The effect of a novel recombination between the homeobox gene *NKX2-5* and the *TRD* locus in T-cell acute lymphoblastic leukemia on activation of the *NKX2-5* gene

Grzegorz K. Przybylski
Willem A. Dik
Piotr Grabarczyk
Jens Wanzeck
Paulina Chudobska
Kacper Jankowski
Anne von Bergh
Jacques J.M van Dongen
Christian A. Schmidt
Anton W. Langerak

Background and Objectives. The NK-like homeobox gene (*NKX2-5/CSX*) plays a crucial role in cardiac development but is not normally expressed in hematopoietic cells. Here, we describe for the first time a fusion between *NKX2-5* and the T-cell receptor delta locus (*TRD*) resulting in *NKX2-5* activation in a case of T-cell acute lymphoblastic leukemia (T-ALL).

Design and Methods. Genomic DNA from a T-ALL patient with an atypical rearrangement, detected by Southern blotting, was analyzed by ligation-mediated polymerase chain reaction (PCR) with *TRD*-specific primers. Expression of *NKX2-5* was analyzed by real-time quantitative PCR in the T-ALL case with the *NKX2-5-TRD* rearrangement, 18 other cases of T-ALL, three T-ALL derived cell lines, two non-hematopoietic cell lines, peripheral blood mononuclear cells from six healthy individuals and sorted thymocyte subsets.

Results. Sequence analysis of ligation-mediated PCR products revealed a novel rearrangement between the third diversity segment of the *TRD* locus (*TRDD3*) and a region on chromosome 5q35.1 located 32 kb downstream of the *NKX2-5/CSX* gene. As a result of this recombination *NKX2-5* was placed under influence of the *TRD* enhancer, resulting in strong ectopic *NKX2-5* expression. High *NKX2-5* expression was also found in the T-cell lines PEER and CCRF-CEM, which harbor an *NKX2-5-BCL11B* rearrangement, and in the embryonic kidney cell line 293. *NKX2-5* was not expressed in any of the major thymocyte subsets, in normal peripheral blood mononuclear cells, or in the majority (17/18) of the other cases of T-ALL.

Interpretation and Conclusions. Our finding of overexpression of yet another homeobox gene in T-ALL further supports the hypothesis that homeobox genes play an important role in malignant transformation of particular types of T-ALL.

Key words: TRD, TRDD3, *NKX2-5*, T-ALL.

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From the Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland (GKP, PC, KJ); Klinik für Innere Medizin C, Universität Greifswald, Sauerbruchstr., Greifswald, Germany GKP, PG, JW, CAS); Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands (WAD, JJMvD, AWL); Department of Cytogenetics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands (AvB).

Correspondence:
Grzegorz K. Przybylski M.D., Ph.D.
Institute of Human Genetics, Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznan, Poland.
E-mail: przybylg@man.poznan.pl

T-cell acute lymphoblastic leukemia (T-ALL) is a rare but aggressive hematologic malignancy of thymocytes arrested at diverse stages of differentiation, caused by a variety of DNA alterations. To become fully malignant a T-cell has to acquire alterations of two or more genes belonging to different functional classes. The DNA lesions in T-ALL can be grouped into four classes: point mutations providing a self-renewal potential (*NOTCH1*); mutations and fusions leading to a proliferative and survival advantage (*ABL1* fusion, *RAS, FLT3*); mutations and deletions leading to loss of cell cycle control (*CDKN2A/B*, *RB1*, *TP53*); and chromosomal translocations resulting in cell differentiation impairment.[1] T-cell receptor gene (*TCR*)-associated translocations in T-ALL are thought to originate during thymocyte development due to VDJ recombinase errors. These translocations are characterized by fusions between *TCR* genes (mainly *TRD*, *TRB* and *TRA*) and a variety of proto-oncogenes such as the homeobox genes *HOX11*, *HOX11L2*, and *HOXA*, the LIM domain only genes *LMO1*, and *LMO2*, and the basic helix-loop-helix-genes *LYL1*, *TAL1*, and *TAL2*. Due to juxtaposition to *TCR* regulatory elements the involved oncogene will be ectopically expressed, resulting in impaired thymocyte differentiation.[1-4] Although many DNA alterations involved in the development of T-ALL have already been described, the list of genes contributing to the malignant transformation of T cells is still growing, and is updated each year. This leads to better understanding of the mechanism of leukemogenesis and will result in better diagnosis and treatment of T-ALL. Due to recent advances in fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR)-based methods, such as ligation-mediated PCR, (cryptic) chromosomal changes in leukemia can now be identified more easily.[5] In this study we describe a case of T-ALL in which we identified a novel chromosomal fusion that involves the cardiac homeobox gene (*NKX2-*

