Kynase
Cdc2	17	38	16	26		*
CDK2	10	34	14	18		*
CDK3	10	33	13	17		*
Syk	9	19	24	3	*	*
SHIP	7	18	10	10	*	
PAG	9	14	4	8	*	
PRP4	3	19	8	2		*
Lyn	4	10	9	7	*	*
Ig-beta	0	22	2	0	*	
Hck	3	8	5	3	*	*
WASP	2	7	4	3	*	
SHP-1	4	7	4	1	*	
Btk	0	11	4	1	*	*
cofilin 1	2	7	5	1		
SgK223	6	1	4	4		*
LANP-L	0	6	5	3		
PKCD	4	3	2	4	*	*
GCET2	5	7	1	0	*	
HSP90B	5	5	0	2		
GSK3A	2	3	4	3	*	*
GSK3B	2	3	4	3	*	*
p38-alpha	2	3	6	1	*	*
hnRNP A1	5	2	3	1		
GRF-1	1	4	2	4		
SPTBN1	0	5	4	1		
SPTBN1 iso2	0	5	4	1		
PLCG2	2	4	3	0	*	
DYRK1A	1	4	3	1		*
Lck	4	2	1	2	*	*
HSP90A	2	4	1	1		
Blk	2	3	2	1	*	*
IRAP	1	4	3	0		
DDX3	4	3	0	0		
PL10	4	3	0	0		
Cbl-b	1	3	1	1	*	
BCAP	1	4	1	0		
Dok3	0	6	0	0	*	
ANXA2	1	2	2	1		
VIM	2	2	2	0		
L-plastin	0	4	2	0		
ENO1	0	2	2	2		
PIK3R1	1	2	2	1	*	*
DYRK1B	1	2	2	1		*
Src	1	2	1	2	*	*
Fyn	1	2	1	2	*	*
Yes	1	2	1	2		*
hnRNP 2H9	2	3	1	0		
hnRNP U	2	3	1	0		
hnRNP U iso2	2	3	1	0		
PAI-RBP1	2	2	2	0		

Table 5. Number of phospho-peptides corresponding to the specific protein is reported.

TMA

We obtained two TMA with MCL cases from the archives of our hospital (figure 23).

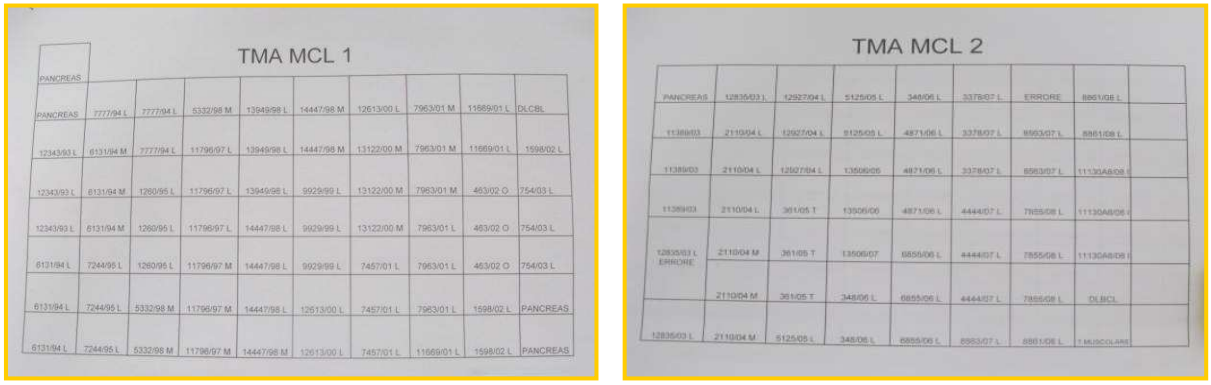


Figure 23. TMA cases (histologic number of each case is reported) with x,y position in the block.

Looking at H&E slides at least one core with MCL was present for all cases.

We've used two cores with normal pancreatic tissue to properly orient the section on the slide.

Two cores of a case of diffuse large B cell lymphoma were placed at the end of each TMA to have a immunohystochemical quality internal control for classic antibodies used in haematopathology.

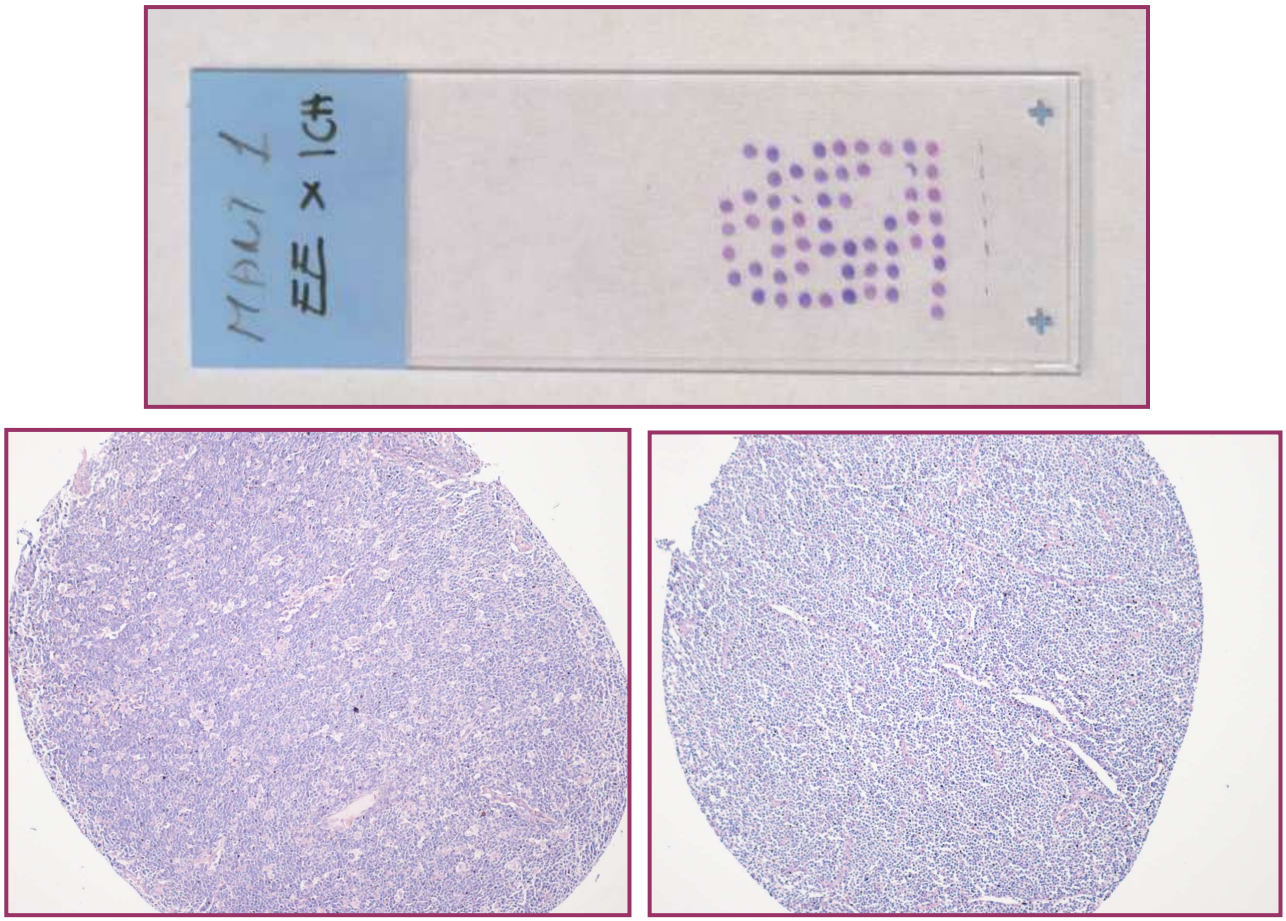


Figure 24. TMA 5 µm whole slides (upper part of the picture) and detail (lower part), coloured with H&E, microscopic 4x image.

S-Phase

Patients were 11 females and 27 males, with a median age of 56 years at diagnosis. No significant survival differences were present between males and females, between older (>60 years) and younger patients and between IPI-low (0-2) and IPI-high (3-5) patients. S-phase analysis showed a S-phase fraction variable from 0 to 53% of cells. Due to the non-normal distribution of this variable, values were natural-log transformed and then tested for normality by means of the Shapiro-Wilk test; this confirmed the lognormal distribution of the S-phase fraction in our series (figure 25).

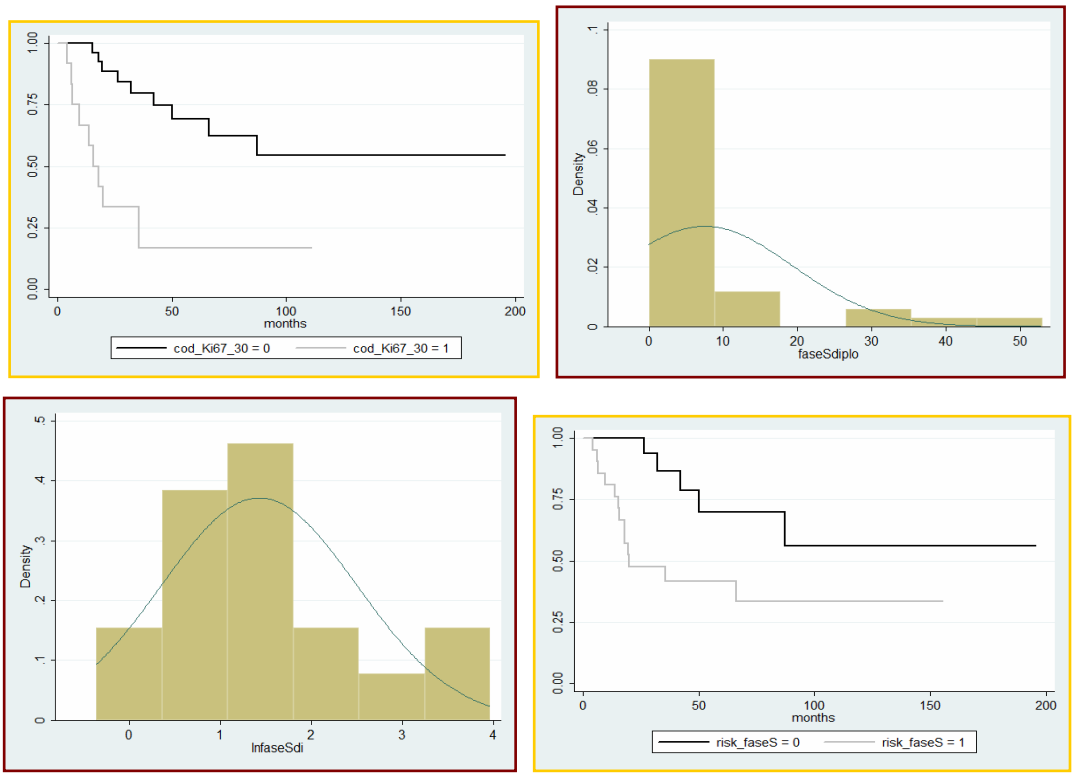


Figure 25. S-phase fraction data (with natural-log transformed) and survival curves of ki67 and S-phase natural logarithm.

