A Correlation of Immunohistochemical and Molecular Detection of Human Papilloma Virussubtypes 16 and 18

Saleh Hussein Bensumaidea[1], Hussain Gadelkarim Ahmed[2], Salim Saeed Bafakeer[3], Mohammed Siddiq Abd-al-Aziz[1], Fawaz D. Alshammari[4]

[1]Department of Histopathology and Cytology, Faculty of Medical Laboratory Science, Sudan University for Science and Technology, Sudan

[2]Department of Pathology, College of Medicine,Molecular Diagnostics and Personalized Therapeutics Unit, University of Hail, KSA; Department of Histopathology and Cytology, FMLS, University of Khartoum, Sudan.

[3]Department of Pathology, College of Medicine, Hadhramout University of Science and Technology, Yemen.

[4]Department of Medical Laboratory, College of applied Medical Science, University of Hail.

Abstract: *Background: Human papillomavirus (HPV) infection involved in the development of more than 90% of cases of cervical cancer. Therefore, the aim of this study was to assess role of IHC for detection of HPV subtypes 16 and 18, as compared to molecular identification.* *Methodology: A total of 84 cervical cancer tissues that were previously diagnosed as having cervical cancer, were investigated for the presence of HPV subtypes 16 and 18. Both immunohistochemistry and molecular (Polymerase Chain Reaction (PCR)).* *Results: In application of molecular (PCR) testing for HPV subtypes 16 and 18; 62/84 (73.8%) were found positive for HPV subtype 16 and the remaining 22/84 (26.2%) were negative. On the other hand, on testing the samples for HPV subtype 18; 23/84 (27.4%) were found positive and the remaining 61/84 (72.6%) were found negative.* *Conclusion: Immunohistochemistry using clone K1H8 (anti-HPV) is considerably lower-priced, and has reasonable specificity to be applied in Yemen for screening for HR_HPV.*

Keywords: HPV, cervical cancer, Immunohistochemistry, PCR

1. Introduction

Human papillomavirus (HPV) is a DNA virus from the papillomavirus family that is capable of infecting keratinocytes of the skin or mucous membranesThere are more than 170 HPV-subtypes which are classified into the alpha, beta, gamma, mu, and nu genera [1]. There are more than 40 HPV-subtypes belonging to the alpha genus that cause sexually transmitted infections. These infections are frequently responsible of many pathological conditions such as, condylomas and anogenital intra-epithelial neoplasia. At least 18 of these subtypes are causative agents of anogenitalcarcinomas [2]. Of the known HPV subtypes, 15 are classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 as probable high-risk (26, 53, and 66), and 12 as low-risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) [3]. High risk HPV types possess two early (E)oncoproteins(E6 and E7) [4]. The HPV genome is composed of six early (E1, E2, E4, E5, E6, and E7) ORFs, two late (L1 and L2) ORFs, and a non-coding long control region (LCR) [5].The E6/E7 proteins inactivate two tumor suppressor proteins, p53 (inactivated by E6) and pRb (inactivated by E7) [6].

Cervical cancer was estimated 527,624 new cases in 2012 and 265,653 deaths worldwide [7]. More than 80% of global invasive cervical cancer cases occur in developing countries and almost present with a higher mortality/incidence ratio [8].Oncogenic types of HPV subtypes are the principal cause of cervical cancer and its precursor lesions [9]. HPV16, 18, 45 and 35 are the most common HPV subtypes in African women with invasive cervical cancer [10].

However, the expression of HPV markers and surrogate markers of HPV infection can be simply estimated by Immunohistochemistry (IHC). Diverse antigens can be used including virus related (capsidic antigens, E5-E6-E7 proteins), and virus induced and/or altered host proteins (p16INK4a, pRb, Cyclin proteins, p-53)[11]. Several authors have stressed that a hallmark of the presence of HPV in cancer could be found in p16 nuclear or cytoplasmic overexpression, so that p16 could be considered a useful surrogate marker for HPV [12,13].

Therefore, the aim of this study was to assess role of IHC for detection of HPV subtypes 16 and 18, as compared to molecular identification.

2. Materials and Methods

A total of 84 cervical cancer tissues that were previously diagnosed as having cervical cancer, were investigated for the presence of HPV subtypes 16 and 18. Both immunohistochemistry and molecular (Polymerase Chain Reaction (PCR)). The diagnosis was based on clinical examination and histological features of the biopsy. The sample included full coverage of patients with cervical cancer.

