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Evaluation of CAMD1 Gene Promoter Hypermethylation in Human Papillomavirus Positive Cytological Samples of Cervical Cancer

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Abstract

Background: Cervical cancer (CC) is the second most common type of cancer among women. A key factor in developing the disease is the human papillomavirus (HPV) infection. Aberrant DNA hypermethylation in gene promoter regions is one of the most well-fined epigenetic alterations in tumors. This study aimed to investigate cell adhesion molecule 1 (CADM1), promoter methylation in HPV serological positive samples in the Iranian population.

Method: Genomic DNA was extracted from cervical smears from patients and healthy patients acting as a control group for this analytical observational case-control investigation. Reverse dot blotting was performed to first identify the presence of HPV DNA infection. After that, each sample was transformed by being treated with sodium bisulfite, and MSP was then used to determine the CADM1 gene's level of methylation.

Results: Among total number of positive HPV samples (n = 52), 63.5% methylated, 23% hemi-methylated, and 13.5% were unmethylated. On the contrary, in the control group (n=38), 11% methylated, 71% hemi-methylated, and 18% were unmethylated ($P < 0.0001$). Furthermore, comparing the methylation status among high, low and high/low patients indicated that methylation was (66.66, 24.24, and 9.09%), (25, 66.66, and 8.33%), and (14.28, 57.14%, and 28.57%), respectively ($P < 0.0001$).

Conclusion: The present study confirmed that the methylation status of CADM1 gene was significantly higher in HPV-positive patients than in patients negative for HPV DNA. Moreover, the CADM1 pattern of the gene was associated with the high-risk subtypes of HPV, not with the low-risk ones. Therefore, CADM1 methylation appeared to be a promising biomarker for future studies.

Keywords: Human papillomavirus, Uterine cervical neoplasms, Hypermthylation, Epigenomics

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Introduction

Cervical cancer is the second women malignancy worldwide, particularly in developing countries.[1] Its prevalence in Iran is low; however, there are risk factors increasing its

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incidence in recent years.[2] It causes cancer in squamous cells in the transitional area of the cervix. The percent of women affected by this disease has increased from 10 to 40 over 30 years ago.[3] Smoking,[4] being young when having your first sexual encounter, having multiple partners, using oral contraceptives for an extended length of time, and most importantly, the human papillomavirus (HPV), which is spread via sex, are risk factors.[5] It can be easily controlled by the screening method like Pap smear from age 20 to 60[6] Vaccination can also be effective against cervical cancer from age 12 up to 25.[7]

HPV is a non-developed, double-stranded DNA virus.[8] Over 200 types of this virus were discovered.[9] Some types are of high risk, and the most popular ones are 16, 18 and 31 in Asia. In addition, some are of low risk, and the most popular ones are 6 and 11. High-risk HPV causes cervix, vagina, penis, anus, head, and neck cancers, and the low-risk one causes anogenital warts and recurrent respiratory papillomata.[10] Years before cancer develops, HPV may alter cervical cells, which are known as CIN1 when one quarter, CIN2 when two quarters, or CIN3 when three quarters are made up of cancer cells.[11] The screening approach may be used to find these alterations. Due to cervical cancer, HPV virus induces hypo methylation in fewer genes[12] or hyper methylation in more suppressor genes or oncogenes.[13]

