Virchows Arch (2005) 447: 909–919
DOI 10.1007/s00428-005-0069-1

REVIEW ARTICLE

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Ancillary techniques in bone marrow pathology: molecular diagnostics on bone marrow trephine biopsies

Received: 18 May 2005 / Accepted: 11 July 2005 / Published online: 18 October 2005

Abstract Pathologic examination of trephine bone marrow (BM) biopsies plays a central role in the diagnosis and staging of haematological neoplasms and other disorders affecting haematopoiesis. Haematopathology has been profoundly influenced by the advent of molecular genetic techniques suitable for paraffin-embedded tissues, and certain applications, such as the determination of B- and T-cell clonality, belong to its standard diagnostic repertoire. Many of these molecular tests can be performed successfully with nucleic acids extracted from BM trephine biopsies, if some technical aspects specific to this template source such as various fixation and decalcification procedures are taken into consideration. The current indications for molecular BM diagnostics range from the confirmation of lymphoma involvement with gene rearrangement analysis, demonstration of tumor-specific translocations in lymphoid and chronic myeloproliferative disorders along to the detection of microorganisms or marrow involvement by soft tissue sarcomas. The availability of quantitative polymerase chain reaction techniques for the investigation of allelic imbalances and gene expression levels in paraffin-embedded material also open new avenues for research and advanced diagnostics. The molecular detection of minimal residual disease in haematological neoplasms, especially in the context of new treatment strategies, will provide future challenges. This article summarizes the current state of the art in molecular diagnostics applied to paraffin-embedded BM biopsies.

Keywords Bone marrow biopsy · Molecular analysis · Clonality · Gene expression · Lymphoma · Haematological neoplasms

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Introduction

Examination of the bone marrow (BM) is of central importance for the diagnosis and staging of haematological disorders. Conventional aspiration cytology, flow cytometric immunophenotyping, cytogenetics and the histopathological examination of trephine BM biopsies, usually obtained from the posterior iliac crest, are currently the standard examination techniques [4, 15, 80]. Traditional indications for a trephine biopsy include all disorders in which marrow aspiration alone is considered unreliable or insufficient, such as staging of malignant lymphoma, chronic myeloproliferative disorders, metastatic disease and any disorder accompanied by marrow fibrosis [21, 68]. However, in many centres, trephine biopsy is considered an integral part of BM examination and is taken in most instances when an aspirate is performed, irrespective of the indication. Traditionally, the examination of BM biopsies was limited to conventional histological and histochemical stains, such as hematoxylin and eosin, Giemsa, NASD-chloroacetate-esterase, as well as iron and reticulin stains. The availability of a broad range of antibodies suitable for paraffin sections as well as the development of molecular techniques applicable to fixed tissues have significantly broadened the possibilities for the examination of trephine biopsy samples. Although an excellent morphology undoubtedly remains the mainstay and basis for the diagnosis of haematological disorders, the thoughtful application of these ancillary methods can improve diagnostic accuracy, can help to obtain a better subclassification of neoplastic disorders and may increase sensitivity for the detection of residual disease. This review focuses on the methodical

possibilities and limits as well as the diagnostic applications of molecular in vitro techniques for the examination of BM trephines. The role of immunohistochemistry in BM diagnostics is addressed in a separate paper.

Relevance of BM fixation and processing for molecular studies

The presence of bone trabecules and the necessity for preservation of the subtle cytological features of haematopoietic cells poses special problems with regard to specimen fixation, decalcification and processing. To obtain optimal morphology, a variety of fixatives, including buffered formalin, Bouin's, mercury-containing solutions such as Zenker's fixative and B5 or a combination of formalin and glutaraldehyde (Schäfer's solution), are used, followed by brief acid-based or EDTA decalcification [62]. However, several of these fixatives have a detrimental influence on the immunoreactivity and/or the preservation of nucleic acids and therefore limit the application of ancillary techniques [74, 77]. In addition to paraffin embedding, plastic embedding of biopsies is still used. Extraction of DNA and even RNA suitable for molecular studies such as polymerase chain reaction (PCR)-based determination of B-cell clonality or detection of fusion transcripts is possible from plastic-embedded BM samples [6, 27]. However, due to the decreasing role of plastic embedding, the techniques described in this review are based on the use of conventional formalin-fixed, EDTA-decalcified, paraffin-embedded BM specimens, but can be equally applied to non-decalcified material such as BM clot preparations. Samples briefly decalcified with acid-based agents can also be used for DNA-based PCR assays [8, 30]. Fixation in buffered neutral formalin for 24 h followed by EDTA decalcification renders both satisfactory morphology as well as good antigen, DNA and even RNA preservation and therefore can be regarded as a good compromise between optimal morphological detail and the availability of the full spectrum of ancillary techniques. Although decalcification with EDTA is more time-consuming than treatment with acid-based agents, application of an ultrasonication bath allows decalcification within 8 to 12 h.

