Quantitative molecular analysis in mantle cell lymphoma

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SUMMARY

A molecular analysis has three major roles in modern oncopathology – as an aid in the differential diagnosis, in molecular monitoring of diseases, and in estimation of the potential prognosis. In this report we review the application of the molecular analysis in a group of patients with mantle cell lymphoma (MCL). We demonstrate that detection of the cyclin D1 mRNA level is a molecular marker in 98 % of patients with MCL. Cyclin D1 quantitative monitoring is specific and sensitive for the differential diagnosis and for the molecular monitoring of the disease in the bone marrow. Moreover, the dynamics of cyclin D1 in bone marrow reflects the disease development and it predicts the clinical course. We employed the molecular analysis for a precise quantitative detection of proliferation markers, Ki-67, topoisomerase IIα, and TPX2, that are described as effective prognostic factors. Using the molecular approach it is possible to measure the proliferation rate in a reproducible, standard way which is an essential prerequisite for using the proliferation activity as a routine clinical tool. Comparing with immunophenotyping we may conclude that the quantitative PCR-based analysis is a useful, reliable, rapid, reproducible, sensitive and specific method broadening our diagnostic tools in hematopathology. In comparison to interphase FISH in paraffin sections quantitative PCR is less technically demanding and less time-consuming and furthermore it is more sensitive in detecting small changes in the mRNA level. Moreover, quantitative PCR is the only technology which provides precise and reproducible quantitative information about the expression level. Therefore it may be used to demonstrate the decrease or increase of a tumor-specific marker in bone marrow in comparison with a previously aspirated specimen. Thus, it has a powerful potential to monitor the course of the disease in correlation with clinical data.

Keywords: mantle cell lymphoma – quantitative PCR – cyclin D1– minimal residual disease – proliferation markers

Kvantitativní molekulární analýza u lymfomu z buněk pláště

SOUHRN

Molekulární analýza zařazená po bok morfologickým vyšetřením plní v moderní onkopatologii 3 hlavní úlohy – diferenciálně diagnostickou, v molekulárním sledování chování nemoci a při stanovení prognostických faktorů. V této práci shrnujeme využití molekulární analýzy u pacientů s lymfomem z buněk pláště (mantle cell lymphoma, MCL). Prokazujeme, že hladina mRNA cyklinu D1 slouží jako spolehlivý molekulární marker pro 98 % pacientů s MCL. Kvantitativní analýza cyklinu D1 je specifickým a citlivým molekulárním nástrojem pro diferenciální diagnózu i pro molekulární sledování onemocnění v kostní dřeni. Sledování dynamiky cyklinu D1 v kostní dřeni navíc odráží dynamiku onemocnění a předpovídá následný klinický průběh nemoci. Molekulární analýzu jsme rovněž využili pro kvantitativní stanovení proliferačních markerů, Ki-67, topoisomerázy IIα a TPX2, jako prognosticky významných molekul. S využitím molekulární analýzy lze reprodukovatelně měřit proliferační aktivitu a techniku lze standardizovat napříč pracovišti. Možnost standardizace a reprodukovatelnost vyšetření je nutnou podmínkou pro využití proliferační aktivity v klinických studiích. Ve srovnání s imunofenotypizací lze shrnout, že kvantitativní PCR je spolehlivý, rychlý, reprodukovatelný, citlivý a specifický přístup, který rozšiřuje diagnostické možnosti hematopatologie. Ve srovnání s interfázní FISH je kvantitativní PCR méně technicky a časově náročná a navíc poskytuje přesnou a reprodukovatelnou informaci o hladině exprese vybraných molekul. Kvantitativní PCR je citlivější a lze ji využít i pro detekci malých změn hladiny mRNA. Kvantitativní PCR tak může sloužit pro sledování úbytku nebo nárůstu sledovaného nádorového znaku ve srovnání s předchozím odběrem a stává se tak účinným nástrojem sledování průběhu onemocnění v korelaci s klinickými informacemi.

