Detection of gene copy number aberrations in mantle cell lymphoma by a single quantitative multiplex PCR assay: clinicopathological relevance and prognosis value

Mantle cell lymphoma (MCL) is a distinctive subtype of lymphoma that represents about 6% of all B-cell non-Hodgkin lymphomas (NHL). At the cytogenetic level, this entity is defined by the t(11;14)(q13;q32) translocation, which occurs in more than 95% of cases. Despite the important role of this translocation, which leads to the overexpression of cyclin D1, it is well established that it is not sufficient for the full transformation of the cells, nor does it explain the aggressiveness of the tumour (Jares et al., 2007). In addition to this primary genetic event, recent genetic and molecular studies, based on fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH) -array experiments, have revealed a multitude of molecular abnormalities (Salaverria et al., 2007; Sander et al., 2008; Schraders et al., 2008). In rare MCL cases without cyclin D1 overexpression, recurrent secondary genetic aberrations have been observed, demonstrating their leading role in oncogenesis (Salaverria et al., 2007). Among these additional oncogenic events, recurrent genomic aberrations that target the coding sequence of proteins involved in cell cycle regulation, senescence, or cellular response to DNA damage have been clearly identified (Jares et al., 2007). For instance, deletion of the 9p21-22 chromosomal region represents one of the most frequent events in MCL, occurring in 15–30% of patients (Jares et al., 2007). This region contains the CDKN2A gene that encodes for p14<sub>ARF</sub>/p15<sub>INK4B</sub> and p16<sub>INK4A</sub>. CDKN2A and MYC aberrations were associated with a high MCL international prognostic index (MIPI). CDK2/MDM2 gains and CDKN2A/TP53 losses correlated with an unfavourable outcome. PCR experiments with frozen and FFPE-tissues indicated that our approach is valid in a routine diagnostic setting, providing a powerful tool that could be used for patient stratification in combination with MIPI in future clinical trials.

Summary

The t(11;14)(q13;q32) is the hallmark of mantle cell lymphoma (MCL). Additional genetic alterations occur in the majority of cases. This study aimed to design a polymerase chain reaction (PCR) assay to determine the incidence and relevance of recurrent gene copy number aberrations in this disease. Forty-two MCL cases with frozen- or paraffin-embedded (FFPE) tissues were selected. Three different quantitative Multiplex PCR of Short Fluorescent Fragments (QMPSF) assays were designed to simultaneously analyse eight genes (CDKN2A, RB1, ATM, CDK2, TP53, MYC, CDKN1B, MDM2), to analyse the 9p21 locus (CDKN2A/CDKN2B) and FFPE tissues. Gains of MYC, CDK2, CDKN1B, and MDM2 were observed in 10% of cases. Losses of RB1, CDKN2A, ATM or TP53 were observed in 38%, 31%, 24% and 10% of cases, respectively. Analysis of the 9p21 locus indicated that, in most cases, tumours displayed a complete inactivation of p14<sub>ARF</sub>/p15<sub>INK4B</sub> and p16<sub>INK4A</sub>. CDKN2A and MYC aberrations were associated with a high MCL international prognostic index (MIPI). CDK2/MDM2 gains and CDKN2A/TP53 losses correlated with an unfavourable outcome. PCR experiments with frozen and FFPE-tissues indicated that our approach is valid in a routine diagnostic setting, providing a powerful tool that could be used for patient stratification in combination with MIPI in future clinical trials.

Keywords: deletions, gains, mantle cell lymphoma, prognosis.
In an effort to gain a more comprehensive understanding of the underlying molecular alterations in MCL, a specific prognostic index (MCL international prognostic index, MIPI) has recently been developed, which classifies patients into low risk [44% of patients, median overall survival (OS) not reached], intermediate risk (35%, median OS of 51 months), and high risk groups (21%, median OS of 29 months) (Hoster et al, 2008). This index is based on simple clinical and biological factors [age, Eastern Cooperative Oncology Group (ECOG) performance status, lactate dehydrogenase (LDH), and white blood cell (WBC) count]; however, these risk groups probably mirror the consequences of the underlying MCL genetic heterogeneity.

We previously developed and validated an inexpensive and sensitive genomic polymerase chain reaction (PCR) assay (Multiplex PCR of Short Fluorescent Fragments, QMPSF) to detect gene copy number abnormalities (GCNA) in various haematological malignancies including diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukaemia (CLL) or CD4+ CD56+ haematodermic neoplasms (Bastard et al, 2007; Jardin et al, 2008, 2009). In the present study, we developed a dedicated MCL QMPSF assay, based on the most frequent recurrent GCNA reported in this setting. We correlated genomic alterations, detected by this assay, to pathological features of the tumour, MIPI, and clinical outcome. Furthermore, due to its high frequency of involvement and its crucial role in MCL oncogenesis, we studied deletions of the 9p21 locus in more detail using the same technical approach and assessed their clinical and pathophysiological relevance. In addition, we developed and validated a specific QMPSF assay applicable in a routine diagnostic setting to formalin-fixed, paraffin-embedded (FFPE) tissue specimens that can be used in combination with the MIPI to integrate GCNA of the most relevant genes.

**Patients and methods**

**Patients and tissue samples**

Forty-two patients diagnosed with MCL followed in a single institution were selected. The diagnosis of MCL was established and confirmed according the criteria of the World Health Organization classification system (Swerdlow et al, 2008). This study was approved by the local institutional review board. Inclusion criteria were the availability of appropriate paraffin-embedded-tissues, available tumour DNA at the time of diagnosis from fresh or frozen tissues, complete clinical data, and complete follow-up. MIPI prognostic score was calculated according to the published formula as follows: 0.03535× age (years) + 0.6978 (if ECOG > 1) + 1.367 × log10(LDH/upper limit of normal) + 0.9393 × log10(WBC count) (Hoster et al, 2008). The combined biological index (MIPIb) was calculated by the addition of 0.02142 × Ki-67 (%) to the MIPI score, as originally reported (Hoster et al, 2008). Details of the main clinical features and treatments of the 42 patients are provided in Table I. Conventional cytogenetic analysis was performed in 40/42 cases. The t(11;14)(q13;q32) was detected in 20/22 cases (91%) with aberrant karyotype.

