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REVIEW

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Chromosome aberrations in B-cell chronic lymphocytic leukemia: reassessment based on molecular cytogenetic analysis

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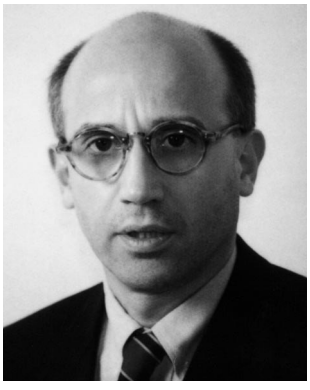
Abstract In B-cell chronic lymphocytic leukemia (B-CLL) clonal chromosome aberrations are detected in approximately 40–50% of tumors when using conventional chromosome banding analysis. Most studies find trisomy 12 to be the most frequent chromosome aberration, followed by structural aberrations of the long arm of chromosomes 13 and 14. Trisomy 12 and the "14q+" marker are associated with shorter survival times, while the patients with 13q abnormalities have a favorable outcome, similar to those with a normal karyotype. The development of molecular cytogenetic techniques has greatly improved our ability to detect chromosome aberrations in tumor cells. Using fluorescence in situ hybridization, chromosome aberrations can be detected not only in dividing cells but also in interphase nuclei, an approach referred to as *interphase cytogenetics*. The prevalence of specific aberrations in B-CLL is currently being reassessed by interphase cytogenetics. By far the most frequent abnormality are deletions involving chromosome band 13q14, followed by deletions of the genomic region 11q22.3-q23.1, trisomy 12, deletions of 6q21-q23, and deletions/mutations of the *TP53* tumor suppressor gene at 17p13. The evaluation of the true incidence of these aberrations now provides the basis for more accurate correlations with clinical characteristics and outcome. Deletions/mutations of the *TP53* gene have been shown to be associated with resistance to treatment and to be an independent marker for poor survival. 11q deletions have been associated with extensive nodal involvement, rapid disease progression, and short survival times. Whether trisomy 12, 13q14, and 6q deletions have a prognostic impact awaits further study. The application of these molecular cytogenetic techniques will also contribute to the identification of the pathogenetically relevant genes that are affected by the chromosome aberrations in B-CLL.

Key words B-cell chronic lymphocytic leukemia · Fluorescence in situ hybridization · *TP53* · 11q22-q23 · Comparative genomic hybridization

Abbreviations *B-CLL* B-cell chronic lymphocytic leukemia · *B-PLL* B-cell prolymphocytic leukemia · *CGH* Comparative genomic hybridization · *FISH* Fluorescence in situ hybridization · *IWCCLL* International Working Party on Chromosomes in CLL · *MCL* Mantle cell lymphoma · *RFLP* Restriction fragment length polymorphism · *SLL* Small lymphocytic lymphoma · *SSCP* Single-strand conformation polymorphism



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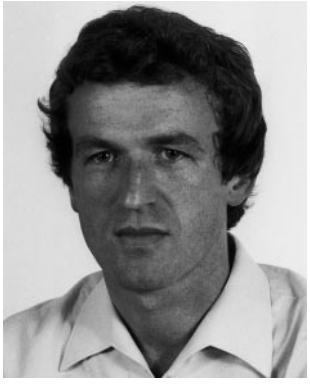

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Methodological aspects of cytogenetic analysis in B-cell chronic lymphocytic leukemia

Chromosome banding analysis (metaphase cytogenetics)

In the 1960s and 1970s most cytogenetic studies of B-cell chronic lymphocytic leukemia (B-CLL) were performed on blood lymphocytes stimulated with phytohemagglutinin, and the vast majority of tumors exhibited a normal karyotype. A review by Mitelman and Levan in 1978 [1] on chromosome aberrations in human neoplasms associated no specific aberration with B-CLL. Specific chromosome aberrations in B-CLL were identified not before the late 1970s when B-cell mitogens were shown to induce leukemic cells from B-CLL tumors to proliferate in culture [2–6]. Among the mitogens that have been used are tetradecanoyl-0-phorbol-13-acetate, Epstein-Barr virus, lipopolysaccharide, pokeweed mitogen, cytochalasin B, anti-human IgM, B-cell growth factor, calcium ionophore (Ca^{2+}), and an anti-CD40 antibody [7–9]. Despite the use of these mitogens chromosome banding analysis has remained difficult in B-CLL. Clonal chromosome aberrations are currently detected in only 40–50% of cases [10–12]. In the cases without clonal abnormalities mitotic cells often stem from nonleukemic T-lymphocytes, as shown by the study of Autio et al. [13] using the technique of sequential immunophenotyping and karyotype analysis.

Fluorescence in situ hybridization (interphase cytogenetics)

The development of molecular cytogenetic techniques has greatly enhanced our ability to detect chromosome aberrations in tumor cells [14, 15]. Delineation of specific DNA sequences in the cells by the technique of in situ hybridization is the basis for this molecular cytogenetic approach. The most popular protocol is fluorescence in situ hybridization (FISH) with genomic DNA probes. Numerical and structural chromosome aberrations that involve changes in the copy number in tumor cells are identified by aberrant signal numbers per cell, while translocation breakpoints are detected by the pattern of spatial distribution of the fluorescence signals. The sensitivity of detection is determined by the probe and the target size. While chromosome banding analysis detects only gross aberrations, i.e., rearranged or deleted subregions several megabasepairs is size, FISH identifies aberrant regions as small as the sequences targeted by the DNA probe(s). To achieve a sufficient hybridization efficiency the cloned DNA fragment should be at least 30–40 kb in size, i.e., DNA fragments cloned in cosmid vectors [15]. Routine diagnosis of heterogeneous tumor material using interphase cytogenetics generally require the DNA probes to be of even higher complexity, i.e., DNA fragments of 80 kb to several hundred kilobases cloned in P1, PAC, BAC, and YAC vectors.

The diagnostic potential of these molecular cytogenetic techniques is not restricted to the study of metaphase chromosomes but, most importantly, includes the analysis of interphase nuclei, referred to as interphase cytogenetics [16, 17]. Interphase cytogenetics has been shown to be of particular advantage in tumors in which metaphase cells are difficult to obtain, or in which metaphase cells are not representative of the leukemic clone. One of these tumors is B-CLL. Given these methodological problems of conventional cytogenetic analysis in B-CLL, it is not surprising that the incidence of specific aberrations is considerably higher when assessed by interphase cytogenetic analysis.

Recurring chromosome aberrations in B-CLL

The first reports on specific chromosome aberrations in B-CLL were published in the late 1970s [2–6, 18]. In 1980 Gahrton et al. [5, 6] showed that trisomy 12 is a recurring aberration in B-CLL; 5 of 11 tumors had trisomy 12. In the following years several investigators confirmed that trisomy 12 is a frequent aberration in B-CLL [19–27]. In the late 1980s another recurring chromosome abnormality was identified: deletions and, less frequently, translocations involving the long arm of chromosome 13, in particular band 13q14 [24, 28–30]. Other chromosome aberrations that have been identified with varying frequencies include deletions of 6q [6, 21, 22, 26, 27], 11q [21, 22, 24, 29, 31] and 17p [26], partial or total trisomy 3 [19, 21, 24], and translocations involving band 14q32 [3, 6, 19, 21, 23, 26, 27, 32]. The most frequently found translocation is the t(11;14)(q13;q32). Another balanced translocation reported in rare B-CLL cases is the t(14;19)(q32;q13) [33, 34]. Partial karyotypes of some of the most frequently recurring chromosome aberrations in B-CLL are shown in Fig. 1.

