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Original Article

KDM4A, KDM4B and KDM4C in non-small cell lung cancer

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Abstract: KDM4A, KDM4B and KDM4D are lysine demethylases which demethylate H3 at lysine K9 and K36 sites, additionally KDM4D also the H1.4 linker histone at K26 lysine. Lysine methylation changes can repress or induce gene expression at specific sites thus influencing cellular functions. We analysed the immunohistochemical expression of KDM4A, KDM4B and KDM4D in a clinical material of 188 patients with lung carcinomas. There were 132 (70%) squamous cell carcinomas, 53 (28%) adenocarcinomas and 3 (2%) large cell carcinomas in the study. Additionally, the trimethylated state of chromatin was detected with an antibody to trimethylated H3K9 residue. Nuclear KDM4A and KDM4D were associated with the presence of lymph node metastases in tumors. Cytoplasmic KDM4A was associated with poor survival of the patients ($P = 0.015$) and with a shorter recurrence free interval ($P = 0.028$). KDM4A and KDM4D appear to have a significant role in the metastatic spread of lung carcinomas. The findings are also in line with their proposed involvement in mechanisms associated with cell proliferation, apoptosis and DNA repair.

Keywords: Lung, cancer, epigenetics, KDM, demethylase

Introduction

Epigenetic mechanisms, in contrast to DNA mutations, do not change the structure of the DNA but still change the expression of genes which are under their influence. Such mechanisms can be grouped in four categories; modification of histone residues by changing the chemical groups associated in amino acids, DNA methylation, microRNA and other RNAs which can change the level of target mRNAs before translation and remodelling proteins. This may move the position of DNA in the nucleosome or change the histone proteins to other subtypes, for instance [1, 2].

The nucleosomeconsists of 1.47 rounds of DNA covered by histone proteins H2A, H2B, H3 and H4 forming an octamer. Between nucleosomes there exists a linker histone H1. Histones have amino acid tails in which individual amino acids can undergo chemical changes such as methylation, acetylation, sumoylation, ubiquitination and phosphorylation. The chemical changes involve specific amino acids i.e. methylation/demethylation involves lysine and arginine, for instance, and phosphorylation serine or threonine [1, 2].

Lysine methylation/demethylation has been found to be important in lysine residues of H3K9, H3K27 and H3K36 where changes in the number of methyl residues affects the expression of genes by loosening or tightening the attachment of DNA to the nucleosome [3]. There are several enzymes which may influence or cause lysine methylation. One main group is the jumonji group or KDM proteins of which there are two subgroups; KDM1 and KDM 2-7 [4, 5]. KDM4A and KDM4B have as a substrate H3K9 and H3K36 and KDM4D H3K9 and H1.4K26 [5].

Lysine may contain maximum of three methylated residues and methylase enzymes can either add or move methyl groups to the amino

Table 1. Histological type, KDM4A, KDM4B, KDM4D and H3 positivity*

PAD	KDM4A				KDM4B				KDM4D				H3	
	nuclear		cytoplasm		nuclear		cytoplasm		nuclear		cytoplasm		nuclear	
SQ	68	41	59	53	53	62	60	55	56	57	60	53	57	53
AD	18	26	22	26	30	19	26	23	23	24	21	25	22	24
LC	18	7	13	12	17	7	18	7	13	11	13	11	13	12
	178		185		188		189		184		183		181	

SQ = Squamous cell carcinoma, AD = Adenocarcinoma, LC = Large cell carcinoma; *Due to detachment or loss of samples from slides the number of cases is lower than the maximal 189.

acid [4, 5]. Methylation of H3K4, H3K36 and H3K79 is generally considered to activate genes while methylation of H3K9, H3K27, H3K56, H4K20 and H1.4K26 causes transcriptional repression [5]. KDM4A and KDM4B remove the tri- and dimethylated marks from H3K9 and H3K36 thus leading to gene repression while KDM4D can only move a methyl group from a trimethylated mark of H3K36 [5].

In non-neoplastic tissues, expression of KDM4C is especially high in the testes and expression in the lung is very low [5]. KDM4A and KDM4B have a generally higher expression in non-neoplastic tissues the highest levels being found in ovary and spleen, but they are moderately expressed also in the lung [5]. In tumor tissues, KDM4A is upregulated in breast cancer [6]. On the other hand, KDM4B is down regulated in prostate cancer [7].

