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ORIGINAL ARTICLE

Lower expression of ATM and gene deletion is more frequent in adrenocortical carcinomas than adrenocortical adenomas

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Abstract Adrenocortical carcinoma (ACC) is a rare endocrine malignancy accounting for approximately 0.02–0.2% of all cancer deaths. The molecular pathogenesis of ACC has been the hot topic of recent reviews but it is still poorly understood. It is imperative to have a better understanding on the pathophysiology of ACC so as to establish precise diagnosis and effective treatment. This study aims to identify the molecular markers between ACCs and adrenocortical adenomas (ACAs). With MLPA, we checked on 10 ACA and 9 ACC tissue samples. The MLPA results showed deletion on chromosomes 18q, 11q, 11p, and 13q and duplication on chromosomes 3q, 4q, 6p, and 19p. There was a significant difference in the number of aberration copies of the ataxia telangiectasia-mutated (ATM) gene located on chromosome 11q22–q23 between ACCs and ACAs. Five out of 9 (56%) ACC specimens had deletion of ATM ($P = 0.011$). RT-PCR result then demonstrated that ATM mRNA level is lower in ACCs than in ACAs ($P < 0.001$). In addition, immunohistochemistry (IHC) study of the 19 ACA and 18 ACC samples confirmed lower expression of ATM protein in ACCs than in ACAs ($P < 0.001$). The study demonstrated that ATM expression was diminished in ACC than in ACA, suggesting an important role of ATM in the tumorigenesis of ACC.

Keywords Ataxia telangiectasia-mutated gene · Adrenocortical carcinomas · Multiplex ligation-dependent probe amplification

Abbreviations

ACC	Adrenocortical carcinoma
ACA	Adrenocortical adenoma
MLPA	Multiplex ligation-dependent probe amplification
IHC	Immunohistochemistry
ATM	Ataxia telangiectasia-mutated
CGH	Comparative genomic hybridization
RT-PCR	Real-time polymerase chain reaction
AT	Ataxia telangiectasia
DSB	Double strand breaks
DDR	DNA damage response
IGF-II	Insulin-like growth factor II gene
Ens@t	European Network Study of Adrenal Tumor

Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with a prevalence of 1–2 incidences per million. Unfortunately, ACC has metastasized in 40–70% of patients at the time of diagnosis [1], and it accounts for approximately 0.02–0.2%

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of all cancer deaths [2]. Patients present with signs of steroid hormone excess (e.g., Cushing's syndrome, Conn's syndrome, and virilization) or an abnormal abdominal mass [3], and the age distribution is reported as bimodal with the first peak in childhood and the second higher peak in the fourth or fifth decade [4, 5]. The molecular pathogenesis of ACC has been the hot topic of recent reviews but it is still poorly understood [4, 6, 7]. It is imperative to have a better understanding on the pathophysiology of ACC so as to establish precise diagnosis and effective treatment.

It is reported that chromosomal changes accumulated during tumor progression may indicate one of the key mechanisms. Recent studies using comparative genomic hybridization (CGH) provide the evidence for chromosomal alterations in the development and progression of ACCs. As reported by Stephan et al., in most of the chromosomes, commonly shared amplifications appearing in >50% of tumors include regions within chromosomes 4, 5, 7, 12, 16q, and 20. Deleted genomic regions within ACC include portions of chromosomes 1, 2q, 3p, 6q, 9p, 10q, 11, 14q, 15q, 17, and 22q [1]. Additional studies have identified gains and high-level amplifications in chromosomes 7, 14, and 19 only in ACC and not in benign adrenocortical neoplasms, suggesting that these events may be late genetic perturbations in tumorigenesis [8].