Similar to conventional paraffin tissues, DNA and RNA extracted from BM biopsies is significantly degraded, and the prevalent fragment size for DNA is below 500 bp. Decalcification procedures, prolonged fixation in comparison to surgical specimens and additives such as glutaraldehyde may further reduce the yield of amplifiable DNA. Nevertheless, we and others have demonstrated that DNA from trephine biopsies can be used for a broad range of PCR assays, and amplification products of more than 500 bp can be achieved [6, 43, 59, 64, 81, 83]. A large number of extraction protocols have been published for paraffin-embedded tissues. In our experience, such standard protocols can also be applied to BM trephines, as long as column-based kits designed for fresh tissue are used with caution, since they may lead to the exclusion of small DNA and RNA fragments. Common to all these procedures, proper digestion with Proteinase K (best performed at elevated temperature overnight) is mandatory to release the maximum amount of DNA from the tissue, whereas purification of DNA extracts is not necessary on a routine basis. Irrespective of the procedure used, control amplification of single copy gene fragment(s) of adequate size—usually in the range of 250–400 bp—is mandatory to check DNA quality, since some tests such as clonality analyses may render false positive results with highly degraded DNA. Control PCRs for amplificates of increasing size in a multiplex format, such as designed by the BIOMED-2 group, may help to get a better estimate of DNA quality [79].

If available, archival, air-dried BM smears can be used as an additional source for DNA and RNA of excellent quality, since they have not been subjected to cross-linking fixatives. Air-dried slides, which are received at many pathology laboratories in addition to trephine biopsies, can even be used for Southern blot analysis, which requires high molecular weight DNA [34].

Although the majority of applications of molecular diagnostics on BM biopsies still use DNA as template source, recent studies have described RNA-based analyses from paraffin-embedded BM samples for a variety of purposes, especially in combination with quantitative techniques such as real-time reverse transcriptase (RT)-PCR (Table 1). The negative effects of fixation, embedding and extraction procedures on RNA integrity, which is less resistant than DNA, have been elucidated [51]. Parameters such as tissue fixation (i.e. type of fixation reagent, duration of fixation) and RNA extraction (i.e. concentration of Proteinase K, duration of sample digestion) have different effects on RNA integrity [40, 41, 55, 73]. However, the mechanisms which lead to cross-linkage between RNA and proteins and to the addition of monomethylol ($-CH_2OH$) groups to all four bases by formalin is largely unknown [51]. The decalcification procedure applied on BM trephines also exhibits a negative effect on RNA integrity [7]. Preferably, the fixed BM trephine should be decalcified in a solution displaying a neutral pH such as EDTA, because an acidic environment augments the process of RNA fragmentation. The degree of RNA fragmentation is variable within different tissues, but the average length of RNA from archival tissues such as BM trephines can be estimated rarely to exceed 200 bp. PCR products which are 100 bp in size or less are amplified more efficiently and thus highly recommended [32, 46, 73].

Indications for molecular examination of BM biopsies

Determination of clonality in the diagnosis of lymphoproliferative disorders

The differential diagnosis of lymphoid infiltrates in the BM is one of the most frequent problems in haematopathology. Although the majority of lymphoid infiltrates can safely be diagnosed as either reactive or neoplastic based on the

Table 1 Common applications of molecular diagnostics on trephine bone marrow biopsies

Diagnostic application	Method	References
B-cell clonality	IgH PCR	[8, 9, 22, 27, 59, 61, 77]
	Microdissection and IgH PCR	[43–45]
	Quantitative RT-PCR for κ and λ light chains	[48]
T-cell clonality	TCR gamma PCR	[30, 81, 82]
	TCR gamma PCR with RNase protection assay	[35]
Cyclin D1 overexpression in B-NHL	Quantitative RT-PCR	[71, 72]
BCR–ABL fusion transcripts in CML	Quantitative RT-PCR	[3, 6, 7, 40, 41]
C-kit mutations in systemic mastocytosis	Sequencing, allele-specific probes, microdissection	[70, 76]
Mycobacterial infection	PCR	[1, 69]
General methodical studies	DNA PCR	[34, 46, 64, 74, 83]
	RT-PCR	[32, 40, 41, 49, 55]

IgH Immunoglobulin heavy chain gene, *TCR* T-cell receptor gene

extent of involvement, distribution, cytology and immunophenotype, a significant minority of cases cannot be classified with certainty [43, 78]. For some low-grade lymphoma entities such as follicular and marginal zone B-cell lymphoma, the detection of an aberrant immunophenotype may be difficult or impossible in small BM infiltrates. Even flow cytometric immunophenotyping as complementary technique may fail to detect a proportion of cases with BM infiltration, mainly due to sampling error in the presence of focal fibrosis [36, 75]. However, the presence of limited vs disseminated disease—as characterized by the presence of BM involvement—is of crucial importance as prognostic information and for therapeutic decision making.

Detection of B-cell clonality

A variety of approaches have been used for the molecular detection of B-cell clonality in BM specimens. For paraffin-embedded tissues in general, the use of consensus primers against the framework 3 (FR3) or framework 2 (FR2) regions of the immunoglobulin (Ig) heavy chain variable genes in conjunction with primers against the joining region (JH or FR4) [2, 18, 20, 56, 79] is the most frequently chosen approach and will pick up clonality in 50–90% of B-cell non-Hodgkin's lymphoma (B-NHL) cases with a significant BM infiltrate, depending on the type of lymphoma and the method used for the analysis of amplicons [8, 9, 14, 27, 61, 63, 81]. Whereas B-cell chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma show a clonal product in nearly 100% of cases with these primer sets; other entities, especially follicular lymphoma, will show much lower clonality rates. This is due to point mutations and other events during ongoing somatic hypermutation, which alter or abolish primer binding sites. This relatively high false negative rate has to be kept in mind when evaluating molecular results in the setting of suspicious lymphoid BM infiltrates. Acute lymphoid leukemias may show false negative results due to incomplete VDJ rearrangements [79].

