An evaluation of immune system cell infiltrate in the cervical stroma of patients with grade III cervical intraepithelial neoplasia after treatment with intralesional alpha-2B interferon

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Summary

The aim of this study was to characterize infiltrating immune cells in cervical stroma biopsy samples from patients diagnosed with cervical intraepithelial neoplasias (CINs) who were treated with IFN-α 2b. The authors studied 13 volunteers who were diagnosed with Cervical intraepithelial neoplasiaCIN II or III and who received intra-lesional treatment with IFN-α 2b. They collected pre- and post-treatment biopsies from each patient. They also examined the slides under a common optical microscope with a X400 lens for biopsy sample sections that were labeled with immunohistochemistry for T lymphocyte, B lymphocyte, natural killer cell, macrophage, iNOS, and perforin markers. The presence of immune response cells in the lesion was observed after treatment with intralesional IFN-α 2b in patients with CIN II/III changes, a reduction in CD4+ and CD8+ T lymphocyte infiltration in the women who responded well to treatment. However, there was a significant increase in these markers in samples from women who did not respond to treatment. Nonetheless, immunotherapy with IFN-α 2b administered intralesionally in patients with CIN II/III yields favorable results in patients who do not smoke.

Key words: Cervical intraepithelial neoplasia; Immunotherapy; Immune response.

Introduction

Approximately 500,000 women are diagnosed with cervical cancer worldwide every year, and more than 54% of them go on to die from it [1,2]. Diagnosis is most common in adolescents and in women between 45 and 50 years of age, although lesion regression is more common in adolescents, who show a regression rate of 90% [3]. Cervical cancer is associated with human papillomavirus (HPV) infection, the main oncogenic types of which are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 [4]. In Brazil, there are 24,562 cases of cervical cancer per year, with the common HPV serotypes 16 and 18 accounting for 71% of the cases [5]. Indeed employing modern molecular biology techniques, researchers have found that ~90% of cervical cancer biopsies contain HPV viral DNA [6].

Cervical cancer can develop from cervical intraepithelial neoplasia (CIN) lesions. CIN lesions are subdivided into grades 1, 2, and 3, corresponding to mild, moderate, and severe dysplasia, respectively [7]. In CIN I, the dysplasia is confined to the basal third of the epithelium. In CIN II, the dysplasia affects the basal two-thirds of the epithelium, and CIN III is a squamous intraepithelial lesion in which changes to maturation and nuclear anomalies affect more than two-thirds of the epithelium's thickness.

CIN II/III is commonly treated with surgical excision of the lesion by conization (i.e. cold knife conization or loop electrosurgical excision procedure). However, various studies have demonstrated that pregnant women who previously received conization have a higher risk of pre-term delivery. Moreover, stenosis of the cervical orifice after conization can make it difficult for women to conceive [8]. Therefore, new clinical therapies are being studied with the goal of minimizing surgical intervention to maintain the anatomy and physiology of the cervix. Promising results have been reported from studies of immunotherapy with interferon (IFN)-α to treat invasive and intraepithelial lesions of the cervix [9].

A study developed by Ramos *et al.* [10] demonstrated that 60% of the patients who were treated with IFN-α-2b responded well to treatment, and that responders had greater expression of Th1 cytokines (IFN-γ, tumor necrosis factor [TNF]-α, and interleukin [IL]-2) in the cervical stroma than non-responders. Misson *et al.* [11] further found that patients with a good clinical response to IFN-α-2b treatment had elevated blood levels of IL-12.

An efficacious immune response to HPV impedes the infection's persistence, which is attributed to the virus' ability to evade the immune system [4]. The virus infects the basal bed of the epithelium, a location that is difficult for antigen-presenting cells to reach, and because HPV infection does not cause lysis of keratinocytes, the local inflammatory response is diminished. Moreover, the irregular replication cycle of HPV results in antigen presentation difficulties in secondary lymphoid organs [6].

Cellular immunity plays a key role in the local response to HPV. Inflammatory infiltrate of macrophages and CD4+ T cells can be observed in condilomas that spontaneously

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regress. The lympho-proliferative response of CD4+ T cells specific to the E2 antigen is associated with HPV elimination. Conversely, CD8+ T cells specific to the E6 and E7 antigens are found in patients with large lesions or cervical tumors [12]. Antigen presentation is compromised by the presence of viral capsids, which impede the activation of Langerhans cells [13].