5) and the *TCR* δ locus (*TRD*) and results in ectopic activation of *NKX2-5*.

Design and Methods

Samples

A 3-year old male patient (T045) presented with T-ALL with a high blast cell count in both the bone marrow (>90% blasts) and peripheral blood (95% blasts). The patient's immunophenotype was: membrane $CD3^-$, cytoplasmic $CD3^+$, $CD2^+$, $CD5^+$, $CD4^+$, $CD7^+$, $CD10^{dim}$, TdT^+ and $CD1^+$, thus representing a common T-ALL. Eighteen other T-ALL samples were kindly provided by W-D Ludwig (Charité, Berlin, Germany). T-cell lines PEER and CCRF-CEM, with t(5;14)(35;32), were kindly provided by Dr. Roderick A. F. MacLeod (Department of Human and Animal Cell Cultures, Braunschweig, Germany). The T-cell line Jurkat, the embryonic kidney cell line 293 and the epithelial cell line HeLa were obtained from the American Type Culture Collection (Manassas, VA, USA). Mononuclear cells were purified by Ficoll density gradient centrifugation from peripheral blood of six healthy volunteers. $CD34^+CD1a^-$, $CD34^+CD1a^+$, immature single positive ($CD3^-CD4^+$), double positive ($CD3^-CD4^+CD8^+$ and $CD3^+CD4^+CD8^+$) and single positive ($CD3^+CD4^+$ and $CD3^+CD8^+$) thymocyte populations, which represent consecutive T-cell developmental stages were obtained from human thymi as described previously.[6] Briefly, total thymocytes from six donors (aged 6 weeks to 3.5 years; median 11 months) were pooled. $CD34^+$ cells were positively selected using magnetic CD34 beads (Miltenyi Biotec). After this magnetic sorting, the $CD34^+$and $CD34^-$ fractions were labeled with fluorochrome-conjugated monoclonal antibodies for further purification by high speed cell sorting on a FACS DiVa cell sorter (BD Biosciences). All fractions obtained were >90% pure.

Southern blot analysis

TCR gene rearrangements were determined by Southern blot analysis. Briefly, DNA was digested with with *Eco*RI, *Hind*III and *Bgl*II restriction enzymes, size separated in an agarose gel and transferred to a nylon membrane which was subsequently hybridized with ^{32}P-labeled TCRDD1, TCRDD3, TCRDJ1, TCRGJ13, TCRGJ21, TCRBJ2 and TCRBJ1 probes (Dako Corporation, Carpinteria, CA, USA) as described previously.[7-9]

