

Original Article

Soluble fibrinogen-like protein 2 in *condyloma acuminatum* lesionsMei Zeng^{1,2}, Kang Zeng¹¹ Department of Dermatology, Nanfang Hospital, Southern Medical University, Guangzhou, China² Department of Dermatology, Huizhou Municipal Central Hospital, Huizhou, China**Abstract**

Introduction: An imbalance in type 1 (Th1) and type 2 (Th2) T helper cells is associated with the development and recurrence of *condyloma acuminatum* (CA); however, the mechanisms underlying this imbalance remain unknown. We investigated soluble fibrinogen-like protein 2 (sFGL2), which is an effector of regulatory T cells (Tregs), to determine its role in the Th1/Th2 imbalance of CA.

Methodology: Lesion and blood samples were obtained from 30 CA patients, and these samples were compared with genital skin and blood samples from 20 control subjects. Serum sFGL2, mRNA, and protein levels were estimated using ELISA, quantitative real-time polymerase chain reaction, and western blot, respectively. Immunohistochemical analyses were also conducted.

Results: Compared to controls, CA patients had decreased serum sFGL2 levels. However, sFGL2 mRNA and protein levels in the lesions of CA patients were significantly increased. Interleukin (IL)-4 and Foxp3 mRNA levels were upregulated, and interferon (IFN)- γ levels were downregulated in CA patients compared to those in controls. Additionally, the sFGL2 mRNA level positively correlated with Foxp3 and IL-4 levels and negatively correlated with IFN- γ and IL-17 levels.

Conclusion: sFGL2 contributes to the underlying Th1/Th2 imbalance in CA pathogenesis.

Key words: Soluble fibrinogen-like protein 2; Th1/Th2; *condyloma acuminatum*.

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Introduction

Condyloma acuminatum (CA) is a common sexually transmitted disease caused by the human papillomavirus (HPV). About 40 distinct types of HPV-related CA have been identified, while HPV types 6 and 11 account for more than 90% of cases [1]. Since the early 1980s, clinical identification of HPV infections has increased abruptly [2]. It is estimated that about one million new cases of CA are reported each year [3]. Although different therapies have proven successful for the treatment of CA, many issues remain unsolved, such as long latency, a high rate of relapse, and rapid viral growth. Additionally, persistent infection of HPV can lead to the development of various cancers, including anogenital cancers, such as in cervical, vaginal, penile, and anal [4]. Therefore, it is important to study the pathogenesis of CA in order to find more effective treatments.

Previous studies have demonstrated that the T helper (Th) type 1/2 cell imbalance plays an important role in the incidence and recurrence of CA [5,6]. However, the mechanisms underlying this imbalance remain uncharacterized. Moreover, CD4⁺CD25⁺ T regulatory cells (Tregs), which are a unique subset of

CD4⁺ T cells, require a forkhead box protein-3 (Foxp3) for their differentiation and development in the thymus [7,8]. The role of Tregs in CA is very important [9]. Tregs become decreased by Th1-type cytokines and increased by Th2-cytokines [7,10]. However, conflicting results have been reported [11].

Soluble fibrinogen-like protein 2 (sFGL2) is a member of the fibrinogen-related protein superfamily [12] and has two forms with distinct functions. The membrane protein form, FGL2 prothrombinase, exerts pro-coagulative activity [13] and promotes thrombosis [14]. The soluble form is constitutively expressed by the CD4⁺ and CD8⁺ T cells, is secreted by induced Tregs, and serves as a Treg effector. It also inhibits dendritic cell maturation, suppresses Th1 and Th17 immune responses, and enhances Th2 and Treg responses [15-17].

Previous studies have focused on the role of sFGL2 in pathogenic infections, miscarriages, transplant rejections, autoimmune disorders, and tumor formations [18], although none have examined its role in CA. Hence, we sought to evaluate the serum sFGL2 levels in CA patients and its associated protein and mRNA levels in their CA lesions.