Understanding the mechanisms and cells involved in the immune response that leads to CIN regression is fundamental to the ability to develop new therapies. Therefore, the objective of this study was to elucidate the composition of infiltrating cells (i.e., proportions of total T lymphocytes, T helper, T cytotoxic, B lymphocytes, natural killer (NK) cells, and macrophages) and the presence of the immune response mediators perforin and nitric oxide in CINs of patients treated with IFN-α 2B. Observations were compared between treatment responders and non-responders.

Materials and Methods

Setting and patients

A prospective study was carried out at the Maria da Glória outpatient clinic of the Hospital School of the *Federal University of the Triângulo Mineiro* in the discipline of Gynecology and Obstetrics from 2007 through 2009. The group studied consisted of 13 patients, 23–50 years of age, with diagnoses of CIN II or III who had not yet received any treatment. Patients provided information about their age, habits, lifestyles (e.g., smoking, use of drugs, number of sexual partners), contraceptive methods used, history of sexually transmitted diseases, and use of hormone replacement therapy.

All procedures were performed in accordance with the criteria developed by the Ethics Committee (CEP/UFTM Nos. 759 and 1525). The inclusion criteria were: absence of bleeding during the examination; no use of oral antibiotics, vaginal fungicides or creams in the previous 30 days; no sexual activity for two days preceding sample collection; no previous history of treatment for HPV. Colposcopic change is greated than one cm. The exclusion criteria were: immunosuppressant diseases, serious cardiopathies, changes in liver or kidney function, pregnancy, a reported intolerance to IFN, or an absence of a visible lesion at colposcopy.

Application of IFN

Human recombinant IFN-α 2b was used for the therapy. The IFN-α 2b was applied intralesionally at a dose of 3,000,000 IU (flask-ampoule with lyophilic powder diluted in 1.0 ml of diluent before each application). The applications were performed using a 1.0-ml syringe with a 13 × 0.45 needle and administered three times per week on alternate days until a total of 18 applications were delivered.

A vaginal speculum was used to expose the cervix. The medication was applied following antisepsis of the cervix and the vaginal walls with gauze soaked in topical povidone using Sheron forceps. After the first, sixth, 12th, and 18th application of IFN-α 2b, peripheral blood was collected from a vein in the right forearm of each patient.

Evaluation of clinical response

Patients were grouped according to treatment response based on colposcopic examination and histological observation of biopsies. If colposcopy showed disappearance or regression of the lesion and this observation was corroborated by histological examination of the patient's biopsy demonstrating regression to CIN I or no CIN, the treatment was considered successful, and the patient was assigned to the good response group (GR). If no regression of the lesion was observed at colposcopic examination, and the persistence of CIN II/III was confirmed by histological examination of the patient's biopsy, then the treatment was considered to have failed, and the patient was assigned to the bad response group (BR). All patients with persistent CIN II and III were submitted immediately to cold knife conization. The patients were submitted to follow-up with cytology and colposcopy every six months.

Immunohistochemical methods

The biopsy specimens were paraffinized and then four-µm-thick histological slices were cut, mounted on silanized slides, and then subjected to immunohistochemical staining with a polymer detection kit. To characterize immune cell infiltrates and identify cervical stroma markers in biopsy samples, the authors used antibodies specific to the following proteins: CD3, CD8, CD4, CD20, CD68, CD16, perforin, and induced nitric oxide synthase (iNOS).

The slide-mounted slices were held in an incubator at 56 ºC for 24 hours and then deparaffinization in three five-minute submersions in xylol. The tissues were then hydrated in three baths of absolute alcohol and one bath of 80% alcohol, for about ten seconds each. The slides were then hydrated in phosphate buffered saline solution (PBS, pH 7.2) for five minutes. After removal of excess PBS from the slides, drops of 3% oxygenated water were placed on each slice for ten minutes to block endogenous peroxidase activity. After blocking, the slides were washed again in PBS.