Ligation-mediated PCR for the analysis of atypical TRD gene rearrangements

To determine the DNA regions rearranged to the *TRDD2* and *TRDD3* gene segments, ligation-mediated PCR was performed, as described in detail previously.[5] Briefly, aliquots of 1 μg high molecular weight DNA were digested with blunt end restriction enzymes (*Eco*RV, *Dra*I, *Pvu*II, and *Stu*I) and 50 μM of an adaptor was ligated to both ends of the restriction fragments. The ligation was followed by two rounds of PCR with nested adaptor-specific primers and sets of *TRD* specific primers, i.e. D2(-89) (5'-GGCAGCGGGTGGTGATGGCAAA GTG-3') and D2(-41) (5'-AGAGGGTTTTTATACTGATGT-3') for *TRDD2* and D3(+133) and D3(+77) for *TRDD3*, as described previously.[5] The presence of the *NKX2-5-TRDD3* recombination was confirmed by a regular PCR on undigested DNA with primer sets spanning the junctional region: 5' NKX(BP-1950) (5'-CCGGCTGTCCCCATATTTGTGCTGTTAC) or NKX(BP-442) (5'-CAATTCATTCTTAACATGTGGCAG-3') and 3' D3(+240) (5'-AGTTTCA CCCAAGGAAGAAC-3') or J1(+62) (5'-GAGTTACTTACTTGG TTCCAC).

Interphase FISH analysis

FISH analysis was essentially performed as described previously.[10] Briefly, cell slides were prepared from methanol/acetic acid cell suspensions and hybridized with probes flanking *TRA/D*, *IGH* and *NKX2-5* (Figure 3A shows a schematic overview of chromosomes 14 and 5 with the relevant probes plus their colors used in FISH analysis) and subsequently counterstained with DAPI.

Real-time quantitative PCR

To check NKX2-5 activation, real-time quantitative PCR was performed using the ICycler Detection System (Bio-Rad; Hercules, CA, USA) as previously described.[5] NKX2-5 was amplified with primers NKX2-5f433 (5'-TTCCCGCCGCCCCCGCCTTCTAT-3', located in exon 1) and NKX2-5r571 (5'-CGCTCCGCGTTGTCCGCCTCTGT-3', located in exon 2). The number of copies was determined using a standard dilution of a duplex vector containing a fragment of the *NKX2-5* gene and, as a reference, a fragment of the β2-microglobulin gene (*β2MG*). In brief, PCR amplification was performed in a total volume of 25 μL with 1 μL of cDNA, 2.5 pmol of each primer, 4 pmol of dNTP and 1 U of Taq Polymerase (Eppendorf; Hamburg, Germany) in a 1.5 mM $MgCl_2$ PCR buffer (Eppendorf) and 0.1x final concentration of SYBR Green I fluorescent dye (Sigma-Aldrich). After initial denaturation at 95°C for 1 min, 40 three-step cycles consisting of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec in the case of *β2MG*, and 95°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec in the case of *NKX2-5* were performed. The specificity of amplified DNA fragments was confirmed by DNA sequencing, melting curve and gel analysis. The expression of *NKX2-5* was normalized to the expression level of the *β2MG* reference and the results are given as the number of *NKX2-5* copies per 10^5 *β2MG* copies.

Results

Identification of TCR gene recombinations

Southern blot analysis of DNA from patient T045 revealed biallelic *TRG* (*TRGV2/V4-TRGJ2.3* and *TRGV7-TRGJ2.3*) and biallelic *TRB* (*TRBV2-TRBJ1.1* and unidentified) gene rearrangements. Analysis with the TCRDD1 probe revealed a monoallelic *TRD* rearrangement indicated by 8.3 kb *Bgl*II, 9 kb *Hind*III, and >7.5 kb

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*Eco*R1 bands, while the TCRDD3 and TCRDJ1 probes revealed a monoallelic *TRD* rearrangement with 8.3 kb BglII, 4.8 kb *Eco*RI, and 8.2 kb *Hind*III bands. The sizes of these restriction fragments were not in line with any of the known *TRD* gene rearrangements involving *TRDD2* or *TRDD3*[7] therefore suggesting that patient T045 has an atypical *TRD* rearrangement. This suspicion was strengthened by negative results with standardized PCR protocols used for the identification of common *TRD* gene rearrangements as targets for minimal residual disease detection in ALL.[11]

