Definition of MYC genetic heteroclonality in diffuse large B-cell lymphoma with 8q24 rearrangement and its impact on protein expression

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MYC rearrangement can be detected in a subgroup of diffuse large B-cell lymphoma characterized by unfavorable prognosis. In contrast to Burkitt lymphoma, the correlation between **MYC** rearrangement and **MYC** protein expression in diffuse large B-cell lymphoma is less clear, as approximately one-third of rearranged cases show negative or low expression by immunohistochemistry. To better understand whether specific characteristics of the **MYC** rearrangement may influence its protein expression, we investigated 43 *de novo* diffuse large B-cell lymphoma positive for 8q24 rearrangement by FISH, using 14 Burkitt lymphoma for comparison. Different cell populations (clones), breakpoints (*classical vs non-classical* FISH patterns), partner genes (*IGH vs non-IGH*) and immunostaining were detected and analyzed using computerized image systems. In a subgroup of diffuse large B-cell lymphoma, we observed different clones within the same tumor distinguishing the *founder* clone with **MYC** rearrangement alone from other subclones, carrying **MYC** rearrangement coupled with loss/extra copies of derivatives/normal alleles. This picture, which we defined **MYC genetic heteroclonality**, was found in 42% of cases and correlated to negative **MYC** expression ($P=0.026$). *Non-classical* FISH breakpoints were detected in 16% of diffuse large B-cell lymphoma without affecting expression ($P=0.040$). *Non-IGH* gene was the preferential partner of rearrangement in those diffuse large B-cell lymphoma showing **MYC heteroclonality** ($P=0.016$) and/or *non-classical* FISH breakpoints ($P=0.058$). **MYC heteroclonality** was not observed in Burkitt lymphoma and all cases had positive **MYC** expression. *Non-classical* FISH **MYC** breakpoint and *non-IGH* partner were found in 29 and 20% of Burkitt lymphoma, respectively. In conclusion, **MYC genetic heteroclonality** is a frequent event in diffuse large B-cell lymphoma and may have a relevant role in modulating **MYC** expression.

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Diffuse large B-cell lymphoma is the most common mature aggressive B-cell lymphoma. Despite recent therapeutic achievements, approximately 40% of diffuse large B-cell lymphoma cannot be cured, which indicates the need for further studies focusing

on the characterization of the putative genes involved in diffuse large B-cell lymphoma pathogenesis. In this context, **MYC** has a relevant role, and the presence of **MYC** rearrangement, described in approximately 5 to 15% of diffuse large B-cell lymphoma cases, correlates with worse prognosis and poor response to R-CHOP treatment.^{1–5}

A novel commercial monoclonal antibody has improved the detection of **MYC** protein expression by immunohistochemistry in formalin-fixed paraffin-embedded tissues. Using this antibody, Burkitt

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lymphoma with *MYC* rearrangement were found to show intense positive staining for *MYC* protein in the vast majority of the cells, supporting the existence of a strong correlation between gene and protein status.^{6–7} In contrast, in *MYC* rearranged diffuse large B-cell lymphoma, the association between rearrangement and protein expression is less clear, as approximately one-third of cases with this rearrangement show protein staining in < 40% of the tumor cells.^{5,8–12}

Conventional and molecular cytogenetic studies of diffuse large B-cell lymphoma have described cases with *MYC* rearrangement that are characterized by complex karyotypes, revealing the presence of different populations of cells with loss/extra copies of derivatives of the *MYC* rearrangement and/or extra copies of chromosome 8.^{13–18} The occurrence of these alterations and their contribution to protein expression remains unknown.

To further complicate this picture, B-cell lymphomas with *MYC* rearrangement show a great variability in both the location of the breakpoint in the 8q24 region (involving or not the *MYC* coding region) and the partner gene implicated in the translocation. Non-*IGH* loci have been reported as the most common partners in diffuse large B-cell lymphoma.^{4,16,19} In addition, when non-*IG* loci are involved, breakpoints are usually located outside of the *MYC* coding region.^{15,20–25} The effects of the different *MYC* breakpoints and/or rearrangement partners on *MYC* expression are not well understood, but recent studies revealed that the *IG* partner (mainly represented by the *IGH* gene) is a negative predictor of survival in diffuse large B-cell lymphoma.^{19,26} Finally, concomitant *BCL2* and/or *BCL6* rearrangements in so-called double/triple-hit lymphomas have been reported in the context of the diffuse large B-cell lymphoma complex karyotypes.^{1,4,16}

To better understand whether specific characteristics of *MYC* genetic alterations may influence its protein expression, we performed a detailed analysis of *MYC* rearrangement using new computerized imaging systems in a series of *de novo* diffuse large B-cell lymphoma. We described and quantified different populations of cells with *MYC* rearrangement and loss/extra copies of the derivatives of the translocation and/or of the normal allele. Different types of *MYC* breakpoints and their correlation to *IGH* as a translocation partner were also investigated. *MYC* protein staining was evaluated and compared with all findings.