When we looked at ki-67 we find low, intermediate and high proliferative index (figure 26-29), but we decided to classify cases into two groups of low and high with the cut-off of 30% of cells as recently suggested in literature (Kazenberger 2006).

The median of the log-transformed variable (1.30687, corresponding to 3.69% cells in S-phase) was used as cut-off: 21 patients were classified as S-phase-high and 17 as S-phase-low. Ki-67 index was lower than 30% in 27 cases, and higher than 30% in the remaining 11. Linear regression coefficient between Ki-67 and S-phase was 0.20581. OS was calculated according to the method by Kaplan and Meier, and survival curves were compared by means of the log-rank test. The statistical significance of the impact of variables on overall survival (OS) was determined by Cox's Proportional Hazard modelling.

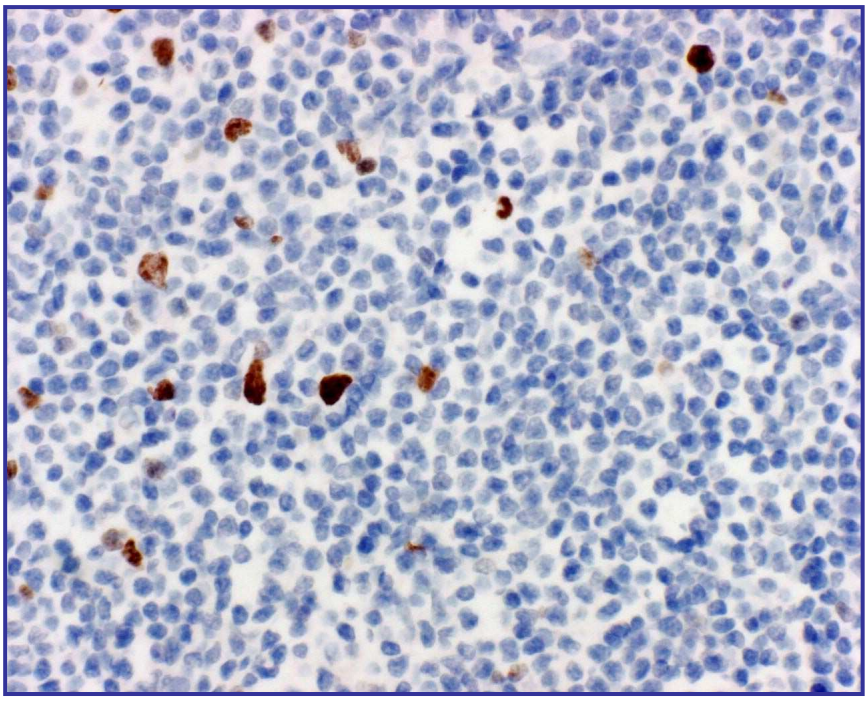


Figure 26. ICH ki-67: very low proliferative index.

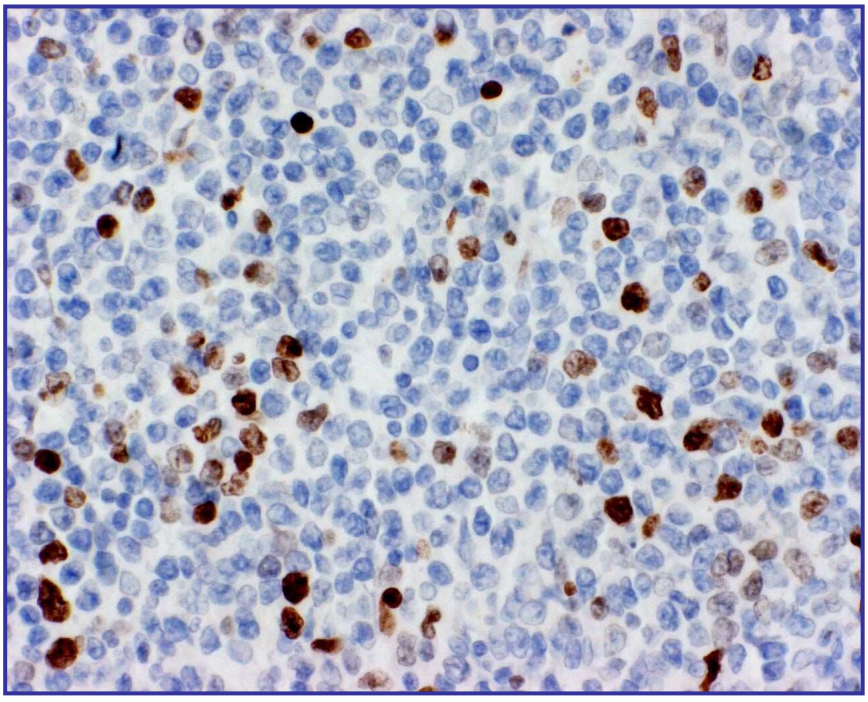


Figure 27. ICH ki-67: intermediate proliferative index.

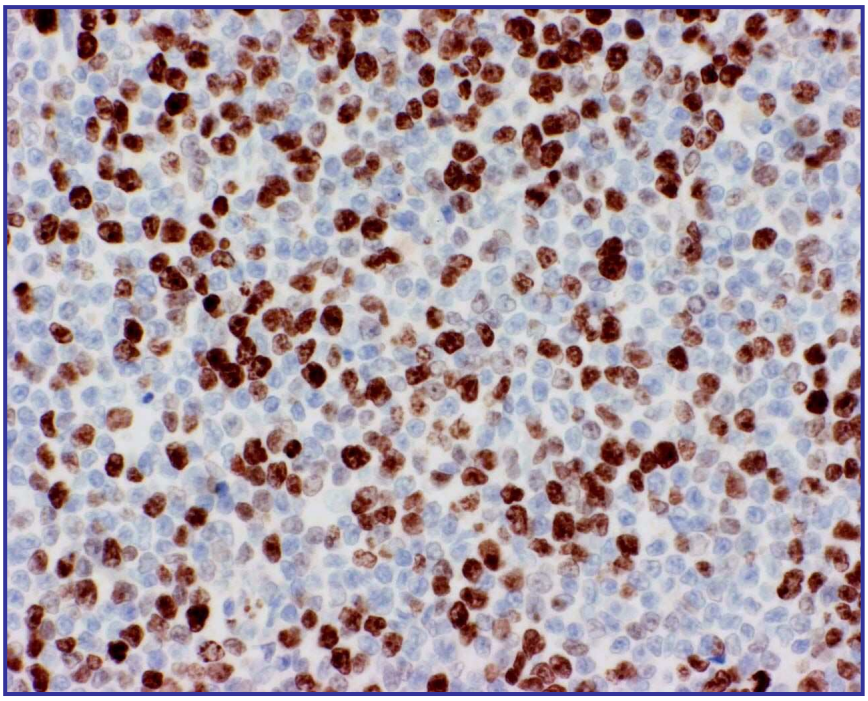


Figure 28. ICH ki-67: high proliferative index.

At the univariate analysis (table 6), both Ki-67≥30% and S-phase-high appeared as statistically significant negative prognostic factors. Also significantly predictive of poorer outcome at the univariate analysis were: blastoid variant, leukemic involvement and a therapeutic regimen not comprising high-doses chemotherapy (HDT) followed by autologous stem cell transplantation (autoPBSCT). At the multivariate analysis only this latter (no HDT plus autoPBSCT) remained independently predictive on survival, while S-phase fraction and blastoid variant showed an interesting trend at the limits of statistical significance (P=0.057 and P=0.058, respectively); Ki-67 labelling index did not remain independently significant at the multivariate analysis.

	unadjusted HR (95% CI)	***P***	***adjusted HR(95% CI)***	***P***
Ki67 ≥ 30%	5.10 (1.95 -13.34)	*0.001*	2.45 (0.57 – 10.6)	*NS*
S-phase-high (ln)	3.28 (1.16 – 9.27)	*0.02*	4.98 (0.95 – 26.1)	*0.057 (NS)*
blastoid variant	3.72 (1.31–10.59)	*0.01*	3.96 (0.99 – 15.7)	*0.051 (NS)*
leukemic involvement	2.81 (1.08 – 7.30)	*0.03*	1.43 (0.47 – 4.34)	*NS*
no-HDT + autoPBSCT	11.39 (1.49–86.9)	*0.02*	26.21 (2.5 – 275)	*0.006*
tetraploid subclones	0.37 (0.1 – 1.34)	*NS*		
IPI ≥ 3	1.65 (0.65 – 4.19)	*NS*		
male sex	0.99 (0.35 – 2.79)	*NS*		
age ≥ 60	1.73 (0.66 – 4.53)	*NS*		
B systemic symptoms	2.18 (0.85 – 5.55)	*NS*		
elevated LDH	1.56 (0.62 – 3.93)	*NS*		

Table 6. Prognostic factors in univariate analysis.

Animal model

Attempts to grow primary MCL cells in NOD/SCID mice were unsuccessful because after 3-4 weeks mice developed GHVD and died. So we inoculated MCL cell lines in rag -/- γ -/- grey mice. We tested different ways of inoculation, intraperitoneal, subcutaneous and intravenous (table 7) and sacrificed mice as soon as they developed a macroscopic mass or when we saw illness symptoms.

n. RES	mouse model	MCL cell line	way of inoculation	findings	age at sacrifice
C	Rag2(-/-) γ chain (-/-)	Jeko-1	subcutaneous (s.c)	mass	54 days
D	Rag2(-/-) γ chain (-/-)	Jeko-1	intraperitoneum (i.p.)	mass	49 days
E	Rag2(-/-) γ chain (-/-)	Jeko-1	intraperitoneum (i.p.)	mass	59 days
I	Rag2(-/-) γ chain (-/-)	Jeko-1	Intravenous (i.v)	illness	70 days
J	Rag2(-/-) γ chain (-/-)	Jeko-1	Intravenous (i.v)	illness	70 days
A	Rag2(-/-) γ chain (-/-)	UPN-1	subcutaneous (s.c)	mass	28 days
B	Rag2(-/-) γ chain (-/-)	UPN-1	subcutaneous (s.c)	mass	28 days
K	Rag2(-/-) γ chain (-/-)	UPN-1	intraperitoneum (i.p.)	mass	29 days
G	Rag2(-/-) γ chain (-/-)	UPN-1	intravenous (i.v)	mass	72 days
H	Rag2(-/-) γ chain (-/-)	UPN-1	intravenous (i.v)	mass	72 days
F	Rag2(-/-) γ chain (-/-)	Maver-1	intraperitoneum (i.p.)	mass	29 days

Table 7. MCL cell lines injected with way of inoculation and age at sacrifice.