Immunostained using avidin biotin technique to detect the HPV (L1 capsid protein) by the use of monoclonal mouse

anti-human papillomavirus clone K1H8 from Dakocompany which is used to demonstrate HPV type 6,11,16,18,31,33,42,51,52,56 and 58. All sections on silanized coated slides were dewaxed in hot plate oven and cleared in two changes of xylene two minutes for each, hydrated through descending grades of ethyl alcohol (100%, 90%, 70%, 50%) and finally to distilled water, 2 minutes for each change. The sections were boiled in the Target Retrieval Solution of Dako (citrate buffer solution pH 6) in a water bath at 95°c for 30 min, then left to cool at room temperature and washed three times with PBS. 3% hydrogen peroxide in methanol were added to each section for 15 min to block endogenous peroxidase activity, and then washed three times with phosphate buffer saline (PBS) pH 7.4 for 3 minutes, then the diluted monoclonal mouse anti-human papillomavirus clone K1H8 from Dako company was added to each slide for 30 minutes, washed in PBS for 3 min., then treated with biotinylated link for 15 minutes, washed in PBS for 3 min, treated in conjugated streptavidin, washed in PBS for 3 min, then treated with 3,3-diaminobenzidinetetrahydrochlorate (DAB) for 10 minutes, washed in PBS for 3 minutes, then stained in Mayer's haematoxyline as counter stain for one minute, then washed and blued in running tap water, hydrated, cleared and mounted in DPX.

Positive and negative controls from known positive and negative samples were treated as the samples.

3. DNA extraction

DNA was extracted according to the steps described in DNA extraction kit purchased from Sacace biotechnologies-Casera –Italy. The pellet obtained from previous steps was treated with 300 μl of Reagent 2 (lysis buffer) in addition 100 μl of sample, vortexed, incubated at 65 °C for 5 min and centrifuged at (12000–16000 g) for 10 min and transfer the supernatant into new tube (sterile 1.5 ml Eppendorf tube) for DNA extraction. Vortexed vigorously sorbent and added 20 μl to each tube, Vortexed for 5–7 sec and incubated all tubes for 3 min at room temperature, then this step was repeated. Then all tubes were centrifugated for 30 sec at 5000 g and used amicropipette with aplugged aerosol barrier tip, carefully removed and discarded supernatant from each tube without disturbing the pellet. Tips was Changed between the tubes. 500 μl of Washing Solution was added to each tube. Vortexed vigorously and centrifuged for 30 sec at 10000 g. Supernatant was removed and discarded from each tube. This step was repeated and incubated all tubes with open cap for 5–10 min at 65°C.The pellet was resuspended in 100 μl of DNA-eluent. Incubate for 5 min at 65°C and vortex periodically. The tubes were centrifuged for 1 min at 12000x g. The supernatant was containing DNA ready for amplification stored at - 20°C until used.

3.1 Polymerase chain reaction (PCR) Amplification of HPV

Type specific primers (primer for HPV 16 and HPV18) were used to detect HPV16 and 18. DNA in cervical malignant lesions. Amplification was performed according to HPV16/18 kit from Sacace-Biotechnologies S.r.l. Caserta – Italy. The final reaction volume of 40 μl containing 20 μl

mix-1(contained in PCR tubes), 10 μl of mix-2 and 10 μlof extracted DNA (sample). Negative control, positive HPV16 DNA and positive control 18 DNA tubes contained 10 μl of DNA buffer, 10 μl of HPV 16 DNA and10μl of HPV18 DNA respectively. Samples and controls were amplified using Gene Amp PCR system 9700.

4. Results

This study investigated 84 tissue samples obtained from patients with cervical cancer their ages ranging from 21-75 with a mean age of 47 years. All of the samples confirmed as positive by immunohistochemical testing using pan anti-human papillomavirus antibody. In application of molecular (PCR) testing for HPV subtypes 16 and 18; 62/84 (73.8%) were found positive for HPV subtype 16 and the remaining 22/84 (26.2%) were negative. On the other hand, on testing the samples for HPV subtype 18; 23/84 (27.4%) were found positive and the remaining 61/84 (72.6%) were found negative.

