Targeting CD30 in Malignant Tissues: Challenges in Detection and Clinical Applications

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Key Words

Anaplastic large-cell lymphoma · CD30 · Immunohistochemistry · Flow cytometry

Abstract

The CD30 transmembrane receptor from the tumor necrosis factor receptor family is expressed in a distinct, yet diverse set of lymphoproliferative disorders and a small subset of normal activated lymphocytes. Therefore, detection of CD30 expression when performed properly according to the standardized methods facilitates diagnosis of Hodgkin lymphoma, anaplastic large cell lymphoma, and other disorders expressing the receptor. More recently, CD30 has also become an attractive therapeutic target. The preliminary observations indicate that the methods currently used to detect CD30 expression, typically immunohistochemistry performed on formalin-fixed, paraffin-embedded tissues, may be suboptimal in regard to identifying CD30 as a therapeutic target since only a limited number of CD30 receptor molecules per cell may be sufficient to achieve therapeutic effect.

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Introduction

CD30, a 120-kDa cytokine receptor and transmembrane glycoprotein, is a member of the tumor necrosis factor (TNF) receptor superfamily. The protein is comprised of an extracellular domain, a transmembrane re-

gion and a cytoplasmic domain. The majority of antibodies against human CD30 recognize epitopes within the extracellular domain. The soluble form of CD30 has a molecular weight (85 kDa) produced through proteolytic cleavage releasing the extracellular domain [1].

Binding of CD30 with its receptor ligand (CD30L) activates TNF receptor-associated factor (TRAF)2 and TRAF5, initiating signal transduction pathways that can lead either to proliferation or apoptosis; CD30-induced apoptosis may lead to the self-regression seen in lymphomatoid papulosis lesions, whereas proliferation may result in anaplastic large-cell lymphoma (ALCL) [2].

The expression of CD30 by malignant lymphocytes affords an opportunity as a therapeutic target. Because CD30 is not found in most normal tissues outside of the immune system or on resting monocytes or lymphocytes, it provides an ideal target for immunotherapy [1–5].

While it has been demonstrated that anti-CD30 antibodies alone display therapeutic efficacy [6], newer agents markedly enhance antibody effectiveness through their conjugation with various cytotoxic agents [7–9]. Therefore, CD30 can serve both as a diagnostic marker for lymphocyte malignancies and as a target for therapy.

CD30 Expression and Detection

In pathological conditions, CD30 expression is found at various concentrations in different lymphomas of B cell or T cell derivation, and also in several reactive condi-

Table 1. Lymphoproliferations with CD30 expression [3]

<i>Primary systemic</i>
Classical Hodgkin lymphoma
ALCL, anaplastic lymphoma kinase positive*
ALCL, anaplastic lymphoma kinase negative*
Subsets of diffuse large B cell lymphoma
Anaplastic variant
Primary mediastinal large B cell lymphoma
Primary effusion lymphoma
Subsets of peripheral T cell lymphoma entities
Epstein-Barr virus-positive B cell lymphoproliferative disorders
Reactive lymphoid hyperplasias
<i>Primary cutaneous</i>
Primary cutaneous CD30-positive lymphoproliferative disorders
Primary cutaneous ALCL*
Lymphomatoid papulosis
Mycosis fungoides, large-cell (transformed) variant
Subsets of nonepidermotropic cutaneous T cell lymphomas
Reactive cutaneous lymphoproliferations

* Entities characterized by strong and homogenous CD30 expression.

tions (table 1) [3]. Strong and homogenous CD30 expression by neoplastic cells occurs primarily in three groups: classical Hodgkin lymphoma, systemic ALCL and primary cutaneous CD30-positive T cell lymphoproliferative disorders [1, 4, 5].

Detection of CD30 is generally accomplished by three methods, utilized singly or in combination: immunohistochemical (IHC) staining, flow cytometry (FC) or, in the case of soluble CD30, enzyme-linked immunosorbent assay. IHC is the most commonly used of these techniques, with FC becoming more frequently utilized, particularly when large amounts of fresh tissue are available.

Immunohistochemistry

For IHC testing, antibody panels directed towards multiple antigens typically applied to formalin-fixed, paraffin-embedded tissues aid in making diagnostic decisions by complementing the morphologic findings. A basic panel used in cases of suspected Hodgkin lymphoma will typically target the antigens depicted in table 2 [10].