All the 3 lines injected (UPN1, JEKO-1 and MAVER1) grown very well in our mice and looking at histology we could see a high grade lymphoma with a very high proliferative index, almost resembling blastoid mantle cell lymphoma (figure 29).

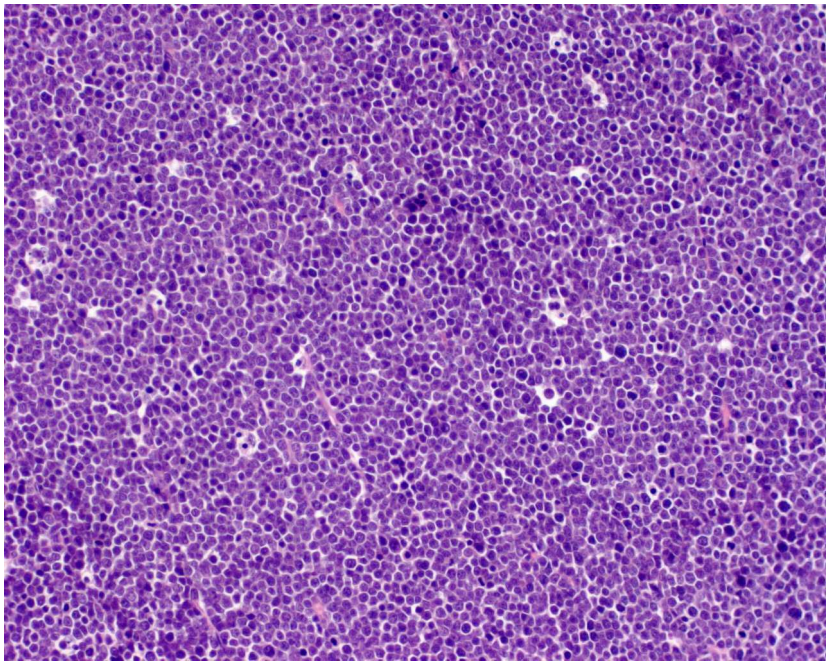


Figure 29. H&E image of tumoral mass of mouse, 10x.

The first two mice we sacrifice, who were injected with UPN-1 MCL cell lines sub cutis almost one month before, developed a huge mass due to a high grade lymphoma infiltrating the abdominal wall and the adrenal gland (or brown fat?), with no involvement of visceral organ nor bone marrow (mouse A, mouse B). The third mouse had received Jeko-1 cells sub cutis too, but developed a small mass after 2 months, which bulge under the epidermidis growing deep down in the abdominal wall, with no involvement of visceral organ nor bone marrow (mouse C). When we've looked at mice injected intraperitoneum, two with Jeko-1 sacrificed after 6 and 7 weeks, one with UPN-1 sacrificed after 2 months and one with MAVER-1 sacrificed after 5 weeks, we've found after 6 week just a big intra abdominal mass without organ involvement (mouse D) while in the mouse sacrificed one week later (mouse E) (figure 30, figure 31) a high grade lymphoma seems to permeate almost all abdominal organ with massive infiltration of scheletric muscle of the abdominal wall, adrenal gland, uterus, the mesentere and the wall of large and small bowel without reaching the mucosa except than in the stomach. One kidney, the pancreas, the spleen, the liver as a mass or growing along with portal tract and bone marrow were involved too (figure 32-33).

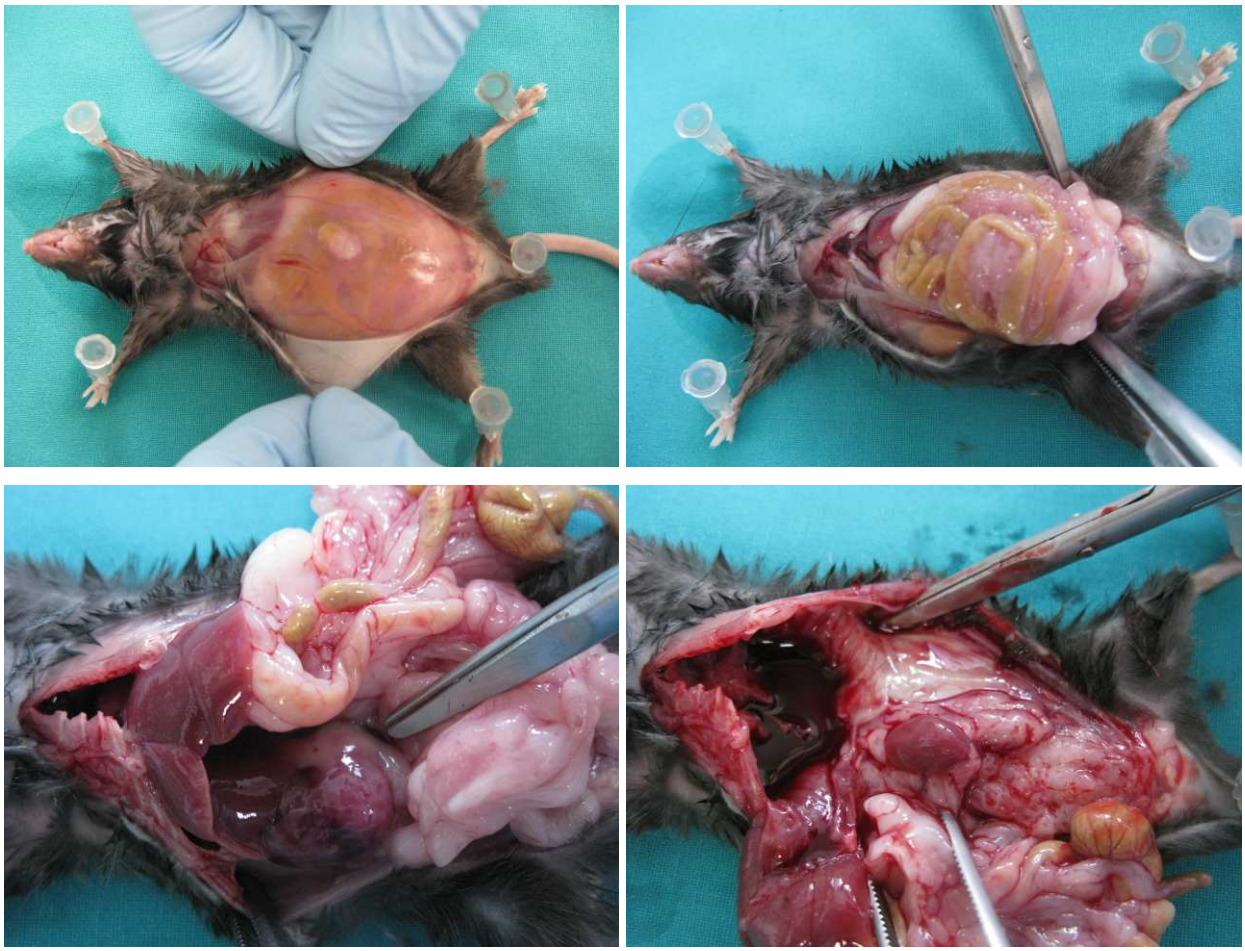


Figure 30. Autopsy performed on mouse E, with evidence of deep infiltration of mesenteric fat (upper images) of one kidney (lower left) while the other kidney was preserved (lower right).

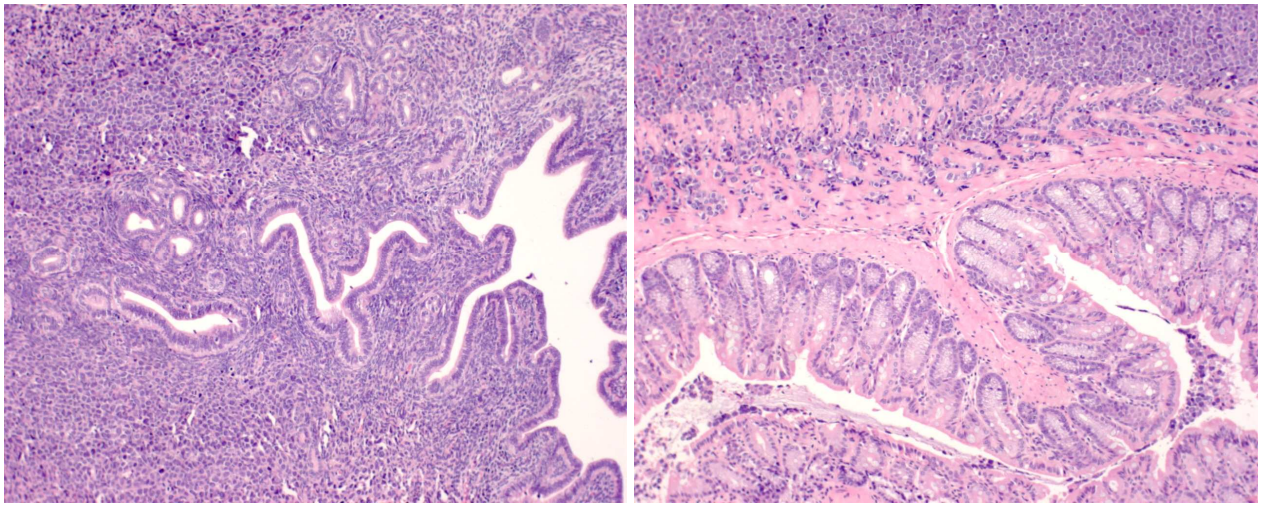


Figure 31. Lymphomatous infiltration of uterus on the left and of muscle bowel wall on the right.

