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Expression of P38 Mitogen-Activated Protein Kinase in Pemphigus Patients and Its Correlation with Disease Subtypes and Activity.

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ABSTRACT:

Objective: In contrast to healthy controls, we examined P38 MAPK levels in pemphigus patients with various subtypes in this study. Furthermore, this marker's association with disease activity and severity was studied.

Methodology: Western blotting analysis was used to detect the P38 MAPK expression level in keratinocytes in 50 samples, 25 of which were from patients with pemphigus disease and 25 of which seemed to be healthy controls. The severity of the disease was assessed using the Pemphigus Disease Area Index (PDAI) score.

Results: The mean age in the pemphigus group was 44.72±12.69 years. Most patients (60%) were female, and female-to-male ratio was 1.5:1. Of the subtypes of pemphigus, P. vulgaris was the most prevalent (76%). Although the extent and activity indices of the P. vulgaris subtype were somewhat higher than those of the P. foliaceus subtype, the differences were not statistically significant. According to the research, P38 MAPK levels were lower in pemphigus patients than in the control group. No significant associations were found between this marker and other disease subtypes or clinical indicators.

Conclusion: P38 MAPK is downregulated in those who have pemphigus. It is neither significantly involved in the classification of pemphigus disease nor in the severity of the disease. Still, it may be implicated in the pathophysiology of pemphigus subtypes and may be a predictive marker of disease progression.

Keywords: P38 MAPK; pemphigus; disease activity; expression; western blot.

1. INTRODUCTION

Pemphigus diseases are blistering autoimmune disorders affecting the mucous membranes and skin (Schmidt et al., 2019). It is caused by pathogenic autoantibodies, which target the cell surface proteins of keratinocytes and cause acantholysis. Acantholysis refers to the intraepidermal clefts caused by keratinocytes in the epidermal stratum spinosum losing their intercellular connections (Porro et al., 2019; Rehman et al., 2021). Various subtypes of pemphigus are distinguished by their clinical presentation, histological characteristics, and the specific antigens that target circulating autoantibodies. These subtypes include paraneoplastic pemphigus (PNP), drug-induced pemphigus, pemphigus vulgaris (PV), pemphigus foliaceous (PF), pemphigus vegetans, pemphigus erythematosus, and pemphigus herpetiformis (Malik et al., 2021;Zahed et al., 2021).

With an incidence of 0.1–0.5/100,000 population, Pemphigus vulgaris (PV) is the most typical representative of pemphigus disorders even in Egyptian population and many Arab countries (Saleh, 2015) PV epidemiology accounts for 65–95% of cases of pemphigus (Kridin & Schmidt, 2021). Most epidemiological studies reveal a female predominance with a peak age between 50 and 60 years (Egami et al., 2020). The development of IgG autoantibodies against transmembrane desmosomal glycoprotein desmoglein (Dsg) 3 and, in some situations, Dsg 1 indicates the etiopathogenesis of PV. Autoantibodies attach to these desmosomal elements, resulting in intraepithelial blister development and acantholysis (Schmitt & Waschke, 2021). Some signaling pathways activated in PV are the known elements in the p53 signalling network, such as p38 MAPK and c-Myc (Rehman et al., 2021;Sajda & Sinha, 2018; Schmitt & Waschke, 2021).

P38 Mitogen-activated protein kinase, or P38 MAPK, is vital in producing pro-inflammatory cytokines and regulates the inflammatory immune response. As a result, it makes P38 MAPK pathway components a target for managing certain inflammatory and autoimmune disorders (Soares-Silva et al., 2016). This protein has been identified as having four isoforms: delta, gamma, beta, and alpha (Nicoletti, 2019). Furthermore, P38 MAPK is activated by the pemphigus autoantibodies that adhere to keratinocytes. In turn, it triggers a cascade of events that

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culminate in blister formation and acantholysis (Soares-Silva et al., 2016).