While there is inevitably some variability in methods from site to site, provided that good technique is used, the staining of CD30 is generally straightforward and reproducible. However, there are many diagnostic pitfalls in lymphoma diagnosis, as described in table 3 [11].

Table 2. Basic Hodgkin lymphoma IHC panel [10, modified]

Antigen	Reactivity in normal lymphoid tissues	Findings in RS cells in CHL
PAX 5	B cells	Most cases (~90%) positive
CD20	B cells	Sometimes positive in a subset
CD3	T cells, NK cells	Negative
CD15	Granulocytes, some histiocytes, epithelial cells and carcinomas, CMV-infected cells	Most cases (~85%) positive
CD30	Activated lymphoid cells	Positive
LCA	Leukocytes	Negative
ALK	None	Negative

RS = Reed-Sternberg; CHL = classical Hodgkin lymphoma; CMV = cytomegalovirus; LCA = leukocyte common antigen.

Table 3. Pitfalls in lymphoma diagnosis [11]

<i>Specimen related</i>
Small size
Crushed or distorted tissue
Poor fixation
Delays in transfer of fresh tissue into fixative
Insufficient fixative volume
Insufficient time in fixative
Poor penetration of fixative into large tissue fragments
Excessive fixation
Formalin pigment deposition
Interference with antigen retrieval
Necrotic tissue
<i>IHC related</i>
Insufficient understanding of antibody reactivity pattern
Inappropriate positive or negative controls
Alterations in reagents or staining equipment
Insufficient scope of the antibody panel
Usage of outdated reagents

The quality of histological interpretation of lymph nodes and other lymphoid tissues depends on the quality of the sections and use of appropriate technique. Fixation is the single most influential factor in the sequence of steps between procurement of the specimen and cover-slipping of the stained slide, as errors in fixation are permanent [12]. Fixatives denature and deactivate enzymes, inhibit the growth of bacteria, and stabilize cells and tissues to protect them from the rigors of the staining process. The length and the type of fixation influence the

quality of IHC staining; underfixation often produces reduced staining in the central region of the tissue, with a stronger reaction in the peripheral areas, while overfixation typically generates the opposite effect [13].

In a well-processed and well-fixed specimen, there should be no shrinkage of the cells, the cell cytoplasm will be well preserved, and there will not be artifactual spaces between the individual cells. All of these imperfections might occur if fixation is delayed. If the fixation is incomplete, the nuclei of the cells may appear muddy or smudged, and the tissue morphology will not be well maintained [12].

Tissue fixation can also be problematic because many antigens suffer a loss of immunoreactivity secondary to the fixation process, and a lack of consistency in fixation techniques between laboratories can markedly influence the outcomes of IHC for a specific antigen. Modifications of the tertiary structure of antigens during fixation may prevent subsequent binding of the antibody, frequently necessitating an additional processing step of ‘retrieval’ of the epitope site.

The common technique of heat-induced epitope retrieval (HIER), when used with inadequate time or with inadequate temperature, can generate a variety of pitfalls that include the creation of new antigenic binding sites, or the exposure of endogenous biotin in formalin-fixed tissues. Moreover, not all antigens can be effectively retrieved by means of HIER, particularly after prolonged formalin fixation. Given the existence of many diverse methods of antigen retrieval, greater attention must be directed toward the standardization of fixation in conjunction with antigen retrieval, and these processes will likely have to be optimized for each specific antigen.

Flow Cytometry

FC is a powerful analytical tool for the analysis of heterogeneous cell populations, and one particular advantage of FC is that its applications are highly amenable to standardization. Additional advantages of FC include the identification of distinct cell populations, the ability to exclude dead cells from the analysis and the detection of weakly expressed cell surface antigens [14]. Furthermore, multi-color analyses can be performed that allow for an accurate definition of the surface antigen profile of specific cells. As the complexity of FC increases, there will be a growing need for standardization of diagnostic methods and antibody panels to support the reliability and reproducibility of these diagnostic assays between laboratories [15, 16].