Materials and methods

Case Selection

A total of 43 *de novo* diffuse large B-cell lymphoma cases characterized by *MYC* rearrangement in 8q24 were retrieved from a series of B-cell lymphomas

investigated for diagnostic purposes during the last 15 years at the Institute of Pathology, Locarno (Switzerland), Laboratories of Pathology from the Hospital Clínic of Barcelona and the Hospital del Mar, Barcelona (Spain), and Institute of Pathology at the University Hospital, Basel (Switzerland). All tumors were diagnosed according to World Health Organization (WHO).²⁷ Formalin-fixed paraffin-embedded tissue was available for all cases. Clinical information and follow-up data were obtained for 25 diffuse large B-cell lymphoma patients. To compare our results with another group of mature aggressive B-cell lymphomas with *MYC* rearrangement, 14 Burkitt lymphoma cases were included in this study.

Immunohistochemistry

Immunohistochemical studies were performed with a panel of monoclonal and polyclonal antibodies using a peroxidase-labeled detection system, standard antigen retrieval protocols and an automated immunostainer (Roche Ventana Medical Systems, Baar, Switzerland and Dako Autostainer, Dako, Copenhagen, Denmark). The panel of antibodies used included CD20, CD79a, CD10, BCL6, MUM1, BCL2 and Ki-67 (Supplementary Table 1). *In situ* hybridization was performed to analyze the presence of Epstein–Barr virus (EBV). For all of these markers, a cutoff of 25% stained cells was used to interpret the results as positive, similar to previously reported studies, with the exception of BCL2, for which a cutoff of 50% was used.⁸ Diffuse large B-cell lymphoma were subclassified into GCB and non-GCB according to the Hans' algorithm.²⁸

MYC protein expression was assessed using the ready-to-use monoclonal antibody (clone Y69) following the manufacturer's protocol (Roche Ventana Medical Systems) and evaluated with a computerized system. Digital images were captured and analyzed with an Aperio Image Scope (Aperio Technologies, Vista, CA, USA). Areas of artifacts, necrosis and other poor quality regions were excluded from analysis. Expression in >40% of tumor cells was used as a cutoff to classify a case as *MYC* positive, as reported in the literature.^{7–9}

Fluorescence *In Situ* Hybridization

FISH was performed on 3- to 4- μ m-thick formalin-fixed paraffin-embedded tissues sections following current recommendations, as previously described.^{29,30} The commercially available *MYC* split signal probe from Dako was applied to all cases (*MYC* FISH DNA Probe, Split Signal, Dako). This probe was designed to hybridize upstream and downstream of the *MYC* breakpoint cluster region with a green probe (that binds to a 652-kb segment centromeric to the *MYC* breakpoint cluster region on chromosome 8q24) and a red probe (that binds to a 418-kb segment telomeric to

Figure 1 Schematic representation of FISH signal patterns using *MYC* split signal probe in a *MYC* normal cell (a), in a *MYC* rearranged cell with classical (b) or non-classical FISH *MYC* breakpoints (c) (probe design and breakpoints definition should be intended using the Dako Split Signal probe). Complex FISH patterns showing additional *MYC* alterations (d). Population carrying *MYC* rearrangement alone (founder clone, b or c) and derived subclones with *MYC* complex pattern (d) resulting in *MYC* genetic heteroclonality (e).

the *MYC* breakpoint cluster region), respectively, with a gap of approximately 94 kb. Colocalization of the probes results in a red/green signal (fusion, 1F) indicating normal *MYC* allele, thus a normal cell display a 2F FISH pattern (Figure 1a). Translocation events in the *MYC* breakpoint cluster region will split one fusion signal in separate green (1G) and red (1R) signals, thus resulting in a cell with 1G1R1F FISH pattern (Figure 1b). As a result of this specific probe design (<http://www.dako.com>), breaks occurring in the sequences centromeric or telomeric to the *MYC* breakpoint cluster region result in 1G2F or 1R2F FISH pattern, respectively (Figure 1c). Cells carrying one of these different FISH signals patterns were considered positive for the presence of *MYC* rearrangement. For simplification, on the basis of FISH pattern, cells with 1G1R1F signals were classified as carrying a *classical FISH MYC breakpoint*, whereas those with 1G2F or 1R2F signals were grouped as having a *non-classical FISH MYC breakpoint*.

