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ORIGINAL ARTICLE

Allelic genotyping reveals a hierarchy of genomic alterations in mantle cell lymphoma associated to cell proliferation

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Abstract Mantle cell lymphoma (MCL) is a distinct subentity of non-Hodgkin lymphoma, characterized by the chromosomal translocation t(11;14)(q13;q32) leading to an overexpression of cyclin D1 in virtually all cases. However, additional cytogenetic aberrations are apparent in the vast majority of MCL. Applying LOH analysis in 52 MCL patient samples, we confirmed frequent alterations in 9p21 (28.6%) and *p53* (28.9%) but also detected allelic losses in 1p21, 9q21, 13q13-14, 13q31-32, 17p13.1, and 17p13.3 in 28–45% of cases and allelic gains in 3q27-28 and 19p13.3 in 14–22% of cases. In addition, losses in the 2p23 and 7q22-35 genomic regions not previously described to be altered in MCL were identified in up to 20% of cases. Applying multivariate analysis, a cluster of genomic aberrations including 1p21, 3q27, 7q22-36, 6p24, 9p21, 9q31, and 16p12 alterations was identified which was closely associated to cell proliferation as determined by Ki67 immunostaining. This proliferation-dependent network of oncogenic alterations complements the previously identified proliferation expression signature described by RNA expression profiling in MCL.

Keywords MCL · LOH analysis · Cell proliferation · 17p13

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Introduction

Mantle cell lymphoma (MCL) has been recognized as a distinct subtype of malignant lymphoma in the current WHO Lymphoma classification system. It represents 6–9% of malignant lymphomas and is characterized by distinctive immunophenotypic and genetic features [13, 26]. The clinical outcome of mantle cell lymphoma is dismal, with a median survival time of only 3 years due to rapidly developing chemoresistance and frequent relapses [13]. However, a minority of patients survive up to 10 years, indicating the clinical and biological heterogeneity of MCL [39].

Morphologically, two subgroups of MCL have been recognized. The classical MCL is composed of small to medium-sized lymphoid cells with small, irregular nuclei and a relatively low proliferative index [2, 48]. The blastoid variant is characterized by either pleomorphic or larger blast-like nuclei with a fine and dispersed chromatin and occasional small nucleoli. This variant displays a higher cell proliferation and a more aggressive clinical course than typical MCL [35]; [48]. Secondary transformations towards

the blastoid variant forms have been observed in 26–70% of MCL patients. Genetically, MCL is characterized by the chromosomal translocation t(11;14)(q13;q32), which corresponds to the juxtaposition of *cyclinD1* to the immunoglobulin heavy chain gene promoter and resulting in the constitutive overexpression of CyclinD1. Recently, it could be demonstrated that the proliferation gene expression signature is a central prognostic factor in MCL [39]. The expression profile of only four genes identified low risk patients with a median survival of up to 10 years. Another genetic signature distinguished Ki-67^{high} from Ki-67^{low} MCL and consisted of 32 genes involved in cellular processes, such as mitotic spindle formation, gene transcription and cell cycle regulation [16]. In addition, Katzenberger et al. show a closed correlation between the Ki67 index of tumor cells and survival in MCL [27].

Despite the fact that *cyclinD1* has been identified as an important oncogene in various solid tumors, the transforming properties of *cyclin D1* seem to be less efficient than other oncogenes [23]. Thus, in *cyclin D1* transgenic animals mice the lymphomagenesis required the cooperation of other additional oncogenes, indicating that additional molecular alterations are necessary for the clinical development and progression of MCL [30].

Accordingly, secondary molecular alterations are frequently detected in MCL. Blastoid variants of MCL have a high incidence of *p53* gene mutations [20, 21], *p16^{INK4a}* deletions or hypermethylation [14, 25, 36]. In addition, shorter survival among MCL patients has been correlated with overexpression of *c-myc* [32] and a high cell proliferation index as measured by immunostaining of other proliferation-associated transcription factors [44]. Applying FISH analysis, a deletion of the 11q14-q24 chromosomal region including the *ATM* gene could be identified in 46% of MCL cases [45]. The detection of mutations of *ATM* in MCL patients suggested a potential susceptibility role in the tumorigenesis of MCL [42]. Another important tumor suppressor region on chromosomal band 13q14 was deleted in 70% of MCL [14, 46].

