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Role of Immunofluorescence antigen mapping in the diagnosis of Epidermolysis Bullosa - A rare genetic blistering disorder

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Abstract
Background: Epidermolysis Bullosa is a rare genetic mechanobullous skin and mucosal fragility with blistering. It is classified into: Epidermolysis (Simplex), lucidolytic (Junctional) and dermolytic (Dystrophic). The disease is extremely debilitating necessitating early detection and treatment. Immunofluorescence mapping is gold standard of diagnosis.
Objective: To determine types and inheritance patterns of cutaneous and extracutaneous Epidermolysis Bullosa by immunofluorescence mapping.
Methods: 5 µm frozen section from lesions /artificial bulla are collected over multispot PTEF coated slides. Incubation done with primary antihuman mouse antibodies (IgG) and with FITC conjugated anti-IgG mouse secondary antibodies. Antibodies used against Collagen IV, Collagen VII, cytokeratin 4, cytokeratin 14, Laminin 332, alpha6 and beta4 integrins. Healthy skin is positive control.
Results: In Simplex type blister and split is above lamina densa with reduced CK14 at base (Dominant). Anti-integrins, laminin 332 and collagen IV all are present at base. In Junctional type, collagen IV present at base, cytokeratin or integrin at roof, and cleavage is at lamina lucida. Laminin 332 is reduced (dominant) or absent (recessive). In Dystrophic type blister is below lamina densa. Collagen IV or VII stains roof of blister. Collagen VII is absent in recessive type and reduced in dominant type.
Limitations: Rare disease
Conclusion: Agreement of clinical diagnosis and mapping is moderate indicating mapping is an essential adjunct.

Keywords: TPO expression, CK-19 expression

Introduction

Epidermolysis Bullosa (EB) is a genetically determined mechanobullous disorder of the skin encompassing a group of conditions that share skin fragility as a common feature. The overall prevalence and incidence of inherited EB in the United States has been recently reported to be 11.1 per one million population and 19.6 per one million live births, respectively, based on data derived from the National (USA) EB Registry from 1986-2002. The prevalence of DDEB and RDEB is 1.49 and 1.35 per one million; corresponding incidence is 2.12 and 3.05 per one million live births, respectively [1].

Pearson classified EB into three major types: Epidermolysis (EBS), lucidolytic (JEB) and dermolytic (DEB), [2] depending on the ultra-structural levels at which the split develops. Different types of bullous disorders manifest in multiple ways with a substantial amount of overlapping symptoms. Some of them can be extremely debilitating or may develop catastrophic sequelae, necessitating their early detection and treatment. [3] Clinical examination provides the dermatologist a gross idea about the nature of the disease. A thorough history about affected family member is helpful for screening and prevention of the disease in the subsequent generations. Immunofluorescence mapping (IFM) remains the gold standard for definitive diagnosis of EB.

Light microscopy (LM) helps only in differential diagnosis from other types of bullous lesions. It has no role in the diagnosis of EB disorders. Advanced technologies like IFM or transmission electron microscopy (TEM) must be implicated to diagnose these disorders.

Though TEM remains the gold standard, it is expensive, time-consuming, technically demanding and not widely available.

There are distinct advantages of IFM that it is less expensive and biopsy samples can be stored at room temperature or in Michel's transport medium [4] for up to 28 days. Therefore, use of IFM not only allows sub classification of EB but also helps detecting the affected protein and aid further prognosis of the disease.

Primary Objectives

To study the IFM findings in the patients of EB

Secondary Objectives

1. To correlate the clinical and antigen mapping findings of EB
2. To classify the different types and subtypes of EB
3. To study inheritance patterns of EB
4. To study the cutaneous features and extracutaneous involvements of the cases of EB

Material and Methods

Study Design: This is a cross sectional, analytic and single institution based study. In view of rarity of the condition we kept our sample size to 44 subjects.

Time span: The data was collected with the help of a proforma containing information regarding patient particulars, history, complaints and other parameters throughout four and a half years. The histopathological and immunofluorescence studies were done along with this. Six months taken to analyses the data.

