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# Indirubin, an acting component of indigo naturalis, inhibits EGFR activation and EGF-induced CDC25B gene expression in epidermal keratinocytes☆

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ABSTRACT

*Background:* Topical indigo naturalis ointment is clinically proved to be an effective therapy for plaque-type psoriasis. Indirubin, as the active component of indigo naturalis, inhibits cell proliferation of epidermal keratinocytes. However, the detailed underlying mechanism is not fully understood.
*Objective:* To further investigate the anti-proliferating effects of indigo naturalis and indirubin on epidermal keratinocytes.
*Methods:* The decreased expression of CDC25B in indigo naturalis- or indirubin-treated epidermal keratinocytes, as revealed by cDNA microarray analysis, was studied. The CDC25B expression was examined under different serum concentrations and compared between primary and immortalized keratinocytes. The activation of EGFR and the effect of EGF on the cell proliferation and CDC25B expression were also investigated in epidermal keratinocytes. RT/real-time PCR and western blot method were used to analyze the CDC25B expression at the mRNA and protein levels, respectively.
*Results:* Indigo naturalis and indirubin were confirmed to down-regulate CDC25B expression significantly at both the mRNA and protein levels. The growth-dependent expression of CDC25B was demonstrated by the increased expression in serum-stimulated and immortalized keratinocytes. The activation of EGF receptor, known to be highly expressed in psoriatic lesions, was inhibited by indigo naturalis or indirubin. The cell proliferation and CDC25B expression of epidermal keratinocytes were induced by EGF alone and confirmed to be inhibited by indigo naturalis or indirubin.
*Conclusion:* Except being a common therapeutic target in various cancers, CDC25B also plays an important role in the hyper-proliferation of epidermal keratinocytes which can be suppressed by anti-psoriatic drug indigo naturalis and its component, indirubin.


## 1. Introduction

Psoriasis is a chronic inflammatory skin disease with a multifactorial genetic basis which affects approximately 2–3% of the population worldwide. It is characterized by hyperproliferation, aberrant epidermal differentiation of epidermal keratinocytes, dilated and hyperplastic dermal blood vessels, and infiltration of T cells into the epidermis resulting in the release of proinflammatory molecules [1]. Genetic studies have reported that patient's immune system might abnormally send out faulty signals and play a key role in the development of psoriasis. Psoriasis is not contagious, although the molecular etiology and genetics remain not yet fully understood [2,3].

Indigo naturalis (also known as Qing Dai in Chinese), prepared from leaves of plants such as *Baphicacavthus cusia*, *Polygonum tinctorium*, *Isatis indigotica* and *Indigofera tinctoria*, has long been used to treat various inflammatory disease and dermatosis [4,5]. Our previous clinical studies found that topical indigo naturalis ointment was a safe and effective therapy for plaque-type psoriasis [6,7]. We also demonstrated that indigo naturalis and one of its major components, indirubin, could inhibit the cell proliferation of cultured epidermal keratinocytes [8]. We have performed cDNA microarray analysis and results showed a profound change in gene expression profile of epidermal keratinocytes after indigo naturalis or indirubin treatment. The down-regulation of CDC25B in epidermal keratinocytes either by indigo naturalis or indirubin was revealed by cDNA microarray analysis.

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CDC25B is a member of the CDC25 family of phosphatases, the main gatekeepers responsible for the dephosphorylations that activate the cyclin-dependent kinases (CDK) at specific stages of the cell cycle [9]. Overexpression of CDC25B has been found in a significant number of tissue samples from various human cancers [10–12]. In addition, the anti-CDC25B autoantibodies detected in serum has recently been considered as a novel prognostic tumor marker [13]. Inhibitors of CDC25B could prevent hyperproliferation and even represent a promising therapeutic approach for cancer therapy [14,15]. Psoriasis, characterized by hyperperliferation of skin epidermal keratinocytes, is a cancer-like disease due to the loss of cell cycle control, may also potentially be treated by inhibitors of CDC25B.

Epithelial growth factor receptor (EGFR) is a major regulator of keratinocyte homeostasis within the epidermis that controls cell proliferation and migration. It has been reported that the EGFR and its endogenous ligands are significantly over-expressed in psoriatic lesions [16]. In addition, a multiplex cytokine assay also demonstrates a 10-fold increase of serum epithelial growth factor (EGF) in psoriatic patients compared with normal controls [17]. These findings suggest that the increased activation of EGFR pathway may contribute to the pathophysiology of psoriasis. The inhibition of the expression or activation of EGFR might be potentially useful to block the progression of psoriasis.

We first established an immortalized epidermal keratinocytes by E6E7 transfection in order to mimic the hyperproliferative property of cells in psoriatic lesion and used in our study. This study aimed to investigate the anti-proliferating effect of indigo naturalis and indirubin on inhibiting the activation of EGFR and EGF-induced CDC25B gene expression in epidermal keratinocytes.