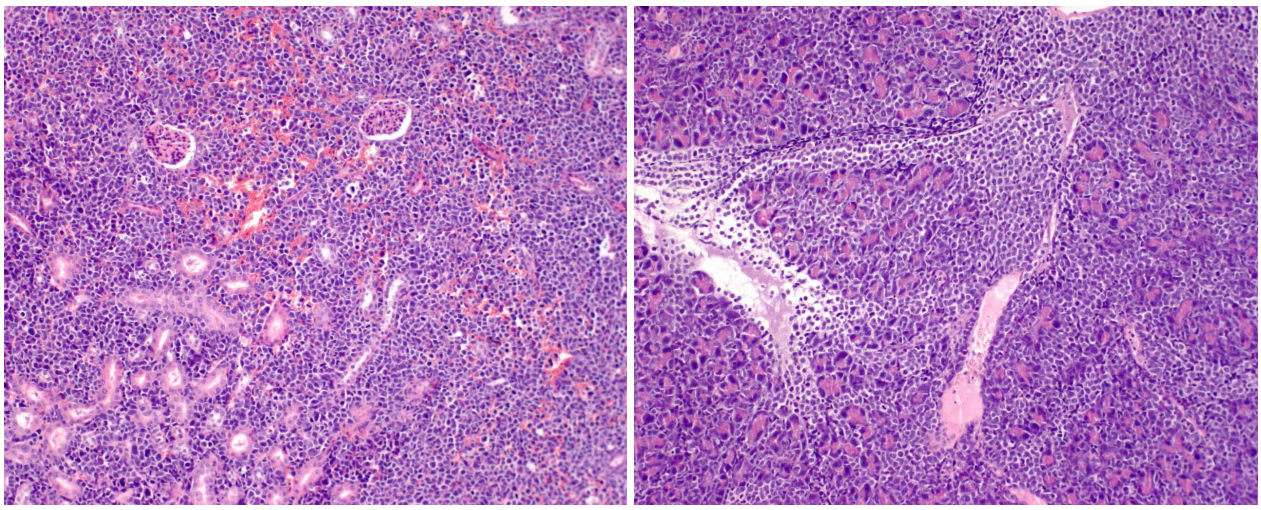


Figure 32. Kidney (left) and pancreatic (right) infiltration.

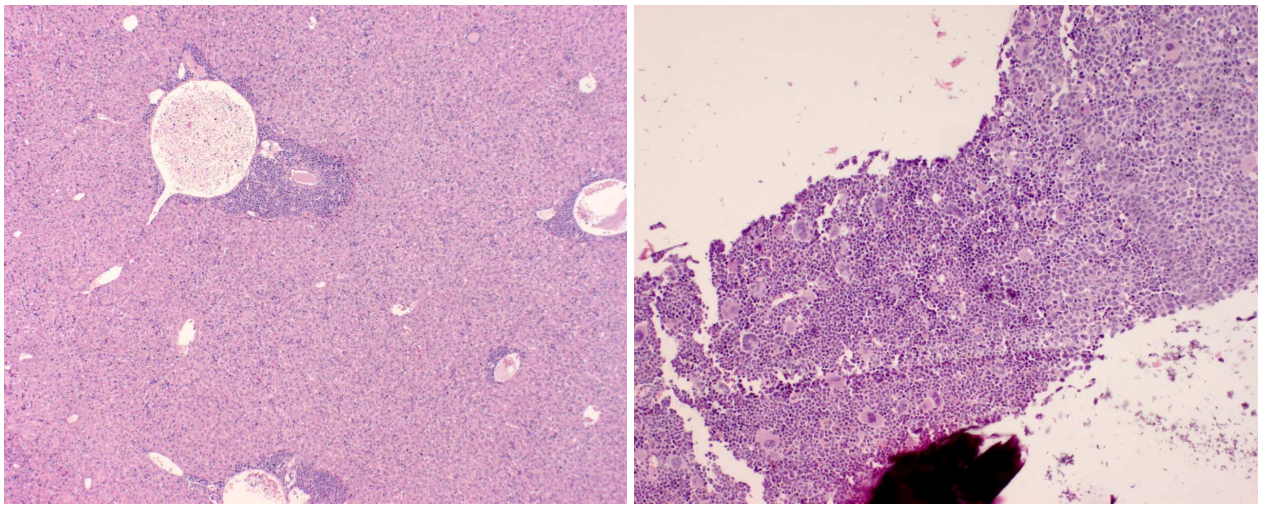


Figure 33. Liver (left) and bone marrow (right) infiltration.

UPN-1 on the other hand, after two months were able to produce an abdominal mass with no organ infiltration. Just one kidney presented a focal subcapsular localization (mouse K) (figure 34). MAVER-1 gave us the same results after 5 weeks from the day of inoculation (mouse F).

Intravascular way of inoculation of UPN-1 cells after more than 2 months produced a subcutis mass and a spleen involvement associate to small subpleural localization (mouse G) (figure 34 and figure 35), but nothing in visceral organs in the other (mouse H).

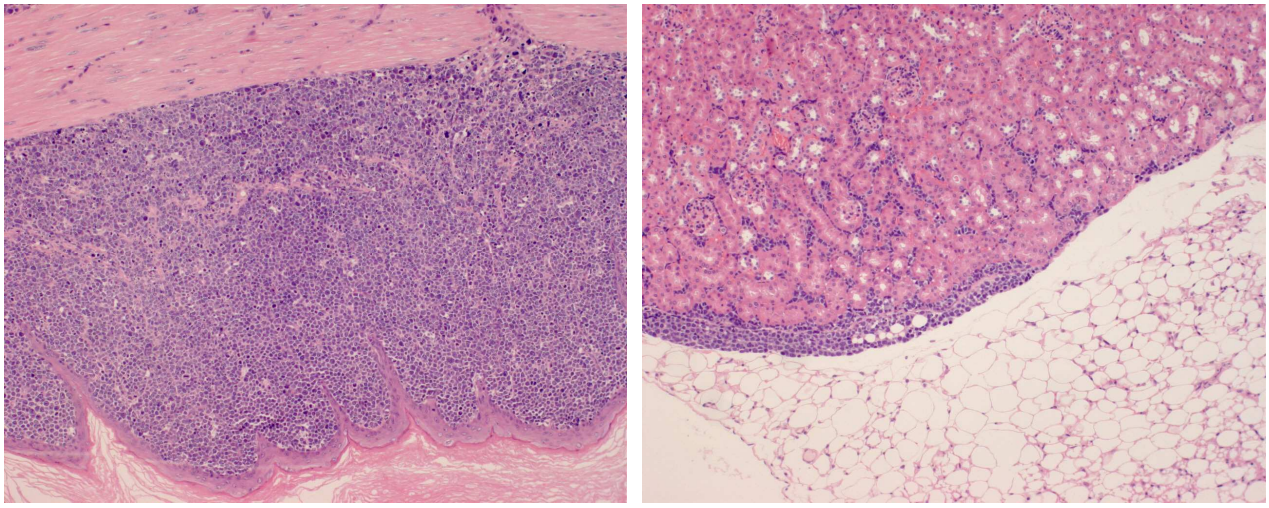


Figure 34. Subcutis mass (left) in mouse G and capsular kidney localization (right) in mouse K.

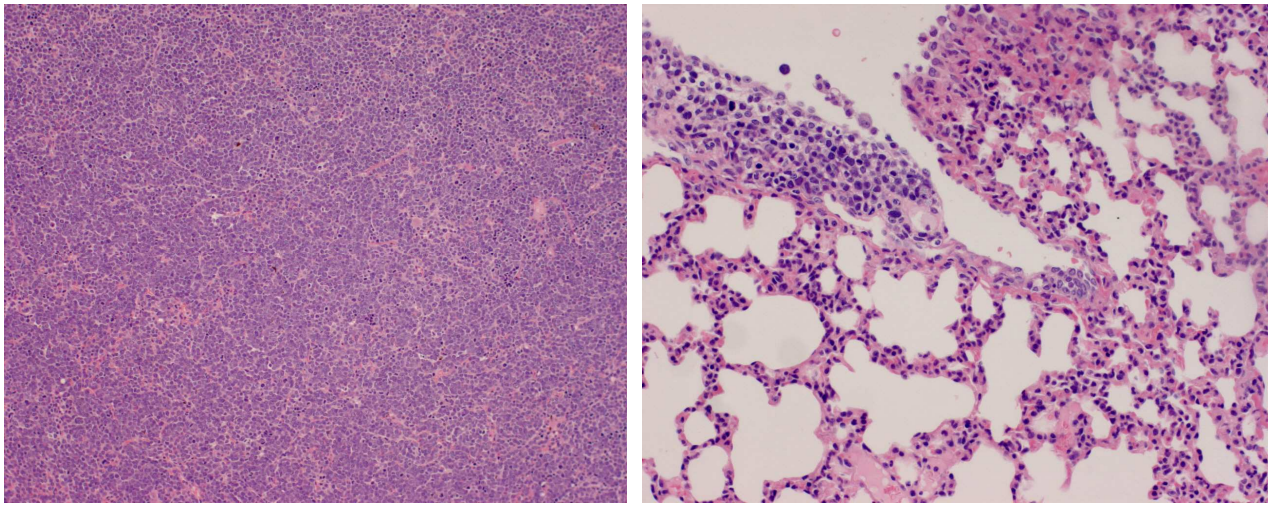


Figure 35. Spleen (left) and subpleural (right) infiltration of mouse G.

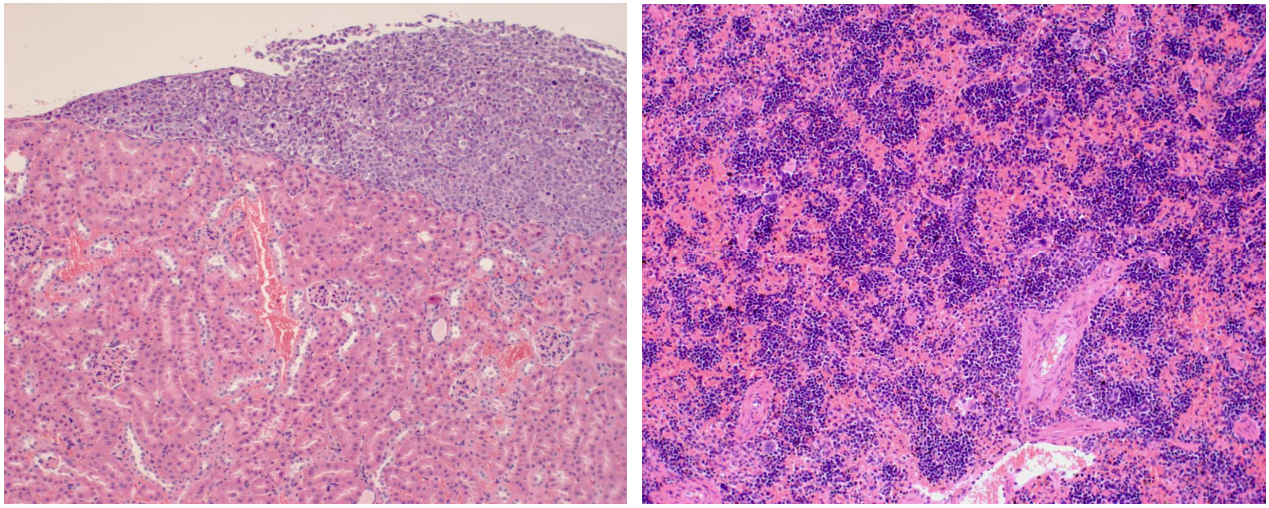


Figure 36. Kidney subcapsular infiltration (left) and normal spleen (right) of mouse I.

