Published in final edited form as:

Am J Dermatopathol. 2015 October ; 37(10): e116–e121. doi:10.1097/DAD.0000000000000323.

Multispectral imaging approach to the diagnosis of a CD20+ cutaneous T-cell lymphoproliferative disorder (CTCLPD): a case report

Katrin A. Salva, MD*, Daniel Bennett, MD*, Jack Longley, MD*, Joan Guitart, MD**, and Gary S. Wood, MD*

*University of Wisconsin, Department of Dermatology

**Northwestern University, Department of Dermatology

Abstract

Expression of the pan B-cell marker CD20 by T-cell lymphoproliferative disorders is exceedingly rare. We present a 52-year old male with a unilesional cutaneous CD20+ T-cell lymphoproliferative disorder. Multispectral imaging analysis of CD3-CD20 double-stained lesional tissue sections allowed a) the visualization of double-positive T lymphocytes in situ with sensitivity superior to that of conventional immunohistochemistry and b) the quantitative assessment of marker co-expression. Here, 23% of CD3+ signals in the patient's lesion were also CD20+, while 38% of CD20+ signals were also CD3+. In contrast, both parameters were below 1 % in the tonsil control. Overall, the percentage of double-positive cells in lesional skin was 35%, while only 0.4 % such cells were detected in the tonsil. This is the first demonstration of aberrant CD20 expression by skin-infiltrating T cells using multispectral imaging.

Keywords

CD20; cutaneous T-cell lymphoproliferative disorder; cutaneous T-cell lymphoma; multispectral imaging analysis

Introduction

Expression of the human B lymphocyte differentiation antigen CD20 is generally restricted to normal and neoplastic B-cells, and therefore provides a valuable tool in distinguishing B-cell from T-cell lymphomas. Furthermore, CD20 represents a therapeutic target in the management of B-cell malignancies such as diffuse large B-cell lymphoma and chronic lymphocytic leukemia. T-cell lymphomas (TCLs) with aberrant CD20 positivity have been occasionally described, however, the vast majority of such cases involve extracutaneous peripheral TCLs [1–3]. CD20-expressing cutaneous T-cell lymphomas (CTCLs) remain exceedingly rare with only 20 cases of CD20+ mycosis fungoides (MF) published to date

Corresponding author: Katrin A. Salva, University of Wisconsin, Department of Dermatology, 1300 University Avenue, 453 MSC, Madison, WI 53706, Phone: 608-263-6276, Fax: 608-263-5223, ksalva@dermatology.wisc.edu.

Conflicts of interest: None to report.

[4]. Here, we present the first case of a CD20+ clinically benign cutaneous T-cell lymphoproliferative disorder. To the best of our knowledge, this is also the first report of utilizing multispectral imaging to aid in the visualization and quantitation of aberrant expression of CD20 by skin-infiltrating T lymphocytes.

Case report

A 52-year old male presented to his dermatologist with a 2-month history of a 2 cm erythematous plaque on the right shoulder. Biopsy revealed epidermal spongiosis with parakeratosis and acanthosis, as well as a dense band-like lymphoid infiltrate with prominent epidermotropism and occasional Pautrier's microabscess-like collections of lymphocytes within the epidermis (Figure 1 B–D). Single-stain immunohistochemistry (IHC) showed that the infiltrate was composed primarily of T-cells (CD3 and CD4+) with fewer CD20+ cells, most of which appeared PAX5– but CD3+ (Figure 1 E–H). These findings suggested the possibility of MF with aberrant CD20 expression, prompting a referral to our Cutaneous Lymphoma clinic for further evaluation and treatment.

