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IMMUNOHISTOCHEMICAL EXPRESSION OF p16$^{(INK4A)}$ PROTEIN IN CERVICAL DYSPLASIA AND CARCINOMA IN PATIENTS ATTENDING FEDERAL TEACHING HOSPITAL, GOMBE, NIGERIA

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ABSTRACT

Aim: This study was conducted to determine and compare the immunohistochemical expression of p16$^{(INK4a)}$ in cervical dysplasia and carcinoma in Gombe, Nigeria.

Materials: Seventy eight cases of cervical neoplasm; comprising 17 cases of cervical intraepithelial neoplasia (CIN), 53 cases of squamous cell carcinoma (SCC), 6 cases of adenocarcinoma and 2 cases of adenosquamous carcinoma. All samples were stained using p16$^{(INK4a)}$ Rabbit monoclonal anti-CDKN2A/p16$^{(INK4a)}$ antibody.

Results: The p16$^{(INK4a)}$ expression was graded as negative, focal and diffuse positivity. The study showed p16$^{(INK4a)}$ expression in CIN I, CIN II and CIN III as 0%, 80.0% and 83.3% respectively. Majority of SCC (88.7%), adenocarcinoma (83.3%) and adenosquamous carcinoma (100%) showed p16$^{(INK4a)}$ expression.

Conclusion: Immunohistochemical detection of p16$^{(INK4a)}$ can be a useful diagnostic marker for all degrees of cervical dysplasias and carcinomas.

Key words: Dysplasia, Carcinoma, Immunohistochemistry, HPV, p16, Neoplasia,

INTRODUCTION

Cervical cancer is one of the major causes of death worldwide, with an estimated incidence of 530,000 new cases identified every year; over 85% of these women are from low and middle income developing countries in South America, sub-Saharan Africa, and the Far East with Nigeria accounting for more than 10% of the cancer burden (WHO, 2013). The incidence of invasive cervical cancer is much lower in the United States; the American Cancer Society stated that in 2010, there were approximately 12,200 new cases, with estimated deaths of 4,210 (Jemal et al., 2010). The level of mortality in Russia is 5.0 per 100,000 (Zaridze, 2000). This is due mainly to the wide screening protocols which allow identification of early asymptomatic forms of cervical cancers. However, early detection of cervical cancer has some challenges. The main screening test for cervical cancer is the cytological smear staining technique developed by Papanicolaou and Traut (1941) and known as Pap test (Gustafsson et al., 1997; Volgareva et al., 2004). Despite evident success, some false-positive and false-negative results have been reported (Volgareva et al., 2004). The association between cervical premalignant and malignant epithelial lesions and human papillomaviruses (HPV) has been well established (Walboomers et al., 1999; Oboma and Avwioro, 2012). More than 200 HPV have been identified (Mendes de Oliveira and Levi, 2016). They have been classified into high-risk (HR-HPV) and low-risk (LR-HPV) based on their association with cervical cancer (Wolf and Ramirez, 2001). HPV infection causes some alterations in gene or protein expression within the infected cells. One of the substances produced by infection with high risk HPV is E7 oncoprotein, which binds to the retinoblastoma gene product (Rb), resulting in its functional inactivation. Since expression of the

cyclin-dependent kinase inhibitor gene p16$^{(INK4a)}$ is under negative feedback of functional Rb, overexpression of p16$^{(INK4a)}$ ultimately occurs in cells infected by high-risk HPV. The p16$^{(INK4a)}$ protein can be detected with immunohistochemical methods, therefore, it can serve as a surrogate marker for high risk HPV, especially as the protein is not expressed in normal cervical squamous epithelium. Overexpression of the cyclin-dependent kinase inhibitor p16$^{(INK4a)}$ is known to be strongly associated with the onset of transforming infections of high-risk (HR) human papillomaviruses (HPV) (Mulvany et al., 2008; Wentzensen and von Knebel Doeberitz, 2007). Histology of colposcopy-guided biopsies is still considered the gold standard in the diagnosis of cervical lesions. However, heamatoxylin and eosin (Avwioro, 2011; Avwioro, 2014) stained histologic assessment of cervical lesions may be complicated by omissions arising from observer variability (Stoler and Schiffman, 2001). Errors in histologic diagnosis can lead to either overtreatment or under treatment of patients (Hwang and Shroyer, 2012). The aim of the study was to come up with a potential biomarker (p16 $^{(INK4a)}$) that can be used as a confirmatory test to improve diagnostic accuracy in the interpretations of cervical lesions, for better management of patients with these lesions.

