Biomedical Sciences and Clinical Medicine 2025;64(1):54-62.
doi: 10.12982/BSCM.2025.08
https://he01.tci-thaijo.org/index.php/CMMJ-MedCMJ/index

Original Article

Open Access

# Detection of JunB and C-FLIP Psoriasis Genes Using the Reverse Transcriptase in-situ PCR Technique

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Received: January 31, 2024;
Revised: July 7, 2024;
Accepted: September 17, 2024



## ABSTRACT

**OBJECTIVE** Psoriasis is a chronic skin disease and considered a multifactorial condition as it is caused by several risk factors; including genetic and environmental interactions. This study aimed to examine localization the expression of *JunB* and *c-FLIP* psoriasis genes at the mRNA cellular level using reverse transcriptase *in-situ* PCR (*in-situ* RT-PCR).

**METHODS** The *in-situ* RT-PCR technique was used to amplify the targeted mRNA from the formalin-fixed and paraffin-embedded tissues of three psoriasis patients. The localization of *JunB* and *c-FLIP* mRNA expression was achieved using *in-situ* RT-PCR along with the immunodetection technique. An immunohistochemistry profiler was used to count the pixels including the percentage contributions.

**RESULTS** Hematoxylin and eosin staining revealed the presence of parakeratosis, acanthosis, spongiosis and inflammatory cells. The staining analysis outcomes from *in-situ* RT-PCR and immunohistochemistry detection showed that the *c-FLIP* genes and *JunB* genes were significantly expressed in the surrounding nucleus area at the cellular level of the psoriasis tissue. *JunB* and *c-FLIP* mRNA scored positively in the the immunohistochemical analysis.

**CONCLUSIONS** RT *in-situ* PCR can be a tool for identifying mRNA cellular gene expression. *JunB* and *c-FLIP* gene expression are significantly present in psoriasis.

**KEYWORDS** psoriasis, RT *in-situ* PCR, *c-FLIP*, *JunB*

## INTRODUCTION

Psoriasis is considered a chronic immune-mediated inflammatory skin disease defined by thickened, flaky skin lesions (1). Psoriasis is thought to be associated with cellular, molecular, and genetic susceptibility to psoriasis (2). Several studies have reported that there is an association between inherited susceptibility genetic variants and environmental risk factors for the development of psoriasis (3). Population based, candidate gene, and genome-wide linkage studies have shown that genetic factors are susceptibility to the development of psoriasis (2).

Numerous studies have identified several psoriasis susceptibility loci, known as psoriasis susceptibility gene 1-9 (PSORS1-9), which con-

tribute to disease susceptibility (4). Among these, PSORS1 is the most strongly associated locus and contains genes such as human leukocyte antigen (HLA)-Cw6, which have been highly correlated with early-onset psoriasis (5). Other important genes include *IL12B* which encodes the p40 subunit of interleukin-12 and interleukin-23 as well as *IL23R*, which plays a crucial role in T-cell differentiation and inflammatory responses (6). Together, these genetic factors underscore the complex and multifactorial nature of psoriasis.

*Psoriasis vulgaris* is considered to be responsible for growth acceleration, altered variation of keratinocytes, possibly by the activation of cellular immune infiltrates and cytokines (7). The pathological differences affecting the skin in psoriasis are caused mainly by an abnormal skin differentiation secondary to the activation of T-cells or antigen-presenting cells, which triggers various chemokines and cytokines that signal keratinocytes to hyperactive proliferation (8). A study suggested that *JunB* is a component of activator protein-1 (AP-1) transcription factor that is located at the PSORS6 locus and is expressed in healthy human skin could regulate the keratinocyte proliferation and differentiation (9). Epidermal keratinocytes have been shown to reduce the production of *JunB* epithelial cells which activate the cytokine release, attracting neutrophils and macrophages to the epithelium leading to phenotypic improvements in psoriasis were identified (10).

Another study reported that deleting *JunB* and *c-Jun* from epidermal keratinocytes could reproduce can reduce psoriasis. In the patients with psoriasis, the *JunB* gene may be involved in the intricate network of signaling pathways in the immune system which lead to inflammation and skin cell proliferation (11).

