GeneChip analyses point to novel pathogenetic mechanisms in mantle cell lymphoma

GeneChip analyses point to novel pathogenetic mechanisms in mantle cell lymphoma

Inga Vater[1], Florian Wagner[2], Markus Kreuz[3], Hilmar Berger[3], José I. Martín-Subero[1], Christiane Pott[4], Jose A. Martinez-Climent[5], Wolfram Klapper[6], Kristina Krause[6], Martin J. S. Dyer[7], Stefan Gesk[1], Lana Harder[1], Alberto Zamo[8], Martin Dreyling[9], Dirk Hasenclever[3], Norbert Arnold[10], Reiner Siebert[1]

1. Institute of Human Genetics, Christian-Albrechts University Kiel, Kiel, Germany
2. German Resource Center for Genome Research (RZPD), Berlin, Germany; current address: ATLAS Biolabs GmbH, Berlin, Germany
3. Institute for Medical Informatics, Statistics and Epidemiology (IMISE), University of Leipzig, Leipzig, Germany
4. Second Medical Department, Christian-Albrechts University Kiel, Kiel, Germany
5. Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain.
6. Institute of Pathology, Section Haematopathology and Lymph Node Registry, Christian-Albrechts University Kiel, Kiel, Germany
7. MRC Toxicology Unit, University of Leicester, Leicester, UK
8. Department of Pathology, University of Verona, Verona, Italy
9. Department of Medicine III, University Hospital Grosshadern/LMU, CCG Leukemia, GSF-National Research Center for Environment and Health, Munich, Germany
10. Department of Gynecology and Obstetrics, Christian-Albrechts University Kiel, Kiel, Germany

Acknowledgements: This study was supported by the Lymphoma Research Foundation (New York) and the EU (LSHC-CT 2004-503351) in the framework of the “European MCL Network”.

Corresponding author:

Dr. rer. nat. Inga Vater

Institute of Human Genetics

Christian-Albrechts University Kiel

Schwanenweg 24

D-24105 Kiel

Germany

Phone: ++49 431 597-3549

Fax: ++49 431 597-1880

E-mail: ivater@medgen.uni-kiel.de

Running Title: GeneChip microarray analyses in mantle cell lymphoma

Key Words: mantle cell lymphoma, microarray, single nucleotide polymorphisms, microtubule-associated proteins, partial uniparental disomy

Summary

The translocation t(11;14)(q13;q32) is the genetic hallmark of mantle cell lymphoma (MCL) but is not sufficient for inducing lymphomagenesis. Here we performed genome-wide 100K GeneChip Mapping in 26 t(11;14)-positive MCL and 6 MCL cell lines. We show that partial uniparental disomy (pUPD) is a recurrent chromosomal event not only in MCL cell lines but also in primary MCL. Remarkably, pUPD affects recurrent targets of deletion like 11q, 13q and 17p. Moreover, we identified 12 novel regions of recurrent gain and loss as well as 12 high-level amplifications and 8 homozygously deleted regions hitherto undescribed in MCL. Interestingly, GeneChip analyses identified different genes, encoding proteins involved in microtubule dynamics, such as *MAP2*, *MAP6* and *TP53,* as targets for chromosomal aberration in MCL. Further investigation, including mutation analyses, fluorescence in situ hybridisation as well as epigenetic and expression studies, revealed additional aberrations frequently affecting these genes. In total, 19 of 20 MCL cases, which were subjected to genetic and epigenetic analyses, and 5 of 6 MCL cell lines harboured at least one aberration in *MAP2, MAP6* or *TP53*. These findings provide evidence that alterations of microtubule dynamics might be one of the critical events in MCL lymphomagenesis contributing to chromosomal instability.

Introduction

Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin lymphoma (B-NHL) genetically characterised by the translocation t(11;14)(q13;q32) that leads to overexpression of cyclin D1 (Williams*, et al* 1992). Based on studies with transgenic mice, it is well established that this chromosomal event alone is not sufficient to result in lymphomagenesis and that secondary genomic alterations are required for malignant transformation (Gladden*, et al* 2006, Lovec*, et al* 1994). In this multistep transformation process, tumour suppressor gene (TSG) inactivation has been shown to be a key mechanism. In MCL, frequently targeted TSGs encode for proteins that inhibit malignant transformation by protecting the genome from DNA damage (*ATM*) or from deregulated cell cycle progression (*CDKN2A/P16, RB1*) or by inducing apoptosis in cells with a disrupted cell cycle control (*TP53*)(Nielander*, et al* 2007).

MCL has been the subject of several array-based comparative genomic hybridisation (arrayCGH) studies, and genomic aberrations in addition to t(11;14)(q13;q32) have been extensively characterised (de Leeuw*, et al* 2004, Kohlhammer*, et al* 2004, Mestre-Escorihuela*, et al* 2007, Rubio-Moscardo*, et al* 2005a, Rubio-Moscardo*, et al* 2005b, Tagawa*, et al* 2005). Except one, all these studies used microarrays consisting of bacterial artificial chromosome (BAC) or P1-artificial chromosome (PAC) clones for which maximal resolution is restricted to 50-200 Kb. Microarrays consisting of short synthetic oligonucleotides provide higher resolution. Some platforms allow genotyping simultaneously, so that loss of heterozygosity (LOH) without changes in DNA copy number is also detectable. This chromosomal event, so called partial uniparental disomy (pUPD), has been recently reported as a frequent mechanism of TSG inactivation in haematological neoplasms and solid tumours (Bignell*, et al* 2004, Bruce*, et al* 2005). In 2006, we could show that pUPD is a recurrent genetic mechanism alternative to chromosomal deletion in MCL cell lines (Nielaender*, et al* 2006). By loss of (part of) one parental chromosome and duplication of the remaining one from the other parent, pUPD results in LOH without chromosomal deletion. As the gene dosage is not altered, pUPD cannot be detected by conventional arrayCGH.

In the present study, we used 100K GeneChip arrays to determine genomic imbalances and pUPD in a series of 26 mantle cell lymphoma and 6 MCL cell lines. Taking advantage of the high resolution of this array, we focused on detection of small homozygous deletions. Moreover, this microarray enables to identify pUPD. The detection of these two genetic alterations has been shown to be a promising strategy to identify novel TSGs (Nielander*, et al* 2007). We identified minimally altered regions targeted by homozygous deletions harbouring novel candidate TSG loci, which might be associated with MCL pathogenesis. Moreover, we could show that pUPD is a recurrent chromosomal event not only in MCL cell lines, but also in MCL primary cases and leads to inactivation of TSGs. This further confirms that pUPD has to be considered as an alternative mechanism of TSG inactivation in MCL. Finally, our study identified genes encoding microtubule-associated proteins to be frequently targeted by chromosomal and epigenetic aberrations in MCL.

Methods and Materials

Tissue samples. DNA-samples and fixed cells were selected from the files of the Institute of Human Genetics, the Second Medical Department and the Institute of Haematopathology, (University Hospital Schleswig-Holstein, Campus, Kiel, Germany). Genomic DNA was extracted from frozen DMSO stocks derived from lymph node (LN), peripheral blood (PB), bone marrow (BM) and spleen (S) or from frozen tissue blocks of the affected LN using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The MCL panel for SNP array comprises 26 primary MCL. The median age of the patients at diagnosis was 66 years (range from 38 to 84 years). A subset of cases of this panel were also used for fluorescence in situ hybridisation (FISH) (n=13), *MAP2* mutation analyses (n=20), *TP53* mutation analyses (n=26) and epigenetic studies (n=20). For the FISH screenings and denaturing high performance liquid chromatography (DHPLC) analyses of the *MAP2* gene, additional 14 and 20 t(11;14)-positive MCL cases from files of the Institute of Human Genetics (University Hospital Schleswig-Holstein, Campus, Kiel, Germany) were included, respectively. Supplementary Table 1 shows all MCL samples included in this study. Tonsils and PB of healthy donors were used as non-tumourous controls. For real time RT-PCR experiments, LN cDNA pooled from 12 Caucasians (ages 20-59) and purchased from the human immune system multiple tissue cDNA panel (Clontech Laboratories, Mountain View, CA) and a tonsil freshly prepared from the Institute of Haematopathology (University Hospital Schleswig-Holstein, Campus, Kiel, Germany) were used as normal controls. The study was performed in the framework of the “European MCL Network”, for which central and local ethics approval was obtained.