Cell adhesion molecule 1 (CADM1) gene is one of the tumor suppressor genes which are hyper methylated in the promoter region, especially during metastasis or invasion.[14] CADM1 produces adherent cellular molecules like immunoglobulin that consists of three immunoglobulin loops[15] that like a scaffold move the immune cells, including keratinocytes.[16] It plays a key role in tumor invading and running away from the immune system.[17] The loosing function of this gene via hyper methylation results in decreasing cellular connections, and then forming tumor and metastasis and invasion to other tissues[18] and causes vascular endothelial repair. In the immune cells like T-cells, mast cells and NK cells enhance the immune function.[19] It functions as a suppressor gene and is as a part of the super immunoglobulin family acting as the transverse glycoprotein membrane.[7] Besides, it stops the growth of squamous cell carcinoma by developing an extracellular receptor complex on the cell surface involving this gene and HER2-ITGA6B4. As a result, it blocks the STAT3 pathway from phosphorylating these proteins.[20] and lowers the expression of the CADM1 gene in ovarian cancer, while increasing the expression of cancer cells in leukemia and lymphoma. The cellular pathway of phosphatidylinositol 3 kinase / AKT and mTOR regulates the cell cycle, cell proliferation, growth, survival, protein synthesis, movement, transcription, angiogenesis, and glucose metabolism, which is hyperactive in 30% of cancers. In ovarian cancer, this gene inhibits cancer invasion by inhibiting the P13K / AKT / mTOR cell pathway by regulating upstream regulators (LXR/RXR, IGF1, IFI44L and C4BPA) and downstream effectors (APP, EDN1, TGFBI and RAP1A); therefore, the gene can be used as a potential agent for cancer treatment.[21]

Three main factors in the pathogenesis of cervical cancer are necessary in which the first and second factors are related to the presence of HPV virus, and the effect of E6/E7 proteins and as a result entrance to cellular genome, and the third factor is the accumulation of genetic damage that is related to the development of tumor.[22] The major change in cervical cancer is methylation which is caused by the addition of methyl group to cytosine in the CPG region, which is activated by the DNA-methyl transferase family. This is activated by HPV virus E6 protein.[23] and two oncoproteins E6 sets a goal for the P107, P130, Rb, protein by arresting the cell cycle, and e7 sets a goal for p53, e6ap, CBP, P300, BAK, hTERT, MAGUK, CIAP, Survivin proteins by cell proliferation blocking apoptosis.[9] Methylation is a clear pattern in cervical cancer, with most genes being hypermethylated and three genes being hypomethylated. When ribosomal DNAs are cytosine in the CpG regions of a hypo-methylated promoter, as is the case in cervical cancer tissues, the result is an excess synthesis of rRNA and the development of the disease.[24]

Table 1. The number of patients and type of HPV in high- and low-risk cervical samples obtained from the affected and control groups in our study

HPV type	High risk	Low risk	High and Low risk	Total (%)
31	4	-	8	12 (23.07%)
52	4	-	2	6 (11.53%)
54	-	5	2	7(13.46%)
62	-	4	-	4(7.69%)
45	2	-	2	4(7.69%)
18	-	-	6	6(11.53%)
43	-	-	4	4(7.69%)
6	-	4	2	6 (11.53%)
11	-	4	4	8(15.38%)
91	-	-	2	2(3.84%)
16	2	-	1	3(5.76%)
58	-	-	2	2(3.84%)
30	-	-	2	2(3.84%)
83	-	-	2	2(3.84%)
67	-	-	2	2(3.84%)
66	2	-	-	2(3.84%)
56	2	-	-	2(3.84%)

HPV: Human papillomavirus

This study aimed to investigate the methylation levels of the CADM1 gene using cytological smears by the qualitative MSP analysis. The focus was on the specific CpG region not described in more detail previously due to examining the significance of the methylation levels as an indicator of metastasis in CIN2 / CIN3 samples. Furthermore, evaluation of the hyper methylation of CADM1 in cytological and non-cancerous cervical samples and analysis of the effect of HPV high-risk type in different degrees of hyper methylation were conducted. Finally, comparing the methylation percentage of the CADM1 gene in high- and low-risk groups and its comparison to the methylation percentage in this cancer in other groups and in different cancers were performed.