MATERIALS AND METHOD

Study Design

This is a study of p16 $^{(INK4a)}$ expression on cases with cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) using immunohistochemical method. The formalin fixed paraffin embedded (FFPE) samples were obtained from the archive of the Department of Histopathology, Federal Teaching Hospital, Gombe. This study was approved by the Ethics and Research Committee of Federal Teaching Hospital, Gombe (NHREC/25/10/2013). The cases studied comprised of diagnostic categories of; Normal, CIN I, CIN II, CIN III and SSC. Information on histological report and age of patients were retrieved from the histopathology register book/cards.

Immunohistochemical Method

Paraffin blocks were sectioned at 3µm thick using rotary microtome (Micro M), 2 sections were picked on sialinized slides (test and negative control), allowed to drain and placed in the oven at 70^{o}C for 60 minutes. Sections were then deparaffinized by passage through 2 changes of xylene, 10 minutes in each. Sections were rehydrated in graded alcohol of decreasing concentration i.e. 100%, 80% and 70% at three minutes interval per change. They were then rinsed in running tap water. Antigen retrieval was performed with the sections placed in the target retrieval solution (0.1M Citrate buffer, pH 6.0) for 60 minutes at temperature of 95^{o}C using water bath method followed by cooling at room temperature for 20 minutes (Enemari et al., 2016). Sections were then rinsed with phosphate buffer (PBS). Sections were treated with Peroxidase block for 10 minutes. They were then washed in the buffer solution and treated with Protein block for 10minutes. Sections were incubated for 60 minutes at room temperature with the primary antibody; Rabbit monoclonal anti-CDKN2A/p16$^{(INK4a)}$ antibody (1:100 dilution, from Abcam Plc, Cambridge UK). After washing thoroughly with PBS, the sections were treated with the secondary antibody (Biotinylated goat anti-rabbit IgG) for 10 minutes. Sections were then washed in PBS buffer solution. The sections were treated with Streptavidin Peroxidase for 10 minutes. Sections were washed in two (2) changes of the buffer solution. A drop of diamino benzidene (DAB) + Substrate mixture (1 drop of DAB to 1ml of DAB substrate mixture) was then spread over the section for seven (7) minutes and then rinsed in PBS. The sections were then counter-stained with haematoxylin for 5-10 seconds before rinsing with running water for three minutes and dehydrated in increasing alcohol concentration and mounted in DPX.

Immunohistochemical Analysis

The positive control in the assay for p16$^{(INK4a)}$ was squamous cell carcinoma tissue according to the recommendation of the manufacturer of the test kits. The negative control was stained in a similar way with the test but with omission of the primary antibody. The status of p16$^{(INK4a)}$ expression was then evaluated by observing the stained slides microscopically. The p16$^{(INK4a)}$ reactivity was graded by determining the percentage of p16$^{(INK4a)}$ immunoreactive cells i.e. brown nuclear and cytoplasmic reactivity (Redman et al., 2008). The staining reactivity was graded as follows:

Negative: 0% to 5% immunoreactive cells.

Positive: Focal/Scattered positivity (5% to 50% immunoreactive cells).

Diffuse positivity (more than 50% of immunoreactive reactive cells).

Statistical Analysis

GraphPad Prism 6 was used in the statistical analysis. The percentage of cases with p16$^{(INK4a)}$ expressions for CIN III and SCC were evaluated using Fisher's exact test. Any p value less than 0.05 was considered statistically significant.

