ORIGINAL PAPER

Histone lysine demethylases as epigenetic modifiers in HPV-induced cervical neoplasia

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Abstract

Epidemiologic data confirmed high-risk human papillomaviruses as etiological agents of cervical cancer. On the other hand genetic and epigenetic modifications are known to play an important role in carcinogenesis. Histone lysine-specific demethylases (KDMs) have been shown to contribute to development and progression of many types of cancer. We evaluated the possible role of KDMs in cervix oncogenesis by analysing their expression in patients with HPV-induced cervical lesions and cancer (86 cervical tissue specimens). HPV DNA was detected and genotyped and KDMs expression levels were investigated by qRT-PCR.

Significantly increased genes expression levels of KDM4C, KDM5C, KDM6A and KDM6B were found in CIN2+ lesions and SCC but KDM4C and KDM5C expression levels in SCC samples were significantly higher than in precancerous lesions. A connection between high-risk HPV infection and KDMs in cervical cancer was revealed following experiments using siRNA to silence E6 and E7 oncogenes.

Results showed KDMs expression pattern is linked to oncogenic transformation in HPV-induced lesions and could represent new potential diagnostic tools and therapeutic targets in oncology.

1. Introduction

Cervical cancer is the third most commonly diagnosed type of cancer and the fourth leading cancer associated mortality in women worldwide [1]. Cervical cancer represents a public health problem in Romania as it has one of the highest incidences in Europe, the mortality rate in 2000-2006 being 21.5% [2]. Human papilloma virus (HPV) is considered the etiologic agent of cervical neoplasia, the infection with high-risk HPV types (hrHPV) being associated with cervical dysplasia and carcinogenesis.

Infection of cervical epithelial cells with HPV results in premalignant lesions, referred as cervical intraepithelial neoplasia (CIN) that can be classified according to their severity into CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia). If left untreated, CIN2 or CIN3 can progress to cervical cancer. The majority of CIN 1 lesions regress spontaneously to normal due to the intervention of cellular immune system; nevertheless, in some cases, these low-grade lesions can progress to high-grade lesions (CIN3) and cancer if left untreated [3]. At present, there are ongoing efforts to improve diagnosis and treatment of cervical high grade lesions.

It is well establish that pathogenesis and development of cancer represents a complex process and is associated with both genetic and epigenetic changes that alter the transcriptional program. Epigenetic regulation of gene expression involves two major mechanisms:

DNA methylation and chromatin remodelling. The structure that compact and organize eukaryotic genomes and is responsible for regulation of processes (such as DNA replication, mitosis and gene expression) is a complex of DNA, histone proteins and associated macromolecules and is called chromatin. The basic unit of chromatin is the nucleosome composed of DNA wrapped around an octamer of histone, which is comprised of two copies each of H2A, H2B, H3, and H4. Histones are proteins consisting of a globular domain and an unstructured tail that suffers post-translational modifications leading to regulation of processes such as transcription, cell cycle progression and DNA repair [4].

An important role in gene transcriptional activation and repression through chromatin regulation plays histone modifications such as methylation, acetylation, phosphorylation and ubiquitination [5]. Once thought to be a stable modification, histone methylation is acknowledged now to be a dynamically process controlled by two types of enzymes that work together to maintain global histone methylation patterns: histone lysine methylases (HMTs) and histone lysine demethylases (KDMs) [6,7].

Histone methylation may occur on different lysine residues and the interplay between HMTs and KDMs regulates the methylation level and contribute to activation or repression of gene expression, depending on the specific lysine residue on which they act [8].

