

POSITIVE ASSOCIATION BETWEEN STAT3 AND KI-67 IN CERVICAL INTRAEPITHELIAL NEOPLASIA

Sheau-Fang Yang,^{1,2} Shyng-Shiou F. Yuan,³ Yao-Tsung Yeh,⁴ Sz-Chi Hung,⁵
Ming-Tsang Wu,⁶ Jiniu-Huang Su,⁷ and Chee-Yin Chai^{1,2}

Departments of ¹Pathology and ⁷Obstetrics and Gynecology, Kaohsiung Medical University Hospital,

²Department of Pathology, Faculty of Medicine, College of Medicine, and

⁶Graduate Institute of Occupational Safety and Health, Kaohsiung Medical University,

³Department of Obstetrics and Gynecology, E-Da Hospital and I-Shou University,

⁴Department of Medical Technology, Fooyin University, and ⁵Department of Obstetrics and Gynecology, Kaohsiung Municipal Min-Sheng Hospital, Kaohsiung, Taiwan.

Signal transducer and activator of transcription 3 (STAT3) has been regarded as an oncogene in many types of cancers. However, its role in cervical carcinogenesis is not well determined. The purpose of this study was to evaluate the expression patterns of STAT3 in cervical intraepithelial neoplasia (CIN), normal cervix (NC), and squamous cell carcinoma (SCC) to explore its possible role in cervical carcinogenesis. Paraffin-embedded sections from 83 patients including 20 CIN 1, 10 CIN 2, 26 CIN 3, and 27 comparative cases of 10 NC and 17 stage Ib SCC were collected in this study. Immunohistochemistry was performed to analyze the expression patterns of STAT3, and the results obtained were categorized by a semiquantitative method and were further correlated with the CIN histopathologic grade and the proliferation marker, Ki-67, using the χ^2 test. Our results showed that nuclear STAT3 expression was predominantly in the squamous epithelial cells, and that high-grade CIN and stage Ib SCC lesions had a higher nuclear STAT3 expression when compared with NC and CIN 1. Furthermore, the nuclear STAT3 expression in CIN was significantly correlated with Ki-67 expression ($p = 0.025$), but not CIN lesion grade. In summary, our results indicate that an altered STAT3 expression in CIN is correlated with cell proliferation but may not have a direct contribution to cervical carcinogenesis.

Key Words: cervical cancer, cervical intraepithelial neoplasia, STAT3
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Cervical cancer is the second most common malignant disease among women worldwide, and is the principal cancer among women in most developing countries [1]. Although it has been considered to be a preventable cancer because of a long preinvasive state, cervical intraepithelial neoplasia (CIN) [2], the

underlying mechanisms of cervical carcinogenesis remain an important issue.

Signal transducer and activator of transcription (STAT) proteins comprise a family of transcription factors latent in the cytoplasm that consists of seven different members: STAT1, 2, 3, 4, 5A, 5B, and 6 [3]. These transcription factors are activated by a series of extracellular signaling proteins such as cytokines, growth factors, and hormones that bind to specific cell-surface receptors, and are consequently phosphorylated to form homo- or heterodimers allowing their translocation to the nucleus to influence different

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Address correspondence and reprint requests to: Dr Chee-Yin Chai, Department of Pathology, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan.

E-mail: cychai@kmu.edu.tw

normal physiologic cell processes including proliferation, differentiation, apoptosis, and angiogenesis [4], and also various pathologic events, such as cell transformation and oncogenesis [5]. Among these STAT family members, STAT3 is more often considered to be an oncogene [6] because STAT3 knockout mice die early in embryogenesis [7]. In addition, constitutively activated STAT3 is observed in a wide range of human cancer cell lines and primary tumors, including carcinoma of the head and neck [8], ovary [9], prostate [10], breast [11], etc. Although accumulating evidence shows that STAT3 pathway may contribute to cervical tumorigenesis [12,13], the potential role of STAT3 in the development of cervical cancer is not clinically established.