One of the largest chromosome banding series was reported by the First and Second International Working Party on Chromosomes in CLL (IWCCLL) [10, 11]. The Second IWCCLL compiled data on 662 patients from 11 institutions [11]. Of the 604 cytogenetically evaluable

Table 1 Comparison of the frequency of specific chromosome aberrations in B-CLL as assessed by chromosome banding[a] (Second IWCCLL) and interphase cytogenetics[b] (Heidelberg Study)

	Chromosome banding		Interphase cytogenetics	
	n	%	*n*	%
Trisomy 12	112/604	19	36/245	15
Structural 13q aberrations	62/604	10	129/245	53
Structural 11q aberrations	49/604	8	48/250	19
Structural 6q aberrations	36/604	6	18/208	9
Structural 17p aberrations	22/604	4	20/243	8

[a]Structural 13q aberrations most frequently involved band 13q14, and those of chromosome 11 most commonly bands 11q13 (14 cases), 11q14 (6 cases), and 11q23 (5 cases)

[b]13q14 deletions were assessed by probes recognizing *RB1* and the *D13S25* locus, 11q deletions by a YAC clone from 11q22.3-q23.1, 6q deletions by two YAC clones mapping to bands 6q21 and 6q27, and 17p deletions by a probe identifying the *TP53* gene at 17p13 (data from this series)

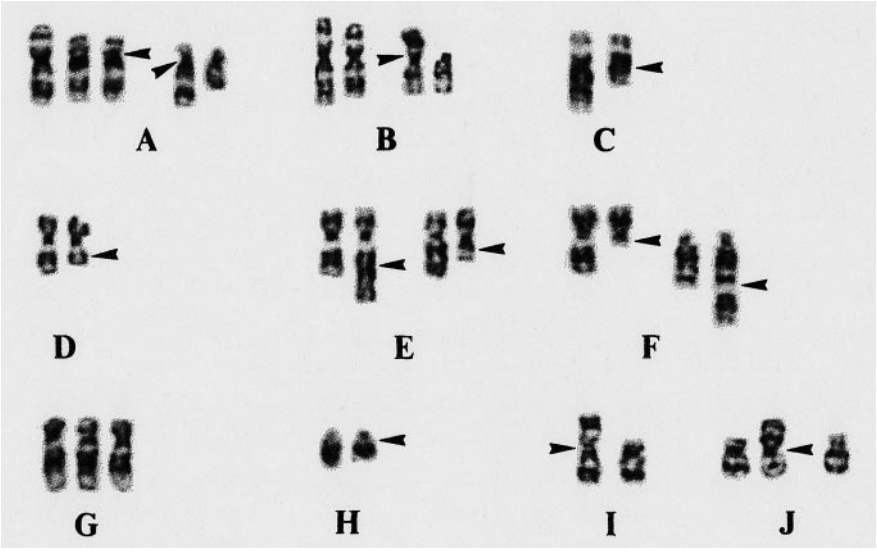

Fig. 1A–J Partial G-banding karyotypes of recurring chromosome aberrations in B-CLL (images were obtained and processed in the Ikaros system, MetaSystems, Altlussheim, Germany). **A**,**B** Unbalanced translocations leading to trisomy or partial trisomy 3q. The commonly duplicated segment comprises the distal region of 3q: t(3;18)(p10;q10), +der(3)t(3;18)(p10;q10) leading to trisomy 3q (**A**) and der(15)t (3;15)(q21;p11) with duplication 3q21qter (**B**). **C** Interstitial deletion del(6)(q15q23). Based on chromosome banding and RFLP analysis, the commonly deleted region in B-CLL and SLL appears to be 6q21-q23. **D**,**E** Structural aberrations of bands 11q21-q23. **D** Interstitial deletion del(11)(q21q23). In a recent FISH study, we delineated the commonly deleted segment of these 11q deletions to a 2- to 3 Mb sized genomic fragment in bands 11q22.3-q23.1. In the Heidelberg interphase cytogenetic study these 11q deletions represent the second most frequent chromosome aberration in B-CLL. **E** A reciprocal translocation t(X;11)(q13;q23) that maps to the commonly deleted region. **F** Translocation t(11;14)(q13;q32). Based on the recent molecular studies the t(11;14) is not a recurring translocation in B-CLL but strongly associated with mantle cell lymphoma. Most B-CLL tumors that are reported to carry this translocation likely represent leukemic MCL. **G** B-CLL tumor exhibiting trisomy 12, which is the most frequent numerical chromosome aberration in this disease. **H** Interstitial deletion del(13)(q12q14). Deletions, and less frequently, translocations involving band 13q14 are the most common chromosome aberration in B-CLL. A new putative tumor suppressor gene is likely located between *RB1* and the *D13S25* locus at 13q14. **I**,**J** B-CLL tumors with an isochromosome i(17)(q10) (**I**) and an unbalanced translocation der(17;18)(q10;q10) (**J**), both leading to loss of 17p13, the site of the *TP53* tumor suppressor gene. FISH and SSCP analyses have shown that deletions or mutations of the *TP53* tumor suppressor gene occur in approximately 8–15% of B-CLL tumors

tumors 311 (51%) had clonal chromosome aberrations. The most common aberration was trisomy 12 (19% of the evaluable cases), followed by structural aberrations of chromosomes 13 (10%), 14 (8%), 11 (8%), 6 (6%) and 17 (4%; Table 1).

For a comprehensive interphase cytogenetic analysis of B-CLL, we and others have designed DNA probes that allow to identify the most important numerical and structural chromosome aberrations in B-CLL. Our set includes DNA probes that permit the diagnosis of trisomy 12 as well as deletions of several genomic regions, such as 13q14, 11q22.3-q23.1, 6q21/q27, and 17p13 (Fig. 2). The frequencies of these specific aberrations are quite different depending on whether they are assessed by chromosome banding or interphase cytogenetic analysis. The Heidelberg interphase cytogenetic study (Table 1) found that the

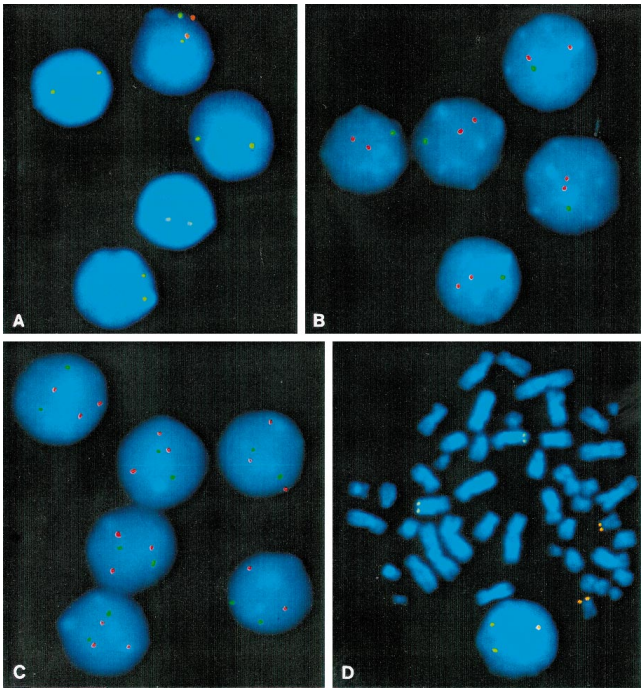

Fig. 2A–D Detection of chromosome aberrations in B-CLL ▶ using interphase cytogenetics. **A** B-CLL tumor with a biallelic deletion of the *D13S25* locus Four of the five nuclei show no *D13S25* signal (detected via Cy-3; *red*) but two copies of the *RB1* gene (detected via FITC; *green*). The nucleus at the top of the image exhibits a normal signal pattern (two signals of both probes). **B** B-CLL tumor with an 11q22.3-q23.1 deletion. All nuclei have only one green signal (internal control in red fluorescence). **C** B-CLL tumor with a trisomy 12. Five of the six nuclei show three signals with a YAC clone from chromosome band 12q13 (detected via Cy-3; *red*). **D** B-CLL tumor with a *TP53* gene deletion. The single nucleus at the bottom of the image exhibits only one *TP53* (*red*) and two control signals (green; YAC probe from band 8q24). The metaphase cell likely does not stem from the leukemic clone because it shows two signals of both DNA probes

