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HDAC1 is indirectly involved in the epigenetic regulation of p38 MAPK that drive the lung cancer progression

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Abstract. – OBJECTIVE: p38 MAPK are a class of protein kinase that may induce or prevent apoptosis in different circumstance. Emerging researches show that it plays a vital role in tumor progression and therefore understanding its dual role in different stages of lung cancer are important to investigate. Also in this study, we planned to understand its upstream target proteins like HDAC1 and uPAR which are responsible for p38 MAPK activation in the pathway.

MATERIALS AND METHODS: We initially develop lung cancer mice model by exposing them to high nicotine content tobacco smoke. The pathological stages of initial and advanced lung cancer are observed and confirmed through histological sectioning. The expression of HDAC1, uPAR and p38 MAPK are observed and analyzed in different stages of lung cancer using immunohistochemistry and Western blotting.

RESULTS: After 4 and 6 months of regular exposure of high nicotine content smoke, the A/J strain mice develop initial and advanced stage of lung cancer. The initial stage cancer develops thick tissue layers with fibrosis whereas advanced stages of lung cancer show more proliferative cells. The expression of HDAC1 and uPAR shows the minimal expression pattern in control and initial stages of lung cancer, but its expression increased in advanced stage of cancer. In case of phospho-p38 MAPK, mild expression was observed almost in every individual cell in the initial stages of cancer, which implies its protective role in preventing advanced stage of cancer. But in advanced stage of lung cancer, we observed dysregulated overexpression of phospho-p38 MAPK.

CONCLUSIONS: The epigenetic regulation of uPAR by HDAC1 confirms its indirect role in regulating p38 MAPK as tumor progress.

Key Words:
HDAC1, uPAR, p38 MAPK, Tobacco smoke.

Introduction

Lung cancer is a most common type of cancer which accounts for leading cancer death worldwide[1]. Due to developed medical practice the mortality rate of lung cancer declines constantly over several decades, but still there is no improvement in the 5-year survival rate of the patients[2]. The reason behind them is that the most of the patients are diagnosed in the late stage of lung cancer and therefore new ways are to be put forward to diagnose in early stages. Several risk factors like smoking, abdominal obesity are associated with epigenetic and genetic aberrations that notably promote lung cancer progression[3]. The development of lung cancer is a multistep process which involves slower changes at genetic and epigenetic level, which alters the behavior of normal lung epithelial cells[4,5]. It was documented that not all cells, but the subsets of lung epithelial cells, especially pulmonary epithelial stem cells are more susceptible to initial transformation[6]. The molecular level changes that happen in tumor initiating cells expand to adjacent cells[7] that inhibits anti-growth signals, apoptosis, controlled angiogenesis, replicative potential and thereby promote metastasis[8,9]. The various molecular changes are associated with different genes which determine their biological behavior and responsiveness of lung cancer cells[6]. The carcinogens are able to introduce epigenetic changes like DNA methylation in lung cancer associated genes like F2RL3 and AHRR which induce their risk[10]. The epigenetic alteration like histone modifications, DNA methylation and the expression of non-coding RNA are able to determine the individual ability to repair

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and to sustain the risk factors. Histone deacetylase 1 (HDAC1) as it names indicate involved in deacetylation of many histone and non-histone protein and thereby involved in the epigenetic regulation of many genes[11]. The overexpression of HDAC1 are able to promote cell proliferation, inhibit apoptosis and cell adhesion in breast, colon and lung cancer[12-14]. The activation of p38 Mitogen-activated protein kinase (MAPK) occurs at the time of stress factors like heat, oxidative, osmotic and in response to TGF-β signalling[15]. The p38 MAPK signalling pathway phosphorylate downstream proteins like protein kinases and transcription factors and thereby play a complex role in cancer progression[16]. Also in the stressful environment the p38 MAPK is reversible acetylated which helps to induce stress response inside the cell[17]. In this research work we try to understand the role of HDAC1 in regulating p38 MAPK.

Materials and Methods

Experimental Animals

To carry out the experiment we choose 3 months old A/J strain of female mice. All animals are carefully maintained in a laboratory condition as approved by the ethical committee of Tongji University School of Medicine. The experiment carried out using the animals and the protocol followed are approved by the Tongji University School of Medicine Ethical Committee. The mice are kept in cages provided with food and water whenever it's needed. The animals that are subjected to experimental handlings are observed regularly twice in a day.