Other primer combinations, such as the recently published BIOMED-2 primer sets, which give a much higher pick-up rate of more than 90% irrespective of lymphoma type, have not been tested sufficiently on BM samples to allow for definitive conclusions [79]. However, primer sets rendering amplicons in excess of 300 base pairs such as the family-specific framework 1 (FR1) region primers should be used with caution on fixed BM samples, because poor DNA quality may result in detection of pseudoclonality due to preferential amplification of rare B cells. The same problem may occur, if semi-nested or nested protocols are used which can give oligoclonal or pseudoclonal results with poor template quality [37, 43]. To avoid false positive results, stringent control of DNA quality and reproducibility of PCR results are mandatory. Repetition of the same PCR reaction with different template concentrations, or repeat extractions, are ways to confirm clonal results [25, 38].

To increase the sensitivity for the detection of small clonal populations in nodular lymphoid infiltrates, we have successfully used microdissection to enrich the target cell population [43–45]. Although this increases the pick-up rate through reduction of the polyclonal background population, it also increases the risk of pseudoclonality due to the small amount of template, and only clearly reproducible bands of identical size should be regarded as evidence for clonality [43, 85]. Since the most frequent diagnostic question is the presence of BM involvement by a malignant lymphoma diagnosed at another site, DNA from the primary tumor can be used for comparative purposes, and clonal products of the same size and sequence will confirm the presence of BM infiltration. However, one has to keep in mind that lymphoid infiltrates in the marrow may be the result of another clonal process, rather than dissemination of an extramedullary lymphoma. This is especially the case in so-called discordant lymphoma, where a BM infiltrate composed of small lymphocytes is found in the setting of nodal or extranodal large cell lymphoma. In a recent study, we were able to show that one third of the cases of discordant lymphoma actually represented two different clonal processes, rather than morphological progression of the same clone (Fig. 1) [45].

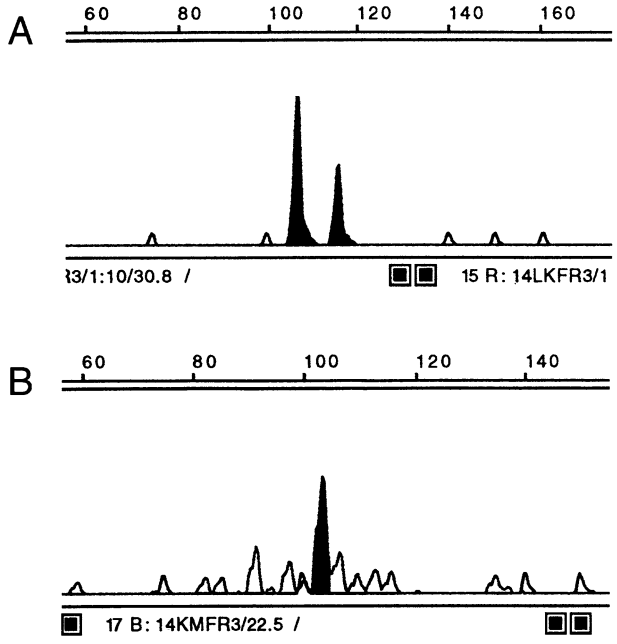

Fig. 1 Genescan analysis of immunoglobulin heavy chain gene PCR products with framework 3 (*FR3*) primers in a patient with diffuse large B-cell lymphoma and so-called discordant bone marrow involvement by small lymphocytes. **a** Lymph node biopsy. **b** Microdissected lymphoid infiltrates from the BM trephine. Genescan analysis shows two clonal products of different size. A distinct clonal origin was confirmed by sequencing

Another application of microdissection in BM biopsies is the clonal analysis of two neoplastic processes with divergent phenotype, such as composite NHL or combinations of Hodgkin's and non-Hodgkin's lymphoma [28, 29, 45]. In addition to nodular infiltrates necessitating discrimination from reactive lymphoid aggregates, some lymphoma subtypes with occasionally very subtle patterns of infiltration such as splenic marginal zone lymphoma or intravascular large B-cell lymphoma may require molecular studies for diagnostic confirmation [22].

There are very few data available concerning the sensitivity of molecular techniques in BM trephines, but it can be assumed from mixing experiments and other data that the sensitivity is the range of 2–10% clonal cells with conventional consensus primers, if sensitive detection methods such as the Genescan technique with fluorescently labelled primers, heteroduplex analysis or high resolution polyacrylamide gels are used. BM trephines may be more representative samples for molecular studies than simultaneously obtained aspirates, since the aspirate is frequently significantly diluted by peripheral blood lymphocytes, a phenomenon similarly observed in flow cytometric immunophenotyping [14, 36].

