Connexin 26 in psoriatic skin before and after two conventional therapeutic modalities: methotrexate and PUVA

**Background:** Direct intercellular signaling, which controls keratinocyte behavior, proliferation and differentiation, occurs through gap junctions. Altered expression of connexins may play a role in the development of psoriatic lesions. **Objectives:** We estimated connexin 26 (Cx26) mRNA in psoriatic patients and investigated whether the standard therapeutic modalities (methotrexate and PUVA) exert their anti-psoriatic activity partially through altering Cx26 mRNA levels. We also detected Cx26 in skin biopsies by immunohistochemistry. RT-PCR measured Cx26 mRNA levels in 24 chronic plaque psoriasis patients. Group A received intramuscular methotrexate and group B was treated by PUVA for ten weeks, each followed by measurement of Cx26 mRNA levels and immunohistochemistry. Twelve healthy volunteers served as controls. **Results:** Cx26 mRNA expression was significantly higher in the patients before treatment than in controls (P<0.001). Post treatment levels were significantly lower than pre-treatment levels (P<0.001), however, significantly higher than in controls (P<0.001). Methotrexate and PUVA caused significant reductions in Cx26 mRNA expression (P=0.002, P=0.028 respectively). Post treatment levels were slightly significantly lower in the methotrexate group than in the PUVA group (P=0.046). The reduction in Cx26 mRNA expression was significantly positively correlated with the clinical improvement of the psoriatic plaque (P=0.002). Immunostaining of Cx26 decreased after treatment. **Conclusion:** Altered expression of the gap junction protein Cx26 may have a role in the development of the psoriatic phenotype. Both methotrexate and PUVA significantly lowered the expression of Cx26 mRNA and protein. **Key words:** psoriasis, gap junctions, connexin 26, methotrexate, PUVA

Experimental studies in rat and mouse embryos have demonstrated changes in connexin expression at different stages of development corresponding with the onset of stratification and differentiation of the epidermis [12]. Wounding of adult rat tail skin was found to be associated with upregulation of Cx26 and downregulation of Cx31.1 and Cx43 in the differentiated cells proximal to the wound edge, suggesting a role for connexins in wound healing [13]. Mutations in connexin genes have been reported in many human disorders [14-17]. Several mutations have been reported in various syndromes with disordered keratinization, such as erythrokeratoderma variabilis, keratitis-ichthyosis-deafness syndrome, palmoplantar keratoderma with sensorineural deafness, Vohwinkel syndrome and oculo-dento-digital dysplasia with curly hair and hyperkeratosis [18-22]. Psoriasis is a common disorder of keratinization. Abnormal epidermal proliferation and differentiation represent one of the major pathogenic abnormalities in this disorder [23]. It results from either an increase in the number of stem cells [24] or transiently amplifying cells [25] entering the cell cycle, in addition to apoptosis resistance [26]. Several biochemical and/or molecular pathways
that regulate cell replication are known to be affected in psoriasis [27]. A wide range of systemic drugs has been developed in recent years for the treatment of psoriasis. Methotrexate (MTX) is one of the classical agents and is still one of the most frequently used systemic treatments for psoriasis worldwide. The mechanism of action is not fully understood, but MTX is suggested to act primarily as an anti-inflammatory and immunosuppressant drug [28]. Psoralen plus UVA (PUVA) phototherapy is also a current mainstay of the treatment of psoriasis, whose target is directly the T cell-mediated immunopathology of psoriasis [29].

Alterations in epidermal gap junctions may play a role in the abnormal proliferation and differentiation seen in psoriasis. Electron microscopic studies have shown a marked increase in gap junctions between psoriatic keratinocytes of the Malpighian layer [30], which decreased following topical calcipotriol treatment [31]. On the other hand, functional studies of gap junctions in non-lesional psoriatic skin, using micro-injection with Lucifer Yellow, showed no increase in dye coupling when compared to normal skin [3]. Furthermore, upregulation of Cx26 has been reported in psoriatic lesions [32-34].

This study was carried out to further verify the possible role of the gap junction protein Cx26 in the pathogenesis of psoriasis by studying its mRNA and protein expression in lesional psoriatic skin compared to normal control skin. Moreover, the study aimed to compare the effects of both methotrexate and PUVA therapy on the mRNA expression of this gap junction protein.

### Subjects and methods

#### Patients

The study was carried out on 24 patients with psoriasis vulgaris (18 males and 6 females). Patients were selected from the outpatient clinic of the Dermatology Department, Faculty of Medicine, Cairo University, during the period from February 2010 till March 2011. Written consent was obtained from the patients before initiation of the study.

