Combined interphase fluorescence *in situ* hybridization elucidates the genetic heterogeneity of T-cell acute lymphoblastic leukemia in adults

Paolo Gorello,[1] Roberta La Starza,[1] Emanuela Varasano,[1] Sabina Chiaretti,[2] Loredana Elia,[2] Valentina Pierini,[1] Gianluca Barba,[1] Lucia Brandimarte,[1] Barbara Crescenzi,[1] Antonella Vitale,[2] Monica Messina,[2] Sara Grammatico,[2] Marco Mancini,[2] Caterina Matteucci,[1] Antonella Bardi,[3] Anna Guarini,[2] Massimo Fabrizio Martelli,[1] Robin Foà,[2] and Cristina Mecucci[1]

[1]Hematology and Bone Marrow Transplantation Unit, University of Perugia, Perugia, Italy; [2]Hematology, Dept. of Cellular Biotechnologies and Hematology, "La Sapienza" University of Rome, Rome, Italy, and [3]Hematology, University of Ferrara, Ferrara, Italy

PG and RLS contributed equally to this manuscript.

Acknowledgments: the authors would like to thank GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto), Dr. G A Boyd for assistance in the preparation of the manuscript and Roche Molecular Systems.

Funding: this work was supported by a grant from IAP (Interuniversity Attraction Poles, University of Leuven, Belgium), AIRC (Associazione Italiana Ricerca sul Cancro), PRIN-MIUR (Programmi di Ricerca Cofinanziati-Ministero per l'Istruzione, l'Università e la Ricerca Scientifica, Italy), Fondazione Cassa di Risparmio di Perugia, Associazione "Sergio Luciani", Fabriano, Italy; and the European Community. BC received a grant by FIRC (Fondazione Italiana Ricerca sul Cancro).

Manuscript received on April 21, 2009. Revised version arrived on June 9, 2009. Manuscript accepted on July 8, 2009.

Correspondence: Cristina Mecucci, Hematology and Bone Marrow Transplantation Unit, Ospedale S.M. della Misericordia, S Andrea delle Fratte, 06156 Perugia, Italy. E-mail: crimecux@unipg.it

The online version of this article has a supplementary appendix.

ABSTRACT

Background

Molecular lesions in T-cell acute lymphoblastic leukemias affect regulators of cell cycle, proliferation, differentiation, survival and apoptosis in multi-step pathogenic pathways. Full genetic characterization is needed to identify events concurring in the development of these leukemias.

Design and Methods

We designed a combined interphase fluorescence *in situ* hybridization strategy to study 25 oncogenes/tumor suppressor genes in T-cell acute lymphoblastic leukemias and applied it in 23 adult patients for whom immunophenotyping, karyotyping, molecular studies, and gene expression profiling data were available. The results were confirmed and integrated with those of multiplex-polymerase chain reaction analysis and gene expression profiling in another 129 adults with T-cell acute lymphoblastic leukemias.

Results

The combined hybridization was abnormal in 21/23 patients (91%), and revealed multiple genomic changes in 13 (56%). It found abnormalities known to be associated with T-cell acute lymphoblastic leukemias, i.e. *CDKN2A-B*/9p21 and *GRIK2*/6q16 deletions, *TCR* and *TLX3* rearrangements, *SIL-TAL1*, *CALM-AF10*, *MLL*-translocations, del(17)(q12)/*NF1* and other cryptic genomic imbalances, i.e. 9q34, 11p, 12p, and 17q11 duplication, del(5)(q35), del(7)(q34), del(9)(q34), del(12)(p13), and del(14)(q11). It revealed new cytogenetic mechanisms for *TCRB*-driven oncogene activation and *C-MYB* duplication. In two cases with cryptic del(9)(q34), fluorescence *in situ* hybridization and reverse transcriptase polymerase chain reaction detected the *TAF_INUP214* fusion and gene expression profiling identified a signature characterized by *HOXA* and *NUP214* upregulation and *TAF_I*, *FNBP1*, *C9orf78*, and *USP20* down-regulation. Multiplex-polymerase chain reaction analysis and gene expression profiling of 129 further cases found five additional cases of *TAF_I-NUP214*-positive T-cell acute lymphoblastic leukemia.

Conclusions

Our combined interphase fluorescence *in situ* hybridization strategy greatly improved the detection of genetic abnormalities in adult T-cell acute lymphoblastic leukemias. It identified new tumor suppressor genes/oncogenes involved in leukemogenesis and highlighted concurrent involvement of genes. The estimated incidence of *TAF_I-NUP214*, a new recurrent fusion in adult T-cell acute lymphoblastic leukemias, was 4.6% (7/152).

Key words: T-ALL, CI-FISH, genomic rearrangements

Citation: Gorello P, La Starza R, Varasano E, Chiaretti S, Elia L, Pierini V, Barba G, Brandimarte L, Crescenzi B, Vitale A, Messina M, Grammatico S, Mancini M, Matteucci C, Bardi A, Guarini A, Martelli MF, Foà R, and Mecucci C. Combined interphase fluorescence in situ *hybridization elucidates the genetic heterogeneity of T-cell acute lymphoblastic leukemia in adults. Haematologica. 2010; 95:79-86. doi:10.3324/haematol.2009.010413*

Introduction

Full genomic characterization is needed to trace pathogenic pathways in individuals with T-cell acute lymphoblastic leukemia (T-ALL), establish the impact of primary and secondary changes and determine how diverse genetic abnormalities interact/co-operate as T-ALL derives from combinations of diverse molecular lesions (multi-step hits). These lesions affect genes implicated in cell proliferation and/or survival (*LCK* and *ABL1*), self-renewal (*NOTCH1*), cell differentiation (*HOX* genes, *MLL*, *LYL1*, *TAL1/2* and *LMO1/2*), and cell cycle control (*CDKN2A/CDKN2B*).[1,2] The main causes of gene deregulation are: (i) oncogene activation with ensuing ectopic or over-expression, which is mainly due to juxtaposition with T-cell receptor loci (*TCRB-HOXA*, *TCRA/D-HOX11*, *TCRA/D-LMO2*, *TCRA/D-LMO1*);[3,4] (ii) gain of function mutations (*NOTCH1* and *JAK1*);[5,6] (iii) tumor suppressor gene haploinsufficiency or inactivation, which is usually the result of deletion (*CDKN2A-B*) and/or loss of function mutation (*PTEN*);[1,7] and (iv) chromosomal translocations producing fusion proteins which are associated with specific subgroups of T-ALL (*CALM-AF10*, *NUP98-*, *MLL-* and *ABL1*-fusions).[1,2]

