RETRACTED ARTICLE: Ras-ERK signalling represses H1.4 phosphorylation at serine 36 to promote non-small-cell lung carcinoma cells growth and migration

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\textbf{ABSTRACT}
Recent papers suggest that oncogenic Ras participate in regulating tumour cells proliferation and metastasis. This work linked Ras with H1.4 modification in non-small-cell lung carcinoma (NSCLC), to better understand the oncogenic effects of Ras. A plasmid for expressing Ras mutated at G13D and T35S was transfected into NCI-H2126 and A549 cells. Phosphorylation of H1.4S36 was determined by immunoblotting. Effects of phosphorylation of H1.4 at serine (S) 36 (H1.4S36ph) on NCI-H2126 and A549 cells were tested by MTT assay, soft-agar colony formation assay, flow cytometry and transwell assay. Chromatin-immunoprecipitation (ChIP) and RT-qPCR were conducted to measure the effects of H1.4S36ph on Ras downstream genes. The catalyzing enzymes participate in H1.4S36ph were further studied. We found that Ras-ERK signalling repressed the phosphorylation of H1.4 at S36. H1.4S36ph functioned as a tumour suppressor, as its overexpression repressed NCI-H2126 and A549 cells viability, colony formation, S-phase arrest, migration and invasion. H1.4S36ph was able to mediate the transcription of Ras downstream genes. Ras-ERK signalling repressed H1.4S36ph through degradation of PKA, and the degradation was mediated by MDM2. In conclusion, Ras-ERK signalling repressed H1.4 phosphorylation at S36 to participate in NSCLC cells growth, migration and invasion. Ras-ERK signalling repressed H1.4S36ph through MDM2-dependent degradation of PKA. This study provides a novel explanation for Ras-ERK’s tumour-promoting function.

\textbf{HIGHLIGHTS:}

1. H1.4S36 phosphorylation is repressed by Ras-ERK activation;
2. H1.4S36ph inhibits the phenotype of NSCLC cells;
3. H1.4S36ph regulates the transcription of Ras downstream genes;
4. Ras-ERK represses H1.4S36ph by MDM2-dependent degradation of PKA.

\textbf{Introduction}

Ras is a proto-oncogene, whose mutation is frequently found in multiple tumour cells. In mammals, the Ras gene family is composed of three subtypes: \textit{HRas}, \textit{NRas} and \textit{KRas}. Different ways of Ras activation result in the genesis of cancer. Among which, point mutation is the most common activation way. Around 17% of cancer patients possess an activating Ras mutation, and \textit{KRas} mutation is the most frequently mutated Ras isoform, accounting for 69% of all cases [1]. Recent studies revealed that the activation of Ras contributes to human cancers due to its critical role in regulating several signalling pathways, such as PI3K, MAPK, ERK and MEK. In non-small-cell lung carcinoma (NSCLC), \textit{KRas} is one of the most frequently mutated genes. \textit{KRas} mutations were observed in around 30% of lung adenocarcinomas [2] and 5% of squamous cell carcinomas [3]. However, the studies focus on how \textit{KRas}-driven NSCLC are lacking.

Histone modification is one of the epigenetic changes that regulate the chromatin structure and, therefore, impact diverse DNA-templated procedures [4]. Accumulating studies have demonstrated that changes in histone modification occurred in different types of human malignant tumours and having value in clinical practice, such as markers for predicting prognosis, assessing therapeutic response to some chemotherapeutic drugs, and emerging therapeutic targets for treating cancers [5–7]. There are five histone families in human, i.e. H1, H2A, H2B, H3 and H4. All of them can undergo posttranslational modifications, such as methylation, acetylation, phosphorylation, etc., in various positions. H1 histone family is the most divergent and heterogeneous sort of histones. Ten variants of H1 have been found in human, i.e. H1.0, H1.1, H1.2, H1.3, H1.4, H1.5, H1oo, H1t, H1x, and testis-specific H1. Different variants of histone H1 subtypes show a clear sequence and functional divergences from one another [8]. Phosphorylation of H1.4 at serine (S) 36 (H1.4S36ph) has
been suggested as a key progress in regulating cell cycle progression [9]. Besides, in vivo characterization of H1.4S36ph has been studied in various diseases, including NSCLC [10–12]. These raise an intriguing question about the role of H1.4S36ph in the onset and progression of NSCLC.