Definition of problem: Epidemiological, clinical and pathological characteristics of EB in different age groups.

Study variables: Demographics, clinical features, routine blood investigations, histopathological and IFM findings.

Period of Study: 5 years

Case, Control required or not: Case is the patients with EB screened and enrolled as per inclusion criteria and both positive and negative controls taken.

Inclusion Criteria: All new cases of EB will be enrolled after signing informed consent.

Exclusion criteria

1. Patients who are unable or unwilling to give informed consent.
2. Moribund patient
3. Cases of immunobullous disease, history of trauma, burn injury, allergic dermatitis, insect bite or bulla formation due to infections like herpes or impetigo.

Parameters and the procedure

Written informed consent was taken from the patients or their parents/guardian. History and clinical findings were recorded in a case record form. Appropriate routine laboratory investigations were done. The data were recorded with in the proforma. A punch biopsy samples of approximately of 3 mm size was taken from lesions for Histopathological examination in 10% buffered formaldehyde solution. For light microscopy examination sections of 4- 5µm thickness from paraffin embedded material and were stained routinely with haematoxylin and eosin (H&E). In suspected cases of EB, a punch biopsy was taken from an artificial bulla which was formed by rubbing with an eraser. The samples were collected in Michel's solution and stored at 2 to 8 degree C till further processed for IFM. Frozen section skin tissue was sectioned in 5µm thickness and mounted over special PTEF coated glass slides. These slides were further incubated with IgG anti-mouse primary antibodies followed by FITC conjugated anti-mouse secondary antibodies and were observed under florescence microscope. Antibodies against Collagen IV, Collagen VII, cytokeratin 4, cytokeratin 14, laminin 332, alpha6 and beta4 integrins were used to delineate the blister level. An exception is the antibody against a6 integrin where rat is the source, and the secondary antibody is anti-rat IgG. The data received from histopathology and IFM are corroborated with the clinical data of each patient. Each skin biopsy examined by two trained pathologists by LM and immunofluorescence microscopy.

Immunofluorescence mapping (IFM)

IFM principle is based on the detection of structural proteins of keratinocytes or the dermo-epidermal junction (DEJ) by using specific polyclonal or monoclonal antibodies. By this method, level of split can be identified by localizing a given antigen and normal, reduced or absent expression of the structural proteins can be also detected. According to the proteins targeted for mutation analysis, we use IgG antibodies against cytokeratin 5, cytokeratin 14, a6 integrin, b4 integrin, laminin 332, and type VII collagen. We also use anti-type IV collagen (present in the lamina densa of the dermoepidermal BMZ) antibodies for better visualization of the level of split formation, especially in patients with DEB. Specific fluorescent signal os secondary antibody at 450 to 490 nm allows the visualization of specific antibody binding under the IF microscope. Original primary and secondary antibodies are diluted and divided into aliquots and kept in the refrigerator. Working dilutions are prepared fresh [Table I]. We have used a special type of Multispot microscope slide with PTFE and specialized coating for IFM imported from Hendley Essex Company. Negative controls were incubated with PBS, instead of the primary antibody. Rest of the procedure remains same as the cases. Positive controls were skin biopsies other than EB cases.

Table I: Antibodies used in the study with dilution factors

1st Antibodies	Dilution	Host	Company	Catalog Number
Cytokeratin 5	1:200	Mouse	Millipore	MAB 3224
Cytokeratin 14	1:200	Mouse	Millipore	MAB 3232
a6 integrin	1:10	Rat	Millipore	MAB 1378
b4 integrin	1:10	Mouse	Millipore	MAB 1964
Collagen VII	1:1000	Mouse	Millipore	MAB 1345
Laminin 5	1:300	Mouse	Millipore	MAB 1949
Collagen IV	1:500	Mouse	Millipore	MAB 3326
2nd Antibodies				
IgG	1:200	Goat anti-mouse	Millipore	AP 124F

Statistical analysis

Results of the study were compiled, tabulated and compared with the known data and inferences were drawn. Data was analyzed by appropriate statistical tests using statistical software i.e. Graph-pad Prism 5 and SPSS. Test of significance of difference was calculated using Chi-square test and ANOVA test. P-value of <0.05 was considered to be statistically significant.