Regarding age, most of the infected patients were at age range 41-50 years constituting 35/84 (41.7%), followed by age group 31-40, 51+ and 21-30 years, representing, 23/84 (27.4%), 18/84 (21.4%) and 8/84 (9.5%), respectively as indicated in Fig 1. For HPV subtype 16, most of cases were observed at age group 41-50 representing 30/62 (48.4%) followed by age range 31-40 years constituting 15/62 (24.2%). For HPV subtype 18, most of cases were observed at age group 41-50 representing 9/23 (39%) followed by age range 51+ years constituting 7/23 (30.4%), as shown in Fig. 2.

Table 1: Distribution of the study subjects by HR-HPV subtypes 16 & 18 and age

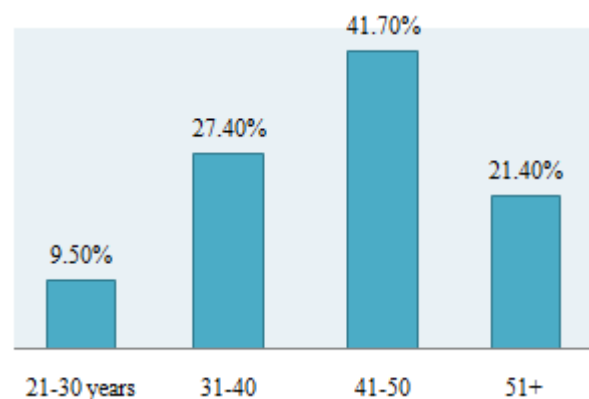| Age group	HPV16	HPV18
21-30 years	7	3
31-40	15	4
41-50	30	9
51+	10	7
Total	62	23

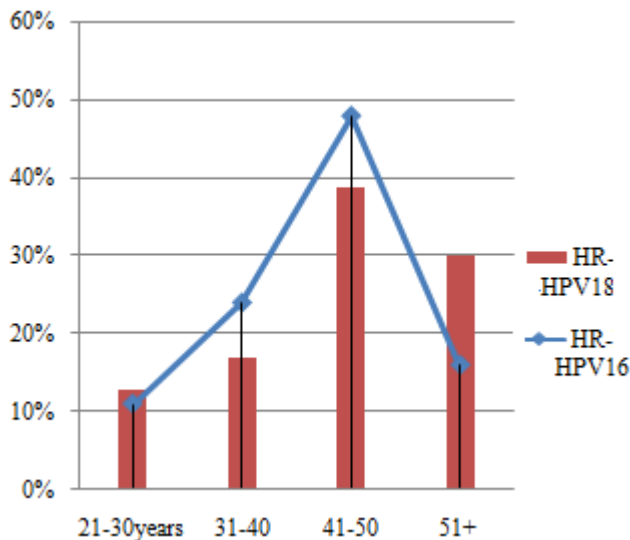**Figure 1:** Description of HR-HPV positive cases by age

Figure 2: Description of HR-HPV subtypes16 and 18 positive cases by age

5. Discussion

Cervical cancer is the second most common cancer that affects women worldwide. In more than 99% of cases of cervical cancer are attributed to a history of persistent

Infection by HR-HPV [14]. HPV-16 and HPV-18 are the most HR-HPV subtypes that frequently occurring in cervical cancer. These two subtypes account for over 70% of squamous cell carcinomas [15]. Therefore, the current study mainly screened cervical cancer tissues for the presence of these two HR-HPV subtypes and evaluated their detection in IHC as compared to molecular detection. Since all of our investigated samples were positive for HPV by immunohistochemical demonstration, the molecular (PCR) testing for HPV subtypes 16 and 18 has showed high correlation particularly with HPV subtype 16 (73.8%) but relatively low with HPV subtype 18; (27.4%). However, such variation is expected since we used an antibody (K1H8) that is capable of detecting more HPV subtypes rather than HPV subtypes 16 and 18.

