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ORIGINAL ARTICLE

Heterogeneous patterns of amplification of the *NUP214-ABL1* fusion gene in T-cell acute lymphoblastic leukemia

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Episomes with the *NUP214-ABL1* fusion gene have been observed in 6% of T-ALL. In this multicentric study we collected 27 cases of *NUP214-ABL1*-positive T-ALL. Median age was 15 years with male predominance. Outcome was poor in 12 patients. An associated abnormality involving *TLX1* or *TLX3* was found in all investigated cases. Fluorescent *in situ* hybridization revealed a heterogeneous pattern of *NUP214-ABL1* amplification. Multiple episomes carrying the fusion were detected in 24 patients. Episomes were observed in a significant number of nuclei in 18 cases, but in only 1–5% of nuclei in 6. In addition, intrachromosomal amplification (small hsr) was identified either as the only change or in association with episomes in four cases and two T-ALL cell lines (PEER and ALL-SIL). One case showed insertion of apparently non-amplified *NUP214-ABL1* sequences at 14q12. The amplified sequences were analyzed using array-based CGH.

These findings confirm that the *NUP214-ABL1* gene requires amplification for oncogenicity; it is part of a multistep process of leukemogenesis; and it can be a late event present only in subpopulations. Data also provide *in vivo* evidence for a model of episome formation, amplification and optional reintegration into the genome. Implications for the use of kinase inhibitors are discussed.

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Introduction

The translocation t(9;22)(q34;q11.2) fusing the *ABL1* to the *BCR* gene is the hallmark of chronic myeloid leukemia. The molecular consequence of this translocation is the production of a constitutively activated tyrosine kinase, BCR-ABL1, which is the target of therapy with the inhibitors of ABL1 kinase activity.[1] The t(9;22) has also been observed in 3 and 25% of childhood and adult B-cell acute lymphoblastic leukemia, respectively, as well as rare cases of acute myeloid leukemia.[2–4]

Besides *BCR*, other *ABL1* fusion partners have been rarely described.[5,6] The *ETV6-ABL1* fusion of the translocation, t(9;12)(q34;p13), also revealed the same biological consequences including uncontrolled proliferation and increased survival of hematopoietic progenitors.[5]

In T-cell acute lymphoblastic leukemia (T-ALL), recent studies have shown the presence of the *NUP214-ABL1* fusion gene in 6% of cases, whereas *BCR-ABL1* and *ETV6-ABL1* are rare ($<1\%$).[5,7–9] The *NUP214-ABL1* fusion gene was first reported on amplified episomes due to extrachromosomal circularization of the 500 kb DNA fragment located between the two genes.[9] Intrachromosomal amplification of the fusion has been reported on chromosome 2 in one patient.[10] More recently, the analysis of the breakpoints of a novel cryptic translocation t(9;14)(q34;q32) revealed an *EML1-ABL1* fusion gene in a single case of T-ALL.[11,12]

Of interest, the *NUP214-ABL1* and *EML1-ABL1* fusions in T-ALL are usually associated with alterations of other genes such as *CDKN2a*, *TLX1* or *TLX3* and *NOTCH1*.[9–12] These observations indicate a multigene contribution to the pathogenesis of T-cell leukemia. It has been postulated that (i) homo- or heterozygous deletion of the tumor suppressor gene *CDKN2a* impairs cell cycle control and favors genetic instability, (ii) activating *NOTCH1* mutations provide T-cell precursors with self-renewal capacity, (iii) ectopic expression of *TLX1* or *TLX3* impairs thymocyte differentiation and (iv) constitutive kinase activity of ABL1 fusions provides proliferative and survival advantages.[13]

Given that tyrosine kinase inhibitors (TKI), such as imatinib, suppress the constitutive kinase activity of ABL1, there is potential for their use in therapies of T-ALL patients with *NUP214-ABL1* and *EML1-ABL1*.[9,11,14]

Here, we report clinical and genetic characteristics of 27 T-ALL cases and genetics of two T-ALL cell lines positive for the *NUP214-ABL1* fusion gene. These data allow us to present a model for *NUP214-ABL1* fusion on episomes, followed by optional re-integration in a chromosome as small amplified sequences (homogeneously stained regions (hsr)).

Materials and methods

Patient samples

Bone marrow, blood, lymph node or pleural fluid samples from 347 T-ALL patients at diagnosis and, if possible, also at relapse were collected from 21 centers in collaboration with the 'Groupe Francophone de Cytogénétique Hématologique' and the Belgian Cytogenetic Group for Hematology and Oncology. Eight T-ALL patients were included from the UK Cancer Cytogenetics Group (Patients 20–27 in Table 1). Although they were published earlier, they were not identified as *NUP214-ABL1*-positive; only the amplification of *ABL1* was reported.[8] Owing to the random selection, these patients were not used in assessing the frequency of the fusion in T-ALL.

Samples were obtained according to the guidelines of the local ethical committees. Diagnosis of T-ALL was based on morphology, cytochemistry and immunophenotyping according to the World Health Organization and European Group for the Immunological Characterization of Leukemias criteria.[15,16]

Cell lines

ALL-SIL and PEER are T-ALL-derived cell lines positive for the *NUP214-ABL1* fusion (DSMZ, Braunschweig, Germany). ALL-SIL displays the following phenotype: CD2−, CD3−, cyCD3+, CD4+, CD5+, CD6+, CD7+, CD8+, CD13−, CD19−, CD34−, TCRalpha/beta-, TCRgamma/delta- and karyotype: 90∼95,XXYY,t(1;13)(p32;q32)x2,+6,del(6)(q25)x2,+8,+8,del(9)(?p23p24)x2,t(10;14)(q24;q11.2)x2,add[17](p11)x2/90∼95,sl,-20,20. This cell line shows expression of TLX1.[17]

PEER displays the following phenotype: CD2−, CD3+, CD4+, CD5+, CD6+, CD7+, CD8+, CD13−, CD19−, CD34−, TCRalpha/beta-, TCRgamma/delta+ and karyotype: 42∼47,XX,der(4)?dup ins(4;4)(?p11;?q21q25),del(5)(q22q31), del(6)(q13q22),del(9)(p11p22),del(9)(q22). The homeobox gene *NKX2-5* is aberrantly expressed in PEER.[18]

Immunophenotyping

Intracytoplasmic CD3, surface CD3, CD2, CD5, CD7, CD4, CD8, and CD1a expression was assessed in each center using standard methods.

