Univerzita Karlova v Praze

1. lékařská fakulta

1. interní klinika- klinika hematologie

Všeobecné fakultní nemocnice v Praze

Autoreferát disertační práce

Clinical implications of minimal residual disease evaluation by polymerase chain reaction in patients with B- cell lymphoproliferative disorders

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Praha 2008

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze
a Akademie věd České republiky

Obor: Molekulární a buněčná biologie, genetika a virologie.

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Školicí pracoviště: 1. interní klinika Všeobecné fakultní nemocnice v Praze

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1. INTRODUCTION

B- cell lymphoproliferative disorders (B-LPD) are clonal proliferations of B- lymphocytes arrested at a specific stage of differentiation. Based on biological behavior these tumors can be divided into so called indolent, intermediate and aggressive lymphomas. Follicular lymphoma (FL), mantle cell lymphoma (MCL), small cell lymphocytic lymphoma (SLL) together with chronic lymphocytic leukemia (CLL) represent most frequent subtypes of indolent B-LPD. FL, CLL/SLL and MCL are typically incurable by standard treatment approaches. Although majority of patients achieves complete remission after first line chemotherapy many patients experience recurrence of disease. Complete clinical remission (CR) is defined as disappearance of the malignant clone under a detectable level when using conventional clinical tests (radiodiagnostic and or laboratory; Cheson et al., 1996; Cheson et al., 1999). Relapses are the primary reason for treatment failure. It is generally accepted that subclinical minimal residual disease (MRD) contributes to relapses of hematological malignancies (Donovan et al., 2000; Gribben, 1994). The situation with no detectable MRD is

called molecular complete remission (MCR, PCR negativity), and is defined as disappearance of previously detectable molecular marker.

Intensive sequential chemotherapy or high dose therapy (HDT) with autologous stem cell transplantation (ASCT) can induce PCR negativity in a significant number of patients with indolent lymphomas (Corradini et al., 1997). It has been also demonstrated that monoclonal antibody anti-CD20 (rituximab) can induce MCR in significant number of patients with lymphomas (Czuczman et al., 1999). Routine monitoring of MRD of patients with B-LPD is however not yet generally accepted.

2. MINIMAL RESIDUAL DISEASE (MRD)

In leukemia CR is defined as presence of less than 5% of blasts in the bone marrow, but that may actually represent up to 10^{10} leukemic cells. In order to cure a patient with a hematological malignancy it is prerequisite to eradicate the malignant clone completely. In another words it is desirable to achieve a MCR (Gribben et al., 1994). MRD can be assessed by several different methods (Table 1). For high sensitivity and relative feasibility

polymerase chain reaction (PCR) is frequently used for MRD evaluation. Monitoring of MRD has been well established in chronic myeloid leukemia (CML). This field gave also rise to MRD nomenclature. Molecular or PCR relapse is a term suggested by Lion described a situation when quantitative PCR detects expansion leukemic clone by 1 log in absence of clinical relapse (Rel) (Lion, 1994).

3. MOLECULAR MARKERS USED FOR MRD MONITORING

We have studied patients with following B-LPDs: FL, MCL, and CLL/SLL. Patients with other lymphomas were frequently evaluated by PCR as well, but not used for our studies.

The hallmark of FL is translocation t(14;18)(q32;q21) involving the *BCL*-2 gene. This aberration can be detected in 80% to 90% of cases. On the chromosome 14 all the breaks in the immunoglobulin heavy chain gene (IgH) occur within the joining (J) segments and on chromosome 18 the majority (50%) of breaks occur in the major breakpoint region (MBR) (Cleary and Sklar, 1985),

followed by the more distant minor cluster region (mcr) (Bakhshi et al., 1987).

Translocation t(11;14)(q13;q32) is characteristic for MCL and can be detected in up to 70 % cases by cytogenetics. This translocation juxtaposes the *BCL-1* gene at 11q13 with the IgH locus at 14q32, leading to heterotopic deregulation of *BCL-1* (Leroux et al., 1991). More than 80% of chromosomal breaks are clustered in 80-110 bp region on the 11q chromosome called „major translocation cluster" (MTC; (Rimokh et al., 1990). The sequences from the above mentioned chromosomal breaks are used as primers for detection by PCR.

In CLL/SLL and or other B-LPD without translocation detectable by PCR the detection of complementarity determining region III (CDRIII) within the IgH rearrangement can be used for PCR monitoring.

