Histochem Cell Biol (1997) 108:391–402

ORIGINAL PAPER

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Fluorescence in situ hybridization as a diagnostic tool in malignant lymphomas

Accepted: 30 July 1997

Abstract Primary and secondary chromosomal abnormalities play an important role in the characterization of biological, pathological, and clinical subgroups of malignant lymphomas. The introduction of fluorescence in situ hybridization (FISH) and the combination of immunophenotyping plus FISH to the diagnosis of lymphatic neoplasms allows the fast and sensitive detection of specific chromosomal changes and provides new insights into the genetic basis of lymphomagenesis. This article reviews the possibilities and limitations of molecular cytogenetic techniques in comparison to cytogenetic and molecular genetic methods and discusses their clinicopathological impact for non-Hodgkin's lymphoma and Hodgkin's disease.

Introduction

During the past two decades, since the first description of a recurrent translocation, t(8;14), in Burkitt's lymphomas by Zech and coworkers in 1976 (Zech et al. 1976), a number of characteristic chromosomal translocations in malignant lymphomas has been identified. The subsequent cloning of the breakpoints involved and the isolation of the affected genes provided insights into the molecular mechansims by which recurrent chromosomal translocations such as t(8;14), t(14;18), t(11;14) or t(2;5) contribute to lymphomagenesis. Finally, comparison of genetic, pathological and clinical features revealed close associations between some chromosomal changes and distinct clinico-pathological entities of lymphomas (Table 1, 2; Offit 1992).

Our current understanding of the biological and clinical significance of genetic alterations in malignant lymphomas relies on the new techniques developed recently. The introduction of banding techniques allowed the exact description of chromosomal aberrations, in particular the precise localization of chromosomal breakpoints. The development of molecular DNA technologies enabled the identification and characterization of genes affected by cytogenetically defined aberrations. The introduction of the polymerase chain reaction (PCR) finally provided a means for highly sensitive detection of clonal aberrations in archival tissue and in samples with a low percentage of tumor cells.

Cytogenetic and molecular genetic analyses have disclosed substantial pathogenetic mechanisms in malignant lymphomas and, in many instances, they have become valuable tools in the diagnostic process (Harris et al. 1994). Nonetheless, their application is, a priori, limited due to methodological considerations. Cytogenetic analysis, for example, requires metaphase spreads and, therefore, viable and proliferating cells are needed. Thus, it cannot be performed on fixed cells. Additionally, the procedure of karyotyping is time consuming and cytogenetic evaluation is in many cases hampered by a low yield or bad quality of tumor cell metaphases. Furtheron, the metaphases analyzed need not necessarily represent the malignant clone in vivo, but could result from culture artifacts developed in vitro. The application of DNA technologies such as Southern blotting and PCR analyses is restricted to the detection of certain structural chromosomal aberrations. Additionally, the quantification of the malignant cells by these methods is nearly impossible. Loss of genetic material, e.g., loss of tumor suppressor genes during the progression of malignant lymphomas, is frequently not detectable by either cytogenetics or molecular genetics because the deletions are often too small to be detected in metaphase chromosomes and because the portion of cells carrying this aberration in the sample is below the detection level of loss of heterozygosity studies.

Recently, some of the methodological limitations of cytogenetics and molecular genetics in the diagnosis of genetic aberrations in malignant lymphomas have been overcome by the introduction of molecular cytogenetic techniques, such as **f**luorescence **i**n **s**itu **h**ybridization

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Table 1 Recurrent chromosomal aberrations in malignant lymphomas (Rabbitts 1994) (*NHL* non-Hodgkin's lymphoma)

Chromosomal aberration	Genes involved		Histopathological subtype
Peripheral B-cell lymphomas			
t(14;18)(q32;q21)	IgH	bcl-2	Follicular NHL
t(11;14)(q13;q32)	bcl-1	IgH	Mantle cell NHL
t(14;19)(q32;q13)	IgH	bcl-3	Chronic lymphocytic leukemia (B-lineage)
t(3;14)(q27;q32)	bcl-6	IgH	Diffuse large cell NHL
t(3;22)(q27;q11)	bcl-6	Igλ	Diffuse large cell NHL
t(2;3)(p12;q27)	Igκ	bcl-6	Diffuse large cell NHL
t(8;14)(q24;q32)	c-myc	IgH	Burkitt's-type NHL, lymphoblastic NHL
t(8;22)(q24;q11)	c-myc	Igλ	Burkitt's-type NHL, lymphoblastic NHL
t(2;8)(p12;q24)	Igκ	c-myc	Burkitt's-type NHL, lymphoblastic NHL
Peripheral T-cell lymphomas			
inv(14)(q11;q32.1)	TCR-α	IgH	Chronic lymphocytic leukemia (T-lineage)
t(14;14)(q11;q32.1)			Prolymphocytic leukemia (T-lineage) Cutaneous T-cell NHL
t(X;14)(q28;q11)	C6.1B	TCR-α	Prolymphocytic leukemia (T-lineage)
i(8)(q10)	–	–	Chronic lymphocytic leukemia (T-lineage) Prolymphocytic leukemia (T-lineage) Cutaneous T-cell NHL Pleomorphic T-cell NHL
t(2;5)(p23;q35)	alk	npm	Large cell anaplastic NHL

Table 2 Correlations of secondary chromosomal aberrations and clinico-pathological features in NHL (Offit and Chaganti 1991; Offit 1992)

Chromosomal aberration	Clinical significance
+7; +12	Transformation to a higher histological grade; decreased survival
1p; 1q21-23; 6q; 17p	Transformation to a higher histological grade; decreased survival
1p32-36; 6q22-24; -11	Bone marrow involvement
6q11-16	B-symptoms
3q21-25; 13q32	Bulky disease
14q22-24	Spleen involvement
2p; 3p; 14	Cutaneous involvement
del(6)(q23); +11	Meningeal involvement
11q	Gastrointestinal involvement

(FISH; Pinkel et al. 1986; Lichter and Ward 1990; Lichter et al. 1990) and **f**luorescence immunophenotyping and **i**nterphase **c**ytogenetics as a **t**ool for **i**nvestigation **o**f **n**eoplasia (FICTION; Weber-Matthiesen et al. 1992, 1993a–c). This article briefly reviews the principles of a diagnosis of genetic aberrations in lymphomas by FISH and FICTION and the potential of these techniques to obtain new insights in lymphoma biology.