However, the ability of CGH to distinguish between different numbers of copies is very limited, and it is difficult to identify which specific gene is involved in the pathogenesis of ACC. To find new candidate genes, we performed multiplex ligation-dependent probe amplification (MLPA) analysis in sporadic ACCs. MLPA, performed by multiplex polymerase chain reaction (PCR) amplification of the hybridized probe, is a robust method for detecting copy number changes in chromosomes. Because of its low cost, reliability, and ease of performance, it has become very popular both as a research and a diagnostic tool and could offer an alternative to CGH studies [9–11]. According to our search in literature, MLPA has never been reported to be used in ACC.

This study aims to identify the molecular markers between ACCs and adrenocortical adenomas (ACAs). We investigated 10 ACA and 9 ACC tissue samples by MLPA analysis and found that several genes could contribute to the tumorigenesis of ACCs. Furthermore, real-time PCR and immunohistochemistry (IHC) were later applied to confirm the previous analysis.

Materials and methods

Subjects and tissues

Nineteen patients (9 women and 10 men; mean age 53.8 ± 14.6 years; range 21–76 years) operatively proven with sporadic adrenocortical tumors were included in this study. The diagnosis was further confirmed morphologically by two experienced pathologists. Of the 19 patients, 9 had ACC (a malignant disease) and 10 were with ACA (a benign lesion). The ACCs were classified according to the most recent Ens@t (European Network Study of Adrenal Tumor) 2008 classification [12].

All of the patients underwent surgery between 2004 and 2009 and were clinically monitored at the Shanghai Clinical Center for Endocrine and Metabolic Diseases in the Ruijin Hospital, Shanghai, P. R. China. The study was approved by the Ethics Committee of Ruijin Hospital, affiliated to Shanghai JiaoTong University School of Medicine and informed consent was obtained from all patients involved.

Conn's syndrome was diagnosed according to the presentations of high blood pressure and/or hypokalemia, with elevated aldosterone and reduced renin level. To confirm the diagnosis, these patients also underwent intravenous salt load test. Cushing's syndrome was diagnosed with increased cortisol and 24 h urinary free cortisol, moreover, plasma and urinary cortisol were unresponsive to the high dose dexamethasone suppression test. Testosterone, follicle-stimulating hormone, luteinizing hormone, estrogen, and dehydroepiandrosterone sulfate were also measured.

The adrenal tumors selected for this study were all sporadic adrenocortical tumors. All the analyzed tumor tissues were derived from the primary lesions. Tumor tissues were snap-frozen in liquid nitrogen immediately after the surgery. Genomic DNA was then extracted from fresh-frozen tumor specimens by standard procedures using a DNA extraction kit (Qiagen) according to the manufacturer's guide. All adrenal tumors were histologically analyzed before DNA extraction to ensure preparation of a population of the tumor cells without cross-contamination with the normal cells.

Histological features, including high mitotic rate, atypical mitoses, high nuclear grade, low percentage of clear cells, necrosis, diffuse architecture of tumor, capsular invasion, sinusoidal invasion, and venous invasion, were carefully analyzed according to the method of Weiss. A score of more than 3 was considered to be malignant [13, 14].

MLPA reaction

MLPA analyses were performed by using SALSA P006 Chromosomal Aberration MLPA kits (MRC-Holland, Amsterdam, The Netherlands; details available at http://www.mlpa.com) according to the manufacturer's protocol. The P006 kit includes 41 genes loci, which were selected because of their reported involvement in cancer. Five μl of DNA samples were heated at 98°C for 5 min. Normal DNA of males

and females for control were included in the same reaction. After the addition of the probe mix, samples were heated for 1 min at 95°C and then incubated for 16 h at 60°C. Ligation of the annealed oligonucleotide probes was performed for 15 min at 54°C in a buffer containing Ligase-65 enzyme. Multiplex PCR was carried out using Cy5-primers, dNTPs and SALSA polymerase. PCR was performed for 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. All the reactions were carried out in PTC-225 DNA Engine Tetrad (MJ Research Inc., San Francisco, CA, USA). PCR products were analyzed using Beckman Coulter CEQ 8800 sequencer (Beckman Coulter, Fullerton, CA, USA).

