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Efficacy of Poly-Gamma-Glutamic Acid in Women with High-Risk Human Papillomavirus-Positive Vaginal Intraepithelial Neoplasia: an Observational Pilot Study

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Poly-gamma-glutamic acid (γ-PGA) is a natural polymer that is synthesized by *Bacillus* species and has been reported to have antitumor activity. The aim of this study was to investigate the effect of γ-PGA on the treatment of vaginal intraepithelial neoplasia (VAIN). A retrospective observational study on γ-PGA therapy for biopsy-proven VAIN was conducted. The efficacy was assessed by evaluating the results of Pap cytology and the viral load of high-risk HPV at three time points: at enrollment, and at the first and second post-treatment visits. Of 17 patients treated with γ-PGA, only 12 patients who had a high-risk HPV infection were included in the analysis. Histology was VAIN1 in seven patients, VAIN2 in two patients, and VAIN3 in three patients. γ-PGA was administered for newly diagnosed VAIN in five (41.7%) patients and persistent VAIN in seven (58.3%) patients for the mean time of 4.5 months. At the first and second post-treatment visits, cytological regression was observed in five (41.7%) and six (50%) patients, respectively. Regarding the HPV load, the overall response rate was 66.7%, and the mean level was 670.6 ± 292.5 RLU at the first follow-up, which was lower than the initial viral load of 1,494.8 ± 434.5 RLU ($p = 0.084$). At the second follow-up, the overall response rate was 58.3%, and the mean viral load level was 924.2 ± 493.7 RLU. γ-PGA may be helpful for the cytological regression and reduction of viral load in patients with high-risk HPV-positive VAIN, suggesting that γ-PGA is a promising treatment option for primary or persistent VAIN.

Keywords: Poly-γ-glutamic acid, vaginal intraepithelial neoplasia, human papillomavirus

Introduction

Vaginal intraepithelial neoplasia (VAIN) is defined as squamous cell atypia without stromal invasion and is a premalignant condition that may develop into invasive squamous cell carcinoma of the vagina. Since the first report by Hummer in 1933 [3], the incidence of VAIN has been reported as 0.2–0.6 per 100,000 women, accounting for 0.4% of intraepithelial neoplasia of the lower genital tract [9, 18]. Although the true incidence of this rare disease is unknown, due primarily to underestimation, it is increasingly diagnosed because of a wider application of cytology screening and the liberal use of colposcopy. Multiple risk factors for cervical, vulvar, or anal neoplasia are closely associated with VAIN as well, such as older age, smoking, low socioeconomic status, multiple sex partners, early age of onset of sexual intercourse, immunosuppression, and a history of genital warts. Above all, the most powerful factors are a history of cervical intraepithelial neoplasia (CIN) or cancer and human papillomavirus (HPV) infection [14]. The development rate of subsequent VAIN after hysterectomy for CIN ranges from 0.9% to 6.8%, and the rate of high-grade VAIN increases to 7.4% in women who underwent hysterectomy for high-grade CIN [17]. In addition, a systemic review involving 232 VAIN cases showed that HPV prevalence was as high as 98.5% in VAIN1 and 92.6% in VAIN2 or VAIN3 [19].

Women between 40 and 60 years of age presenting with

postcoital spotting or unusual vaginal discharge should be evaluated for VAIN risk. However, as most patients are asymptomatic, diagnosis is greatly limited and usually made by abnormal Papanicolaou (Pap) smears taken from the vaginal vault with colposcopic assessment and biopsy [16]. Moreover, owing to the rarity of VAIN, conflicting results have been reported about not only diagnostic strategies and post-treatment surveillance but also optimal management. A broad range of therapeutic modalities include local excision, partial or total vaginectomy, laser ablation, topical application of imiquimod or 5-fluorouracil, cavitational ultrasonic surgical aspiration, and radiotherapy.

Poly-γ-glutamic acid (γ-PGA), a naturally occurring anionic polymer, is mainly produced by bacteria belonging to the genus *Bacillus* and consists of D- and L-glutamic acid units connected by γ-amide linkages between γ-carboxyl and α-amino groups [21]. Because of the beneficial characteristics of γ-PGA, such as the high solubility, safety, biodegradability, and non-toxicity, considerable interest has been recently focused on the availability of γ-PGA in various fields as a promising biomaterial. Our previous work performed in a mouse tumor model was the first study to demonstrate that the oral administration of γ-PGA displays antitumor activity by initiation of the innate immune response [11, 13]. In the present study, we aimed to investigate the clinical efficacy of γ-PGA in women with VAIN.