Cytogenetics

Cells were cultured and harvested in the different centers following standard methods, and karyotypes were analyzed and described according to the International System for Human Cytogenetic Nomenclature.[19]

Fluorescence in situ *hybridization*

Each sample was tested with the LSI *BCR/ABL1* dual color ES probe (Vysis, Ottignies, Belgium) either locally (38%) or centrally at the UCL in Brussels (62%). Cases with an abnormal hybridization pattern (more than 2 *ABL1* signals per nucleus, asymmetry of the signals, aberrant localization of the signals on metaphase cells) were further characterized using BAC and fosmid probes selected from http://www.ensemble.org (Chori BACPAC Resources, Oakland, USA). These probes included differentially labeled *ABL1* break-apart BAC probes covering the 5′ part and the 3′ part of *ABL1* (RP11-57C19 and RP11-83J21 clones, respectively). *NUP214* break-apart fosmid probes (G248P89679D11 flanking the 5′ part and G248P87560C9 flanking the 3′ part of *NUP214*) were used to detect breakpoints on *NUP214*. BAC clone RP11-544A12 covering *NUP214* was used to assess its colocalization with *ABL1*. A set of probes targeting genes located at 9q34 (*SET, GPR107, ASS, FUBP3, LAMC3, NTNG2, VAV2*) was used as described earlier to delimit 9q34 amplifications.[9] *TLX1/TLX3* rearrangement and *CDKN2a* (p16) deletion screening was performed using commercial probes (Dako, Glostrup, Denmark; Abbott, UK). A minimum of 200 nuclei were examined in each sample. When an abnormality was present at a low percentage, 500 to 1000 nuclei were examined.

Reverse transcriptase PCR

Reverse transcriptase PCR (RT-PCR) for the *NUP214-ABL1* fusion transcript and *TLX1* or *TLX3* expression was performed in the different centers according to the local protocols. *NUP214-ABL1* positivity was centrally validated using semi-nested RT-PCR with the following primers in five cases:

NUP214 ex23 F: 5′-AGTCAGGCACCAGCTGTAAAC-3′;
NUP214 ex29 F: 5′-AGGGAGGCTCTGTCTTTGGT-3′;
NUP214 ex31 F: 5′-AGAGGGGGAGGTTTCCTCAGT-3′;
NUP214 ex32 F: 5′-GCCAAGACATTTGGTGGATT-3′ combined with:
ABL1 ex3 R: 5′-TAACTAAAGGTGAAAAGCTCCGG-3′ (first round)
and *ABL1* ex2-3 R: 5′-GTGAAGCCCAAACCAAAAAT-3′ (second round).
Primers used for *TLX1* expression: *TLX1* ex2 F: 5′-GCGTCAAC AACCTCACTGGCC-3′;
TLX1 ex3 R: 5′-GTGGAAGCGCTTCTCCAGCTC-3′.
Primers used for *TLX3* expression: *TLX3* ex2 F: 5′-GCGCATC GGCCACCCCTACCAGA-3′;
TLX3 ex3 R: 5′-CCGCTCCGCCTCCCGCTCCTC-3′.

We used the following PCR conditions: denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s (*NUP214-ABL1*) or 30 s (*TLX1* and *TLX3*) and a final cycle of 7 min at 72 °C.

Chromosomal copy number change analysis

For case no. 15, chromosomal copy number change analysis was performed on the diagnosis and the first relapse samples using the GeneChip Human Mapping 250 K NspI single nucleotide polymorphism array (http://www.Affymetrix.com) according to protocols provided by the manufacturer (Affymetrix Inc., Santa Clara, CA, USA).

Array-based comparative genomic hybridization (array CGH) was performed on two *NUP214-ABL1*-positive T-ALL cell lines, ALL-SIL and PEER, using the Human Genome CGH Microarray

Table 1 Clinical and genetic characteristics of 27 *NUP214-ABL1*-positive T-ALL patients