MLPA data analysis

Data analysis was performed with fragment analysis module. The Gene Scan data of sizes and peak height of multiplex PCR products were exported to an Excel file. All the expected MLPA products were normalized by dividing each peak height by the combined peak height of all peaks in that lane (relative peak height). The relative copy number for each probe was expressed as a ratio of the relative peak height for each locus of the sample to that of the normal sex-matched control. The reference median peak heights were obtained from normal tissue samples (both males and females), each of which was analyzed at least thrice independently. There were no significant differences in the outcome of normalized areas and heights in most of the samples. A difference was considered significant if the both ratios calculated from areas and heights were less than 0.75 (loss) or higher than 1.3 (gain). Then, the statistical analysis was performed with the SPSS 16.0 software package. Fisher's test was used to evaluate the difference between ACCs and ACAs. P value <0.05 was considered to be statistically significant.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen) and reverse transcribed from an Random Primers (Promega) according to the manufacturer's instructions. Real-time PCR was performed by an Roche LightCycler 480 system using SYBR® Premix Ex Taq™ (Takara). The conditions of real-time PCR were conducted as follows: denaturation at 95°C for 10 s, 40 cycles at 95°C for 10 s, 60°C for 30 s. A melting curve was built in the temperature range of 60–95°C at the end of the amplification. PCRs were performed in triplicate, and GAPDH was amplified as an internal control. The primers sequences used for real-time PCR were designed as follows: GAPDH (accession no. NM_002046.3) forward primer 5′-ATGGGGAAGGT GAAGGTCG-3′ and reverse primer 5′-GGGGTCATTGA TGGCAACAATA-3′; GAPDH (accession no. NM_000 051.3) forward primer 5′-ACGTTACATGAGCCAGCA AAT-3′ and reverse primer 5′-GAAAATGAGGTGGATT AGGAGCA-3′.

Immunohistochemistry

During the process of IHC, the following steps were taken to minimize sampling variability: all samples were (1) run in parallel and each treated identically, (2) examined under identical conditions, and (3) repeated at least twice. All samples were done by the same pathologist. At the time of examination, the pathologist was blinded to the diagnosis and other data. IHC was performed on formalin-fixed paraffin embedded tissue sectioned at 4 μm onto the slides. Slides were deparaffinized and re-hydrated. Then, the sections were immersed in sodium citrate buffer adjusted to pH 6.0 and heated in a microwave oven for 5 min at 100°C, next treated with 0.3% H_2O_2 for 30 min to inhibit peroxidases, then blocked with 2% normal rabbit serum for 30 min. The slides were incubated overnight in a solution of ataxia telangiectasia-mutated (ATM) monoclonal antibody [Abcam Rabbit monoclonal to ATM (ab32420)] diluted to 1:100 with the same buffer. The primary antibody binding was demonstrated with standard avidin–biotin-peroxidase complex technique (vector pk-7200). The slides were developed using diaminobenzidine and counterstained with hematoxylin. B cell chronic lymphocytic leukemia tissue, known to absent ATM protein, was used as the positive control. Negative controls were incubated with no primary antibody. The ATM expression was scored according to the proportion of positive cell numbers of ATM staining. The results were assigned to four groups (3+, >60%; 2+, 30–60%; +, <30%; −, negative). The data was then entered into a ZEISS Axioplan 2 microcomputer, and statistical analysis was carried out using SPSS 16.0 software package. The Kruskal–Wallis test was applied to evaluate the relationship among the percentage of ATM immunoreactivity between ACAs and ACCs.

