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review

Polymerase chain reaction procedures in the diagnosis of lymphoproliferative disorders

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Immunohistochemistry and molecular genetic techniques greatly contribute to our understanding of lymphoproliferative disease. The majority of lymphomatous lesions can be diagnosed by morphology alone but additional diagnostic tests have to be employed when cell lineage and clonality are not obvious. Morphologic distinction of Hodgkin's from non-Hodgkin's lymphoma, or of inflammatory lesions from malignant lymphoma, can be challenging. Non-random chromosomal translocations may help to recognize lymphoma subgroups with distinct biological characteristics. This review focuses on the polymerase chain reaction (PCR) techniques that have become an important diagnostic tool applicable to limited cellular material and paraffin-embedded tissues. The effectiveness of PCR in rearrangement analyses of T cell receptor g and immunoglobulin heavy chain genes is well documented. Some of the known translocations, such as t(14;18) or t(2;5) can be routinely assessed by genomic PCR or by reverse-transcription PCR. Those involving the bcl-1, bcl-6, and c-myc genes require more elaborate and sophisticated PCR procedures. Molecular genetic analyses by PCR, besides their immediate diagnostic value, bear the potential to identify new criteria for lymphoma diagnosis in conjunction with cytomorphology and immunophenotyping.

Key words: lymphoproliferative disorders-diagnosis; polymerase chain reaction; lymphoma-diagnosis

Introduction

Clonality of B cell proliferations, B or T lineage assignment, and maturation stage of lymphoid tumor cell populations are mainly established by immunophenotyping. This method largely fails to determine clonality in T cell tumors and to assign lineage to either very immature cells, which do not yet express these markers, or abnormally activated cells with loss of surface antigen expression. Lymphoma tissues may also be difficult to classify based on morphology and immunohistochemistry alone when the malignant clone is obscured by reactive lymphoid cells. The discovery of immunoglobulin (IG) gene

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rearrangements and the application of molecular probes for these genes has opened a new avenue to the diagnosis and biological understanding of lymphoproliferative disease. The discovery that rearrangement processes in T cell receptor (TCR) genes parallel the recombination events in immunoglobulin genes, led to the development of molecular tools applicable to the search for clonality in T-lymphoproliferative lesions.[1] Cloning of molecular breakpoints for tumor-specific chromosomal translocations permits the identification of a malignant clone using molecular probes for analysis of DNA simply extracted from lymphoid tumor tissue.[2] Data from molecular investigations with such probes now provide information about the origin of tumor lymphocytes as well as clues to molecular mechanisms involved in blastic transformation and tumor progression.

Gene rearrangement analyses by Southern blot procedures have become a valuable adjunct in the diagnosis of lymphoproliferative disorders.[3] This approach has serious limitations. The DNA has to be cut with several restriction enzymes. Hybridization with several probes is often required to obtain reliable and reproducible results. Large quantities (usually more than 20 µg) of high molecular weight DNA have to be extracted from fresh / fresh-frozen tissue or cell suspensions for these studies. The whole procedure takes several days, often weeks. Application of the polymerase chain reaction (PCR) techniques overcomes these limitations.[4] Specific DNA fragments can be rapidly amplified with oligonucleotide primers binding to both ends of a gene sequence of interest. Genomic DNA amplification techniques are not restricted to the analysis of fresh-frozen tissue samples with their limited morphological quality. DNA extracted from the more readily available formalin-fixed, paraffin-embedded tissue samples, even tiny areas of interest from a stained section or single cells with approximately 10 pg of DNA provide enough template for amplification.

This review focuses on well characterized PCR protocols for routine analysis of B and T cell neoplasms. Diagnostically important chromosomal aberrations will be considered and the advantages and limitations of molecular genetics in lymphoma diagnosis are discussed.

IG and TCR gene rearrangement

Mechanisms and methods

The only known rearranging genes are lymphocyte receptor genes for antigen, coding for either IG or TCR chains. Rearrangement involves random assembly of different variable (V), sometimes diversity (D), and joining (J) gene segments, which are discontinuously spread out within a chromosomal location in germ line configuration.[5] The recombinase activity is at least in part initiated by products of two recombinase activating genes, RAG1 and RAG2. Expression of these genes strictly correlates with V(D)J recombinase activity. Their transcripts occur in pre-B and pre-T cells, and are re-expressed during B cell selection in germinal centers where they may be involved in V gene replacement of rearranged IG genes.[6] A set of conserved nucleotides, heptamers and nonamers flanks the germline V, D, and J segments. They function as recombination signal sequences for V-D, D-J, or V-J joining, and are recognized by a recombinase enzyme system. These signal sequences are separated by a nonconserved spacer. The spacer situated 3' of the V or D gene segment is 21-23 bp long (about two turns of the DNA helix). It is 11-12 bp long (about one turn of the double helix) when located 5' of the D or J gene segment. Flanking sequences with a one-turn spacer signal can only rearrange to a two-turn signal. This probably ensures joining of

