Integration of transcriptome and methylome analysis of aldosterone-producing adenomas

Masanori Murakami¹, Takanobu Yoshimoto¹, Kazuhiko Nakabayashi², Kyoichiro Tsuchiya¹, Isao Minami¹, Ryotaro Bouchi¹, Hajime Izumiyama^{1,3}, Yasuhisa Fujii⁴, Kosei Abe², Chiharu Tayama², Koshi Hashimoto^{1,5}, Takayoshi Suganami^{6,7}, Ken-ichiro Hata², Kazunori Kihara⁴ and Yoshihiro Ogawa^{1,8}

¹Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan, ²Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan, ³Center for Medical Welfare and Liaison Services, Departments of ⁴Urology, ⁵Preemptive Medicine and Metabolism and ⁶Organ Network and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan, ⁷Japan Science and Technology Agency, PRESTO, Tokyo 102-0076, Japan and ⁸Japan Science and Technology Agency, CREST, Tokyo 102-0076, Japan

Correspondence
should be addressed
to T Yoshimoto
Email
tyoshimoto.mem@tmd.ac.jp

Abstract

Objective: The pathophysiology of aldosterone-producing adenomas (APA) has been investigated intensively through genetic and genomic approaches. However, the role of epigenetics in APA is not fully understood. In the present study, we explored the relationship between gene expression and DNA methylation status in APA.

Methods: We conducted an integrated analysis of transcriptome and methylome data of paired APA-adjacent adrenal gland (AAG) samples from the same patient. The adrenal specimens were obtained from seven Japanese patients with APA who underwent adrenalectomy. Gene expression and genome-wide CpG methylation profiles were obtained from RNA and DNA samples that were extracted from those seven paired tissues.

Results: Methylome analysis showed global CpG hypomethylation in APA relative to AAG. The integration of gene expression and methylation status showed that 34 genes were up-regulated with CpG hypomethylation in APA. Of these, three genes (*CYP11B2*, *MC2R*, and *HPX*) may be related to aldosterone production, and five genes (*PRRX1*, *RAB38*, *FAP*, *GCNT2*, and *ASB4*) are potentially involved in tumorigenesis.

Conclusion: The present study is the first methylome analysis to compare APA with AAG in the same patients. Our integrated analysis of transcriptome and methylome revealed DNA hypomethylation in APA and identified several up-regulated genes with DNA hypomethylation that may be involved in aldosterone production and tumorigenesis.

European Journal of
Endocrinology
(2015) 173, 185–195

Introduction

Acquired epigenetic changes, such as DNA methylation at CpG dinucleotides, play a role in a variety of physiologic and pathophysiologic conditions (1). It has been shown that global DNA hypomethylation is associated with chromosomal instability in oncogenic processes (2). In addition, aberrant DNA methylation patterns have been shown to alter transcriptional regulation in specific genes, such as tumor suppressor genes, and to thereby lead to

tumor development (2). Recent studies have also revealed aberrant DNA methylation status in adrenocortical adenoma and carcinoma (3, 4, 5). However, the pathophysiologic significance of differentially methylated genes in aldosterone-producing adenoma (APA) is unknown.

APA is a subtype of primary aldosteronism (PA), which is the most common form of endocrine hypertension (6, 7) and is closely associated with severe cardiovascular

complications relative to primary hypertension (8). From the therapeutic viewpoint, this classification is important because APA is curable by unilateral adrenalectomy. To understand the pathogenesis of APA, it is critical to know how tumorigenesis occurs in the adrenal cortex via autonomous aldosterone production independent of the systemic renin angiotensin system.

Although approaches to dissecting the pathogenesis of PA have been relatively limited, recent studies with new generation exome sequencing and transcriptomic techniques have provided clues to the molecular mechanisms that underlie autonomous aldosterone production in PA (9). Indeed, somatic mutations of a potassium channel (*KCNJ5*) (10), ATPases (*ATP1A1* and *ATP2B3*) (11, 12), and a voltage-dependent calcium channel (*CACNA1D*) (11, 13) as well as the up-regulation of genes related to steroidogenesis and calcium signaling pathways (9, 14, 15, 16) may result in the sustained opening of membrane voltage-dependent calcium channels and the activation of the downstream calcium signaling pathway, which thereby leads to the up-regulation of *CYP11B2*, a rate-limiting enzyme in the aldosterone biosynthetic pathway, and thus to the production of autonomous aldosterone. With regard to tumorigenesis of APA, a couple of studies have suggested the involvement of the Wnt/ β -catenin and sonic hedgehog signaling pathways (17, 18), but their detailed pathogenic roles are poorly understood. Importantly, APA mostly develops in middle-aged adults in a sporadic manner. It is therefore conceivable that in addition to genetic factors, acquired and environmental factors such as epigenetics may all play a role in the molecular pathogenesis of APA.

In the present study, to gain insight into the role of epigenetic change in the pathogenesis of APA, we performed an integrated analysis of transcriptome and methylome of APA by comparing APAs with their adjacent adrenal glands (AAG). The study demonstrated global DNA hypomethylation in APA and identified several genes that are up-regulated with DNA hypomethylation and are thus potentially associated with the pathophysiology of APA through a DNA methylation mechanism.