Material and Methods

Specimens and study population

In this analytical observational case-control study, cytological smears from the uterine cervix were obtained from 91 female patients. In this research, 90 Pap smear samples were examined; 52 of the samples showed abnormal cervical appearance, whereas 38 of the samples showed normal Pap smear findings with negative pathology. The applicable samples were gathered between January 2021 and July 2021 at the Boali Laboratory in Zanjan. The age range of the samples was between 23 and 70 years old. All patients were informed about the study, and all of them filled the form, which was prepared for this study. National Committee for Ethics in Biomedical Research of Islamic Azad University-Zanjan Branch approved this study (reference number: IR.IAU.Z.REC.1399.061). All data concerning the patients' personal details, age, previous treatment for HPV infection, including surgical or destructive treatment of the cervix, comorbidities, and so forth, were investigated. Moreover, all individuals provided written informed consent. The specimens were stored at -70 ºC until they were studied.

HPV genomic DNA extraction and serological identification

DNA extraction and deposition were performed by two methods of deposition and column from Pap smear or warts of the affected and healthy individuals using the DNA Technology kit. The extracted DNA's quality and quantity were prepared in accordance with industry standards. In cytological samples of the cervix, the HPV genotyping test seeks to identify high- and low-risk HPV. In the first stage, 100µl of DNA liquid sample or the desired tissue (in case of warts) is cut into pieces and prepared, and added 300µl lysing buffer in it. The samples mixed by vertex

Table 2. Percentage and number of affected and control groups based on methylated, unmethylated and hemi-methylated genotypes, and assessment of methylation in both groups

Sample type	M/M	U/U	M/U	M+M/U	Analysis type	*P*- value	95% CI	Odd ratio
Affected (52)	33(63%)	7(13%)	12(23%)	45(86%)	M, U, M/U	<0.0001	5.9-7	12.2
Normal (38)	4(11%)	27(71%)	7(18%)	11(29%)	M+M/U , U	<0.0001	4-7.8.	16.19

M/M: Methylated/Methylated (Fully- methylated alleles); U/U: Unmethylated/ Unmethylated, (Un-methylated alleles); M/U: Methylated/ Unmethylated, (Hemi methylated alleles); CI: Confidence interval

machine, then put them in a hot plate at 65ºC , that followed by centrifugation (13000 PM, 30 second). Then, 400µl of precipitation solution was added, mixed by vortexing, then centrifuged for 15 minute. In this stage, sediment was observable and we took out the liquid above the sediment and added 500 µl wash bugger solution and centrifuged that for 5 minute. Washing stage repeated by 300µl of washing buffer, then put in 65ºC in hot plate. Finally samples were eluted by adding of 50 µl dilution solution and putting in hot plate for ten minute.

In the next step, 8 different marked micro tubes used for low- and high-risk samples, then put 12 µl oil inside head of the micro tubes, put 19 µl master mix, and 2.5 µl of low risk primer in 4 micro tube, and 2.5 µl of high risk primer in 4 other tube and then poured 1 µl taq polymerase enzyme and 2.5 µl of sample and mixed well, finally placed in a PCR machine for 3.5 hours. In the next step, it is hybridized using operon kit. The samples were taken out of the PCR machine and hybridized. To examine the quality of the isolated DNA, GAPDH was used to amplify it. The high-risk sheets were affixed to the high-risk areas for each sample, and the low-risk sheets were attached to the low-risk sections. The sheet's top had a control extension bar that showed if the hybridization was done correctly; below it, another band in the high-risk gates and low-risk GAPDH was identified as beta-globulin. High-risk and low-risk strips were adhered to the reverse dot blot sheet for the purpose of determining the HPV type. Each strip tested positive for some HPVs in the low-risk portion and some in the high-risk part. Besides, the type of HPV in each sample was determined.

Bisulfite conversion and genotyping by methylation specific PCR (MSP)