Cellular FLICE-like Inhibitory Protein (*c-FLIP*) is a regulatory protein that plays a substantial role in the development of psoriasis, characterized by inflammation and the rapid proliferation of skin cells. A study has shown that the overexpression of *c-FLIP* in certain skin cells could inhibit their programmed cell death and increase the survival of the cells (12).

The *c-FLIP* is a significant antiapoptotic protein that triggers or inactivates signaling proteins for cytoprotection. The *c-FLIP* plays an essential role the development of psoriasis as it is involved in necrosis and autophagy. Compared to the non-lesional skin, the level of *c-FLIP* protein mRNA is higher in lesional skin among psoriasis subjects (13). Studies have reported that the expression of *c-FLIP* and *JunB* genes has a significant multi-functional role in various signaling pathways in psoriasis in animal models (14, 15). Thus *JunB* and *c-FLIP* are considered to be important markers for the level of the susceptibility to posriosis.

In the *in-situ* PCR the targeted gene could be amplified in a fixed cell without distortion of the subcellular components; however, there was a low sensitivity to detecting the gene expression. The reverse transcriptase *in-situ* PCR (RT *in-situ* PCR) method, however, can be used on tissue samples and consists of a combination of reverse transcription of mRNA into cDNA, PCR of cDNA templates with specific primers which can achieve the immunodetection of PCR (16, 17).

In Malaysia, a hospital-based study reported 9.5% of 5,607 dermatological patients were diagnosed with psoriasis; however, the incidence varied with age, gender and ethnicity (18). To the best of our knowledge, there appears to be a lack of studies on genetic factors related to the susceptibility to the development of psoriasis among Malaysians. Detection of *JunB* and *c-FLIP* genes is essential to determining the important mechanisms of keratinocyte proliferation in psoriasis, as they could be genetic markers for psoriasis. Hence, this study was initiated to determine the gene expression of *JunB* and *c-FLIP* using *in-situ* RT-PCR.

## METHODS

### Ethics statement

The use of archived leftover diagnostic tissues for *in-situ* RT-PCR analysis for research purposes and anonymized patient data analysis has been approved by the ethical committee of the University College of MAIWP International, with the reference (J-160277E). All work has been carried out in compliance with the Helsinki Declaration.

### Subjects

Three diagnosed psoriasis subjects were selected based on clinical and histopathological skin biopsy. The psoriasis severity index score was used for assessment of the biopsy site and

recorded. The psoriasis severity index scores were high (> 10) in many of the subjects. Diagnoses in the patients were conducted by histopathological examination by identifying the thinning of the suprapapillary plate, intermittent parakeratosis, perivascular infiltration of lymphocytes, and the occasional presence of neutrophil aggregates in the epidermis. Patients with other dermatological conditions such as eczema, allergic contact dermatitis and parapsoriasis were excluded. Figure 1 shows the flow chart of the methodology of this study.

**Sample preparation and fixation**

*Psoriasis vulgaris* was diagnosed using the formalin-fixed and paraffin-embedded (FFPE) tissue sections. The FFPE tissue sections were obtained from Lablink (M) Sdn. Bhd. Kuala Lumpur, Malaysia. The morphological changes of the psoriasis tissue were observed using hematoxylin and eosin (H&E) staining. A 50µL of RNase-free DNase I was used to digest the DNA which was then incubated for 20 minutes at 37°C. Diethyl pyrocarbonate water was used to wash the slides, which were rinsed with absolute alcohol for one minute then air-dried and kept for further processing.

**One-step RT *in-situ* PCR assay**

The Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) was used to synthesize the first strand of cDNA. The *Tfl* DNA polymerase was used to synthesize the second strand of cDNA and for DNA amplification. The primers were designed using Primer3 and validated and confirmed using the Basic Local Alignment Search Tool (http:/blast.ncbi.nlm.nih.gov/). *c-FLIP* forward: 5'- TGTTGATGCTTTGACTTTC-3', reverse: 5'-AGGGGTCTAC ATGGCAACTG-3' and *JunB* forward primers: 5'-AGAGACAATACAGGCCGCTG -3' and reverse: 5'- TATTTGACCCGCCCAAGC AT-3' were used for *in-situ* RT-PCR.