The authors performed antigen retrieval using a Pascal pressurized chamber. Briefly, they added 45 ml of buffer (for CD4 TRIS/EDTA, pH 9.0; for all others citrate, pH 6.0) to the chamber and subjected the slides to three minutes of boiling followed by at least 30 minutes of recovery to allow the chamber's pressure and temperature to decrease. After antigen retrieval, they again washed the slides in PBS buffer three times, five minutes per wash before incubating the slices with primary antibody. Each primary antibody was diluted in buffer with 10% bovine serum albumin to the concentration recommended in the manufacturer's specifications as follows: CD3 [1:3.200], CD8 [1:200], CD4 [1:300], CD20 [1:6.000], CD68 [1:3.000], CD16 [1:50], perforin [1:100], and iNOS [1:500]. The slides were incubated with primary antibody for about 18 hours in a wet chamber at 3–4 ºC.

Following the primary antibody incubation period, the slides were allowed to warm to room temperature for about 15 minutes. The slides were then washed in PBS and dried. The authors applied post-primary penetration enhancing reagent from the polymer detection kit to each slide, and maintained the slides at room temperature for 30 minutes in a moist chamber. They washed the slides three times, five minutes per wash, in PBS and then added the polymer reagent (also from the kit) and allowed the slices to incubate with the polymer reagent at room temperature for 30 minutes in a moist chamber. After three five-minute washes in PBS, labeling was visualized by exposing the slides to a chromogenic solution for five minutes. The authors then rinsed the slides in tap water and dipped them in Harris's hematoxylin for two seconds to add a nuclear counterstain. Finally, they immersed the slides in three baths of absolute alcohol for around ten seconds each to remove excess water, one bath of phenicated xylol to remove excess alcohol, and three baths of xylol for five minutes each to clarify the slides (diaphanization). The stained specimens were covered with coverslips and viewed under a light microscope.

Table 1. — *Clinical characteristics, histological diagnosis of biopsy, and IFN treatment response by case.*

Patient	Age (years)	Smoker	Parity	Initial diagnosis	Final diagnosis	IFN treatment outcome
1	30	No	4	CIN II	HPV infection	Response
2	50	No	3	CIN II	CIN I	Response
3	23	No	0	CIN III	CIN I	Response
4	36	Yes	7	CIN III	HPV infection	Response
5	23	No	4	CIN II	HPV infection	Response
6	28	No	0	CIN II	HPV infection	Response
7	31	Yes	1	CIN II	CIN II	Failure
8	25	Yes	4	CIN II	CIN III	Failure
9	38	Yes	3	CIN III	CIN II	Failure
10	45	Yes	5	CIN III	CIN III	Failure
11	30	Yes	1	CIN III	CIN II	Failure
12	48	Yes	2	CIN III	CIN III	Failure
13	34	No	0	CIN II	CIN II	Failure

Immunohistochemical analysis

The authors evaluated pre- and post.treatment cervical stroma biopsy specimens for total population of T lymphocytes ($CD3^+$), T helper lymphocytes ($CD4^+$), T cytotoxic lymphocytes ($CD8^+$), B lymphocytes ($CD20^+$), NK cells ($CD16^+$), macrophages ($CD68^+$), and the presence of iNOS-expressing cells and perforin. Initially, they observed the cells at low magnification (×100) to evaluate their general distribution. Next, they examined them more closely (×400 magnification); for pre-treatment biopsies, they examined the subjacent stroma below the CIN II or CIN III lesion and for post-treatment biopsies, they examined the CIN or HPV infection site to obtain a final score. Lymphoid cell quantity was scored on a 0–3 scale as follows: 0, absence of inflammatory cells; 1, sparse inflammatory cells; 2, a moderate number of inflammatory cells; and 3, numerous inflammatory cells [14].

Statistical analysis

An electronic database was developed for the statistical analysis. The variables were analyzed using GraphPad Prism 5.0 software. The values were submitted to Student's *t* test. The differences were considered statistically significant at $p < 0.05$.

Results

The mean age of the 13 patient participants was 33.9 ± 9.1 years (range, 23–50). The age, parity, smoking status, initial and final diagnoses, and clinical response to treatment of each patient are summarized in Table 1. Overall, about half (6/13; 46.15%) of the patients were multiparous (≥ two births) and about half (7/13; 53.85%) were smokers. At initial diagnosis, 7/13 patients (53.85%) had CIN II and 6/13 (46.15%) had CIN III.

