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Clinical and molecular characterization of diffuse large B-cell lymphomas with 13q14.3 deletion

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Background: Deletions at 13q14.3 are common in chronic lymphocytic leukemia and are also present in diffuse large B-cell lymphomas (DLBCL) but never in immunodeficiency-related DLBCL. To characterize DLBCL with 13q14.3 deletions, we combined genome-wide DNA profiling, gene expression and clinical data in a large DLBCL series treated with rituximab, cyclophosphamide, doxorubicine, vincristine and prednisone repeated every 21 days (R-CHOP21).
Patients and methods: Affymetrix GeneChip Human Mapping 250K NspI and U133 plus 2.0 gene were used. MicroRNA (miRNA) expression was studied were by real-time PCR. Median follow-up of patients was 4.9 years.
Results: Deletions at 13q14.3, comprising *DLEU2*/*MIR15A*/*MIR16*, occurred in 22/166 (13%) cases. The deletion was wider, including also *RB1*, in 19/22 cases. Samples with del(13q14.3) had concomitant specific aberrations. No reduced *MIR15A*/*MIR16* expression was observed, but 172 transcripts were significantly differential expressed. Among the deregulated genes, there were *RB1* and *FAS*, both commonly deleted at genomic level. No differences in outcome were observed in patients treated with R-CHOP21.
Conclusions: Cases with 13q14.3 deletions appear as group of DLBCL characterized by common genetic and biologic features. Deletions at 13q14.3 might contribute to DLBCL pathogenesis by two mechanisms: deregulating the cell cycle control mainly due *RB1* loss and contributing to immune escape, due to FAS down-regulation.

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introduction

Deletions at 13q14.3 are common in chronic lymphocytic leukemia (CLL) [1] and are also present in other B-cell malignancies [2–6], including *de novo* and transformed diffuse large B-cell lymphomas (DLBCL) [6–10]. We have recently shown that 13q14.3 deletions are absent in immunodeficiency-related DLBCL [11, 12], suggesting that the lesion might contribute to the immune escape of lymphoma cells. In CLL, the minimal deleted region (MDR) includes the two microRNAs (miRNAs), *MIR15A* and *MIR16* [13, 14]. Very recently, Klein et al. [15] showed that MDR- or miR-15a/16-1-deficient mice develop CLL and CD5 negative non-Hodgkin's lymphomas resembling DLBCL. In the mouse model, the loss of two miRNAs determines an increased cell proliferation due to a lack of down-regulation of cell cycle-related genes [15]. While the role of deletions at 13q14.3 has been extensively studied in CLL [1, 13, 14], its presence in DLBCL has not been analyzed in detail. Therefore, we analyzed the biological and clinical characteristics of DLBCL patients with 13q14.3 deletions as detected by genome-wide DNA profiling in a large series of cases.

patients and methods

tumor panel

DNA was extracted from 166 frozen tumor biopsies of DLBCL, taken at diagnosis as previously described [9, 16]. Consecutive cases were selected based upon the availability of frozen material and for having a fraction of malignant cells in the pathologic specimen representing >70% of overall cellularity as determined by morphologic and immunophenotypic studies. Cases of primary mediastinal large B-cell lymphoma, human immunodeficiency virus-related DLBCL and posttransplant DLBCL were excluded. Seventy-five percent (124/166) of the patients were treated with rituximab, cyclophosphamide, doxorubicine, vincristine and prednisone repeated every 21 days (R-CHOP21) and had follow-up data. The cell of origin was determined in 109/166 cases: in 49/109 (45%) with gene expression (GEP) [17] and in 60/109 (55%) with immunohistochemistry (IHC) according to the algorithm by Hans et al. [18]. The study was approved by the Bellinzona ethical committee.

