CHAPTER 8

SERRATION PATTERN ANALYSIS FOR DIFFERENTIATING EPIDERMOLYSIS BULLOSA ACQUISITA FROM OTHER PEMPHIGOID DISEASES

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Published in Journal of the American Acadamy of Dermatology, 2018;*78*(*4*):*754*-9.

ABSTRACT

BACKGROUND

Direct immunofluorescence (DIF) microscopy of a skin biopsy specimen is the reference standard for the diagnosis of pemphigoid diseases (PDs). Serration pattern analysis enables the differentiation of epidermolysis bullosa acquisita (EBA) from other PDs using DIF microscopy alone. However, practice gaps need to be addressed in order to implement this technique in the routine diagnostic procedure.

OBJECTIVE

We sought to determine and optimize the technical requirements for serration pattern analysis of DIF microscopy and determine interrater conformity of serration pattern analysis.

METHODS

We compared serration pattern analysis of routine DIF microscopy from laboratories in Groningen, The Netherlands and Lübeck, Germany with 4 blinded observers. Skin biopsy specimens from 20 patients with EBA and other PDs were exchanged and analyzed. Various factors were evaluated, including section thickness, transport medium, and biopsy specimen processing.

RESULTS

The interrater conformity of our 4 observers was 95.7%. Recognition of serration patterns was comparable in samples transported in saline and in Michel's medium and with section thicknesses of 4-, 6-, and 8-μm.

LIMITATIONS

Limitations include our small sample size and the availability of 20 samples that were compared retrospectively.

CONCLUSION

DIF serration pattern analysis is not restricted by variation in laboratory procedures, transport medium, or experience of observers. This learnable technique can be implemented as a routine diagnosticmethod as an extension of DIF microscopy for subtyping PD. (J Am Acad Dermatol. https://doi.org/10.1016/j.jaad.2017.11.029.)

CAPSULE SUMMARY

- Epidermolysis bullosa acquisita can be differentiated from other pemphigoid diseases by direct immunofluorescenc serration pattern analysis of a skin biopsy specimen.
- In the current study, high interrater conformity was shown independent of laboratory procedures, transport medium, or experience.
- Serration pattern analysis can be implemented as a routine diagnostic technique as an extension of direct immunofluorescence microscopy.

In patients with pemphigoid diseases (PDs), direct immunofluorescence (DIF) microscopy of a skin biopsy specimen is considered the reference standard for diagnosis.[1] PDs are autoimmune bullous diseases of the skin and mucosa that are characterized by circulating and tissue-bound autoantibodies against structural proteins in the epidermal basement membrane zone (EBMZ). PD includes the subtype epidermolysis bullosa acquisita (EBA), which is characterized by autoantibodies against type VII collagen.[1,2] Differentiating EBA from other PDs is relevant for both the treatment and prognosis of patients. The diagnosis of PD can be challenging because of the heterogeneous clinical presentation and histopathology.[1,3] For the diagnosis of EBA, in-vivo bound linear immunodepositions can be detected along the EBMZ in a skin biopsy specimen using DIF microscopy and serration pattern analysis[4] or detected by direct immunoelectron microscopy.[5-7] Circulating autoantibodies against type VII collagen can be detected in serum by indirect immunofluorescence microscopy on saltsplit skin (SSS), immunoblot, or enzyme-linked immunosorbent assay.[3,6,8,9] However, labeling of autoantibodies to the dermal side (floor) of the artifical split in indirect immunofluorescence microscopy SSS is also seen in patients with anti-laminin-332 mucous membrane pemphigoid and anti-p200/laminin γ1 pemphigoid.[10-12] In addition, the technique of DIF microscopy using salt-split patient skin may destruct the tissue sample and cannot differentiate EBA from other PDs.[6] Furthermore, the serologic diagnosis of EBA is hampered by the low sensitivity of these immunoserologic tests, with no detectable circulating autoantibodies and possible misdiagnosis in approximately 50% of patients with EBA.[7,13,14] Although the concept of DIF serration pattern analysis was described in 2004 and incorporated into standard textbooks, its use in routine DIF microscopy was limited, perhaps by perceived hurdles of high technical requirements.[4,15,16] Consequently, EBA is likely underdiagnosed, especially in seronegative patients.