Cell lines. A panel of 6 MCL cell lines was studied: GRANTA-519, HBL-2, UPN-1, REC-1, MAVER-1 and JEKO-1. A compilation of studies characterising these cell lines is shown in Supplementary Table 2.

Microarrays. The GeneChip Human Mapping 100K array set (Affymetrix, Santa Clara, CA, USA) has been used according to the protocol provided by the manufacturer (Affymetrix, Santa Clara, CA) (http://www.affymetrix.com). Microarrays were washed and stained with the Fluidics Station 450 (Affymetrix, Santa Clara, CA) and scanned with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) using GeneChip Operating System (GCOS; Affymetrix, Santa Clara, CA) version 1.4. The BRLMM algorithm (2006) was used with default parameters (score threshold=0.5, prior size=10000 and DM threshold=0.17) to genotype MCL tumour samples in combination with Hapmap reference arrays (2006). The genotyping call rates of the hybridised SNP chips ranged from 96.06% to 99.88% (median = 98.59%) for the 50K XbaI array and from 94.54% to 99.77% (median = 98.92%) for the 50K HindIII array. Ninety HapMap samples provided by Affymetrix (30 CEPH trios) were used as normal reference arrays (http://www.affymetrix.com/support/technical/sample_data/hapmap_trio_data.affx). A complete list of Affymetrix reference samples is shown in Supplementary Table 3.

Copy Number Analysis. Copy number analysis was performed using the CNAG program v2.0 (Nannya*, et al* 2005). The optimised reference selection method implemented in CNAG was used (Nannya*, et al* 2005). Thus CNAG selected a gender-specific reference set out of the 90 controls individually for each array. XbaI and HindIII

arrays were combined for the analysis. Segmentation of raw copy number data was performed using the Hidden Markov Model (HMM) approach provided by CNAG. HMM parameters were adjusted individually for each array due to differences in hybridisation quality and tumour cell content. Starting with default parameters, the mean levels of HMM states were adjusted manually to increase smoothing for samples with higher noise while increasing sensitivity for low noise samples. With regard to outliers and technical artefacts, HMM segments with aberrant copy number were considered as copy number aberration only if they consisted of at least 10 consecutive SNPs. High level amplifications were defined as aberrations with HMM copy number ≥ 5. For male cases, the call of high level amplifications on chromosome X was copy numbers ≥ 4.

Liberal screening for homozygous deletions. Homozygous deletions were defined as aberrations with copy number = 0.

Sensitive screening for homozygous deletions. In this more sensitive approach for detecting homozygous deletions, the Copy Number Analysis Tool (CNAT; Affymetrix, Santa Clara, CA, USA) version 2.0 was used to calculate the copy number (CN), applying a 0.5Mb genome smoothing filter. The data set was screened for regions with a copy number ≤ 0.6 in a minimum of two adjacent SNPs of which at least one showed a "NoCall".

***LOH analysis*.** A HMM based method (Beroukhim*, et al* 2006) implemented in the dChip program (Lin*, et al* 2004, Zhao*, et al* 2004) (Build date: Apr 11 2007) was used to infer regions with LOH from tumour samples. The *HMM considering haplotype (LD-HMM)* (Beroukhim*, et al* 2006) method was selected for the LOH calculations to account for linkage disequilibrium (LD)-induced SNP dependencies. The LOH *call threshold* was set to the default value of 0.5. An empirical haplotype correction (Beroukhim*, et al* 2006) was applied. Thus, the genotypes of putative tumour-associated LOH regions were compared with the genotypes observed in euploid reference samples. If the genotypes in the respective region were highly concordant between the tumour sample and at least 5% of the normal reference samples the LOH region was rejected by dChip.

***Partial uniparental disomy*.** Partial UPD regions represent genomic regions in which the LOH is not caused by altered copy number. A LOH region determined by dChip was called UPD if the copy number analysis using CNAG revealed no aberrations within that region. If a LOH region was partially affected by copy number aberrations, subregions with normal copy number were called UPD if they comprised at least 50 neighbouring SNPs.

***Copy number polymorphisms*.** As an attempt to distinguish copy number aberrations from copy number variations (CNVs) present in healthy individuals, aberrant regions were compared to published data included in the "Database of Genomic Variants" (http://projects.tcag.ca/variation/). Regions showing overlap ≥50% with known genomic variants were classified as CNVs.

***Interphase FISH*.** The commercially available locus-specific identifier (LSI) IGH/CCND1 dual colour probe (Abbott/Vysis, Downers Grove, IL) was applied to detect translocation t(11;14)(q13;q32). Differentially labeled bacterial artificial chromosome (BAC) clones, fosmid clones or commercial centromere probes (Abbott/Vysis)

were applied to verify copy number results from 100K GeneChip analyses and to perform FISH screenings. A summary of the used FISH assays is shown in Supplementary Table 4. FISH was performed as published elsewhere (Martin-Subero*, et al* 2002). 100 nuclei were evaluated per hybridisation whenever possible.

***Delineation of homozygous deletions*.** Polymerase chain reaction (PCR)-based methods were used to confirm homozygous deletions detected in the SNP array data of the MCL cell lines. To identify novel candidate TSGs, regions of homozygous loss were delineated by PCR. Primer sequences, polymerase enzymes and PCR conditions are summarised in Supplementary Table 5.

***Mutation analyses*.** The coding exons and exon/intron-boundaries of *MAP2* were amplified and analysed by DHPLC as previously described (Arnold*, et al* 1999). Sequences and annealing temperatures of primer pairs are shown in Supplementary Table 5. PCR products which showed aberrant chromatograms were subjected to direct sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit and the Genetic Analyser ABI PRISM 310 system (Applied Biosystems, Foster City, CA). *TP53* mutation analysis was performed as previously described (Gross*, et al* 2001). Sequence variations were checked and named according to the R12 release of the IARC TP53 Database (Petitjean*, et al* 2007) to determine whether they are rare polymorphisms or deleterious.

***Methylation studies*.** Sodium bisulfite conversion of genomic DNA was performed with EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The methylation status of MAP2 was evaluated by methylation-specific PCR (MSP) (Herman*, et al* 1996) as well as bisulfite sequencing (Frommer*, et al* 1992). For bisulfite sequencing, the whole CpG island upstream of the MAP2 gene was amplified after DNA bisulfite treatment, ligated into a pCR2.1-TOPO vector and transformed into competent E.coli (TOP10) using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Insertion of the DNA fragments was tested by PCR of the colonies. Cloned DNA was amplified from 10 positive colonies using vector-specific primers followed by direct sequencing using Big Dye Terminator v1.1 Cycle Sequencing Kit and the Genetic Analyser ABI PRISM 310 system (Applied Biosystems, Foster City, CA). Primer sequences for MSP and bisulfite sequencing, the used polymerase enzymes and PCR conditions used in the analyses are shown in Supplementary Table 5. To control the result of bisulfite conversion and the specificity of the MSP reactions, we used CpGenome Universal Methylated and CpGenome Universal Unmethylated DNA (Millipore, Billerica, MA).

