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Original Article

Early detection of cervical tumor cell of origin through Oncogene-induced senescent HPV-positive cells

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Abstract

Human Papilloma Virus (HPV) is an oncogenic virus and is the most common cause of cervical cancer. HPV has been shown to induce senescence. Cellular senescence is involved in cancer progression and tumorigenesis. Identification and isolation of cells of tumor origin before tumorigeneses is an important step in cancer prevention and treatment. This study aimed to investigate the early cervical atypical senescent cytological preneoplastic change in non-menopausal women. Cervical smears of 121 patients were randomly selected and included in the study which cytopathologically diagnosed as atypical squamous cells of undetermined significance (AS-CUS) in correlation to HPV status, parakeratosis (PK), p16 immunostaining, enlarged Squamous cells nuclei (ES) and inflammatory cells infiltration (ICI). Results revealed that out of the total 121 patients, 32 cases (26%) were positive for high-risk HPV (HR-HPV), 26 cases (22%) were positive for low-risk HPV (LR-HPV) and 63 (52%) were negative for HPV. HPV infections were significantly associated with age groups ($p<0.026$), PK ($p = 0.043$), p16 ($p = 0.001$), ES ($p = 0.002$) and ICI ($p = 0.049$). The positive immunostaining expression of p16 was only noticed in two HR-HPV patients. ES cells were found in 9.5% of HPV-negative cases, 27% of LR-HPV cases and 40.5% of HR-HPV cases. High PK cell positivity was seen only in HR-HPV. High ICI scores were seen in 40.6% of HR-HPV patients, 26.9 % of LR-HPV and 17.4 % of negative HPV patients. It was concluded that high PK positivity, high ICI score, positive p16 immunostaining and ES were correlated with HR-HPV in non-menopausal women. These findings could provide potential diagnostic clues for HPV-harboring senescent cells as a strategy for reducing HPV risk of cervical cancer development and identifying the cell of tumor origin, which could be beneficial for improving the utility of senolytic agents and immunotherapy in clinical practice.

Keywords: Atypical squamous cells, Cellular senescence, Cervix, HPV, p16 immunostaining, Parakeratosis

1. Introduction

Malignant tumors remain a largely incurable disease. Tumor-initiating cells also known as cancer stem cells, are the only cells that can make a new tumor. Cancer stem cells “cell of tumor origin" if identified and isolated scientists might be able to turn cancer into a manageable chronic disease. As a consequence, cancer therapy drugs actually focus on tumor-initiating cells and their relevant targets [1]. Carcinoma of the cervix is one of the most common and preventable causes of female genital tract malignancies [2], and the commonest type of squamous cell origin [3]. Cervical Cytology primarily detects abnormality in epithelial cells that leads to carcinoma of the cervix which ranges from different grades of cervical intraepithelial neoplasia to invasive carcinoma [4]. The Bethesda System reports the abnormality of epithelial cells which comprises either squamous cell abnormalities or glandular cell abnormalities. The abnormalities of squamous epithelial cells include atypical squamous cells of undetermined significance (AS-CUS), atypical squamous cells cannot exclude high-grade squamous intraepithelial lesions (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC). Atypical cervical squamous cells are the most common and prevalent form of all abnormal cervical cytology diagnoses and interpretations [5]. Cervical cytological smear screening method is essential because of its simplicity, effectiveness, rapidity and inexpensiveness technique [6]. HPV infection is the primary causative factor for cervical cancer [7]. HPV 16 and 18 are the commonest high-risk group types associated with cervical cancer while HPV 6 and 11 (Low-risk types) are rarely associated with cancer but are usually related to benign lesions [8, 9]. P16, is a cyclin-dependent kinase inhibitor, has an important role in cell cycle control by reducing cell progression from G1 phase to S phase in normal healthy cells. It is usually expressed in low concentration, while it is overexpressed in cervical cancer and HSIL [10]. Studies reported that immunostaining with p16 have an important diagnostic role in the differentiation between low and high-grade cervical dysplasia, as well as in differentiating transient HPV infection from persistent

