Original Article

Gene Expression Changes in the Pituitary Gland of Rats Exposed to Electromagnetic Pulses*

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Abstract

Objective We examined alterations in the expression of tumorigenesis-related genes in the pituitary gland of rats exposed to electromagnetic pulses (EMP).

Methods The global gene expression profiles of the pituitary gland in EMP-exposed and control groups were detected by cDNA microarray analysis. We then validated and further investigated the reduced expression of two tumorigenesis-related genes, *Pten,* and *Jund*, by assessing their mRNA and protein expression by quantitative real-time-PCR, western blotting, and immunohistochemistry in the pituitary gland of rats 6 months after exposure to EMP.

Results EMP exposure induced genome-wide gene expression changes in the rat pituitary gland. There was decreased expression of the *Pten* and *Jund* mRNAs and proteins in EMP-exposed rats compared with in unexposed control animals.

Conclusion EMP exposure alters the expression of tumorigenesis-related genes in the pituitary gland. These tumorigenesis-related genes are potentially involved in the development of pituitary gland tumors in rats.

Key words: Tumorigenesis; Electromagnetic pulses; Pituitary gland; Pten; Jund

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INTRODUCTION

Pituitary tumors are common intracranial neoplasms that account for 10%-15% of all brain cancers in humans[1-4]. Several potential pathogenic factors have been suggested to be involved in tumorigenesis of the pituitary gland, including hormones, growth factors, receptors, cell cycle regulators, and signal transduction pathways[5-6]. It has also been reported that epigenetic mechanisms affect gene expression in the transformation of a normal cell into one with a propensity for uncontrolled growth[2,7]. However, the principal etiology of pituitary gland tumors remains unclear. Exposure to electromagnetic pulses (EMP) is unavoidable in our daily activities and can cause adverse biological and health effects, including tumorigenesis of the pituitary gland[8-9]. Inactivation of anti-oncogenes and/or altered expression of proto-oncogenes are reported to result in abnormal

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cell proliferation leading to tumorigenesis[10-15]. Inactivation of anti-oncogenes such as Pten, p16, and MEN1[16-17], and/or up-regulation of proto-oncogenes such as Ras, PTTG, and c-jun[18-20] have been suggested to be involved in the development of pituitary gland tumors. In the present study, a single high dosage of EMP (100 kV · m^{-1} with 300×10^3 pulses) was used to induce tumorigenesis in the pituitary gland. The expression of the tumorigenesis-related genes *Pten* and *Jund* was investigated in the early stage of adenoma development, 6 months after EMP exposure.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the Public Health Service policy on the Humane Care and Use of Laboratory Animals, and were approved by the Fourth Military Medical University Animal Care and Use committee. Eight-week-old female Wistar rats weighing 100-120 g were provided by the Animal Center of the Fourth Military Medical University. Ninety-two rats were randomly assigned among the experimental groups (Table 1). All rats were anesthetized by intra-peritoneal injection of sodium pentobarbital (45 mg/kg body weight), and their pituitary glands were excised and kept frozen for the cDNA microarray, quantitative (q)RT-PCR, and western blotting, or were fixed for hematoxylin-eosin staining and immunohistochemistry (IHC).

Table 1. Number of Rats Used in Each Experiment

		Number of EMPs after EMP Treatment			
		0 (control)	10×10^3	100×10^3	300×10^3
6 Months	Microarray	6	-	-	6
	qRT-PCR	6	6	6	6
	WB	6	6	6	6
	IHC	6	-	-	6
2 Years	Hematoxylin-Eosin Staining	5	-	-	5
	Gross Appearance	5	-	-	5

Note. EMP: electromagnetic pulse; WB: western blotting; IHC: immunohistochemistry.