Methodology

Patients and specimens

Thirty CA patients and 20 control subjects were enrolled, All the CA patients and control subjects had undergone analysis of the sFGL2 protein by western blotting (clinical characteristics of the CA patients are shown in Table 1). This study was approved by the hospital ethics committee (approval number: LLBA201805A), and informed consent was obtained from all patients or their guardians prior to their enrolment. All CA patients (20 males and 10 females; aged 16–53 years; 31.73 ± 9.03 ; median age: 31.5 years) were recruited from the Huizhou Municipal Central Hospital (Guangdong, China) and were diagnosed based on CA criteria [19]. All patients had no medical history of systemic antiviral drugs or immunomodulators for two weeks prior to enrolment. The control subjects (13 males and seven females; aged 14–39 years; 27.20 ± 7.51 ; median age: 28 years) had genital skin tissues surgically removed in the plastic surgery clinic of our hospital. Individuals with

autoimmune or severe systemic disorders or other infectious diseases were excluded from the study.

Lesion and skin collection

CA lesions (or normal genital skin samples) and 2.5 mL of venous blood were collected from patients (or the control subjects) when they received laser treatment (or the operation). The age ($t = 1.856$, $p = 0.07$)

and gender ($\chi^2 = 0.015$, $p = 0.903$) between the two groups were not significantly different.

Serum collection and enzyme-linked immunosorbent assay (ELISA) for serum sFGL2

Peripheral blood samples were clotted overnight at 4 °C, and then centrifuged for 20 minutes at 2000 ×g. Sera were collected and stored at –80 °C. Serum levels of sFGL2 were measured using ELISA kits and calculated using a standard curve (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

Table 1. Clinical characteristics of CA patients.

Participant Number	Gender	Age (years)	Site of the lesions	HPV genotype	Western (Yes/No)
1	F	35	Vulva	11	Yes
2	F	36	Vulva\vagina \cervical	11,16,45	Yes
3	F	36	Vulva	6,52,58	Yes
4	M	16	Urethra meatus	11	Yes
5	M	19	Perianal area\penis	11,68	Yes
6	M	21	Scrotum	11	Yes
7	F	40	Vulva/ cervical/ Perianal area	6,39,51	Yes
8	M	23	Perianal area	6,51,52	Yes
9	M	24	Perianal area	6,11	Yes
10	F	42	Vulva	6,11	Yes
11	M	24	Penis/ perianal area	6	Yes
12	M	25	Penis	6	Yes
13	F	50	Vulva/ cervical	6,11,52,53,56,66	Yes
14	M	26	coronal sulcus	6	Yes
15	M	26	Perianal area	11,45	Yes
16	M	27	Perianal area	11	Yes
17	M	27	Penis/ veneris	6,11	Yes
18	M	27	Penis/ veneris	6	Yes
19	M	31	coronal sulcus/veneris/ Foreskin/ perianal area	6,18,53	Yes
20	M	31	Penis	6	Yes
21	F	51	Perianal area /vulva	6,16	Yes
22	M	32	Perianal area	11,58,81	Yes
23	M	33	Perianal area	6,18	Yes
24	M	33	veneris	11,45	Yes
25	M	34	Scrotum/ coronal sulcus	6,56	Yes
26	F	53	Vulva\vagina/ Cervical	11	Yes
27	M	35	Perianal area	11,59	Yes
28	M	35	Perianal area	6	Yes
29	F	35	Vulva	6,45	Yes
30	F	25	Vulva/cervical	11,51,73	Yes

F: female, M: male.

Flow cytometry analysis for CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood

The number of CD4⁺CD25⁺Foxp3⁺ Tregs in the peripheral blood of the patients with CA and the control subjects was determined by flow cytometry at the Central Laboratory of Southern Hospital affiliated with Southern Medical University.