Recently, a growing number of literature have reported that oncogenic Ras alters histone modification pattern and thus participate in regulating tumour cells proliferation and metastasis [13–15]. The present work attempted to link oncogenic Ras with H1.4S36ph in NSCLC, in order to better understand the oncogenic effects of Ras on NSCLC.

Materials and methods
Plasmids and antibodies

The coding regions of human H1.4, Ras and PKA, MDM2 were amplified by PCR. The PCR products of H1.4 and Ras were placed into a pEGLFP-N1 plasmid (Clontech, Palo Alto, CA), PKA was inserted into a pCMV-HA plasmid (Clontech, Palo Alto, CA), and MDM2 was inserted into His-tagged (Invitrogen, Carlsbad, CA). The pEGLFP-RasG13D/T35S construct was mutated using site-directed mutagenesis. The pEGLFP-H1.4S36E construct was made by using the TakaRa MutanBEST Kit (Takara, Dalian, China). siRNAs specific against MDM2 were from GenePharma (Shanghai, China).

Anti-H1.4S36ph antibody (Catalog number: PA5-31007) was acquired from Thermo Fisher Scientific (Waltham, MA). Primary antibodies specific for H1.4 (Catalog number: ab116393), p-ERK1/2 (Catalog number: ab214362), ERK1/2 (Catalog number: ab54230), HA (Catalog number: ab18181), GFP (Catalog number: ab183734), PKA (Catalog number: ab75993), IgG (Catalog number: ab238004), β-actin (Catalog number: ab8227) and MDM2 (Catalog number: ab38618) were acquired from Abcam (Cambridge, MA). Anti-His antibody (Catalog number: PTGX-HIS-2H5) was obtained from ProteoGenix (Schiltigheim, France). Goat anti-rabbit (ab7090) and goat anti-mouse (ab97040) IgG H&L (HRP) secondary antibodies were from Abcam (Cambridge, UK).

Cell culture and transfection

Human NSCLC cell lines NCI-H2126 (ATCC® CCL-256™) and A549 (ATCC® CCL-185) were bought from ATCC (Manassas, VA). The cells were subcultured in 75 cm² flask and maintained at 37 °C in an atmosphere with 5% CO². The complete growth medium for NCI-H2126 cells culture was made by adding 5% fetal bovine serum (FBS, Gibco, Grand Island, NY), 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 mM sodium selenite, 10 mM hydrocortisone, 10 mM β-estradiol and 2 mM L-glutamine (all Sigma-Aldrich, St. Louis, MO) into DMEM:F12 medium (Gibco). The complete growth medium for A549 cells was made by adding 10% FBS into F-12K medium (ATCC).

Transfection was executed in 6-well plates when the confluence of cells reached ~80%. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was utilized for transfection as recommended by the manufacturer. MG132, a proteasome inhibitor, was purchased from MedChemExpress (Monmouth Junction, NJ). Cells were administrated with 25 μM MG132 for diverse times.

Cell viability assessment

The cells were placed into 96-well plates (5000 cells per well). After 48 h of incubation, 20 μL 5 mg/mL MTT solution (Sigma) was added and the plates were incubated for 4 h at 37 °C. The optical density (OD) value was recorded by using an ELISA reader (Bio-Rad, Hercules, CA) at 570 nm.

Colony formation assay

The colony formation capacity of cells in agarose-coated plates was detected as previously described [16]. After 2 weeks of incubation, colony number from five randomly selected fields was counted microscopically.

Transwell assay

The cells were placed in the upper chamber of the transwell system (Costar, Boston, MA) and cultured in non-serum medium. The lower chamber was filled with complete growth medium. 12 h later, the cells in the lower side were stained by 0.5% crystal violet (Sangon Biotech, Shanghai, China). The stained cells were then eluted with acetic acid, and the OD-values of the sample were recorded by the ELISA reader (Bio-Rad) at 570 nm. The invasion of cells was analyzed as the same with migration assay, except the transwell system was pre-coated with Matrigel (Millipore, Bedford, MA).

Cell cycle assay

The cells were washed twice with precooled phosphate buffer saline (PBS). The cells were then fixed with 70% ethanol overnight at 4 °C. After rising with precooled PBS, cells were incubated in a staining solution containing 50 μg/mL propidium iodide (PI), 100 μg/mL RNAse A, 0.2% Triton X-100 (all from Sigma). The staining procedure was performed in the dark for 30 min on ice. 20,000 cells per sample were determined by using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). The proportion of cells in G0/G1, S and G2/M phases was analyzed by Modfit LT software (Becton Dickinson, San Jose, CA).