appropriate gene segments. Thus, one V and one J can recombine, but more than one D segment can join. TCR Vβ and Jβ heptamers, however, are virtually indistinguishable from those found next to immunoglobulin genes suggesting that the recognition devices for IG and TCR gene rearrangements are very similar. This explains the observation that T cells have occasionally D-J rearrangements of the IG heavy chain genes. Most commonly, the coding joint stays in the chromosome and a circular DNA molecule containing the signal joint and intervening sequences is excised. Intervening DNA stretches are retained if the two segments are joined in an opposite transcriptional orientation (conversion). At the coding ends, the joining is commonly imprecise. This happens through differential trimming of recombining gene termini by exonucleases and through duplication of one or two nucleotides at the recombination cleavage sites (P-nucleotides). Introduction of up to 15 nucleotides between V-D, D-D, D-J or V-J junctions in every possible random sequence generates non-template (N-) diversity. The enzyme terminal desoxynucleotidyl transferase (TdT) probably mediates addition of these nucleotides. N diversity contributes most significantly to the variability of the immune receptors, but it may also result in the generation of stop codons at the coding junctions.[7] In B cells, affinity maturation of the IG receptor to antigen in germinal centers is managed by extensive base substitutions in the rearranged V segments. Most of these mutations accumulate in the three complement-determining regions (CDRs). This process of somatic hypermutation apparently does not occur in TCR genes. Rearrangements involve different TCR and IG chains at different stages of lymphocyte ontogeny as defined by immunophenotyping. The first step in B cell development involves the D to J joining of the IG heavy chain genes (incomplete rearrangement) which precedes the V to DJ joining (complete rearrangement) forming a functional V region gene on one allele of the pre-B cell with cytoplasmic IGμ expression. Later during B cell ontogeny, the IG light chain (IGL) genes rearrange. Immature B cells, expressing either μk or μλ on the surface, result from the successful completion of heavy and light chain gene rearrangements. Similarly, a stepwise rearrangement of the immune receptor genes is found in cells committed to T lineage. Ninety to 95% of mature T cells carry the TCRαβ receptor on their surface, the remainder having TCRγδ chain heterodimers. Studies of precursor T-cell leukemia suggest that the TCRδ genes rearrange before the TCRγ genes. It is known that an incomplete DJ joining of the TCRβ chains is the next event and that a complete rearrangement of this gene locus follows. Finally, the TCRα chains rearrange. T cells expressing the TCRγδ receptors may not have rearranged their TCRα chain genes on both alleles, since the TCRd locus is nested between the Va and the Ja segments on chromosome 14q11, and rearrangement of these segments would result in a deletion of the TCRδ locus.

As a result of the maturation of lymphocyte progenitor cells, individual T cells with uniquely rearranged TCR genes and individual B cells with uniquely rearranged IG genes arise. Antigenic stimulation generates a polyclonal or oligoclonal lymphoproliferation under control of the immune system. Clonal populations will emerge if immune surveillance fails to control the lymphoproliferation. Clonality indicates autonomous growth of tumor cells which is an important diagnostic criterion for malignant lymphomas. TCR and IG gene rearrangement studies are therefore extremely valuable in the diagnosis of lymphoproliferative disease.

PCR primers for IG and TCR genes

PCR amplification requires less than 1 μg of template DNA. Degradation of the DNA as

present in formalin-fixed, paraffin-embedded material, stained sections or archival cytology smears does not seriously affect the reaction as long as the cell morphology is sufficiently preserved. Therefore, even small areas of interest in a stained tissue section can be scraped off and used as DNA source.[8] Rearranged IGH genes of single lymphoid cells from sections of fresh-frozen or fixed tissue specimens have been successfully amplified with V gene family-specific and consensus J region primers.[9] Prolonged over a week long with formalin or with B5 generally results in a less reliable amplification when compared to shorter formalin fixation times. Certain fixatives, such as Bouin's solution, or decalcification of tissue with ethylenediaminetetraacetic acid (EDTA) results in extensive DNA degradation so that, at best, only very short sequences can be amplified. Visualization of PCR amplification products on a high percentage agarose or a polyacrylamide gel normally identifies a clonal population, and rarely is Southern blot analysis or sequencing of the gene products necessary for routine applications. Because of its speed and simplicity, the PCR analysis is given priority for the diagnostic assistance in a difficult case. The rate of false-negative results is, however, higher than in Southern blot studies, so that the latter technique is still valuable in the case of unsatisfactory PCR results.

