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Endokrynologia Polska
DOI: 10.5603/EP.a2017.0047
Tom/Volume 68; Numer/Number 5/2017
ISSN 0423–104X

Preliminary studies on the differential expression of adrenocorticotropic hormone receptor in adrenal adenomas

Wstępne badania dotyczące zróżnicowania ekspresji receptorów kortykotropiny w gruczolakach nadnerczy

Dongliang Hu, Tongzu Liu, Xinghuan Wang

Department of Urology, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, China

Abstract

Introduction: The ACTH receptor (ACTHR) is primarily expressed in the adrenal cortex. Previous studies focused on the regulatory function of ACTHR in glucocorticoid secretion, but research on adrenal tumours is rare. The aim of this study was to evaluate ACTHR expression in common adrenal adenomas and investigate its influence on adrenal tumorigenesis using adrenocortical H295R cells.

Materials and methods: Real-time polymerase chain reaction and western blot were used to detect the expression of ACTHR in 18 aldosterone-producing adenomas, 16 cortisol-producing adenomas, 9 non-functional adenomas, and 12 normal adrenal samples. Lentiviral vector pLVX-mCMV-ACTHR-ZsGreen was transfected into the H295R cells to increase ACTHR expression. WST-1 and cell count were applied to evaluate cell proliferation at different ACTHR levels. TUNEL staining was used to measure cell apoptosis.

Results: Compared with normal adrenal samples, the aldosterone-producing adenoma samples had higher ACTHR mRNA and protein levels. However, the mRNA and protein levels of ACTHR in non-functional adenomas and cortisol-producing adenomas were lower than those in the normal adrenal samples. Proliferative activity in the experimental cells was higher than that in the control cells in the first three days. The proliferative activity peaked in the second day. However, this trend was reversed in the fourth day and became more apparent with time. By contrast, TUNEL staining showed that ACTHR overexpression did not induce a significant difference between the two groups.

Conclusions: Differential ACTHR expression may be determined by different types of adrenocortical tumours. ACTHR is more likely to have induced the proliferation rather than the apoptosis of H295R cells. **(Endokrynol Pol 2017; 68 (5): 512–518)**

Key words: *adrenocorticotropic hormone receptor, Adrenal tumour, proliferation, H295R cells*

Streszczenie

Wstęp: Receptor kortykotropiny (*adrenocorticotropic hormone receptor*, ACTHR) występuje głównie w korze nadnerczy. Prowadzono wcześniej badania dotyczące regulacyjnej funkcji ACTHR w wydzielaniu glikokortykoidów, lecz rzadko obejmowały one chorych z guzami nadnerczy. Badanie przeprowadzono w celu oceny ekspresji ACTHR w najczęstszych gruczolakach nadnerczy i zbadania jej wpływu na rozwój tych nowotworów w komórkach kory nadnerczy ludzkich linii H295R.

Materiał i metody: Do wykrycia ekspresji ACTHR w 18 gruczolakach wydzielających aldosteron, 16 gruczolakach wydzielających kortyzol, 9 gruczolakach nieczynnych hormonalnie i 12 próbkach prawidłowych nadnerczy użyto reakcji łańcuchowej polimerazy w czasie rzeczywistym oraz metody *Western blot*. W celu zwiększenia ekspresji ACTHR transfekowano wektor lentiwirusowy pLVX-mCMV-ACTHR-ZsGreen do komórek H295R. Proliferację komórek przy różnych poziomach ekspresji ACTHR oceniono na podstawie WST-1 oraz liczby komórek. W celu zmierzenia apoptozy komórek zastosowano barwienie metodą TUNEL.

Wyniki: W porównaniu z próbkami prawidłowych nadnerczy w próbkach gruczolaków wydzielających aldosteron stwierdzono wyższą ekspresję mRNA i białka ACTHR. Jednak ekspresja mRNA i białka ACTHR w gruczolakach nieczynnych hormonalnie oraz w gruczolakach wydzielających kortyzol była niższa niż w próbkach prawidłowych nadnerczy. W ciągu 3 pierwszych dni aktywność proliferacyjna w komórkach eksperymentalnych była wyższa niż w komórkach kontrolnych. Aktywność proliferacyjna osiągnęła maksymalną wartość w drugim dniu. Jednak w czwartym dniu ta tendencja uległa odwróceniu, co było wyraźniejsze w miarę upływu czasu. Z kolei barwienie metodą TUNEL wykazało, że nad ekspresja ACTHR nie spowodowała istotnej różnicy między grupami.

