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ORIGINAL ARTICLE

Higher Expression of Toll-like Receptors 3, 7, 8, and 9 in Pityriasis Rosea

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Background: Pityriasis rosea (PR) is a common papulosquamous skin disease in which an infective agent may be implicated. Toll-like receptors (TLRs) play an important role in immune responses and in the pathophysiology of inflammatory skin diseases. Our aim was to determine the possible roles of TLRs 3, 7, 8, and 9 in the pathogenesis of PR. **Methods:** Twenty-four PR patients and 24 healthy individuals (as controls) were included in this case control study. All recruits were subjected to routine laboratory investigations. Biopsies were obtained from one active PR lesion and from healthy skin of controls for the detection of TLR 3, 7, 8, and 9 gene expression using real-time polymerase chain reaction. **Results:** This study included 24 patients (8 females and 16 males) with active PR lesions, with a mean age of 28.62 years. Twenty four healthy age- and sex-matched individuals were included as controls (8 females and 16 males, with a mean age of 30.83 years). The results of the routine laboratory tests revealed no significant differences between both groups. Significantly elevated expression of all studied TLRs were detected in PR patients relative to healthy controls ($p < .001$). **Conclusions:** TLRs 3, 7, 8, and 9 might be involved in the pathogenesis of PR.

Key Words: Immunity, innate; Toll-like receptors; Reverse transcriptase polymerase chain reaction; Pityriasis rosea

Although pityriasis rosea (PR) was identified some time ago, and much has been done to describe and diagnose the rash, little is known regarding PR's etiology. There are many studies supporting the theory that PR is caused by an infectious agent.[1] These studies were based on several facts, including the resemblance of PR's rash to viral exanthemas, the rare recurrences of PR suggesting lifelong immunity after a single episode,[2] the occurrence of seasonal variation, and the clustering in some communities, as well as the appearance of flu-like symptoms in some patients.[3] Some evidence suggests a relationship between human herpes virus (HHV) 6 and 7 and PR.[4,5]

Toll-like receptors (TLRs) are a group of pattern recognition receptors that are involved in mechanisms of host defense against a wide range of pathogenic microorganisms.[6] TLRs 3, 7, 8, and 9 are intracellular TLRs, in which they sense virus-derived pattern molecules and respond with the induction of antiviral genes, such as type I interferon.[7] Expression of TLRs 3, 7, and 9 has previously been detected in blood lymphocytes from patients with PR.[8]

The aim of this study was to evaluate the possible role of TLRs 3, 7, and 9 in the pathogenesis of PR.

MATERIALS AND METHODS

The current case control study was approved by the Research Ethical Committee Office (REC), Department of Dermatology, Faculty of Medicine, Cairo University. All participants provided full informed written consent prior to this study.

This study included 24 patients with classic active PR and 24 age- and sex-matched and apparently healthy individuals serving as controls. All participants were recruited between May 2012 and January 2013 from the dermatology outpatient clinic of Kasr Al Ainy Hospital.

All patients in the PR group had classical findings of PR and

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were in the active stage of the disease (having active lesions appearing within the same week of their visit to the clinic). All participants were otherwise healthy and immunocompetent, none had a recent history of immunization, and none were on systemic steroids or other immunosuppressive therapy.

All recruits were subjected to routine laboratory investigations including hemoglobin (Hb), white blood cells count, erythrocyte sedimentation rate first hour (ESR1), erythrocyte sedimentation rate second hour (ESR2), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and urea and creatinine levels.

A 4-mm punch biopsy was obtained from the active edge of one of the lesions from each patient and from the apparently healthy skin of the trunk from each control. The biopsies were stored at –70°C until used for measurement of TLR mRNA.

Detection of TLR 3, 7, 8, and 9 gene expression levels using real-time polymerase chain reaction

Total RNA was extracted from skin tissue (TRIzol reagent, Invitrogen, Karlsruhe, Germany), and the quantity and purity of the extracted RNA were assessed by measuring absorbance at 260 nm and the ratio of absorbance at 260 nm to absorbance at 280 nm (A260/A280), using an ultraviolet spectrophotometer (NanoDrop Inc., Wilmington, DE, USA). Only samples with an A260/A280 ratio under 1.8 were considered valid for real-time polymerase chain reaction (RT-PCR). Reverse transcription of 1 μg of RNA into cDNA was performed (First Strand cDNA Synthesis Kit, TaKaRa, Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions.