Jeko-1 intravascular injection produced bone marrow involvement and subcapsular kidney infiltration in one mouse (mouse I) (figure 36), nothing in visceral organs in the other (mouse J)

ICH

Immunohistochemistry performed on TMA showed expression of CD20, CD5, cyclinD1 (figure 37). As some cases turned out to be sox11 just weakly positive or negative we performed those antibody on whole slides obtained from paraffin block to avoid fixation problems.

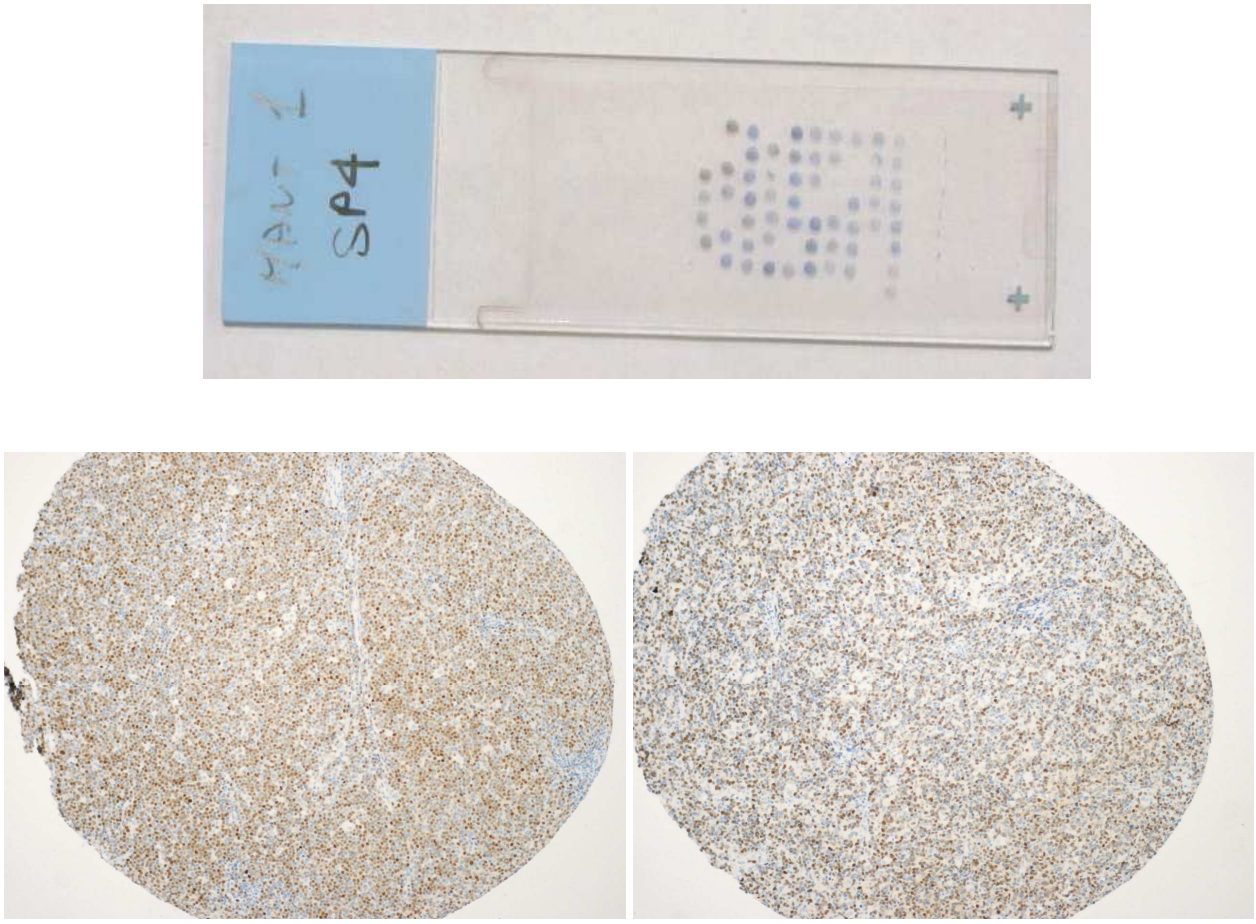


Figure 37. Cyclin D1 expression in whole TMA and in detail.

ICH performed on lymphoid mass grown in mice showed strong positivity for CD20, cyclin D1, sox 11 and a high proliferative index. CD5 turned out to be negative, as it is not expressed in MCL cell lines before injection either.

About new antibodies, tested first on reactive lymph node or tonsil to check the right dilution and the better retrieval method, we can say that Pragmin, GCET2 and CDC2 turned out to be expresses in tumoral mass of the first four mice we sacrificed (figure 38-41, table 8). As we plan to sacrifice

24 mice we than decide to wait for all of them and build a TMA with mice donor blocks and test new antibodies on it.

mouse	RES 9 A	RES 9 B	RES 9 C	RES 9 D
MCL cell line	UPN1	UPN1	JEKO-1	JEKO-1
ph STAT3	neg	neg	neg.	neg.
Pragmina (DKFZp..)	+	+	+	+
PRKCD	neg		neg.	+
GCET2	+	+/-	+	+
CdK2	--/-/+	--/-/+	--/-/+	--/-/+
CDC2	+	+	+	
PRPF4 beta	neg	neg		neg
CD3	neg	neg	neg.	neg.
CD5	neg	neg		+/-
CD20	+	+	++	+
CD7	neg	neg		neg.
CD10	+/-	+/-	++/+/-	+
ki67	> 80%	> 80%	> 80%	80%
sox11	+	+	+	+
sp4	+	+	+	+

Table 8. ICH on lymphomatous mass of the first 4 mice sacrificed.

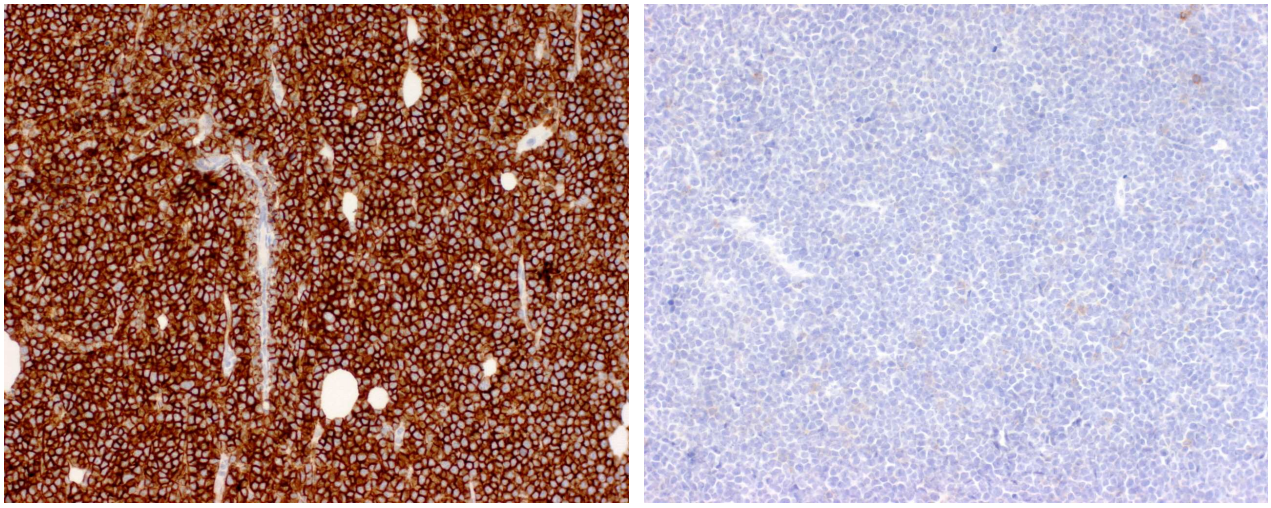


Figure 38. ICH on mouse B lymphoid mass: CD20 (left) and CD5 (right).

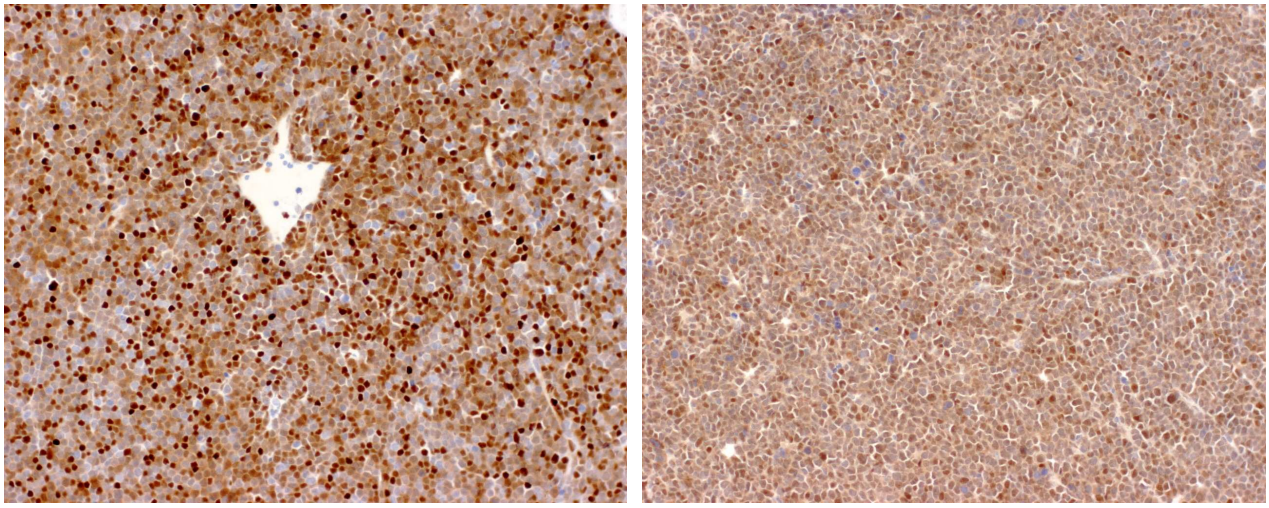


Figure 39. ICH on mouse B lymphoid mass: Cyclin D1 (left) and SOX 11 (right).