**Inclusion and exclusion criteria**

The only inclusion criterion was psoriasis on more than 30% body surface area, justifying treatment with either methotrexate or PUVA therapy. Children below 12 years of age as well as pregnant and lactating females were excluded. Careful history taking, routine laboratory investigations, general examination, skin examination and ophthalmologic examination were carried out to exclude patients with any form of liver disease, kidney disease, severe anemia, bone marrow suppression, cataract or photosensitive disorders.

**Groups**

Patients were divided into two groups.

**Group 1: Methotrexate group.** This group included 12 patients (10 males and 2 females). They received 25 mg weekly intramuscular injections of methotrexate. Folic acid in an oral dose of 5 mg/day was given to the patients, except on the day of the methotrexate injection. Monitoring of the patients was done by carrying out liver function tests, kidney function tests and complete blood analysis weekly for the 1st month then every 2 weeks.

**Group 2: PUVA group.** This group included 12 patients (8 males and 4 females). They received PUVA sessions 3 times weekly. The patients received 0.7 mg/kg 8-methoxypsoralen (MOP) 2 hours before phototherapy. Each patient was instructed to use sunscreens and protect the eyes during the sessions. Males were instructed to cover the genitalia during the sessions. If thick scales were present, topical keratolytics were given. Monitoring of the patients was done by liver function tests and ophthalmologic examination every 4 weeks.

PUVA light was delivered by a UV cabin (PUVA 1000, Waldmann, GmbH Germany) equipped with an integrated UV radiometer equipped with F85/100-W fluorescent lamps which emit UV light in the wavelength range of 315-400 nm with a peak emission at 355 nm. The initial UV A dose was dependant on the skin type, and it was 2 J/cm² for skin types IV and V and 1 J/cm² for skin type III. The dose of UVA was increased by 0.5 J/cm² every other session until mild erythema occurred, then the dose was fixed. Each patient was subjected to the following. The PASI score [35] was documented before treatment and at the final follow up. Laboratory investigations were done weekly for the first month, then every two weeks. The percentage reduction of PASI score was calculated. A 4 mm punch skin biopsy was obtained from a particular psoriatic plaque (lesional skin) before the initiation of therapy and at the end of the ten weeks from an adjacent area to the previous one, from all patients. Each biopsy was divided into two portions, one part was stored at -70 ºC for quantitative RT-PCR measurement of Cx26 mRNA, the other was fixed in 10% formol saline and processed to obtain paraffin blocks for immunostaining.

**Controls**

Twelve healthy volunteers served as controls (six males and six females). Consent was obtained from each control before obtaining the 4 mm punch skin biopsy for quantitation of the gene expression of Cx26 as well as its protein by immunostaining.

#### Methods

**RNA extraction**

Total RNA was extracted from the skin biopsy using an SV total RNA extraction kit provided by Promega Corporation, Madison, WI, USA. RNA purity and quantity was measured by spectrophotometer at 260 nm.

**Primers sequence**

Two sets of primers were used for amplification of Cx26 and β-actin. The sequences are illustrated in **table 1**.

**RT-PCR for Cx26 mRNA**

Reverse transcription was carried out on 1 µg of RNA, 0.25 µg random primers, 0.1 mM/L dNTPs mixture, 40 units of RNase inhibitor, 200 units of superscript II reverse transcriptase in 1×PCR buffer (10 mM/L Tris-HCL, 1.
Table 1. Sequences of the primers

<table>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Cx 26 F</td>
<td>5'-TCACTGCAAACCTCTCCCTCTC-3'</td>
</tr>
<tr>
<td>Cx 26 R</td>
<td>5'-TCTGATGTCCTCCTTCCCCCTCCCTCCCT-3'</td>
</tr>
<tr>
<td>B-actin F</td>
<td>5'-TACGCCGTTTGGGTTTACCGGGG-3'</td>
</tr>
<tr>
<td>B-actin R</td>
<td>5'-TTGGCCCTTGGGTTTACGGGGG-3'</td>
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5 mM/L MgCl2 and 50 mM/L KCl, pH 8.3. The reaction was carried out at 37 °C for 1 h followed by 5 min at 95 °C to destroy the enzyme. Five microliters of cDNA were added to the following PCR mixture: 2.5 μL of dNTPs (10 mM each), 5 μL of 10×PCR buffer containing 25 mM MgCl2, 1 μL of forward and reverse primers (30 pmol each), 2.5 U of Taq DNA polymerase and completed with nuclease free water to a final volume of 50 μL. Samples were denatured at 94 °C for 3 min followed by 35 cycles of amplification, each consisting of 1 min at 94 °C, 1 min at 55 °C and at 72 °C for 1 min, with a final elongation of 10 min at 72 °C. RT-PCR for β-actin, a housekeeping gene, was performed to confirm integrity of RNA.