**Immunohistochemistry and proliferation index**

Histopathological and immunohistochemical analysis was performed from paraffin-embedded tissues on conventional slides. In addition to markers used for case selection (all cases were CD20 and CCND1 positive), specific immunohistochemical staining was performed for p53 (DAKO, Copenhagen, Denmark) and Rb-P (Ozyme, St Quentin, France) proteins. Cases were considered to be p53 positive when >50% of cells were positive. Rb-P nuclear expression was analysed using a semi-quantitative count. Staining with the MIBI antibody, which detects the Ki-67 antigen (DAKO), was performed to evaluate the proliferation index (PI). In all cases, mitotic index was analysed by counting the number of mitotic figures/mm².

**QMPSF assays design, validation and sensibility assessment**

QMPSF is a sensitive method for the detection of genomic deletions or duplications. It is based on the simultaneous

### Table I. Clinical characteristics and treatments of the MCL patients studied.

| Clinical features at diagnosis | No. of cases/all cases (%) |
|------------------------------|---------------------------|
| Male gender                  | 27/42 (64%)               |
| Age, years                   | 67.5 [34–76]              |
| MIPI score                   |                           |
| Low (<5)                     | 9/42 (21%)                |
| Intermed (5–7–6)             | 14/42 (33%)               |
| High (>6)                    | 19/42 (45%)               |
| ECOG PS >1                   | 8/42 (19%)                |
| Stage III                    | 10/42 (24%)               |
| Stage IV                     | 32/42 (76%)               |
| LDH >normal value            | 26/42 (62%)               |
| Splenomegaly                 | 20/40 (50%)               |
| Leucocyte count >10×10⁹/l    | 12/42 (29%)               |
| Bone marrow involvement      | 29/41 (71%)               |
| Histological subtype         |                           |
| Classic                      | 36/42 (86%)               |
| Blastoid variant             | 6/42 (14%)                |
| First line treatment         |                           |
| Anthracycline containing regimen | 31/42 (74%)           |
| Single-agent therapy         | 7/42 (16%)                |
| Radiotherapy                 | 1/42 (2%)                 |
| Rituxinab combined with chemotherapy | 13/42 (31%)        |
| Intensified chemotherapy with autologous stem transplantation | 12/42 (29%) |

MIPI, mantle cell lymphoma international prognostic score; ECOG, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase.
amplification of short genomic fragments using dye-labelled primers under quantitative conditions (patent FR 020924) (Casilli et al., 2002; Tournier et al., 2004). Tumour DNA was extracted from 42 lymph nodes with the use of the standard proteinase K/RNase treatment and salting-out extraction procedure. PCR products were analysed on a sequencing platform used in the fragment analysis mode, where both peak heights and areas are proportional to the quantity of template present for each target sequence. We designed a dedicated QMPSF assay that contained the following target genes: MYC (8q24), TP53 (17p13), CDKN2A (9p21), CDK2 (12q13) RB1 (13q14), ATM (11q22-23), MDM2 (12q14) and CDKN1B (12p13). Two control DNA were used [commercial DNA (Roche Diagnostics, Meylan, France), and a DNA extracted from a reactive lymph node] to calculate the mean normal/ tumoural peak height ratio. CECR1, located at 22q11, was chosen as reference gene and the SEMA4F (2p11) amplicon was used as endogenous DNA quality control. Primer pairs were designed so as not to encompass single nucleotide polymorphisms, for each of these 10 genes to generate PCR fragments ranging from 150 to 250 base pairs. In addition, we designed a dedicated QMPSF assay to analyse the 9p21 locus in more detail. This assay contained 10 primer pairs that cover a 2.8 Mb region and five genes, (Telomere > MIR31/MTAP/ CDKN2A/CDKN2B/DMRTA1 > Centromere), located within this locus (Fig 1). The complete primer sequences used to design the assays are indicated in Table SI. PCR conditions have been described previously (Jardin et al., 2008). Briefly, PCRs were run from 100 ng of genomic DNA in a final volume of 25 μl with 0.16 mmol/l of each deoxynucleoside triphosphate, 1.5 mmol/l MgCl2, 1 unit of thermoprime Plus DNA polymerase (ABgene, Epsom, UK), 5% dimethyl sulphoxide and 0.5–1.6 μmol/l of each primer, one primer of each pair carrying a 6-carboxyfluorescein label. After an initial denaturation for 3 min at 94°C, 20 cycles were performed consisting of denaturation, 94°C for 15 s, annealing 90°C for 15 s (ramping 3°C/s) and extension 70°C, 15 s (ramping 3°C/s, followed by a final extension step for 5 min at 70°C).

To assess the sensitivity of the assays, we performed analysis of a MCL cell line (REC), serially diluted in normal DNA (Fig 1A and B). Our QMPSF assay demonstrated that, in addition to the t(11;14), this cell line is characterized by the presence of additional genomic aberrations, including CDKN1B and RB1 heterozygous deletions, CDKN2A homozygous loss, and MYC, CDK2, and ATM gains. In contrast, TP53 and MDM2 remained in germline configuration. These findings were subsequently confirmed by FISH using DNA probes designed to detect genomic aberrations involving MYC, TP53, ATM and CDKN2A (data not shown). Serial dilution with non-tumoural DNA indicated that our assay is a very sensitive approach, able to detect gene copy number gains and losses in samples comprised of as little as 30% of tumoural DNA (Fig 1). Reproducibility of the two QMPSF assays was assessed with the MCL cell line by five PCR experiments in four serial dilutions (30–50–70–90%) (data not shown). The mean 95%-CI for each primer set ranges from 0.01 to 0.03, demonstrating a high reproducibility rate of the QMPSF method.

To avoid false positive diagnosis, we finally chose to use thresholds of 0.7 and 1.2 to retain gene copy losses and gains, respectively. These thresholds had also been validated in our previous study on DLBCL, where they proved to be able to identify most genetic unbalances detected by array CGH (Jardin et al., 2008).