Results

Clinical characteristics

Nine patients were pathologically diagnosed with ACC and 10 patients with ACA (Table 1). Six out of the 10 ACA patients were diagnosed with Conn's syndrome. The other four ACA patients were diagnosed with Cushing's syndrome. In the ACC group, one patient presented with the extra secreting of aldosterone, while six presented with hypercortisolemia and two with non-functional carcinoma. Two cases of ACC group were also found to have virilization. Six of 9 (67%) of the ACC patients already had

Table 1 The clinical characteristics of ACAs and ACCs patients in MLPA analysis

	ACAs	ACCs
Number of patients	10	9
Sex (M/F)	4/6	5/4
Age (average ± SD)	49.6 ± 15.7	55.4 ± 12.0
Presentations		
Incidentaloma (%)	0	2 (22%)
Conn's syndrome (%)	6 (60%)	1 (11%)
Cushing's syndrome (%)	4 (40%)	6 (67%)
Virilization (%)	0	2 (22%)
Tumor size (mm)	32.5 ± 21.4	75.9 ± 35.6

metastatic spread at the time of diagnosis, including metastasis to the lung, kidney, liver, and lymph nodes. Not surprisingly, three ACC patients underwent a second operation due to the recurrence of the tumor lesions on the adrenals. Weiss scores of nine ACCs were also presented (Table 2).

MLPA

MLPA analysis suggested that all 9 malignant and 10 benign adrenocortical tumors have multiple copy number losses or gains (Fig. 1). The losses or gains of the genes represented the deletion or the duplication of the chromosome, where the genes are located. In this study, deletion showed on chromosomes 18q, 11q, 11p, and 13q, while duplication was found on chromosomes 3q, 4q, 6p, and 19p. The most obvious difference between ACCs and ACAs was ATM gene locus on chromosome 11q22–q23. In ACCs, loss of ATM gene was detected in 5 out of the 9 specimens (56%), however, none of the 10 ACA samples showed alteration of ATM gene ($P = 0.011$, Fisher's test). The number of aberrations in ACAs and ACCs ranged from 1 to 15 and 5 to 25, and the average copy number changes were 7.0 and 11.7, respectively. Specifically, the mean number of gains and losses per tumor was both 3.5 in ACAs, with the range of 1–8 and 0–7. By contrast, the average number of gains per tumor was 5.8 in ACCs, indicating 0.1 lower than that of losses per tumor, and the respective ranges of gains and losses were 3–13 and 2–12 relatively.

RT-PCR

RT-PCR analysis of tumor RNA was carried out to verify mRNA differences in 29 ACAs and 13 ACCs. As a result, ATM mRNA level was significantly lower in ACCs than in ACAs, which was in accordance with the MLPA genetic copy number alteration analysis ($P < 0.001$, Student's t test; Fig. 2).

Immunohistochemistry

Immunohistochemical analysis was performed on 37 samples (19 ACAs, 18 ACCs) to determine the expression of ATM protein in adrenocortical tumors. Strong positive staining of ATM protein was found in the nuclei of the adrenocortical cells (Fig. 3). In ACAs, 95% of the specimens showed the staining of 3+, 2+, and +. However, none of ACCs were stained with 3+, while 14 out of 18 (78%) of the carcinomas showed a negative staining. The Kruskal–Wallis test indicated significantly lower ATM protein expression in ACCs, again confirming the results of MLPA and RT-PCR ($P < 0.001$, K–W test; Table 3).

Discussion

With the method of MLPA, we found that all the samples including ACCs and ACAs had genetic aberrations. The method of MLPA could detect more genetic copy numbers alteration than CGH because MLPA had a higher resolution (40 bp) than CGH (10–20 Mb). MLPA was more

Table 2 The clinical characteristics of ACC patients

Patient no.	Sex (M/F)	Age (yr)	Operation times	Tumor size (mm)	Metastatic location	Hormonal pattern	Weiss score
1	M	72	2	70 × 60 × 60	Kidney	NS	5
2	M	40	1	250 × 120 × 100	Lymph node	GC	4
3	M	60	2	100 × 100 × 100	None	GC	4
4	F	46	2	50 × 50 × 50	None	GC	4
5	F	64	1	80 × 60 × 90	Lung	GC+A	6
6	F	70	1	80 × 80 × 80	None	GC+A	5
7	M	60	1	60 × 60 × 60	Lung	NS	5
8	M	55	1	100 × 110 × 100	Kidney, liver, lung	GC	8
9	F	38	1	58 × 54 × 42	None	MC	4