Results

Distribution of cases of Epidermolysis bullosa

In our study we got total 44 cases among which 20 cases were EBS, 10 cases were JEB, 12 cases were DEB and rest two cases (1 EBS and another 1 DEB) remained unclassifiable on the genotypic nature based on clinical ground and IFM.

EBS was found to be the most common form of EB disorder constituting 45% (20 out of 44 cases) followed by DEB 27% (12 cases) and JEB 23% (10 cases). 2 cases (5%) remain unclassified following IFM. [Figure 1].

Among the 21 EBS cases 20 cases were autosomal dominant (AD) type. Rest 1 case was EBS unclassified. 9 JEB cases were autosomal recessive (AR) type 1 JEB was AD type. Among 13 DEB cases 10 were AR and 2 were AD and another 1 remain unclassified.

In our study we considered the IFM findings as the gold standard final diagnosis as we do not have facilities of TEM or molecular diagnosis at the genomic level. Only thing we have done that the corroboration first degree relatives by pedigree analysis.in autosomal dominant disorders one of the parent is carrying the defective gene allele and probality of affection of their son or daughter is 1:2. In cases of autosomal recessive disorders both the parents carry the defective gene allele and probality of affection of their son or daughter is 1:4. [Figure 2].

Histological Types

In all 21 patients with EBS using the antibody against integrins or laminin 332 (present in the lamina lucida) and collagen 4 (present in the lamina densa), the base of the blister was stained. In 12 of 20 cases, staining for cytokeratins 5 and 14 labeled mainly the floor of the blister. Remnants of basal keratinocytes present at roof were also positive. In EBS cases the split occurs above the lamina densa of basement membrane. All the AD cases (20) show reduced expression of CK14 antibody at the base of blister. The EBS case which remain unclassified (1) showed normal intensity of CK14 at the base. [Table II & III].

Table II: Antibody expression in Epidermolysis Bullosa cases

Lesions Antibody	EBS (AD) (n=20)	EBS Unclassified (n= 1)	JEB (AD) (n=1)	JEB (AR) (n=9)	DEB (AD) (n=2)	DEB (AR) (n=10)	DEB Unclassified (n=1)
CK 5	N	N	N	N	N	N	N
CK 14	R	N	N	N	N	N	N
LAMININ 5	N	N	R	A	N	N	N
COLLAGEN VII	N	N	N	N	R	A	N
COLLAGEN IV	N	N	N	N	N	N	N
INTEGRIN α6	N	N	N	N	N	N	N
INTEGRIN β4	N	N	N	N	N	N	N
Lesions Antibody	**EBS (AD) (n=20)**	**EBS Unclassified (n= 1)**	**JEB (AD) (n=1)**	**JEB (AR) (n=9)**	**DEB (AD) (n=2)**	**DEB (AR) (n=10)**	**DEB Unclassified (n=1)**
CK 5	N	N	N	N	N	N	N
CK 14	R	N	N	N	N	N	N
LAMININ 5	N	N	R	A	N	N	N
COLLAGEN VII	N	N	N	N	R	A	N
COLLAGEN IV	N	N	N	N	N	N	N
INTEGRIN α6	N	N	N	N	N	N	N
INTEGRIN β4	N	N	N	N	N	N	N

N= Normal expression, R= Reduced expression, A= Absent

Table III: Immunofluorescence Antigen Mapping findings

Lesion	Antibody						
	CK 5	**CK 14**	**Collagen IV**	**Collagen VII**	**Laminin 332**	**Integrin α6**	**Integrin β4**
EBS, AD (n=20)	Floor 3+	Floor 1+ R	Floor 3+	Floor 3+	Floor 3+	Floor 3+	Floor 3+
EBS, Unclassified (n=1)	Floor 3+	Floor 3+	Floor 3+	Floor 3+	Floor 3+	Floor 3+	Floor 3+
JEB, AD (n=1)	Roof 3+	Roof 3+	Floor 3+	Floor 3+	Floor 1+ R	Roof 3+	Roof 3+
JEB, AR (n=9)	Roof 3+	Roof 3+	Floor 3+	Floor 3+	A	Roof 3+	Roof 3+
DEB, AD (n=2)	Roof 3+	Roof 3+	Roof 3+	Roof 1+ R	Roof 3+	Roof 3+	Roof 3+
DEB, AR (n=10)	Roof 3+	Roof 3+	Roof 3+	A	Roof 3+	Roof 3+	Roof 3+
DEB, Unclassified (n=1)	Roof 3+	Roof 3+	Roof 3+	Roof 3+	Roof 3+	Roof 3+	Roof 3+