New genomic techniques such as interphase fluorescence *in situ* hybridization (FISH), and array-comparative genomic hybridization have greatly improved the detection of genomic abnormalities in T-ALL.[8] They bypass the inherent difficulties of poor normal cell proliferation in T-ALL and failure of conventional cytogenetics to identify cryptic molecular events such as the *NUP214-ABL1* fusion, t(9;14)(q34;q32)/*EML1-ABL1*, *C-MYB* duplication or translocation, and cryptic del(17q)/*NF1*.[9-13] In T-ALL gene expression profiling, assessing over- and under-expression of a myriad of genes, has elucidated distinct signatures that are associated with over-expression of *LYL1*, *HOX11*, *TAL1*, *LMO1*, *LMO2*, *HOXA* and *HOX11L2* oncogenes.[14,15]

In the present study, we developed a newly designed strategy, i.e. combined interphase FISH, to increase our knowledge on the genetics of each individual case.

Design and Methods

Patients

This study recruited 152 adult patients (111 males, 41 females; median age 30; range, 17-64 years) with T-ALL who were enrolled in two consecutive Italian multi-center GIMEMA (*Gruppo Italiano Malattie Ematologiche dell'Adulto*) studies (LAL-0496 and LAL-0904) except for one 18-year old male patient who was enrolled in the AIEOP LLA 2000 protocol (*Online Supplementary Methods*). Combined interphase FISH was done on 23 cases for whom results of mutational analysis (*NOTCH1* and *FBW7*) (*Online Supplementary Methods*) and multiplex-polymerase chain reaction (PCR) analysis (*BCR-ABL1*, *PBX-E2A*, *SIL-TAL1*, *MLL-AF4*, *MLL-ENL*, *NUP98-RAP1GDS1*) were available.[16] The other 129 patients underwent a newly designed multiplex-PCR analysis or gene expression profiling.

Combined interphase fluorescence in situ hybridization

Specific DNA clones, ranging in size from 59 to 215 Kb, for genes/loci that have been implicated to date in T-ALL were selected (Table 1).[3,17-19] Clones were labeled with spectrum orange and green (Vysis, IL, USA) for double-color break-apart tests or combined split FISH tests and applied on bone marrow and/or peripheral blood samples (*Online Supplementary Methods*).

The *SIL-TAL1* fusion was investigated using a SIL-TAL1 FISH DNA probe (Dako Italia, Milan, Italy). In cases with 9q34/*ABL1* deletion FISH was done with the LSI BCR-ABL1 ES dual color probe (Vysis, IL, USA) and four clones for the 9q34 band, centromere to telomere as follow: RP11-216B9, RP11-550J21, RP11-143H20, RP11-544A12. Abnormal hybridization patterns were: (i) a split signal (one fusion signal and separate green and red signals); (ii) duplication/trisomy (three fusion signals); (iii) deletion/monosomy (one fusion signal); and (iv) partial deletion (one fusion signal and one orange or one green signal). Cut-offs were the upper limit of 500 normal peripheral blood nuclei for each assay: split, duplication/trisomy patterns were considered positive when found in more than 5% of interphase cells; monosomy/deletion/partial deletion patterns were considered positive when found in more than 10% of cells.

Comparative genomic hybridization

Comparative genomic hybridization was performed in patient n. 6 to elucidate the chromosome 6q rearrangement and in patients n. 14 and 17 to investigate *TCRB* involvement in putative unbalanced translocations (*Online Supplementary Methods*).

Molecular studies

Reverse transcriptase PCR was performed as described elsewhere.[20] The following primers were used to investigated *TAF_I* and *NUP214*: TAF_540F (exon 6) (5'-GAAGAGGCAGCATGAGGAAC-3') + NUP_2916R (exon 20) (5'-TACTTTGGGCAAGGATTTGG-3') for the first amplification round and TAF_747F (exon 7) (5'-TGACGAAGAAGGGGATGAGGAT-3') + NUP_2601R (exon 18) (5'-ATCATTCACATCTTGGACAGCA-3') for the nested PCR. Nested-PCR was used to monitor minimal residual disease in two patients for whom material was available. The TAF_540F/NUP_2916R and TAF_747F/NUP_2601R primers were also added to our multiplex-PCR for diagnostic screening of a cohort of 96 cases.[16] Isoform-specific PCR was done using TAFa_283F (5'-GAAACCAAGACCACCTCCTG-3') and TAFb_38F (5'-AGCTCAACTCCAACCACGAC-3') primers (*Online Supplementary Methods*). A new reverse transcriptase PCR for *SIL-TAL1* was set up (*Online Supplementary Methods*). Real-time quantitative PCR was performed to corroborate gene expression profile findings (*Online Supplementary Methods*).

Gene expression profiling and statistical analysis

We used the oligonucleotide arrays HGU133 Plus 2.0 gene chips, Affymetrix. Unsupervised clustering was performed as previously described[21,22] and the distance between two genes was computed as one minus the correlation between standardized expression values across samples. Supervised analyses included analysis of variance (ANOVA) and *t* tests. ANOVA (p value <0.01) was used to compare the following subgroups: (i) five samples with normal or heterogeneous combined interphase FISH findings (patients n. 1, 5, 9, 15 and 16 of Table 2); (ii) two samples with the *HOX11* rearrangement; (iii) four samples, defined as "*HOXA*"-positive because each had one of the following fusions inducing *HOXA* gene over-expression: *CALM-AF10*, *MLL-ENL*, *MLL*-translocation with an unknown partner and *TCRB-HOXA*;[15,23-25] (iv) three *SIL-TAL1*-positive samples; and (v) four *TAF_I-NUP214*-positive samples. Patients n. 2, 12, 17, 18, 19 and 21 were excluded from supervised analysis, since each had a different aberration. t-tests (p value <0.01, fold

change difference >1.5) were performed between the *TAF_I-NUP214* and "*HOXA*" subgroups and *TAF_I-NUP214*-positive patients and the other T-ALL patients.