Molecular-cytogenetic investigations for the detection of translocations in non-Hodgkin's lymphomas

A number of recurrent chromosomal aberrations are closely associated with the tumorigenesis of distinct pathological subtypes of non-Hodgkin's lymphomas (NHL; Harris et al. 1994; Table 1). Many of these recurrent aberrations are primary abnormalities, i.e., they play a major role during the initial phase of lymphomagenesis. Other alterations are considered as secondary abnormalities, which are acquired during lymphoma progression (Offit 1992; Johansson et al. 1995).

Primary aberrations are predominantly translocations. A translocation is defined as an exchange of genetic material between two chromosomes. Some of the most important translocations in NHL are t(14;18), t(8;14), t(11;14), and t(2;5).

Translocation t(14;18)(q32;q21)

Cytogenetically, the t(14;18)(q32;q21) translocation can be detected in 80–85% of follicular NHL, 30% of diffuse large cell NHL and sporadically in Burkitt's lymphoma (Offit and Chaganti 1991; Offit 1992). Among the diffuse large cell NHL, t(14;18) characterizes a subgroup of patients with poor prognosis (Offit et al. 1994a).

On the molecular level, the t(14;18) translocation juxtaposes the IgH locus in 14q32 next to the bcl2 gene in 18q21, leading to overexpression of the Bcl-2 protein, which prevents cells from undergoing apoptosis. The breakpoints in 14q32 are mainly located in the J (joining) region of the IgH gene, whereas 50–60% and 25% of the breakpoints in 18q21 cluster in the 150 bp-span-

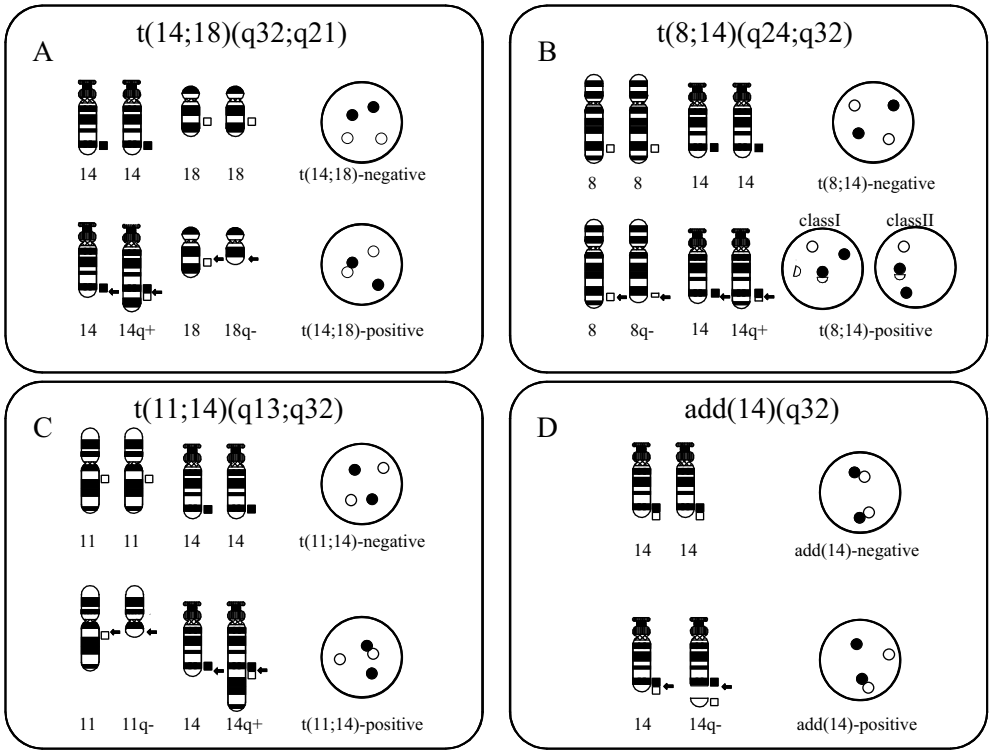

Fig. 1A–D Schematic representation of interphase fluorescence in situ hybridization (FISH) assays for the detection of the most frequent chromosomal translocations in B-cell non-Hodgkin's lymphoma. Ideograms representing the expected localization of signals by two-color FISH assays in t(14:18) (**A**), t(8;14) (**B**), t(11;14) (**C**), and add (14) (**D**) negative and positive metaphase and interphase cells. *Black:* Probe hybridizing centromeric to the breakpoint region of the IgH locus in 14q32, *white:* probes hybridizing telomeric to the bcl-2 (**A**), bcl-1 (**C**) or IgH (**D**) locus or spanning the breakpoint region of the c-myc gene in 8q24 (**B**)

ning major breakpoint and the 500 bp-containing minor cluster region, respectively (Cleary and Sklar 1985; Cleary et al. 1986; Bakhshi et al. 1987).

We recently developed a FISH system for the detection of t(14;18) in interphase nuclei by means of two YACs hybridizing to the bcl-2 gene and the IgH gene (Fig. 1A; Silverman et al. 1990; Choi et al. 1993). Thus, cells carrying the translocation are indicated by the colocalization of a single bcl-2 and IgH signal (Poetsch et al. 1996). In our study, 28 follicular NHL were investigated by cytogenetics, PCR, and FISH. The t(14;18) translocation was detected in 64% of the samples by PCR, in 86% by cytogenetics, and in 100% by FISH. The results showed that, at the time of diagnosis, FISH is the most sensitive technique for detecting a t(14;18) translocation. In cytogenetically t(14;18)-negative NHL, the percentage of FISH-positive nuclei was considerably lower than in cytogenetically positive cases. In the latter, the proportion of positive metaphase cells was higher than the percentage of positive interphase cells. This suggests a growth advantage of t(14;18)-positive cells in vitro. Remarkably, by FISH the t(14;18) was also detected in 2–5% of cells derived from lymphoid tissue affected with non-neoplastic lymphoproliferation. By FICTION we were able to assign the t(14;18)-carrying cells exclusively to the B-lymphocyte compartment (Poetsch et al. 1996; Siebert et al. 1997). The FICTION technique is discussed later in this review.