ID	*Sex/Age*	*Immuno-phenotype*	*WBC (× 10e9/l)*	*Marrow blasts (%)*	*Karyotype*	*Additional molecular findings*		*Response/ Relapse OS (months)*	*NUP214-ABL1 fusion analysis*			
						TLX1/ TLX3[a]	*CDKN2a*[a,b]		*FISH*			*RT–PCR*
									Pattern of ampl.	*Nuclei with ampl.*	*Signals/ nuclei*[c]	
1	Male/28	Mature	NA	10	46,XY,del(7)(p11p15)[3]/46,XY[17]	*TLX3*	ND	Relapse (7) OS (7)	Episomes hsr insertion	<1% 5% 33%	NA	ND
2	Female/36	Cortical	89.2	70	46,XX,t(10;14)(q24;q11)[6]/46,XX[27]	*TLX1*	ND	Relapse (45) OS (52)	Episomes	2%	6 (5) [3–15]	+
3	Male/3	pre-T	195	61	48,XY,+7,+8[3]/46,XY[17]	*TLX1*	ND	CR1 OS (73+)	Episomes	1%	NA	+
4	Male/45	Cortical	NA	NA	47,XY,+?8[2]/46,XY[11]	ND	ND	Relapse (12) OS (15)	ND	/	/	+
5	Female/14	Cortical	52.6	90	46,XX[29]	*TLX3*	ND	CR1 OS (125+)	No abnormality	/	/	+
6	Female/6	NA	330	94	45,XX,dic(14;19)(p11;p11)[16]/46,XX[4]	*TLX3* (93%)	ND	CR1[a,d] OS (6)	Episomes	80%	NA	ND
7	Female/28	Cortical	38	93	46,XX,t(7;10)(q35;q24)[10]/46,XX[5]	*TLX1*	ND	Relapse (10) OS (17)	hsr	90%	/	+
8	Male/22	Cortical	67	89	46,XY,t(7;10)(q35;q24),inv(14)(q11q32)[5]/46,XY[10]	*TLX1*	+/+	CR1 OS (77+)	Episomes	80%	27(25) [20–45]	+
9	Male/7	Cortical	38.8	85	88~92,XXYY[7]/46 XY[12]	*TLX3*	ND	CR1 OS (62+)	Episomes	4%	NA	+
10	Male/18	Cortical	4.0	90	46,XY[3]	*TLX1* (80%)	ND	CR1 OS (40+)	Episomes[e]	1%	5 (5) [3–6]	+
11	Male/11	pre-T	37.1	77	46,XY,del(1)(p22p32)[15]/46,XY[15]	*TLX3*	+/+	CR1 OS (94+)[d]	Episomes	10%	10(6) [3–26]	+
12	Male/26	Cortical	4.1	85	47,XYY	*TLX3* (82%)	−/−(95%)	Relapse x2 (9, 18) OS (19)	Episomes	5%	12 (9) [3-30]	+
					relapse: 47,XYY,del(11)(q13q23)[18]/47,XYY[2]							
13	Male/5	pre-T	10.0	26	46,XY,del(6)(q21q25)[15]/46,XY[2]	*TLX3*	−/−	Relapse (22), CR2 OS (23+)	Episomes	9%	10(8) [3–35]	+
14	Male/8	pre-T	14.4	95	49~50,X,-Y,+6,+13,+14,+19,+20,+22[cp15]/46,XY[8]	*TLX3*	+/+	Relapse (14), CR2 OS (24+)[d,f]	Episomes	81%	20(15) [3–42]	ND
15	Male/17	pre-T	83.1	80	46,XY[20]	*TLX3*	−/−	OS (30)[d,f]	Episomes hsr	28% 55%	20 (25) [10–50]	+
					relapse 1: 46,XY,inv(9)(q32q34)[15]/46,XY[5]	ND	ND	Relapse 1 (18)	Episomes hsr	1% 95%		
					relapse 2: 46,XY,inv(9)(q32q34)[1]/47,idem,del(1)(p?12p31), ?der(19),+20[7]/47,idem,del(1)(p?12p31),-2,-7,?der(19), +20,+mar1,+mar2[2]	ND	ND	Relapse 2 (27)	Episomes hsr	0% 78%		
					relapse 3: 47,XY,del(1)(p?12p31),inv(9)(q32q34),+20[4]/47, idem,-2,add(7)(p11), add(8)(q23),-10,?der 19),+mar1,+mar2[cp7]	ND	ND	Relapse 3 (29)	Episomes hsr	0% 23%		

Table 1 (*Continued*)

ID	Sex/Age	Immuno-phenotype	WBC (× 10e9/l)	Marrow blasts (%)	Karyotype	Additional molecular findings		Response/ Relapse OS (months)	NUP214-ABL1 fusion analysis			
						TLX1/ TLX3[a]	CDKN2a[a,b]		FISH			RT–PCR
									Pattern of ampl.	Nuclei with ampl.	Signals/ nuclei[c]	
16	Male/12	pre-T	18	44	46,XY,der 2).ish(wcp2+,wcp9p+),der(5)t(5;9)(q33;q31), del(9)(q12q33),der(9) del(9)(p13p22)t(2;9)(q22;q21),der(11) t(9;11)(q?;p15),inv(14)(q11q32)[4]/46,XY[1]	*TLX3*	+/−	CR1[f,g] OS (12+)	Episomes hsr	/	/	+
17	Male/40	pre-T	47.6	75	46,XY[20]	*TLX1*	+/+	CR1 OS (19+)[d,f]	Episomes	90%[h]	16(13) I3–25I	+
18	Male/48	Cortical	53.8	89	47,XY,t(3;8)(p25;q12),t(7;10)(q35;q24),+8[15]/46,XY[3]	*TLX1*	+/−	CR1 OS (3+)[f]	Episomes	85%	19(11) I5–35I	+
19	Male/9	NA	394	NA	47,XY,t(5;14)(q33;q32),del(9)(q13q22),+12[8]/ 46,XY[2]	*TLX3* (36%)	−/− (90%)	Relapse (8), CR2[i] OS (86+)	Episomes	88%	>30	ND
20	Male/11	NA	111	96	Failure	*TLX3* (90%)	−/− (81%)	NA (1) OS (1)	Episomes	67%	I5–20I	ND
21	Female/15	NA	18	84	47,XX,t(2;9)(p2?3;q1?),t(4;6)(q3?;q1?),+8[5]	ND	−/− (95%)	Relapse (10) OS (12)	Episomes	91%	NA	ND
22	Male/12	NA	18.5	92	46,XY[20]	*TLX3* (92%)	−/− (94%)	Relapse (10) OS (13)	Episomes	47%	6	ND
23	Female/14	NA	2.6	87	96,<4n>,XXXX,+X,+11,+14,+16,del(16)[4],inc[cp5]	*TLX3* (88%)	+/− (7%)	CR1 OS (86+)	Episomes	82%	>30	ND
24	Male/42	NA	90	95	45,X,-Y,t(10;14)(q24;q11)[8]	*TLX1* (87%)	+/− (21%) −/− (12%)	Relapse (18) OS (24)	Episomes	94%	NA	ND
25	Male/2	NA	204	NA	46,XY[8]	*TLX1*	−/− (72%)	CR1 OS (10+)	Episomes	87%	I6–30I	ND
26	Male/18	NA	46	98	45,XY,der(1)t(1;9)(p36;q?),del(6)(q?2),r(7),-9[20]	ND	+/− (92%)	CR1 OS (30+)	Episomes	82%	>30	ND
27	Male/36	NA	114	NA	46,XY,?add(3)(p21),t(10;14)(q24;q11),-12, add(12) (p?1),+mar,inc[cp5]	*TLX1* (84%)	−/− (94%)	NA OS (1+)	Episomes	12%	NA	ND