Immunohistochemical analyses of cutaneous sFGL2 and Foxp3

Formalin-fixed skin was embedded in paraffin and dewaxed in xylene. Sections were then incubated with peroxidase-blocking reagent and labeled with anti-sFGL2 (1:200 dilution, Abcam, Cambridge, UK) and anti-Foxp3 (1:400 dilution, Abcam, Cambridge, UK) antibodies at 4 °C overnight. Antibody binding for sFGL2 and Foxp3 was revealed by 3,3'-diaminobenzidine staining. The sFGL2 and Foxp3 expressions were scored semi-quantitatively using a volume density optical (OD × mm²) model analysis (Image-Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA) in 20 fields at 400× magnification.

qRT-PCR for measuring cutaneous sFGL2/interferon-γ/interleukin-4/Foxp3/interleukin-17 mRNA expression

Total RNA was purified using TRIzol reagent (Takara, Kusatsu, Japan) according to the manufacturer's instructions. RNA was transcribed into cDNA using First-Strand Synthesis SuperMix for qRT-PCR (Takara, Kusatsu, Japan). qRT-PCR was performed using the SYBR green quantitative kit (Takara, Kusatsu, Japan). After an initial denaturation (15 seconds at 95 °C), amplification was performed for 45 cycles (15 seconds at 94 °C and 45 seconds at 55 °C). All qRT-PCR testing was performed in triplicate. The specificity of the product was verified using the melting curve analysis. The qRT-PCR primer

sequences are listed in Table 2. The expression of mRNA was normalized to that of β-actin and was calculated against control genital skin tissues using the 2-ΔΔCt method [20].

Western blot analysis of the sFGL2 protein

Cellular proteins were solubilized in lysis buffer and quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). SDS-PAGE was performed using 12% glycine gels, loading equal amounts of proteins per lane. Following electrophoresis, separated proteins were transferred onto a polyvinylidene difluoride membrane and blocked with 5% non-fat milk. The membrane was incubated with antibodies against sFGL2 (1:2,000) and β-actin (1:1,000, Abcam) in 5% non-fat milk overnight at 4 °C and then with a goat anti-rabbit IgG monoclonal antibody conjugated with horseradish peroxidase. The semi-quantitative analysis results are expressed as optical volume densities (OD × mm²) and normalized to β-actin expression.

Statistical analyses

SPSS 17.0 statistical software package (IBM, Chicago, IL, USA) was used for data analyses. All data are presented as mean ± standard error (SE) or median (interquartile range). The two independent sample t (or t') test or Wilcoxon rank sum test were used to compare the continuous variables between two groups. Gender was tested by chi-square test. Correlations between two variables were calculated using Pearson product-moment correlation or Spearman's rank correlation test. A p-value of 0.05 to less was considered significant (two-sided).

Table 2. Primer sequences.

Gene name		Primer sequence: 5' to 3'
β-Actin	Sense	ACCGTGAGAAGATGACGCAGA
	Antisense	GCCAAGTCCAAACGAAGAATT
sFGL2	Sense	GCAGAGGGAAATGCGAAG
	Antisense	CTGCCTGGGTCTCCGTTG
Foxp3	Sense	CAAGTGCTTTGTGCGGGTG
	Antisense	TCGTCCATCCTCCTTTCCT
Interferon-γ	Sense	AGAAGGAAGATGATTGTGACG
	Antisense	AAACAGATGAATACCAGGCTA
Interleukin-4	Sense	CCCCTCTGTTCTTCCTGCTA
	Antisense	TGTCTGTTACGGTCAACTCGG
Interleukin-17	Sense	CACCCCGATTGTCCACCA
	Antisense	GTTTAGTCCGAAATGAGGCTGT

sFGL2: soluble fibrinogen-like protein 2; Foxp3: Forkhead box protein-3.

Results

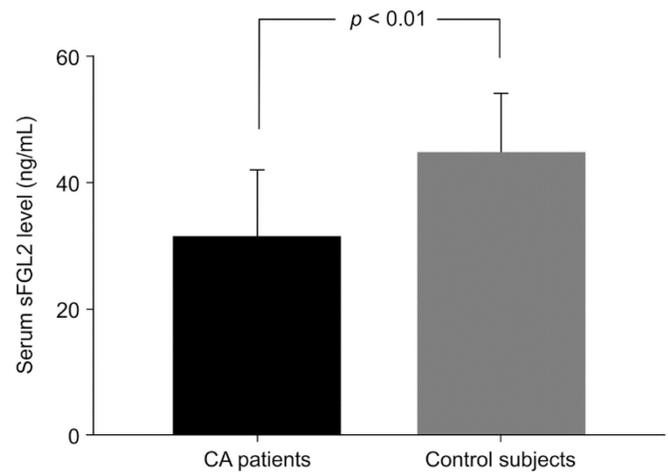
sFGL2 levels and CD4⁺CD25⁺Foxp3⁺ Tregs in the peripheral blood of CA patients and controls

