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ORIGINAL ARTICLE

Genomic deletion and promoter methylation status of *Hypermethylated in Cancer 1* (*HIC1*) in mantle cell lymphoma

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Abstract Mantle cell lymphomas (MCL), characterized by the t(11;14)(q13;q32), frequently carry secondary genetic alterations such as deletions in chromosome 17p involving the *TP53* locus. Given that the association between *TP53*-deletions and concurrent mutations of the remaining allele is weak and based on our recent report that the *Hypermethylated in Cancer 1* (*HIC1*) gene, that is located telomeric to the *TP53* gene, may be targeted by deletions in 17p in diffuse large B-cell lymphoma (DLBCL), we investigated whether *HIC1* inactivations might also occur in MCL. Monoallelic deletions of the *TP53* locus were detected in 18 out of 59 MCL (31%), while overexpression of p53 protein occurred in only 8 out of 18 of these MCL (44%). In *TP53*-deleted MCL, the *HIC1* gene locus was co-deleted in 11 out of 18 cases (61%). However, neither *TP53* nor *HIC1* deletions did affect survival of MCL patients. In most analyzed cases, no hypermethylation of the *HIC1* exon 1A promoter was observed (17 out of 20, 85%). However, in MCL cell lines without *HIC1*-hypermethylation, the mRNA expression levels of *HIC1* were nevertheless significantly reduced, when compared to reactive lymph node specimens, pointing to the occurrence of mechanisms other than epigenetic or genetic events for the inactivation of *HIC1* in this entity.

Keywords Mantle cell lymphoma · del(17p) · *TP53* · *HIC1*

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Introduction

Mantle cell lymphoma (MCL), an aggressive subtype of B-cell non-Hodgkin lymphomas (B-NHL), comprises 5–10% of human B-cell lymphomas approximately [1]. The clinical course is heterogeneous, with survival times ranging from a few months to more than 10 years [2–4]. The hallmark genetic feature of this neoplasm is the translocation t(11;14)(q13;q32) that juxtaposes the *cyclin D1* gene and the enhancer region of the immunoglobulin heavy chain genes, leading to constitutive overexpression of *cyclin D1* [5–8]. Although the deregulation of *cyclin D1* is thought to be the primary genetic alteration in the pathogenesis of MCL, additional oncogenic events are required to promote tumor progression [9]. Various studies that applied genome-wide techniques to unravel secondary chromosomal aberrations in MCL, resulted in the characterization of frequently deleted regions and subsequently in the identification of particular tumor suppressor genes (e.g.,

ATM, *BIM*, *CDKN2A*). However, in many deleted regions, candidate tumor suppressors have not been identified so far [7, 10–15].

One critical tumor suppressor gene that is frequently targeted by deletions in the short arm of chromosome 17 is the transcription factor *TP53*. In MCL, *TP53* gene deletions are almost invariably associated with a loss of the whole arm of chromosome 17p [7, 10, 12]. Whether, therefore, loss of the *TP53* locus represents the most important biological consequence of 17p deletions in MCL, remains unknown. We recently reported that the tumor suppressor candidate *Hypermethylated in Cancer 1* (*HIC1*), which is located in the chromosome band 17p13.3 telomeric to the *TP53* locus, is frequently inactivated by concurrent deletion and promoter hypermethylation in diffuse large B-cell lymphoma (DLBCL) [16]. To investigate whether inactivation of *HIC1* also occurs in MCL, we performed a detailed analysis of the chromosome 17p deletion status on the basis of conventional cytogenetic analysis, fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR)-based loss of heterozygosity (LOH) analysis, and quantitative real-time (RT-qPCR) approaches. Our results demonstrate that *HIC1*, although frequently co-deleted with *TP53* in 17p13.3, is—in contrast to DLBCL—infrequently hypermethylated in MCL suggesting that deletions of 17p might have different biological consequences in MCL and DLBCL.

Material and methods

Tumor specimens and cell lines

Fifty-nine MCL specimens, referred to the Institute of Pathology, University of Würzburg, were classified according to the criteria of the World Health Organization (WHO) classification system [17]. *Cyclin D1* overexpression or the presence of the t(11;14)(q13;q32) were identified in all cases by immunohistochemistry or FISH analysis, respectively. Genomic DNA was extracted from formalin-fixed and paraffin-embedded tissue (7 out of 59), from fresh-frozen tissue (18 out of 59), and from leukemic blood specimens (34 out of 59) as previously described [16, 18].

Among these 59 MCL patients, 47 were male and 12 female. The median age at diagnosis was 66 years (range 48 to 97 years), and median survival was 2.2 years. Of 59 MCL, 45 tumors were obtained at the time of primary diagnosis, while 14 lymphoma samples were relapsed MCL. Our study was approved by the local Ethics Committee of the University of Würzburg, Germany. The therapy was heterogeneous but, in most patients CHOP-based (without addition of Rituximab).

The human MCL cell lines used in this study were GRANTA-519, HBL2, MINO, REC1, and JVM2. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were maintained in Roswell Park Memorial Institute-1640 cell culture medium (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (PAA, Pasching, Austria). DNA and RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according the manufacturer's protocol.

Chromosome banding analysis

Chromosome spreads were prepared from lymphocyte short-term cultures in 46 of 59 MCL cases according to standard protocols [19]. After Giemsa–trypsin banding, the karyotypes were established according to the International System for Human Cytogenetic Nomenclature (ISCN) [20]. Identical structural aberrations, or genetic gains, in two or more metaphases and identical genetic losses in at least three metaphases were defined as clonal.

Immunohistochemistry

Immunohistochemical staining for the p53 protein was assessed on freshly cut slides from formalin-fixed and paraffin-embedded tissues using the DO-7 antibody (1:50; DAKO, Glostrup, Denmark), as previously reported [21]. The staining for p53 was considered positive when ≥20% of tumor cells showed strong nuclear reactivity.