DNA extraction, array comparative genomic hybridization analysis and data mining

DNA samples were analyzed using the GeneChip Human Mapping 250K NspI (Affymetrix, Santa Clara, CA), as previously described [5, 9, 12, 16]. Data mining was carried out as previously reported [5, 9, 12, 16]. Briefly, the modified Bayesian Piecewise Regression method [19] was used to estimate the copy number (CN) starting from raw CN values obtained with Affymetrix CNAT 4.01. After normalization of each profile to a median log2 ratio of zero, thresholds for loss and gain defined as six times the median absolute deviation symmetrically ~0 with an associated P value <0.001 after Bonferroni multiple test correction. The recurrent minimal common regions (MCR) were defined using the algorithm by Lenz et al. [20]. Differences in frequencies between subgroups were evaluated using a Fisher's exact test followed by multiple test correction.

gene expression

GEP data, obtained using the Affymetrix GeneChip U133 plus 2.0, were available in 54/166 cases [17]. Data were analyzed using Partek Genomics Suite 6.4 (Partek, St. Louis, MO). Signal intensities were normalized by Partek RMA. Statistical differences were calculated by analysis of variance analysis and a false discovery rate of 0.2 was applied. The Gene Set Enrichment Analysis [21] was used to identify pathways and regulating mechanisms possibly explaining the observed GEP profile differences (http://www.broadinstitute.org/gsea). Criteria for statistical significance were: at least three genes of overlap, P value <0.05.

miRNA arrays

In 32/166 cases, miRNA expression levels were analyzed, as previously reported [16]. Total RNA including miRNA was extracted from cryopreserved tissues with mirVana miRNA isolation Kit (Ambion, Austin, TX). The reverse transcription was carried with 300 ng of total RNA with Megaplex RT Primers and enzyme kit as suggested by manufacturer (Applied Biosystems, Foster City, CA). The quantitative real-time PCR was carried out on 7900HT Fast Real-Time PCR System (Applied Biosystems, CA). To enhance assay sensitivity, a preamplification step of 12 cycles was introduced using Megaplex PreAmp Primers. The preamplified complementary DNA (cDNA) loaded onto the 384-well format TaqMan microRNA assays plates (Taqman human microRNA A array V2.0, AB1, CA). The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeds the fixed threshold of 0.1 with automatic baseline using the RQ Manager 1.2 software (Applied Biosystems, CA). Data were normalized with the average expression U6 small nuclear RNA (quadruplicate in each 384-well plate). The ΔCt values of all the miRNAs measured were calculated and exported from RQ Manager 1.2 directly. The differential miRNA expression between cases with and without del(13q14.3) was carried out using random variance Student's t-test.

validation of genome-wide DNA profiling

Real-time PCR on genomic DNA was done using the TaqMan Copy Number Assays Hs03857853_cn (Applied Biosystems, Rotkreuz, Switzerland) targeting the *DLEU2* locus normalizing using the TaqMan Copy Number Reference Assay RNase P (Applied Biosystems, Switzerland) and a DNA sample known to be diploid for the *DLEU2* locus. Reactions were run in quadruplicate in 96-well plates with 5 ng of DNA per reaction on a StepOnePlus instrument and analyzed with CopyCaller software v.1.0 (Applied Biosystems, Switzerland).

immunohistochemistry

RB1 expression was evaluated using specific antibody (MK-15-1S; Medical and Biological Laboratories Co., Nagoya, Japan). Immunohistochemical stainings were carried out on formalin-fixed paraffin-embedded tissues using the avidin-biotin-peroxidase complex method and a semiautomated immunostainer (Ventana System and/or Lieca Bond) as described [22]. Nuclear (RB1) stains were scored as neg, + (<50) and ++ (>50%) of tumor cells.

analysis of clinical data

The median follow-up was computed by the reverse Kaplan–Meier method [23]. Overall survival (OS), progression-free survival, disease-free survival and response criteria were defined according to Cheson et al. [24]. Actuarial survival probabilities were computed by the life-table method. Survival curves were estimated by the Kaplan–Meier method, and differences between curves were evaluated by the log-rank test. Binomial exact 95% confidence intervals (95% CI) were calculated for percentages. Associations in two-way tables were tested for statistical significance using either the χ^2 test or Fisher's exact test (two-tailed test), as appropriate [25]. All tests were two-sided, and the P value for significance was ≤0.05. Statistical analysis was conducted using the Stata11 (StataCorp, College Station, TX).

results

A MCR comprising the 13q14.3 locus was identified in 22/166 (13%) DLBCL. The lesion extended for ~560 000 bp and contained seven transcripts: *DLEU2*, *TRIM13*, *KCNRG*, *MIR15A* and *MIR16*, *DLEU1* and *ST13P4*. The deletion detected by genome-wide DNA profiling was validated by real-time PCR on genomic DNA in 4/4 cases. The deleted region was often wider, including also the *RB1* gene in 19/22 (86%) and *DLEU7* in 21/22 (95%) cases (Figure 1). Two patients, not bearing the del(13q14.3), were affected by copy neutral loss of heterozygosity (LOH), i.e. LOH without changes in DNA CN. Due to the low patient number affected by this event, we did not perform any further analyses for these cases.