Gene copy number abnormalities detected by QMPSF

GCNA frequencies and distributions of the eight targeted genes are shown in Table II and detailed, for each individual case, in

Statistic analysis

Overall survival (OS) was measured from the time of diagnosis to the date of death or last living follow-up. Actuarial survival analysis was performed according to the method described by Kaplan-Meier, and curves were compared by the log rank test. A multivariate analysis using a Cox model was conducted to assess the independent prognostic influence of the MIP1 and QMPSF results. Fisher’s exact test was used to evaluate the unequal distribution of the different genetic abnormalities between the blastoid or common variants, as well as to correlate protein expression and their allelic status. Correlation between mitotic index and gene copy number status was calculated according the non-parametric Mann–Whitney test. Correlation between QMPSF ratio obtained with matched DNA extracted from frozen tissues and FFPE tissues was established using the Pearson coefficient (R). Analyses were performed using STATVIEW® and SEM software. The limit of significance for all analyses was defined as a P value of 0.05.

Results

QMPSF assay design and sensibility assessment

To assess the practicability of our quantitative PCR assay, we performed experiments using DNA from a MCL cell line (REC), serially diluted in normal DNA (Fig 1A and B). Our QMPSF assay demonstrated that, in addition to the t(11;14), this cell line is characterized by the presence of additional genomic aberrations, including CDKN1B and RB1 heterozygous deletions, CDKN2A homozygous loss, and MYC, CDK2, and ATM gains. In contrast, TP53 and MDM2 remained in germline configuration. These findings were subsequently confirmed by FISH using DNA probes designed to detect genomic aberrations involving MYC, TP53, ATM and CDKN2A (data not shown). Serial dilution with non-tumoural DNA indicated that our assay is a very sensitive approach, able to detect gene copy number gains and losses in samples comprised of as little as 30% of tumoural DNA (Fig 1).

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Table SII. An overall of 69 GCNA was observed in 42 cases (range 0–6/case), including 50 losses and 19 gains. RB1 was the most frequently deleted gene (38%), followed by CDKN2A (31%), and ATM (24%). Both ATM and RB1 were deleted in 19% of cases, a

Fig 1. Quantitative multiplex PCR of short fluorescent fragments (QMPSF) experiments with the REC mantle lymphoma cell line. To assess the sensitivity of the QMPSF assays dedicated to MCL and 9p21 locus analysis, DNA of a tumoural cell line is serially diluted in normal DNA (x-axis). Corresponding QMPSF ratio is indicated (y-axis) for each individual set of primers with corresponding targeted genomic regions. The curve obtained was used to deduce the most reliable minimal QMPSF ratio cut-offs. (A) QMPSF assay dedicated to MCL, showing a homozygous deletion of CDKN2A, a heterozygous loss of RB1 and CDKN1B, a gain of ATM, CDK2, and MYC. TP53 is in germline configuration, as indicated by the stability of the QMPSF ratio, in all DNA dilutions. SEMA4F and CECR1 ratios are used as references genes. By definition, the CECR1 ratio is equal to 1 (not shown in the figure). (B) QMPSF assay, dedicated to the analysis of the 9p21 locus, confirms the complete loss of CDKN2A (including exon 1α and exon 1β). In contrast, for the remaining sets of primers, a QMPSF ratio of 0Æ5 indicated that the REC cell line harbours only one copy each of MTAP, MIR31, and DMRTA1. (C) QMPSF electrophoregrams are indicated in green and orange, respectively. Amplicons are separated and identified by their respective expected sizes (x-axis). Heights of the peak (fluorescence, y-axis) are proportional to the amount of corresponding target DNA.
higher frequency than expected \((P = 0.001)\) (Table SII). \(CDK2, MYC, CDKN1B,\) and \(MDM2\) displayed an equal frequency of copy gains (approximately 10%). Some of these genes were almost exclusively gained (\(MYC, CDK2\)), lost (\(RB1, ATM, TP53, CDNK2A\)), or displayed both GCNA patterns (\(MDM2, CDKN1B\)).

**Analysis of the 9p21 locus by a dedicated QMPSF assay**

Subsequently, we more accurately analysed the 9p21 locus using a specifically designed QMPSF assay (Fig 2). Similarly, we assessed the usefulness of the dedicated 9p21 QMPSF assay in the REC cell line. As expected, this assay confirmed \(CDKN2A\) deletion and gave a more accurate picture of the 9p21 deletion genomic structure. In this cell line, the QMPSF ratio of primer pairs located within \(CDKN2B\) and \(CDKN2A\) (including exon 1\(a\) and exon 1\(b\)) was null, indicating a complete loss of these genes and therefore a complete inactivation of \(p15^{ARF}, p15^{INK4B},\) and \(p16^{INK4a}\). In addition, a partial deletion of \(CDKN2B\) (exon 1) was observed in seven cases, and a heterozygous deletion of \(CDKN2A/CDKN2B\) was observed in three cases (Fig 2). It is of note that a \(MIR31\) deletion was consistently observed in combination with \(CDKN2A\) loss. In contrast, only one case (Case MCL19, Fig 2) showed a homozygous deletion of \(MTAP\) (exon 8) without any detectable \(CDKN2A\) deletion. Our data indicated that a majority of centromeric breakpoints in these deletions were located between the first exon of \(CDKN2B\) and \(DMRTA1\) (or centromeric to \(DMRTA1\)). In addition, most telomeric deletion boundaries were located between \(MIR31\) and \(CDKN2A\) exon 3 (or telomeric to \(MIR31\)).

**Correlation with histological subtype, mitotic index and immunostaining**

Six cases (14%) displayed histological features of the blastoid variant (Table I). QMPSF assays indicated that these tumours had a median of 2 GCNA/case (range 1–5). Notably, higher frequencies of \(CDKN2A/CDKN2B\) homozygous losses and \(CDKN1B\) gains \((P = 0.04\) and \(0.005,\) respectively) were observed in this subtype (Table II and Table SII).

