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Running title:

Interobserver variation in CD30 IHC interpretation

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Abstract

CD30 immunohistochemistry (IHC) in malignant lymphoma is used for selection of patients in

clinical trials using brentuximab vedotin, an antibody drug-conjugate targeting the CD30

molecule. For reliable implementation in daily practice and meaningful selection of patients

for clinical trials, information on technical variation and interobserver reproducibility of

CD30 IHC staining is required. We conducted a 3-round reproducibility assessment of CD30

scoring for categorized frequency and intensity, including a technical validation, a "live

polling" pre- and post-instruction scoring round, and a web-based round including individual

scoring with additional IHC information to mimic daily diagnostic practice. Agreement in all

three scoring rounds was poor to fair (κ=0,12 to 0,35 for CD30 positive tumor cell

percentage, and κ=0,16 to 0,41 for staining intensity), even when allowing for one category

of freedom in percentage of tumor cell positivity (κ=0,30 to 0,61). The first round with CD30

staining performed in 5 independent laboratories showed objective differences in staining

intensity. In the second round, about half of the pathologists changed their opinion on CD30

frequency after a discussion on potential pitfalls, highlighting hesitancy in decision-making.

Using fictional cut-off points for percentage of tumor cell positivity, agreement was still

suboptimal (κ=0,35 to 0,60). Lack of agreement in cases with heterogeneous expression is

shown to influence patient eligibility for treatment with brentuximab vedotin both in clinical practice and within the context of clinical trials, and limits the potential predictive value of the relative frequency of CD30 positive neoplastic cells for clinical response.

Keywords: CD30, immunohistochemistry, malignant lymphoma, interobserver variation

Introduction

IHC characterization is an integral part of daily pathology practice for classifying and subtyping various malignancies, including malignant lymphomas. In recent years, targeted therapies related to specific proteins expressed on tumor cells have prompted the use of IHC for the detection or measurement of these specific molecules as predictive markers for treatment outcome. Examples include human epidermal growth factor receptor 2 (HER2) assessment as a predictive marker for decision making in breast cancer treatment with targeted therapy against HER2(1), PDL-1 staining on tumor cells and tumor-associated histiocytes in relation to PD1 inhibitory treatment in melanoma patients (2), and increasingly, selection of patients with diffuse large B-cell lymphoma (DLBCL) for treatment choices within and outside clinical trials based on IHC algorithms for cell-of-origin classification(3).

In recent years, CD30 has gained attention as a molecule of interest for targeted therapy of hematological malignancies. CD30 is a type I transmembrane protein with 6 cysteine-rich pseudo-repeat motifs in its extracellular domain, and contains a cytoplasmic

tail with several tumor necrosis factor receptor-binding sequences that are able to activate nuclear factor κ-B and extracellular signal-regulated kinase signaling pathways(4). CD30 can be specifically targeted by brentuximab vedotin(5), a CD30 antibody drug-conjugate, that has shown high efficacy in classical Hodgkin lymphoma (CHL) and anaplastic large cell lymphoma (ALCL), malignant lymphomas with often strong and homogeneous IHC expression of CD30. Other lymphoma classes, such as diffuse large B-cell lymphoma (DLBCL) and various T-cell lymphoma subtypes (especially extranodal NK/T-cell lymphomas and enteropathy-associated T-cell lymphomas (EATL)), may express CD30, albeit with heterogeneous staining intensity and percentage of positive tumor cells, and currently the efficacy of treatment with brentuximab vedotin is actively being explored in these lymphoma types (6, 7). However, there is no consensus on CD30 cutpoints or the staining pattern that should be observed, and widely variable criteria are used (8, 9).