4. AIMS OF THE STUDY

The presented study focused on the impact of minimal residual disease detection and monitoring in patients with B-LPDs. I

have concentrated on patients with following diagnoses: follicular lymphoma, mantle cell lymphoma and chronic lymphocytic leukemia or small lymphocytici lymphoma.

THE AIMS OF THIS WORK CAN BE SUMMARIZED AS FOLLOWS:

1. **Introduction of molecular methods into regular evaluation of patients with B- cell lymphoproliferative disorders both as a part of diagnostic process as well as during follow up as part of evaluation of treatment efficiency.**
2. **Evaluation of the impact of molecular remission after treatment on clinical outcome in monitored patients.**
3. **Evaluation of the impact of particular therapeutic approach on both attainment of molecular remission and in turn on clinical outcome.**

5. METHODS

5.1. Patient samples

Between November 1999 and July 2002 there were 945 samples (lymphnodes, bone marrow aspirates, peripheral blood and also peripheral blood progenitor cells) submitted for PCR detection of molecular markers (translocation t(14;18) and translocation t(11;14) and also CDRIII rearrangement) from 432 patients. Three hundred fifty were newly referred patients.

The following tissues were collected at times of disease activity (diagnosis, progression or relapse): LN, BM and/or PB. BM and PB samples were tested during the follow up period and peripheral blood progenitor cells (PBPC) were tested prior to transplantation.

5.2. Molecular techniques

We have used the following techniques of molecular biology: PCR (qualitative), comparative duplex PCR (quantitative), realtime PCR (quantitative), and sequencing.

PCR: The DNA was extracted using standard procedures with precautions to avoid cross-contamination. Detection of translocations t(14;18) or t(11;14) was performed using our modification of so called touchdown PCR technique that has been described previously (Cerny et al., 2004). Samples in which the PCR did not detect translocation were further assayed for the presence of CDRIII rearrangement.

Each reaction contained positive (CDRIII, MBRA, or MTCC positive DNA) and negative control (normal DNA isolated from PB of healthy individuals). PCR products were visualized on agar gel stained with ethidium bromide. Under these conditions, the sensitivity of CDRIII detection was 10 positive cells in 10^{4-5} normal cells, the PCR detection of t(14;18) or t(11;14) had higher sensitivity (10 or less positive cells in 10^6 normal cells).

Comparative duplex PCR: Serial dilutions of consecutive DNA samples were submitted to the same duplex PCR. We have used ras and β-globin as normalizing markers in co-amplification with primers for disease-specific markers. Amplification products were analysed on 10% polyacrylamide gels stained with ethidium bromide and the amount of PCR products was determined by gel

densitometry using gel documentation system and gel analysis software, as described in (Slavickova et al., 2005; Slavickova et al., 2000).

Real-time PCR: The real-time PCR quantification was performed using the LightCycler and the t(14;18-mbr) Quantification Kit of Roche Molecular Biochemicals exactly as recommended by the manufacturer.

Sequencing: Amplified PCR products were separated by electrophoresis on 3% Metaphor agarose gels stained with ethidium bromide. The products of PCR amplification were extracted from gel slices using the crush and soak technique, then purified with Microcon-100 (Millipore, Foster City, CA, USA) purification columns sequenced using usual PCR primers with BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed on ABI Prism 310 Genetic Analyzer (PE BioSystems) according to the manufacturer's instructions. The sequence data were assigned on the basis of their similarity to BLAST sequences and confirmed by homology to each germ-line sequence as summarized in (Cerny et al., 2004).

6. RESULTS

As mentioned above between November 1999 and July 2002 there were 945 samples submitted for PCR detection of molecular markers from 432 patients (350 were new referrals). The actual histological subtypes and molecular markers are summarized in Figure 1, Tables 2 and 3. Our institution has a large referral base and these results include all referrals. They include patients who were suspected to have a B-LPD, but were diagnosed either with a different malignancy or malignancy was ruled out completely. This explains the relatively low percentage of positive results (Table 2). These results also include referrals whose samples were sent only one time and such patients could not have been part of monitoring and were also often lost to follow up.