Ki-67 antigen is a nuclear protein, which is tightly associated with somatic cell proliferation [14]. Detailed cell cycle analyses have revealed that this antigen is expressed in all active phases of the cell cycle (G1, S, G2/M) but not in the resting phase (Go) [15,16], indicating that the Ki-67 antigen could be used as a marker for cells of the "growth fraction". In many cancers, antibodies against the human Ki-67 protein pave the way for the immunohistochemical assessment of cell proliferation [17]. In CIN lesions, Ki-67 labeling index in basal and parabasal layers of the cervix shows a progressive rise with increasing grade, suggesting that Ki-67 immunoquantitation is a sensitive biologic indicator of progression of seemingly low-grade CIN [18,19].

In the present study, we focused on examining the expression patterns of the activated form of STAT3, usually expressed in the nucleus, CIN lesions, normal cervix (NC), and cervical squamous cell carcinoma (SCC), and correlated the results with promising parameters to assess the potential role of STAT3 in cervical carcinogenesis.

MATERIALS AND METHODS

Surgical specimens

Between January 1996 and December 2002, 120 tissue samples of CIN were obtained from the Department of Pathology, Kaohsiung Medical University Hospital. For the purpose of this study, 56 CIN cases (mean age, 45.9 ± 11.8 years; range, 21–86 years) undergoing loop electroexcision procedure with free surgical margin were identified. In addition, another 10 cases with normal cervical tissue retrieved by total

hysterectomy for uterine leiomyomas, and 17 cases with International Federation of Gynecology and Obstetrics (FIGO) [20,21] stage Ib FIGO grade 2 SCC receiving radical hysterectomy without vascular invasion and metastases were chosen for comparison with the CIN cases. According to the WHO grading histopathologic classification [22], the 56 CIN cases were divided into three groups: 20 CIN 1, 10 CIN 2, and 26 CIN 3 cases. All the patients were followed up for 24–94 months, and remained free of persistent or recurrent lesions. Each tissue specimen was routinely embedded in paraffin wax after 10% formalin fixation, and cut into several 3 μ m thick sections for conventional hematoxylin and eosin staining and immunostaining. For accurate histopathologic diagnosis, repeated confirmations were made by two gynecologic pathologists.

Immunohistochemistry

Three micrometer thick sections from representative tissue blocks were cut, deparaffinized with xylene rinse and rehydrated into distilled H₂O through graded alcohol. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 15 minutes. Endogenous peroxidase activity was quenched by 10 minutes of incubation in a 3% hydrogen peroxide/methanol buffer. The slides were then incubated with primary rabbit polyclonal STAT3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:200 for 1 hour at room temperature. Slides were washed three times in phosphate buffer solution and further incubated with a biotinylated secondary antibody for 20 minutes at room temperature. Antigen-antibody complexes were detected by the avidin-biotin-peroxidase method using diaminobenzidine as a chromogenic substrate (DAKO Corp., Carpinteria, CA, USA). Finally, the slides were counterstained with hematoxylin and then examined by light microscopy. Ki-67 expression was assessed using purified antihuman monoclonal antibody (1:75; DAKO).

Evaluation of immunohistochemical staining

The percentage of immunoreactive dysplastic cells for STAT3 and Ki-67 was graded by a four-tiered semiquantitative system: Score 1, $\leq 25\%$ positive cells; Score 2, 26–50% positive cells; Score 3, 51–75% positive cells; and Score 4, $\geq 76\%$ positive cells (Figure 1). More than 1,000 cells expressed in three to four