EMP Exposure

Rats were restrained in Plexiglas boxes and exposed to EMPs for 50 min. Three alternative doses of EMP (low: 10×10^3, medium: 100×10^3, and high: 300×10^3 pulses) were delivered with a field intensity of 100 kV · m^{-1}. Rats in the control group were placed in the exposure chamber but were not exposed to EMPs. The EMPs were generated by a spark gap pulse generator (devised at the Department of Mechanical Engineering, Southeast University, Nanjing, Jiangsu Province, China), which generates a short duration higher peak voltage pulse from a basic lower pulse voltage of longer duration. Then, the EMP was transmitted to a GTEM cell (Gigahertz Transverse Electromagnetic Cell) via a coaxial cable.

Histological Analysis

Two years after EMP treatment, ten EMP-treated rats and ten control rats were used for histological analysis. Five EMP-treated rats and five control rats were anesthetized with intra-peritoneal injection of sodium pentobarbital (45 mg/kg body weight) and then decapitated, and the gross appearance of the pituitary gland was observed. In addition, five EMP-treated rats and five control rats were perfused and fixed, and then the pituitary glands were sectioned for hematoxylin-eosin staining.

RNA Isolation and Microarray Analysis

For this study, pituitary glands were excised from six EMP-exposed and six control rats, 6 months after EMP exposure. Each gland was homogenized in an appropriate volume of TRIzol (Invitrogen, USA) and left at room temperature (RT) for 5 min. Chloroform (0.2 mL/mL TRIzol; Invitrogen, USA) was then added, vigorously mixed for 15 s, left at RT for 2-3 min, and centrifuged at 12 000 × g for 15 min at 4 °C. The top, aqueous phase in each sample was carefully transferred into a new tube. An equal volume of isopropanol was then added, mixed thoroughly and centrifuged at 12 000 × g for 15 min at 4 °C. The cell pellet in each tube was washed in 70% ethanol, dissolved in diethylpyrocarbonate-treated water and the total RNA was extracted. The quality and quantity of RNA were determined by the absorbance at 260 nm (8 453 ultraviolet-visible spectrophotometer; Robbins Scientific, USA).

Five micrograms of each RNA sample was mixed with 5 μL of T7 promoter primer for 10 min at 65 °C, chilled on ice for 5 min and reverse transcribed to cDNA for 1 h at 4 °C using a reaction buffer containing 5× first strand buffer, 0.1 mol/L dithiothreitol, dNTPs, random hexamers, Moloney Murine Leukemia Virus reverse transcriptase, and RNAse OUT (Agilent Technologies). All cDNA samples were transcribed into linear cRNA in a solution containing 4× transcription buffer, 0.1 mol/L dithiothreitol, NTPs, amino allyl-UTP, T7 RNA

polymerase, inorganic pyrophosphatase, and RNAse OUT (Agilent Technologies, USA). One microgram of cRNA from each EMP-exposed and control sample was then labeled with Cy3 or Cy5 dye (dissolved in sodium bicarbonate buffer; Amersham, USA), respectively, for 1 h. The reaction was terminated by the addition of hydroxylamine. The quantity of purified cRNA (RNeasy Mini Kit; Qiagen, USA) was determined using an 8 453 ultraviolet-visible spectrophotometer (Robbins Scientific, USA) and was then hybridized onto rat oligo microarray slides (Agilent Cat. No. G4130A, USA) for 17 h at 60 °C.

The slides were washed twice with 6× saline-sodium citrate/0.005% Triton X-102 for 10 min at RT, then washed twice with 0.1× saline-sodium citrate/0.005% Triton X-102 and stored at 4 °C. The excess buffer was removed and scanned for the intensity of the Cy3 and Cy5 dyes at 532 and 635 nm, respectively (GenePix 4000B; Axon Instruments; pixel resolution 5 mm, 100% laser power, and photomultiplier tube voltage 600 V). The 16-bit TIFF color image features were extracted (GenePix Pro 3.0; Axon Instruments software package) and were overlaid with their respective gray scale images. The ratio of color: gray was defined by accessing the gene list file described by the location of each gene on the microarray. The ratio was then subtracted from the median of the background intensity. Relative expression levels were calculated by global normalization between EMP-exposed and control samples using Spotfire Decision Site 8.0 software.