For the entire series, the median mitotic index (MI) was 3–18/mm\(^2\) (range 0–27). Analysis of GCNA distribution according to this index indicated that tumours that displayed...
a MI ≥ 3/mm² (n = 21) were characterized by a higher rate of GCNA (median number/case = 2, compared to 1 in the low MI group, \( P = 0.007 \)); this high MI group was also characterized by a higher frequency of CDKN2A losses and MYC gains (\( P < 0.05 \)). This result contrasted isolated CDKN2B losses (p13\(^{nloc} \)) which were not associated with a high mitotic index and were not observed in blastoid variants (Table II and Table SII). Similarly, a high Ki-67 index (≥50%), assessed in 39 cases, correlated with a higher number of GCNA (median number/case = 2, as compared to 1 in patients with low Ki-67 index, \( P = 0.02 \)), including a higher frequency of CDKN2A deletions (\( P < 0.01 \)) (Table II and Table SII). Patients with a Ki-67 index <10% (n = 15) did not display TP53 loss or CDK2 gain and only one case (MCL7, Table SII) displayed CDKN2A deletion. For this case, the two QMPSF assays (Fig 2 and Table SII) showed a monoaallelic loss, suggesting that CDKN2A may not be fully inactivated, or alternatively, that only a minor subclone may display a biallelic inactivation.

Two of four cases (Cases MCL5 and MCL6, Table SII) with TP53 allelic loss still displayed p53 protein expression. Direct sequencing of the highly conserved exons 5–8 (central core domain) showed that both cases harbour TP53 missense mutations located within exon 7 (codon 248, CGG > CAG) and exon 8 (codon 273 GGT > CAT), respectively (data not shown). Together, these data suggest a complete loss of p53 protein function via deletion of one allele and mutation of the other. In contrast, the two cases that lacked significant p53 protein expression did not display mutations of the screened regions. It is notable that cases with TP53 biallelic inactivation were also characterized by CDKN2A losses, indicating that alterations in the CDKN2A locus and TP53 inactivation are not mutually exclusive (Table SII). Finally, Rb-P protein expression greater than 20% was more frequently observed in patients with CDKN2A losses (Table II, \( P = 0.04 \)).

Application of a dedicated assay for paraffin embedded tissues

In order to evaluate the potential of the QMPSF assay for widespread application in a routine diagnostic setting, we secondarily developed a dedicated assay. In the initial assay, the lengths of the amplicons were longer than 140 base pairs. These amplicons showed delayed or no amplifications with DNA obtained from FFPE. For the new assay, we chose primers that led to the amplification of PCR products shorter than 140 base pairs, a size which appeared reliable and fit for quantification in this setting. Considering the results of our first MCL-dedicated QMPSF assay, we decided to focus on the detection of two genes, CDKN2A and TP53, whose GCNA are relevant not only in MCL but also in a wide spectrum of tumours.

A very good correlation was observed between the QMPSF ratios obtained by the complete eight-gene assay and the modified two-gene assay (\( R^2 = 0.93 \), \( P < 0.0001 \), data not shown). Subsequently, we compared QMPSF ratios obtained with DNA extracted from frozen and FFPE in 28 samples (Table SII). In 2/28 cases, we failed to amplify extracted DNA. However, in the 26 remaining cases, a very good correlation was obtained for CDKN2A (\( R^2 = 0.85 \)) and, to a lesser extent, for TP53 (\( R^2 = 0.57 \)) (Fig 3). Finally, using a threshold of 0.7 to retain gene copy loss and 0.5 to retain biallelic loss, a concordance between frozen and FFPE tissues was obtained in all studied samples, except one (Case MCL13, QMPSF ratio = 0.72 and 0.63 in frozen and FFPE tissues respectively, Fig 3 and Table SII). Using these thresholds, the three tumour categories, including tumours with homozygous/heterozygous deletions or in germline configuration, appeared to be clearly separated (Fig 3). These results indicated that the modified QMPSF assay focusing on TP53 and CDKN2A may represent a reliable molecular tool that can be extensively used routinely in both frozen and FFPE tissues.

Of note, we designed a multiplex PCR assay that included additional targets, such as CDK2 and MYC, giving an overall good concordance between frozen and FFPE tissues (data not shown, primer sequences available upon request). However, this assay clearly required a higher DNA quality that can not be constantly obtained from all FFPE tissues samples. Furthermore, in this setting, PCR product sizes need to fall within a narrow window (between 90–110 bp) (Fig 3), limiting the number of analysed genes. With shorter PCR products we observed a competitive PCR that precludes subsequent quantitative analysis. By contrast, longer fragment analysis with DNA extracted from FFPE tissues was impaired by DNA integrity. For these technical limitations a multiplex assay including the eight genomic regions initially studied with frozen tissues was not obtained with sufficient reliability. Therefore, for this study and future developments, we focused on our analysis on the ‘two hits’ TP53/CDKN2A assay.

Gene copy number abnormalities, MIPI and prognosis

According to the MIPI score, the number of GCNA tended to be higher in the high/intermediate risk group (median = 1, range 0–6) than in the low risk group (median = 0, range 0–3, \( P = 0.07 \)). More specifically, MYC gains were exclusively observed in the high risk group (\( P = 0.04 \)), and CDKN2A deletions were observed in patients with the highest MIPI (Table SII). Similarly, MYC gains and CDKN2A losses appeared more frequently in patients with a high MIPI (Table II). No significant correlation between GCNA and ECOG, WBC count, LDH level or Ann Arbor staging was demonstrated. However, regarding the limited number of cases, GCNA distribution analysis indicated that CDKN2A deletion was more frequently detected in patients with a high performance status (ECOG > 1, \( P = 0.08 \)), a high LDH level (\( P = 0.17 \)) and a high WBC count (\( >10 \times 10^9/l \), \( P = 0.14 \)). Similarly, CDK2 gain was mainly observed in patients with
Table II. Gene copy number aberrations in MCL detected by QMPSF in the overall population and according to their clinical and histopathological features.

