Genetic lesions of the *TRAF3* and *MAP3K14* genes in classical Hodgkin lymphoma

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Summary

Hodgkin and Reed/Sternberg (HRS) cells in classical Hodgkin lymphoma (cHL) show constitutive activation of nuclear factor (NF)-κB. Several genetic lesions contribute to this deregulated NF-κB activity. Here, we analysed two further NF-κB regulators for genetic lesions, the inhibitory factor TRAF3 and the key signalling component of the alternative NF-κB pathway, MAP3K14 (NIK). Single nucleotide polymorphism (SNP) array analysis of cHL cell lines revealed a uniparental disomy of the long arm of chromosome 14 associated with a biallelic deletion of *TRAF3* located on this chromosome in cell line U-HO1. Cloning of the deletion breakpoint showed a 123 371 bp deletion. No inactivating mutations of *TRAF3* were found in six other cHL cell lines or in microdissected HRS cells from seven cHL. However, in primary cHL samples interphase cytogenetic analyses revealed signal patterns indicating monoallelic deletion of *TRAF3* in 3/20 other cases. SNP array analysis revealed a gain of copy number for *MAP3K14* in three cHL cell lines. Gains of *MAP3K14* were detected in 5/16 cases of primary cHL. In conclusion, in rare instances, HRS cells harbour inactivating mutations of the *TRAF3* gene and recurrently show gains of *MAP3K14*, indicating that more components of NF-κB signalling show genetic lesions in HRS cells than previously known.

Constitutive activation of the nuclear factor (NF)-κB signalling pathway is a hallmark of the Hodgkin and Reed/Sternberg (HRS) tumour cells in classical Hodgkin lymphoma (cHL) (Bargou *et al*, 1996). This activity is essential for the survival of the HRS cells, as inhibition of NF-κB results in cell death of cHL cell lines (Bargou *et al*, 1997). Signalling through cell surface receptors may partly cause this NF-κB activity: HRS cells express the tumour necrosis factor receptor (TNFR) family members CD30, CD40, RANK and BCMA, and ligand-expressing cells are found in the vicinity of HRS cells in the lymphoma microenvironment (Küppers, 2009). In the about 40% of cases of Hodgkin lymphoma (HL) that harbour Epstein–Barr virus (EBV)-infected HRS cell clones, the EBV-encoded oncogene latent membrane protein 1 (LMP1) is expressed, and this factor also causes NF-κB activation by mimicking an activated CD40 receptor (Kilger *et al*, 1998). Importantly, however, also genetic lesions in HRS cells play a key role in the deregulated NF-κB activation. Inactivating mutations in the *NFKBIA* gene, encoding the main negative regulator of NF-κB, i.e. IκBα, have been found in several cHL cell lines and about 10–20% of primary cHL cases (Cabannes *et al*, 1999; Emmerich *et al*, 1999; Jungnickel *et al*, 2000; Lake *et al*, 2009). In one study, mutations were found in *NFKBIE* (IκBε) in one of six HL cell lines and

one of six primary cHL (Emmerich *et al*, 2003). Furthermore, genomic gains of the gene encoding the NF-κB factor REL are present in 30% of cHL (Joos *et al*, 2000; Martin-Subero *et al*, 2002a). The most frequent genetic lesion in the NF-κB pathway in HRS cells affects the NF-κB inhibitor A20, encoded by the *TNFAIP3* gene. This tumour suppressor is inactivated in about 40% of cHL (Kato *et al*, 2009; Schmitz *et al*, 2009). Notably, EBV infection and *TNFAIP3* mutations appear to be largely alternative pathogenetic mechanisms, as about 70% of EBV-negative cases, but only 15% of EBV-positive cases, harbour *TNFAIP3* mutations (Schmitz *et al*, 2009).

