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DNA methylation analysis of WIF1 and DKK4 in cervical precancerous lesions and cancer

I. Gladchuk, N. Rozhkovska, N. Kashtalian, A. Volyanska, G. Lavrynenko

Odessa National Medical University

Abstract

Epigenetic gene silencing by excessive methylation was found in many tumors. Methylation of tumor suppressor genes is often found in cervical cancer, which is an important pathogenetic factor of oncologic process. The objective of this study was to identify gene methylation of *WIF1* and *DKK4* events and their usefulness in distinguishing cancer and precancerous lesions from normal cervical cells that could eventually be added to standard diagnostic testing for cervical precancer. We examined methylation index of *WIF1* and *DKK4* genes in the tissue specimen of 42 women: CIN I in 9 cases, CIN II in 11 cases and CIN III in 10 cases, 7 samples included invasive cancer and control group of 5 normal cases. In analysis of methylation level of *WIF1* and *DKK4* genes aberrant methylation of *WIF1* gene was found in women with CIN+ and aberrant methylation of *DKK4* gene was found in all women. There was a trend of methylation level increase according to the severith of the precancerous lesions with the maximum mnethylation levels in cases of invasive cervical cancer. Methylation indexes determination in *WIF-1* and *DKK-4* genes is a perspective method of cervical cancer diagnostics.

Key words: cervical intraepithelial neoplasia, cervical cancer, gene silencing, methylation, WIF1, DKK4, tumor suppressor genes

Oncogenesis is a result of both genetic and epigenetic changes. In the context of normal cell functioning epigenetic DNA modification is the most common way of chromatin

structure regulation and gene expression control. Coaction of DNA methylation, giston modification and ferments which control these modifications plays a role of control of a variety of gene expression in different cell types on different stages of development. Exclusion of one or several of these control mechanisms is a common occurrence in pathological states. In oncologic processes chromatin reorganisation together with DNA methylation and transcriptional repressive giston modifications lead to transcriptional blockage of tumor suppressor genes [9].

Epigenetic gene silencing by excessive methylation was found in many tumors [9]. Methylation of tumor suppressor genes is often found in cervical cancer, which is an important pathogenetic factor of oncologic process [21, 22]. Genes coding main regulation mechanisms of oncogenic Wnt/β-catenin pathway are often blocked though excessive methylation of their promoter regions in cervical cancer [19, 20].

For this study, we selected a set of two tumor suppressor genes *WIF1* and *DKK4* that, based on a survey of the literature, had previously been shown to be or could be potential targets for aberrant DNA methylation in cervical cancer. The objective of this study was to identify gene methylation events useful in distinguishing cancer and precancerous lesions from normal cervical cells that could eventually be added to standard diagnostic testing for cervical precancer.

Materials and Methods

We examined methylation index of *WIF1* and *DKK4* genes in the tissue specimen of 42 women, who underwent excisional treatment. All women were high oncogenic type HPV positive. Tissue samples were hystopathologically proven to include CIN I in 9 cases, CIN II in 11 cases and CIN III in 10 cases. Besides 7 samples included invasive cancer and in control group of 5 cases no pathology was found.

Methylation analysis was performed by quantitative pyrosequencing method using Qiagen laboratory set and та 7 pmol of specific sequencing primers to the genes tested according to the methodics (Qiagen). Quantitative analysis was performed on PyroMark Q24 equipment using Pyro Q-Cp software. Software automatically calcutated Cp methylation degree and showed its percentage for each methylation site. Average methylation of all sites was calculated for each gene.

For amplyfication and sequincing we used the following primers:

DKK4-F-B ATAGATTTGAAGGGATTTGTTGAAGTTT

DKK4-R CAAAACCAACTCAACCCCAACAAAAC

DKK4-S CTAAACTAACAACTCAACAC

WIF1-5´-F-B GAGTGATGTTTTAGGGGTTT

WIF1-5´-R CCTAAATACCAAAAAACCTAC

WIF1-5´-S AAACTACATTCACAATAC

In analysis we used benchmark level of 15% methylation, cases with over 15% gene methylation were considered as aberrant methylation [18].

Results

In analysis of methylation level of *WIF1* and *DKK4* genes aberrant methylation of *WIF1* gene was found in women with CIN+ and aberrant methylation of *DKK4* gene was found in all women (Tab. 1).