Wnioski: Zróżnicowanie ekspresji ACTHR może wynikać z różnych typów guzów nadnerczy ocenianych w badaniu. Wydaje się, że ACTHR pobudza proliferację, a nie apoptozę komórek H295R. **(Endokrynol Pol 2017; 68 (5): 512–518)**

Słowa kluczowe: *receptor kortykotropiny, guz nadnerczy, proliferacja, komórki H295R*

Introduction

Adrenal diseases characterised by hormonal disorders or neoplasms are caused by adrenal tumours or hyperplasia. Pathological findings indicate that most adrenal diseases are diagnosed with benign adenoma. By contrast, adrenocortical carcinoma is extremely rare. However, different diseases determine distinct treatments. Surgeons need to confront different preoperative preparations and surgical risks. Therefore, the

Dongliang Hu, Department of Urology, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, China, e-mail: hdlhdl2008@163.com, tel: 86–027–67813104

Table I. *Perioperative data*
Tabela I. *Dane przedoperacyjne*

	APA	CPA	NFA	NA
Number of patients	18	16	9	12
Age (y)	46.2 ± 6.5	48.1 ± 7.1	45.9 ± 8.3	50.3 ± 7.7
Gender (n)				
Male	10	7	5	7
Female	8	9	4	5
BMI [kg/m^2]	25.4 ± 3.4	25.8 ± 4.5	24.9 ± 4.1	23.8 ± 3.6
Laterality (n)				
Left	11	9	5	6
Right	7	7	4	6
Operation time (min)	52.1 ± 10.3	53.6 ± 13.1	49.5 ± 14.8	59.4 ± 15.2
Tumor size [cm]	1.9 ± 0.3	2.1 ± 0.6	2.8 ± 0.8	—

APA: aldosterone-producing adenoma; CPA: cortisol-producing adenoma; NFA: non-functional adenoma; NA: normal adrenal

pathogenesis of adrenal tumours must be investigated to discover new treatments.

The adrenocorticotropic hormone receptor (ACTHR, also called MC2R) is a G-protein coupled, seven-transmembrane receptor that transmits the adrenocorticotropic hormone (ACTH) signal to induce related hormone production in adrenocortical cells by the cAMP//PKA pathway [1]. This receptor is primarily expressed in the adrenal cortex [2]. Previous studies focused on the regulatory function of ACTHR in glucocorticoid secretion, but research on adrenal tumours is rare. Recent documents have shown that ACTH is crucial in adrenal cell growth and differentiation; ACTH is even considered as a proliferation inducer [3]. So far, a few studies have experimented at tissue level [2, 4]; however, studies at the cellular level are limited. Therefore, we explored the effects of ACTHR on adrenal tumour formation at the tissue and cellular levels using human NCI-H295R adrenocortical cells.

Materials and methods

Tissue collection

From July 2013 to August 2014, a total of 55 patients underwent retroperitoneoscopic adrenalectomy because of adrenal tumour (Table I). There were 29 men and 26 women, aged 34 to 65 years (mean 47). Of these 55 patients (31 on the left side, 24 on the right side), 18 aldosterone-producing adenomas (APAs), 16 cortisol-producing adenomas (CPAs), and nine non-functional adenomas (NFAs) were collected from patients with clinically and pathologically confirmed diseases. Twelve normal adrenal (NAs) samples were obtained from patients who underwent adrenalectomy along with nephrectomy because of renal carcinoma or renal pelvic carcinoma. All samples were collected by surgical resection. Immediately after surgery, samples were snap-frozen in liquid nitrogen and experiments were performed. ACTHR expression was determined by western blot and real-time polymerase chain reaction (PCR).

The study was approved by the Ethical Committee of Zhongnan Hospital of Wuhan University and carried out in compliance with the Helsinki declaration. Participants signed an informed consent prior to enrolment in the study.