The affected and healthy cervical specimens determined HPV type in the previous step and were loaded onto 1.5% agarose gel; the concentration and quality of the sample were determined by measuring the sample purity in a UV spectrophotometer, and subsequently, 1-2 µg of DNA was bisulfite-treated using an EpiTect Bisulfite kit. This process converts the non-methylated cytosine to uracil, while the methylated cytosine remains unchanged. In brief, 20 µl of the selected samples mixed by modification reagents in kit, and then put in PCR for 10 minute at 98 ºC, and then incubated at 60 ºC for 60 minute. Then, the bisulfite treatment, the converted DNA was held at 20ºC for further investigation after employing binding buffer and elution buffer samples that were ready for the following step. The bisulfite-treated DNA was used as a template for MSP. Using conversion specific primers during MSP-analysis, the methylation status was then analyzed. Bisulfite modified DNA was amplified with CAMD1 gene-specific primers for methylated and unmethylated sequences: methylated: (forward) - 5′- AGT ATT TTA TTA GTT GTT CGT TCG G-3′ (reverse) - 5′- TAC CTA TAA AAA TCA ATA CCG CGA C -3′ and unmethylated: (forward) - 5′- AGT ATT TTA TTA GTT GTT TGT TTG G -3′, (reverse) - 5′ -ACA CCT ACC TAT AAA AAT CAA TAC CAC A -3′. PCR amplification was carried out (in duplicate for each primer pair) in a 25 µl mixture containing: 8.5 µl water, 12.5 µl PCR buffer (10×), 1 µl (0.5 µM) of each of forward and reverse primers, 2 µl (100 ngr) of bisulfite modified DNA. The reaction was heated at 94ºC for 3 minutes and then amplified for 40 cycles (94ºC/ 45 seconds, 52ºC/ 45 seconds and 72ºC/ 1 minute), followed by a final 5-minute extension at 72ºC. The final PCR products were analyzed on 2% agarose gel with safe stain, and visualized under ultraviolet light. Distilled water was used as negative control.

Table 3. Percentage and number of affected individuals with fully methylated, unmethylated and hemi-methylated in high- and low-risk groups

HPV affected samples =52	(MM)=33	(MU) =12	(UU) = 7	Analysis type	*P*-value	Odd ratio
High risk	22(66.66%)	3(25%)	1(14.28%)	M, M/U	0.0006	6.0075
Low risk	8(24.24%)	8(66.66%)	4(57.12%)	M,U	0.0051	6.184
High and low risk	3(9.09%)	1(8.33%)	2(28.56%)	M/U , U	0.9689	1.0294
High and low risk	3(9.09%)	1(8.33%)	2(28.56%)	M+M/U , U	0.0358	3.750

MM: Methylated/Methylated (Fully- methylated alleles); MU: Methylated/ Unmethylated (Hemi-methylated alleles); UU: Unmethylated/ Un methylated (Un-methylated alleles)

Statistical analysis

Calculations were made about the methylation, hemi-methylation, and unmethylation status of the control and patient groups. The Statistical Package for Social Sciences was used for all statistical analyses (SPSS, version 20).The association between hyper-methylation of the CADM1 gene and the risk of HPV was estimated by computing odds ratios (ORs) and 95% confidence intervals (CI) by means of the Pearson's chi-square. Statistical significance was defined as $P < 0.05$.

Results

Comparison of the HPV subtypes in high- and low-risk groups

By considering age, a family history of cancer, menstruation or menopausal circumstances, and the early or late stages of the histopathology of the treatment process, this research attempted to assess the methylation alterations of the CADM1 gene in cervical cancer. For the patients, the prevalence of high-risk and malignant HPV was 42.30 %, low-risk and non-cancerous HPV was 38.46 %, and both forms of HPV were 19.23 % (Table 1). In the high-risk group, HPV types 52, 31, and 18 were the most common, and among low-risk individuals, types 6, 11, and 54 were the most common type of HPV, and among positive individuals, an equal ratio of 0.02 was observed for both high- and low- risk HPV types. HPV types were as follows: HPV type 31 with 12 (23.07%), HPV types 6,18 and 52 with 6 (11.53 %), HPV type 54 with 7(13.46%), HPV types 43, 45 and 62 with 4 (7.69%), HPV type 11 with 8 (15.38%), HPV type 16 with 3 (5.76%), HPV type 30, 56, 58, 66, 67, 83 and 91, with 2(3.84%) (Table 1).