In selected cases, *MYC* was also evaluated by means of another break-apart probe (Vysis *MYC* Break Apart FISH Probe Kit, Abbott Molecular, IL, USA), which was similarly designed with a dual color strategy, but covering a larger region at 8q24 with a gap of approximately 1.6 Mb. To identify the *IGH* locus as a potential partner for *MYC* rearrangement, an *IGH/MYC* dual fusion probe (Vysis LSI *IGH/MYC*, CEP 8, Tri-color Dual Fusion Translocation Probe, Abbott Molecular) was used in cases with available material. FISH for *BCL2* and *BCL6* using split signals probes were performed for most diffuse large B-cell lymphoma cases (*BCL2* and *BCL6* FISH DNA Probe, Split Signal, Dako).

Only cases with at least 90% hybridization efficiency were considered. FISH signal patterns were interpreted following the criteria of Ventura et al.³¹ Ten tonsil sections were used as negative controls. The cutoff used to establish the presence of

rearrangements with the split signal and dual fusion probes was 5%.^{29,31}

All FISH experiments were evaluated with a conventional fluorescence microscope by two expert operators (AV and VM). In addition, all hybridizations performed with the *MYC* Dako split signal probe were analyzed with the Bioview image-based quantification system (BioviewDuet™, NesZiona, Israel). A minimum of 100 cells with clear non-overlapping nuclei were selected and automatically scored for each case. Tissue matching was performed for the H&E, FISH and immunohistochemistry slides to ensure that the same tumor areas were evaluated.

Statistical Analysis

Categorical data were compared using Fisher's exact test with two-sided *P*-values, and for ordinal data, nonparametric tests were used. Statistical analysis was performed using the SPSS software package version 21.0 (SPSS, Chicago, IL, USA).

Results

Clinical and Pathological Characteristics of Diffuse Large B-Cell Lymphoma

Forty-three patients, including 31 males and 12 females, with a mean age of 68 years (range of 38–93 years) were examined in this study. One (2%) patient had HIV infection, 22/43 (51%) had extranodal presentation and 9/25 (36%) cases died of disease. All cases showed CD20 and/or CD79a expression. CD10 was positive in 32/42 (76%), *BCL6* in 36/40 (90%) and MUM1 in 12/34 (35%). *BCL2* expression was detected in 34/41 (83%) cases. Thirty-two of 40 (80%) cases had a GCB immunophenotype. A high proliferation index (Ki-67 > 90%)

Table 1 Detailed FISH patterns using MYC split signal probe and MYC expression in diffuse large B-cell lymphoma with MYC genetic heteroclonality

Case	MYC rearrangement		MYC rearrangement +extra normal allele		MYC rearrangement +extra/loss derivatives		MYC rearrangement +extra normal allele +extra derivatives		No MYC rearrangement		MYC IHC % Of cells
	% Of cells	Pattern	% Of cells	Pattern	% Of cells	Pattern	% Of cells	Pattern	% Of cells	Pattern	
1	27%	1G1R1F	36%	1G1R2F	—	—	—	—	37%	2F	97%
2	32%	1G1R1F	55%	1G1R2F	—	—	—	—	13%	2F	NE
3	46%	1G1R1F	45%	1G1R2F	—	—	—	—	9%	2F	35%
4	10%	1G1R1F	90%	1G1R3F	—	—	—	—	—	—	26%
5	18%	1G1R1F	10%	1G1R2F	50%	2G2R1F	—	—	22%	2F	8%
6	30%	1G1R1F	23%	1G1R2F	14%	1G2R1F	16%	1G2R2F	17%	2F	92%
7	22%	1G1R1F	22%	1G1R2F	—	—	46%	1G2R2F	10%	2F	86%
8	9%	1G1R1F	28%	1G1R2F	—	—	27%	2G1R2F	36%	2F	35%
9	19%	1G1R1F	22%	1G1R2F	—	—	53%	2G1R2F	6%	2F	3%
10	7%	1G1R1F	5%	1G1R2-4F	—	—	57%	1G2-3R2-4F	31%	2F	13%
11	5%	1G1R1F	49%	1G1R3-6F	—	—	17%	1G2R3-6F	11+18%	2F+3-6F	92%
12	20%	1G1R1F	—	—	22%	2G1R1F	35%	2G1R2F	23%	2F	8%
13	60%	1G1R1F	—	—	25%	2G1R1F	—	—	15%	2F	24%
14	20%	1G1R1F	—	—	80%	1G2R1F	—	—	0%	2F	98%
15	9%	1G1R1F	—	—	61%	1G0R1F	—	—	30%	2F	20%