Translocation t(8;14)(q24;q32)

By cytogenetics, the t(8;14)(q24;q32) translocation is found in the majority of Burkitt's-type lymphomas/leukemias, and sporadically too in other high-grade B-cell lymphomas. The t(8;14) juxtaposes the c-myc gene in 8q24 next to the IgH locus in 14q32, resulting in overexpression of the transcription factor c-Myc. As the 8q24 breakpoints are distributed over a region of about 600 kb, the detection of t(8;14) and the variant translocations t(2;8)(p11;q24) and t(8;22)(q24;q11) by molecular genetic techniques is limited (Pelicci et al. 1986; Neri et al. 1988; Shiramizu et al. 1991; Joos et al. 1992a). Therefore, we and others established interphase FISH approaches for the detection of t(8;14). Ried et al. (1992) described a triple-color FISH assay using a chromosome 8 plasmid DNA library (painting probe) in combination with two probes hybridizing to either side of the c-myc breakpoint in 8q24. Lishner et al. (1993) established a double color FISH system for the detection of the t(8;14) using plasmids hybridizing to the joining region of the IgH locus and to the c-myc gene, respectively. Veronese et al. (1995) reported a double color approach by use of two differently labelled YACs spanning the breakpoint region in 8q24. By this assay, the breakage in 8q24 results in the dissociation of a two color YAC signal. Since this approach detects the breakage in the c-myc locus, it is suitable to detect both classical and variant Burkitt's translocations. The affected partner chromosome is, in this approach, identified by additionally employed centromeric or telomeric probes for the corresponding chromosome. However, due to the spatial separation of the probes applied on the derivative chromosome, the resulting hybrid signal may be hard to detect reliably in interphase cells due to chromatin decondensation.

We established another FISH system employing one probe hybridizing to the IgH constant region in 14q32 and a second probe consisting of different clones spanning the c-myc locus in 8q24. Within interphase nuclei, a t(8;14) translocation should lead to a split of the c-myc probe and, in addition, to the colocalization of one of the split signals with the 14q32 probe (Fig. 1B). This approach is similar to that described by Joos et al. (1992b). Based on these stringent criteria, the cut-off level was set at 2%. If only one criterion, namely the colocalization of one 8q24 signal with one 14q32 signal alone (without signal split for the c-myc probe) was used a cut-off limit of 11% was determined. We investigated nine Burkitt's-type lymphomas/leukemias and one Burkitt-like lymphoma by this FISH approach. Cytogenetically, nine of these ten tumors contained a translocation, t(8;14)(q24;q32). In interphase FISH, all tumors met the less stringent criteria. Except for the Burkitt-like NHL, all cases were also positive according to the stringent criteria for the detection of t(8;14). In cytogenetically positive cases, the percentage of metaphases containing the t(8;14) translocation was significantly higher than the percentage of interphase nuclei found to harbor t(8;14) by FISH. Thus, as for t(14;18)-positive cells, t(8;14)-positive cells appear to have a growth advantage in vitro (R. Siebert et al. submitted).

Translocation t(11;14)(q13;q32)

The chromosomal translocation t(11;14)(q13;q32) is the hallmark of mantle cell lymphoma (MCL) in which it can be detected cytogenetically in about 75% of cases (Raffeld and Jaffe 1991; Banks et al. 1992). The t(11;14) juxtaposes the IgH locus in 14q32 next to the bcl1 locus in 11q13 and, thus, leads to deregulation of the cell cycle regulatory protein cyclin D1, which is encoded by the CCND1 gene localized at the telomeric border of the bcl1-locus (Motokura et al. 1991; Motokura and Arnold 1992). MCL has the worst prognosis among all low-grade NHL (The Non-Hodgkin's Lymphoma Classification Project 1997). In some instances, however, the pathological differentiation between MCL and other low-grade B-cell NHL is difficult. Therefore, the detection of t(11;14) is of essential diagnostic value for the risk-adjusted management of patients with MCL (Weisenburger and Armitage 1996). As the 11q13 breakpoints are scattered over a region of more than 120 kb bordered distally by the CCND1 gene and proximally by the major translocation cluster (MTC), the application of molecular genetic techniques is limited (Tsujimoto et al. 1984, 1985; Meeker et al. 1989, 1991; de Boer et al. 1993; Rimokh et al. 1994). So far, only a small series of MCL has been studied for the presence of t(11;14) by use of interphase FISH (Zucca et al. 1995; Bigoni et al. 1996; Monteil et al. 1996; Coignet et al. 1996). In this series, different single- and double-color FISH assays were applied. In the published single-color FISH approaches a t(11;14) translocation was indicated by the splitting of a YAC DNA probe spanning the bcl1 locus (Bigoni et al. 1996) or by splitting of a chromosome 11 painting probe (Zucca et al. 1995). Nevertheless, these signal splits do not necessarily designate cells carrying a t(11;14) translocation but only a bcl1 rearrangement. Additionally, trisomy 11 has to be ruled out. Furthermore, the detection of signal splits of a bcl1 YAC may be unreliable as the part of the YAC remaining on the aberrant chromosome 11 may be too tiny and, thus, too hard to detect in the interphase nuclei of MCL. On the other hand, bcl1-containing YACs have been found to be highly unstable (Szepetowski et al. 1995). Therefore, rearrangements of such YACs leading to loss of parts of their inserts hybridizing proximal to the MTC have to be considered. Additionally, in the routine diagnostic scenario, a proof for the juxtapositioning of parts of bcl1 and IgH loci has to be provided. The latter proof is also required for those double-color FISH assays using probes flanking the 11q13 breakpoint (Coignet et al. 1996).