CADM1 gene methylation distribution

The analysis of samples by MSP showed that from 52 patients, the number of methylated, unmethylated, and hemi-methylated individuals were 33 (63%), 7 (13%), and 12 (23%), respectively. In contrast, by the evaluation of control individuals, the number of methylation, unmethylation, and hemi-methylation was 4 (11%), 27 (71%), and 7 (18%), respectively. Besides, the total number of methylated individuals and unmethylated in patients was 45 (86%); however, healthy individuals was only 11 (29%) ($P < 0.0001$). Comparison between methylated with unmethyled groups, as well as methylated with hemi-methyl groups and methyl plus hemi-methylated with unmethyled, and methyl with hemi-methyl groups differences was statically significant (Table 2). Results showed that methylation was more significant in the group than in the healthy group. Seven times as many people in the afflicted group were methylated than in the healthy group. Additionally, there were four times as many methylated people in the healthy group as there were in the afflicted group. The number of hemi-methylated individuals in the patients was 1.5 times more than that in healthy individuals. Thus, the total number of methylated and unmethylated individuals in affected individuals was four times more than that in healthy individuals.

Evaluation of methylation status between high- and low-risk groups

Among the patients, 26 samples (50%) had the high-risk HPV type, 20 (38.46%) had the low-risk HPV type, and 69 (11.53%) had both high- and low-risk HPV types (Table 3). Among high-risk samples, 22 (66.66) were methylated homozygotes, 3 (25%) were homozygous, and 1

(14.28%) was an unmethylated homozygote. Among low-risk people, 8 (24.24%), 8 (66.66%), and 4 (57.14%) were methylated, methylated and methylated homozygotes, respectively. Among the positive individuals for two types of high- and low-risk HPV, 3 (9.09%), 1 (8.33%), and 2 (28.57%) were methylated, homozygous, and unmethylated, homozygotes, respectively. In comparison between methylated and umethylated individuals with the high-risk group ($P = 0.0051$, OR= 6.1842), the importance of methylation in the high-risk group was indicated. Similarly, the analysis of the hemi-methylated and non-methylated groups ($P = 0.9689$, OR = 1.0294) were observed. In this group, no real significance ($P = 0.0006$, OR = 6.0075) was found in the comparison between the methylated and hemi-methylated groups, which is significant and demonstrates the significance of methylation. Furthermore, it was shown that methylation was important in this group when comparing the methylated and hemi-methylated groups with the non-methylated group ($P = 0.0358$, OR = 3.7500). The highest percent in the high-risk group was related to homozygous methylation. The highest rate of hyper-methylation was observed in 52 (23.07%) and 31 (11.53%) individuals with HPV type (Table 3).

Discussion

Hypermethylation of the CADM1 gene promoter was found in high frequency in HPV infected samples, counting for 63% (3 of 52 samples), compared with non-HPV infected samples, counting for 11% (4 of 38 samples). Furthermore, the hypermethylation of CADM1 gene was strongly associated to the HPV infection, particularly, high-risk HPV type infected compared with low-risk and or non-HPV group, 66.66% were methylated in the high-risk and 24.24% in the low-risk groups. In the high- and low-risk groups, 9.09% were methylated. Among 52 positive Pap smear samples for cervical cancer, some were for high-risk HPV types, such as 31, 52, 45, 16, 18, 56, and 66, and some for low-risk HPV types, such as 6, 11, 67, 43, 91, 54, and 62. Therefore, some were infected with both low- and high-risk types. Among the high-risk groups, strains 31, 52, and 18 had the highest rates, and among the low-risk groups, strains 11, 6, and 54 had the highest rates of infection. These high- and low-risk strains have been observed in previous studies in Asian communities.