Molecular characterization of the atypically rearranged TRD allele

Ligation-mediated PCR with *TRDD2* primers showed a distinct PCR band of 1000 bp in the *Pvu*II digest that differed in size from the germline band (*data not shown*). Direct sequence analysis of the patient-specific *Pvu*II band, followed by a homology search using BLASTn (National Center for Biotechnology Information, *http://www.ncbi.nlm.nih.gov/BLAST/*) revealed a signal joint type recombination between *TRDD2* at 14q11.2 and a region located just downstream of the *IGHV4-34* variable gene segment of the immunoglobulin heavy chain locus at 14q32.33 (GenBank accession number: DQ084548). The sizes of the *Eco*RI, *Hind*III and *Bgl*II restriction fragments for the *TRDD2-IGHV4-34* rearrangement matched those observed in Southern blotting and thereby confirmed the recombination. The *TRDD2-IGH4-34* recombination was further confirmed by a regular PCR on undigested DNA. At the chromosomal level the rearrangement between the *TRD* and *IGH* loci results from an inv14(q11;q32), which is a known recurrent chromosomal aberration in B- and T-cell malignancies.[12] Ligation-mediated PCR with *TRDD3*-specific primers yielded weak bands representing the *TRDD3* germline sequence and stronger additional bands of 500 bp, 1500 bp and 800 bp in the *Dra*I, *Pvu*II and *Stu*I digests, respectively (Figure 1A). Sequence analysis of these bands revealed a novel rearrangement between *TRDD3* and a region on chromosome 5q35.1 located 32 kb downstream of *NKX2-5/CSX* and 36 kb upstream of BCL2/adenovirus E1B 19kDa interacting protein gene (*BNIP1*) (*GenBank accession number: DQ084549*) Figure 2). The sizes of the *Eco*RI, *Hind*III and *Bgl*II restriction fragments for the *NKX2-5-TRDD3* rearrangement matched those observed in Southern blotting and thereby confirmed the recombination (Figure 2). The *NKX2-5-TRDD3* recombination was further confirmed by a regular PCR on undigested DNA with primers spanning the junctional region: NKX(BP-442), located on chromosome 5, 442 bp upstream of the break, and D3(+240), located on chromosome 14, 240 bp downstream of *TRDD3* (Figure

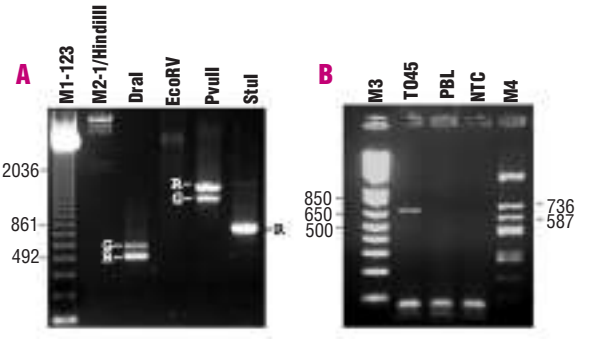

Figure 1. Amplification of the *NKX2-5-TRD* breakpoint region. A. Ligation-mediated PCR. Genomic DNA isolated from the T-ALL case T045 was digested with *Dra*I, *Eco*RV, *Pvu*II or *Stu*I and, upon ligation of an adapter, amplified by nested PCR with primers specific for the *TRDD3* segment: D3(+133) and D3(+77). Distinct bands of 500 bp, 1500 bp and 800 bp, differing from germline, were obtained in the *Dra*I, *Pvu*II and *Stu*I digests, respectively. M1 and M2: size markers, G: germline fragment, R: rearrangement. B. Regular PCR. Undigested DNA was amplified by PCR with two specific primers, NKX(BP-442) and D3(+240), spanning the break region. A 700 bp band, representing the *NKX2-5-TRDD3* rearrangement, was amplified in the T-ALL case (T045) but not in peripheral blood leukocytes (PBL) from a healthy individual. M3 and M4: size markers; NTC: no template control.

