RETRACTED ARTICLE: K-Ras$^{G12V/Y40C}$-PI3K/AKT pathway regulates H1.4$^{S35ph}$ through PKA to promote the occurrence and development of osteosarcoma cancer

Jingzhe Zhang, Wanguo Liu, Hang Dong and Wenjun Wang

Department of Orthopedics, China-Japan Union Hospital of Jilin University, Changchun, China

ABSTRACT

Background: Osteosarcoma is prevalent in children and adolescents. H1.4 modification is involved in various types of cancers. Ras pathway is often activated in human cancers. Herein, we explored the effects of Ras pathway through H1.4$^{S35ph}$. Methods: Osteosarcoma cancer cell line MG-63 was transfected with Ras gene with G12V and Y40C site mutation. The phosphorylation of H1.4$^{S35}$ and AKT was detected by Western blot. Cell viability, cell colonies and migration were analyzed by MTT assay, soft-agar colony formation assay and Transwell assay, respectively. The expression of Ras pathway downstream factors and PKA was detected by qRT-PCR. The relationship between Ras and downstream factors was detected by ChIP. The cell cycle progression was measured by flow cytometry.

Results: Transfection with Ras$^{G12V/Y40C}$ decreased H1.4$^{S35ph}$ expression while switched on p-AKT$^{Ser473}$. Ras$^{G12V/Y40C}$ increased cell viability, colony numbers and migration while H1.4$^{S35E}$ (H1.4$^{S35ph}$ overexpression) led to the opposite results. The regulation of Ras$^{G12V/Y40C}$ and H1.4$^{S35E}$ on Ras downstream factors was contrary to each other. Results demonstrated a positive relationship between PKA with H1.4$^{S35ph}$ with Ras$^{G12V/Y40C}$ down-regulated both. However, PKA and MDM2 revealed negative regulation with Ras$^{G12V/Y40C}$ transfection up-regulated MDM2.

Conclusion: Ras$^{G12V/Y40C}$-PI3K/AKT signal pathway decreased H1.4$^{S35ph}$ through down-regulation of PKA while up-regulation of MDM2 in MG-63 cells.

HIGHLIGHTS

1. H1.4$^{S35ph}$ is regulated by K-Ras$^{G12V/Y40C}$-PI3K/AKT in MG-63 cells;
2. Overexpression of H1.4$^{S35ph}$ regulates MG-63 cell growth;
3. H1.4$^{S35ph}$ regulates Ras downstream factors;
4. K-Ras$^{G12V/Y40C}$-PI3K/AKT activity induces PKA degradation to down-regulate H1.4$^{S35ph}$;
5. K-Ras$^{G12V/Y40C}$-PI3K/AKT activity involves in PKA degradation via MDM2.

Introduction

Osteosarcoma, as one aggressive malignant, is prevalent in children and adolescents [1]. There are various treatment methods for osteosarcoma, such as preoperative chemotherapy, wide surgical excision, careful pathologic mapping of the resected tumor, and postoperative chemotherapy based upon the percentage of necrosis of the tumor [2,3]. Sadly, patients with high-grade osteosarcoma is characterized by metastasis normally have poor prognosis and outcomes [4]. This terrible situation made the need for improving understanding and looking for novel therapeutic methods for osteosarcoma becomes urgent.

Osteosarcoma phenotypes were influenced by different reasons. For example, the previous report demonstrated that cancer stem cell phenotypes are tightly closed with drug resistance and carcinogenesis [4]. Recently, epigenetics shed a light on understanding osteosarcoma causing reason and treatments [5]. Histone modification, as one of the most important epigenetics forms, has brought deep-going impact on the understanding of the molecular mechanisms that lead to various diseases, including osteosarcoma [6]. It is well-known that eukaryotic DNA is wrapped by histone octamers which were made of four different kinds of histones, H2A, H2B, H3 and H4, with a linker histone H1 [7]. There are multiple H1 variants, such as H1.2, H1.3, H1.4, and H1.5 which participated in the product of compact chromatin [8]. Among all these identified H1 subtypes, H1.4 is one of the most abundant informs. Most of the H1 variants contain a conserved hydrophobic globular domain and own less conserved N- and C-terminal tails compared to histones which involved in the chromatin building [9]. Increasing evidence suggested that dysregulation of H1.4 modification was involved in many types of cancers, such as breast cancer T47D-MTVL cells [10,11]. Moreover, H1.4 can go through
posttranslational modifications, including phosphorylation. For example, research from Chu et al. found that histone H1.4 was phosphorylated at Ser-35 by protein kinase A (PKA) during mitosis [12]. Therefore, we intended to investigate whether H1.4\(^{535}\)p was involved in osteosarcoma.