Materials and Methods

Study Participants

After approval by the institutional review board from the Korea University Guro Hospital, an observational pilot study was conducted. Data from patients who received γ-PGA treatment for histologically confirmed VAIN of any grade at the Korea University Guro Hospital from January 2012 to March 2013 were retrospectively reviewed. Study inclusion required that patients had eligible medical records regarding γ-PGA use, a positive result on HPV testing before the treatment, a minimum of 12 months of follow-up from the time of initial diagnosis, and at least one evaluation after the end of γ-PGA use.

Experimental Design

In a cervical screening, colposcopic assessment was performed for abnormal findings on Pap smear or positive results on HPV testing, or as otherwise clinically indicated. All patients in the present study underwent a physical examination, Pap smear, HPV testing, and punch biopsy at enrollment, followed by a Pap smear and HPV testing every 3–6 months and colposcopic examination, if indicated, after the diagnosis of VAIN. On histopathology, VAIN was graded according to the depth of epithelial involvement as follows: low-grade VAIN (VAIN1) and high-grade VAIN (VAIN2–3). If patients had multifocal lesions of different grades, the highest grade of VAIN was described as the final histologic diagnosis. γ-PGA was given under the conditions of either newly diagnosed VAIN or persistent VAIN. The latter was defined as abnormal results on Pap smear and HPV testing at least two consecutive times despite first-line treatment for initially biopsy-confirmed VAIN. As described previously [11], γ-PGA molecules were isolated from *Bacillus subtilis* var. *chungkookjang* derived from *chungkookjang*, a Korean traditional fermented soybean food, and provided by BioLeaders Corporation (Daejeon, Korea). A dose of 500 mg γ-PGA was orally administered twice daily for at least 3 months in a viscous liquid formulation.

Pap Smear and HPV Testing

Pap smear and HPV testing were carried out separately in a two-step process. First, cytology specimens were collected using a liquid-based system with a broom-type sampling device (SurePath, BD Corp., Franklin Lakes, NJ, USA). Slides were prepared based on the manufacturer's instructions and interpreted by cytopathologists according to the Bethesda 2001 System. Then, a modified soft, cone-shaped cervical brush (Cervical Sampler, Digene Corp.) was used to obtain samples from the cervix or vaginal vault for HPV testing by means of Hybrid Capture 2 (HC2; Digene Diagnostics, Gaithersburg, MD, USA). HC2 is an *in vitro* nucleic acid hybridization assay that can detect 13 high-risk types of HPV DNA (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Specimens containing the target DNA hybridize with a specific HPV RNA probe, leading to a reaction with alkaline phosphatase conjugated antibodies, which is detected with a chemiluminescent substrate. The emitted light resulting from the substrate split is measured by a luminometer, and the intensity of the light is described in relative light units (RLU), which allows for semiquantitative determination of viral DNA quantity in the specimen. RLUs less than the mean value of the positive cut-off (1 pg HPV DNA/ml) were considered to be negative. Colposcopic assessment was performed in the routine manner using saline and green filter application. The cervix and upper vagina were examined after the application of 5% acetic acid followed by Lugol's solution, and then gynecologists biopsied all lesions that were suspicious for VAIN or CIN.

Patients' demographic characteristics were reviewed, including age, previous or current morbidities, and medico-surgical history for the treatment of other anogenital neoplasias, such as CIN or cervical cancer, vulva intraepithelial neoplasia (VIN), or vulva cancer. The treatment effect of γ-PGA was assessed by comparing the initial Pap smear and HPV testing results with those performed at the first and second post-treatment visits, which were scheduled at 0–3 months and 6–12 months from the end of γ-PGA use, respectively. Cytological changes were classed as regression, persistence, or progression. Regression or progression was defined as an improvement or a worsening of at least one grade on Pap smear, and persistence was diagnosed when the follow-up was

the same cytology compared with baseline. For the analysis of the response to HPV infection, not only was the mean viral load calculated but the rates of cases assigned one of the following categories were also measured: complete response (negative on follow-up), partial response (more than 30% reduction in viral load), stable response (less than 30% reduction or increase), and progression (more than 30% increase).

Statistical Analysis

Continuous data were compared between baseline and follow-up using the Wilcoxon signed rank test. The SPSS package ver. 20.0 statistics software was used. A *P*-value of <0.05 was considered statistically significant.