An incubation chamber was made from *in-situ* coverslips, and the sections were stabilized by an *in-situ* adapter in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) along with *in-situ* blocks. The slides were placed in the incubator for the reverse transcription process at 45°C for 45 minutes. The cycling conditions of *in-situ* RT-PCR are as follows: initial denaturation, denaturation, annealing, elongation and final elongation steps were performed for 94°C for 2 minutes, 94°C for 45 seconds, 53°C for 40 seconds, 68°C for 1 minute, and 68°C for 4 minutes for 34 cycles.

**Immunodetection of the PCR products**

In order to detect the PCR signal, digoxigenin-labelled PCR products were prominent when submerged in 40-µL anti-digoxigenin and diluted in BSA with PBS for 30 minutes at room temperature. The slides were counterstained according to the standard procedure and examined under a light microscope to analyze the images. The specificity of the RT *in-situ* PCR was checked using positive and negative controls. The negative control was digested with DNase and with the exclusion of the reagent primers digoxigenin-11-dUTP and anti-digoxigenin.

**Scoring tissue samples**

An automated digital application of the immunohistochemistry (IHC) image analysis algorithm was used to assess the staining intensity in tissue sections. The IHC staining intensity of the nuclear-type molecule was evaluated on a scale of "negative (no staining), low positive (< 30% cell staining), positive (30-60% cell staining) and high positive (> 60 % cell staining) (19). The scoring process was automated and used with the ImageJ program based on the macro compatibility that generates a histogram profile of the 3,3′-Diaminobenzidine images.

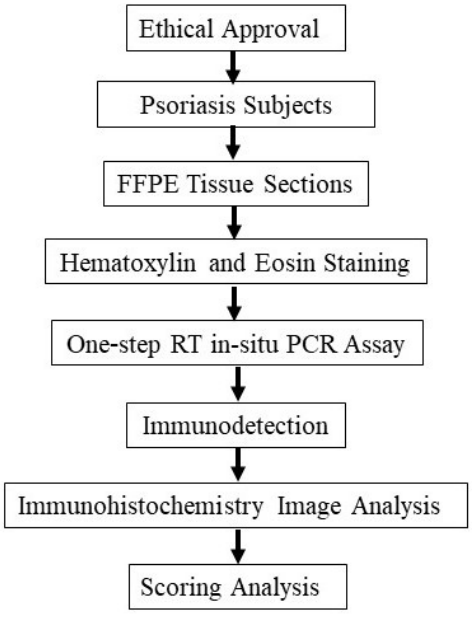


**Figure 1.** Flowchart of the methodology

## RESULTS

Figure 2A shows the acanthosis along the even elongation of the network of the blood vessels in the epidermis. In contrast, the superficial dermis shows an accumulated inflammatory response comprised primarily of small T-lymphocytes (Figure 2B). Spongiosis and parakeratosis are seen in the microphotograph of the hematoxylin and eosin staining of psoriasis tissue section in Figure 2C and Figure 2D, respectively.

The tissue sections treated with proteinase K and DNase I were excluded in the positive control. No signal was detected in the negative control section, while signals in the cells were observed in the positive control but the signals were restricted to the nuclei. This could be due to the exclusion of DNase I in the positive control sections. The cycling conditions and the concentration of the reagents were optimized to detect the *JunB* and *c-FLIP* genes. Figure 3 shows microphotographs of sections through skin tissue affected by psoriasis with the exclusion of digoxigenin-11-dUTP (Fig. 3A), anti-digoxigenin (Figure 3B), primer (Figure 3C) and an intense nuclear signal which was observed in the positive control with the exclusion of DNase I treatment (Figure 3D).

A mild brown color staining was observed in the *c-FLIP* mRNA gene expression (Figure 4A). Using higher magnification, the gene localization was observed clearly. Figure 4B shows the brown signal in nucleus areas (Figure 4B). The IHC analysis showed a positive score with more than 30.00% contribution (36.29%). The *JunB* mRNA was established in all the tissue sections. The gene localization was clearly observed around the nucleus areas, which are marked with an arrow in Figure 4B. The scoring analysis for *JunB* and *c-FLIP* genes is shown in Table 1. There is a higher positive score in *c-FLIP* (24.94%) compared to the *JunB* (36.61%) mRNA. Figure 5 shows the histogram of the scoring analysis for *c-FLIP* mRNA and *JunB* mRNA in tissue samples.