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infections [11, 12]. Persistent of DNA damage signals promote p16 activation via the Ets family transcription factor. P16, is an important cell cycle stopper, thereby leading to non-reversible cell cycle arrest [13]. Parakeratosis (PK) is one of important morphological HPV infection-related changes with koilocytosis, dyskeratosis, nuclear hyperchromasia, multinucleation, and keratohyalin granules [14]. Cellular senescence is involved in cancer progression and tumorigenesis [2]. Failure of immune system to eliminate senescent cells and their gradual accumulation in the tissue could lead to adverse effects. Senescence-associated secretory phenotype (SASP) is a characteristic cellular senescence phenotype that includes multiple factors, for example proteases, chemokines, cytokines, interleukins, and growth factors [15]. SASP accumulation components could promote tumorigenesis and remodel the microenvironment of the tumors [16]. The mechanisms and role of SASP and senescence-related phenotypes have been increasingly reported to be associated with many diseases [17]. The chemokines that are released from senescent cells as SASP factors stimulate the activation of immune cells, such as macrophages and NK cells that can scavenge senescent cells [18]. Many reported studies suggested that SASP factors related to aging-associated with cancer and inflammatory lesions [19]. According to the above issues, the objective of this study was to understand the cause of cervical cancer initiation, by screening and identifying the early atypical cytological change in cell morphology (cervical epithelial cell abnormalities) in correlation to HPV infection and p16 immunostaining.

2. Materials and Methods

2.1. Patient selection

One hundred and twenty-one cervical smears were randomly selected and included in this study from Alzahrawi laboratory in Erbil, Kurdistan region of Iraq. Inclusion Criteria: Cases were cytopathologically diagnosed as atypical squamous cells of undetermined significance (ASCUS) and were interpreted according to the 2015 Bethesda System [20], during the period from January 2022 to January 2024. All cases were from non-menopausal women and had conventional types of cervical smear cytological preparation. Exclusion criteria were: individuals who received treatment before taking the sample.

2.2. Sample collection, DNA extraction and HPV genotyping

Biological samples of cervical (swab) were collected for detection and characterization of HPV genotyping, encompassing high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 53, 73, 69 and 44), and low risk (6, 11, 42, 61 and 70) groups of HPV. Only one positive HPV gene cases were included in the study. Polymerase chain reaction (PCR) was used to determine the presence of HPV DNA by PCR-based genotyping kit, the Roche LINEAR ARRAY HPV assay was used in this study.

2.3. Cytomorphology and Immunostaining

P16 immunostaining positive results showed cells with confined nuclear with/without cytoplasmic staining, categorized into three groups: Flake, Patchy, and Unicellular. The Flake group was defined by cells' distribution as flake clusters of positive cells; the Patchy group was defined by positive cells distributed as clusters of a few cells the Unicellular group was defined by single positive cells. Negative results were defined as confined cytoplasmic staining [20]. Enlarged Squamous cell nuclei (ES) were considered positive when squamous cells enlarged (more than 3 times the area of the normal intermediate cell nucleus) with bland, smooth nuclei with mild contour irregularity, or mild hyperchromasia, while squamous cells with "bland nuclear enlargement" only nuclear enlargement without nuclear membrane irregularity or significant hyperchromasia called negative [21]. Features of PK which are defined as eosinophilic parabasal keratinocytes with a nuclear/cytoplasmic ratio of 30% to 50% were explored by observing the number of PK in the whole slide [22]. For quantitative evaluation of immune/ inflammatory cells, three areas at low magnification were selected. The selected areas were those in which it had been confirmed at high magnification. Lymphocytes, neutrophils and macrophages were then counted by independent investigators. These observers were blinded to each other and also not provided with any clinical information on the outcome of the patients. The average counts for each of the three areas were divided into two groups showing high and low inflammatory (immune) cell infiltration (ICI) [23].

2.4. Statistical analysis

To evaluate how likely the observed difference between the sets of parameters investigated in this study arose by chance, the data were analyzed using Chi-square goodness of fit test. Moreover, Fisher's exact test also was used in the analysis of contingency tables of parameter studies. Contingency tables were formed and used to analyze counts of categorized parameters to determine if there is association between variables. The associations were considered to be extremely statistically significant (p <0.05), which indicates that the two parameters are dependent variables.

Correlation coefficients were estimated between studied parameters using Statistical Analysis System User's Guide. Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. The USA.

3. Results

3.1. Patients' characteristics

The age of the 121 patients included in this study ranged from 19 to 49 years with an average of 33.4 ± 6.36 years. A total of 44 (36%), 51 (42%) and 26 (22%) patients were classified as group 1 (of 19 – 29yrs), group 2 (of 30 – 39yrs), and group 3 (of 40 – 49yrs), respectively. Results revealed that out of the total 121 patients, 32 cases (26%) were positive for high-risk HPV (HR-HPV), 26 cases (22%) were positive for low-risk HPV (LR-HPV) and the rest 63 cases (52%) were negative for HPV (Table 1).