These developments imply that the role of the pathologist to support selection of patients for treatment will further increase in this field. Building on the experience with major reproducibility issues and variable cut-off point definitions for predictive IHC markers both in solid tumors (10, 11) and lymphoma (12), similar challenges may be expected for CD30 testing. Before meaningful implementation of predictive scoring for CD30 in daily practice, this aspect should be evaluated, especially since variations will likely influence eligibility for inclusion in clinical trials and may preclude meaningful correlative studies. Therefore, we performed a 3-round formal validation study including aspects of technical reproducibility/interlaboratory variability, interobserver variability and learning effects.

Materials and methods

Tissue microarray

Tissue microarrays (TMAs) were constructed using 20 archival formalin-fixed and paraffin-embedded (FFPE) patient samples originating from one pathology laboratory of various lymphoid malignancies to cover various staining intensities and positive tumor cell frequencies for CD30 and known pitfalls, including 12 cases of DLBCL, 3 cases of EATL, and 1 case each of mediastinal grey zone lymphoma, adult T-cell lymphoma/leukemia (ATLL), peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), and ALK1-negative ALCL. Two representative 1,0 mm cores were processed using standard procedures (13). Five-micrometer sections were cut and sent to 5 pathology laboratories in The Netherlands for staining with CD30 antibodies using local protocols for routine diagnostic procedures.

Immunohistochemistry interpretation

In all assessments, the percentage of CD30-positive neoplastic cells and the intensity of staining were visually estimated. Positive tumor cells were scored in percentage classes: no expression, >0-2%, 3-10%, 11-20%, 21-30%, 31-50%, and >50%. Staining intensity was scored as no expression, heterogeneously negative-weak, uniformly weak, heterogeneously weak-strong, and uniformly strong.

For round 1 of technical validation and IHC interpretation, each core of the TMA was assessed by the local pathologist of the laboratory that performed the staining procedure (n=5).

Round 2 was performed during a national workshop on CD30 as a therapeutic target in hematological malignancies, in which 25 medical professionals, including (hemato)pathologists, hemato-oncologists, and dermatologists, participated in a live polling system for 6 cases using on screen photographs of CD30 staining in 3 cases of DLBCL, 2 cases of PTCL-NOS, and 1 case of EATL, representative of the spectrum of frequency and intensity of staining. All participants of round 1 were present in scoring round 2. This was followed by a presentation on pitfalls of CD30 IHC interpretation by one of the authors (LK), after which the exact same scoring procedure was directly repeated. The pitfalls discussed comprised CD30-positivity in reactive cells, technical issues, and the interpretation of cases with tumor cells that show the same size as reactive surrounding cells.

Round 3 consisted of 20 cases presented as representative photographs of the HE-slides, CD30 IHC and relevant diagnostic IHC markers. Participants, who had all attended the national workshop, scored the CD30 IHC stain in a series consisting of representative areas of 13 cases of DLBCL, 2 cases of PTL-NOS, 2 cases of EATL, one case of ALK1-negative ALCL, and one case of extranodal NK-/T-cell lymphoma, nasal type. All cases were revised beforehand (LK, DJ) according to the latest criteria.

Statistical analysis

Interrater agreement was quantified by means of kappa coefficients and percentage of pairs in agreement. Overall kappa coefficients for exact agreement and multiple raters were calculated in STATA 14 (14) for percentage positivity and intensity. Confidence intervals were obtained using a bootstrap procedure. Percentage agreement and two-rater kappa coefficients were calculated in R version 2.3.5 (15) for each pair of raters. The average kappa

and average percentage agreement were calculated together with their range to show the variability in agreement between different pairs of raters. Kappa coefficients and percentage agreement for percentage positivity allowing for 1 category of freedom were calculated in R for each pair of raters. The average of the kappa coefficients and their range were calculated. Finally, kappa coefficients and percentage agreement were calculated for positivity using fictional cut-off points of 2% and 10%. We categorized kappa's as poor (<0,40), fair (0,40-0,75) or excellent (>0,75).

Results

An overview of the results of the 3 scoring rounds is represented in Table 1.