Interphase fluorescence in situ hybridization

Interphase FISH with *TP53*- and *HIC1*-locus-specific probes, using BAC clones RP11-199F11 and RP11-667K14, respectively, was performed according to previously published protocols [16]. The BAC clones were obtained from the libraries RPCIB753 and RPCIP704 of the RZPD German Resource Center for Genome Research (Berlin, Germany).

To determine the cutoff level for each probe, control studies with five reactive lymph node specimens were carried out. The cutoff ratios for RP11-199F11 and RP11-667K14 were 11.7% and 11.8%, respectively. Signal visualization of at least 100 intact nuclei per case was accomplished on a Leica fluorescence microscope (Leica Microsystems, Bensheim, Germany). Illustrations were documented using the ISIS imaging system (MetaSystems, Altlussheim, Germany).

Real-time quantitative PCR

To detect genomic losses of the *HIC1* and *TP53* loci in the tumor specimens, RT-qPCR using genomic DNA was performed as previously described [16]. All measurements were done in triplicates. Presence or loss of the genomic *HIC1* or *TP53* region was defined relative to the β-2-

microglobulin (*B2M*) locus (15q21) and calculated with the standard curve method using DNA prepared from peripheral blood mononuclear cells of healthy volunteers. The cutoff ratios for deletion were determined by analyzing DNA of five reactive lymph nodes. The cutoff ratios for *HIC1*/*B2M* and *TP53*/*B2M* were 0.74 and 0.76, respectively.

HIC1 mRNA expression was determined by TaqMan qRT-PCR. Reverse transcription of 1 μg total RNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All measurements were done in triplicates and calculated with the standard curve method using cDNA of reactive lymph node specimen and *β-Actin* as endogenous control. Primers for *HIC1* amplification were 5′-CTGGATCCGCCGT CAGC-3′ (sense) and 5′- TGTCCAGCATCGTCTGC-3′ (antisense). To amplify *β-Actin*, the following oligonucleotides were used: 5′-GACGAGGGCGTGCTGGT GGG-3′ (sense) and 5′-GATGCCTCTCTTGCTCTGGGC-3′ (antisense).

LOH analysis

Normal and tumor DNA were analyzed for loss of heterozygosity (LOH) of the *TP53* (D17S1678) and *HIC1* (D17S831) gene loci as previously reported [18].

Methylation analysis of *HIC1*

DNA was bisulphite-modified as previously described [18]. Methylation-specific PCR (MSP) was carried out according to published protocols [22]. Primers used to amplify the 5′-untranslated region (exon 1A) of the *HIC1* gene were also reported previously [16].

Statistical analysis

The Kaplan–Meier method was used to estimate overall survival of the patients. The statistical significance of associations between the *TP53* or *HIC1* status and survival was determined by using the log rank test. A p value of <0.05 was considered statistically significant. For all analyses, the SPSS software V12.0 (SPSS, Chicago, IL, USA) was used.

Results

Deletions of the *TP53* gene locus are not strictly associated with altered p53 protein expression

To define the deletion status of *TP53* in the chromosome band 17p13.1, fluorescence in situ hybridization (FISH) with the *TP53*-locus-specific probe RP11-199F11 was performed in 51 out of 59 MCL cases. In addition, tumor and normal DNA of 24 out of 59 MCL could be analyzed for loss of heterozygosity (LOH) of *TP53* using the microsatellite marker D17S1678. A monoallelic deletion of the *TP53* gene was observed in 18 out of 59 MCL (31%) (Table 1).

In conventional cytogenetic analyses of 46 out of 59 MCL, a deletion in the short arm of chromosome 17 [del (17p)] corresponded with loss of the *TP53* gene in 6 out of 18 samples, while in 8 out of 18 tumors, the monoallelic *TP53*-deletion, as detected by FISH and/or LOH, was not visible by chromosome banding. In four cases that harbored a deletion of the *TP53*-locus by FISH, no banding analyses were available (Table 1). Interestingly, conventional cytogenetic analysis revealed a del(17p) in two MCL, without affecting the *TP53* locus as shown by FISH and LOH (Table 1).

To check for possible inactivating mutations of the second, non-deleted *TP53* allele, we performed immunohistochemical staining of the p53 protein in 51 of 59 cases. An overexpression of the p53 protein was detected in 18 out of 51 MCL. Whereas eight of these cases harbored *TP53* gene deletions, in the remaining ten MCL samples, accumulation of p53 protein occurred without corresponding deletion of the *TP53* locus. Vice versa, no p53 overexpression was observed in 33 of 51 MCL. Of those, 25 MCL did not harbor a deletion of the *TP53* gene, while in 8 out of 33 tumors, a monoallelic *TP53*-deletion was detected (Table 1). Focusing on the eight MCL harboring a *TP53* deletion without detectable p53 overexpression, three of these cases carried a del(17p) by conventional cytogenetic analysis, while in three additional tumors, a del(17p) was not visible by chromosome banding. In the remaining two MCL, no chromosome banding data was available, and the *TP53* deletion was defined only on the basis of FISH and LOH analyses (Table 1).

In summary, deletion of the *TP53* locus in concert with overexpression of p53 protein suggesting p53 inactivation was evident in 8 of 59 MCL (14%). Of note, in 3 of 8 MCL a del(17p), as indicated by conventional cytogenetic analysis, was not associated with overexpression of p53-protein. Moreover, in 2/59 MCL samples a del(17p) occurred without affecting the *TP53*-locus, pointing to the existence of another tumor suppressor gene in chromosome 17p.