3.2. HPV Genotypes

The result of the PCR revealed that HPV genotype type 16 had the highest frequency for HR-HPV cases, while HPV type 6 genotype had the highest frequency for LR-HPV cases (Figure 1). In this study, 14 HR-HPV genotypes were assayed, and the resulting frequencies are shown in Figure 2. Moreover, 5 LR-HPV were assayed. The result revealed that seven out of 32 (21.88%) patients of HR-HPV were of genotype 16, followed by 4/32 (12.50%) patients of genotype 53 (Figure 2). Meanwhile, the result of PCR showed that 13/26 (50%) patients of LR-HPV were of genotype 6, followed by 8/26 (30.77%) patients of

Table 1. Associations of HPV with other parameters.

Category	HPV Negative	Low risk	High risk	Total	*p* value
Age group (yrs.):					
19 – 29	19	11	14	44	0.026
30 – 39	23	13	15	51	
40 – 49	21	2	3	26	
PK					
No cell	62	23	23	108	0.043
<3 cells	1	3	4	8	
3 – 9 cells	0	0	4	4	
More than 9 cells	0	0	1	1	
p16:					
Negative	63	26	30	119	0.001
Positive	0	0	2	2	
Cell Nuclear Size:					
Non ES	57	19	19	95	0.002
ES	6	7	13	26	
ICI:					
Low	52	19	19	90	0.049
High	11	7	13	31	
Total / HPV	63	26	32	121	

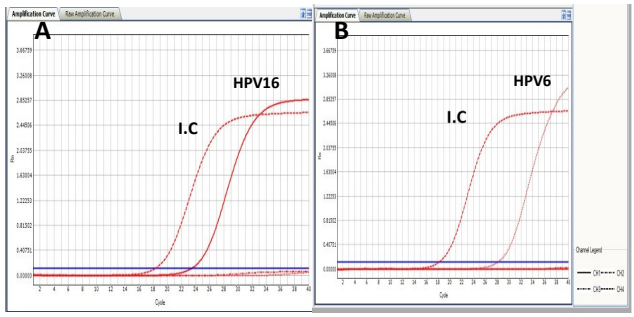
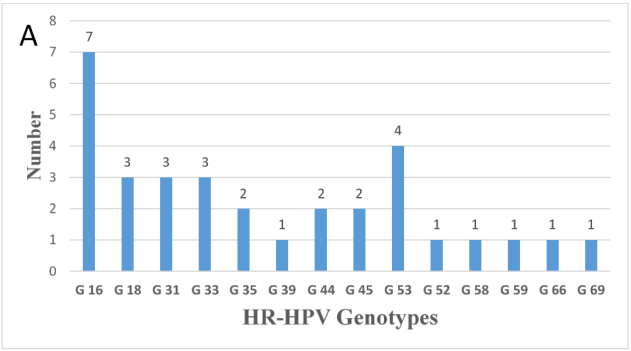


Fig. 1. HPV genotype PCR detection (Real-time PCR).



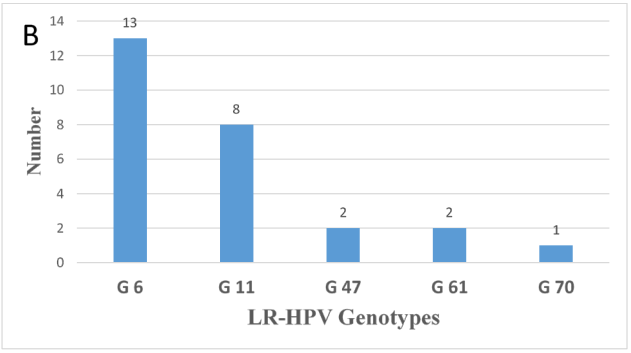


Fig. 2. (A) Distribution and prevalence of HR-HPV genotypes and (B) Distribution and prevalence of LR-HPV genotypes.

genotype 11 (Figure 2).