IHC of p16INK4A (p16) has been extensively used for the detection of high-risk HPV in cervical squamous lesions, and some studies have proved that this method has a higher sensitivity than HPV-ISH in squamous lesions [16, 17]. Nonetheless, Milde-Langosch et al. reported p16 expression in 41% of HPV-negative carcinomas [18], and Houghton et al. reported that p16 immunoreactivity in unusual types of carcinoma does not reflect HPV infection [19]. To the best of our knowledge, there is a lack of studies that have concurrentlyassessed HPV-PCR genotyping, and p16 immunoreactivity in carcinom tissues (-Yemelyanova2009) [20]. In an earlier study, Tase et al. identified HPV DNA in 42.5% of Formali Fixed Paraffin Embedded (FFPE) sections from carcinoma cases using ISH with mixed probes [21]. Using RNR-RNA ISH, Milde-Langosch et al. found HPV-16 and -18 E6/E7 oncogene expressions in 62.3% of FFPE sections from adenocarcinoma cases [18].However, multiple HPV infections were expected in this study. Multiple infections have been considered less common in adenocarcinoma than in squamous cell carcinoma. Zielinski,

et al. found multiple subtypes in 6% of adenocarcinoma [22], and Pirog et al. reported 7.6% [23]. These findings suggest that invasive adenocarcinoma growth may be triggered by the actions of a single HPV type, rather than by the synergetic actions of multiple HPVs. However, the molecular negative results in this study may be attributed to multiple infections with subtypes other than 16 and 18 which are known positive with the used antibody in present study.

It is assumed the most reliable method to detect a biologically relevant HPV infection, is the detection of viral mRNA expression carried out by quantitative PCR (qPCR) techniques on fresh-frozen–derived samples[24]. Although this method may show valuable in research, it is logistically difficult to present into a routine pathology service where diagnostic procedures are based on the assessment of FFPE tissue[25].

In the present study, we used anti-human papillomavirus, clone K1H8 (anti-HPV) which reacts with a non-conformationalinternal linear epitope of a major capsid protein of HPV-1, which is broadly expressedamong the different HPV subtypes [26]. Anti-HPV was found to be immunoreactive with FFPEHPV-infected tissues which were demonstrated by Southern blot hybridization to be infected with HPV type6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58. Positive immunostaining was largely confined to the nuclei of infected cells. Occasionally, thecytoplasm of koilocytotic cells was observed to be immunoreactive[27].

The major limitation of this study is the application of molecular analysis only to HPV subtypes 16 and 18. Future prospect: Further studies are needed to evaluate all HPV subtypes that are expected to be positive K1H8, this in addition to the evaluation of P16 in this context.

In conclusion Immunohistochemistry using clone K1H8 (anti-HPV) is considerably lower-priced,and has reasonable specificity to be applied in Yemen for screening for HR_HPV.The K1H8 antibody reacts with a nonconformational,internal, linear epitope of a majorcapsid protein of HPV that is specific for HPV subtypes 16 and 18 E6 Protein is suitable choice for detection of these common HR-HPB subtypes.

References

[1] de Villiers EM. Cross-roads in the classification of papillomaviruses. Virology2013; 445: 2–10.
[2] Nicolas Berthet, Michael Falguières, Claudia Filippone, et al. Resequencing Microarray Technology for Genotyping Human Papillomavirus in Cervical Smears.PLoS One. 2014; 9(11): e109301.
[3] Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ . "Epidemiologic classification of human papillomavirus types associated with cervical cancer". N. Engl. J. Med. 2003;348 (6): 518–27.
[4] Ault KA. "Epidemiology and Natural History of Human Papillomavirus Infections in the Female Genital tract". *Infectious Diseases in Obstetrics and Gynecology* 2006;**2006**: 1–5.

Paper ID: 20111401

2489

[5] Ganguly N, Parihar SP. "Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis". *Journal of biosciences* 2009; **34** (1): 113–123.

[6] Chaturvedi, Anil; Maura L. Gillison. "Human Papillomavirus and Head and Neck Cancer". In Andrew F. Olshan. *Epidemiology, Pathogenesis, and Prevention of Head and Neck Cancer* (1st ed.). New York: Springer 2010. ISBN 978-1-4419-1471-2.

[7] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 [Internet] International Agency for Research on Cancer: Lyon, France; 2013.

[8] WHO/ICO Information Centre on HPV and Cervical Cancer, (HPV Information Centre): *Human Papillomavirus and Related Cancers in Ethiopia. Summary Report 2010*. Available from: http://www.hpvcentre.net, accessed on 01/06/2011.

[9] Faridi R, Zahra A, Khan K, Idrees M. Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer. Virol. 2011;8:269.

[10] Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM. Human papillomavirus typedistribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. Int J Cancer. 2007;121(3):621–632.