The detection rate can be increased by addition of primers for common translocations, namely the t(14;18)(q32;q21) involving the bcl-2 locus in follicular lymphoma and the t(11;14)(q13;q32) involving the bcl-1/CCND1 locus in mantle cell lymphoma. However, extremely sensitive tests such as nested PCR, which can pick up rare cells carrying the translocation, should be avoided in the diagnostic setting. A detection of the t(14;18) without morphological correlate is not sufficient for a diagnosis of malignancy on its own, since rare benign B cells carrying this translocation are a frequent finding in elderly patients [52, 53]. Positive results, especially for bcl-2 rearrangements, obtained in BM samples should always be compared to the primary diagnostic tissue by appropriate techniques such as sequencing to confirm the clonal identity of the detected translocation.

Alternative methods for detection of B-cell clonality such as the use of primers against the kappa light chains can be employed analogously to the common Ig heavy chain assays [33]. More recently, a promising technique for BM biopsies based on the quantitative detection of kappa and lambda mRNA transcript ratio has been described [48].

Detection of T-cell clonality

Since BM involvement by T-cell NHL (T-NHL) is distinctly less frequent compared to B-cell lymphoma, only a limited number of studies have dealt with the molecular detection of T-cell clonality in BM trephines [30, 35, 44, 81, 82]. Nevertheless, molecular studies may provide significant help in certain diagnostic settings. The higher number of reactive T-cells in normal BM, the frequent difficulty to identify an abnormal immunophenotype in T-cell neoplasms and the subtle pattern of infiltration characteristic for certain T-NHL such as T-cell proliferations of large granular lymphocytes (T-LGL) or hepatosplenic T-cell lymphoma are potential reasons to study T-cell clonality in BM trephines [30, 82]. The most frequently used strategy for detection of T-cell clonality in paraffin-embedded tissues are various primer combinations directed at the T-cell receptor gamma locus (TCRγ), less commonly against the TCRβ genes, since the latter locus is more complex and requires a large number of primers [2, 57, 79]. The vast majority of T-cell neoplasms of both α/β, as well as γ/δ, phenotype show TCRγ rearrangements. Since somatic hypermutation of rearranged TCR genes does not occur, mispriming is less of a problem than in B-cell lymphomas. However, due to the restricted repertoire of germline genes, pseudoclonality due to diverse rearrangements of identical length may cause false positive results, especially with a limited number of target cells. Therefore, analysis of PCR products by either Genescan or heteroduplex analysis is mandatory, and each reaction should be run at least in duplicate [79].

General issues of clonality determination

One needs to point out that demonstration of B- or T-cell clonality in the BM should not be equated with malignancy. Leaving the technical problem of pseudoclonality aside, true lymphocyte clones may be detected in a variety of fairly common benign or premalignant disorders, such as monoclonal gammopathy of unknown significance (MGUS), in patients with subclinical clonal B-cell populations with a B-

CLL phenotype or occasionally in patients with nodular lymphoid infiltrates [26, 43]. This underlines that a diagnosis of malignancy should only be made if appropriate clinical, morphological and phenotypical findings are present.

The identification of a clonal population in the presence of a morphologically suspicious lymphoid infiltrate with standard molecular techniques has to be discerned from the detection of minimal (residual) disease for staging or follow-up purposes. The presence of minimal numbers of clonal cells in morphologically negative BM samples of lymphoma patients, identified through highly sensitive techniques such as nested PCR or with clone-specific primer sets, usually does not carry the same impact on patient management as overt BM involvement, but may help to identify patients at higher risk for relapse or progression. For a more detailed review of the topic of minimal residual disease, especially in the context of new treatment options and the technical issues involved such as tumor-specific primers and quantitative PCR, the extensive haematological literature should be consulted [11, 17, 23].

Practical issues of DNA studies in trephine BM biopsies

DNA is extracted either from serial paraffin sections or microdissected tissue fragments with xylene followed by a graded alcohol series. The resulting pellet is digested overnight with Proteinase K in an adequate amount (25–200 μl) of buffer at 55°C and continuous shaking. If the supernatant is clear, Proteinase K is inactivated by boiling, and the resulting crude extract is used directly as template for PCR. Since quantitation of DNA by photometric measurement is of limited usefulness, it can be helpful to perform two PCR reactions in parallel, using two different DNA concentrations (e.g. 1–2 μl undiluted and diluted 1:10). As mentioned above, DNA extracted from trephine BM biopsies may show increased degradation, and it is crucial to check amplification efficiency with a control primer set. The size of the control product should be larger than the fragment size achieved with primer sets routinely used for clonality determination, e.g. at least 250–270 bp. Most diagnostic DNA PCR assays in our laboratory are carried out as single-step PCR with 40 cycles of amplification. For clonality determination, AmpliTaq Gold (Perkin Elmer) has shown better amplification efficiency. Separation of fluorescently labelled PCR products for clonality determination is performed in our laboratories by automated fragment length analysis using an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA). Alternatively, polyacrylamide gels (with or without heteroduplex analysis) may be used, but agarose gel electrophoresis alone is clearly insufficient for product analysis. Currently used primer sets are listed in Table 2. Although the clonality pick-up rate in NHL varies with different primer sets, a fairly good concordance between different laboratories has been reached in multi-centre trials, if stringent quality controls are performed and more than one primer set is used [12].

Quantitation of gene expression

In addition to using the ratio of Ig light chain mRNAs as a surrogate marker for B-cell clonality [48], the recent advent of real-time RT-PCR for the precise quantitation of mRNA transcripts holds significant promise for BM diagnostics.