Agarose gel electrophoresis
All the PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized by UV transilluminator. Gene expression of Cx26 produced sharp bands at 179 bp and the β-actin at 150 bp.

Quantitation of the PCR product
The PCR products were then quantitated by using a quantitation kit (from Promega Corporation, Madison, WI, USA). This method depends on purification of the PCR using the Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantitation consisted of DNA quantitation buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase and DNA. All these were incubated at 37 °C for 10 min. Then, 100 μL of Enliten L/L reagent was added. Immediately, the reaction was read using a luminometer. The same steps were done on DNAs of known concentrations provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the concentrations. Then, the readings of the amplified PCR products of the five different genes after using the luminometer were read from the standard curve. The results are expressed as μg/mg wet tissue [36].

Immunohistochemical study
Mouse anti-connexin 26 monoclonal antibody (Invitrogen Corporation, Carlsbad, CA catalogue number 33-5800) was used for localization of Cx26 in skin tissues. This antibody can be used to specifically detect the Cx26 protein. No cross-reactivity with the closely related Cx26 protein has been observed.

Immunostaining with connexin antibody required pre-treatment by boiling in 10 Mm citrate buffer pH 6 for antigen retrieval. This was done for 10 min and left to cool at room temperature for 20 min. Immunostaining was completed by the use of ultravision detection system (catalogue number TP-015-HD), purchased from Lab Vision Thermo Scientific. Counterstaining was done using Mayer’s hematoxylin (catalogue number TA-060-MH). Negative control was used as reference; this was done by following the steps of immunostaining but omitting the step of the Cx26 primary antibody. Positive immunoreactivity appeared as brown deposits. The area percentage of positive Cx26 immunostaining was measured at magnification ×400 in 10 non-overlapping fields in every specimen for all patients and controls. Image analysis was done using an image analyzer computer system.

Statistical methods
Data were statistically described in terms of range, mean ± standard deviation (SD), median, frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables between the main study groups was done using the Mann Whitney U test for independent samples. Within each group, comparison of quantitative variables between before and after values was done using the Wilcoxon signed rank test. For comparing categorical data, the Chi-square (χ²) test was performed. Yates correction equation was used instead when the expected frequency was less than 5. Correlation between various variables was done using the Spearman rank correlation equation. A probability value (p value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) statistical program [37].

Results

Patient and control data
The study included 24 patients, 18 males (75%) and 6 females (25%). Their ages ranged from 15-60 years with a mean of 38.13±13.8 years. Patients were divided into 2 groups. The first group, group (1), received methotrexate therapy. They included 12 patients, 10 males (83.3%) and 2 females (16.7%) and their ages ranged from 18-60 years with a mean of 42±14.96. The second group, group (2), received PUVA therapy. They included 12 patients, 8 males (66.7%) and 4 females (33.3%) and their ages ranged from 15-56 years with a mean of 38.2±3.53 years. Twelve controls were included in the study, 6 males (50%) and 6 females (50%). Their ages ranged from 23-54 years with a mean of 39±2.61 years. Both patients and controls were age (p=0.737) and sex (p=0.157) matched. Similarly, both patient groups were age (p=0.394) and sex (p=0.223) matched.

Results of the evaluation of PASI score (table 2)
Both patient groups were statistically homogenous as regards pre-treatment PASI score (p=0.394). The post treatment PASI score was significantly lower than the pre-treatment PASI score in both the methotrexate group (p=0.002) and the PUVA group (p=0.002). However, no


Table 2. PASI score and the percentage of clinical improvement in the biopsied psoriatic plaque

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (Methotrexate)</th>
<th>Group 2 (PUVA)</th>
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<tbody>
<tr>
<td>PASI score (pre treatment) (Mean±SD)</td>
<td>12.71±7.4</td>
<td>10.13±4.8</td>
</tr>
<tr>
<td>PASI score (post treatment) (Mean±SD)</td>
<td>4.89±2.6</td>
<td>5.77±4.1</td>
</tr>
<tr>
<td>Percentage of reduction in PASI score (Mean±SD)</td>
<td>59%±8.3</td>
<td>43%±19.6</td>
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Table 3. Results of the quantitative PCR measurement of Cx26 mRNA expression