Physical examination of the lesion revealed a 2 cm erythematous plaque with a central hemorrhagic crust (Figure 1 A). Review of systems was unremarkable, and except for pruritus of the affected area, the patient denied other symptoms. Medical history was notable for type 2 diabetes, hypertension, hyperlipidemia and epilepsy, which necessitated the longstanding use of divalproex sodium, simvastatin, hydrochlorothiazide, metoprolol succinate, clonazepam, mesalamine, thioridazine, glyburide, pioglitazone and folic acid. Complete blood count with differential showed mild lymphocytosis (3600/μl) in the absence of other pathological findings. Re-biopsy demonstrated features similar to the previous biopsy, showing most notably a dermal band-like infiltrate composed mainly of lymphocytes, many of which had slightly large, hyperchromatic nuclei with occasional mitoses, and slightly spongiotic epidermis with focal clusters of lymphocytes. Molecular analysis of T-cell receptor gamma chain and immunoglobulin heavy chain gene rearrangements did not detect a dominant T-cell or B-cell clone. Extended immunophenotyping was inconclusive, showing the following profile: CD2+, CD3+, CD4+, CD5+, CD7+, CD8 (<10%), CD30 (<1%). A CD3-CD20 dual stain was performed to corroborate the tentative diagnosis of CD20+ CTCLPD.

Materials and Methods

CD3-CD20 dual staining of formalin-fixed paraffin-embedded (FFPE) lesional tissue and FFPE tonsil control tissue was performed using CD3 (clone 2GV6, Ventana, Tucson, AZ), CD20 (clone L26, Ventana), the Ventana BenchMark XT automated stainer, and ultraView DAB and Red detection kits (Ventana) in accordance with the manufacturer's protocols.

The double-stained slides were analyzed with the Nuance multispectral imaging system (Perkin Elmer, Waltham, MA). Multispectral image cubes of relevant areas on each slide were acquired in brightfield mode at 20nm intervals from 420 to 720nm, using a full CCD frame at 1×1 binning. A spectral library containing the characteristic wavelength emission curves of diaminobenzidine (DAB), Red and hematoxylin (HE) was created by sampling

spectra from tissue sections stained with single chromogens. Green, red and blue pseudocolors were assigned to visualize DAB, Red and HE, respectively. Using the spectral library as reference, the Nuance 3.0.2 software measured the amount of target signal per pixel, subsequently generating pseudo-colored composite images, which were further unmixed into individual components. Nonspecific background staining was eliminated by setting appropriate thresholds for each component (pixels with signals equal to or below background were ignored). The software determined the amount of CD3-CD20 co-localization within each image, and a co-localization mask was applied to visualize the double-positive pixels. Data were displayed as percentage of CD20-positivity within CD3 positivity and vice versa. To calculate the percentage of double-positive cells, the previously captured image cubes were used to train inForm 1.4 software (a pattern-recognition image analysis software, part of the Nuance system software package) to distinguish epithelium versus non-epithelial areas and to segment the cells into nuclear and cytoplasmic compartments. Subsequently, inForm determined the percentage of double-positive cells within each image cube.

Results

Figure 1 I shows a low-power image of CD3-CD20 double-stained lesional skin, demonstrating strong expression of both markers by the infiltrating cells. The brown DAB stain (CD3) and the red stain (CD20) appeared to be co-localized in at least some areas of the lesion. Figure 2 A depicts a representative area of putative double-positivity at higher magnification. The difficulty of discerning the stains by the unaided eye prompted us to subject the slide to multispectral imaging analysis. The computer-generated image composite of user-assigned false colors (green replacing DAB, red replacing pink) facilitated the interpretation of the dual stain by significantly increasing the visual contrast between the two chromogens (Figure 2 B). The system also allowed us to view CD3 and CD20 expression individually (Figures 2 C and D, respectively) and to capture the image of both markers without the counterstain (Figure 2 E). Marker co-localization was also visualized by applying a “co-localization mask” to the composite, highlighting all pixels containing both chromogens in yellow (Figure 2 F). Additional illustrative examples are shown in Figure 3 A–I. By comparison, normal tonsil control did not demonstrate significant double-positivity (Figure 3 J–L).

Quantitative data provided by the Nuance software revealed that 23 % of the CD3+ signals in the patient’s lesion were also positive for CD20, whereas CD3 was detected within 38 % of CD20+ signals. In contrast, both parameters were below 1 % in the tonsil tissue. Moreover, inForm software analysis showed that lesional skin contained 35 % double-positive cells, while only 0.4 % such cells were detected in the tonsil.

