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ORIGINAL ARTICLE

A cohort retrospective study of high-risk HPV recurrence in Greek women after cervical lesion treatment through detection of viral E6/E7 mRNA expression

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Summary

Purpose: *Our aim was to detect and evaluate potential alterations in the postoperative status of E6/E7 HPV mRNA in women treated for cervical intraepithelial lesions (CIN) and if so, to evaluate its potential use as a prognostic tool to identify patients with increased risk of treatment failure or recurrent disease.*

Methods: *Our study retrospectively analyzed 101 women with an abnormal Pap smear, or in some cases with histological reports or molecular analysis requiring colposcopic evaluation. Thin-prep cytological samples were collected before colposcopy and histology (when necessary). After treatment, all women were scheduled for colposcopy in six months. The cytological material was analyzed with CLART-2 HPV-DNA test and HPV-PROOFER E6/E7 mRNA test.*

Results: *Concerning demographics, no significant correlations were found for smoking, condom use or vaccination status. It seems that the only statistically significant correlation with actual severity came from the mRNA-test after treatment. This shows that clinical cases with more severe CIN may have higher chances of unsuccessful treatment. At the first post-op visit, 83.5% of HPV mRNA-positive women had a negative HPV mRNA-test while only 60.4% of HPV DNA-positive women became negative. There were 12 HPV-mRNA positive patients both before and after treatment, 3 of whom had a negative HPV DNA test, meaning that, if based only on HPV-DNA results, they would have been managed wrongly as successfully treated patients. Our study shows that E6/E7 mRNA detection has particularly high specificity and positive likelihood ratio for the prediction of treatment failure in comparison with HPV DNA-testing.*

Conclusions: *E6/E7 mRNA overexpression seems to be a promising candidate as an indicator-biomarker to determine the success of treatment.*

Key words: *cervical cancer, cervical intraepithelial neoplasia, human papilloma virus, HPV-mRNA testing, loop electrosurgical excision procedure, treatment*

Introduction

Although the popularity of cervical cancer screening based on Pap smear has significantly increased during the last decades, cervical cancer is still the third most common malignant disease among women all over the world [1]. Human papillomavirus, especially its high-risk subtypes (hr-HPV) has been recognized as the main etiologic agent of cervical cancer. Even though most HPV infections are transient, often appearing and disappearing without cytologic abnormalities, long-term persistent infection with hr-HPV subtypes is strongly associated with progression to high-grade lesions and cervical cancer. In fact, 2 HPV subtypes, 16 & 18, are currently considered responsible for approximately 70% of all cervical cancers worldwide [2].

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The oncogenic activity of hr-HPV subtypes is associated with the transformation properties of two viral oncoproteins, E6 and E7, that are usually expressed at low levels during transient HPV infections [3]. However, in persistent infections, the HPV genome is integrated into the host genome and activates the mechanisms of E6 & E7 mRNA transcripts over-expression, leading to deregulation of cellular division and differentiation and gradually to the development of cervical cancer through inhibition of tumor suppressor proteins p53 and pRb [4,5].

The strategy of cervical cancer prevention is based on the identification and treatment of high-grade cervical intraepithelial lesions (HSIL), usually via conization, which has both diagnostic and therapeutic roles [6]. Cold-knife conization (CKC) and loop electrosurgical excision procedure (LEEP) are the most common approaches with overall high rates of success [7,8]. Despite these procedures, treatment failure within 2-3 years in terms of residual/recurrent HSIL cases requiring subsequent re-excisional therapy may occur in approximately 5-30% of the cases [9,10]. Women treated for cervical lesions are at higher risk of cervical or other lower-genital tract HPV-related diseases over time compared to the general population due to HPV integration in the host genome. Indeed, the risk of cervical cancer remains elevated for years following treatment of cervical intraepithelial neoplasia in comparison with the average population [11-13], thus confirming the need for a careful and close monitoring of such women, at least for 10 years postoperatively [14], usually with a combination of cytology, HPV-DNA testing, colposcopy, patient demographics (e.g. smoking habits, sexual activity) and surgical characteristics (e.g. surgical margins, lesion size, histological grade) aimed at the early detection of patients at high-risk for residual/recurrent disease after treatment.