Table 2 Sequences of primers commonly used in our laboratories

Primer	Sequence 5′–3′	Product size
B-cell clonality		
FR3, forward	ACACGGCYSTGTATTACTGT[a]	~70–130 bp[b]
FR2a, forward	TGGRTCCGMCAGSCYYCNGG[a]	~230–270 bp[b]
Bcl2 (MBR), forward	TTAGAGAGTTGCTTTACGTGGCCTG	~100–250 bp
JHa, reverse[c]	ACCTGAGGAGACGGTCACC	–
T-cell clonality		
Vγ11, forward	TCTGGRGTCTATTACTGTG[a]	–
Vγ101, forward	CTCACACTCYCACTTC[a]	–
Jg11, reverse	CAAGTGTTGTTCCACTGCC	~60–80 bp (Vγ11 + Vγ101)[b]
Jp11, reverse	GTTACTATGAGCYTAGTCC[a]	~70–90 bp (Vγ11 + Vγ101)[b]
BCR–ABL		
b2, forward	ATCCGTGGAGCTGCAGATG	b2a2 (111 bp), b2a3 (118 bp)
b3, forward	GAGTCTCCGGGGCTCTATGG	b3a2 (98 bp), b3a3 (103 bp)
e1, forward	AGATCTGGCCCAACGATGGCGA	e1a2 (73 bp), e1a3 (79 bp)
a2, reverse	TCAGATGCTACTGGCCGCTGA	–
a3, reverse	TGTGATTATAGCCTAAGACC	–

MBR Major breakpoint region of bcl-2 in follicular lymphoma
[a]These primers contain sites with multiple ("degenerate") nucleotides to allow annealing to different VH (or TCR J) genes
[b]Gives the approximate size range for a product derived from a normal polyclonal population
[c]Alternatively, a mix of multiple JH primers (JH 1-2-4-5 and JH3 and JH6) can be employed

By using microdissected cells from paraffin sections including decalcified BM biopsies, we could show that determination of cyclin D1 levels with real-time PCR, using intron-spanning primers (exon 1/2) amplifying a 70-bp mRNA fragment and a corresponding specific TaqMan probe (Perkin Elmer) is an excellent tool for the differential diagnosis of small B-cell neoplasms [71]. Mantle cell lymphomas could be reliably discerned from other lymphoma subtypes by virtue of their constantly high cyclin D1 levels caused by the t(11;14)(q13;q32) translocation (Fig. 2). Hairy cell leukemia, which shows up-regulation of cyclin D1 by another, so far unknown mechanism, showed cyclin D1 levels intermediate between mantle cell lymphoma and other B-NHL. In multiple myeloma, real time RT-PCR was able to discriminate three groups with high, intermediate and low to negative cyclin D1 levels. All cases with high mRNA levels showed the presence of a t(11;14) by fluorescence in situ hybridization (FISH), whereas most cases with intermediate values contained a trisomy 11, pointing to a role of gene dosage for cyclin D1 levels. Cases with low to negative cyclin D1 did not show alterations of the CCND1 (cyclin D1) locus on 11q13 by FISH [72]. This example demonstrates the enormous potential of quantitative gene expression analysis in archival tissues. However, the pitfalls as well as the biological and technical limitations of these techniques have to be kept in mind if they are applied for diagnostic purposes [32, 49, 51, 73, 74]. The problem of normalization has not been solved satisfactorily to date, since many so-called "housekeeping" genes can show some variation in expression levels depending on cell type and conditions. In addition, the normalization against multiple housekeeping genes recommended by some investigators may be impractical in the diagnostic setting with sometimes very limited archival material. Furthermore, quantitative RT-PCR assays may show variable sensitivity to fixation-mediated degradation or alterations of secondary RNA structure, which necessitates stringent controls for comparisons among different mRNA species. Nevertheless, the investigation of differential expression ratios using the delta/delta Ct method is feasible in paraffin-embedded BM biopsies if results are rigorously controlled, and the expression levels of the target genes are within the linear range of the standard curve.