| Clinical and histopathological features | Genes | Allelic status | Germ-line | Gain | Loss | Germ-line | Gain | Loss | Germ-line | Gain | Loss | Germ-line | Gain | Loss | Germ-line | Gain | Loss | Germ-line | Gain | Loss | Germ-line | Gain | Loss |
|----------------------------------------|-------|----------------|-----------|------|------|-----------|------|------|-----------|------|------|-----------|------|------|-----------|------|------|-----------|------|------|-----------|------|------|
| Overall population, n = 42 (%)         | MYC   | Loss           | 0         | 38   | 4    | 0         | 38   | 4    | 10       | 32   | 0    | 4         | 38   | 0    | 13         | 29   | 0    | 3         | 34   | 5    | 4         | 34   | 4    |
|                                        | CDK2  | (90.5)         | (9.5)     | (90.5) | (9.5) | (38.1)   | (61.9) | (23.8) | (76.2)   | (9.5) | (90.5) | (30.9)   | (69.1)| (71) | (80.9)    | (11.9)| (9.5) | (80.9)    | (9.5) |      |
|                                        | RB1   | Germ-line      | 14        | 22   | 0    | 10       | 26   | 0    | 1         | 35   | 0    | 8         | 28   | 0    | 3         | 31   | 2    | 4         | 29   | 3    |
|                                        | ATM   | Germ-line      | 6         | 7    | 0    | 2        | 11   | 0    | 3         | 10   | 0    | 10        | 0    | 0    | 1         | 8    | 4    | 1         | 11   | 2    |
|                                        | TP53  | Germ-line      | 5         | 3    | 0    | 6        | 0    | 3    | 3         | 0    | 5*   | 1         | 0    | 3    | 3*        | 0    | 5    | 1         |      |      |
|                                        | CDKN2A| Germ-line      | 0         | 3    | 3    | 2        | 4    | 0    | 0         | 6    | 0    | 3         | 3    | 0    | 5*        | 1    | 0    | 3         | 3*   | 0    | 5         | 1    |      |
|                                        | CDKN1B| Germ-line      | 0         | 3    | 3    | 2        | 4    | 0    | 0         | 6    | 0    | 3         | 3    | 0    | 5*        | 1    | 0    | 3         | 3*   | 0    | 5         | 1    |      |
|                                        | MDM2  | Germ-line      | 0         | 3    | 3    | 2        | 4    | 0    | 0         | 6    | 0    | 3         | 3    | 0    | 5*        | 1    | 0    | 3         | 3*   | 0    | 5         | 1    |      |
|                                        |       | Germ-line      | 0         | 3    | 3    | 2        | 4    | 0    | 0         | 6    | 0    | 3         | 3    | 0    | 5*        | 1    | 0    | 3         | 3*   | 0    | 5         | 1    |      |

Unequal distribution of the different genetic abnormalities between the different MCL groups are highlighted in bold with asterisk (Fisher exact test, \(P < 0.05\)).

MIPI, mantle cell lymphoma international prognostic score; MIPIb, biological MIPI.
poor performance status \( (P = 0.07) \) and \( \text{TP53} \) deletion in patients with elevated LDH value \( (P = 0.07) \).

In this series, a high-risk MIPI \( (P = 0.33) \), elevated LDH level \( (P = 0.22) \), age >60 years \( (P = 0.059) \), WBC count \( >10 \times 10^9/\text{l} \) \( (P = 0.08) \), performance status >1 \( (P = 0.15) \), and high Ki-67 index \( (\geq 50\%, P = 0.14) \), tended to be predictive of the outcome. It is of note that, using a cut-off of 6 \( \leq 3 \) (rather than the proposed cut-off of 6 \( \leq 18 \)), we defined a high risk group \( (n = 15) \) with a 3-year OS of 30%, as compared to a 3-year OS of 71% in patients with MIPI <6 \( \leq 3 \) \( (P = 0.01) \). Furthermore, in this series the blastoid variant correlated with an unfavourable outcome (3-year OS = 0% vs. = 62%, \( P < 0.0001) \).

The prognostic value of the QMPSF assay, performed with DNA extracted from frozen tissues, was analysed for each individual gene. With a median follow-up of 22 months, both \( \text{CDK2} \) (3-year OS = 0%) and \( \text{MDM2} \) (3-year OS = 0%) gains and both \( \text{CDKN2A} \) (3-year OS = 20%) and \( \text{TP53} \) (3-year OS = 25%) losses correlated to a shorter OS \( (P < 0.0001, P = 0.0007, P = 0.004, \) and \( P = 0.03, \) respectively). In a multivariate analysis, \( \text{CDKN2A} \) deletions remained predictive of the outcome independently of the MIPI \( (P = 0.007) \). The combination of four abnormalities, namely \( \text{TP53} \) and \( \text{CDKN2A} \) losses and \( \text{CDK2} \) and \( \text{MDM2} \) gains, was subsequently used to build a QMPSF score. Scoring each of these GCNA as +1, three groups with distinct outcomes were identified:
group I (n = 26, score = 0, 3-year OS = 68%), group II (n = 11, score = 1, 3-year OS = 38%), and group III (n = 5, score >1, 3-year OS = 0%) (Fig 4). Finally, this QMPSF score identified one third of patients (score ≥ 1) with an unfavourable prognosis.

**Discussion**

In this study, we developed a QMPSF assay specifically designed for the analysis of MCL. Following previous works performed in the setting of CLL or DLBCL, the present data illustrates the great flexibility of this approach and demonstrates its potential for a widespread application in the routine diagnosis of B-cell malignancies (Bastard et al, 2007; Jardin et al, 2008). Results obtained by a single PCR assay, giving the GCNA frequency of eight target genes, are in complete accordance with those obtained by FISH or CGH (Jares et al, 2007; Bea et al, 2008). Our study confirms that deletions of RB1, CDKN2A, or ATM occurs in at least 60% of MCL, constituting some of the most frequent secondary genetic events following the t(11;14) translocation. Deletions of ATM and RB1 are significantly coupled and represent the most
frequent deletion combination, occurring in 19% of cases. This combination has been observed with the exact same frequency (19%) by FISH in an independent and larger series of 95 MCL cases (Sander et al, 2008). This delineates a homogeneous, genically-defined MCL sub-group, independent of the cell of origin, as defined by the IGHV mutational status, as concomitant deletions of RBL and ATM can be observed in both mutated and unmutated cases (Sander et al, 2008).