Cell culture

H295R adrenocortical cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM/F12 medium supplemented with 2.5% Nu Serum (BD Biosciences, Bedford, MA), and 1% ITS^{+1} (Sigma-Aldrich, St. Louis, MO). The cells were grown at 37°C in a humid atmosphere of 95% air/5% CO_2. Transfection or treatment was initiated 24 h after reseeding the cells (about 80～90% confluence).

Lentiviral vector constructs and transfection

Lentiviral vector pLVX-mCMV-ACTHR-ZsGreen (Biowit, Shenzhen, China) was used to increase ACTHR expression. The virus containing the expression vector but lacking the ACTHR cDNA was also transfected into the H295R cells to act as the negative control group. Normal H295R cells, without any virus, were used as a blank control. According to the manufacturer's directions, cells were infected by virus (multiplicity of infection = 20) and 8 μg/mL polybrene was added. The infected cells were grown at 37°C in a humid atmosphere of 95%

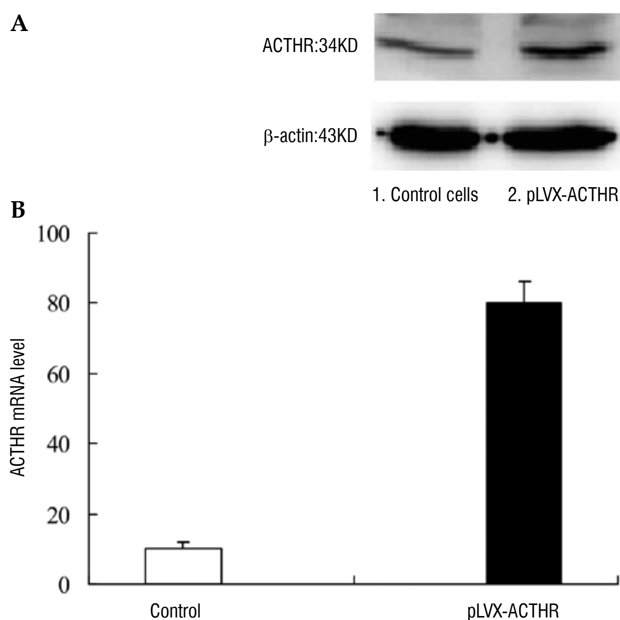

Figure 1. *Lentiviral vector pLVX-ACTHR effectively increased ACTHR expression at protein level (**A**) and mRNA level (**B**). The protein and mRNA levels of ACTHR in the experiment cells increased by 1.4-fold (ratio of relative intensities: 0.54 ± 0.06 and 0.39 ± 0.07) (P = 0.028) and 8-fold (P < 0.01), respectively, compared with those in the control cells*

Rycina 1. *Wektor lentiwirusowy pLVX-ACTHR skutecznie zwiększył ekspresję ACTHR, zarówno na poziomie białka (**A**), jak i mRNA (**B**). Ekspresja białka i mRNA ACTHR w komórkach eksperymentalnych zwiększyła się odpowiednio 1,4-krotnie (stosunek względnej intensywności: 0,54 ± 0,06 i 0,39 ± 0,07) (p = 0,028) i 8-krotnie (p < 0,01) w porównaniu z ich ekspresją w komórkach kontrolnych*

