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Aberrant G protein-receptor expression is associated with DNA methylation in aldosterone-producing adenoma

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ABSTRACT

This study aimed to evaluate the methylation levels of G protein-coupled receptor (GPCR) related genes and the effects of methylation on mRNA expression levels in aldosterone-producing adenoma (APA). DNA methylation array and transcriptome analysis were applied in non-functioning adrenocortical adenoma (NFA) and APA. We investigated 192 GPCR-related genes and found hypo-methylation in the promoter region of 66 of these genes in APA. An integration study between microarray and methylation analysis revealed that *HTR4*, *MC2R*, *TACR1*, *GRM3*, and *PTGER1* showed hypo-methylation and up-regulation of mRNA in APA. qPCR analysis showed that *HTR4* and *PTGER1* expression was 9.3-fold and 6.6-fold higher in APAs than in NFAs, respectively, whereas expression of the other genes was not different between the groups. Methylation of *HTR4* and *PTGER1* at positions −229 and −666 from the transcription start site, respectively, showed a significant inverse correlation with their mRNA levels. Methylation levels were not associated with *KCNJ5* or *ATP1A1* mutations in human adrenal samples. We demonstrated an increased incidence of CpG island demethylation of GPCR-related gene in APA. The expression of two receptors, *HTR4* and *PTGER1*, showed a strong association with DNA methylation.

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1. Introduction

Primary aldosteronism (PA) is the most frequent cause of secondary hypertension associated with autonomous excess aldosterone production and secretion (Funder et al., 2016). Patients with PA have an increased risk of cardiovascular disease compared with essential hypertension with similar blood pressure and risk profiles (Milliez et al., 2005, Mulatero et al., 2013). Therefore, the elucidation of autonomous excess aldosterone production machinery leads to detect therapeutic target for PA, and it is important to improve health and extend life span.

PA is mainly classified with aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism. APA is a form of secondary hypertension potentially curable by adrenalectomy (Funder et al., 2016). Recent research shows that 50–80% of APAs contain somatic mutations of *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, or *CTNNB1*, resulting in over-production of aldosterone (Akerstrom et al., 2016, Azizan et al., 2013, Beuschlein et al., 2013, Choi et al., 2011). On the other hand, it is well known that ectopic or aberrant membrane receptors such as G protein-coupled receptors (GPCR) are expressed in APAs and are associated with acceleration or suppression of aldosterone production by agonists or antagonists, respectively (Duparc et al., 2015, Perraudin et al., 2006, Ye et al., 2007, Zwermann et al., 2009). The regulation of the membrane receptor may be a therapeutic target for aldosterone production in APA. However, the mechanism of ectopic or aberrant membrane receptor expression in APA has not been elucidated.

Berger et al. defined an epigenetic trait as a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (Berger et al., 2009). DNA methylation is a well-studied epigenetic modification that occurs mostly on the 5-carbon of cytosine residues in CpG dinucleotides. Hypomethylation of CpG in the promoter region potentiates gene transcription by facilitating the binding of transcription factors (Sen et al., 2016). Alteration of DNA methylation can influence the development or progression of some disorders including cancer, adenoma, and life style-related diseases (Maqbool et al., 2016). In fact, we and others have demonstrated that CYP11B2, the rate-limiting enzyme for aldosterone production, is demethylated in APA (Howard et al., 2014, Yoshii et al., 2016). In adrenal tissues, DNA methylation is likely to be involved in fetal adrenal development

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and adrenocortical cancer progression via its regulation of transcription (Legendre et al., 2016, Slieker et al., 2015).

Taken together, the transcription of GPCR or GPCR-related genes might be regulated by their DNA methylation in adrenal cells, however DNA methylation levels of GPCR or GPCR-related genes has not been studied. Therefore, we hypothesized that the expression of GPCRs might be regulated by DNA methylation in adrenal cells. The aims of our study were to clarify the DNA methylation levels of GPCR or GPCR-related genes in APA and the effects of DNA methylation on mRNA expression levels in APA.

2. Methods

2.1. Patients and samples

The inclusion criteria for patients and tissue samples have been described in a previous report (Yoshii et al., 2016). Briefly, the diagnosis of PA was based on the guidelines of the Japan Endocrine Society (Nishikawa et al., 2011). PA was diagnosed if the aldosterone renin ratio (aldosterone, ng/dL; plasma renin activity, ng/mL/hr) was greater than 20. The captopril challenge test, furosemide-upright test, and saline infusion test were performed to confirm the diagnosis. Adrenal venous sampling was performed for subtype diagnosis in all patients. Non-functioning adenoma (NFA) was diagnosed by radiological findings and endocrinological results showing cortisol or aldosterone excess as previously reported (Oki et al., 2012). Twelve NFA and 35 APA samples were obtained by surgery and stored at −80 °C until used for DNA methylation and quantitative polymerase chain reaction (qPCR) analysis. The clinical characteristics of the patients were shown in our previous report (Yoshii et al., 2016). The genotypes of the APAs included 5 *ATP1A1* mutations, 21 *KCNJ5* mutations, and 9 wild types. Five of 12 NFA and 19 of 35 APA samples were used for microarray analysis. This study was approved by the ethics committee of Hiroshima University, and written informed consent was obtained from all patients.