One of the most widely studied phosphatidylinositol 3’-kinase (PI3K)/protein kinase B (AKT) pathway is frequently activated and plays important roles of oncogenic in various human cancers [13]. The Ras oncogene product K-Ras exerts as a switch by cycling between GTP-bound active and GDP-bound inactive forms, by which to control cell proliferation and differentiation [14]. However, the exact epigenetic regulation mechanism about how Ras-PI3K/AKT functions in tumors is not well illuminated. Further, whether Ras-PI3K/AKT could influence the phosphorylation levels of H1.4\(^{535}\) and how their effects on cell viability, colony and migration in osteosarcoma cells would be unveiled in our study.

**Materials and methods**

**Cell culture and materials**

The osteosarcoma MG-63 cells (ATCC\(^{®}\) CRL-1427) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in the medium of ATCC-formulated Eagle’s Minimum Essential Medium (EMEM, Cat. No. 30–2003) supplemented with heat-inactivated fetal bovine serum (FBS, Life Science, UT, USA) at a concentration of 10%. Cells were maintained at 37°C with 5% CO\(_2\).

MG132 (Cat. No: M7449) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO). Then, MG132 was diluted into 25 μM and was added to the transfection cells and kept for different treatment time.

The antibodies used in this study were listed as follows. Anti-H1.4 (ab105522), anti-p-AKT\(^{Ser473}\) (ab81283), anti-AKT (ab8805), anti-HA (ab18181), anti-GFP (ab13970), anti-AKT (ab187515), anti-GAPDH (ab181602), anti-β-actin (ab227387), anti-His (ab213204), anti-MDM2 (ab38618), anti-CYR61 (ab228592), anti-IGFBP3 (ab76001), anti-NT5E (ab81720), anti-GDF15 (ab206414) and anti-CARD16 (ab168243) antibodies were obtained from Abcam (Cambridge, MA, USA). Antibodies specific for H1.4\(^{535}\)p (PA5-31907) and WNT16B (orb163331) were obtained from Invitrogen (Carlsbad, CA, USA) and Biorbyt (San Francisco, CA, USA), respectively.

**Plasmid construction and siRNA**

Empty-pEGFP-N1 vector, pEGFP-K-Ras\(^{WT}\), pEGFP-K-Ras\(^{G12V/Y40C}\) (with G12V and Y40C site mutation) plasmids were purchased from Clontech (Palo Alto, CA, USA). MG-63 cells were transfected with empty-pEGFP-N1 vector, pEGFP-K-Ras\(^{WT}\), pEGFP-K-Ras\(^{G12V/Y40C}\) plasmids. The coding region of human H1.4, MDM2-His and MDM2-MU were amplified from MG-63 cDNA by the method of RCR. Then, the specific gene products were screened out by using gene with HA-tag or his-tag through Western blot. pEGFP-K-Ras\(^{G12V/Y40C}\) plasmids were obtained by site-directed mutagenesis. siRNAs, which were using interference RNA to silence the goal RNA (Mouse double minute 2 homolog (MDM2)) and the plasmids with overexpression of PKA were obtained from Shanghai GenePharma (Shanghai, China). The pEGFP-H1.4\(^{535}\)p was constructed using the TaKaRa MutanBEST Kit (TaKaRa, Shiga, Japan), as recommended by the manufacturer.

**Transfection**

The MG-63 cells at the density of 5 × 10\(^5\) cells/well were cultured in 6-well plates for 12 h. Then, these cells were transfected with plasmids or siRNA using Lipofectamine 2000 (Invitrogen). Then qRT-PCR and/or western blot were used to determine the transfection efficiency after 48 h of transfection.