Abbreviations: F, fusion signal (normal MYC allele); G, green signal (MYC centromeric derivative); IHC, immunohistochemistry; NE, not evaluable; R, red signal (MYC telomeric derivative).

Clones dimensions are intended as the percentage of tumor cells carrying the specific pattern or staining, both evaluated by computerized strategy. For simplification, only cases with classical FISH MYC breakpoint are listed (full raw data available in Supplementary Table 2).

Table 2 Clinical, pathological and genetic features in diffuse large B-cell lymphoma regarding MYC genetic heteroclonality

	MYC genetic (n = 43)	
	Heteroclonality (n = 18)	No heteroclonality (n = 25)
Clinical		
> 60 years	15/18 (83%)	15/25 (60%)
Gender (M:F)	14:4	17:8
Dead of disease	4/11 (36%)	5/14 (26%)
Extranodal location	11/18 (61%)	11/25 (44%)
Immunohistochemical		
CD10+	15/18 (83%)	17/24 (71%)
BCL6+	17/18 (94%)	19/22 (86%)
MUM1+	3/15 (20%)	9/19 (47%)
GCB phenotype	15/17 (88%)	17/23 (74%)
BCL2+	16/18 (89%)	18/23 (78%)
MYC+*	8/17 (47%)	20/25 (80%)
Genetics		
IGH/MYC+**	6/14 (43%)	18/22 (82%)
Double/triple hit	12/15 (80%)	15/21 (71%)

*P = 0.026, **P = 0.016.

was observed in 25/41 (61%) cases. EBV infection was present in 1/24 (4%) case.

Twenty-eight of 42 (67%) cases were classified as positive for MYC expression, which was observed in a range of 42–98% tumor cells, with mean and median values of 77% and 86%, respectively. Fourteen cases (33%) were classified as negative for MYC expression, with a range of 3–35% stained cells and mean and median values of 18% and 17%, respectively.

Genetic Characteristics of Diffuse Large B-Cell Lymphoma

According to FISH patterns of MYC translocation, two subgroups of diffuse large B-cell lymphoma were identified (all raw data available in Supplementary Table 2). Twenty-five of 43 (58%) diffuse large B-cell lymphoma showed a single pattern of rearrangement characterized by one copy of a normal MYC allele and two derivatives (Figures 1b and 1c), which was observed in 40–98% of the tumor cells (mean: 71% and median: 73%). We termed this population of cells as 'MYC founder clone' (ie, cells showing MYC rearrangement as the sole abnormality). In contrast, 18/43 (42%) cases had the MYC founder clone (5–60% of cells; mean: 22% and median: 20%) in combination with two or more clones harboring MYC rearrangement coupled with additional alterations (extra copies of the normal allele, extra copies of derivatives, extra copies of both, or deletion of one of the derivatives; Figure 1d). We called this complex distribution of MYC rearrangement MYC genetic heteroclonality (MYC HC) (Figure 1e). Table 1 shows the FISH patterns details of cases characterized by MYC HC. No clinical or pathological differences were observed in the diffuse large B-cell lymphoma according to these two subgroups (detailed in Table 2).