ampl., amplification; FISH, Fluorescent *in situ* hybridization; hsr, homogeneously stained regions; RT-PCR, Reverse transcriptase PCR; T-ALL, T-cell acute lymphoblastic leukemia ND, not done; NA, non available.
[a]% of nuclei with the abnormal pattern.
[b]By FISH (+/+ = no deletion, +/− = heterozygous and −/− = homozygous deletion).
[c]Mean (median) Irangel.
[d]allo-transplantation in CR1.
[e]10% on non cultured cells.
[f]Imatinib given in addition to chemotherapy.
[g]*NUP214-ABL1* still detectable by RT-PCR after 12 months.
[h]Microdeletion at 9q34.
[i]allo-transplantation in CR2.

244 K (Agilent, Santa Clara, CA, USA) with a resolution of 6.4 kb, following manufacturer instructions. The array CGH data were analyzed using the software CGH analytics 3.4.40 (Agilent, Santa Clara, CA, USA).

Results

Screening of T-ALL samples for the presence of *NUP214-ABL1* using Fluorescent *in situ* hybridization (FISH) with the *BCR-ABL1* ES probe (Vysis) and/or RT-PCR identified 19 positive cases. Eight cases, reported earlier as amplification of ABL1 and subsequently identified as NUP214-ABL1-positive were included in this series.[8]

The relevant clinical and biological characteristics of these 27 T-ALL patients are given in Table 1.

Characteristics of the patients

Median age at diagnosis was 15 years (2–48 years) with a male predominance (21M:6F). The same male/female ratio was also observed in the whole group of T-ALLs selected for this retrospective study. Immunophenotype was mature in one patient, cortical in nine cases, pre-T in seven patients and not specified in ten cases. Complete remission was achieved in all patients apart from one with early death (no. 20). Ten patients relapsed before completion of treatment (including maintenance therapy), two relapsed off treatment and one died from infection in complete remission. Kaplan–Meier estimated survival at 5 years was 49 ± 11% with a median follow-up of 7.1 years (reverse Kaplan–Meier). Structural and numerical chromosomal aberrations were detected in 21 cases, including six T-ALL with 10q24/*TLX1* rearrangements and four with trisomy 8. Cytogenetic aberrations of the long arm of chromosome 9 were observed in five cases. Case nos. 16 and 19 presented with del(9)(q12q33) and del(9)(q13q22), respectively. Additionally, case no. 16 had unbalanced translocations of chromosome 9q material onto chromosome 2, 5 and 11. Case no. 15 harbored an inv(9)(q32q34) at relapse, case no. 21 a balanced t(2;9)(p2?3;q1?) and case no. 26 an unbalanced der(1)t(1;9)(p36;q?). Rearrangement of *TLX1* or *TLX3* or their ectopic expression were detected by FISH or RT-PCR, respectively, in all 24 investigated cases confirming the association of *NUP214-ABL1* with aberrations of *TLX1* and *TLX3* in T-ALL.[9–12]

Deletion of *CDKN2a* was found in 14 out of the 18 analyzed cases by FISH (nine homozygous, four heterozygous and one having both patterns).

Characterization of the NUP214-ABL1 amplification

FISH analysis using the *BCR-ABL1* probe demonstrated episomal amplification of *ABL1* in 24 of the 26 analyzed cases (Figure 1 and Table 1). Using break-apart probes for *ABL1* and *NUP214*, we demonstrated that this amplification contained only the 3′ part of *ABL1* and the 5′ part of *NUP214*. *LAMC3* located between these genes was also coamplified. RT-PCR confirmed the presence of a *NUP214-ABL1* fusion transcript in all investigated cases ($n = 15$). The percentage of nuclei with episomal amplification was highly variable among patients (<1–94%). Furthermore, the number of episomes per nucleus varied from 3 to 30 or more within the same sample. Samples with *NUP214-ABL1* amplification in a high percentage of nuclei tended to have a higher number of episomes per nucleus (Table 1).

Interestingly, case no. 10 showed a higher percentage of nuclei with episomal *NUP214-ABL1* amplification when frozen non-cultured cells rather than cultured Carnoy fixed cells were investigated (10 versus 1%).