There are few studies on KDM demethylases in lung tumors. This study was undertaken to investigate the immunohistochemical expression of KDM4A, KDM4B and KDM4D in a set of 188 lung carcinomas. The results were associated with tumor histology, parameters describing the spread of the tumors, and survival of the patients. As an additional marker, the antibody to H3 trimethylated state was used.

Material and methods

Clinical characteristics

The representative samples of primary lung tumors were collected from 189 patients, diagnosed and treated for non-small cell lung carcinoma (NSCLC) during 1978-1996 in the University Hospital of Kuopio. All clinical data from the patients' files were re-evaluated and the stage of the disease was recorded according to TNM classification. Experienced histopathologists re-examined and classified the sections of the primary tumors according to WHO classification (YS), being unaware of the clinical data [8]. There were 115 (61%) squamous cell carcinomas (SCC), 49 (26%) adenocarcinomas and 25 (13%) large cell carcinomas in the study. The mean age of the patients was 62.5 years. There were 170 men and 19 females in the study material.

Treatment and follow-up

The patients were treated mainly with radical lobectomy or radical pulmectomy. Palliative operation or only explorative thoracotomy was performed in < 5% patients. None of the patients received preoperative radio- or chemotherapy. Postoperative radiotherapy and chemotherapy was given to 42/189 (22%) and 6/189 (3%) patients, respectively. The patients were followed-up by a senior physician according to a routine protocol. Time between the radical operation and any documented sign (radiological or clinical) of recurrence was considered as disease-free survival (DFS). For overall survival (OS), corrected survival rates were used, i.e. only deaths due to NSCLC were considered as outcome events and all other deaths as censored events.

Immunohistochemistry

The 4 μm thick slides, were dehydrated in graded alcohols, and paraffin was removed with xylene. All the antibodies were purchased from Abcam laboratories. The KDM4A antibody (ab-104831) was a mouse monoclonal antibody, KDM4B and KDM4D were rabbit polyclonal antibodies (ab103129 and ab93694, respectively) and the anti H3 antibody was directed against the trimethylated H3K9 residue (ab-8898). The dilutions of the primary antibodies were 1:1000, 1:200, 1:1000 and 1:2000, respectively. In staining with KDM4A, KDM4B and KDM4D the samples were pre-treated in microwave owen in EDTA buffer in pH 8.0 for 2x5 minutes. After application with primary antibody the HRP (Dako) kit was used for further processing. For the anti-H3 antibody, cit-

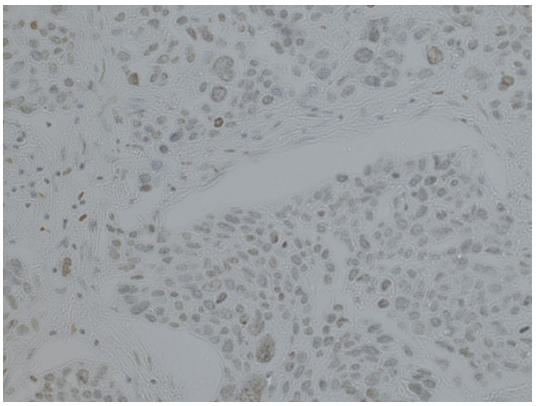

Figure 1. In a case of a squamous cell carcinoma mainly nuclearKDM4A staining can be detected.

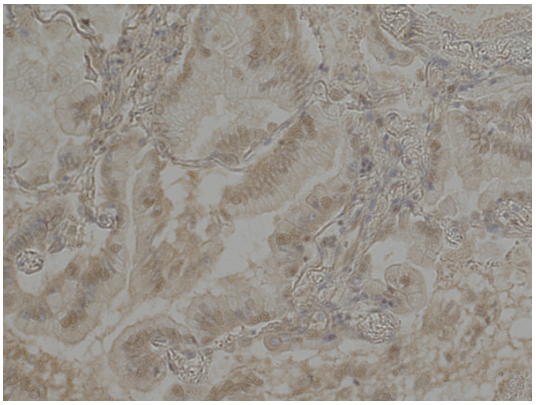

Figure 2. In a case of an adenocarcinoma of a lung showing lepidiformic growth pattern both nuclear and cytoplasmic immunostaining for KDM4B can be seen.

rate buffer was used and after incubation with the primary antibody, ABC-complex was used. Diaminobenzidine was used as a chromogen, and the slides were slightly counterstained with haematoxylin-eosin.