The serum level of sFGL2 in patients with CA (31.05 ± 10.89 ng/mL) was decreased significantly compared to the control subjects (44.22 ± 9.85 ng/mL) ($t' = 3.693$, $p = 0.001$; Figure 1). However, the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the peripheral blood did not differ between the CA and control subjects (median 3.29% (interquartile range 3.63%) and median 4.42% (interquartile range 1.22%) respectively; $Z = -1.365$, $p = 0.172$).

sFGL2 and Foxp3 expression in CA lesions and control skin tissues

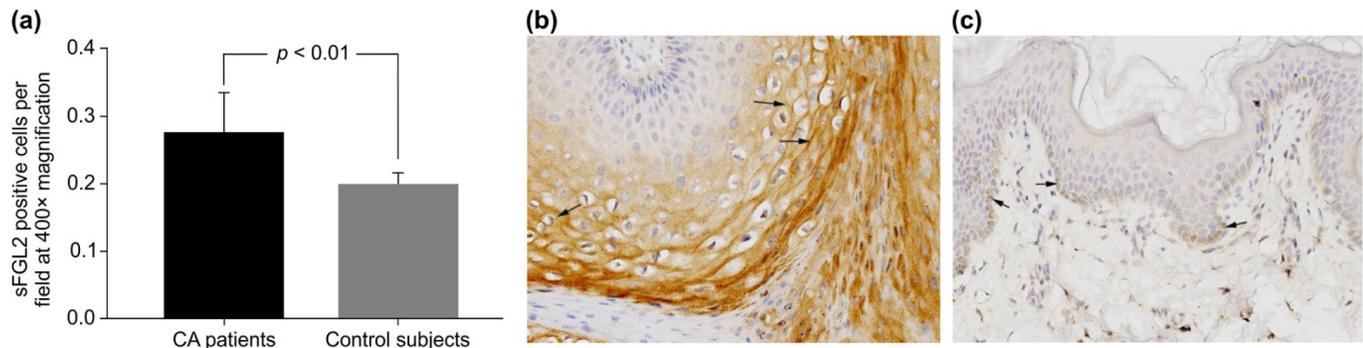
Immunohistochemical staining at 400× magnification showed that sFGL2-positive areas were present in the cytoplasmic compartments of infected epidermal cells. The relative expression of sFGL2 was

Figure 1. Serum soluble fibrinogen-like protein 2 (sFGL2) levels.



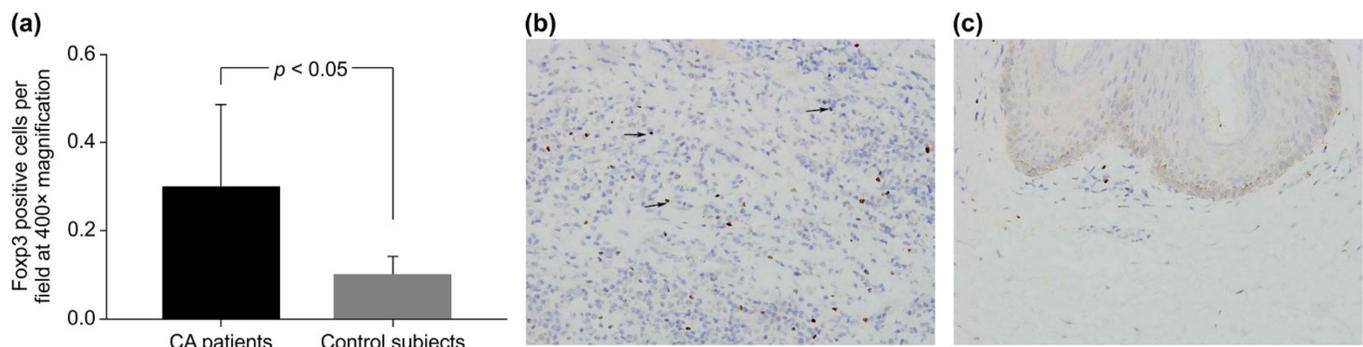
The serum sFGL2 level was significantly lower in the patients with condyloma acuminatum (CA) than in the control subjects ($t' = 3.693$, $p < 0.01$). Data are expressed as mean ± SD; $n = 30$ for CA patients; $n = 20$ for control subjects.