Intrachromosomal *NUP214-ABL1* amplification was detected in four cases (case nos. 1, 7, 15 and 16) and in the 2 cell lines. Patient no. 1 displayed intrachromosomal amplification of the *NUP214-ABL1* fusion on chromosome 10 (Figure 1d) or at 9q34 in different cells. These intrachromosomal amplifications represented small homogeneously stained regions (hsr) as described by conventional cytogenetics. Furthermore, this case exhibited episomes in less than 1% of nuclei and an intrachromosomal insertion of 9q34 sequences at 14q12 in 33% of nuclei. This intrachromosomally inserted fragment was apparently not amplified (Figure 1e and f). FISH analysis demonstrated that this insertion included the 5′ part of *NUP214* (Figure 1e), the 3′ part of *ABL1* that encode the tyrosine kinase domain (Figure 1f) and *LAMC3* located between both genes (not shown), but neither the 5′ part of *ABL1* nor the 3′ part of *NUP214*. This insertion corresponded to the genomic sequence of one single episome.

Case no. 7 displayed an intrachromosomal amplification of the *NUP214-ABL1* fusion at the original 9q34 site without detectable episomes (not shown). This small hsr was first overlooked by FISH but detected retrospectively when, following the RT-PCR results, the FISH data were reassessed.

FISH analysis of patient no. 15 showed *NUP214-ABL1* amplification in 83% of the nuclei. It is noteworthy that this amplification was present both as episomes and hsr in 34 and 66% of the cells with amplification, respectively (Figure 2a). In the first relapse and subsequent samples, the percentage of cells with hsr in the nuclei with *NUP214-ABL1* amplification increased to 99% and 100%, respectively. In the samples at diagnosis and first relapse, *NUP214-ABL1* amplification was also demonstrated by single nucleotide polymorphism array analysis, and the amplified region exactly matched the episome segment (Figure 2) as described earlier.[9]

In case no. 16, episomes and hsr coexisted in the context of a very complex karyotype that displayed several copies of the 9q34 region.

No FISH aberrations were found in case no. 5, although it was positive for the *NUP214-ABL1* fusion by RT-PCR. Either an alternative mechanism is involved in the formation of the *NUP214-ABL1* fusion or the episomes were lost during cell culture and cytogenetic processing as observed in case no. 10.

Characterization by FISH showed that patient no. 17 carried a cryptic microdeletion of one chromosome 9 at 9q34 corresponding to a region slightly larger in size than one episome. This was demonstrated by FISH with specific probes showing that the 5′ part of *ABL1* was also deleted.

In both cell lines, ALL-SIL and PEER, intrachromosomal amplification of *NUP214-ABL1* was found at 9q34 (Figure 1g), whereas no episomal forms of the fusion were detected. Array CGH indicated that these small hsr contained sequences corresponding only to complete *NUP214-ABL1* episomes (3′*ABL1-LAMC3*-5′*NUP214*) (Figure 1h).

Discussion

This detailed characterization of 27 T-ALL cases and two cell lines, positive for the *NUP214-ABL1* fusion gene, revealed heterogeneous patterns of amplification of this fusion. The mechanism of formation and the clinical significance of such rearrangements remain largely unknown.