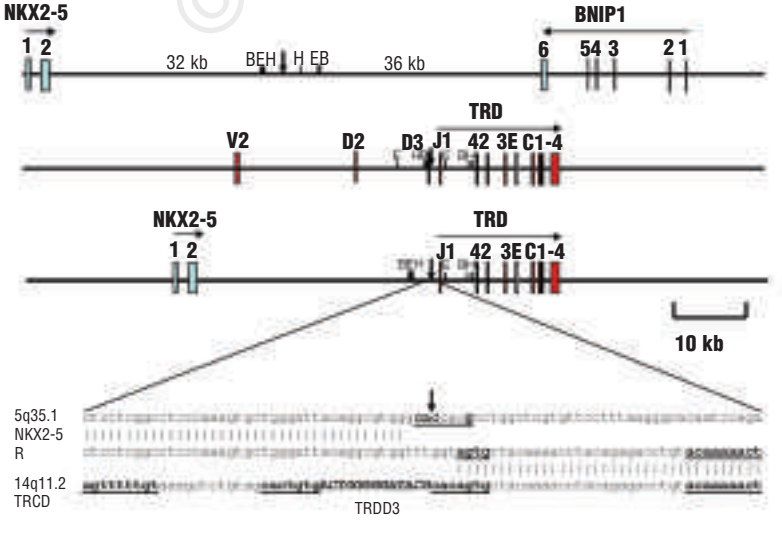

Figure 2. *NKX2-5-TRD* rearrangement. The breaks, indicated by arrows, are located between the *NKX2-5* and *BNIP1* genes on chromosome 5q35.1 and downstream of the *TRDD3* segment on chromosome 14q11.2. V: variable, D: diversity, J: joining, C: constant, E: enhancer. The *Bgl*II (B), *Eco*RI (E) and *HindIII* (H) restriction sites surrounding the breaks in *NKX2-5* and *TRAD* are indicated. The sequence of *TRDD3* is printed in bold capitals, the recombination signal sequences (RSS) are underlined. Nucleotides of the RSS-like sequence located near the 5q35.1 break that match the consensus heptamer RSS sequence are in bold.

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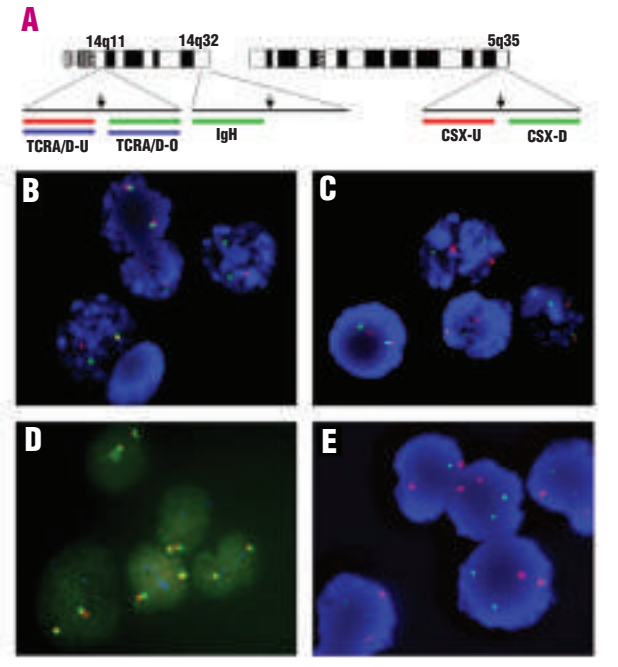

Figure 3. Interphase FISH analysis of case T045. A. Schematic overview of chromosomes 14 and 5 with the relevant probes plus their colors used in FISH analysis. B. Use of TCRA/D-U (red) and TCRA/D-D (green) shows a fusion on one allele and a split of signals on the other allele. C. Upon hybridization with TCRA/D-U (red) and IgH (green) one fusion signal was found, confirming the *TRDD2-VH4-34* fusion observed by PCR. D. Hybridization with the CSX-U (red) and CSX-D (green) probes together with the TCRA/D-U probe (blue) shows two *NKX2-5/CSX* fusions without co-localization with the *TRA/D* locus. E. Use of CSX-U (red) and IgH (green) probes does not show fusion of the *NKX2-5/CSX* and *IGH* genes.