However, the effectiveness of this combination is limited due to many parameters. For example, some women treated for CIN2+ lesions with free surgical margins could be at risk of disease recurrence due to a possible multifocal lesion. On the other hand, most women with positive resection margins (which suggest an incomplete excision of the lesion) do not develop recurrent disease over time, thus indicating the limited usefulness of this marker [15]. Additionally, colposcopy has been shown to add little information about the detection rate of residual/recurrent HSIL cases [16,17]. Also, although Pap testing is currently considered the most widely accepted follow-up procedure after conization, its reported false-positive rates after treatment cannot be neglected [18-20] while at the same time cervical changes after CIN treatment can make cytology evaluation difficult especially in case of cervical stenosis [21]. Furthermore, it is known that persistent positivity of HPV-DNA typing is considered a prognostic index of recurrent disease in patients treated for CIN2+ [15]. HPV detection, and particularly genotyping, has an adequate high rate of sensitivity and specificity, for accurately predicting treatment failure, thus allowing intensified monitoring activity [15]. However, in cases of treated HSIL patients, HPV is usually integrated into the host genome in a way that "hides" or "discontinues" its genetic DNA sequence, which makes viral detection difficult for most HPV-DNA tests and leads to false negative results.

Therefore, an accurate test able to successfully predict clinical outlook as well as reduce the follow-up period and unwanted issues including anxiety, psychosexual outcomes and overall health costs, would be particularly helpful. Molecular detection of HPV mRNA molecules could be a promising candidate test due to its capability to detect viral mRNA which corresponds to a persistent HPV infection with viral integration and not just viral detection that could be the result of a transient HPV infection.

In our study, we used one HPV-mRNA typing methodology as prognostic index of recurrent HPV infection in women treated for CIN in our Gynecology Department, combined with other parameters including cytology, colposcopy, histology and HPV-DNA typing. Our initial aim was to detect and evaluate potential alterations in the status of this HPV biomarker after treatment for CIN and if so, to evaluate its potential use as a prognostic tool to identify patients with increased risk of treatment failure or recurrent disease through persistent biomarker positivity. Such findings could play a vital role in guiding the medical staff in their preoperative decisions regarding treatment and possibly determine the intensity of follow-up visits after treatment.

Methods

Ethical approval

All participating patients have given their written informed consent and were informed regarding the purpose of the study. The study protocol has been approved by the Aristotle University bioethics research committee on human research.

Study population, design and collection

Our study retrospectively analyzed 101 female patients who visited the Colposcopy Clinic of the 2nd Department of Obstetrics & Gynecology, Medical Faculty,

Aristotle University of Thessaloniki at Hippokration General Hospital (Thessaloniki, Greece), between January 2014 and December 2018, with an abnormal Pap smear, either conventional or liquid-based (LBC) requiring colposcopic evaluation. In some cases, the referral was based on other suspicious findings such as histological reports or HPV DNA testing. Women were recruited in the study based on the following criteria: (a) aged 18 years or older, (b) without previous cervical cancer or precancerous lesions, (c) no history of cervical lesion treatment (d) non-pregnant (e) having at least one LBC sample sent for molecular analysis prior to treatment. The initial number of patients was 140 but 39 of them were excluded from our study sample since they failed to meet one or more of the previously mentioned inclusion criteria (usually due to absence of preoperative LBC sampling).