In all 10 JEB cases, immunoreactivity for collagen 4 stained the floor of the blister, while cytokeratin or integrin labeling occurred exclusively at the roof, indicating the cleavage level at the lamina lucida. Primary defects in laminin 332 evidenced by reduced staining with laminin 332 was seen in 1 case (AD) and absent staining in 9 cases (AR). Collagen XVII defect may be assumed based on the level of blistering. In cases with collagen XVII defect, staining for laminin 332 and integrins labeled the floor and roof of the blister with normal intensity, respectively. [Table II & III].

In all 13 DEB cases, the blistering occurred below the lamina densa. The antibodies against collagen 4 or 7 stained the roof of the blister. Expression of type VII collagen was absent (AR) and reduced (AD) in 10 and 2 cases,

respectively. 1 DEB case show split at the level of sub lamina densa with normal intensity of collagen VII expression at the roof of blister by IFM. [Table II & III].

Age and Sex distribution

Overall gender distribution: Out of 44 cases, 24 cases (54.5%) were male and 20 cases (45.5%) are female. Male: female ratio is 1.2:1 which reflected an overall male preponderance. [Figure 3].

For EBS cases male: female ratio is 1.6:1. For JEB cases male: female ratio is 1.5:1. For DEB cases male: female ratio is 1: 1.6. So overall male preponderance is seen in EBS and JEB cases whereas female preponderance was seen in DEB cases.

Overall age distribution: The youngest were two 2 months old boy and girl and 42 years old male was the oldest. The mean age of the study population was 104 ± 114 months (mean ± SD). [Table 3]. Mean age for EBS was 166 ± 128.9 months (mean ± SD). Mean age for JEB was 60 ± 77.6 months (mean ± SD). Mean age for DEB was 41.6 ± 39.8 months (mean ± SD). So it is observed that more severe variants are characterized by earlier clinical manifestations. [Table IV].

History of Consanguinity: Among EB patients 30 of them (68%) were born of consanguineous marriage and the association is statistically significant (*p*-value is 0.015893 which is $p < 0.05$). Amongst them 18 are EBS, 7 are JEB, 5 are DEB cases. [Figure 4]. Rest 14 patients do not have any history of parental consanguinity. [Figure 4].

History of involvement of other family members: A positive family history was associated with all the sub-types of EB. [Table IV].

Duration of disease: The mean duration of illness in cases of EBS is 93.2± 85.9 months, JEB is 57 ± 77.6 months, DEB is 36.4 ± 37.8 months and considering the age of onset recorded as described by the patients or parents. [Table IV].

Nature of bulla: Most cases of JEB and DEB presented with tense bulla whereas 5 cases of EBS (25%) presented with flaccid bulla. Rest of the EBS associated with tense bulla [Table 4]. Association of type of bulla with disease category is statistically insignificant (*p*-value is 0.38735).

History of drug intake: No association found between history of drug intake and appearance of bulla.