Analysis of the clinical parameters at diagnosis of the whole cohort was comparable to previously published DLBCL populations [26]. To characterize patients affected by del(13q14), we compared their clinical parameters to those without the aberration (Table 1). Patients bearing the aberration had more favorable prognostic features at time of diagnosis with an international prognostic index (IPI) ≥2 in 54% versus 80% (7/13 versus 83/104; $P = 0.036$). Among 124 patients treated with R-CHOP21, no remarkable differences were observed regarding response to treatment and relapse rate. With a median follow-up of 4.9 years (25th–75th percentiles ranging from 4 to 7 years), the 5-year OS was 64% (95% CI 34% to 83%) for patients with del(13q14.3) and 76.5% (95% CI 67% to 84%) for cases without the loss ($P = 0.2821$) (Figure 2). Survival was not influenced by the length of the deleted regions.

We evaluated by real-time PCR the level of expression of the two miRNAs, *MIR15A* and *MIR16*, between 6 cases with and 26 without del(13q14.3). No statistical difference was observed.

GEP analysis comparing 7 cases with del(13q14) versus 47 without, revealed 172 transcripts with a significantly differential expression and 44 with more than twofold change (supplemental Table S1, available at *Annals of Oncology* online). Only four transcripts were significantly underexpressed: *RB1* (3.5-fold) and *FAS* (3.2-fold), calponin 2 (*CNN2*; 1.9-fold) and cDNA *DKFZp686F2044* (1.2-fold). The search for groups of functionally related transcripts revealed an overrepresentation of genes involved in the cell cycle (Table 2). Also, the most significant overlap with the chemical and genetic perturbations gene sets was with the 'BRCA2–Pearson Correlation Coefficient network', representing genes whose expression positively correlate with that of BRCA2 across a compendium of normal tissues ($P = 5.16E-10$). In accordance with the real-time data, no statistical overlap was identified with genes possibly regulated by miRNAs. BCL2, previously reported up-regulated in CLL with del(13q14.3) [27], was not up-regulated in DLBCL cases bearing the aberration. Reduced expression of RB1 expression in cases with 13q14.3 loss was validated by IHC (Figure 3) on 26/166 cases, including 4 cases bearing the deletion.

In order to identify lesions associated with del(13q14.3), we compared the genomic profiles of samples diploid for 13q14.3 (further designated as wild type) with those affected by del(13q14.3) (Figure 4). Del(13q14.3) was never observed as single aberration. We observed that the profile of samples with del(13q14.3) had more commonly concomitant aberrations on different chromosomes (Fisher's exact test $P < 0.05$; $q < 0.2$): del(5q33.3), del(17p) (including *TP53*), del(18p11.32), del(18p) and del(19p13.3-p13.2) (Table 3). Since a trend for a higher frequency of 18q gain was observed among patients