Deletion of CDKN2A/CDKN2B represents one of the most frequent secondary genetic alterations in MCL, observed in approximately one third of patients at the time of diagnosis. Our study confirms that CDKN2A deletions correlate with a higher mitotic index, the blastoid variant subtype, a higher expression of the phosphorylated RBL1 form, and display a strong prognosis value. Finally, our study suggests that CDKN2A inactivation constitutes a major component of the underlying molecular basis for the MIPI. Given that p14ARF interacts with MDM2, blocking the MDM2-mediated degradation of p53, it has been suggested that CDKN2A locus alterations and TP53 alterations may be mutually exclusive (Pinyol et al, 2000). Two cases, with both biallelic TP53 inactivation and CDKN2A homozygous losses, are clearly identified in this study, suggesting that tumour cells may nevertheless benefit from this combination. Despite the fact that CDKN2A deletion correlates with a higher expression of the phosphorylated, and thus inactivated, RBL1 form (confirming that CDKN2A inactivation impacts mainly the G1/S transition regulation), this observation suggests that cells may also take advantage of alternative functions of the p14ARF/p53 pathway, such as the well-established p14ARF-related ribosome regulation function (Rizos et al, 2006).

Using a PCR assay specifically designed to analyse the 9p21 locus, we provided more information regarding the fine molecular structure of the deletion involving this locus. MIR31 is constantly co-deleted with CDKN2A, suggesting that its deletion does not represent, by itself, an essential oncogenic event. However, the exact consequences of the biallelic loss of this microRNA in tumour cells remain unknown. On the contrary, the isolated observation of a biallelic deletion of MTAP without CDKN2A copy aberration suggests that this gene may play a specific role in MCL oncogenesis. Such a pattern has been previously reported in one of 52 MCL cases (including seven patients with CDKN2A/MTAP co-deletions) (Marce et al, 2006). MTAP is involved in the activation of the intrinsic mitochondrial-dependent apoptotic pathway. Patients with MTAP deletions have a significantly shorter overall survival than patients with wild type MTAP, suggesting that a down regulation of the intrinsic mitochondrial-dependent apoptotic pathway may be a factor in chemoresistance (Marce et al, 2006). Our dedicated 9p21 QMPSF also indicated that most 9p21 genomic alterations are characterized by a large deletion of more than 2.8 Mb, involving the centromeric and telomeric boundary markers (namely MIR31 and DMRAT1). This indicates that additional genes near the 9p21 locus are also frequently deleted in MCL. Interestingly, it has been recently suggested that the genomic alteration of bands 9p24, 9p23, 9p22, and 9p21 detected by CGH, which contain a cluster of genes involved in differentiation blockage or apoptosis, can identify patients with shorter survival (Blenk et al, 2008). A QMPSF assay focusing on the analysis of the 9p21-24 bands may therefore constitute a relevant tool to predict precise MCL outcome and to identify distinct pathogenic pathways. At the clinical level, MIPI has been proposed as a novel clinical index in patients with advanced stage MCL (Hoster et al, 2008). Several molecular features have previously been identified to be correlated to the aggressiveness of the tumour and to a less favourable outcome. These include the blastoid variant subtype, deletion of CDKN2A, inactivation of TP53, or activation of MDM2 (Pinyol et al, 1997; Rubio-Moscardo et al, 2005; Jares et al, 2007). Here, we identify four GCNA, namely CDKN2A/TP53 losses and CDK2/MDM2 gains, which strongly correlate to an unfavourable outcome and a shorter OS. Interestingly, the QMPSF score based on these genomic abnormalities remained predictive of the prognosis of patients within the high-risk MIPI group, suggesting that the MIPI can be redefined by our molecular tool.

Recently, a predictive model (including MYC, RAN, and TNFRS10) has been proposed by Hartmann et al (2008), based on the expression of five genes determined by quantitative reverse transcriptase PCR (qRT-PCR). The authors demonstrated that a slightly modified qRT-PCR-based test in a low-density array format is applicable to FFPE tissue specimens using shorter amplicons (Hartmann et al, 2008). A limitation to the qRT-PCR approach is that the gene expression level is largely dependent on the amount of tumoural cells contained in the tissues. Here we propose a complementary strategy, based on the analysis of recurrent genomic alterations. At the genomic level, we demonstrated, by dilution experiments, that using relatively stringent cut-offs still enables the detection of GCNA in a wide range of tumour cells, and therefore constitutes a robust approach. However, both approaches will still remain limited by the fact that MCL are characterized by clonal heterogeneity (Sander et al, 2008). The presence of minor subclones with distinct genomic alterations probably remains undetectable at the time of diagnosis. However, it has been recently shown that subclones usually represent more than 30% of the tumoural contingent, representing therefore a sufficient amount of cells detectable by QMPSF in most of MCL cases (Sander et al, 2008).

It was recently demonstrated that the Ki-67 index provided strong prognostic information in advanced-stage MCL, this marker remaining relevant in the era of anti-CD20 therapy (Katzenberger et al, 2006; Determann et al, 2008). Gene expression profiling of MCL demonstrated that 50% of the genes associated with inferior outcome belonged to a ‘proliferation signature’ that is highly expressed in dividing cells (Rosenwald et al, 2003). This proliferation signature appears mainly under the influence of CCND1 mRNA abundance and CDKN2A gene copy number status (Rosenwald et al, 2003).
Given the fact that we observed a strong association between the presence of GCNA, especially CDKN2A loss and the Ki-67 index, our results suggest that the dedicated MCL QMPSF assay can provide information regarding the genomic basis of the proliferation signature, partially explaining its prognostic value.

Validation of our molecular predictor in an independent set of patients homogeneously treated by chemotherapy and rituximab is warranted to confirm its clinical relevance. Because this technical approach is highly flexible, we also plan to design a second-generation assay that will incorporate candidate genes, such as BCL2 and CDK4, with potential prognostic value.