Comparative genomic hybridization (CGH) and array-based genomic analysis revealed amplifications in 3q, 7p, 8q, 12q, 18q, 9q and deletions in 6q, 1p, 11q, 10p, 17p, 9p, and 13q as frequent secondary genetic alterations [1, 5, 19, 28, 41, 44]. Those genetic alterations were more frequently found in blastoid than in classical MCL [4, 31].

Despite these important observations, little is known about the functional interaction of secondary genomic aberrations in MCL. To determine the impact of chromosomal imbalances on tumor cell proliferation, a comprehensive genomic allelotyping was performed in 52 MCL patient samples (blastoid and classical) using 87 microsatellite-primers evenly distributed over the whole genome. Applying PCR-based LOH analysis, tumor DNA and normal control samples of individual patients were analyzed for deletions or amplifications of distinct genomic regions.

Materials and methods

Tumor samples Fifty-two MCL patients with a median age of 66.5 years were investigated in this retrospective study. All tumors were reviewed by a member of the European MCL Pathology panel (G.O.) and classified according to the criteria of the WHO lymphoma classification. *Cyclin D1* overexpression or t(11;14)(q13;q32) translocation were identified by Northern blot analysis or cytogenetic FISH analysis, respectively. Genomic DNA was extracted from formalin fixed tumor tissue and/or buffy coat from leukemic samples as previously described [25]. After Ficoll separation of lymphocytes (buffy coat) and granulocytes (pellet), DNA was extracted applying the Nucleospin^{R} Blood XL kit (Machery Nagel, Dueren, Germany). Purity of cell compartments (>95%) was confirmed by cytospin analysis. Control DNA was obtained from granulocytes or whole blood (non-leukemic cases).

LOH analysis DNA of 52 morphologically confirmed MCL and control cells were comparatively analyzed for genomic imbalances by semi-automated allelotyping applying 87 fluorescence-labeled microsatellite markers evenly distributed throughout the human genome (supplement Table 1; RER/LOH assay kit, PE Biosystems, Foster City, CA; Table 1) labeled with either FAM (blue), TET (green),

Table 1 Overview of frequently altered genomic regions in MCL and association to other genomic alterations

Frequently altered genomic loci	Associated to alterations
1p13	7q, 8q, 13q13-33, 15q25, 17p13.1
2p23	4q, 6q, 8q
3p25-pter	17q23
3q22-23	7q, 8q, 13q31
3q27-q28	7q, 8q, 13q31
4q31.1	1p
6p24	19p13, 17p13.1
6q23-26	2p, 8q, 13q, 17p13.2
7q	1p, 9p21, 3q
8q	15q25, 13q13-33
9p21	7q, 17p13.1, 17p13.2, 13q13-33, 16p12
9q	3q, 15q, 17p12-13, 17p13.2, 17p13.3, 17q23
11q14	4q
12q	
13q13-33	1p, 3q, 6q, 8q, 9p21
15q25	8q, 1p, 9q, 9p21
16p12	8q, 15q, 9p21

or HEX (yellow). PCR was performed with 25 ng genomic template DNA in a 20 µl reaction volume according to the manufacturer's protocol. After an initial AmpliTaq gold (Applied Biosystems) activation step at 95°C for 10 min, 35 PCR cycles were performed at 95°C for 10 s, 55 for 30 s and 72°C for 2 min followed by a final extension at 72°C, 10 min. Amplification products were pooled and analyzed on an ABIPrism310 DNA sequencer (PE Biosystems). Analysis of peak height and fragment size was performed with the Genescan Fragment Analysis and Genotyper software (PE Biosystems). Based on previous standardization experiments, allelic imbalance was diagnosed if the peak ratio of both alleles differed by more than three standard deviations between lymphoma and normal peripheral blood cells. Allelic imbalances were evaluated by calculating the ratio between the allele ratio of the healthy sample and that of the diseased sample considering a score higher than 1.35 as an amplification and lower 0.67 as loss of heterozygosity [43].