Lysine methylation is a complex chromatin modification due to the number of methyl groups that can be added per residue; thus lysine methylation can occur in a mono- (me1), di- (me2) and trimethylated (me3) state. Also, the degree of methylation and the position of lysine being modified are associated with different consequences: for instance the H3 lysine 4 monomethylation (H3K4me1) labels enhancers, while H3K4me3 are found mainly at the transcriptional start site of active genes, opposed to H3K9me3 which is associated with repression [9]. Generally, methylated H3K4, H3K36 and H3K79 are considered activating marks whereas methylation of H3K9, H3K27 and H4K20 are often associated with gene silencing [10-13]. Lately, it has been shown that histone lysine demethylases play an important role in development and progression of numerous types of cancer. Many studies revealed a KDM altered pattern of expression, presenting a distinct profile in cancer samples vs. normal, in different type of cancer such as lung, colon, breast, prostate and retinoblastomas [14-16]. KDMs deregulation has been associated with cancer aggressiveness, this observation proposing them as molecular tumour markers. Taking into account the specific lysine residue on which KDMs act, leading to gene activation/repression, demethylases could play distinct role in cancer progression acting either as putative oncogenes or tumour suppressors.

An important role in malignant transformation in cervical cancer is played by viral E6 and E7 oncoproteins; they are expressed in hrHPV-transduced cells to inactivate tumor suppressor proteins p53 and pRb, leading to cell cycle disorder, telomerase activation and cell immortalization. Viral oncogenes' expression is necessary for the induction and maintenance of the transformed phenotype [17]. Many studies have shown that the expression of E6 and E7 viral oncogenes is essential but not sufficient for cancer development. On the other hand, reports have demonstrated that both genetic changes and epigenetic modifications play important roles in cervical carcinogenesis [18]. Until recently it was shown that E6 and E7 oncoproteins can associate with enzymes that modulate histone acetylation and thus, regulate the transcriptional capacity of host cell chromatin [19-21]. Moreover it was reported that E7 HPV16 induce epigenetic and transcriptional alterations and this is caused by transcriptional induction of the KDM6A and KDM6B histone 3 lysine 27 (H3K27) specific demethylases [22].

Given the continuing interest regarding KDMs potential role in oncogenesis, we aimed to evaluate the expression of selected histone lysine demethylases in samples from cervical precursor lesions and cancer and to link KDMs with HPV infection.

2. Materials and Methods

Patients and specimens

A total number of 86 tissue cervical specimens from women with high-risk HPV-induced dysplastic cervical lesions comprising of 30 patients with CIN1 (20-59 years old, median: 32.5) and 34 CIN2+ patients (21-63 years old, median: 37), 22 tissue specimens from patients with squamous cervical carcinoma (SCC) (22–77 years old, median: 49) were included in the study. CIN2+ was defined histologically as CIN2, CIN3 and adenocarcinoma *in situ*. Also 30 cervical specimens from women (20-48 years old, median: 32) with normal biopsies who underwent hysterectomy for other causes than cervical cancer (and without HPV infection) were included in the control group. Cervical samples obtained from surgical or biopsy specimens were immediately preserved in RNA latter and stored at $-80°C$ until use. Informed consent was obtained from all subjects. The study was in agreement with the guidelines approved by Ethic Committee.

HPV detection and genotyping

Viral testing was performed with Linear Array (LA) HPV Genotyping Test (Roche Molecular Biochemicals, Mannheim, Germany), according to manufacturer instructions. Briefly, this test uses amplification of target DNA by the PCR and nucleic acid hybridization for HPV detection and genotyping. The kit is designed to amplify an approximately 450 bp sequence within the polymorphic L1 region of the HPV genome from thirty seven anogenital HPV DNA genotypes [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108]. An additional primer pair targets the human β-globin gene (268 bp amplicon) as a control for cell adequacy, extraction and amplification.

Cell culture and siRNA transfection

The human-derived cervical carcinoma cell line HeLa used in this study was obtained from the American-Type Culture Collection (ATCC). Cells were maintained in DMEM-F12 (1: 1) medium containing 10% fetal calf serum at $37°C$ in a humidified 5% CO2 environment. HeLa cells ($1.5x10^5$) were used for transfection with 75 nM specific E7HPV18-siRNA and E6HPV18-siRNA (*Ambion; Applied Biosystems*). Cells treated with siRNA control, cells with transfection medium as well as untreated cells were used as controls and were harvested 24 hours after transfection. QRT-PCR was used to generate quantitative data about E7 and E6 HPV18 knockdown in transfected HeLa cells using the following primer:

E7HPV18-F: 5'-CCGAGCACGACAGGAACGACT-3'
E7HPV18-R: 5'-TCGTTTTCTTCCTCTGAGTCGCTT-3';
E6HPV18-F: 5'- GCGACCCTACAAGCTACCTG-3'and
E6HPV18-R: 5'- GTTGGAGTCGTTCCTGTCGT-3'.