Deletion of *Hypermethylated in Cancer 1 (HIC1)* gene in the chromosome band 17p13.3 occurs in addition to the loss of *TP53* gene locus in MCL

Because we had observed an inactivation of *HIC1* by monoallelic deletion and concurrent promoter hypermethylation of the remaining allele in diffuse large B-cell

Table 1 *TP53* and *HIC1* status in mantle cell lymphoma

Case no.	17p copy number (cytogenetics)	TP53 copy number (FISH)	TP53 copy number (LOH)	TP53 copy number (pPCR)	TP53 summarized deletion status	p53 over-expression	HIC1 copy number (FISH)	HIC1 copy number (LOH)	HIC1 copy number (qPCR)	HIC1 summarized deletion status	HIC1 Methylation (normal cell)	HIC1 Methylation (tumor cell)
1	2	n.a.	2	2	No	−	2	2	2	No	n.a.	n.a.
2	2	2	2	2	No	n.a.	n.a.	2	2	No	U	U
3	1	1	1	1	Yes	−	1	1	1	Yes	U	U
4	2	2	2	n.a.	No	−	1	2	1	Yes	n.a.	n.a.
5	2	2	2	2	No	n.a.	2	2	n.a.	No	U/m	U/m
6	1	1	1	1	Yes	+	1	1	1	Yes	n.a.	n.a.
7	2	2	2	2	No	+	2	2	n.a.	No	n.a.	n.a.
8	2	2	2	n.a.	No	−	2	2	n.a.	No	n.a.	n.a.
9	2	2	2	n.a.	No	−	2	n.a.	n.a.	No	n.a.	n.a.
10	2	2	2	n.a.	No	−	2	n.a.	n.a.	No	n.a.	n.a.
11	2	2	2	n.a.	No	−	n.a.	2	n.a.	No	n.a.	n.a.
12	1	2	2	2	No	+	1	2	1	Yes	n.a.	n.a.
13	2	2	2	n.a.	No	+	2	2	n.a.	No	n.a.	n.a.
14	2	1	1	n.a.	Yes	−	1	1	n.a.	Yes	U/M	U/M
15	1	2	n.a.	2	No	−	1	n.a.	1	Yes	n.a.	n.a.
16	1	1	1	n.a.	Yes	−	2	2	n.a.	No	n.a.	n.a.
17	2	2	2	2	No	−	1	1	1	Yes	U	U
18	2	2	n.a.	n.a.	No	−	2	2	n.a.	No	n.a.	n.a.
19	2	1	n.a.	1	Yes	+	2	2	2	No	U/M	U/m
20	2	2	n.a.	n.a.	No	+	2	n.a.	n.a.	No	U/m	U
21	2	2	n.a.	n.a.	No	n.a.	1	n.a.	n.a.	Yes	U	U/m
22	2	2	n.a.	n.a.	No	−	2	n.a.	n.a.	No	n.a.	n.a.
23	2	2	n.a.	2	No	−	2	2	2	No	U	U
24	2	2	n.a.	2	No	+	2	n.a.	2	No	n.a.	n.a.
25	1	1	n.a.	1	Yes	+	1	1	2	Yes	U	U
26	2	2	n.a.	2	No	n.a.	2	n.a.	2	No	U/m	U/m
27	2	2	n.a.	2	No	n.a.	2	n.a.	2	No	U/m	u/M
28	2	2	n.a.	2	No	−	2	n.a.	2	No	U/m	U/m
29	2	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
30	2	2	n.a.	2	No	+	2	n.a.	2	No	n.a.	n.a.
31	2	2	n.a.	2	No	n.a.	2	n.a.	2	No	U/m	U/M
32	2	2	n.a.	2	No	−	n.a.	2	2	No	U/M	U/m
33	2	n.a.	2	n.a.	No	−	n.a.	2	n.a.	No	U/m	U/m
34	2	n.a.	1	n.a.	Yes	n.a.	n.a.	1	n.a.	Yes	n.a.	n.a.
35	2	n.a.	1	n.a.	Yes	+	n.a.	1	n.a.	Yes	n.a.	n.a.
36	2	n.a.	1	n.a.	Yes	−	n.a.	2	n.a.	No	n.a.	n.a.
37	2	2	n.a.	2	No	+	2	n.a.	2	No	n.a.	n.a.
38	2	2	n.a.	2	No	+	2	n.a.	2	No	n.a.	n.a.

39	2	2	n.a.	2	No	+	2	n.a.	2	No	n.a.	n.a.
40	2	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
41	2	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
42	1	1	n.a.	2	Yes	n.a.	2	n.a.	2	No	n.a.	n.a.
43	1	1	n.a.	n.a.	Yes	−	1	n.a.	n.a.	Yes	n.a.	n.a.
44	2	1	n.a.	n.a.	Yes	+	2	n.a.	n.a.	No	n.a.	n.a.
45	2	1	n.a.	1	Yes	+	1	n.a.	1	Yes	n.a.	n.a.
46	2	1	n.a.	n.a.	Yes	−	1	n.a.	n.a.	Yes	n.a.	n.a.
47	n.a.	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
48	n.a.	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
49	n.a.	2	n.a.	2	No	−	1	n.a.	1	Yes	n.a.	n.a.
50	n.a.	1	n.a.	2	Yes	+	2	n.a.	2	No	n.a.	n.a.
51	n.a.	1	n.a.	1	Yes	−	2	n.a.	2	No	n.a.	n.a.
52	n.a.	1	n.a.	n.a.	Yes	+	1	n.a.	1	Yes	n.a.	n.a.
53	n.a.	1	n.a.	1	Yes	−	1	n.a.	n.a.	Yes	n.a.	n.a.
54	n.a.	2	n.a.	2	No	−	2	n.a.	2	No	n.a.	n.a.
55	n.a.	2	2	2	No	−	1	1	1	Yes	U	U
56	n.a.	n.a.	2	n.a.	No	−	n.a.	2	n.a.	No	U	U
57	n.a.	2	n.a.	n.a.	No	−	2	n.a.	n.a.	No	n.a.	n.a.
58	n.a.	n.a.	2	2	No	−	n.a.	2	2	No	U/M	U/M
59	n.a.	n.a.	2	n.a.	No	+	n.a.	2	n.a.	No	U	U