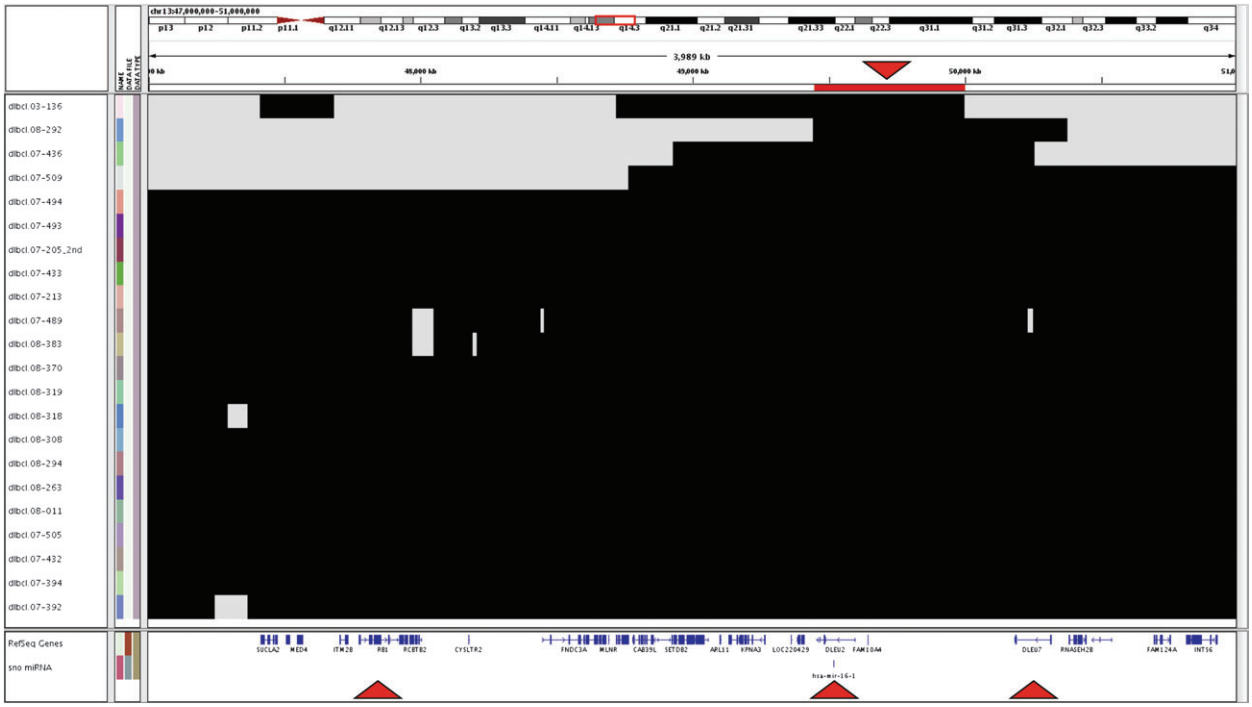

Figure 1. High-resolution analysis of the del(13q14.3). Ideogram of chromosome 13 with a zoom on 4 Mb encompassing *RB1*, *MIR15A*/*MIR16* and *DLEU7*. The red line and the triangles on the top indicate the minimal common region; the red triangles below indicate the mapping localization of *RB1*, *MIR15A*/*MIR16* and *DLEU7*. Each line represents one of the 22 diffuse large B-cell lymphomas cases with del(13q14.3); black, deletion, gray, diploid status.

Table 1. Clinical characteristics according to the presence of del(13q143)

Parameter	13q14 wild type ($n = 143$)		del(13q14) ($n = 22$)	
	Value	Percent	Value	Percent
Clinical characteristics				
Median age (range), years	64 (18–87)		66 (38–83)	
Age > 60	85/134	63	13/19	68
Gender (m : f)	62 : 69	47 : 53	9 : 10	47 : 53
ECOG PS >1	30/124	24	4/19	21
LDH > UNL	68/115	59	8/15	53
Stage III/IV	84/127	66	10/18	56
>1 Extranodal sites involved	35/119	29	3/17	18
IPI > 1	83/104	80	7/13	54
Bulky disease	26/113	23	4/15	27
B symptoms	42/124	35	4/18	22
BM involvement	24/117	20	3/16	19
GCB	45/92	48	10/17	62
Consensus cluster				
B-cell receptor/proliferation cluster	29/45	64	6/7	86
Oxidative phosphorylation cluster	1/45	2	0/7	0
Host response cluster	15/45	33	1/7	14
HCV infection	11/78	14	1/10	10
Outcome				
CR	95/120	79	13/17	76
PR	16/120	13	3/17	18
SD or PD	9/120	8	1/17	6
Median follow-up (range), months	21 (1–123)		25 (8–73)	
Relapses	32/127	25	5/18	28
Deaths	33/131	25	2/18	11

Except for IPI ≥ 2 ($P = 0.036$), no statistical differences have been observed between the two groups.
m, male; f, female; ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; UNL, upper normal limit; IPI, international prognostic index; BM, bone marrow; HCV, hepatitis C virus; GCB, germinal center like diffuse large B-cell lymphomas; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

with del(13q14) (8/23, 35%, versus 25/143, 17%; $P = 0.08$), we evaluated the concomitant presence of del(18p) and gain of 18q, suggestive for an i(18q): this event occurred statistically more frequent among cases with del(13q14) than cases without the lesion (5/23, 21% versus 2/144, 1%; $P < 0.001$). Moreover, 18q gains with a CN > 4 were also mainly observed in patients with del(13q): 3/23 (13%) versus 1/143 (0.07%). Since *RB1* was both down-regulated and target of genomic losses, we evaluated also the genomic status of the remaining gene, FAS, showing over twofold down-regulations: a deletion was observed in 6/22 (27%) cases with del(13q14.3) versus 8/144 (5%) without del(13q14.3) ($P = 0.001$).