It has been recently demonstrated that MCL genomes display a high number of partial uniparental disomies, demonstrating that this molecular mechanism, leading to a loss of heterozygosity without gene copy number variation, is crucial to MCL oncogenesis (Bea et al., 2008). Currently, the QMPSF assays are not designed to detect such a mechanism but could be redesigned to detect some target genes frequently involved by this process, such as TP53 (Bea et al., 2008).

In conclusion, we developed a reliable PCR assay that enabled the delineation of distinct MCL oncogenic pathways with strong prognostic impact. This represents a powerful tool that can be used for patient stratification in combination with the MIPI and could be relevant in future clinical trials. This robust molecular and genetic predictor may become, in the near future, an essential tool for the management of patients with MCL. Finally, this approach may also represent a molecular indicator useful in identifying patients that may benefit from targeted therapies, such as CDK inhibitors (including flavopiridol) or inactivation of the alternative de novo AMP synthesis (Kouroukis et al., 2003; Lacrima et al., 2005; Marcé et al., 2006).

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Authorship

FJ designed the project, analysed the data and wrote the manuscript. JMP diagnosed selected specimens, analysed the data and revised the paper. PR performed some PCR experiments, analysis data and wrote the paper. FP performed most of the PCR experiments and designed PCR assays. MC performed immunohistochemistry experiments performed DNA extraction and acquisition of the data. DP performed molecular experiments. PB performed data analysis. HL and OC performed histopathological analysis. CH analysed the data and revised the paper. AW analysed data and revised the paper. CB supervised the project, analysed QMPSF data and revised the paper. HT supervised the project, analysed clinical data and revised the paper.

References

Bastard, C., Raux, G., Fruchart, C., Parmentier, F., Vaur, D., Penther, D., Troussard, X., Nagib, D., Lepretre, S., Tosi, M., Frebourg, T. & Tilly, H. (2007) Comparison of a quantitative PCR method with FISH for the assessment of the four aneuploidies commonly evaluated in CLL patients. *Leukemia*, 21, 1460–1463.

Bea, S., Ribas, M., Hernandez, J.M., Bosch, F., Pinyol, M., Hernandez, L., Garcia, J.L., Flores, T., Gonzalez, M., Lopez-Guillermo, A., Piris, M.A., Cardesa, A., Montserrat, E., Miro, R. & Campo, E. (1999) Increased number of chromosomal imbalances and high-level DNA amplifications in mantle cell lymphoma are associated with blastoid variants. *Blood*, 93, 4365–4374.

Bea, S., Salaverria, I., Armengol, L., Pinyol, M., Fernandez, V., Hartmann, E.M., Jares, P., Amador, V., Hernandez, L., Navarro, A., Ott, G., Rosenwald, A., Estivill, X. & Campo, E. (2008) Uniparental disomies, homozygous deletions, amplifications and target genes in mantle cell lymphoma revealed by integrative high-resolution whole genome profiling. *Blood*, 113, 3059–3069.

Blenk, S., Engelmann, J.C., Pinkert, S., Weniger, M., Schultz, J., Rosenwald, A., Muller-Hermelink, H.K., Muller, T. & Dandekar, T. (2008) Explorative data analysis of MCL reveals gene expression networks implicated in survival and prognosis supported by explorative CGH analysis. *BMC Cancer*, 8, 106.

Casilli, F., Di Rocco, Z.C., Gad, S., Tournier, I., Stoppa-Lyonnet, D., Frebourg, T. & Tosi, M. (2002) Rapid detection of novel BRCA1 rearrangements in high-risk breast-ovarian cancer families using multiplex PCR of short fluorescent fragments. *Human Mutation*, 20, 218–228.

Determann, O., Hoster, E., Ott, G., Wolfram Bernd, H., Loddenkemper, C., Leo Hansmann, M., Barth, T.E., Unterhalt, M., Hiddemann, W., Dreyling, M. & Klapper, W. (2008) Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood*, 111, 2385–2387.

Hartmann, E., Fernandez, V., Moreno, V., Valls, J., Hernandez, L., Bosch, F., Abrisqueta, P., Klapper, W., Dreyling, M., Hoster, E., Muller-Hermelink, H.K., Ott, G., Rosenwald, A. & Campo, E. (2008) Five-gene model to predict survival in mantle-cell lymphoma using frozen or formalin-fixed, paraffin-embedded tissue. *Journal of Clinical Oncology*, 26, 4966–4972.

Hoster, E., Dreyling, M., Klapper, W., Gisselbrecht, C., van Hoof, A., Kluin-Nelemans, H.C., Pfeundtschuh, M., Reiser, M., Metzner, B., Einsle, H., Peter, N., Jung, W., Wormann, B., Ludwig, W.D., Duhrsen, U., Eimermacher, H., Wandt, H., Hasford, J., Hiddemann, W. & Unterhalt, M. (2008) A new prognostic index (MIPI) for patients with advanced-stage mantle cell lymphoma. *Blood*, 111, 558–565.

Jardin, F., Ruminy, P., Kerckaert, J.P., Parmentier, F., Piquenot, J.M., Quief, S., Villenet, C., Buchonnet, G., Tosi, M., Frebourg, T., Bastard, C. & Tilly, H. (2008) Detection of somatic quantitative genetic alterations by multiplex polymerase chain reaction for the prediction of outcome in diffuse large B-cell lymphomas. *Haematologica*, 93, 543–550.
Jardin, F., Callanan, M., Penther, D., Ruminy, P., Troussard, X., Kerckaert, J.P., Figeac, M., Parmentier, F., Rainville, V., Vaida, I., Bertrand, P., Duval, A.B., Picqueton, J.M., Chaperot, L., Marolleau, J.P., Plumas, J., Tilly, H. & Bastard, C. (2009) Recurrent genomic aberrations combined with deletions of various tumour suppressor genes may deregulate the G1/S transition in CD4+ CD56+ haematodermic neoplasms and contribute to the aggressiveness of the disease. *Leukemia*, 23, 698–707.