Klíčová slova: lymfom z buněk pláště – kvantitativní PCR – cyklin D1– minimální residuální choroba – proliferační markery

Cesk Patol 2011; 47(3): 101–105

Mantle cell lymphoma (MCL) is a distinct entity of B-cell non-Hodgkin lymphomas (B-NHLs) arising as a clonal proliferation of B cells at a specific stage of differentiation. MCL accounts for 3–10 % of B-NHLs. From the clinical aspect MCL is an aggressive disease combining unfavorable features of low grade and high grade lymphomas. It has an adverse prognosis with a rapid progression, frequent relapses and it is refractory to conventional B-NHL therapy (1). Therefore, it is important to establish a correct diagnosis and to responsibly monitor the course of the disease.

In this report we review the results of our studies (2–4) using real-time reverse transcription PCR (RQ-RT-PCR) to demonstrate the applications of molecular analysis for the differential diagnosis, disease monitoring and prognostication in a group of patients with MCL.

Quantitative measurement of cyclin D1 mRNA – a potent diagnostic tool to separate MCL from other B-cell lymphoproliferative disorders

The diagnosis of MCL is based on a combined morphological, immunohistochemical and genetic examination. The morphology

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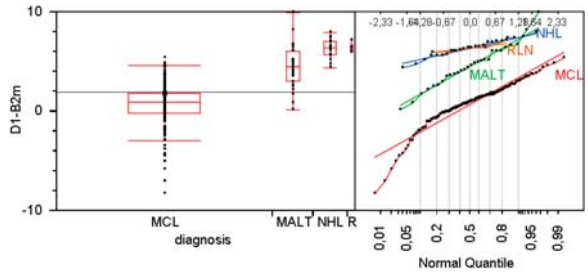

Figure 1: Normalized cyclin D1 values in primary tumors
Box plot graph demonstrates the distribution of the cyclin D1 values normalized to the β-2-microglobulin expression.

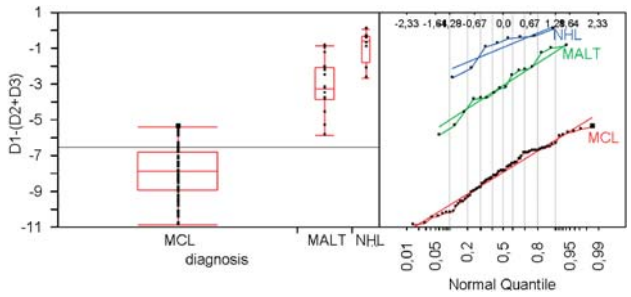

Figure 2: Normalized cyclin D1 values in primary tumors
Box plot graph demonstrates the distribution of the cyclin D1 values normalized to the combination of cyclin D2 and cyclin D3.

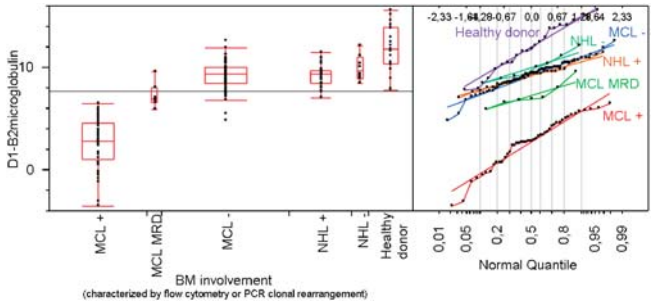

Figure 3: Normalized cyclin D1 values in bone marrow specimens according to the bone marrow involvement
Box plot graph demonstrates the distribution of the cyclin D1 values normalized to the β-2-microglobulin expression.

