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The PCAF/WSTF/MOF Complex Regulates H3K9ac and H4K16ac in Breast Cancer Cells

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Abstract In order to study WSTF (Williams syndrome transcription factor) regulation mechanism involved in Ras signal related to breast cancer cells. Western blot was used to detect WSTF phosphorylation and histone modification levels. GST pull-down was conducted to testify the interaction between WSTF, PCAF and MOF. Acetyltransferase activity of PCAF and MOF was tested via HAT activity assay. ChIP and Real-time PCR were applied to confirm gene expression. In vivo tumor growth analysis was used to test tumor formation capability. Results revealed an interaction between WSTF, PCAF and MOF. WSTF phosphorylation increased following Ras activation with enhancement of the association between WSTF and PCAF while the association between WSTF and MOF was attenuated. The changes resulted in an increase of PCAF activity and decrease of MOF, and upregulation of H3K9ac and downregulation of H4K16ac followed by gene expression changes and enhancement of tumor formation. In conclusion, WSTF involved in regulation of PCAF and MOF, meanwhile, tumor formation was affected as a consequence of changes of H3K9ac, H4K16ac and tumor related gene expression.

Keywords: *breast cancer, WSTF, MOF, PCAF*

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1. Introduction

Histone modifications are altered by abnormal signaling pathways in tumor cells [1,2]. Such issues have been reported in our previous studies, such as the Ras-PI3K pathway-specific regulation of acetylation of histone H3 lysine residue 56 (H3K56ac) in breast cancer cells [3,4]. These histone modifications that are altered by abnormal regulation in tumor cells can affect the expression of tumor-related genes by regulating the state of chromatin, thereby regulating the biological characteristics of tumor cells such as proliferation, migration and tumorigenicity, and ultimately affecting the occurrence and development of tumors [5,6,7]. Although these problems have been widely concerned by researchers, histone modification is a process of coordinated regulation, and many modifications in tumor cells often change the same or opposite at the same time [8,9,10]. Therefore, only one site modification is detected in the process of disease diagnosis and treatment, and its effect is limited [11,12]. We need to analyze the modification changes of multiple interrelated sites for specific types of tumors in order to more effectively apply the research results to clinical practice.

Williams syndrome transcription factor (WSTF) is a multifunctional protein closely related to the pathogenesis and clinical symptoms of Williams syndrome [13,14]. It is mainly involved in chromatin remodeling, transcription and translation [15]. However, it is not clear whether WSTF is related to other diseases except Williams syndrome.

In this report, we found that WSTF interacts with both the acetyltransferases MOF and PCAF, as measured in cells and mouse samples. After being regulated by Ras-MAPK signaling, the phosphorylation level of WSTF is altered, and the interaction with MOF and PCAF is also altered, thereby affecting H4K16ac and H3K9ac levels, downstream gene expression, and tumorigenicity of cells.

2. Materials and Methods

2.1. Cell Lines

Human MCF-7 cells cells were obtained from the Cell Culture Center of Peking Union Medical College. The

cells were maintained in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% Penicillin/Streptomycin (Solarbio, China) at 37°C with 5% CO_2. All animal studies were conducted with a North China University of Science and Technology Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals.

2.2. Antibodies

Antibodies against H3 (Abcam, USA, ab1791, 1:1000), Anti-Histone H4 (acetyl K16) (Abcam, USA, ab109463, 1:1000), Anti-Histone H4 (Abcam, USA, ab61255, 1:500), H3K9ac, MOF (Abcam, USA, ab72056), PCAF (Abcam, USA, ab176316, 1:1000) were purchased from Abcam (Shanghai, China). PV-9000 Polymer Detection System for Immuno-Histological Staining was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China).

2.3. Transfection of Gene Expression Plasmids

The expression plasmids of *H-Ras*, *WSTF*, *PCAF* and *MOF* were constructed by our laboratory, and the template was HeLa cell cDNA. PCR products were cloned into HA, Flag or EGFP tagged plasmids, respectively.