by far most common aberration is deletion of band 13q14 (53%), followed by deletion of 11q22.3-q23.1 (19%), trisomy 12 (15%), deletion of 6q21 (9%), and deletion of 17p13 (8%). These differences in frequencies between metaphase and interphase cytogenetic analysis most likely do not result from patient selection but from differences in the methods used, with interphase cytogenetics revealing the real incidence of chromosome aberrations.

At present data are scarce regarding the critical genomic regions of these numerical and structural aberrations in B-CLL. Except for the *TP53* gene at 17p13 no candidate gene affected by these aberrations has so far been identified. Based on chromosome banding analysis the critical regions have been narrowed to the level of a single or a few chromosome bands for some of the recurring aberrations. In contrast, FISH will not only allow more accurate evaluation of the frequency of these aberrations but will also be instrumental in identifying the pathogenetically relevant genes. Using FISH with contigs of DNA clones, the aberrations, especially deletions, can be systematically delineated to the resolution level of several kilobases.

The specific aberrations in B-CLL are discussed in more detail below in the order of the frequency in which they have been detected in our ongoing interphase cytogenetic study.

Deletions/translocations involving chromosome band 13q14

Chromosome banding analysis

Structural abnormalities of chromosome 13 were first described as a recurrent aberration in smaller chromosome banding studies in the late 1980s [24, 28, 29]. In the more recent series they are almost invariably the most common structural chromosome aberration in B-CLL. The majority of these abnormalities are deletions while a few cases appear as balanced translocations on the resolution level of chromosome banding. These deletions and translocations commonly involve band 13q14 (Fig. 1H) [24, 27–30].

Molecular analysis

Chromosome band 13q14 is the site of the retinoblastoma tumor suppressor gene (*RB1*), which has led to speculation about a pathogenetic role of this gene in B-CLL. *RB1* encodes a nuclear phosphoprotein that links cell cycle control to transcriptional activity [35]. Loss of function resulting from inactivation of both alleles by mutation or deletion appears to be involved in the pathogenesis of retinoblastoma and of a variety of other solid tumors. Indeed, it has been shown by molecular techniques that deletions of *RB1* are found in B-CLL tumors exhibiting 13q14 aberrations [36–38].

However, some findings argue against a role for *RB1* in B-CLL. Liu et al. [39] studied the structure of *RB1* in 18 B-CLL tumors in further detail using single-strand conformation polymorphism (SSCP) analysis. Nine of these tumors had monoallelic *RB1* deletion by quantitative Southern blot analysis, but no structural abnormalities of *RB1* were found in the 18 cases by SSCP. In 17 of the 18 tumors RB1 protein was detected by immunofluorescence analysis revealing no obvious alteration of the protein concentration. More recent studies suggest a pathogenetic role for the genomic region containing the *D13S25* locus distal to *RB1* at 13q14 [39, 40]. Deletions of this region have been shown to be more frequent than *RB1*, and to be homozygous in a substantial number of B-CLL tumors [39–41]. Furthermore, reciprocal translocations involving band 13q14 are more frequently associated with deletions of *D13S25* than with *RB1*. Because *D13S25* is located several hundred kilobases distal to *RB1*, the existence of a new tumor suppressor gene has been postulated [40].

Subsequent studies aimed at delineating the critical region more precisely [42–45]. Devilder et al. [42] initially located the critical region between *D13S25* and the more distal marker *D13S294*, while in our study the region mapped between *D13S25* and *RB1* [43]. The results of our study are supported by those of Liu et al. [44] who found a critical region involving the marker *D13S319* located between *RB1* and *D13S25*. Bullrich et al. [45] identified a minimal deletion region between *D13S25* and the marker *206XF12* located less than 550 kb proximal of *D13S25*. In this study the deletion did not cluster in a single genomic region, and the potential significance of several different loci was discussed.

Toward the identification of the pathogenetically relevant gene, several groups recently constructed high-resolution physical maps of DNA fragments cloned in cosmids, PACs and BACs spanning several hundred kilobases at the *RB1–D13S25* interval [46–50]. These studies performed a detailed deletion mapping using various methods such as microsatellite and quantitative Southern blot analysis or FISH in large series of B-CLL tumors. Kalachikov et al. [46] identified a commonly deleted segment of approximately 300 kb around the *D13S272* marker. Deletions of this segment were found in 84 of 156 (54%) B-CLLs tested. Furthermore, 23 expressed sequences were mapped to this critical region. Liu et al. [47] even narrowed the critical region down to a 10-kb interval immediately centromeric to *D13S272*. Mutation analysis of two candidate genes, *Leu1* and *Leu2*, located in this region failed to show mutation of the second allele. Bouyge-Moreau et al. [48] delineated a 550-kb critical region starting telomeric of *D13S272* and overlapping with the segment described by Kalachikov et al. [46].

We constructed a 1.4-Mb contig of DNA fragments covering the *D13S273–D13S25* interval [50]. Using FISH with probes for *RB1* and *D13S25*, we first analyzed a series of 322 B-CLLs and 30 mantle cell lymphomas (MCL). The frequency of 13q14 deletion was 51% in B-CLL and even 70% in MCL (Table 1). We subsequently performed a detailed deletion mapping study with representative clones from the contig map in the tumors which exhibited deletion of *D13S25* but not of *RB1*, and vice versa. This allowed us to delineate a 400-kb commonly deleted segment starting 100 kb centromeric of *D13S272*. In two tumors the deletion did not involve the entire 400-kb

region but only a 120-kb segment at *D13S272* and an 80-kb segment located 240 kb distal of *D13S272*, respectively [50]. Within the genomic segment at *D13S272* we identified several cDNA fragments [50]. Two of these, ep272–3 t5 and ep272–3 t4, correspond to the *Leu1* and *Leu2* genes, respectively, which were described in the study by Liu et al. [47]. The latter study had already excluded these two genes as the relevant gene since no intragenic mutation were detected.

Garcia-Marco et al. [51] reported on a high frequency of deletions of the *BRCA2* gene in B-CLL. Deletions of the *BRCA2* gene that maps to band 13q12 were found independently of deletions of the *D13S25* locus at 13q14. The existence of such noncontiguous deletions has not been confirmed by others [50, 52]. We analyzed 105 B-CLLs by FISH for deletions of *BRCA2* and a 600-kb region encompassing the gene [50]. Deletions were detected in three cases, and only one of these deletions occurred without 13q14 deletion.

