Assessment of Cervical Cancer Molecular-Based Screening Tools; HPV-DNA Detection versus E6/E7 mRNA Testing; First Report of a Prospective Cohort Study among Iranian Women

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Abstract

Background: Human papillomavirus (HPV) has been found as the most considerable causes of cervical cancer. Recently, several molecular methods have been introduced to increase the accuracy of the screening programs and decrease the mortality rate. Among these methods, mRNA-based methods have more advantages as they assess the expression level of HPV E6 and E7 oncopgenic mRNAs. This study aimed to evaluate the results of HPV RNA- and DNA-based methods among Iranian women population with normal cytology results.

Methods: Overall, 4640 women were enrolled referred to the Gynecology Oncology Ward of Vali-e-Asr Hospital, private and academic clinics, Tehran, Iran from Jan 2016 to Apr 2018. To assess the HPV-DNA infection INNO-LiPA® HPV Genotyping Extra-II kit was used. For HPV-RNA assessment, Aptima HPV Assay and in house HPV-RNA genotyping methods were applied.

Results: The positivity rates of HPV infection according to DNA- and RNA-based methods were 18.0% and 11.2%, respectively (P<0.001). The positive predictive value, negative predictive value, specificity and sensitivity of DNA-based method in contrast with RNA-based method were 59.2% (56.6-61.6), 99.4% (99.0-99.6), 91.7% (90.8-92.6) and 95.2% (93.0-96.9) respectively.

Conclusion: At the present study for prognosis of cervical cancer, RNA-based method seemed to be more specific in contrast to DNA-based method. Patient follow up and further studies will be conducted in order to clarify the clinical sensitivity and specificity of the two methods.

Keywords: Cervical cancer; Human Papillomavirus (HPV); Intraepithelial lesion; Malignancy

Introduction

Human papillomavirus (HPV) has been found as the most considerable causes of cervical cancer (1). This virus can be divided into high-risk (HR) and low-risk (LR) genotypes according to the
clinical pathogenicity (2). High-risk genotypes include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 (3, 4). Although the majority of the infections could be resolved spontaneously, persistent infections with HR-HPV genotypes could lead the patients toward cervical cancer and death (5). The high mortality rate from cervical cancer could be declined according to screening programs (6). This issue has raised attention in our country as well. Therefore, National Association of Iranian Gynecologists and Obstetricians (NAIGO) with Iranian Society for Colposcopy and Cervical Pathology and Armin Pathobiology Laboratory (APL) planned a screening-based program study to assess the cervical cancer molecular-based screening tools.

Recently, several molecular assays in combination with cytology testing have been introduced to increase the accuracy of cervical cancer screening (7, 8). Among the molecular-based assays, RNA-based methods have more advantages as they assess the expression level of the HPV E6 and E7 mRNAs (9-11). Since there is little data in studies regarding the prevalence of oncogenic HPV E6 and E7 mRNAs and no comparative studies with DNA-based method in our region, we compared HPV RNA- and DNA-based methods among Iranian women population.

In this prospective cohort study, for the first report, we assessed liquid-based cytology samples of individuals referred to APL for routine screening tests including cytology assessment and HPV RNA testing. Moreover, HPV-DNA-based detection was performed to compare with the RNA-based method.

Materials and Methods

Study participants and design
The design of Cervical Cancer Screening Study in Iranian Women (CCSSIW) is based on a three-year follow up of Iranian women aged between 25 to 65 yr with negative for intraepithelial lesion and malignancy (NILM) results of cytology testing according to Bethesda 2014 system. The study protocol conforms to the ethical guidelines of the 1975 declaration of Helsinki and Ethics Committee of Tehran University of Medical Sciences approved the study design.

All the participants showed no history of immunological abnormalities or disorders, types of cancer including cervical cancer, precancerous changes of the cervix, radiotherapy, chemotherapy, hysterectomy, HPV vaccination, HR-HPV infections and abnormal cytology. During the sampling, none of the participants was pregnant. For the first report, we determined our cohort population for further follow-up studies and compared the HPV-DNA- and -RNA-based detection results.