Table IV: Distribution of different variables in Epidermolysis Bullosa

Lesions Variables	EBS	JEB	DEB	P value
Mean age (months)	**166 ± 128.9**	**60 ± 77**	**41.6 ± 39.8**	
Family History				
Present	21	10	13	
Absent	0	0	0	
History of Consanguinity				
Present	18	7	5	*p*-value is.015893. The result is significant at $p < .05$.
Absent	3	3	8	
Nature of Bulla				
Flaccid	5	1	1	The *p*-value is.38735. The result is *not* significant at $p < .05$.
Tense	16	9	12	
History of Drug Intake				
Present	**0**	**0**	**0**	
Absent	**20**	**10**	**12**	

Site distribution

All the patients show cutaneous involvement with maximum patients showing involvement of lower limb. 95 % EBS patients were showing the involvement of upper and lower limb. Almost 100% JEB patient showed involvement of face, scalp and trunk without any involvement of hand and feet. 85% DEB patients show involvement of face, scalp and trunk along with generalized involvement including limbs in 60% cases. Unclassified cases show predominantly scalp and trunk involvement. [Table V]. Mucosal involvement was present in 57% EBS patients, 90% JEB patients and 77% of DEB patients but disease association with the mucosal involvement is statistically not significant (*p*-value is 0.143402). Nail and hair involvement on the other hand has statistically significant disease association (*p*-value is < 0.00001). [Table VI].

Table V: Distribution of lesions in Epidermolysis Bullosa

Site of lesions	No. of EB patients	EBS, AD	EBS, Unclassified	JEB, AD	JEB, AR	DEB, AD	DEB, AR	DEB, Unclassified
Cutaneous	44	20	1	1	9	2	10	11
Hands	25	19	0	0	0	0	6	0
Feet	28	19	0	1	0	2	6	0
Face & neck	I9	0	0	0	9	0	10	0
Scalp	32	9	1	1	9	1	10	1
Trunk	22	0	1	0	9	1	10	1
Mucosal (oral, nasal, conjunctiva, genital)	31	11	1	0	9	0	10	0
Nails & Hair	23	3	0	0	9	0	10	1
Dental enamel defect	11	0	1	0	8	0	2	0
Dyspigmentation	20	8	0	0	4	1	6	1

Table VI: Pattern of involvement in Epidermolysis Bullosa

	EBS, AD (n=20)	EBS, Unclassified (n=1)	JEB, AD (n=1)	JEB, AR (n=9)	DEB, AD (n=2)	DEB, AR (n=10)	DEB, Unclassified (n=1)	p value
Cutaneous involvement								
Present	20	1	1	9	2	10	1	
Absent	0	0	0	0	0	0	0	
Mucosal involvement								
Present	11	1	0	9	0	10	0	The *p*-value is 0.143402. The result is *not* significant at *p* <.05.
Absent	9	0	1	0	2	0	1	
Involvement of nails and hair								
Present	3	0	0	9	0	10	1	The *p*-value is < 0.00001. The result is significant at *p* <.05.
Absent	17	1	1	0	2	0	0	
Dental enamel defect								
Present	0	1	0	8	0	2	0	The *p*-value is < 0.00001. The result is significant at *p* <.05.
Absent	20	0	1	1	2	8	1	
Depigmentation								
Present	8	0	0	4	1	6	1	The *p*-value is 0.380005. The result is *not* significant at *p* <.05.
Absent	12	1	1	5	1	4	0	

Dental enamel defect is mostly seen with the patients of JEB (80%) and it also shows statistically significant disease association (*p*-value is < 0.00001). Depigmentation may be associated with repeated bulla formation and scarring commonly seen in DEB patients (58%). It does not have any significant disease association (*p*-value is 0.380005) with type of EB. Exuberant granulation tissue formation is the characteristic features of JEB patients and identified in 70% cases. Mitten deformities" or pseudosyndactyly is present in 65% of AR cases of DEB.

Pattern of mucosal involvement: DEB and JEB cases are more prone to develop mucosal involvement. Most frequent site is oral mucosa in DEB patients (91%) without any statistically significant association (*p*-value is 0.314048). Nasal mucosal involement seen in JEB patients (90%) and the association is statistically significant (*p*-value is.000012). EBS patient shows oral mucosal involvement only in 55% cases. [Table 6]. Conjunctiva involvement and genital involvement also showed significant disease association with *p*-value is 0.00006 and 0.000017 respectively. [Table VII].

Clinico histopathological concordance with reference to immunofluorescence mapping: In our study, the average concordance between clinic Histo pathological diagnosis and IFM is 54.5% and discordance is 45.5%. Out of 21 cases of clinically diagnosed EBS, 13 are confirmed by IFM and thus concordance in EBS is 62%. Concordance is even lower in DEB (61.5%) and JEB (50%).