Copy numbers are indicated by 2 (normal) and 1 (deleted). The immunohistochemical staining for p53 was considered positive when ≥20% of tumor cell nuclei were stained distinctively from the background (+). Methylation status of *HIC1* is indicated by U (unmethylated product), u (weakly unmethylated product), M (methylated product) and m (weakly methylated product).
FISH Fluorescence in situ hybridization, *LOH* loss of heterozygosity analysis with microsatellite markers, *qPCR* quantitative real-time PCR approach

lymphomas (DLBCL) [16], we examined, whether the deletion of *HIC1*, located in the chromosome band 17p13.3 telomeric to *TP53*, might also be associated with the pathogenesis of MCL.

The deletion status of *HIC1* was examined in 59 MCL, using FISH with the *HIC1* locus-specific probe RP11-667K14 (in 32 out of 59 fresh-frozen and leukemic blood samples) or LOH analysis with the microsatellite marker D17S831 (on DNA from 10/59 MCL samples extracted from leukemic blood) or by using both methods when appropriate material was available (in 17 out of 59 MCL). A monoallelic deletion of RP11-667K14 by FISH and/or loss of heterozygosity of D17S831 by LOH analysis was observed in 18 out of 59 cases (31%) (Table 1). Deletion of both the *HIC1* and *TP53* loci simultaneously occurred in only 11 of these MCL. By conventional cytogenetic analysis, a del(17p) was detected in 4 out of 11 MCL samples, indicating complete loss of chromosome 17p in these cases (Table 1, Fig. 1a–c).

In 7 out of 18 *HIC1*-deleted MCL, no loss of the *TP53* gene was observed. Two of these cases were shown to harbor a del(17p) by cytogenetic analysis without concomitant loss of the *TP53* locus, indicating a del(17p), with a breakpoint telomeric to the chromosome band 17p13.1, harboring the *TP53* locus (Table 1, Fig. 1a,f–g).

In 41 out of 59 MCL, no deletion of *HIC1* was detected, but seven of these cases carried a monoallelic *TP53* deletion. Two of these MCL cases harbored a del(17p) by conventional cytogenetics, which suggests a deletion of chromosome 17p, excluding the telomeric part of the region containing the *HIC1* gene (Table 1, Fig. 1a,d–e).

To validate the deletion pattern of *HIC1* and *TP53* with an alternative technique, we used quantitative PCR (qPCR) in 33 MCL to determine the *HIC1* and *TP53* deletion status in correlation to the reference gene beta-2-microglobulin (*B2M*). By this approach, 22 of 33 samples were shown to harbor a normal *TP53* and *HIC1* status, as indicated by *HIC1*/*B2M* and *TP53*/*B2M* ratios ranging between 0.95 and 1.06, comparable to the results of FISH and LOH experiments (Table 1, Fig. 2). Monoallelic deletion of the *TP53* gene without loss of the *HIC1* locus was observed in 3 out of 33 MCL (nos. 19, 25 and 51) by qPCR (Table 1, Fig. 2). In 5 out of 33 tumors (nos. 12, 15, 17, 49 and 55), which exhibited one FISH signal with the *HIC1*-specific probe, *HIC1* deletion was observed by qPCR, as indicated by *HIC1*/*B2M* ratios between 0.19 and 0.31, while a normal status was detected for the *TP53* gene (Table 1, Fig. 2). A concurrent deletion of both the *HIC1* and *TP53* gene loci was found in 3 out of 33 MCL (nos. 3, 6, and 45), confirming the results of FISH and LOH experiments (Table 1, Fig. 2).