According to the epidemiological and molecular studies, persistent infection with one of at least 13 high-risk types of HPV, including 16/18/33/31/35/39/45/51/52 /56/58/59/68 from the alpha papilloma virus family is necessary to cause cervical cancer.[25] Due to the early expression of E6/E7 viral genes, oncoproteins are overexpressed as a result. This condition, known histologically as CIN II, may progress to cervical cancer, if left untreated. For cancer to start, there must be epigenetic alterations, including methylation of CpG sites. Seven human genes-SOX1, PAX1, mir-124, MAL, FAM19a4, CADM1, and EPB41l3-have been shown to exhibit hypermethylation in precancerous alterations of cervical cancer.[26] The same methylation of tumor suppressor genes, such as MAL and CADM1, turns the primary cancer into metastatic cancer.[27] The downregulation of the CADM1 gene is through the loss of the signal pathway of the Rb suppressor tumor.[28]

Several studies examined the relationship between CADM1 gene methylation and HVP. In another study conducted by Mersakova et al., three CpG islands of the CADM1 gene were analyzed by pyro sequencing, and $P < 0.001$ in CIN1, 2 at the third CpG site compared with the first and second CpG was obtained, indicating the significance of methylation at this point. Furthermore, the methylation percentage of the CADM1 gene was 88.6%, 68%, 80%, and 89% for inflammatory, CIN1, CIN2, and squamous cell carcinoma groups, respectively. By increasing the lesion severity, the percentage of methylation of this gene increased.[29] In another study conducted by Wiyada Dankai et al., after extraction and bisulfite and quantitative MSP, the rate of hypermethylation of this gene changed from 25 to 86.63 in the first group of positive HPV 18/16

to the second group of positive HPV 16/18/52/58. In other words, the hyper methylation rate of the CADM1 gene changed to 95.45 with increasing high-risk HPV from 18.16 to 58.52.18; however, 69% in the positive group had 18/16 HPV.[30] In another study conducted by Remco et al. on non-small lung cells in fibrosis and metastasis samples whose pathology was positive, it was concluded that 64, 47, and 74% of the patient samples were in m1, m5, and m9 areas, respectively, compared with the surrounding healthy tissues, which were 65, 23, and 43%, respectively. Most non-small cell lung tumors showed methylation at these locations. In contrast to the 38 percent of neighboring healthy cells and the 67% of non-small lung cells, dense methylation at two or more locations was found in 66% of tumor cells.[31] In one investigation by Hassan et al., nested PCR was used to analyze the DNA methylation in the CADM11 gene in 100 paraffin samples of ovarian cancer after DNA sequencing. Thus, 75% of patients were infected with HPV 18 hyper-methylation.[32]

The strength of the study was that despite the small statistical population of the patient and the control group, we obtained significant results about carcinogenic strains of the virus and its percentages in the patient group almost similar to those obtained in Asia. Additionally, the non-invasive MSP approach for CADM1 hyper methylation detection and HPV type in Pap test samples might be seen as a good option for non-invasive biomarkers that could perhaps be employed for diagnostic and prognosis reasons for Iranian patients. All the clinicopathologic information of the patients were not available completely, the analysis of the correlation between hypermethylation of CADM1 and gene expression alternation was not detected in this research.

Conclusion

Regarding the high percentage of methylation, studies conducted so far have investigated cervical cancer and CIN3 lesions of the CADM1 gene, since this gene is potentially located in the cell junctions of epithelial cells in different places. Moreover, it acts as a tumor suppressor gene in the differentiation and apoptosis of damaged cells, and its promoter hypermethylation has been used in most studies with over 50% in cervical cancer, especially advanced lesions, which are regarded as diagnostic biomarkers in tissues with HPV in tissues obtained from Pap smear and HPV typing.

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Conflict of Interest

None declared.