This study aimed to investigate the role played by P38 MAPK in patients with pemphigus disease. Moreover, study the correlation between P38 MAPK expression levels and disease activity and clinical forms of the disease.

Patients and Methods

Study design and populations

Fifty participants were included in this randomized case-control study, including 25 patients with pemphigus disease belonging to various clinical subtypes, and 25 volunteers appeared to be healthy and matched in age and sex. Patients were recruited from the Dermatology and Venereology Department's in-patient ward and its outpatient clinic, Main University Hospital, Alexandria University, from February 2021 to June 2021. Patients with any type of pemphigus, both sex, with age ranging from 9 years old up to 66 years old were involved in the study. All participants provided written informed consent and were advised to stop the study at any time and that all information would be kept private and secure.

Ethical approval:

All procedures were performed in compliance with relevant laws and institutional guidelines of Medical Research Institute The ethics committee of the medical research institute is constituted and operating according to ICH GCP guidelines and applicable local and institutional regulations and guidelines which govern IRB operation (E/C.S/N T50/2019). The Ethical Guideline (Guideline number 4 and 5) of the Medical Research Institute, Alexandria University, approved collecting keratinocyte samples.

Pemphigus Disease Area Index (PDAI)

The Pemphigus disease area index (PDAI) score was used to determine the extent of the illness in each patient (Rosenbach et al., 2009).

Keratinocyte Sampling and processing

Subjects were pre-anaesthetized under occlusion by pridocaine for 20 minutes. Then, samples were collected from the perilesional area by skin scrapping using a sterile surgical curette sterilized in an autoclave overnight. After skin scraping, samples were preserved in a falcon containing sterile culture media supplemented with penicillin-streptomycin at 100 IU/ml concentration as a transport medium (Rasmussen et al., 2013).

Keratinocyte culture

Sterile flat-bottomed 96-well tissue culture plates were used as vehicles for lyophilized collagen type IV (Sigma, Cat No. C5533), which targets the attachment of epithelial cells. The collagen type IV was provided as a liquid mother concentration of 1 mg/ml in 0.25% acetic acid and was then freshly dispensed into each well at a final concentration of 0.25 mg/ml culture media. (ratio 1:5). Collagen-coated plates were refrigerated overnight and then sterilized by UV light in a biohazard hood.

1. Keratinocyte isolation: Falcons containing keratinocytes obtained by scrapping were vortexed and centrifuged at 3000 rpm for 4 minutes at room temperature. The culture medium was discarded, and then 3 ml of sterile normal saline was added, vortexed, and then re-centrifuged at 3000 rpm for 4 minutes at room temperature. Keratinocyte pellets were re-suspended in 0.5 ml complete tissue culture media (Beeley, 1994).

2. Assessment of keratinocyte count and keratinocyte harvest: keratinocytes were re-suspended and counted using a hemocytometer after staining with trypan blue 0.2% for estimation of cell viability according to this equation:

Viability= no. of living cells/total no. of cells

Briefly, 200 ul of keratinocytes suspension in supplemented culture media at a final concentration of 5 x 10^4/ml were dispensed into the collagen-coated wells and kept for propagation in a humidified CO_2 incubator with an atmosphere of 5% CO_2, 95% humidity and 37°C. The plate was initially checked the following day for cell attachment and confluence using an inverted microscope and then propagated for 7-10 days. Cell confluence was rechecked at the end of the incubation time (at least 75-80%). The collagen was trypsinized to obtain a single-cell suspension using trypsin-EDTA (0.5 ml/well). Keratinocyte pellets were then harvested and washed twice. Finally, cells were re-suspended in 1 ml sterile normal saline to obtain the final count of the cells (5x10^4/ml) and frozen at -20□□C until further use (Figure 1).

Figure 1: The human epidermal keratinocyte culture

Figure 1: **The human epidermal keratinocyte culture.** A10x photo from the culture plate demonstrating individual keratinocytes (indicated by white arrows).