Transfection efficiency was calculated according to manufacturer instructions (Silencer siRNA Starter Kit; *Ambion*):

% knockdown = 100 – 100 X 2–ΔΔCt E7/E6 HPV18, where ΔCt it is defined as follows: ΔCt = Ct E7/E6 HPV18– Ct GAPDH; and ΔΔCt: ΔΔCt = ΔCt E7/E6 HPV18– ΔCt Negative Control #1 [23].

RNA Extraction

Total RNA was isolated from cervical samples using TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, than purified through on-column DNase I treatment with RNase-free Dnase (*Qiagen*) and afterwards the samples were stored at -80ºC until used. RNA quantity and quality were determined using a NanoDrop Technologies ND-1000 spectrophotometer and Agilent 2100 bio-analyser (*Agilent Technologies, Inc, Santa Clara, CA*). RNAs (with an RNA integrity number >7.5) were used to generate cDNAs.

Quantitative Real-time PCR

1 µg of each isolated RNA was revers-transcribed using Transcriptor First Strand cDNA Synthesis Kit (*Roche*) according the manufacturer instructions and the cDNAs were stored at -20°C until use. Real-time PCR was performed using FastStart SYBR Green Master (*Roche*) according the manufacturer instructions on Applied Biosystems 7300 Real Time PCR system (*Applied Biosystems, Foster City, CA*). GAPDH gene was used as references and each sample was analysed in duplicate. Each gene expression levels were calculated using double normalization method ($\Delta\Delta$Ct).

Statistical analysis

All statistical data were expressed as mean ±standard deviation (SD) from at least two separate experiments and were analysed using Statistical Program for Social Sciences (SPSS) 20.0 software (*SPSS, Chicago, IL*) and GraphPad Prism 5.0 (*Graph Pad Software Inc., San Diego, CA, USA*). One way analyses of variance test, two-tailed Student's t-test were used. P values <0.05 were considered statistically significant.

3. Results

In order to highlight the role of KDMs in cervical oncogenesis, we investigated the samples that presented high-risk genotype HPV 18 in single or co-infection and analysed them according to specific lysine residue they target (table 1).

Table 1. Summary of KDMs expression levels in cervical samples consistent with lesion severity.

	CIN1 min ÷ max (mean ± SD)	CIN2+ min ÷ max (mean ± SD)	SCC min ÷ max (mean ± SD)	p-value
KDM1B	-1.924 ÷ -1.234 (-1.631 ± 0.3353)	-1.072 ÷ 0.7917 (0.0255 ± 0.5738)	-2.462 ÷ 1.415 (-0.4139 ± 1.237)	p>0.05
KDM2B	-1.915 ÷ -0.3703 (-1.056 ± 0.661)	-1.626 ÷ 0.9783 (-0.12 ± 0.8356)	-0.9121÷1.466 (0.1101 ± 0.8431)	**0.0440**[*]
KDM3C	0.3425 ÷ 1.285 (0.9412 ± 0.4289)	2.288 ÷ 4.302 (3.010 ± 0.6360)	0.5027 ÷ 2.369 (1.389 ± 0.5307)	**0.0001**[*]
KDM4C	-0.1866 ÷ 0.6863 (0.193 ± 0.3595)	-0.1565 ÷ 1.349 (0.5960 ± 0.5088)	0.2649 ÷ 3.320 (1.413 ± 0.8283)	**0.0023**[*]
KDM5C	-0.4816 ÷ -0.1204 (-0.2869 ± 0.2496)	0.8489 ÷ 2.234 (1.526 ± 0.4458)	1.024 ÷ 2.559 (1.806 ± 0.6902)	**0.0041**[*]
KDM6A	-4.883 ÷ -2.615 (-3.269 ± 0.9254)	-2.766 ÷ -0.7857 (-1.792 ± 0.6934)	-1.722 ÷ 0.5178 (-0.6625 ± 0.7192)	**0.0004**[*]
KDM6B	-2.604 ÷ -1.330 (-1.678 ± 0.6192)	-1.361 ÷ 0.6442 (-0.5434 ± 0.6184)	-1.972 ÷ 2.032 (-0.1720 ± 1.052)	**0.0225**[*]

All evaluated parameters are expressed as absolute values in means ± standard deviation, also shown are minimum and maximum values. P-value was calculated using ANOVA test and values <0.05 were considered statistically significant and represented with [*].