RT-qPCR

Total RNA was extracted by RNAiso Plus RNA extraction reagent (Takara, Dalian, China). Recombinant DNase I (Takara) was used to avoid DNA contamination. PrimeScript RT Master Mix and TB Green™ Premix Ex Taq™ II (both Takara) were utilized for cDNA synthesis and qPCR. The qPCR condition was 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and then 72 °C for 20 s. Relative expression of targeted genes was standardized to β-actin and calculated by the 2⁻ΔΔCt method.
Immunoblotting

Proteins were extracted by RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentration was detected by Enhanced BCA Protein Assay Kit (Beyotime). After separating by sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferring onto polyvinylidene fluoride membranes (Millipore, Bedford, MA), proteins were probed by primary antibodies at 4°C overnight. Subsequently, incubation with secondary antibodies for 1 h at room temperature, and the positive bands were developed by BeyoECL Plus kit (Beyotime). The intensity of bands was analyzed by Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD).

Chromatin-immunoprecipitation (ChIP)

3 × 10⁶ cells per sample were fixed with 1% formaldehyde at 37°C for 10 min. Glycine solution with a final concentration of 0.125 M was added to stop crosslinking. After washing with precooling PBS for twice, cells were lysed with SDS Lysis Buffer and sonicated in an ultrasonic bath (Bioruptor, Tosho Denki, Yokohama, Japan). The samples were then immunoprecipitated with anti-H1.4S36ph and anti-PKA antibodies overnight. The control immunoprecipitations were performed using anti-IgG antibody. 60 μL protein A-agarose/salmon sperm DNA beads (Millipore) were added into the sample. After washing with salt wash buffer as previously described [17], the DNA was eluted from the beads in 100 μL 10% SDS, 100 μL 1 M NaHCO₃, 800 μL ddH₂O. RT-qPCR was carried out to examine the enrichment of target mRNA.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was done by SPSS 20.0 software (IBM, New York, NY). Statistical difference was analyzed by ANOVA followed by Duncan post-hoc multiple comparisons. A p value of < .05 was considered as a significant result.

Results

H1.4S36 phosphorylation is regulated by Ras-ERK pathway

It is well-established that, Ras mutation is a switch in activation of ERK signalling. Our finding was consistent with this fact, as ERK1/2 phosphorylation was clearly promoted in NCI-H2126 cells after transfection with RasG13D/T35S expression vector (Figure 1(A)). In the meantime, H1.4S36ph was suppressed by transfection with RasG13D/T35S expression vector as relative to transfection with an empty pEGFP-N1 plasmid.
Same trends were observed in A549 cells, that ERK1/2 phosphorylation was induced while H1.4S36ph was repressed (p < .01) by transfection with RasG13D/T35S expression vector (Figure 1(D–F)). It seems that activation of Ras-ERK signalling is capable of reducing the H1.4S36ph.

**H1.4S36ph inhibits the phenotype of NSCLC cells**

Next, the effects of H1.4S36ph on NCI-H2126 and A549 cells phenotype were studied to evaluate the role of H1.4S36ph in the onset and progression of NSCLC. As results are shown in Figure 2(A,B), transfection of cells with RasG13D/T35S expression vector significantly increased OD-values in MTT assay, indicating Ras-ERK activation enhanced both NCI-H2126 and A549 cells viability. However, the enhanced viability was flattened by transfection with H1.4S36E (a mimicked plasmid for expressing H1.4S36ph) with a gradually increased amount (p < .05 or p < .01). Similar trends were observed in Figure 2(C,D), the enhanced colony number by Ras-ERK activation was attenuated by H1.4S36E (p < .01). Ras-ERK activation led to more cells arrest in S-phase, while transfection with H1.4S36E attenuated the arrest (Figure 2(E,F)). Also, the migrating and invasive capacities enhanced by Ras-ERK activation were weakened by H1.4S36E (p < .05 or p < .01, Figure 2(G–J)).
The effects of H1.4S36ph on the transcription of Ras downstream genes, including CYR61, IGFBP3, WNT16B, NT5E, GDF15 and CARD16, were further studied to decode how H1.4S36ph reversed Ras-ERK’s function. RT-qPCR data in Figure 3(A) displayed that, transfection of RasG13D/T35S expression vector significantly up-regulated CYR61 (p < .01), IGFBP3 (p < .01) and WNT16B (p < .01), whereas down-regulated NT5E (p < .01), GDF15 (p < .001) and CARD16 (p < .01) at mRNA level. The alterations of these genes made by Ras-ERK activation were all attenuated by transfection with H1.4S36E (p < .05 or p < .001). Besides, ChIP results in Figure 3(B) displayed that the enrichment of H1.4S36ph on the differentially expressed genes (*p < .05; **p < .01; ***p < .001).