***Expression studies*.** *MAP2* and *MAP6* expression in MCL cell lines was analysed by SYBR Green based real-time RT-PCR using the iCycler iQ multi-color-real-time PCR detection system (Biorad, Hercules, CA). Total RNA was isolated from cultured cells with NucleoSpin RNA/Protein Kit (Macherey-Nagel, Düren, Germany), high quality was confirmed with the Experion automated electrophoresis system (Biorad, Hercules, CA) and cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For the real-time PCR of *MAP2* we used QuantiTect SYBR Green PCR Kit and the QuantiTect Primer Assays of *MAP2* (Hs_ MAP2_1_SG) and *MAP2* alternative transcripts (Hs_MAP2_va.1_SG) (Qiagen). These two primer assays are able to detect all known transcript variants of *MAP2*. For *MAP6* real time PCR we used the QuantiTect Primer

Assay Hs_ MAP6_1_SG. Normalisation for the quantity of cDNA was done by performing simultaneous real-time RT-PCR for hypoxanthine phosphoribosyltranferase 1 (*HPRT1*), β-glucuronidase (*GUSB*) and glucose-6-phosphat-dehydrogenase (*G6PD*) with appropriate QuantiTect Primer Assays (Hs_HPRT1_1_SG, Hs_GUSB_1_SG, and Hs_G6PD_1_SG) (Qiagen). The thermal profile for the SYBR Green-based PCRs consisted of 15min Taq polymerase activation at 95°C followed by 45 cycles of PCR at 94°C for 15s (denaturation), 55°C for 30s (annealing), and 72°C for 30s (extension). Following amplification, a melting curve analysis was performed to verify the correct product by its specific melting temperature (Tm). Melting curve analysis consisted of a denaturation step at 95°C for 1min, lowered to 55°C for 30s, and followed by 80 cycles of incubation in which the temperature is increased to 95°C at a rate of 0.5°C/10s/cycle with continuous reading of fluorescence. Results were analysed by iCycler iQ Optical Software Version 3.0a and ratios were calculated by ΔΔCT method.

Results

GeneChip array data

In this study, 26 MCL from 20 male and 6 female patients have been subjected to 100K GeneChip Mapping analyses. The characteristic translocation t(11;14)(q13;q32) was confirmed by cytogenetics and/or FISH. Based on FISH, the tumour cell content varied from 21% to 95% (mean 75%) (Supplementary Table 1). In addition, a panel of six t(11;14)-positive MCL-derived cell lines was studied. Results of the LOH analysis based on this 100K GeneChip array data have already been published for 5 of these MCL cell lines (Nielaender*, et al* 2006).

Gains and losses. Copy number analyses identified a pattern of genomic imbalances typical for MCL, including low-level gain and monoallelic loss of regions which are known to be recurrently affected in MCL. A genomic overview of gains and losses detected in the investigated MCL primary cases is given in Figure 1. 236 and 204 genetic events leading to monoallelic loss were identified in the primary MCL and MCL cell lines, respectively (mean of 9 per MCL and 34 per MCL cell line). In addition to deletions, 206 low-level copy gains were detected in the primary MCLs (mean of 8 per MCL) and 181 in the MCL cell lines (mean of 30 per MCL cell line). By overlapping genomic segments showing copy number changes, we delineated the minimal altered regions (MARs) as well as minimal peak regions within these MARs, which were determined by imbalances of single cases (Supplementary Figure 1). Chromosomal regions which were affected in at least 4 primary MCL or MCL cell lines are summarised in Supplementary Table 6. Frequently affected regions of chromosomal gain were identified in 1q23.3-q25.1, 2p25.1, 2q32.3, 3q27.3-3q28, 4q35.1-q35.2, 7p22.3-p15.3, 7p12.1, 7q21.11, 8p23.3, 8q22.1-q24.21, 10p15.3, 10p12.1-p11.22, 11q13.3-q21, 12q13.3-q14.1, 13q31.3, 14q11.2, 15q21.3, 17q23.2, 18q21.2-q22.3 and 19p12. Genomic loss was frequently detected in 1p33-p32.3, 1p31.1-p21.1, 2q13, 2q24.1-q31.2, 2q37.1, 3p14.2-p12.2, 6q23.3-q27, 8p23.2-p21.2, 9p24.3-p21.2, 9q13-q31.2, 10p14-p13, 11q22.3-q23.2, 12p13.2-p13.1, 13q12.3-q13.1, 13q14.2-q14.3, 13q22.2-q31.1, 13q34, 14q32.12, 15q11.2, 17p13.3-p12,

22q11.22, 22q13.2-q13.33, and Xp22.33. Some of these regions contain known CNVs, also present in healthy individuals. These CNVs are marked in Supplementary Table 6. In addition to common alterations previously reported in MCL, 100K GeneChip mapping identified 5 regions frequently targeted by chromosomal gain as well as 7 regions of recurrent genomic loss, which have not been reported yet. Some of these novel regions contain candidate genes showing typical tumour suppressor or oncogene properties such as *FHIT* in 3p13, *CDKN1B* in 12p13.1, *MYCBP* in 13q22.3, or *CDKN2AIP* and *ING2* in 4q35.1 (Supplementary Table 6).

***High-level amplifications*.** Regions showing a copy number ≥ 5 were classified as high-level amplified. Amplicons included genes previously described as being amplified or overexpressed in MCL like *BCL2* (18q21.33), *GPC5/miR-17-92* (13q31.3) and *BMI1* (10p12.2) (Bea*, et al* 2001, de Leeuw*, et al* 2004, Rubio-Moscardo*, et al* 2005b). Novel detected regions of genomic amplification in MCL involved chromosomes 4p14, 6p21.2, 8p23.1, 8p23.1-p22, 8q24.21, 11q13.4-q13.5, 11q14.1, 11q22.3, 11q23.1-q23.2, 17p11.2, 18q12.2 and 18q12.2-q12.3 (Supplementary Table 7). Noteworthy, most of the detected high-level amplifications (~90%) were observed in the MCL cell lines. There were only 2 primary MCL cases showing this kind of aberration. Each of these newly detected amplified regions was observed in only one sample with the exception of 11q13.4-q13.5, which was detected in 3 MCL cell lines. This region harbours 9 genes including *MAP6* (microtubule-associated protein 6), *SERPINH1* (serine proteinase inhibitor 1), *GDPD5* (glycerophosphodiester phosphodiesterase domain 5), *RPS3* (ribosomal Protein S3), *UVRAG* (UV radiation resistance associated gene), *WNT11* (wingless-type *MMTV* integration site family), *MOGAT2* and *DGAT2* (mono- and diacylglycerol O-acyltransferases), as well as the hypothetical protein *LOC283212*. In addition, this chromosomal region is coding for 2 snoRNAs (*U15A and U15B*).

***Homozygous deletions*.** Two different approaches were used for the identification of candidate homozygously deleted regions in the MCL GeneChip data. In the liberal screening of the MCL cell lines, 11 homozygous deletions were identified in 8 different chromosomal regions and except the CNV all of them were proven by PCR and/or FISH (Table 1). Applying the sensitive screening method, 19 additional candidate homozygous deletions all affecting different loci were identified in MCL cell lines. Four of them could be validated by PCR and/or FISH, two were consistent with known CNVs (Table 1). The additional 13 regions suggestive for homozygous loss could not be confirmed. Noteworthy, this evaluation criteria detected every previously reported homozygous deletion in MCL cell lines, including TSG loci in 1p32.3 *(INK4C/P18)*, 2q13 *(BIM)*, 9p21.3 *(INK4C/P16)*, and 13q14.2 *(RB1)* (Nielander*, et al* 2007). Nevertheless, 43% of the regions suggestive for homozygous deletion represented false positive findings. However, four PCR-proven homozygously deleted regions and two CNVs detected by the sensitive screening were not detected by the liberal approach, most likely due to smoothing of the data. Table 1 displays the verified regions of homozygous loss in the MCL cell lines detected by both approaches. Moreover, the borders of 5 novel deleted regions were delineated by PCR to identify potential TSGs (Table 1).

In the 26 primary MCL cases 15 and 6 regions met the criteria for homozygous deletion in the sensitive and liberal approach, respectively. Confirmation by PCR-based methods was not performed due to contamination of the tumour samples by normal surrounding tissue. FISH was only applied for verification of deletions exceeding ~100 kb (i.e. the insert size of the used BAC, PAC or fosmid clones) which were not classified as CNVs. Homozygous deletion could be confirmed in 9p21.3 and in Xp22.33. In Table 2, regions of homozygous loss, which were detected by GeneChip data analysis of the MCL primary cases, are summarised with regard to size, CNVs, target genes and FISH verification.

Loss of heterozygosity in regions with normal copy number. 100K GeneChip data of the primary MCL cases was also subjected to LOH analyses in order to identify regions of pUPD. A median number of 1.6 pUPDs (ranging from 0 to 5) was identified in the samples. As previously reported for MCL cell lines (Nielaender*, et al* 2006), regions frequently affected by pUPD in primary MCL are known to be commonly targeted by deletions in MCL, e.g. 11q and 13q (Figure 2A). Some of the detected regions showing pUPD are known to harbour common TSGs, like *TP53* in 17p13.1.