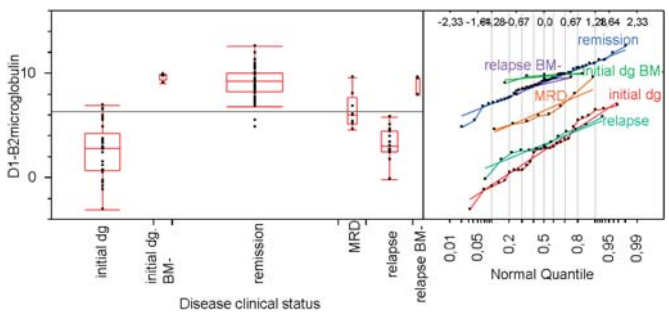

Figure 4: Normalized cyclin D1 values in bone marrow according to the disease clinical status
Box plot graph demonstrates the distribution of the cyclin D1 values normalized to the β-2-microglobulin expression.

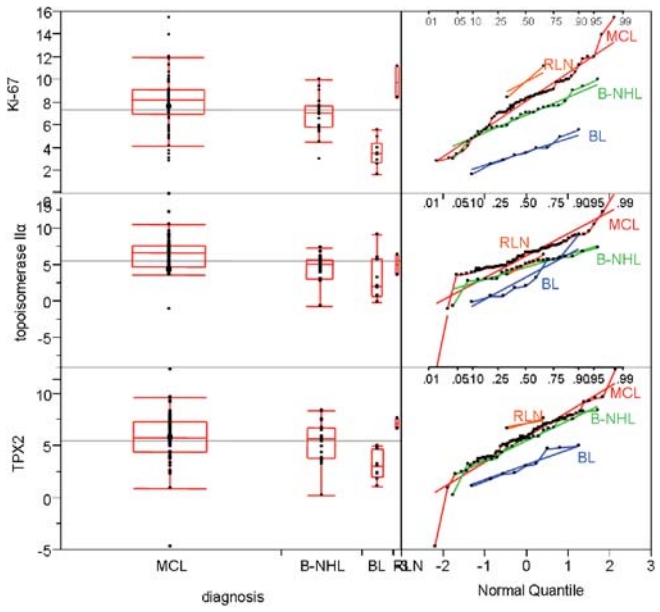

Figure 5: Normalized proliferation markers values in primary tumors
Box plot graph demonstrates the distribution of the proliferation markers values normalized to the β-2-microglobulin expression.

Figure1-5:
The lower the value is the higher is the actual expression level. Boxes represent values between the 25th and 75th percentile with the median, whiskers represent the 10th and 90th percentile and outlying values are represented by dots. The normal quantile plot shows both the difference in the values (vertical position) and the variances (slopes) for each group. The normality is judged by how well the points follow a straight line. The standard deviations are the slopes of the straight lines. Lines with steep slopes represent the distribution with greater variances.

and immunophenotype are not sufficient to make a definitive diagnosis in a significant proportion of patients, especially in cases with atypical morphology and immunophenotype. Therefore, a genetic or a molecular support is often required for the MCL diagnosis.

Genetically, MCL is characterized by a reciprocal translocation t(11;14)(q13;q32) leading to the *CCND1* gene transcriptional deregulation and the overexpression of its protein product, **cyclin D1** (5–8). The characteristic translocation t(11;14) is specifically detected using interphase fluorescence in situ hybridization (FISH) in up to 95 % of patients with MCL and therefore it is recommended by WHO classification (9–12). FISH provides an important molecular support for the differential diagnosis, especially for cases with a variable morphology and/or immunophenotype. Despite the high percentage of MCL patients, in which FISH demonstrates t(11;14), FISH is not a convenient and sensitive technique for molecular monitoring of the disease and it does not allow quantitative monitoring of the disease.

A PCR-based technique should be used for such monitoring. Therefore, we employed a quantitative PCR detection of cyclin D1 mRNA in our study (2). We detected a cyclin D1 mRNA overexpression in 98 % of MCL with no cross positivity in other B-NHLs and reactive lymph nodes (Figure 1). We obtained a PCR amplifiable marker for 98 % of patients with MCL. It represents a great improvement in comparison with techniques previously used for MCL molecular diagnosis (13,14). However, to correctly recognize MCL, a reliable cut-off limit of the cyclin D1 mRNA level must be established by examination of other B-NHL and reactive lymph nodes specimens.