In each case, both the cytoplasmic and nuclear expression was evaluated from the four array slides containing the samples from different areas from various parts of the tumor. The evaluation was based on a semi quantitative scale with 0-1% = 0, 1-25% = 1, 25-50% = 2, 50-75% = 3 and over 75% = 4. The sum of the four evaluations was calculated and the results were divided in two groups according to the median value obtained from the sum divided by four. Thus the cases were finally divided to two groups showing either negative to weakly positive staining or moderate to strongly positive staining.

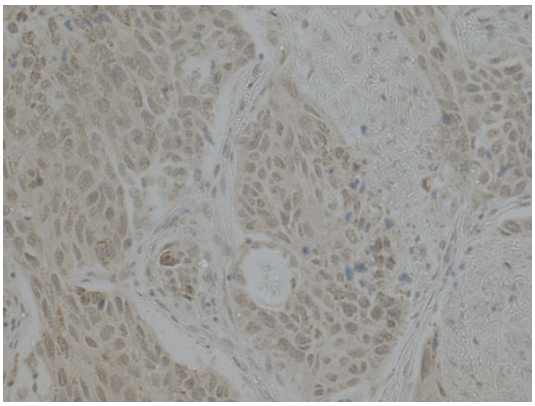

Figure 3. In a case of a squamous cell carcinoma of the lung, nuclear immunoreactivity for KDM4C can be seen.

Results

KDMs and H3 in tumors

The results are compiled in **Table 1**. In all tumors KDM4A nuclear positivity (**Figure 1**) was associated with simultaneous H3 positivity ($P<0.001$) but the association was not found with cytoplasmic positivity ($P = 0.371$). On the other hand, KDM4B nuclear positivity (**Figure 2**) was not related significantly with H3 expression ($P = 0.551$) while cytoplasmic expression did ($P < 0.001$). In the case of KDM4D, both nuclear and cytoplasmic positivity, were associated with H3 ($P < 0.001$ for both). KDM4A nuclear positivity associated also with KDM4D nuclear positivity ($P < 0.001$) and KDM4D cytoplasmic positivity (**Figure 3**) with KDM4A nuclear ($P < 0.001$) and cytoplasmic ($P = 0.001$) positivity. Moreover, KDM4B cytoplasmic positivity was related with KDM4D nuclear positivity ($P < 0.001$) and KDM4B cytoplasmic positivity ($P < 0.001$). Nuclear KDM4A expression associated with cytoplasmic KDM4B positivity ($P < 0.001$) and cytoplasmic KDM4A positivity with both nuclear ($P = 0.003$) and cytoplasmic KDM4B ($P < 0.001$) positivity.

There were significantly more cases with nuclear KDM4A expression in squamous cell carcinomas as compared to adenocarcinomas ($P = 0.019$) (**Table 1**). Similarly, large cell carcinomas had more nuclear KDM4A positivity compared to adenocarcinomas ($P = 0.023$). Squamous cell carcinomas showed less nuclear KDM4B expression as compared to adenocarcinomas and other tumors together ($P = 0.017$).