The concordance of individual diagnosis (Clinico histopathological) is statistically significant. [Table VIII]. The agreement between Clinical diagnosis [6] and IFM was found to be moderate (Cohen's kappa κ=0.4291) [Table IX]. IFM is therefore essential adjunct for proper categorical diagnosis of the level of split as well as inheritance pattern.

Table VII: Pattern of mucosal involvement in Epidermolysis Bullosa

	EBS, AD (n=20)	EBS, Unclassified (n=1)	JEB, AD (n=1)	JEB, AR (n=9)	DEB, AD (n=2)	DEB, AR (n=10)	DEB, Unclassified (n=1)	p value
Oral mucosal involvement								
Present	11	0	0	7	0	10	0	The *p*-value is.314048. The result is *not* significant at *p* <.05.
Absent	9	1	1	2	2	0	1	
Nasal mucosal involvement								
Present	1	0	0	9	0	4	0	The *p*-value is.000012. The result is significant at *p* <.05.
Absent	19	1	1	0	2	6	1	
Conjunctival involvement								
Present	0	1	0	7	0	9	0	The *p*-value is.00006. The result is significant at *p* <.05.
Absent	20	0	1	2	2	1	1	
Genital involvement								
Present	0	1	0	8	0	9	0	The *p*-value is.000017. The result is significant at *p* <.05.
Absent	20	0	1	1	2	1	1	

Table VIII: Clinico histopathological concordance with Immunofluorescence Mapping

			Clinical Diagnosis		
			Correct	**Incorrect**	**Total**
Final Diagnosis (IFM)	EBS	Count	13	8	22
		% within clinical diagnosis	62	38	100
	JEB	Count	5	5	10
		% within clinical diagnosis	50	50	100
	DEB	Count	8	5	13
		% within clinical diagnosis	61.5	38.5	100

Table IX: Agreement between Clinical diagnosis and Immunofluorescence Mapping

IFM Diagnosis				
Clinical Diagnosis		EBS (n=21)	JEB (n=10)	DEB (n=13)
	EBS	13	3	4
	JEB	0	5	1
	DEB	8	2	8

Discussion

In the present study majority of the cases were in the age range of 6-14 years (48% or 24 cases). The mean age of the study population was 104 ± 114 months (mean ± SD). Male: female ratio is 1.2:1 which reflected an overall male preponderance. Barzegar *et al*. done IFM on 95 referred patients, comprising 49 females and 46 males, aged 5 days–45 years (average 9.53 years) from October 2011 to October 2013 in the immunofluorescence laboratory of the Shohadae Tajrish Educational Hospital. [6]

In our study EBS was found to be the most common type of EB constituting 45%, followed by followed by DEB 27% and JEB 23%. 2 cases (5%) remain unclassified following IFM. 95% of EBS cases were autosomal dominant type with 5% EBS remaining unclassified genotypic ally. 90% of JEB cases were autosomal recessive type and rest 10% were autosomal dominant. 77% of JEB cases were autosomal recessive type, 15% were autosomal dominant and rest were unclassified. A study by Barzegar *et al*. done on 95 patients where 13 (14%) were diagnosed as EBS, 14 (15%) as JEB, and 62 (65%) as DEB. Diagnosis was not made in five (5%) patients as their specimens were suboptimal (i.e., they contained no blister). One patient with Kindler syndrome was diagnosed based on the characteristic clinical findings, including ectropion, photosensitivity, progressive poikiloderma, and marked cutaneous atrophy especially at acral sites [7]. Findings of our study is different possibly due to different genetic makeup of our study population and no such previous data of our population is available in the literature.

This study of our experience regarding the use IFM to diagnose and classify EB is the first series reported from the eastern India. It supports and confirms the feasibility of IFM as an accurate diagnostic method for EB disease with both phenotypic and genotypic classification in almost 95% cases. We applied the IFM method with the above mentioned panel of antibodies for the diagnosis of EB and its subtypes.