Expression of the target gene TLR was quantified using the comparative threshold cycle (Ct) method, with the amount of target mRNA normalized to an internal control (glyceraldehyde phosphate dehydogenase [GAPDH]). RT-PCR was performed using a commercial kit (Light Cycler Fast Start DNA SYBR-Green I Kit, Roche Applied Sciences, Mannheim, Germany) in accordance with the provided protocol. Briefly, 10 μL amplification mixtures were prepared, containing the equivalent of 8 ng of reverse-transcribed RNA, along with 300 M primers (sequences of the PCR primer pairs used for each gene are shown in Table 1). Reactions were run on a cetection system (ABI Prism 7900 HT, Applied Biosystems, Foster City, CA, USA). PCR parameters were one cycle at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds and 60°C for 1 minute. Data were analyzed and quantified using ABI Prism sequence detection system software (Sequence Detection Software v1.7, PE Applied Biosystems). Relative expression of studied genes was calculated using the comparative threshold cycle method, and all values were normalized to GAPDH genes.[9] The level of expression of each target gene was normalized relative to the expression of GAPDH mRNA in that sample using the ΔCt method. Relative differences in gene expression between groups were determined using the comparative Ct (ΔΔCt) method, and fold expression was calculated as $2^{-\Delta\Delta Ct}$, where ΔΔCt represents ΔCt values normalized relative to the mean ΔCt of control samples.

Table 1. Sequence of primers used for real-time PCR

		Primer sequence
TLR3	Forward	5'- GCATTTGTTTTCTCACTCTTT-3'
	Reverse	5'-TTAGCCACTGAAAAGAAAAAT-3'
TLR7	Forward	5'-AAACTCCTTGGGGCTAGATG-3'
	Reverse	5'-AGGGTGAGGTTCGTGGTGTT-3'
TLR8	Forward	5'-CTGTGAGTTATGCGCCGAAGA-3'
	Reverse	5'-TGGTGCTGTACATTGGGGTTG-3'
TLR9	Forward	5'-CGCCCTGCACCCGCTGTCTCT-3'
	Reverse	5'-CGGGGTGCTGCCATGGAGAAG-3'

PCR, polymerase chain reaction; TLR, Toll-like receptor.

Statistical methods

Data were described in terms of mean ± standard deviation (± SD), frequencies (number of cases) and relative frequencies (%) when appropriate. Comparisons between groups were done using T-tests for all quantitative variables, and chi-square (χ^2) tests for categorical data (sex distribution). Correlations between various variables were calculated using Pearson moment correlation equations for linear relationships. $p < .05$ was considered statistically significant. All statistical calculations were done using the computer program Microsoft Excel (Microsoft Corporation, New York, NY, USA) and SPSS ver. 19 for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

This case control study included 24 patients (8 females [33.3%] and 16 males [66.7%]) with classic active PR developing within the 2 weeks prior to recruitment. Their age ranged from 19 to 45 years with mean ± SD of 28.62 ± 8.30. Twenty-four apparently healthy age- and sex-matched individuals ($p = .370$ and $p > .990$, respectively) were also included in this study as controls. The controls included eight females (33.3%) and 16 males (66.7%), and their age ranged from 19–46 years with mean ± SD of 30.83 ± 8.59.

Routine laboratory data

Comparing routine laboratory findings between the patient and control groups revealed no significant differences (using

Table 2. Summary of descriptive data from routine laboratory investigations in pityriasis rosea patients and controls

Variable	Patient (n=24)	Control (n=24)	p-value
Hb (mg)	12.69±1.27 (10.8–14.9)	12.97±1.31 (10.8–14.9)	.431
WBC (mm^3)	6.77±1.63 (4.8–9.8)	6.46±1.60 (3.9–9.8)	.512
ESR1 (mm/hr)	14.01±6.27 (6–27)	12.04±5.36 (6–25)	.251
ESR2 (mm/hr)	26.25±7.62 (15–40)	23.37±7.60 (15–40)	.198
ALT (U/L)	19.41±10.40 (10–47)	21.91±12.12 (10–47)	.081
AST (U/L)	20.41±8.37 (10–40)	21.87±7.90 (12–40)	.126
Urea (mg/dL)	28.29±9.89 (15–48)	24.70±8.62 (15–50)	.092
Creatinine (mg/dL)	0.88±0.23 (0.5–1.3)	0.89±0.28 (0.5–1.5)	.436

Values are presented as mean±standard deviation (range).
Normal routine laboratory reference values: hemoglobin (Hb; male: 13–18 mg/female: 12–17 mg), white blood cells (WBCs; 4,000–12,000 mm^3), erythrocyte sedimentation rate first hour (ESR1; up to 8 mm/hr), erythrocyte sedimentation rate second hour (ESR2; up to 18 mm/hr), alanine aminotransferase (ALT; 5–40 U/L), aspartate aminotransferase (AST; 7–56 U/L), urea (20–50 mg/dL), creatinine (0.5–1.5 mg/dL).