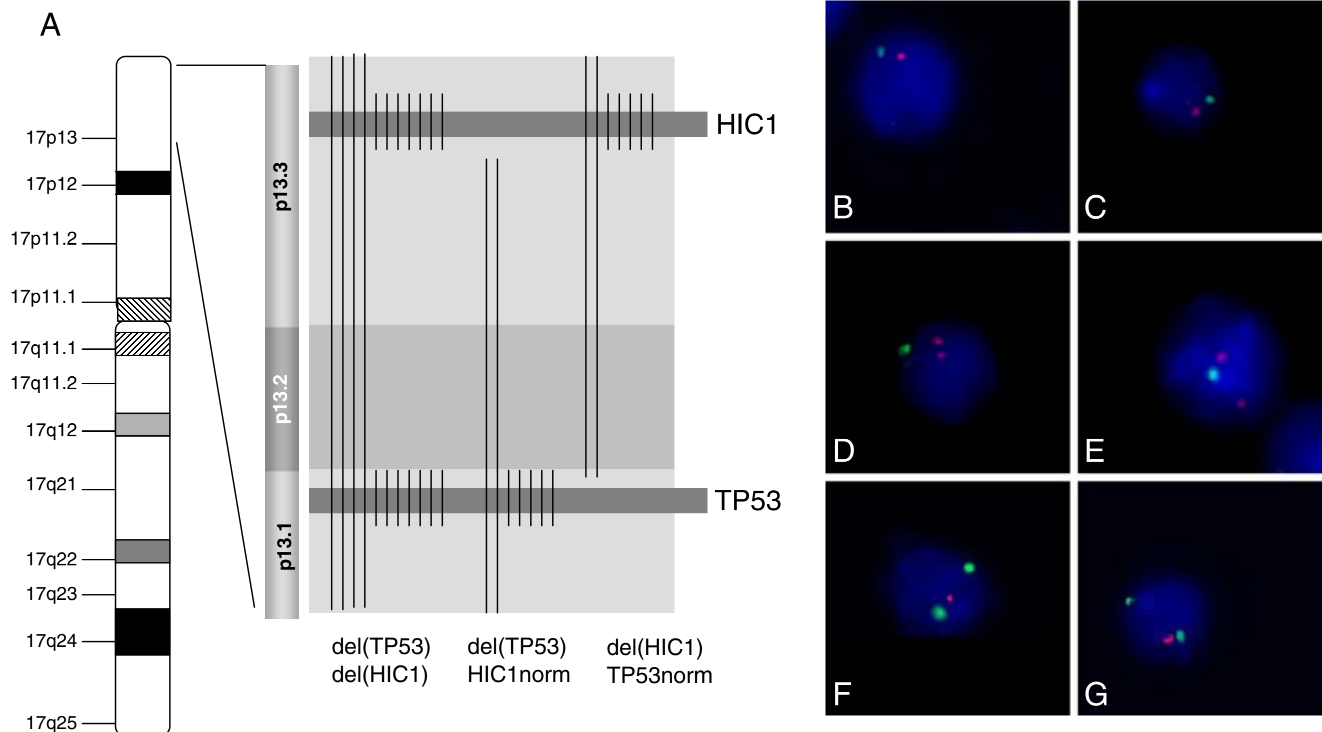

Fig. 1 Chromosome 17p deletions in MCL. Whole arm deletion of chromosome 17p, including *TP53* and *HIC1*, in 11/59 MCL (**a**), as indicated by fluorescence in situ hybridization using the *TP53*-specific probe RP11-199F11 (*green*) and the *HIC1*-specific probe RP11-667K14 (*red*) (**b**–**c**). Partial deletion of chromosome band 17p13.1 to 17p13.3 including only the *TP53*-locus in 7 out of 59 MCL (**a**), as seen by loss of *one green signal* (*TP53*) in contrast to two *HIC1*-signals (*red*) (**d**–**e**). Deletion of chromosomal band 17p13.3, including *HIC1* without loss of *TP53* in 7/59 MCL (**a**), as indicated by one *HIC1*-signal (*red*) instead of two *TP53*-signals (*green*; **f**–**g**)

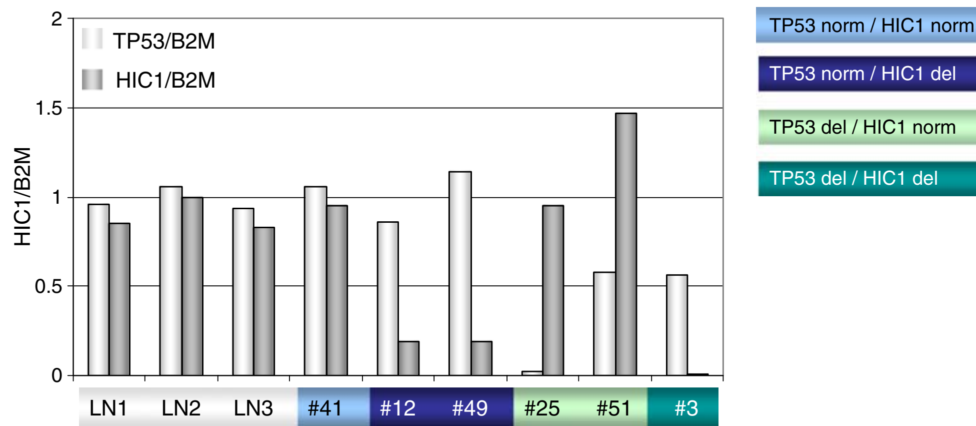

Fig. 2 Genomic deletions of the *HIC1* and *TP53* loci detected by quantitative real-time PCR. Three reactive lymph node samples show *HIC1/B2M* and *TP53/B2M* ratios close to 1, indicating wild type configuration of both loci (LN1–LN3, *grey*), as also presented for MCL no. 41 (*light blue*). Two MCL (nos. 12, 49) show a *HIC1/B2M* ratio below the cutoff level indicating a deletion of the *HIC1* locus, while the *TP53/B2M* ratio indicates wild-type configuration of the *TP53* locus (*dark blue*). Two MCL (nos. 25, 51) show evidence of a *TP53* deletion in the presence of two copies of *HIC1* (*light green*). One MCL (no. 3) harbors a co-deletion of the *HIC1* and *TP53* loci (*dark green*)

Monoallelic deletion of the *HIC1* gene locus is not associated with hypermethylation of the *HIC1* promoter

Because monoallelic deletion of the *HIC1* gene was previously described to be associated with promoter methylation of the remaining *HIC1* allele in DLBCL [16], we investigated the *HIC1* exon 1A promoter in a subset of MCL. Hypermethylation of exon 1A, which codes for the 5′ untranslated region of the transcript, has been described as a mechanism for *HIC1* gene inactivation in mouse lymphomas, and in human tumors and was associated with decreased *HIC1* mRNA expression [23–25].