Given the involvement of numerous components of NF-κB signalling in the pathogenesis of cHL, we investigated whether two further factors might also be affected by genetic lesions in HRS cells, namely TNFR-associated factor 3 (TRAF3) and MAP3K14 (also called NIK). TRAF3 is a negative regulator of the alternative NF-κB signalling pathway that mainly functions by inhibiting the kinase MAP3K14 (Liao *et al*, 2004; Zarnegar *et al*, 2008), but that may additionally have inhibitory functions in the canonical NF-κB pathway (Zarnegar *et al*, 2008). MAP3K14 is the main activator of the alternative NF-κB pathway which functions by phosphorylating the NF-κB factor p100 and thereby inducing its proteolytic processing into the active form p52 (Vallabhapurapu & Karin, 2009). Inactivating mutations and gene deletions of *TRAF3* have already been identified in a fraction of chronic lymphocytic leukaemias, other low-grade lymphomas, and multiple myelomas (Annunziata *et al*, 2007; Keats *et al*, 2007; Braggio *et al*, 2009; Nagel *et al*, 2009; Rossi *et al*, 2011). That *TRAF3* represents a tumour suppressor gene is supported by the recent demonstration that B cell-specific deletion of this gene in mice causes the development of B cell lymphomas (Moore *et al*, 2011). For *MAP3K14* (*NIK*), genomic gains and other genetic alterations were found in multiple myelomas and in splenic marginal zone lymphomas (Annunziata *et al*, 2007; Keats *et al*, 2007; Rossi *et al*, 2011). Therefore, we analysed single nucleotide polymorphism (SNP) array data of six cHL cell lines with regard to these two gene loci and searched for mutations in the *TRAF3* gene by amplification and sequence analysis of its coding exons from cell lines and microdissected HRS cells. Moreover, we performed fluorescence *in situ* hybridization (FISH) on primary HRS cells to detect aberrations targeting *TRAF3* or *MAP3K14*.

Materials and Methods

Patient samples and cell lines

Six cHL cell lines L428, HDLM-2, KM-H2, L1236, SUP-HD1, and U-HO1 were analysed previously using SNP 6.0 microarrays (Schmidt *et al*, 2010). These six lines were used for mutational analysis together with the cHL cell line L591 and the DEV line, which is derived from a nodular lymphocyte predominant HL (Maggio *et al*, 2002; Schumacher *et al*, 2010). Lymph node samples from seven cHL patients were collected from the Senckenberg Institute of Pathology at the University of Frankfurt for *TRAF3* sequencing. Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms (FICTION) analyses were performed on lymph node sections from 21 primary cHL cases (Table I). These samples were provided by the Department of Pathology (Haematopathology Section and Lymph Node Registry) at the Christian-Albrechts-University Kiel. The institutional review boards of the Universities of Frankfurt and Kiel approved the study.

SNP array analysis

The genome-wide human SNP array 6.0 (Affymetrix, Santa Clara, CA, USA) was used as described before (Schmidt *et al*, 2010). In detail, 500 ng of DNA were hybridized to the SNP arrays according to the manufacturer's instructions. Thereafter, the microarrays were washed and stained with the Fluidics Station 450 (Affymetrix) and scanned with the GeneChip Scanner 3000 (Affymetrix) using the Command Console software (Affymetrix). The Birdseed v2 algorithm was used for genotyping. Copy number analysis, loss of heterozygosity (LOH) analysis and segmentation was calculated using Genotyping Console software version 3.0.2 (Affymetrix).

Table I. Patient data for the interphase cytogenetic collective.

Case	Age (years) at diagnosis	Gender	Subtype	EBV	*TRAF3* deletion	*MAP3K14 (NIK)* gain
1	10	M	MC	+	No	No
2	67	M	MC	+	Yes	Yes
3	66	M	NS	−	No	Yes
4	71	M	MC	−	No	No
5	13	F	NS	+	No	Yes
6	14	M	MC	−	No	No
7	31	F	NS	−	No	No
8	34	F	MC	na	No	na
9	42	F	MC	+	No	No
10	16	M	MC	+	No	No
11	48	F	MC	+	No	na
12	64	M	NS	−	Yes	Yes
13	12	M	MC	+	No	No
14	15	F	MC	−	Yes	na
15	60	M	MC	+	na	na
16	12	M	na	+	No	Yes
17	19	M	NS	−	No	No
18	36	F	NS	+	No	No
19	54	F	NS	−	No	No
20	14	M	MC	+	No	na
21	65	F	MC	−	No	No

EBV, Epstein–Barr virus; M, male; F, female; MC, mixed cellularity; NS, nodular sclerosis; na, not analysed.