Round 1

IHC for CD30 on a TMA containing 20 lymphoma cases and 2 staining control tissues was performed in 5 pathology laboratories according to routine procedures using automated staining protocols (DAKO Autostainer platform n=2, Ventana Medical Systems Benchmark platform n=3) and anti-CD30 antibody clone Ber-H2 (Ventana Ber-H2 (790-4858) n=3, DAKO Ber-H2 (IR602) n = 1, DAKO Ber-H2 (M0751) n=1)). Slides were scored according to local guidelines. Despite the use of the same antibody clone, the staining results varied dramatically (Figure 1), resulting in pairwise agreement of 46% and a κ of 0,35 for percentage of positive tumor cells and pairwise agreement of 56% and a κ of 0,47 for staining intensity. Overall, there was a minor difference in agreement between the pathologists scoring slides stained in the DAKO automated platform (percentage positivity; pairwise agreement 56%/κ=0,46 and intensity; pairwise agreement 83%/κ=0,79) and those scoring the Ventana platform stained slides (percentage positivity; pairwise agreement

42%/κ=0,31 and intensity; pairwise agreement 49%/κ=0,39). Different laboratory techniques could not systematically explain the staining and scoring results.

Round 2

In round 2, pilot scoring of CD30 was performed "real life' using a live polling system with 22 medical professionals. Based on 6 cases, agreement for all participants was poor both for quantitative results (pairwise agreement 33%/κ=0,17) and for assessment of staining intensity (pairwise agreement 53%/κ=0,36). Reproducibility was still poor when allowing for one category of freedom in CD30 tumor cell positivity class (overall pairwise agreement 63%/κ=0,33, for (hemato)pathologists pairwise agreement 62%/κ=0,30). The same slides were re-scored after a presentation on pitfalls (LK), with 17 medical doctors of the first scoring round participating. 16 of the 17 participants changed their scores for 1 to 5 cases (mean 2,9 cases changed) with one or more categories in either direction or not scoring at all (Figure 2). 14 of the 17 participants changed their interpretation of staining intensity in 1-6 cases, but with a substantially lower mean of 1,8 cases changed. Overall, the changes in interpretation between the 2 rounds before and after instruction resulted in similar suboptimal agreement scores.

Round 3

Round 3 was designed to mimic a true diagnostic situation. Information on classifying lymphoma diagnosis and scanned images of H&E stained slides and relevant IHC as support for recognition of tumor cells (CD20, CD3) were provided. All cases were scored by 15

participants, including 5 academic hematopathologists, 6 pathologists with a special interest in hematopathology and 4 residents with basic training in hematopathology. The distribution of percentage classes of CD30-positivity per tumor varied substantially among the individual participants, showing that some pathologists have a systematic tendency for higher scores of CD30-positivity than others (Figure 3). Exact pairwise agreement in CD30 positive tumor cell percentage and staining intensity were 33% (κ=0,20) and 74% (κ=0,36), respectively, and therefore no substantial improvement from round 2 was reached. In contrast to scoring round 2, allowing for one category of freedom in CD30 positive tumor cell percentage led to an improvement of reproducibility to fair agreement (pairwise agreement 71%/κ=0,50). Agreement levels were not dependent on the level of training or experience in years of practice of the participants.

Using fictional cut points of 2% and 10% positivity fair agreement was reached (2% cut-off: pairwise agreement 78%/κ=0,48; 10% cut-off: pairwise agreement 76%/κ=0,52) (Table 2). A 2% cut-point classified 5/20 cases as positive by all participants, whereas for the 10% cut-off 6/20 cases were scored with complete agreement (3 cases CD30 negative and 3 cases CD30 positive). For implementation of CD30 scoring as a tool for treatment decisions, discordant decisions around the cut off points are most relevant. Using dichotomized cut-points for (virtual) trial inclusion, the opinion on inclusion or not differed from the majority opinion in up to 46% of the pathologists (mean 2,15 participants for the 2% cut point, mean 2,25 for the 10% cut point). As an example, in case 15, 11/15 pathologists considered the tumor CD30-positive using a 2% cut point and 4/ 15 pathologists considered the tumor

CD30-positive with a 10% cut point (Figure 4), emphasizing the ambiguity in interpretation especially in tumors with relatively few CD30-positive tumor cells.