Each major EB type is diagnosed by determination of the ultra-structural level within which blisters develop following minor traction to the skin. For making a correct diagnosis, skin biopsies were obtained from freshly induced blisters [8]. Blister was induced by rubbing the intact skin before taking the biopsy. In milder EB types (EBS and dominant DEB), prolonged rubbing beyond the tolerance of the patient is needed. In a study Barzegar *et al.* recommended inducing blister on specimens after taking the biopsies by saltsplit method. This new method, seemed to be a more efficacious and produced less discomfort for patients [6].

Subtypes of EB were previously defined on the basis of transmission electron microscopic findings, and clinical phenotype. In our study, IFM was done in 44 cases of EB to subcategorize them.

In patients with EBS split formation occurs in epidermis by cytolysis of basal keratinocytes (intraepidermal). CK 5 and collagen IV showed normal expression in all the cases. CK 14 showed reduced expression in cases which were finally diagnosed as EBS, AD type. All antibodies (CK 5, CK 14, laminin 332 /laminin 5, collagen VII, collagen IValpha6 and beta 4 integrins) were present at the base of the blister. In AR type EBS CK 14 staining is completely absent. In patient with EBS with muscular dystrophy plectin is absent and in rare variant of EBS Ogna plectin is markedly reduced [9]. In our study we have not included anti-plectin antibody. In EB subtype with pyloric atresia (either EBS or JBS) plectin or alpha6 beta4 integrins are reduced or absent. In our study no clinical history of pyloric atresia was present in the patients and all patients show normal alpha6 beta4 integrins expression.

All DEB subtypes are caused by the mutation in type VII collagen which is the main anchoring fibril of epidermis and dermis. The level of cleavage occurs in sublamina densa. Collagen 17 and laminin 332 stains seen at the roof of the blister. In our study we have not used the antibody against collagen XVII but we have used the antibody against laminin 332. Immunostaining for type IV collagen occurs on the roof of the blister indicating dermolytic DEB. In our study Collagen VII showed reduced expression in 2 cases which were finally diagnosed as DEB, AD type. Collagen VII expression was completely absent in 10 cases and diagnosed as severe generalized DEB, AR type [10].

The main target protein of JEB are type XVII collagen (BP180 or $BPAG_2$) and laminin 332 (laminin 5) [11]. Collagen XVII is seen at the roof of the blister whereas other antibodies are seen at the floor. In severe Herlitz form of JEB, mutation occurs in laminin 332 gene which is absent in IFM. In case of non-Herlitz form laminin 332 expression is reduced. Type IV collagen localizes to the blister floor in all forms of JEB. In our study expression of laminin 332 was absent in 9 cases, all of which were later diagnosed to be JEB, AR type. Expression of laminin 332 was reduced in 1 case which was later diagnosed to be JEB, AD type. All the cases of EB were compared with a positive control which showed normal expression of CK 5, CK 14, laminin 332, collagen VII and collagen IV. Consistent with our results, in a series of 77 patients suspected of having EB Berk *et al*. done IFM and showed that all five JEB cases due to collagen XVII defect, cleavage occurred between normally expressed laminin 332 chains at base and b4 integrin at top. However, in JEB cases due to laminin 332 mutations, when antibody against laminin 332 was used, fluorescence was absent or was seen on the roof and or floor of the blister with reduced intensity [12].

Genetically JEB is linked to a mutation in exon 39 of the LAMA3 gene, which is specific to the LAMA3A isoform and leads to a short truncation within the N-terminal laminin alpha3 chain [13]. Different underlying mutation known in JEB are in LAMA3, LAMAB3, or LAMAC2 genes responsible for producing different patterns in IFM. Thus IFM may help to predict which of these three genes harbor the pathogenetic laminin 332 mutation in a case of JEB. Future studies in this area may be done based on mutational

analysis results. In summary, there appear to be two types of JEB with overlap with laryngo-onycho-cutaneous (LOC), one with ocular lesions and exon 39 mutations and one with distal mutations on the other allele.

A similar study revealed that in patients of EBS, the split was formed within the epidermis and there was reduced expression of CK 14 [8]. In another study, in the cases of JEB, the expression of laminin 5 was reduced or absent and in a blister they appeared on the roof or floor depending on the subtype of JEB [11].