The level of cyclin D1 mRNA below the established cut-off limit was detected in 2 % of our MCL specimens (2). It may represent cyclin D1 negative MCLs that were described in 5–10 % of MCL cases and which still share the MCL morphology, immunophenotype and a unique gene expression signature. In the rare MCL cases with low cyclin D1 expression the alternative D type cyclins, cyclin D2

or cyclin D3, are upregulated (15). The low cyclin D1 mRNA that was detected in these cases may also be caused by a posttranscriptional mechanism inducing an increased stability of the cyclin D1 transcript and thus resulting in an enhanced translation of cyclin D1 or by a translational mechanism leading to the cyclin D1 protein expression independently from the mRNA overexpression (16–18). We assume that such an alternative cyclin D1 deregulation occurred in our MCL specimens with low cyclin D1 mRNA because we observed variant t(11;14) or complex chromosomal changes by FISH in these cases. A polymorphism or a mutation of the affected allele preventing the assay from detecting the mRNA overexpression can not be excluded either. These rare discordant results emphasize that it is very important to correlate the clinical data, morphology, immunophenotype and molecular profile to reach a reliable conclusion. However, we were able to recognize 98 % of MCL tumors from other B-NHLs and reactive hyperplasia by measuring the cyclin D1 expression level making the RQ-RT-PCR system a very specific approach for the MCL differential diagnosis.

In extranodal lymphomas, the cyclin D1 mRNA expression analysis is more complicated, since the extranodal lymphoma specimens generally contain epithelial cells and as epithelial cells are physiologically cyclin D1 positive (19). Thus, the traditionally used normalizing transcript, β-2-microglobulin, can not distinguish the cyclin D1 expression of the contaminating non-neoplastic epithelial cells. To use cyclin D1 as a molecular marker also for differential diagnostics in extranodal lymphomas an improved normalizing transcript is required. To silence the influence of the background variability and to enhance the ability to detect cyclin D1 mRNA specifically in the lymphocytic population we tested additional normalizing transcripts, alternative D-type cyclins (cyclin D2 and D3) and CD19 as a B cell specific molecule (2). Using the alternative D-type cyclins, cyclin D2 and cyclin D3 as normalizing transcripts we reached a 99 % specificity to correctly distinguish MCL from extranodal marginal zone lymphomas of mucosa associated lymphoid tissue (MALT) lymphomas (Figure 2). In one case of extranodal MALT lymphoma we observed cyclin D1 expression slightly above the established cut-off limit. This case showed three FISH signals for the *CCND1* locus, which indicates that the extra copy of the *CCND1* gene resulted in an increased gene dosage leading to the increased mRNA level.

Thus, using the refined cyclin D1 normalization enables the distinction of the MCLs from other B-NHLs, including cases arising in extranodal sites.

Quantitative monitoring of cyclin D1 expression in MCL: a molecular marker for minimal residual disease monitoring and a predictor of the disease outcome

In patients with MCL, bone marrow (BM) involvement is frequently detected at the time of primary diagnosis. For the BM molecular monitoring PCR-based methods are generally used as they allow the detection of a tumor specific marker with the sensitivity 10^{-5}–10^{-6} (20). So, the PCR-based techniques are the most sensitive to detect a minimal number of tumor cells and also qualify as a methodology to detect minimal residual disease (MRD). For patients with MCL, PCR negativity in BM predicts a durable clinical remission, and a molecular relapse, defined as PCR positivity, is followed by a clinical relapse. Likewise, molecular monitoring of the disease after the BM transplantation predicts the outcome, and to reach the PCR negativity is recommended as one of the therapeutical aims (13,21–23). Thus, it is clinically relevant to monitor MCL in BM using molecular targets.