discussion

We have analyzed the genomic profiles of DLBCL samples bearing or not the del(13q14.3), showing that cases carrying the deletion were characterized by distinct genetic features, which might affect cell cycle regulation and contribute to immune escape of the lymphoma cells.

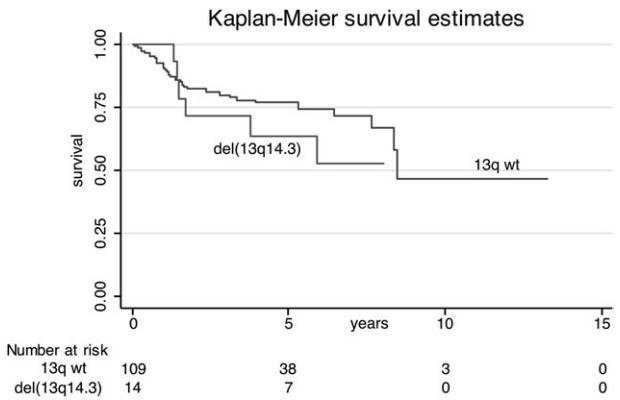

Figure 2. Overall survival of diffuse large B-cell lymphomas patients treated with rituximab, cyclophosphamide, doxorubicine, vincristine and prednisone repeated every 21 days according to 13q14 loss (14 versus. 108 cases; $P = 0.28$).

The 13q14.3-deleted region was large and comprised not only the *MIR15A*, *MIR16*, *DLEU2* but almost always also *RB1* and *DLEU7*. Similarly to what reported by Li et al. [28],

Table 2. Deregulated pathways in diffuse large B-cell lymphomas with del(13q14.3) based upon Gene Set Enrichment Analysis

Pathway	Collection	Number of genes in overlap	*P* value	
Interphase	GO biologic process	4	0.0220	*CDC7*, *TIMELESS*, *POLE*
Tubulin binding	GO molecular function	3	0.0353	*TUBGCP5*, *CEP290*, *CENPJ*
Cell cycle phase	GO biologic process	6	0.0491	*CDC7*, *TIMELESS*, *POLE*, *RB1*, *NUMA1*, *MSH5*
Genes involved in centrosome maturation	Reactome	5	0.0112	*NUMA1*, *TUBGCP5*, *PCNT*, *CENPJ*, *CEP290*
Genes involved in activation of the prereplicative complex	Reactome	3	0.0183	*MCM6*, *POLE*, *CDC7*
Genes involved in G2/M transition	Reactome	5	0.0207	*NUMA1*, *TUBGCP5*, *PCNT*, *CENPJ*, *CEP290*

GO, Gene Ontology.

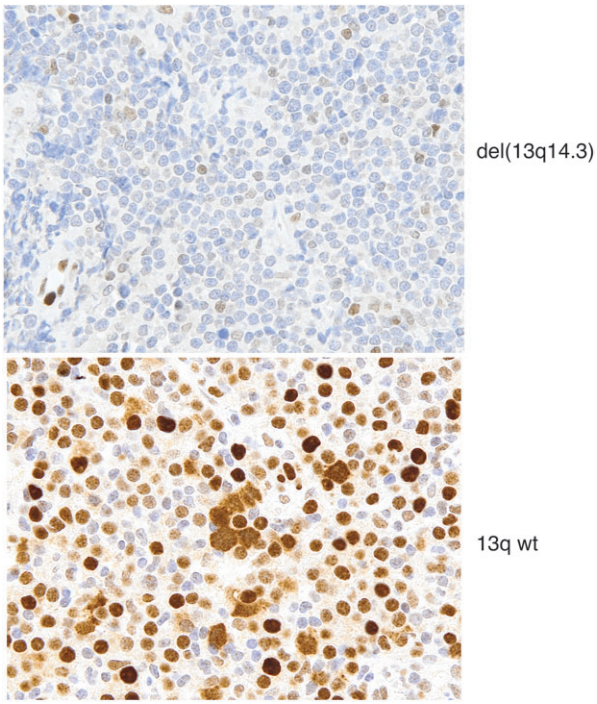

Figure 3. RB1 staining in diffuse large B-cell lymphomas with and without *RB1* genomic loss at 13q14.3.