Real-time qRT-PCR

RNA was extracted from each rat pituitary gland as described above. Total RNA (1 μg) was reverse transcribed into first strand cDNA using Moloney Murine Leukemia Virus reverse transcriptase and oligo(dT) primers (Invitrogen) in a reaction volume of 20 μL, according to routine reverse transcription methods. PCR was then conducted using primers specific for *Pten* and *Jund*, while the housekeeping gene Gapdh was used as a constitutive control for normalization (Table 2). Real-time PCR was performed in 20 μL buffer containing 10 μL of SYBR Premix Ex Taq (IQ5 Real-Time PCR System; BIO-RAD). The reaction was first incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 62 °C for 30 s, and 72 °C for 20 s. Each PCR was performed in triplicate. The cumulative fluorescence of the *Pten* and *Jund* products was normalized to that of Gapdh from the same sample. The relative quantities were expressed as "fold-increase" over the Gapdh controls. Amplified products were also identified by melting point analysis. The fractional cycle at which the amount of amplified target became significant was calculated automatically by the thermocycler. At least two independent experiments were conducted using the PCR products form each pituitary gland, and each PCR experiment was performed in triplicate.

Table 2. Primers Used for Real-time PCR

Gene	Primers (5' to 3')	
Pten	Forward	GACGACAATCATGTTGCAGCA
	Reverse	GCCTTTAAAAACTTGCCCCG
Jund	Forward	TCAAGACCCTCAAAAGCCAGA
	Reverse	TGTTGACGTGGCTGAGGACTT
Gapdh	Forward	GGCACAGTCAAGGCTGAGAAT
	Reverse	AGCCTTCTCCATGGTGGTGAA

Western Blotting

Total protein lysate (50 mg), isolated in the presence of the protease inhibitor phenylmethylsulfonyl fluoride, was denatured in sodium dodecyl sulfate (SDS) sample buffer, separated by electrophoresis on an 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. The membrane was dried and subsequently incubated with monoclonal rabbit anti-Pten (1:200; R & D Systems, USA) or polyclonal rabbit anti-Jund (1:100; Santa Cruz Biotechnology, USA), and mouse anti-β-actin antibody (1:1 000; Abcam, Cambridge, UK) in 5% bovine serum albumin or in 5% non-fat dry milk in Tris-buffered saline/Tween 20 overnight at 4 °C. After three washes in Tris-buffered saline/Tween 20, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:500; ZSGB-Bio, China). Immunodetection was performed using the ECL Plus detection system with autoradiography on Hyperfilm (Amersham Life Science, USA). The optical density of the appropriately sized bands was measured using Kodak 1D Image Analysis Software (Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY, USA). The relative expression of Pten and Jund in the rats exposed to different doses of EMP was calculated as the ratio with the optical density of the corresponding protein in control rats. All target protein expression was normalized to that of β-actin.

Immunohistochemistry and Image Analysis

The abundance and distribution of the Pten and Jund proteins in the pituitary glands excised from six EMP-exposed and six control rats were characterized