Deletions of chromosome bands 11q22.3-q23.1

Chromosome banding analysis

In a compilation of data from the Catalog of Chromosome Aberrations in Cancer [53] one of the most common structural aberrations resulting in loss of chromosomal material in the categories of lymphoproliferative disorders and non-Hodgkin's lymphomas were deletions affecting the region 11q21-q25, most frequently chromosome band 11q23 (Fig. 1D,E). The frequency of this type of aberration has probably been substantially underestimated in most chromosome banding studies in B-CLL. The Second IWCCLL [11] reported fewer than 5% of tumors to have structural abnormalities involving various bands on 11q other than 11q13. More recent series on 544 and 480 B-CLL tumors studied by conventional cytogenetic analysis [54, 55] also do not report 11q deletions as a recurrent aberration. Evidence for the significance of chromosomal loss from 11q came from smaller chromosome banding studies in B-CLL [21, 22, 24, 29, 31, 56, 57]. In the studies by Fegan et al. [56] and Neilson et al. [57] 11q deletions were among the most common chromosome aberrations and were associated with disease progression and reduced survival. Hernandez et al. [58] analyzed 609 patients with various B-cell chronic lymphoproliferative disorders, including 423 cases classified as typical B-CLL, B-CLL mixed cell type, and atypical B-CLL. Although 11q deletions were the most common structural aberration in these tumors, the prevalence was only 6% (25 of 423 cases).

Molecular analysis

The recurrent loss of chromosome material from 11q21-q25 suggests that a novel tumor suppressor gene is located in this region. So far only few data have been obtained for molecular characterization of the genomic region affected by these deletions. A study by Kobayashi et al. [59] used FISH to analyze 15 hematological neoplasms (mostly acute myeloid leukemias and myelodysplastic syndromes, three non-Hodgkin's lymphomas), and a non-Hodgkin's lymphoma cell line with 11q deletions. In 14 of 16 tumors a commonly deleted segment at 11q23.1 containing the *NCAM* (neural cell adhesion molecule) gene was found. The *BCL1* locus at 11q13 and the *MLL* gene at 11q23.3 were located outside the critical region. However, the resolution of the deletion map was limited because the probes applied were scattered along a large genomic region.

To further delineate the commonly deleted segment in chromosome bands 11q21-q25 we recently performed a molecular cytogenetic study of 43 tumors classified as B-CLL (n=40) and MCL (n=3) [60]. As probes for FISH we selected 17 representative clones from a contig map of YACs encompassing bands 11q14.3-q23.3 [61]. Because overlapping YACs were applied, it was possible to systematically delineate the extent of the deletions at the molecular level. We identified a single critical region of 2–3 Mb in bands 11q22.3-q23.1 where all deletions and translocations clustered. This genomic fragment contains the genes coding for *ACAT1*, *NPAT*, *ATM*, *DDX10*, *RDX*, and *FDX1*. Two of these genes, *RDX* (radixin) and *ATM* (ataxia telangiectasia mutated), appear as potential tumor suppressor genes. *RDX* has homology to the neurofibromatosis-type 2 gene (*NF2*) [62]. Evidence for a growth suppressor function of *ATM* comes from murine knockout models [63] and from studies of human tumors [64, 65]. Mice deficient for *ATM* develop T-cell neoplasms [63]. We recently delineated a similar 11q22-q23 deletion cluster in T-prolymphocytic leukemia [64]. In cases exhibiting deletion of one *ATM* allele we detected small intragenic deletions as well as nonsense and missense mutations of the second allele, indicating that the gene may have tumor suppressor function. It is tempting to speculate that *ATM* is also the target gene of the 11q22-q23 deletions in B-CLL.

We subsequently screened more than 200 B-CLL tumors for 11q deletion by FISH using a genomic clone from the critical segment [66] (Fig. 2B). 11q deletions were found in 43 of 214 (20%) tumors and were the second most frequent aberration following 13q14 deletions. 11q deletions were even more frequent than trisomy 12, which in most chromosome banding studies is the most common aberration (see data from this series in Table 1).

Trisomy 12

Chromosome banding analysis

Trisomy 12 has been the most common numerical chromosome abnormality in all banding studies in B-CLL (Fig. 1G). The frequency ranges from approximately 7% to more than 25% [12]. Only few B-CLL cases have been reported that on banding analysis exhibit a partial trisomy 12 likely pointing to the critical region of this aberration [8, 26, 67]. The segment that was duplicated in all cases includes bands 12q13-q21.2, indicating that this region contains the

Table 2 Detection of chromosome aberrations in B-CLL by FISH

	Location	*n*	FISH		Banding
			n	%	(%)
Trisomy 12					
Perez Losada et al. 1991 [69]	Helsinki	15	2	13	7
Anastasi et al. 1992 [70]	Chicago	50	18	36	23
Raghoebier et al. 1992 [71]	Leiden	67	8	11	ND
Döhner et al. 1993 [8]	Heidelberg	45	8	18	11
Escudier et al. 1993 [72]	Houston	117	41	35	6
Que et al. 1993 [73]	London	183	21	12	9
Criel et al. 1994 [74]	Brugge, Leuven	111	16	14	12
Arif et al. 1995 [75]	Hiroshima	42	8	19	ND
Matutes et al. 1996 [54]	London, others	544	97 (18)[a]		
Döhner et al. 1997 [66]	Heidelberg	214	32	15	ND
13q14 deletion					
Stilgenbauer et al. 1993 [37]	Heidelberg	35	11	31	11
Arif et al. 1995 [75]	Hiroshima	42	12	29	ND
Döhner et al. 1997 [66]	Heidelberg	214	96	45	ND
11q22.3-q23.1 deletion					
Döhner et al. 1997 [66]	Heidelberg	214	43	20	ND
17p13 (*TP53*) deletion					
Döhner et al. 1995 [80]	Heidelberg	100	17	17	ND
Döhner et al. 1997 [66]	Heidelberg	214	20	10	ND
6q21 deletion					
Stilgenbauer et al. 1996 [79]	Heidelberg	208	18	9	ND
9p21 (*CDKN2, p16*) deletion					
Schröder et al. 1995 [107]	Heidelberg	50	0	0	ND

[a]Data combined from chromosome banding and FISH analysis

gene(s) involved in the pathogenesis of B-CLL tumors carrying the trisomy. Restriction fragment length polymorphism (RFLP) studies show that trisomy 12 results from duplication of one homolog, rather than from loss of one homolog and triplication of the remaining one [68].

Molecular analysis

Numerous studies have been published on the incidence of trisomy 12 using interphase cytogenetics [8, 54, 69–75] (Table 2). All studies comparing conventional chromosome banding techniques and interphase cytogenetics find the frequency of trisomy 12 higher when assessed by FISH using DNA probes recognizing the repetitive sequences of the centromeric and pericentromeric region. The frequencies of trisomy 12 in FISH studies ranges from 11% to more than 30% in two studies from the United States [70, 72] (Table 2). This variation likely results from patient selection, depending on whether the respective centers are secondary or tertiary referral centers for B-CLL patients. However, differences may also be due to variable geographical distribution of this chromosome aberration. Of the 245 B-CLL tumors that we analyzed in Heidelberg 36 (15%) exhibited trisomy 12 (Fig. 2C). As shown in Table 1, trisomy 12 was only the third most common chromosome aberration when assessed by interphase cytogenetics.

Deletions of 6q

Chromosome banding analysis

Deletions of the long arm of chromosome 6 are among the most common chromosome aberrations in lymphoid neoplasms. The Second IWCCLL [11] found structural aberrations of chromosome 6 in 6% of the evaluable B-CLL tumors. The chromosome bands 6q15 and 6q23 were most commonly involved in structural aberrations (Fig. 1C).