Cervical exfoliated cell samples, used for both cytology testing and HPV biomarker analysis, were collected by brushing from the cervical surface before the colposcopic evaluation and punch biopsy (when necessary). Two main protocols were followed: a) when both cytology and colposcopy were suggestive of high grade disease (CIN 2+), punch biopsies could be skipped and treatment was scheduled directly (see and treat) and b) when cytology and colposcopy were not clearly suggestive of CIN2+, punch biopsies were obtained and, if suggestive of high grade lesion then treatment was scheduled for the next appointment. LEEP was used in most cases, due to its ease of use and the advantage of cervical tissue sampling for histological evaluation [22]. Very few cases were treated with other methods such as CKC or laser cone. Excision was performed mainly under local anesthesia and always under colposcopic guidance aiming to remove the entire lesion as well as the transformation zone. Afterwards, all women were discharged with a scheduled post-op follow-up visit for colposcopy in six months.

The cytological material both before and 6 months after treatment was collected in PreservCyt/ThinPrep solution (Hologic Inc, USA). Molecular analyses were performed by the Molecular Cytopathology Laboratory of the Clinic. Punch biopsies and the excised cones were sent for detailed histological evaluation to the Pathology Lab of the Hospital in order to evaluate the grade of the lesion, the excision margin status and involvement of glandular crypts.

Cytological evaluation

The latest LBC technology was used for the Papanicolaou (*Pap*) test. Thin-layer slides were prepared using the Thin Prep 2000 Processor (*Hologic Inc*) according to the manufacturer's instructions. The prepared slides were stained by the Pap method and assessed by the cytopathologist of the clinic according to the criteria set out in the third edition of the Bethesda System for Reporting Cervical Cytology (2015) [23]. The findings were categorized as (a) negative for intraepithelial lesion or malignancy (NILM), (b) atypical squamous cells of undetermined significance (ASC-US), (c) atypical squamous cells of undetermined significance without excluding high-grade squamous intraepithelial lesions (ASC-H), (d) low-grade squamous intraepithelial lesions (LSIL), (e) high-grade squamous intraepithelial lesions (HSIL), and (f) squamous cervical carcinomas (SCC).

HPV DNA typing test

The *CLART HPV-2* genotyping assay (Genomica, Spain) was used. This methodology uses biotinylated primers that amplify a 450 bp fragment within the HPV L1 region. Amplicons are detected by hybridization in a low-density microarray containing triplicate DNA probes specific to 35 types. Semi-quantitative results can be obtained in an automatic reader. All the experimental steps from DNA extraction to microarray reading were performed according to the manufacturer's instructions. In our study, the HPV DNA test was considered positive when at least one of the following HPV types were detected: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82, which are all high-risk and probably high-risk HPV types. Nucleic acid concentration and test quality were assessed using a Nanodrop Lite spectrophotometer (Thermo Scientific, USA).

HPV E6/E7 mRNA typing test

From each Thin Prep sample, 5 millilitres were used for RNA extraction using the NucliSens miniMAG platform, according to the manufacturer's instructions (BioMérieux, France). Nucleic acid concentration and test quality were assessed using a Nanodrop Lite spectrophotometer (Thermo Scientific, USA). Real-time nucleic acid sequence-based amplification (NASBA) and detection assay HPV-PROOFER were performed for the qualitative detection of E6/E7 mRNA of HPV types 16, 18, 31, 33, and 45 according to the manufacturer's instructions (*Pretect, Norway*). All the steps were executed in ready-to-use 8-strips with caps and, after one incubation cycle, NASBA was carried out at 41˚C in the NUCLISENS EasyQ Analyzer.

Statistics

Data analysis was carried out with SPSS software (Version No.24). The statistical significance of the association was tested with chi-square (x^2) test (association was significant if p value was <0.05).

Results

Demographics

Most of our patients were Caucasian (91%) with an age of 28-67 years (median 44.3 years). 65 out of 101 women (64.4%) were smokers with an average consumption of 12 cigarettes per day and 93 out of 101 (92.0%) had at least one vaginal delivery. The mean age at first sexual intercourse of our population was 17.2 years and 70 out of 101 women (69.3%) had more than 4 lifetime sexual partners. In terms of condom usage during sex, the majority denied taking any prophylactic measure (77.2%). Also, only 3 of them were vaccinated

(2 with Gardasil-4 and 1 with Cervarix), with the vaccination taking place after becoming sexually active.