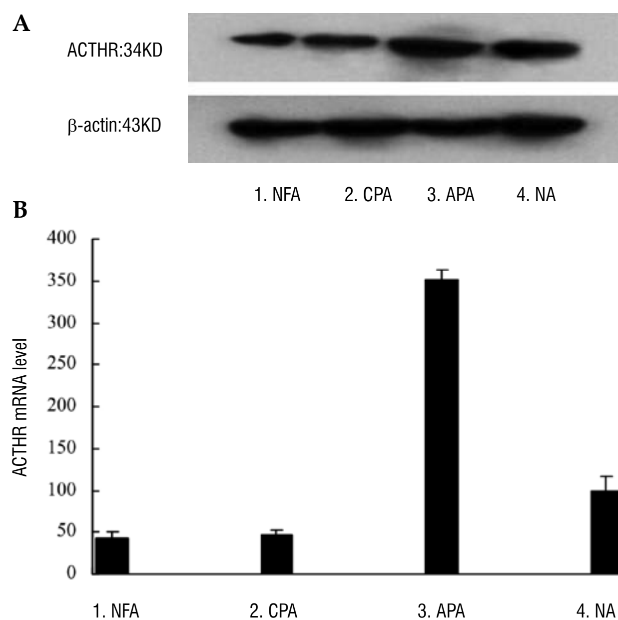

Figure 2. *The expression of ACTHR at protein (**A**) and mRNA levels (**B**) in adrenal tumors and normal adrenal. At the protein level, the relative intensities of theses four kinds of tissues were 0.28 ± 0.06; 0.31 ± 0.05; 0.81 ± 0.04 and 0.49 ± 0.04 (P = 0.017), respectively. At the gene level, NA mRNA level was considered as 100 so as to facilitate comparison. It was 2.16 and 2.31 times higher than the CPA and NFA groups. The mRNA level of ACTHR was 3.52-fold higher in APA than that in NA (P = 0.002)*

Rycina 2. *Ekspresja ACTHR na poziomie białka (**A**) i mRNA (**B**) w guzach nadnerczy i nadnerczach prawidłowych. Na poziomie białka względna intensywność w czterech ocenianych w badaniu typach tkanek wynosiła odpowiednio 0,28 ± 0,06; 0,31 ± 0,05; 0,81 ± 0,04 i 0,49 ± 0,04 (p = 0,017). Na poziomie genu w celu ułatwienia porównań przyjęto ekspresję w prawidłowych nadnerczach (*normal adrenal*, NA) mRNA jako 100. Była ona 2,16- i 2,31-krotnie wyższa niż w gruczolakach wydzielających kortyzol (cortisol-producing adenoma, CPA) i gruczolakach nieczynnych hormonalnie (non-functioning adenoma, NFA). Ekspresja mRNA ACTHR była 3,52-krotnie wyższa w gruczolakach wydzielających aldosteron (*aldosterone-producing adenoma*, APA) niż w prawidłowych nadnerczach (p = 0,002)*

air/5% CO_2. Medium was changed 24 h later. Cells were harvested to perform subsequent assays according to different experiments 48 h later.

Real-time PCR

Total RNA was extracted using a Total RNA Miniprep Kit (Axygen, Union, CA) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). To ACTHR primers, we refer to the previous publication [5]: forward, 5′-CCCAGAAAGTTCCTGCTT-3′, reverse, 5′- ATATCTCCTCCGGCAAAA-3′; (GAPDH) forward, 5′-GTGAAGGTCGGAGTCAACG-3′, reverse, 5′-TGAGGTCAATGAAGGGGTC-3′. Real-time PCR was carried out using the Rotor Gene 3000 sequence detection system (Qiagen, Venlo, Holland). A 25 μL total volume consisted of 2 μL of cDNA, 12.5 μL of SYBR Green qPCR Master Mix (2×; MBI Fermentas, Lithuania), and 0.3 μM forward and reverse primers. The cycling conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. All samples were tested in triplicate under these conditions, and the mRNA levels of ACTHR were standardised against GAPDH.

Western blot

Each sample was lysed in RIPA buffer. Protein concentration was measured using a standard BCA protein assay kit (Beyotime, Haimen, China). Each sample was electrophoresed on 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The blots were probed with anti-ACTHR (2 μg/mL, polyclonal, Abcam, UK) and anti-β-actin (Santa Cruz, CA). Expression was detected using an Ultra enhanced chemiluminescence kit (BioInd, Israel). Proteins were quantified using the software Quantity One (Bio-Rad, Hercules, USA).

Proliferation assays

To measure proliferation, 2×10^3 cells were seeded in 96-well plates. After 24 h of transfection, the proliferation rates of the experimental and control cells were studied with the WST-1 proliferation assay (Beyotime, Haimen, China). Absorption values were detected every 24 h in the next four days. Cell numbers were counted daily. Results are expressed as the average of at least three independent experiments performed in duplicate.