**Cell viability**

MTT (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich) was used for detecting cell viability. After cells were cultured for 48 h, 20 μL of 5 mg/mL MTT was administrated to each well, then cells were cultured for another 4 h at 37°C. Afterward, we used 100 μL DMSO (Sigma-Aldrich) to lyse formazan crystal and then read the value at 570 nm by a multiwell spectrophotometer (Emax, Molecular Devices, Sunnyvale, CA, USA).

**Reverse transcription polymerase chain reaction (RT-PCR)**

RT-PCR was referred to what was described in the literature [15]. Total RNA was obtained and isolated from MG-63 cells using Trizol (Invitrogen) reagent. DNase-I-treated total RNA was supplied for first-strand cDNA synthesis by M-MuLV reverse transcriptase (Fermentas, York, UK) and oligo-dT primers (Invitrogen). QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used to amplify the target sequence. GAPDH was used as an internal control for detecting RNA expression based on triplicate experiments.

**Soft-agar colony formation assay**

Soft agar assay was performed for measuring the cell colony formation ability [16]. These cells firstly suspended in full culture medium with 0.35% low-melting agarose, then cells transferred into solidified 0.6% agarose in six-well culture plates (103 cells/well). The number of the colonies was checked three weeks later using microscopically (40×).

**Transwell migration assay**

Cell migration was evaluated by using a modified two-chamber migration Transwell (Costar-Corning, NY, USA) with a pore size of 8 μm. Cell suspension 100 μL (around 2 × 10\(^5\) cells/mL) without serum was added to upper Transwell. Then 600 μL culture medium with 10% FBS was added in the lower compartment of 24-well Transwell. MG-63 cells were maintained for 24 h at 37°C with humidified air with 5% CO\(_2\).
The cells that migrated into the lower transwell were fixed with methanol, stained with 0.5% crystal violet, and microscopically inspected. Cells at the upper surface of the filter were removed by a cotton swab. The after cells had been washed with acetic acid, OD value at 570 nm was measured.

**Zymography assay**

The proteolytic activity of MMP-2 and MMP-9 in the culture medium was measured by gelatin zymography assay. MMP Zymography assay kit (Applygen, Beijing, China) was utilized in this process. The proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Western blot**

Western blot was used in our study to detect the all proteins expression. Protein was obtained from MG-63 cells using radio-immunoprecipitation assay (RIPA) lysis buffer (Cat. No: R0010, Solarbio, Beijing, China) supplemented with protease inhibitors (Thermo Fisher Scientific, Rockford, IL, USA). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used for determining proteins concentrations. The western blot system was established using a Bio-Rad Bis-Tris Gel system following the manufacturer’s instructions. Primary antibodies were prepared in 5% blocking buffer and diluted according to the product instruction. These primary antibodies were incubated in the membrane and maintained at 4°C overnight at the recommended concentration. Then, for second antibody incubation, incubated with horseradish peroxidase (HRP) conjugated secondary antibody. Detection was performed by capturing the signals and analyzing the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

**Flow cytometric analysis of cell cycle distribution**

MG-63 cells were cultured until cell liquid reaches 75–80% confluence, then cells were washed by PBS to remove the non-adherent cells. Collected cells were all adherent cells and then fixed with cold 70% ethanol. Then, cells were washed with PBS again and after that, stained cells with 4, 6-diamidino-2-phenylindole (DAPI) (Partec, Münster, Germany) and keep the cells in the darkness for 30 min. Flow cytometry was used for detecting cell cycle distribution. The percentage of cells in different cell cycle stages was calculated.

**Chromatin immunoprecipitation (ChiP)**

MG-63 cells were cultured and transfected, then cells were fixed in 1% formaldehyde, lysed, and sonicated. Then 5 mg chromatin was immunoprecipitated with Dynabeads. After that, purified DNA was used for PCR amplification at the CYR61, IGF835, WNT16B, NTSE, GDF15, CARD16 promoter. The detailed process could refer to the literature [17].

**Statistical analysis**

All the data were analyzed by Graphpad 6.0 statistical software (GraphPad, San Diego, CA, USA). Data were present as mean± standard deviation (SD). The statistical analyses were performed using the one-way ANOVA followed by Duncan post-hoc multiple comparisons. p Values of <.05 was considered significant.