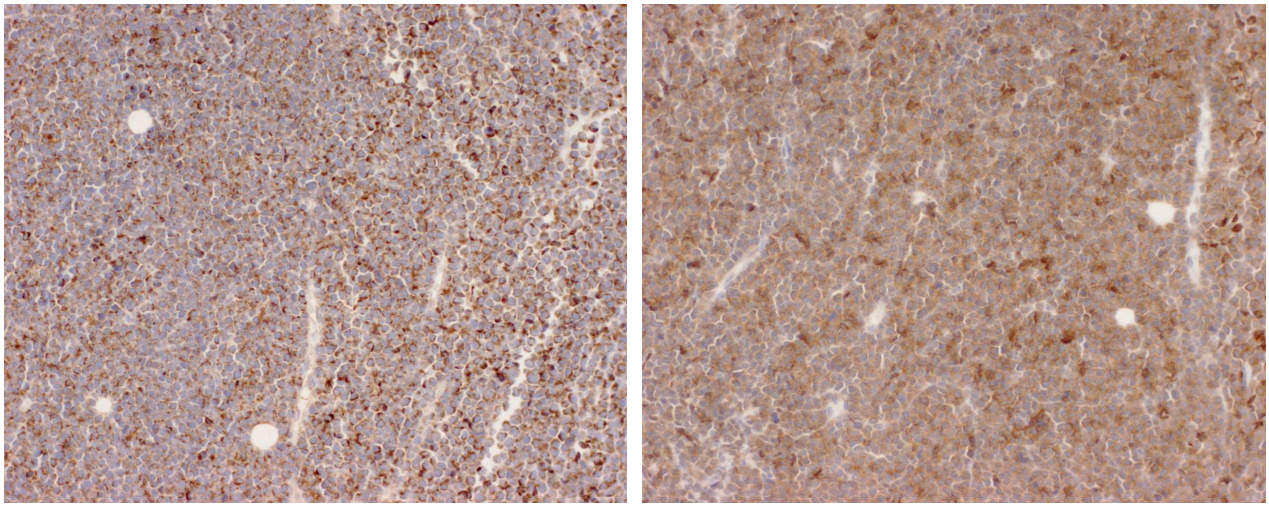


Figure 40. ICH on mouse B lymphoid mass: Pragmin (left) and GCET2 (right).

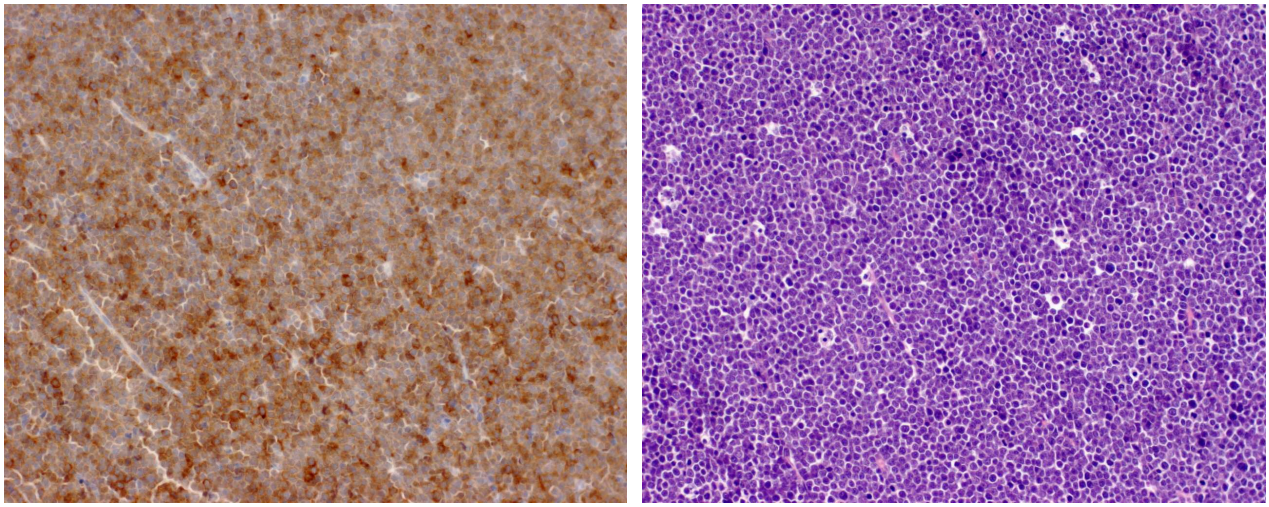


Figure 41. ICH (left) on mouse B lymphoid mass for Cdc2, H&E corresponding image (right).

DISCUSSION

Candidate kinase identification

Bioinformatics was used to obtain information on the phosphorylation sites and the presence of kinase-specific motifs for the identified phosphoproteins. As mentioned above, most of the identified proteins contained the typical MAPK1 and CK2 phosphorylation motifs, although casein kinase 1 and novel or atypical PCK members (epsilon and zeta) were also commonly found.MAPK1 is involved in a wide variety of cellular processes such as proliferation, differentiation, and transcription regulation. The activation of this kinase requires its phosphorylation by upstream kinases. Interestingly, it has been reported that the constitutive activation of MAPK1 plays a key role in B-cell lymphoproliferative disorders (Ogasawar 2003), but further validation is still lacking. CK2 is a ubiquitous serine–threonine kinase that regulates many relevant biologic processes, and is considered an oncogene (Seldin 2005). CK2 is known to interact with p27 (Tapia 2004, and in yeast CK2 depletion blocks cell cycle by an increase in p27 homolog Sic1 (Tripodi 2007). Since p27 degradation is a known event in MCL pathogenesis, whose effect is to relieve its inhibition on cyclin/Cdk complexes, it is tempting to speculate that it might be favored by CK2 activity. Interestingly, CK2 also interacts with CD5 (Raman 1998), which is typically expressed in MCL. It has been demonstrated that CK2 is involved in the pathophysiology of multiple myeloma, suggesting that it might play a crucial role in controlling survival and sensitivity to chemotherapeutics of malignant plasma cells (Piazza 2006). Novel members of the PKC family are also potentially interesting therapeutic targets (MacKay 2007). PKCzeta can activate both NF-kB and Stat6 pathways (Moscat 2006). Of interest, PKCzeta has been found to be active in MCL cells (Leseux 2008), and PKCepsilon has been shown to be a very potent inducer of Cyclin D1 expression (Soh 2003). We were able to construct a network showing the involvement of the identified phosphoproteins in some important signal transduction pathways, based on the published literature. Hereafter, some of the phosphoproteins belonging to signal transduction pathways that are relevant to tumorigenesis (including NF-kB, mTOR, and mitochondrial signalling) are briefly discussed.

NF-kB signaling related phosphoproteins

NF-kB is a transcription factor present in all cell types that is involved in cellular responses to stimuli such as stress, cytokines, free radicals, UV irradiation, and bacterial or viral antigens (Okamoto 2007). NF-kB is responsible for increased proliferation of cells and tumour growth by transcriptional activation of anti-apoptotic proteins. The nuclear translocation of NF-kB is controlled by two main pathways, namely the classical and the alternative. The classical NF-kB

pathway involves the proteasome degradation of the inhibitory molecule IkBa, while the alternative NF-kB pathway induces p100 proteasome processing and p52 generation through the activation of NF-kB-inducing kinase (NIK). Gene expression profiling studies have shown that the transcripts involved in NF-kB signaling pathways are overe-xpressed in MCL (Martinez 2003) and that its inhibition induces death in MCL cells (Pham 2003, Shishodia 2005). Interestingly, both cyclin D1 and Bcl-2 are regulated by NF-kB (Shishodia 2002, Baichwal 1997). We found that several proteasome subunits (PSMA1,PSMA2, PSMA4, PSMA5, PSMB2, PSMB3, and PSMB4), the proteasome activator PA28 (PSME1, PSME2), and ubiquitin (UBB) are phosphorylated and highly expressed in MCL cell lines. Our results are consistent with a new MCL therapeutic strategy based on proteasome inhibition, recently approved by the FDA (Leonard 2006). Among the phosphoproteins involved in the classical NF-kB pathway, we identified Importin alpha3 (KPNA3), which contributes to NF-kB nuclear transfer (Fagerlung 2005). We also identified hsp90 (HSP90AB1), a protein involved in the alternative NF-kB pathway. This protein has been recently shown to be upregulated in MCL by antibody-microarray technology (Gobrial 2005). Accordingly, it has been demonstrated that HSP90AB1 protects NIK from autophagy-mediated degradation (Qing 2007). It is of interest to note that 17-allylamino-17- demethoxy-geldanamycin (17-AAG), an hsp90 inhibitor, induced G0/1 cell cycle arrest, and cell death in MCL cell lines (Georgakis 2006). We also identified basic transcription factor 3 (BTF3), which acts as a transcription factor and a modulator of apoptosis. This is in agreement with other findings showing that BTF3 regulates the transcription of NF-kB (and its target genes) (Kusumawidijaja 2007). We also found, as a phosphorylated protein, the retinoblastoma binding protein 4 (RBBP4) which is transcriptionally controlled by NF-kB (Pacifico 2007) and belongs to the HDAC complex that binds to the tumor suppressor retinoblastoma protein Rb.

mTOR signaling-related phosphoproteins

The mTOR is a large and highly conserved kinase that integrates growth factor stimulation, energy, and nutrient availability to modulate translation of proteins responsible for cellular growth and proliferation (Petroulakis 2006). mTOR inhibitors have demonstrated efficacy against lymphoma cells, and in particular in MCL (Costa 2007); moreover, the phosphoinositide-3 kinase (PI3K)-AKT pathway has been identified as a key player in MCL by gene expression profiling (Rizzatti 2005) as well as Western blotting and biochemical studies (Rudelius 2006). Among the phosphorylated proteins, we identified eukaryotic translation initiation factor 2 (EIF2S1). Previous data provided evidence that EIF2S1 phosphorylation is involved in the induction of PI3K that activates mTOR signalling (Kazemi 2007). We also identified eukaryotic translation initiation factor 3 (eIF3), (eIF3S1 and eIF3S2) as a phosphorylated protein. eIF3 binds to the 40S ribosomal subunit and

interacts with other initiation factors and mRNA, regulating the rate of translation initiation. Recently, it has been demonstrated that eIF3 also acts as a scaffold for the mTOR/raptor complex and its substrate (S6 kinase1), allowing the coordination of protein translation 8Holz 2005). Interestingly, phosphorylation of eIF3 was found to be dependent on mTOR kinase activity (Ahlemann 2006, Harris 2006). In addition, increased levels of the eIF3 have been detected in a wide variety of human tumours and have been suggested to be prognostic for poor clinical outcome (Zhang 2007). eEF2 was also identified. Along these lines, it has been reported that mTOR signalling also regulates the translation elongation process through eEF2 phosphorylation (Redpath 2007). eEF2 is a GTP-binding protein that mediates the translocation step of elongation. When phosphorylated, eEF2 loses its ability to bind to ribosomes and is thus inactivated. FK506 binding protein 4 (FKBP4, also known as FKBP52) was one of the phosphorylated proteins correlated with copy number gains. FKBP4 is another potential regulator of mTOR, as homologs (like FKBP12 and FKBP51) have been recently demonstrated to be important players in mTOR signalling (Bai 2007).