MCF-7 cells were seeded in 6-well plates (2×10^5/well) and incubated overnight. The cells at 50% confluence were transfected with plasmids using the Lipofectamine ^{2000} transfection reagent (Invitrogen, USA) and incubated. Forty-eight hours post-transfection, the cells were collected. Overexpression and silencing efficiency were tested by western blot and/or real time PCR. The experiments were repeated 3 times.

2.4. Chromatin IP, ChIP

The experimental procedure was performed according to the instructions of Chromatin IP Assay Kit (Upstate) [16]. The main steps were as follows: formaldehyde was added to the culture dish that grew an appropriate number of cells to cross-link the intracellular substances. After adding the lysis buffer, the DNA was ultrasonically broken to 300-500 bp fragments, and then ChIP Dilution and Protein A / G beads were added and mixed. After the control was retained, specific antibodies were added to the experimental group and mixed at 4°C for 6 h. After washing with buffer solution, decrosslinked at 65 °C and extracted DNA for PCR.

2.5. Reverse Transcription and Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was performed as described previously [17]. Total RNA from cultured cells (include si-Notch1, NC and Mock cell lines) was isolated using TRIzol® LS Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) from each sample was used as a template to produce cDNA with PrimeScript First-strand cDNA Synthesis kit (Takara). Notch1 mRNA levels were analyzed by quantitative real-time PCR (qPCR) with an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). All PCR reactions were finished as follows: initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 62°C for 40 sec, annealing at 94°C for 1 min and extension at 60°C for 3 min.

2.6. Western Blot

Total cell lysates were obtained from MCF-7 cells RIPA buffer (Beyotime Institute of Biotechnology P0013B, Haimen, Jiangsu, China). Protein concentrations in the samples were determined by the BCA protein assay kit (Pierce, Rockford, IL USA). Cell lysate was loaded and run on a 10% SDS-PAGE, and the protein was transferred to a PVDF membrane (Millipore, Billerica, MA, USA) using the BioRad Semi-dry transfer system (BioRad, Hercules, CA, USA). The membrane was incubated with the primary antibody followed by the secondary alkaline phosphatase-conjugated the goat anti-rabbit (ZB-2301 dilution 1:5000, Zhongshan Golden Bridge, China) and goat anti-mouse antibodies (ZB-2305 dilution 1:5000, Zhongshan Golden Bridge, China). Dilution was performed 1000-fold for all of the antibodies. Protein expression levels in patient samples were examined by Western blot and quantified with ImageJ software. Protein expression levels in tumor samples were normalized to those of paired normal tissues ($P < 0.01$, Pearson's chisquare test).

2.7. Co-immunoprecipitation (Co-IP) and *In vitro* HAT Activity Assay

Co-IP was performed as described previously [18]. Briefly, cells were suspended with buffer and fragmented by sonication. Then, the cell lysates were reacted with normal IgG or different antibodies. The complex reacted with Protein A/G agarose beads. Next, the beads were washed with buffer and the deposited proteins were freed by boiling. *In vitro* HAT activity assay: The mixture containing MOF or PCAF and wild-type or mutant WSTF obtained by Co-IP reacted with nucleosome and acetyl coenzyme A in the presence of HAT detection buffer at 30°C for 0.5 h, and then acetylated protein was detected by Western Blot.