**Results**

**H1.4**

H1.4 was negatively regulated by RasG12V/Y40C

Overwhelming evidence suggested that PI3K/AKT pathway was closely involved in osteosarcoma [18,19]. We constructed empty vector pEGFP-N1, RasWT, and RasG12V/Y40C plasmids and transfected them into MG-63 cells. Results showed that transfection with RasG12V/Y40C decreased the phosphorylation level of H1.4 (p < .01, Figure 1(A)). Then, the result of Western blot validated that transfection with RasG12V/Y40C could activate PI3K/AKT pathway (Figure 1(B)). Hence, we can conclude that RasG12V/Y40C regulated H1.4 and acted as a switch on PI3K/AKT pathways.

**H1.4**

H1.4 was involved in regulation of Ras pathway in osteosarcoma cancer cell phenotype

Increasing studies pointed out that histone modification play an important roles in cancer cell incidence and development [20].

![Figure 1.](image-url) H1.4 was negatively regulated by RasG12V/Y40C-PI3K/AKT pathway. (A) The H1.4 levels and (B) the phosphorylation of AKT were measured using Western blot under different groups by transfection with empty-pEGFP-N1 (pEGFP-N1), pEGFP-K-RasWT (RasWT), pEGFP-K-RasG12V/Y40C (RasG12V/Y40C) plasmids in MG-63 cells. Data presented as mean± standard deviation (SD) of three triplets experiments, **p < .01.
In order to identify the functions of histone modification of H1.4S35ph, we constructed H1.4S35E plasmids to induce the phosphorylation of H1.4S35. Simultaneously, we co-transfected the mimicked H1.4S35E plasmids and RasG12V/Y40C plasmids into MG-63 cells. Thereafter, we detected cell viability, colony ability and migration. Interestingly, results showed that transfection with RasG12V/Y40C significantly enhanced cell viability (p<.001) while co-transfection with RasG12V/Y40C and H1.4S35E significantly decreased the cell viability in a dose-dependent manner (p<.01, Figure 2(A)). Since cell viability decreased the most after transfection with H1.4S35E plasmids at the amount of 2μg, 2μg of H1.4S35E was chosen in the subsequent experiments. Similarly, transfection with RasG12V/Y40C significantly enhanced colony number (p<.01, Figure 2(B)) and cell migration (p<.01, Figure 2(C)) compared with transfection with empty vector. On the other side, co-transfection with RasG12V/Y40C and H1.4S35E reduced colony numbers (p<.05, Figure 2(B)) and migration (p<.05, Figure 2(C)) significantly. Consistently, the proteolytic activity of MMP-2 and MMP-9 was enhanced by transfection with RasG12V/Y40C while was reduced to the basic level by co-transfection with RasG12V/Y40C and H1.4S35E (Figure 2(D)). Altogether, results demonstrated that RasG12V/Y40C promoted cancer cell development while H1.4S35E inhibited cell phenotype.

**H1.4S35ph participated in the regulation of the downstream factors of Ras pathway**

Ras-PI3K/AKT pathway was complex regulation progress which can regulate several downstream factors [21]. In order to investigate more about the underlying mechanism about how Ras pathway achieved their functions, we explored several downstream factors CYR61 [22], IGFBP3 [23], WNT16B [24], NT5E [25], GDF15 [26], CARD16 [27], which participated tumor cell growth and metastasis. Then, we observed that RasG12V/Y40C pathway up-regulated the expression of CYR61 (p<.01), IGFBP3 (p<.01), WNT16B (p<.01) and decreased the expression of NT5E (p<.01), GDF15 (p<.01), GARD16 (p<.01, Figure 3(A)). In contrary, co-transfection with RasG12V/Y40C and H1.4S35E led to the opposite results, which indicated that H1.4S35ph might play crucial roles in the regulation of these downstream factors. The same trend was observed in the protein levels of these factors, which was tested by Western blot analysis (Figure 3(B)). Furthermore, ChIP assay results demonstrated that transfection with RasG12V/Y40C significantly down-regulated H1.4S35ph level at these genomic loci (p<.05 or p<.01, Figure 3(C)), which indicated that down-regulation of H1.4S35ph was reduced at the promoters of these genes as along with activation of PI3K/AKT signaling. Therefore, we inferred that the Ras-PI3K/AKT downstream factors were influenced by phosphorylation of H1.4S35.
Overexpression of PKA regulated H1.4S35ph expression and decreased osteosarcoma cell growth

