

Immunohistochemical Study of a Correlation between Pemphigus Vulgaris Activity Score and Stem Cell Control

Heba A. S. Bazid¹, Rehab Monir Samaka², Samar Saeed Shaban Abo Hegazy^{*1}, Iman Seleit¹

Departments of ¹Dermatology, Andrology & STDs and ²Pathology, Faculty of Medicine, Menoufia University, Egypt

***Corresponding Author:** Samar Saeed Shaban Abo Hegazy, **Mobile:** (+20)01011264404, **Email:** samar.saeed1142013@gmail.com

ABSTRACT

Background: Pemphigus vulgaris (PV) is a potentially life-threatening autoimmune blistering disease. PV autoantibodies disrupt desmosomal adhesion and cause acantholysis. Previous researches have shown that stem cells are indirectly involved as a result of desmoglein deficiency. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) as a follicular stem cell marker was evaluated in aim to correlate its intensity of expression with disease severity.

Objective: To correlate LGR5 intensity of expression with disease severity.

Patient and methods: This prospective cross sectional study was carried out on 20 PV patients. Patients were subjected to complete history taking, general, dermatological examination and assessment of disease severity by the Pemphigus Vulgaris Activity Score (PVAS), histopathological and immunohistochemical expression of LGR5 were done.

Results: All studied cases showed positive cytoplasmic basal LGR5 expression in patchy manner. 75% of cases had mild intensity of expression, 15% had moderate intensity and their Histo (H) score ranged from 50-130 with Mean \pm SD 110 \pm 18.92. There were no significant correlation between PVAS scores "skin, mucosa and total involvement" and H score of LGR5 expression.

Conclusion: The current study could shed a new light on the disease and its correlation with stem cells, LGR5 as a stem cell marker could be related to the healing process in PV. However, it didn't correlate PVAS scores either in skin, mucosa or total involvement.

Keywords: LGR5, Pemphigus vulgaris, Stem cells, Skin, Autoimmune.

INTRODUCTION

Pemphigus Vulgaris is an autoimmune disease that affects humans and domestic animals ⁽¹⁾. PV is characterized by loss of cell-cell adhesion between basal and adjacent suprabasal keratinocytes in mucous membranes and skin ⁽²⁾. It manifests as loss of keratinocyte cohesion triggered by autoantibody binding to desmoglein3 (Dsg3), an intercellular adhesion molecule of mucous membranes, epidermis, and epidermal stem cells. Here we describe a so far unknown signaling cascade activated by PV antibodies, it extends from a transient enhanced turnover of cell surface exposed, nonkeratin-anchored Dsg3 and associated Plakoglobin (PG), through to depletion of nuclear PG, and as one of the consequences, abrogation of PG-mediated c-Myc suppression, which in turn influences the delicate balance of stem cell recruitment; proliferation and terminal differentiation in keratinocytes ⁽³⁾.

To detect stem cells that maintain tissue homeostasis, we used marker of stem cell populations within the hair follicle. LGR5 positive cells comprise an actively proliferating and multipotent stem cell population in mouse hair follicles that maintains all cell lineages of the hair follicle, appendages and epidermis during wound healing ⁽⁴⁾. LGR5 has been detected in actively cycling cells in the bulge and

secondary germ of telogen hair follicles, as well as the lower outer root sheath of anagen mouse hair follicles ⁽⁵⁾. The aim of this study was to correlate LGR5 expression with disease severity.

PATIENTS AND METHODS

Study Design: A total of 20 PV patients were examined in this prospective cross sectional study. Patients were selected from the Dermatology Outpatient Clinic, Menoufia University Hospital during the period between February 2019 and April 2020. The diagnosis of PV was made on the basis of the patient's history and the typical clinical features.

Ethical considerations: Prior to collection of samples, written informed consent was obtained from all studied subjects and the Local Ethics Committee of Research involving human subjects in Faculty of Medicine approved this study; record number: 2/2019 DERM, in agreement with the Declaration of Helsinki (World Medical Assembly).

All the patients underwent: Complete history taking: Personal history that included: age and Sex. Present history that included: duration and course of the disease. Drug history: To exclude drug induced pemphigus. General examination: Full general examination was done.

All PV cases that had newly erupted lesions were



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included in this study. Any case with any of the followings was excluded from the study: Dermatological, systemic autoimmune or inflammatory diseases.