Western blotting

Western blotting was carried out in Faculty of Medicine, Alexandria University. Keratinocyte lysates were prepared by repeated freezing-thawing cycles (5x) and then used to perform SDS PAGE electrophoresis (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) to separate the crude extract into their constituting bands (Laemmli, 1970). Keratinocyte isolates were subjected to 12.5% polyacrylamide gel and stained by commassie brilliant blue. The lysates were dissolved in SDS lysis buffer (4% 2-mercaptoethanol, 10 % glycerol, 2% SDS, 0.001%

bromophenol blue and 1M Tris-base (pH 6.8). The prepared solutions were heated at 95^{o}C for 10 minutes. They were then subjected to SDS/PAGE using the Laemmli buffer system (Mahmood & Yang, 2012). After electrophoresis, the proteins were transferred to PVDF (Hydrophilic polyvinylidene fluoride) membranes at 10 V for 35 min using a Bio-Rad semi-dry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA).

The PVDF membrane was stained using Ponceau S stain to determine the p38 MAPK band in all samples, followed by washing for 5 min by TBS (tris-buffered saline) or de-ionized H_2O. The membrane was incubated with blocking buffer overnight (5% non-fat dry milk and 0.5ml Tween 20), followed by washing with phosphate buffer saline (PBS). Following that, probing of specific anti-P38 MAPK was performed by incubation for 2 hours with recombinant rabbit monoclonal primary antibody (Phospho-p38 MAPK (dilution 1:500), washing four times with PBS, then incubation for 1 hour with HRP conjugated secondary antibody (dilution 1:25), washing for six times using PBS. Finally, strips were incubated with the substrate tetramethyl benzedine (TMB) for 20 minutes, and then colour bands were demonstrated and quantified using the Canon CamScan euro-immune Germany. This work was conducted in the Faculty of Medicine,Alexandria University

Statistical analysis

Version 20.0 of the IBM SPSS software program was used to analyze the data (Armonk, NY: IBM Corp). Number and percent were used to determine the qualitative data. Range, mean and standard deviation were used to describe quantitative data. At the 5% level, the results' significance was assessed. The chi-square test was used to compare the categorical variable results between the two groups in the study. The student t-test was utilized for quantitative variables with a normal distribution, whereas the Mann-Whitney test was employed for abnormally distributed data. The Kruskal-Wallis test was used to compare more than two groups. The Spearman coefficient was utilized to evaluate the correlation analysis.

Results

Demographic and clinical patient characteristics

The study population comprised 31 (62%) females and 19 (38%) males, with the patient group consisting of 15 females and 10 males with a female-male ratio of 1.5:1. The mean age of pemphigus patients and control was (44.72±12.69, 44.12±10.54 years) respectively. There was no statistically significant difference between patients and control groups regarding age and gender (P=0.856; 0.771, respectively). None of our study population had a family history of pemphigus.

The distribution of pemphigus patients according to subtype showed a predominance of P. Vulgaris in 76%, followed by P. Foliaceus in 16%, while IgA pemphigus was detected only in 8% of the patients.

Pemphigus disease activity index and treatment duration

The skin and mucosal activity assessments were the only ones to evaluate disease activity. Table 1 shows the distribution of measurements based on PDAI, such as damage, extent and activity of skin in 25 patients and the treatment duration.

Table 1: Descriptive analysis of the disease activity indexes and treatment duration.

	Min. – Max.	**Mean ± SD.**
Damage index	0.0–13.0	7.20±3.40
Extent index	3.0–18.0	10.16±4.33
Activity index	6.0–40.0	22.68±10.56
Treatment duration (years)	0.08–20.0	6.02 ± 5.86

Min: minimum; Max: maximum; SD: Standard deviation

Pemphigus disease activity index and Subtypes analysis

(Table2) presents the PDAI, including damage, extent and activity in subtypes of pemphigus patients. A slightly higher damage index was observed in the P. Folaceous subtype, while the extent and activity were slightly higher in the P. vulgaris subtype; however, the differences were insignificant.