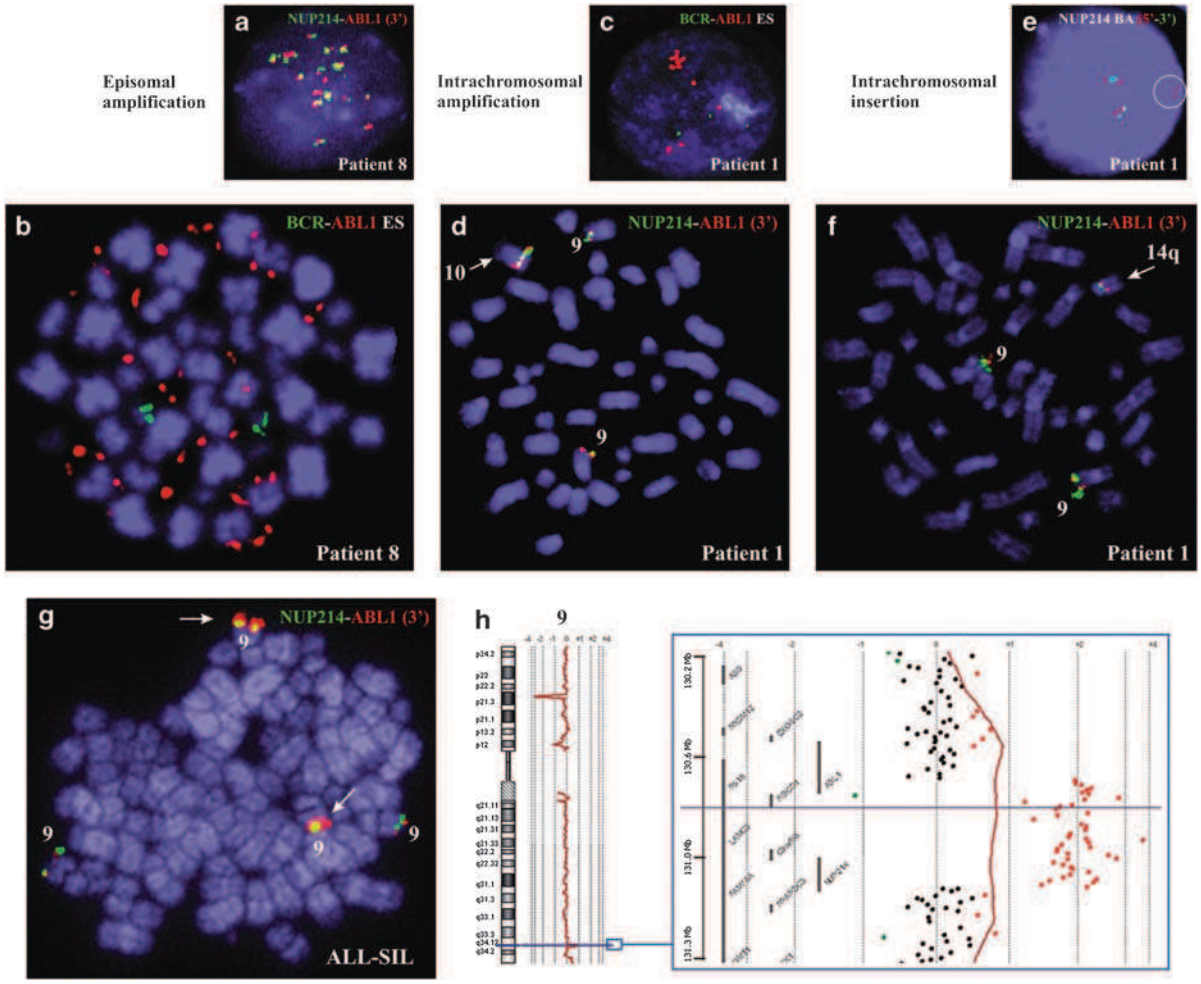

Figure 1 Different genomic patterns of the *NUP214-ABL1* fusion gene detected by FISH in both interphase and metaphase cells. Top: typical presentation of *NUP214-ABL1* as extrachromosomal episomes, intrachromosomal amplification and intrachromosomal insertion. (**a**) Nucleus from patient no. 8 showing coamplification of *NUP214* and *ABL1* probes. *LAMC3* located between these genes is also coamplified (data not shown). (**b**) Episomes in patient no. 8 seen as small dots between chromosomes using the *BCR-ABL1* ES probe set (Vysis, Ottignies, Belgium). (**c**) Nucleus of patient no. 1 showing a clustered amplification of the fusion (marked by a white circle) as revealed by the *BCR-ABL1* ES probe set (Vysis, Ottignies, Belgium). (**d**) Corresponding findings on a metaphase from patient no. 1 using differentially labeled probes for the 3′ part of *ABL1* and for *NUP214*. The small hsr was seen either on a chromosome 10 as illustrated or at 9q34 (data not shown). (**e**) 33% of nuclei in patient no. 1 showed a non-amplified intrachromosomal insertion corresponding to the 5′ part of *NUP214* (as illustrated by a white circle) and 3′ part of *ABL1* (not shown). (**f**) Corresponding findings on a metaphase using differentially labeled probes for the 3′ part of *ABL1* and for *NUP214*. The small insertion is located at 14q12. *LAMC3* also colocalized at 14q12 (not shown). Bottom: FISH and array CGH data from the cell line ALL-SIL. (**g**) *NUP214-ABL1* fusion was intrachromosomally amplified at 9q34 without detectable episomes. On this tetraploid metaphase, two chromosomes 9 show amplified *NUP214* and *ABL1* signals (arrows) as compared with normal signals on the two remaining chromosomes 9. (**h**) Array CGH data: on the left, hybridization pattern along chromosome 9 showing deletion at 9p21–p22 and amplification at 9q34; on the right, enlargement of the 9q34 region indicating that the amplification corresponds to 9q sequences located between 3′*ABL1* and 5′*NUP214*, the exact episomal sequence. Array CGH, array-based comparative genomic hybridization; FISH, Fluorescent *in situ* hybridization; hsr, homogeneously stained regions.

Both *ABL1* and *NUP214* genes are located at 9q34 in the same transcriptional orientation, with *NUP214* located more telomeric than *ABL1*. Consequently, the simplest way to generate a *NUP214-ABL1* fusion would be through a translocation, t(9;9)(q34;q34), leading to 5′*NUP214*-3′*ABL1* and the reciprocal 5′*ABL1*-3′*NUP214* fusions on the two derivative chromosomes 9. This translocation would be cytogenetically cryptic because of the close vicinity of both genes within the terminal region of 9q, but could be detected by FISH using the break-apart probes. Interestingly, this translocation has not yet been observed in any cases studied so far. The presence of multiple episomes is, at least at diagnosis, the most common and characteristic form of amplification of the *NUP214-ABL1* fusion gene. However, we also found small hsr, containing the exact sequences harbored by episomes, at various chromosomal localizations in the absence or presence of *NUP214-ABL1* episomes. Amplification of the *NUP214-ABL1* fusion gene was the hallmark of all cases. This is in accordance with the observation that the NUP214-ABL1 protein is less potent than BCR-ABL1 and EML1-ABL1 in transforming hematopoietic cells to growth factor independency.[20]

We present a model in which the formation of the episome is the primary event generating the *NUP214-ABL1* fusion gene (Figure 3). In some cases (for example as found in patient no. 17) this process is associated with a microdeletion at 9q34, but generally the 9q34 region is intact. Secondary circularization of the deleted or copied-and-excised fragment takes place, resulting in generation of the *NUP214-ABL1* fusion gene