PKA was an important factor in the process of H1.4 phosphorylation during mitosis [12]. PKA overexpression was achieved by transfection MG-63 cells with PKA. qRT-PCR and Western blot results demonstrated that transfection with PKA-HA markedly up-regulated the expression of PKA (Figure 4(A,B)). Then, the effects of PKA overexpression on cell viability, migration and cell cycle were studied. We found that co-transfection with RasG12V/Y40C and a pCMV increased cell viability (p<.001, Figure 4(C)), migration (p<.001, Figure 4(D)) and cell percentage in S stage (Figure 4(E)). However, the promoting effects were reversed by co-transfection with RasG12V/Y40C and PKA as evidenced by decreasing cell viability (1lgl, p<.01, Figure 4(C)), migration (1lgl, p<.01, Figure 4(D)) and scaled down cell percentage in S stage (Figure 4(E)). Above all, these results demonstrated that overexpression of PKA could enhance H1.4S35ph and inhibit MG-63 cell growth.

Activation of Ras-PI3K/AKT induced PKA degradation to decrease H1.4S35ph expression

To identify the underlying mechanism of how PKA influences the phosphorylation of H1.4S35, RT-PCR and Western blot were performed to verify in which step PKA expression was altered. Results showed that transfection with RasG12V/Y40C only influences the expression of PKA in protein level (Figure 5(B)) but not in RNA level (Figure 5(A)). Therefore, we inferred that RasG12V/Y40C affected the expression of PKA at the translation level. Further experiment confirmed our inference that the influence of RasG12V/Y40C on PKA and H1.4S35ph revealed the similar changing trend (Figure 5(C)). ChIP assay results demonstrated that the recruitment of PKA to the genes was repressed when cells were transfected with RasG12V/Y40C (p<.05 or p<.01, Figure 5(D)). To validate this possible mechanism, the proteasome inhibitor MG132 was added and we found that MG132 administration impaired the inhibition effects induced by transfection with RasG12V/Y40C (Figure 5(E)). Then, we detected the phosphorylation of H1.4S35 in a time-dependent manner without MG132. Western blot results showed that the longer the transfection time (24, 48, 51, 54 and 60 h), the lower expression level of H1.4S35ph was found (Figure 5(F)). Then, further experiments were performed to determine precisely the effects of MG132 on the expression of H1.4S35ph. Results showed that the phosphorylation of H1.4S35 was enhanced with the increasing treatment time of MG132 (0, 3, 6 and 12 h) (Figure 5(G)). Altogether, these studies suggested that RasG12V/Y40C pathway affected the phosphorylation level of H1.4S35 was through PKA.

Ras-PI3K/AKT activation degraded PKA via MDM2

MDM2 is an important factor which plays crucial roles in histone modification in many different cells [28]. The next experiment was designed to explore whether MDM2
participated in the regulation of PKA in H1.4S35ph. We found that the following co-transfection with RasG12V/Y40C and PKA with tagged HA, the expression level of PKA was downregulated with the increasing level of MDM2 (Figure 6(A)). Then no transfection with PKA, results showed that the endogenous expression of PKA demonstrated downregulating trend with the increasing level of MDM2 (Figure 6(B)), which indicated that there might exist negative regulation between PKA and MDM2. Afterward, we found that co-transfection with RasG12V/Y40C and PKA with tagged HA, MDM2 downregulated PKA expression while MDM2 mutant failed to downregulate PKA (Figure 6(C)), which suggested that it was MDM2 that could decrease PKA. Removed transfection with PKA, similar results were shown in Figure 6(D), which strengthened the conclusion in Figure 6(C). Next, we directly detected the effects of transfection with RasG12V/Y40C on the expression of MDM2 and H1.4S35ph. Upregulation of MDM2 and downregulation of PKA was simultaneously found by transfection with RasG12V/Y40C (Figure 6(E)). Next, we knockdown of MDM2 (Figure 6(F)), and then determined the effects of co-transfection with RasG12V/Y40C and si-MDM2 (Figure 6(G)) on the expression of H1.4S35ph. We found that MDM2 silence upregulated the expression of H1.4S35ph which indicated that Ras-PI3K/AKT might downregulate H1.4S35ph through regulating MDM2.