Figure 6A shows the detection of *c-FLIP* mRNA in a psoriasis sample at the higher magnification of 40x. The arrow indicates that the presence of brown color between abundant quantities of nucleus areas. There is a presence of the *c-FLIP* genes in the dermis of the psoriasis tissue section. From the IHC profiler analysis, more than 30.00% were observed in the positive score analysis for *c-FLIP* mRNA in the psoriasis tissue section.

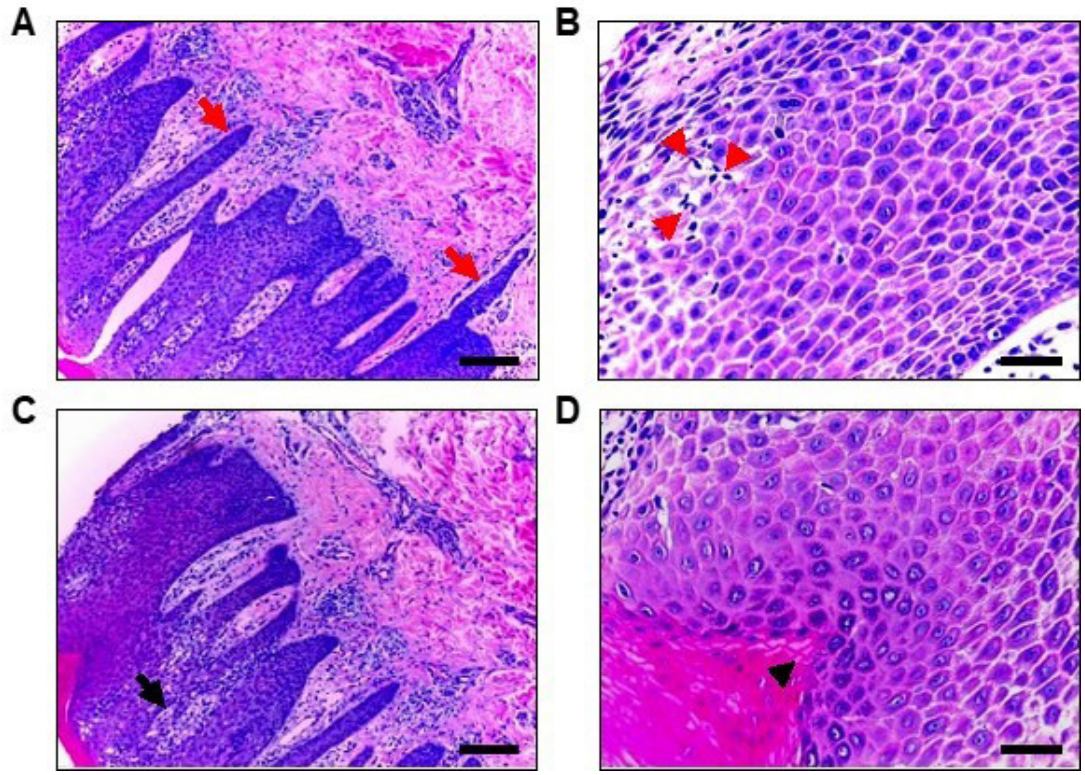


**Figure 2.** H&E staining of skin sections from psoriasis tissue. (A) acanthosis with irregular hyperplasia (red arrow), (B) infiltration of inflammatory cells (red arrowhead), (C) spongiosis with intraepidermal vesicle (black arrow), (D) parakeratosis with abnormal retention of nuclei (black arrowhead). The scale bars denote 50 μm.