From the group of *H3K4 demethylases* we determine KDM1B and KDM5C expression levels. KDM1B expression increased from CIN1 (mean=-1.631 ± 0.3353) to CIN2+ (mean=0.0255 ± 0.5738) and a statistically significant difference between the two groups (p=0.004) (figure 1a) was found. No statistically significance (p>0.05) regarding the expression levels between SCC samples (mean=-0.4139 ± 1.237) and CIN2+ was observed.

KDM5C expression levels were proportionally with the severity of lesions. KDM5C levels were found increased in CIN2+ lesions (mean= 1.526 ± 0.4458, p=0.016) and significantly increased in SCC cases (mean= 1.806 ± 0.6902, p=0.0025) as compared with CIN1 cases (mean= -0.2869 ± 0.2496) (figure 1b).
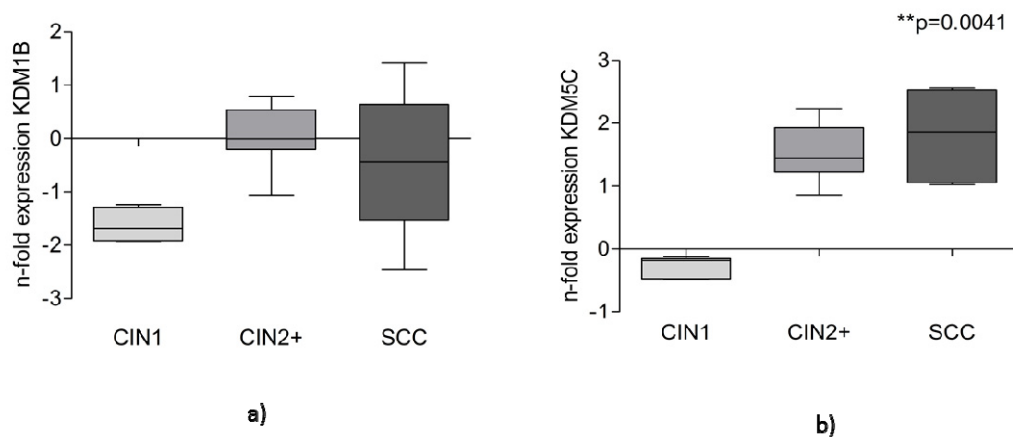

Figure 1. (a) KDM1B and (b) KDM5C expression levels in cervical lesions and cancer.
All results were expressed as the means ± SD. *p< 0.05, **p< 0.001, ***p<0.0001.

Regarding *H3K9 demethylases* (KDM3C and KDM4C) expression profile, our results showed that KDM4C presented an increased expression levels in SCC samples (mean= 1.413 ± 0.8283) in contrast with CIN1 (mean= 0.193 ± 0.3595, p=0.0029) and CIN2+ samples (mean= 0.5960 ± 0.5088, p=0.0165) (figure 2 a). Meanwhile, KDM3C display a slightly different pattern of expression. Highest levels of expression were obtained in CIN2+ samples (mean= 3.010 ± 0.6360), whereas in CIN1 (mean= 0.9412 ± 0.4289) and in SCC samples (mean= 1.389 ± 0.5307) lower levels of expression were observed. There was a statistically significant difference between CIN2+ tissue samples and those with SCC (p=0.0002); also between CIN2+ and CIN1 groups a statistically difference was noted (p=0.0007) (figure 2b).