Subjects and methods

Subjects

Adrenal tissues were obtained from seven patients with APA who underwent adrenalectomy at the Tokyo Medical and Dental University Hospital. All of the patients satisfied the diagnostic criteria of PA based on the standard sodium

chloride loading test, captopril test, and/or posture test. Adrenal vein sampling with adrenocorticotrophic hormone (ACTH) stimulation was performed and demonstrated unilateral aldosterone hypersecretion in all seven of the cases. The adrenal venous sampling (AVS) data are shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Successful adrenal vein cannulation was determined if the selectivity index (SI) was >5 . The SI was defined as the ratio of plasma cortisol concentration in the adrenal vein to that in the inferior vena cava. The lateralization index (LI) was defined as the ratio of plasma aldosterone to cortisol in the dominant side to that in the nondominant side. The contralateral (CL) ratio was defined as the ratio of plasma aldosterone to cortisol in the nondominant side to that in the inferior vena cava. CL suppression was defined as a CL value of <1 (19). The criteria for unilateral subtype by AVS after cosyntropin administration (a bolus infusion of 250 μ g cosyntropin) included the following: i) an LI of ≥ 2.6 and/or ii) a CL ratio of <1 and/or iii) unilateral plasma aldosterone concentration (PAC) in the adrenal vein of $\geq 14\,000$ pg/ml according to the Japan Endocrine Society guideline (20). The clinical and laboratory features of the patients studied are summarized in [Table 1](#). APA and AAG tissues were macroscopically dissected and stored at -80°C . The study protocol was approved by the Ethics Committee for Human Research at the Tokyo Medical and Dental University (no. 1371). Written informed consent was obtained from all of the participants.

Extraction of genomic DNA and total RNA

Genomic DNA and total RNA samples were isolated from a pair of APA and AAG tissues from each of the seven patients using an All Prep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Detection of somatic mutations of *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* by PCR and direct sequencing

Total RNA samples from APA and AAG were reverse transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan). In order to sequence *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D*, 1.0 μ l of cDNA was subjected to PCR using TaKaRa Ex Taq Hot Start Version (Takara-bio, Shiga, Japan). The primers used for the PCR and DNA sequencing (10, 11, 12, 13, 21, 22, 23, 24) are shown in [Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article. The PCR products were purified using electrophoresis (2% agarose gel) and a

Table 1 Clinical and genetic features of the APA patients^a.

Case no.	Age	Sex	Tumor size (mm)	Somatic mutations	SBP (mmHg)		DBP (mmHg)		Number of antihypertensive drugs		Serum potassium (mEq/l)		Potassium chloride supplementation (mEq/day)		PAC (pg/ml)		PRA (ng/ml per h)	
					Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	47	F	15	KCNJ5 p.G151R	131	100	84	70	1	0	3.1	4.2	32	0	260	139	0.4	1.3
2	42	F	14	KCNJ5 p.G151R	130	120	65	80	2	0	2.6	4.2	32	0	598	75	0.2	1.9
3	34	F	20	KCNJ5 p.L168R	160	128	107	93	1	0	3.4	4.3	24	0	232	61	0.1	0.1
4	49	F	15	KCNJ5 p.G151R	147	135	91	57	1	0	3.7	4	24	0	448	48	0.1	0.1
5	43	F	17	KCNJ5 p.G151R	173	123	104	79	2	1	3	4.3	16	0	665	67	1.1	1.5
6	52	F	18	KCNJ5 p.G151R	134	120	90	80	3	1	3.6	5	0	0	675	67	0.2	0.4
7	65	M	13	ATP1A1 p.V332G	122	121	75	73	2	1	3.2	4.7	0	0	283	52	0.2	0.9

APA, aldosterone-producing adenoma; SBP, systolic blood pressure; DBP, diastolic blood pressure; PAC, plasma aldosterone concentration.

^aRelevant data were collected pre- and post-surgery. Pre indicates the value obtained before surgery, and post indicates the value obtained after the removal of the APA. Each case number corresponds to the number presented in the heat map figures.

QIAEX II Gel Extraction Kit (Qiagen) before being directly sequenced by an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems).

Gene expression microarray analysis

RNA samples obtained from each pair of seven APA and AAG tissues were subjected to microarray analysis using a SurePrint G3 Human GE 8x60K Microarray (Agilent, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. Total RNA samples (100 ng) were amplified and labeled with Cy3 using a Low Input QuickAmp Labeling Kit (Agilent). The cRNAs obtained were fragmented for 30 min at 60 °C in the dark, and 600 ng of them were hybridized on the microarray for 17 h at 65 °C. After washing, the slides were scanned with the Agilent Microarray Scanner G2505B. Feature Extraction Software (version 10.7.3.1; Agilent) was used to calculate signal intensities from the scanned images. Raw intensity data were imported into the Subio Platform Software version 1.16 (<https://www.subio.jp/products/platform>), normalized (75th percentile global normalization), and subjected to further analysis, including hierarchical clustering and the selection of differentially expressed genes. The present analysis mainly focused on up-regulated genes in APA, such as those described in previous studies based on the comparison between APA and AAG (15, 16).

Quantitative real-time PCR

Total RNA samples used for microarray analysis were reverse transcribed into cDNA using ReverTra Ace (Toyobo). Quantitative real-time PCR (qPCR) was performed with a StepOnePlus Real-time PCR System using Fast SYBR Green Master Mix Reagent (Life Technologies, Carlsbad, CA, USA) as described previously (25). *ACTB* was used as an internal reference gene. The primers used for the qPCR are described in [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article.

DNA methylation profiling analysis

DNA samples obtained from seven paired APA and AAG tissues were used for methylation profiling analysis. One microgram of DNA was bisulfite-converted using an EpiTect Plus DNA Bisulfite Kit (Qiagen). The converted DNA (300 ng) was assayed with an Infinium Human-Methylation450 BeadChip Array Kit (Illumina, San Diego, CA, USA). The BeadChips assess the methylation status at >485 000 individual CpG sites, covering 99% of RefSeq

genes and 96% of CpG islands. Each DNA sample first underwent an overnight isothermal whole-genome amplification step. Amplified DNA was fragmented, precipitated, and resuspended. Samples were hybridized to BeadChips overnight at 48 °C in a hybridization oven (Illumina). Hybridized arrays were processed through a single-base extension reaction on the probe sequencing using dinitrophenol- or biotin-labeled nucleotides, with subsequent immunostaining. The BeadChips were then coated, dried, and imaged on an Illumina HiScan (Illumina). β values, the ratio of the normalized intensity of the methylated bead type to the combined normalized locus intensity, ranging from 0 (unmethylated) to 1 (completely methylated), were calculated using GenomeStudio version 2011.1 (Illumina).

