Methods

In this prospective investigative study, 44 consecutive EB patients (27 males and 17 females) were recruited at a single tertiary care center in New Delhi between July 2013 and December 2015. After obtaining informed consent, a detailed clinical history as well as a cutaneous and systemic examination were made. Baseline clinical photographs were taken from all patients. A skin biopsy was taken from normal-appearing skin after rubbing, mostly on the thigh, as recommended [5], and transferred in Michel medium for IHC and IFM antigen mapping. The Institutional Ethics Committee approved the study (IEC No.: IESC/T-147/04.04.2014) which was conducted in accordance with the declaration of Helsinki.

Chemicals

All the primary antibodies used for the study were mouse monoclonal antibodies against cytokeratin-14 (KRT-14), laminin-332 (LAM-332), and type IV (COL-IV) and VII (COL-VII) collagens. Unfortunately, antibodies against other affected proteins like cytokeratin-5, integrin-α_6 and -β_4, plectin and type XVII collagen could not be procured due to administrative reasons. Fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit antimouse IgG was used as a secondary antibody. Supplementary table 1 depicts the clone, supplier name, and dilutions of the respective antibodies.

IFM Antigen Mapping

The biopsy material was washed in phosphate-buffered saline (PBS) and mounted in optimal cutting temperature media. The lesional and control tissues were cut into 7-μm frozen sections on a cryostat set at –25°C and placed on poly-L-lysine-coated glass slides. Slides were fan dried for 10 min and placed in a moist chamber at room temperature. Sections were washed in PBS (3 serial washes × 5 min) and incubated with the primary antibodies for 1 h. All sections were again washed in PBS buffer (3 serial washes × 5 min) and incubated with a secondary antimouse FITC conjugate for 30 min. After a final wash in PBS, the slides were mounted in

buffered glycerine mixture and viewed using a Nikon Eclipse E200 fluorescent microscope (Nikon, Tokyo, Japan) and photographed**.**

Immunohistochemistry

IHC was done using the avidin-biotin indirect method. Initially, IHC was tried on formalin-fixed paraffin-embedded tissue sections. However, due to inconsistent and unsatisfactory results (Fig. 2), probably due to loss of antigens [4], we modified the technique and used fresh frozen skin tissue sections as prepared for IFM. Sections were washed in PBS (3 serial washes × 5 min), and endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 5 min followed by incubation with the primary antibodies for 1 h. All sections were again washed in PBS buffer (3 serial washes × 5 min) followed by sequential incubations with biotinylated-linked secondary antibody and peroxidase-labeled streptavidin according to the manufacturer's protocol (Dakocytomation LSAB+ System-HRP kit). Final incubation was done with 3,3'-diaminobenzidine peroxidase substrate to give a brown stain followed by counterstaining with hematoxylin.

Positive and negative controls were run simultaneously in each batch. Table 1 depicts the interpretation of staining patterns for IHC and IFM.

DNA-Based Mutational Analysis

Mutational screening was performed using whole-exome capture sequencing in all patients as described previously [6]. At the time of analysis only 30 cases had a confirmed genetic diagnosis that included 4 EBS (1 *KRT5* and 3 *KRT14*), 8 JEB (1 *LAMA3*, 4 *LAMB3*, 2 *ITGB4*, and 1 *COL17A1*), and 18 DEB (*COL7A1*) cases.

Statistical Analysis

The quantitative characteristics of the patients were summarized as means ± SD and qualitative characteristics as proportions (%). Chance-adjusted agreement (kappa coefficient) was calculated between IFM, IHC, and genetic diagnosis along with 95% confidence intervals. Sensitivity, specificity, and predictive values of correct diagnosis with IHC and IFM

were calculated taking genetic diagnosis as the gold standard. All analyses were implemented on Stata version 14.1. A p value <0.05 was considered significant.