Laser microdissection and pressure catapulting of HRS cells

Five micrometer frozen lymph node section of cHL biopsies were fixed on membrane-covered slides (PALM, Bernried, Germany) and stained with anti-CD30 antibody (BerH2; Dako, Hamburg, Germany). Single $CD30^+$ HRS cells were microdissected from dried sections using laser microdissection and pressure catapulting (PALM) into 20 µl of HiFi polymerase chain reaction (PCR) buffer (Roche Appplied Science, Mannheim, Germany), and pooled into groups of 10 or 20 cells.

Amplification and sequencing of TRAF3

Cells in HiFi buffer were digested with 0·25 mg/ml of proteinase K for 2 h at 50°C, followed by an incubation at 95°C for 5 min to inactivate the proteinase. The coding exons of *TRAF3* (exons 3–12) of the primary cases were amplified in a nested PCR (with exception of exon 6, which was amplified in a non-nested PCR) with the primers displayed in Supporting Information Table SI. The exons were multiplex-amplified in two batches (exons 3, 4, 5, 6, 7 together and exons 8, 9, 10, 11, 12 together) in a first round of PCR using first round primers and 40 cycles of amplification. The PCR program consisted of the following steps: 95°C for 120 s, 40 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 90 s, followed by 72°C for 5 min. The reaction mix contained 3 mmol/l $MgCl_2$, 1 mol/l betain, 0·2 mmol/l of each dNTP, 1× HiFi buffer, and 2 µmol/l of each primer. Semi-nested amplification of the individual exons was performed in separate reactions using identical conditions with internal primers and 40 cycles in the second round of PCR. For the HRS cell lines, the *TRAF3* coding exons were analysed in a one-round PCR with identical conditions and second round primers. PCR products were gel-purified and directly sequenced from both sides on an ABI 3130 sequencing apparatus (Applied Biosystems, Weiterstadt, Germany), using the PCR primers for sequencing.

Long distance PCR

For analysing the deletion of *TRAF3* in the U-HO1 cell line, long distance PCR was performed using HiFi-polymerase mixture (Roche) and multiple primer combinations (not shown). A PCR product was obtained with primers TRAF3-Up-8-F (5′-AGTGGCACCTCTCACACCTTCATTTG-3′) and TRAF3-Down-3-R (5′- GAAGCTGCCCCTGAGTGACTTGCT TC-3′). The PCR product was gel-purified and directly sequenced from both sides with internal sequencing primers TRAF3-Up-7bS-F (5′-ATGTCTCACATGGCAGCAGGCAA-GAG-3′) and TRAF3-Down-2aS-R (5′-ACACTGCAGGTGCC ATGTGTGCACAG-3′) on an ABI 3130 sequencing apparatus (Applied Biosystems).

FICTION studies for aberrations of the TRAF3 and MAP3K14 genes

FICTION analyses for the detection of copy number alterations or translocations altering the *TRAF3* and *MAP3K14* gene loci were performed as described previously (Martin-Subero *et al*, 2002b). In detail, cryo-preserved lymph node sections from tumour tissue of cHL patients were fixed in acetone and incubated with a primary monoclonal antibody BER-H2 against CD30 (BER-H2 cell culture supernatant). The sections were washed with PN buffer (1 mol/l $NaH_2PO_4 \times 2H_2O$; 1 mol/l Na_2HPO_4, pH 8·0) and the primary antibody detected with Alexa-594-conjugated rabbit anti-mouse secondary antibody diluted 1:50 (Molecular Probes, Leiden, The Netherlands) for the identification of the $CD30^+$ HRS cells. For the immunophenotyped sections the corresponding FISH probes were used: for *TRAF3* break apart assay, two probes partially spanning the gene were combined with BAC clone RP11-236F13, labelled with spectrum orange, and BAC clone RP11-877O9, labelled with spectrum green; for the *MAP3K14* break apart assay, two colocalized probes spanning the gene (BAC clones CTD-3040P21 and RP11-666C2, both labelled with spectrum green) were combined with flanking, colocalized probes (CTD-3158P6 and CTD-2191O10, both labelled with spectrum orange).