Discussion

CD20 is a 33kD transmembrane phosphoprotein believed to function as an ion channel, thereby allowing the influx of calcium necessary for B cell activation, proliferation and differentiation [5, 6]. High-level (bright) CD20 expression is a feature of most normal and neoplastic B lymphocytes, whereas T cells, basophils, plasma cells as well as non-

hematopoietic cells are usually CD20⁻. Nevertheless, low-level (dim) CD20-expression by bone marrow-derived T cells and peripheral blood T cells is well documented [7–9]. CD20-expressing CD3⁺ cells have also been observed in bone marrow specimens from patients with multiple myeloma and acute/chronic myelogenous leukemia [7]. T-cell malignancies, predominantly extracutaneous T-cell lymphomas classified as peripheral T-cell lymphoma not otherwise specified, occasionally express CD20 [1–3, 10, 11]. However, CD20⁺ CTCLPDs are extraordinarily rare [4]. The 20 cases reported in the English literature can be summarized as follows: a) all patients suffered from various stages of MF, b) 50% experienced a progressive clinical course with tumor formation and/or large cell transformation, c) monoclonal rearrangement of the TCR- γ gene was identified in 14 cases, d) the infiltrate included CD3⁺ lymphocytes which also expressed CD20 (all cases) but lacked at least one typical B cell marker like Pax5 or CD79a (14 cases) [4].

Early-stage MF commonly poses a diagnostic challenge due to the overlap of clinical and microscopic features with a wide range of other lymphoproliferative disorders as well as inflammatory dermatoses. Accurate assessment therefore depends on the correlation of histopathologic, immunologic and molecular parameters with clinical findings. In our case, molecular studies did not demonstrate clonality and the extended immunohistochemical profile was inconclusive. In the view of these findings, and given the resolution in response to two triamcinolone acetonide injections as well as the lack of new lesions at 2-year follow-up, we favor the diagnosis of a cutaneous T-cell lymphoproliferative disorder lacking true malignant potential over that of MF. The patient's medications included several agents known to induce pseudolymphomatous skin reactions (clonazepam, divalproex sodium, hydrochlorothiazide, and thioridazine hydrochloride), however, these drugs were long-term agents without apparent temporal association with the current skin lesion. Thus, the underlying cause of the lymphoproliferative reaction described here remains undetermined.

Conventional IHC represents the gold standard for the detection of antigens in frozen and paraffin-embedded skin biopsies. Nearly all previously published reports of CD3-CD20 double-positive lymphocytes in lesional skin were based on serial single IHC stains, supplemented by a CD3-CD20 dual stain in five cases [4, 12]. Dual immunofluorescence was performed in one case, and one investigator used flow cytometry to demonstrate CD3-CD20 co-expression by T-cells isolated from an involved lymph node [4, 10]. Ideally, the chromogens used for traditional microscopic observation of co-localization should provide optimal contrast between the individual colors and the mixed-color component. However, the few chromogen combinations that fulfill these requirements, such as beta-galactosidase activity in turquoise and alkaline phosphatase (AP) activity in red, are considered problematic due to technical challenges with resulting low sensitivity and/or inconsistent results [13]. The combination of horseradish peroxidase (HRP) activity in brown and AP activity in red has the advantage of ensuring two sensitive and efficient visualization procedures that produce a crisp microscopic appearance, but the unaided human eye cannot reliably discern red and brown in areas of color overlap because a distinct red-brown mixed color is lacking [13]. Nevertheless, red-brown is frequently used to demonstrate co-localization, as exemplified by all previously published images of CD3-CD20 double-positive cells in MF biopsies [4, 12]. In our case, the CD3-CD20 stain was difficult to interpret based on light microscopy alone: the infiltrate consisted predominantly of densely

packed cells which stained strongly positive for both CD3 and CD20, and we were unable to differentiate the two chromogens in areas of presumed double positivity (Figure 1). To overcome this drawback, the double-stained slide was subjected to multispectral imaging (MI). MI systems measure light transmission at multiple wavelengths across the entire visible spectrum, allowing the accurate unmixing of spatially co-localized colors irrespective of their visual properties [13, 14]. Here, MI analysis enabled us to visualize CD3-CD20 double-positivity and to quantitatively assess the percentage of double-positive T-cells in the biopsied skin section. Our previous work showed that MI is useful for the detection of low-level expression of CD30 on neoplastic cells in MF lesions that are deemed CD30 by standard light microscopic observation [15]. The current report highlights an additional advantage of MI analysis over conventional light microscopy in the assessment of protein expression by T cells.