Using DIF microscopy, 2 serrated patterns of linear immunodeposits (immunoglobulin G [IgG], IgA, and complement component 3) along the EBMZ can be identified in PD: the u-serrated pattern in EBA and in the more rare bullous systemic lupus erythematosus and the n-serrated pattern in all other PDs, including bullous pemphigoid and mucous membrane pemphigoid. The u-serrated pattern is recognizable by finger-like structures or spikes (Fig. 1a and b) and is pathognomonic for EBA/ bullous systemic lupus erythematosus, where immunodeposits are located below the lamina densa associated with type VII collagen as part of anchoring fibrils. The linear n-serrated pattern (Fig. 1c and d) can be seen in all other PDs, and the linear deposits are located on or above the lamina densa.[4,17] By comparing various technical factors of routine DIF microscopy procedures of 2 laboratories - including the use of saline versus Michel's transport medium, cryosection thickness, and conformity among observers with different levels of expertise - this study sought to optimize the DIF microscopy serration pattern analysis for implementation in the routine diagnostic procedure.

MATERIALS AND METHODS

Twenty perilesional skin biopsy specimens obtained from patients with suspected PD and positive findings of linear IgG immunodeposition along the EBMZ by DIF microscopy were analyzed. Archived biopsy specimens of patients diagnosed with EBA (n=10) based on a linear u-serrated IgG immunodeposition pattern (100%) were included. These patients with EBA were either seropositive with circulating autoantibodies against type VII collagen confirmed by

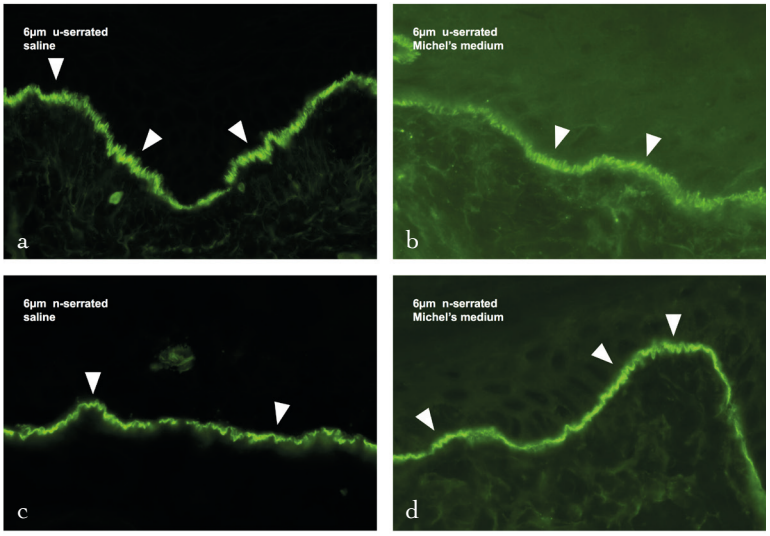

Fig. 1 Direct immunofluorescence microscopy serration pattern analysis of u-serrated and n-serrated patterns in patients with pemphigoid diseases. Linear u-serrated immunoglobulin G deposition patterns along the epidermal basement membrane zone in patients with epidermolysis bullosa acquisita with biopsy specimens transported in saline (**A**) and Michel's medium (**B**). Note the distinct spikes or "growing grass" in the u-serrated patterns (*arrowheads*). Linear n-serrated immunoglobulin deposition patterns along the epidermal basement membrane zone in patients with other pemphigoid diseases with biopsy specimens transported in saline (**C**) and Michel's medium (**D**). Note a continuous curved linear deposition with closed arches at the top (*arrowheads*). All 6-µm cryosections were cut and processed in the Lübeck laboratory. (Original magnification, ×400.)

enzyme-linked immunosorbent assay or immunoblot (60%) or seronegative (40%). Biopsy specimens of patients who were diagnosed with other PDs (n=10) were included based on a linear (n-serrated) IgG immunodeposition pattern along the EBMZ. For comparison, biopsy specimens transported in saline (Groningen) and Michel's transport medium (Lübeck) were exchanged between the 2 laboratories and processed according to routine diagnostic procedures for DIF microscopy of each laboratory (see below). This type of study with a retrospective reanalysis of leftover patient materials for diagnostic purposes is exempted from the review by the medical ethical committees in The Netherlands and Germany.

DIRECT IMMUNOFLUORESCENCE MICROSCOPY

Four observers with different grades of experience independently examined routine 6-µm sections for serration pattern analysis, including a dermatopathologist (GD) and dermatologist (MJ) from Groningen, and a dermatologist (ES) and resident in dermatology (AV) from Lübeck. Sections were presented at random (JM and IA) to the observers and the observed immunodeposition patterns were classified as linear n-serrated, linear u-serrated, or undetermined. The intensity of fluorescence of the IgG immunodeposition was graded as weak (+), moderate (++), or bright (+++). To evaluate the influence of section thickness on serration pattern analysis, 4-, 6-, or 8-µm thick sections from all biopsy specimens were cut in the Groningen laboratory and randomly examined by 2 observers (GD and MJ).