Tab. 1

Structure of methylation indexes of *WIF1* and *DKK4* genes

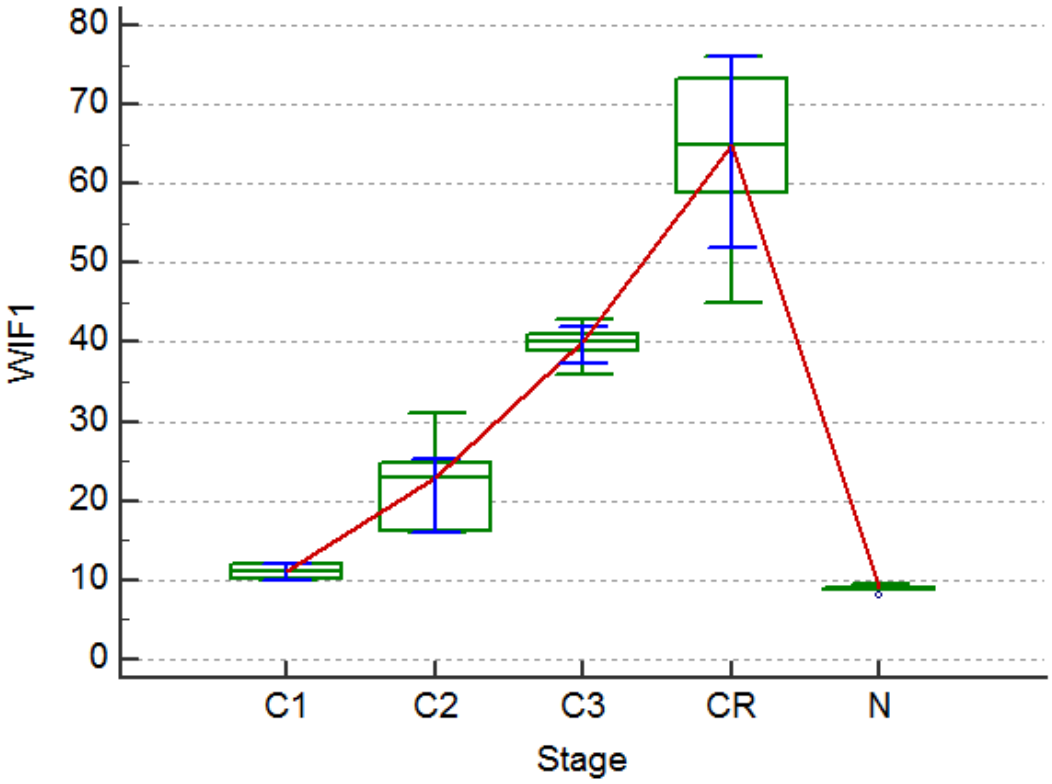
Gene	Disease				
	Norm n=5 M±m	CINI n=9 M±m	CINII n=11 M±m	CINIII n=10 M±m	Cancer n=7 M±m
WIF1	8,9±0,24	11,08±0,35*	21,43±1,56*	39,4±1,06*	63,57±4,08*
DKK4	31,2±0,97	37,43±1,63*	37,82±1,12*	46,7±1,74*	65,71±2,65*