Integration of gene expression array and DNA methylation BeadChip data

To integrate the DNA methylation profiling data and gene expression array data, the average β values were calculated for the six functional gene regions (transcription start site (TSS) 1500, TSS200, 5'-UTR, the first exon, gene body, and 3'-UTR) among the 31 240 GenBank accession numbers listed as UCSC_REFGENE_ACCESSION in the probe annotation provided by Illumina (HumanMethylation450_15017482_v.1.1.csv). The normalized signal intensity and flag data obtained by the SurePrint G3 Human GE 8x60K Microarray and the average β values just described were merged using GenBank accession numbers for matching (20 424 accession numbers were common between the two platforms).

Gene ontology analysis of differentially methylated genes

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to determine enriched gene ontology (GO) terms and KEGG pathways within the selected gene groups (26).

Data deposition

The data used in the present publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through Gene Expression Omnibus (series accession no. GSE60044 for DNA methylation array and gene expression array).

Statistical analysis

The fold change in the qPCR data that compared APA and AAG was analyzed using Wilcoxon's signed rank tests. The microarray data that compared APA and AAG were analyzed using paired *t*-tests, and *P* values were calculated. Differentially expressed genes (*P* value of <0.05) were selected for GO analysis. Adjusted *P* values were also calculated with Benjamini and Hochberg false discovery rate correction for the reference.

Results

Patient information

Table 1 shows the clinical and endocrinological characteristics of the seven patients with APA before and after adrenalectomy. All of the patients exhibited hypertension with hypokalemia, low plasma renin activity (PRA), and high PAC. After adrenalectomy, they all showed reduced blood pressure with normalization of PAC and serum potassium levels. PRA values and/or PRA:PAC ratio were normalized after adrenalectomy in all of the patients except for cases 3 and 4, whose PRA measurements were only available after surgery and who were not available for follow-up. All of the resected adrenal specimens revealed only one solitary adrenal nodule that was histologically diagnosed as adrenocortical adenoma. We examined previously reported somatic mutations of *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* in all of the APA specimens; five out of seven of the cases carried a glycine to arginine substitution at codon 151 (p.G151R), and one case carried a leucine to arginine substitution at codon 168 (p.L168R) in *KCNJ5*. One case with no mutation in *KCNJ5* carried a valine to glycine substitution at codon 332 (p.V332G) in *ATP1A1*. We found no mutation in *ATP2B3* and *CACNA1D* in all seven of the patients. We therefore concluded that all of the cases examined represented a florid phenotype of PA; all of them had distinct solitary APA and were hypokalemic, and most of them had a somatic *KCNJ5* mutation (exclusively the women). The present results are compatible with previous reports that somatic mutations of *KCNJ5* are common among Japanese APA patients (27, 28).

Transcriptome analysis

Global changes in gene expression associated with APA were evaluated and compared to those with their paired AAG using an Agilent SurePrint G3 Human GE 8x60K

Figure 1

Heat map representation of differentially expressed genes in APA as compared to AAG tissues from the seven patients. Genes expressed with more than a twofold change or less than a 0.5-fold change ($P < 0.05$) in APA as compared to AAG were selected (1730 genes) and analyzed by average linkage clustering. The clustering feature clearly shows differential gene expression pattern between APA and AAG. The heat map color indicates the expression value of each gene in the corresponding sample based on the $\log_2$ of signal strength (see color bar).

Microarray. Unsupervised hierarchical clustering analysis revealed differential gene expression patterns between APA and AAG (Fig. 1). We identified 277 genes that were up-regulated (more than twofold, $P < 0.05$ by paired t -test) in APA (the fold change was the ratio of average expression between the APA and AAG groups) (Supplementary Table 3). To validate the transcriptome analysis, qPCR analysis was performed for seven genes (*CTNND2*, *CYP11B2*, *MC2R*, *ATP2B3*, *HOXA7*, *HOXA9*, and *HOXB9*). The qPCR data obtained were roughly comparable to the microarray data (Fig. 2). The genes listed (Supplementary Table 4, see section on supplementary data given at the end of this article) included those that have been described in previous transcriptome studies of PA, such as *PCP4*, *CLRN1*, *HTR4*, *MYB*, *ALDH1A2*, and *CYP11B2* (15, 16). We identified several genes that were up-regulated in APA that were not reported previously (Supplementary Table 4). These included the *HOX* gene family, which has been shown to play a crucial role in adrenal zonation (29). A heat map representation of the *HOX* gene family revealed increased expression of *HOXA7*, *HOXA11*, and *HOXB9* in APA relative to AAG (Fig. 3).

GO analysis of differentially up-regulated genes in APA showed that GO terms such as 'metal ion transport', 'cation transport', and 'calcium ion transport' were enriched with significant P values (Supplementary

Table 5A, see section on supplementary data given at the end of this article). KEGG pathway analysis revealed significant involvement of the 'Ca signaling pathway' (Supplementary Table 5B).