H1.4S36 phosphorylation is mediated by PKA

Next, how H1.4S36 was phosphorylated in NCI-H2126 cells was studied. PKA was previously described as a kinase responsible for H1.4 phosphorylation [9]. In the current study, PKA in NCI-H2126 cells was overexpressed by transfection with a PKA expression vector (PKA-HA). As seen in Figure 4(A), PKA expression was overexpressed after transfection with PKA-HA as relative to transfection with an empty vector. Of note, transfection of cells with PKA-HA recovered H1.4S36ph expression even under Ras-ERK activated condition (Figure 4B), confirming the catalysis of PKA in H1.4S36 phosphorylation. We additionally found that PKA exhibited the same anti-tumour effects to H1.4S36ph. As compared to Ras+ pCMV group, cell viability, colony formation, S-phase cell rate, migration and invasion were all reduced in Ras + PKA group (Figure 4(C–G)).

Ras-ERK pathway represses H1.4S36 phosphorylation through degradation of PKA

Next, whether Ras-ERK pathway represses H1.4S36 phosphorylation through regulating PKA was tested. By transfection of cells with RasG13D/T35S expression vector, the mRNA level of PKA was unaffected (Figure 5(A)), whereas both endogenous (Figure 5(B)) and exogenous (Figure 5(C)) protein levels of PKA were clearly down-regulated as compared to transfection with pEGFP-N1. The result shows that Ras mutation is capable of degradation of PKA protein. Besides, PKA could regulate the transcription of Ras downstream genes. As results are shown in Figure 5(D), the enrichment of PKA was declined at these genomic loci (p < .05, p < .01 or p < .001). Subsequently, a proteasome inhibitor MG132 was utilized to treat cells and the expression of PKA protein was recovered even under Ras-ERK activated condition (Figure 5(E)), suggesting PKA degenerated in a proteasome-dependent way. Furthermore, the declined expression of H1.4S36ph, made by Ras mutation, was recovered by addition with MG132 (Figure 5(F,G)). Therefore, we preliminary conclude that Ras-ERK pathway represses H1.4S36 phosphorylation through degradation of PKA.

Ras-ERK pathway degrades PKA through modulation of MDM2

Further, how PKA was degraded by Ras-ERK signalling was studied. Considering MDM2 is a widely known protein in driving proteasome-mediated degradation [18], we focused on MDM2 for further investigation. Results in Figure 6(A,B) revealed that transfection of cells with an MDM2 expression vector (MDM2-His) remarkably repressed the endogenous and exogenous protein levels of PKA. However, such repressed PKA expression was not observed when MDM2 was mutated (Figure 6(C,D)). More interestingly, Ras-ERK activation induced a remarkably up-regulation of MDM2 (Figure 6(E)). The above results raised the question of whether the reduction in PKA levels following Ras-ERK activation is a consequence of an up-regulation of MDM2. To test the authenticity of the hypothesis, MDM2 expression in the cell was
silenced by siRNA transfection (Figure 6(F)). The silence of MDM2 recovered H1.4S36ph expression even under Ras-ERK activated condition (Figure 6(G)), suggesting Ras-ERK pathway degrades PKA through modulation of MDM2.

**Discussion**

Ras-ERK signalling has been well studied for about three decades [19]. Ras mutation and ERK activation frequently appear in the majority of human cancers, including NSCLC. Thus, scientists around the world struggle to study the contribution of Ras-ERK signalling in the onset and progression of human cancers. A small fraction of these scientists focused on the relationship between Ras-ERK signalling and histone modification and found that Ras exerts oncogenic functions possibly by regulating different histone modification patterns. For instance, Peláez et al. demonstrated that activation of MEK signalling through Ras mutation induced the proliferation and metastasis of colorectal cancer through regulating H3 three-methylation at lysine 27 (H3K27me(3)) [13]. Likewise, Yamada et al. reported that histone deacetylases (HDACs) are critical in regulating Ras-ERK signalling-driven NSCLC cells [14]. The present work demonstrated that Ras-ERK signalling repressed the phosphorylation of H1.4S36, a modification that is known to be implicated in regulating cell cycle progression [9]. We additionally found that H1.4S36ph functioned as a tumour suppressive factor, as its overexpression significantly repressed NCI-H2126 and A549 cells.
viability, colony formation, S-phase arrest, migration and invasion. Besides, H1.4S36ph was able to mediate the transcription of Ras downstream genes. These pieces of evidence suggested that Ras-ERK signalling promoted NSCLC cells growth, migration and invasion by repressing H1.4S36. Moreover, we revealed that Ras-ERK signalling repressed H1.4S36ph through MDM2-dependent degradation of PKA.