2.2. DNA genotyping and methylation analysis

Genomic DNA was extracted from NFA or APA samples by DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Genotyping of the adenomas for *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, and *CTNNB1* was performed as previously described (Kishimoto et al., 2016). DNA methylation levels were determined using the Infinium Human-Methylation450 BeadChip kit (Illumina, San Diego, CA, USA), as previously described (Yoshii et al., 2016). Ninty-nine percent of the RefSeq genes and 96% of the CpG islands were covered. Methylation levels were shown as β values, which were then used to estimate the methylated signal intensity (Dedeurwaerder et al., 2011). The average β values were expressed as 0 to 1, representing completely non-methylated to completely methylated values, respectively.

2.3. RNA extraction, qPCR assay, and microarray analysis

Total RNA extraction and cDNA synthesis were performed as previously described (Kishimoto et al., 2016). PCR primers for *HTR4* (5-hydroxytryptamine receptor 4), *MC2R* (melanocortin 2 receptor), *TACR1* (tachykinin receptor 1), *GRM3* (glutamate metabotropic receptor 3), *PTGER1* (prostaglandin E receptor 1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) are shown in Table S1. Expression levels were assessed using SYBR-green-based gene expression assays (Takara Bio SYBR Premix EX Taq; Takara Bio Inc., Shiga, Japan). Gene expression levels were determined as arbitrary units normalized against *GAPDH* expression. Microarray analysis was performed using the SurePrint G3 Human Gene Expression 8 × 60K v2 array (Agilent Technologies Inc., Santa Clara, CA, USA).

2.4. Statistical analysis

Results were expressed as mean ± S.D. The differences were considered significant at $P < 0.05$. Analyses were performed using SPSS for Windows (release 24.0; SPSS Inc., Chicago, IL, USA). First, *t*-test was applied for the differences of DNA methylation or mRNA expression levels by DNA methylation array and transcriptome analysis, respectively. Significantly higher and lower DNA methylation levels of the genes in APA were denoted as “hyper-methylation” and “hypo-methylation”, respectively. Significantly higher and lower mRNA expression levels of the genes in APA were expressed as “up-regulation” and “down-regulation”, respectively. Second, the expression levels of *HTR4*, *MC2R*, *TACR1*, *GRM3*, and *PTGER1* mRNA detected by qPCR were analyzed by the *t*-test after logarithmic transformation, because they did not fit a normal distribution. The relationships between DNA methylation and mRNA expression levels or plasma aldosterone levels were analyzed by Spearman's test. Finally, differences of DNA methylation levels among APA genotypes were analyzed by one-way ANOVA followed by Bonferroni comparisons.

3. Results

3.1. Methylation levels of GPCR or GPCR-related genes in APA

We identified 192 GPCR or GPCR-related genes from the Gene Set Enrichment Analysis website (http://www.broadinstitute.org/gsea/index.jsp). From this list of genes, 185 had as DNA methylation lesion in the promoter region up to −1500 bases from transcription start site (TSS). Methylation at these sites was compared between NFAs (n = 12) and APAs (n = 35). APAs showed hypomethylation in 66 genes and hypermethylation 14 genes (Table 1 and Table S2). Six genes showed a mixture of hypo- and hypermethylation in APAs (Table 1 and Table S2). Ninety-nine genes showed no differences in DNA methylation between NFAs and APAs.

3.2. Integration analysis between methylation level and mRNA expression

Results of the integration study between microarray and methylation analysis are shown in Table 1. We focused on genes with an inverse association between methylation and gene expression, because this indicates that DNA methylation directly regulates the expression of these genes. *HTR4*, *MC2R*, *TACR1*, *GRM3*, and *PTGER1* showed hypomethylation and upregulation of mRNA in APAs (Table 1 and Fig. S1). Nine genes showed higher mRNA expression in APAs than NFAs (Table S3), but no differences in DNA methylation between APA and NFA. Thus, some GPCR and GPCR-related genes

Table 1
Relationship between DNA methylation and mRNA expression among GPCR in APA.

mRNA expression in APA compared with NFA	DNA methylation in APA compared with NFA		
	Hypo-methylation	Mixed	Hyper-methylation
up-regulation	5	0	2
not significant	61	6	12
down-regulation	0	0	0

A *t*-test was applied for the differences of DNA methylation or mRNA expression levels by DNA methylation array and transcriptome analysis. Significantly higher and lower DNA methylation levels of the genes in APA were denoted as “hyper-methylationis” and “hypo-methylation”, respectively. Significantly higher and lower mRNA expression levels of the genes in APA were expressed as “up-regulation” and “down-regulation”, respectively. The specific gene symbols were shown in Table S2. NFA, non-functioning adrenocortical adenoma; APA, aldosterone-producing adenoma; GPCR, G protein-coupled receptor.

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