By use of cosmids hybridizing distal of the CCND1 gene in 11q13 and proximal breakpoint region in 14q32, a recent double-color FISH approach provides a reliable one-step system for the detection of t(11;14) in interphase cells (Monteil et al. 1996). Unfortunately, this latter assay suffers from a low sensitivity, as the mean proportion of false-positive control cells has been reported to be 8%; Considering the standard deviation of 3.7% the cut-off level for detecting a t(11;14) translocation has to be set at 19%, which is not acceptable for routine use.

We also established a double-color FISH assay for the detection of the translocation using a YAC probe spanning the breakpoint region in 11q13 and a cosmid probe hybridizing proximal to the J_H region in 14q32. Cells with a t(11;14) translocation show a colocalization of the signals for IgH and bcl1 (Fig. 1C). According to our control studies, samples containing more than 10% of cells with this signal constellation can be diagnosed as carrying a clonal t(11;14). We investigated the feasibility and sensitivity of this approach in a series of 15 MCL. All 11 MCL found to carry the t(11;14) translocation by chromosome analysis were positive in our FISH assay. Additionally, two of four MCL lacking a clonal t(11;14) by chromosome analysis were shown to carry this aberration in 14% and 37% of interphase nuclei, respectively. Southern blot data indicate that our FISH assay reliably detects t(11;14) independent of the location of the breakpoints within the bcl1 region (R. Siebert et al., submitted). The addition of a third probe hybridizing distal of the breakpoint in 14q32 might even improve the sensitivity and specificity of our approach, as a t(11;14) translocation would be indicated by two criteria, namely, splitting of the signals for the IgH locus and colocalization of one of these signals with a bcl1-signal (Döhner et al. 1997). Nevertheless, no data on the feasibility and reliability of this approach for routine detection of t(11;14) in MCL have been published so far.

Other recurring translocations with breakpoints in 14q32

The most frequent structural chromosomal aberrations in B-cell lymphomas are translocations involving the IgH locus in 14q32. In addition to the translocations t(14;18),

t(8;14) and t(11;14) described above, there exist other primary chromosomal aberrations with breakage in 14q32, e.g., the translocations t(14;19)(q32;q13) and t(3;14)(q27;q32) (Table 1). They result in the juxtaposition of the IgH gene next to the bcl3 gene in 19q13 and the bcl6 (laz-3) gene in 3q27, respectively (Baron et al. 1993; Michaux et al. 1996). The t(3;14) translocation can be detected in different subtypes of B-cell lymphomas. In diffuse lymphomas with a large cell component, the molecular counterpart of this translocation, a bcl6-rearrangement, has been reported to characterize a subgroup of patients with frequent extranodal sites of disease and a favorable prognosis (Offit et al. 1994a).

In principle, all translocations with breakpoints in the IgH gene can be detected by FISH systems similar to those described for t(14;18), t(8;14) and t(11;14), using probes for each of the affected chromosomal regions. In addition, a screening for 14q32 aberrations by FISH is possible by use of two differently labelled probes hybridizing to either side of the IgH locus (Taniwaki et al. 1995). By this approach, cells with two normal chromosomes 14 display two-color signals for the intact IgH genes. Translocations disrupting the IgH-locus lead to a dissociation of one two-color signal, which is detectable in interphase cells (Fig. 1D). Ueda et al. (1996) have used this approach in a comparative cytogenetic/molecular cytogenetic study of 70 B-cell lymphomas. While translocations affecting 14q32 were detected by cytogenetics in 23% of the cases, 41% were positive by FISH.

Translocation t(2;5)(p23;q35)

A number of cytogenetic studies have identified the t(2;5)(p23;q35) translocation as a recurrent chromosomal aberration in Ki1-positive large cell anaplastic lymphomas, frequently as the sole aberration (Mason et al. 1990; Schlegelberger et al. 1994a). The t(2;5) leads to disruption of the npm gene in 5q35 and to an alk/npm fusion gene encoding a protein with tyrosine kinase activity (Morris et al. 1994). So far, two molecular cytogenetic approaches for the detection of this translocation by interphase FISH have been published. Weber-Matthiesen et al. (1996) used two differently labelled YAC DNA probes mapping to either side of the breakpoint in the npm gene (Lu-Kuo et al. 1994). The intact npm gene is represented by a two-color signal, whereas a disruption of this gene leads to a dissociation of the two signals (Fig. 2). Less than 1% of control cells displayed the signal constellation suggestive for a t(2;5) translocation. In cytogenetically positive large cell anaplastic lymphomas and lymphoma cell lines the derivative signal constellation was observed in 8–87% of total cells. Matthew et al. (1997) reported an approach for the detection of t(2;5) using probes immediately centromeric to the npm gene and an alk-probe located telomeric to the chromosome 2 breakpoint. In this system, a t(2;5) translocation is indicated by the colocalization of each signal. The false-positive rate of this approach was reported to be 2–7%.

Molecular cytogenetic investigations for the detection of numerical chromosomal aberration in NHL

Numerical chromosome aberrations, too, are found in NHL samples. Though frequently considered as secondary aberrations, some have been observed regularly as sole cytogenetic aberrations, suggesting their role as primary abnormalities (Mitelman 1994). Trisomy 3, for example, seems to play a role in the pathogenesis of some low-grade NHL subtypes, as it has been recurrently observed as the sole aberration in MALT- and peripheral T-cell lymphomas (Wotherspoon et al. 1995; Schlegelberger and Feller 1996). Among the latter group, an additional chromosome 3 has been observed predominantly in Lennert and T-zone lymphomas as well as in cases of angioimmunoblastic lymphadenopathy with dysproteinemia (AILD; Schlegelberger and Feller 1996). By cytogenetic analyses, trisomy 3 has been observed in 41% of peripheral T-cell lymphomas as compared to 78% by interphase FISH. According to the FISH studies, the pathogenetic role of trisomy 3 in T-cell lymphomas may be even more pronounced than suggested by cytogenetic data. Additionally, a combined cytogenetic and FISH analysis with centromeric probes for chromosomes 3 and X in 36 cases of AILD revealed in 47% of samples the existence of unrelated (independent) tumor cell clones (Schlegelberger et al. 1994b).

