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Resistance to renal denervation therapy — Identification of underlying mechanisms by analysis of differential DNA methylation

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ABSTRACT

Background: Factors causing resistance to renal denervation (RDN) for treatment of arterial hypertension are not known. In the current study, we sought to determine mechanisms involved in responsiveness to renal denervation therapy in patients with difficult-to-control and resistant hypertension.

Methods and results: We evaluated the differential CpG methylation of genes in blood samples isolated from patients of a recently described cohort of responders or non-responders to renal denervation using microarray technique and measured protein levels of identified downstream effectors in blood samples of these patients by ELISA.

Our analysis revealed up to 6103 methylation sites differing significantly between non-responders and responders to renal denervation therapy. Software based analysis showed several of these loci to be relevant for arterial hypertension and sympathetic nervous activity. Particularly, genes involved in glutamate synthesis, degradation and glutamate signaling pathways were differently methylated between both groups. For instance, genes for glutamate dehydrogenase 1 and 2 central to glutamate metabolism, genes for ionotropic (AMPA, NMDA) and metabotropic glutamate receptors as well as glutamate transporters revealed significant differences in methylation correlating with responsiveness to RDN. To underline their potential relevance for responsiveness to RDN, we measured plasma protein levels of norepinephrine, a downstream effector of the glutamate receptor pathway, which were significantly lower in non-responders to RDN.

Conclusions: The present study describes novel molecular targets potentially contributing to reduction of blood pressure after RDN in some patients. Identifying patients with a high responsiveness to RDN could contribute to an individualized therapy in drug resistant hypertension.

1. Introduction

Catheter-based renal sympathetic denervation (RDN) is a novel treatment option in patients with resistant hypertension [1]. However, RDN is an invasive procedure with the inherent risks of side effects. The net clinical benefit of this procedure is subject of ongoing clinical trials [2], and clear determinants to predict beneficial outcome of this intervention are warranted.

During RDN, sympathetic outflow to the kidney is reduced by inflicting damage to the renal peri-vascular nervous system [3]. Since the first description of interventional renal denervation, several experimental and clinical studies suggested a beneficial effect on arterial hypertension [4–6]. RDN was also suggested to have effects on pathophysiological settings other than arterial hypertension such as ventricular arrhythmias, glucose metabolism or insulin sensitivity [7,8]. While the underlying mechanisms are not entirely characterized, in the setting of catecholamine dependent ventricular tachycardia, modulation of cardio-cardiac reflexes of sympathetic origin may account for the observed effects [9–12]. Now, experimental and clinical research tries to define indicators, which can predict RDN success, and clinical markers to identify patients responding to the procedure.

Although arterial hypertension is a multifactorial disease, dysregulation of sympathetic/parasympathetic activity is a central mechanism of its pathophysiology. Accordingly, sympathetic nerve activity is considered a major contributor to the pathophysiology of arterial

Abbreviations: ABPM, ambulatory blood pressure monitoring; AF, atrial fibrillation; BP, blood pressure; BRS, baroreflex sensitivity; GFR, glomerular filtration rate; NE, norepinephrine; RDN, renal denervation therapy; PVI, pulmonal vein isolation.

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hypertension [13]. Dysbalance of sympathetic activity may be a result of changes in signaling tailored by epigenetic changes in expression of molecules associated with the sympathetic signaling transduction [14]. For example in patients suffering from postural tachycardia syndrome, it was demonstrated that expression of the norepinephrine transporter is inhibited by suppressive histone-methylation [15]. These pathophysiological considerations render the sympathetic regulatory system a potential candidate to understand mechanisms of resistance to blood pressure regulation. Recently, we described a novel approach to identify patients as responders or non-responders to RDN by measuring baroreflex sensitivity (BRS) obtained from non-invasive recordings as surrogate measure for underlying sympathetic tone [16]. We were able to demonstrate that impaired BRS was a strong and significant predictor of response to RDN [16]. Epigenetic regulation is discussed as one of the emerging mechanisms affecting arterial hypertension [17]. To uncover possible targets, which affect responsiveness to RDN, we performed microarray analysis of responders and non-responders in our BRS patient collective and determined differential methylation possibly resulting in decreased DNA transcription.

2. Materials and methods

2.1. Ethics

Patients' samples were obtained after patients gave written and informed consent. The study was approved by the local ethics committee and conforms with the ethical principles outlined in the Declaration of Helsinki.