Proliferation index and p53 expression status The proliferation (Ki67) index and p53 expression were assessed by immunohistochemistry as previously described [14, 35]. P53 overexpression was defined as strong p53 staining of >20% of tumor cell nuclei.

TP53 mutation status MCL patient samples were screened for mutations in *p53* exons 5–9 by PCR-SSCP analysis coupled with direct PCR sequencing as previously described [50].

Data analysis For statistical analysis Chi-square tests, Fisher's exact *t*-test and odds ratio analysis were performed. An association was assumed significant if $p<0.05$. The NCBI data base http://www.ncbi.nlm.nih.gov/genome/guide/human/d and http://www.ncbi.nlm.nih.gov/entrez/query was searched for potential candidate genes. The human (*Homo sapiens*) Genome Browser Gateway http://genome.cse.ucsc.edu/cgi-bin/hgGateway was used to screen for microRNA locations.

Results

Identification of genomic imbalances In 560 out of 4,367 analyses (12.8%) genomic imbalances were identified including 378 deletions (67.5%) and 182 amplification (32.5%). The median number of losses and gains per patient was 7.3 and 3.5. In all patients, at least one genomic alteration was detected, with a median number of 11 genomic imbalances per patient. Number of genomic imbalances was not significantly different in blastoid or classical MCL as well as those with low and high proliferation.

Imbalances of genomic regions The frequencies of identified genomic imbalances are summarized in Fig. 1. In 13 of 87 microsatellite markers, a significant rate (>20%) of allelic losses were detected corresponding to nine of the 37 analyzed chromosomal arms (24.3%). The most frequently detected allelic losses involved chromosomal bands 1p21 (28%); 2p23 (20.4%); 6q26 (20.9%), 7q35 (20.6%); 9q21 (42.1%), 9q31-33 (24.2%); 9p21 (28.6%); 13q13-14 (26.5%); 13q31-32 (25%), 17p13.1 (45.2%); TP53 (28.9%), and 17p13.3 (50%; Fig. 1).

A significant rate (>10% of patients) of allelic gains were detected in 9.7% of chromosomal bands corresponding to