Table 4. Findings from NordiQC for CD30 IHC staining [17]

Criteria for optimal staining			
– At least weak to moderate and distinct membranous staining of activated interfollicular and perifollicular B and T cells in the tonsil			
– At least weak to moderate, predominantly membranous and dot-like cytoplasmic staining of the majority of the Hodgkin cells in Hodgkin lymphoma			
– Moderate to strong, predominantly membranous staining of the majority of the neoplastic cells in the embryonal carcinoma; weak background staining due to necrosis was accepted			
– Strong cytoplasmic staining in the plasma cells in all specimens			
– No or only a weak background staining			
Current and previous findings for CD30 testing			
	2004	2009	2011
Participating labs, n	74	126	172
Adequate results	92%	78%	77%

The decision to employ FC is often dictated by the sample state, since FC can only be utilized with fresh tissue. Furthermore, the biopsy tissue sample is often small, necessitating usage of the entire sample only for the histopathologic analysis [15]. These limitations restrict the number of specimens that can be analyzed by FC and, in many centers, it is not routine practice to acquire unfixed tissue, even when lymphoma is suspected clinically [15]. Therefore, while FC is the diagnostic tool of choice for the analysis of blood, bone marrow and fluids, the analysis of solid tissues often comes down to a question of how much tissue is actually optimal and most cost efficient to obtain a diagnosis.

The Consequences of Method Variation

NordiQC, an independent scientific organization that promotes the quality of IHC, assessed CD30 staining amongst 172 laboratories in 2011, their third such study. Based on their criteria for optimal staining (table 4), it was revealed that only 77% (133/172) of the sites achieved adequate staining [17]. These data, compared with the previous findings from 2004 and 2009 when the number of participating labs was much smaller, show a decreased frequency of staining considered to be adequate (table 4).

Fig. 1. Classical Hodgkin lymphoma (image courtesy of Dr. Daniel A. Arber, Stanford University Medical Center).

Fig. 2. Primary cutaneous ALCL (image courtesy of Dr. Daniel A. Arber, Stanford University Medical Center).

The prevalent feature in the inadequate results was negative or only very weak staining of the expected cells. In 90% of the results (35 out of 39) a too weak or a false-negative staining was observed and, in the remaining 10%, both weak and false-positive staining were seen. Thus, poor staining was seen in virtually all cases and characterized by weak or equivocal membranous staining. The primary causes of insufficient staining were too low concentrations of the primary antibody and/or a too short time of HIER. The false-positive staining appeared most likely to be caused by a too high titer of the primary antibody and/or inadequate washing steps. In a few cases, contamination with another primary antibody occurred [17].

In a broader sense, this study reveals that the relative quality of staining for CD30 is low, at least in some laboratories. Specimen handling, in particular the timing of its fixation, tissue sectioning and fixative selection also impact the results with regard to both cell morphology and IHC staining

Interpretation of NordiQC Findings

It was concluded from the NordiQC study that the monoclonal antibodies BER-H2, 1G12, 15B3, CON6D/B5 and JCM182 are all useful for the detection of CD30. HIER, preferably in an alkaline buffer, is mandatory to provide an optimal staining reaction for CD30, and tonsil is recommended as a positive control [17].

Fig. 3. Epstein-Barr virus-positive lymphoproliferative disorder (image courtesy of Dr. Daniel A. Arber, Stanford University Medical Center).

Interpretation of the IHC stains is predominantly dependent on the tissue handling and staining methods used. Cells that have been crushed during the surgical procedure or tissue sampling frequently show nonspecific staining regardless of the antibody used. Similar artifacts may be observed in necrotic regions, as necrotic and apoptotic cells often show false-positive staining due to oxidative enzymes [13].

Fig. 4. Diffuse large B cell lymphoma (image courtesy of Dr. Dennis D. Weisenburger, City of Hope National Medical Center).

Fig. 5. Angioimmunoblastic T cell lymphoma (image courtesy of Dr. Marsha C. Kinney, University of Texas Health Science Center, San Antonio).

The extent of immunoreactivity in IHC should be assessed based upon: (1) the extent or proportion to which the target antigen is immunoreactive, and (2) the intensity of the immunoreactivity [18]. While there is no universal IHC scoring system at the present time, estimates of the positive cells in a given sample are sometimes expressed in percentage form. Specific examples are discussed in figures 1–8.