The CDR3 region of the VH gene segments, formed by the D gene segment and the V-D as well as the D-J junctions with the variable nucleotide deletions/insertions, can be amplified by PCR with primers that bind to consensus gene regions in IGH V framework regions (FR1, 2 or 3) and FR4 in the J regions.[10] Only complete VDJ rearrangements with the V and J sequences in the right orientation are amplified. Clonal incomplete DJ rearrangements, readily detectable by Southern blot using appropriate J and C region gene probes, cannot be amplified by PCR since the V regions are separated from the J segments by intervening sequences which are too long to be amplifiable. The use of other primers residing in the FR2 region is helpful in those cases where the FR3 primers fail to bind. V gene family-specific primers derived from the FR1 region are used in separate PCR reactions or combined in one so-called multiplex PCR, but they require a template DNA of reasonably good quality to allow for the longer PCR products (about 350 bp as compared to 120 bp when CDR3 region primers are used).

Similar to Southern Blot analyses, a background smear of amplified DNA is detected when polyclonal B cells are analyzed, since the length of the amplified fragment varies between individual B cells due to the different D gene sequences and the diversity of the flanking N regions. PCR products of one or two predominant sizes separated from the polyclonal background smear by gel electrophoresis are evidence of a clonal B cell population. This implies that the sensitivity of the (quantitative) PCR approach to recognize clonal immune receptor rearrangements at least matches the sensitivity of Southern Blot analyses.

TCR rearrangements are detected by an approach based on the same principles as the IGH gene analysis.[11] Mostly TCRγ gene rearrangements have been studied in T cell lymphomas. Even though it is impossible to find consensus primers for all the different V and J regions, the limited repertoire of these genes allows the use of primer mixes in a multiplex PCR which identifies most rearrangements of this gene. Given the lack of D region genes in the TCRγ locus, the size variability of the amplified V gene segments is based on the imprecise V-J joining only and therefore considerably lower than in IGH V segments. Another strategy is to use consensus primers and differentially cut the amplification products with restriction enzymes or determine the differences of the individual PCR products by gradient gel electrophoresis or single-strand conformational polymorphism (SSCP) analyses.

Primer sequences have also been established for the amplification of TCRβ genes. Several primers have been developed which recognize a set of different V, D and J region genes. Even though a substantial number of cases with clonal TCRβ gene rearrangements are missed, this methods seems to be useful as an additional test for clonally expanded T cells expressing the αβ heterodimer. TCRδ PCR has widely been used for the detection of clone-specific rearrangements in acute lymphoblastic leukemia. Primers established from the unique sequences of the VDJ or VDDJ junctions of leukemia blasts are clone-specific.[12] With such a clone-specific, quantitative PCR approach a sensitivity level of one in 10^6 cells can be reached which is well suited for minimal residual disease (MRD) detection. Amplification of TCRδ gene sequences has not been performed in a large series of lymphoma cases since the TCRδ genes are frequently deleted in mature T cell neoplasms.

Consensus Vα and Jα region primers for genomic amplification of TCRα gene recombinations are not used. Reverse transcribed cellular RNA can be amplified using C region oligonucleotides and degenerate 5' end primers for the cDNAs in a so-called RT-PCR.[13] Cloning and sequencing of the amplification products or separation of the fragments on a gradient gel or by SSCP analysis allows the detection of a predominant, clonal rearrangement. The PCR procedures for TCRα gene rearrangement detection are hence more sophisticated than TCRγ/δ PCR, require cell suspensions or fresh/fresh-frozen tissue and may not be widely applicable for routine diagnostic purposes.

PCR detection of IGH and TCR rearrangements in lymphoproliferative disease

The sensitivity and specificity of the PCR approach for the detection of T and B cell clonality has been tested extensively in the past. In several large series encompassing several hundred B cell non-Hodgkin's lymphoma (NHL) specimens 70-80% of cases studied with IGH PCR revealed clonal amplification products. Serial dilution experiments of clonal with polyclonal DNA suggest a clonal detection limit of 1% or lower. False positive results seem to be exceedingly rare when cross-contamination is avoided and appropriate controls are run with the clinical samples. Even though the amount of target DNA needed for successful amplification is low, it is advisable to use DNA concentrations corresponding to more than a hundred B cells. This helps to avoid preferential primer binding which may lead to a pseudoclonal amplification result with the IGH primers.[14] Immunohistochemical analysis of the specimen which should always be done prior to molecular analysis, provides an estimate of the proportions of T and B lymphocyte populations in the lymphoma tissue. Additional use of primers directed against the FR2 region of the IGH variable gene results in a higher detection rate of clonality (greater than 85%) in B-cell lymphoma cases than the employment of FR3-region primers only (about 75%). This is explained by a lack of primer binding due to deletions and extensive hypermutations occuring predominantly in the FR3 and less so in the FR2 region sequences. Alternatively, the consensus V region primers may not anneal to some rare or unknown VH genes participating in the IGH rearrangement. With FR3 primers, low-grade B-NHL with the exception of centroblastic/centrocytic lymphomas and those of mucosa-associated lymphoid tissue (MALT) nearly always have detectable clonal PCR products. The detection rate in high-grade B cell lymphomas ranges from 75 to more than 80%. The percentage of positive cases tends to be lower among centroblastic lymphomas (60 -70%) but not in large cell anaplastic lymphomas of B-type (B-LCAL) [Griesser, unpub-