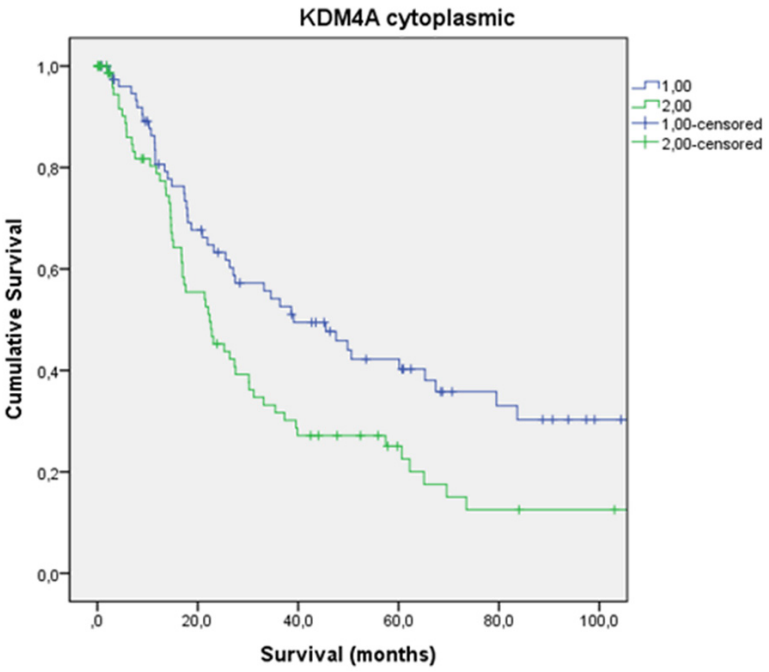

Figure 4. In squamous cell carcinoma, cases with strong cytoplasmic KDM4A positivity showed a poor survival compared to those with weak staining (1 = weak immunoreactivity, 2 = strong immunoreactivity, P = 0.015).

Other associations were not found to be significant.

Association of the KDMs and H3 with the size and the presence of lymph node metastases

Nuclear (P = 0.66) or cytoplasmic (P = 0.67) KDM4A expression did not associate with the size (T1-2/T3-4) of the tumors. There were significantly more nuclear KDM4A expression in the tumors with lymph node metastasis than in tumors with no metastasis (P = 0.049). The same tendency was observed with cytoplasmic expression (P = 0.054). There were only two cases with metastases in distant sites. Consequently, statistical assessment for the M status was not considered relevant.

Nuclear (P = 0.40) or cytoplasmic (P = 0.09) KDM4B expression did not correlated with tumor size. Also nuclear (P = 0.75) or cytoplasmic (P = 0.53) expression was not associated with lymph node metastases.

With KDM4D, nuclear (P = 0.66) or cytoplasmic (P = 0.19) expression was not associated with tumor size. As with KDM4A, KDM4D nuclear expression associated with the presence of lymph node metastases (P = 0.009) but the same relationship was not noted with cytoplasmic expression (P = 0.20).

As for H3K9, its expression did not associate with tumor size (P = 0.52), but it showed a near significant association (P = 0.052) with a positive lymph node status.

Association of the KDMs and H3 with survival and recurrence

Nuclear KDM4A was not associated with survival (P = 0.137, log rank). However, cytoplasmic expression of KDMA4 was associated with poor survival (P = 0.015, log rank) (**Figure 4**). Nuclear KDM4A expression was not associated with time of relapse (P = 0.59) but cytoplasmic KDM4A expression did (P = 0.028).

Nuclear or cytoplasmic KDM4B expression was not associated with survival (P = 0.22 and P = 0.53, respectively). Neither did these factors associate with recurrence (P = 0.059 and P = 0.34, respectively).

Nuclear or cytoplasmic KDM4D expression was not associated with survival (P = 0.20 and P = 0.82, respectively) and were not related with recurrence (P = 0.22 and P = 0.75, respectively).

H3 expression was not associated with survival (P = 0.88) or the time of recurrence (P = 0.53).

Squamous cell carcinomas or adenocarcinomas did not separately associate with survival. However, adenocarcinomas with strong cytoplasmic KDM4A staining had a poor prognosis (P = 0.048).

When evaluated survival by Cox regression analysis including tumor grade, histology, gender or TNM state, none of the KDMs or H3 had independent prognostic value.

Discussion

Lysine demethylases are divided in two subfamilies, the flavin dependent KDM1 subfamily

and the 2-oxoglutarate dependent JmjC subfamily [4]. These enzymes remove the N associated methyl residues from lysine which can be mono- di- or trimethylated or, of course, lack the methyl groups altogether [4]. Lysine methylation in histone H3 protein affects the accessibility of DNA leading to changes in gene expression. This prompted us to study the expression of three lysine demethylases, KDM4A, KDM4B and KDM4C, in lung cancer samples and correlate the results with the clinicopathological parameters and survival. Additionally, we analysed the methylation state in the tumors with H3 antibody directed against 3-trimethylated lysine.