Methylome analysis

We examined the genome-wide CpG methylation pattern in APA and AAG using Illumina Infinium Methylation 450 BeadChips. The CpG methylation patterns of APAs were distinct from those of the AAGs as calculated by the mean β value for the entire probe set (485 764 probes) with unsupervised hierarchical clustering analysis (representative data from TSS200 probes set are shown in Fig. 4A). Scatter plotting of the average β values indicated invariable CpG hypomethylation status in APA relative to AAG (Fig. 4B). We next compared the distribution of CpG methylation status between APA and AAG by calculating the average $\Delta\beta$ values at each functional gene region (TSS1500, TSS200, 5'-UTR, the first exon, gene body, and 3'-UTR). The average $\Delta\beta$ values in more than half of the seven pairs, ≥ 0.1 and ≤ -0.1 , were defined as hypermethylation and hypomethylation respectively. Although CpG hypermethylation was observed sparsely (0.06–0.22% of the functional gene regions tested; Fig. 4C), 3.2–10.8% was CpG hypomethylated in APA relative to AAG (Fig. 4C). These observations suggest that modest but global CpG hypomethylation at the entire functional gene region is a distinct characteristic of APA relative to AAG.

Figure 2

Confirmation of microarray result by qPCR. Relative mRNA levels were calculated for each of the seven APA–AAG pairs and are presented as box plots. * $P < 0.05$.

Figure 3

Heat map representation from microarray analysis of *HOX* gene expression. The heat map color indicates the expression value of each gene in the corresponding sample based on the $\log_2$ of signal strength (see color bar).

Integration of the transcriptome and methylome analysis

To explore the relationship between increased gene expression and CpG hypomethylation at the promoter regions (TSS200 and TSS1500), we integrated the transcriptome and methylome analysis. We found 34 genes

that were up-regulated with CpG hypomethylation in APA relative to AAG (Table 2). *CLRN* was counted twice in the table, because two alternative promoters of *CLRN* were hypomethylated in the TSS1500/TSS200 region in APA relative to AAG. They included genes related to aldosterone production (*CYP11B2*, *MC2R*, and *HPX*), tumorigenesis (paired related homeobox 1 (*PRRX1*), RAS oncogene family (*RAB38*), fibroblast activation protein (*FAP*), glucosaminyl *N*-acetyl transferase 2, I-branching enzyme (I blood group) (*GCNT2*), and ankyrin repeat and SOCS box containing 4 (*ASB4*)), and differentiation (calmodulin-like protein 3 (*CALML3*)); among these, *CYP11B2* and *MC2R* are known to be key regulators of steroidogenesis in APA (9). On the other hand, *HPX* is known to down-regulate AT1R expression, which may play a role in the responsiveness of endogenous angiotensin II to aldosterone production (30). *PRRX1* (31, 32), *RAB38* (33), *FAP* (34, 35), *GCNT2* (36), and *ASB4* (37) have been associated with tumor progression. By contrast, *CALML3* has been documented as a marker in well-differentiated tissues (38, 39).

Discussion

The present study was designed to explore the pathogenesis of APA through the integration of transcriptome and methylome analysis. In the study, we compared APA with AAG in the same patient rather than comparing APA and adrenal glands from different nephrectomized patients. The comparison between APA and AAG may have minimized the noise that can be caused by inter-individual variation (15). Although the pair comparison has been employed in transcriptome analyses in the past (15), the present study is the first methylome analysis to compare APA and AAG in the same patient. We identified a number of genes with increased gene expression and CpG hypomethylation at their promoter regions.

In the present study, we found several genes to be up-regulated with CpG hypomethylation, some of which may be involved in autonomous aldosterone production and in tumorigenesis. It is known that the activation of G protein-coupled receptors (GPCRs), such as AT1R and *MC2R*, up-regulates *CYP11B2*, which thus increases aldosterone production (9). In the present study, we found that *CYP11B2* is up-regulated with CpG hypomethylation at its promoter region. A series of transcriptome studies have shown that *CYP11B2* is up-regulated in APA (9, 14, 15, 16). We also found *MC2R* and *HPX* to be up-regulated with CpG hypomethylation, and this may be associated with autonomous aldosterone production in

Figure 4

(A) Hierarchical clustering heat map of hypomethylated probes in TSS200 using the genome-wide DNA methylation platform (Illumina Infinium HumanMethylation450 BeadChip). Green indicates unmethylated genes, whereas red indicates methylated genes. (B) Scatter plotting of average β values (all probes) of APA with AAG, indicating the global tendency of

hypomethylation in APA. (C) The number of differentially methylated genes divided into each functional region in APA as compared to AAG. Genes that show average $\Delta\beta$ values of ≥ 0.1 and ≤ -0.1 in more than half of the seven pairs are defined as hypermethylated (or hypomethylated).

APA. Indeed, MC2R, a member of the melanocortin receptor family, is activated by ACTH, and it plays a role in aldosterone production in APA (40). On the other hand, HPX is an acute phase protein that is responsible for angiotensin II responsiveness in humans through the down-regulation of AT1R (30). This is consistent with the notion that most APAs are not responsive to angiotensin II stimulation (41) but are responsive to ACTH in terms of aldosterone production (42). Further studies are required to validate this hypothesis. Collectively, these observations suggest that autonomous aldosterone production in APA is mediated at least partly via a DNA methylation mechanism.

It is also important to understand the mechanism of adrenal tumorigenesis in APA. A previous study suggested that aberrant activation of the PKA and/or Wnt pathways as a result of genetic mutation is involved in the pathogenesis of adrenocortical adenoma and carcinoma (43). However, the detailed mechanisms of tumorigenesis in APA have not yet been elucidated. In the present study, we identified several genes that are potentially involved in tumorigenesis through an epigenetic mechanism. For

instance, PRRX1, a homeobox protein, was identified as an epithelial-mesenchymal transition inducer in thyroid and colorectal cancer (31, 32). The increased expression of RAB38 is associated with a poor prognosis and migration of gliomas (33). FAP is an integral membrane serine peptidase associated with proliferation and invasion in osteosarcoma (35). GCNT2 is related to breast cancer metastasis (36). ASB4 is highly expressed in hepatocellular carcinoma and induces cell migration and invasion (37). CALML3, an epithelial-specific calcium-sensing protein, has also been identified as being an up-regulated and promoter-hypomethylated gene. CALML3, which is expressed at high levels in normal epithelium, has also been shown to be down-regulated during malignant transformation, and it thus assumed to be a biomarker of terminally differentiated epithelium (38, 39). It has been shown that specific DNA hypomethylation at gene promoter regions plays a crucial role in tumorigenesis through the up-regulation of oncogenes, such as *Rras* in gastric cancer and *cyclin D2* in pancreatic cancer (44). It is conceivable that the up-regulated genes with DNA hypomethylation are possibly involved in tumorigenesis in APA.