Mutation analyses of *TP53* in one MCL sample displaying pUPD in the short arm of chromosome 17 (Figure 2B) revealed a homozygous missense mutation of a single nucleotide (g.14490 T>A) in exon 8, affecting a DNA binding domain (Figure 2C). This mutation has been reported to be deleterious for *TP53*-DNA interaction (http://www-p53.iarc.fr). FISH analysis using a *TP53* locus-specific probe confirmed a normal gene dosage of two copies (Figure 2D).

Involvement of genes encoding microtubule-associated proteins in MCL

Remarkably, 100K GeneChip data and further investigation of candidate genes identified genes encoding different microtubule-associated proteins such as *MAP2, MAP6* and *TP53* to be recurrently affected by chromosomal aberrations in MCL. Although it is widely known that MCL karyotypes show high complexity, alterations in microtubule organisation have not been reported so far.

***MAP6* gene.** High-level amplification of the *MAP6* gene was confirmed in all 3 MCL cell lines MAVER-1, JEKO-1 and HBL-2 by FISH using a BAC clone spanning the *MAP6* locus. A combined analysis of FISH and R-banding revealed the existence of 2 derivative chromosomes 6 harbouring the amplified 11q13.5 region in MAVER-1 (Figure 3). A FISH screening on interphase nuclei of 27 primary MCL identified one case with 5 gene copies, although the control gene locus in 11q22.3 was also involved. To investigate if gene dosage affects *MAP6* expression, we performed real-time RT-PCR in the 6 MCL cell lines. Higher expression of *MAP6* was detected in JEKO-1, GRANTA-519 and REC-1 compared to a freshly prepared tonsil, which was used as normal control (Supplementary Figure 2).

***MAP2* gene.** Homozygous deletion of *MAP2* detected in the MCL cell line UPN-1 was confirmed by PCR and FISH. In addition, the REC-1 cell line showed a heterozygous deletion of part of the long arm of chromosome 2,

including the *MAP2* gene. A FISH screening of the additional MCL cell lines and 27 primary cases failed to detect further cases with chromosomal loss affecting the MAP2 gene.

Mutation analysis of *MAP2* was performed in 40 primary MCL and 5 MCL cell lines by DHPLC of the coding exons and the exon/intron boundaries followed by sequencing aberrant fragments. The DHPLC screening of the *MAP2* gene identified a G>A transition in one MCL case (MCL 16). This mutation affected the first position of a coding triplet in exon 5 causing an amino acid change from glutamic acid (E) to lysine (K) (Figure 4). There is no evidence for a SNP at this DNA sequence position in NCBI Build database and DHPLC analyses failed to detect this alteration in 50 DNA samples (100 alleles) derived from peripheral blood of healthy individuals (data not shown).

To investigate the DNA methylation status of the CpG island within the *MAP2* promotor (Figure 5A), bisulfite-treated DNA probes of 5 MCL cell lines and 20 MCL cases were subjected to MSP. In 18 of the 20 MCL cases the pattern of PCR products indicated partial DNA hypermethylation, including the MCL 16 with the *MAP2* mutation. The DNAs of the MCL cell lines JEKO-1 and HBL-2 were also partially hypermethylated and the MSP pattern of the REC-1 cell line indicated virtually complete DNA methylation of the *MAP2* CpG island (Figure 5B). Noteworthy, REC-1 has only one *MAP2* allele due to a heterozygous deletion of part of chromosome 2. The MCL cell line GRANTA-519 showed only a weak PCR product of methylated DNA and the additional two MCL cases and the MCL cell line MAVER-1 seemed to be completely unmethylated. In contrast to the distribution in MCL, 8 non-tumourous controls (4 tonsils and 4 PB) showed a MSP pattern indicating an unmethylated status of *MAP2* promotor. These findings could be confirmed by bisulfite sequencing (Figure 5C-D, Supplementary Figure 3).

To investigate how DNA methylation status of the *MAP2* CpG island correlates with *MAP2* gene expression in MCL cell lines we performed real-time RT-PCR. Two different primer assays were used to detect all known *MAP2* transcript variants (Figure 5E). In UPN-1 and REC-1, no expression of *MAP2* transcripts could be detected. Real-time RT-PCR results of HBL-2 indicated reduced expression of all *MAP2* transcripts compared to control LN tissue. In GRANTA-519 and MAVER-1 the expression of *MAP2* transcripts was similar or minimally reduced whereas JEKO-1 showed a considerable higher expression of all *MAP2* transcripts compared to the LN tissue (Figure 5E). Similarly results were obtained using a freshly prepared tonsil as reference (data not shown).

***TP53* gene.** Due to its influence on microtubule dynamics, the *TP53* gene was subjected to mutation analyses in all samples of the MCL GeneChip panel. One case showing a homozygous *TP53* mutation was already analysed as part of the pUPD investigation. In six of the additional 25 primary cases mutations within the coding sequence of *TP53* were detected using DHPLC followed by sequencing aberrant fragments. Detected mutations include 6 different point mutations and one microdeletion of one single base pair (Table 3). In all these cases copy number and LOH analyses of the 100K GeneChip data indicated genomic loss of the second allele (Supplementary Table 6). Three point mutations and one microdeletion affecting the coding sequence of *TP53* gene were detected in MCL cell lines UPN-1, HBL-2, MAVER-1 and JEKO-1 (Table 3). In all these cell lines the

second *TP53* allele was deleted. Both REC-1 and GRANTA-519 exhibited the wild type allele but the latter showed a heterozygous deletion of *TP53* gene locus. Some of the MCL cell line data has been already reported elsewhere (Amin*, et al* 2003, Camps*, et al* 2006, M'Kacher*, et al* 2003, Zamo*, et al* 2006).

Discussion

100K GeneChip microarray data

In the present study, we performed 100K GeneChip microarray analyses of 26 primary MCL and 6 MCL cell lines. The use of short synthetic oligonucleotides as arrayed elements allowed the detection of genomic imbalances with high resolution (approx. 24kb) and moreover genotyping was performed simultaneously to identify regions of pUPD. Overall, the identified genomic imbalance pattern was consistent with those previously described by cytogenetics or arrayCGH studies in MCL (de Leeuw*, et al* 2004, Kohlhammer*, et al* 2004, Mestre-Escorihuela*, et al* 2007, Pinyol*, et al* 2007, Rubio-Moscardo*, et al* 2005b, Tagawa*, et al* 2005).

Using the GeneChip mapping technique, we not only delineated previously reported alterations but also identified novel regions of recurrent genomic imbalance in MCL. Taking advantage of the high resolution of the used SNP arrays, we focused on detecting high-level amplifications and small regions of homozygous loss. In this way, novel regions harbouring potential oncogenes or candidate TSG were identified, which might be involved in tumourigenesis. Moreover, we introduced two useful bioinformatic approaches for identifying homozygous losses in 100K GeneChip data and point out advantages and disadvantages.

In line with a recent study of 5 MCL cell lines (Nielaender*, et al* 2006), LOH analysis of primary MCL tumor tissue demonstrated that pUPD is a recurrent genetic mechanism in MCL tumourigenesis. Genomic distribution of detected pUPD in the analysed primary MCL showed that recurrently affected regions such as 11q and 13q are commonly targeted by deletions in MCL. Furthermore, we could explicitly demonstrate TSG inactivation by pUPD targeting the *TP53* gene locus in 17p13.1. A homozygous missense mutation affecting a DNA binding domain was detected. This mutation is frequent in lymphomas (28%) and has been reported to be deleterious for *TP53*-DNA interaction (http://www-p53.iarc.fr). *TP53* gene inactivation by chromosomal deletion is a common chromosomal event in MCL and is associated with poor prognosis (Rubio-Moscardo*, et al* 2005b). In this study, we could show that pUPD is an alternative mechanism to chromosomal deletion leading to homozygosity of a TSG inactivating mutation. Thus, pUPD seems to be a critical genetic event in MCL pathogenesis.