The significance of CD20 expression by T lymphocytes is yet to be elucidated. The increase in CD20+ T-cells in the lymph nodes of monkeys upon stimulation with simian immunodeficiency virus as well as the expression of cell activation antigens CD38 and HLA-DR in a patient with CD20+ lymphoma/leukemia support the hypothesis that CD20 acts as a T-cell activator [3, 16, 17]. Interestingly, the number of circulating CD20dim normal T cells increases with age, and it has been suggested that these cells might give rise to CD20+ T-cell lymphomas [9, 10]. The efficacy of anti-CD20 antibodies such as rituximab in the therapy of various B-cell malignancies raises the possibility that recalcitrant CD20+ MF might also be amenable to this treatment [4]. CTCLPDs do not necessitate aggressive intervention, however, rare occurrences of lymphoma following the apparent resolution of a drug-induced benign lymphoproliferations have been observed [18]. The cases of CD20+ MF described so far indicate that CD20 expression might be associated with a poor clinical outcome [4]; however, our case shows that CD20 expression can also be associated with clinically benign CTCLPDs.

Acknowledgments

Sources of funding: This study was supported in part by Merit Review funding from the Department of Veterans Affairs (GS Wood) and the NIH T32AR055893 grant (KA Salva).

We thank the University of Wisconsin Translational Research Initiatives in Pathology laboratory, in part supported by the UW Department of Pathology and Laboratory Medicine and UWCCC grant P30 CA014520, for use of its facilities and services.

References

1. Buckner CL, Christiansen LR, Bourgeois D, et al. CD20 positive T-cell lymphoma/leukemia: a rare entity with potential diagnostic pitfalls. *Ann Clin Lab Sci.* 2007; 37(3):263–267. [PubMed: 17709692]
2. Kamata M, Sugaya M, Miyagaki T, et al. A case of CD20-positive peripheral T-cell lymphoma treated with rituximab and multiagent chemotherapy. *Int J Dermatol.* 2014; 53(1):24–26.
3. Rahemtullah A, Longtine JA, Harris NL, et al. CD20+ T-cell lymphoma: clinicopathologic analysis of 9 cases and a review of the literature. *Am J Surg Pathol.* 2008; 32:1593–1607. [PubMed: 18753947]
4. Harms KL, Harms PW, Anderson T, et al. Mycosis fungoides with CD20 expression: report of two cases and review of the literature. *J Cutan Pathol.* 2014; 41:494–503. [PubMed: 24467775]