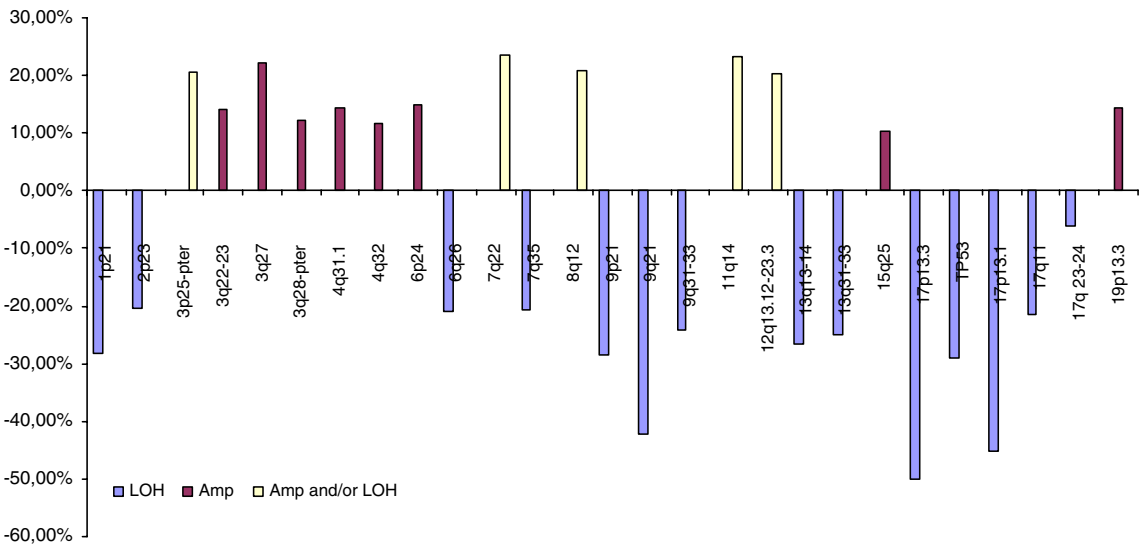

Fig. 1 Frequent genomic alterations in MCL samples

five of 37 analyzed chromosome arms (13.5%). Allelic gains were most frequently detected on chromosomal bands 3q27 (22.2%), 4q31.1 (14.2%), 6p24 (15%), 19p13.3 (14.2%; Fig. 1).

Allelic losses and/or gains of 5 additional frequently altered loci (3p25-pter, 7q22, 8q12, 11q14, 12q13-23) were identified (Fig. 1).

Morphology, proliferation index, and genetic imbalances Detailed statistical analysis (odds ratio, chi square tests) revealed a cluster of genomic alterations of chromosomal bands 1p21, 3q27, 6p24, 7q35, 9p21, 9q31-33, and 16p12 which was closely associated with a high proliferation index (Fig. 2). Alterations of 7q35 were mainly associated to alterations of loci of the proliferation cluster (9p21; 1p; 3q) but also to 17p13.1 (Fig. 2; Table 1). Genomic alterations of 3q27, 6p24, and 4q31.1 were significantly more frequent in samples with blastoid morphology.

p53 alterations *p53* alterations, *p53* mutations and/or alterations in P53 protein expression, were found in 47% of analyzed samples. These alterations were directly correlated to genomic imbalances in 17p13.1 ($p<0.05$). Genomic alterations in 17p13.1 were highly associated to blastoid morphology and loci of the proliferation-associated cluster like 1p21, 6p24, 9p13-21, and 9q31-33 (Table 2, Fig. 3).

Discussion

MCL is characterized by the t(11;14)(q13;q32) chromosomal translocation leading to constitutive overexpression of *cyclinD1* [49]. However, this translocation alone is not sufficient to promote MCL development but additional genomic alterations appear to be essential for malignant transformation [30]. To identify secondary genetic alterations involved, several groups have applied different techniques such as cytogenetic analysis, comparative genomic hybridization or DNA microarray [1, 5, 11, 19, 28, 44]. In this study, we used a comprehensive method of allelotyping for the detection of genomic gains and losses in MCL [24].

Our data confirmed previously described MCL genomic alterations. Allelic losses and gains were encountered most frequently in chromosomal bands 1p21, 9p21, 9q21, 13q13-14, 13q31-32, 17p13.1, and 17p13.3.

One of the most frequently altered locus in the present study (9q21; 42%) harbors various genes involved in DNA repair and maintenance of chromosome stability, but also the B-cell-associated tyrosine kinase which was described as a potential therapeutic target by genomic and expression profiling [38]. Alterations of 9q21-q22 have recently been described as a novel marker of inferior outcome in MCL [40]. We could demonstrate a borderline association between genomic alterations of 9q21-q22 and *p53* ($p=0,052$). Such *p53* mutations have been described to be associated with variant cytology and predict a poor prognosis [20]. In the present study, alterations of the 9q21 locus were not directly associated to proliferation, but to the proliferation-associated cluster of alterations (Table 1).

The 9p21 locus is one member of the proliferation-associated cluster, which harbors the *INK4* tumor suppressor gene cluster. Inactivation of tumor suppressor genes *p15 (INK4b)* and *p16(INK4a)* has previously been shown to be involved in secondary transformations of indolent lymphoma [14], [15, 17] and specifically in the blastoid variant of MCL [14, 37]. Accordingly, in the present study, LOH of 9p21 was closely associated with cell proliferation but also *p53* alterations (Figs. 2 and 3). The previously described rare *p16 (INK4a)* promoter methylation may represent an additional mechanism of gene-specific cell cycle dysregulation in MCL [25]. 9p21 alterations were indirectly associated to other