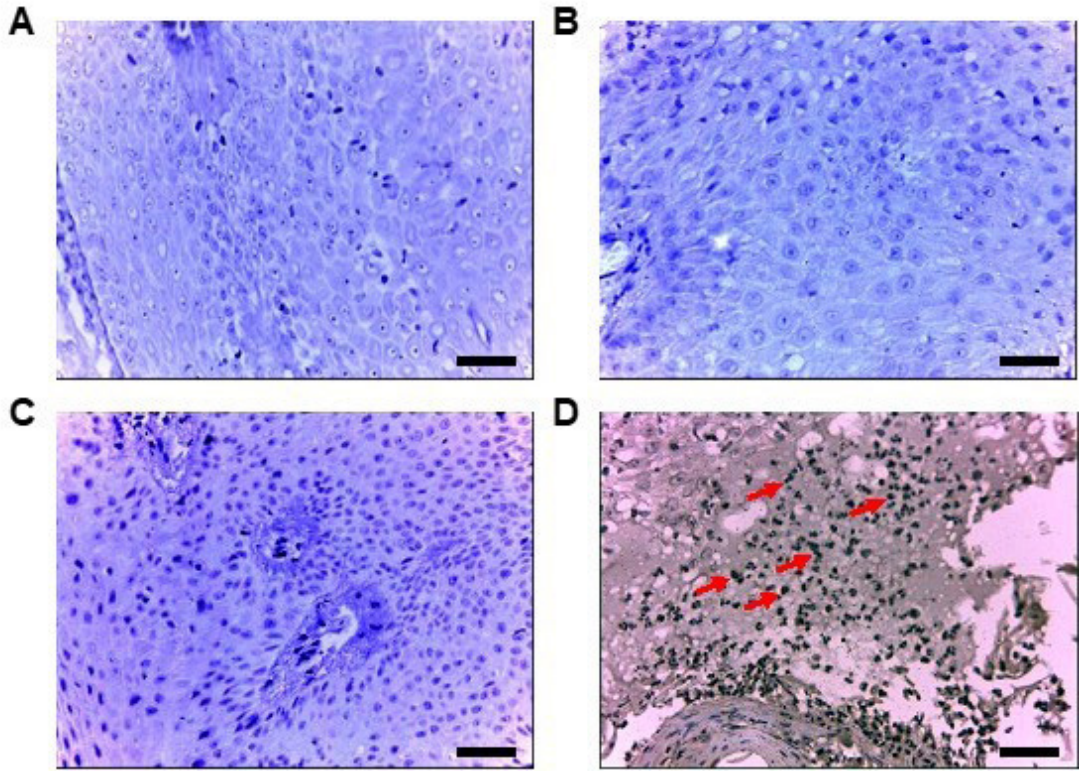


**Figure 3.** Microphotograph of sections through skin tissue affected by psoriasis. (A) The skin tissue showed the negative control with lack of mRNA signal and the exclusion of digoxigenin-11-dUTP, (B) Skin tissue showed negative control with lack of mRNA signal with the exclusion of anti-digoxigenin, (C) The skin tissue section showed negative control with lack of mRNA signal with the exclusion of primer, (D) The tissue section showed intense nuclear signal (red arrow) in the positive control with the exclusion of DNase I treatment. The scale bars denote 50 μm.

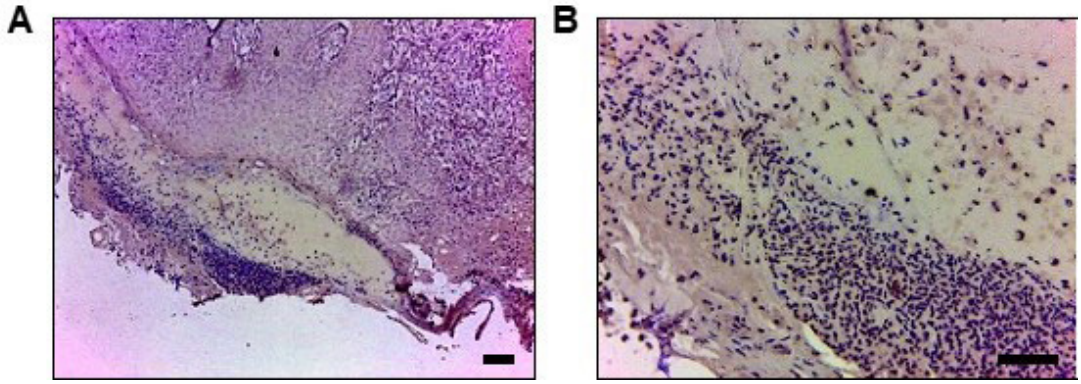


**Figure 4.** Detection of *c-FLIP* mRNA in psoriatic skin lesion. (A-B) *in-situ* PCR reveals that *c-FLIP* mRNA (brown coloration) is strongly expressed in the nucleus areas of the epidermis. The scale bars denote 50 μm.