Results

Patients

The clinical and hematologic features of the 23 patients investigated in the preliminary study are summarized in *Online Supplementary Table S1*. Tables 2 and 3 show these patients' cytogenetic and molecular findings. Eight (34.8%) had a normal karyotype, six (26.1%) had an abnormal karyotype and karyotype analysis was unsuccessful in the remaining nine cases (39.1%). Multiplex PCR detected *SIL-TAL1* in one patients and *MLL-ENL* in another. *NOTCH1* and *FBW7* mutations were found in 11 and four cases, respectively (Table 2).

Combined interphase fluorescence in situ hybridization

Combined interphase FISH detected diverse, multiple genomic aberrations, finding the aberrant clone in 10-98% of analyzed cells. The hybridization was abnormal in 21/23 patients, with 13 of these patients having two or more genetic changes (Tables 2 and 3). Abnormalities included *CDKN2A-B* deletions, del(6)(q16)/*GRIK2*, *TCRB*, *TCRA/D TLX3* (Figure 1A), and *MLL* translocations, and *CALM-AF10*.[26] Combined interphase FISH detected a cryptic del(1)(p32)/*SIL-TAL1* in three cases and PCR confirmed the fusion in all, using only our new specific primers (*Online Supplementary Results and Online Supplementary Figure S1*). A del(17)(q12)/*NF1* was detected in patients # 9 and 19.

Combined interphase FISH also unraveled new underlying cytogenetic mechanisms. In patient # 6, three separate hybridization signals were obtained from clone RP11-32B1 encompassing *C-MYB* (Figure 1B) and flanking clones RP11-557H15 and RP11-448D5. This finding, which is indicative of partial 6q trisomy and was validated by comparative genomic hybridization, may underlie *C-MYB* over-expression. In cases # 14 and 17 *TCRB* clones showed deletion of the *3′TCRB* flanking probe (Figure 1C), indicating unbalanced translocations. In patient # 14, comparative genomic hybridization detected loss of 7q33-qter and gain of the 10q24-qter suggesting that a trisomic 10q was implicated in an unbalanced translocation with the *TCRB* gene. A double-color experiment combining RP11-1220K2, flanking *5′TCRB*, and RP11-107I14, flanking *3′HOX11*, demonstrated that *TCRB* and *HOX11* were juxtaposed (Figure 1D). In patient # 17, comparative genomic hybridization revealed that the entire 9q arm was trisomic, but lack of biological material precluded further studies.

Combined interphase FISH detected nine cryptic imbalances: dup(17)(q11.2)/*NF1*, dup(9)(q34)/*ABL1-NOTCH1*, dup(11p), dup(12)(p13)/*ETV6*, del(5)(q35)/*TLX3*, del(12)(p13)/*ETV6*, del(7)(q34)/*TCRB*, del(14)(q11)/*TCRA/D*, and del(9)(q34)/*ABL1*. In two cases with *ABL1*/9q34 deletion, the LSI BCR-ABL ES dual-color probe proved that the deletion extended centromeric to *ABL1* (Figure 1E). Four additional 9q34 probes defined its endpoints centromerically, between RP11-216B9 and RP11-550J21, within the *TAF_I* gene and telomerically, between RP11-143H20 and RP11-554A12 (Figure 1F) within the *NUP214* gene. These findings suggest that the *5′TAF_I* and the *3′NUP214* were juxtaposed.

Table 1. Combined interphase-FISH assay genes and genomic clones.

Gene/map	Centromeric	Spanning	Telomeric
TCRB/7q34	RP11-1220K2		RP11-556I13
TCRAD/14q11	P11-242H9		RP11-447G18
TAL1/1p32+	RP11-332M15		RP11-346M5
TLX3/5q35	RP11-182E4 RP11-453D13		RP11-117L6 CTB-31E20 RP11-266N12
GRIK2/6q21		RP1-258B3	
C-MYB/6q23	RP11-557H15	RP1-32B1	RP11-448D5
CDKN2A/9p21		RP11-149I2	
ABL1/9q34	RP11-57C19		RP11-83J21
NOTCH1/9q34	RP11-83N9		RP11-251M1
LMO2/11p13	RP11-313G13		RP11-60G13
LMO1/11p15	RP11-782G4		RP11-1065L8
CALM/11q14	RP11-90K17		RP11-12D16
NUP98/11p15	RP11-348A20		CTD-3234F16
MLL/11q23	RP11-832A4		
ETV6/12p13	RP11-418C2		RP11-861M13
NF1/17q12	RP5-926B9		RP11-434C1
LCK/1p34	RP11-22K3		RP5-1002G3
TAL2/9q32	RP11-287A8		RP11-68H10
HOX11/10q24	RP11-108L7		RP11-576E23
HOXA/7p15	RP1-170O19		RP11-107I14
C-MYC/8q24	RP11-1136L8		RP1-167F23
CCND2/12p13	RP11-388F6		RP11-460A11
TCL1/14q32	RP11-1070N10		RP11-320N7 RP11-952P19
LYL1/19p13	RP11-1078F11		RP11-963I8
OLIG2/21q22	RP11-94I18		RP11-76I24

+TAL1 assay detects 1p32/TAL1 translocations but not cryptic del(1)(p32)/SIL-TAL1. The SIL-TAL1 FISH DNA probe, sub-deletion signal (Dako Italy) was applied in all cases.