Discussion

Biomarker assays as a selection tool for treatment with targeted compounds should be technically robust and interpretation should be reproducible. In this study, we show that the results for CD30 staining on FFPE biopsy samples of malignant lymphomas are variable between 5 laboratories, in which this procedure is part of routine lymphoma work-up. Although all results were fully adequate for diagnostic classification purposes, this variation resulted in major differences in quantitative and qualitative assessment of CD30 data. These results are in line with a quality monitoring study by NordiQC, showing that only 179/252 (71%) of laboratories tested were able to produce an optimal CD30 staining according to well-described criteria, supporting the notion that staining heterogeneity is a factor that cannot be ignored in the broader pathology community (13). Technical variation for IHC and its impact on standardization of biomarker scoring has also been demonstrated for other membranous, cytoplasmic and nuclear markers in lymphoma (16). As a consequence, we still advise central processing of biopsy samples for treatment selection in the context of clinical trials, including those employing CD30-targeting drugs. However, as tissue fixation and subsequent tissue processing protocols inevitably vary considerably between laboratories, at least some variation will remain inherent to IHC-based assays. It will not be possible to define universally optimized staining procedures as a gold standard for determining CD30 positive tumor cell percentage and intensity.

Variation in CD30 positive tumor cell percentage scoring and intensity assessment cannot only be explained by technical differences between laboratories. Also when assessing CD30 positive tumor cell percentage and intensity from the same digitalized slides and under the same circumstances, agreement between pathologists is still poor to fair at best. The difficulty in decision-making was emphasized by the high percentage of participants who showed a high level of intra-observer variability when scoring the same cases twice at the "real time validation" effort. Indeed, even experienced hematopathologists in this audience were hesitant to provide their scores in the second round after a presentation on pitfalls in interpretation. These results highlight that the same slides can be interpreted in different ways, even by the same pathologist, and interpretation can be influenced by the mention of potential pitfalls. A possible weakness of this "real life validation" effort is the rather artificial setup. In daily practice, IHC stains are never assessed outside their context of clinical information, morphology and a panel of diagnostic immunohistochemical stains to provide information on architectural distribution and cellular properties of tumor cells and reactive cell populations. Therefore, in the third validation round, H&E slides and essential additional images of diagnostic IHC slides were provided to mimic a real life situation. The agreement did not improve substantially, however. Although the exact agreement in quantifying CD30-positive neoplastic cells was still suboptimal, allowing for one category of freedom in this category improved agreement substantially to fair.

Our study showed that quantifying CD30-positive tumor cells is variable amongst pathologists. This phenomenon may not pose excessive problems for the majority of patients to be included in clinical trials based on a dichotomized score as these currently include classes that are uniformly CD30 positive (ALCL; uniform CD30-positivity in 100%, and

classical Hodgkin lymphoma; uniform CD30-positivity in 100%, and DLBCL, uniform CD30-posivity in 19% in the relapse setting (based on the files of the Amsterdam Comprehensive Cancer Center Database, D. de Jong, personal communication). For heterogeneously CD30 positive lymphoma classes that are increasingly considered for targeted treatment, the situation may be.