RESULTS

There were a total of 78 cervical squamous neoplastic lesions, comprising 17 cases of Cervical Intraepithelial Neoplasm (6 – CIN I (7.7%), 05 - CIN II (6.4%) and 06 - CIN III (7.7%)) and 61 cases of Carcinoma (53 Squamous Cell Carcinoma; large cell non-keratinizing and large cell keratinizing (68.0%), 6 cases of Adenocarcinoma (7.7%) and 2 cases of Adenosquamous carcinoma (2.5%)). Five cases of non-neoplastic (normal) cervical tissues were included as controls. The distribution of cases with cervical dysplasia and carcinoma is presented in Table 1. The age distribution of the patients studied showed that the youngest patient was 27 years old on presentation while the oldest patient was 80 years old. CIN I was seen in patients less than 40 years old (66.6%), while CIN II occurred in patients up to 50 years old (80.0%). CIN III, on the other hand was found both in patients 31-40 years and above. The highest number of patients with squamous cell carcinoma of the cervix was seen in patients between ages of 41-50 years (39.6%). Other types of cervical carcinomas were found in patients but mostly at the age of above 40 years up to 80 years of age. The age distribution of patients with cervical dysplasia and carcinoma is shown in Table 2.

Table 1: Distribution of patients with cervical dysplasia and carcinoma

	CIN I	CIN II	CIN III	Squamous Cell Carcinoma	Adenocarcinoma	Adenosquamous
Cases	06	05	06	53	06	02
%	7.7	6.4	7.7	68	7.7	2.5

Table 2: Age Distribution of Patients with cervical dysplasia and carcinoma

Age	CIN I	CIN II	CIN III	SCC	Adenocarcinoma	Adenosquamous
21-30	2 (33.3)	2 (40.0)	0 (0)*	4(7.5)	0 (0)	0 (0)
31-40	2 (33.3)	0 (0)	3 (50.0)	10 (18.9)	3(50.0)	0 (0)
41-50	1 (16.7)	2 (40.0)	0 (0)	21 (39.6)	0 (0)	0 (0)
51-60	1 (16.7)	0 (0)	1(16.7)	5 (9.4)	0 (0)	1(50.0)
61-70	0 (0)	1 (20.0)	1(16.7)	9 (17.1)	2(33.3)	1(50.0)
71-80	0 (0)	0 (0)	1(16.7)	4 (7.5)	1(16.7)	0 (0)
Total	06	05	06	53	06	02

*percentage is written in parenthesis, Squamous Cell Carcinoma (SCC)

Immunohistochemical expression of $p16^{(INK4a)}$ in the cervical epithelial cells was characterized by focal and diffuse nuclear and cytoplasmic staining. There was no noticeable difference in the intensity of staining between the different epithelial layers. Clear and distinctive positive staining was observed both in some dysplastic cells (CIN II and CIN III) and carcinomas. In all the five (5) samples from patients with normal or non-neoplastic cervical tissues, $p16^{(INK4a)}$ expression was observed to be negative (Table 3 and Figure 1A). Immunohistochemical expression of $p16^{(INK4a)}$ amongst patients with dysplastic cervical samples showed that all six (6) CIN I samples were negative for $p16^{(INK4a)}$ immunostaining expression (Table 3 and Figure 1B). CIN II samples showed focal expression in the 80% of the samples observed (Table 3 and Figure 1C), while a sample, representing 20% was negative. CIN III samples showed $p16^{(INK4a)}$ immunostaining in 83.3% of the samples observed (focal expression 66.6% and diffuse expression 16.7%) and negative expression in 16.7% as in Table 3 and Figure 1D. Cervical samples from patients with squamous cell carcinomas (large cell non-keratinizing and large cell keratinizing) showed $p16^{(INK4a)}$ immunostaining in 88.7% of the samples observed (diffuse expression in 64.2% and focal expression in 24.5%) and negative expression of 11.3% (Table 3, Figures 1E&F). Out of the 6 samples from patients with adenocarcinoma of the uterine cervix, $p16^{(INK4a)}$ immunostaining was observed in 83.3% (focal expression in 50.0%, diffuse expression in 33.3%) and negative expression in 16.7% of the samples (Table 3 and Figure 1G). All the two samples from patients with Adenosquamous carcinoma of uterine cervix showed focal expression of $p16^{(INK4a)}$ immunostaining (100%) Table 3 and Figure 1H