Molecular analysis

Based on loss of heterozygosity detected by RFLP analysis, at least two independent regions of commonly deleted segments have been identified in malignant lymphomas, one at 6q21-q23 and another at 6q25-q27 [76]. Deletions of 6q21-q23 were associated particularly with small lymphocytic lymphoma (SLL), the lymphomatous counterpart of B-CLL [76, 77]. Using RFLP analysis Gaidano et al. [78] found 6q deletions in 4 of 100 B-CLL tumors. However, the latter study used DNA probes that localize in band 6q27, which according to the study by Offit et al. [76] and to our molecular cytogenetic data is likely not contained within the critical region involved in B-CLL/SLL [79]. We performed an interphase cytogenetic study on 208 B-CLL

cases using two YAC clones mapping to bands 6q21 and 6q27 [79]. Among these tumors we identified 18 cases (9%) that exhibited a 6q deletion (Table 1). All deletions were found by the YAC probe mapping to 6q21, whereas the 6q27 region was deleted in only 6 tumors. Thus 6q deletions in B-CLL tumors occur at a frequency of approximately 9% and preferentially involve the 6q21-q23 region. At present no candidate gene of pathogenetic significance in B-CLL has been identified on 6q.

Deletions of the *TP53* tumor suppressor gene at band 17p13

Chromosome banding analysis

Structural aberrations involving chromosome 17 most commonly result in loss of material from its short arm, the site of the *TP53* tumor suppressor gene [26, 80] (Fig. 1I,J). The Second IWCCLL [11] found 4% of the evaluable tumors to have structural aberrations of chromosome 17. Only one single-center study using conventional banding analysis has reported a higher incidence of chromosome 17 aberrations [26]: 5 of 31 (16%) evaluable cases had abnormalities of chromosome 17, all leading to loss of the short arm.

Molecular analysis

Evidence for a role of *TP53* in lymphoid malignancies including B-CLL came from a study demonstrating *TP53* gene mutations in these diseases by SSCP analysis and by sequencing of the polymerase chain reaction amplified fragments [81]. This study found *TP53* gene mutations in 6 of 40 (15%) B-CLL tumors. Three subsequent SSCP studies in B-CLL reported *TP53* gene mutations at frequencies ranging from 10% to 15% [78, 82, 83] (Table 3).

Point mutations coupled with deletion of the second allele is one of the characteristics of a recessively acting tumor suppressor gene such as *TP53*. Allelic loss of the *TP53* gene has so far been demonstrated most commonly using RFLP analysis. In our study we applied FISH using a *TP53* genomic probe to screen for allelic deletions of the *TP53* gene in a large series of patients with chronic B-cell leukemias (Fig. 2D) [80]. Of the 100 cases of B-CLL and its variants, B-cell prolymphocytic leukemia (B-PLL) and Waldenström's macroglobulinemia, we identified 17 (17%) tumors that exhibited a *TP53* gene deletion. We have extended this interphase cytogenetic analysis to 243 B-CLL tumors; in this series monoallelic *TP53* gene deletions were detected in 20 (9%) cases (Table 1). The lower frequency than in our initial study is most likely due to patient selection. In the present series we only included lymphoid leukemias with the morphological and immunophenotypical characteristics of B-CLL and did not include cases, for example, of B-PLL, which had a high frequency of *TP53* gene deletions. Thus the data from both the FISH and the SSCP studies indicate that mutations of the *TP53* gene occur in approximately 9-15% of B-CLL tumors (Table 3). As is discussed below, the presence of a *TP53* gene mutation has strong implications for the clinical course of the disease.

Table 3 Detection of *TP53* gene mutations in B-CLL using single strand conformational polymorphism (SSCP) or fluorescence in situ hybridization (FISH) analysis

	Method	Mutations *n*	%
Gaidano et al. 1991 [81]	SSCP	6/40	15
Fenaux et al. 1992 [82]	SSCP	4/39	10
El Rouby et al. 1993 [83]	SSCP	8/53	15
Gaidano et al. 1994 [78]	SSCP	10/100	10
Döhner et al. 1995 [80]	FISH	11/90	12
Döhner et al. 1997 [66]	FISH	20/214	9

Translocations involving the *IgH* locus at band 14q32

Structural aberrations of chromosome 14 in B-CLL most commonly involve band 14q32, the site of the immunoglobulin heavy chain (*IgH*) gene. Balanced translocations are frequently reported with chromosome band 11q13, the site of the *CCND1* gene [84, 85] (Fig. 1F). The t(11;14)(q13;q32), however, has recently been associated with MCL [86]. It is likely that many cases of B-CLL that have been reported to carry this translocation represent in fact cases of leukemic MCL. Furthermore, initial molecular studies also suggest a role for *CCND1* in the pathogenesis of B-CLL [87–89]. The breakpoints of the translocation were originally cloned from two B-CLL tumors and one large cell lymphoma cell line [87, 88]. However, both B-CLL cases were later reclassified as MCL [90]. Almost no subsequent molecular studies have found a role for *CCND1* in B-CLL [78, 91–94]. Gaidano et al. [78] studied 100 B-CLL tumors that were classified according to the criteria of the International Workshop on Chronic Lymphocytic Leukemia [95] and the National Cancer Institute-sponsored Working Group [96]. Leukemic cells of all patients coexpressed CD19 and CD5 and also were $CD23^{+}$. No rearrangements of *CCND1* were detected using Southern blot analysis. Lymphoproliferative disorders exhibiting the translocation t(11;14) should therefore be carefully examined for the morphological and immunophenotypical characteristics of MCL.

A pathogenetic role was also suggested for *BCL2* at 18q21. Adachi et al. [97, 98] have reported on B-CLL tumors exhibiting a *BCL2* rearrangement. While the breakpoints of t(14;18) in follicular lymphoma occur in the major breakpoint region (mbr) or in the minor cluster region (mcr) within the 3' portion of *BCL2*, the breakpoints in these B-CLL tumors have been localized at the 5' end, with preferential juxtaposition of *BCL2* to the immunoglobulin light chains. However, subsequent studies show that *BCL2* rearrangements are rare events in B-CLL [78, 93, 99–101].

Another translocation involving band 14q32 is the t(14;19)(q32;q13) [33, 34, 102]. The breakpoint on 19q13 has been shown to involve the *BCL3* gene [103–105] which encodes a transcriptional coactivator, the I-κB-like protein [106]. Among 4487 cytogenetic analyses of lymphoproliferative disorders (the diagnoses were not further specified in the study), Michaux et al. [102] found 6 cases showing the t(14;19) translocation, including one case with a variant translocation. Five of these cases were classified as B-CLL. The cytogenetic analysis was complemented by Southern blot analysis using probes for the *BCL3* locus in 1150 cases. Rearrangements were detected in all 6 cases with the t(14;19); however, no additional cases were identified.

Thus, rearrangements of these *BCL* proto-oncogenes in B-CLL are either rare events or are likely associated with other types of lymphoproliferative disorders. From the review of these molecular genetic data, the "14q+" marker, which was previously described in many chromosome banding studies of B-CLL, does not appear to be a frequent aberration in this disease.

Other chromosome aberrations

Chromosome 3

Total or partial trisomy 3 has been reported to occur at low frequency in B-CLL [19, 21, 24]. In our B-CLL series (unpublished data) we identified few tumors that exhibited partial trisomy 3. Based on chromosome banding analysis, the commonly duplicated segment comprises the distal region of the long arm (Fig. 1A,B).

9p21 (CDKN2, p16)

After the initial reports on the frequent loss of another putative tumor suppressor gene, *CDKN2* (*p16*), in various human cancers, we studied 50 cases of chronic B-cell leukemias by FISH using a pool of cosmids covering approximately 250 kb of the *CDKN2* region at 9p21 [107] (Table 2). We detected no deletions of the gene in these 50 tumors. This finding is consistent with the data from chromosome banding studies which show no structural aberrations of 9p in B-CLL. In agreement with this, studies using Southern blot analysis have either found no deletion of *CDKN2* or have found them only as rare events [108–110].