One of the alternatives to improve reproducibility of CD30 assessment as a treatment selection tool may be automated image-analysis-based scoring. In a phase 2 study of brentuximab vedotin in relapsed/refractory DLBCL with variable CD30 expression, all responding patients had quantifiable CD30 by computer-assisted assessment of IHC (8), albeit that there was no statistical correlation between response and level of CD30 expression. Staining intensity of CD30 was not considered in this study. However, interpretation of IHC stainings, irrespective of conventional "manual" assessment or computer-assisted scoring, is complicated by the difficult differentiation of CD30 staining in neoplastic cells versus non-malignant CD30-positive cells in the tumor microenvironment, such as various populations of resting CD8-positive T-cells, activated T-cells, activated reactive B-cells and NK-cells (17). In particular if the cut-off point for CD30-positivity for study eligibility is set at a very low percentage, such as 1 or 2%, reactive CD30-positive cells may easily influence decision-making. In a study in PTCL, CD30 IHC was shown to be highly correlated with mRNA levels using an IHC scoring system incorporating both staining intensity and percentage of positive tumor cells (18). Measurement of CD30 mRNA as an alternative assay may be technically more complicated and expensive, however and also using this technique CD30-positive tumor cells cannot be distinguished from CD30-positive surrounding reactive cells. Flow cytometry (FACS) has the advantage of a quantitative assay,

allows for multiple-marker staining and is often more sensitive than IHC. However, fresh tissue suspensions, necessary for this technique, are not always available and the cell membrane of the large tumor cells of CD30 positive T- and B-cell lymphomas is often vulnerable and easily shed when preparing cell suspensions for FACS, precluding use in daily practice (19). Another way to evaluate CD30 is the detection of soluble CD30 in the peripheral blood. Soluble CD30 is the extracellular domain of CD30 that is released into the circulation after proteolytic cleavage near the cell membrane, and can be detected by enzyme-linked immunosorbent assay (ELISA)(20). Soluble CD30 levels have shown to be correlated to disease burden in ALCL(21), clinical features and prognosis in CHL(22), but the levels of soluble CD30 are not correlated to clinical response to brentuximab vedotin in relapsed/refractory DLBCL(8). These alternative methods for CD30 quantification therefore all seem to have more disadvantages than benefits, and conventional visual scoring of CD30 IHC by pathologists thus remains an important method to be optimized.

The role of staining intensity of CD30 in the clinical response to treatment with brentuximab vedotin is unclear. The only study correlating CD30 expression to this response did not consider staining intensity(8). The study showing high correlation between CD30 IHC and CD30 mRNA levels(18) did consider both staining intensity and percentage of positive tumor cell, indicating that staining intensity might be very relevant in assessing this marker. This study was however restricted to peripheral T-cell lymphomas, and there is no evidence that this type of CD30 IHC scoring or mRNA expression are correlated to clinical response to brentuximab vedotin.

In summary, reproducibility of the IHC CD30 stain is suboptimal, in part by variation in staining methods and patterns between different pathology laboratories, but also due to interobserver variation between pathologists. These differences could potentially influence patient eligibility for clinical trials with antibody-drug conjugate brentuximab vedotin, and also hamper the correlation of the amount of CD30-positive neoplastic cells to the degree of clinical response to this treatment.