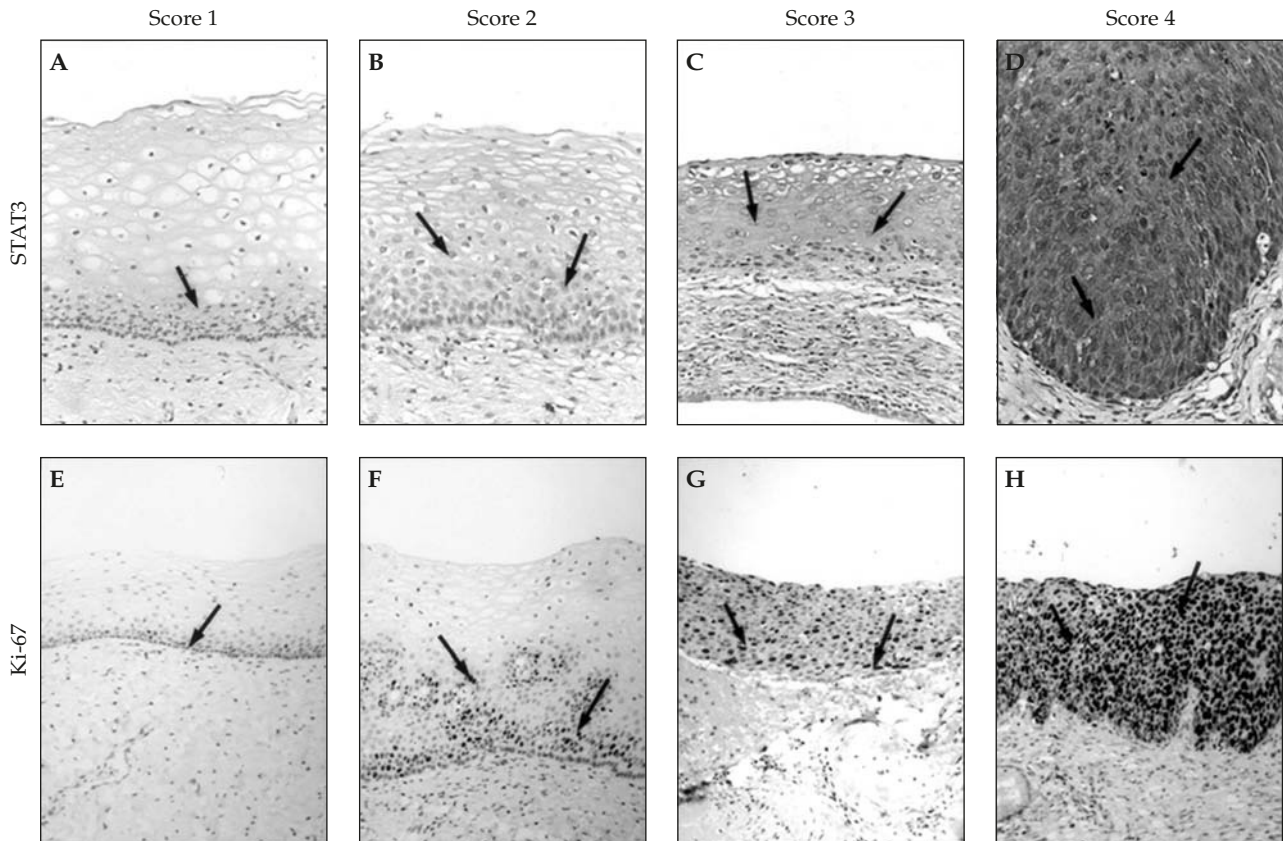


Figure 1. Scoring systems for signal transducer and activator of transcription 3 (STAT3) (A–D) and Ki-67 (E–H) immunostaining. Arrows indicate positive cells (original magnification, 200×).

different high-power field areas were analyzed for each sample. Sections of breast cancer tissue were used as a positive control for STAT3 immunostaining, while sections of tonsil tissue were used as a positive control for Ki-67 immunostaining. Negative controls were obtained by substituting the primary antibody with the immunoglobulin fraction of non-immune rabbit serum (for STAT3) and nonimmune mouse serum (for Ki-67). The immunostaining was determined for each specimen, estimated by two independent pathologists. The rare cases with discordant scores were reevaluated and scored on the basis of the consensual opinion.

Statistical analysis

STAT3 and Ki-67 staining statuses were classified as low (Scores ≤ 2) or high (Scores >2) expression before statistical evaluation. All analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The correlation of STAT3 expression with the histopathologic grading of CIN and with Ki-67 expression

was analyzed using the χ^2 test. A *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

Expression patterns of STAT3 and Ki-67 in CIN, NC, and SCC

The results from the immunohistochemical analysis are summarized in Table 1. The expression of STAT3 was predominantly present in the nucleus of squamous epithelium and leukocytes, and focally present in the cytoplasm of keratinocytes, leukocytes, and parenchymal cells (Figure 1A–D). In comparison with the adjacent normal epithelium and NC (Figure 3F), the nuclear expression of STAT3 was increased in CIN (Figures 2A, B and 3G–I) and SCC lesions (Figure 3J). In addition, a strong immunostaining (Score 4) of STAT3 was observed in 42.3% of CIN 3 cases and 23.5% of SCC cases, but was not observed in any comparative NC and CIN 1 cases (Table 1).