2.8. *In vivo* Tumorigenesis Assay in Mice

In vivo tumorigenesis assay in mice was performed as described previously [18]. Fifteen female BALB/C nude mice in a SPF grade aged 6 weeks (body weight 18-20 g,) were randomly divided into three groups (pEGFP-N1 + Flag-WSTF group, $Ras^{G12V/T35S}$++$Flag\text{-}WSTF^{WT}$ group and $Ras^{G12V/T35S}$++$Flag\text{-}WSTF^{S158A}$ group). Subconfluent monolayer cells were used, they were detached from the bottom of the flask with trypsin and suspended in sterile PBS with density of (1-2) $\times10^8$/ml. The mice in three groups received injection of MCF7 cell, and MCF7 cell stably expressing pEGFP-N1+Flag-WSTF, $Ras^{G12V/T35S}$++$Flag\text{-}WSTF^{WT}$ and $Ras^{G12V/T35S}$++$Flag\text{-}WSTF^{S158A}$ stable expressing MCF7 cells respectively. After injection, the mice were marked with picric acid on different location of the body, and then continued to be

fed at SPF level. General conditions of nude mice were recorded every day. Subcutaneous tumor: 0.2 mL of each suspension was subcutaneously injected into the right breastpad of the nude mice. The subcutaneous tumor formation, dynamic observation tumor size and weight of nude mice were observed. Long and perpendicular dimension (a, b) of the tumor were measured by using a caliper, the volume of the tumor was calculated according to the formula: $v = a \times b^2/2$. After 5 weeks, mice were killed by cervical dislocation.

3. Results

3.1. Ras-MAPK (Mitogen-activated protein Kinase) Pathway Regulates H3K9ac and H4K16ac

Human breast cancer cells MCF7 transfected with p-EGFP-N1-Ras$^{G12V/T35S}$ plasmid activated the Ras-MAPK pathway [19,20], and the activity was shown by the level of phosphorylated ERK1/2 (P-ERK1/2). 48 h after transfection, the total protein sample was detected by Western blot, and it was found that the phosphorylation of serine residue at 158th position of WSTF (P-WSTF^{S158}) was up-regulated, and H3K9ac was up-regulated, while H4K16ac was down-regulated, which could be inhibited by MAPK pathway-specific inhibitor AZD6244 ($P<0.05$) (Figure 1).

3.2. The WSTF Interacts with both PCAF and MOF

It has been reported that WSTF interacts with PCAF to regulate H3K9ac. We verified this interaction by GST pull-down experiment and detected that WSTF can also bind to MOF ($P<0.05$) (Figure 2).

3.3. WSTF Phosphorylation Levels Regulate Intermolecular Interactions and Histone Acetylase Activity

WSTF$^{-/-}$ MCF7 cells were transfected with wild type (WT), simulated dephosphorylation modification (S158A) and simulated phosphorylation modification (S158E) mutant plasmids. 48 h later, it was found that phosphorylation at 158 site of WSTF promoted its binding to PCAF and the acetylase activity of PCAF. Dephosphorylation at 158 site of WSTF promoted its interaction with MOF and the acetylase activity of MOF ($P<0.05$) (Figure 3).