Genes encoding microtubule-associated proteins as targets of chromosomal aberrations

Interestingly*,* analysis of the 100K GeneChip data identified different genes encoding microtubule-associated proteins (MAPs) to be involved in chromosomal alterations in MCL. MAPs are cellular proteins that are associated with microtubules and alter their dynamics. Microtubule dynamic property is crucial for the assembly of the mitotic spindle and the attachment and movement of chromosomes along the spindle (Zhai*, et al* 1996). Microtubule-targeting drugs suppressing microtubule dynamics are widely used as cancer chemotherapeutic agents (Jordan and Wilson 2004). In addition to their direct involvement in the physical process of mitosis, microtubules also serve as scaffolds for signalling molecules (Mollinedo and Gajate 2003). The family of MAPs includes products of

oncogenes, tumour suppressors and apoptosis regulators, suggesting that alteration of microtubule dynamics and changes in the scaffolding properties of microtubules may be critical events in tumourigenesis and tumour progression (Bhat and Setaluri 2007). Until now, alterations in microtubule organisation have not been reported in MCL(Jares*, et al* 2007).

In this study, a homozygous deletion of the *MAP2* locus was identified in the MCL cell line UPN-1. Real time RT-PCR revealed absence of *MAP2* expression in UPN-1 and REC-1. REC-1 harbours a heterozygous deletion of part of the long arm of chromosome 2 and epigenetic studies showed complete DNA methylation of the CpG island of the remaining *MAP2* allele. Moreover, the DNAs of the MCL cell lines JEKO-1 and HBL-2 were also partially hypermethylated. MSP analysis demonstrated partial hypermethylation in 90% of 20 investigated primary MCL. In one of these cases showing partial hypermethylation, a point mutation affecting the coding sequence of *MAP2* was identified. These findings suggest that DNA hypermethylation is a frequent mechanism leading to *MAP2* gene inactivation in MCL. MAP2 protein participates in the stabilisation of microtubules and is predominantly expressed in neurons, where it is essential for the regulation of organelle transport within axon and dendrites (Sanchez*, et al* 2000). Moreover, MAP2 was the first protein shown to copurify and interact directly with the regulatory subunit of the protein kinase A (PKA), also known as cAMP-dependent protein kinase (cAPK) (Theurkauf and Vallee 1982, Vallee*, et al* 1981). Among other cellular effects, PKA-catalysed phosphorylation modulates cell growth, cell division and actin cytoskeleton rearrangements. MAP2 protein operates as a A-kinase anchoring protein (AKAP) and targets the PKA to microtubules. Attachment to microtubules occurs through its tubulin-binding domain (Hirokawa 1994, Serrano*, et al* 1984). MAP2 also harbours a conserved binding site for phosphatase PP2A, although direct binding of the phosphatase has yet to be reported. PP2A represents a family of heterotrimeric serine/threonine phosphatases implicated in the regulation of a plethora of cellular processes such as apoptosis, transcription, translation, DNA replication, signal transduction, protection against tumourigenesis and cell division (Janssens*, et al* 2005). In 2005, Soltani et al. reported that *MAP2* expression is associated with prognosis in melanoma. A five-year clinical follow-up study showed longer disease-free survival of patients whose primary tumors express abundant *MAP2* as compared with patients with weak or no *MAP2* expression. Moreover, exogenous expression by adenovirus leads to cell cycle arrest, growth inhibition and apoptosis in metastatic melanoma cells (Fang*, et al* 2001, Soltani*, et al* 2005). Thus, lack of *MAP2* expression might be also associated with MCL pathogenesis.

The p53 protein is also associated with microtubules *in vitro* and *in vivo* (Giannakakou*, et al* 2000) and has been reported to regulate other microtubule-associated proteins (Johnsen*, et al* 2000, Mirza*, et al* 2002, Murphy*, et al* 1999). *TP53* gene inactivation by chromosomal deletions or by mutations is a common genetic alteration in MCL. In 2003, Galmarini et al. (Galmarini*, et al* 2003) demonstrated that microtubule protein composition was altered in *TP53* mutants (mut-p53) and dynamic instability of microtubules was significantly increased. Mutation analyses in this report identified 7 primary MCL cases to harbour homozygous mutations in the coding sequence of *TP53.* In

all these cases, the second allele got lost by chromosomal deletion or pUPD. Similarly, *TP53* was shown to be homozygously mutated in 4 of 6 investigated MCL cell lines (Amin*, et al* 2003, Camps*, et al* 2006, M'Kacher*, et al* 2003, Zamo*, et al* 2006). Galmarini et al. (Galmarini*, et al* 2003) also reported that the *MAP6* (also called *STOP*) protein and its corresponding mRNA-expression were increased in the mut-p53 cells, than in the wt-p53 cells suggesting negative transcriptional regulation of *MAP6* by p53 protein. Interestingly, our MCL GeneChip data showed high-level amplification of *MAP6* in the three MCL cell lines MAVER-1, JEKO-1 and HBL-2. Real-time RT-PCR detected higher expression of *MAP6* in JEKO-1, GRANTA-519 and REC-1 compared with non-tumourous tonsil tissue. The apparent lack of correlation between *MAP6* gene dosage and expression in MAVER-1 and HBL-2 might be caused by putative non-neuronal transcripts variants that cannot be detected with the primers used. According to this, the murine homologue shows non-neuronal transcript variants, which lacks whole exons (Bosc*, et al* 2003). So far, no human non-neuronal transcript variant has been identified. High-level amplification of *MAP6* was frequently detected in MCL cell lines but in none of the investigated primary MCL. As cell lines are frequently derived from cases with advanced disease, this findings might indicate that *MAP6* amplification is associated with disease progression. In line with this hypothesis, a chromosomal rearrangement of another MAP gene, i.e. *MAP4*, was recently identified as secondary alteration in a large B-cell lymphoma (DLBCL), which was present at relapse but not at initial diagnosis (Murga Penas*, et al* 2006). According to our GeneChip data, it is widely assumed that cell lines generally harbour an increased number of chromosomal aberrations compared to the primary tumour cells.

Our study provides evidence that alterations of microtubule dynamics might be critical in MCL tumourigenesis and tumour progression. Nineteen of the 26 primary MCL from the SNP array panel and 5 of the 6 MCL cell lines harboured a genetic or epigenetic defect in at least 1 of the 3 microtubule-associated genes *MAP2, MAP6* and *TP53*. 6 primary cases were not analysed by methylation-specific methods. Only 1 case and 1 MCL cell line, in which all 3 gene loci were analysed, did not show any of the investigated alterations.

The complexity of the mitotic spindle requires fine tuning of the dynamics of all microtubules for proper function. MAPs can either stabilise or destabilise microtubules. Changes in levels of expression have been reported to correlate with aggressiveness of cancer cells or their sensitivity to microtubule-targeting agents. Although plenty of studies exist about microtubule associated proteins in neurons, where they play a critical role in neurite outgrowth and dendrite development, their mechanisms of operation in mitotic spindle regulation are rather unclear. Mitotic spindle organisation is a fine tuning process and investigation of the involved proteins is difficult due to low dosage. In contrast to abundant gene expression in brain tissue, expression of *MAP2* and *MAP6* is hardly detectable in other kinds of tissues. In our study, genomic alterations, such as *MAP6* amplification or *MAP2* and *TP53* inactivation, provide evidence for the involvement of microtubule-associated genes in MCL tumourigenesis. Alterations in microtubule dynamics and mitotic spindle organisation might contribute to the karyotype complexity and chromosomal instability which are characteristic features of MCL. Supporting this

hypothesis, a recent study underlined the high expression level of centrosome-associated gene products in blastoid MCL (Neben*, et al* 2007). MCL has one of the worst prognoses among all lymphomas. There is no therapy that can be considered as standard. Resistance against microtubule-targeting chemotherapeutic agents may be the consequence of changes in microtubule dynamics.