GC glucocorticoid secretion, *A* androgen secretion, *MC* mineralocorticoid secretion

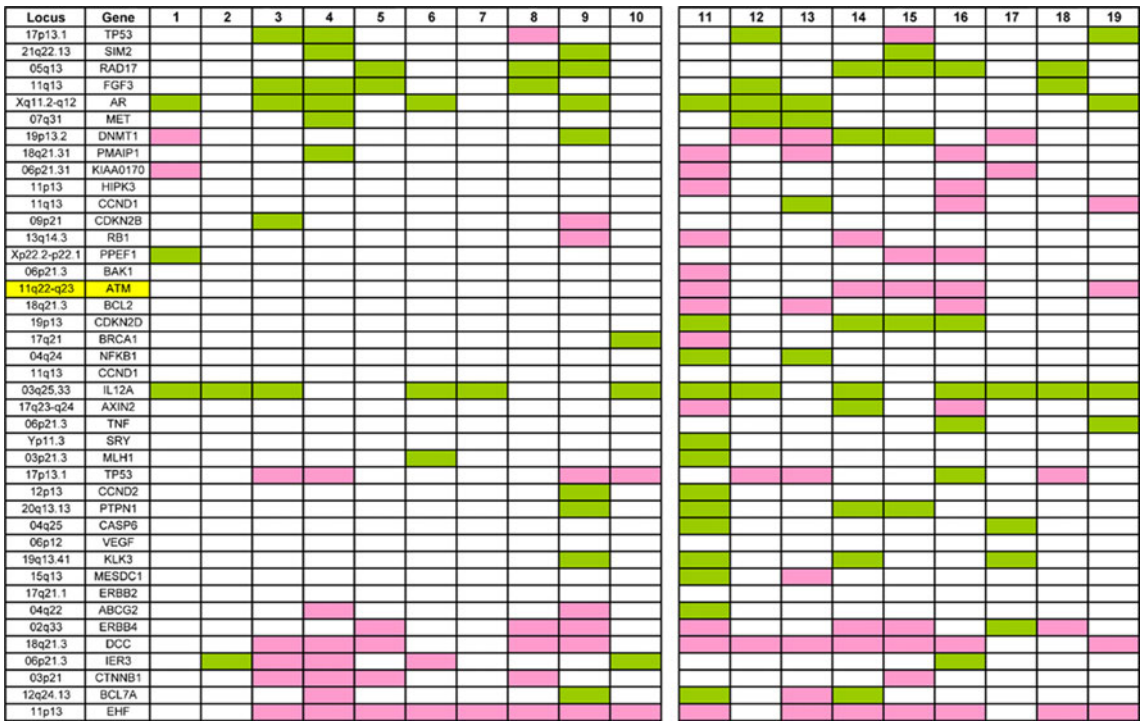

Fig. 1 Summary of genetic aberrations of 19 benign and malignant adrenocortical tumors detected by SALSA P006 Chromosome Aberration Kits with a total of 41 genes. The *first line* indicates the numbers of the patients. The left column shows 10 adrenocortical adenoma patients, while the right column demonstrates 9 adrenocortical carcinoma patients. Colors used: *green* gain; *red* loss; *white* no genetic aberration. ATM is emphasized in *yellow*