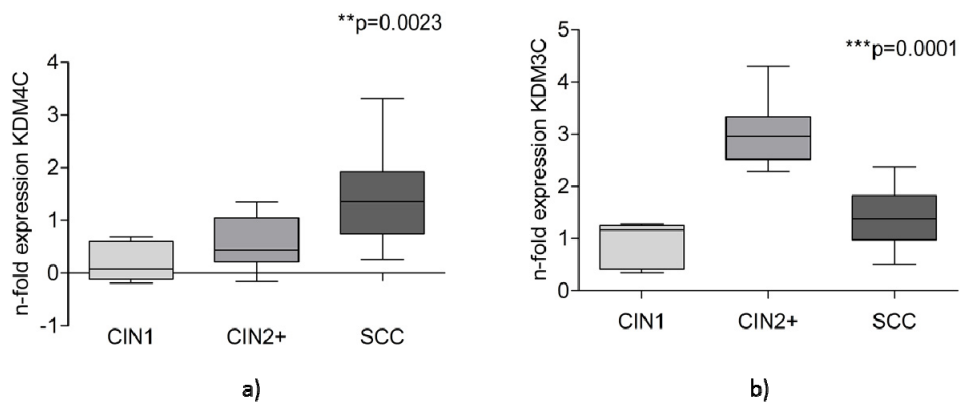**Figure 2.** H3K9 demethylases − KDM4C (a) and KDM3C (b) expression levels in cervical samples.
All results were expressed as the means ± SD. *p< 0.05, **p< 0.001, ***p<0.0001.

Analysing the expression of a *H3K36 demethylase*, KDM2B, we found that overall this gene present an ascending pattern but no statistically significant differences was noticed (figure 3). Statistically difference between CIN1 (mean=-1.056 ± 0.661) and CIN2+ cases (mean=-0.12 ± 0.8356) for KDM2B expression levels was observed (p=0.0237) as well as comparing CIN1 and SCC cases (p=0.0350).
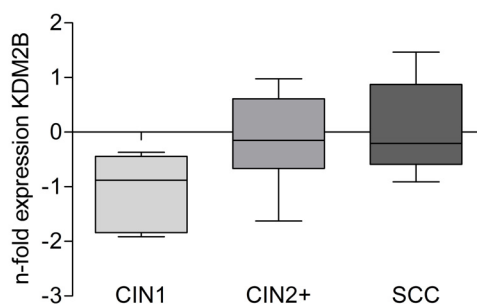

Figure 3. KDM2B levels in cervical samples.
All results were expressed as the means ± SD.

Investigating the expression levels of H3K27 *demethylases* (KDM6A and KDM6B) in study groups we noted that both presented an increased expression in CIN2+ and SCC cases (figure 4a and 4b). Analysing KDM6A demethylase, significant data were found when comparing CIN1 with SCC (p=0.0016) respectively with CIN2+ (p=0.0062). Also, differences were noted between CIN2+ and SCC cases (p=0.0082).

KDM6B levels were found increased in CIN2+ lesions (mean=-0.5434 ± 0.6184, p=0.0162) and SCC cases (mean= -0.1720 ± 1.052, p=0.0143) compared with CIN1 samples (mean=-1,678 ± 0.6192).
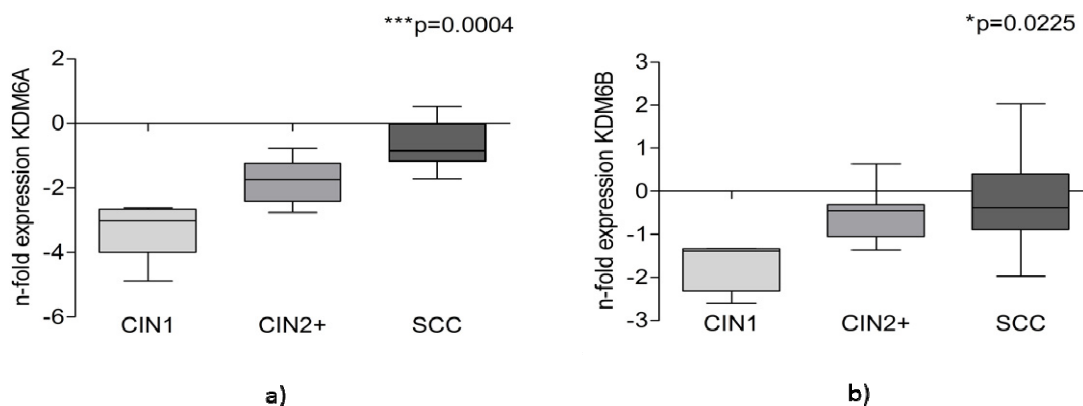

Figure 4. (a) KDM6A and (b) KDM6B expression levels in cervical samples.
All results were expressed as the means ± SD. *p< 0.05, **p< 0.001, ***p<0.0001.