BCL2 and BCL6 rearrangements were detected in 19/36 (53%) and in 13/34 (38%) cases, respectively. Overall, double hit characterized 19/33 (58%) cases (12 MYC and BCL2; 7 MYC and BCL6) and triple hit characterized 5/33 (15%) cases. The presence of

Table 3 Clinical, pathological and genetic features in diffuse large B-cell lymphoma regarding the type of FISH MYC breakpoints

	MYC breakpoints (n = 43)	
	Classical FISH pattern (n = 36)	Non-classical FISH pattern (n = 7)
<i>Clinical</i>		
> 60 years*	23/36 (72%)	7/7 (100%)
Gender (M:F)	25:11	6:1
Dead of disease	7/20 (35%)	2/5 (40%)
Extranodal location	19/36 (53%)	3/7 (43%)
<i>Immunohistochemical</i>		
CD10+	28/36 (78%)	4/6 (67%)
BCL6+	32/36 (89%)	4/4 (100%)
MUM1+	10/30 (3%)	2/4 (50%)
GCB phenotype	28/35 (80%)	4/5 (80%)
BCL2+	28/35 (80%)	6/6 (100%)
MYC+**	21/35 (60%)	7/7 (100%)
<i>Genetics</i>		
IGH/MYC+***	22/30 (73%)	2/6 (33%)
Double/triple hit	23/31 (74%)	4/5 (80%)
MYC genetic heteroclonality	15/36 (42%)	3/7 (43%)

*P = 0.057; **P = 0.040; ***P = 0.058.

double hit or triple hit was associated with a trend toward a worse prognosis compared with other cases carrying MYC as a single hit ($P = 0.055$); all patients who died of disease showed a double/triple hit. Double hit and triple hit were found in MYC HC (7/14, 50% and 4/14, 29%, respectively) and also in cases without MYC HC (12/19, 63% and 1/19, 5%, respectively).

The classical FISH MYC breakpoint was found in 36/43 (84%) diffuse large B-cell lymphoma, whereas the non-classical FISH MYC breakpoint was detected in 7/43 (16%) cases. Four of the seven cases with non-classical FISH pattern showed a break centromeric to the MYC breakpoint cluster region, whereas the remaining three had a break telomeric to the MYC breakpoint cluster region. Cases with non-classical FISH MYC breakpoint were also analyzed using the Abbott MYC break-apart probe, confirming the presence of the rearrangement in all cases. No difference was observed in the mean number of MYC rearranged cells between cases with classical and non-classical FISH pattern (74%, range: 40–100%, in classical FISH MYC breakpoint, and 81%, range: 46–100%, in non-classical FISH MYC breakpoint cases). The comparison of the clinical and pathological characteristics of the diffuse large B-cell lymphoma with both types of MYC breakpoints is summarized in Table 3.

In 36 cases, the presence of an IGH/MYC dual fusion was studied. Twenty-four cases (67%) presented the specific translocation t(8;14), whereas 12 cases had non-IGH rearrangements. The IGH/MYC translocation was observed in the majority of cases with a classical FISH MYC breakpoint (22/30, 73%), but only in 2/6 (33%) cases with a

non-classical FISH MYC breakpoint ($P = 0.058$, Table 3). An IGH/MYC translocation was observed in 18/22 (82%) cases without MYC HC, but in 6/14 (43%) cases with MYC HC ($P = 0.016$, Table 2). Sixteen of 22 (73%) cases with IGH/MYC translocation and 6/8 (75%) cases without an IGH/MYC showed double/triple hit. The presence of an IGH/MYC translocation did not significantly correlate with overall survival or other clinical–pathological features (Supplementary Table 3).

Correlation Between MYC Expression and MYC Genetic Status in Diffuse Large B-Cell Lymphoma

Nine of 14 (64%) cases with lower MYC expression (< 40% of stained cells, classified as MYC immunohistochemistry negative) had MYC HC ($P = 0.026$) (Table 1) and the MYC founder clone was in the range of 7–60% (mean: 22%, median: 18%). Five cases with low expression and without MYC HC showed the MYC founder clone in the range of 46–91% (mean: 73% and median: 74%).

In contrast, only 8/28 (29%) cases with positive MYC expression had MYC HC. In these cases, the MYC founder clone was in the range of 5–33% of cells (mean: 21% and median: 23%). The remaining cases with positive expression but no MYC HC showed the MYC founder clone in the range of 40–98% of cells (mean and median: 71%). FISH and immunohistochemistry images of two cases with MYC HC are shown in Figures 2a–d.

Owing to the high chromosomal complexity involving MYC in heteroclonal cases, it was not possible to establish specific correlations between other single clones or a specific combination of these clones and MYC protein expression. Notably, the FISH pattern with loss of red signal (corresponding to the derivative chromosome carrying MYC telomeric portion) characterized 5/14 (36%) cases with lower MYC expression ($P = 0.003$).