In this cross-sectional study, more than 8000 Iranian women were visited in the Gynecology Oncology Ward of Vali-e-Asp Hospital, private and academic clinics from Jan 2016 to Apr 2018. Among them, 7584 women accepted to participate in the study and were referred to APL for routine screening of cervical cancer and assessment with RNA-based methods.

All study participants provided informed consent. Regarding our inclusion criteria and exclusion of the participants with missing data, 4640 cases were selected. The age of the participants ranged from 25 to 65 years. The cytological evaluation of the participants in the last twelve months from the date of sampling showed no abnormal results. The results of cytology, RNA- and DNA-based testing were performed blinded to the outcomes of each test.

Sample collection and preparation
ThinPrep® (TP) PreserCyt solution (Hologic, Inc. USA) was used for cervical specimen collection. The samples were subjected to a simultaneous cytology and HPV molecular analysis of DNA and RNA testing. Before the cytology testing, sufficient amount of the samples was taken and kept in -24 °C until DNA and RNA isolation were performed.

Cytology assessment
Cytology testing of the samples was performed by at least two different cytopathologists using
routine evaluation of the TP Pap test slides, prepared using TP 2000 system (Hologic, Inc. USA). Thereafter, slides fixation and staining were undertaken manually according to TP stain user’s manual. Regarding the Bethesda 2014 system, the results were categorized negative for intraepithelial lesion and malignancy (NILM); atypical squamous cells of undetermined significance (ASC-US); atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H); low-grade squamous intraepithelial lesion (LSIL); high-grade squamous intraepithelial lesion (HSIL) and glandular cells abnormalities. The NILM patients were selected as the cohort population for further studies. In addition to the patients with other cytological results, patients with unsatisfactory cytological results were excluded.

DNA isolation and high-risk HPV DNA Genotyping

The HPV DNA isolation was performed using QIAamp DNA blood mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The HPV-DNA-based detection and genotyping were performed using the INNO-LiPA® HPV Genotyping Extra-II kit (Fujirebio Europe, N.V. Belgium). Since we assessed the HR-HPV genotypes, all acquired genotypes except HR-HPV genotypes were considered as negative results.

Aptima HPV Assay (AHPV)

The Aptima HPV testing was performed using the Panther system (Hologic, Inc. USA) according to HPV Aptima handbook instructions. This method assesses the HPV viral E6/E7 mRNA from 14 HR-HPV genotypes including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. This assay cannot distinguish between HR-HPV types. According to the handbook of AHPV, it would be possible to observe cross reactivity with HPV types 26, 67, 70 and 82. Thereby positive Aptima cases with DNA genotyping results of these types were returned to negative.

RNA isolation and HPV RNA genotyping of types 16, 18 and 45

The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s recommendation. Moreover, on-column DNase digestion was performed. The in-house HPV-RNA genotyping method was performed on Aptima positive samples, infected with at least two HR-HPV genotypes including types 16 and/or 18 and/or 45 according to our DNA-based method. In addition, the results of the patients infected with only one type of HR-HPV according to DNA-based method were considered for HR-HPV-RNA as well. The sequence of the primers and probes for RNA genotyping of HPV-RNA-types; 16, 18 and 45 were extracted from previous study (12) with some alterations; Rox and Cy5 were used to label HPV-18 and -45 probes, respectively. All probes were labeled with 3'-BHQ. The Quantifast® Pathogen RT-PCR+IC kit and Rotor-Gene Q 5plex HRM System (Qiagen, Hilden, Germany) were used to perform one-step and multiplex detection of HPV-RNA genotypes. The PCR reactions were performed with initial step at 50 °C for 20 min followed by denaturation step at 95 °C for 5 minutes. Cycling steps were performed at 95 °C for 15 sec followed by 60 °C for 30 sec and repeated for 45 times. Acquiring on green, orange, red and yellow was performed at the end of each repeat.