DIF LABORATORY PROCEDURES

In the Groningen routine DIF microscopy laboratory procedure, perilesional biopsy specimens were placed in saline in polypropylene vials, stored overnight in saline (for 12-16 hours) and processed the next day. Biopsy specimens were then placed in aluminium vials, snap frozen in liquid nitrogen, and stored at -80°C until processing. Temperature of cryostate (Leica CM3050S; Leica, Wetzlar, Germany) and microtome knife was regulated between as low as -20°C and -35°C to prevent compression and deformation of the sections. For biopsy fixation, first a small wetted piece of filter paper was applied to the fixation table, a circle of Tissue-Tek O.C.T. Compound (Sakura, Alphen aan den Rijn, The Netherlands) was added to the filter paper, frozen, and filled with another drip of O.C.T. Compound for fixation of the biopsy specimen (Supplemental Figs. 1 and 2, available online at http://www.jaad.org). The biopsy specimen was only partially embedded, with the base in O.C.T. Compound and the top of the specimen protruding for cutting (Supplemental Figs. 2 and 3, available at http://www.jaad.org). Cryosections were cut with tungsten carbide conventional microtome knives (Type C, 160 mm; Spikker Specials, Zevenaar, The Netherlands). The microtome knives were standard wedge-shaped with a bevel angle of 20° and were resharpenable (Supplemental Fig. 3). Cryosections were mounted on Polysine (Thermo Fisher Scientific, Waltham, MA) glass slides sideways with no pressure applied (Supplemental Video, available at http://www.jaad.org). The sections were stained for 30 minutes in a moist chamber at room temperature using fluorescein isothiocyanate-labeled Fc-specific goat $F(ab')_2$ antibody against human IgG (Protos 311; Protos Immunoresearch, Burlingame, CA). SlowFade Antifade Reagent (Thermo Fisher Scientific) was added to inhibit photobleaching. Sections were examined with a Leica DM2000 microscope and Leica HCX PL Fluotar 40×/0.75 dry objective and 10× ocular (total magnification, ×400; Leica).

The Lübeck routine DIF microscopy laboratory procedure only showed minor differences compared to the Groningen procedure. Biopsy specimens were either kept in Michel's medium or snap frozen at -20°C until processing. Biopsy specimens were fixated by completely embedding the material in Tissue-Tek O.C.T. Compound, and cryosections were cut using disposable microtome blades S35 (Feather, Osaka, Japan). Sections were stained using a monoclonal mouse antihuman IgG-fluorescein isothiocyanate antibody (Bio-Rad, Hercules, CA) and examined with a Olympus BX40 microscope and 40×/0.75 dry objective (UPlan FI; Olympus, Tokio, Japan). Photographs, videos, and extended methods are available at http://www.jaad.org.

For additional automated serration pattern recognition, all slides were photographed with total magnification ×400 with standardized settings and processed with Leica Application Suite imaging software. The automated technique by Shi *et al.*[18] was developed based a trainable inhibitionaugmented COSFIRE filter with detection of the distinctive spikes (ridge endings) found in u-serrated patterns, similar to recognition of fingerprints.

DIF LABORATORY PROCEDURES

Data were analyzed using SPSS software (version 23; IBM, Armonk, NY). Qualitative data were expressed as frequencies and percentages. Statistical analysis of recognition rates of serration pattern analysis was performed using the McNemar test. The Cochran Q test was used for comparison of recognition rates between 4-, 6-, and 8-μm sections. Conformity among 4 observers (inter- and intraobserver variability) was assessed of routine serration pattern analysis of 6-μm sections and of fluorescence intensity of IgG staining and was calculated with 2-mixed

intraclass correlation coefficient (ICC [3,4]). All tests were 2-tailed, and P<0.05 was considered statistically significant.

RESULTS

The primary outcome for diagnostic purposes in daily practice was the recognition rate of serration patterns of routinely cut 6-µm sections (n=20) from both laboratories (Fig. 1). The mean recognition rate by 4 observers was 97.5% and the intraclass correlation coefficient ICC [3,4] for the conformity among 4 observers (inter- and intraobserver variability) was 0.957 (95% confidence interval 0.916- 0.981). Moreover, no erroneous serration patterns were classified in the complete experimental setup, and differences only occurred in the classification of "undetermined" immunodepositions (Supplemental Table I, available at http://www.jaad.org). Sections were classified as n-serrated in 48.8%, u-serrated in 43.8%, and undetermined in 7.5%. Of interest was 1 specific case classified by all observers as undetermined with a homogeneous immunodeposition of IgG instead of a linear immunodeposition (Supplemental Table I, case GR5). In retrospect, this biopsy specimen proved to have been obtained from a patient with porphyria cutanea tarda who was initially misdiagnosed with mechanobullous EBA (seronegative) and is the subject of current research.