by IHC. The glands were fixed in 10% neutral buffered formalin, paraffin processed and 5-μm sections were prepared on microscope slides. The tissue sections were deparaffinized in xylene and rehydrated through a graded series of alcohol, washed, and incubated with 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity. After washing with 0.01 mol/L phosphate-buffered saline (pH 7.4) the sections were incubated with normal goat serum for 15 min at RT to block nonspecific binding sites. Monoclonal rabbit anti-Pten (1:200; R & D Systems, USA) or polyclonal rabbit anti-Jund (1:100; Santa Cruz Biotechnology, USA) antibodies were used to incubate the sections for 24 h at 4 °C. The negative control was incubated in buffer without primary antibody. The sections were then rinsed and incubated with biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories, USA) for 1 h at 37 °C. After rinsing, the sections were labeled with 3, 3' diaminobenzidine, counterstained with hematoxylin, and rinsed in xylene. The sections were mounted with coverslips using Permount, and examined at 40× magnification under a light microscope (AHV-3; Olympus, Japan). All cells containing brown-yellow granules were considered to show immunoreactivity. Image analysis was performed using a Quantiment 570 image analyzer (Leica, German) with Quic software (Olympus, Japan). According to a previous report[21], a 250-μm × 250-μm region was selected at random in the section, and in this region, the average intensity and immunoreactive area of Pten and Jund were individually detected. The mean for each rat was derived from the data from four sections. In the immunoreactive density results, the smaller the gray density value is, the denser the immunoreactivity is.

Statistical Analysis

The data were expressed as mean±SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) using SPSS (version 13.0) for Windows (SPSS, Inc., Chicago, IL). Statistical significance was set at $P<0.05$.

RESULTS

EMP Exposure Induces the Development of Pituitary Adenomas

To establish a model of tumorigenesis in the pituitary gland, we exposed rats to different doses of EMP (low: 10×10^3, medium: 100×10^3, and high: 300×10^3 pulses at 100 $kV\cdot m^{-1}$). Two years after exposure, 72% of the rats in the high-dose group had developed a tumor, whereas tumors were found in 61.7 and 31.2% of rats in the medium- and low-dose groups, respectively, and in none of the control, unexposed rats. The average tumor size in the high-dose animals was 4×3×3 mm^3 and the tumor appeared darker than the surrounding tissue (Figure 1A). Hematoxylin-eosin staining revealed that, compared with normal control cells (Figure 1B), tumor cells were characterized by a large nucleus of irregular size and shape with a prominent nucleolus. Furthermore, the cytoplasm was scarce and intensely colored or, in contrast, was pale (Figure 1C). Our data indicated that exposure to EMP induced tumorigenesis in the pituitary gland.

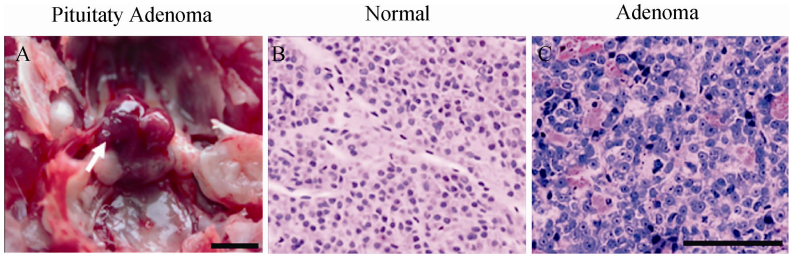

Figure 1. High-dose EMP (300×10^3 pulses at 100 $kV\cdot m^{-1}$) induced the development of pituitary adenomas. A: Gross appearance of a pituitary adenoma in an EMP-exposed rat, 2 years after exposure. The white arrow indicates the adenoma. Scale bar: 5 mm. B and C: Hematoxylin-eosin stained sections showing a comparison of the morphological features between normal pituitary gland section (B) and adenoma section (C). In C, the cells were of irregular size and shape, and some had prominent nucleoli; the cytoplasm was either scarce and intensely colored or pale. Scale bar: 50 μm.

Many transcription factors are differentially expressed in EMP-induced pituitary adenomas. We used a microarray to identify global variations in gene expression in the pituitary gland at an early stage of adenoma development, 6 months after exposing rats to high-dose EMP. The microarray

comprised 22 575 probe sets representing over 20 000 well-characterized rat genes or expressed sequence tags. We found that 402 genes were differentially expressed (defined as a fold difference of >2.0 or <0.5) between EMP-exposed rats and controls. Among these, 128 were up-regulated and 274 were down-regulated (Figure 2A). The relative percentage distribution of the known gene ontology groups is represented in Figure 2B. The largest ontology group of known genes that were altered included several families of transcription factors (34%) and those were involved in signaling/transport (15%). Altered genes that code for receptors accounted for 10% of the total and this was closely followed by several genes involved in regulation of the cell cycle (9.4%). There were seven other groups: DNA repair (6.3%), cytoskeleton (6.3%), proto-oncogene and anti-oncogene groups (5.5%), apoptosis (3.9%), immune function (3.9%), inflammatory response (3.1%), and protein synthesis (3.1%). The details of the fold-changes in the expression level of several important genes and their *P* values are given in Table 3.