TAF_I-NUP214 fusion

Reverse transcriptase PCR gave an 802 bp product in case # 3 and a 643 bp in case # 4; the amplification products detected by nested PCR were 280 bp and 121 bp, respectively. Cloning experiments and sequence analysis showed that nucleotide 813 (exon 7) of *TAF_I* was fused to nucleotide 2389 (exon 17) of *NUP214* in patient # 3 and nucleotide 813 (exon 7) of *TAF_I* was fused to nucleotide 2548 (exon 18) of *NUP214* in patient # 4 (Figure 2A). These two samples were used as positive controls when screening 96 additional T-ALL patients. Multiplex reverse transcriptase PCR found the *TAF_I-NUP214* fusion transcript in three out of these 96 patients (Figure 2B). In each of the five patients with *TAF_I-NUP214* fusion, isoform-specific reverse transcriptase PCR detected *TAF_Iα-NUP214* and *TAF_Iβ-NUP214* fusion transcripts (Figure 2C). Molecular cloning in patients # 3, 4, X, and Y showed both isoforms had the same *TAF_I-NUP214* fusion point. The *TAF_I-NUP214* fusion was found during minimal residual disease monitoring in both patients for whom material was available (*Online Supplementary Table S2*).

Gene expression profiling showed over-expressed HOXA *genes in patients with T-cell lymphoblastic leukemia with the* TAF_I-NUP214 *fusion*

Gene expression profiles were evaluable in 24 samples (22/23 samples from patients undergoing combined interphase FISH and 2/3 samples from patients found to be *TAF_I-NUP214*-positive by multiplex-PCR). Unsupervised analysis selected 3557 probe sets, corresponding to 2795 genes, but did not reveal any clear subgroups. ANOVA selected 406 probe sets, corresponding to 372 genes, and recognized five specific clusters, i.e. samples with heterogeneous combined interphase FISH findings, *HOX11*,

Table 2. Conventional cytogenetics, molecular and combined interphase-FISH findings in 23 patients with T-ALL.

Patient mutations	Karyotype	Multiplex RT-PCR	NOTCH1/FBW7	Combined interphase-FISH
1	46,XY,add(19)(q13)[10] 46,XY[6]	Neg	No/no	trisomy 17q12/*NF1*
2	46,XY[15]	Neg	No/no	t(7;9)(q34;q32)/*TCRB-TAL2* del(6)(q16)/*GRIK2* del(9)(p21)/*CDKN2A-B**
3	46,XY[15]	Neg	Ex34/no	del(9)(q34)/*TAF-I-NUP214*
4	46,XY[15]	Neg	Ex34/no	del(9)(q34)/*TAF-I-NUP214* del(6)(q16)/*GRIK2* del(12)(p13)/*ETV6*
5	n.e.	Neg	No/ex9	del(9)(p21)/*CDKN2A-B**
6	46,XY[15]	Neg	No/no	t(7;11)(q34;p13)/*TCRB-LMO2* del(6)(q16)/*GRIK2* trisomy 6q23/*C-MYB*
7	n.e.	Neg	Ex27-34/ex8	inv(7)(p15q34)/*TCRB-HOXA*
8	46,XY[15]	SIL-TAL1	No/no	del(1)(p32)/*SIL-TAL1* del(9)(p21)/*CDKN2A-B*
9	n.e.	Neg	Ex26-34/no	del(17)(q12)/*NF1*
10	n.e.	Neg	No/no	t(7;10)(q34;q24)/*TCRB-HOX11* del(9)(p21)/*CDKN2A-B** del(12)(p13)/*3'ETV6*
11	48,XY,+8,+8[2]/48,idem,del(11)(q23)[10] 46,XY[2]	*MLL-ENL*	Ex26/no	MLL-translocation
12	n.e.	Neg	No/ex8-9	t(7;11)(q34;p13)/TCRB-LMO2 del(9)(p21)/CDKN2A-B*
13	n.e.	Neg	No/no	del(5)(q35)/TLX3 del(6)(q16)/GRIK2 MLL-translocation
14	n.e.	Neg	Ex27-34/no	der(7)t(7;10)(q34;q24)/*TCRB-HOX11* del(9)(p21)/*CDKN2A-B**
15	46,XY,der(13;14)(q10;q10)c[20]	Neg	Ex26/no	normal
16	46,XY[20]	Neg	No/ex8	normal
17	46,XX,del(6)(q21),del(14)(q22)[10] 46,XX[10]	Neg	Ex34/no	del(6)(q16)/*GRIK2* del(7)(q34)/*3'TCRB* del(9)(p21)/*CDKN2A-B* trisomy 9q34/*ABL1-NOTCH1* del(14)(q11)/*TCRA-D*
18	47,XY,der(7)t(2?;7)(q23?;q32),+mar[14] 46,XY[6]	Neg	No/no	del(7)(q34)/*TCRB*
19	46,XY,del(6)(q25)[5] 46,XY[19]	Neg	Ex34/no	del(5)(q35)/*TLX3* trisomy 11p13-15/*NUP98-LMO1-LMO2* del(17)(q12)/*NF1*
20	47,XX,del(9p),t(10;11)(p13;q14), +der(11)t(10;11)[15]	Neg	Ex34/no	del(9)(p21)/*CDKN2A-B* t(10;11)(p13,q14)/*CALM-AF10* trisomy 11p13/*LMO2*
21	n.e.	Neg	Ex26/no	5q35/TLX3-rearrangement el(9)(p21)/*CDKN2A-B* trisomy (12)(p13)/*ETV6*
22	n.e.	Neg	No/no	del(9)(p21)/*CDKN2A-B** el(1)(p32)/*SIL-TAL1* t(7;11)(q34;p15)/*TCRB-LMO1* 14q11/TCRAD-translocation
23	46,XY[15]	Neg	No/no	del(1)(p32)/*SIL-TAL1*

*n.e., not evaluable; neg, negative; ex, exon; * biallelic CDKN2A-B deletion.*

"*HOXA*", *TAL1* and *TAF_I-NUP214* (Figure 3A). The *TAF_I-NUP214* cluster was characterized by over-expression of *HOXA3*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10* (*P* value <0.005) suggesting a similarity with the "*HOXA*" cluster, despite less up-regulation (Figure 3B). t-testing highlighted *TAF_I* down-modulation and higher *NUP214* expression in *TAF_I-NUP214*-positive patients than in "*HOXA*" patients (Figure 3C). *FNBP1*, *C9orf78* and *USP20* at the 9q34 deleted region were significantly (*P*<0.01) down-regulated in the four *TAF_I-NUP214*+ samples (Figure 3C).