Discussion

Overwhelming evidence validated the importance of epigenetic modifications in human cancers, including osteosarcomas [29]. Histone modification is one of the most important forms of epigenetic modifications [30]. The post-translational modifications of histones have an impact on most of the DNA-dependent processes, such as replication, transcription and repair. Simultaneously, the researches on modification of core histones are much more than the linker histone H1 [31]. On the other side, because of Ras-PI3K/AKT might play important roles in osteosarcoma, our study initially investigated the
possible regulatory relationship between K-Ras and PI3K/AKT and the phosphorylation of H1.4\(^{S35}\) in osteosarcomas cells. H1.4 is one common H1 variant, and H1.4\(^{S35}\) was N-terminal phosphorylation of H1.4 at serine 35 by PKA [12]. In the current study, K-Ras\(_{G12V/Y40C}\) plasmids were obtained by site-directed mutagenesis and results showed that K-Ras\(_{G12V/Y40C}\) decreased the expression of H1.4\(^{S35}\), which hinted us that K-Ras might achieve its functions via regulation of H1.4\(^{S35}\). In addition, further results demonstrated that K-Ras was a switch of PI3K/AKT, which stand the same line with the previous study that Ras was a switch of PI3K/AKT [14]. Through this cascade reaction, K-Ras\(_{G12V/Y40C}\)-PI3K/AKT-H1.4\(^{S35}\), we might
draw a big picture of Ras-PI3K/AKT and H1.4S35ph: Ras-PI3K/AKT downregulated the phosphorylation of H1.4S35ph.

Considering that Ras-PI3K/AKT was often activated in human cancers exerting carcinogenesis effects, we next explored the effects of Ras-PI3K/AKT on cell viability, colony ability and migration [13]. Results demonstrated that RasG12V/Y40C-PI3K/AKT activated cell viability, colonies ability and migration in MG-63 cells, which was consistent with the previous study that hyper-activation of PI3K/AKT promoted disease development, including the progression of tumorigenesis, proliferation, invasion, cell cycle progression in osteosarcoma [32]. On the other side, we found that co-transfection with RasG12V/Y40C with H1.4S35ph impaired the promoting effects of RasG12V/Y40C-PI3K/AKT, which suggested that overexpression of H1.4S35ph inhibited osteosarcoma development. This is the first time that the effects of H1.4S35ph on cancer cell were investigated.

There are several downstream factors of RasG12V/Y40C-PI3K/AKT regulating cell growth or metastasis. CYR61, IGFBP3,
WNT16B, NT5E, GDF15 and CARD16 are all important downstream factors which are involved in the regulation process of Ras pathways. Interestingly, we found that RasG12V/Y40C-PI3K/akt demonstrated the contrary results compared with RasG12V/Y40C-PI3K/AKT with H1.4S35Ph. This changing trend spoke the same words with what we obtained in the results before, which all pointed out that overexpression of H1.4S35Ph will block the effects induced by RasG12V/Y40C-PI3K/akt. CHIP assay results suggested that RasG12V/Y40C-PI3K/AKT could bind to the promoter of these downstream genes.

Former study proved that phosphorylation of H1.4S35 was achieved by PKA during mitosis [12]. Therefore, we further investigated how PKA regulated the expression of H1.4S35Ph. The results of overexpression of PKA correspond to the higher accumulated level of H1.4S35Ph which suggested that there might be a positive relationship between PKA and H1.4S35Ph. The following experiments were performed to determine our inference. As expected, we found that overexpression of PKA significantly decreased cell viability, migration and the percentage of S cell stage. These findings indicated that the role of H1.4S35Ph might through regulating PKA.