Table 1. Nuclear expression of signal transducer and activator of transcription 3 (STAT3) and Ki-67 in normal cervix (NC), cervical intraepithelial neoplasia (CIN), and cervical squamous cell carcinoma (SCC)

	NC (n=10) n (%)	CIN 1 (n=20) n (%)	CIN 2 (n=10) n (%)	CIN 3 (n=26) n (%)	SCC (n=17) n (%)
STAT3 (%)					
≤ 25	10 (100)	6 (30)	6 (60)	11 (42.3)	4 (23.5)
26–50	0 (0)	9 (45)	1 (10)	1 (3.9)	4 (23.5)
51–75	0 (0)	5 (25)	2 (20)	3 (11.5)	5 (29.4)
≥ 76	0 (0)	0 (0)	1 (10)	11 (42.3)	4 (23.5)
Ki-67 (%)					
≤ 25	10 (95)	19 (95)	5 (50)	2 (7.7)	0 (0)
26–50	0 (0)	1 (5)	3 (30)	1 (3.9)	2 (11.8)
51–75	0 (0)	0 (0)	2 (20)	6 (23.1)	4 (23.5)
≥ 76	0 (0)	0 (0)	0 (0)	17 (65.4)	11 (64.7)

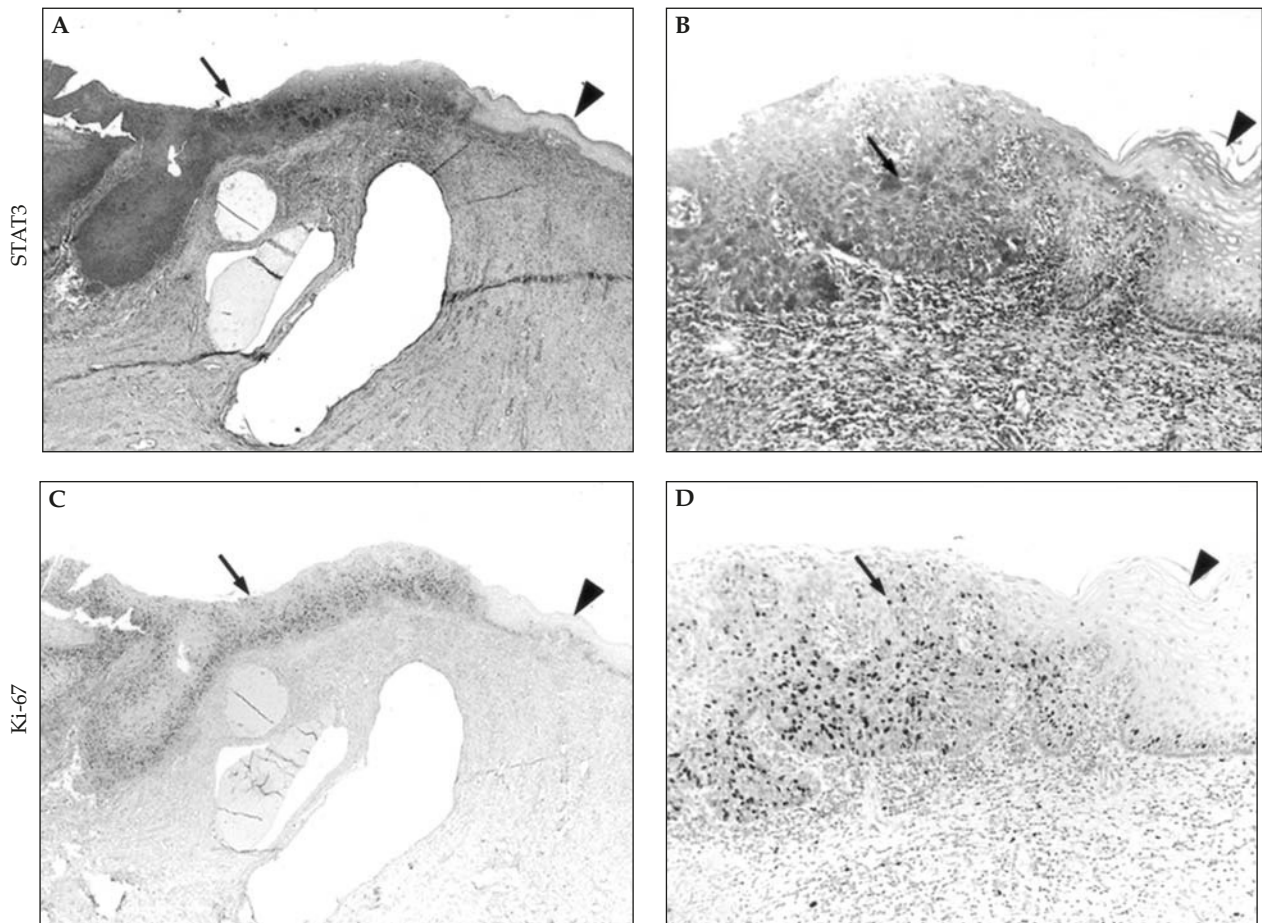


Figure 2. (A, B) Signal transducer and activator of transcription 3 (STAT3) and (C, D) Ki-67 immunostaining in cervical intraepithelial neoplasia 3 and adjacent normal cervical epithelium. The dysplastic cells (arrows) show an intense nuclear staining of STAT3 and Ki-67, while the adjacent normal squamous epithelium (arrowheads) shows relatively low nuclear STAT3 and Ki-67 staining (original magnification: A and C, 40 \times ; B and D, 200 \times).