Table 1. Overview of the scoring results of the 3 scoring rounds

	N	Percentage of tumor cell positivity								Quantification of positivity			
		κ				% agreement				κ		% agreement	
		Exact		1 cat. freedom		Exact		1 cat. freedom		Exact		Exact	
		Multi-rater (95% CI)	Range for two-raters	Mean for two-raters	Range for two-raters	Mean for two-raters	Range for two-raters	Mean for two-rater	Range for two-rater	Multi-rater (95% CI)	Range for two-raters	Mean for two-raters	Range for two-raters
Round 1 overall	6	**0.34 (0.21–0.47)**	**[0.13 , 0.65]**	**0.61**	**[0.25, 0.91]**	**46**	**[27, 71]**	**75**	**[50, 94]**	**0.41 (0.25–0.57)**	**[0.09, 0.79]**	**56**	**[22, 83]**
Round 2.1 overall	17	**0.17 (0.096-0.26)**	**[-0.25, 0.79]**	**0.33**	**[-0.60, 1.0]**	**33**	**[0, 83]**	**63**	**[33, 100]**	**0.36 (0.22-0.50)**	**[-0.17, 1.0]**	**53**	**[17, 100]**
Pathologist/resident pathology	13	0.22 (0.14-0.31)	**[-0.25, 0.79]**	0.30	[-0.20, 1.0]	37	[0, 83]	62	[33,100]	0.32 (0.18-0.45)	[-0.17,1.0]	49	[17,100]
Round 2.2 overall	17	**0.14 (0.089-0.197)**	**[-0.39, 1.0]**	**0.34**	**[-0.42, 1.0]**	**29**	**[0, 100]**	**59**	**[0, 100]**	**0.40 (0.28-0.53)**	**[-1.0, 1.0]**	**56**	**[0,100]**
Pathologist	13	0.12 (0.054-0.18)	[-0.39, 0.79]	0.31	[-0.33, 1.0]	26	[0, 83]	57	[0, 100]	0.39 (0.24-0.54)	[-1.0, 1.0]	55	[0, 100]
Round 3 overall	15	**0.20 (0.13-0.27)**	**[-0.16, 0.57]**	**0.50**	**[-0.03, 0.92]**	**33**	**[0, 65]**	**71**	**[25, 95]**	**0.16 (0.095-0.23)**	**[-0.15, 0.76]**	**37**	**[5, 85]**
>2% tumor cell positivity	15	0.49 (0.29, 0.69)	[-0.07, 1.0]	x	x	78	[40, 100]	x	x	x	x	x	x
>10% tumor cell positivity	15	0.51 (0.33, 0.70)	[0.11, 0.9]	x	x	76	[45, 95]	x	x	x	x	x	x

Table 2. Pairwise agreement and κ using cut-off values for percentage tumor cell positivity

	N	>2% tumor positivity		>10% tumor positivity	
		% agreement	κ	% agreement	κ
Round 3 overall	**15**	**78**	**0.49 (0.29, 0.69)**	**76**	**0.51 (0.33, 0.70)**
Pathologist	11	80	0.49 (0.27, 0.71)	78	0.54 (0.34, 0.74)
• Academic pathologist	5	85	0.57 (0.30, 0.85)	82	0.60 (0.35, 0.85)
• Non-academic pathologist	6	76	0.35 (0.14, 0.56)	73	0.45 (0.24, 0.66)
Resident	4	71	0.43 (0.15, 0.70)	75	0.45 (0.12, 0.77)

Legends to the Figures

Figure 1. CD30 immunohistochemistry performed on TMA in 5 different laboratories

An overview of the different CD30 immunohistochemistry slides shows apparent differences in staining intensity in some of the cores. A and B are stained by the Ventana Benchmark stainer with a Ventana Ber-H2 antibody, C is stained by the same machine, but with a DAKO Ber-H2 antibody, and D and E are stained by the DAKO Autostainer, using a DAKO Ber-H2 antibody.

Figure 2. Intraobserver variation in the interpretation of percentage tumor cell positivity

In round 2, a substantial part of the participants changed their opinion on percentage tumor cell positivity for the same slide only 15 minutes after scoring it for the first time, sometimes even changing several scoring categories.

Figure 3. The distribution of scoring CD30 tumor cell positivity percentage

For scoring round 3, the results per individual participants are depicted, emphasizing individual variation and the tendency of some participants to easily score higher tumor cell positivity than others.

Figure 4. Case example

This case shows the pictures that were evaluated and scored by the participants of a diffuse large B-cell lymphoma with CD30 (A), H&E (B), CD20 (C) and CD3 (D), showing considerable variation in assessment, especially using the fictional 2% and 10% cut-off for CD30 positivity.

Online Supporting Information

Supplemental data – Round 3: the photographs of CD30 immunohistochemistry and additional slides and diagnosis information per case are depicted together with a graph of scoring categories for tumor cell percentage positivity and the number of participants scoring these categories

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