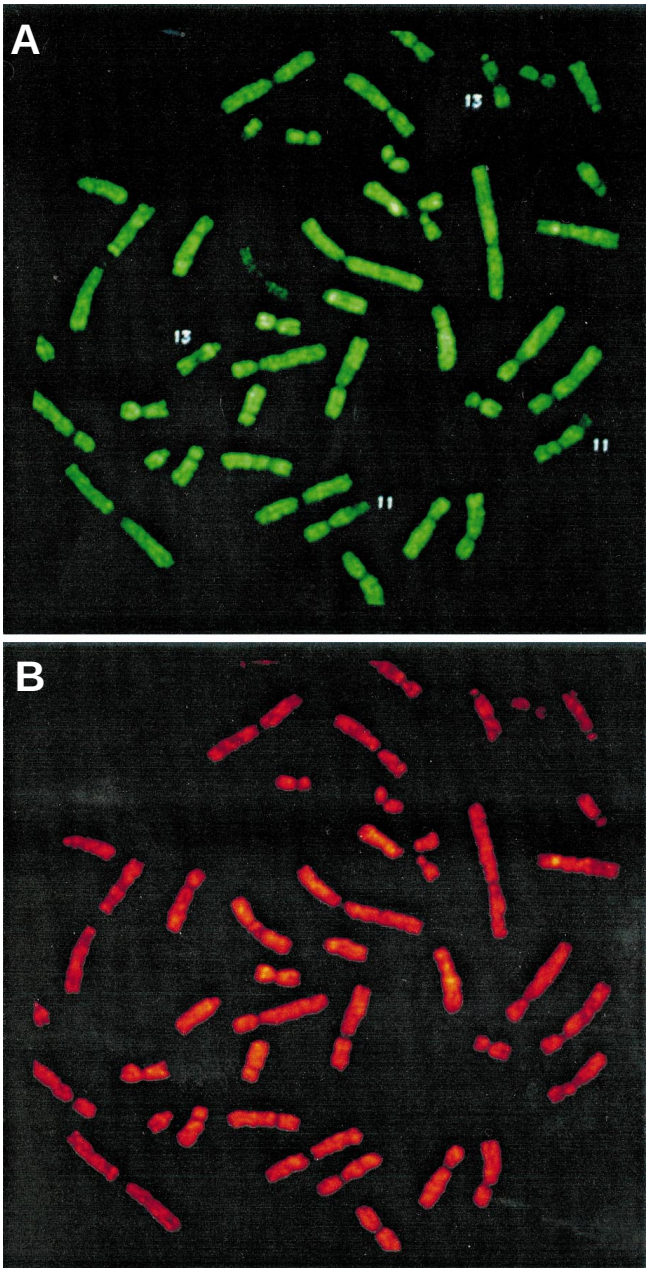

Fig. 3A,B Normal metaphase cell hybridized simultaneously with DNA from a B-CLL tumor detected via FITC (**A**, *green*) and a control DNA detected via rhodamine (**B**, *red*). The hybridizations are visualized using different fluorescence filter sets. Whereas with the control DNA (**B**, *red*) all regions containing euchromatin are stained more or less homogeneously, weaker staining of interstitial sequences on the long arm of chromosome 13 and the distal part of chromosome 11 is clearly visible. In this tumor, deletions of these chromosome regions were present that were also confirmed by FISH using region-specific DNA probes

Comparative genomic hybridization analysis

In contrast to FISH, the novel technique of comparative genomic hybridization (CGH) [111] allows comprehensive screening for the presence of chromosomal imbalances in a tumor genome and does not depend on the knowledge of candidate regions that are altered in a specific tumor [111–113]. For CGH the whole genomic DNA of the tumor of interest is hybridized as probe to well-defined (normal) metaphase cells under suppression conditions (reverse in situ hybridization, reverse painting). Hybridization of genomic DNA results in a more or less homogeneous staining of all chromosomes. Chromosome regions that are overrepresented (e.g., trisomies or DNA amplifications) or underrepresented (e.g., monosomies or deletions)

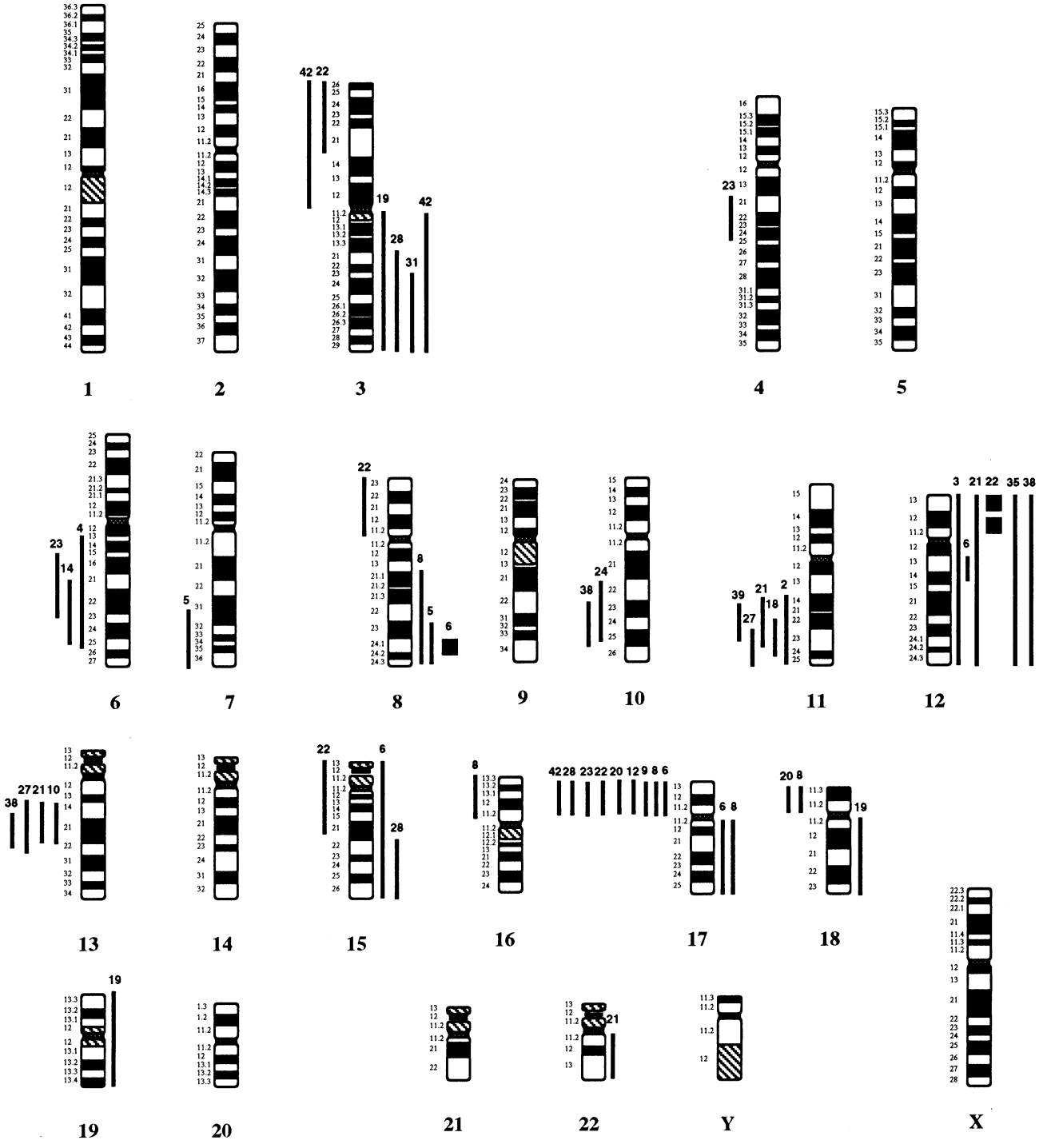

Fig. 4 Summary of chromosomal imbalances detected in 42 patients with chronic B-cell leukemias using CGH. Cases 6, 9, and 28 were classified as B-PLL; all other cases were B-CLL. *Lines left of ideograms* Loss of chromosomal material; *lines right of ideograms* gain of chromosomal material; *black squares* high-level DNA amplifications. These data were obtained using the software application ISIS (MetaSystems, Altlussheim, Germany)