The authors found that 6/13 patients (46.15%) responded well to treatment with IFNα-2b; conversely, 7/13 patients (53.85%) had therapeutic failure. Of the six patients who responded well to the treatment, four (66.67%) were multiparous, and five (83.3%) were non-smokers (Table 1). Of the seven patients whose therapy failed, four (57.14%) were multiparous and only one (14.28%) was a non-smoker.

Relative to their pre-treatment assessments, the GR patients showed shifts toward greater expression (from 0–1 to 2–3 on the Georgiannos protocol) of the CD3 and CD68 markers following treatment with IFN-α 2b (Table 2). Expression of iNOS was stable from pre- to post-treatment in the GR patients, but two BR patients' Georgiannos biopsy scores went from 2–3 pretreatment to 0–1 posttreatment (Table 2). The GR and BR patients showed opposite pre- to post-treatment patterns in CD4 (Figure 1) and CD8 (Figure 2) positivity. BR patients showed a strong trend ($p = 0.05$) toward greater expression of the CD4 marker post-treatment, relative to pre-treatment. And post-treatment, we observed a weak trend ($p = 0.09$) of BR patients having greater CD4 marker expression than GR patients. Meanwhile, GR patients showed a strong trend ($p = 0.056$) toward a reduction in expression of the CD8 marker from pre- to post-treatment (Figure 2). In post-treatment, the authors observed significantly lower CD8 expression in GR patients than in BR patients ($p = 0.03$). They did not observe evidence of treatment effects on expression of CD16, CD20, iNOs, and perforin (Table 2).

Discussion

In this study, the authors observed that 6/13 patients (46.15%) had responded to treatment with IFN-α 2b as evidenced by a disappearance of the high-grade lesion. The remaining seven patients (53.85%) experienced therapeutic failure, meaning that they observed high grade squamous intraepithelial lesion (HSIL) in the initial and final diagnoses.

Since the early 1980s, and particularly in the last decade, numerous studies examining IFN therapies for treatment of gynecological cancers have achieved varying responses [15, 16]. IFN's actions, both as an antiproliferative and as an immunoregulatory, are now a focus of interest for many investigators. In studies with IFN-α, CIN remission rates vary from 30% to 80% [17-19]. However, Byrne *et al.* [20], using a topically applied IFN-α gel on CINs, and Frost *et al.* [21], applying IFN-α 2b intralesionally, obtained results

Table 2. — *Analysis of distribution of expression of cellular markers and products in GR and BR patients.*

Marker	Score	Good response		Poor response	
		Before x/n (%)	After x/n (%)	Before x/n (%)	After x/n (%)
CD3	0 - 1	4/6 (66.7)	2/6 (33.3)	4/7 (57.1)	4/7 (57.1)
	2 - 3	2/6 (33.3)	4/6 (66.7)	3/7 (42.9)	3/7 (42.9)
CD4	0 - 1	3/6 (50.0)	4/6 (66.7)	6/7 (85.7)	2/7 (28.6)
	2 - 3	3/6 (50.0)	2/6 (33.3)	1/7 (14.3)	5/7 (71.4)
CD8	0 - 1	1/6 (16.7)	4/6 (66.7)	5/7 (71.4)	2/7 (28.6)
	2 - 3	5/6 (83.3)	2/6 (33.3)	2/7 (28.6)	5/7 (71.4)
CD16	0 - 1	5/6 (83.3)	4/6 (66.7)	5/7 (71.4)	6/7 (85.7)
	2 - 3	1/6 (16.7)	2/6 (33.3)	2/7 (28.6)	1/7 (14.3)
CD20	0 - 1	4/6 (66.7)	5/6 (83.3)	5/7 (71.4)	5/7 (71.4)
	2 - 3	2/6 (33.3)	1/6 (16.7)	2/7 (28.6)	2/7 (28.6)
CD68	0 - 1	3/6 (50.0)	2/6 (33.3)	4/7 (57.1)	2/7 (28.6)
	2 - 3	3/6 (50.0)	4/6 (66.7)	3/7 (42.9)	5/7 (71.4)
iNOS	0 - 1	4/6 (66.7)	4/6 (66.7)	4/7 (57.1)	6/7 (85.7)
	2 - 3	2/6 (33.3)	2/6 (33.3)	3/7 (42.9)	1/7 (14.3)
Perforin	0 - 1	6/6 (100)	6/6 (100)	6/7 (85.7)	6/7 (85.7)
	2 - 3	0/6 (0.0)	0/6 (0.0)	1/7 (14.3)	1/7 (14.3)