3.3. Associations of HPV with other parameters

The positive immunostaining expression of p16 was only noticed in two HR-HPV cases (as single cells with positive nuclear staining). While all others were negatively stained. Regarding squamous cell nuclear size, 6 out of 63 (9.5%) of HPV negative cases, 7 out of 26 (27%) LR-HPV cases and 13 out of 32 (40.5%) HR-HPV cases presented with features of ES cells with enlarged smooth, bland nuclei with mild contour irregularity, or mild hyperchromasia, (P=0.002) significant difference. Squamous differentiation was also evaluated using the emergence of PK and compared the prevalence between the three groups. Out of the 63 negative HPV, only1.6% (1/63) showed PK cell positivity, compared to 11.5% (3/26) of patients with LR-HPV and 28.1% (9/32) of women with HR-HPV exhibited significant PK positive (P =0.043). The number of PK per slide ranged from 1-15. Notably, 3.1% (1/32) of patients with HR-HPV featured more than nine PK and 12.5% (4/32) of patients with HR-HPV featured 4-8 PK per slide. Those patients may develop cervical cancer in the future if some measures will not be taken. Whereas, no LR-HPV and negative HPV patients had more than three PK per slide (Figure 3).

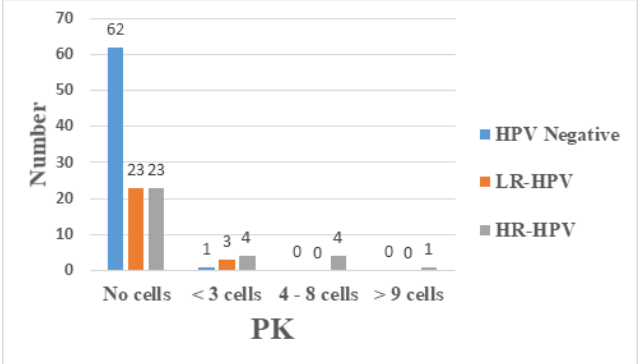


Fig. 3. Distribution of the groups across PK.

Thirteen cases out of 32 (40.6%) HR-HPV patients, 7 out of 26 (26.9 %) LR-HPV and 11 out of 63 (17.4 %) negative HPV patients had high inflammatory (immune) cell infiltration scores and the neutrophil was the predominant inflammatory cell (Figures 4 and 5).

3.4 The correlation coefficients among studied parameters

A highly significant strong correlation coefficients were observed between HPV with age groups, PK cells, p16 and cell nuclear size. Moreover, p16 was highly correlated with cell nuclear size and ICI. Cell nuclear size was highly correlated with ICI. This indicated that associations of HPV with other parameters investigated were dependent variables, and showed relationships between them (Table 2).

4. Discussion

This study compared the cervical cytological findings in non-menopausal women with AS-CUS, “atypical squamous cells, of undetermined significance" in correlation to human papillomavirus HPV analysis, patient age, PK, immunostaining expression of p16, squamous cell nuclear size and the inflammatory (immune) cells infiltration score. Moreover, the management of women with AS-CUS remains a clinical challenge and the diagnoses are highly subjective and may lead to misdiagnosis with a variety of causes including HPV infection, other pathogenic infections, atrophy, air dryness, intrauterine device and tumor-related factors [24]. Our results agreed with previous studies conducted in which HPV type 16 prevalence was reported to be the most common HPV genotype in HR-HPV group and that HPV type 6 prevalence was reported to be the most common HPV genotype in LR-HPV group [25]. The positive immunostaining expression of p16 was only noticed in two HR-HPV patients defined as single positive cells. ES cells were found in 9.5% of HPV-negative cases, 27% LR- HPV cases and 40.5% of HR-HPV cases. The association between parakeratosis in the cytological investigation revealed higher PK in HR-HPV (28.1%) than LR-HPV (11.5%) and negative HPV (1.6%) groups of patients. High inflammatory (immune) cell infiltration scores were seen in 40.6% of HR-HPV patients, 26.9 % of LR-HPV and 17.4 % of negative HPV patients. Cervical cytology is a minimally invasive procedure and plays an important role in the diagnosis of cervical lesions. HPV DNA combined with cytology has been applied to improve the sensitivity for the diagnosis of different cervical lesions. The expression levels of p16 were significantly higher in the HSIL groups and cervical cancer compared with the LSIL and Benign groups. Immunostaining occur-

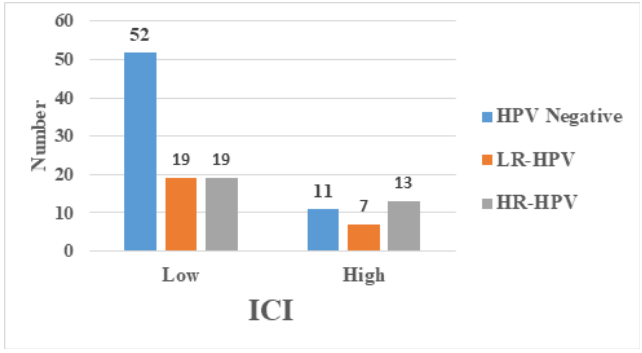


Fig. 4. Distribution of the groups across ICI.