M± M-average, m — average error

*significance of difference with control group $p<0,05$

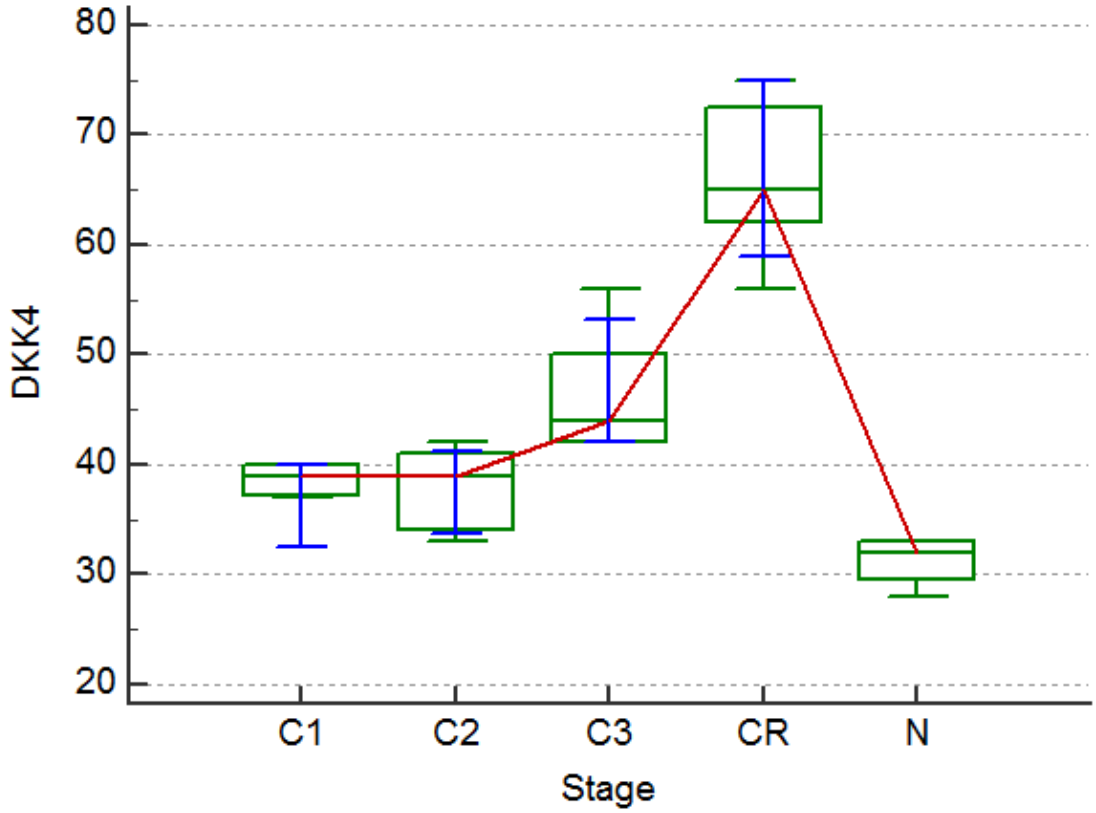
Methylation index increased in all patients with the increase of pethology severity with the maximum level in invasive cervical cancer. This trend is very persistent in *WIF1* gene methylation. Despite the initial aberrant methylation of *DKK4* gene, the increased methylation trend was persistent here, too. Besides almost similar methylation levels of both genes in cervical cancer were found.

The tendency of increase in methylation level corresponding to the disease progression is very clear for *WIF1* gene: in CIN I methylation increases 1,2 times compared to norm, in CIN II methylation increases 1,9 times compared to CIN I and 2,4 times compared to norm, in CIN III — 1,84 times compared to CIN II and 4,4 times compared to norm, in invasive cancer — 1,6 times compared to CIN III and 7,1 times compared to norm (Pic. 1).



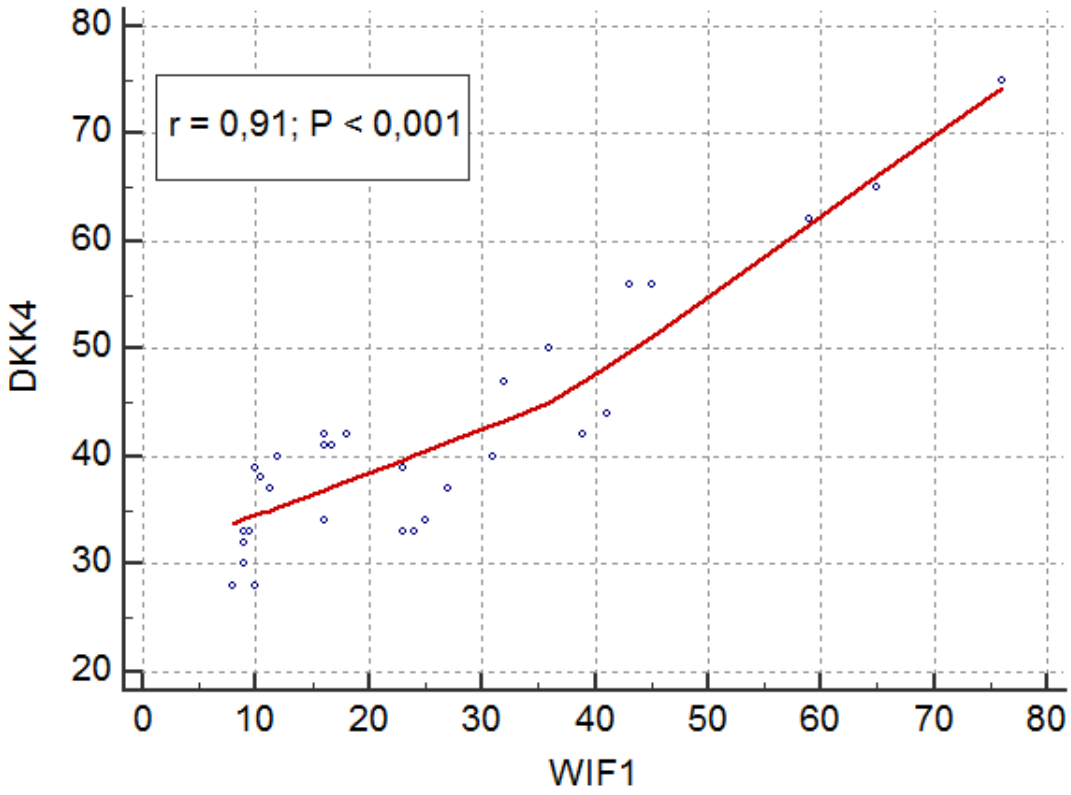
Pic. 1. Result of one factor dispersion analysis ANOVA of *WIF1* gene methylation on different stages of the disease