Clinical Study	M Murakami and others	Transcriptome and methylome analysis of APAs	173:2	192
----------------	-----------------------	--	-------	-----

Table 2 List of up-regulated and hypomethylated genes in the TSS1500/TSS200 region in APA as compared to AAG^a.

Accession no.	Gene symbol	Fold change	P value	Adjusted P value	Mean $\Delta\beta$ value	
					TSS1500	TSS200
NM_174878	CLRN1	16.47	<0.001	0.008	-0.095	-0.231
NM_052995	CLRN1	15.18	<0.001	0.007	-0.144	0.000
NM_000486	AQP2	8.691	0.002	0.039	-0.058	-0.071
NM_022449	RAB17	6.710	<0.001	0.020	0.014	-0.122
NM_000498	CYP11B2	5.315	<0.001	0.003	-0.067	-0.136
NM_152321	ERP27	4.055	0.002	0.047	0.000	-0.103
NM_000529	MC2R	3.708	0.001	0.024	-0.123	-0.040
NR_002763	CPS1-IT1	3.444	0.002	0.047	-0.101	0.000
NM_052913	TMEM200A	2.997	<0.001	0.014	-0.070	-0.150
NM_033066	MPP4	2.967	0.013	0.117	-0.038	-0.150
NM_006902	PRRX1	2.946	0.001	0.032	-0.056	NA
NM_022337	RAB38	2.894	<0.001	0.007	-0.063	-0.074
NM_002722	PPY	2.815	0.001	0.036	-0.142	-0.100
NM_016529	ATP8A2	2.694	0.004	0.062	-0.049	NA
NM_004460	FAP	2.684	0.038	0.214	-0.030	-0.122
NM_001080444	IGSF5	2.597	<0.001	0.009	-0.044	-0.126
NM_152349	KRT222	2.535	0.004	0.059	-0.071	-0.029
NM_032342	TMEM246	2.529	<0.001	0.002	-0.185	-0.013
NM_001012980	SATL1	2.508	0.001	0.034	-0.023	-0.105
NR_002835	HAS2-AS1	2.416	0.021	0.154	-0.080	-0.014
NM_032735	BEST3	2.201	0.010	0.104	-0.001	-0.150
NM_000613	HPX	2.071	<0.001	0.013	-0.042	-0.126
NM_014799	HEPH	1.929	0.000	0.008	-0.013	-0.104
NM_005549	KCNA10	1.902	0.014	0.123	-0.100	-0.088
NM_004118	FOXS1	1.840	0.038	0.215	-0.088	-0.175
NM_033500	HK1	1.743	0.001	0.034	-0.054	-0.039
NM_001491	GCNT2	1.675	0.006	0.081	-0.063	-0.154
NM_016511	CLEC1A	1.652	0.032	0.196	-0.103	-0.029
NM_016116	ASB4	1.647	0.002	0.039	-0.010	-0.041
NM_001080410	FBXO41	1.640	0.001	0.029	-0.104	0.000
NM_001001657	OR2Y1	1.612	0.038	0.213	-0.182	-0.186
NM_002042	GABRR1	1.554	0.027	0.180	-0.070	-0.131
NR_026878	FOXD2-AS1	1.539	0.025	0.171	-0.051	-0.068
NR_005185	CALML3	1.515	0.001	0.036	-0.134	-0.053

TSS, transcription start sites; APA, aldosterone-producing adenoma; AAG, adjacent adrenal glands.

^aThe list includes genes whose expression level was increased by >1.5-fold with statistical significance ($P < 0.05$) and that were hypomethylated in the TSS1500 or TSS200 region in APA as compared to AAG. Hypomethylation is defined as an average $\Delta\beta$ value of < -0.1 in more than half of seven pairs. The mean $\Delta\beta$ value of TSS1500 and TSS200 of each gene are shown for the reference.

The present transcriptome analysis identified many genes that are up-regulated with no inverse correlation with DNA methylation in APA relative to AAG. They included those that have been reported previously (*PCP4*, *CLRN1*, *HTR4*, *MYB*, and *ALDH1A2*), which suggests the validity of the present study. It is noteworthy that some of the *HOX* family genes (*HOXA7*, *HOXA11*, and *HOXB9*) are up-regulated in APA relative to AAG. A previous report demonstrated that 23 out of 39 *HOX* cluster genes are expressed in human adrenal glands and that *HoxB2*, *B4*, *B5*, and *B9* are expressed in order from the capsule to the inner zona reticularis in the rat adrenal cortex (29). Because some *HOX* genes are involved in cellular differentiation and tumorigenesis (45), it is interesting to

speculate that aberrant *HOX* gene activation contributes to tumorigenesis in APA.

Evidence has suggested a role for DNA methylation in the pathogenesis of benign and malignant tumor formation; DNA hypomethylation occurs in the early stage of colon cancer, and the accumulation of aberrant DNA methylation in the promoter regions could contribute to malignant tumorigenesis (46). In this regard, it is noteworthy that the global DNA hypomethylation in APA is distinct from that reported in adrenocortical carcinoma (ACC); global hypomethylation accompanied by hypermethylation in the promoter regions of tumor-suppressor genes is a hallmark of ACC (3, 4, 5). Differences in DNA methylation status between APA and ACC may

contribute to the differences in their benign and malignant characteristics.