In order to illustrate a relationship between high-risk HPV infection and histone lysine demethylases expression in cervical cancer, siRNA technique was used as experimental model to silence E7 and E6 HPV18. For this purpose, specific siRNAs for the viral oncogenes were designed and used to transfect HeLa cell line. Different siRNAs concentrations (35, 75 and 100 nM) and different incubation times (24h, 48h after transfection) were tested. The optimal siRNA concentration (75 nM) and the period of treatment (24 hours) were established according to the growth state of the negative control and to the mRNA expression level of E6

and E7. E6 and E7HPV 18 mRNA expressions by RT–PCR in HeLa cells 24 h after transfection were performed in order to estimate the knockdown percentage (54.8% for E6 gene and 63.2 % for E7). Histone lysine demethylases 'expression levels in siRNA treated cells compared with controls were quantified by RT–PCR (figure 5).
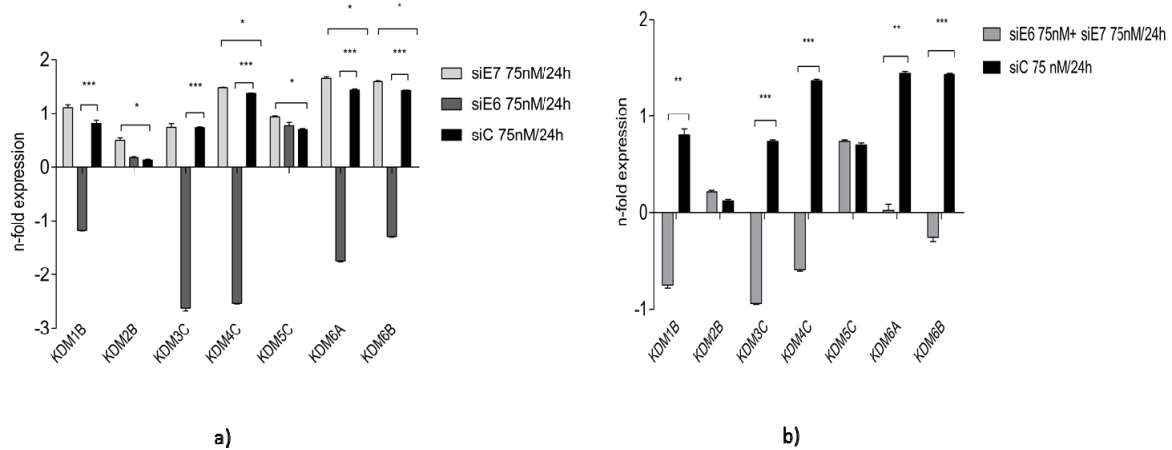

Figure 5. KDMs expression levels in siRNA treated HeLa cells.
(a) Analysis of KDMs expression levels measured by RT-PCR following transfection of HeLa cells with E7HPV18- siRNA and with E6HPV18- siRNA; (b) Analysis of KDMs expression levels measured by RT-PCR following transfection of HeLa cells with E7HPV18- siRNA and E6HPV18- siRNA at the same time. GAPDH gene was used as reference. The normalized values (ΔCt) from all samples were then compared with control HeLa- untreated cells, in each group ($\Delta\Delta$Ct). The results were expressed as Log10 ($2^{-\Delta\Delta Ct}$). All results were expressed as the means ± SD. *p< 0.05, **p< 0.001, ***p<0.0001.