lished]. Using both FR3/FR4 and FR2/FR4 primer sets we also detected clonal IGH rearrangements in 2 of 12 cases of nodular sclerosing and 4 of 8 mixed cellularity Hodgkin's disease (HD) in a recent study.[15] Among 7 samples of nodular lymphocyte predominant HD, only one case with a high-grade B cell lymphoma component showed clonal IGH rearrangement. Detection of B-cell clonality thus helps to distinguish B-NHL from lymphocyte predominant HD and favors a B-NHL over nodular sclerosing HD, but it is certainly not a reliable criterion in the differential diagnosis of mixed cellularity HD and T-cell rich B-cell lymphoma (TCRBL). The finding of more than three clonal PCR products indicates that more than one B-cell clone is present. Such biclonal or oligoclonal rearrangements are detectable in rare cases of follicular center cell-derived lymphomas and acute lymphoblastic leukemias (ALL).[16] The majority of B-NHL, however, has only one predominant clonal band in routine PCR examinations detectable with simple size selection procedures on agarose or non-denaturing polyacrylamide gels. Amplification of short PCR products with FR3 and FR2 primers is as effective with DNA extracted from well-preserved formalin-fixed tissues as with cellular DNA from fresh/fresh frozen samples. Even though clonality is detected in nearly all B-cell lymphoma cases by Southern Blot but missed by PCR in 10-20% of cases, we have seen rare cases that were PCR-positive and Southern Blot negative.[16] Primers established from the unique sequences of the VDJ junctions of leukemic lymphoma cells are clone-specific. With such a allele-specific primers PCR detection of MRD reaches a sensitivity level of one in 10^6 cells.[17]

TCRγ PCR findings have been reported for a few hundred cases of T-NHL. Clonality is found in more than 85% of the lymphoma samples with no false-positive results. A combined investigation of T cell neoplasms with primers for both, TCRγ and TCRβ, may lead to even superior results. Our studies on 48 LCAL cases has shown that TCRγ-PCR helps to identify T lineage in more than half of the tumor cell populations lacking surface expression of T cell markers in routinely-fixed samples.[15,18] Investigations of ALL samples were mostly performed for MRD detection. A positive result in bone marrow-derived DNA seems to have clinical relevance in predicting relapse. Detection of circulating tumor cells, however, appears clinically insignificant for lymphoma patients in otherwise complete remission.[19]

Biclonal or oligoclonal TCRg rearrangements are sometimes detected by PCR in angioimmunoblastic lymphadenopathy (AIL)-like T-cell lymphomas and rarely in cutaneous T-cell lymphomas. A considerable proportion of $CD3^-$, $CD56^+$ natural killer cell-like large granular lymphocyte (NK-LGL) leukemias/lymphomas lack TCR rearrangements by PCR as well as Southern blot studies. At least some cases from female patients can be studied for clonality with alternative PCR methods, such as analyses for methylation patterns of the human androgen receptor genes.[20]

Unexpected results in rearrangement analyses by PCR

Failure to detect clonal rearrangements can have technical reasons. A clonal cell population may not be well represented in the specimen used for DNA extraction; the DNA may be severely degraded and not even suitable for PCR amplification; DNA preparations from routinely processed tissue may contain PCR inhibitors. In the latter two instances PCR amplification of ubiquitous genes will fail in control experiments. Some AIL-type T-NHL and stage I and II mycosis fungoides may not contain molecularly detectable T cell clones. The absence of a clonal gene rearrangement in the presence of a histologic pic-

ture typical for malignant lymphoma is diagnostically irrelevant. Examinations can be repeated in subsequent biopsies or specimens taken from a different site if molecular genetical support of the diagnosis is crucial.