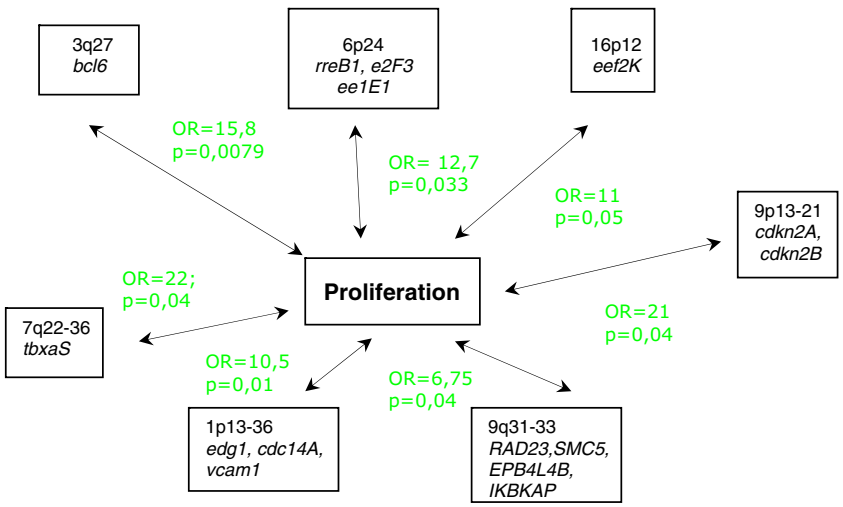

Fig. 2 The proliferation-associated cluster of frequent genomic alterations. Associations were determined as significant if $p<0.05$

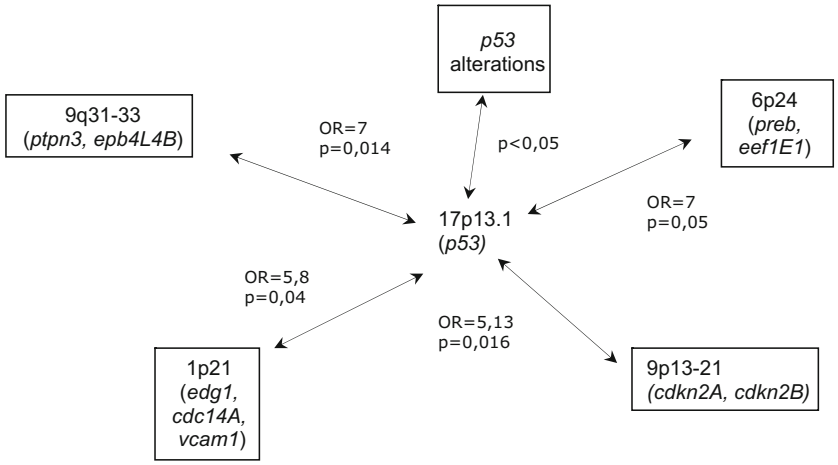

Fig. 3 The cell proliferation cluster of frequently genomic alterations in MCL. An association was determined significant if $p \leq 0.05$. *p53* alterations include mutations in *p53* and alterations in P53 protein expression

members of the proliferation cluster (Table 1). However, alterations of the genetic loci 3q27 and 1p13-36, members of the proliferation cluster, were not associated to each other and thus may represent alternative signal pathways linked to 7q alterations (Table 1). Other members of the proliferation-associated cluster are constituted by alterations in 9q31-33, 6p24, and 16p12 (Fig. 2) harboring potential interesting candidate genes such as *rad23*, the *e2f* transcription factor3, *eef1E1* (eukaryotic translation elongation factor 1 epsilon), and the *eef1E1* kinase.

We could also show frequent alterations (amplification and LOH) in the 12q13 locus. This locus potentially involves the *cdk4*, *mdm2*, *rarg*, *atf*, and *cd63* genes [4]. Those genes were reported to be differentially expressed in MCL as compared to normal B-cell populations [22]. In addition, Hernandez et al. reported that *cdk4* and *mdm2* gene alterations mainly occur in MCL with wild-type *INK4a/ARF* locus and may contribute to the higher proliferation and more aggressive behavior of the tumor [22]. However, in our series, only a tendency towards reverse correlation between 9p21 and 12q13 alterations has been detected (data not shown).