Considering the breakpoint type observed by FISH, MYC protein staining was positive in all cases (7/7) with a non-classical FISH MYC breakpoint (MYC expression in the range of 42–97% tumor cells; mean: 85%, median: 93%), and in 21/35 (60%) cases with the classical breakpoint (MYC expression in the range of 43–98%; mean: 74%, median: 79%). The 14 cases classified as negative for MYC expression had all the classical FISH MYC breakpoint ($P = 0.040$).

No difference was observed in MYC protein expression according to IGH or non-IGH genes as the MYC translocation partner. Sixteen of 25 (64%) cases with positive MYC expression and 8/11 (73%) cases with negative expression showed IGH/MYC translocation. Cases with IGH/MYC translocation had a similar number of MYC stained cells (43–97%, mean: 73%, median: 75%)

Figure 2 FISH and immunohistochemistry images of two diffuse large B-cell lymphoma captured by digital system (Bioview and Aperio, respectively); (**a**, **b**) case with *MYC* genetic heteroclonality and positive MYC expression; (**c**, **d**) case with *MYC* genetic heteroclonality and negative (low) MYC expression; (**e**) schematic example for tumoral growth and clonal evolution leading to FISH pattern observed in (**c**) (case #9 of Table 1).

than those with non-IG partner (42–98%, mean: 84%, median: 93%).

Clinical, Pathological and Genetic Characteristics of Burkitt Lymphoma

The control group comprised 14 Burkitt lymphoma patients, 10 males and 4 females, with a mean age of 41 years (range: 7–83). Extranodal presentation was observed in 10/13 (77%) patients. All samples were positive for CD20 and CD10. BCL6 was also positive in 12/13 (92%) cases and MUM1 expression was present in 6/11 (55%) cases. The proliferation index (Ki-67) was higher than 90% in 12/13 (92%) cases, and EBV infection was detected in 3/12 (25%) cases. MYC expression was evaluable in 11 of 14 cases, and all cases were positive, showing a range of 57–95% stained cells (mean: 83%, median: 87%). MYC rearrangement by FISH analysis was found in a range of 65–95% tumor cells (mean: of 83%, median: 85%). MYC HC was not observed in any Burkitt lymphoma case. All Burkitt lymphoma were characterized by a uniform population carrying the MYC rearrangement without additional alterations (all raw data available in Supplementary Table 2).

Ten of 14 (71%) Burkitt lymphoma cases showed a *classical FISH MYC breakpoint* and 4 of 14 (29%) a *non-classical FISH MYC breakpoint*. Among the latter cases, two showed a break in the region centromeric to MYC breakpoint cluster region, and two in the telomeric one (similarly to diffuse large B-cell lymphoma, these cases were confirmed using the MYC break-apart probe from Abbott Molecular). The cases with a *classical FISH MYC breakpoint* showed the rearrangement in 65–95% of cells (mean: 87%, median: 91%), and those with a *non-classical FISH MYC breakpoint* in 67–79% of the cells (mean and median: 72%). Cases with the *classical FISH MYC breakpoint* had MYC expression in 57–95% of cells (mean: 87%, median: 92%), and in those with a *non-classical FISH MYC breakpoint*, protein expression was observed in 60–84% of cells (mean: 73%, median: 75%). An IGH/MYC dual fusion probe was used in 10 cases, and 8 (80%) demonstrated the specific translocation t(8;14)(q24;q32). Seven of eight (88%) cases with IGH/MYC carried the *classical FISH MYC breakpoint*. No clinical or pathological differences according to type of MYC breakpoint was observed in Burkitt lymphoma cases.

Discussion

Diffuse large B-cell lymphoma represents an exceedingly heterogeneous disease both at the clinical and biological levels. Despite the corroborating role of MYC alterations as a prognostic marker for diffuse large B-cell lymphoma,^{1–5} its assessment represents a major challenge in clinical practice. A novel commercial monoclonal antibody has improved the detection of MYC protein expression,^{6–7} however,

immunohistochemistry assessment and quantification still remains difficult because of highly variable MYC expression.^{11–12} In this regard, image digital analysis and computerized assisted interpretation may prove to be a valid aid. Applying this strategy (Aperio) to our series of 43 MYC rearranged diffuse large B-cell lymphoma, we observed high (>40%, immunohistochemistry positive) and low (<40%, immunohistochemistry negative) MYC protein expression in 88% and 12% cases, respectively, confirming previous observations.^{5,8–10} Interestingly, even if all of the investigated diffuse large B-cell lymphoma samples had a MYC translocation, great differences were observed in terms of the frequency of MYC expressing cells: in MYC immunohistochemistry positive cases, the median number of stained cells was 86%, whereas it was 17% in MYC immunohistochemistry negative cases. To better understand the possible role of the MYC gene in the variability of MYC protein expression, we performed a detailed investigation of MYC rearrangement in the whole tumoral context, as well as in single-cell details.