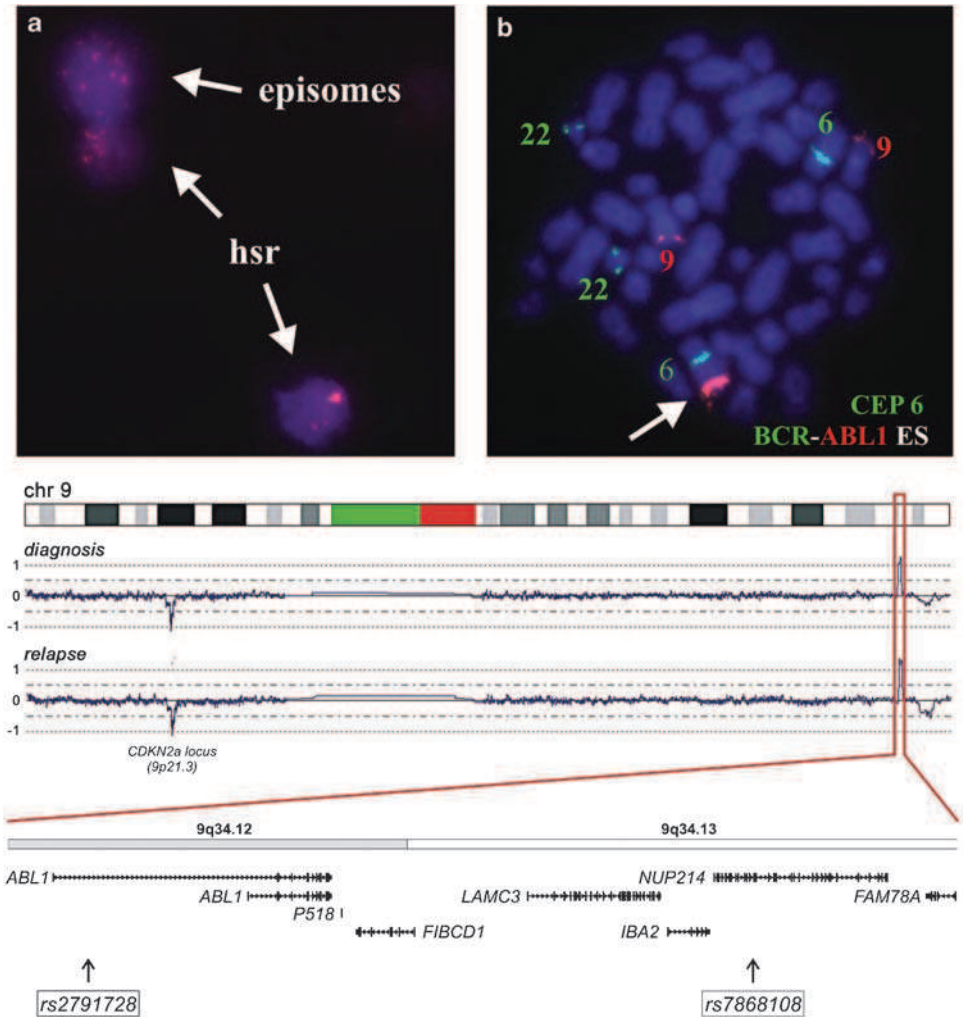

Figure 2 FISH and SNP array results of patient no. 15. Top: (**a**) At diagnosis, both episomal amplification as well as intrachromosomal amplification (hsr) of the *NUP214-ABL1* fusion are present. (**b**) At relapse, hsr is the only form of amplification. Bottom*:* SNP array analysis performed on diagnosis and relapse samples. Apart from the homozygous deletion of the tumor suppressor gene *CDKN2a* at 9p21 and the observed loss of sequences at 9q34.2q34.3, the *NUP214-ABL1* amplification was detected (boxed). The amplified region was flanked by SNP rs2791728 and rs7868108, which exactly matches the episome content as described earlier.[9] SNP,single nucleotide polymorphism; FISH, Fluorescent *in situ* hybridization; hsr, homogeneously stained regions.

containing episome. During cell division, episomes will segregate unequally due to the absence of a centromeric structure. From an oncogenic viewpoint, cells containing the highest number of the *NUP214-ABL1* fusion containing episomes will be preferentially selected. In some cases, reintegration of episomes into the genome may occur. This increases the stability of the fusion gene through successive rounds of cell division, while the asymmetrical distribution of episomes at mitosis probably results in more loss of cells as a result of variation in oncogenic potential. The findings in patient no. 15 are an *in vivo* example of this model as both episomes and hsr were present at disease presentation and, during disease progression, the episomes disappeared with an associated increase in the percentage of cells with hsr to 100%. This model is supported by the observation of frequent intrachromosomal integrations of the Epstein–Barr Virus (EBV) episomal sequences in Epstein–Barr Virus-positive cell lines.[21]

The exact mechanisms by which episomes reintegrate into chromosomes remain to be explored. Some hypotheses have been proposed for the reintegration of double minutes, which are large acentric fragments that can multiply and form hsr in given stress situations.[22–24]

The presence of multiple episomes detected by FISH with an *ABL1* probe is most characteristic for *NUP214-ABL1* T-ALL. But, as seen in this series, the percentage of nuclei with multiple episomes may be low (<5%) and escape FISH detection. In this study, 6 of the 24 cases displayed episomes in 5% of nuclei or less. Our experience with patient no. 10 showed that episomes may be lost during the cell culture performed to obtain metaphases for karyotyping. Another explanation may be that *NUP214-ABL1* fusion is a late event in the development of T-ALL, which at the time of diagnosis may be present only in a minor subpopulation. The *NUP214-ABL1* containing subpopulation has the potential to increase its oncogenic potential through episomal amplification of the fusion gene. Accordingly, we observed that the copy number of episomes is particularly high (>30 copies) in cases having a predominant *NUP214-ABL1* clone (Table 1). The presence in some patients of a low percentage of nuclei with *NUP214-ABL1* amplification, together with a high percentage of cells with *TLX1* or *TLX3* and *CDKN2a*