Complete IGH rearrangements are a feature of B-cells, complete TCR rearrangements highly characteristic of T- cells. Cross-lineage rearrangements detected in Southern blot experiments mostly represent incomplete erroneous immune receptor gene rearrangements or, more rarely, translocation events involving the IGH or TCR gene locus. The detection of clonal IGH rearrangements by PCR has an advantage over the Southern blot procedure since only complete VDJ rearrangements will be amplified enzymatically, but not incomplete rearrangements or rearrangements resulting from chromosomal translocations involving the IGH gene locus. Incomplete cross-lineage IGH rearrangements in neoplastic T cells, which are detectable in Southern Blot analyses, will not be amplified by PCR which makes lineage assignment to the B cell series straightforward. However, complete TCR cross-lineage rearrangements may be detectable by PCR in cases of common ALL and pre-B lymphoblastic lymphoma. In addition to the IG genes, several TCR loci frequently undergo rearrangement and these illegitimate rearrangements may be complete. This renders genotypic lineage determination in such cases by Southern blot procedures or PCR unreliable.

In AIL-type T cell lymphomas IGH rearrangements are not infrequently detected by PCR and are likely to be due to the existence of a true B cell clone coexisting with the neoplastic T cell population. Recently we found some cases among TCRBL samples with clonal IGH as well as TCRγ rearrangement in PCR studies. Morphology and immunohistochemical results suggested that these cases were indeed composite lymphomas with a high-grade B-cell and a low-grade T-cell component.[21,22]

The occurrence of clonal TCR rearrangements is diagnostically challenging in lymphoproliferative T cell disorders which are clinically considered non-malignant. Activated T cell clones especially in lymphoproliferations of the skin may expand to a point where they become detectable by rearrangement studies but remain localized and controlled by the immune system. Clonal cutaneous lymphoproliferations such as lymphomatoid papulosis (LYP) lesions, however, may coincide with or transform into a malignant T cell lymphoma. A clonal TCR rearrangement is of limited value for the differential diagnosis between LYP and a cutaneous LCAL. T lymphocyte clones may also expand due to persistent antigen exposure. These lymphoproliferations are mainly oligoclonal but it was shown that single reactive cytotoxic T cell clones may be detectable in healthy, elderly individuals.[23]

Molecular genetical studies require a dedicated laboratory with sufficient volume of samples and significant test experience. Failure to detect clonality, or the finding of unexpected or illegitimate TCR rearrangements is rare under these circumstances. Diagnostic problems arise particularly when molecular results are not correlated with histological and immunophenotypical findings. Problems with unexpected results are mostly avoided when molecular studies in a routine laboratory setting are restricted to diagnostically challenging cases and the search for minimal disease.

Common chromosomal translocations in malignant lymphomas

Different from stepwise rearrangement processes during B cell ontogeny, chromosomal translocations involving bcl-2, bcl-1, bcl-6 and c-myc can be viewed as an accident during B cell development and activation. They most frequently involve the IGH gene

locus on chromosome 14q32 and sometimes the IGL gene loci. In the majority of cases involving bcl-1 and bcl-2, translocations can be pinpointed to an early stage of B cell differentiation before the heavy chain genes have completed their rearrangement. Translocations of the bcl-6 gene can involve a number of chromosomes besides those harboring immune receptor genes. These chromosomal abnormalities not only serve as clonal markers but also aid in the classification of lymphoma subtypes.[24] Bcl-2/JH recombination is highly characteristic of germinal center cell derived lymphomas and bcl-1/JH recombination is mainly detected in mantle zone cell-derived lymphomas. The t(11;14) is not observed together with the t(14;18) translocation and bcl-2 rearrangements are undetectable in mantle cell lymphomas. The finding of one of these translocations is therefore useful in the differential diagnosis of follicular lymphomas and mantle cell lymphomas with a nodular growth pattern. Bcl-1 rearrangement analyses also aid in the identification of blastic variants of mantle cell lymphoma.[25] Among the high-grade NHL, c-myc rearrangements are a constant feature in Burkitt's lymphoma and bcl-6 translocation is frequently detected in centroblastic lymphomas. The only common and consistent translocation in T-lineage lymphomas results in the NPM/alk fusion which is almost exclusively found in T- LCAL.

Molecular probes are being generated through isolation and sequence analysis of regions flanking the chromosomal breakpoints. These probes are useful for the detection of chromosomal translocations in Southern blot analyses if the breakpoints are clustered and not spread out over a large chromosomal region. After identification and cloning of specific chromosomal breaks their sequences can be analyzed and tumor-specific PCR primers flanking the breakpoint be designed. This PCR approach is highly suitable for molecular follow-up studies in a particular patient since amplification products are only generated from cellular DNA carrying this translocation. Fusion gene transcripts from reciprocal chromosomal translocations are detectable by PCR amplification of cDNA (RT-PCR).