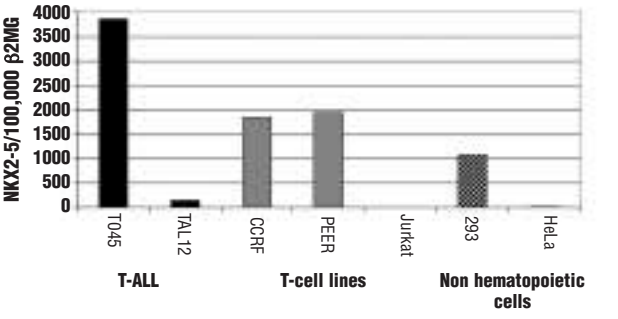

Figure 4. *NKX2-5* expression. Expression of the *NKX2-5* gene was measured by real-time quantitative PCR using SYBR-Green fluorescent dye. The expression of *NKX2-5* was normalized to the expression level of the *β2MG* reference and the result was given as the number of *NKX2-5* copies per 10^5 *β2MG* copies. No *NKX2-5* expression was found in peripheral blood mononuclear cells from six healthy individuals, various thymocyte subsets ($CD34^+CD1A$, $CD34^+CD1A^+$, $CD3^-CD4^+$, $CD3^-CD4^+CD8^+$, $CD3^+CD4^+CD8^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) or in 17 of 18 cases of T-ALL (not included in the diagram).

1B). Additionally, the rearrangement was amplified and subsequently sequenced with two other primer sets: NKX(BP-1950)/D3(+240) and NKX(BP-442)/J1(+62), altogether spanning a region starting 1950 bp upstream and ending 1000 bp downstream of the break (*data not shown*). Directly downstream from the break on chromosome 5 a recombination signal sequence (RSS)-like motif was found (Figure 2), which was most likely recognized by the VDJ recombinase, followed by introduction of double strand DNA breaks and subsequent joining of the upstream 5q35.1-derived DNA fragment to the RSS located 3' from *TRDD3*. This is consistent with type 1 V(D)J-mediated chromosomal aberrations, typical of T-ALL.[13] As a result of the recombination *NKX2-5* was joined head to tail to the 3' region of the *TRA/D* locus probably placing it under influence of the *TRD* enhancer (Figure 2).

Cytogenetic analysis of the recombinations detected by ligation-mediated PCR

FISH analysis with probes for the involved loci on chromosomes 5 and 14 was performed to confirm the presence of the observed recombinations (Figure 3A). Although a clear split-signal was found upon hybridization with a *TRA/D* probe set (Figure 3B), this was only found to result in fusion of the *TRA/D* locus with *IGH* (Figure 3C), representing the *TRDD2-IGHV4-34* recombination. However, fusion of neither the *TRA/D* and *NKX2-5* loci, nor the *NKX2-5* and *IGH* loci was observed (Figure 3D,E), suggesting that the *NKX2-5-TRDD3* recombination as observed by Southern blotting and PCR is probably the result of a complex recombination event, in which yet other chromosome regions are involved. Karyotypic data to support this belief are, unfortunately, lacking.