Mice Exposed to Tobacco Smoke

To initiate lung cancer, the mice are exposed to tobacco smoke with high nicotine content as described by Witschi et al[18]. Following exposure, the mice were maintained in an animal chamber with a periodic 12 hours' light dark cycle. The tobacco smoke was given in alternative days and the dosage range per day was given for continuous 7 hours. The inhalation of tobacco smoke was continued up to 4 months in developing an initial tumor and subjected to 6 months of exposure for developing advanced lung cancer. Following the full term of tobacco smoke exposure, the mice are given with a recovery period of 1 week.

Histological Imaging

The lung tissue developed with initial and advanced stage of lung cancer is dissected out and fixed with 10% formaldehyde solution. After fixation, the tissue is washed in distilled H_2O and subjected to gradual dehydration using isopropyl alcohol. The processed tissues are then infiltrated with paraffin wax. Using microtome, the sections are cut into 7 µm size and placed on the slide. After dewaxing and processing the sections are stained with haematoxylin and eosin. Finally, the slides are mounted with DPX (distyrene, a plasticizer, and xylene) for permanent visualization.

Immunohistochemistry

The processed sections are incubated with 3% H_2O_2 solution in methanol, which helps to block the cellular endogenous peroxide activity. Antigen retrieval was performed using 10 mM citrate buffer (pH 6.0; 95°C for 7 min) which helps to expose the antigen. After antigen retrieval the slides are incubated with 4% BSA (Bovine Serum Albumin) which act as a blocking solution. The primary antibody (anti-HDAC1 antibody, Abcam (ab19845, Pudong, Shanghai, China) or anti-uPAR antibody, Abcam (ab103791, Cambridge, MA, USA) or anti-phospho p38 MAPK antibody, Abcam (ab4822, Cambridge, MA, USA) in 1% BSA solution is allowed to incubate for overnight at 4°C. After washing with 1X PBS (phosphate-buffered saline) the slides are incubated with secondary antibody and placed in room temperature for 1 hour. After washing the non-specific binding of antibody with 1X PBS (three times) the slides are developed with DAB solution which gives brown colour signals.

Western Blotting

The dissected sample tissue is homogenized along with a 2X protein sample buffer in ice cold condition. The prepared cell extract is subjected to heat in boiling water bath for 10 min to obtain the protein samples. The samples are loaded with equal concentration (70 µg) of protein samples and subjected to SDS-PAGE at 50 V for 3 hours. The separated proteins are transferred to polyvinylidene difluoride (PVDF) membrane and subjected to blocking for 1 hours using 4% BSA in TBST (Tris-buffered saline and Tween 20). After blocking, the membranes were incubated overnight with primary antibody (anti-HDAC1 antibody, Abcam (ab19845, Pudong, Shanghai, China) or anti-uPAR antibody, Abcam (ab103791, Cambridge, MA, USA) or anti-phospho p38

MAPK antibody, Abcam (ab4822, Cambridge, MA, USA) at 4°C. After incubated with secondary antibody the membrane is developed with DAB (3'-diaminobenzidine) solution, to obtain the signals.

Statistical Analysis

The experimental data are obtained by repeating the experiments for at least three times. The statistical differences between the groups are expressed as standard errors of the mean. The statistical significant are evaluated using Student's *t*-test for independent samples and level of significance was analysed using ANOVA followed by Tukey's Post-Hoc test for multiple comparisons. We deemed significant level of p-value < 0.05.

Results

Induction of Lung Cancer in A/J Strain Mice

To understand the link between HDAC1 and p38_MAPK in lung cancer, we initially induced lung cancer in female A/J mouse strain. The mice are divided into three groups and each group are having 10 mice in total. The group 1 mice act as a control which are exposed to non-nicotine content smoke. Group 2 and 3 mice are exposed to high nicotine content tobacco smoke for 4 and 6 months, respectively as described in materials and methods. After 4 months of exposure, group 2 mice developed initial stage of lung cancer and after 6 months of exposure the group 3 mice develop adverse stage of lung cancer. The initial and advanced stages of cancer development in experimental mice are analysed and confirmed through histological imaging (Figure 1A-C). The haematoxylin and eosin (HE) stained control lung tissue shows thin epithelial tissue with uniformly organized tissue pattern (Figure 1A). The mice lungs exposed to 4 months of high nicotine smoke develop hard tissue like fibrosis (Figure 1B) and the mice exposed to 6 months of time show magnitude of proliferative cells with hardened tissue in isolated pockets (Figure 1C).