Table 2: Disease indices in pemphigus patients with different subtypes

	Subtypes			**F**	**P-value**
	P. vulgaris (n = 19)	**P.foliaceus (n = 4)**	**IgA Pemphigus (n = 2)**		
Damage index (Mean ± SD.)	7.05 ± 3.61	8.50 ± 3.32	6.0 ± 0.0	0.413	0.667
Extent index (Mean ± SD.)	10.63 ± 4.67	9.0 ± 3.16	8.0 ± 2.83	0.484	0.623
Activity index (Mean ± SD.)	23.11 ± 11.75	22.50 ± 6.56	19.0 ± 5.66	0.127	0.881

SD: Standard deviation F: F for One-way ANOVA test p: p-value for comparing between the studied groups

*: Statistically significant at $p \leq 0.05$

P38 MAPK expression level in pemphigus

The expression level of p38 MAPK in pemphigus patients and control determined by western blotting (Figure 2). The mean expression level of P38 MAPK in pemphigus patients was 0.25 ± 0.33, while in control, it was 0.34 ± 0.17, which revealed a highly significant decrease in P38 MAPK expression levels in pemphigus patients compared with those in normal controls (P = 0.004).

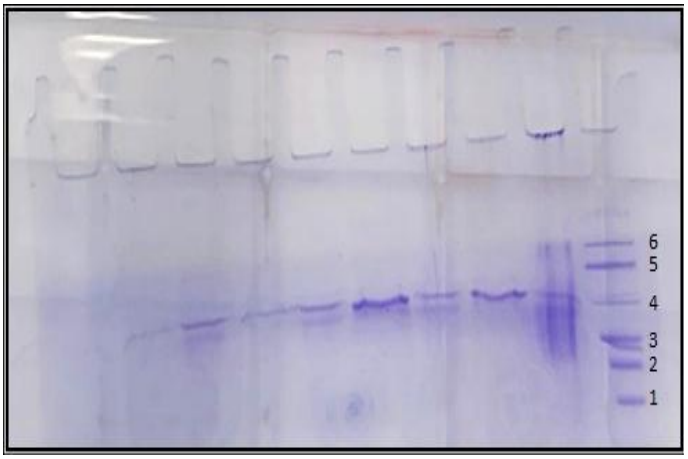

Figure (2A): A photo for the electrophoretic behavior of keratinocyte crude extracts

P38 MAPK band was located nearby the 4th band of 50 kD confirming its molecular identification.

The ladder used is thermo scientific ™ PageRuler ™ Plus Prestained Protein are a mixture of nine recombinant proteins ranging from 10kDA to 250kDa

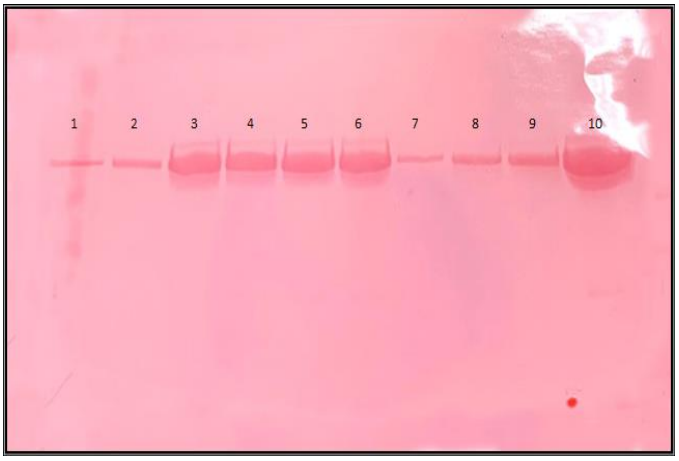

Figure (2B) The immunological identity of the p38 MAPK bands formed after immunostaining with specific antibodies and HRP-conjugates.Lanes (3,4,5,6,10) represent the P38 MAPK identification in keratinocyte extracts of control subjects, while the rest represent pemphigus patient