Figure 2. Serum soluble fibrinogen-like protein 2 (sFGL2)-positive cells.



Condyloma acuminatum (CA) lesions and control skin tissues were stained for sFGL2 using 3,3'-diaminobenzidine (DAB) and examined microscopically at a magnification of 400×. (a) The expression of sFGL2 was significantly higher in the CA patients than in the control subjects ($t = 3.605$, $p < 0.01$). Data are expressed as mean ± SD; $n = 30$ for CA patients; $n = 20$ for control subjects. sFGL2 expression was evident in the CA lesion (b) (400×; see arrows), while weak expression was observed in (c) the control skin tissues (400×).

Figure 3. Foxp3-positive cells.



Condyloma acuminatum (CA) lesions and control skin tissues were stained for Foxp3 using 3,3'-diaminobenzidine (DAB) and examined microscopically at a magnification of 400×. (a) The expression of Foxp3 was significantly higher in the CA lesion tissue than in the control skin tissue ($t = 2.283$, $p < 0.05$). Data are expressed as mean ± SD; $n = 30$ for CA patients; $n = 20$ for control subjects. Foxp3 expression was evident in the (b) CA lesion (400×, see arrows) while weak expression was observed in the (c) control skin tissue (400×). 3,3'-diaminobenzidine (DAB) staining was used for all experiments.

significantly higher in the patients with CA (0.27 ± 0.06) than that in the control subjects (0.20 ± 0.02) ($t = 3.605$, $p = 0.001$) (Figure 2a–c). Immunohistochemical staining at $400\times$ magnification showed that Foxp3-positive areas were present in the nuclei of infected epidermal cells. The relative expression of Foxp3 was significantly higher in the patients with CA (0.30 ± 0.19) than that in the control (0.10 ± 0.04) subjects ($t = 2.283$, $p = 0.048$) (Figure 3a–c).

sFGL2, IFN- γ , IL-4, Foxp3, and IL-17 mRNA levels in the CA lesions and the control skin tissues

The relative mRNA level of sFGL2 was significantly higher in the CA lesions (5.14 ± 2.47) compared to that in the control skin tissues (1.71 ± 1.74) as detected by qRT-PCR ($t = 4.291$, $p = 0.001$) (Figure 4). The levels of sFGL2 mRNA positively correlated with that of Foxp3 ($r = 0.622$, $p = 0.006$) and IL-4 ($r = 0.654$, $p = 0.003$) in the patients with CA (Figure 5a, b). The levels of sFGL2 mRNA negatively correlated with IFN- γ ($r = -0.817$, $p = 0.001$) and IL-17 ($r = -0.863$, $p = 0.001$) in the patients with CA (Figure 5c, d). The levels of IL-4 (2.73 ± 1.76) and Foxp3 (6.05 ± 2.67) mRNA were significantly higher in patients with CA than those in controls (1.53 ± 1.35 and 1.33 ± 0.98 , respectively) ($t = 2.436$, $p = 0.021$; $t = 6.075$, $p = 0.001$, respectively). The IFN- γ levels decreased significantly in the patients with CA (0.59 ± 0.21) compared to the control subjects (1.39 ± 1.01) ($t = -3.278$, $p = 0.003$). IL-17 level also decreased in the patients with CA (1.26 ± 1.06) compared to the control subjects (1.51 ± 1.21); however, this difference was not significant ($t = -0.612$, $p = 0.545$).