In conclusion, our study demonstrates that 100K GeneChip microarray analyses is a useful strategy to analyse MCL genomes with regard to genomic imbalances, particularly homozygous deletions, as well as pUPD. Moreover, we identified novel candidate TSG and oncogene loci which might harbour genes involved in MCL pathogenesis. Interestingly, different genes encoding microtubule-associated proteins could be identified as targets of chromosomal aberrations in MCL. Our findings suggest that alteration of microtubule dynamics is a critical genetic event in MCL.

References

(2006) Affymetrix (2006) BRLMM: an improved genotype calling method for the genechip human mapping 500k array set. *Tech. rep., Affymetrix, Inc. White Paper*.

Amin, H.M., McDonnell, T.J., Medeiros, L.J., Rassidakis, G.Z., Leventaki, V., O'Connor, S.L., Keating, M.J. & Lai, R. (2003) Characterization of 4 mantle cell lymphoma cell lines. *Arch Pathol Lab Med,* **127,** 424-431.

Arnold, N., Gross, E., Schwarz-Boeger, U., Pfisterer, J., Jonat, W. & Kiechle, M. (1999) A highly sensitive, fast, and economical technique for mutation analysis in hereditary breast and ovarian cancers. *Hum Mutat,* **14,** 333-339.

Bea, S., Tort, F., Pinyol, M., Puig, X., Hernandez, L., Hernandez, S., Fernandez, P.L., van Lohuizen, M., Colomer, D. & Campo, E. (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. *Cancer Res,* **61,** 2409-2412.

Beroukhim, R., Lin, M., Park, Y., Hao, K., Zhao, X., Garraway, L.A., Fox, E.A., Hochberg, E.P., Mellinghoff, I.K., Hofer, M.D., Descazeaud, A., Rubin, M.A., Meyerson, M., Wong, W.H., Sellers, W.R. & Li, C. (2006) Inferring loss-of-heterozygosity from unpaired tumors using high-density oligonucleotide SNP arrays. *PLoS Comput Biol,* **2,** e41.

Bhat, K.M. & Setaluri, V. (2007) Microtubule-associated proteins as targets in cancer chemotherapy. *Clin Cancer Res,* **13,** 2849-2854.

Bignell, G.R., Huang, J., Greshock, J., Watt, S., Butler, A., West, S., Grigorova, M., Jones, K.W., Wei, W., Stratton, M.R., Futreal, P.A., Weber, B., Shapero, M.H. & Wooster, R. (2004) High-resolution analysis of DNA copy number using oligonucleotide microarrays. *Genome Res,* **14,** 287-295.

Bosc, C., Andrieux, A. & Job, D. (2003) STOP proteins. *Biochemistry,* **42,** 12125-12132.

Bruce, S., Leinonen, R., Lindgren, C.M., Kivinen, K., Dahlman-Wright, K., Lipsanen-Nyman, M., Hannula-Jouppi, K. & Kere, J. (2005) Global analysis of uniparental disomy using high density genotyping arrays. *J Med Genet,* **42,** 847-851.

Camps, J., Salaverria, I., Garcia, M.J., Prat, E., Bea, S., Pole, J.C., Hernandez, L., Del Rey, J., Cigudosa, J.C., Bernues, M., Caldas, C., Colomer, D., Miro, R. & Campo, E. (2006) Genomic imbalances and patterns of karyotypic variability in mantle-cell lymphoma cell lines. *Leuk Res,* **30,** 923-934.

de Leeuw, R.J., Davies, J.J., Rosenwald, A., Bebb, G., Gascoyne, R.D., Dyer, M.J., Staudt, L.M., Martinez-Climent, J.A. & Lam, W.L. (2004) Comprehensive whole genome array CGH profiling of mantle cell lymphoma model genomes. *Hum Mol Genet,* **13,** 1827-1837.

Fang, D., Hallman, J., Sangha, N., Kute, T.E., Hammarback, J.A., White, W.L. & Setaluri, V. (2001) Expression of microtubule-associated protein 2 in benign and malignant melanocytes: implications for differentiation and progression of cutaneous melanoma. *Am J Pathol,* **158,** 2107-2115.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. & Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A,* **89,** 1827-1831.

Galmarini, C.M., Kamath, K., Vanier-Viornery, A., Hervieu, V., Peiller, E., Falette, N., Puisieux, A., Ann Jordan, M. & Dumontet, C. (2003) Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *Br J Cancer,* **88,** 1793-1799.

Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonny, M.V. & Fojo, T. (2000) p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat Cell Biol,* **2,** 709-717.

Gladden, A.B., Woolery, R., Aggarwal, P., Wasik, M.A. & Diehl, J.A. (2006) Expression of constitutively nuclear cyclin D1 in murine lymphocytes induces B-cell lymphoma. *Oncogene,* **25,** 998-1007.

Gross, E., Kiechle, M. & Arnold, N. (2001) Mutation analysis of p53 in ovarian tumors by DHPLC. *J Biochem Biophys Methods,* **47,** 73-81.

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. & Baylin, S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A,* **93,** 9821-9826.

Hirokawa, N. (1994) Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr Opin Cell Biol,* **6,** 74-81.

Janssens, V., Goris, J. & Van Hoof, C. (2005) PP2A: the expected tumor suppressor. *Curr Opin Genet Dev,* **15,** 34-41.

Jares, P., Colomer, D. & Campo, E. (2007) Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. *Nat Rev Cancer,* **7,** 750-762.

Johnsen, J.I., Aurelio, O.N., Kwaja, Z., Jorgensen, G.E., Pellegata, N.S., Plattner, R., Stanbridge, E.J. & Cajot, J.F. (2000) p53-mediated negative regulation of stathmin/Op18 expression is associated with G(2)/M cell-cycle arrest. *Int J Cancer,* **88,** 685-691.

Jordan, M.A. & Wilson, L. (2004) Microtubules as a target for anticancer drugs. *Nat Rev Cancer,* **4,** 253-265.

Kohlhammer, H., Schwaenen, C., Wessendorf, S., Holzmann, K., Kestler, H.A., Kienle, D., Barth, T.F., Moller, P., Ott, G., Kalla, J., Radlwimmer, B., Pscherer, A., Stilgenbauer, S., Dohner, H., Lichter, P. & Bentz, M. (2004) Genomic DNA-chip hybridization in t(11;14)-positive mantle cell lymphomas shows a high frequency of aberrations and allows a refined characterization of consensus regions. *Blood,* **104,** 795-801.

Lin, M., Wei, L.J., Sellers, W.R., Lieberfarb, M., Wong, W.H. & Li, C. (2004) dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. *Bioinformatics,* **20,** 1233-1240.

Lovec, H., Grzeschiczek, A., Kowalski, M.B. & Moroy, T. (1994) Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. *EMBO J,* **13,** 3487-3495.

M'Kacher, R., Bennaceur, A., Farace, F., Lauge, A., Plassa, L.F., Wittmer, E., Dossou, J., Violot, D., Deutsch, E., Bourhis, J., Stoppa-Lyonnet, D., Ribrag, V., Carde, P., Parmentier, C., Bernheim, A. & Turhan, A.G. (2003) Multiple molecular mechanisms contribute to radiation sensitivity in mantle cell lymphoma. *Oncogene,* **22,** 7905-7912.

Martin-Subero, J.I., Harder, L., Gesk, S., Schlegelberger, B., Grote, W., Martinez-Climent, J.A., Dyer, M.J., Novo, F.J., Calasanz, M.J. & Siebert, R. (2002) Interphase FISH assays for the detection of translocations with breakpoints in immunoglobulin light chain loci. *Int J Cancer,* **98,** 470-474.

Mestre-Escorihuela, C., Rubio-Moscardo, F., Richter, J.A., Siebert, R., Climent, J., Fresquet, V., Beltran, E., Agirre, X., Marugan, I., Marin, M., Rosenwald, A., Sugimoto, K.J., Wheat, L.M., Karran, E.L., Garcia, J.F., Sanchez, L., Prosper, F., Staudt, L.M., Pinkel, D., Dyer, M.J. & Martinez-Climent, J.A. (2007) Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood,* **109,** 271-280.

Mirza, A., McGuirk, M., Hockenberry, T.N., Wu, Q., Ashar, H., Black, S., Wen, S.F., Wang, L., Kirschmeier, P., Bishop, W.R., Nielsen, L.L., Pickett, C.B. & Liu, S. (2002) Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene,* **21,** 2613-2622.