## 2. Materials and methods

### 2.1. Cell culture

Human epidermal keratinocytes were isolated from skins of donors by enzymatic digestion method [18]. Skin samples were obtained with informed consent, under the approval of the Institutional Review Board of Chang Gung Memorial Hospital in Tao-Yuan, Taiwan. Briefly, skin was incubated in dispase (type II, 2.5 mg/ml in 1× PBS) for 2 h at 37 °C to dissociate the epidermis from dermis. The epidermal layer was then incubated in 0.25% trypsin for 30 min at 37 °C. Primary keratinocytes released after enzyme digestion were grown in KGM (Gibco, NY, USA) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. In the present study, keratinocytes were infected with recombinant E6E7 containing retrovirus and selected by G418. The established immortalized keratinocytes acquired highly increased proliferative capacity, properly served as an experimental cell model for the psoriasis study, were used for the experiments.

### 2.2. Extraction and preparation of indigo naturalis and indirubin

Indigo naturalis powder was purchased from Guang Sheng Trading (Taipei, Taiwan). The fingerprints and quantity analysis of standard samples of indirubin, indigo, and tryptanthrin were established and confirmed by Dr. Yann-Lii Leu at Department of Traditional Chinese Medicine, Chang Gung University, Tao-Yuan, Taiwan [8]. A voucher specimen (SF-1) is deposited in the herbarium of Chang Gung University. The powder of indigo naturalis was dissolved in dimethyl sulfoxide (DMSO) in a proportion of 1:10 (w/v), then sterilized by filtration (pore size 0.2 μm), and stored at −20 °C for experiments. Purified indirubin was purchased from Alexis (Lausen, Switzerland) and dissolved in DMSO to make stock solution.

### 2.3. MTT assay

Primary epidermal keratinocytes or immortalized keratinocytes were seeded in 24-well plates for MTT assay. Cells reached 60–70% confluency were used in experiments for 24 h incubation and cells at 20–30% confluency were used in experiments for 3 d incubation. EBM (0.5 ml) containing 0.05 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) purchased from Sigma (MO, USA) was added to each well and incubated for 2 h. After removing MTT solution, 0.5 ml DMSO was added into each well to extract the blue products by horizontal shaking. The cell viability was read at 570 nm with a reference filter of 630 nm.

### 2.4. RT/real-time PCR

Total RNA was extracted from keratinocytes by acid guanidinium thiocyanate–phenol–chloroform extraction method [19], and complementary (c)DNA was synthesized using 1 μg total RNA in a 20 μl RT reaction mix containing 0.5 μg/μl of random primers, 0.1 mM dNTP, 0.1 M DTT and 5× first strand buffer. Real-time PCR was performed using an SYBR Green I technology and MxPro-Mx3000P QPCR machine (Stratagene, CA, USA), and a master mix was prepared with Smart Quant Green Master Mix with dUTP & ROX Kit (Protech, Taipei, Taiwan). Relative gene expressions between experimental groups were determined using MxPro software (Stratagene, CA, USA) and GAPDH was used as an internal control. All real-time PCRs were performed in triplicate, and changes in gene expressions were reported as multiples of increases relative to the controls. The following primers were used: GAPDH: 5′-GAGGGGCCATCCACAGTCTT-3′ (forward) and 5′-TTCATTGACCTCAACTACAT-3′ (reverse), and CDC25B: 5′-CATCTCACCAGAAACGAT-3′ (forward) and 5′-CCGCCTTCATATTCATAG-3′ (reverse).

### 2.5. Western blot analysis

Cells were washed twice with 1× PBS and harvested in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF, and 1% Triton X-100) followed by sonication. Cell lysates were centrifuged at 13,000 × $g$ for 10 min, and the supernatants were retained. The protein concentration of the cell extracts was determined by the Bradford assay (Bio-Rad Laboratories, CA, USA). Protein extracts were separated by SDS-PAGE using 10% polyacrylamide gels and blotted onto polyvinylidenedifluoride (PVDF) membranes. Subsequently, membranes were blocked with 1% BSA and 1% goat serum in 1× PBS for 30 min at room temperature. After blocking, membranes were incubated with the primary antibodies for 2 h at room temperature, washed and further incubated with goat anti-mouse/rabbit immunoglobulin G (IgG) conjugated with HRP-conjugated secondary antibodies (Cell Signaling Technology, MA, USA) for 1 h. Antigen–antibody complexes were detected by ECL blotting analysis system (Millipore corporation, MA, USA). The following primary antibodies were used: EGFR antibody (Neo Marker, MI, USA), phosphorylated EGFR antibody (Cell Signaling Technology, MA, USA), CDC25B (Sigma, MO, USA), and tubulin (NeoMarker, MI, USA) was used as the sample loading control.

### 2.6. Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis of group differences was performed using Student's $t$-test. A value of $^{*}P < 0.01$ was considered statistically significant.