a reduced expression of *MIR15A* and *MIR16* was not demonstrated in DLBCL cases bearing the del(13q14.3). At least two types of deletions affecting 13q14.3 have been described in CLL [29–32]: one smaller, encompassing the *DLEU2/MIR15A/MIR16* locus, more often biallelic and associated with lower expression of *MIR15A* and *MIR16*; a second type, larger, comprising also *RB1*, and usually monoallelic. Here, in DLBCL, deletions appeared similar to the second type of lesion observed in CLL, monoallelic, affecting *RB1* and with apparently no changes in *MIR15A* and *MIR16* expression levels. Genes such as *RB1* or *SETDB2*, *PHF11* and *RCBTB1*, as recently proposed [30], could be the transcripts targeted by del(13q14.3) in a subgroup of CLL as well in DLBCL.

The comparison of the clinical parameters between patients with and without del(13q14.3) revealed a lower incidence of unfavorable IPI but without differences in outcome for patients treated with R-CHOP21. On the converse, in Burkitt lymphoma, del(13q14.3), present in ~35% of the cases, has been reported to be associated with poorer survival [10].

In CLL, the size of the deletion affecting the 13q14 region has been suggested to determine differences in prognostic features at the time of diagnosis and in treatment outcome [29–31]. Here, the vast majority of patients had the large type of deletions; thus, we could not compare their clinical parameters to those of the cases bearing the larger deletion. We only evaluated the impact in survival of the length of the aberration and no statistically significant differences were found, but, again, the number of cases with small deletions was very small.

GEP analysis revealed mostly up-regulated genes in DLBCL with del(13q) when compared with the remaining cases. The reason remains unknown. As already discussed, this was unlikely due to a direct increase of expression of *MIR15A* and *MIR16* target genes since neither reduced expression of the two miRNAs was observed nor a statistical enrichment of their target genes was demonstrated. However, intriguingly, in accordance to what recently reported in the mouse model [15], the differentially expressed genes were enriched of factors involved in cell cycle regulation, which could play a role in lymphomagenesis. Among the 13q− DLBCL, *RB1* was the most significant down-regulated transcript, which can largely be explained by the loss of its locus, similarly to what reported in CLL [33]. The 3.5-fold reduction of its transcript in patients bearing the lesion might have an important pathogenetic effect. It is also worth of mentioning that the deletions always comprised *DLEU7*. Recently, Palamarchuk et al. [34] have identified this gene as a tumor suppressor gene in CLL since it represents a potent inhibitor of the nuclear factor κB signaling. Thus, its loss could play an important role in DLBCL too since a constitutive activation of this pathway is relevant in a subset of DLBCL patients [35].

The other down-regulated gene in DLBCL with del(13q14.3) was *FAS*. This gene codes for the tumour necrosis factor receptor superfamily, member 6 (TNFRSF6/CD95). Interestingly, down-regulation of *FAS* can protect the cell from apoptosis, contributing to immune escape [36]. This would partially explain the notion that del(13q14.3) is