Mechanisms and methods

The bcl-2 oncogene is located on chromosome 18q21. The breakpoints on chromosome 18 in the t(14;18) translocation, which is very characteristic of germinal center cell derived lymphomas irrespective of the growth pattern and blast cell content, are clustered around two regions.[26,27] In about 70% of cases the breakpoint occurs in the major breakpoint region (MBR) which is located in the 3' untranslated region of bcl-2 exon III. In most of the remaining cases it occurs in the minor cluster region (mcr) located more than 20 kb 3' of the mbr. The breakpoint on chromosome 14q32 is found within the JH gene cluster. Translocation of the bcl-2 gene is found rarely in chronic B-lymphocytic leukemia involving predominantly IGL gene loci rather than the IGH gene locus; the breaks on chromosome 18 tend to be outside and usually 5' of the mbr/mcr. The bcl-2α and bcl-2β proteins are involved in cell death regulation and have been shown to block apoptosis. Bcl-2 overexpression as a result of the translocation t(14;18) may lead to inadequate survival of B cells and render them susceptible to additional genetic aberrations.

The bcl-1 locus resides on chromosome 11q13 and is involved in the translocation t(11;14). The chromosome 11 sequences join within the J region cluster of the IGH gene locus on chromosome 14q32. Again, one predominant breakpoint region is described (major translocation cluster, mtc) on chromosome 11.[28] At least two more sites have been identified where breakpoints occur within chromosome 11q13. Aside from occasional

cases of B-CLL and multiple myelomas this translocation is characteristic of mantle zone derived B-cell lymphomas where it is found in about 50% of the mantle cell lymphoma cases with mtc probes. With the additional use of probes for the minor breakpoint cluster regions the percentage may be even higher than 70%. In contrast to the bcl-2 oncogene, the bcl-1 locus does not harbor an oncogene but likely is linked to a regulatory gene sequence of PRAD1, which is telomeric to the breakpoint region. The translocation leads to constitutive expression of the gene product cyclin D1 which promotes passage through the G1 phase of the cell cycle.

Translocations involving the c-myc locus on chromosome 8q24 are somewhat more complex.[29] They are a constant finding in sporadic and endemic Burkitt' lymphoma cases and most frequently involve the IGH genes in a t(8;14). Rarely the IGκ genes on chromosome 2p12 or IGλ genes on chromosome 22q11 can also be translocated to the myc locus. The breakpoint on chromosome 8 in the t(8;14) generally lies 5' or within the c-myc gene, whereas in variant translocations t(2;8) or t(8;22) involving IGL chain loci the break occurs 3' of the c-myc gene at distances up to 300 kb. Translocations t(8;14) are characteristic of Burkitt's lymphoma. Two different breakpoints occur depending on the type of Burkitt's lymphoma. In most endemic cases (eBL), the break occurs more than 20 kb upstream of the myc locus and involves either the J_H or the switch m region on chromosome 14. In most sporadic types (sBL) exon I of the c-myc gene or 5' flanking sequences are involved, and more often the switch m or switch g regions than the J region take part on chromosome 14. Translocation into the JH or JL region suggest that the translocation occurs at a pre-B cell stage whereas translocation into the IG switch sequences potentially take place throughout the B cell differentiation process. Expression of c-myc is high in proliferating cells and rapidely induced in quiescent cells on mitogenic stimuli. In addition to mediating cell proliferation, c-myc is also implicated in blocking the cellular programs of differentiation. Highly proliferating cells with a differentiation block are prone to apoptosis. Translocation of c-myc gene generally results in constitutive expression of this otherwise tightly regulated oncogene. Besides translocations, mutations in certain cluster regions of the c-myc gene have a similar effect and are detected in more than 50% of Burkitt's lymphomas.[30]

Chromosomal alterations affecting the bcl-6 gene at band 3q27 are a frequent recurrent abnormality in high-grade B-cell lymphomas.[31] Several partner chromosomes are involved including the sites of the IGH genes at 14q32, the IGk genes at 2p11 and the IGλ genes at 22q11. Bcl-6 seems to function as a transcription factor that binds specific DNA sequences and represses transcription from linked promoters.[32] High Bcl-6 expression is restricted to mature B-cells inside the germinal centers and has been shown to be a key regulator of germinal center formation and B-cell immune response. Chromosomal translocations usually disrupt the gene in and around the first exon leaving the coding domain intact. On the partner chromosome the bcl-6 coding domain is juxtaposed to a heterologous promoter resulting in a new chimeric transcript which encodes a normal bcl-6 protein. The promoter substitution prevents bcl-6 downregulation and blocks post-germinal center maturation of B-cells. Bcl-6 rearrangements are highly specific for high-grade B-NHL of centroblastic type where they are detected in 35% of cases. Additionally they are found in a small fraction of follicular lymphomas. Additionally, in about 70% of the high-grade B-NHL and 50% of follicular lymphomas the bcl-6 gene is affected by multiple, often biallelic mutations clustering within the 5' non-coding sequences.[33] It has been suggested that those large cell lym-

phomas only with bcl-6 rearrangement may represent de-novo high-grade B-NHL whereas those with bcl-6 and/or bcl-2 alterations may be secondary high-grade lymphomas with a less favorable prognosis.[34,35]