Mollinedo, F. & Gajate, C. (2003) Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis,* **8,** 413-450.

Murga Penas, E.M., Kawadler, H., Siebert, R., Frank, M., Ye, H., Hinz, K., Becher, C., Hummel, M., Barth, T.F., Bokemeyer, C., Stein, H., Trumper, L., Moller, P., Marynen, P., Du, M.Q., Yang, X., Hansmann, M.L. & Dierlamm, J. (2006) A novel fusion of the MALT1 gene and the microtubule-associated protein 4 (MAP4) gene occurs in diffuse large B-cell lymphoma. *Genes Chromosomes Cancer,* **45,** 863-873.

Murphy, M., Ahn, J., Walker, K.K., Hoffman, W.H., Evans, R.M., Levine, A.J. & George, D.L. (1999) Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. *Genes Dev,* **13,** 2490-2501.

Nannya, Y., Sanada, M., Nakazaki, K., Hosoya, N., Wang, L., Hangaishi, A., Kurokawa, M., Chiba, S., Bailey, D.K., Kennedy, G.C. & Ogawa, S. (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res,* **65,** 6071-6079.

Neben, K., Ott, G., Schweizer, S., Kalla, J., Tews, B., Katzenberger, T., Hahn, M., Rosenwald, A., Ho, A.D., Muller-Hermelink, H.K., Lichter, P. & Kramer, A. (2007) Expression of centrosome-associated gene products is linked to tetraploidization in mantle cell lymphoma. *Int J Cancer,* **120,** 1669-1677.

Nielaender, I., Martin-Subero, J.I., Wagner, F., Martinez-Climent, J.A. & Siebert, R. (2006) Partial uniparental disomy: a recurrent genetic mechanism alternative to chromosomal deletion in malignant lymphoma. *Leukemia,* **20,** 904-905.

Nielander, I., Bug, S., Richter, J., Giefing, M., Martin-Subero, J.I. & Siebert, R. (2007) Combining array-based approaches for the identification of candidate tumor suppressor loci in mature lymphoid neoplasms. *APMIS,* **115,** 1107-1134.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P. & Olivier, M. (2007) Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat,* **28,** 622-629.

Pinyol, M., Bea, S., Pla, L., Ribrag, V., Bosq, J., Rosenwald, A., Campo, E. & Jares, P. (2007) Inactivation of RB1 in mantle-cell lymphoma detected by nonsense-mediated mRNA decay pathway inhibition and microarray analysis. *Blood,* **109,** 5422-5429.

Rubio-Moscardo, F., Blesa, D., Mestre, C., Siebert, R., Balasas, T., Benito, A., Rosenwald, A., Climent, J., Martinez, J.I., Schilhabel, M., Karran, E.L., Gesk, S., Esteller, M., deLeeuw, R., Staudt, L.M., Fernandez-Luna, J.L., Pinkel, D., Dyer, M.J. & Martinez-Climent, J.A. (2005a) Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood,* **106,** 3214-3222.

Rubio-Moscardo, F., Climent, J., Siebert, R., Piris, M.A., Martin-Subero, J.I., Nielander, I., Garcia-Conde, J., Dyer, M.J., Terol, M.J., Pinkel, D. & Martinez-Climent, J.A. (2005b) Mantle-cell lymphoma genotypes identified with CGH to BAC microarrays define a leukemic subgroup of disease and predict patient outcome. *Blood,* **105,** 4445-4454.

Sanchez, C., Diaz-Nido, J. & Avila, J. (2000) Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. *Prog Neurobiol,* **61,** 133-168.

Serrano, L., Avila, J. & Maccioni, R.B. (1984) Controlled proteolysis of tubulin by subtilisin: localization of the site for MAP2 interaction. *Biochemistry,* **23,** 4675-4681.

Soltani, M.H., Pichardo, R., Song, Z., Sangha, N., Camacho, F., Satyamoorthy, K., Sangueza, O.P. & Setaluri, V. (2005) Microtubule-associated protein 2, a marker of neuronal differentiation, induces mitotic defects, inhibits growth of melanoma cells, and predicts metastatic potential of cutaneous melanoma. *Am J Pathol,* **166,** 1841-1850.

Tagawa, H., Karnan, S., Suzuki, R., Matsuo, K., Zhang, X., Ota, A., Morishima, Y., Nakamura, S. & Seto, M. (2005) Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM. *Oncogene,* **24,** 1348-1358.

Theurkauf, W.E. & Vallee, R.B. (1982) Molecular characterization of the cAMP-dependent protein kinase bound to microtubule-associated protein 2. *J Biol Chem,* **257,** 3284-3290.

Vallee, R.B., DiBartolomeis, M.J. & Theurkauf, W.E. (1981) A protein kinase bound to the projection portion of MAP 2 (microtubule-associated protein 2). *J Cell Biol,* **90,** 568-576.

Williams, M.E., Swerdlow, S.H., Rosenberg, C.L. & Arnold, A. (1992) Characterization of chromosome 11 translocation breakpoints at the bcl-1 and PRAD1 loci in centrocytic lymphoma. *Cancer Res,* **52,** 5541s-5544s.

Zamo, A., Ott, G., Katzenberger, T., Adam, P., Parolini, C., Scarpa, A., Lestani, M., Menestrina, F. & Chilosi, M. (2006) Establishment of the MAVER-1 cell line, a model for leukemic and aggressive mantle cell lymphoma. *Haematologica,* **91,** 40-47.

Zhai, Y., Kronebusch, P.J., Simon, P.M. & Borisy, G.G. (1996) Microtubule dynamics at the G2/M transition: abrupt breakdown of cytoplasmic microtubules at nuclear envelope breakdown and implications for spindle morphogenesis. *J Cell Biol,* **135,** 201-214.

Zhao, X., Li, C., Paez, J.G., Chin, K., Janne, P.A., Chen, T.H., Girard, L., Minna, J., Christiani, D., Leo, C., Gray, J.W., Sellers, W.R. & Meyerson, M. (2004) An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. *Cancer Res,* **64,** 3060-3071.

Table 1: Homozygous deletions in MCL cell lines

locus[2]	liberal screening [start-stop in bp[1]]	sensitive screening [start-stop in bp[1]]	PCR-based confirmation and delineation [start-stop in bp[1]]	FISH confirmation	affected cell lines	target genes
1p32.3	not detected	50 837 181-57 453 350	(Mestre-Escorihuela, *et al* 2007)	not done	UPN-1	*CDKN2C/P18*
2q13	111 616 112-112 182 931	111 616 112-112 155 057	(Tagawa, *et al* 2005)	not done	JEKO-1	*BIM*
2q34	210 048 358-210 472 004	210 048 358-210 472 004	210 079 601-210 462 202	confirmed	UPN-1	*MAP2*
2q37.3	not detected	237 396 643-237 486 004	237 272 264-237 637 712	confirmed	UPN-1	no genes
9p21.3	21 948 524-22 102 599	21 948 524-22 090 176	(de Leeuw, *et al* 2004, Kohlhammer, *et al* 2004, Tagawa, *et al* 2005)	not done	GRANTA-519, REC-1, MAVER-1	*CDKN2A/P16, CDKN2B/P15*
9p21.2	27 316 780-27 716 911	27 248 185-27 716 731	27 287 093-27 940 630	not done	MAVER-1	*MOBKL2B, IFNK, C9ORF72*
9p21.1	28 761 537-30 316 115	28 761 537-30 316 115	confirmed but not delineated by PCR	not done	MAVER-1	no genes
12p13.1	not detected	13 617 759-13 618 078	13 616 272-13 652 667	not done	REC-1	*GRIN2B*
13q14.2	not detected	47 812 793-47 817 924	(Pinyol, *et al* 2007)	not done	UPN-1	*RB1*
13q33.1	101 552 480-101 943 751	101 552 480-101 943 751	confirmed by FISH not delineated by PCR	confirmed	JEKO-1	*FGF14*
18q22.1	62 853 023-63 491 358	63 097 606-63 491 358	62 390 461-64 346 179	confirmed	UPN-1	*c18orf4*
22q11.22	20 994 635-21 479 136	20 994 635-21 479 136	CNV[2]	not done	GRANTA-519, HBL-2	*IGL*
Xp22.31	not detected	6 492 343-6 543 186	CNV[2]	not done	JEKO-1	no genes
Xq28	not detected	153 981 072	CNV[2]	not done	GRANTA-519	no genes