Trisomy 12 is a hallmark of B-lymphocytic leukemia and other small lymphocytic lymphomas. Due to the low proliferation rate of the tumor cells in these chronic B-cell lymphomas, cytogenetic analysis is frequently unsuccessful. In contrast, FISH with a chromosome 12 centromeric probe allows a rapid detection of this aberration in peripheral blood and bone marrow (Anastasi et al. 1992).

Molecular cytogenetics for the detection of deletions in NHL

In contrast to primary chromosomal alterations, which occur during the initial steps in tumorigenesis, secondary aberrations are associated with tumor progression. In malignant lymphomas, secondary aberrations are mostly not specific for distinct pathological subgroups. Nevertheless, some are closely associated with certain clinical and prognostic features (Table 2). Whereas the vast majority of primary aberrations in lymphomas seem to be translocations, deletions of tumor suppressor genes seem to be the predominant and most important secondary genetic changes (Johansson et al. 1995).

Deletions of the tumor suppressor genes p53 and p16

Molecular and immunohistochemical investigations stress the prognostic impact of the inactivation of the p53 tumor suppressor gene in a variety of malignant neoplasias. In malignant lymphomas, the association of p53 in-

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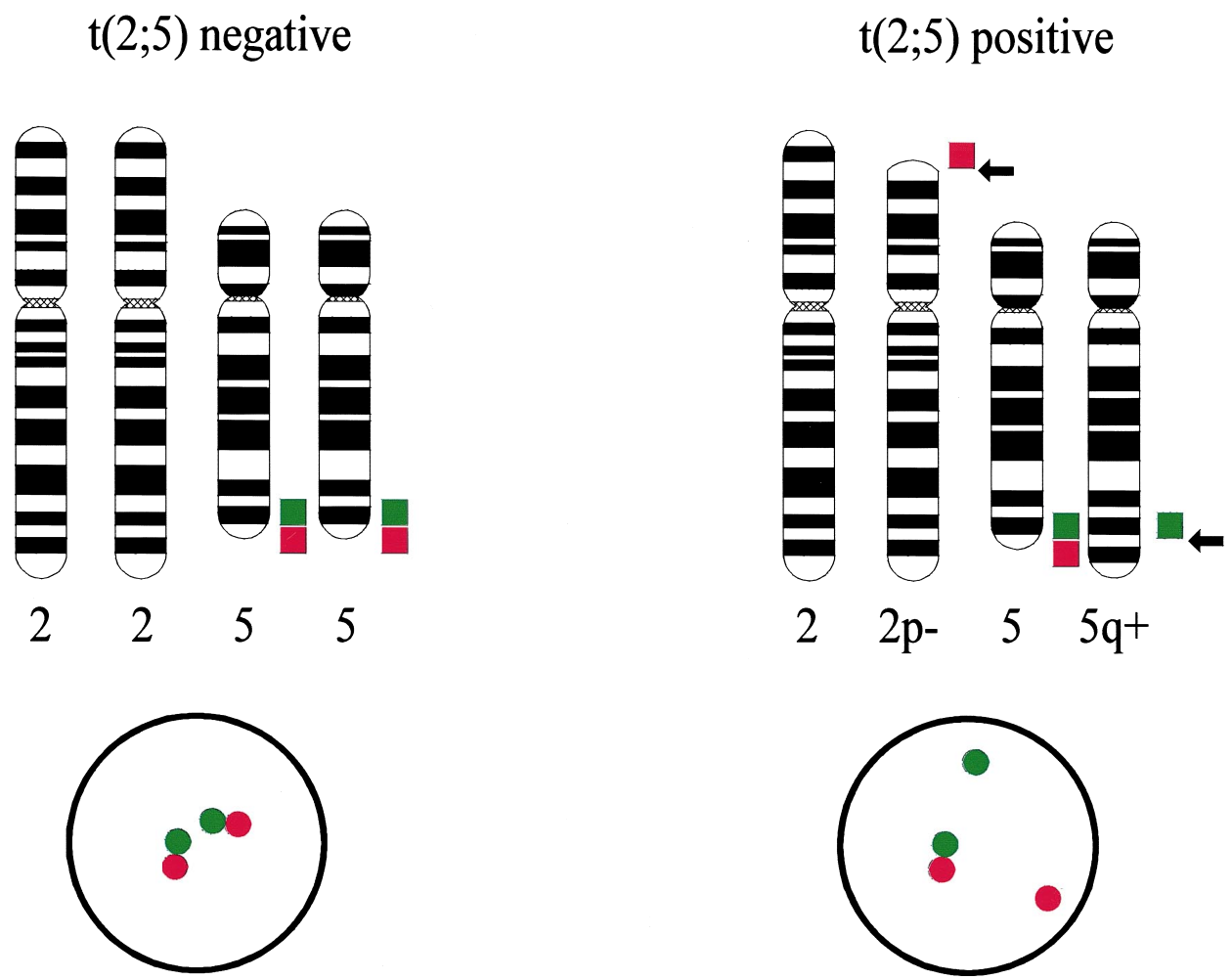
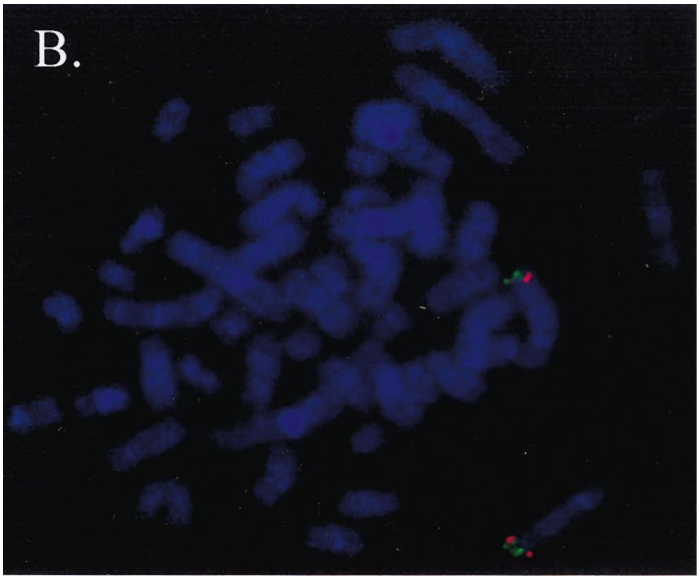
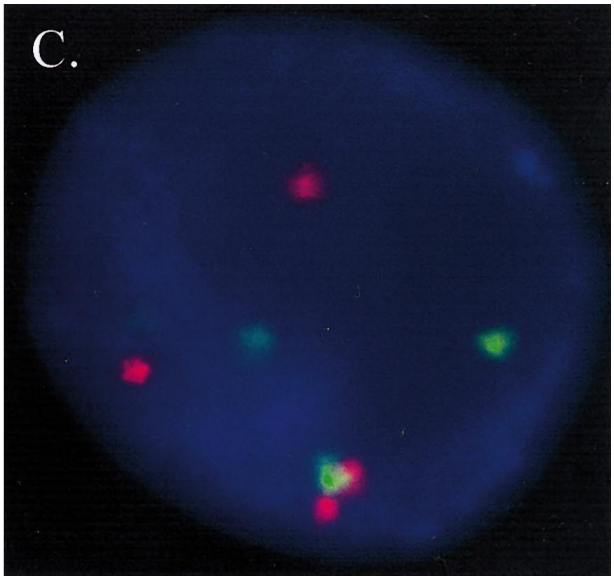