Because the somatic mutation of *KCNJ5* is common among Japanese APA patients (27, 28), it is important to investigate whether the presence of a *KCNJ5* somatic mutation affected the transcriptome and methylome analysis. In the present study, only six out of the seven cases had the *KCNJ5* somatic mutation, which did not allow us to compare clinical characteristics between the patients with and without the mutation in a statistically appropriate manner. Further studies are required to elucidate the pathophysiological significance of a *KCNJ5* somatic mutation in gene expression and DNA methylation in APA.

Recently, Howard *et al.* (47) compared APA with normal adrenal tissue and/or nonfunctioning adrenocortical adenoma obtained from different patients, and they reported global DNA hypomethylation in APA. They also identified *CYP11B2* as being an up-regulated gene with CpG hypomethylation in APA. Their data are roughly consistent with the present data, but there are some differences. For example, *HTR4*, a GPCR that is up-regulated in APA (14), was also up-regulated in both studies, although CpG hypomethylation of *HTR4* was only detected by Howard *et al.* Such differences may have resulted from variations in the experimental designs, ethnic differences between the study subjects, and so on.

There are some limitations in the present study. First, because we dissected AAG macroscopically as described previously (15), the AAG samples contained not only zona glomerulosa but also other zones of adrenal cortex and medulla. Our analysis did not represent the pure comparison between APA and zona glomerulosa, the site of physiological aldosterone production. The down-regulated genes in APA may simply reflect the incorporation of other zones of adrenal cortex and medulla, so we therefore focused our analysis mainly on the up-regulated genes in APA in order to minimize this influence in the present study. For strict comparison between APA and zona glomerulosa, further study using *in situ* methodology, such as laser-capture microdissection, would be required. Secondly, whether AAG really represents the normal adrenal gland under the influence of hyperaldosteronism is an equivocal matter; however, Wang *et al.* (15) stated that transcriptome comparison with paired APA–AAG could have less variation, because these tissues are exposed to the same hormonal milieu. Although the first and second limitations in the present study should be taken into account, the intra-individual comparison of transcriptome and methylome between the tumors and

their background tissues in our study has some significance in considering the pathophysiology of APA. Third, the relatively small sample size of seven patients is a further limitation of the present study. Further study with larger samples will be required, especially to evaluate the relationships between methylome and the presence or absence of a somatic *KCNJ5* mutation.

In summary, we found several genes that were up-regulated with CpG hypomethylation in APA. The data from the present study support the notion that DNA methylation status is closely associated with autonomous hormone production and tumorigenesis in APA. The combination of methylome and transcriptome analysis provides a powerful method for elucidating the pathogenesis of APA and thus contributes to the development of novel diagnostic and therapeutic strategies for PA.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-0148>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (grant number 24591110 to T Yoshimoto). This study was partially supported by “Advancing Care of Primary Aldosteronism in Japan Study” – Japan Primary Aldosteronism Study (JPAS) – from Japan Agency for Medical Research and development, AMED.

Acknowledgements

The authors thank all of the other staff members of the Molecular Endocrinology and Metabolism Department at Tokyo Medical and Dental University.

References

- 1 Jaenisch R & Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* 2003 **33** (Suppl) 245–254. (doi:10.1038/ng1089)
- 2 Das PM & Singal R. DNA methylation and cancer. *Journal of Clinical Oncology* 2004 **22** 4632–4642. (doi:10.1200/JCO.2004.07.151)
- 3 Rechache NS, Wang Y, Stevenson HS, Killian JK, Edelman DC, Merino M, Zhang L, Nilubol N, Stratakis CA, Meltzer PS *et al.* DNA methylation profiling identifies global methylation differences and markers of adrenocortical tumors. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** E1004–E1013. (doi:10.1210/jc.2011-3298)

Clinical Study	M Murakami and others	Transcriptome and methylome analysis of APAs	173:2	194
----------------	-----------------------	--	-------	-----