As previously described, we detected a high frequency of LOH in the *p53* locus, which has been reported as a marker of poor prognosis associated with blastoid variants of MCL [20, 21]. Accordingly, Tamaru et al. concluded that overexpression of *p53* and *p27KIP1* may be linked to a cellular mechanism involved in the development of the variant form of MCL [47]. Mutant *p53* alleles were also correlated to proliferation signature and worse overall survival in comparison to wild-type cases [20]. In line with these observations, LOH in 17p13.1 harboring *p53* were associated to members of the proliferation-associated cluster in our study.

Table 2 Associations between genetic alterations in 17p13.1 and genetic alterations of other loci

Locus/morphology (number of analyzed samples)	Alterations in 17p13.1 (nr; %)	Odds ratio	*p* value
1p21(D1S206) (42)			
13 +	+(11; 84.6%)	5.8	0.04
29 −	−(14; 48%)		
6p24 (D6S1574) (42)			
10 +	+(9; 90%)	7	0.05
32 −	−(18; 56%)		
9p13-21 (D9S285, D9S171, D9S273) (50)			
20 +	+(17; 85%)	5.13	0.016
30 −	−(15; 50%)		
9q31-33 (D9S1677) (37)			
10 +	+(6; 60%)	6.6	0.014
27 −	−(5; 18.5%)		
17p13.2 (D17S938, D17S516) (45)			
23 +	+(14; 60.8%)	15.5	0.014
22 −	−(2; 8.3%)		
Morphology (33)			
10 + (blastoid)	+(6; 60%)	33	0.01
23 − (classical)	−(1; 4.3%)		

+ Presence of alterations, − no alteration

We could also identify a distinct frequently altered chromosomal region on 17p13.3, with several candidate tumor suppressor genes such as *tusc5*, *ovca2*, *mnt/rox*, or *hic1* which were described to be altered in other cancers [10, 29]. Interestingly, a functional cooperation between *hic1* and *p53* has been described. *Hic1* was involved in certain feedback regulation of *p53* by histone deacetylase *sirt1* [7, 8].

The frequently altered loci of chromosomal arm 13q31-33 harbor miRNA, small non-coding RNAs thought to be involved in physiologic and developmental processes by negatively regulating expression of target genes (http://microrna.sanger.ac.uk/). Ota et al. identified a new gene *C13orf25* in 13q31-32 which contains seven mature microRNAs in its untranslated region [34]. Additionally, O'Donnel et al. showed that *c-myc* activates expression of a cluster of six miRNAs on chromosome 13. Furthermore, expression of *e2f1*, another target of *c-myc* promoting cell cycle, is negatively regulated by two miRNAs of this cluster [33].

Another important tumor suppressor region on chromosomal band 13q14, deleted in up to 70% of MCL, has been suggested relevant for the pathogenesis of mantle cell lymphomas [14, 46]. In CLL deletions of 13q13-14 have been reported to be strongly associated clinical outcome [3, 12]. Accordingly, genetic alterations of 13q14 have been associated with inferior overall survival in MCL [28]. In this region, miR-15a and miR-16a have been reported to be frequently deleted and/or down-regulated in CLL patients [6]. Expression of miR-15a and miR-16a has been inversely correlated to BCL2 expression in CLL and characterized as negative regulator of *bcl2* at posttranscriptional levels [9].

In summary, most of the frequent genomic alterations demonstrated in MCL in the present study are associated with dysregulation of the cell cycle machinery and interfere with the cellular response to DNA damage. Systematic genotyping identified a proliferation-associated cluster involving 1p21, 6p24, 9p21, and 9q31 loci. These results support and complement the proliferation signature previously defined by RNA expression profiling [18, 39]. Additional studies of this genomic region will provide further insights into the molecular pathogenesis of MCL.

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