PCR amplification of clonally rearranged *immunoglobulin (Ig)* genes or specific translocations is currently utilized by several groups of authors for the molecular monitoring of B-NHL patients (23–26). However, this approach is time consuming and technically demanding and it does not appear to be entirely sufficient. For patients with MCL, PCR can detect t(11;14) in just 30–50 % of patients carrying t(11;14) in the major translocation cluster and the clonality PCR detection is not specific for MCL differential diagnosis. Using a combined strategy detecting t(11;14) and the clonally rearranged *IgH* gene PCR yielded an amplifiable target in 73 % of MCL cases (13). Thus, the PCR amplifiable marker was not obtained in a significant proportion of patients with MCL, which limits the implication of the currently used techniques for MRD monitoring.

In our study (3) we tested whether a cyclin D1 expression, as a consequence of the characteristic t(11;14), may be used as a sensitive, reliable and efficient molecular marker for the molecular monitoring in patients with MCL. Measuring the cyclin D1 mRNA level in a group of control BM specimens obtained from patients with other than MCL B-NHLs and healthy donors we established a cut-off limit. We observed cyclin D1 above the cut-off limit specifically in BMs infiltrated with MCL (Figure 3), which was defined by flow cytometry or by PCR of clonally rearranged *Ig* genes. In the MRD specimens we showed the cyclin D1 expression level to be slightly above the cut-off limit corresponding to the minimal BM tumor load. The cyclin D1 quantitative monitoring was presented as a reliable and sensitive approach for the BM analysis which provides the MRD-PCR target for all patients with a cyclin D1 overexpression in the primary tumor (98 % in our study). The cyclin D1 mRNA monitoring approach therefore overruns the previously described MRD monitoring, providing a molecular marker for 73 % of patients with MCL only (13), and it may replace the complex, time-consuming and labor-intensive MRD detection using the clonal *Ig* rearrangement.

We observed that the cyclin D1 expression level in BM correlated with the clinical status of the disease (Figure 4)(3). A high cyclin D1 expression level was detected at the time of initial diagnosis, was low at the time of clinically determined remission, and the disease relapse was accompanied by the cyclin D1 increase. Slightly increased cyclin D1 levels, corresponding to the minimal residual infiltration, were shown at the time of partial remission. Thus, the correlation presents the cyclin D1 mRNA level measuring as a marker for the molecular monitoring of the MCL in BM. Moreover, the cyclin D1 level dynamics reflects the disease development as it was shown by the individual cyclin D1 level monitoring in patients with more than one sequential BM aspirate available during the disease course. Actually, BM specimens aspirated closely before the clinically apparent relapse showed increasing cyclin D1 levels regardless of whether these patients were at that time still in clinical remission. The cyclin D1 increase was followed consecutively by a clinical relapse.

However, the data are preliminary due to a limited number of BMs aspirates taken at the time before relapse. Currently, there is no standard protocol for timing the BM aspiration at specific intervals during the MCL treatment. Furthermore, there is an ethical limitation to aspirate BM at the time of the disease remission. Thus, we analyzed BMs after the start of treatment to monitor the response and then BMs at the time of relapse. There were few BMs available at the time before the clinical relapse and thus some crucial samples were missing in this study to verify whether or not the cyclin D1 increase may herald the relapse.

Because a predictive significance of the BM PCR monitoring was recently described for patients with MCL (23) and we demonstrate that cyclin D1 is a reliable MCL molecular marker for monitoring BM involvement (3), the predictive significance of the cyclin D1 level dynamics may be assumed. Therefore, despite the invasiveness of the BM aspiration and despite the economical cost the BM analysis is recommended. To definitively establish the prognostic value of the cyclin D1 dynamics in patients with MCL, which is strongly assumed, it is very important to study the cyclin D1 expres-

sion level at particular time points during the disease progression. Well designed prospective clinical trials need to be performed to build a basis for the use of MRD monitoring as a routine clinical tool, to define BM sampling time points and critical MRD levels in patients with MCL. The kinetics of the therapy response, which may be followed using the cyclin D1 quantitative approach in patients with MCL, is probably more important than the absolute MRD data obtained at a single time point. Therefore, we recommend to aspirate and to quantitatively analyze BM more frequently at defined time intervals after treatment in patients with MCL.