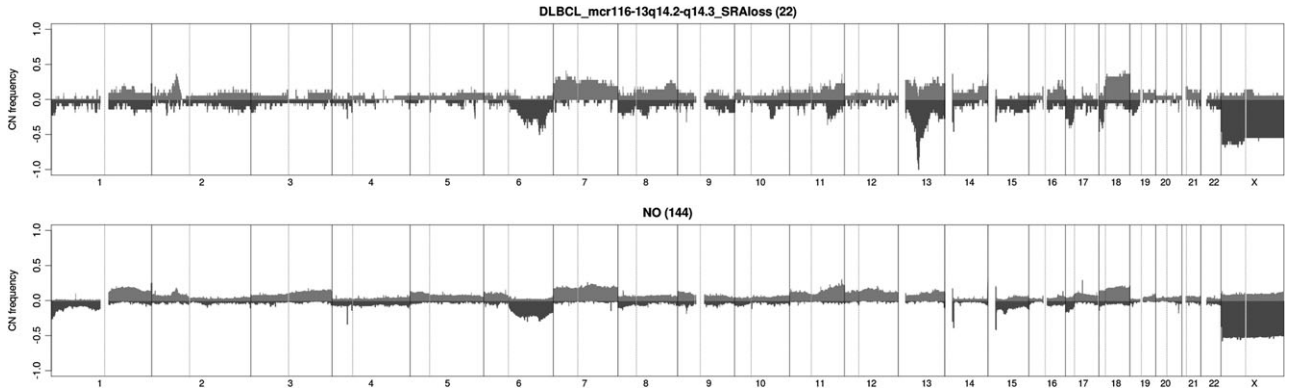

Figure 4. Frequency of DNA gains and losses observed in 166 diffuse large B-cell lymphomas patients according to the presence of del(14q14.3). Upper panel: frequency plot of gains and losses in 22 patients with del(13q14.3). Lower panel: frequency plot of gains and losses in 144 patients without del(13q14.3). *Y*-axis, percentage of cases showing the aberrations. Chromosome X has not been compared due to the presence of mixed male and female population. Light gray (up), gains; Dark gray (down), losses.

Table 3. Association between presence of del(13q14.3) and other concomitant genomic lesions among 166 cases of diffuse large B-cell lymphomas as evaluated by applying Fisher's exact test (*P* value) followed by multiple test correction (*q* value)

Associated region	del(13q14.3), %	No del(13q14.3), %	*P* value	*q* value
del(18p11.32)	11/23 (48)	19/143 (13)	0.0004	0.0056
del(18p)	7/23 (30)	8/143 (6)	0.0012	0.0160
del(17p) (TP53)	8/23 (35)	15/143 (10)	0.0051	0.0567
del(5q33.3)	5/23 (22)	6/143 (4)	0.0087	0.0755
del(19p13.3-p13.2)	7/23 (26)	11/143 (8)	0.0162	0.1148

underrepresented in immunodeficiency-related DLBCL [11, 12], in which the need to escape immunity is reduced. Similarly to RB1, the significant deregulation of *FAS* appeared, at least partially, due to DNA losses since its genomic locus was significantly more commonly deleted in 13q− DLBCL than in the remaining cases. Other features specific of immunodeficiency-related DLBCL cells or of the particular microenvironment might determine GEP changes similar to those observed here in these series of DLBCL from immunocompetent hosts.

In DLBCL, del(13q) was never the sole lesion, differently from CLL, in which the lesion is often isolated [31, 32, 37]. A series of genomic lesions appeared associated with the occurrence of del(13q14.3), including gains of the long arm of chromosome 18 with losses of the corresponding short arm suggestive of the presence of i(18q) and losses of the short arm of chromosome 17. Interestingly, we have observed a similar pattern of concomitant lesions (13q−/18q+/17p−) in splenic marginal zone lymphomas [5], indicating that a series of genes mapping on these chromosomes might be required for lymphoma pathogenesis. The observed pattern of concomitant genomic lesions could also be linked with an increased genomic instability in these patients, in accordance with cell cycle deregulation. In DLBCL, gain of 18q is more common among activated B-cell-like DLBCL than in germinal center B-cell-like DLBCL, and the *BCL2* and *NFATC1* genes have been suggested as the involved genes [20]. Here, the presence of del(13q.14.3) was not associated with a specific DLBCL subtype. None of the genes mapping on 18q, including *BCL2* or *NFATC1*, appeared to be overexpressed in our series of DLBCL cases with del(13q14.3): although this might be due to sample size, we cannot rule out that other transcripts not investigated by the Affymetrix U133 plus 2.0 array could be altered. Del(17p11.2-p13.3), which includes the *TP53* gene, is one of the most common lesions in cancers. In DLBCL, only the presence of somatic mutations and not the simple loss of 17p is associated with a poor outcome [37], maybe explaining the presence of this lesion in association with del(13q14.3) and an apparently good outcome.

In conclusion, we have described that DLBCL cases bearing del(13q14.3) present distinct genetic features that might affect cell cycle regulation and might contribute to immune escape of the lymphoma cells.

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disclosure

The authors declare no conflict of interest.

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