activation and histological transformation to low-grade lymphomas in high-grade lymphomas has been well documented (LoCoco et al. 1993). Additionally, a recent FISH analysis of 100 patients with B-CLL, B-PLL or Waldenström's disease, clearly showed the clinical importance of deletions of the p53 gene in low-grade B-cell lymphomas. In this study, Döhner and coworkers (1995) reported a poorer response to treatment with purine analogs and a shorter survival in patients with deletions of the p53 gene, as detected by FISH.

The cyclin-dependent kinase 4 and 6 inhibitor p16 is a negative regulator of the cell cycle. p16 is a physiological antagonist of the cyclin D1 protein, which is involved in the pathogenesis of MCL via t(11;14). Similarly to p53, p16 is inactivated in a variety of malignant tumors. In acute lymphoblastic leukemia (ALL, deletions of the p16-gene, which is located in chromosomal region 9p21, can be detected in up to 80% of cases. Though the prognostic impact of p16 deletions in hematologic disorders has not been well established, recent FISH analyses suggest a role of p16 inactivation in the transformation of low-grade into high-grade lymphomas (Siebert et al. 1996).

Deletions in the long arm of chromosome 6

Deletions of the long arm of chromosome 6 (6q) are the most frequent secondary chromosomal changes in malignant lymphomas and are related to poor survival (Offit and Chaganti 1991; Tilly et al. 1994). Cytogenetic and molecular investigations suggest the regions 6q25-27, 6q23, and 6q21 to harbor tumor suppressor genes involved in the progression of different subtypes of lymphomas (Gaidano et al. 1992; Offit et al. 1993). In a recent comparative analysis of 39 B-cell lymphomas, we were able to detect deletions in 6q23-24 in 33% of cases by cytogenetics, but in 57% of cases by FISH (Zhang et al. 1997). In contrast to the cytogenetic study by Offit and coworkers (1994b), which suggested 6q21-23 to be the common region of deletion in low-grade B-cell lymphomas lacking a t(14;18) translocation, we frequently detected by FISH deletions in 6q23-24 in lymphomas carrying this translocation.

◀ **Fig. 2A–C** Interphase FISH for the detection of the t(2;5) translocation. **A** Ideogram representing the expected localization of signals by two-color FISH in t(2;5)-negative (*left*) and -positive (*right*) metaphase chromosomes and interphase cells. *Red color* YAC 939F4 mapping telomeric of the breakpoint in 5q35. *Green color* YAC 756A7 mapping centromeric of the breakpoint in 5q35. Interphase cells lacking a t(2;5) translocation show two red-green hybrid signals. Cells carrying a t(2;5) translocation display one red-green hybrid signal and isolated red and green signals indicative of the translocation. **B** Metaphase cell without t(2;5) showing a colocalization of single red and green signals in chromosome region 5q35. **C** Interphase nucleus of a cytospin preparation of cerebrospinal fluid from a patient with Ki1-positive anaplastic large T-cell lymphoma. The cell contains four copies of chromosome 5 with two red-green hybrid signals and isolated pairs of red and green signals indicative of two derivative chromosomes 5