BAC clones (Invitrogen, GmbH, Darmstadt, Germany) were cultured overnight in LB medium. BAC DNA was isolated using the PhasePrep™ BAC DNA Kit (Sigma-Aldrich, Munich, Germany) and labelled by random priming using the Bioprime DNA Labelling System (Invitrogen) and fluorescent-dUTPs (Enzo Life Sciences GmbH, Lörrach, Germany) according to the instructions of the supplier. Labelled probes were purified using the Amicon Ultra-0.5, Ultracel-30 Membrane, 30 kDa (Millipore, Cork, Ireland).

Slides were analysed using a Zeiss fluorescence microscope (Göttingen, Germany) equipped with appropriate filter sets (AHF, Tübingen, Germany) and documented using an ISIS imaging system (MetaSystems, Altlussheim, Germany). For each case 5–20 large, $CD30^+$ cells were evaluated. Each case was evaluated independently by two observers. Ploidy level of the analysed cases was estimated, taking mean signal numbers of the centromeric probes CEP6 (Schmitz *et al*, 2009), CEP10 (unpublished), CEP16 (Lamprecht *et al*, 2010) and CEP17 (unpublished) (CEP probes; Abbott/Vysis, Downers Grove, IL, USA). Considering the complexity of HRS cells, the cut-offs for breakpoint events or copy number alterations was arbitrarily set to 30%; thus an alteration was counted if the observed signal pattern differed from the estimated ploidy level of the case in at least 30% of HRS cells/case. For FISH analysis of cHL cell lines, the same *MAP3K14* probe as for FICTION experiments was applied and the signals evaluated according to the same criteria.

Validation of L1236 genomic sequencing data

Custom whole genome sequencing of the cell line L1236 was performed (Illumina HiSeq technology, GATC Biotech AG, Koblenz, Germany) using paired-end libraries with 300 bp insert size yielding an overall coverage of 23-fold. Data were analysed using the CREST (clipping reveals structure) tool (Wang *et al*, 2011). For validation of next generation sequencing data, DNA sequences were downloaded from the University of California, Santa Cruz (UCSC) Genome Browser (www.genome.ucsc.edu) and primers were designed using the PRIMER3 v.0.4.0 software (http://frodo.wi.mit.edu/primer3/). The junction fragment on chromosome 17 in L1236 was amplified using the forward primer 5′-ATGGGGT TTTACCATGTTGG-3′ and reverse primer 5′-CCTCACTT CCATTTTTGGGATA-3′. The PCR product was sequenced using both the forward and reverse primers by standard Sanger sequencing.

Results

Using the high resolution SNP 6.0 array to analyse the status of the *TRAF3* and *MAP3K14* genes in six cHL cell lines, we identified LOH without copy number loss of the complete long arm of chromosome 14 in the U-HO1 cell line, which we interpreted as uniparental disomy of chromosome 14 (UPD14). In this chromosome we delineated an approximately 123 kb-sized homozygous deletion restricted to the *TRAF3* gene (Fig 1). Moreover, the SNP array indicated LOH regions spanning the *TRAF3* gene in the L428 and KM-H2 cell lines. To search for inactivating mutations in the *TRAF3* gene in HL cell lines we amplified and sequenced all coding exons of *TRAF3* (exons 3–12). Besides the six cHL cell lines used in the SNP array analysis we also included the EBV^+ cHL cell line L591, and cell line DEV, the only line established from a nodular lymphocyte predominant HL, as the tumour cells of this HL subtype show strong NF-κB activity, too (Brune *et al*, 2008). For cell line U-HO1 no amplificate was obtained for any of the exons, thus confirming a biallelic deletion of *TRAF3* in this line (Table II). By testing various combinations of primers located upstream and downstream of the deletion, we finally obtained a 2·6 kb amplificate carrying the deletion breakpoints. Sequence analysis of this amplificate showed a 123·371 kb long deletion from intron 1 of *TRAF3* to a position 8163 bp downstream of the last codon of the gene (Fig 1). The other six cHL cell lines and DEV were unmutated. A homozygous single nucleotide exchange in exon 12 was identified in cell line KM-H2 (Table II). As no germline DNA is available for this line, we cannot clarify whether this is a somatic point mutation – that might combine with partial uniparental disomy as LOH of the region was observed using the SNP array – or a novel single nucleotide polymorphism. Nevertheless, as this is a silent nucleotide exchange, it most likely does not have functional consequences.