<table>
<thead>
<tr>
<th>Patients groups</th>
<th>Cx26 mRNA (µg/gm tissue) (before treatment) (Mean ± SD) Median</th>
<th>Cx26 mRNA (µg/gm tissue) (after treatment) (Mean ± SD) Median</th>
<th>Percentage of reduction in Cx mRNA (Mean ± SD) Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Methotrexate)</td>
<td>(2,894.58±643.7) 3,016.5</td>
<td>(2,224.08±322.4) 2,099.9</td>
<td>(20.7%±14.9) 18.80%</td>
</tr>
<tr>
<td>Group 2 (PUVA)</td>
<td>(2,892.08±725.9) 3,126</td>
<td>(2,579.42±528.5) 2,099.9</td>
<td>(9.2%±12.3) 9.3%</td>
</tr>
<tr>
<td>All patients</td>
<td>(2,893.33±670.9) 3,026.5</td>
<td>(2,401.75±464.9) 2,294.5</td>
<td>(14.9%±14.6) 13.3%</td>
</tr>
</tbody>
</table>

Results of the quantitative PCR measurement of Cx26 mRNA expression

Summary of the quantitative PCR measurement of Cx26 mRNA expression before and after treatment as well as the percentage of reduction in Cx26 mRNA expression in the patients as a whole, as well as in each patient group, are illustrated in table 3. The mean Cx26 mRNA expression in the control group was 393.92±74.689 µg/gm tissue and the median was 373.50 µg/gm tissue. Cx26 mRNA expression was significantly higher in the patients (as a whole) before treatment than in controls (p<0.001). Although post treatment expression in the patients (as a whole) was significantly lower than pre-treatment levels (p<0.001), post treatment levels were still significantly higher than in controls (p<0.001).

Both patient groups were statistically homogenous as regards pre-treatment Cx26 mRNA expression (p=1.000). Post treatment Cx26 mRNA expression was significantly lower than pre-treatment expression in both the methotrexate group (p=0.002) and the PUVA group (p=0.028). However, post treatment levels were still significantly higher than controls in both groups (p<0.001). Post treatment levels of Cx26 mRNA were lower in the methotrexate group than in the PUVA group and the difference was slightly significant (p=0.046). There was no statistically significant difference between both patient groups as regards the percentage of reduction in Cx26 mRNA expression (p=0.65).

When evaluated in the patients as a whole, the percentage of reduction in Cx26 mRNA expression was not significantly correlated with the percentage of reduction in PASI score (r=0.368, p=0.077).

Immunohistochemical results

Immunohistochemical results (Cx26 immunostaining) were compared to negative controls, these showed negative immunostaining in the nuclei, which appeared completely bluish. The only brown colour was the melanin pigment that was present supranuclear, only in the basal cell layer (figures 3-4). Examination of a control specimen revealed very weak positive Cx26 immunostaining. (figure 2). In specimens from psoriatic patients before treatment, strong positive immunostaining was evident. A weaker reaction was detected in specimens from psoriatic patients after treatment, but it was still stronger than the control.

Morphometric results

The mean area percentage of positive Cx26 immunostaining in control sections (6.15±1.56) was significantly lower when compared to both groups (1, 2) of psoriasis patients (32.46±8.43 and 31.56±13.44 respectively) before treatment (P<0.001). Also, there was significant difference between the control group and the psoriatic groups after treatment (18.16±11.41 and 22.11±9.87 respectively (P<0.001). At the same, time there was a significant difference in both groups before and after treatment. The percentage reduction in positivity after treatment was greater in the group treated with methotrexate (14.3%±9.9 vs 9.45%±5.3) (table 4), (figures 2-4).

Figure 1. An agarose gel electrophoresis (2%) stained with ethidium bromide showing expression of Cx26 mRNA by RT-PCR at 179 bp. M: molecular DNA marker (1,000, 750, 500, 300, 150, 50 bp). Lanes 1 and 2: highly positive expression from samples before treatment. Lanes 3 and 4: weak positive expression from samples after treatment. Lane 5: control sample.
Table 4. Morphometric results of Cx26 immunostains

<table>
<thead>
<tr>
<th>Patients groups</th>
<th>mean area % of positive Cx26 immunostaining (before treatment) (Mean ± SD)</th>
<th>mean area % of positive Cx26 immunostaining (after treatment) (Mean ± SD)</th>
<th>Percentage of reduction in Cx26 immunostain positivity (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Methotrexate)</td>
<td>32.46±8.43</td>
<td>18.16±11.41</td>
<td>14.3%±9.9</td>
</tr>
<tr>
<td>Group 2 (PUVA)</td>
<td>31.56±13.44</td>
<td>22.11±9.87</td>
<td>9.45%±5.3</td>
</tr>
</tbody>
</table>

Discussion

This study revealed a significant increase in Cx26 mRNA expression and its protein by immunostaining in lesional psoriatic skin when compared to normal skin (P<0.001). Our results are in accordance with the results of Rivas et al. [32] and Labarthe et al. [33], who used cDNA differential display and northern blot techniques to demonstrate increased expression of Cx26 mRNA in lesional psoriatic skin.