**Table 1.** Scoring analysis for *c-FLIP* mRNA and *JunB* mRNA

| Score | Percentage contribution (%) | |
|---|---|---|
| | ***c-FLIP* mRNA** | ***JunB* mRNA** |
| High positive | 24.9492 | 8.4785 |
| Positive | 32.7576 | 36.2920 |
| Low positive | 30.0147 | 36.6186 |
| Negative | 12.2785 | 18.6110 |

The *JunB* mRNA shows a positive score in the IHC scoring analysis and shows the presence of the *JunB* gene in the psoriasis tissue sections. Figure 6B shows the presence of brown color at higher magnification.

The observed prominence of the brown color indicates the presence of *JunB* mRNA. This shows that the *JunB* genes play an essential role in the development of psoriasis growth. Depending upon the cell-cycle stage and the external conditions,

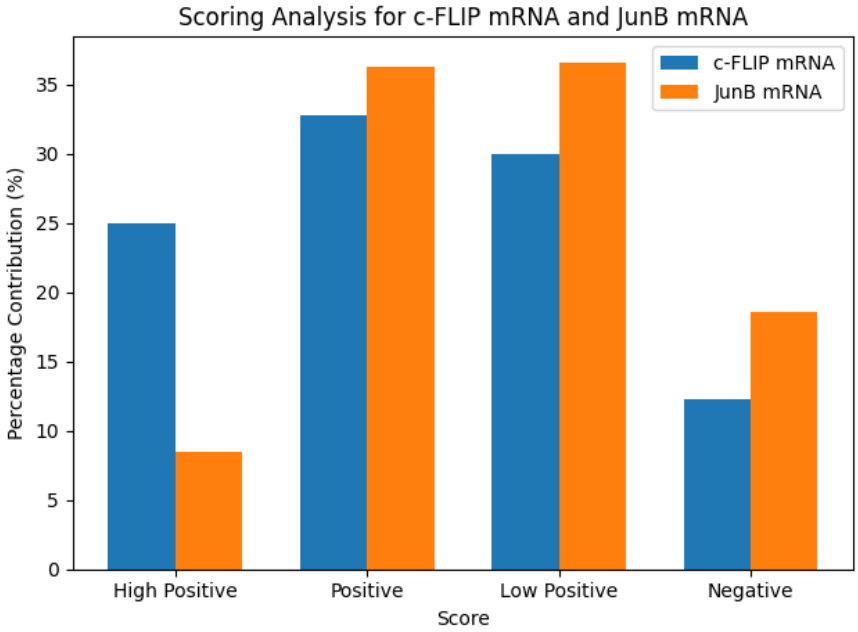


**Figure 5.** Scoring analysis for *c-FLIP* mRNA and *JunB* mRNA in tissue samples. The bar chart represent the percentage contribution of each score category (high positive, positive, low positive, and negative) for both *c-FLIP* mRNA (blue bars) and *JunB* mRNA (orange bars). The scoring was based automated analysis using ImageJ. The results show variability in the expression levels of both *c-FLIP* mRNA and *JunB* mRNA across different intensity levels, with notable differences in low positive and positive categories.