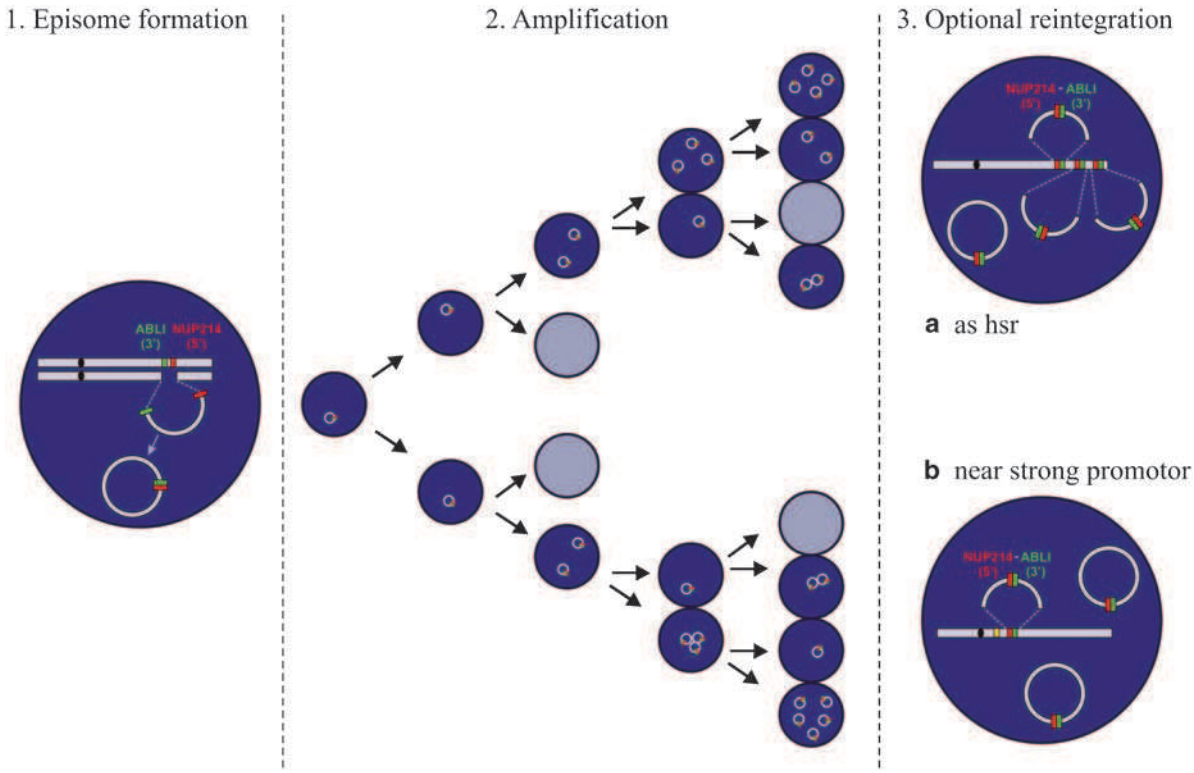

Figure 3 Model for *NUP214-ABL1* fusion on episomes followed by amplification and/or optional reintegration. (1) Episome formation may have originated from a microdeletion at 9q34. Secondary circularization of the deleted fragment creates an episome containing the *NUP214-ABL1* fusion. (2) Unequal segregation of episomes during cell division leads to selection of cells containing the highest number. (3) Optionally, secondary episomal reintegration into chromosomes may produce a more stable form of the fusion. (**a**) Reintegration of numerous episomal sequences may be clustered within a chromosomal region (small hsr) (**b**) Alternatively, reintegration may place the episome within the vicinity of a highly active promoter, increasing the fusion gene transcription rate without genomic amplification. hsr, homogeneously stained regions.

abnormalities supports the secondary nature of the *NUP214-ABL1* fusion (Table 1).

Insertion of one episome into chromosome 14 as the sole anomaly in 33% of metaphases in patient no. 1 is unexpected because of the apparent lack of amplification. One could hypothesize that the integration of this episome brought the *NUP214-ABL1* fusion into the vicinity of a highly active promoter increasing the transcription rate of the fusion (Figure 3b). Unfortunately, due to lack of material we could neither verify this hypothesis nor map the exact site of insertion.

Intrachromosomal reintegration could confer stability to the *NUP214-ABL1* fusion. Of interest, patient no. 15 presented only with intrachromosomal amplification (hsr) of *NUP214-ABL1* at the time of relapse, and hsr was the only form of the fusion seen in patient no. 7 and in cell lines. We therefore recommend the use of FISH to distinguish hsr from episomes in a clinical setting. RT-PCR is useful for the detection of low percentages of cells with amplified *NUP214-ABL1* fusion, which may be missed with FISH, and to follow the response of the NUP214-ABL1-positive clone to the treatment, but has probably limitations in the assessment of the residual disease during follow-up as in some cases only a proportion of leukemic cells have this abnormality.[12]

The *NUP214-ABL1* fusion protein is sensitive to TKI such as imatinib, dasatinib or nilotinib;[9,14] therefore, these drugs may have potential in the treatment of this subgroup of T-ALL patients. However, the low incidence of *NUP214-ABL1*-positive T-ALL patients makes it difficult to conduct randomized trials to evaluate the benefit of introducing TKI into the standard treatment regimens. Importantly, as episomes have the property to easily self-amplify, the risk of resistance to therapy is increased by the selection of those cells with the greatest number of episomes. Additionally, *NUP214-ABL1* appears to be a relatively late event in the multistep process of T-ALL leukemogenesis indicating that TKI would have to be given in addition to drugs which target the earlier genetic defects. The schedule of administration of TKI with respect to the delivery of chemotherapy may be of great importance as TKI may potentially decrease the number of cycling cells available to incorporate the cytotoxic drugs and thus decrease their efficacy, as experienced in early trials with FLT3 inhibitors in AML.[25]

In conclusion, the observations reported in this study lead us to recommend an optimized screening of T-ALL patients for the presence of the *NUP214-ABL1* fusion gene, which may occur only in subpopulations. They also show *in vivo* evidence for a model of episome formation, amplification and optional reintegration into the cellular genome. As patients expressing *NUP214-ABL1* may potentially benefit from TKI treatment, understanding the oncogenic contribution of this fusion is important to the design of new therapeutic schemes.

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