Labarthe et al. [33], using immunofluorescence, revealed abundant expression of Cx26 in all living layers of the interfollicular epidermis at the centre of psoriatic plaques. Non-lesional skin from psoriatic patients revealed no Cx26 expression in the interfollicular epidermis, similar to that of healthy controls. Using immunohistochemistry, Luke et al. [34] detected prominent expression of Cx26 in lesional psoriatic epidermis, mainly in suprabasal non-proliferating (Ki67 –ve) cells of the interfollicular epidermis. Cx26 was absent from the interfollicular epidermis of normal skin and non-lesional psoriatic skin. The above mentioned studies demonstrated a graduated increase in the expression of Cx26 as they progressed from non-lesional psoriatic skin to a fully mature psoriatic plaque. This indicates a role of Cx26 in the evolution of the fully mature psoriatic phenotype.

Cx26 expression was prominent in suprabasal non-proliferating cells of viral warts, vaginal and buccal epithelium, similar to its expression in lesional psoriatic skin.

It appeared in a patchy distribution in the basal epidermis within 24 hours of tape stripping and proceeded to a more extensive distribution in basal and suprabasal epidermis by 48 hours [34]. Cx26 appeared to be expressed mainly by non-proliferating cells, suggesting that upregulation of Cx26 may be a feature of keratinocyte differentiation, which accompanies hyperproliferation rather than being a feature of proliferation itself [34]. An upregulation of Cx26 was also detected in cornoid lamellar keratinocytes obtained from a porokeratosis lesion. These cornoid lamellae represent a histological manifestation of an aberrant keratinocyte differentiation [38].

Cellular communication via connexins has been implicated in the three functions that are significantly altered in psoriatic keratinocytes. These include: regulation of cell growth [13, 32], cell migration [13, 39] and cell differentiation [40, 41].

The Cx26 gap junction may play a role in the transfer of metabolites between cells in different layers of acanthotic hyperplastic epithelia. It is suggested that Cx26 may change the gap junction permeability, facilitating in some way the regenerative pathway of keratinocyte maturation that is transient in tape-stripped skin and in wound healing, but persistent in psoriasis [34].

Moreover, it has been found that Cx26 is upregulated in basal cell carcinomas [42]. Its expression declined in

![Figure 2. Immunohistochemical analysis of Cx26 in control sample, stained with mouse anti-human monoclonal antibodies to Cx26. Control specimen shows very weak immunostaining in the nuclei of the epidermal cells or the cytoplasm of the prickle cells and the superficial cells.](image1)

![Figure 3. Immunohistochemical analysis of Cx26 in psoriatic patients, stained with mouse anti-human monoclonal antibodies to Cx26. A) Psoriasis specimen before methotrexate treatment, showing the skin epidermis. Strongly positive Cx26 immunostaining can be seen in some nuclei, strong immunostaining is present in the cytoplasm of all epidermal cells. B) Psoriasis specimen from a patient after treatment with methotrexate, showing epidermis of the skin. There is a decrease in the positivity of Cx26 immunostaining that can be seen in the nuclei, with scanty positive Cx26 immunostaining in the cytoplasm of some of the epidermal cells.](image2)
On an experimental in vitro level, UVA radiation was found to decrease gap junction mediated intercellular communication (GJIC) in normal human keratinocytes and this was associated with internalization of Cx43, the most abundant gap junction protein in normal human keratinocytes [50, 51]. No similar studies have discussed the effect of UV radiation on Cx26 expression. Similarly, no previous in vitro studies have examined the effect of methotrexate on Cx26 expression.

Post treatment levels of Cx26 mRNA were significantly higher than in controls. This may add to the understanding of psoriasis as a chronic disorder with a relapsing nature. Although there was no statistically significant difference between the two patient groups as regards the percentage of reduction in Cx26 mRNA, post treatment levels in the methotrexate group were slightly significantly lower than in the PUVA group (p=0.046). Further studies on larger numbers of patients are needed to further verify this finding. This finding further substantiates the possible role of Cx26 in the development of the psoriatic phenotype. Further studies are needed to identify the exact mechanism by which methotrexate and PUVA therapy reduces Cx26 mRNA expression.

Disclosure. Acknowledgment: We would like to thank Dr. Ola Abouzeid, lecturer of dermatology, Faculty of Medicine, Cairo University, for her great effort and help in the collection of the patients. Financial support: none. Conflict of interest: none.

References