Cell apoptosis

Apoptosis was assayed using a One Step TUNEL Apoptosis Assay Kit (Beyotime, Haimen, China) according to the producer's protocol. In brief, the cells were fixed with 4% paraformaldehyde after 48 h of transfection, permeabilised with 0.1% Triton X-100 in sodium citrate, and incubated for 60 min at 37°C in a humidified atmosphere in the dark after adding the TUNEL reaction mixture. Finally, the samples were analysed under a fluorescence microscope.

Statistical analysis

All experiments were repeated at least three times. Measurement data was analysed for statistical significance using *t* tests and ANOVA (SPSS 15.0 software, Chicago, IL). Differences were considered significant at $p < 0.05$.

Results

ACTHR overexpression by lentiviral vector

When ACTHR expression vector was used to transfect H295R cells, real-time PCR and western blot showed that the protein and mRNA levels of ACTHR in the transfected cells increased by 1.4 and 8 fold ($p < 0.01$), respectively, compared with those in the control cells (Fig. 1). The ratio of relative intensities (Western blot) in the two groups was 0.54 ± 0.06 and 0.39 ± 0.07, respectively ($p < 0.05$).

Expression of ACTHR in common adrenal tumours

ACTHR expression in APA was higher than that in NA regardless of the protein or mRNA levels. The mRNA level of ACTHR was 3.52-fold higher in APA than that in NA ($P < 0.05$). Moreover, the protein levels in APA and NA were 0.81 ± 0.04 and 0.49 ± 0.04 ($P < 0.05$), respectively (Fig. 2). The mRNA and protein expression levels of ACTHR were decreased in CPA and NFA compared with those in NA. At the gene level, the former were 2.16 and 2.31 times higher than the latter, respectively (Fig. 2). At the protein level, these values were 0.49 ± 0.04 vs. 0.28 ± 0.06 and 0.49 ± 0.04 vs. 0.31 ± 0.05, respectively ($p < 0.05$).

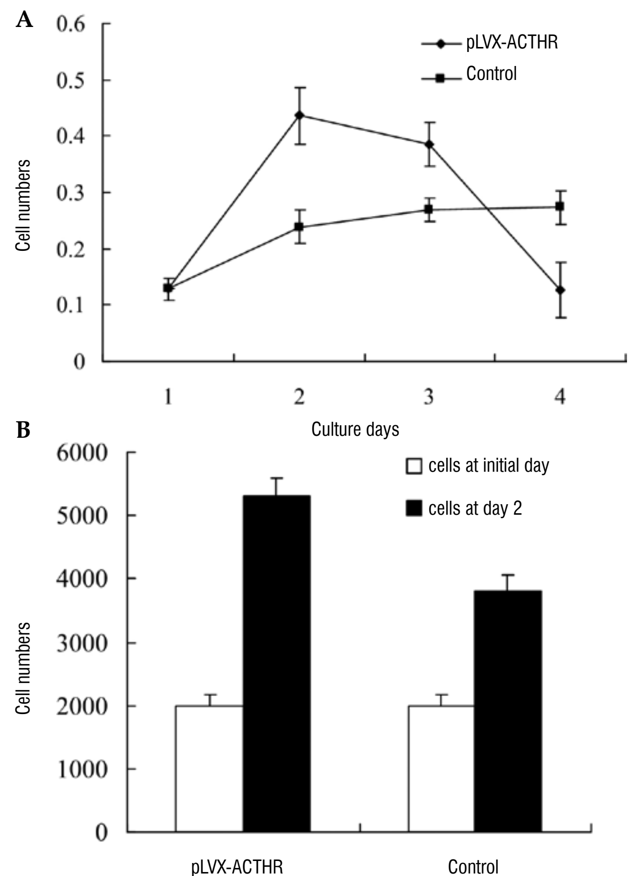

Figure 3A. *Time-dependent proliferation in the ACTHR overexpression H295R cells and control cells. The absorption value of the experimental and control cells were detected in the next 4 d every 24 h, respectively;* **B.** *Effect of ACTHR on cell number after 2 days (peak stage). The numbers of experimental and control cells peaked at 5.3 ± 0.21 x 10^3 and 3.8 ± 0.24 x 10^3 in the second day, respectively ($P = 0.032$)*