Statistical Analysis

The descriptive statistics were calculated by determining the mean (SD) and shown by frequency (percentage). The degree of agreement between the two sets of data were assessed by calculation of the agreement percentage and the Kappa statistic. According to Altman’s classification method, agreement ranges from zero to one (poor: k<0.2; fair: 0.2≤k<0.4; moderate: 0.4≤k<0.6; good: 0.6≤k<0.8; very good: k≥0.8) (13). Specificity, sensitivity, negative predictive value (NPV), positive predictive value (PPV) and 95% confidence interval (95% CI) for HPV-DNA detection in contrast with HPV-RNA detection were calcu-
lated. In this study, the specificity shows probability that an HPV-DNA-based result will be negative when the HPV-RNA-based result is not positive (true negative rate). The sensitivity shows probability that an HPV-DNA-based result will be positive when the HPV-RNA-based result is positive (true positive rate). The PPV shows probability that the HPV-RNA-based result is positive when the HPV-DNA-based result is positive. The NPV shows probability that the HPV-RNA-based result is not positive when the HPV-DNA-based result is negative. Statistical analyses were performed using the IBM SPSS Statistics for Windows (Ver. 21.0, Released 2012, IBM Corp, Armonk, New York, USA) and STATA statistical package for Windows (Ver. 13, Released 2013, StatCorp LP, College Station, Texas, USA).

Results

The mean age of the participants was 36.9 with standard deviation of ±8.1 years. The positivity rates and agreement percentages between DNA- and RNA-based methods are illustrated in Table 1. Moreover, 34 samples (0.7%) were positive for APHV and negative for HR-DNA genotyping method. According to Aptima’s handbook nine out of 34 of RNA positive results were returned to negative due to possibility of cross reactivity with HPV types; 26, 67, 70 and 82.

Table 1: Agreement between HR-HPV detection by RNA- and DNA-based methods stratified by age groups

| Age groups (n) | Positivity Rate | RNA⁺/DNA⁺ | RNA⁺/DNA⁻ | RNA⁻/DNA⁺ | RNA⁻/DNA⁻ | %Agreement | Kappa | P-value |
|----------------|----------------|-----------|-----------|-----------|-----------|------------|-------|---------|
| All (4640)     | 519            | 835       | 3780      | 341       | 25        | 494        | 92.11 | 0.69    | < 0.001 |
| Row (%)        | (11.2)         | (18.0)    | (99.34)   | (40.84)   | (0.66)    | (59.16)    |       |         |         |
| 25-30 (986)    | 162            | 249       | 732       | 92        | 5         | 157        | 90.16 | 0.71    | < 0.001 |
| Row (%)        | (16.4)         | (25.3)    | (99.32)   | (36.95)   | (0.68)    | 63.05      |       |         |         |
| 31-35 (1453)   | 183            | 296       | 1149      | 121       | 8         | 175        | 91.12 | 0.68    | < 0.001 |
| Row (%)        | (12.6)         | (20.4)    | (99.31)   | (40.88)   | (0.69)    | 59.12      |       |         |         |
| 36-40 (976)    | 95             | 151       | 819       | 62        | 6         | 89         | 93.03 | 0.67    | < 0.001 |
| Row (%)        | (9.7)          | (15.5)    | (99.27)   | (41.06)   | (0.73)    | (59.94)    |       |         |         |
| 41-45 (504)    | 35             | 68        | 434       | 35        | 2         | 33         | 92.66 | 0.6     | < 0.001 |
| Row (%)        | (6.9)          | (13.5)    | (99.54)   | (51.47)   | (0.46)    | (48.53)    |       |         |         |
| 46-50 (357)    | 20             | 35        | 320       | 17        | 2         | 18         | 94.68 | 0.63    | < 0.001 |
| Row (%)        | (5.6)          | (9.8)     | (99.38)   | (48.57)   | (0.62)    | (51.43)    |       |         |         |
| 51-55 (198)    | 14             | 18        | 178       | 6         | 2         | 12         | 95.96 | 0.73    | < 0.001 |
| Row (%)        | (7.1)          | (9.1)     | (98.89)   | (33.33)   | (1.11)    | (66.67)    |       |         |         |
| 56-60 (121)    | 8              | 15        | 106       | 7         | 0         | 8          | 94.21 | 0.67    | < 0.001 |
| Row (%)        | (6.6)          | (12.4)    | (100)     | (46.67)   | (0.00)    | (53.33)    |       |         |         |
| 61-65 (45)     | 2              | 3         | 42        | 1         | 0         | 2          | 97.76 | 0.79    | < 0.001 |
| Row (%)        | (4.4)          | (6.7)     | (100)     | (33.33)   | (0.00)    | (66.67)    |       |         |         |