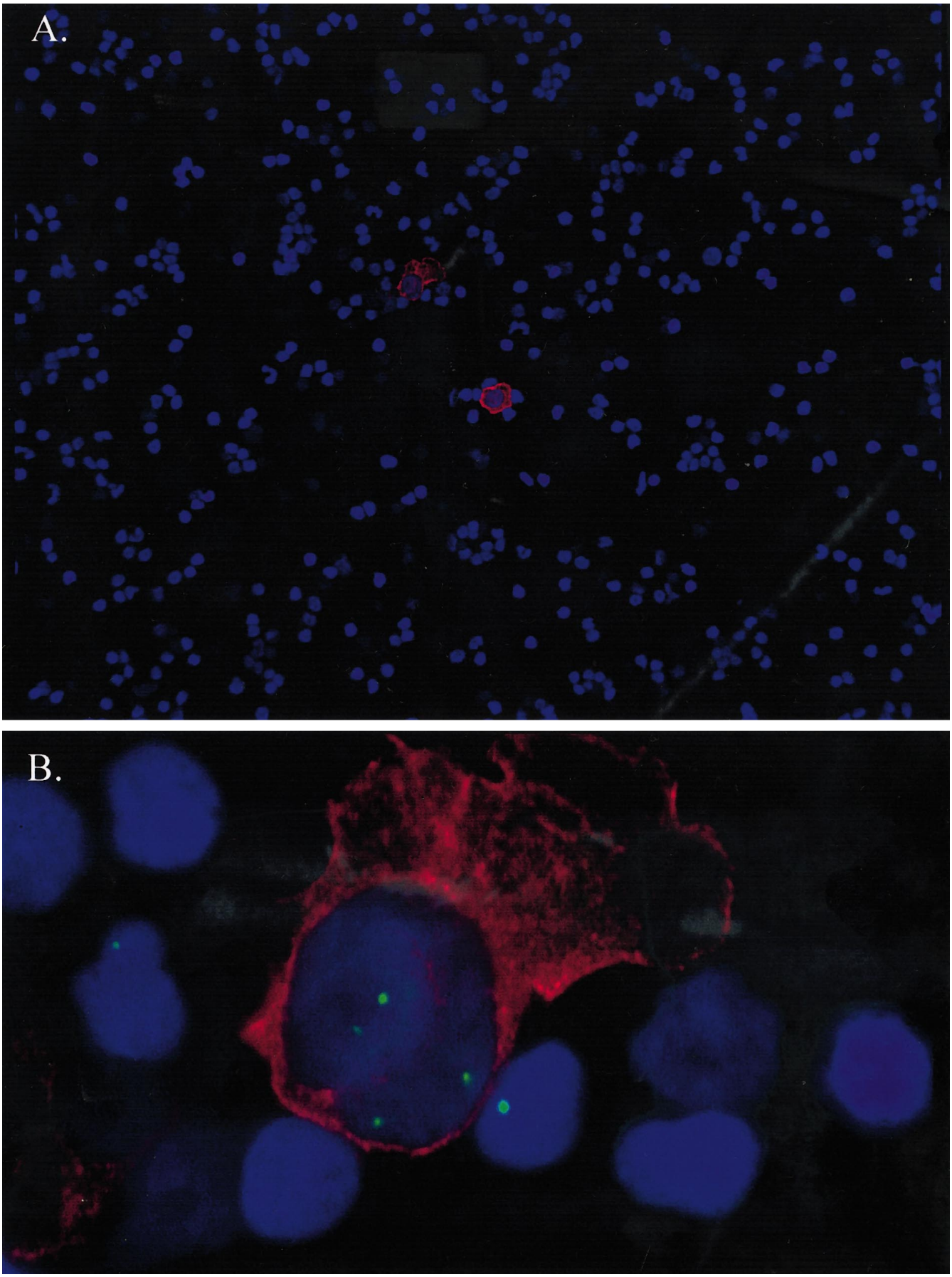
Combined immunophenotyping and molecular cytogenetics in Hodgkin's disease (FICTION technique)

Although very powerful in certain fields of research and diagnostics, FISH has one crucial disadvantage: it is not possible by FISH to unequivocally correlate particular genetic aberrations with particular cell populations in the tissue studied. This disadvantage is shared with all other genetic analysis techniques. In cytogenetics, for example, the cytoplasm and the cell membranes of mitotic cells must be completely eliminated to obtain analyzable metaphases. Thus, all morphological and immunophenotypical details of the analyzed cells are destroyed. The same problem arises in molecular genetic studies: Southern blot and PCR techniques are suitable for detecting DNA alterations; however, neither technique can determine which cells in the tissue actually carry the alteration.

These difficulties have been overcome by a new method combining fluorescence immunophenotyping and fluorescence in situ hybridization. This technique is called FICTION (Weber-Matthiesen et al. 1992, 1993a–c, 1995a–c, 1996). By means of the FICTION-technique, cells can be immunophenotyped and, at the same time, be evaluated for chromosome aberrations. FICTION can study all cells on a slide no matter whether they are in mitosis or in interphase. FICTION studies are possible on cytospin slides, smears, imprints, and cryostat sections.

The FICTION technique has been described comprehensively elsewhere (Weber-Matthiesen 1996). Here, we give a brief introduction to the procedure to explain the principle behind the method. Slides are fixed in acetone and then immunophenotyped by an indirect fluorescence detection system. Secondary antibodies conjugated with fluorescent dyes are used to visualize antigen-specific monoclonal antibodies. Immunophenotyping is followed by a fixation step to preserve the fluorescent immunostaining during the harsh hybridization procedure. After fixation, in situ hybridization is performed using appropriate, for example centromeric, DNA probes. Visualization of the hybridized probes is also done with fluorescence-labeled antibodies. As in the well-known FISH technique, the number of hybridization signals agrees with the number of copies of a given chromosome in the cells studied. Under the fluorescence microscope, the immunophenotype and the hybridization signals can be evaluated simultaneously.

A number of FICTION studies have recently been performed on Hodgkin's disease. A typical feature of Hodgkin's disease is the presence of Hodgkin and Reed-Sternberg (HRS) cells in lymph nodes of affected patients. The proportion of HRS cells in affected lymph nodes is usually very low, often far below 1%. Because they are so extremely rare and because HRS cell-like cells also exist in other diseases, it has been doubted for a long time whether HRS cells actually represent the malignant cells in Hodgkin's disease. In about 30% of cases studied with cytogenetic techniques, aberrant clones,


A.
B.

mostly with hyperploid chromosome numbers, were detected. With classical cytogenetic techniques it was impossible to define the cell type of the aberrant cells. They could correspond to HRS cells or to other, as yet unidentified, cell populations. This question can now be studied by means of the FICTION technique. All HRS cells strongly express the CD30 antigen, whereas the majority of the other cells in the lymphoid tissue are CD30 negative. With FICTION, cytospin slides can be immunophenotyped for CD30 and hybridized with appropriate DNA probes to show the hyperploidy typically found in cytogenetic analyses. Fig. 3 demonstrates the application of FICTION to a cytospin slide prepared from lymph node cells from a patient with Hodgkin's disease. Immunophenotyping was performed for CD30 and in situ hybridization was done using a chromosome 1 centromere-specific DNA probe. Fig. 3B clearly shows a strongly CD30-positive Hodgkin cell containing four copies of chromosome 1. From a FICTION study of 30 cases of Hodgkin's disease, we now know that only the CD30-positive HRS cells contain numerical chromosome aberrations, whereas CD30-negative lymph node cells regularly have normal numbers of hybridization signals (Weber-Matthiesen et al. 1995a).