Dermatological examination: Determination of affected sites, Nikolsky sign, Asbo-Hansen sign and presence of mucosal lesions. Assessment of the disease severity of PV patients was done by PVAS. PVAS was based on: The extent of lesions: Total number of lesions and different anatomical regions in skin and all mucous membrane, The presence of Nikolsky's sign and the lesion type according to the healing process. PVAS score components are: Skin involvement: which is presented by the sum of the number of skin lesions (N), distribution in different body areas (D) and presence of: for bulla or erosions is 1, for crusts is 0.5 and for pigmentation is 0. Mucous membrane involvement (m): which is presented by the sum of the number of mucous membrane lesions (N), distribution (D), is weighed by type of the lesion (T). The mucous lesions type coefficient for bulla or erosions is 1 and for ulceration is 0.5 ⁽⁶⁾.

Tissue preparation: Five mm punch biopsies were taken under local anesthesia from lesional newly erupted blisters within either 1-2 days (recent) or 3-4 days (old) of all PV patients ⁽⁷⁾. Biopsies were fixed in 10% of neutral formalin and subjected to Pathology Department, Faculty of Medicine Menoufia University for routine processing and preparation of haematoxylin and eosin (H & E) stained slides to confirm diagnosis and for histological assessment. Biopsies subjected to routine tissue processing ending with paraffin embedded blocks formation, several paraffin sections, each 4 μ thick, were cut from each block, one of them was stained by H & E to evaluate pathological changes and the other sections were cut on positive charged slides for immunohistochemical staining procedure.

Immunohistochemical staining: LGR5 mouse polyclonal Anti- GPR49/LGR5 antibody (cat. no. YPA2037; Chongqing Biospes Co/China) was used. It was received in a single vial containing 1*TBBS (pH 7.4), 1%BSA, 40% Glycerol. Preservative: 0.05% Sodium Azide. IHC was performed using the RNAscope Formalin-Fixed and Paraffin-Embedded (FFPE) assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) ⁽⁸⁾. The primary antibody was applied to the slides, by dropping in tissue sections and then incubated horizontally in a humidity chamber over night at room temperature. 1-2 drops of biotinylated goat polyvalent secondary antibody were applied, incubated for 10 minutes at room temperature. Detection of bound antibody was accomplished using a modified labelled streptavidin peroxidase reagent for 10 min, followed by rinsing in phosphate buffered saline (P.B.S). one drop of

3,3Diaminobenzidine tetrahydrochloride (DAB) and one drop of 0.6% hydrogen peroxide were used as chromogen substrate, and applied for 1-10 min. Slides were counterstained with Mayer haematoxylin for 5–10 min. Negative control slides were prepared by omitting the primary antibodies from the staining procedure. The positive control was human liver tissue.

Immunohistochemical interpretation: Expression of LGR5 in epidermis was assessed in all studied cases, with regard to positivity, intensity of staining, pattern of expression (nucleocytoplasmic or cytoplasmic) and localization of LGR5 ⁽⁹⁾. The H score was used to evaluate positive cases, with both the intensity and the percentage of positivity measured using the following formula:

$H \text{ score} = 3x (\% \text{ of strong intensity}) + 2x (\% \text{ of moderate intensity}) + (\% \text{ of slight intensity})$.

The score ranges between 0 to 300 ⁽¹⁰⁾. The scores were classified into two categories: low (< 150) and high (\geq 150).

Ethical approval:

The study was approved by the Ethics Board of Menoufia University and an informed written consent was taken from each participant in the study.

Statistical Methodology:

Results were statistically analyzed by IBM personal computer and Statistical Package for the Social Sciences (SPSS) version 25 program U.S.A. Two types of statistics were done. The description of data was in the form of mean (\bar{x}) SD for quantitatively data, and frequency and proportion for qualitative data. Standard student-t test (t) and Chi-Squared (χ^2) were used. P value less than 0.05 was considered a significant level.

RESULTS

Clinical examination of PV cases:

Patients were 13 females (65%) and 7 males (35%), with a female to male ratio of 1.85:1. The age of the selected cases ranged between 30 and 59 years with mean of 42.5 ± 9.17 . Regarding the disease severity, PVAS ranged from 4.5-13 with mean \pm SD value of 8.07 ± 2.63 with skin domain ranged from 3-11 with mean \pm SD value of 6.25 ± 2.31 and mucosal domain ranged from 1-3 with mean \pm SD value of 1.82 ± 0.54 .