5. Anolik J, Looney RJ, Bottari A, et al. Down-regulation of CD20 on B cells upon CD40 activation. *Eur J Immunol.* 2003; 33(9):239802409.
6. Maloney DG. Anti-CD20 antibody therapy for B-cell lymphomas. *N Engl J Med.* 2012; 366:2008. [PubMed: 22621628]
7. Algino KM, Thomason RW, King DE, et al. CD20 (pan-B cell antigen) expression on bone-marrow-derived T cells. *Am J Clin Pathol.* 1996; 106(1):78–81. [PubMed: 8701937]
8. Hultin LE, Hausner MA, Hultin PM, et al. CD20 (pan-B cell) antigen is expressed at a low level on a subpopulation of human T lymphocytes. *Cytometry.* 1993; 14:196–204. [PubMed: 7679964]
9. Storie I, Wilson GA, Granger V, et al. Circulating CD20dim T-lymphocytes increase with age: evidence for a memory cytotoxic phenotype. *Clin Lab Haematol.* 1995; 17(4):323–8. [PubMed: 8697727]
10. Sen F, Kang S, Cangiarella J, Kamino H, Hymes K. CD20 positive mycosis fungoides: a case report. *J Cutan Pathol.* 2008; 35:398–403. [PubMed: 18261116]
11. Warzynski MJ, Graham DM, Axtell RA, et al. Low level CD20 expression on T cell malignancies. *Cytometry.* 1994; 18:88–92. [PubMed: 7523045]
12. Hagen JW, Schaefer JT, Magro CM. CD20+ mycosis fungoides: a report of three cases and review of the literature. *Am J Dermatopathol.* 2013; 35:833. [PubMed: 24247573]
13. Van der Loos, CM. Royal Microscopy Handbook no 45. BIOS Scientific Publishers; Oxford, UK: 1999. Immunoenzyme multiple staining techniques.
14. Huang W, Hennrick K, Drew S. A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Hum Pathol.* 2013; 44(1):29–38. [PubMed: 22944297]
15. Salva KA, Sundram U, Krathen M, et al. Analysis of protein expression in situ using multispectral imaging is superior to conventional immunohistochemistry (IHC): a new paradigm for patient selection for targeted therapy. *JID.* 2013; 133(S):169.
16. Murayama Y, Mukai R, Sata T, et al. Transient expression of CD20 antigen (pan B cell marker) in activated lymph node T cells. *Microbiol Immunol.* 1996; 40:467. [PubMed: 8839435]
17. Takami A, Saito M, Nakao S, et al. CD20-positive T-cell chronic lymphocytic leukemia. *Br J Haematol.* 1998; 102:1327. [PubMed: 9753064]
18. Bocquet H, Bagot M, Roujeau JC. Drug-induced pseudolymphoma and drug hypersensitivity syndrome (drug rash with eosinophilia and systemic symptoms: DRESS). *Semin Cutan Med Surg.* 1996; 15(4):250–257. [PubMed: 9069593]

Figure 1.

A: Clinical appearance of the lesion at presentation in our institution. **B:** Low-power view of the original biopsy shows dense dermal lymphoid infiltrate extending into the epidermis (H&E, original magnification x100). High-power images (x400) of the infiltrate demonstrate pleomorphic lymphocytes with hyperchromatic, slightly angular nuclei (**C**) and Pautrier's microabscess-like clusters of lymphocytes in the epidermis (**D**). Selected immunohistochemical stains (DAB, all images are magnified x100): CD4 (**E**), CD3 (**F**), PAX-5 (**G**) and CD20 (**H**). **I:** Double-stain (ultraView DAB and Red, hematoxylin counterstain, x100) showing CD3 (brown) and CD20 (pink) expression.

Figure 2.

The multispectral imaging system uses the unique spectral curves of each chromogen to convert the standard light microscopic (LM) image into a pseudo-colored composite image, which can be unmixed into individual components. **A:** Standard LM image of a representative area of the CD3-CD20 double-stained section. **B:** Computer-generated composite depicts CD3, shown here in the user-assigned pseudocolor green, and CD20, shown in red; these colors were chosen to facilitate the visual differentiation of the markers. The counterstain is shown in blue, it appears darker in areas of spatial overlap with the green pseudocolor. **C:** CD3 only. **D:** CD20 only. **E:** CD3 and CD20, the counterstain is eliminated. **F:** Yellow co-localization mask indicates areas of CD3-CD20 double-positivity.

Figure 3.

A, D Standard LM images of representative areas of the CD3-CD20 double-stained section (ultraView DAB and Red, hematoxylin counterstain, original magnification 400x). **B, E:** Corresponding pseudo-colored composite images, showing CD3 (green), CD20 (red) and counterstain (blue). **C, F:** The yellow color highlights areas of CD3-CD20 co-localization. **G:** Close-up of infiltrating epidermal cells (original magnification 900x), corresponding pseudo-colored composite image (**H**) and co-localization mask (**I**) demonstrate CD3-CD20 double-positivity.