Quantitative PCR detected a significant (*P*<0.05) down-modulation of *TAF_I* transcripts and a significant over-expression (*P*<0.001) of the *NUP214* 3' region in *TAF_I-NUP214*-positive patients compared with all other clusters (*data not shown*). Gene expression profiling analysis, validated in 33 additional cases of T-ALL (*data not shown*), detected two cases with a *TAF_I-NUP214* signature confirmed by FISH (*Online Supplementary Table S2*) and by reverse transcriptase PCR (*data not shown*).

Discussion

Remarkable progress was made in the understanding of the genetics of T-ALL when conventional cytogenetics were combined with new technologies such as FISH, reverse transcriptase PCR, and microarray analysis. In reviewing reports we found that although at least 25 recurrent genes/loci had been identified as playing roles in T-ALL leukemogenesis, few data were available on concomitant molecular events in each individual patient. The results presented here show how we successfully set up a combined interphase FISH assay that was suitable for in-depth characterization of T-ALL in clinical and research laboratories. It detected cryptic aberrations and elucidated preferential and forbidden associations of multiple genetic events involved in the pathogenesis of each case. Combining at least six molecular assays in one slide, it provided major savings in time, costs and biological material.

This open, combined interphase FISH showed an abnormal hybridization pattern in 91% of T-ALL patients, 56% of whom had multiple rearrangements even though karyotyping and multiplex-PCR had shown genetic defects in only 26% and 8.7% of patients, respectively. It reliably detected typical, recurrent abnormalities such as 9p21/*CDKN2A* and 6q16/*GRIK2* deletions, *SIL-TAL1*, *CALM-AF10*, and *TCR*-rearrangements and found, for the first time in two adult patients, the monoallelic del(17)(q12)/*NF1*, which had already been reported in three children with T-ALL.[13] This finding confirms that *NF1*, a tumor suppressor gene, plays a role in the onset/development of diverse hematologic malignancies. Molecular lesions such as the *NOTCH1* mutation, but not the characteristic del(9)(p21)/*CDKN2A/B* or del(6)(q16)/*GRIK2* were also observed in these two patients.

In association with comparative genomic hybridization, combined interphase FISH revealed new cytogenetic mechanisms underlying typical T-ALL molecular lesions. In one case *MYB* duplication, due to a partial 6q22q25 trisomy, was associated with a *TCRB-LMO2* rearrangement and del(6q)/*GRIK2*, confirming previous reports of *MYB* duplication being present together with other genetic lesions.[12] A hitherto unknown deletion of the *TCRB* 3' flanking region, which corresponded to an unbalanced der(7)t(7;10)(q34;q24) translocation, produced the *TCRB-HOX11* rearrangement.

Focusing on del(9)(q34)/*ABL1*, one of the four recurrent cryptic chromosome imbalances detected by combined interphase FISH (Tables 2 and 3), we found del(9)(q34) produced *TAF_I-NUP214* as previously described in acute myeloid leukemia and in 5.4% of cases of pediatric T-ALL.[20,27] *TAF_I* (official name *SET*), encodes for TAF_Iα and TAF_Iβ isoforms which are localized to the nucleus and have different N-terminal sequences deriving from an alternative first exon. They are chromatin remodeling proteins

Table 3. Summary of the 57 aberrations detected by CI-FISH in 21/23 adult T-ALL patients.

PATIENTS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
GENE																						
*HOXA**							T															
TCRB		T				T	T			T		T		T			L (3')	L				T
TCRAD																	L					T
SIL-TAL1								L														L
TLX3													L						L		T	
GRIK2		L		L		L							L				L					
C-MYB						G																
CDKN2AB		L			L			L		L		L		L			L			L	L	L
ABL1			L	L													G					
*TAL2**		T																				
NOTCH1																	G					
*HOX11**										T				T								
NUP98																			G			
LMO2						T						T							G	G		
LMO1																			G			T
CALM																				T		
MLL											T		T									
ETV6				L						L (3')											G	
NF1	G								L										L			

G, gain; L, loss; 3' refers to the DNA clone mapping at the 3' flanking region; T, translocation; * tested only as candidate in cases with *TCR* rearrangements.

involved in DNA replication and transcription.[28,29] NUP214, an FG nucleoporin interacting with hCRM1 in nucleus-cytoplasmic traffic, is localized at the cytoplasmic side of the nuclear pore complex. It also rearranges with *ABL1* in T-ALL bearing episomal or intrachromosomal amplification.[9] Remarkably, NUP214 like NUP98, another nucleoporin with a putative nucleus-cytoplasmic shuttling function, is involved in the pathogenesis of T-ALL and myeloid malignancies.

As observed in children, *TAF_I-NUP214*-positive T-ALL adults had a specific gene expression signature with *HOXA* gene cluster over-expression which was not as marked as in the so-called "*HOXA*" cluster T-ALL, i.e. patients with *CALM-AF10*, *MLL*-translocation and *HOXA* rearrangements (Figure 3). The gene expression signature was also characterized by *NUP214* up-regulation and *TAF_I* down-regulation. Furthermore, down-regulation of *FNBP1*, *C9orf78*, and *USP20*, mapping within the cryptic del(9)(q34), suggested haploinsufficiency of these genes.