Figure 2: Western blotting analysis of the protein expression of p38 MAPK in the pemphigus and control groups. Lanes (3,4,5,6,10) represent the P38 MAPK identification in keratinocyte extracts of control subjects, while the rest represent pemphigus patients. (a): A photo of the electrophoreticbehavior of keratinocyte crude extracts, (b): The immunological identity of the p38 MAPK bands formed after immunostaining with specific antibodies and HRP (Horse Reddishperoxidase) conjugates.

Demographic and clinical features in relation to p38 MAPK expression levels

Table 3 shows the association of P38 MAPK expression levels with pemphigus patients' characteristics. No significant differences were observed regarding gender and the three subtypes of pemphigus.

Table 3: Association between P38 MAPK and pemphigus patients' characteristics and subtypes

Variables		N	P38 MAPK expression level		Test of Sig.	P-value
			Mean ± SD.	Median (Min. – Max.)		
Gender	Male	9	0.242± 0.299	0.106 (0.091– 0.953)	U=64.00	0.677
	Female	16	0.248± 0.360	0.107 (0.085–1.270)		
Subtypes	Pemphigus vulgaris	19	0.238± 0.327	0.106 (0.085–1.270)	H=1.462	0.482
	Pemphigus foliaceus	4	0.343± 0.471	0.110 (0.104–1.050)		
	IgA pemphigus	2	0.128± 0.030	0.128 (0.107– 0.150)		

SD: Standard deviation; U: Mann Whitney test; H: H for Kruskal Wallis test

p: p-value for comparing between the studied groups; *: Statistically significant at $p \leq 0.05$

The correlation between p38 MAPK expression and the patient's characteristics (demographic and clinical) was also examined to elucidate its role and value in pemphigus further. We found a non-significant correlation between p38 MAPK expression and age, disease indices and treatment duration (Table 4).

Table 4: Correlation between P38 MAPK and different parameters in the cases group

Variables	P38 MAPK	
	r	P-value
Age (years)	-0.307	0.135
Damage index	0.307	0.135
Extent index	0.219	0.293
Activity index	0.212	0.308
Treatment duration (years)	-0.021	0.919

r: spearman correlation coefficient

Discussion

The expression of p38 MAPK in pemphigus and its relationship to clinical variables still need to be determined since there is not enough evidence available now, even though p38 MAPK plays a significant role in inflammatory disorders, including pemphigus. When keratinocyte cell-cell adhesions are lost, pemphigus vulgaris (PV) and pemphigus foliaceous (PF) occur, the activity of p38 MAPK has been extensively researched (Soares-Silva et al., 2016). By assessing p38 MAPK levels in several pemphigus subtypes and connecting them to disease activity, this study aims to shed additional light on the role of this signaling molecule in PV patients.

According to the results of this study, pemphigus vulgaris made up 76% as the most prevalent subtype, which is comparable to those of studies by Porro et al. (2019) and Banciu et al. (2022) who found that PV accounts for almost 70% of cases of pemphigus. P. foliaceus and IgA pemphigus are known to have sporadic incidence with some estimates for P. folaceous of about 20%. The IgA pemphigus is a very rare subtype where Didona et al. (2019) defined it as a case report.

Patients in this study varied in age from 43 to 66 years. This finding was consistent with reports from Kumar et al. (2019) who found that most patients belonged to the 41–60 age range and from Kridin and Schmidt (2021) who also reported that pemphigus can occur at any age range, with most patients being between 45 and 65 years old.