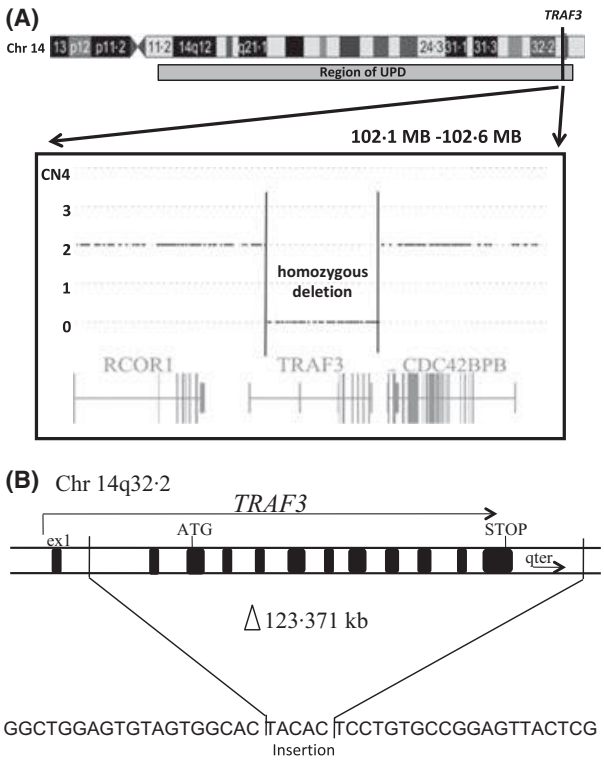

Fig 1. Structure of the *TRAF3* deletion in the cHL cell line U-HO1. (A) Display of the SNP array results for the U-HO1 cHL cell line with a UPD of the complete long arm of chromosome 14 and homozygous deletion of the *TRAF3* gene. (B) Structure of the *TRAF3* gene and DNA sequence around the 123·371 kb long deletion from a position within intron 1 of *TRAF3* to a position downstream of the last exon. At the breakpoint of the deletion there is a 5 bp insertion.

To check for copy number alterations or translocations of the *TRAF3* gene in primary cHL cases we performed combined immunofluorescence staining for CD30 and fluorescence *in situ* hybridization (FICTION) on lymph node sections (Figure S1). Using the cut-off described above, FICTION detected *TRAF3* losses in 3/20 (15%) cases, but also gains were detected in 7/20 (35%) cases (Table I). No homozygous deletions were identified although it must be noted that small bi-allelic losses of several kb are beyond the resolution of FICTION and thus might be missed.

We extended the study by amplifying and sequencing of the coding exons of *TRAF3* from pools of microdissected HRS cells from seven primary cases of cHL. However, no mutations were found (Table II). In several lines and primary cases, we observed a known SNP in exon 5 of *TRAF3*. Thus, one of seven HL cell lines, but none of seven primary cases of cHL showed inactivating mutations of the *TRAF3* gene.

SNP arrays of the *MAP3K14* gene identified a gain of copy number in three of the six cHL cell lines (L428, HDLM-2, U-HO1). Moreover, the use of FISH on the six cell lines identified a complex signal pattern indicating the presence of a breakpoint in 17q21.31 region in HDLM-2 and L1236

Table II. Mutation analysis of the *TRAF3* gene in HL cell lines and isolated HRS cells.

Cell line/case	HL subtype	Age at diagnosis (years), gender	EBV	Mutations	SNPs*	SNP array†
Cell line						
L428	NS		−			LOH
L1236	MC		−		Exon 5, heterozygous	
L591	NS		+		Exon 5, homozygous	nd
KM-H2	MC		−	Silent mutation or SNP in exon 12‡		LOH, gain
HDLM-2	NS		−		Exon 5, homozygous	
SUP-HD1	NS		−			Gain
U-HO1	NS		−	123 kb homozygous deletion, beginning in intron 1§		Homozygous deletion
DEV	NLPHL		−		Exon 5, homozygous	nd
Primary case						
A	MC	23, f	−		Exon 5, heterozygous	nd
B	MC	21, f	−			nd
C	MC	62, f	+		Exon 5, heterozygous	nd
D	MC	34, m	+			nd
E	NS	51, m	−			nd
F	NS	70, f	−			nd
G	NS	24, m	−		Exon 5, heterozygous	nd

HL, Hodgkin lymphoma; EBV, Epstein–Barr virus; SNP, single nucleotide polymorphism; MC, mixed cellularity; NS, nodular sclerosis; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma:, m, male; f, female; nd, not done; LOH, loss of heterozygosity.