Results

Study Patients

During the study period, 33 patients were diagnosed with VAIN. Of 17 patients treated with γ-PGA for VAIN, 12 who met the inclusion criteria were included in the analysis. The median age of the patients was 44 (range, 29–69) years (Table 1), and histologic diagnosis was VAIN1 in seven (58.3%) patients, VAIN2 in two (16.7%), and VAIN3 in three (25%). All five (41.7%) cases had a history of premalignant or malignant disease in the anogenital tract, and a medico-surgical history for those diseases consisted of cervical conization in three (25%) and concurrent chemoradiation with or without radical hysterectomy in two (16.7%). One patient had undergone a simple trachelectomy (cervicectomy) due to a high-grade lesion on the cervix that remained after hysterectomy for uterine leiomyoma. At the time of VAIN diagnosis, combined abnormalities of VIN with or without CIN was confirmed by colposcopic biopsy in two (16.7%) patients. The median duration of γ-PGA treatment was 3 (range, 1–9) months. Approximately half of the patients (41.7%) received γ-PGA as a first-line therapy for newly diagnosed VAIN, but the other half (58.3%) were treated with other treatment modalities, including radiation, laser therapy, and medication using topical cream or oral pills, in which single γ-PGA therapy was applied for the treatment of persistent VAIN.

Table 1. Basic characteristics of the VAIN patients.

Variables	*N* = 12
Age (years)	44 (29–69)
Biopsy-proven diagnosis	
VAIN1	7 (58.3%)
VAIN2	2 (16.7%)
VAIN3	3 (25%)
Previous history of anogenital neoplasia	
CIN2	1 (8.3%)
CIN3	1 (8.3%)
CIS	1 (8.3%)
Cervical cancer	2 (16.7%)
Medico-surgical history for anogenital neoplasia	
Cervical conization	3 (25%)
Trachelectomy	1 (8.3%)
Radical hysterectomy with CCRT	1 (8.3%)
CCRT	1 (8.3%)
Comorbidities of anogenital neoplasia	
VIN2 with CIN2	1 (8.3%)
VIN3	1 (8.3%)
Duration of γ-PGA use (months)	3 (1–9)
Indication of γ-PGA treatment	
Newly diagnosed VAIN	5 (41.7%)
Persistent VAIN	7 (58.3%)
First-line treatment in patients with persistent VAIN	
Radiation ± medication[a]	3 (25%)
Laser therapy + medication[a]	1 (8.3%)
Medication[a]	3 (25%)

Data are given as median (range) or case number (%).

VAIN, vaginal intraepithelial neoplasia; VIN, vulvar intraepithelial neoplasia; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; CCRT, concurrent chemo-radiation

[a]Combined therapy with topical imiquimod cream and systemic polysaccharide pills

Cytological Regression on γ-PGA Treatment

The initial cytology was atypical squamous cells of undetermined significance (ASCUS) in five (41.7%) and low-grade squamous intraepithelial lesion (LSIL) in seven (58.3%; Table 2) cases. Within three months from the end of γ-PGA treatment, Pap smears were performed in all patients except for one who was followed-up by HPV testing alone. The first post-treatment evaluation showed that cytological diagnosis was changed to normal in three cases (25%), ASCUS in three cases (25%), and LSIL in five cases (41.7%), which corresponded to cytological regression in 41.7%, persistence in 25%, and progression in 25%. At the second post-treatment visit, the cytology results were normal in five cases (41.7%), ASCUS in one case (8.3%), LSIL in three cases (25%), and high-grade squamous intraepithelial lesion in one case (8.3%), which coressponds to cytological regression in 50%, persistence in 8.3%, and progression in 16.7%, when compared to baseline cytologies. We excluded

Table 2. Initial and follow-up results of cervical Pap cytology.

Variables	*N* = 12
Initial cytology	
ASCUS	5 (41.7%)
LSIL	7 (58.3%)
Cytology at the first post-treatment visit	
Regression	5 (41.7%)
Persistence	3 (25%)
Progression	3 (25%)
Not determined	1 (8.3%)
Cytology at the second post-treatment visit	
Regression	6 (50%)
Persistence	1 (8.3%)
Progression	3 (25%)
Not determined[a]	2 (16.7%)

[a]Cytology test was not performed in one case, and additional treatment was performed for the progressive disease after the end of γ-PGA treatment in one case.
ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion.

a patient from the analysis of the second follow-up results because she received radiotherapy for VAIN2 after the first post-treatment evaluation

High-Risk HPV Viral Load Reduction on γ-PGA Treatment

Before γ-PGA treatment, the mean DNA load of high-risk HPV was 1494.8 (range, 9.1-5477.1) RLU (Table 3). At the first post-treatment visit, the mean viral load was 670.6 (range, 0-3263.36) RLU and tended to be lower than the initial viral load, which was of borderline statistical significance ($p = 0.084$). Meanwhile, the viral load at the second follow-up was 924.2 (range, 0-4970) RLU without a significant difference ($p = 0.328$). The rate of complete response, defined as disappearance of high-risk HPV, was 16.7% and 33.3% at the first and second follow-up, respectively. In addition, the overall response (complete and partial response) was shown in more than half of the patients at both first and second follow-ups (66.7% and 58.3%). Between the two groups of newly diagnosed VAIN and persistent VAIN, the overall response rate at the first follow-up was higher in the persistent VAIN group as follows: 60% (three of five cases) versus 71.4% (five of seven cases). Cytological response was well correlated with the change in viral load as six cases of cytological regression, one of persistence, and three of progression at

Table 3. Initial and follow-up results of HPV load, evaluated using the hybrid capture assay.