Quantitative PCR of proliferation markers (Ki-67, topoisomerase IIα, and TPX2) in MCL

The molecular analysis may not only assist in achieving the diagnosis and the monitoring of the disease but it may also provide important prognostic information. Even thought MCL belongs to the group of indolent lymphomas and thus it is refractory to therapy, its clinical behavior is generally aggressive with the median overall survival 3–5 years (27,28). Even so, there exists a small cohort of patients with a relatively favorable condition and a survival of more than 10 years with no need of any therapy (28–31). As MCL patients are elderly people (6th and 7th decade) and the current therapy is toxic with severe side effects, the clinical variability in the group of patients with MCL calls for prognostic factors predicting the clinical course (31). Such prognostic factors will allow the discrimination between patients with the indolent and the aggressive course of the disease as well as to choose an approach to the patient respecting the individual risk. A prognostic index specific for patients with MCL (MCL international prognostic index, MIPI) was recently introduced as the most efficient prognosticator for patients with MCL (32).

In addition to clinical and laboratory parameters (age at diagnosis, Eastern Cooperative Oncology Group score, serum lactate dehydrogenase level and leukocyte number) MIPI includes the first marker respecting the cell cycle biology, a proliferation activity. The proliferation activity was described to be the most effective prognostic factor even superior to other clinical, laboratory and histological characteristics (29,33). Multivariate analyses demonstrated three key molecules which are involved in the active proliferation, Ki-67, topoisomerase IIα and TPX2, to be prognostically relevant in patients with MCL (33–39). All current studies have used the immunohistochemistry (IHC) for detection of the protein expression. IHC allows a correlation between the morphology and the protein expression, but a standard, objective and reproducible quantification is complicated (40). Because the length of the survival of MCL patients depends upon the quantitative differences in the progression from the G1 to S phase of the cell cycle (41) a standardized quantitative measurement is needed for using the proliferation activity as the most important biological prognostic factor in clinical trials (33,37).

Therefore, we designed a simple, reliable, reproducible and routinely applicable methodology for a quantitative PCR measurement of the expression level of proliferation markers, Ki-67, topoisomerase IIα and TPX2 (4)(Fig. 5). We found a correlation between the mRNA level and a semi-quantitatively evaluated protein expression by IHC indicating a reliability of the RQ-RT-PCR approach to effectively measure the proliferation activity. The RQ-RT-PCR technique is a potent tool for clinical trials because it combines the speed and ease of PCR-based systems with an accurate and reproducible quantification.

CONCLUSION

The molecular analysis broadens the spectrum of investigative tools in contemporary pathology. In this review we demonstrated the importance of such an analysis in a group of patients with MCL. Molecular methods provide a support for the differential diagnosis which is particularly important in the difficult B-NHLs differential diagnostics. Moreover, by using a molecular marker obtained by the primary tumor molecular analysis it is possible to monitor the course of the disease at the sensitive molecular level, including MRD detection. Molecular monitoring is becoming a part of treatment protocols, it is used for therapy guiding, monitoring of the therapy response and for establishing the efficiency of new therapeutic approaches (42). Recently, the PCR-based monitoring has been reported to be clinically important for patients with MCL and it is recommended to be implemented in the MCL therapy (23).

The molecular analysis is helpful also to establish prognostically important molecules. Because "The Lymphoma/Leukemia Molecular Profiling Project"(42) suggests incorporation of the quantitative assessment of the tumor proliferation rate into clinical trials in MCL, the PCR-based system established in this study represents a potent approach to be used as a standard methodology in routine clinical practice. It may be assumed that a precise quantitative evaluation of prognostic factors will allow the stratification of patients and to guide the therapy according to the individual disease risk.

The increasing role of the molecular analysis in modern medicine is obvious and therefore it becomes an integral part of the diagnostic process as we have illustrated in patients with MCL.

ACKNOWLEDGMENT

This work is supported by the Research Project
VZ MZOFNM2005/6704.

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