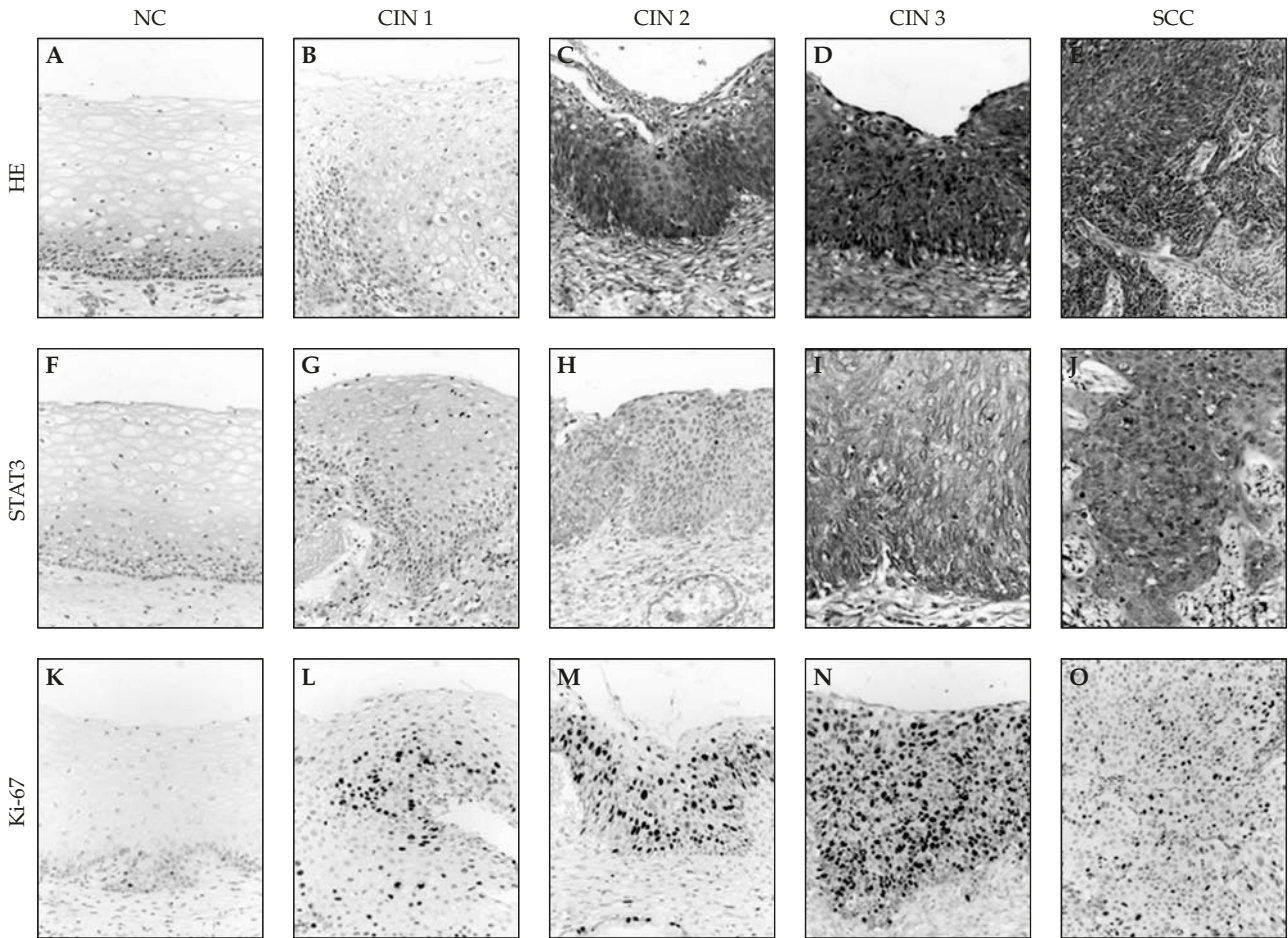


Figure 3. (A–E) Ordinary hematoxylin and eosin (HE) staining; (F–J) immunostaining of signal transducer and activator of transcription 3 (STAT3); and (K–O) Ki-67 in normal cervix (NC), cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) (original magnification, 200×).

Ki-67 staining was predominantly seen in the nuclei of the squamous epithelial cells (Figure 1E–H). As expected, with increased CIN histopathologic grading, the Ki-67 nuclear expression was increased and extended from the basal layer to the superficial layer (Figure 3L–N). The majority of CIN 3 and SCC cases exhibited strong Ki-67 expression (Table 1, Score 4), while none of the NC, CIN 1, and CIN 2 cases expressed strong Ki-67 nuclear expression (Table 1).

Correlation of nuclear STAT3 expression with the histopathologic grading of CIN and Ki-67 expression

Although an intense nuclear STAT3 immunostaining was present in the dysplastic cells from the basal layer to the superficial layer (Figure 3), and a high nuclear STAT3 expression (Score >2) was observed in 25%, 30%, and 53.9% of CIN 1, 2, and 3 lesions (Table 2),

Table 2. Correlation between nuclear signal transducer and activator of transcription 3 (STAT3) expression and cervical intraepithelial neoplasia (CIN) lesion grade

Lesion	n	High STAT3 expression (n = 22) n (%)	p*
CIN 1	20	5 (25)	0.112
CIN 2	10	3 (30)	