References

1. Catarino R, Petignat P, Dongui G, Vassilakos P. Cervical cancer screening in developing countries at a crossroad: Emerging technologies and policy choices. *World J Clin Oncol.* 2015;6(6):281-90. doi: 10.5306/wjco.v6.i6.281.
2. Momenimovahed Z, Salehiniya H. Cervical cancer in Iran: integrative insights of epidemiological analysis. *Biomedicine (Taipei)*. 2018;8(3):18. doi: 10.1051/bmdcn/2018080318.
3. Song B, Ding C, Chen W, Sun H, Zhang M, Chen W. Incidence and mortality of cervical cancer in China, 2013. *Chin J Cancer Res.* 2017;29(6):471-6. doi: 10.21147/j.issn.1000-9604.2017.06.01.
4. Roura E, Castellsagué X, Pawlita M, Travier N, Waterboer T, Margall N, et al. Smoking as a major risk factor for cervical cancer and pre-cancer: results from the EPIC cohort. *Int J Cancer.* 2014;135(2):453-66. doi: 10.1002/ijc.28666.
5. Ghebre RG, Grover S, Xu MJ, Chuang LT, Simonds H. Cervical cancer control in HIV-infected women: Past, present and future. *Gynecol Oncol Rep.* 2017;21:101-8. doi: 10.1016/j.gore.2017.07.009.
6. Sanford NN, Sher DJ, Butler S, Xu X, Ahn C, D'Amico AV, et al. Cancer screening patterns among current, former, and never smokers in the United States, 2010-2015. *JAMA Netw Open.* 2019;2(5):e193759. doi: 10.1001/jamanetworkopen.2019.3759.
7. Athanasiou A, Bowden S, Paraskevaidi M, Fotopoulou C, Martin-Hirsch P, Paraskevaidis E, et al. HPV vaccination and cancer prevention. *Best Pract Res Clin Obstet Gynaecol.* 2020;65:109-24.doi: 10.1016/j.bpobgyn.2020.02.009.
8. McBride AA. Mechanisms and strategies of papillomavirus replication. *Biol Chem.*