Cytological findings

We collected cervical samples from a total of 112 women, before and after treatment. However, 11 women had to be excluded from the study since either their preoperative or postoperative samples were unsatisfactory for molecular analysis due to insufficient quantity of extracted RNA.

Before treatment, most women, 56.5% (n=57), had high grade disease (HSIL or ASC-H) results, whereas 32.7% (n=33) were diagnosed with LSIL. A total of 6.9% (n=7) of the examined women had ASCUS and 3% (n=3) had normal cytological results. In one case, Ca was diagnosed (1%). Six months after treatment, most women had normal Pap test (58.4%, n=59) and almost one fourth (24.8%, n=25) had ASCUS. Only 17 women showed intraepithelial cervical abnormalities, 13 LSILs and 4 HSILs. The above results are summarized in Table 1.

Colposcopic findings

Before treatment, colposcopy was performed in all 101 cases. In 20 patients (19.8%), no suspect lesions were detected. Similar numbers of patients were diagnosed either with low-grade or high-grade disease (43 and 37 patients, respectively) and one patient was diagnosed with SCC. In CIN2+ cases (38), a specimen was taken by colposcopically directed biopsy which histologically confirmed the colposcopic evaluation in all cases.

During the 6-month follow-up, no suspect lesions were observed in most of these patients (88.1%) and either LSIL or HSIL was detected in only 14 patients (11 and 3, respectively). It should be noted that the colposcopists were aware of the preoperative cytology and biomarker results. All the above results are summarized in Table 1.

Histological findings

As previously mentioned, all CIN2+ cases included in this study were histologically confirmed by punch biopsy before any treatment. The most common therapeutic approach was LEEP, followed by CKC (85.15% and 11.88%, respectively). During treatment, 68 out of 101 patients (68.3%) were histologically diagnosed with CIN2+. Detailed histological results are presented in Table 2.

HPV DNA testing

Before treatment, all patients were subjected to HPV-DNA testing which showed that 93 out of 101 (92.0%) were positive for viral DNA. The most commonly detected HPV types were HPV-16 (49.5%) and HPV-31 (20.8%) followed by HPV-51, HPV-58 and HPV-18 (10.9%, 10.9% and 9.9%, respectively).

Within the 6-months follow-up after treatment, 59 out of 101 treated patients had a negative HPV DNA test (58.4%). HPV-16 was the most commonly detected HPV type among HPV-positive patients

Table 1. Cytological and colposcopic diagnosis before and after treatment

Cytological diagnosis	*Before treatment n (%)*	*After treatment n (%)*
NILM	3 (3.0)	59 (58.4)
ASC-US	7 (6.9)	25 (24.8)
ASC-H	4 (4.0)	0 (0.0)
LSIL	33 (32.7)	13 (12.9)
HSIL	53 (52.5)	4 (4.0)
SCC	1 (1.0)	0 (0.0)
Total	101 (100)	101 (100)
Colposcopic diagnosis	*Before treatment n (%)*	*After treatment n (%)*
Negative	20 (19.8)	87 (86.1)
CIN1/HPV/LSIL	43 (42.6)	11 (10.9)
CIN2/CIN3/HSIL	37 (36.6)	3 (3.0)
SCC	1 (1.0)	0 (0.0)
Total	101 (100)	101 (100)

Table 2. Histological diagnosis of the excised lesion during treatment

Histology during treatment	*n (%)*
Negative	17 (16.8)
CIN1/HPV/LSIL	15 (14.9)
CIN2/CIN3/HSIL	66 (65.3)
SCC	3 (3.0)
Total	101 (100)