Chromosomal translocations may result in a novel fusion transcript that can be detected by RT-PCR. This is the case in LCALs with the translocation t(2;5) (p23; q35).[36] The nucleolar phosphoprotein nucleophosmin (NPM) gene on chromosome 5q35 is translocated to the anaplastic lymphoma kinase (alk) on chromosome 2p23 in about 60% of adult and over 80% of childhood LCAL of T- or 0-type.[37] The result is a chimeric protein with the amino terminus of the gene coding for NPM and the carboxy terminus coding for alk. Replacement of the 5' alk sequences switches the transcription regulation of the catalytic sequences of alk to the NPM promoter. Regulatory sequences of the NPM gene, which is transcriptionally most active before the cell entry into the S phase, may activate the alk gene. This kinase gene is physiologically not transcribed in lymphocytes. The fusion protein of the two genes could potentially phosphorylate intracellular substrates which are normally under control of lymphoid lineage-specific kinases only. This deregulation eventually triggers the malignant transformation of T-lineage cells.

PCR detection of chromosomal translocations

The molecular detection of these abnormalities is most reliably done by conventional Southern blot technique that identifies abnormal restriction fragment gel mobilities. The use for routine analysis, however, is limited since the variability of the breakpoints requires the application of several probes and DNA restriction with several enzymes. This also explains why rearrangements are less frequently detected by PCR, which is performed with a limited set of primers (since not all the sequence data for the exact breakpoint locations are available) and can only amplify short DNA stretches in routine applications. On the other hand, once a translocation is detected by the PCR approach in individual patients, it is far more sensitive as a clonal marker than the Southern Blot procedure.

Translocations t(14;18) involving the IgH gene locus and the bcl-2 gene region are detected by PCR with consensus primers for the JH region of the mbr or the mcr region.[38-40] Sensitivity and specificity are enhanced if sets of outer and inner primers are sequentially applied in the so-called nested PCR reaction. Alternatively, Southern blotting of PCR products with subsequent probing can be done using a radioactive-labeled internal oligonucleotide.

Chromosomal rearrangements of the bcl-1 gene locus have been amplified by PCR using mtc breakpoint oligonucleotides.[41,42] This approach is not reliable enough for routine diagnostic purposes since various minor breakpoint sites are also involved in mantle cell lymphomas. Designing, testing and using many different primer sets for these additional breakpoint locations is impracticable in a routine laboratory setting.

A limited number of cell lines and some Burkitt's lymphoma samples have been analyzed by PCR using primers for the switch region of the μ heavy chain gene, and sequences flanking and inside exon I of c-myc. With this approach it is possible to detect some of the translocations t(8;14) in sBL cases.[43] With new generations of Taq polymerases it has become possible to amplify sequences of more than 10 kb length. Using primers for IG constant region genes and primers flanking sequences 5' to breakpoint cluster regions it is possible to amplify a considerable proportion of those c-myc and bcl-6 translocations by long-distance (LD-) PCR that were formerly only detectable by

Southern blot.[44] However, template DNA has to be extracted from fresh/fresh-frozen tissue samples or cell suspensions and routinely-processed specimens cannot be analyzed.

The presence of chimeric mRNA from the NPM/alk fusion is detected by RT-PCR.[45] Even though RNA extracted from routinely-processed specimens is strongly degraded reverse transcribed cDNA fragments of 300 bp are readily amplifiable. This leads to the detection of fusion transcripts in 40-60% of T-LCAL cases by RT-PCR.

Advantages and drawbacks of translocation analyses by PCR

Most of the experience with the detection of translocations by PCR in malignant lymphoma has been accumulated for the t(14;18) abnormality. About 80% of the Southern blot-positive follicular lymphomas are PCR positive suggesting that PCR analysis for bcl-2/IGH translocation is a good molecular marker for the t(14;18) abnormality in follicular lymphomas. The PCR technique is more sensitive in the detection of qualitative rather than quantitative abnormalities such as clonal immune receptor gene rearrangement on a background of polyclonal cells. It is therefore well suited for analysis of minimal disease in lymphomas carrying the t(14;18). Reports about positive PCR results in follicular hyperplasia should be considered when looking for MRD.[46,47] The (overly) high sensitivity may be achieved by using high amounts of template DNA (> 1μg) in the reaction and very efficient primers and cycling conditions. Similarly, the high degree of sensitivity may also be responsible for some reports about surprisingly frequent detection of t(14;18) PCR products in Hodgkin's lymphomas. False-positive PCR results have to be avoided in MRD detection. DNA from the original tumor should be run in parallel when looking for minimal infiltration. Identity of the amplification products is confirmed by identical size on polyacrylamide gels and/or by sequencing. A quantitative PCR approach would also help to establish the relative number of cells in the sample which contain the rearrangement, but this approach usually goes beyond routine analysis.