Relative to MYC rearrangement, regardless of the specific type of FISH pattern, the percentage of rearranged cells in our diffuse large B-cell lymphoma cases ranged from 40% to 98%. Using a computerized imaging system (Bioview), we were able to specifically describe and quantify all the different populations of cells within the same tumor, distinguishing the MYC founder clone (MYC rearrangement alone) from other populations of cells with MYC complex patterns, showing the MYC translocation coupled to further alterations involving MYC.

Reproducing the hypothesis of a clonal evolution model with a founding clone and multiple subclones, as described for other hematologic malignancies,^{32,33} we hypothesize that during tumor growth, the first pathogenetic event (MYC translocation) is followed by subsequent changes involving the MYC derivatives or the normal allele (gain/loss or extra copies, respectively). The expansion of these descending cells results in new subclones similar to the progenitor (sharing MYC translocation) but also different (carrying additional modifications) (Figure 2e). This picture defines the presence of MYC genetic heteroclonality/HC, distinguishing this precise status from the more general concept of genetic heterogeneity. Results from conventional cytogenetic studies (<http://cgap.nci.nih.gov/Chromosomes/CytList>) reinforce this hypothesis, and our findings confirm that FISH is a valid approach to characterize the presence of MYC HC in tissue samples.

MYC HC was observed in 42% of diffuse large B-cell lymphoma and correlated with lower MYC expression. In contrast, the vast majority of cases with MYC expression (80%) did not show MYC HC. In cases with MYC HC, the MYC founder clone represented a minor population, corresponding to a median of 20% of tumor cells. Interestingly, in the

Figure 3 Putative model to describe the impact of the presence of *MYC* genetic heteroclonality on MYC protein expression in diffuse large B-cell lymphoma based on the percentage of rearranged and stained cells. M, median.

heteroclonal cases with lower expression, the dimensions of the founder clone and of the MYC stained population nearly overlapped (median 18% and 20%, respectively) (Figure 3a). Similarly, in cases without *MYC* HC and positive for MYC expression, the dimension of the *MYC* founder clone and the number of overexpressing cells were very similar (71% vs 74%) (Figure 3d). In Burkitt lymphoma, this phenomenon was even more evident, that is, all cases lacked *heteroclonality*, had positive expression and showed the *MYC* founder clone as a major population (85% and 87%, respectively). In other words, this precise quantification and comparison revealed that in about two-thirds of diffuse large B-cell lymphoma cases with *MYC* rearrangement, the relationship between founder and expressing clones was strong: the smaller or bigger the first, the smaller or bigger the second. In contrast, in the subgroup of cases without *MYC* HC and lower MYC expression (corresponding to about 10% of diffuse large B-cell lymphoma), the dimension of the *MYC* founder clone was completely different from the expressing population, with the former exceeding by six times the latter (median dimension: 74% vs 12%) (Figure 3b); the opposite was observed in the subgroup of cases with *MYC* HC but positive MYC expression (about 20%) wherein the number of expressing cells highly outnumbered that of the founder clone (median 23% vs 92%, respectively) (Figure 3c). All these data suggest that MYC protein expression may be affected by the presence of *heteroclonality* (see a putative model in Figure 3).

From a biological point of view, changes in the synthesis of MYC protein may be related to primary events affecting the founder clone itself, or to secondary events occurring in the derived subclones during tumor growth. In fact, the majority of *MYC* HC showed a reduced expression, indicating the occurrence in subclone of modifications at the

post-transcriptional/translational level or strictly affecting *MYC* gene itself. The fact that five cases with low MYC expression were characterized by loss of the red derivative (which is the one that contains the coding portion of *MYC* juxtaposed to the promoter of the partner gene) may support this hypothesis. In contrast, in those HC cases still expressing MYC protein, the subclones are likely not influenced by changes and remain capable of maintaining the expression. Although the mechanisms responsible for the loss of MYC expression are still unclear, the presence of *heteroclonality* may explain the low MYC expression in 10–30% of diffuse large B-cell lymphoma with *MYC* rearrangement.^{5,8–12,26} Further studies using combined method of FISH and immunofluorescence (such as FICTION technique) may help to characterize more precisely the relationship between MYC gene and protein status in all these different clones.