in the test genome can be detected by a stronger or a weaker staining of the respective target regions in the metaphases (Fig. 3). Because signal inhomogeneities can also be caused by experimental parameters, an internal standard is introduced by cohybridization of normal genomic DNA. Signal inhomogeneities of diagnostic relevance are identified by comparison of the differentially visualized signal intensities of the test and control DNAs along the chromosomes.

The results of our CGH study of 28 patients with chronic B-cell leukemias show the potential of this method for diagnosis of genetic alterations in B-CLL [114]. Whereas many of the aberrations detected by CGH were already well known to occur in this disease, a gain of ma-

terial on chromosome arm 8q that was identified in 3 of 28 patients had not been described before. Comparison of the CGH data with banding results revealed a high proportion of cases (13 of 28) in which additional imbalances were detected by CGH. Among these there were 6 tumors that had a normal karyotype on banding analysis. This was almost half of the cases with normal karyotypes (n=13) included in this study. Other discrepancies were based on a failure of preparing metaphase cells or on complex karyotypes, in which not all overrepresented regions could be assigned to specific chromosome bands by banding studies. Figure 4 summarizes the CGH data of 42 chronic B-cell leukemias (39 B-CLL, 3 B-PLL).

In two of the complex cases reported in our initial study [114] high copy number amplifications of chromosomal subregions were identified (chromosome bands 8q24, 12p11–12 and 12p13), a finding that is rare in chronic B-cell leukemias [115]. CGH has proven to be a sensitive method for the detection of high-level DNA amplifications, and high incidences of such amplifications have recently been reported in various subtypes of lymphoproliferative disorders [116–120]. In addition to identifying amplified sequences, CGH also provides information for localization within the genome. The involvement of candidate proto-oncogenes mapping to the respective bands has been tested in the two B-CLL tumors by Southern blot and interphase FISH analyses. These studies demonstrated amplification of the proto-oncogene *MYC* (8q24) and the cell cycle gene encoding cyclin D2 (*CCND2*; 12p13) [114, 117].

Delineation of critical genomic regions by CGH has provided important information for selecting locus-specific DNA probes to be used for rapidly screening large numbers of B-CLL cases by interphase cytogenetics.

Correlations of chromosome aberrations with prognosis and clinical characteristics

Correlations based on chromosome banding studies

The First IWCCLL [10] observed a correlation between the karyotype and overall survival in 391 patients with B-CLL. Patients whose leukemic cells had a normal karyotype had a better survival (median 15 years) than those whose cells had clonal aberrations (median 7.7 years). Furthermore, patients with complex abnormalities had an worse outcome than those with single aberrations. However, multivariate analysis confirmed neither the presence of clonal aberrations nor the number of clonal aberrations to be independent prognostic variables.

Trisomy 12 was the first specific chromosome aberration in B-CLL that univariate analysis associated with both shorter treatment-free interval [121] and shorter overall survival [22] (Table 4). The First and Second IWCCLL [10, 11] evaluated the survival of patients with single chromosome abnormalities. Among these patients those with trisomy 12 had the poorest survival. However, one should consider that such an adverse prognostic impact of trisomy 12 has not been confirmed by other studies from single institutions [20, 21, 26, 27].

Other aberrations that have been associated with inferior survival, are translocations involving chromosome band 14q32, leading to the formation of a "14q+" chromosome [10, 21], abnormalities of chromosome 17 [55], and 11q deletion [57]. The First IWCCLL [10] reported that patients with a 14q+ had shorter survival times than those with trisomy 12. However, this 14q+ aberration commonly resulted from the reciprocal translocation t(11;14)(q13;q32) that is associated with MCL. Excluding these cases, the 14q+ aberration is probably not a frequent chromosome abnormality in B-CLL. A recent analysis of 480 newly diagnosed, untreated B-CLL patients discovered abnormali-

Table 4 Correlations of specific chromosome aberrations with the clinical characteristics and outcome in patients with B-CLL

	Chromosome banding	Interphase cytogenetics
Trisomy 12	Atypical morphology [26, 54, 58]	Atypical morphology [54, 72–74]
	Stronger SmIg + FMC7 expression [54, 73, 74]	Stronger SmIg + FMC7 expression [54, 73, 74]
	Advanced stages [53, 70]	Advanced stages [54, 72]
	Shorter treatment-free interval [22, 121]	
	Shorter survival times [10, 11, 22, 25]	Shorter survival times [72]
	No difference in survival [20, 21, 27, 54]	
13q aberrations	Favorable prognosis [10, 11, 57]	No data
11q aberrations	Disease progression [56, 57]	Extensive lymphadenopathy [66]
		Advanced stages [66]
		Shorter treatment-free interval [66]
	Shorter survival times [57]	Shorter survival times [66]
17p aberrations		Shorter treatment-free interval [80]
	Shorter survival times [55]	Shorter survival times [80, 83][a]
		Resistance to treatment [80, 83][a]

[a]Analysis of the *TP53* gene by SSCP analysis

ties of chromosome 17 to be associated with poor survival [55]. This has been the only cytogenetic finding with independent prognostic value. Finally, in a series of 84 B-CLL patients, Neilson et al. [57] found 11q deletions to be associated with rapid disease progression and shorter survival. In contrast, patients with structural aberrations of chromosome 13 seem to have a favorable outcome. In the IWCCLL studies these patients had survival times similar to those with a normal karyotype [10, 11].

Correlations based on interphase cytogenetics

Evaluation of the incidence of specific aberrations in B-CLL by the novel molecular cytogenetic techniques will provide the basis for more accurate correlations with prognosis. However, at present only few interphase cytogenetic studies on larger patient series are available that allow a meaningful statistical analysis. In addition, one of the drawbacks of these studies is that only single aberrations are evaluated for their prognostic significance, not taking into account the impact of additional chromosome aberrations.

Trisomy 12

Only a single large interphase study has so far been published that evaluated the prognostic impact of trisomy 12 [72]. This study observed no significant difference in survival probabilities between patients with (*n*=41) and those without trisomy 12 (*n*=76) as assessed by FISH. However, when the data of conventional chromosome banding analysis were included in the analysis, median survival in patients with trisomy 12 was significantly shorter than that in patients with a normal karyotype (7.8 versus 14.4 years). In addition, the patients with trisomy 12 were more likely to have been treated previously and to have advanced Binet stages than those without trisomy 12. The response to treatment with fludarabine was similar in patients with trisomy 12 and those with diploid karyotypes, but there was a trend for earlier disease progression in the trisomy 12 group.