Histopathological examination:

The epidermis of all cases showed suprabasal acantholysis, spongiosis and blistering. The blister cavity contained acantholytic cells, eosinophils, neutrophils, and some lymphocytes (**Figures 1 and 2**).

Some of cases showed regenerative changes in the form of multiplication of basal cell layer.

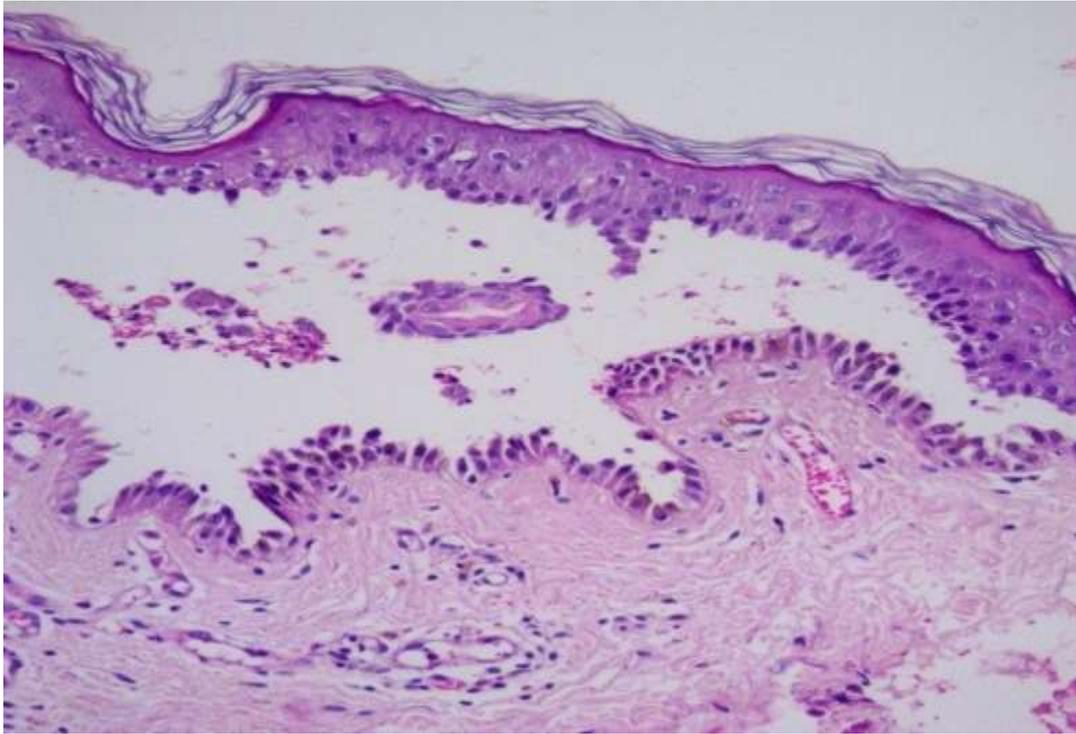


Figure (1): A case of PV (early lesion) showed suprabasal blister with the floor formed of basal cell layer (row of tomb stone) and the roof formed of suprabasal layers exhibiting spongiosis. The blister cavity contains acantholytic cells and an avulsed eccrine sweat gland. Dermis showed perivascular lymphocytic infiltrate (H&E X 200).