What is the relationship between PKA and H1.4S35Ph? The next experiment was done to unveil this question. RasG12V/Y40C does not influence PKA level in RNA form but in protein form, which inferred that the effects of PKA might be exert after transcription. Transfection with RasG12V/Y40C has a similar influence trend on PKA and H1.4S35Ph. After transfection with RasG12V/Y40C without MG132 administration, the expression of H1.4S35Ph was inhibited in a time-dependent manner. Then after added MG132 which maintained a high level of PKA before, the accumulated level of H1.4S35Ph also increased in a time-dependent manner in MG-63 cells. Altogether, results showed that RasG12V/Y40C decreased the expression of H1.4S35Ph through down-regulation of PKA.

Then, the next question is by which PKA was regulated in this whole progression? DMD2 was reported to be involved in H1 modification and also plays important roles in various tumors [33]. We asked whether MDM2 participated in the process of PKA on H1.4S35Ph. Firstly, we found that with the increasing level of MDM2, the accumulated level of PKA was down-regulated, which indicated that there might be a negative relationship between MDM2 and PKA. Then, transfection with RasG12V/Y40C inhibited the expression of H1.4S35Ph while enhanced the expression of MDM2, which revealed that RasG12V/Y40C inhibited the expression of H1.4S35Ph through upregulation of MDM2. Next, we confirmed this conclusion cause that silence of MDM2 enhanced the expression of H1.4S35Ph. Above all, the cascade might be MDM2 negatively regulated PKA, PKA positively regulated H1.4S35Ph, which was negatively modulated by RasG12V/Y40C-PI3K/AKT. This result stands by the conclusion that MDM2 was upregulated in osteosarcoma [34]. Our result provides an explanation to connect RasG12V/Y40C-PI3K/AKT with MDM2 and further with H1.4S35Ph.

The schematic diagram describing the contents of this study was provided in Figure 7. This study was performed in MG-63 cells since this cell line is a suitable transfection host. However, it is well-known that there are no functional p53 in MG-63 cells and MDM2 is the transcriptional target of p53 [35,36]. So, further studies are still needed to investigate whether the revealed regulation can be reproduced in other types of osteosarcoma cells.

In one word, our study unveiled the underlying mechanism of how RasG12V/Y40C-PI3K/AKT regulated the expression of H1.4S35Ph in MG-63 cells. Through this study, we understand how histone H1 modification could influence in the development of osteosarcoma cells and also it provided a network for connecting the vital pathway RasG12V/Y40C-PI3K/AKT with histone modification in osteosarcoma cells.

Acknowledgement

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Disclosure statement

Authors declare that there are no conflict of interests.

References

[1] Luetke A, Meyers PA, Lewis I, et al. Osteosarcoma treatment – where do we stand? A state of the art review. Cancer Treat Rev. 2014;40:523–532.
[2] Moore DD, Luu HH. Osteosarcoma. Cancer Treat Res. 2014;162:65–92.
[3] Yang Z, Li X, Han W, et al. Galangin suppresses human osteosarcoma cells: an exploration of its underlying mechanism. Oncol Rep. 2017;37:435–441.
[4] Brown HK, Tellez-Gabriel M, Heymann D. Cancer stem cells in osteosarcoma. Bull Cancer. 2011;98:E62–68.
[5] Esteller M. Cancer epigenomics: DNA methylation and histone-modification maps. Nat Rev Genet. 2007;8:286–298.
[6] Dmochowska A, Ekkawi K. Histone modification patterns and epigenetic codes. Biochimica et Biophysica Acta. 2009;1790:863–868.
[7] Christophorou MA, Castelo-Branco G, Halley-Stott RP, et al. Citrullination regulates pluripotency and histone H1 binding to chromatin. Nature. 2014;507:104–108.
[8] Lawrence MDB, Coutin N, Choi JK, et al. Histone acetylation, not stoichiometry, regulates linker histone binding in Saccharomyces cerevisiae. Genetics. 2017;207:347–355.
[9] Scaffidi P. Histone H1 alterations in cancer. Biochim Biophys Acta. 2016;1859:533–539.
[10] Izquierdo-Bouldstridge A, Bustillos A, Bonet-Costa C, et al. Histone H1 depletion triggers an interferon response in cancer cells via activation of heterochromatin repeats. Nucleic Acids Res. 2017;45:11622–11642.
[12] Chu CS, Hsu PH, Lo PW, et al. Protein kinase A-mediated serine 35 phosphorylation dissociates histone H1.4 from mitotic chromo-

[13] Aoki M, Fujishita T. Oncogenic roles of the PI3K/AKT/mTOR Axis. Curr Top Microbiol Immunol. 2017;407:153–189.