Variables	*N* = 12
Mean viral load (RLU)	
Before γ-PGA treatment	
Mean ± SE	1,494.8 ± 434.5
Median range	1,066.7 (9.1–5,477.1)
At the first post-treatment visit	
Mean ± SE	670.6 ± 292.5[a]
Median range	89.1 (0–3,263.36)
At the second post-treatment visit	
Mean ± SE	924.2 ± 493.7[b]
Median range	9.4 (0–4,970.6)
Viral load change at the first post-treatment visit	
Complete response	2 (16.7%)
Partial response	6 (50%)
Stable	1 (8.3%)
Progression	3 (25%)
Viral load change at the second post-treatment visit	
Complete response	4 (33.3%)
Partial response	3 (25%)
Stable	1 (8.3%)
Progression	3 (25%)
Not determined	1 (8.3%)

SE, standard error; RLU, relative light unit.
[a]In comparison with the initial viral load, $p = 0.084$ based on the Wilcoxon signed rank test.
[b]In comparison with the initial viral load, $p = 0.328$ based on the Wilcoxon signed rank test.

the second follow-up exhibited complete or partial response, stable response, and progressive response, respectively, in viral load.

Discussion

In the present pilot study, we detailed our experience with γ-PGA treatment in patients with HPV-positive VAIN. Our data showed that cytological regression was achieved in approximately half of the patients, and the overall response rate with respect to HPV infection was more than 50% until 6−12 months after γ-PGA use. To the best of our knowledge, this is the first study to evaluate the treatment efficacy of γ-PGA in patients with premalignant or malignant disease.

Although the natural course of VAIN has not been fully clarified, it is considered to be a premalignant disease that

can progress to invasive vaginal carcinoma in a range of up to 9% [1]. In addition, one of the largest studies that reviewed 127 cases of VAIN with a minimum follow-up of 12 months reported that overall 11% of patients experienced recurrence or persistence, regardless of dysplasia grade or treatment status [22]. Despite such a noticeable risk of recurrence or progression, the lack of consensus in relation to the optimal treatment of VAIN is evidenced by the various approaches taken by individual providers, which varies from expectant management to total vaginectomy. Although there have been no randomized controlled trials to compare treatment methods for VAIN, a recent meta-analysis concluded by reviewing articles published from 1948 to 2012 that all modalities involving vaginectomy, brachytherapy, laser ablation, and medication had good success rates for disease clearance of 45–100% [8]. Among these modalities, surgery is the cornerstone of treatment, particularly for high-grade VAIN, because it may produce longer disease-free survival [8] and allow histological confirmation of diagnosis. However, surgical treatment is related to various complications, such as vaginal scarring and consequent shortening or stenosis that may adversely affect sexual activity, as well as significant morbidities, such as genito-urinary fistula and the need for skin grafts. In addition, similar toxicities of brachytherapy have also limited the application of radiation as a first-line treatment option in spite of the high cure rate reported in several observational studies [20]. On the other hand, interest in medical management with topical agents (trichloroacetic acid, 5-fluorouracil, imiquimod, *etc*.) has recently been increasing owing to the advantages of low morbidity, easy application, wide coverage of multifocal lesions in the vagina, and acceptance of outpatient management. Nevertheless, this option still causes adverse effects, such as vaginal discharge, burning sensation, dyspareunia, and vaginal ulcers, and a high recurrence rate and an inconsistent cure rate among the retrospective studies are other issues [8].