[11] Pannone G, Santoro A, Papagerakis S, et al. The role of human papillomavirus inthe pathogenesis of head and neck squamous cell carcinoma: an overview. *Infectious Agents and Cancer 2011*, **6**, 4.

[12] Gillespie MB, Rubinchik S, Hoel B, Sutkowski N. Human papillomavirus and oropharyngeal cancer: what you need to know in 2009. *Curr Treat Options Oncol 2009*, **10**, 296-307.

[13] Goon P, Stanley MA, Ebmeyer J, et al. HPV and head and neck cancer: a descriptive update. *Head and Neck Oncology 2009*, **1**, 36.

[14] Walboomers JMM, Jacobs MV, Manos MM, et al: Human papillomavirus is a necessary cause of invasive cancer worldwide. J Pathol 1999, 189(1):12–19.

[15] Munoz N, Bosch FX, Castellsague X, et al: Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 2004, 111(2):278–285.

[16] Iaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D and von KnebelDoeberitz M. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001; 92: 276-84.

[17] Kong CS, Balzer BL, Troxell ML, Patterson BK and Longacre TA. p16INK4A immunohistochemistry is superior to HPV in situ hybridization for the detection of high-risk HPV in atypical squamous metaplasia. Am J SurgPathol 2007; 31: 33-43.

[18] Milde-Langosch K, Riethdorf S, Kraus-Pöppinghaus A, Riethdorf L and Loning T. Expression of cyclin-dependent kinase inhibitors p16MTS1, p21WAF1, and p27KIP1 in HPV-positive and HPV-negative cervical adenocarcinomas. Virchows Arch 2001; 439: 55-61.

[19] Houghton O, Jamison J, Wilson R, Carson J and McCluggage WG. p16Immunoreactivity in unusual types of cervical adenocarcinoma does not reflect human papillomavirus infection. Histopathology 2010; 57: 342-50.

[20] Yemelyanova A, Vang R, Seidman JD, Gravitt PE and Ronnett BM. Endocervical adenocarcinomas with prominent endometrial or endomyometrial involvement simulating primary endometrial carcinomas: utility of HPV DNA detection and immunohistochemical expression of p16 and hormone receptors to confirm the cervical origin of the corpus tumor. Am J SurgPathol 2009; 33: 914-24.

[21] Tase T, Okagaki T, Clark BA, Manias DA, Ostrow RS, Twiggs LB and Faras AJ. Human papillomavirus types and localization in adenocarcinoma and adenosquamous carcinoma of the uterine cervix: a study by in situ DNA hybridization. Cancer Res 1988; 48: 993-8.

[22] Zielinski GD, Snijders PJ, Rozendaal L, Daalmeijer NF, Risse EK, Voorhorst FJ, Jiwa NM, van der Linden HC, de Schipper FA, Runsink AP and Meijer CJ. The presence of high-risk HPV combined with specific p53 and p16INK4a expression patterns points to high-risk HPV as the main causative agent for adenocarcinoma in situ and adenocarcinoma of the cervix. J Pathol 2003; 201: 535-43.

[23] Pirog EC, Kleter B, Olgac S, Bobkiewicz P, Lindeman J, Quint WG, Richart RM and Isacson C. Prevalence of human papillomavirus DNA in different histological subtypes of cervical adenocarcinoma. Am J Pathol 2000; 157: 1055-62.

[24] Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, SnijdersPJ,PawlitaM,etal.A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 2007;121:2465–72.

[25] Andrew G. Schache, TriantafilosLiloglou, Janet M. Risk et al. Evaluation of Human Papilloma Virus Diagnostic Testing in Oropharyngeal Squamous Cell Carcinoma: Sensitivity, Specificity, and Prognostic Discrimination. Clin Cancer Res October 1, 2011 *17;* 6262.

[26] Iwasaki T, Sata T, Sugase M, Sato Y, Kurata T, Suzuki K, Ohmoto H, Iwamoto S, Matsukara T. Detectionof capsid antigen of human papillomvavirus (HPV) in benign lesions of female genital tract using the anti-HPV monoclonal antibody. J Pathol 1992; 168:293.

[27] Prendiville W, Davies P. The Health Professional's HPV Handbook: Human Papillomavirus And Cervical Cancer, Volume 1. United Kingdom: Taylor & Francis 2004;12.