In order to establish the incidence of *TAF_I-NUP214* in

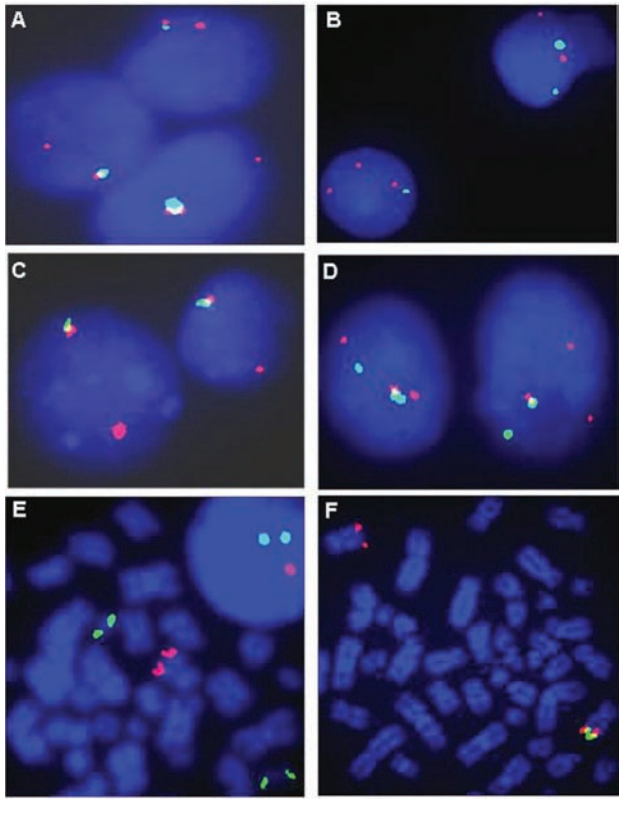

Figure 1. Representative FISH detection of A) *TLX3* unbalanced translocation in patient n. 21: the *TLX3* assay gives one fusion and one orange signal. (B) Trisomy of 6q including *C-MYB* in patient n. 6: clone RP1-32B1 spanning *C-MYB* gives three orange signals. The green signal corresponds to RP1-258B3 for *GRIK2* which, in this case, underwent monoallelic deletion. (C and D) Unbalanced der(7)t(7;10)/*TCRB-HOX11* in patient n. 14: the *TCRB* assay gives 1 fusion and 1 orange signal (C) and the double-color assay combining RP11-1220K2 (green) and RP11-107I14 (orange) results in one fusion, two orange and one green signal (D). Panels E and F) Cryptic del(9)(q34) in patient n. 3: metaphase FISH with the LSI BCR-ABL1 produces two green and one orange signals (E); a double-color experiment with RP11-544A12 orange and RP11-143H20 green shows one fusion signal on normal 9 and an orange signal on del(9)(q34) (F).

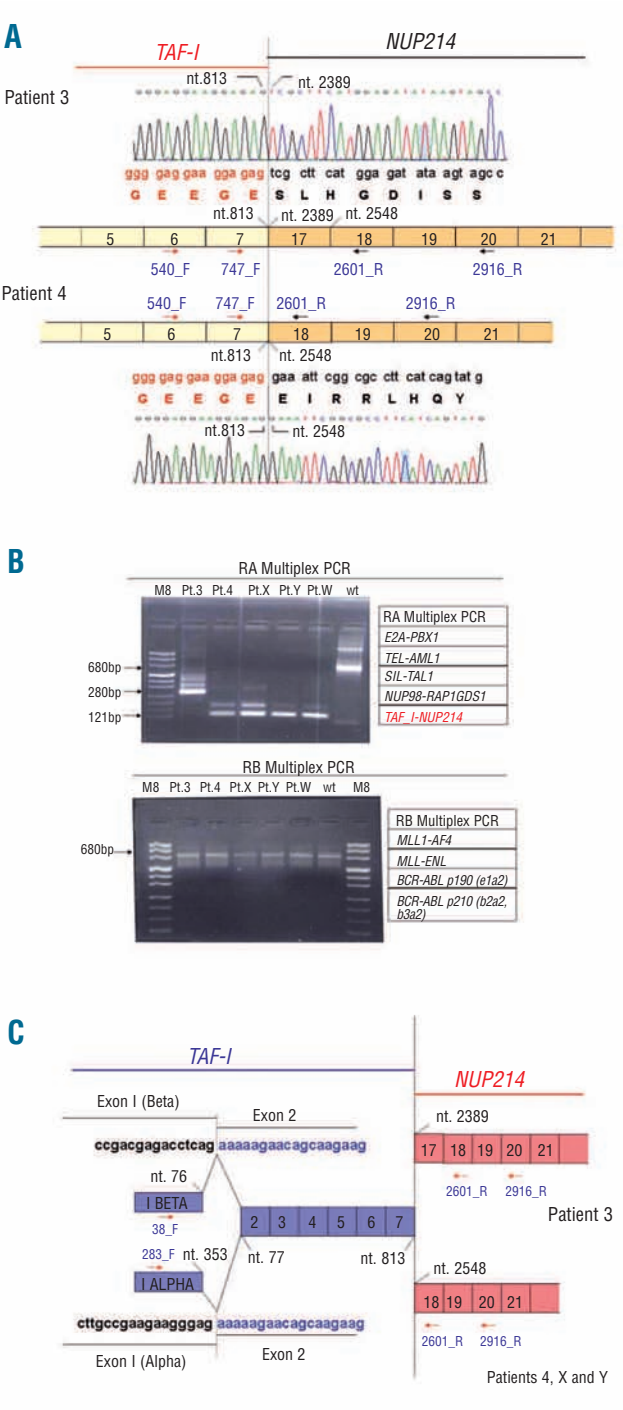

Figure 2. Characterizing *TAF_I-NUP214* fusion transcripts. Panel A) *TAF_I-NUP214* fusion transcript cloning and sequencing in patients 3 (upper schema) and 4 (lower schema). Arrows indicate the primers used in PCR amplification. Panel B) Multiplex PCR screening study used specific primers for E2A control gene amplification (680 bp). RA multiplex PCR: Upper gel includes oligonucleotides listed in right boxes. *TAF_I-NUP214* fusion transcripts were confirmed in patients 3 (280 bp) and 4 (121 bp) and detected in another three patients, i.e. X (121 bp) and Y (121 bp) and W (121bp). RB multiplex PCR: Lower gel includes oligonucleotides listed in right boxes. Only the control gene was amplified. Panel C) TAF_I nucleotide 353 (exon Iα) is fused in-frame to *TAF_I* nucleotide 77 (exon 2) in the *TAF_Iα/NUP214* isoform. TAF_I nucleotide 76 (exon Iβ) is fused in-frame to TAF_I nucleotide 77 (exon 2) in the *TAF_Iβ-NUP214* isoform. Sequence numbers refer to GenBank accession NM_003011.1 for *TAF_I* and NM_005085.2 for *NUP214*. The *TAF_I* exon I_ sequence is reported as NM_001122821.1.