T-test) (Table 2). In the patient group, 14 patients had anemia (low Hb), none of the patients had leukocytosis or leucopenia, 18 patients showed elevated ESR1 and ESR2, none of the patients had elevated ALT or AST, and none showed elevated urea or creatinine levels. In the control group, 14 controls had anemia (low Hb), two controls showed leucopenia and none had leukocytosis, 15 controls showed elevated ESR1 and ESR2, none of the controls had elevated ALT or AST, and none showed elevated urea or creatinine levels.

Histopathologic findings among PR patients included focal parakeratosis, absent granular cell layer, mild acanthosis, mild spongiosis, papillary dermal edema, perivascular and superficial dermal interstitial infiltrate of lymphocytes and histiocytes, and focal extravasation of erythrocytes as previously described,[10] confirming the diagnosis of PR.

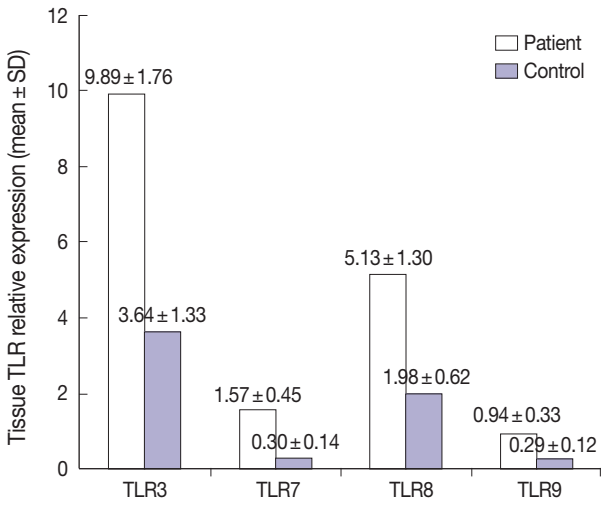

Fig. 1. Mean Toll-like receptors (TLRs) relative expression in patient and control groups. SD, standard deviation.

TLRs expression

Summary of the descriptive data of different studied TLRs in both groups were presented in Fig. 1. Comparing the TLR 3, 7, 8, and 9 expression levels between the patient and control groups revealed that the mean levels of all studied TLRs were significantly higher in patients relative to healthy controls ($p < .001$ for all, using T-test) (Fig. 1).

Our results revealed nonsignificant correlations between the studied TLRs in the patient group (using Pearson correlation test).

DISCUSSION

The current study highlights the possible roles played by different TLR pathways in the pathogenesis of PR. In this study, a significantly elevated expression of the studied TLRs 3, 7, 8, and 9 was documented in PR cases relative to healthy controls.

The results of the current study are in accordance with another,[8] which found the expression of TLRs 3, 7, and 9 to be significantly elevated in the peripheral blood (PB) lymphocytes of patients with PR relative to normal controls. However, unlike in our study, that study did not find elevated levels in the skin. This discrepancy between skin and PB lymphocytes may support the concept of PR being induced through a systemic insult to the body, most likely an infective agent, with the primary and early burden on the immune system.

Our results revealed non-significant correlations among the studied TLRs in the patient group. This could suggest that each TLR can play an individual role in the pathogenesis of PR.

TLRs 3, 7, 8, and 9 have been shown to be involved in responses to viral infection,[11] but no studies have referred to their involvement with HHV. The use of TLRs 3, 7, 8, and 9 analogs as antiviral therapy supports the role of TLR in viral infections.[12,13]

The possible role of TLRs in PR can be attributed to several mechanisms. Vercammen *et al.*[14] demonstrated that TLR 3 triggering activates specific signaling pathways that mount an effec-

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tive immune response through the induction of cytokines and other pro-inflammatory mediators. These mediators may participate in enhancing inflammation in the affected area of the PR. Renn *et al*.[15] also reported that after the stimulation of TLR 3, and to a lesser extent TLRs 7 and 8, langerhans cells are stimulated, which were found to be increased in lesions of PR; langerhans cells produce large amounts of chemoattractants, which may participate in the inflammation present in PR.

However, the fact that TLRs are involved with the initiation of innate and acquired immune responses for many other pathogens precludes us from asserting that their high expression in PR points to an infective etiology.

In conclusion, the current study indicated that TLRs 3, 7, 8 and 9 have a positive role in PR. Studying the link between HHV 6 and 7 and TLRs in cases of PR is part of our ongoing research in order to report a possible link between the TLRs and HHV in the pathogenesis of PR and to detect possible triggering factors for HHV reinfection or reactivation in PR.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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