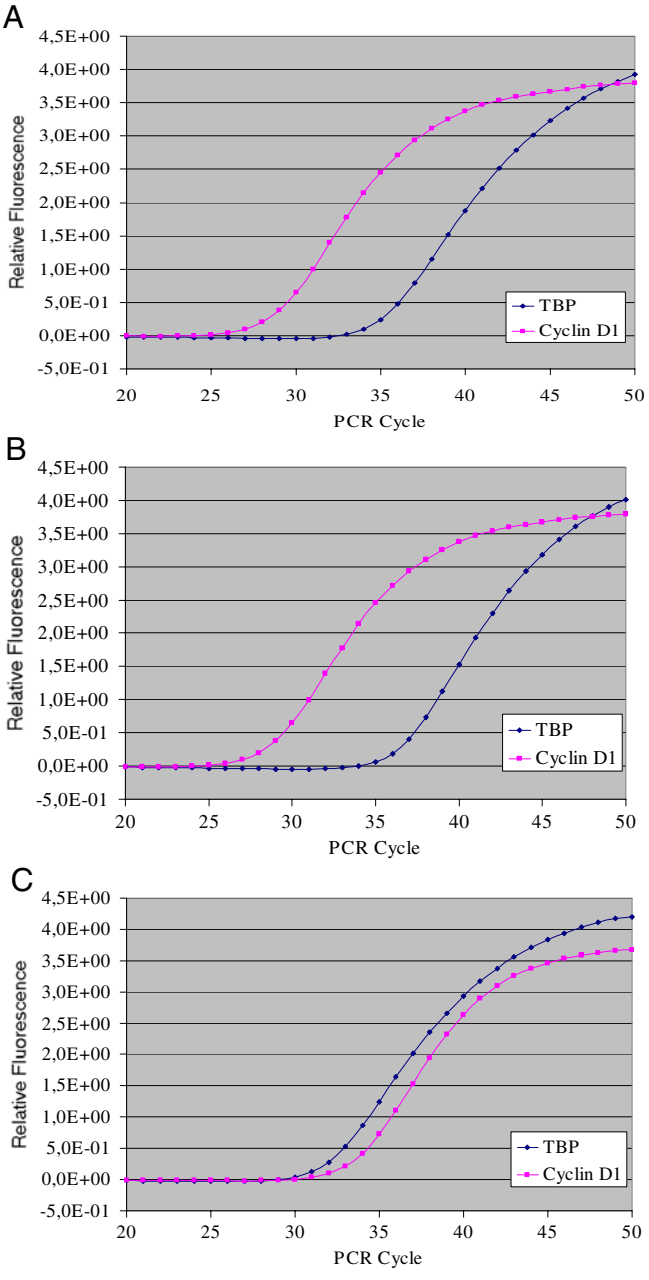

Fig. 2 Amplification plots of cyclin D1 and housekeeping gene TBP of microdissected tumor cells from a patient with mantle cell lymphoma. **a** Lymph node biopsy. **b** BM biopsy involved by lymphoma. **c** Reactive control lymph node. Whereas the curves for cyclin D1 and TBP are almost superimposed in the control tissue, both lymph node and BM biopsy show an identical left shift of the cyclin D1 curve with significantly lowered Ct, indicating significant overexpression of cyclin D1 mRNA

Molecular diagnostics in myeloproliferative disorders

The rapidly expanding knowledge of the molecular alterations in myeloproliferative diseases and their importance for patient management have resulted in an increasing amount of work aimed at identifying these genetic alterations in trephine biopsy samples. A model disease where molecular studies are of significant practical relevance is chronic myelogenous leukemia (CML), which is characterized by the hallmark Philadelphia chromosome caused by the t(9;22) translocation resulting in a breakpoint cluster region–Abelson (BCR–ABL) fusion. Already more than 10 years ago, up to two-decade-old archival tissues were demonstrated to be suitable for detection of BCR–ABL hybrid genes and fusion transcripts [3]. In accordance with these data, the successful detection of BCR–ABL transcripts also has been shown in other reports [40, 41]. Predominantly, these studies were conducted to confirm that these molecular techniques are applicable to archival tissues, i.e. proof of principle. The transfer of these techniques to the daily routine, however, represents a challenging step.

Histomorphological evaluation of the patients' BM trephine in synopsis with clinical data usually enables the diagnosis of CML. In CML, the initial diagnostic workup is often accompanied by application of metaphase cytogenetics or FISH analysis performed on BM aspirates, i.e. fresh specimens. In more than 95% of typical CML as well as in up to 30% of adult and 5% of childhood acute lymphoblastic leukemia (ALL), a reciprocal chromosomal translocation t(9;22), can be detected [13, 19]. This translocation exhibits an underlying molecular anatomy with either a major BCR (M-BCR), a minor BCR (m-BCR) or rarely a micro BCR (μ-BCR). In CML, the M-BCR typically encodes for the fusion transcript b2a2 or b3a2, whereas in ALL, the m-BCR mainly encodes for e1a2 [19]. In some cases of chronic neutrophilic leukemia, e19a2 fusion transcripts were detected as the result of a breakpoint in the μ-BCR [19]. Although several rare types of fusion transcripts have been described (e.g. b2a3, e1a3) [47], the most frequently detected types are indeed b2a2, b3a2 and e1a2 (Fig. 3). However, there is no complete concordance between the type of BCR–ABL fusion transcript and the disease type, either CML or ALL. Rare cases of typical CML can exhibit transcripts derived from the m-BCR [66]; conversely, some ALL cases contain M-BCR fusions [31]. The fusion transcripts of the M-BCR and m-BCR encode for chimeric proteins, the $p210^{BCR–ABL}$ and the $p190^{BCR–ABL}$ protein, respectively. This causes aberrant expression and constitutive activation of the ABL tyrosine kinase with uncontrolled proliferation of the affected neoplastic cells [19]. In cases of ALL, the detection of BCR–ABL fusion transcripts is associated with an aggressive course and poor survival [13].

Although CML and ALL typically can be diagnosed by histomorphological evaluation and in ALL additional immunohistochemistry, pathologists may be confronted with the need to investigate the BCR–ABL rearrangement in patients presenting with myeloproliferative disorders in case of (1) small BM biopsies with haematopoiesis that is not representative (2) leukemic infiltrates without available metaphase cytogenetics and (3) the existence of cryptic translocations that cannot be detected by conventional cytogenetics [10]. In addition, the embedded BM trephine may be the only available material when cytogenetics has not been conducted initially. Especially since the introduction of a specific tyrosine kinase inhibitor against the BCR–ABL protein (imatinib mesylate, formerly STI 571), which has shown marked activity in patients with CML and Ph1+ ALL, the detection of a BCR–ABL transcript and its type should accompany both initial diagnosis and the monitoring of tyrosine kinase inhibitor-based therapies [13, 24].