Table 3. HPV DNA and HPV mRNA results before and after treatment

HPV DNA test result	*Before treatment n (%)*	*After treatment n (%)*
Negative	8 (8.0)	59 (58.4)
Positive	93 (92.0)	42 (41.6)
Total	101 (100)	101 (100)
HPV mRNA test result	*Before treatment n (%)*	*After treatment n (%)*
Negative	22 (21.8)	89 (88.1)
Positive	79 (78.2)	12 (11.9)
Total	101 (100)	101 (100)

Table 4. Spearman's rho statistical correlations

			Correlations				
Correlations			*Cytology after treatment*	*Colposcopy after treatment*	*DNA test after treatment*	*mRNA test after treatment*	*Histology during treatment*
Spearman's rho	Cytology after treatment	Correlation Coefficient	1.000	0.539**	0.256**	0.258**	0.049
		Sig. (2-tailed)	-	0.000	0.010	0.009	0.627
	Colposcopy after treatment	Correlation Coefficient	0.539**	1.000	0.049	0.024	0.035
		Sig. (2-tailed)	0.000	-	0.628	0.814	0.727
	DNA test after treatment	Correlation Coefficient	0.256**	0.049	1.000	0.424**	0.092
		Sig. (2-tailed)	0.010	0.628	-	0.000	0.360
	mRNA test after treatment	Correlation Coefficient	0.258**	0.024	0.424**	1.000	0.261**
		Sig. (2-tailed)	0.009	0.814	0.000	-	0.008
	Histology during treatment	Correlation Coefficient	0.049	0.035	0.092	0.261**	1.000
		Sig. (2-tailed)	0.627	0.727	0.360	0.008	-

** Correlation is significant at the 0.01 level (2-tailed)

Table 5. Sensitivity, specificity and predictive values for HPV-DNA and HPV-mRNA tests after treatment

Threshold: CIN2+ confirmed with cytology, colposcopy or histology	*HPV-DNA test %*	*HPV-mRNA test %*
Sensitivity (SV)	94.44	81.25
Specificity (SP)	84.34	98.82
Positive predictive value (PPV)	56.67	92.86
Negative predictive value (NPV)	98.59	96.55

(13.9%) followed by HPV-31 (4.0%) and HPV-66 (3.0%). The above results are summarized in Table 3.

HPV mRNA testing

Before treatment, only 22 out of 101 patients (21.8%) had a negative HPV-mRNA test while E6/E7 mRNA was detected in 79 patients (78.2%). In fact, HPV-16 was detected in almost half of the cases (48.5%) followed by HPV-31 (18.8%). Almost one every ten HPV-mRNA positive women was found positive for HPV-18 (9.9%) and approximately one out of twenty was found positive either for HPV-33 or HPV-45 (5.9% and 5.0%, respectively). In six patients, co-infections from two HPV types were detected mostly involving either HPV-16 or HPV-31.

After treatment, most women had a negative HPV mRNA test (88.1%) and a mere 11.9% was found positive for viral mRNA (12 out of 101) with HPV-16 detected in most cases (8 out of 12) followed by HPV-18 (2 out of 12). Those 12 women had the same positive HPV-mRNA result before and after treatment. All the above results are presented in Table 3.

Statistical analysis

Concerning demographics, most parameters failed to predict the postoperative positivity of biomarkers or the severity of the excised cone, meaning that social and sexual characteristics cannot be used as a preoperative predictive tool to estimate the risk of recurrent cervical disease. No significant correlations were found for smoking, condom use or vaccination status (p values 0.224, 0.797 and 0.618, respectively).

Although crosstabulation gives a detailed picture of the common distribution between the pairs of variables we have examined previously, the chi-square test is not so reliable since too many cells of the table have required expected frequencies below 5 (the sample is not so large and certain categories are not represented well). Consequently, considering that the values of all variables under examination are ordered (low/high value means low/high severity), we applied Spearman's ρ (rho) non-parametric correlation coefficient. The coefficient has values in the interval [-1, +1] with values close to -1 or to +1 showing strong negative or positive correlation. As we can see from the last column (or row), the only statistically significant correlation with actual severity (histology during treatment) comes from the mRNA test after treatment (ρ=0.261, p=0.008). This probably shows that

severe cases have higher chances of unsuccessful treatment. The mRNA test after treatment is also significantly correlated with cytology after treatment (ρ=0.258, p=0.009) and with DNA after treatment (ρ=0.424, p<0.001) while a high correlation exists between cytology and colposcopy after treatment (ρ=0.539, p<0.001).