MYC HC was observed with similar frequencies in cases showing *MYC* rearrangement with *classical* or *non-classical FISH breakpoints* (42% and 43% of cases, respectively), thus indicating that the breakpoint does not have a role in clonal evolution. Interestingly, *MYC* HC cases were primarily partnered with a non-*IGH* rearrangement, whereas the most recurrent partner of cases without *MYC* HC was the *IGH* gene, similar to Burkitt lymphoma in our series, suggesting that *IGH-MYC* fusion represents a crucial driver that remains stable during tumoral expansion without evolving in subclones. Overall, the *IGH* partner does not seem to influence protein expression in our series of diffuse large B-cell lymphoma, but in cases without HC it was associated with higher MYC expression, corroborating findings of a recent study.²⁶

Despite the fact that no peculiar clinical-pathological features have been observed in diffuse large B-cell lymphoma with *MYC* HC, all the aforementioned characteristics may not only reflect the

complex biology of this disease, but also may provide a useful tool for defining diffuse large B-cell lymphoma subgroups. In particular, the recognition of *MYC HC* may be a diagnostic clue for daily practice as it is absent in Burkitt lymphoma.

Further to *genetic heteroclonality*, the FISH approach we used to investigate the *MYC* genetic status at the single-cell level allowed the determination of the presence of different breakpoints that may affect *MYC* rearrangement. In this context, it is important to underline that FISH is an indirect method for breakpoint description and is probe related; therefore, the terms '*classical* vs *non-classical FISH MYC breakpoint*' that we defined in this study should be intended only referring to *MYC* split signal probe that we used.

In our cohort, we observed that the most common breakpoints occur in, or close to, the *MYC* breakpoint cluster region (*classical FISH MYC breakpoint*) both in diffuse large B-cell lymphoma (84%) and in Burkitt lymphoma (71%), and in these cases the preferential translocation partner was the *IGH* gene. Non-classical disruptions seems to affect a significant subgroup of patients (16% of diffuse large B-cell lymphoma and 29% of Burkitt lymphoma), especially those diffuse large B-cell lymphoma with *MYC* rearrangements involving a *non-IGH* gene ($P=0.058$), confirming literature data.^{15,24–25,34} Of note, in our cohort all 14 diffuse large B-cell lymphoma with *non-classical FISH breakpoint* had a positive *MYC* expression, whereas all 7 cases with low expression had a *classical* break ($P=0.040$), thus indicating that a *non-classical* break does not influence protein expression and again confirming that loss of *MYC* expression may be related to more complex post-transcriptional/translational mechanisms. No correlation was observed between clinical–pathological characteristics and different types of breakpoints, but *non-classical FISH breakpoints* seemed more frequent in diffuse large B-cell lymphoma patients older than 60 years; larger studies are needed to assess the possible biological reasons for this observation.

Finally, we investigated the potential relationship between *MYC* rearrangement and the concomitant presence of *BCL2* and/or *BCL6* rearrangements by virtue of their common negative prognostic value for diffuse large B-cell lymphoma. In accordance with the literature, double hit and triple hit were frequently observed in our cohort (58% and 15%, respectively) and indicated a poor prognosis,³⁵ but the presence of double hit or triple hit was neither correlated with *MYC HC* nor with the type of FISH *MYC* breakpoints or partners, confirming the biological complexity of this disease.

In conclusion, *MYC* status reflects complex genomic alteration mechanisms in diffuse large B-cell lymphoma that may specifically rebound on *MYC* rearrangement itself (such as *non-classical breakpoints* and/or different partners) or globally to its distribution in tumoral cell populations. Our study

defines, for the first time, the presence of *MYC genetic heteroclonality* in a large series of diffuse large B-cell lymphoma positive for *MYC* rearrangement. *MYC HC* is a frequent event in diffuse large B-cell lymphoma and has a relevant role in modulating *MYC* protein expression. Prospective studies of larger cohorts are needed to corroborate these findings and to understand their potential clinical relevance.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)