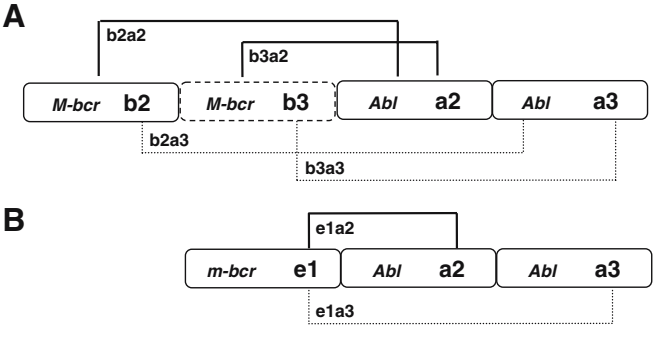

Fig. 3 Schematic illustration of the BCR and ABL hybrid genes fused in the M-BCR (**a**) and m-BCR (**b**). *Drawn lines* in the M-BCR (b2a2, b3a2) and m-BCR (e1a2) represent the most common fusion transcripts derived from the BCR–ABL hybrid genes. *Dotted lines* indicate rare but optional detectable fusion transcripts (b2a3, b3a3, e1a3)

Given the considerable extent of RNA degradation in paraffin-embedded tissues, the detection of BCR–ABL transcripts is more efficient when the product amplified by RT-PCR is less than 100 bp. In addition, for the specific detection of a particular BCR–ABL transcript, one primer should be placed directly into the fusion region. Primers suitable for RT-PCR for amplification of either b2a2 or b3a2 fusion transcripts have been previously described [16]. Although not particularly designed for the application in archival tissues, the accuracy of these assays has been successfully reproduced in our hands in archival BM trephines of patients with proven Ph-chromosome positive CML and ALL.

RT-PCR assays should be able to detect the three most common BCR–ABL transcripts. For the daily routine in a diagnostic molecular pathology laboratory, at least three RT-PCR reactions per case (+RT in duplicate, single −RT reaction as the case-specific control) per given BCR–ABL fusion type as well as adequate positive control reactions for all types have to be performed, giving a total of at least 12 RT-PCR reactions for one case. However, rare BCR–ABL fusion transcripts will not be detectable by such an approach. Multiplex RT-PCR approaches could reduce time and costs by enabling the detection of several potential BCR–ABL fusion types in a single reaction. A variety of multiplex RT-PCR assays including commercially available kits have been described by different laboratories [54, 67, 84]. However, their usefulness is restricted to fresh, unfixed samples such as peripheral blood or BM mononuclear cells because of the large amplicons generated by these assays. Unfortunately, a multiplex PCR approach often leads to a reduced sensitivity due to primer interference along with suboptimal PCR reaction conditions [60]. We have recently described a simple, robust and reliable multiplex RT-PCR, the usefulness of which for daily routine has been extensively demonstrated (Fig. 4) [7]. In addition, the detection of rare transcripts such as e1a3 is enabled by using the same forward primers and placing an additional reverse primer in ABL exon a3 (see Table 2). However, for diagnostic purposes it is highly recommended to initially establish BCR–ABL fusion type-specific RT-PCR assays separately and only consecutively design a multiplex RT-PCR along with optimization of reaction conditions.

The quantification of BCR–ABL fusion transcripts in peripheral blood mononuclear cells (PBMC) and BM aspirates of patients with CML and ALL using real-time RT-PCR has been shown to be important for monitoring the response to therapeutical intervention such as BM transplantation, peripheral blood stem cell tranplantation

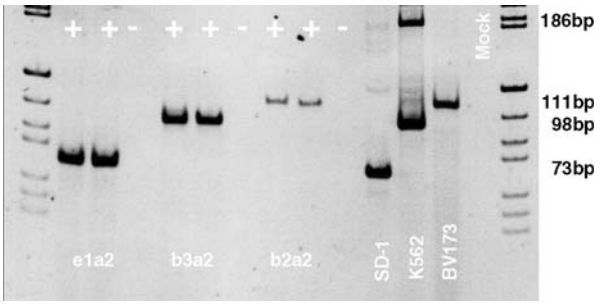

Fig. 4 Representative gel-electropheresis demonstrating BCR–ABL positivity in bone marrow trephines of patients with Ph+ CML (b3a2, b2a2) and Ph+ ALL (e1a2). Each case was analysed in duplicate by a multiplex RT-PCR along with a negative control. BCR–ABL positive cell lines (SD-1, K562, BV173) serve as positive control. Note that these primer design and primer mix respect the inevitable fragmentation of RNA in archival tissues by generating PCR products that are as small as required for an efficient RT-PCR approach. The two PCR products obtained in the K562 cell line derived from the exon-specific binding of the forward primers (b3–a2=98 bp, b2–[b3]–a2=186 bp, respectively). DNA marker, pBR322/Bsu RI. Note that it is digitally inverted