Table 2 shows the PPV, NPV, specificity and sensitivity, with 95% CIs of HPV-DNA-based method in contrast with HPV-RNA-based method.

Among the HR-HPV-DNA positive individuals, 559 (66.9%) individuals were infected with one type. The combination of HR-HPV-DNA and -RNA genotyping methods (excluding the 25 samples according to negative results in HR-HPV-DNA genotyping method) revealed that 354 (70.9%) individuals were infected with one HR-HPV-RNA type. Table 3 demonstrates the distribution of HR-HPV genotypes in all and mono-infected individuals within HR-HPV-DNA and -RNA infected cases.
Table 2: Positive Predictive Value (PPV), Negative Predictive Value (NPV), sensitivity and specificity, with 95% CIs for HPV-DNA detection in contrast with HPV Aptima assay

| Age groups | PPV (95% CI) | NPV (95% CI) | Specificity (95% CI) | Sensitivity (95% CI) |
|------------|--------------|--------------|----------------------|---------------------|
| All        | 59.2 (56.6-61.6) | 99.4 (99.0-99.3) | 91.7 (90.8-92.6) | 95.2 (93.0-96.9) |
| 25-30      | 63.1 (58.4-67.5) | 99.3 (98.4-99.7) | 88.9 (86.5-90.9) | 96.9 (92.9-99.0) |
| 31-35      | 59.1 (54.9-63.2) | 99.3 (98.7-99.7) | 90.5 (88.7-92.0) | 95.6 (91.6-98.1) |
| 36-40      | 58.9 (52.9-64.7) | 99.3 (98.4-99.7) | 93.0 (91.1-94.6) | 93.7 (86.6-97.7) |
| 41-45      | 48.5 (40.4-56.7) | 99.5 (98.3-99.9) | 92.5 (89.8-94.8) | 94.3 (80.8-99.3) |
| 46-50      | 51.4 (39.5-63.3) | 99.4 (97.7-99.8) | 95.0 (92.1-97.0) | 90.0 (68.3-99.8) |
| 51-55      | 66.7 (46.9-81.9) | 98.9 (96.1-99.7) | 96.7 (93.0-98.8) | 85.7 (57.2-99.8) |
| 56-60      | 53.3 (35.8-70.1) | 100 | 93.8 (87.7-97.5) | 100 (63.1-100) |
| 61-65      | 66.7 (22.4-93.3) | 100 | 97.7 (87.7-99.9) | 100 (15.8-100) |

Table 3: The distribution of HR-HPV genotypes in all and mono-infected individuals among HR-HPV-DNA and -RNA infected cases