In literature, gender predilection has predicted diverse outcomes. There is an average female-to-male ratio of 1.4:1, with a range of 1.1 in Finland to 5.0 in the United States (Kutlubay et al., 2021; Porro et al., 2019). The current study revealed a majority of females (60%–15 cases) with a female-to-male ratio of 1.5:1, this study finding is comparable to Porro et al. (2019). However, some exceptions with a male predominance (3:1, 3:2) were also available (Bockus & Scofield, 2009).

This study used the Pemphigus disease activity index (PDAI) as a clinical indicator of disease activity. The ranges of the results were as follows: activity index (6–40), extent index (3–18), and damage index (0–13). Moreover, the course of treatment might last anywhere from 0.08 to 20 years (Rosenbach et al., 2009).

Protein phosphorylation may be found using techniques such as mass spectrometry, enzyme-linked immunosorbent assay (ELISA), pro-Q Diamond dye, western blotting, and isotopic labelling. Western blotting is the most used technique among them because of its high level of resolution, and specificity (Han et al., 2022). In this study, the western blotting technique was used to determine the expression level of p38MAPK in keratinocyte extracts. According to the Western blotting examination, pemphigus patients' expression level of phosphorylated p38 declined in comparison to the control group ($P = 0.004$). The impairment of P38 MAPK observed in the present study was in accordance with Sevilla et al. (2021) who stated that most of the anti-inflammatory action of glucocorticoids and the resulting suppression of disease activity is mainly attributed to inhibition of P38 MAPK.

Although P38 MAPK results were significantly impaired in pemphigus patients, three of our study population had elevated p38 MAPK compared to both their pemphigus partners as well as the corresponding healthy controls. In fact, persistent elevation of P38 MAPK in keratinocyte extracts of these patients was the expected research outcome despite being treated for long by corticosteroids. Profound analysis of these cases revealed that two of them were in relapse, and one was being tapered, indicating an early sign of regaining disease activity. However, this explanation is still in need of further investigations and intimate follow-up. Elevated P38 MAPK is in accordance with Sobeih et al., [26] who reported that P38 MAPK and MAPKAPK2 were upregulated in patients with PV in comparison to controls. In fact, persistent elevation of P38 MAPK in keratinocyte extracts of these patients was the expected research outcome despite being treated for long time by corticosteroids.

The three pemphigus subtypes and gender have statistically negligible variations in the P38 MAPK expression level. Furthermore, the expression of P38 MAPK and age, as well as the duration of treatment, did not significantly correlate. These non-significant correlations may be due to small sample size

P38 MAPK and disease activity indices did not significantly correlate in the current study. On the other hand, Sobeih et al. (2020) found a positive correlation between the severity of the disease and P38 MAPK, suggesting that P38 MAPK might be utilized as a marker to monitor the progression of the disease. In addition Fransiska et al, concluded that inhibition of P38 MAPK prevents the loss of intracellular adhesions in keratinocytes and also can restore the cohesion of these cells (Vielmuth et al., 2018).

Limitations

The study's small sample size explains the disease's rarity. This small sample size may have several drawbacks. First, it might cause the need for notable differences in disease activity amongst the many subtypes involved. Second, small sample size could be needed to identify a difference between groups, resulting in inefficiency in terms of time and resources. Therefore, to guarantee accurate and legitimate statistical results, it is essential to have a sufficient sample size.

Another limitation was the difficulty in finding naïve patients who didn’t receive any immunosuppressive medications or to stopped their treatment prior to their inclusion in the study which consequently impaired the expression of P38 MAPK in the patient group.

Conclusion

To sum up, we assessed the P38 MAPK levels in several pemphigus disease subtypes and related it to clinical indicators of the disease Our study's findings supported earlier findings that P38 MAPK expression and activity are downregulated in individuals with pemphigus as a result of corticosteroid treatment.

Although P38 MAPK is not substantially engaged in the classification of pemphigus disease or its severity, it may be

implicated in the pathophysiology of different pemphigus subtypes and may serve as a prognostic biomarker of the course of the disease.

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