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

Katzenberger, T., Petzoldt, C., Holler, S., Mader, U., Kalla, J., Adam, P., Ott, M.M., Muller-Hermelink, H.K., Rosenwald, A. & Ott, G. (2006) The Ki67 proliferation index is a quantitative indicator of clinical risk in mantle cell lymphoma. *Blood*, 107, 3407.

Kouroukis, C.T., Belch, A., Crump, M., Eisenhauer, E., Gascoyne, R.D., Meyer, R., Lohmann, R., Lopez, P., Powers, J., Turner, R. & Connors, J.M. (2003) Flavopiridol in untreated or relapsed mantle-cell lymphoma: results of a phase II study of the National Cancer Institute of Canada Clinical Trials Group. *Journal of Clinical Oncology*, 21, 1740–1745.

Lacrima, K., Valentini, A., Lamberti, C., Taborelli, M., Rinaldi, A., Zucca, E., Catapano, C., Cavalli, F., Gianella-Borradori, A., Maccallum, D.E. & Bertoni, F. (2005) In vitro activity of cyclin-dependent kinase inhibitor CYC202 (Selicibib, R-rosocvitine) in mantle cell lymphomas. *Annals of Oncology*, 16, 1169–1176.

Marce, S., Balague, O., Colomo, L., Martinez, A., Holler, S., Villamor, N., Bosch, F., Ott, G., Rosenwald, A., Leoni, L., Esteller, M., Fraga, M.F., Montserrat, E., Colomer, D. & Campo, E. (2006) Lack of methylthioadenosine phosphorylase expression in mantle cell lymphoma is associated with shorter survival: implications for a potential targeted therapy. *Clinical Cancer Research*, 12, 3754–3761.

Pinyol, M., Hernandez, L., Cazorla, M., Balbin, M., Jares, P., Fernandez, P.L., Montserrat, E., Cardesa, A., Lopez-Otin, C. & Campo, E. (1997) Deletions and loss of expression of p16INK4a and p21Waf1 genes are associated with aggressive variants of mantle cell lymphomas. *Blood*, 89, 272–280.

Pinyol, M., Hernandez, L., Martinez, A., Cobo, F., Hernandez, S., Bea, S., Lopez-Guillermino, A., Nayach, I., Palacin, A., Nadal, A., Fernandez, P.L., Montserrat, E., Cardesa, A. & Campo, E. (2000) INK4a/ARF locus alterations in human non-Hodgkin’s lymphomas mainly occur in tumors with wild-type p53 gene. *American Journal of Pathology*, 156, 1987–1996.

Rizos, H., McKenzie, H.A., Ayub, A.L., Woodruff, S., Becker, T.M., Scull, L.L., Stahl, J. & Kefford, R.F. (2006) Physical and functional interaction of the p14ARF tumor suppressor with ribosomes. *Journal of Biological Chemistry*, 281, 38080–38088.

Rosenwald, A., Wright, G., Wiestner, A., Chan, W.C., Connors, J.M., Campo, E., Gascoyne, R.D., Grogan, T.M., Muller-Hermelink, H.K., Smeland, E.B., Chiorazzi, M., Giltnane, J.M., Hurt, E.M., Zhao, H., Averett, L., Henrickson, S., Yang, L., Powell, J., Wilson, W.H., Jaffe, E.S., Simon, R., Klausner, R.D., Montserrat, E., Bosch, F., Greiner, T.C., Weisenburger, D.D., Sanger, W.G., Dave, B.I., Lynch, J.C., Vose, J., Armitage, J.O., Fisher, R.I., Miller, T.P., LeBlanc, M., Ott, G., Kvaloy, S., Hole, H., Delabie, J. & Staudt, L.M. (2003) The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell*, 3, 185–197.

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

Salaverría, I., Zettl, A., Bea, S., Moreno, V., Valls, J., Hartmann, E., Ott, G., Wright, G., Lopez-Guillermino, A., Chan, W.C., Weisenburger, D.D., Gascoyne, R.D., Grogan, T.M., Delabie, J., Jaffe, E.S., Montserrat, E., Muller-Hermelink, H.K., Staudt, L.M., Rosenwald, A. & Campo, E. (2007) Specific secondary genetic alterations in mantle cell lymphoma provide prognostic information independent of the gene expression-based proliferation signature. *Journal of Clinical Oncology*, 25, 1216–1222.

Sander, S., Bullinger, L., Leupolt, E., Benner, A., Kienle, D., Katzenberger, T., Kalla, J., Ott, G., Muller-Hermelink, H.K., Barth, T.F., Moller, P., Lichter, P., Dohner, H. & Stilgenbauer, S. (2008) Genomic aberrations in mantle cell lymphoma detected by interphase fluorescence in situ hybridization. Incidence and clinicopathological correlations. *Haematologica*, 93, 680–687.

Schraden, M., Jares, P., Bea, S., Schoenmakers, E.F., van Krieken, J.H., Campo, E. & Groenen, P.J. (2008) Integrated genomic and expression profiling in mantle cell lymphoma: identification of gene-dosage regulated candidate genes. *British Journal of Haematology*, 143, 210–221.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, A., Thiele, J. & Vardiman, J.W. (eds), (2008) *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues*. IARC, Lyon.

Tournier, I., Paillerets, B.B., Sobol, H., Stoppa-Lyonnet, D., Liderau, R., Barrois, M., Mazoyer, S., Coulet, F., Hardouin, A., Chompret, A., Lortholary, A., Chappuis, P., Bourdon, V., Bonadona, V., Maugard, C., Gilbert, B., Nogues, C., Frebourg, T. & Tosi, M. (2004) Significant contribution of germline BRCA2 rearrangements in male breast cancer families. *Cancer Research*, 64, 8143–8147.

**Supporting information**

Additional Supporting Information may be found in the online version of this article:

*Table SI.* Sequences and locations of primers used in the QMPSF assays.

*Table SII.* Details of clinical characteristics, treatments, histopathological features and QMPSF features of the patients with mantle cell lymphoma.

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