| HR-HPV genotypes | All infected cases | Mono-infected cases |
|------------------|--------------------|---------------------|
|                  | DNA+ n (%) | RNA+ n (%) | DNA+ n (%) | RNA+ n (%) |
| 16               | 218 (26.1) | 103 (19.8) | 117 (20.9) | 79 (22.3) |
| 18               | 72 (8.6)   | 24 (4.6)   | 21 (3.8)   | 11 (3.1)  |
| 45               | 69 (8.3)   | 22 (4.2)   | 27 (4.8)   | 12 (3.4)  |
| 31               | 476 (57.0)* | 370 (71.4)* | 53 (9.5)   | 39 (11.0)* |
| 33               | 3 (0.5)    | 2 (0.6)    | 3 (0.5)    | 2 (0.6)   |
| 35               | 15 (2.7)   | 16 (4.5)   | 15 (2.7)   | 16 (4.5)  |
| 39               | 46 (8.2)   | 35 (9.9)   | 46 (8.2)   | 35 (9.9)  |
| 51               | 40 (7.1)   | 27 (7.6)   | 40 (7.1)   | 27 (7.6)  |
| 52               | 44 (7.9)   | 21 (5.9)   | 44 (7.9)   | 21 (5.9)  |
| 56               | 42 (7.5)   | 20 (5.7)   | 42 (7.5)   | 20 (5.7)  |
| 58               | 35 (6.3)   | 24 (6.8)   | 35 (6.3)   | 24 (6.8)  |
| 59               | 20 (3.6)   | 13 (3.7)   | 20 (3.6)   | 13 (3.7)  |
| 66               | 57 (10.2)  | 27 (7.6)   | 57 (10.2)  | 27 (7.6)  |
| 68               | 39 (7.0)   | 28 (7.9)   | 39 (7.0)   | 28 (7.9)  |
| Total            | 835 (100)  | 519 (100)  | 559 (100)  | 354 (100) |

Abbreviations: h, Higher distribution in contrast to obtained percentage of HR-HPV-DNA positive cases; n, Number; *, Distribution of other HR-HPV genotypes (31, 33, 35, 39, 51, 52, 56, 58, 59, 66 and 68) obtained after subtracting the summation of acquired data from HPV types-16, -18, -45

Discussion

Up to now, several methods have been introduced for HR-HPV detection and cervical cancer screening using nucleic acid testing of viral particles (14). Generally, we can look at these methods from two different aspects: the power of prognosis of the cell transformation and cervical cancer screening, and the power of detection of viral particles that determines the presence or absence of HPV (15).

To date, six HPV tests have been approved by U.S Food and Drug Administration (FDA) (16, 17) and several comparative studies evaluate these methods (11, 18-22). AHPV, the only mRNA-based FDA approved HPV test, has similar clinical sensitivity and improved specificity for screening and prediction of moderate to
severe cervical dysplasia compared to other DNA-based methods (23). Although these methods have been studied and compared in numerous studies, some certain limitations in our region, including availability, dependency on instruments with sophisticated technology and possible lack of affordability by some clinical centers exist. Therefore, comparing one of these methods with one of widely used commercially approved HPV detection and genotyping kits would be practical. Accordingly, the CCISIW study was designed. This primary report can provide an overview of the prospective cohort study. According to our results, the positivity rate of AHPV among NILM individuals is 11.2%. Recently in our region, the Cobas HPV test, another FDA approved method, was used and the positivity rate of HPV in the NILM group was 13.5% (102 cases out of 753) (24). Considering the improved specificity of AHPV in contrast to the DNA-based methods this issue may be obtained.

The positivity rate of HR-HPV-DNA using the INNO-LiPA® HPV Genotyping Extra-II kit among our population is 18%. Since this method uses the PCR reaction for amplifying the 65 bps of L1 region, it is expected that the power of detection and sensitivity of this method is higher than AHPV, which uses the S/CO calculation, based on E6/E7 mRNA detection and designed in order to predict the cell transformation and cervical cancer screening.

Table 1 confirms this idea and reveals that among the HR-HPV-DNA positive individuals, only 59.16% of the patients were positive for AHPV as well. This pattern has also been repeated in all age groups. The comparison of the two methods using chi-square test showed significant differences in all age categories ($P<0.001$). The concordance between the two methods was performed using the Kappa statistic, which did not show very good agreement (Table 1). Table 2 reveals that PPV of the DNA-based method in contrast to AHPV is ranged between 48.5 to 66.7%. If we consider the AHPV as the golden standard for prediction of moderate to severe cervical dysplasia, this finding would indicate that the DNA-based method has lower clinical specificity and higher sensitivity. Moreover, the NPV of the DNA-based method in contrast to AHPV is ranged between 99.3% and 100%. This issue suggests this assay has high precision for identifying the negative cases of AHPV. Although the DNA-based method was more powerful than RNA-based method for detection of HPV particles, 25 (0.54%) of the individuals with RNA positive results were found negative according to DNA-based method. This issue suggests that during the integration process of HPV genome, the L1 region, which is not obligatory for cell transformation, may be deleted or disrupted. Although this percentage of L1 disruption among our cohort population is negligible, this number is increased (4.82%) among the Aptima positive individuals and might be debatable.