Tendency to increase in methylation level according to the disease progression is also found in *DKK4* gene, but methylation increase from norm to CIN II is insubstantial, though is clear for CIN III and invasive cancer: in CIN III methylation intesity increases 1,2 compared to CIN II and 1,5 times compared to norm, in cervical cancer methylation level increases 1,4 times compared to CIN III and 2,1 times compared to norm (Pic. 2).



Pic. 2. Result of one factor dispersion analysis ANOVA of *DKK4* gene methylation on different stages of the disease

Despite both genes being key regulators of Wnt/catenin signaling pathway high correlation level (r=0,91; p<0.001) of methylation indexes of *WIF1* and *DKK4* genes in precancerous and malignant pathology of cervix was found, with noted maximum correlation on high levels of hypermethylation in invasive cervical cancer (Pic. 3).



Pic 3. Scatter-graph of correlation of methylation level of *WIF1* and *DKK4* genes in CIN and cervical cancer

Discussion

Genes coding functional inhibitors of Wnt signaling pathway are commonly reported in literature as aberrantly methylated in cervical cancer [1, 3, 4, 5, 14, 20, 23, 24]. Despite that data on clear determination of perspective biomarkers and prognostic factors in cervical cancer is lacking [1].

DNA methylation in gene *WIF1* locus is aberrant in cervical cancer which is reported in literature [3, 17, 21] and is characteristic not only for squamous cancer of cervix but for adenocarcinoma [20].

Van Der Meide et al. determine methylation index of *WIF1* gene in cervical cancer on the level of 54% [20] and Siegel et al. on the level of 46% [18], which is lower than our data of 63,57%. This difference could be partially explained by methodology, and partially by low amount of cervical cancer cases and absence of stratification by the stage of cancer and presense of methastases, that can explain different methylation levels.

Nowadays there is no data on *WIF-1* and *DKK-4* gene methylation in cervical precancerous lesions [18], and on *DKK-4* gene methylation in cervical cancer. According to

literature data literature *DKK-4* is connected to colorectal cancer [13, 15, 16] and hepatoma [2, 12]. Though role of gene methylation of other genes in DKK family was proven for cervical cancer. Van Der Meide et al. found aberrant methylation of *DKK-3* in adenocarcinoma of cervix [20], Jiang et al. describe increased expression of *DKK-1* in serum of patients with CIN and cervical cancer [7], endometrial cancer [6] and other gynecological cancers according to the stage and presense of metastases [9], Lee et al. describe *DKK-1* silencing in cell lines of cervical cancer [11].

Ko et al. report that *WIF-1* can effectively coregulate proapoptosis activity in cervical cancer through combination with *DKK1* [10]. This could also be the reason for methylation levels correlation for *WIF1* and *DKK4* genes in our data.

Conclusion

Methylation indexes determination in *WIF-1* and *DKK-4* genes is a perspective method of cervical cancer diagnostics. Calculation of methylation index of *WIF-1* gene can be used in diagnostics of significant precancerous lesions of cervix (CIN II+) and their differential diagnostics, calculation of methylation index of *DKK-4* gene is a perspective method of differential diagnostics of CIN III in biopsy metherial on preexcisional stage.

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