Aim 1: We have noticed a significant increase of diagnostic samples submitted for PCR evaluation during my presence the molecular biology laboratory. This together with the fact that the molecular biology laboratory has been maintaining international accreditation (namely by the Royal College of Pathologists of Australasia Quality Assurance Programs Pty Ltd) I see as one of

positive outcome of work related to my thesis. The awareness of molecular testing and monitoring of MRD increased rapidly among the clinicians and thus I feel that aim one of my PhD work was accomplished.

Aim 2: Patients with indolent B-LPDs who achieve MCR have better prognosis as measured by PFS or OS (see Figure 2). Not attaining MCR was found to be a strong predictor for disease recurrence and shorter OS (HR for PFS 3.489 (1.7071-7.1309), p=0.001, and HR for OS 4.6848 [1.6071-13.6567], p= 0.005).

Aim 3: Rituximab based therapy induces MCR in high number of patients, which can be further improved by ASCT and those patients then have an excellent outcome (see Figure 2). Patients with PCR positive PBPC harvest are at high risk of relapse after ASCT.

Secondary observations: Prognosis of patients with biallelic IgH is similar to patients with single allele IgH rearrangement. True biclonal disorders are very rare, but biclonalities should be ruled out as they may have prognostic and therapeutic implications. The presence of additional IgH rearrangement may decrease the sensitivity of PCR and should be considered for purposes of

quantitative MRD monitoring. Biclonality ought to be ruled out by a combination of at least two methods.

7. CONCLUSSIONS

The achievement of MCR is a realistic goal in patients with B-LPDs and should be the aim of treatment especially for young patients and patients that have unfavorable prognostic factors. Our observations suggest that MCR is a strong predictor of prolonged survival. We have also seen that more intensive therapy (rituximab therapy followed by ASCT) ensures superior outcome. Similar observations were recently reported by a randomized, multicenter phase III trial in patients with FL, which was the first trial to prospectively explore the value of MCR as a prognosis predictor. Similar to our experience it also determined that more intensive treatment showed improved outcome (Ladetto et al., 2008).

While awaiting the data from other ongoing studies to mature and quantify the relevance of MRD and OS, efforts should be focused on making flow cytometry and/or molecular

monitoring of MRD by PCR routinely available to most centers and further optimizing and standardizing these sensitive and sophisticated methods. An upcoming challenge will be to revise the current response criteria accordingly. The evaluation of MRD and its physiologic relevance will need to be incorporated in the guidelines for the management of lymphoproliferative disorders.

TABLES and FIGURES

TABLE 1. Sensitivity of different molecular biology techniques used for MRD detection (Dolken, 2001).

Method	Sensitivity	Tumor cells/ total cells	Features
Cytology, cytochemistry and histology	5%	5 / 100	standard clinical procedure, low sensitivity
Flow cytometry (FACS)	1-5 % (0,1%)	1-5 / 100 (to 1000)	Lack of tumor-specific antigens and antibodies
Immuno-histochemistry	1-5 % (0,1%)	1-5 / 100 (to 10 000)	Lack of tumor specific antigens and antibodies
Southern blot	1%	1 / 100	Time consuming, laborious, low sensitivity
Standard cytogenetics	5-10 %	5-10 / 100	Labor intensive, high quality metaphases
Fluorescence in situ hybridization (FISH)			Interphase FISH: false positive results, labor intensive, no need for metaphases
S-FISH („single color")	5-10 %	5-10 / 100	
D-FISH („double color")	1-3 %	1-3 / 100	
PCR	10^{-2}-10^{-3}	1 / 10^4 to 10^6	Sensitivity depends on the total amount and quality of DNA or mRNA (cDNA)
Nested PCR	10^{-3}-10^{-5}	1 v 10^7	Very sensitive; false-positive results due to contamination or carry-over are serious problems

Table 2: The number of PCR positive and negative results in submitted material. (LN- lymphnode; BM- bone marrow; PB- peripheral blood; PBPC- peripheral blood progenitor cells; IgH-CDRIII- immunoglobulin heavy chain gene- complementarity determining region III; MBR- major breakpoint region; MTC_ major translocation cluster; other tissues such as spleen, skin or gastric biopsies are not included).