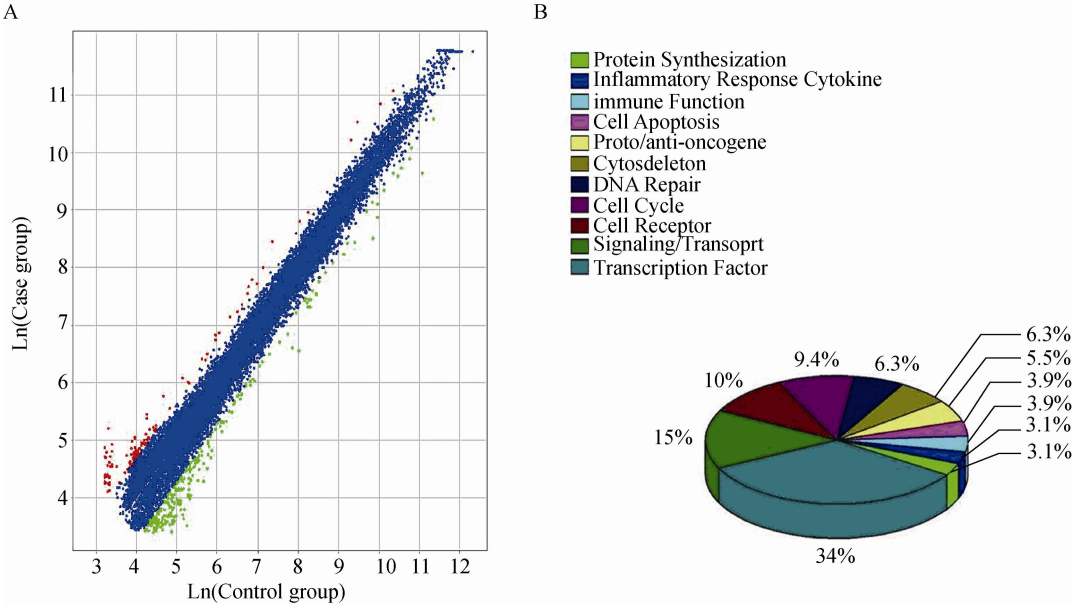

Figure 2. Microarray analysis of gene expression changes. A. Scatter plot comparing ratios of signal values from two replicate microarray hybridizations with Cy3-dUTP-labeled and Cy5-dUTP-labeled mRNAs from the pituitary glands of rats exposed to high-dose EMP and control animals, respectively. Data from the images of dye-swapping experiments were plotted as the mean intensity after normalization of genes/expressed sequence tags spotted in triplicate. The threshold values for >2-fold change (red) and <0.5-fold change (green) are indicated. B. Pie chart showing the relative distribution (percentage) of the gene ontology groups identified by microarray analysis of the pituitary glands of rats exposed to high-dose EMP compared with control rats.

Table 3. Gene Expression Changes Induced by EMP Exposure

Gene Symbol	GenBank Accession Number	Description	EMP Exposure	
			P value	Fold Change
CDK2	NM_019296	cyclin-dependent kinase 2	0.0006	0.47
Dnmt3a	NM_001003957	DNA methyltransferase 3 alpha	0.00004	0.48
Pten	NM_031606	phosphatase and tensin homolog	0.0000	0.47
Jund	NM_138875	Jun D proto-oncogene	0.06	0.48

Pten and Jund mRNAs were Downregulated in EMP-exposed Rats

We selected two tumorigenesis-related genes, *Pten,* and *Jund*, as targets to validate the microarray findings of alterations in tumorigenesis-related gene expression in EMP-exposed pituitary gland. By qRT-PCR, the mRNA expression levels of Pten and Jund in the EMP-exposed pituitary gland were significantly decreased in a dose-dependent manner compared with those in control animals (Figure 3A).