Another issue, which has been discussed very controversially by different investigators was the question, whether the t(2;5) translocation is a recurrent and possibly pathognomonic feature in Hodgkin's disease. This discussion had arisen from the consistent finding of alk/npm fusion gene transcripts by reverse transcription-PCR (RT-PCR) in 11 of 13 cases of Hodgkin's disease by Orscheschek et al. (1995). As discussed above, the alk/npm fusion gene resulting from the t(2;5)(p23;q35) translocation is found almost exclusively in a part of large cell anaplastic lymphoma. Another study by Southern blot analysis also pointed to a possible role of npm gene rearrangements in Hodgkin's disease (Bullrich et al. 1994). One other RT-PCR study had revealed the translocation, too, but only in 3 of 72 cases of Hodgkin's disease (Yee et al. 1996). However, following the principles of Hodgkin's disease, contradictory results were published simultaneously by others, who reported more than 100 cases of Hodgkin's disease, all of which lacked the t(2;5) translocation in RT-PCR studies (Ladanyi et al. 1994; Weiss et al. 1995; Wellmann et al. 1995).

We have looked into this problem with the FICTION approach (Weber-Matthiesen et al. 1996). While RT-PCR is a powerful means for detecting genetic alterations at the level of mRNA, DNA in situ hybridization is able to reveal underlying alterations in the genome. Using two YAC DNA probes mapping to either side of the breakpoint in the npm gene at 5q35 (Lu-Kuo et al. 1994), we investigated whether the translocation can be detected by molecular cytogenetics in HRS cells. In order to identify the rare HRS cells, we combined the interphase cytogenetic study with CD30 immunostaining (FICTION technique). Thirteen cases of Hodgkin's disease were studied by this; 15–45 HRS cells (mean: 27) were analyzed per case. In some cases, we detected more than two red/green double signals for the intact npm gene, which is in accordance with the above-mentioned cytogenetic and molecular cytogenetic reports typically showing hyperploid karyotypes in Hodgkin's disease. In 12 of the 13 cases, red and green signals were always closely associated, indicating that in these cases the distal npm fragment was not shifted away from chromosome 5, e.g., by the t(2;5) translocation, in any of the analyzed cells. This also held true for one case in which cytogenetic analysis had revealed a derivative chromosome 5. Only in one case (mixed cellularity, 26 years, female, cytogenetic data not available) was the situation ambiguous. CD30-negative cells (200 per case) were also studied for the presence of t(2;5). The percentage of cells with the typical signal constellation was far below 1%, i.e., below the detection limit. We could conclude from this study, that the t(2;5) translocation is not a primary event in the development of Hodgkin's disease (Weber-Matthiesen et al. 1996).

FICTION is also well suited to overcome the detection limit of FISH. The detection of tumor clones with aneuploid chromosome numbers by means of FISH is restricted to cases with relatively high percentages of aberrant cells. At least 1–2% of the cells analyzed must be trisomic to define the presence of an aberrant clone. In the case of monosomy, more than 5–10% of the cells on the slide must show the chromosome loss. The reason is that normal specimens hybridized with centromeric DNA probes also show considerable proportions of cells with one or three signals. This is due to unspecific or ineffective in situ hybridization. Consequently, in samples with low numbers of tumor cells it is impossible to differentiate true aneuploidy from artificial hybridization results. This problem arises, for example, in most cases of Hodgkin's disease, where the malignant HRS cells rarely comprise more than 1–2% of the total. The same holds true if residual tumor cells after therapy have to be detected. FICTION is able to overcome this problem. True aneuploidy is confirmed if numerical aberrations are detected exclusively within cells with a tumor cell-associated immunophenotype. This way, a low percentage of tumor cells does not limit interphase cytogenetic studies by means of FICTION if tumor cells and normal cells have different immunophenotypes.

◀ **Fig. 3A, B** Fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation (FICTION) analysis of a cytospin preparation from a lymph node affected with Hodgkin′s disease. **A** Low power magnification: identification of very few CD30-positive (red fluorescence) Hodgkin and Reed-Sternberg cells. **B** One of the CD30-positive cells displayed in **A** at high power magnification. The cell contains four green signals for the centromeric probe of chromosome 1, indicative of tetrasomy 1. Signals in the surrounding CD30-negative cells are out of focus

Conclusion

FISH and FICTION are easy-to-handle, rapid, and highly sensitive tools for the genetic analysis of tumor cells on a single cell level, allowing the morphology and im-

munophenotype of aberrant cells to be studied. Recently, many important tumor genetic data have been obtained using these techniques. In addition to the data summarized in this review, FISH and FICTION have been applied to other diagnostic questions, e.g., the detection of chimerism after sex-discordant bone marrow transplantation or of minimal residual disease. Nonetheless, one should not overlook the fact that molecular cytogenetics is only able to detect defined chromosomal aberrations searched for by a specific assay. Thus, a number of other genetic alterations may be missed which would have been disclosed by conventional cytogenetic analyses. Therefore, FISH and FICTION should not replace conventional cytogenetic studies but provide additional tools in cases in which cytogenetic analyses are impossible or of insufficient sensitivity.

Acknowledgements The authors' own studies have been supported by the Wilhelm-Sander-Stiftung (grant 95–003–1) and the Deutsche Krebshilfe (grants W10/92Schl1, W87/94/Schl2, and 10–0992-Schl3).

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