Biopsy spceimens transported in Michels's transport medium or frozen at -20°C showed a higher background fluorescence in the dermis compared to biopsy specimens that were transported in saline (Fig. 1). However, no significant difference was seen in the recognition of serration patterns comparing 6-µm sections of samples transported in Michel's transport medium from the Lübeck laboratory to samples transported in saline from the Groningen laboratory (p=0.49). For interpretation of these data, fluorescence intensity of IgG staining of the random selected biopsies was classified as 11 (11.3%), 21 (48.7%), and 31 (40.0%). The classification of fluorescence intensity of IgG staining among the four observers showed a high conformity (ICC [3,4] 0.836). The overall serration pattern recognition rate of sequentially cut 4-, 6-, and 8-µm sections (n=60) was 95.8%. No statistical significant differences in recognition rates were seen between the groups with 4-, 6-, and 8-µm sections (p=0.368, Supplemental Fig. 4, available at http://www.jaad.org) or between the 2 observers (p=0.063).

DISCUSSION

The implementation of DIF microscopy serration pattern analysis in the routine diagnostic procedure is hampered by presumed technical hurdles, such as the inability to use Michel's medium for transport, the need of cutting thin (<4-µm) sections, the requirement of highmagnification ocular objectives (×600), and the need of a trained pathologist. We found in 2 independent laboratories that not a single technical factor limits the performance in routine diagnostics. Using DIF microscopy serration pattern analysis and recognizing the pathognomonic u-serrated pattern may increase the number of diagnosed cases of EBA substantially and is the most informative and cost-effective diagnostic test for PD.[13,14] This learnable technique can be trained online with DIF photos of serrated patterns at our website (nversusu.umcg.nl). In

more detail, a higher background fluorescence was observed in biopsy specimens transported in Michel's medium compared to saline (Fig. 1).[19] A decreased signal to noise ratio was mainly observed in 8-µm cryosections, which may lead to false-negative fin ings in cases with low staining intensities. The additionally performed automated pattern recognition based on the DIF microscopy photographs of all slides showed a recognition rate of 90% of u-serrated patterns, confirming that the spikes or ridge-endings of the u-serrated patterns are distinctive recognizable features.[4,18] The recognition rate decreased with thicker 8-µm sections of biopsy specimens transported in Michel's medium because of the loss of details of spikes with the higher background fluorescence.

EXCHANGE OF EXPERIENCES IN TWO LABORATORIES

Although the various technical factors did not critically impair the DIF microscopy serration pattern recognition, the exchange of experiences of observers and laboratory technicians taught us the following on optimizing the DIF procedure. The nondisposable wedge-shaped microtome blade with a bevel angle is optimal; however, cutting 4-µm cryosections was also possible with disposable microtome blades. Only partially embedding the biopsy specimen tissue in O.C.T. Compound improved the cutting characteristics, resulting in more stretched sections that are better microphotographed within the depth of field (Supplemental Figs. 2 and 3; and Supplemental Video). In contrast, details can be lost in overexposed areas and micrometering was needed to focus in 8-µm cryosections. The use of saline transport medium and cutting thinsections decreases background staining, which is necessary to distinguish serrated patterns in specimens with low amounts of IgG deposition. The limiting factor for saline transport is the required transport time to be <48 hours from physician to (reference) laboratory.[19] Alternatively, Michel's medium allows transport times longer than 48 hours. We investigated in both laboratories preincubation of biopsy specimens in saline before storage in
Michel's medium with various time intervals, and also washing of biopsy specimens with saline and various buffers after storage in Michel's medium. However, these alternative detours did not decrease background fluorescence.

In conclusion, serration pattern analysis in routine DIF microscopy can differentiate EBA from other PDs and is essential for a definitive diagnosis of EBA in patients who have no detectable circulating autoantibodies in serum. The high recognition rate and conformity among various observers in this study show that serration pattern analysis is not critically restricted by variations in technical laboratory procedures, transport media, and the experience of the observers. This easily learned technique can be implemented as a routine diagnostic method as an extension of DIF microscopy for the diagnosis and subtyping of PDs.

ACKNOWLEDGEMENTS

We thank Chenyu Shi, Jiapan Guo, George Azzopardi, and Nicolai Petkov for sharing their contributions in the development of automated serration pattern analysis.

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