CIN 3	26	14 (53.9)	

* χ^2 test.

respectively, there was no statistically significant correlation between nuclear STAT3 expression and CIN lesion grade (Table 2, $p = 0.112$). In comparison with Ki-67 expression, we found that in 31 cases with high nuclear STAT3 expression, 20 and 11 cases revealed high and low nuclear Ki-67 expressions, respectively.

While in 52 cases with low STAT3 expression, 20 and 32 cases revealed high and low Ki-67 expressions, respectively. Intriguingly, nuclear STAT3 expression was significantly correlated with Ki-67 expression in CIN ($p=0.025$).

DISCUSSION

It has been demonstrated that introduction of STAT3 small interfering RNA (siRNA) into SCC cells leads to an inhibition of tumor growth, and STAT3 siRNA-transfected SCC cells had an impaired tumor growth in nude mice [23], implicating that an altered STAT3 expression may play an important role in cervical carcinogenesis. Consistently, in the present study, we also found that nuclear STAT3 expression was increased along with CIN lesion grade (Tables 1 and 2), although not reaching statistical significance (Table 2, $p=0.112$). Intriguingly, nuclear STAT3 expression was significantly associated with nuclear Ki-67 expression in CIN ($p=0.025$). Therefore, we suggest that an altered STAT3 expression might contribute to cervical cancer development through its association with cell proliferation.