TP53 gene deletion

In our study 17 of 100 cases (17%) with chronic B-cell leukemias exhibited a monoallelic *TP53* deletion [80]. The presence of the deletion had strong implications for the clinical course of the disease. Patients with a *TP53* deletion had significantly shorter survival times than those without a deletion. Furthermore, *TP53* gene deletion predicted for nonresponse to treatment with purine analogs. None of the 12 patients with a *TP53* gene deletion responded to therapy with fludarabine or pentostatin, compared to 20 of 36 (56%) patients without a deletion. Multivariate analysis identified *TP53* gene deletion as the strongest prognostic factor for survival, followed by known prognostic factors in B-CLL such as age, Rai stage, and hemoglobin level. We have extended this series to 243 B-CLL patients. Figure 5 presents the survival probabilities of patients with (*n*=20) and without (*n*=223) *TP53* gene deletion. The difference between the two curves is highly significant ($P<0.001$). This strong predictive value of a *TP53* gene mutation has also been shown in a SSCP study of 53 patients [83]. *TP53* gene mutation occurred at a similar frequency and, in agreement with our interphase cytogenetic study, was the strongest independent prognostic factor. Thus *TP53* gene deletion/mutation is the first aberration shown to have an independent prognostic impact in B-CLL.

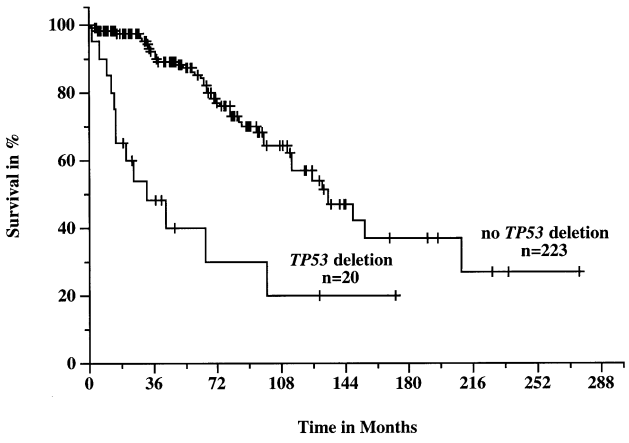

Fig. 5 Survival probabilities from the time of diagnosis of 243 B-CLL patients with (*n*=20) and without (*n*=223) *TP53* gene deletion. The difference between the two curves is highly significant ($P<0.001$)

11q deletions

More recently we have identified the 11q22.3-q23.1 deletion as a second chromosome aberration which in multivariate analysis is of independent prognostic significance [66]. In this study 43 of 214 (20%) B-CLL cases exhibited an 11q deletion. Presence of the 11q deletion was associated with a characteristic clinical picture. Patients with 11q deletions were younger and had more advanced Rai stages. Notably, 11q deletions were associated with extensive lymphadenopathy as assessed by the extent of peripheral lymph node involvement, the frequency of mediastinal or abdominal lymphadenopathy, and the largest lymph node diameter measured (Fig. 6). Patients with an 11q deletion had a more rapid disease progression, as shown by a shorter treatment-free interval (9 months vs 43 months; $P<0.001$). The prognostic effect of 11q deletion on survival strongly depended on the age: median survival in patients aged under 55 years was significantly shorter in the deletion group (64 months vs 209 months; $P<0.001$), while there was no significant difference in those aged 55 years or older (94 months vs 111 months; $P=0.82$). Figure 7 shows the survival probabilities of 250 patients with (*n*=48) and without (*n*=202) 11q deletions. The difference

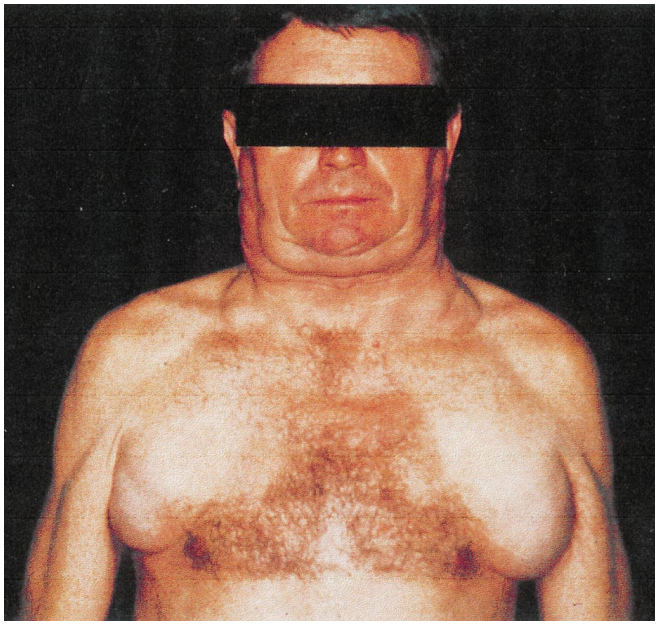

Fig. 6 Patient whose leukemic cells exhibited a deletion of the genomic region 11q22.3-q23.1. 11q deletion is associated with extensive nodal involvement and inferior survival

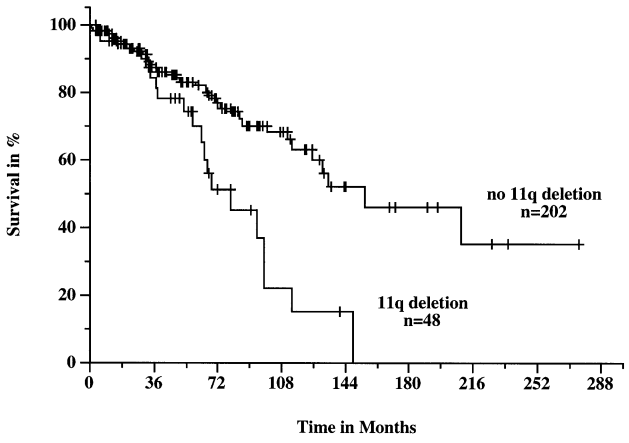

Fig. 7 Survival probabilities from the time of diagnosis of 250 B-CLL patients with (n=48) and without (n=202) 11q deletion. The difference between the two curves is significant (P<0.001)

between the two curves is significant (P<0.001). Thus by using interphase cytogenetics we identified a new clinical subset of B-CLL characterized by extensive lymph node involvement. In younger B-CLL patients 11q22.3-q23.1 deletion is an important predictor of survival.

Conclusion

The use of molecular cytogenetic and molecular genetic techniques led to identification of two new independent prognostic markers, deletion of the *TP53* tumor suppressor gene and deletion of the genomic region 11q22.3-q23.1, which should now be evaluated prospectively in large clinical trials. To obtain a more comprehensive view on the chromosome aberrations in B-CLL, it will be necessary to screen the tumors for a number of other critical regions. Thus, it will be important to design a disease-specific DNA probe-set that can be applied for interphase cytogenetics or for the recently developed technique of matrix-based CGH [122] in large patient series. The results of such studies should provide unambiguous data on the impact of single chromosome aberrations in B-CLL.

For interphase cytogenetic analysis to become a routine clinical test in the work-up of B-CLL patients [123] it will be important to demonstrate that specific chromosome aberrations provide prognostic information in addition to that obtained by the clinical prognostic markers, for example, disease stage. In particular, it would be of great clinical relevance if specific chromosome aberrations could be used as prognostic markers for early disease progression in patients with Rai stage 0–2 or Binet stage A. Because the prognostic impact may also depend on the age of the patient, it is important to assess the prognostic significance of these aberrations in different age groups, especially in younger B-CLL patients who are candidates for experimental treatment approaches such as high-dose chemotherapy and radiotherapy followed by autologous or allogeneic hematopoietic stem cell transplantation.

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