Following E7HPV18-siRNA treatment, all KDMs except KDM1B were increased (p<0.05). By contrast, KDMs levels in E6HPV18-siRNA treated cells presented a decrease expression as compared with controls, most notably in the case of KDM3C and KDM4C (p<0.0001). A greater influence of E6 oncogene on KDMs was noticed. Concomitant treatment with E6 and E7HPV18-siRNA leads to a reduce expression of KDM1B, KDM3C, KDM4C and KDM6B (p<0.0001) but in a moderate manner compared with E6 siRNA alone.

4. Discussion

The present study shows a differential pattern of expression for KDMs in precancerous and cancerous cervical lesions as compared with controls. Results indicate that among histone lysine demethylases some correlates better with lesions severity. We found that KDM4C, KDM5C, KDM6A and KDM6B genes expression significantly increased in high grade lesions (CIN 2+) and SCC.

KDMs deregulation has been associated with cancer aggressiveness, this observation proposing them as molecular tumour markers. As KDMs target specific lysine residue leading to gene activation/repression, KDMs could play distinct role in cancer progression acting either as putative oncogene or tumour suppressors.

Our results showed a significantly increased KDM4C expression levels in SCC samples compared with precancerous lesions (p= 0.0010) proposing it as a suitable tumour marker. KDM4C /GASC1/JMJD2C/ is a histone demethylase that is mainly regarded as oncogene due to its role in demethylating heterochromatic H3K9me3/2. Generally H3K9 methylation marks have been associated with transcriptional repression; moreover global loss of H3K9 methylation was observed in several types of malignancies. High expression of KDM4C gene was found associated with oncogenic progression in esophageal squamous cell carcinoma; also gene

overexpression was noted in breast carcinoma, in prostate, squamous cell carcinoma and metastatic lung sarcomatoid carcinoma and some cases of lymphoma [14; 24-25].

Furthermore, another good marker for server lesions and SCC from our data seems to be KDM5C whose expression levels were found increased in CIN2+ lesions and significantly increased in SCC cases. Interestingly KDM5C role in the pathogenesis of HPV-associated cancers was very recently identified by Smith et al. (2010) who found that KDM5C is recruited by the E2 protein to repress the transcription of the oncoproteins E6 and E7 through the HPV LCR [26]. Also a significantly increased expression in CIN2+ and SCC cases was noted for H3K27 demethylases KDM6A and KDM6B.

On the other hand, H3K4 demethylase KDM1B could represent a potential biomarker for HPV productive infection vs transforming one, as its expression increased from CIN1 to CIN2+ (p=0.004). H3K4me3/2 and H3K4me2/1 residues are very often found on attractively transcribed genes and, by removing active histone marks, H3K4 histone demethylases function primarily as transcriptional repressors. Studies have shown an aberrant overexpression of LSD1 in many types of cancers; for example in prostate cancer a high expression of LSD1 gene was considering a predictive marker for aggressive tumour biology and tumour recurrence during therapy [27]. Given the high KDM3C expression levels observed in CIN2+ and the significant difference between this group of patients and CIN1 (p=0.0007), H3K9 demethylases might represent a potential biomarker for discriminating between productive vs transforming infection. The involvement of KDM3C in carcinogenesis has been suggested by a number of recent studies. For example, reduced levels of KDM3C were found in breast tumors, indicating a putative tumour suppressor role of this demethylases [28].

Another element of interest from this study is consisted by our experimental results that link the expression of some histone lysine demethylase and neoplastic transformation induced by high-risk viral genes E6 and E7, in particular the alleged connection between the expression of E6 oncogene and some histone demethylases (particularly for KDM5C, KDM6A, KDM6B and KDM1B). This possible link between E6 and KDMs expression might also explain the KDMs elevated levels noted in advanced lesions and in cervical cancer.

5. Conclusions

These findings point that KDMs display a differential expression pattern in HPV-induced cervical lesions and cancer, making them possible candidates as tumour markers in cervical carcinogenesis but their role in the mechanism of tumour initiation and progression remains to be solved. Also KDMs could represent new potential diagnostic tools as well as therapeutic targets in oncology, and compounds able to inhibit could be of considerable interested novel anticancer agents.

6. Acknowledgements

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