Table 3: Immunohistochemical expression of p16$^{(INK4a)}$ in normal, dysplastic and neoplastic cervical tissues

		p16(INK4a) immunostaining		
		Negative	Positive	
	N		Focal	Diffuse
Normal	5	5(100)*	0(0)	0(0)
CIN I	6	6(100)	0(100)	0(0)
CIN II	5	1(20)	4(80)	0(0)
CIN III	6	1(16.7)	4(66.6)	1(16.7)
SCC	53	6(11.3)	13(24.5)	34(64.2)
Adenocarcinoma	6	1(16.7)	3(50.0)	2(33.3)
Adenosquamous	2	0(0)	2(100)	0(0)

*percentage is written in parenthesis

Table 4: Number of cases of CIN III and SCC in relation to expressions of p16$^{(INK4a)}$

	p16(INK4a) Expression		
	Negative	Positive	N
CIN III	1	5	6
SCC	6	47	53
*P value = 0.5483			59

CIN-Cervical Intraepithelial Neoplasia;
SCC-Squamous Cell Carcinoma

Diffuse and focal p16$^{(INK4a)}$ staining was observed in CIN III and SCC. The difference of p16$^{(INK4a)}$ expression in CIN III and SCC was not statistically significant (p value is equal to 0.55).

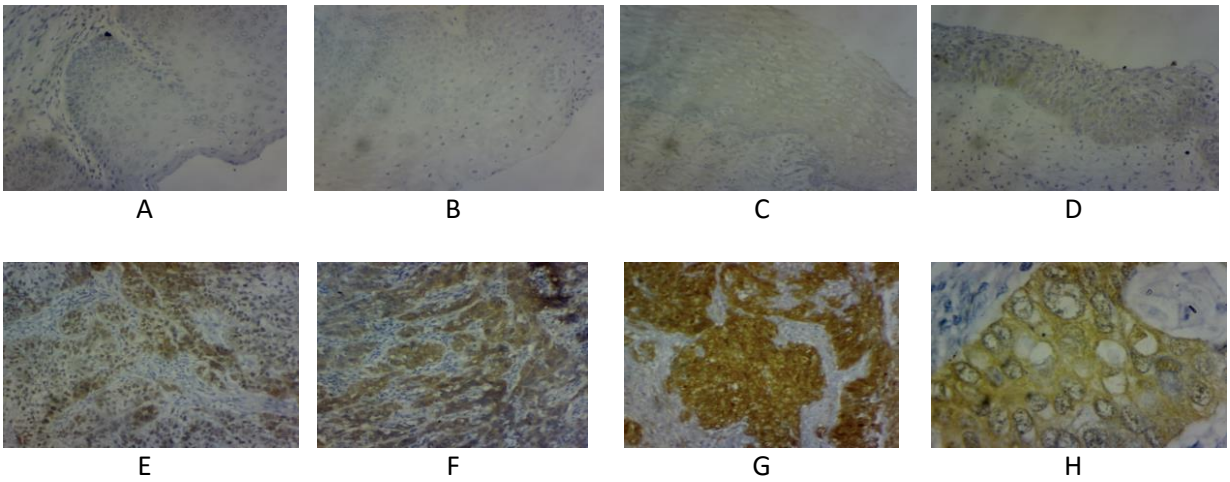

Figure 1. Immunohistochemical expression of p16$^{(INK4a)}$ monoclonal antibody in cervical tissues;
A-Normal epithelium, B-CIN I, C-CIN II, D-CIN III, E-Large cell keratinizing, F-Large cell non-keratinizing, G-Adenocarcinoma and H-Adenosquamous carcinoma. Mag.x400.

DISCUSSION

Knowledge and attitude of majority of the population on cervical screening in Gombe, Nigeria is poor. There are also not enough screening centres in the area which are almost non existence in the rural areas. These have contributed to the high number of patients with high grade (SCC, Adenocarcinoma and Adenosquamous) cervical lesions (78.2%) as reported in table 1. Oche et al., (2013) in a study reported a high incidence of cervical cancer among health workers in Sokoto, but a very low Pap smear tests among the study subjects. Only 10% of the study subjects had done Pap smear (22 out of 220). This was attributed to the fact that some of the subjects felt they were not at risk, while some were not aware of the availability of the service in the hospital, others were just not comfortable exposing themselves in the presence of male doctors. Ahmed et al., (2013) in a similar study reported a fair knowledge of cervical cancer and cervical screening among market women in Zaria (43.5%), however, their knowledge of the risk factors was poor. They found a general good attitude to cervical cancer screening (80.4%), but the level of practice (Pap smear uptake) was low (15.4%). This poor attitude to Pap