CIN may be unchanged, regress to normal or to a lesser grade of CIN, or progress to a higher grade of CIN or invasive carcinoma [24]. In Figure 2, we show that the CIN lesions expressed a higher STAT3 nuclear expression than the adjacent normal cervical epithelium as well as the comparative NC, although no statistically significant correlation was observed between nuclear STAT3 expression and CIN lesion grade (Table 2, $p=0.112$). These results suggest that nuclear STAT3 expression, possibly activated STAT3, may be involved but does not play a critical role, at least in CIN development. Intriguingly, we found that the positive rate of squamous epithelial cells with strong STAT3 nuclear immunostaining increased from CIN 1 to CIN 3, but decreased in SCC lesions (Table 1), suggesting that an altered subcellular location of STAT3 may be an early alteration in cervical carcinogenesis as observed in SCC of the head and neck [25]. In addition, although we have previously demonstrated that the expression of phospho-STAT3 at serine residue 727 in CIN was significantly correlated with CIN lesion grade and cell proliferation [26], the expression of phospho-STAT3 (ser727) also decreased in stage Ib SCC as compared to CIN 3 (data not shown), implying

that other factors or mechanisms may be involved in the transition from CIN to cervical cancer; this requires further investigation.

In conclusion, our results indicate that the nuclear expression of STAT3 might partially imply a higher cell proliferation index but may not have a direct contribution to the progression of CIN. In addition, an altered STAT3 expression may be involved merely in the early events of cervical cancer development.

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子宮頸上皮內贅瘤中，STAT3 的表現 和 Ki-67 的表現呈現正相關

楊曉芳^{1,2} 袁行修³ 葉耀宗⁴ 洪思齊⁵

吳明蒼⁶ 蘇鈞煌⁷ 蔡志仁^{1,2}

高雄醫學大學附設醫院 ¹病理科 ⁷婦產部

高雄醫學大學 ²醫學院醫學系 ⁶職業安全衛生研究所

³義大醫院 婦產部

⁴輔英科技大學 醫事技術系科

⁵高雄市立民生醫院 婦產科

STAT3 (signal transducer and activator of transcription 3) 在很多的癌症中被認為是一種致癌基因，然而它在子宮頸癌的致癌機轉中究竟扮演何種角色仍屬未知。本研究的目的即在評估 STAT3 在子宮頸上皮內贅瘤 (CIN)、正常子宮頸及子宮頸癌中的表現量以探討其在子宮頸癌致癌機轉中所可能扮演的角色。本實驗共蒐集了 83 個病例，包括 56 個 CIN 的病例，其中有 20 個 CIN 1、10 個 CIN 2 和 26 個 CIN 3 病例，以及 10 個正常子宮頸和 17 個 Ib 期的子宮頸鱗狀細胞癌的病例一起做比較。這些病例的石蠟切片經 STAT3 的免疫組織化學染色後再用半定量的方法判讀紀錄染色的結果，其結果和 CIN 組織病理的分級以及常用來表示細胞增生的標記 Ki-67，以 χ^2 test 和 Fisher's exact test 做統計分析比較。結果顯示：STAT3 主要表現在鱗狀上皮細胞的細胞核上，且組織病理分級高的 CIN 和鱗狀細胞癌的表現量比正常子宮頸上皮和 CIN 1 的表現量為高。同時，STAT3 在細胞核的表現量和 Ki-67 在細胞核的表現量呈現有意義的正相關 ($p = 0.025$)，但其表現量和 CIN 的組織病理分級並無關聯性。我們的結果指出：STAT3 在 CIN 中的表現量多寡和細胞增生的程度有關，但其在子宮頸癌致癌機轉中可能沒有直接的貢獻。

關鍵詞：子宮頸癌，子宮頸上皮內贅瘤，STAT3

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通訊作者：蔡志仁醫師

高雄醫學大學附設醫院病理科

高雄市807三民區自由一路100號