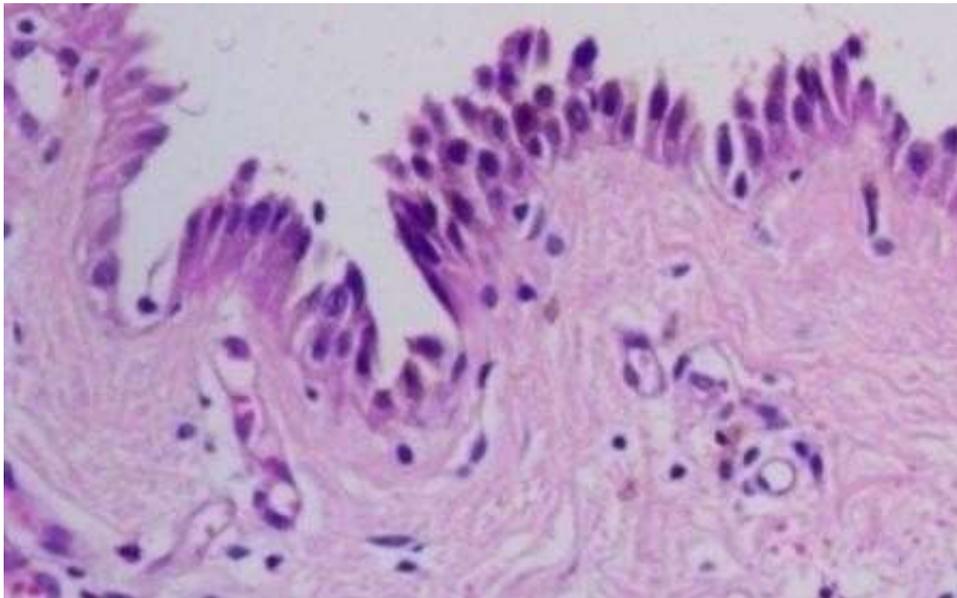


Figure (2): Higher magnification of the previous image showed the floor of the blister cavity formed of the basal cell layer (row of tomb stone). Dermis showed perivascular lymphocytic infiltrate (H&E X 400).

LGR5 immunohistochemical expression in PV cases:

All PV cases showed positive cytoplasmic basal expression in patchy distribution. Negative LGR5 expression was noted in the roof of blister cavity (**Figures 3 and 4**).

Cases which show regenerative changes showed the same expression of LGR5 in basal cell layer.

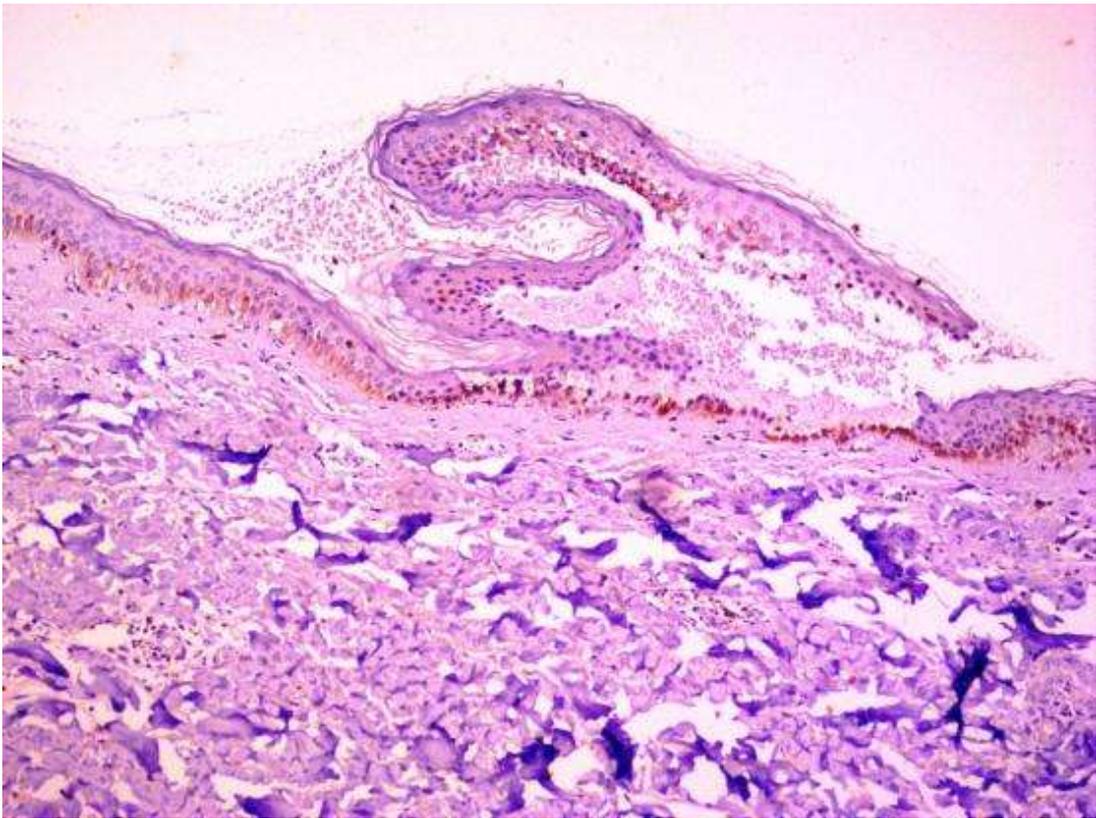


Figure (3): A case of PV showed moderate patchy expression of LGR5 in basal cell layer. However, negative LGR5 expression was noted in the roof of the blister cavity (LGR5 IHC $\times 40$).