There are no previous studies on the expression of KDM4A, KDM4B or KDM4D in lung tumors and only a few studies in clinical materials of other tumors. In addition to demethylation these demethylases appear to have important functions related to carcinogenesis [1, 2]. KDM4A overexpression, for instance, leads to instability of chromosomal regions 1q12, 1q21, and Xq13.1 while not affecting general chromosomal instability [8]. In colon cancer cell lines, KDM4A interacts with p53 and its down regulation leads to increased expression of p21 and puma protein but to a decreased level of blc2 thus leading to an increased apoptosis [9]. Overexpression of KDM4A leads to increased proliferation and its knockdown abrogated proliferation in colon cancer cell lines [9]. KDM4A appears also to promote AP1 inducing genes leading to increased expression of FOS and JUN [10]. Knockdown of KDM4A inhibited the formation of lymph node metastasis and in squamous cell carcinomas; KDM4A was increased in cases with lymph node metastases [10]. This is in line with our study where both nuclear and cytoplasmic KDM4A expression was associated with lymph node metastases in lung carcinomas in breast tumors, KDM4A expression was associated with tumor grade and higher TNM classification and its expression was higher in carcinomas than in normal breast tissues [11, 12]. Our study did not show any association between tumor size and KDM4A expression in lung carcinomas; neither did we found any association between tumor grade and KDM4A.

In breast tumors, KDM4A stimulates ERα activity thus promoting their oestrogen dependent growth [12]. In addition to ERα, KDM4A appears to activate androgen receptor thus making it putatively important also in prostate cancer tumor growth [13]. In line with this, KDM4A was found to be overexpressed in prostate carcinoma [14]. The association of KDM4A with hormone receptors makes its impact different for tumor growth in hormone receptor dependent carcinomas compared to lung carcinomas which may explain the different KDMA's association with tumor size as compared with lung carcinoma. In our study KDM4A was, however, associated with the presence of lymph node metastases and cytoplasmic expression of KDM4A was related with a worse survival and a shorter recurrence free interval. Our results suggest that KDM4A expression promotes tumor spread in lung carcinoma thus also contributing to a shorter survival of the patients.

Like KDM4A, KDM4B promotes AR directed signalling and prevents its ubiquitination [15]. It also associates with ERα receptor and coordinates ERα induced gene transcription through its enzymatic activities thus influencing breast carcinogenesis [16]. Surprisingly, KDM4B is, however, down regulated in prostate carcinoma [7]. In colon cancer, KDM4B stimulates beta-catenin and TCF4 promoting their expression and thus contributing to colon cancer carcinogenesis [17]. In colon cancer, knockdown of KDM4B induced DNA damage, cell cycle arrest and apoptosis [18]. KDM4B is overexpressed in DNA damage and contributes to the resistance of irradiation induced DNA damage [19]. In gastric cancer, knockdown of KDM4B induces apoptosis and abrogates proliferation and elevates the levels of p53 and p21 [20]. Its level is increased both in gastric cancer and in bladder cancer and regulates the expression of CDK6 [20, 21]. Toyokawa et al have also studied lung carcinomas and found a 38% nuclear expression in them [21]. They showed that KDM4B expression promotes growth by relieving G1-S transition [21]. In our cases, nuclear expression was found in 46% of cases which is in the same level as they found. In our studies, squamous cell carcinomas showed a more pronounced expression of KDM4B. Such association with histology was not reported earlier [21]. In our material the expression of KDM4B was not related with any the TNM status of the tumors or patient survival or tumor recurrence.

Thus, in our material, KDM4B seems to have less impact on lung cancer spread than KDM4A.

KDM4D activates proliferation partly through a p53 mediated mechanism and is a pro survival gene [22]. Like the other KDMs, it also interacts with AR [13]. KDM4D is also associated with repair of double stranded DNA break where it phosphorylates ATM substrates [23]. In our study, nuclear KDM4D associated with the presence of lymph node metastases, but it did not influence patient survival in our material. The results thus implicate, that KDM4D plays a role in the metastatic spread of lung carcinomas.

H3 antibody positivity marks a trimethylated state of the chromatin. Approximately a half of the tumors were positive. There was a positive association between KDM4A nuclear, KDM4B cytoplasmic and both KDM4D nuclear and cytoplasmic positivity possibly suggesting an activation of these factors in the presence of trimethylated lysine residues. Increased H3K27 trimethylation has been associated with a better prognosis in lung carcinoma, but we did not find any such association which, however, detects the general trimethyated state of lysines also in other sites [24].

In summary, our results show that KDM4A and KDM4D play a role in spread of the lung carcinomas. Further, cytoplasmic KDM4A positivity associates with patient survival. These results are in line with the supposed role of KDMs in epigenetic regulation of cancer cells, affecting proliferation, apoptosis and DNA repair mechanisms.

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Disclosure of conflict of interest

None.

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