smear may reflect the possibility of failure to identify some of the cancer precursor lesions much earlier before they develop into high grade lesions. The youngest patient in this study was 27 years and the oldest was 80 years old. There were four (4) patients with cervical intraepithelial lesions (2-CIN I and 2- CIN II) and four (4) with SCC in the age group between 21-30 years. This is an indication that some young women become sexually active early and have the risk of contracting HPV virus. None of the women in this study was pregnant. This is similar to the work of Avwioro et al., (2009) in South Western Nigeria where they did not observe any case of cervical cancer in pregnancy. A national study in Malaysia in 2004 found that the median age for the first sexual activity was 23 years and some had sex before the age of 20 years (Cruez, 2007). This finding suggests that younger women should have cervical screening as soon as they become sexually active. All of the CIN I cases were $p16^{(INK4a)}$ negative. The high percentage of negativity of $p16^{(INK4a)}$ in CIN I may be due to latent or subclinical HPV infection with low viral load that may be insufficient for $p16^{(INK4a)}$ expression (Tan et al., 2010). Ishikawa et al., (2006) found that overexpression of $p16^{(INK4a)}$ in CIN I was more common in cases with HPV 16 and HPV 52 infection. The other possible reason for lower expression of $p16^{(INK4a)}$ in low grade lesions may be because a certain percentage of CIN I is thought to be caused by low risk HPV types. Previous studies indicated that viral oncoprotein of low-risk HPV such as HPV-6 have no effect on $p16^{(INK4a)}$ because the affinity of HPV-6 E7 protein for cellular pRb is ten-fold lower than that of HPV-16 E7 for pRb (Sano et al., 1998). Studies also suggest that persistent infection by specific viral type, especially HPV 16 and 18 has the greatest tendency to result in CIN II or CIN III (Dalstein et al., 2003 and Schlecht et al., 2001). The study shows that (88.7%) SCC lesions show $p16^{(INK4a)}$ expression, this further emphasizes the important causal relationship between HPV and cervical cancer. However, a few patients with cervical cancer had $p16^{(INK4a)}$negativity. Nieh et al., (2005) showed that a proportion of cervical cancer cases in their study had neither HPV infection nor $p16^{(INK4a)}$expression. The possible explanation for the absence of expression in these high grade lesions could be methylation of the $p16^{(NK4a)}$promoter resulting in silencing of the $p16^{(INK4a)}$ gene (Ferreux et al., 2003). Our study shows no significant statistical difference in $p16^{(INK4a)}$ expression between CIN III and SCC ($p = 0.55$).

CONCLUSION

Overexpression of the protein $p16^{(INK4a)}$ is a characteristic of dysplastic and neoplastic lesions of cervical epithelium. The expression of $p16^{(INK4a)}$ positivity increases in the order: CIN II – CIN III – Invasive carcinomas. Some samples analysed stained poorly or were not stained at all with $p16^{(INK4a)}$ antibody. In other words, $p16^{(INK4a)}$ negative cervical neoplasms and carcinomas do exist as reported by Valgareva et al.,(2004). All normal cervical tissues were negative for $p16^{(INK4a)}$ expression. The number of $p16^{(INK4a)}$ positive cells increase with the advancement of the stage of CINs and carcinomas. This is an indication that immunohistochemical detection of $p16^{(INK4a)}$ can be used as a specific diagnostic marker of all degrees of cervical dysplasia and carcinomas.

REFERENCES

Ahmed SA, Sabitu K, Idris SH, Ahmed R (2013). Knowledge, attitude and practice of cervical cancer screening among market women in ZARIA, Nigeria. Nigerian Medical Journal. 53: 316-319.

Avwioro OG (2011). Histochemical uses of haematoxylin — A review JPCS 1, 24-34

Avwioro OG (2014). Histochemistry and Tissue Pathology. Claverianun Press, Ib, 4th ed., Nigeria

Avwioro OG, Mustapha KT, Avwioro TO, Owolagba GK, Bankole JK (2009). Cervical cancer in pregnancy is not a common condition in Western Nigeria Journal of Medical Laboratory Science 17 (1), 23-24

Cruez AF (2007). Malaysian youth are involved in sexual activities before the age of 20- Lekhraj Rampal New Straits Times: Press Holding Ltd. Co.