similar to a placebo. Dunham *et al.* [18] performed a study on 14 patients with CIN, seven of which were controls and seven of which were treated twice a week for a month with intralesional injections of IFN-α 2b. They observed an improvement in six out of seven patients in the study group, including two complete cures. Only 3/7 patients in the control group showed spontaneous improvement, but there were no complete cures. Koilocytosis, a cellular characteristic of HPV infection, disappeared in all of the study group cases treated, but two cases of the control cases. Both

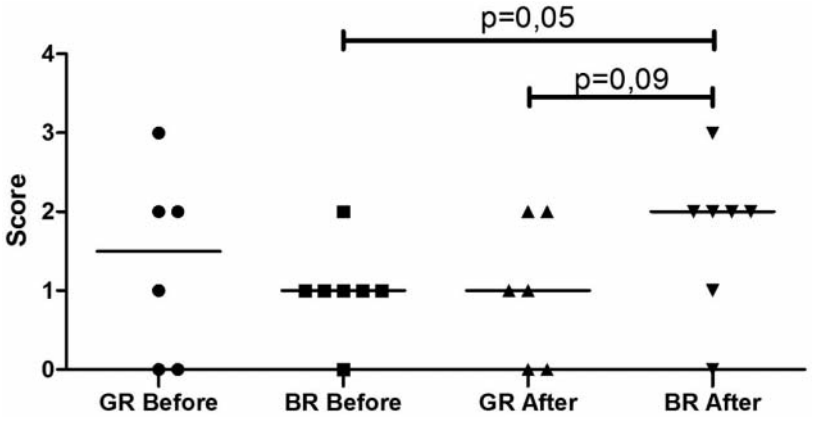

Figure 1. — Representation of the distribution of the T helper lymphocyte marker CD4+ in GR and BR patients, before and after treatment with IFN-α 2b, based on immunohistochemistry, scored according to Georgiannos' classification rubric.

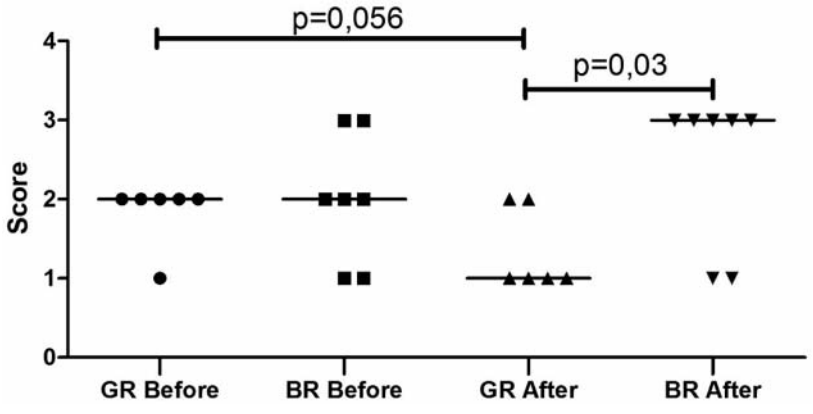

Figure 2. — Representation of the distribution of the T helper lymphocyte marker CD8+ in GR and BR patients, before and after treatment with IFN-α 2b, based on immunohistochemistry, scored according to Georgiannos' classification rubric.

groups were monitored by colposcopy, cervical smears, and biopsies for histology.

There is evidence indicating that both systemic and local immune responses play an important role in progression of CIN [22, 23]. However, local immunity may be more important and efficient than systemic immunity in controlling HPV infection and the development of CINs given that patients with a cellular immune deficiency develop cervical lesions more frequently than the general population [22, 23]. Arany and Tyring [24] analyzed the local immune response of patients with HPV who were treated with IFN. The biopsies of patients who did not respond to IFN treatment had markedly lower levels of Langerhans cells, leading to reduced expression of major histocompatibility complex (MHC) class II molecules and, therefore, reduced activation of CD4+ T cells. There was a drop in the expression of MHCI as well, with a decrease in levels of CD8+ T cells. However, the biopsies of the patients who responded well to treatment were verified to have NK cells (CD16), macrophages, and activated CD4 T cells present, with recruitment of these T cells against HPV-infected cells, demonstrating that a cellular immune response was occurring.