HDAC1 Overexpression as Lung Cancer Progress

To investigate the role of HDAC1 in relation to tumor progression, we analyzed the HDAC1 expression in control, initial and advanced stage of lung cancer. The immunohistochemistry data against anti-HDAC1 protein shows a very minimal expression pattern in control tissue (Figure 2A). Also the expression of HDAC1 shows no significant overexpression in the initial stage of lung cancer (Figure 2B) but it is extensively overexpressed in advanced stage of lung cancer (Figure 2C).

HDAC1 Regulate Urokinase Plasminogen Activator Receptor (uPAR) Level as Tumor Progress

The epigenetic regulation of GM3 by HDAC1 is important for the activation of uPAR; recent works show that deacetylated GM3 is involved in the activation of uPAR/integrin signaling[19]. Here, to determine the epigenetic regulation of GM3 by HDAC1, the subsequent activated product uPAR are analyzed. Our results show overexpression of HDAC1 is associated with

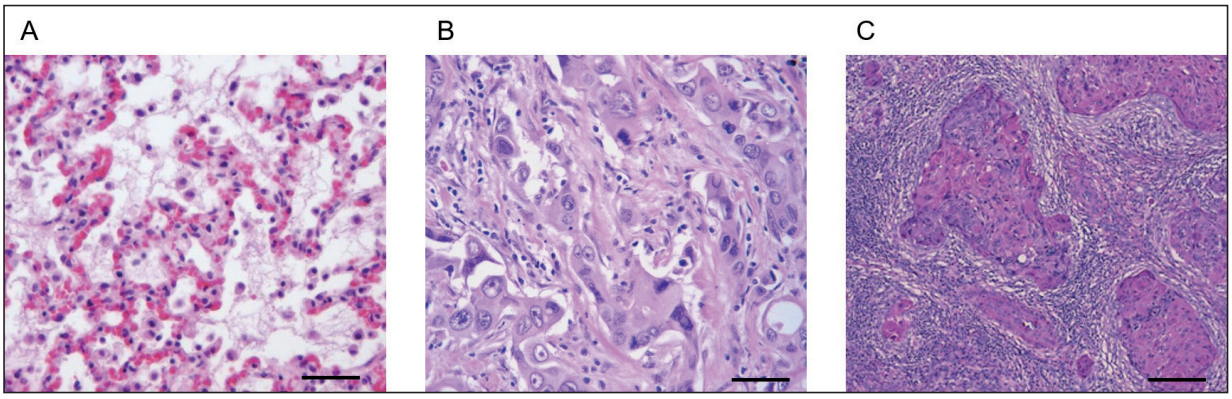

Figure 1. Tumor progressing and pathological changes that are observed using histological sectioning. ***A.*** Lung tissue from control mice shows well organized regular tissue structures. ***B.*** Mice exposed to high nicotine tobacco smoke for 4 months show initial lung cancer with developing fibrosis. ***C.*** Mice exposed to high nicotine tobacco smoke for 6 months develop advanced stage of lung cancer with more proliferative cells. Scale bar – 50 µm.