In combination with cytology, colposcopy and HPV DNA after treatment, it seems that the HPV mRNA test is the only statistically significant correlation with actual severity (histology during treatment) (ρ=0.261, p=0.008). This shows that clinical cases with more severe intraepithelial lesions may have higher chances of unsuccessful treatment and follow up. All Spearman's rho correlations are summarized in Table 4.

For the 6-month follow-up, we calculated the sensitivity (SV), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) for both the HPV-DNA test and the HPV-mRNA test using CIN2+ confirmed either with cytology, colposcopy or punch biopsy during the follow-up visit. We found that HPV-mRNA testing has higher SP (98.82%) and PPV (92.86%) for CIN2+ recurrent lesions after treatment compared with HPV-DNA testing (84.34% and 56,67%, respectively) while the NPV values were similar (96.55% vs 98.59%). All the values are shown in Table 5.

Discussion

The interpretation of cytological and colposcopic results requires a high level of professional knowledge and experience, and there is a certain subjectivity according to different cytopathologists or colposcopists. Furthermore, the accuracy of a Pap test is also related with other factors, such as the sampling procedure and the preservation time of the specimen. That's why in the last decade studies have focused on molecular HPV-related biomarkers whose detection is both reliable and accurate while at the same time able to eliminate discrepancies among different analysts (cytopathologists or colposcopists), thus proving to be ancillary tools for effective and efficient cervical pathology prevention and diagnosis. Contrary to previous studies focusing on HPV DNA testing [24-28], this study investigated the potential role of HPV-mRNA testing after treatment of CIN as a possible predictor of treatment failure.

Generally, the literature is very limited regarding the possible role of HPV-biomarkers, others than HPV DNA testing, in the prediction of treatment failures. This study investigated firstly if alterations in E6/E7 mRNA expression levels of the five most important high-risk HPV types (16,18,31,33,45) actually occur after treatment of cervical lesions and secondly if those alternations can be considered a potential predictor of treatment failure and disease recurrence.

This study verifies that there is an important decrease in the positivity rates of HPV-related biomarkers. Based on previous similar studies [24-28], such an outcome was expected for HPV DNA testing. However, there are limited data regarding HPV mRNA testing in treated women [29-33]. Of course, the negativity of HPV mRNA testing, as in the case of HPV DNA testing, could be logically assumed due to removal of the "abnormal" tissue. Thus, the persistent positivity of HPV mRNA testing after treatment underlying possible CIN recurrence could be suggestive of incomplete or unsuccessful treatment mainly due to the failure of the surgeon to achieve free margins.

Concerning demographics, most parameters failed to predict the postoperative positivity of biomarkers or the severity of the excised cone, meaning that social and sexual characteristics cannot be used as a preoperative predictive tool to estimate the risk of recurrent cervical disease.

As shown in our results, only 12 out of 101 treated women had a positive mRNA test postoperatively. In almost all cases HPV-16 or HPV-18 were detected in a 4:1 ratio. Wheeler et al [34] found that HPV-16 was the most prevalent single HPV genotype in the general population and it was 3-4 times more prevalent than HPV18, a ratio consistent with ours, although our population is specific and not general. HPV16 & HPV-18 are key parameters in the 2015 interim clinical guidance since their detection defines the clinical management of those women [35]. Kang et al [36] concluded that persistent infection, especially with HPV-16 and HPV-18, should be considered a risk factor for developing residual/recurrent CIN2-3, which is confirmed in our study, which shows that persistent infection with HPV-16 or HPV-18 is the most commonly detected in treated women.