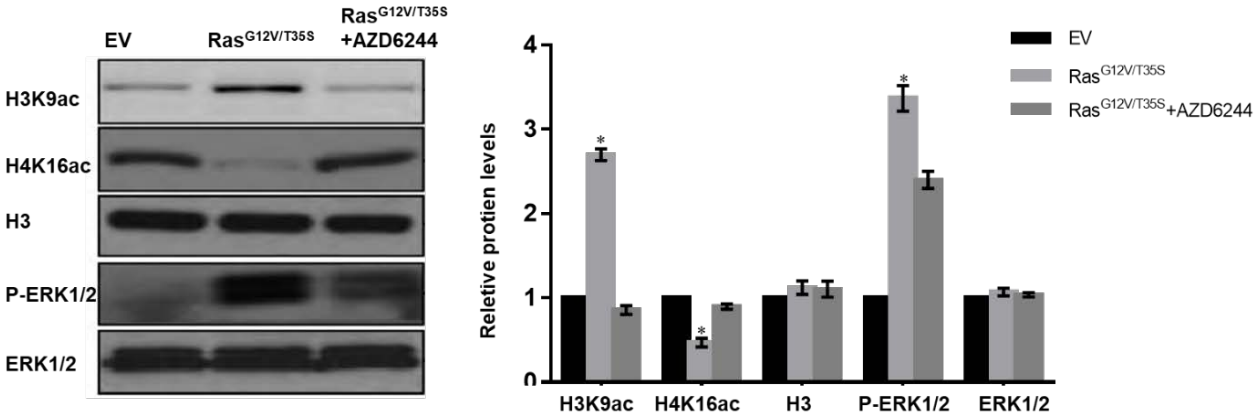

Figure 1. Western blot the expression of proteins. The Ras$^{G12V/T35S}$ expression vector (p-EGFP-N1-Ras$^{G12V/T35S}$) was transiently transfected in MCF-7 cells, the empty vector (EV) was used as a negative control. 24 h post-transfection, some of the cells were treated with 20 μM AZD6244 (MAPK pathway-specific inhibitor) (Ras$^{G12V/T35S}$+ AZD6244) for 48 h. Then cells were lysed and total protein was collected. Protein expression levels of H3K9ac, H6K16ac, P-ERK1/2, H3 and ERK1/2 in each group were analyzed by Western blot assay. Quantification of the protein band was analyzed by Image Pro Plus software. The H3 and ERK1/2 serve as an internal reference. Each set of experiments were independently repeated three times and the results were expressed as mean ± standard deviation using the *t*-test, * $P<0.05$.