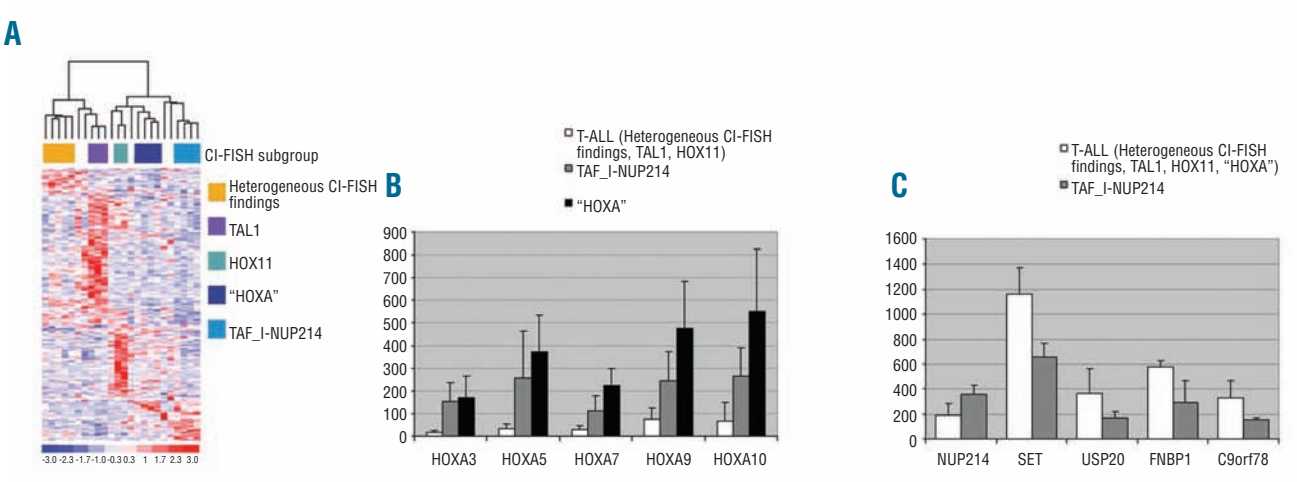

Figure 3. Gene expression profiling A) GEP of 24 adult T-ALL samples. Samples bearing *TAF_I-NUP214* are characterized by a specific signature. Each row represents a probe set, each column represents a single sample. The color scale indicates the relative levels of expression: dark blue the lowest levels of expressions, red the highest levels of expression. Red indicates high expression and blue low expression. B) Expression of *HOXA* cluster genes resulted from ANOVA ($P<0.005$). Panel C) Expression levels of genes differentially expressed between *TAF_I-NUP214* and T-ALL samples.

adult T-ALL we screened 129 additional patients, finding five positive cases: 3/96 screened by multiplex PCR and 2/33 screened by gene expression profiling. Thus, 7/152 adults with T-ALL carried *TAF_I-NUP214*, giving an estimated incidence of 4.6%, which is the same as in children.[27] This subgroup of adult T-ALL has an immature phenotype and one or more additional genomic abnormalities: *NOTCH1* mutations in four cases, del(12)(p13)/*ETV6* in two cases, del(11)(p13)/*LMO2* plus del(11)(q14)/*CALM* in two cases, del(5)(q35)/*TLX3*, del(6)(q16)/*GRIK2*, and del(9)(p21)/*CDKN2A-B* in one case each. Therefore, from a pathogenic point of view, in T-ALL, *TAF_I-NUP214* cooperates with various different concomitant molecular events, all of which concur to determine a poor response to induction therapy. In fact, four of the six patients for whom follow-up information was available died within 12-24 months of diagnosis due to refractory disease or relapse; one patient, who has been followed up for 3 months at the time of writing, had *TAF_I-NUP214* detectable by reverse transcriptase PCR after induction therapy, indicating that molecular remission had not been achieved and that the patient's prognosis was presumably poor. Indeed the only one of these six patients with sustained complete remission for 29 months had undergone allogeneic bone marrow transplantation (*Online Supplementary Table S2*). Interestingly, six of the seven cases studied for TAF_I isoforms, carried both the *TAF_Iα-NUP214* and *TAF_Iβ-NUP214* fusion transcripts. Both fusion transcripts retained the TAF_I amino terminal region which is essential for protein dimerization and, consequently, chromatin remodeling activity. Further studies will help to clarify the roles and interrelationships of the oncogenic *TAF_I-NUP214* isoforms.

In conclusion, combined interphase FISH is a powerful, flexible method as the assay can be extended beyond the panel of 25 genes that were tested in the present study. It provides in-depth molecular characterization in at least 90% of adults with T-ALL, and might be proposed in clinical laboratories as a surrogate for more advanced, expensive technologies. In the research setting, its integration with mutational analysis, PCR, and gene expression profiling in prospective studies of large consecutive T-ALL series will accelerate our understanding of the biology of T-ALL leukemogenic pathways and the design of a genome-based classification.

Authorship and Disclosures

CM designed the study, supervised all the results and wrote the paper. PG designed and supervised the molecular analysis (LE, SG, LB) and drafted the paper. RLS designed and supervised the FISH analysis (VP and BC) and drafted the paper. EV and CaM performed the mutational analysis. GLB and CaM performed the CGH analysis. SC and MM performed the GEP analysis. AV, AB, MM, MFM, AG, RF, and BC provided clinical, immunophenotypic and cytogenetic information on patients.