Analysis of NKX2-5 expression

Real-time quantitative PCR revealed very high expression of *NKX2-5* in the sample from patient T045 ($3854/10^5$ *β2MG*) and in the T-ALL-derived cell lines, CCRF-CEM and PEER, with the t(5;14)(q35;q32) resulting in the *NKX2-5-BCL11B* rearrangement (1885 and $1935/10^5$ *β2MG* respectively) (Figure 4). NKX2-5 was also expressed in the embryonic kidney cell line 293 ($1072/10^5$ *β2MG*) and weakly in the epithelial cell line HeLa ($4/105$ *β2MG*). No expression was detected in the Jurkat T-cell line without a translocation involving the *NKX2-5* gene or in 17 of 18 other T-ALL cases. Only in 1/18 other cases of T-ALL was weak expression of *NKX2-5* ($152/10^5$ *β2MG*) detected. *NKX2-5* was not expressed in normal peripheral blood mononuclear cells from six healthy individuals nor in sorted human thymocyte subsets: double negative ($CD34^+CD1A^-$ and $CD34^+CD1A^+$), immature single positive ($CD3^-CD4^+$), double positive ($CD3^-CD4^+CD8^+$ and $CD3^+CD4^+CD8^+$) and mature single positive ($CD3^+CD4^+$ and $CD3^+CD8^+$). Expression of the homeobox genes *HOX11* and *HOX11L2*, which is frequently observed in T-ALL, was absent in case T045 (*data not shown*).

Discussion

In this study we identified a novel chromosomal translocation in T-ALL that juxtaposes the *TRD* locus to

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the homeobox gene *NKX2-5* resulting in ectopic expression of the latter. The *NKX2-5/CSX* gene, a *Drosophila* tinman homolog, encodes a conserved homeobox transcription factor that is expressed during murine embryogenesis in myocardiogenic progenitor cells and is absolutely essential for correct cardiac development.[14] In humans, *NKX2-5* mutations were found to cause non-syndromic, congenital heart disease.[15] Recently, cells expressing *NKX2-5* were found in the non-hematopoietic mononuclear cell fraction of bone marrow in both mice and human.[16] It was shown that *NKX2-5*-expressing cells are mobilized into the peripheral blood after myocardial infarction. To date, there is only one study that reports on a chromosomal aberration involving *NKX2-5*. Using FISH analysis, Nagel *et al*. found t(5;14)(q32.1;q32.2) juxtaposing the *NKX2-5* gene to the *BCL11B* gene in two T-ALL derived cell lines (CCRF-CEM and PEER) and their early passage divergent subclones (MKB-1 and BE-13).[17] In contrast to what was found in patient T045, the breaks in CCRF-CEM and PEER were localized upstream of *NKX2-5*. Nevertheless the t(5;14)(q32.1;q32.2) also resulted in *NKX2-5* expression in both affected cell lines and their subclones. In line with our results, the authors did not observe *NKX2-5* expression in 18 additional T-ALL-derived cell lines without t(5;14)(q32.1;q32.2) and in 13 other, non-T-ALL, hematopoietic cell lines. We showed that *NKX2-5* is not expressed in either peripheral blood mononuclear cell or in thymocytes at various stages of T-cell development and is, therefore, truly ectopic for the expressing leukemic T-cell clone in case T045.

In summary, we identified a novel chromosomal aberration in T-ALL and characterized it at the molecular level. This aberration results in juxtaposition of the homeobox gene *NKX2-5* to the *TRD* locus. The *NKX2-5-TRD* fusion results in ectopic activation of *NKX2-5*. Our finding of overexpression of yet another homeobox gene in T-ALL further supports the hypothesis that ectopic expression of homeobox genes may contribute to leukemogenesis in primary T-ALL. The biological consequences of ectopic expression of *NKX2-5* in hematopoietic cells remain to be elucidated.

GKP: conception, design of the project, analysis and interpretation of the data, drafting the article; PG: analysis and interpretation of the data, drafting the article; JW: detection of the NKX2-5-TRD rearrangement by LM-PCR, analysis of the data; PC: detection of the IGH-TRD rearrangement by LM-PCR, analysis of the data; KJ: measurement of the NKX2-5 expression by real-time quantitative PCR, analysis of the data; CAS: conception, design of the project, analysis and interpretation of the data; AvB: FISH analysis, analysis of data; WAD: conception, design of the project, analysis and interpretation of the data, thymocyte subset isolation, drafting the article; JJMvD: conception, design of the project, analysis and interpretation of the data; AWL: conception, design of the project, analysis and interpretation of the data, drafting the article. All authors critically revised the manuscript and approved the final version to be published. The authors declare that they have no potential conflicts of interest.

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