In addition to the importance of HPV nucleic acid testing, awareness about the distribution of HPV genotypes would help achieve high throughput implementation and monitoring of HPV vaccination programs (25).

HPV types 16, 18 and 45 had higher carcinogenic potential in contrast to other HR-HPV genotypes (26, 27). Accordingly, in our study we assessed the distribution of these types based on DNA and RNA methods. Table 3 represents the distribution of HR-HPV genotypes in all and mono-infected individuals within HR-HPV-DNA and RNA infected cases.

Although the distribution of HPV types 16, 18 and 45 in all HR-HPV-DNA infected individuals is higher in contrast to all HR-HPV-RNA infected individuals, the distribution of other HR-HPV genotypes is vice versa. In order to assess the importance of HR-HPV genotypes in more detail, we evaluated the HR-HPV genotypes distribution of two different groups among mono-infected cases (Table 3).

After recalculating the HR-HPV-DNA genotyping result of the study conducted in Iranian patients (28), the dominant HR-HPV genotype among mono-infected cases of NILM group, was type 16 with distribution of 20% (6 out of 30). In our study, the dominant HR-HPV genotype among mono-infected individuals of both positive groups was type 16. In the mentioned study
(28), the second dominant HR-HPV genotype among mono-infected cases was type 66 with distribution of 16.7% (5 out of 30). Although the second dominant HR-HPV genotype among mono-infected cases of HR-HPV-DNA positive individuals of our study was type 66, this issue was not observed among mono-infected cases of HR-HPV-RNA positive individuals. Other observed HR-HPV genotypes regarding their distribution were types 18 (6.7%), 33 (6.7%), 39 (6.7%), 51 (6.7%), 52 (6.7%), 56 (6.7%), 58 (6.7%), 68 (6.7%), 31 (3.3%) and 59 (3.3%). According to sample size of the mentioned study, HR-HPV types 35 and 45 were not observed among the mono-infected cases.

As it is obvious in table 3 the distribution of HPV types 16, 31, 33, 35, 39, 51, 58, 59 and 68 is higher in the HR-HPV-RNA mono-infected individuals in contrast to HR-HPV-DNA mono-infected patients. If we consider the Aptima positive patients as high-risk group for development of cervical cancer, these types may have more integration potential in human DNA genome and should be studied more precisely in further studies. Moreover, this issue suggests, 9-valent HPV vaccine should be replaced with the quadrivalent HPV vaccine in order to cover more HR-HPV genotypes in our region.

One limitation of our study was the method used for HR-HPV-RNA genotyping. Since this method only evaluated the E6/E7 mRNA of HPV types 16, 18 and 45, we were not able to assess those patients infected with at least two HR-HPV genotypes except HPV types 16, 18 and 45. In this study we determined the other integrated HR-HPV genotypes acquired by DNA-based method for Aptima positive individuals as well and there was no method for checking the possibility rate of L1 deletion in HR-HPV genotypes separately.

Conclusion

Our findings for cervical cancer screening indicate the RNA-based method seemed to be more specific in contrast to DNA-based method. Although the DNA-based method could be applied for detection of broad ranges of HPV-infected individuals and also can determine the most negative cases of AHPV, false negative results may be acquired due to the HPV genome disruption during the integration process in some cases. In order to evaluate the clinical sensitivity and specificity of DNA- and RNA-based methods, following up our cohort population needs to be conducted.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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