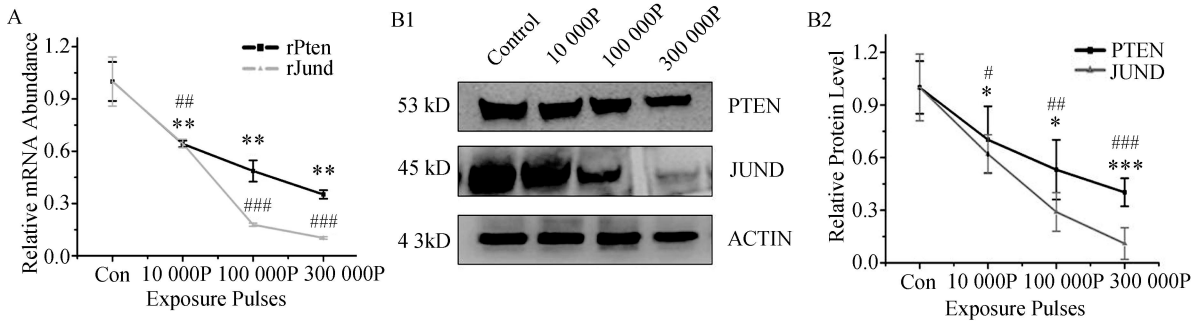

Figure 3. mRNA and protein expression of Pten and Jund in low-, medium-, and high-dose EMP-exposed and control pituitary glands. A. qRT-PCR validation of the microarray data for *Pten* and *Jund*. Both genes exhibited a dose-dependent decrease in expression upon EMP exposure. Target gene expression was normalized to that of the reference gene (Gapdh) and is shown relative to the expression level in control animals (designated as 1). The values represent the mean±SEM of three independent experiments. ###, ***: *P*<0.001; ##, **: *P*<0.01. B1. Protein expression of Pten and Jund, relative to β-actin, from pituitary gland whole cell lysates on western blots. B2. Quantification of the western blotting data. In accordance with the mRNA data, the Pten and Jund proteins exhibited a dose-dependent decrease in expression upon EMP exposure. Target protein expression was normalized to that of the reference protein (β-actin) and is shown relative to the expression level in control animals (designated as 1). The values represent the mean±SEM of three independent experiments. ###, ***: *P*<0.001; ##: *P*<0.01; #, *: *P*<0.05 by one-way ANOVA.

Pten and Jund proteins were Downregulated in EMP-exposed Rats

Changes of the expression of Pten and Jund in the pituitary gland of EMP-exposed rats were detected by western blotting and IHC. We detected a dose-dependent decrease in the expression of both proteins (Figure 3B). Furthermore, by IHC in EMP-exposed rats, Pten immunoreactivity was located in the cytoplasm, while Jund immunoreactivity was concentrated in the nucleus (Figure 4A). Similar to the control, both proteins were evenly distributed in the pituitary gland of EMP-exposed animals. However, for both proteins, the area of immunoreactivity was significantly decreased (*P*<0.05) in EMP-exposed rats compared with that in controls (Pten: 32.4%±1.6% *vs.* 40.6%±5.5%; Jund: 28.0%±2.4% *vs.* 33.7%±1.8%) (Figure 4B). Accordingly, the average gray density of immunoreactivity for both proteins was significantly increased (*P*<0.05) in EMP-exposed rats compared with that in controls (Pten: 158.0±21.2 *vs.* 125.8±6.3; Jund: 131.4±7.0 *vs.* 109.2±9.5) (Figure 4C). The downregulated density of immunoreactivity of both Pten and Jund in EMP-exposed rats was in accordance with our qRT-PCR results.