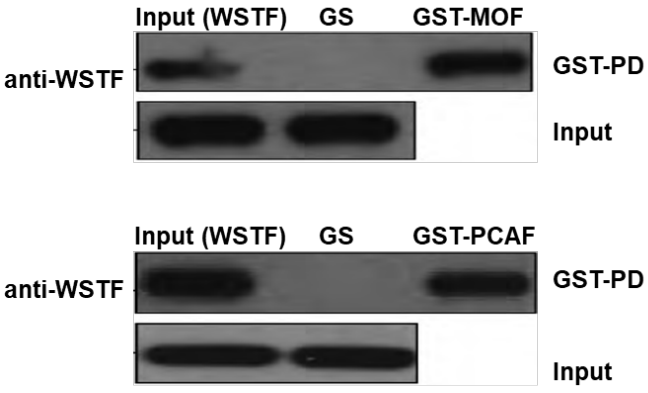

Figure 2. GST pull-down combining with Western blot detection

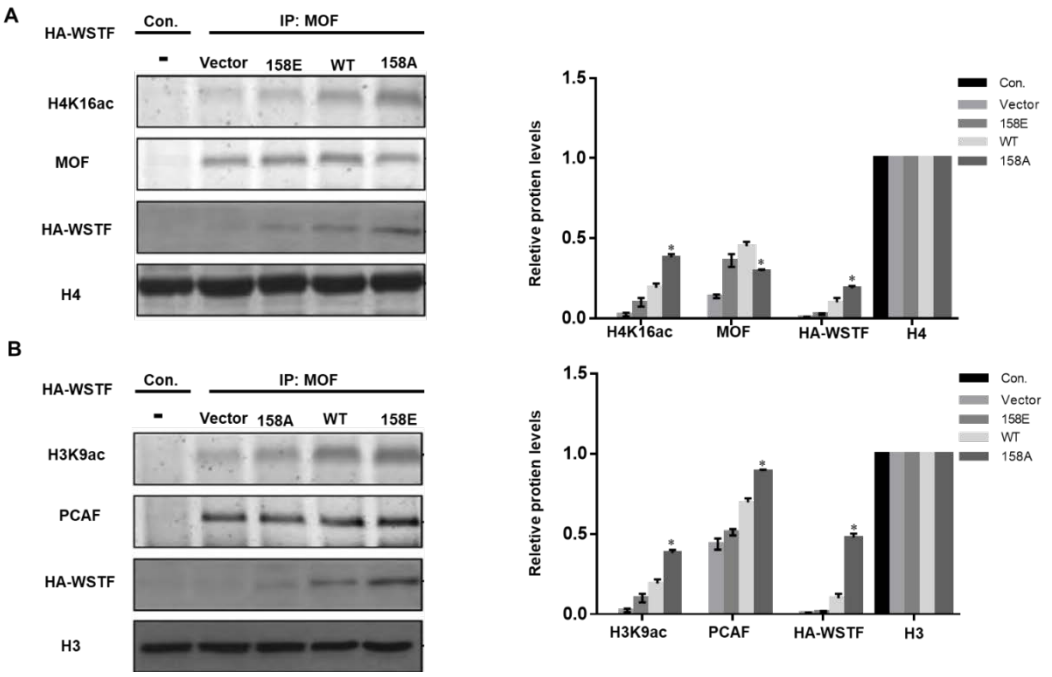

Figure 3. Co-IP and in vitro HAT activity assay. MCF-7 cells were transfected with equal doses of $WSTF^{S158E}$, $WSTF^{WT}$ and $WSTF^{S158A}$ plasmids. Immunoblotting was performed with antibodies as indicated (left panel). MCF7 cells were transfected with or without empty vector as control

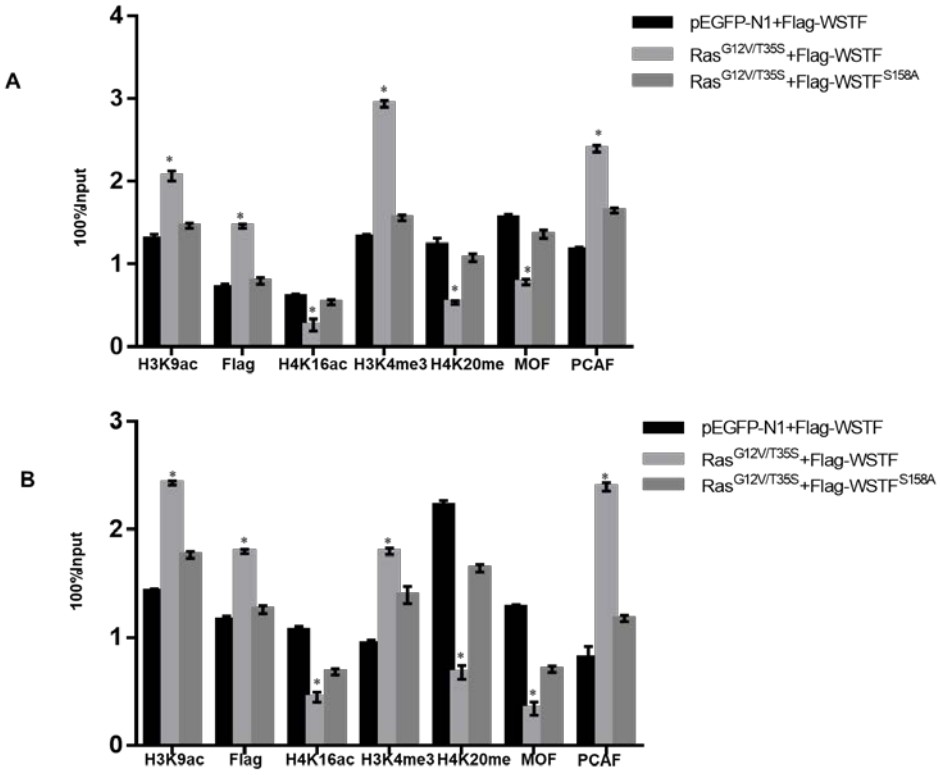

Figure 4. ChIP combined with Real-time PCR experiment. $Ras^{G12V/T35S}$ -expressing plasmids were co-transfected with $WSTF^{S158A}$ -expressing plasmid into MCF7 cells. Empty p-EGFP-N1 and Flag-WSTF plasmids were co-transfected used as a control

3.4. Detection of WSTF-regulated Ras-MAPK Downstream Tumor-associated Genes by ChIP and Real-time PCR.

After MCF7 cells were transfected with $WSTF^{WT}$, $WSTF^{S158A}$ and $Ras^{G12V\ /\ T35S}$ plasmids for 48 h, it was found that genes such as *Brk* and *p21* were directly regulated by WSTF phosphorylation level and MOF, PCAF, H3K9ac and H4K16ac ($P<0.05$) (Figure 4).