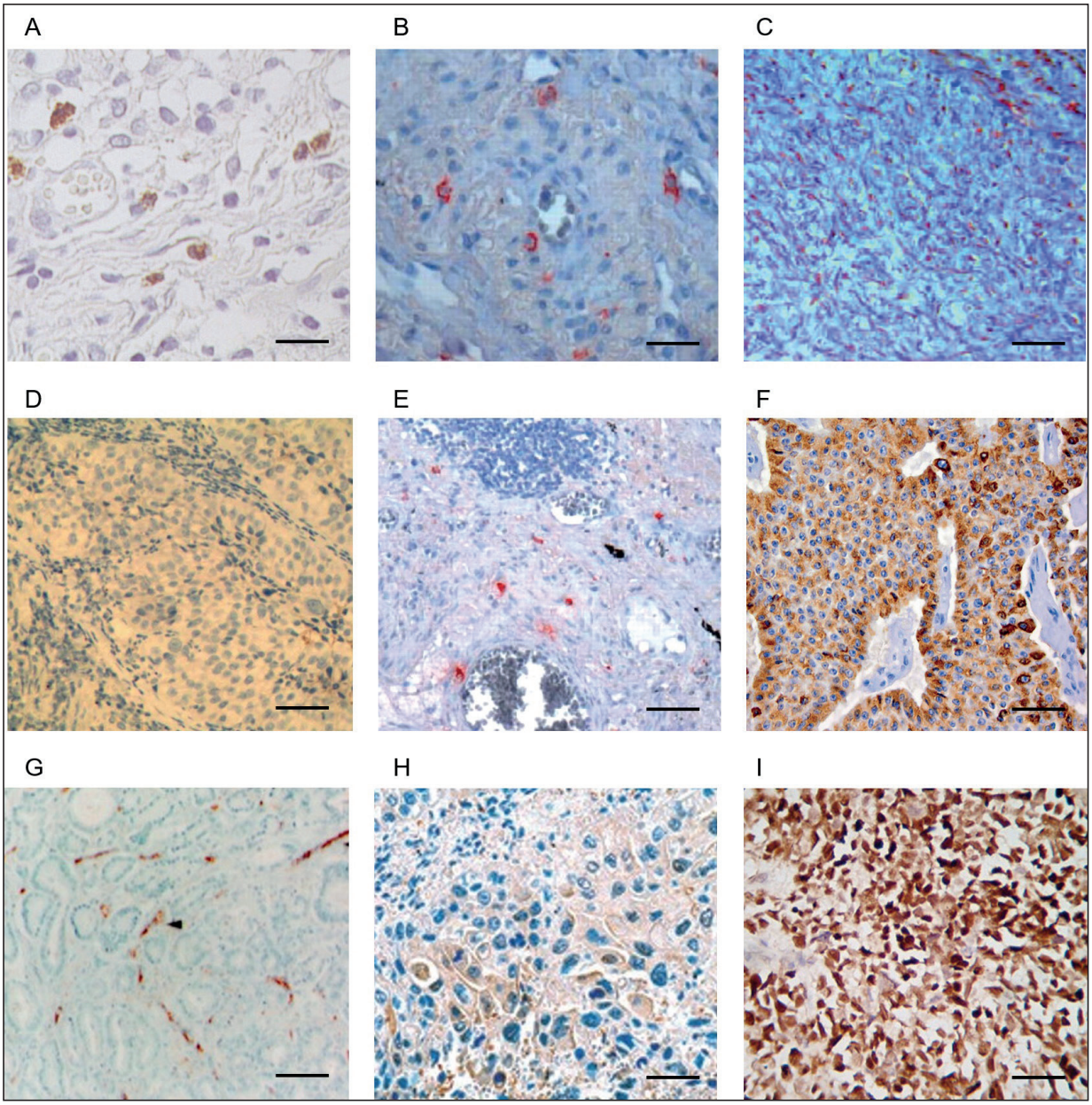

Figure 2. Expression studies of HDAC1, uPAR and p38 MAPK in different stages of lung cancer. ***A.*** Control lung tissue with minimal expression of HDAC1. ***B.*** Initial stage lung cancer tissue showing minimal expression of HDAC1. ***C.*** Advanced stage lung cancer tissue showing overexpression of HDAC1. ***D.*** Control lung tissue showing minimal expression of uPAR. ***E.*** Initial stage lung cancer tissue showing minimal expression of uPAR. ***F.*** Advanced stage lung cancer tissue showing overexpression of uPAR. ***G.*** Control lung tissue with minimal expression of p38 MAPK. ***H.*** Initial stage lung cancer tissue showing minimal expression of p38 MAPK. ***I.*** Advanced stage lung cancer tissue showing overexpression of p38 MAPK.

the expression of uPAR (Figure 2A-F). The expression of uPAR is maintained at the minimum level as that of HDAC1 expression in control and initial stage of cancer (Figure 2D and 2E). But as a tumor developed in advanced stages, the signaling mechanism of uPAR shows enhanced level (Figure 2F).

Dual Role of p38 MAPK as Tumor Protective and Inducer

The activation of P38 MAPK is by means of dual phosphorylation that are regulated by cytokines, TGF-β signaling, G protein-coupled receptor[15,20]. We investigate the indirect link between HDAC1 and phospho-p38 MAPK and observed

that in control lung tissue p38 MAPK expression is in reduced level (Figure 2G). But as tumor initiate the mild expression of phospho-p38 MAPK was initiated in almost major population of cells in the tissue (Figure 2H), which implies its protective role in determining the cell fate. The high level of proliferative cells in advanced stage of cancer along with phospho-p38 MAPK overexpression was observed as tumor progress to the next level (Figure 2I).