2.2. Patients

The study includes 5 randomly selected responders as well as age-, gender-, glomerular filtration rate (GFR)-, treatment- and blood pressure-matched non-responders of a previously described cohort with resistant arterial hypertension undergoing RDN [16]. Briefly, inclusion criteria were defined as follows: patients were 18 years or older, had an office based BP of ≥160 mm Hg, a mean systolic BP of ≥130 mm Hg on ambulatory blood pressure monitoring (ABPM) were on at least 3 antihypertensive drugs, had a GFR of ≥45 ml/min^{-1}/1.73 m^{-2} and no known secondary cause of high BP except sleep apnea or chronic kidney disease. Identically to our previous report [16], response to RDN was defined as reduction of mean systolic arterial blood pressure of 10 mm Hg or greater 6 months after RDN.

2.3. Sample collection

In patients undergoing RDN, blood was taken from the arterial femoral access at the beginning of the procedure. Blood samples were then immediately transferred to blood collection tubes containing EDTA (Sarstedt, Nuembrecht, Germany). Blood samples for DNA analysis were immediately frozen as a whole and stored at −80 °C. For isolation of EDTA plasma, samples were spun down at room temperature at 1250 ×*g* for 10 min. Plasma samples were then collected and stored at −80 °C.

2.4. Norepinephrine (NE) enzyme linked immunosorbent assays (ELISA)

EDTA plasma samples were thawed on ice, resuspended and analyzed using a NE ELISA (Labor Diagnostika Nord, Nordhorn, Germany) following the manufacturer's protocol. All samples were analyzed in duplicate and measured on a microplate reader (Bio-Rad Laboratories, Munich, Germany) at 450 nm. n = 5 samples were analyzed for responders and nonresponders.

2.5. BRS-PRSA

Assessment of cardiac BRS was carried out as described before [16]. Briefly, patients underwent simultaneous 30-min high-resolution electrocardiographic recordings (1.6 kHz in orthogonal XYZ leads) and noninvasive continuous arterial BP monitoring using a finger hotoplethysmographic device (Finapres; TNO-TPD Biomedical Instrumentation, Amsterdam, the Netherlands). The recordings were analyzed according to standardized conditions by an experienced technician blinded to the clinical status of the patient. Cardiac BRS was assessed from the series of RR intervals and systolic BP values by phase-rectified signal averaging (PRSA). For BRS-PRSA, increases of systolic BP (BP) are identified within the BP time series. Subsequently, segments of RR intervals around BP are identified and averaged. The resulting bivariate PRSA signal shows RR oscillations related to increases of systolic BP, whereas heart rate variability due to other causes is eliminated by the averaging process.

2.6. Methylation array

Total DNA was isolated from frozen 2.8 ml EDTA blood samples (n = 5) for responders and nonresponders respectively using the QIAamp DNA Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol.

500 ng of total DNA were bisulfite converted for methylation analysis according to the manufacturer's protocol using the EZ DNA Methylation Kit (Zymo Research, Irvine CA, USA). Bisulfite converted DNA was then applied to Infinium Human Methylation450 BeadChip (Illumina, San Diego CA, USA) and analyzed using an Illumina iScan microarray scanner (Illumina, San Diego CA, USA) according to the manufacturer's protocol. Genes differing in methylation with high significance were further analyzed for association with regulatory pathways, networks and disease. Data analysis was performed using GenomeStudio software (Illumina, San Diego CA, USA) and QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). The pathways shown were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®,QIAGEN Redwood City, www.qiagen.com/ingenuity). Methylation analysis was performed at the Department of Medical Genetics, Microarray Facility, Tuebingen, Germany.

2.7. Statistical analysis

Preliminary statistical analysis of methylation data was carried out in the programming environment “R” using the packages “lumi” and “methylumi”. Resulting β-values were normalized and converted to M-values using logit-transformation. M-values were analyzed in “R” using the “limma” package. For methylation array statistical analysis, genes were considered significantly different between both groups (responder vs. nonresponders), if β-values of at least an absolute 10% were observed. Methylation sites meeting this requirement and showing an uncorrected p-value of $p \leq 0.05$ were considered for pathway analysis. For ELISA and BRS-PRSA values, statistical analysis was performed with SPSS (version 22, IBM Deutschland GmbH, Ehningen, Germany). Concentrations obtained in the ELISA-assay were compared using Mann–Whitney U-test.

3. Results

The patients' characteristics of “responders or nonresponders to RDN” are depicted in Table 1. There was no significant difference regarding factors important for the pathogenesis of arterial hypertension such as gender, body mass index or diabetes. Comparing patients responding to RDN with nonresponders, we were able to identify a large number of sites with differential CpG-methylation. Criteria for selection of methylation sites for further analysis of genes are given in