Rycina 3A. *Proliferacja komórek H295R z nadekspresją ACTHR i komórek kontrolnych w zależności od czasu. Wartość absorpcji w komórkach eksperymentalnych i kontrolnych mierzono w ciągu 4 następnych dni co 24 godziny;* **B.** *Wpływ ACTHR na liczbę komórek po 2 dniach (maksimum). Liczba komórek eksperymentalnych wzrosła w drugim dniu do maksymalnej wartości, wynoszącej odpowiednio 5,3 ± 0,21 x 10^3 i 3,8 ± 0,24 x 10^3 ($p = 0,032$)*

ACTHR and cell proliferation in H295R cells

H295R cells with high ACTHR expression level were cultured for five days, and cell numbers were counted daily. As shown in Figure 3, the absorption values (OD) increased as the number of days increased in the experimental and control cells. The proliferation of cells overexpressing ACTHR presented a double trend. Proliferative activity in the experimental group was higher than that in the control group in the first three days. This activity peaked on the second day. However, this trend was reversed on the fourth day and became more apparent with time. By contrast, this trend steadily increased in the control cells (Fig. 3). Meanwhile,

the numbers of experimental and control cells peaked at $5.3 \pm 0.21 \times 10^3$ and $3.8 \pm 0.24 \times 10^3$ on the second day, respectively ($p < 0.05$). This result demonstrates the marked influence of high ACTHR expression on cell proliferation.

ACTHR and cell apoptosis

To study the effect of ACTHR on apoptosis, experimental and control cells were cultured for 48 h, and the percentage of apoptotic cells was measured by TUNEL staining. However, no significant differences were observed between the two groups ($p > 0.05$).

Discussion

Unlike other tumours, adrenal neoplasm has many types and complex cell components. In addition, distinct zones secrete different hormones, and the cell characteristics and physiological functions differ. For example, Hornsby et al. reported that adrenal cell proliferation and continuous remodelling usually occurs in the zona glomerulosa (ZG) and zona fasciculate (ZF), and cell death usually occurs in the zona reticularis [6]. In terms of ACTHR, most authors understand its effects indirectly by ACTH stimulation. Payet et al. injected ACTH into rats and found a 16-fold increase in the mitosis rate in ZG, but ACTH had no effect on proliferation in the ZF, which developed marked hypertrophy [7]. Mazzuco et al. revealed that chronic stimulation by ACTH induces a phenotypic change in glomerulosa cells to fasciculata cells [8]. Thus, distinct cell features determine their differences in external stimulation.

In recent years, many authors explored the possible correlation of some genetic alteration with adrenal tumours, for example potassium channel (KCNJ5) in APA and protein kinase A catalytic subunit (PRKACA) in CPA. The majority of them focused on detecting somatic gene mutations in tissue specimens, such as KCNJ5 (p.G151R) and PRKACA (p.L206R). However, the overall prevalence of KCNJ5 or PRKACA mutations was just 40%, and the number of samples was not very large [9, 10]. The conclusions varied with gender, age, pathologic type, and different nationality. The genetic and cellular studies still need to be investigated more thoroughly.

We studied common adrenal tumours in a clinical setting and found that ACTHR expression increased in APA. Based on our previous study, we inferred that the possible pathogenic mechanisms may include the following factors and cell components. First, steroidogenic factor-1 (SF-1) is an important transcription factor that is required for adrenal cell proliferation and steroid hormone secretion. As a target gene, the ACTHR promoter region has abundant SF-1 binding sites. After binding with these sites, SF-1 can promote ACTHR expression [11]. Our previous research confirmed that SF-1 increases in APA [12]. Therefore, SF-1 overexpression in APA may promote ACTHR expression and aldosterone secretion. Second, although angiotensin II is the main regulator of aldosterone, ACTH also has an important function in this process. For APA patients, high ACTHR expression increases aldosterone production despite the normal plasma ACTH concentrations in these patients. Third, ACTHR is crucial in adrenal growth and maintenance. Otherwise, ACTHR mutation often causes adrenal atrophy [13]. Thus, the effect of ACTHR on cell growth may lead to tumorigenesis. Fourth, as the main cell component of APA, ZG has a potential tendency to proliferate and differentiate, and shows high expression of related biological indicators, including ACTHR [14].