Because leukemic involvement is frequent in MCL [26], we preferentially focused on the analysis of DNA extracted from leukemic peripheral blood of 20 MCL patients, which facilitates the comparison of the *HIC1* methylation status between malignant MCL cells and normal granulocytes. Three MCL (nos. 21, 27, 31; 15%) showed aberrant *HIC1*-methylation in the tumor DNA, in contrast to DNA obtained from granulocytes of the same patient sample (Fig. 3a, Table 1). In only one of these cases, *HIC1* methylation was associated with a concomitant deletion of the *HIC1* locus (Table 1). In 14 out of 20 cases (70%), no alteration of exon 1A promoter methylation was observed in the lymphocyte DNA when compared to DNA from nonmalignant granulocytes. The majority of these cases showed unmethylated DNA in both normal and tumor cells (in 8 out of 14 cases, Fig. 3b, Table 1), while weakly methylated DNA of both tumor and normal cells was detected in 6 out of 14 MCL (Fig. 3c, Table 1). In 3 out of 20 cases (15%), *HIC1* methylation was observed in DNA of granulocytes, while in lymphocytes from the same patient, the *HIC1*-promoter appeared to be less methylated (Fig. 3d, Table 1).

To ensure that the epigenetic *HIC1* profile of MCL cells in leukemic peripheral blood reflects that obtained from MCL cells in lymph nodes, we analyzed DNA extracted from lymph node specimens in addition to the leukemic peripheral blood in 3 out of 20 cases. No differences in the *HIC1* methylation status were observed when comparing

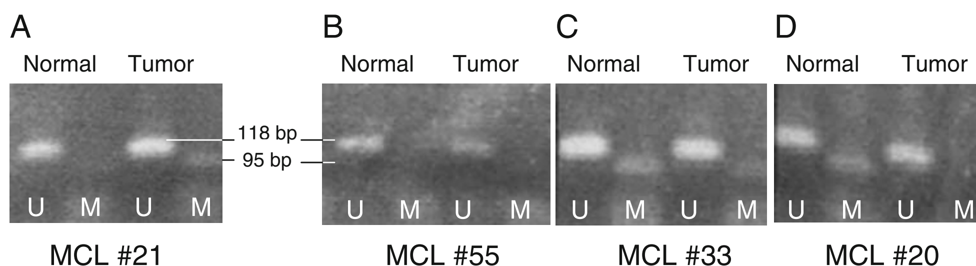

Fig. 3 Methylation-specific PCR of the 5′-UTR of *HIC1* in granulocytes (normal) and lymphocytes (tumor) of MCL patients. Samples containing methylated DNA produced PCR amplicons with the M (methylated) primer set, while samples without methylation yielded PCR products when the U (unmethylated) primer set was applied. Promoter methylation of *HIC1* in tumor cells, while normal cells showed unmethylated *HIC1*-sequence in MCL no. 21 (**a**). Neither normal, nor tumor cells harbored methylated DNA product in MCL no. 55 (**b**). Slightly methylated DNA in normal and tumor cells in MCL no. 33 (**c**). Methylation of the *HIC1*-promoter in normal, but not in tumor cells in MCL no. 20 (**d**)

DNA extracted from lymph node and peripheral blood in these MCL (data not shown).

Reduced *HIC1* mRNA levels in MCL cell lines are not associated with promoter hypermethylation

To investigate the possible role of *HIC1* in MCL pathogenesis in more detail, we studied the five MCL cell lines GRANTA-519, HBL2, MINO, REC1, JVM2, first, to determine the methylation status of *HIC1* and, second, to assess *HIC1* gene expression level. It has to be emphasized that none of the cell lines harbor homozygous deletion of chromosome 17p, as assessed by 500K SNP array analysis (E. Hartmann, unpublished data). While GRANTA-519, HBL2, and MINO presented with monoallelic deletions in the short arm of chromosome 17, including *TP53* and *HIC1* gene locus, REC1 and JVM2 showed a normal diploid status of 17p. None of the cell lines presented with an aberrant methylation of the *HIC1* exon 1A promoter, comparable to those results obtained from reactive lymph node specimens and in the majority of the primary MCL tumors (Fig. 4a). However, expression levels of *HIC1* mRNA were significantly reduced in MCL cell lines, in contrast to the normal lymphoid tissue samples ($p=0.001$, Fig. 4b).

Monoallelic deletion of the *HIC1* locus is not associated with survival of MCL patients

HIC1 and *TP53* inactivations have recently been reported to be negative prognostic markers in diffuse large B-cell lymphoma. To analyze the clinical course of MCL patients according to their *HIC1* and *TP53* status, we compared the patients' survival data of 34 out of 59 cases with a deletion of either *HIC1* or *TP53*, as well as of MCL patients with overexpression of p53 protein. Given the caveat of a small patient series, neither *TP53* deletions nor deletions of the *HIC1* gene locus were associated with inferior survival of MCL patients (Fig. 5a,c). Overexpression of p53 protein, however, clearly predicted for inferior outcome compared to tumors from MCL patients lacking expression of the protein ($p\leq0.0001$, Fig. 5b).

Discussion

MCL is a well-defined subtype of B-cell non-Hodgkin lymphoma, characterized by the translocation t(11;14)(q13;q32), which is present in virtually all cases. In addition to this translocation, MCL tumor cells harbor various secondary chromosomal alterations [27]. One critical regulator of cell survival is the tumor suppressor gene *TP53*, located in chromosome band 17p13.1, a genomic region frequently deleted in MCL [7]. On the basis of the two-hit hypothesis, the inactivation of a tumor suppressor gene, e.g., by concurrent deletion and mutation/hypermethylation of the second allele, is considered a prerequisite for tumor progression [28, 29]. The association of *TP53* gene deletions and mutation of the remaining non-deleted *TP53*-allele, however, has been reported to be only weak in MCL [30, 31]. This is also confirmed in our series, in which 18 out of 59 MCL (31%) harbored a monoallelic *TP53* deletion, while only eight of these cases (44%)