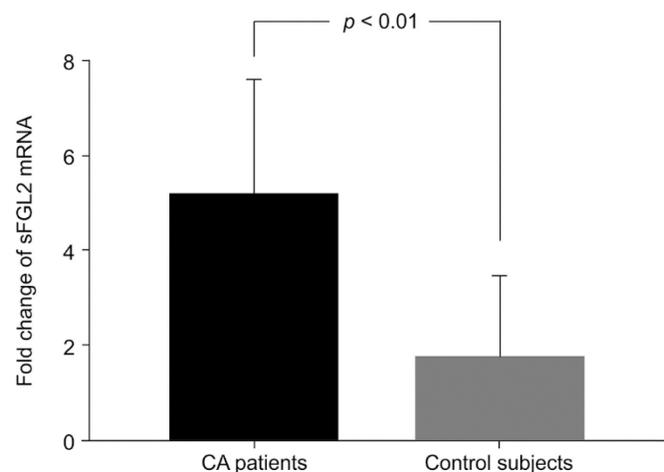
Cutaneous sFGL2 level

The results of the western blot analysis showed that the relative cutaneous sFGL2 protein level increased significantly in the patients with CA (0.47 ± 0.26) when compared with the control subjects (0.21 ± 0.15) ($t = -2.547$, $p = 0.018$) (Figure 6a, b).

Discussion

Recent studies on the pathogenesis of CA focused on the abnormal proliferation and apoptosis of keratinocytes [21,22], but our study mainly explores the immunological pathogenesis of CA. sFGL2, a novel immunoregulatory molecule, is secreted primarily by T cells, especially Tregs [18]. To our knowledge, this study is the first to investigate sFGL2 levels in patients with CA. Our results showed that, compared to the control subjects, the patients with CA had significantly increased cutaneous sFGL2 levels, whereas their serum

Figure 4. Serum soluble fibrinogen-like protein 2 (sFGL2) mRNA levels.



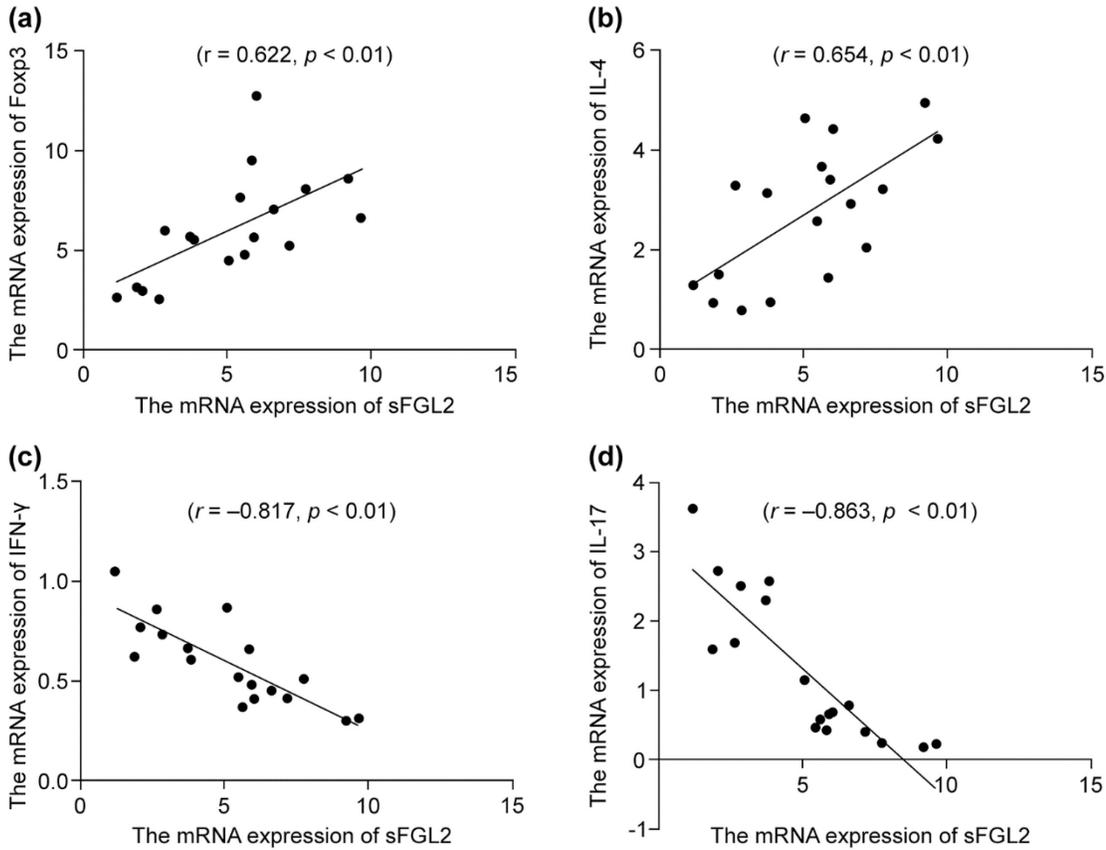
The sFGL2 mRNA level was significantly higher in the *condyloma acuminatum* (CA) lesions than in the control skin tissues as detected using quantitative real-time polymerase chain reaction (qRT-PCR) ($t = 4.291$, $p < 0.01$). Data are expressed as mean \pm SD; $n = 30$ for CA patients; $n = 20$ for control subjects.