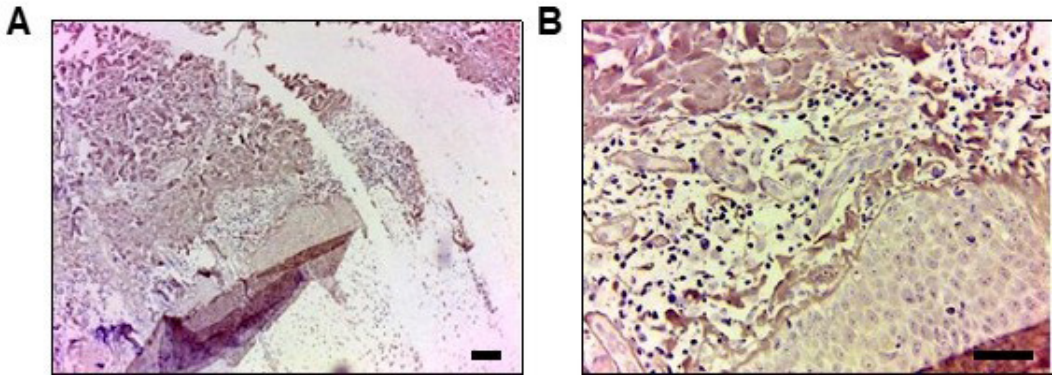


**Figure 6.** Detection of *JunB* mRNA in psoriatic skin lesion. (A-B) RT *in-situ* PCR technique, the reaction product of *JunB* mRNA (brown coloration) is predominantly located in the nucleus areas of the dermis. The scale bars denote 50 μM

*JunB* can have either cell division-promoting or preventing activities. *JunB* genes are expressed in the skin tissues that act on keratinocytes to encourage skin proliferation.

## DISCUSSION

Expression of *c-FLIP* and *JunB* genes on histological skin sections from psoriasis subjects was determined in this study using *in-situ* RT-PCR. The *in-situ* RT-PCR can be used as a tool for identifying pathological and physiological changes in psoriasis for a specific gene. In light of the results, we strongly conclude that *c-FLIP* and *JunB* genes could be a biomarker for psoriasis, particularly among Malaysians.

Pathogenesis of psoriasis is multifactorial as it involves dysregulated inflammation and genetic predispositions. The cause of psoriasis remains unclear; however, several risk factors play an important role in the development of psoriasis (20). The histology of psoriasis is characterized by parakeratosis, the complex interplay among epidermal keratinocytes, immune cells, inflammatory mediators and hyperplasia (6). Studies have suggested that microRNA (miRNA) could regulate cell-differentiation and proliferation,

cytokine responses of keratinocytes, activation and survival of T-cells and the crosstalk between immunocytes and keratinocytes (21). Although the differential expression profile of mRNAs is involved in the development of psoriasis, the biomarkers are not yet used in clinical applications. Studies to determine the role of mRNAs in psoriasis which might lead to prospect insights into diagnosis, pathogenesis and treatment for psoriasis are much needed. For that reason, this study focused on localizing the cellular mRNA of *JunB* and *c-FLIP* gene expression from human psoriasis patients using *in-situ* RT-PCR.

*In-situ* RT-PCR is considered as a valuable tool for detecting the cell-types in tissue sections (22). The *in-situ* PCR technique was used in this study as it is capable of yielding good results despite low-RNA quantity. The results show that *in-situ* RT-PCR could be considered as a valuable method to detect mRNA expression at the cellular level. *In-situ* RT-PCR combined with immunodetection could be used to determine the localization of *c-FLIP* and *JunB* mRNA expression. The expression of *c-FLIP* and *JunB* genes in the pathogenesis of psoriasis were explored in this study. The *c-FLIP* mRNA showed a positive score using *in-situ* RT-PCR and immunohistochemical methods. The visibility of the brown color indicated the presence of the *c-FLIP* mRNA in our study. This confirms that the *c-FLIP* genes could play a vital role in the development of psoriasis growth (23). The data show that epithelial depletion of *c-FLIP* in keratinocytes could be a necessary step in the drastic death of the cells.

In comparison to RT-qPCR, which offers high sensitivity and precise quantification of gene expression, *in-situ* RT-PCR has the advantage of preserving tissue architecture, thus enabling gene expression detection within specfic skin layers. This is critical for understanding localized diseases like psoriasism where spatial distribution of gene expression, such as *c-FLIP* and *JunB*, is key to predicting disease severity (24), while RT-qPCR lacks spatial information due to tissue homogenization (25). RT *in-situ* PCR was chosen for this study becase it provides valuable insighlts into the localization of gene expression within psoriatic lesions, offering a better understanding of the disease's pathology and its potential role in predicting disease severity (26).