Mitochondrial signaling-related phosphoproteins

Mitochondria play important roles in cellular energy metabolism, free radical generation, and apoptosis. It is well known that defects in mitochondrial signaling contribute to the development and progression of cancer. Among the phosphoproteins related to the mitochondrial signaling, cofilin (CFL1) was highly expressed and phosphorylated in MCL. It is interesting to note that the phosphorylation of CFL1 negatively regulates its function on actin by inhibiting the depolymerization activities (Moriyama 1996). More importantly, it has been reported that p21 is involved in inhibition of CFL1 phosphorylation (Lee 2004), and p21 is indeed downregulated in aggressive variants of MCL (Pinyol 1997). Accordingly, unphosphorylated CFL1 can accumulate in mitochondria and induce apoptosis (Chua 2003, Wang 2006). These findings help to explain the high level of phospho-cofilin detected in MCL. Heat shock 110 kDa protein (HSPH1) was also identified, which is over-expressed in a variety of human tumours (Kai 2003). In HeLa cells, it suppresses apoptosis by inhibiting the translocation of Bax to mitochondria (Yamagishi 2006). Remarkably, HSPH1 is bound directly to tubulin alpha (TUBA1A) (Saito 2003), which is another phosphoprotein that we found to be highly expressed in MCL. The activation of Bax is also suppressed by heat shock protein 5 (HSPA5) 70 kDa, which was also highly expressed in MCL cell lines. Recently, it has been demonstrated that HSPA5 prevents apoptosis by inhibiting cytochrome c release from mitochondria (Lee 2001). It is interesting to note that the IRES-dependent translation of the antiapoptotic HSPA5 is enhanced by autoantigen-La (SSB)(Kim 2001). SSB enhances internal ribosome entry site (IRES)-dependent translation of X-linked inhibitor of apoptosis protein (XIAP) (Holcik 2000). SSB is a key protein in RNA biogenesis, and is dephosphorylated and

cleaved during apoptosis (Rutjes 1999). SSB was among the phosphorylated proteins which are highly expressed in MCL, in agreement with a recent report exploring MCL proteome by antibody microarrays (Ghobrial 2005).

Correlation with SNP-chip analysis

While thorough analysis of all positively correlated proteins is beyond the scope of the present investigation, a few comments are merited. It has already been mentioned that FKBP4 (located on 12p13) is involved in mTOR signalling and displayed recurrent copy number gains in MCL cell lines. Another protein showing strong correlation with copy number is ST13 (also known as HIP, located on 22q13), a molecule showing similarities to BID, and probably involved in the regulation of apoptosis through interaction with BAG- 1 and Hsp70 (Caruso 2006). Interestingly, both these molecules appear to be involved in regulation of mitochondrial apoptosis, a pathway not widely explored in MCL.

PhosphoScan

We describe the results obtained from the application of PhosphoScan to MCL cells. This is to our knowledge the first use of this technique in MCL; we report a large amount of data with precise identification of phosphorylation sites for each protein, some of which novel. PhosphoScan analysis identifies the tyrosine-activated phosphoproteome of MCL cell lines and identifies B-cell receptor signalling as the most active pathway. PhosphoScan analysis identified 421 unique tyrosine-phosphorylated peptides, corresponding to 341 proteins, ranked by overall abundance in table 1. Interestingly, several identified proteins mapped to cytogenetic loci that have been reported to be altered in MCL (Bea 2009, Rubio-Moscardo 2005, Vater 2009). Among these, 4 several activated protein kinases were identified (figure 42), that are therefore appealing as potential therapeutic targets. The three most represented phospho-peptides are cyclin-dependent kinases Cdc2, CDK2 and CDK3. Cdc2 (CDK1) is actually part of the proliferation signature able to predict prognosis in MCL (Rosenwald 2003) and has also been validated at the immunohistochemical level (Hui 2005). CDK2 gains have been detected in MCL in a variable proportion of cases (Rubioi-Moscardo 2005, Vater 2009, Jardin 2009), and have been associated with a poor prognosis (Jardin 2009). CDK3 is less studied compared to other cyclin-dependent kinases, but its locus has also been reported as subject to gains (Vater 2009, Togawa 2005). Its involvement in MCL is a novel finding, although the role of CDK3 as an oncogene has been demonstrated in other models (Cho 2009, Zheng 2008). Syk and many other BCR pathway members were then found. Many kinases belonging to this pathway (e.g. Syk, Lyn, Hck, Btk, PKC-delta), were among the most represented phosphopeptides identified by the PhosphoScan approach, and this was confirmed when looking for non-random

enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Ogata 1999) compared to the hypothesis of random distribution.

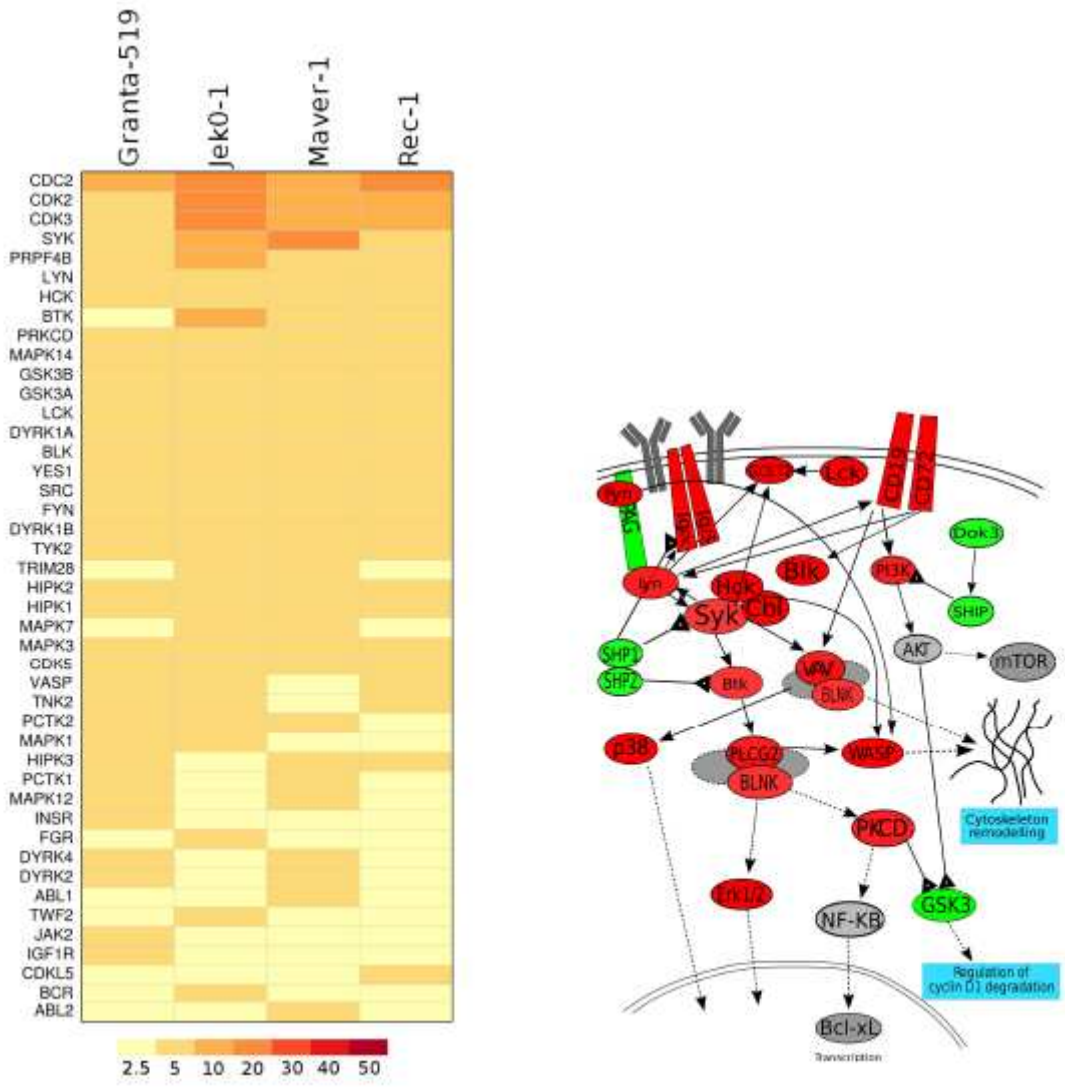


Figure 42. On the left list of protein kinases identified by the PhosphoScan approach, ranked by overall abundance in the four MCL cell lines analyzed. Color intensity proportional to the number of peptides (see figure for color corresponding to the average number of peptides per cell line). On the right simplified diagram showing some of the identified BCR pathway members. In red, activating members; in green, inhibiting members; for some members the precise final effect is not clear; in grey, other proteins known to be present but not identified. Full arrows: direct interaction/activation; dotted arrows: indirect interaction/activation.