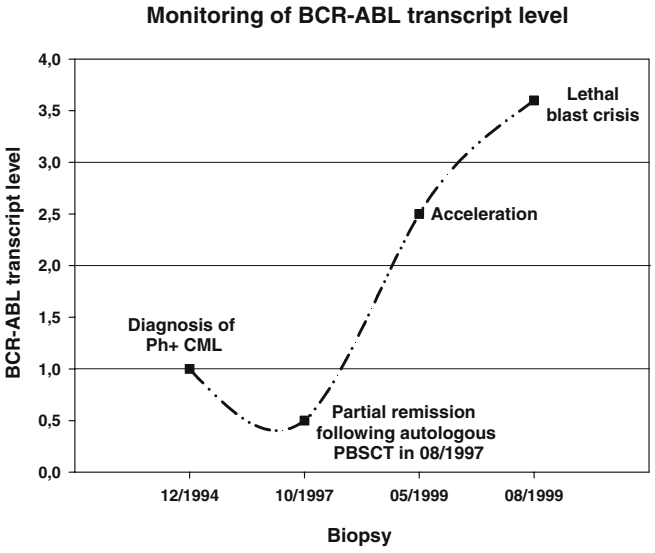

Fig. 5 Retrospective monitoring of BCR–ABL transcript levels in archival bone marrow trephines of a patient with Ph+ CML initially diagnosed in 1994. Declining levels could be correlated to a histopathologically demonstrable partial remission in 10/1997 after an autologous PBSCT in 08/1997. The phase of acceleration diagnosed in 05/1998 was accompanied with a 3.0-fold increase of BCR–ABL transcripts. The patient died in the blast crisis 3 months later after another increase of the BCR–ABL transcript level

(PBSCT), interferons and tyrosine kinase inhibitors [19]. Furthermore, the levels of BCR–ABL transcripts prior, during and after therapeutic intervention seem to predict the clinical outcome of the patients, and thus qualitative and quantitative detection of these transcripts from PBMC became a standard in clinical studies [65]. To transfer our knowledge from qualitative detection to quantitative monitoring of BCR–ABL transcripts, we investigated whether archival BM trephines of patients with proven CML are suitable for determination of the BCR–ABL transcript level at a given time point of the disease [6]. Retrospectively, we retrieved sequential BM trephines that have been taken from ten patients during the course of the disease, including more than 10-year-old plastic-embedded specimens. We were able to demonstrate that monitoring of BCR–ABL transcript levels in both plastic- and paraffin-embedded BM trephines is feasible in an accurate and sensitive manner. Moreover, the intra-individual BCR–ABL transcript level could be properly correlated with the histomorphological features at a given time point during the course of the disease. One representative time course of a CML is shown to demonstrate the BCR–ABL transcript levels in good correlation with therapeutic interventions and histopathological findings (Fig. 5).

Although the detection of BCR–ABL fusion transcripts currently is the most relevant molecular diagnostic assay in myeloproliferative disorders, several other applications have been described in the last years, e.g. suppressor gene methylation in myelodysplastic syndromes and expression of aberrant transcripts in chronic myeloproliferative disorders based on DNA and RNA extracted from paraffin-embedded trephines. Using either laser capture microdissection of immunostained BM sections or special PCR techniques for enrichment of the mutated allele, several groups demonstrated the occurrence of the activating c-kit mutation D816V in mast cells and lymphoid aggregates of patients with systemic mastocytosis [70, 76].

Another potential application of molecular diagnostics on BM trephines is the detection of the recently described activating point mutation V617F in the JAK2 gene, which is found in a high percentage of chronic myeloproliferative disorders other than CML [5, 39, 42, 50].

Other applications of molecular tests in BM pathology

Several other applications for molecular testing on BM biopsies have been described, and many assays performed on aspirates can be adapted to trephine biopsies. For example, the detection of tumor-specific fusion transcripts can also be applied to recurrent translocations of soft tissue tumors involving the BM, such as the t(11;22)(q24;q12) EWS/FLI-1 and t(21;22)(q22;q12) EWS/ERG translocations of Ewing sarcoma. In the future, minimal residual disease quantitation by real-time RT-PCR will play an increasing role for monitoring these patients under therapy [86]. In non-neoplastic disorders, detection of infectious agents such as *Mycobacteria* spp., especially in immunosuppressed patients, can be facilitated by molecular testing [1, 69].

Conclusions

In conjunction with other techniques such as immunohistochemistry and FISH [58, 72], the application of molecular genetics to paraffin-embedded BM trephines has significantly expanded the scope of histopathologic bone marrow examination. Some tests, such as PCR-based determination

of clonality, can help to resolve common diagnostic problems such as the nature of nodular lymphoid BM infiltrates in the setting of NHL. If the technical aspects of BM fixation and processing are observed and common pitfalls such as poor template quality avoided, the success rates for molecular studies in BM trephines are equal to other routinely fixed, archival tissues. The combination of excellent morphological detail and preservation of marrow architecture with the phenotypical and genetic information obtained from ancillary techniques makes the BM trephine biopsy a valuable resource for modern diagnostics and research in haematopathology.

Acknowledgements We thank Mrs. Birgit Geist for her excellent technical support. This study was supported in part by the Deutsche Forschungsgemeinschaft (DFG), grant FE 597/1 to FF, LQ-M and MK.

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