sFGL2 levels decreased. We speculate that this is caused by the HPV infection stimulating sFGL2 secretion, thereby attracting Tregs to the skin leading to an increase in the cutaneous sFGL2 and a decrease in the serum sFGL2, which is consistent with a previous study [23].

The levels of Th1 cytokines are decreased while those of Th2 are increased in the patients with cervical intraepithelial and invasive neoplasia [24]. In our study, IL-4 mRNA levels were significantly higher while the IFN- γ levels were lower, in the CA patients compared to the control subjects; this is consistent with the results reported by Singh [25] and Xie *et al.* [26]. The changes in the IL-4, Foxp3, IFN- γ , sFGL2, and IL-17 levels and the correlations between sFGL2 and IL-4/Foxp3/IFN- γ suggests that an imbalance of Th1/Th2 occurs in patients with CA and that sFGL2 may play an important role in this imbalance. sFGL2 can cause polarization of Th1 to Th2, as shown by Ai *et al.* [27]. A Th2 polarized profile with decreased production of IL-2 and IFN- γ and increased production of IL-4 and IL-10 has been reported in sFGL2-treated allogeneic cultures [28]. In addition, downregulation of sFGL2 increases T cell proliferation, promotes Th1 cell polarization, and inhibits Treg activity [15,29].

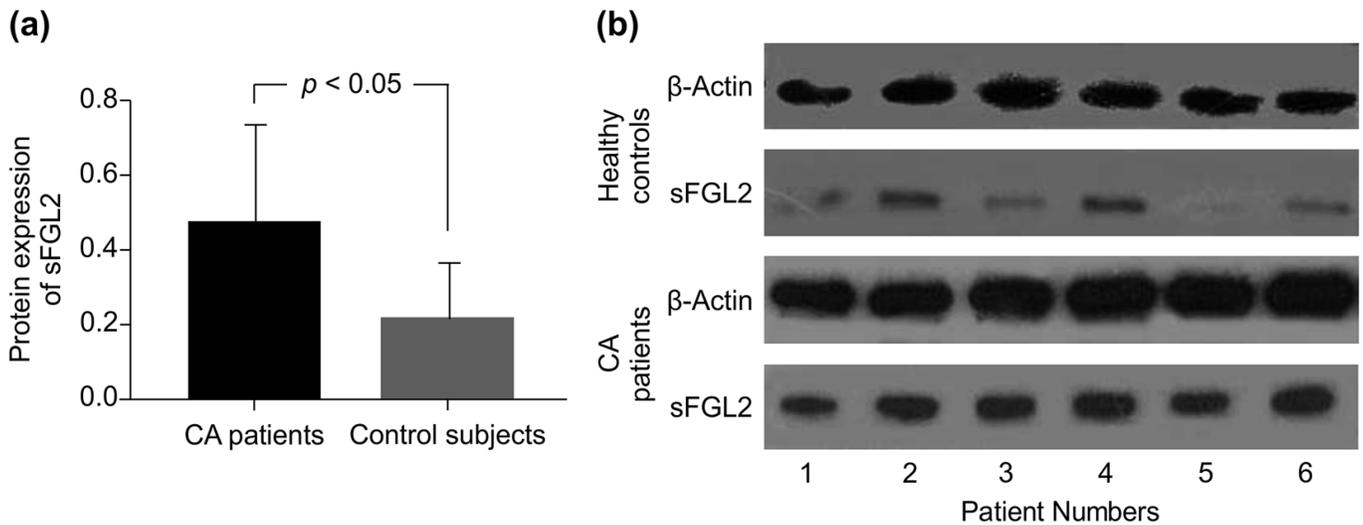
A previous study [5] reported that Foxp3-expressing cells were present in the infected epidermal site of patients with CA, and the number tended to increase in larger warts. Interestingly, we also found that the expression of Foxp3 was upregulated in the CA lesions.