H1.4 is a highly abundant and widely expressed subtype of histones. Within the human H1.4 N-terminal tail, there are 10 serine residues that can be phosphorylated, including S2, S27, S36, S41, S55, S58, S102, S104, S172, S187. Among which, H1.4S36ph is the most commonly assigned by using proteomic discovery mass spectrometry. In general, histone serine phosphorylation is an important posttranslational modification involved in the cellular response to DNA damage [20]. For instance, histone H2AX phosphorylation was found to be a potential marker for DNA damage, as it can be generated during DNA replication [21]. Likewise, phosphorylation of histone H4T80 is able to trigger DNA damage checkpoint recovery [22]. Considering DNA damage is an initial event in the onset of human cancers [23], it is not surprising that H1.4S36ph exerts potent anti-tumour activities.

Figure 5. Ras-ERK pathway represses H1.4S36 phosphorylation through degradation of PKA. (A) NCI-H2126 cells were transfected with empty pEGFP-N1 vector or RasG13D/T35S. mRNA levels of PKA was tested by RT-qPCR. (B) Co-transfection of cells with RasG13D/T35S and pEGFP-N1, and then protein levels of PKA were examined by immunoblotting. (C) Transfection of cells with empty pEGFP-N1 vector or RasG13D/T35S, and then protein levels of PKA and H1.4S36ph were tested by immunoblotting. (D) ChIP was performed to see the enrichment of PKA on the differentially expressed genes. (E) MG132 was used, and the expression of PKA was examined by immunoblotting. (F) Expression of H1.4S36ph was examined by immunoblotting at 0-60 h following transfection. (G) MG132 was used, and the expression of H1.4S36ph was examined. \( p < 0.05; \quad \*\*\*\* p < 0.001.\)

CYP61, IGFBP3, WNT16B, NT5E, GDF15 and CARD16 are six tumour-associated genes and Ras downstream genes. In NSCLC, CYP61 was found to be down-regulated [24], and acted as a tumour suppressor [25]. The same as CYP61, IGFBP3 is also a tumour suppressor, as its overexpression induced apoptosis [26] and inhibited proliferation, migration and invasion of tumour cells [27,28]. Of contrast, WNT16B, NT5E, GDF15 and CARD16 are reported to be oncogenes in various human cancers [29–32]. In the present study, the transcription of these genes was regulated by H1.4S36ph, further linked the relationship between H1.4S36ph and Ras-driven NSCLC cells.

Histone phosphorylation is mediated by various catalytic enzymes. The phosphorylation of H1.4 was largely unknown, but a recent paper demonstrated that H1.4 phosphorylation at S36 was catalyzed by PKA [9]. This fact was also confirmed in this study, that PKA was observed to be a kinase for H1.4S36ph. Besides, PKA exerted similar anti-tumour functions as H1.4S36ph. It has been previously suggested that PKA is a key contributor in mitotic regulation and regulate numerous cellular responses, including cell proliferation, cell-cycle progression, metabolism and cell death [33,34]. Taken together, it seems that H1.4S36ph inhibited NSCLC cells
growth and migration by enhancing PKA activity. Moreover, overexpression of MDM2 led to PKA degradation. Ras-ERK signalling could degrade PKA by regulating MDM2, indicating Ras-ERK inhibited PKA in a proteasome-dependent manner. It is well-known that MDM2 plays a significant role in proteasome-mediated degradation [18]. However, whether MDM2 directly inhibited PKA function or by mediating other factors are still unclear, which needed to be further studied.

In short, our results demonstrated that Ras-ERK signalling repressed H1.4 phosphorylation at S36 to participate in NSCLC cells growth and migration. Ras-ERK signalling repressed H1.4S36ph through MDM2-dependent degradation of PKA (Figure 7). This study provides a novel explanation for Ras-ERK’s tumour-promoting function.

Disclosure statement
No potential conflict of interest was declared by the authors.

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