At the first post-op follow-up visit, 83.5% of the HPV mRNA-positive women had a negative HPV mRNA test result while only 60.4% of the HPV DNA-positive women became negative. However, in 19.6% of the treated women with positive HPV-DNA tests both pre- and postoperatively, the genotyping results were different between the two molecular analyses of the same patient. In most cases, the main high-risk HPV type that was detected preoperatively, was also detected postoperatively along with other HPV types that were detected for the first time. Also, in some cases, the HPV types postoperatively were completely different from the ones detected before treatment. However, due to the

fact that a HPV-DNA test can only detect viral DNA without providing information on HPV oncogenic activity revealed by HPV-mRNA testing, it could not be explained whether the postoperative result was a re-infection with the same HPV type from a sexual partner (possibly as a multiple co-infection with other HPV types) after treatment or if it is sign of residual persistent infection and possibly of incomplete excision of the pathological tissue.

Concerning the 12 patients with positive mRNA test both before and after treatment, 3 of them had negative HPV DNA test pre- and postoperatively, meaning that, if based only on HPV-DNA typing results as ancillary tool, these three patients would have been considered "negative for high-risk HPV" and thus would be managed clinically as successfully treated patients. However, in all these three cases, E6/E7 viral mRNA from HPV-16 was detected, meaning that these cases should be managed as patients with incomplete treatment. Our study shows that E6/ E7 mRNA detection by NASBA has particularly high SP (98.82%) and PPV (92.86%) for the prediction of treatment failure, which is in agreement with previous studies showing that mRNA testing has significantly higher SP and PPV than DNA testing [37,38]. Molden et al [39] have shown that NASBA as an alternative mRNA testing has significant differences from DNA testing, especially for HPV-16, as also shown in our study. The negativity of the HPV DNA tests in the 3 women with positive mRNA test for HPV-16 could be explained by the fact that during the viral genome integration into the human genome sequence, it is highly likely that the L1 viral region of HPV-16 is "broken" or "mis-continued" making it difficult for the primers (PGMY09/11) that are used by most commercial HPV DNA typing tests (including ours) to amplify the target-fragment of the HPV L1 locus, resulting in a negative HPV-DNA result, as suggested in other studies [40].

In all 12 patients, the excised cone was confirmed as CIN2+. However, mRNA positivity was significantly lower in histologically negative and CIN1 patients, in concordance with previous studies [37,38,41], since contrary to HPV-DNA testing that identifies transient infections with no clinical significance, HPV-mRNA testing detects persistent infections that could lead to lesion progression [42,43].

In combination with cytology, colposcopy and HPV DNA testing after treatment, it seems that the HPV mRNA test is the only statistically significant correlation with actual severity (histology during treatment) (ρ=0.261, p=0.008). This probably shows that clinical cases with more severe intraepithelial lesions may have higher chances of unsuccessful treatment.

Conclusion

Although the number of patients (n=101) was adequate for our study, a larger sample is required in order to draw more universal conclusions since as treatment failure of CIN is used as the end-point for assessment of this HPV-biomarker, which is a rare condition in everyday clinical practice in our Clinic, due to the fact that most women (with the exception of some severe cases) that visit our Clinic are initially CIN-1 cases that undergo a series of consecutive colposcopical follow-ups and if their clinical findings become suggestive of CIN-2+, then CIN treatment is scheduled. However, this monitoring process requires time, resulting in low number of cases (n=101) in the 5-year period (2014-2018). As more patients requiring treatment will be recruited and more post-op follow-ups will be conducted, more treatment failures will be diagnosed, and the accuracy of HPV-mRNA testing will be estimated more precisely. E6/E7 mRNA overexpression for HPV types 16,18,31,33, and 45 seems to be a promising candidate as an indicator-biomarker to determine the success of the treatment and the intensity of post-op follow-up visits.

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Conflict of interests

The authors declare no conflict of interests.

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