Figure 5. Correlation of sFGL2 mRNA levels with IFN- γ , IL-4, Foxp3, and IL-17 mRNA levels in the CA lesions.



Serum soluble fibrinogen-like protein 2 (sFGL2) mRNA level positively correlated with that of (a) Foxp3 ($r = 0.622, p < 0.01$) and (b) IL-4 ($r = 0.654, p < 0.01$) in the patients with CA. The level of sFGL2 mRNA negatively correlated with that of (c) IFN- γ ($r = -0.817, p < 0.01$) and (d) IL-17 ($r = -0.863, p < 0.01$) in the patients with CA.

Figure 6. Expression of the soluble fibrinogen-like protein 2 (sFGL2) protein.



(a) The cutaneous sFGL2 level was increased significantly in the patients with *condyloma acuminatum* (CA) compared with that in the control subjects ($t = -2.547, p < 0.05$). Data are expressed as mean \pm SD; $n = 30$ for CA patients; $n = 20$ for control subjects. (b) Representative western blots of sFGL2 expression.

This may be due to the ability of HPV to create an immunosuppressive environment by recruiting immunosuppressive cell types, such as Tregs to the sites of infection [30]. In our study, the level of sFGL2 mRNA was positively correlated with Foxp3. In clinical settings, serum and tissue sFGL2 levels are a surrogate for Treg activity in patients with chronic viral hepatitis C [23]. Our results indicated that sFGL2 may also be a surrogate for Treg activity in patients with CA [31].

Previous studies have demonstrated that a high level of sFGL2 might be harmful to the host's immune integrity, and thus, may facilitate viral replication and expansion [18,32]. Our results revealed that cutaneous sFGL2 increased significantly in the CA patients, which may explain the high recurrence rate of CA as the increased sFGL2 may inhibit the overall immune system, thereby promoting replication and expansion of the virus and leading to an intractable status. Furthermore, sFGL2 may decrease the antiviral responses of the body and so, inhibiting its expression in tissues may strengthen the antiviral immune response [33]. Therefore, it may be possible to inhibit the replication and expansion of the HPV virus by antagonizing the expression of sFGL2 in patients with CA.

Conclusion

We detected Serum, mRNA, and protein levels of sFGL2 in 30 CA patients and 20 control subjects. The expression of sFGL2 increased in the CA lesions compared with the control tissue, and its expression correlated negatively with Th1 cytokines and positively with Th2 cytokines. This suggests that sFGL2 plays an important role in CA pathogenesis. However, this study was limited to the analysis of sFGL2 in clinical specimens without *in vitro* models; hence, further analysis on upstream and downstream pathways of sFGL2 are required.

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Authors' contributions

Conceived and designed the experiments, contributed reagents/materials/analysis tools: Kang Zeng. Performed the experiments, analyzed the data, wrote the paper: Mei Zeng.

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Corresponding author

Dr. Kang Zeng -- Medical Master Degree
 Department of Dermatology, Nanfang Hospital, Southern Medical University
 1838 Guangzhou Avenue North Road
 Guangzhou 510515, China.
 Tel: +86 02061641981
 Fax: 02061641989
 Email: npfkzk@126.com

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