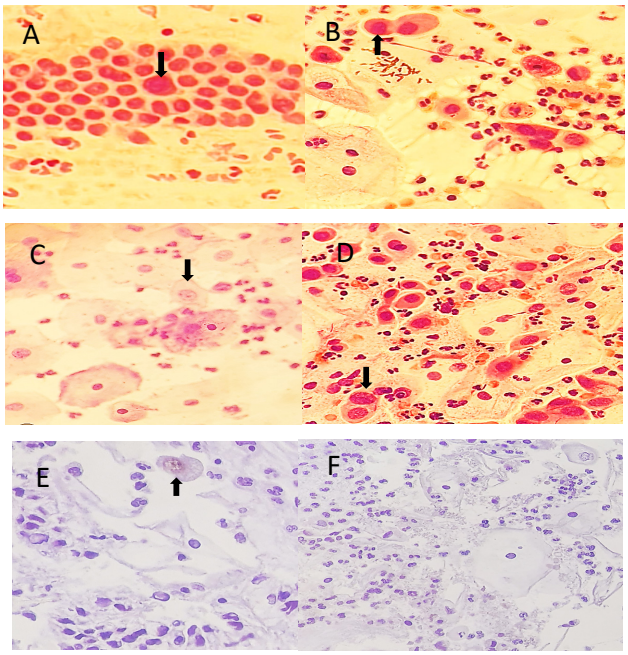


Fig. 5. (A) cervical smear with single atypical morphology cell (AS-CUS) that is obviously noticed (arrow). (B) HR-HPV cervical smear showing squamous cells with PK (arrow). (C) LR-HPV cervical smear showing ES with nuclear enlargement without nuclear membrane irregularity or significant hyperchromasia (arrow) with low inflammatory cell infiltration. (D) HR-HPV cervical smear showing ES cells were considered positive with mild contour irregularity and mild hyperchromasia (arrow)with high inflammatory cell infiltration. (A.B.C&D with H&E stain, x400). (E) HR-HPV cervical smear showing single squamous cell with positive nucleus stained (arrow). (F) LR-HPV cervical smear showing no expression (E&F. p16 immunostain x400).

Table 2. Correlation coefficients among investigated variables.

Variables	Correlation coefficients (p-value)				
	HPV	Pk	p16	Cell nuclear size	ICI
Age groups	-0.23 ** (0.010)	-0.15 NS (0.086)	0.04 NS (0.640)	-0.12 NS (0.192)	-0.16 NS (0.071)
HPV	-	0.37 ** (0.0001)	0.19 * (0.0349)	0.32 ** (0.0003)	0.24 ** (0.0087)
Pk		-	0.61 ** (0.0001)	0.60 ** (0.0001)	0.58 ** (0.0001)
P16			-	0.25 ** (0.0061)	0.53 ** (0.0001)
Cell nuclear size				-	0.53 ** (0.0001)