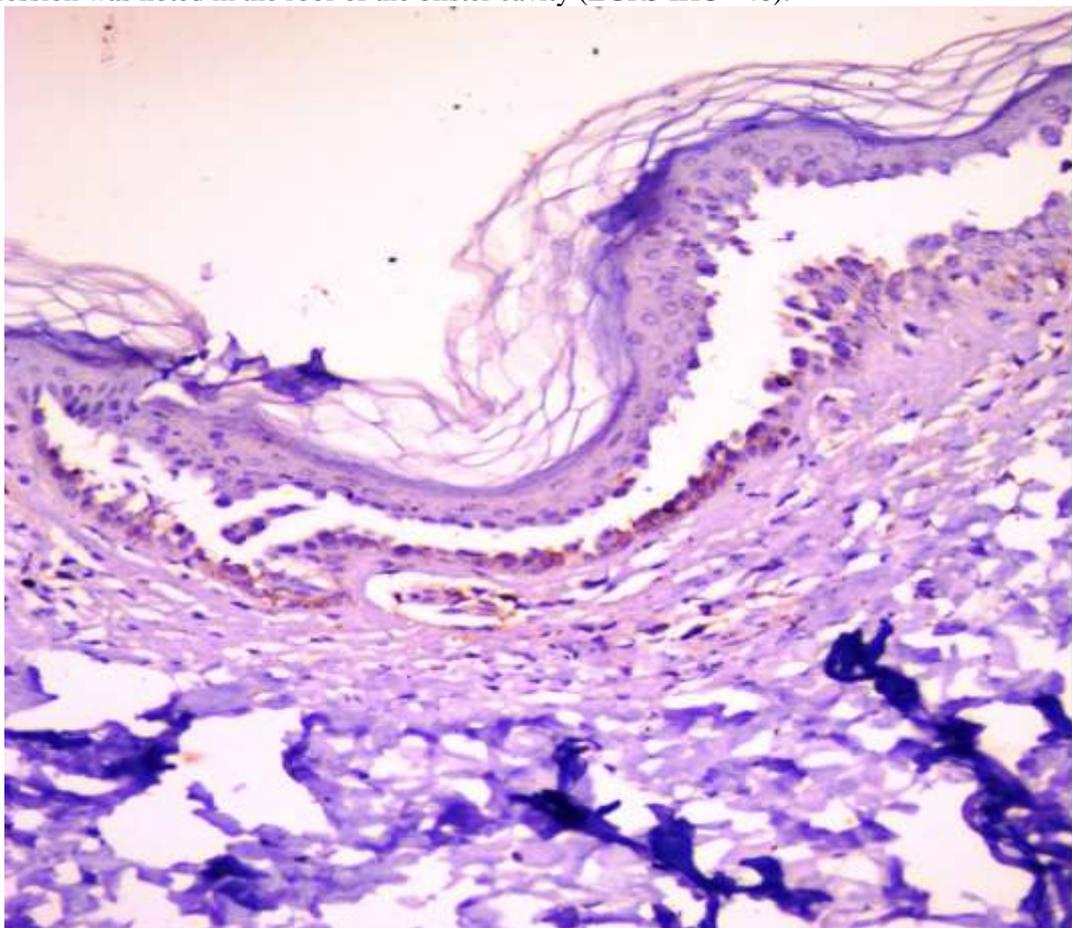


Figure (4): A case of PV showed mild patchy basal expression of LGR5. Negative LGR5 expression was noted in the roof of the blister cavity (LGR5 IHC $\times 100$).

LGR5 expression intensity:

Regarding intensity of expression, 15 cases (75%) had mild intensity and 5 cases (25%) had moderate intensity, LGR5 H score ranged from 50-130 with mean \pm SD 110 \pm 18.92 (Table 1).