- 4 Fonseca AL, Kugelberg J, Starker LF, Scholl U, Choi M, Hellman P, Akerstrom G, Westin G, Lifton RP, Bjorklund P *et al.* Comprehensive DNA methylation analysis of benign and malignant adrenocortical tumors. *Genes, Chromosomes & Cancer* 2012 **51** 949–960. (doi:10.1002/gcc.21978)
- 5 Barreau O, Assie G, Wilmot-Roussel H, Ragazzon B, Baudry C, Perlemoine K, Rene-Corail F, Bertagna X, Dousset B, Hamzaoui N *et al.* Identification of a CpG island methylator phenotype in adrenocortical carcinomas. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** E174–E184. (doi:10.1210/jc.2012-2993)
- 6 Hannemann A & Wallaschofski H. Prevalence of primary aldosteronism in patient's cohorts and in population-based studies – a review of the current literature. *Hormone and Metabolic Research* 2012 **44** 157–162. (doi:10.1055/s-0031-1295438)
- 7 Plouin PF, Amar L & Chatellier G. Trends in the prevalence of primary aldosteronism, aldosterone-producing adenomas, and surgically correctable aldosterone-dependent hypertension. *Nephrology, Dialysis, Transplantation* 2004 **19** 774–777. (doi:10.1093/ndt/gfh112)
- 8 Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME & Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *Journal of the American College of Cardiology* 2005 **45** 1243–1248. (doi:10.1016/j.jacc.2005.01.015)
- 9 Zennaro MC, Jeunemaitre X & Boulkroun S. Integrating genetics and genomics in primary aldosteronism. *Hypertension* 2012 **60** 580–588. (doi:10.1161/HYPERTENSIONAHA.111.188250)
- 10 Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A, Men CJ *et al.* K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* 2011 **331** 768–772. (doi:10.1126/science.1198785)
- 11 Azizian EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, Maniero C, Garg S, Bochukova EG, Zhao W *et al.* Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nature Genetics* 2013 **45** 1055–1060. (doi:10.1038/ng.2716)
- 12 Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, Penton D, Schack VR, Amar L, Fischer E *et al.* Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nature Genetics* 2013 **45** 440–444 (444e1–2). (doi:10.1038/ng.2550)
- 13 Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, Fonseca AL, Korah R, Starker LF, Kunstman JW *et al.* Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nature Genetics* 2013 **45** 1050–1054. (doi:10.1038/ng.2695)
- 14 Ye P, Mariniello B, Mantero F, Shibata H & Rainey WE. G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism. *Journal of Endocrinology* 2007 **195** 39–48. (doi:10.1677/JOE-07-0037)
- 15 Wang T, Satoh F, Morimoto R, Nakamura Y, Sasano H, Auchus RJ, Edwards MA & Rainey WE. Gene expression profiles in aldosterone-producing adenomas and adjacent adrenal glands. *European Journal of Endocrinology* 2011 **164** 613–619. (doi:10.1530/EJE-10-1085)
- 16 Azizian EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, Happerfield L, Marker A, Hoffman GJ & Brown MJ. Microarray, qPCR, and KCNJ5 sequencing of aldosterone-producing adenomas reveal differences in genotype and phenotype between zona glomerulosa- and zona fasciculata-like tumors. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** E819–E829. (doi:10.1210/jc.2011-2965)
- 17 Boulkroun S, Samson-Couterie B, Golib-Dzib JF, Amar L, Plouin PF, Sibony M, Lefebvre H, Louiset E, Jeunemaitre X, Meatchi T *et al.* Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. *Endocrinology* 2011 **152** 4753–4763. (doi:10.1210/en.2011-1205)
- 18 Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, Samson-Couterie B, Zennaro MC, Plouin PF, Skah S *et al.* WNT/ β -catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Human Molecular Genetics* 2014 **23** 889–905. (doi:10.1093/hmg/ddt484)
- 19 Young WF, Stanson AW, Thompson GB, Grant CS, Farley DR & van Heerden JA. Role for adrenal venous sampling in primary aldosteronism. *Surgery* 2004 **136** 1227–1235. (doi:10.1016/j.surg.2004.06.051)
- 20 Nishikawa T, Omura M, Satoh F, Shibata H, Takahashi K, Tamura N, Tanabe A & Task Force Committee on Primary Aldosteronism, The Japan Endocrine Society. Guidelines for the diagnosis and treatment of primary aldosteronism – the Japan Endocrine Society 2009. *Endocrine Journal* 2011 **58** 711–721. (doi:10.1507/endocrj.EJ11-0133)
- 21 Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, Mulatero P, Samson-Couterie B, Hahner S, Quinkler M *et al.* Prevalence, clinical, and molecular correlates of KCNJ5 mutations in primary aldosteronism. *Hypertension* 2012 **59** 592–598. (doi:10.1161/HYPERTENSIONAHA.111.186478)
- 22 Akerstrom T, Crona J, Verdugo AD, Starker LF, Cupisti K, Willenberg HS, Knoefel WT, Saeger W, Feller A, Ip J *et al.* Comprehensive re-sequencing of adrenal aldosterone producing lesions reveal three somatic mutations near the KCNJ5 potassium channel selectivity filter. *PLoS ONE* 2012 **7** e41926. (doi:10.1371/journal.pone.0041926)
- 23 Mulatero P, Tauber P, Zennaro MC, Monticone S, Lang K, Beuschlein F, Fischer E, Tizzani D, Pallauf A, Viola A *et al.* KCNJ5 mutations in European families with nonglucocorticoid remediable familial hyperaldosteronism. *Hypertension* 2012 **59** 235–240. (doi:10.1161/HYPERTENSIONAHA.111.183996)
- 24 Murthy M, Azizian EA, Brown MJ & O'Shaughnessy KM. Characterization of a novel somatic KCNJ5 mutation delI157 in an aldosterone-producing adenoma. *Journal of Hypertension* 2012 **30** 1827–1833. (doi:10.1097/HJH.0b013e328356139f)
- 25 Itoh M, Suganami T, Satoh N, Tanimoto-Koyama K, Yuan X, Tanaka M, Kawano H, Yano T, Aoe S, Takeya M *et al.* Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2007 **27** 1918–1925. (doi:10.1161/ATVBAHA.106.136853)
- 26 Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC & Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biology* 2003 **4** P3. (doi:10.1186/gb-2003-4-5-p3)
- 27 Taguchi R, Yamada M, Nakajima Y, Satoh T, Hashimoto K, Shibusawa N, Ozawa A, Okada S, Rokutanda N, Takata D *et al.* Expression and mutations of KCNJ5 mRNA in Japanese patients with aldosterone-producing adenomas. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** 1311–1319. (doi:10.1210/jc.2011-2885)
- 28 Kitamoto T, Suematsu S, Matsuzawa Y, Saito J, Omura M & Nishikawa T. Comparison of cardiovascular complications in patients with and without KCNJ5 gene mutations harboring aldosterone-producing adenomas. *Journal of Atherosclerosis and Thrombosis* 2015 **22** 191–200. (doi:10.5551/jat.24455)
- 29 Neville SE, Baigent SM, Bicknell AB, Lowry PJ & Gladwell RT. Hox gene expression in adult tissues with particular reference to the adrenal gland. *Endocrine Research* 2002 **28** 669–673. (doi:10.1081/ERC-120016984)
- 30 Krikken JA, Lely AT, Bakker SJ, Borghuis T, Faas MM, van Goor H, Navis G & Bakker WW. Hemopexin activity is associated with angiotensin II responsiveness in humans. *Journal of Hypertension* 2013 **31** 537–541 (discussion 542). (doi:10.1097/HJH.0b013e32835c1727)
- 31 Takahashi Y, Sawada G, Kurashige J, Uchi R, Matsumura T, Ueo H, Takano Y, Akiyoshi S, Eguchi H, Sudo T *et al.* Paired related homeobox 1, a new EMT inducer, is involved in metastasis and poor prognosis in colorectal cancer. *British Journal of Cancer* 2013 **109** 307–311. (doi:10.1038/bjc.2013.339)
- 32 Hardin H, Guo Z, Shan W, Montemayor-Garcia C, Asioli S, Yu XM, Harrison AD, Chen H & Lloyd RV. The roles of the epithelial–mesenchymal transition marker PRRX1 and miR-146b-5p in papillary