Mardegan *et al.* [25] found that 62.5% of patients treated with intralesional IFNα-2b responded to the treatment as evidenced by a complete histological disappearance of the high-grade lesion. Flow cytometry experiments indicated that concentrations of IL-6 and TNF-α in patients' vaginal secretions during treatment (at sixth application) were significantly higher in patients with failed therapy than in therapy responders [25]. Conversely, using the hybrid capture technique, they found a significant drop in viral load, before versus after treatment, in treatment responders. In a larger study of the same therapy, Ramos and collaborators (2010) [10] obtained a satisfactory clinical response in 60% of patients with high-grade CINs. They found that the Th1 (IFN-γ, TNF-α, IL-2) immune response was related to reduction in CIN grade after treatment with IFNα-2b in patients who responded well. There was also a significant drop in the viral load of high-risk HPV (measured using the hybrid capture technique) in patients who responded to treatment [10].

Based on quantification of cytokines in the blood of patients with high-grade CINs treated with intralesional IFN-α 2b, Misson and colleagues (2011) [11] observed a 50% therapeutic response rate. Only 16.6% of those who responded well were smokers, while 66.6% of those with therapy failure were smokers. They therefore associated the use of tobacco with the induction of failure. In the group of patients with successful therapy, they observed a significant increase in Th1-profile cytokines, which stimulated a drop in Treg-profile cytokines. Their analysis of the cytokines, via enzyme-linked immunosorbent assay (ELISA), showed that the average concentration of IL-12 in the blood of patients with successful therapy was significantly elevated on the 12th day, relative to baseline, and that this increase was associated with an agglomeration of the immune cells that produce a mixture of pro-inflammatory and regulatory cytokines.

Despite the various aforementioned studies cited, which evaluated the production of cytokines both systemically and locally in lesions of patients treated with IFN-α, few studies have determined which immune cells might participate in the regression or persistence of a high-grade lesion. Therefore, knowing the intensity and the different local infiltrates of the immune cells is fundamental to understanding the mechanisms involved in cervical tumorigenesis. More specifically, to the authors' knowledge, there are no published studies that have revealed the relationship between local immune response before and after treatment with IFN.

Maluf *et al.* [26] have reported that there are strong indicators of the presence of T lymphocytes (CD3+) in CIN III lesions in patients with a recurrence who had received conization; based on immunohistochemistry studies using Georgiannos' criteria, they found that 100% of the women with recurrent CIN III had a high level of CD3+ lymphocytes. Using the same technique and the same evaluation criteria, Silva *et al.* [27] analyzed 60 histological samples (20 control, 20 CIN III, and 20 invasive carcinoma) and identified the inflammatory infiltrate of CD3+ and CD8+ lymphocytes in all three groups, with the invasive carcinoma patients showing the highest levels. They were unable to affirm anything with respect to the cytotoxic capacity of these lymphocytes.

In this study, the authors demonstrated weak perforin labeling in all 13 cases, independent of clinical response, which may confirm the inactivity of these lymphocytes. Perforin is present in T (CD8+) and NK cells, where the formation of pores in the targeted cell may facilitate the entry of toxic enzymes (granzymes) into carcinogenic cells or into cells infected with certain viruses; or they may make the cell unable to eliminate ions and water, causing it to die [28].

Understanding the molecular mechanisms that regulate signal transduction as mediated by IFN-α, the escape mechanisms that are active in cancer cells, and the tumor's resistance mechanisms against IFN-α are of great value to developing new therapeutic strategies based on IFN-α, in order to expand this cytokine's therapeutic effects. Therefore, the authors conclude that the population of immune response cells in lesions can be changed by treatment with intralesional IFN-α 2b in patients with CIN II/III. There is a general reduction in infiltration of CD4+ and CD8+ T lymphocytes in women who respond well to treatment, whereas women who do not respond to treatment show increases in the infiltration of these lymphocytes. The presents findings indicate that immunotherapy with intralesional IFN-α 2b in patients with CIN II/III yields favorable results in patients who do not smoke.

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