Dalstein V, Riethmuller D, Prétet JL, Le Bail Carval K, Sautière JL, Carbillet JP (2003). Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. International Journal of Cancer. 106:396-403.

Enemari JO, Avwioro OG, Osadolor HB, Idoko J, Yaro JD, Odigie BE (2016). A comparison of the effect of commercial pH buffers on epitope retrieval of 'HER-2/neu' protein. African Journal of Cellular Pathology 6;1-5

Ferreux E, Lont AP, Horenblas S, Gallee MP, Raaphorst FM, von Knebel Doeberitz M (2003). Evidence for at least three alternative mechanisms targeting the p16 INK4A /cyclin D/Rb pathway in penile carcinoma, one of which is mediated by

high-risk human papillomavirus. Journal of Pathology. 201:109-18.

Gustafsson L, Pontén J, Zack M, Adami HO (1997). International incidence rates of invasive cervical cancer after introduction of cytological screening. Cancer Causes and Control. 8(5):755–763.

Hwang SJ, Shroyer KR (2012). Biomarkers of Cervical Dysplasia and Carcinoma. Journal of Oncology. 2012: 9.

Ishikawa M, Fujii T, Saito M, Nindl I, Ono A, Kubushiro K (2006). Overexpression of $p16^{(INK4a)}$ as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. Internal Journal of Gynecological Cancer. 16:347-53.

Jemal A, Siegel R, Xu J, Ward E (2010). Cancer statistics 2010. CA Cancer Journal for Clinicians. 60(5):277–300.

Mendes de Oliveira C, Levi JH (2016). The biological impact of genomic diversity in cervical cancer development. Acta Cytologica. 60(4):396.

Mulvany NJ, Allen DG, Wilson SM (2008). Diagnostic utility of $p16^{(INK4a)}$: a reappraisal of its use in cervical biopsies. Pathology. 40:335–344.

Oboma YI, Avwioro OG (2012) Prevalence of cervical Human Papilloma Virus Infection among women in Bayelsa State Nigeria Continental J. Biomedical sciences 6;1-3

Oche MO, Kaoje AU, Gana G, Ango JT (2013). Cancer of the cervix and cervical screening: Current knowledge, attitude and practices of female health works in Sokoto, Nigeria. International Journal of Medicine and Medical Sciences. 5(4):184-190.

Papanicolaou GN, Traut HF (1941). The diagnostic value of vaginal smears in carcinoma of the uterus. American Journal of Obstetrics Gynecological. 42:193–206

Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T (1998). Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. American Journal of Pathology. 153:1741-8.

Schlecht NF, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA (2001). Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. Journal of the American Medical Association. 286:3106-14.

Stoler MH, Schiffman M (2001). Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. Journal of the American Medical Association. 285(11):1500–1505.

Tan GC, Norlatiffah S, Sharifah NA, Razmin G, Shiran MS, Hatta AZ, Paul-Ng HO (2010). Immunohistochemical study of $p16^{(INK4a)}$ and surviving expressions in cervical squamous neoplasm. Indian Journal of Pathology and Microbiology. 53(1):1-6.

Volgareva G, Zavalishina L, Andreeva Y, Frank G, Krutikova E, Golovina D, Spitkovsky D, Ermilova V, Kisseljov F (2004). Protein p16 as a marker of dysplasia and neoplastic alterations in cervical epithelial cells. BMC Cancer. 4:58.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. Journal of Pathology. 189(1):12–19.

Wentzensen N, von Knebel Doeberitz M (2007). Biomarkers in cervical cancer screening. Disease Markers. 23:315–330.

WHO (2013). World Health Organisation guidance note: comprehensive cervical cancer prevention and control: a healthier future for girls and women. http://*www.who.int*

Wolf JK, Ramirez PT (2001). The molecular biology of cervical cancer. Cancer Investigation. 19(6):621–629.

Zaridze DG (2000). Epidemiology and etiology of malignant tumours. In Carcinogenesis Edited by: Zaridze DG. Moscow: Nauchny Mir, 21 – 56.