[1] NCBI Build 35, [2] According to Database of Genomic Variants, CNV: Copy Number Variation

Table 2: Putative and confirmed homozygous deletions in MCL primary cases

locus[1]	sensitive screening [start-stop in bp[1]]	liberal screening [start-stop in bp[1]]	number of affected MCL	FISH confirmation	candidate genes
1p21.2	100 823 226-100 823 636	not detected	1	not done (<100kb)	no genes
3p26.3	1 510 440-1 511 278	not detected	1	not done (CNV[2])	no genes
4q22.1	90 356 219-90 356 586	not detected	1	not done (CNV[2])	no genes
5q22.2	111 710 742-111 711 216	not detected	1	not done (CNV[2])	*EPB41L4A*
9p24.1	7 501 166-7 503 589	not detected	1	not done (CNV[2])	no genes
9p23	12 742 340-12 750 718	8 916 956-12 974 756	1	not done (CNV[2])	*PTPRD, TYRP1*
9p21.3	not detected	21 762 317-22 801 336	2	homozygous deletion verified	*CDKN2A/P16, CDKN2B/P15*
9p21.2	27 692 974-27 693 855	not detected	1	not done (<100kb)	no genes
9p21.1	not detected	30 425 441-31 302 460	1	not done (CNV[2])	no genes
11q22.1	100 654 097-100 773 657	100 546 098-102 615 349	1	no assay applicable due to repetitive sequences	*BIRC2, BIRC3*
11q22.3	109 749 455-109 749 570	not detected	1	not done (CNV[2])	*TMED*
12p13.1	13 617 759-13 618 078	not detected	1	not done (<100kb)	*GRIN2B*
22q11.22	21 099 653-21 337 561	21 099 653-21 479 136	1	not done (CNV[2])	*IGL*
Xp22.33	677 050-2 528 646	677 050-2 561 008	2	homozygous deletion verified	PAR1 genes
Xp21.2	29 303 376-29 351 134	not detected	1	not done (CNV[2])	*IL1RAPL1*
Xp21.1	33 396 094-33 415 950	not detected	1	not done (CNV[2])	no genes
Xq21.33	98 159 771-98 164 282	not detected	2	not done (<100kb)	no genes

[1] NCBI Build 35, [2] According to Database of Genomic Variants, CNV: Copy Number Variation, PAR1: pseudoautosomal region 1

Table 3: *TP53* mutation analyses in MCL

MCL primary cases	*TP53* mutation	second abberation in 17p13.1
MCL 4	g.12178 delC - p.122X	deletion
MCL 6	g.14487 G > T - p.R273L	deletion
MCL 8	g.14490 T > A - p.V274D	pUPD
MCL 15	g.13203 G > A - p.R175H	deletion
MCL 17	g.14070 C > T - p.R248L	deletion
MCL 19	g.12108 G > T - p.E62X	deletion
MCL 21	g.13091 G > C - p.A138P	deletion
MCL cell lines	***TP53* mutation**	**second abberation in 17p13.1**
UPN-1	g.14525 G>A - p.E286K	deletion
MAVER-1	g.14511 A>G - p.D281G	deletion
JEKO-1	g.12096 delC - p.58X	deletion
HBL-2	g.14511 A>G - p.D281G	deletion

g.: genomic sequence position, p.: protein sequence position (http://www-p53.iarc.fr)

Titles and legends to figures

Figure 1: Genome-wide detection of copy number changes in MCL primary cases. Proportion of gains and losses analysing GeneChip data of 26 MCL primary cases are displayed from 1pter to Xqter. Green columns indicate chromosomal gain, whereas red columns indicate loss of genetic material.

Figure 2: Partial uniparental disomy (pUPD) in MCL. A) Genome-wide distribution of pUPD in 26 primary MCL cases detected by 100K GeneChip Mapping (Affymetrix). Proportion of MCL cases showing pUPD is displayed in turquoise. Chromosomes are shown from 1pter (left) to Xqter (right). **B)** Detection of pUPD in the short arm of chromosome 17 in MCL 8. The profile of genomic imbalances is given as black dots with a value of 2 indicating a balanced status. Chromosome 17 is shown from pter (left) to qter (right). Heterozygous calls are given as green dots and the estimated LOH region is overlaid in grey. A blue arrow indicates chromosomal location of the *TP53* gene. **C)** FISH analysis using a locus-specific probe consisting of BAC clones labelled in spectrum orange (*TP53)* and spectrum green (control locus in 17q21.2). Signal constellation indicates a normal gene dosage of two copies in the MCL 8. **D)** Homozygous point mutation affecting exon 8 of *TP53* in the same MCL which shows pUPD in 17p13.1.

Figure 3: High level amplification in 11q13.5. A) Genomic profiles of part of chromosome 11 in 3 MCL cell lines. Copy number is given as black dots with a value of 2 indicating a balanced status. Chromosomal region in the long arm is shown from centromeric to telomeric. The minimal altered region is indicated by blue vertical bars. **B-D)** Interphase FISH of the MCL cell lines using a locus-specific probe for the *MAP6* gene (spectrum green) and a control locus in 11q22.3 (spectrum orange). **E-F)** R banding followed by FISH on metaphases of MAVER-1 using the described *MAP6* probe. High level amplification was shown to affect two derivative chromosomes 6 [a:der(6)(6pter?6q22::11q13?::11q22?11q23::11q22->11q23::?),b:der(6)(?::6p21?6q22::11q13?11q14::11q23? amp)].

Figure 4: Homozygous deletion in 2q34. A) Genomic profile of part of the chromosome 2 in the MCL cell line UPN-1. Copy number is given as black dots with a value of 2 indicating a balanced status. Chromosomal region in the long arm is shown from centromeric to telomeric. A red arrow indicates the homozygously deleted region. A Genome Browser extract (NCBI Build 35) displays gene content and the chromosomal position of primers (red bars) for delineation of the minimal affected region. **B)** Multiplex-PCR of the target gene and a control locus confirmed the homozygous deletion of *MAP2* gene but not of the adjacent coding sequences of RNAz s59616 and C2orf21. **C)** DHPLC chromatograms of the primary MCL 16 (red) and a healthy control (blue). **D)** Sequencing of the aberrant fragment revealed a heterozygous mutation in exon 5 of *MAP2* changing amino acid composition of the protein (E163K). Here, the reverse strand is displayed showing a C>T transition.

Figure 5: Epigenetic and expression studies of *MAP2*. A) Genome Browser extract (NCBI Build 35) displaying location of *MAP2* CPG island. **B)** Methylation specific PCR (MSP) of REC-1, two primary MCL and controls. Methylation specific amplification was verified using universal methylated (MC) and universal unmethylated (UC)

DNA as controls. Genomic DNA (gC) was tested to exclude unspecific amplification. **C)** Part of the analysed *MAP2* CpG island sequences investigating bisulfite-treated DNA of REC-1 and a tonsil. The included CpG dinucleotides 5-11 are marked by black bars. **D)** Bisulfite genomic sequencing of the *MAP2* CpG island in the MCL cell line REC-1 and two primary MCL. A tonsil and peripheral blood of healthy donors were used as controls. Every column represents a CpG dinucleotide and every row represents one of 10 sequenced clones. Black and white spots indicate methylated and unmethylated CpGs, respectively. Grey spots indicate CpG dinucleotides which failed to be analysed. **E)** Real-time Reverse Transcription (RT-) PCR. Two primer assays were applied to investigate all known *MAP2* transcripts. Primer position is displayed by black arrows. Expression of *MAP2* transcripts was examined in 6 MCL cell lines and compared to *MAP2* expression in lymph node tissue of healthy individuals. Each value has been normalised to the average expression of three housekeeping genes. In UPN-1 and REC-1 no *MAP2* expression was detected, thus $\log_2$ ratio was not calculable (N/C).