*JunB* is located in the PSORS6 locus, and the downregulation of *JunB* in the epidermis plays an important role in the pathogenesis of psoriasis (10, 14). All of the psoriasis slide sections showed the presence of the *JunB* gene expression. The histological visualization shows the presence of the *JunB* genes. A study reported that in the tissue extracts of psoriasis lesions expression of *JunB* mRNA was observed. In addition, a gene profiling study noticed increased *JunB* mRNA among Japanese subjects with psoriasis (26). A lack of information on *c-FLIP* and *JunB* protein expression in the epidermis precluded determination of the impact on the severity of the disease.

While the specific mechanisms by which the *JunB* gene contributes to psoriasis are still being elucidated, its involvement highlights the importance of genetic factors in the development and progression of the condition (27, 28). Researchers continue to investigate how alterations in *JunB* expression and function might be targeted for potential therapeutic interventions. Further research is needed to understand the precise mechanism of *JunB* involvement in psoriasis and how it could be leveraged for more effective treatments. Despite significant progress in detecting psoriasis lesions with altered expressions of protein-coding mRNAs, more information from transcriptome studies is needed to understand the cellular processes that are activated in these lesions, including suggesting novel disease mechanisms, biomarkers and possible drug targets. Table 2 shows various studies of psoriasis in different populations using skin biopsies (14, 29-33). A lack of information on *c-FLIP* and *JunB* protein expression in the epidermis precluded determining the impact on the severity of the disease.

This study must be interpreted with consideration of several limitations. Firstly, the sample size is low and outcomes need to be confirmed with larger samples along with control subjects. Secondly, only *c-Flip* and *JunB* genes were focused on, while other activation genes such as HLA, PSOR1 interleukin and antigens in various miRNAs need to be analyzed further. In addition, further assessment is recommended to evaluate the relationship between the gene expression of *c-FLIP*, *JunB* proteins and both the psoriasis area and the severity index score to determine the severity of the psoriasis.

**Table 2.** Studies of gene expression in skin biopsies in various populations

| No | Author, year, reference | Country | Type of study | Samples (psoriasis/controls) | Sample | Gene/protein | Outcomes |
|---|---|---|---|---|---|---|---|
| 1. | Haider et al., 2006 (14) | USA | Observational | 26 (26/0) | Skin biopsy | *JunB* | ↑*JunB* |
| 2. | Park et al., 2009 (29) | Korea | Case–control | 63 (54/9) | Skin biopsy | *JunB*, *c-Jun* and S100A8 | ↑S100A8 ($p < 0.05$) |
| 3. | Kiafar et al. 2020 (30) | Iran | Observational | 20 (20/0) | Skin biopsy | Thioredoxin reductase | ↓TrxR ($p < 0.01$) |
| 4. | Dilek et al. 2016 (31) | Turkey | Case–control | 75 (50/25) | Skin biopsy | Inducible nitric oxide and myeloperoxidase | ↑MPO ($p < 0.05$) |
| 5. | Yldirim et al. 2003 (32) | Turkey | Case–control | 44 (22/22) | Skin biopsy | Malondialdehyde | ↓MDA skin ($p < 0.01$) |
| 6. | El-komy et al., 2020 (33) | Egypt | Case–control | 40 (20/20) | Skin biopsy | miRNA-155, miRNA-210, and miRNA-20b | ↑miRNA-155, ↑miRNA-210, and ↑miRNA-20b ($p < 0.05$) |
| 7. | Current Study | Malaysia | Observational | 3 (3/0) | Skin biopsy | *c-FLIP* and *JunB* | ↑c-FLIP and ↑*JunB* |

We suggest that, with the incorporation of the optimization steps, the *in-situ* RT-PCR method could be suitable for visualizing a single cell expressing a specific gene on a tissue section. The mRNA detection is very important to detect the substandard cells at the cellular stage of psoriasis to to facilitate earlier treatment of patients.

## CONCLUSIONS

This study clearly demonstrates the presence of *JunB* and *c-FLIP* gene expression using *in-situ* RT-PCR on histological skin sections. *In-situ* RT-PCR shows promise as a valuable method for exploring the relationships between pathological and physiological changes in psoriasis, specifically with respect to gene expression. Further research is needed to validate these findings and explore their correlation with psoriasis severity.

## ACKNOWLEDGMENTS

None

## FUNDING

This research received a grant from Tun Ahmad Sarji Research Endowment Fund: UCMI/TNCPP/RMC/Geran/2022(47)

## CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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