DISCUSSION

Several researchers have examined the gene expression profiles in human pituitary tumors[22-24] and rodent pituitary adenomas[25-27]. In rats, 588 genes were shown to be linked to spontaneously occurring and age-related pituitary adenomas[25]. Deregulation of various genes involved in cell cycle regulation has been reported to be a frequent event in pituitary tumors[28-32]. Wood et al.[27] reported decreased transcription levels of the cell cycle regulator genes cyclin-dependent kinase 2, cyclin A, and p57. Similar observations were made in the current study: the pituitary glands of rats 6 months after exposure to EMP exhibited not only alterations in the genome-wide gene expression patterns, particularly in four functional groups of genes (Figure 1A), but also a 0.47-fold change in the expression of the cyclin-dependent kinase 2 gene (Table 3).

Abnormal DNA methylation is common in many types of cancers. It is reported to mediate the silencing of multiple genes involved in cell cycle regulation and is suggested to be an important epigenetic modification in the pathogenesis of pituitary tumors[33]. DNA methylation is maintained by three DNA methyltransferases: DNMT1 maintains methyltransferase activity, DNMT3a is mostly involved in de novo methylation, and DNMT3b is a putative mediator of epigenetic control through histone modification of gene expression in pituitary cells[34]. In the current study, there was a 0.48-fold decreased expression of DNMT3a in EMP-exposed rats compared with control rats (Table 3) implying a potential epigenetic dysregulation in the pituitary

glands of rats 6 months after exposure to EMP; this suggests that DNA methylation is potentially involved in the development of tumorigenesis induced by EMP.