Positive result	Diagnostic material			
	LN	BM	PB	PBPC
IgH-CDRIII	28	183	74	26
t(14;18)-MBR	3	44	9	1
t(11;14)-MTC	3	14	7	2
Negative	27	362	117	42
Total tested	61	603	207	71

Table 3: Detection of molecular markers in particular diseases. (CLL/SLL- chronic lymphocytic leukemia or small lymphocytic lymphoma; FL- follicular lymphoma, MCL- mantle cell lymphoma; DLCL- diffuse large cell lymphoma; NHL-US- nonhodgkin lymphoma, unspecified; MALT- mucosa associated lymphoid tissue lymphoma; other- other diagnosis; IgH-CDRIII- immunoglobulin heavy chain gene- complementarity determining region III: MBR- major breakpoint region; MTC- major translocation cluster).

Disease	Percentage of detected molecular markers		
	CDR3	t(14;18)-MBR	t(11;14)-MTC
CLL/SLL	88.7	0	0
FL	42.2	20.6	0
MCL	65.5	0	26.0
DLCL	34	0	0
NHL-US	12.5	3.1	0
MALT	7.1	0	0
Other	19.6	0.9	0

Figure 1. Overview of diagnoses from samples submitted for PCR evaluation. CLL/SLL- chronic lymphocytic leukemia or small lymphocytic lymphoma; FL- follicular lymphoma, MCL- mantle cell lymphoma; DLCL- diffuse large cell lymphoma; MALT- mucosa associated lymphoid tissue lymphoma; NHL-US- nonhodgkin lymphoma, unspecified; other- other diagnosis.

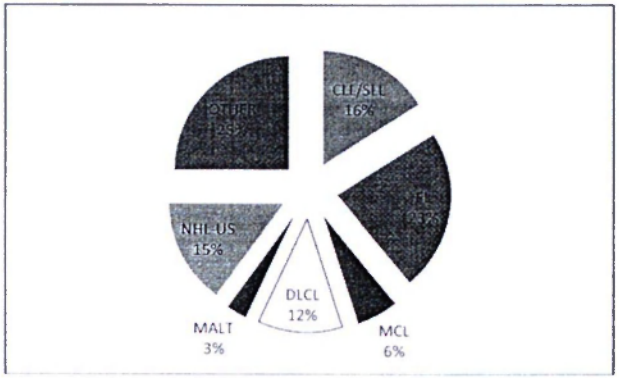
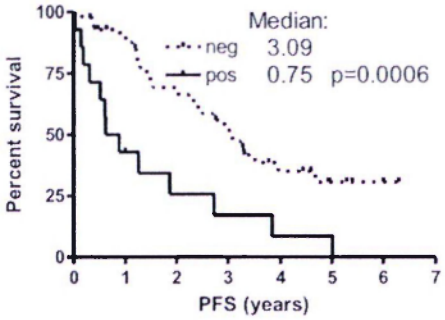


Figure 2: Progression free survival (A) and overall survival (B) based on PCR status after therapy. (neg- PCR negative, pos- PCR positive, PFS- progression free survival, OS- overall survival).

A



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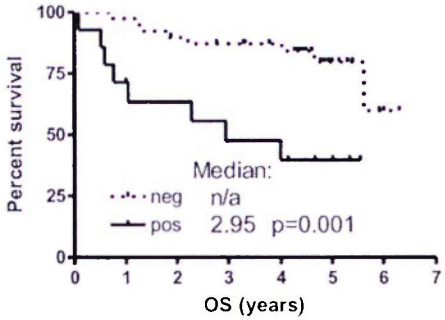
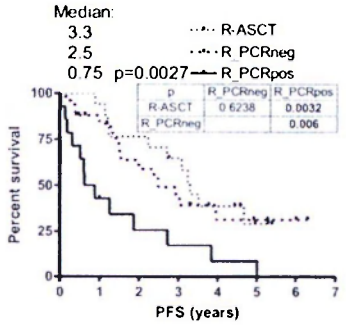


Figure 3: Progression free survival (A) and overall survival (B) based on type of therapy and PCR result after completion of treatment. (R_PCRneg- PCR negative after rituximab, R_PCRpos- PCR positive after rituximab, R-ASCT- rituximab followed by autologous stem cell transplantation, PFS- progression free survival, OS- overall survival).

A



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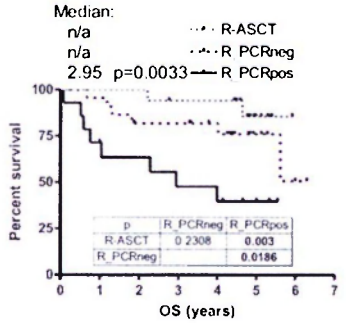

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