*The SNP in exon 5 is a T to C exchange in codon 129 at position 98 234 of Genbank entry NC_000014.8 (rs1131877).

†SNP arrays were performed for six cHL cell lines.

‡The silent mutation or SNP in KM-H2 is located in codon 472 at position 128 016 of Genbank entry NC_000014.8.

§The deletion in U-HO1 extends from position 103 257 420 bp from pter to 103 380 791 bp from pter (GRCh37/hg19).

(Figure S1). Notably, in an ongoing next generation sequencing study of the L1263 cell line we detected a chromosomal duplication (chr17:43.113.852-55.965.648 bp) containing the *MAP3K14* locus and confirming the presence of a chromosomal breakpoint in its vicinity. PCR amplification and subsequent Sanger sequencing of the joined fragments in L1236 indeed showed the existence of the junction (chr17:55.965.648-CTGTCA-43.113.852) created by the duplication of the (chr17:43.113.852-55.965.648) segment (Fig 2). The breakpoint is located outside of *MAP3K14* but results in a gain of one copy of the gene. FICTION analysis of cryo-preserved cHL biopsies detected no translocations for the *MAP3K14* gene but gains of one to three copies of the 17q21.31 region over the estimated ploidy level were identified in 5/16 (31%) of the primary cases. The observed gains may be, at least in some of these cases, caused by duplication of the *MAP3K14* region as exemplified by the L1236 cell line. To what extent these recurrent gains result in elevated expression of MAP3K14 and contribute to NF-κB hyperactivation remains unknown.

Losses of *MAP3K14* were less frequent and detected in 4/16 (25%) cases. In one of the cases with *MAP3K14* loss we observed a subpopulation of cells with a putative homozygous deletion. The relevance of this is unclear. Interestingly, 2/3 *TRAF3* deletions were detected in the same cases as *MAP3K14* gains and for the third *TRAF3*-deleted case no *MAP3K14* copy number data was available (Table I). This might suggest a co-occurrence of these alterations in the deregulation of the alternative NF-κB pathway in cHL.

Discussion

We describe for the first time deletions and LOH of the *TRAF3* gene in cHL, including a biallelic deletion in the cHL cell line U-HO1. We also detected frequent gains of the *MAP3K14* gene in HL cell lines and primary cases. Such gains of *MAP3K14* have recently already been identified by comparative genomic hybridization of isolated HRS cells (Steidl *et al*, 2010), so that our work validates and extends these findings. Interestingly, in addition to the *TRAF3* deletion, U-HO1 also carries destructive mutations of the *TNFAIP3* gene (Schmitz *et al*, 2009). HDLM-2, for which we detected a gain of the *MAP3K14* gene, also carries inactivating mutations of the *TNFAIP3* gene (Schmitz *et al*, 2009). A similar situation has also been seen for L428 with *NFKBIA* and *NFKBIE* mutations, and for KM-H2 with *TNFAIP3*, *CYLD* and *NFKBIA* mutations (Emmerich *et al*, 1999, 2003; Jungnickel *et al*, 2000; Schmitz *et al*, 2009; Schmidt *et al*, 2010). Thus, the present study further validates that often more than one NF-κB regulator is affected by genetic lesions in HRS cells, which indicates that a single aberration in the NF-κB pathway may often not be sufficient to overactivate this well-regulated signalling pathway as strong as the HRS cells apparently need for their survival.