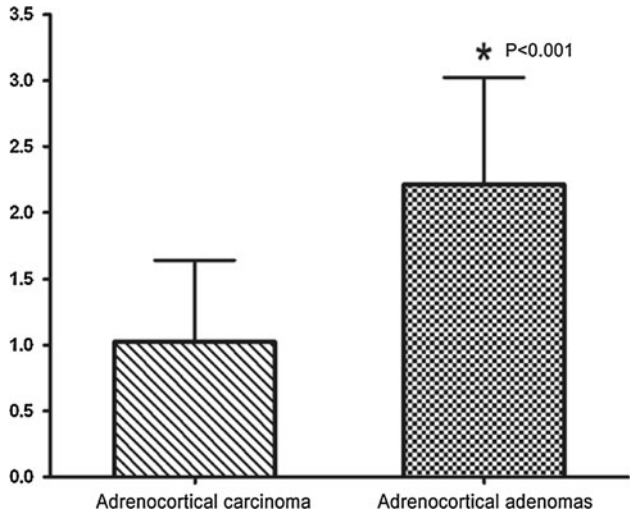

Fig. 2 RT-PCR analysis of 13 ACC and 29 ACA samples. ATM mRNA level was significantly lower in ACCs than in ACAs ($P < 0.001$)

sensitive to detect the gains or losses of small chromosome parts than CGH.

Several hereditary tumor syndromes are associated with the formation of benign or malignant adrenocortical tumors. Mutations of the p53 tumor suppressor gene have been implicated because of the association of ACC with Li-Fraumeni syndrome and confirmed by mutational analyses [15–18]. The insulin-like growth factor II gene (IGF-II) is involved in the pathogenesis of both familial ACCs, as is the case in Beckwith–Wiedemann syndrome, as well as in sporadic ACCs [19–21]. Dysregulation or rearrangement at 11p15.5 results in significant up-regulation of IGF-II in ACC, resulting in an autocrine stimulatory loop.

In our study, the most obvious difference between the two groups was ATM gene. ATM gene is associated with ataxia telangiectasia (AT). AT is a rare, autosomal recessive disorder characterized by increased genetic instability, radiosensitivity, neurodegeneration, oculocutaneous telangiectasia, immune defects, and cancer predisposition [22]. ATM gene consists of 66 exons encoding a large protein kinase that orchestrates the recognition and repair of radiation-induced DNA double strand breaks (DSB) [23–26]. In addition, it regulates cell cycle and acts against DNA damage response (DDR). It is found that potent DDR is present in early lesions and persists in tumors in somatic models [27]. A DDR pathway is responsible for p53 stabilization, apoptosis, and senescence. This response is caused by DNA replication stress, replication fork collapse, which may follow hyperproliferation, leading to the recruitment of the serine-threonine kinase ataxia telangiectasia-mutated (ATM) to the damaged chromosomal sites [28]. What's more, ATM phosphorylates the histone variant H2AX (hereafter termed γH2AX) and p53 binding