In the current study, we used oral γ-PGA for the treatment of primary or persistent VAIN and found that the response rate was acceptable in terms of cytological regression and viral-load reduction. PGA products (BioLeaders Corpo., Daejeon, Korea) were approved as health functional foods by the Korean Food and Drug Administration in 2006, and determined to be generally safe as a food ingredient by the US Food and Drug Administration in 2010. Since γ-PGA was first discovered as a capsule of *B. anthracis* in 1937 [15], *B. subtilis* has been most widely investigated for biosynthesis of γ-PGA. In addition, a bacterium isolated from *chungkookjang*, *B. subtilis (chungkookjang),* has been recently reported as a powerful producer of γ-PGA that has a high molecular mass [2]. Moreover, γ-PGA can be used in various applications, and in medical fields, it has been studied as a drug delivery platform, biological adhesive, and tissue engineering agent [4]. However, the further role of γ-PGA seems to remain enigmatic, and little is known of its clinical efficacy in a practical setting. In a previous work, Kim *et al*. [11] showed that oral administration of high molecular mass γ-PGA had antitumor activity, generating significant natural killer cell-mediated cytotoxicity and interferon-γ secretion in a HPV-16 E7-expressing murine tumor model. In the next step, they first demonstrated that the mechanism underlying the antitumor activity was the initiation of the innate immune response mediated by Toll-like receptor 4 [13]. Finally, the current pilot study was designed to assess the clinical efficacy of γ-PGA in women with vaginal premalignant lesions. No data regarding this issue have been published to date.

Herein, we reported a cytological regression rate of 42–50% after an average period of 3 months of γ-PGA treatment. Meanwhile, data from retrospective studies on the natural history of VAIN showed a regression rate with no treatment of 75–87% in all grades of VAIN [1, 22] and up to 91% in low-grade VAIN [16]. In another study, however, the regression rate was only 39% in VAIN1 and 33% in VAIN2 [7]. Such a considerable disparity among published studies might be largely attributed to differences in defining regression, persistence, or progression, as well as to the patients' characteristics. In our study, seven of 12 women had an intractable VAIN that persisted even after a prior treatment, which may have adversely affected the response to γ-PGA therapy.

In addition to the difficulty in interpreting the cytological response, there is no clear recommendation for post-treatment surveillance. Traditionally, cytology and colposcopic examination have taken center stage in the diagnosis and follow-up of lower genital neoplasia, but recent evidence based on large cohort studies highlights the vital role of HPV testing. Systemic reviews documented that overall HPV prevalence in low- and high-grade neoplasia was 100% and 90.1% in the vagina, 84.1% and 92% in the cervix, 67.8% and 85.3% in the vulva, and 91.5% and 93.9% in the anus, respectively, indicating that HPV prevalence is consistently high through the entire lower genital tract [5, 10, 12]. In a similar fashion, a case-control study reported that HPV-positive patients were at 202 times increased risk for the development of VAIN than HPV-negative patients;

in particular, a higher viral load resulted in a 126 times increased risk [14]. Lastly, colposcopic examination is technically limited in VAIN cases owing to the anatomical structure of the vagina and the high proportion of the cases involving a history of prior treatment, such as radiation or radical hysterectomy. Thus, we believe that HPV testing would be meaningful and beneficial as a follow-up tool for detecting persistent or recurrent VAIN. A prospective study by Frega *et al*. [6] evaluated the diagnostic performance of cytology smear and HPV testing compared with colposcopic examination in 15 patients who were treated with CO_2 laser for high-grade VAIN. As a result, regression from positive to negative for HPV infection occurred in 47% and 15% at 6 and 12 months of follow-up, respectively, and thereby the accuracy for detecting persistent or recurrent VAIN had a sensitivity of 90% and specificity of 78%. Unlike their study, we divided the HPV testing results according to the extent of the change in viral load; hence, the complete and overall response rates seem to be acceptable: 17% and 33%, and 67% and 58% at 0−3 month and 6−12 months follow-up, respectively. Although the mean viral load was fairly lower at follow-up than the baseline, the difference was only of borderline statistical significance, possibly due to the small sample size. Furthermore, the present study did not provide evidence to answer important questions about the optimal dose and duration of γ-PGA use as well as the duration of effect on viral load, so further studies will be required to focus on these issues.

The present study has several limitations based on being a retrospective study. Because patients were not assigned to a placebo group, the efficacy of γ-PGA treatment could not be genuinely determined. Second, cytology and HPV testing results at follow-up were not compared with histologic diagnosis. In summary, given the improvement in cytology and HPV infection status, γ-PGA appears to be efficient for HPV-positive VAIN, suggesting that it can serve as a promising treatment option for primary or persistent VAIN. Although our data do not support the concept that γ-PGA can replace surgery, there may be a role for γ-PGA in selected patients, so it is necessary to confirm its potential effect for VAIN through further large prospective studies. Currently, we have initiated a phase II trial to determine the efficacy and safety of γ-PGA in patients with CIN1 compared with placebo (NCT01826045). The results of this trial are keenly anticipated and will be important in proposing further studies on γ-PGA treatment in patients with VAIN.

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