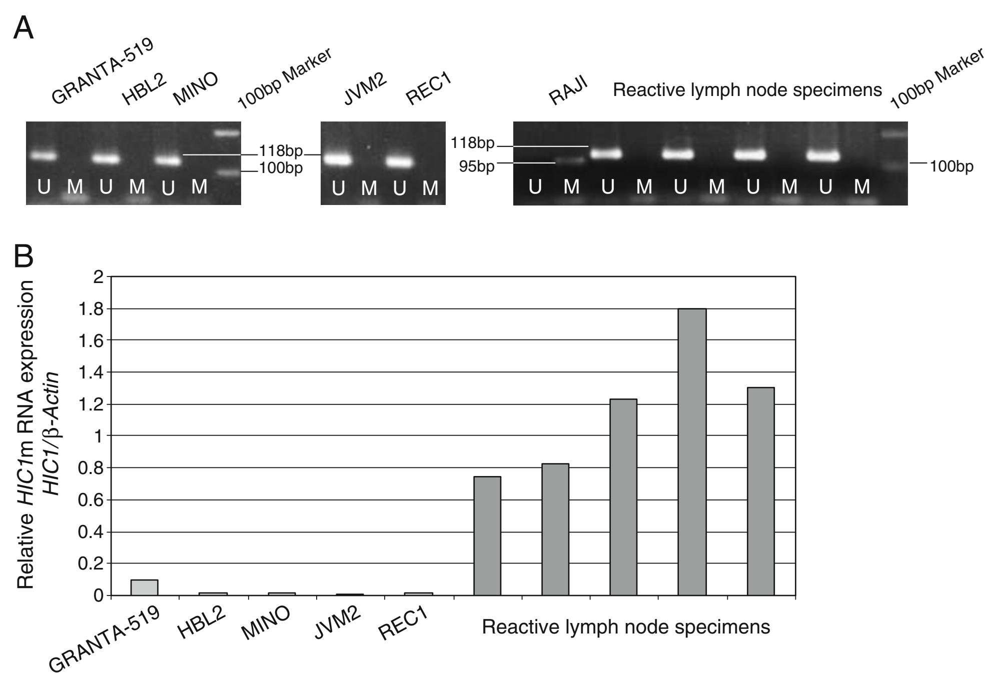

Fig. 4 *HIC1* promoter methylation and mRNA expression in MCL cell lines. None of the analyzed MCL cell lines showed aberrant methylation pattern of the *HIC1* exon 1A promoter, with amplification of the U (unmethylated) primer set, in line with the unmethylated *HIC1* promoter in reactive lymph node specimens. As a control RAJI cell line was used, yielding PCR product when the M (methylated) primer set was applied (**a**). In contrast to reactive lymph-node specimens, the *HIC1* mRNA expression was significantly reduced in all MCL cell lines (**b**)

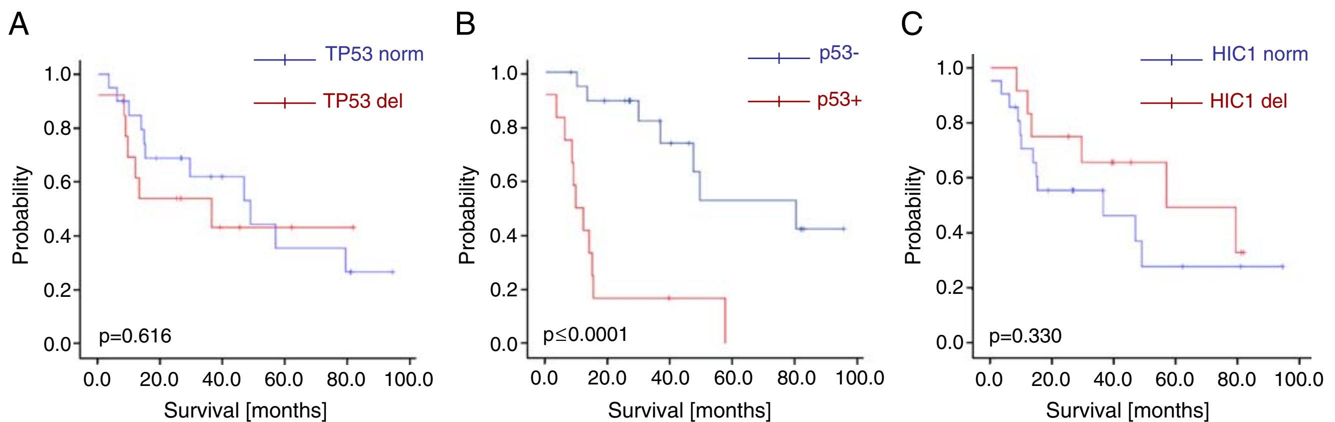

Fig. 5 Survival analysis of MCL patients according to the *TP53* and *HIC1* status. Kaplan–Meier survival curves for patients with monoallelic deletion of *TP53* (**a**), overexpression of p53 protein (**b**) and for MCL patients with monoallelic deletion of *HIC1* (**c**). *Norm* Normal, *del* deleted, *p53+* overexpression of p53-protein, *p53−* no overexpression of p53-protein

showed an overexpression of p53 protein suggesting the inactivation of the remaining *TP53* allele by gene mutation. The finding that 10 out of 18 MCL showed monoallelic *TP53* deletions without concomitant overexpression of p53 protein might be pointing to the existence of another candidate tumor suppressor gene on chromosome 17p in MCL. Because no mutational analysis of the *TP53* gene had been performed in this study, we cannot entirely exclude the occurrence of complete *TP53* inactivation in these cases by immunohistochemical analysis of p53 protein expression, given its false-negative results, especially in the context of non-sense and truncating mutations of the *TP53* gene [32, 33]. However, we consider it unlikely that this scenario accounts for the lack of p53 protein expression in the majority of cases, as most of the *TP53* mutations occurring in MCL are missense mutations which do not result in truncated p53 protein [34, 35]. In addition, it has been reported that discordances between the results of p53 immunohistochemistry and mutational analysis of the *TP53* gene can also be due to alternative mechanisms of p53 stabilization, leading to overexpression of p53 protein without concomitant *TP53* gene mutation. In contrast, none of the previously published MCL cases lacking p53-overexpression showed mutation of the TP53 gene [34–36].