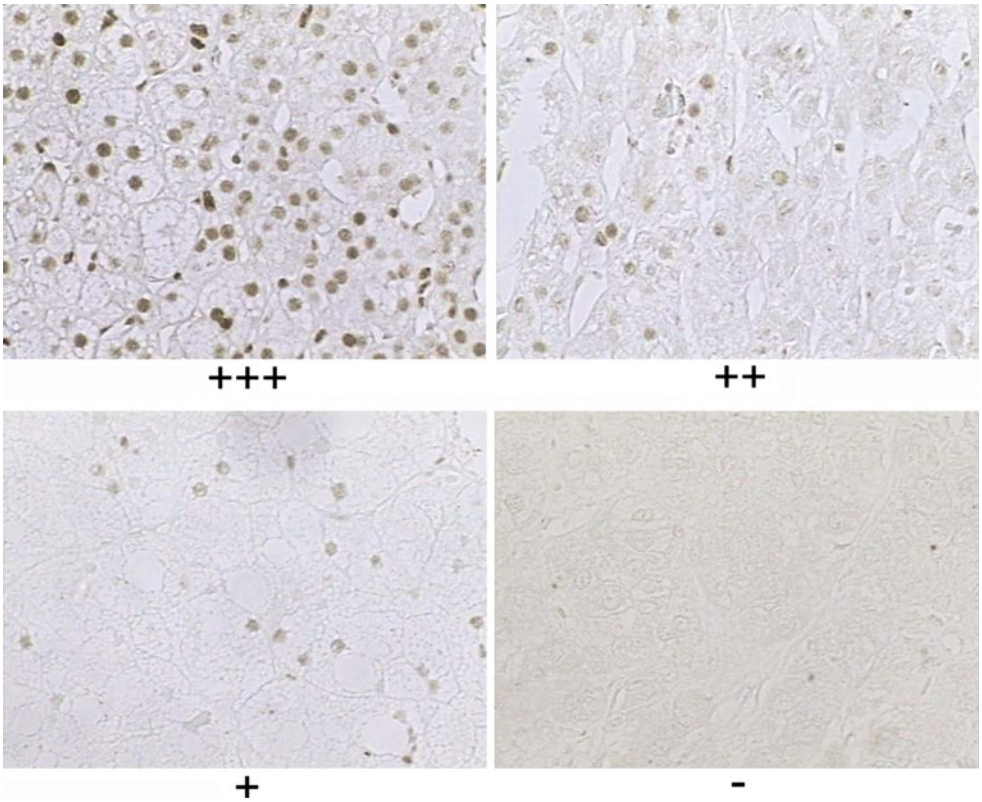

Fig. 3 Immunohistochemistry of ATM nuclear protein in adrenocortical tumors. Indexes were labeled as follows: Negative staining cells, index as (−); <30% positive staining cells, index as (+); 30–60% positive staining cells, index as (2+); >60% positive staining cells index as (3+). Magnification, ×200

Table 3 Immunohistochemistry of the ATM gene revealed that the majority of ACAs showed positive staining (3+, 2+, and + accounted for 95%)

	ACAs (%)	ACCs (%)	*P* value
3+	6 (32)	0 (0)	<0.001
2+	9 (47)	2 (11)	
+	3 (16)	2 (11)	
−	1 (5)	14 (78)	
N	19	18	

However, none of ACCs were stained as 3+ and 78% of ACCs were of negative staining ($P < 0.001$)

protein 1 (53BP1), which are also recruited to DSBs [29, 30]. Hence, DDR signaling likely plays a critical role in blocking progression to tumors.

According to the literature, ATM has been associated with increased risk of development of lymphoma, leukemia, and breast cancer [31, 32]. However, none of the studies about ATM expression in adrenocortical tumors was reported. In our study, MLPA results were in concord with the previous CGH study on ACC. A decreased copy number of ATM was identified in 56% of the ACCs, whereas this finding was not detected in the ACAs. Furthermore, ATM mRNA and protein expression was found to be significantly lower in ACCs than in ACAs. This data indicate the connection between the loss of copy number of the ATM gene and its low expression with ACC, showing the importance of this gene in the tumorigenesis. No doubt that a larger number of samples should be included to the further investigation of ACC and more in vitro experiments need to be conducted to give a precise state of mechanism of the disease.

Yet, there are still some limitations of our study. First, with a prevalence of 1–2 incidences per million of ACC, we have only collected <20 ACC samples during the past years. As a result, the tissues used in this study were relatively small: 10 in MLPA, 13 in RT-PCR, and 19 in IHC. Second, in the ACA group of MLPA, non-functional tumors were not included. Non-functional adrenal tumors were usually small and demonstrated benign on CT scan. These patients were followed-up according to the clinical guideline of adrenal incidentaloma [33], so we lacked this kind of tissue, which resulted in only Conn's and Cushing's syndrome in the ACA group.

To conclude, high frequency of deletion of ATM is detected in sporadic ACC. RT-PCR and IHC analysis then confirmed the lower expression of ATM in ACCs than in ACAs, suggesting that ATM might play a role in the tumorigenesis of ACC.

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Conflict of interest The authors declare that they have no conflict of interest.

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