Table (1): LGR5 immunohistochemical expression in PV cases (No= 20).

LGR5 Expression	No (%)
Intensity	
Mild	15 (75)
Moderate	5 (25)
Strong	0 (0)
H score	
Mean \pm SD	110 \pm 18.92
Median	115
Range	50 – 130

No: Number **SD:** Standard deviation **H score:** Histo-score

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5.

Correlation of PVAS score and LGR5 H score:

There was no significant correlation between PVAS scores either in skin, mucosa or total involvement and H score of LGR5 intensity of expression (Table 2).

Table (2): Correlation of PVAS score and H score of LGR5 expression.

PVAS score	H score of LGR5	
	r	P value
Skin	0.231	0.328
Mucosa	0.215	0.361
Total	0.236	0.317

r: correlation coefficient. **P:** p value for comparing between the 2 scores

PVAS: pemphigus vulgaris activity score.

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5.

DISCUSSION

Pemphigus vulgaris (PV) is a potentially life-threatening autoimmune blistering disease ⁽¹¹⁾. It is a rare disease with a yearly incidence of approximately one in 100,000 people ⁽¹²⁾. It increases to 2-3 per 100,000 people in Ashkenazi Jewish population and in people of Mediterranean decent ⁽¹³⁾.

It is divided into two subgroups according to the type of autoantibodies: the mucosal-dominant type with mucosal erosions but minimal skin involvement; and the mucocutaneous type with extensive skin blisters and erosions in addition to mucosal involvement ⁽¹⁴⁾. The PV autoantibodies disrupt desmosomal adhesion and cause acantholysis. Previous studies showed indirect involvement of stem

cells secondary to plakoglobin and c-myc which are affected secondary to desmoglein depletion ⁽²⁾.

There has been a long debate on the mechanisms by which PV autoantibodies disrupt desmosomal adhesion and cause acantholysis. It was suggested that, PV antibodies that target Dsg 3 and PG, act through the depletion of nuclear PG, and as one of the consequences, abrogation of PG-mediated c-Myc control that will result in disturbed proliferation and terminal differentiation of epidermis ⁽⁷⁾. Modulation of available PG at the plasma membrane, secondary to antibody binding, may affect the ongoing differentiation process. Also, it is a regulator of Wnt pathway ⁽⁵⁾. However, no previous studies have been done to evaluate stem cell markers in PV, we aimed by this study to correlate LGR5 expression which was assessed by H score and disease severity which was assessed by PVAS score.

LGR5 positive cells comprise an actively proliferating and multipotent stem cell population in mouse hair follicles that maintains all cell lineages of the hair follicle, appendages and epidermis during wound healing ⁽⁴⁾.

The current study showed that all studied PV cases had positive LGR5 cytoplasmic basal expression in patchy distribution. 75% of cases had mild intensity of expression and 25% had moderate intensity. LGR5 was expressed in the proliferated base in some of blister cavities and some cases had regenerative changes in the form of multiplication of basal cell layer.

No previous researches assessed the epidermal expression of LGR5. Therefore, we aim to explain its role in the healing of PV lesions. This could be explained as during wound healing, the imbalance in homeostasis of hair follicle stem cells (HFSCs) causes LGR5-SC to expand and migrate to the infundibulum and interfollicular epidermis (IFE) ⁽¹⁵⁾.

There was no significant correlation between H score of LGR5 and PVAS scores either in skin, mucosa, or total involvement. Searches in English literatures failed to find similar data and explanation. And so, we assume that LGR5 contributes towards tissue regeneration and healing of PV lesions but didn't correlate the disease severity. We need further studies to assess the correlation between stem cells and the severity index of PV in either in skin, mucosa or involvement.

This might be clarified as follows: stem cells fuel tissue development, renewal, and regeneration, and these activities are controlled by the local stem cell microenvironment, the "niche". Wnt signals emanating from the niche can act as self-renewal factors for stem cells in multiple mammalian tissues. Wnt operates in stem cell control and, in doing so, identify an integral program for tissue renewal and

regeneration. If the Wnt pathway is inhibited, tissue renewal is crippled ⁽¹⁶⁾.

CONCLUSION

The current study could shed a new light on the disease and its correlation with stem cells, LGR5 as a stem cell marker could be related to the healing process in PV. However, it didn't correlate PVAS scores either in skin, mucosa or total involvement.

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