Figure 1 shows that CD30 expression in this Hodgkin lymphoma case is quite strong and specific. In the cutaneous ALCL shown in figure 2, all of the large cells are positively stained. There is variable CD30 staining in the Epstein-Barr virus-positive lymphoproliferative disorder shown in figure 3. The lighter cells would be classified as weakly positive and this case would be considered to have 30% positive staining. Figure 4 shows a case of diffuse large B cell lymphoma that would be classified negative for CD30, with perhaps only rare weakly positive cells, comprising less than 10%. Background staining that is not membrane staining is typically ignored and it is important that staining be continuous around the cell perimeter. While there are clearly scattered, large atypical cells that strongly express CD30 in this angioimmunoblastic T cell lymphoma (fig. 5), this reactivity would be judged as less than 10% positive. The CD30 staining in this case of follicular lymphoma (fig. 6) shows nonspecific granular staining and may detect dendritic cells that

have captured soluble CD30. The extranodal NK/T cell lymphoma in figure 7 would be scored as approximately 30% positive, since negative tumor cells are evident. The top panel in figure 8 demonstrates staining of control tissue with an optimally calibrated antibody (Ber-H2) and HIER in an alkaline buffer. Activated B and T cells show a strong and predominantly membranous staining reaction. The lower half of the image, showing the same field, reveals only weak and diffuse membranous staining, the result of an antibody concentration that is too dilute.

Conclusion: The Importance of Assay Standardization

IHC and FC assays are complex diagnostic analyses used to aid in the accurate identification and biologic characterization of cell types in neoplastic and nonneoplastic diseases. These tests can also provide important prognostic information and, therefore, may be critical for the appropriate and effective treatment of patients.

These assays require expertise in the selection of the appropriate tissue fixation and processing methods, preparation of the slides, selection of controls, pretreatment, detection systems and reagents, as well as extensive training in test selection and the interpretation of results. Quality control and quality adherence standards are crit-

Fig. 6. Follicular lymphoma, grade 3 (image courtesy of Dr. Daniel A. Arber, Stanford University Medical Center).

Fig. 7. Extranodal NK/T cell lymphoma (image courtesy of Dr. Marsha C. Kinney, University of Texas Health Science Center, San Antonio).

Table 5. Summary of key findings

Key messages

- CD30 provides a potential target for immunotherapy with restricted expression in normal human tissues
- Pitfalls in the detection of CD30 may lead to incorrect interpretations and even affect the diagnostic process:
 - Adequate tissue fixation is essential for satisfactory outcomes
 - HIER, preferably in an alkaline buffer, is mandatory for the optimal staining of CD30
 - The most frequent cause of inadequate CD30 staining is a too low concentration of the primary antibody
 - Tonsil is recommended as a positive control
- Standardization of detection methods will be critical for reliable and reproducible results

ical to ensure the accuracy of the tests and their inter-laboratory reproducibility [18, 19].

These efforts will likely be aided, to some degree, by the development of automated staining systems and by the increased use of commercial staining kits, but standardization remains a challenge due in large part to the presence of uncontrollable factors such as tissue fixation and variable processing conditions [20, 21].

The development and adoption of reference methods and materials by the manufacturers of IHC reagents and test systems could greatly improve the usefulness of these

Fig. 8. Optimized (top) and insufficient (bottom) staining of positive control (tonsil) tissue (images from NordiQC).

tests by both the pathologist and clinician. The extended use of such materials, together with consensus guidelines, could not only improve clinical outcomes, but might also reduce pressures favoring regulatory intervention and control [20].

For the reproducibility of IHC staining techniques, current reagents and protocols are less than satisfactory, and significant further improvement will be dependent

upon resolution of the problems of sample preparation and the scientific challenges associated with developing new and better fixatives. However, the logistical and economic obstacles to replacing formalin with something better, even if it becomes available, are formidable [22]. The key opportunities and challenges facing pathologists and laboratories are summarized in table 5.

‘...cancer is still diagnosed by the surgical pathologist with his/her microscope using methods that essentially are unchanged over 150 years...’ [22]

Future Therapeutic Applications

The development of monoclonal antibodies targeting the CD30 molecule constitutes a promising therapeutic option, and one that could potentially be combined with other innovative treatment modalities in appropriate patients [23]. A CD30-specific antibody conjugated with a

cytotoxic agent has recently been approved for Hodgkin lymphoma and ALCL, and it is possible that other difficult-to-treat disorders that express CD30, such as nasopharyngeal carcinoma, mycosis fungoides and other lymphoproliferative diseases, may benefit from such an approach [5, 9, 24]. While detection of CD30 expression clearly indicates that CD30-targeted therapy may be effective, it is likely that the sensitivity of IHC and other methods currently used is insufficient to identify all cases in which this kind of therapy may be potentially beneficial.

Acknowledgements

We thank the following participants in a roundtable discussion regarding CD30 biology: Daniel Arber, MD, Adam Bagg, MD, Joan Guitart, MD, Marsha Kinney, MD, Michael Tetzlaff, MD, PhD, and Sa Wang, MD.

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