It is even better not only to detect chromosomal abnormalities with high sensitivity but also to identify the cells carrying the genetic abnormality. This is possible using another fast molecular diagnostic approach: the detection of translocations by non-radioactive, usually fluorescence-based, in-situ hybridization of chromosomes (FISH). The probes detect chromosomal DNA either from metaphase spreads or non-mitotic cells.[48] The latter so-called interphase cytogenetic analysis can be done even on previously stained cells. Simultaneous immunophenotyping of the cells helps to focus the interpretation of the results on the tumor cell population.[49] Genomic DNA probes flanking the breakpoint on both sides and derived from different chromosomes are labelled with two different fluorochromes.[50] The two signals would be located on different chromosomes in a metaphase spread or found far apart in interphase cells if the translocation is absent. Joining of genetic material from the two chromosomal localizations, in contrast, leads to a doublet fluorescence signal after hybridization. Results from FISH analysis are usually available within two days, which favourably compares to the Southern blot procedures. When more translocation-specific probes become available, molecular cytogenetics promises to be a fast and simple technique for the detection of nonrandom chromosomal abnormalities. Though less sensitive than PCR detection, FISH is potentially of higher specificity in tracing single tumor cells carrying a specific anomaly. The hybridization efficiency of FISH probes in sections from archival material depends on the fixative and the age of the paraffin blocks. A negative

result in these instances is diagnostically not helpful.

Conclusions

Molecular genetic analysis has become routine for laboratories involved in lymphoma diagnosis. If precautions against sample contamination are taken, the PCR technique generates reliable results within one or two days when applied to routinely processed biopsy samples and cytology specimens containing only a hundred cells. Standard PCR procedures include assessment of lymphocyte clonality using primers for TCRγ and IGH genes as well as the search for a t(14;18) in B cell neoplasms of putative follicular center cell origin. If the results are unsatisfactory, additional primer combinations may be used and/or fresh/fresh-frozen tissue has to be used for DNA studies with Southern Blot techniques. Since no primer sequences suitable for PCR amplification of TCRα and IGL rearrangements are available, these tests are performed by Southern blotting. This is also the method of choice for an in-depth analysis of chromosomal translocations involving the c-myc, bcl-1, bcl-6 or bcl-2 loci, for example in the setting of prospective clinical studies. RT-PCR is successfully applied for the detection of t(2;5) translocations in LCALs because of the fusion transcripts generated by this abnormality. Limitations of the molecular techniques are well recognized and can be handled in an experienced laboratory. The diagnostic process should begin with the cytomorphological evaluation of the specimen. Even if microscopic examination does not lead to a conclusive diagnosis, it provides a hypothesis for immunohistochemical and, ultimately, molecular genetic testing. Molecular test results have to be viewed in the context of morphology and immunohistochemistry. The detection of a clonal lymphocyte population in clinical samples is always abnormal but it should never be considered a proof of malignancy. Similarly, genomic instability may result from abnormal lymphocyte activation or destabilizing influences upon chromosomal DNA leading to translocations. Some of these translocations seem to be necessary but are not sufficient for lymphomagenesis. Thus, the detection of translocations in rare cells by a highly sensitive PCR procedure should not be taken as evidence of malignant lymphoma out of the cytomorphological context. On the other hand, a PCR result can be negative for clonality for many reasons besides the absence of a clonal lymphocyte population, such as lack of primer binding, suboptimal PCR conditions or problems related to the extraction of suitable template DNA or RNA.

Keeping the limitations in mind, immune receptor gene rearrangement studies are well suited to define lineage and clonality of a lymphoproliferation. Analyses of chromosomal translocations, in addition, provide information about the lymphoma subtype, its biologic behavior and mechanisms of lymphomagenesis. Furthermore, the high sensitivity and specificity of the PCR-based molecular tests are most helpful for monitoring lymphoma therapy, identification of MRD and early diagnosis of relapse. In an optimal laboratory setting, cytomorphologic, immunophenotypic and molecular results are interpreted jointly by individuals with expertise in all three methods in order to achieve the best diagnosis possible for the patient.

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