3.5. *In vivo* Tumorigenic Experiment in Mice to Verify the Tumorigenic Ability of Cells

Compared with the co-transfection group of $Ras^{G12V/T35S}$ and WSTF wild-type plasmids, the tumors grew faster and larger in size compared with the co-transfection group of $Ras^{G12V/T35S}$ and $WSTF^{S158A}$ plasmids ($P<0.05$) (Figure 5).

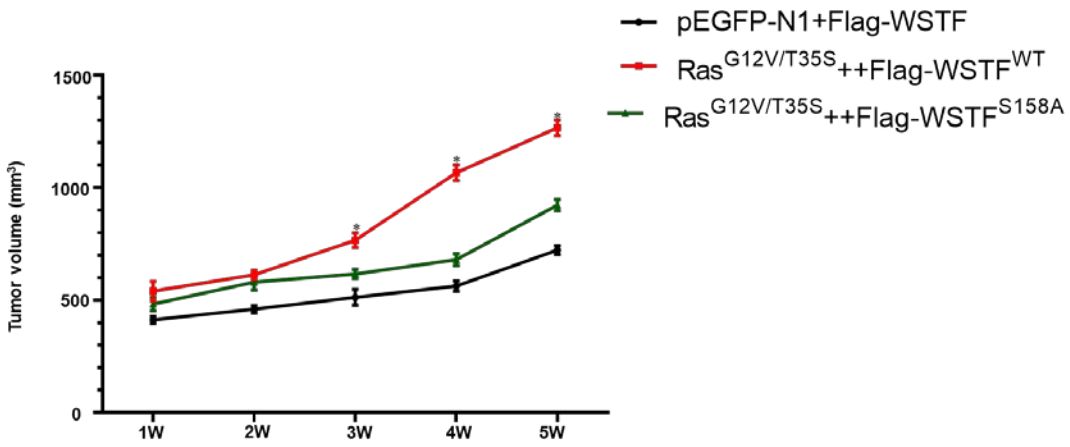

Figure 5. Experimental mice into tumor. *In vivo* tumor growth analysis was performed as described in Materials and methods

4. Discussion

Abnormal regulation of histone modification has been reported in many tumors, but the detailed mechanism is still unclear, especially the synergistic regulation between different sites [21,22,23]. We identified a bridge molecule WSTF, which is essential for the functional relationship between histone acetyltransferase MOF and PCAF [24,25]. After Ras-MAPK pathway activation, WSTF mediated the change of affinity between MOF and PCAF and their substrate-histone H3 and H4. This H4K16ac / H3K9ac regulation mode based on WSTF phosphorylation may be more accurate and rapid than the regulation mode based on PCAF/MOF translation level and post-translation modification.

Our results showed that WSTF was involved in the transcriptional regulation of tumor-related genes downstream of Ras-MAPK pathway. We speculated that WSTF may regulate the transcription of specific genes by recruiting PCAF/MOF into the regulatory region of target genes. This result shows the necessity of bridging molecules in the regulation of signal transduction by complex pathways in tumors. Moreover, our experimental results also suggest that the diversity of Ras signaling pathway function may involve specific epigenetic regulation.

WSTF gene can be detected in all Williams syndrome patients [26,27]. Our report is the first to identify the function of WSTF in malignant tumors and the functional complex PCAF/WSTF/MOF. For the first time, WSTF is also the first kinase molecule reported to form functional complexes with two important histone acetyltransferases.

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Conflict of Interest

All authors declare no conflicts of interest in this study.

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