Western Blotting Analysis of HDAC1, uPAR and p38 MAPK

The expression levels of HDAC1, uPAR and phospho-p38 MAPK in immunohistochemistry are further investigated using Western blotting techniques. We identified a significant correlation between the immunohistochemistry and Western blotting data. The expression of HDAC1 and uPAR is in low level in control and initial lung cancer, but its expression increased in advanced stage of lung cancer (Figure 3). The pattern of phospho-p38 MAPK shows a constant increase in initial cancer, and it subsequently overexpressed in advanced stage of lung cancer (Figure 3).

Discussion

In general, p38 MAPK has a function similar to a tumor suppressor protein that negatively regulate cell proliferation and induce apoptosis[21,22]. The acetylation of p38 MAPK enhances its ATP binding ability, thereby activatingits kinase property[23]. In this work we induce lung cancer using high nicotine containing tobacco smoke which is able to induce lung cancer as like it is. Therefore, it mimics more accurate pathological conditions when compared to knocking out single gene or inducing lung cancer using various carcinogenic chemicals. In initial stage of lung cancer, fibrosis was observed (Figure 1B), which may be due to replacement of impaired cells along with cellular turnover[24]. A high index of proliferative cells is a sign of advanced stage cancer (Figure 1C) which are observed in our study after 6 months of high nicotine exposure[25]. The increase expression of uPAR upon cancer progression implies that HDAC1 target GM3 and thereby activates its downstream target like uPAR[19]. The inhibitory level of HDAC1 expression in the initial stage of lung cancer (Figure 2B) along with minimal expression of uPAR (Figure 2E) when compared with advanced stage of lung cancer correlate with recent experimental findings that HDAC1 depletion favors pancreatic cancer stem cell elimination[26]. A recent work[27] shows the important aspect of HDAC inhibitors like trichostatin, scriptaid, entinostat as a therapeutic strategy to inhibit endometrial cancer. The minimal expression of HDAC1 in initial stages of lung cancer aids in transactivation of uPAR at latter stage of lung cancer which are revealed with siRNA studies against HDAC1[28]. The results demonstrate the coordinate action of HDAC1 and uPAR expression in regulating the lung cancer. We also found out that increased expression of HDAC1 and uPAR promotes cellular proliferation in advanced stage of lung cancer (Figure 2A-F). The minimal expression of p38 MAPK in the initial stage of lung cancer was observed throughout the tissue layer (Figure 2H), which may be due to protective measures in preventing abnormal cell cycle regulation. The pharmacological suppression of p38MAPK signaling pathway plays a positive role in treating acute lungs injury[29] and this, taken together with our results, implies its protective role when expressed in minimal level. The abnormal increased expression of p38 MAPK shows the dysregulated level, which is usually observed in advanced stages of cancers including lung cancer[30].

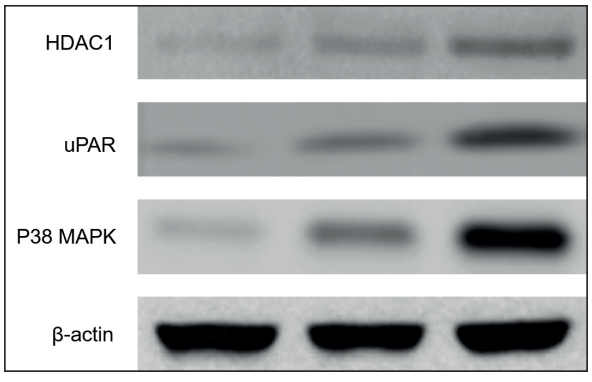

Figure 3. Western Blotting analysis for HDAC1, uPAR and p38 MAPK expression. Lane 1: shows the expression profile of HDAC1 in control, initial stage and advanced stage lung cancer. Lane 2: shows the expression profile of uPAR in control, initial stage and advanced stage lung cancer. Lane 3: shows the expression profile of p38 MAPK in control, initial stage and advanced stage lung cancer. For loading control β-actin was used.

Conclusions

We showed that the higher expression of HDAC1 in advanced stage of lung cancer regulates its downstream protein in its pathway

through epigenetic regulation. The epigenetic regulation of uPAR by HDAC1 significantly affects the p38 MAPK activation pathway as tumor progress.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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