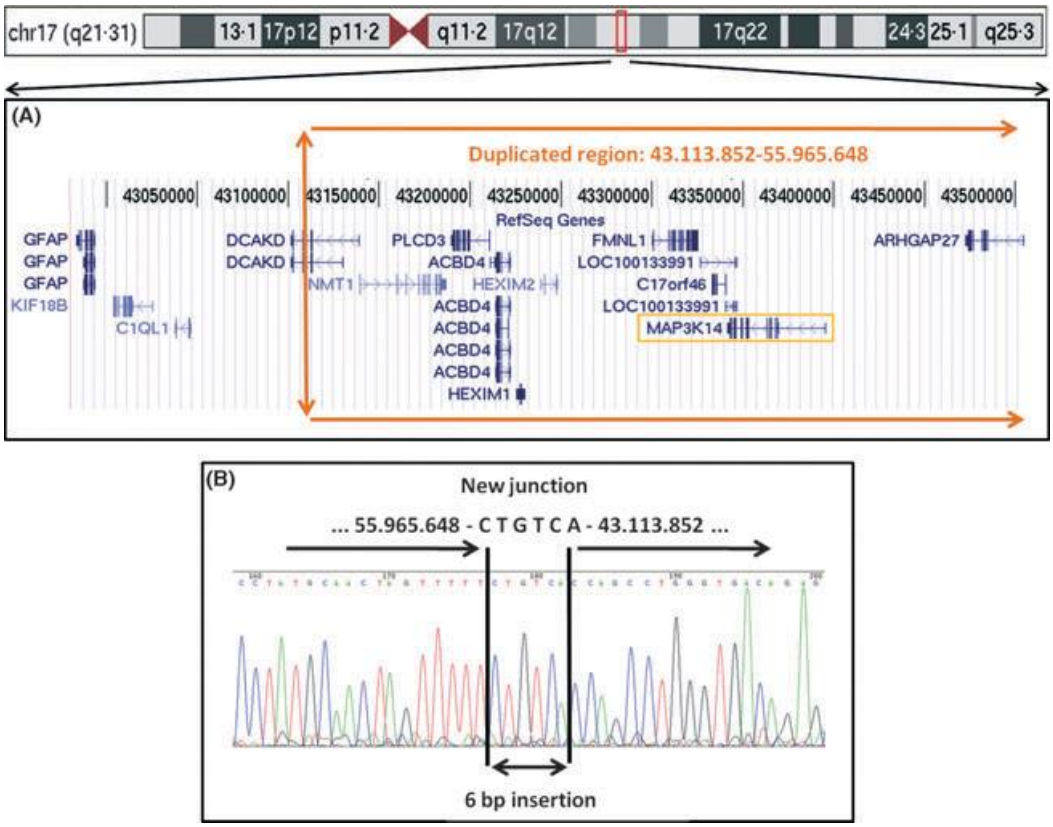

Fig 2. Structure of the *MAP3K14* duplication in the cHL cell line L1236. (A) Schematic presentation of the chr17:43.113.852-55.965.648 duplication resulting in gain of copy number of the *MAP3K14* (*NIK*) gene (box) in the L1236 cell line. (B) Sanger sequencing of the new junction created as a consequence of the duplication. A 6 bp insert is located between the joined fragments (chr17:55.965.648-CTGTCA-43.113.852).

Acknowledgements

This work was supported by the Wilhelm Sander foundation (2005.168.2), the Deutsche Krebshilfe, Mildred Scheel-Stiftung (107562), as part of the "Molecular mechanisms of malignant lymphomas" consortium, and the KinderKrebsInitiative (KKI) Buchholz/Holmseppensen. M. G. was supported by a FEBS long-term fellowship and a "Support for International Mobility of Scientists" fellowship of the Polish Ministry of Science and Higher Education. We thank Philip Abstoß, Gwen Lorenz, Reina Zühlke-Jenisch and Dorit Schuster for excellent technical assistance. We thank Martina Vockerodt for providing DNA of cell line L591.

Author contributions

CO, MG, RK and RS designed the work. CO, MG, AM, IV, SG, and JR performed experiments. WK and MLH performed pathological evaluation and provided lymphoma samples. MS evaluated next generation sequencing data. RK, RS and MG wrote the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. FICTION and FISH photomicrographs.

Table SI. Primers for the amplification and sequencing of the *TRAF3* gene.

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