* (P<0.05), ** (P<0.01), NS: Non-Significant

red with a unicellular pattern. p16 overexpression is used as a good indicator for HR- HPV persistent infections in vivo [20]. Another study correlated immune-expression positivity of P16 in cervical cytology and HR-HPV status in 15 out of 17 cases [26]. Many studies reported that the incidence of cervical smears diagnosed as "atypical squamous cells of undetermined significance" (ASC-US) was lower in postmenopausal patients than in the younger age [27]. Carcinoma of the cervix is one of the most common gynecological malignancies. The rate of morbidity and mortality is still high. Results showed that patients in the low-risk group demonstrated more active immune response and more abundant inflammatory (immune) cell infiltrations compared to patients in the high-risk group. In vitro studies showed IL-1α gene expression was increased in cervical cancer tissues and cells [2]. Still, cervical cytological examination remains the gold standard for cervical lesions and cancer screening. In a recent study, the cervical smear samples were compared and analyzed between women with or without premalignant cervical lesions depending on absence or presence of parakeratosis (PK). PK prevalence was compared between the groups, patients with premalignant cervical lesions had higher prevalence of PK positivity (PK ≥ 1; 46% vs. 7%) than those without cervical lesions [22]. A positive HPV result was more frequently identified in cases with parakeratosis than in cases without parakeratosis [14]. Cervical Cells with bland nuclear enlargement are relatively common in perimenopausal women they represented 15.0% of all AS-CUS cases. The study hypothesizes that some dysregulation of nuclear size occurs as a normal consequence of aging, whether this is due to acquired mutation in DNA or reducing estrogen levels injury, or other causes. The molecular mechanisms of benign nuclear enlargement certainly need more investigations, to know the cause for enlarged nuclei not only in reactive conditions but also in malignant proliferations [21]. HPV can induce senescence. Senescence represents cellular stress response. Senescent cells accumulate in premalignant conditions as a product of oncogene hyperactivation by HPV-driven transformation and the risk of development of cervical cancer in the future [28]. Many recent studies have shown that senescent cells secrete growth factors, chemokines and inflammatory cytokines that alter the local tissue environment. Senescence-associated secretory phenotype (SASP) tends not to be transient but to persist, thereby inducing cancer initiation and progression or only chronic inflammation [29]. Senolysis means the elimination of senescent cells has attracted increasing attention which is essential for controlling SASP including HPV cervical cancer development [28, 29]. These findings help to deepen the insights into the pathogenesis and etiology of cervical cancer, which could be beneficial for immunotherapy and prognostic prediction in clinical practice [2]. Gene expression is a multifaceted process and complex, with intricate regulatory mechanisms. Results showed a complicated interaction of environmental and genetically driven changes in expression with age. Aging is a complex process, characterized by a progressive decline in an organism's regulatory biological function and changes in phenotypic characteristics leading to increased chance of developing disease [30]. Genome instability, and epigenetic alterations, are accompanied by changes in gene expression. Identification of genes that are differentially expressed with age has proved useful in identifying pathways whose behavior is modified by age, as well as identifying biomarkers of aging and therapeutic targets. Meanwhile, results revealed that changes in the mean and variance of gene expression with age have consequences for healthy aging and disease development [30]. Viral oncogenes E6 and E7 appeared to modify epithelial function through distinct pathways, thus contributing to cervical cancer progression. There were significant levels of HPV gene expression in epithelial cells than in macrophages and CD8+ T cells, suggesting heterogeneity of HPV gene expression in HPV tumor cells [31]. Therefore, focusing on energetic cancer stem cells (cells of tumor origin) in cancer therapy could directly hit the target. They might be able to turn malignancy into a treatable and manageable disease. Hence, selecting effective screening methods and improving the diagnosis of cervical premalignant lesions is of great importance in reducing the incidence of cervical cancer.

Findings of this work noticed some cells with atypical morphology which may undergo in future more changes that cause the initiation step in the cancer process (altering the DNA). Some cell(s) showed resistance/tolerance to HPV, while other cell(s) showed abnormality in their shape and size. Nevertheless, the body's repair systems can replace damaged sections of DNA,which allow the cell to recover under normal circumstances. Genomic variations in DNA; make cells within tissue, tissues within organs, organs within individuals; and among individuals unique and offer the opportunity for either resistance/tolerance or sensitivity to cancer. Therefore, the result of this study stimulates and initiates several hypotheses and questions to be addressed, including is there a specific age at which specific gene switched on/off its expression in cell(s) under specific initiating risk factors and how long does it take for that to initiate cancer. All these questions should be answered for early detection, prevention and control of cervical cancer [32].

5. Conclusion

Cervical cytological screening with ancillary methods remains the mainstay for early detection and prevention of cervical cancer in women. HR-HPV causes several cervical atypical and senescence cytological-related changes, focusing on energetic cancer-initiating cells (tumor cell of origin) or cancer stem cells in cancer therapy could directly hit the target and stop the process of cervical tumorigeneses.

Conflict of interests

The author declares no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval

The study protocol was approved by the scientific and ethics committee of the College of Pharmacy, Hawler Medical University, Erbil, Iraq (No.5/HMU).

Availability of data and material

The author guarantees that the data of this research will be provided at the request of other researchers.

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