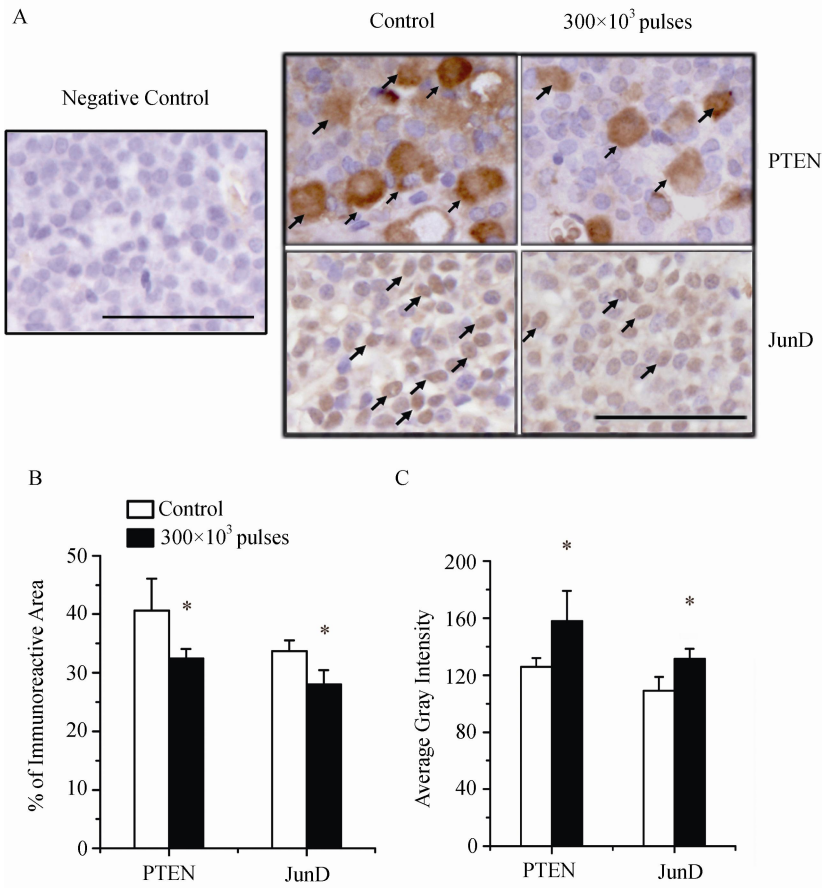

Figure 4. IHC analysis of EMP-induced pituitary adenomas. A. IHC showing a decrease in the protein expression of Pten and Jund in the pituitary glands of rats exposed to high-dose EMP compared with that in control animals. Black arrows indicate the Pten-immunoreactivity or Jund-immunoreactivity cell. Scale bars: 50 μm. The negative control section was incubated without primary antibody. To quantify the data, the percentage immunoreactive area (B) and the average gray density of the immunoreactive regions (C) in the pituitary adenomas and normal pituitary gland cells (control) from (A) were calculated. The values represent the mean±SEM. *: $P<0.05$.

PTEN is reported to be involved in the regulation of signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation, and cell growth[35-39]. Loss of PTEN activity due to mutations, deletions, or promoter methylation silencing has been shown to occur at a high frequency in many primary and metastatic human cancers[40-42]. More importantly, several recent studies have suggested that epigenetic events can lead to down-regulation of PTEN. Gravina et al.[43] used drugs to modulate PTEN expression epigenetically during anti-androgenic therapy for human prostate cancer. Additionally, PTEN repression mediated by microRNA miR-26a in gliomagenesis has provided a new epigenetic mechanism in tumorigenesis[44]. In addition, Pten promoter methylation appeared more frequently in multiple cerebral cavernous malformations[45]. These reports provide ample evidence that DNA methylation is a critical epigenetic mechanism involved in the abnormal expression of Pten in tumorigenesis. The results from the current microarray, qRT-PCR, western blotting, and IHC experiments showed that both the mRNA and protein expression of Pten were significantly down-regulated in the pituitary glands of rats 6 months after EMP exposure. In human pituitary tumors, Pten mRNA is similarly down-regulated, but it is mainly localized in the nucleus[46]. In contrast, our IHC data from rats showed that Pten protein was primarily distributed in the cytoplasm. If a shift in

Pten from nucleus to cytoplasm is correlated with increased neoplasia[47-49], the differential expression of Pten mRNA and protein between human and rat pituitary tumors may imply that there are differences in epigenetic Ptenmodulation in different species.

Jund is a recent addition to the jun proto-oncogene family. It has similar functions to the other jun proteins in trans-activating the AP-1-responsive promoter in conjunction with c-Fos[50]. Thus, Jund can activate or repress a diverse collection of target genes, which are pivotal to the regulation of cell growth[51-52]. Altered expression and protein–protein interactions of Jund are reportedly involved in the modulation of tumor angiogenesis, cellular differentiation, proliferation, and apoptosis[53-56]. Recent reports in multiple endocrine neoplasia type 1 (MEN1), a hereditary tumor syndrome that includes pituitary tumors, have indicated several functions for the causative nuclear protein Men1 in the regulation of transcription, serving either as a repressor or as an activator[57]. Men1 interacts with Jund, changing it from an oncoprotein into a tumor-suppressor protein, putatively by recruitment of histone deacetylase complexes. The regulation of Jund gene expression is complex, including control at the transcriptional and post-transcriptional levels, post-translational modifications, and protein–protein interactions, together suggesting that the regulation of Jund expression might depend on multiple epigenetic modifications. The results of the current study indicated that both mRNA and protein expression of Jund were significantly suppressed in the pituitary gland of EMP-exposed rats, suggesting the contribution of epigenetic processes to pituitary tumorigenesis.

Taken together, the present study indicated that the pituitary gland in rats exposed to EMP exhibited genome-wide changes in gene expression and suggested that EMP exposure may play a role in pituitary tumorigenesis. Our findings provide a new understanding of the etiology of pituitary tumors.

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