By contrast, low ACTHR levels were present in CPA and NFA. The reasons for this result may be summarised as follows. Cortisol, which is mainly produced from ZF, is regulated by ACTH, but has a negative feedback action to ACTH secretion. We inferred that the hypersecretion of glucocorticoid in CPA suppressed ACTH level and reduced ACTHR expression because ACTHR is positively regulated by ACTH [15]. In addition, other pathways (e.g. MRAP1 and AP-1) may be involved in the hypersecretion of glucocorticoid [16, 17]. To date, the expression of ACTHR in CPA was the most controversial issue. Different authors arrived at diverse conclusions from the 1990s until now [2, 18, 19]. On the one hand, CPA has more complicated pathologic types and influence of internal environment than other adrenal tumours. Therefore, there may be selective differences in different publications. On the other hand, different experimental methods and fewer samples also lead to diverse conclusions. Unlike adrenal functional adenomas, NFA did not produce steroid hormone, and the main cell components of NFA were not determined. Thus, NFA probably has a different pathogenesis from functional adenomas. We could not exclude the possibility of tumour type differences or other distinct pathways involved in tumour formation, except ACTHR. Similar to adrenal carcinoma and adenoma, benign and malignant neoplasms have different pathogenic mechanisms even though they occur in the same organ. For example, many authors detected several indicators in adrenal benign and malignant tumours, but they found completely inconsistent expression levels of Ki-67, IGF-II, P450scc, etc. between the two tumour types [20, 21].

To further explore the effect of ACTHR, we performed subsequent cellular research using H295R cells, which are commonly applied in experiments. The results of this study showed that high ACTHR expression initially increased and then decreased the proliferative

activity of H295R cells. Proliferative activity in the experimental group was higher than that in the control group in the first three days. This activity peaked on the second day. However, this trend was reversed on the fourth day and became more apparent with time. This result agreed with previous studies on the double effect of ACTH stimulation [22]. Many scholars proved that the effects of ACTH on adrenocortical hormones and cell growth were mainly reflected in the early stage (from 24 h to 72 h) after stimulation [23]. In this study, we found that this promoting function of ACTHR was time dependent. It may be determined by ACTH signal pathway characteristics and cell growth inhibitory influence during the later high-density growth environment. However, TUNEL apoptosis staining revealed no obvious difference between the two groups. Tumours are often caused by the imbalance between proliferation and apoptosis. Thus, ACTHR may be more likely to induce the proliferation than the apoptosis of H295R cells.

Tumours are commonly caused by multiple factors/ /steps. In this study, we preliminary detected the expression and possible pathogenic influence of ACTHR in common adrenal tumours. Most adrenal tumours have endocrine functions, so the internal environment of an organism and interplay between the environment and organism must be investigated in further research. In-depth studies on ACTHR are expected to become a novel breakthrough in understanding adrenal tumorigenesis and hormone secretion.

Conclusions

Adrenal diseases characterised by hormonal disorders or neoplasms are caused by adrenal tumours or hyperplasia. Tumours are often caused by the imbalance between proliferation and apoptosis. We detected the ACTHR levels in common adrenal tumours and investigated its influence on adrenal tumorigenesis using adrenocortical H295R cells. There were differential expressions of ACTHR in these tumours. Differential ACTHR expression may be determined by different types of adrenocortical tumours. ACTHR showed more obviously an effect on H295R cell proliferation than the apoptosis.

Acknowledgements

This study was supported by the grants from the National Natural Science Foundation of China (No. 81200579).

Disclosure Statement: All the authors have no conflict of interest.

Support grants: This study was supported by the grants from the National Natural Science Foundation of China (No. 81200579).

Authors' contributions:

DH participated in the design of the study, performed the statistical analysis, reviewed the literature, and drafted the manuscript. **TL** participated in the design and coordination of the study, performed the tests, and collected the data. **XW** conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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