The authors reported no potential conflicts of interest.

References

1. De Keersmaecker K, Marynen P, Cools J. Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia. Haematologica. 2005;90(8):1116-27.
2. Van Vlierberghe P, Pieters R, Beverloo HB, Meijerink JP. Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia. Br J Haematol. 2008;143(2):153-68.
3. Cauwelier B, Dastugue N, Cools J, Poppe B, Herens C, De Paepe A, et al. Molecular cytogenetic study of 126 unselected T-ALL cases reveals high incidence of TCRbeta locus rearrangements and putative new T-cell oncogenes. Leukemia. 2006;20(7):1238-44.
4. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. Proc Natl Acad Sci USA. 1991;

88(10):4367-71.
5. Weng AP, Ferrando AA, Lee W, Morris JP 4th, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-71.
6. Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, Knoops L, et al. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med. 2008; 205(4):751-8.
7. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007;13(10):1203-10.
8. Strefford JC, Worley H, Barber K, Wright S, Stewart AR, Robinson HM, et al. Genome complexity in acute lymphoblastic leukemia is revealed by array-based comparative genomic hybridization. Oncogene. 2007;26(29):4306-18.
9. Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R, et al. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. Nat Genet. 2004;36(10):1084-9.
10. De Keersmaecker K, Graux C, Odero MD, Mentens N, Somers R, Maertens J, et al. Fusion of EML1 to ABL1 in T-cell acute lymphoblastic leukemia with cryptic t(9;14)(q34;q32). Blood. 2005;105(12):4849-52.
11. Lahortiga I, De Keersmaecker K, Van Vlierberghe P, Graux C, Cauwelier B, Lambert F, et al. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. Nat Genet. 2007;39(5):593-5.
12. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, et al. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. Blood. 2007;110(4):1251-61.
13. Balgobind BV, Van Vlierberghe P, van den Ouweland AM, Beverloo HB, Terlouw-Kromosoeto JN, van Wering ER, et al. Leukemia-associated NF1 inactivation in patients with pediatric T-ALL and AML lacking evidence for neurofibromatosis. Blood. 2008;111(8):4322-8.
14. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell. 2002;1(1):75-87.
15. Soulier J, Clappier E, Cayuela JM, Regnault A, Garcìa-Peydrò M, Dombret H, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood. 2005;106(1): 274-86.
16. Elia L, Mancini M, Moleti L, Meloni G, Buffolino S, Krampera M, et al. A multiplex reverse transcriptase-polymerase chain reaction strategy for the diagnostic molecular screening of chimeric genes: a clinical evaluation on 170 patients with acute lymphoblastic leukemia. Haematologica. 2003; 88(3):275-9.
17. Clappier E, Cuccuini W, Cayuela JM, Vecchione D, Baruchel A, Dombret H, et al. Cyclin D2 dysregulation by chromosomal translocations to TCR loci in T-cell acute lymphoblastic leukemias. Leukemia. 2006; 20(1):82-6.
18. Sinclair PB, Sorour A, Martineau M, Harrison CJ, Mitchell, WA, O'Neill E, et al. A fluorescence in situ hybridization map of 6q deletions in acute lymphoblastic leukemia: identification and analysis of a candidate tumor suppressor gene. Cancer Res. 2004;64(12):4089-98.
19. http://www.ncbi.nlm.gov/project/mapview /map_search, Build 35.3
20. Rosati R, La Starza R, Barba G, Gorello P, Pierini V, Matteucci C, et al. Cryptic chromosome 9q34 deletion generates TAF-Iα/CAN and TAF-Iβ/CAN fusion transcripts in acute myeloid leukemia. Haematologica. 2007;92(2):232-5.
21. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA. 1998;95(25):14863-8.
22. Chiaretti S, Tavolaro S, Ghia EM, Ariola C, Matteucci C, Elia L, et al. Characterization of ABL1 expression in adult T-cell acute lymphoblastic leukemia by oligonucleotide array analysis. Haematologica. 2007;92(5): 619-26.
23. Ferrando AA, Armstrong SA, Neuberg DS, Sallan SE, Silverman LB, Korsmeyer SJ, et al. Gene expression signatures in MLL-rearranged T-lineage and B-precursor acute leukemias: dominance of HOX dysregulation. Blood. 2003;102(1):262-8.
24. Dik WA, Brahim W, Braun C, Asnafi V, Dastugue N, Bernard OA, et al. CALM-AF10+ T-ALL expression profiles are characterized by overexpression of HOXA and BMI1 oncogenes. Leukemia. 2005;19(11): 1948-57.
25. Bergeron J, Clappier E, Cauwelier B, Dastugue N, Millien C, Delabesse E, et al. HOXA cluster deregulation in T-ALL associated with both a TCRD-HOXA and a CALM-AF10 chromosomal translocation. Leucemia. 2006;20(6):1184-7.
26. La Starza R, Crescenzi B, Krause A, Pierini V, Specchia G, Bardi A, et al. Dual-color split signal fluorescence in situ hybridization assays for the detection of CALM/AF10 in t(10;11)(p13;q14-q21)-positive acute leukemia. Haematologica. 2006;91(9):1248-51.
27. Van Vlierberghe P, Van Grotel M, Tchinda J, Lee C, Beverloo HB, van der Spek PJ, et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. Blood. 2008;111(9):4668-80.
28. Miyaji-Yamaguchi M, Okuwaki M, Nagata K. Coiled-coil structure-mediated dimerization of template activating factor-I is critical for its chromatin remodelling activity. J Mol Biol. 1999;290(2):547-57.
29. Nagata K, Saito S, Okuwaki M, Kawase H, Furuya A, Kusano A, et al. Cellular localization and expression of template-activating factor I in different cell types. Exp Cell Res. 1998;240(2):274-81.