2017;398(8):919-27. doi: 10.1515/hsz-2017-0113.
9. Tommasino M. The human papillomavirus family and its role in carcinogenesis. *Semin Cancer Biol.* 2014;26:13-21. doi: 10.1016/j.semcancer.2013.11.002.
10. Heidegger I, Borena W, Pichler R. The role of human papilloma virus in urological malignancies. *Anticancer Res.* 2015;35(5):2513-9.
11. Klingelhutz AJ, Roman A. Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. *Virology.* 2012;424(2):77-98. doi: 10.1016/j.virol.2011.12.018.
12. Yin FF, Wang N, Bi XN, Yu X, Xu XH, Wang YL, et al. Serine/threonine kinases 31(STK31) may be a novel cellular target gene for the HPV16 oncogene E7 with potential as a DNA hypomethylation biomarker in cervical cancer. *Virol J.* 2016;13:60. doi: 10.1186/s12985-016-0515-5.
13. Lin H, Ma Y, Wei Y, Shang H. Genome-wide analysis of aberrant gene expression and methylation profiles reveals susceptibility genes and underlying mechanism of cervical cancer. *Eur J Obstet Gynecol Reprod Biol.* 2016;207:147-52. doi: 10.1016/j.ejogrb.2016.10.017.
14. Lafage-Pochitaloff M, Gerby B, Baccini V, Largeaud L, Fregona V, Prade N, et al. The CADM1 tumor suppressor gene is a major candidate gene in MDS with deletion of the long arm of chromosome 11. *Blood Adv.* 2022;6(2):386-98. doi: 10.1182/bloodadvances.2021005311.
15. Giangreco A, Hoste E, Takai Y, Rosewell I, Watt FM. Epidermal CADM1 expression promotes autoimmune alopecia via enhanced T cell adhesion and cytotoxicity. *J Immunol.* 2012;188(3):1514-22. doi: 10.4049/jimmunol.1003342.
16. Li Y, Wang G, Wang Q, Zhang Y, Cui L, Huang X. Exosomes secreted from adipose-derived stem cells are a potential treatment agent for immune-mediated alopecia. *J Immunol Res.* 2022;2022:7471246. doi: 10.1155/2022/7471246.
17. Overmeer RM, Henken FE, Snijders PJ, Claassen-Kramer D, Berkhof J, Helmerhorst TJ, et al. Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J Pathol.* 2008;215(4):388-97. doi: 10.1002/path.2367.
18. Baci D, Bosi A, Parisi L, Buono G, Mortara L, Ambrosio G, et al. Innate immunity effector cells as inflammatory drivers of cardiac fibrosis. *Int J Mol Sci.* 2020;21(19):7165. doi: 10.3390/ijms21197165.
19. Kawano S, Ikeda W, Kishimoto M, Ogita H, Takai Y. Silencing of ErbB3/ErbB2 signaling by immunoglobulin-like Necl-2. *J Biol Chem.* 2009;284(35):23793-805. doi: 10.1074/jbc.M109.025155.
20. De Santis MC, Sala V, Martini M, Ferrero GB, Hirsch E. PI3K signaling in tissue hyper-proliferation: From overgrowth syndromes to kidney cysts. *Cancers (Basel).* 2017;9(4):30. doi: 10.3390/cancers9040030.
21. Pal A, Kundu R. Human papillomavirus E6 and E7: The cervical cancer hallmarks and targets for therapy. *Front Microbiol.* 2020;10:3116. doi: 10.3389/fmicb.2019.03116.
22. Yuan BF. 5-methylcytosine and its derivatives. *Adv Clin Chem.* 2014;67:151-87. doi: 10.1016/bs.acc.2014.09.003.
23. Williams K, Christensen J, Helin K. DNA methylation: TET proteins-guardians of CpG islands? *EMBO Rep.* 2011;13(1):28-35. doi: 10.1038/embor.2011.233.
24. Wu EQ, Liu B, Cui JF, Chen W, Wang JB, Lu L, et al. Prevalence of type-specific human papillomavirus and pap results in Chinese women: a multi-center, population-based cross-sectional study. *Cancer Causes Control.* 2013;24(4):795-803. doi: 10.1007/s10552-013-0162-8.
25. Cuschieri K, Ronco G, Lorincz A, Smith L, Ogilvie G, Mirabello L, et al. Eurogin roadmap 2017: Triage strategies for the management of HPV-positive women in cervical screening programs. *Int J Cancer.* 2018;143(4):735-45. doi: 10.1002/ijc.31261.
26. Wentzensen N, Sherman ME, Schiffman M, Wang SS. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol Oncol.* 2009;112(2):293-9. doi: 10.1016/j.ygyno.2008.10.012.
27. Zhang W, Xie HY, Ding SM, Xing CY, Chen A, Lai MC, et al. CADM1 regulates the G1/S transition and represses tumorigenicity through the Rb-E2F pathway in hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int.* 2016;15(3):289-96. doi: 10.1016/s1499-3872(16)60099-1.
28. Meršaková S, Holubeková V, Grendár M, Višňovský J, Ňachajová M, Kalman M, et al. Methylation of CADM1 and MAL together with HPV status in cytological cervical specimens serves an important role in the progression of cervical intraepithelial neoplasia. *Oncol Lett.* 2018;16(6):7166-74. doi: 10.3892/ol.2018.9505.
29. Dankai W, Khunamornpong S, Siriaunkgul S, Soongkhaw A, Janpanao A, Utaipat U, et al. Role of genomic DNA methylation in detection of cytologic and histologic abnormalities in high risk HPV-infected women. *PLoS One.* 2019;14(1):e0210289. doi: 10.1371/journal.pone.0210289.
30. van den Berg RM, Snijders PJ, Grünberg K, Kooi C, Spreeuwenberg MD, Meijer CJ, Pet al. Comprehensive CADM1 promoter methylation analysis in NSCLC and normal lung specimens. *Lung Cancer.* 2011;72(3):316-21. doi: 10.1016/j.lungcan.2010.10.005.
31. Hassan ZK, Hafez MM, Kamel MM, Zekri AR. Human papillomavirus genotypes and methylation of CADM1, PAX1, MAL and ADCYAP1 genes in epithelial ovarian cancer patients. *Asian Pac J Cancer Prev.*

2017;18(1):169-76. doi: 10.22034/APJCP.2017.
18.1.169.