In addition to ten MCL that harbored monoallelic *TP53* deletions without concurrent overexpression of p53 protein in our series, a deletion of the short arm of chromosome 17 was detected in two MCL, without affecting the *TP53* gene locus. This finding points to the loss of genomic material telomeric to the *TP53* locus, possibly inactivating other tumor suppressor genes, which is consistent with the previous finding of novel recurrently deleted regions in 17p13.3, as reported for MCL [37], but also for DLBCL [16] and various leukemia subtypes [38]. These findings provide support for the hypothesis that genomic deletions involving the chromosomal band 17p13 may not always affect *TP53* itself and raises the question whether other tumor suppressor genes might be targeted by deletions in this genomic region. The chromosomal band 17p13.3 harbors several candidate genes, such as *LOST1*, *DPH2L1*, *OVCA2*, *MNT/ROX*, or *HIC1*, which had been described to be altered in various cancer types [39–42]. Because the tumor suppressor candidate *HIC1* had previously been reported to be frequently inactivated in DLBCL [16], as well as in acute lymphocytic leukemia, in chronic myelogenous leukemia [43], and in acute myeloid leukemia [44], we focused our investigation in MCL on this candidate.

Monoallelic deletion of the *HIC1* gene was observed in 18 out of 59 MCL analyzed. Interestingly, *HIC1* deletions were detected in 7 out of these 18 cases that did not show evidence of a concurrent genomic loss of the *TP53* locus. Two of these cases harbored a del(17p), as indicated by cytogenetic analyses, suggesting a partial deletion of chromosome 17p telomeric to *TP53* gene, as had been recently described in DLBCL [16], as well as in myeloid and lymphoid leukemia [38].

Because *HIC1* has been reported to be frequently inactivated by promoter hypermethylation, but not by mutation [23, 42], we focused our investigation on the methylation status. However, methylation of the *HIC1* exon 1A promoter was only rarely encountered in MCL, in contrast to our previous findings in DLBCL [16]. The lack of *HIC1* hypermethylation in the MCL cell lines GRANTA-519, HBL2, MINO, JVM2, and REC1 further supports our findings that aberrant promoter methylation of *HIC1* is an infrequent event in MCL. Nevertheless, the mRNA expression levels of *HIC1* were significantly reduced in the MCL cell lines studied when compared to those observed in reactive lymphoid tissues, indicating that the inactivation of *HIC1* is not necessarily correlated with epigenetic or genetic events affecting the gene locus, but may be due to alternative mechanisms of *HIC1* inactivation. Of note, the reduction of *HIC1* mRNA expression, independent of *HIC1* promoter hypermethylation, has been recently reported in

hematological malignancies [45]. In keeping with previous findings about a possible role of *HIC1* in the differentiation of solid tumors, Britschgi et al. reported a decrease of *HIC1* expression level in primary human acute myeloid leukaemia (AML) when compared with terminally differentiated granulocytes. Although the mechanism by which *HIC1* is inactivated may not be related to genetic or epigenetic alterations, Britschgi and colleagues suggested that the repression of retinoic acid receptor signaling in leukaemia possibly causes downregulation of *HIC1* mRNA expression [45].

Although not epigenetically inactivated, the MCL subset studied here presented with a high proportion of monoallelic deletions of the *HIC1* gene locus. The heterozygous loss of *HIC1* function in mice has been reported to result in earlier tumor formation and increased aggressiveness in *TP53*-altered mice [46], and a potential synergistic effect of *TP53* and *HIC1* inactivation with respect to clinical outcome could be demonstrated in DLBCL patients [16]. Although limited by the small number of cases and the heterogenous treatment of MCL patients, neither the deletion of *HIC1* nor the deletion of *TP53* was associated with inferior overall survival. These results are in line with previous reports described for loss of *TP53* [31] as well as for 17p deletions in MCL in general [10, 12, 15]. In contrast, deletion of chromosome 17p has also been reported to be of prognostic value in B-NHL [47] and specifically in MCL [11]. Because deletions of the *TP53* or the *HIC1* gene locus alone were not associated with the clinical outcome of MCL patients in our study, we cannot entirely exclude the presence of other tumor-relevant candidate genes in chromosome 17p, beyond *TP53* and *HIC1*.

Interestingly, overexpression of p53 by immunohistochemistry, possibly indicating an underlying *TP53* gene mutation, was associated with significantly shorter overall survival of MCL patients in our series in accordance with reports about *TP53* mutations in MCL [34, 35]. The impaired survival of MCL patients with tumors showing overexpression of p53 protein may therefore be explained by the dominant-negative effect of *TP53*-mutations that may drive the remaining wild-type p53 into the mutant conformation resulting in a complete *TP53* inactivation [48].

In conclusion, our study provides further evidence that deletions in chromosome 17p are only weakly correlated with overexpression of p53 protein, indicating that the *TP53* gene may not be the only target of this genomic alteration.

Although we demonstrate that in a subset of MCL, chromosome 17p deletions affect the genomic region around the *HIC1* locus, the inactivation of *HIC1* by promoter methylation of exon 1A was only infrequently observed in MCL. However, in MCL cell lines without hypermethylation of the *HIC1* promoter, the mRNA expression levels were significantly reduced, indicating alternative mechanisms for the inactivation of *HIC1* in this tumor. Because reexpression of *HIC1* was shown to be feasible in AML with downregulated *HIC1* mRNA [45], it will be of interest to study a possible benefit of MCL patients restoring *HIC1* function.

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