[14] Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008;9:517–531.

[15] Bustin SA. Quantification of mRNA using real-time reverse trans-

[16] Horibata S, Vo TV, Subramanian V, et al. Utilization of the soft agar colony formation assay to identify inhibitors of tumorigen-

[17] Schulz S, Haussler S. Chromatin immunoprecipitation for ChIP-

[18] Dong Y, Liang G, Yuan B, et al. MALAT1 promotes the prolifer-

[19] Zhao G, Cai C, Yang T, et al. MicroRNA-221 induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma. PloS One. 2015;10:e022790.

[20] Wang R, Xin M, Li Y, et al. The functions of histone modification enzymes in cancer. Curr Biol. 2014;14:23–45.

[21] Harada H, Omi M, Sato T, et al. Pea3 determines the isthmus region at the downstream of Fgf8-Ras-ERK signaling pathway. Develop Growth Differ. 2015;57:657–666.

[22] Xu W, Chou CL, Israel DD, et al. PGF(2alpha) stimulates FP prostano
dioid receptor mediated crosstalk between Ras/Raf signaling and Tcf transcriptional activation. Biochem Biophys Res Commun. 2009;381:625–629.

[23] Guo L, Costanzo-Garvey DL, Smith DR, et al. Cell non-autonomous regulation of hepatic IGF-1 and neonatal growth by kinase suppressor of Ras 2 (KSR2). Sci Rep. 2016;6:32093.

[24] Johnson LM, Price DK, Figg WD. Treatment-induced secretion of WNT16B promotes tumor growth and acquired resistance to chemotherapy: implications for potential use of inhibitors in cancer treatment. Cancer Biol Ther. 2013;14:90–91.

[25] Wang N, Xiang X, Chen K, et al. Targeting of NTSE by miR-30b and miR-340 attenuates proliferation, invasion and migration of gallbladder carcinoma. Biochimie. 2018;145:58–67.

[26] Li C, Wang J, Kong J, et al. GDF15 promotes EMT and metastasis in colorectal cancer. Oncotarget. 2016;8:860–872.

[27] Karasawa T, Kawashima A, Usui F, et al. Oligomerized CARD16 promotes caspase-1 assembly and IL-1β processing. FEBS Open Bio. 2015;5:348–356.

[28] Minsky N, Oren M. The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression. Mol Cell. 2004;16: 631–639.

[29] Rao-Bindal K, Kleinerman ES. Epigenetic regulation of apoptosis and cell cycle in osteosarcoma. Sarcoma. 2011;2011:679457.

[30] Kimura H. Histone modifications for human epigenome analysis. J Hum Genet. 2013;58:439–445.

[31] Tessarz P, Kouzarides T. Histone core modifications regulating nuclease structure and dynamics. Nat Rev Mol Cell Biol. 2014; 15:703–708.

[32] Zhang J, Yu XH, Yan YG, et al. PI3K/Akt signaling in osteosarcoma. Clinica Chimica Acta Int J Clin Chem. 2015;441:182–192.

[33] Zhang K, Dent SY. Histone modifying enzymes and cancer: going beyond histones. J Cell Biochem. 2005;96:1137–1148.

[34] Senturk JC, Bohlman S, Manfredi JJ. Mdm2 selectively suppresses DNA damage arising from inhibition of topoisomerase II inde-
pendent of p53. Oncogene. 2017;36:6085–6096.

[35] Wang Y, Wei Y, Zhang H, et al. Arsenic trioxide induces apoptosis of p53 null osteosarcoma MG63 cells through the inhibition of catalase. Med Oncol. 2012;29:1328–1334.

[36] Feeley KP, Adams CM, Mitra R, et al. Mdm2 is required for survival and growth of p53-deficient cancer cells. Cancer Res. 2017;77: 3823–3833.