This analysis showed that (using a cut off value of more than five total peptides), the most enriched pathway was hsa04662:B cell receptor signalling pathway (17.16-fold enrichment). Many of the proteins belonging to the most enriched pathways were either present in different pathways (redundant) or are functionally connected. Manual annotation by literature search revealed that several of these, in the topmost part of the ranking, were connected to BCR signalling, even if not present in the canonical KEGG BCR pathway (figure 42). BCR pathway activation in cell lines is

somehow intriguing since it is present in absence of an appropriate antigen stimulation, and is therefore probably self-sustained by tumour cells, either by side-by-side activation or by auto-activation. The activation of the BCR pathway in MCL has been hypothesized in a previous paper based on cytogenetic and RNA studies (Rinaldi 2006), but to our knowledge this is the first protein-based and data driven study that supports this hypothesis. Another proteomic study focusing only on the plasma membrane (Boyd 2009) showed an abnormal association of PKCbeta to the cell membrane in MCL leukemic cells, indirectly supporting an active BCR signalling. Recent studies have shown the importance of tonic BCR signalling in DLBCL (Chen 2008, Davis 2010) and BCLL (Gobessi 2009), with a basal activation of phospho-Syk residue Y352, while Y525 was detected only after BCR cross-linking. The presence of significant basal levels of phospho-Syk Y525 and Y323, with no detectable phospho-Syk Y352 in basal conditions in MCL cells are not concordant with what has been reported in B-CLL and DLBCL (Gobessi 2009), and suggest a different pattern of activation of BCR signalling in MCL. A recent report of a phase 1/2 clinical trial of fostamatinib disodium, the first clinically available oral Syk inhibitor, in patients with recurrent B-cell non-Hodgkin lymphoma, showed that only 1 in 9 MCL showed some response (Friedberg 2010). Several explanations might be possible for this low response rate, including off-target effects (Davis 2010), evolution into BCR-independent clones and different patterns of Syk activation.

TMA

One of the major benefits of TMA is that we can test many antibody with low technical costs.

Moreover we chose cases with good follow-up so we could be able to make clinical correlation of what we find. Unlucky using old blocks sometimes we have problems due to fixation so we have to test weak antibodies on whole sections.

Sox11 seems to be a good sensible antibody for MCL (Ek 2008), but sometimes in our cases showed a weak positivity on TMA slides. So we'll try to perform on whole block section to verify its positivity and make correlation with other clinical parameter.

S-phase

Although limited by the relatively small number of cases and by its retrospective nature, our study support the hypothesis that S-phase fraction is a prognostic marker of overall survival, at least in univaried analysis; so we purpose to test it, whenever possible, as it can represent a more objective way to estimate the proliferative index in MCL and probably prognosis too.

Animal model

Rag -/-γ-/- mice in our hands are good models al least for blastoid variant of MCL lymphoma. They are easy to growth and a relatively small quantity of cells, $5x10^6$ cells, are necessary to develop a

huge tumoral mass. What we can see at microscope is a high grade lymphoma (figure 43) that keeps morphological and phenotypical characteristics of the cell line of origin.

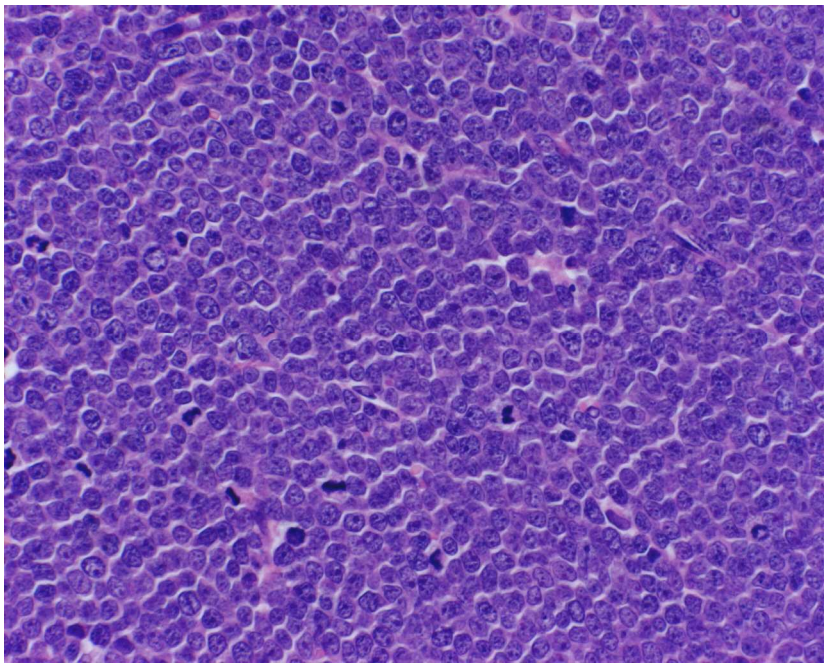


Figure 43. High power view of a lymphoid mass in a mouse.

It is very interesting to observe how lymphoma growth in different way of inoculation. When we inject cells sub cutis usually mice develop a huge mass that bulge under the skin and infiltrate skeletal muscle of the abdominal wall. In the mouse we sacrificed after 2 months, tumoral cells had the time to infiltrate almost all abdominal organs.

When we use intraperitoneal form of inoculation, in one case we obtain a really peculiar way of diffusion that predominantly growth into mesenterial fat reaching wrapping all small and large bowel (figure 44), infiltrating the mucosa just in the stomach (figure 45). This is what usually happens in lymphomatoid poliposis probably due to some local microenvironment specificity.

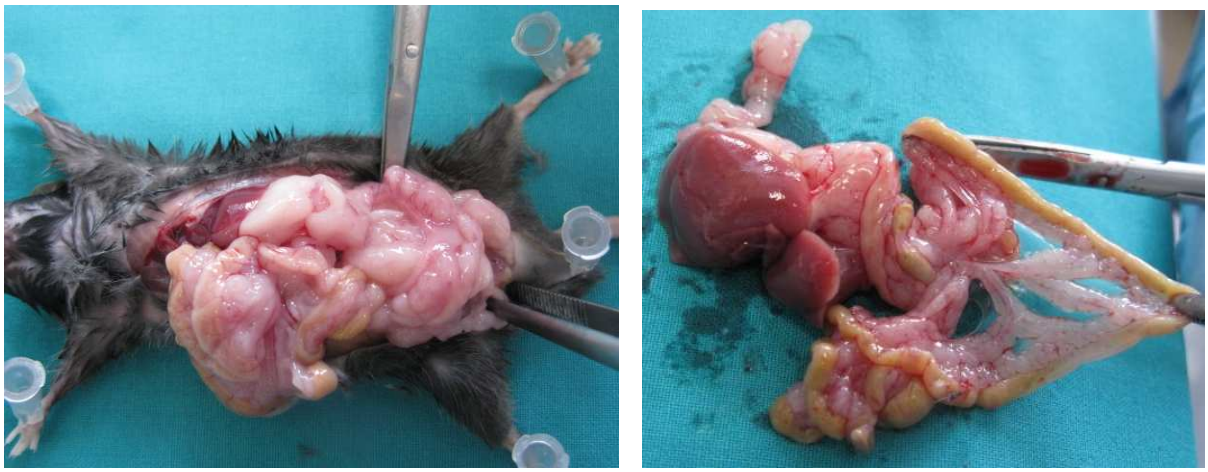


Figure 44. Mesenteric macroscopic involvement.

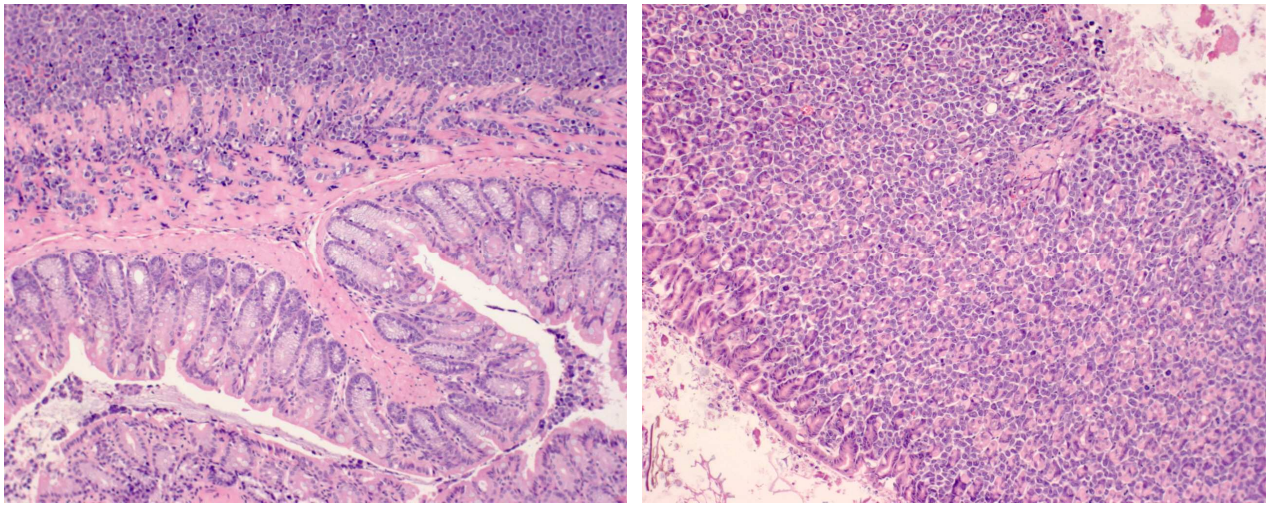


Foto 45. Microscopic infiltration of the muscle wall of the bowel (left) and of gastric mucosa (right).

In four cases we find kidney sub capsular lymphoid infiltration, either as small foci or macroscopic evident nodules. The lymphatic drainage in mice has been recently studied by Harrell et al. (Journal of Immunology 2008) and turned out to be similar to rat's one that has been studied in early 70ies (Tynies 1971); it seems to be no evidence of a lymphatic sub-capsular drainage, so it would be interesting to understand how tumoral cell reach that site.

In two mice which were injected intravenous we weren't able to find a real tumour mass. We decided to sacrifice them because they looked confused, no more in good half. Probably they had microscopic or macroscopic localization in the central nervous tissue, that we haven't explored yet, but surely we'll do in the next mice we'll sacrifice.

Immunohistochemistry performed on the main mass of the first four mice we sacrificed shows positivity for CD20, CD10, cyclinD1, Pragmin, GCET2 and CDC2.

As long as almost all mice are developing tumoral masses we'll build a tissue micro array with mice cases to perform and study ICH on it.

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THANKS TO

All these study and the future ones too wouldn’t have been possible without the good team job of all staff of the group coordinate by dott. Alberto Zamò.

Thus I’d like to thank very much dott. Anna Bertolaso, dott. Daniela Cecconi, dott. Irene Dalai, dott. Marta Franchini, dott. Claudia Parolini, dott. Serena Pedron, dott. Chiara Pighi and dott. Stefano Barbi.