A rare subtype of JEB called JEB-LOC syndrome with musculoskeletal involvement and growth failure was reported in a 5 years old girl, who was misdiagnosed as polyarticular juvenile idiopathic arthritis (JIA). Biopsy from mechanically induced bulla showed split at lamina lucida level and DIF was negative. IFM confirmed the sub epidermal split and displayed linear staining of BMZ with monoclonal antibodies against type 4, type 7 collagen, laminin 332 on the dermal side while BP 230 on the epidermal side [14].

Corroboration with Clinical Findings

Clinical diagnosis is done by using clinical diagnostic matrix developed by Prof V.K. Yenamandra and Celia Moss [5]. In our patients with EBS onset was at or shortly after birth. All the patients gave positive family history. 68% of patients had history of Consanguinity. Blister are mostly distributed over hand, feet, elbow, knee, legs and scalp. There is no involvement of face and neck region. Mucosal involvement except oral mucosal involvement (55%) and dental enamel defect are completely absent. 15% cases show nail dystrophy and hair fall. In localized EBS blisters may not develop until late childhood or even early adulthood [15]. The most common subtype of localized EBS is previously known as Weber-Cockayne disease. The usual distribution of blisters are in palms and soles. EBS Dowling– Meara (DM) is frequently associated and marked morbidity and in minority of patients may result in death during early infancy.

In our JEB patients blisters are present in face, neck, scalp and trunk without any involvement of hand and feet. All the cases show nail dystrophy and hair fall. All AR cases show mucosal and genital blisters. Only one clinical finding that is characteristic of all subtypes of JEB the presence of enamel hypoplasia, manifested as localized or more extensive thimble-like pitting of some or all of the tooth surfaces. This is cannot be diagnostic tool until after the primary teeth have erupted. 80% of our patients with permanent tooth show dental enamel defect. Incidence of JEB Herlitz 20% of all type of JEB. Blisters are present at birth and involve all skin surfaces. Pathognonic finding of JEB is exuberant granulation tissue, which is arises within first several month to 1-2 year of life. Granulation tissue formation is identified with 70% of our cases with higher age. In rest it is absent possibly due to lower age. This may involve not only skin but also upper air ways leads to airway obstruction. Overproduction of granulation tissue in conjunctiva causes blindness [13]. Ocular granulation tissue, however, seems to be particularly germane to mutations in LAMA3A.

All the patients of RDEB show generalised blister at birth involving skin surfaces, mucosal surfaces and genitalia. 20% patients show dental enamel hypoplasia. 65% patients show pseudosyndactyly ("mitten deformities"). The disease is progressive and often time mutilating. There is scarring of skin, corneal blisters or scarring, profound growth retardation, multifactorial anemia, failure to thrive. Recurrent esophageal blistering and erosions, leading to progressive dysphagia and esophageal stricture formation, is common among these patients. 100% of our patients presented with oral mucosal involvement.

Unfortunately, because of lack of genetic analysis and transmission electron microscopy, we are not able to compare our results with an independent standard to measure the diagnostic accuracy of our work. IFM results for different patients within one family are matching with one another. In addition, our IFM results are consistent with the clinical findings and mode of inheritance on pedigree analysis. For instance, all mitten hand deformity cases are diagnosed as DEB, or those with subungual granulation tissue has JEB blisters on IFM. Dominant inheritance is seen mostly in EBS cases and small number of JEB and DEB cases diagnosed by IFM.

Conclusion

Our study highlights the importance of IFM as it is a practical and rapid method to diagnose and classify EB. Large number of patients with AR EB in our part of the country are due to a high rate of consanguineous marriages. There for, establishment of IFM is an essential alternative to genetic mapping and transmission electron microscopy. Genetic counselling can be done after the identification of genetic mutation based on IFM diagnosis also. Chance of recurrence in AR diseases is 25% whereas chance of recurrence in AD diseases is 50%. Prenatal diagnosis is done by fetoscopy, amniocentesis and chorionic villous sampling.

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