- thyroid carcinoma progression. *American Journal of Pathology* 2014 **184** 2342–2354. (doi:10.1016/j.ajpath.2014.04.011)
- 33 Wang H & Jiang C. RAB38 confers a poor prognosis, associated with malignant progression and subtype preference in glioma. *Oncology Reports* 2013 **30** 2350–2356. (doi:10.3892/or.2013.2730)
 - 34 Wikberg ML, Edin S, Lundberg IV, Van Guelpen B, Dahlin AM, Rutegard J, Stenling R, Oberg A & Palmqvist R. High intratumoral expression of fibroblast activation protein (FAP) in colon cancer is associated with poorer patient prognosis. *Tumour Biology* 2013 **34** 1013–1020. (doi:10.1007/s13277-012-0638-2)
 - 35 Ding L, Ye L, Xu J & Jiang WG. Impact of fibroblast activation protein on osteosarcoma cell lines. *Oncology Letters* 2014 **7** 699–704. (doi:10.3892/ol.2014.1788)
 - 36 Zhang H, Meng F, Wu S, Kreike B, Sethi S, Chen W, Miller FR & Wu G. Engagement of I-branching $\{\beta\}$ -1, 6-N-acetylglucosaminyltransferase 2 in breast cancer metastasis and TGF- $\{\beta\}$ signaling. *Cancer Research* 2011 **71** 4846–4856. (doi:10.1158/0008-5472.CAN-11-0414)
 - 37 Au V, Tsang FH, Man K, Fan ST, Poon RT & Lee NP. Expression of ankyrin repeat and SOCS box containing 4 (ASB4) confers migration and invasion properties of hepatocellular carcinoma cells. *Bioscience Trends* 2014 **8** 101–110. (doi:10.5582/bst.8.101)
 - 38 Brooks MD, Bennett RD, Weaver AL, Sebo TJ, Eckert SE, Strehler EE & Carr AB. Human calmodulin-like protein CALML3: a novel marker for normal oral squamous mucosa that is downregulated in malignant transformation. *International Journal of Dentistry* 2013 **2013** 592843. (doi:10.1155/2013/592843)
 - 39 Bennett RD, Pittelkow MR & Strehler EE. Immunolocalization of the tumor-sensitive calmodulin-like protein CALML3 in normal human skin and hyperproliferative skin disorders. *PLoS ONE* 2013 **8** e62347. (doi:10.1371/journal.pone.0062347)
 - 40 Arnaldi G, Mancini V, Costantini C, Giovagnetti M, Petrelli M, Masini A, Bertagna X & Mantero F. ACTH receptor mRNA in human adrenocortical tumors: overexpression in aldosteronomas. *Endocrine Research* 1998 **24** 845–849. (doi:10.3109/07435809809032695)
 - 41 Wisgerhof M, Brown RD, Hogan MJ, Carpenter PC & Edis AJ. The plasma aldosterone response to angiotensin II infusion in aldosterone-producing adenoma and idiopathic hyperaldosteronism. *Journal of Clinical Endocrinology and Metabolism* 1981 **52** 195–198. (doi:10.1210/jcem-52-2-195)
 - 42 Stowasser M, Klemm SA, Tunny TJ & Gordon RD. Plasma aldosterone response to ACTH in subtypes of primary aldosteronism. *Clinical and Experimental Pharmacology & Physiology* 1995 **22** 460–462. (doi:10.1111/j.1440-1681.1995.tb02044.x)
 - 43 Lerario AM, Moraitis A & Hammer GD. Genetics and epigenetics of adrenocortical tumors. *Molecular and Cellular Endocrinology* 2014 **386** 67–84. (doi:10.1016/j.mce.2013.10.028)
 - 44 Feinberg AP, Ohlsson R & Henikoff S. The epigenetic progenitor origin of human cancer. *Nature Reviews. Genetics* 2006 **7** 21–33. (doi:10.1038/nrg1748)
 - 45 Haria D & Naora H. Homeobox gene deregulation: impact on the hallmarks of cancer. *Cancer Hallmarks* 2013 **1** 67–76. (doi:10.1166/ch.2013.1007)
 - 46 Hammoud SS, Cairns BR & Jones DA. Epigenetic regulation of colon cancer and intestinal stem cells. *Current Opinion in Cell Biology* 2013 **25** 177–183. (doi:10.1016/j.ceb.2013.01.007)
 - 47 Howard B, Wang Y, Xekouki P, Faucz FR, Jain M, Zhang L, Meltzer PG, Stratakis CA & Kebebew E. Integrated analysis of genome-wide methylation and gene expression shows epigenetic regulation of CYP11B2 in aldosteronomas. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E536–E543. (doi:10.1210/jc.2013-2558)

Received 9 February 2015

Revised version received 15 April 2015

Accepted 7 May 2015