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SHORT COMMUNICATION

Obstructed defecation—an enteric neuropathy? An exploratory study of patient samples

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Abstract

Purpose Although various strategies exist for chronic constipation therapy, the pathogenesis of chronic constipation is still not completely understood. The aim of this exploratory experimental study is to elucidate alterations of the autonomous enteric nervous system at the molecular level in patients with obstructed defecation, who represent one of the most predominant groups of constipated patients.

Methods Full-thickness rectal wall samples of patients with obstructed defecation were analyzed and compared with controls. Differential gene expression analyses by RNA-Seq transcriptome profiling were performed and gene expression profiles were assigned to gene ontology pathways by application of different biological libraries.

Results Analysis of the transcriptome showed that genes associated with the enteric nervous system functions were significantly downregulated in patients with obstructed defecation. These affected functions included developmental processes and synaptic transmission.

Conclusions Our results therefore indicate that obstructed defecation may represent an enteric neuropathy, comparable to Hirschsprung disease and slow-transit constipation.

Keywords Chronic constipation · Obstructed defecation · Enteric nervous system · Enteric neuropathy · Transcriptome profiling, RNA-Seq

Introduction

Chronic constipation is highly prevalent and results in a considerable socioeconomic burden. Sixteen percent of adults suffer from chronic constipation independent of age, while more than 30% of ≥ 60-year-old adults are affected, causing annual indirect costs in the USA of about USD 230 million [1]. Slow-transit constipation (STC) and normal-transit constipation with and without obstructed defecation (OD) represent the three types of chronic constipation, of which 25–50% of patients suffer from OD [2]. OD is characterized by an incomplete evacuation of the rectum, whereas STC is defined by reduced colonic motility and decreased stool frequency. OD patients often show an internal rectal prolapse or a rectocele in dynamic radiologic imaging causative for incomplete evacuation of the rectum. Though these patients represent a predominant subgroup of constipated patients, the pathogenesis of OD still remains unknown. Vice versa in STC, immunohistochemical studies have shown hypoganglionosis,

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leading to its classification as an enteric neuropathy, comparable to the aganglionosis in Hirschsprung disease [3].

As few immunohistochmical studies have arisen, describing changes of the autonomous enteric nervous system (ENS) in OD patients [4], the aim of the present study is to evaluate the involvement of ENS in patients with OD on a transcriptomic level via RNA-Seq with functional annotation analysis.

Material and methods

Patients

Full-thickness rectal wall samples from three female patients (mean age 61.33 ± 10.69), suffering from refractory OD (Cleveland Clinic Constipation Score 16 ± 1.73, Altomare ODS Score 13.45 ± 5.73), and recto-rectal intussusception and rectocele in dynamic defecography, who underwent STARR procedure (stapled transanal rectal resection), were included. Patients with pathological colon transit time were excluded. Pathological inconspicuous full-thickness rectal wall tissue from patients with non-obstructing rectal adenoma or carcinoma, who did not receive preoperative radiation or chemotherapy, served as controls. OD was ruled out by Altomare ODS Score. Tissue samples were collected after surgical resection, frozen instantly in liquid nitrogen, and stored at − 80 °C for further analysis. The study was approved by an institutional review board (Ethics committee, University Würzburg, Germany, approval number 23/12). All patients gave their informed, written consent.

Transcriptome profiling

RNA was isolated from frozen whole rectal wall samples according to the manufacturer's instructions (RNAeasy Mini Plus Kit, Qiagen, Hilden, Germany) and checked for quality and quantity with Agilent's 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, USA).

cDNA libraries were prepared using Illumina's TruSeqTM RNA Sample Preparation v2 protocol, and sequencing was performed using an Illumina HiSeqTM 2500. cDNA libraries were checked with Bioanalyzer DNA 1000 chips. A sequencing run was carried out following the manufacturer's instructions with a loading amount of 8.5 pmol cDNA per lane.

Mapping was performed with TopHat2 (v2.0.5, default settings) against human reference genome assembly "hg19" with the annotation from Gencode "v17." Gene quantification was calculated with a python script, "rpkmforgenes.py" from the Sandberg Laboratory (http://sandberg.cmb.ki.se/rnaseq/) at readcount and RPKM level (=reads per kilobase of exon model per million mapped reads according to Mortazavi et al. [5] using uniquely mapped reads). Differential gene expression profiling was carried out exclusively based on readcount quantification via the DESeq2 package developed for RNA sequencing data [6]. In order to reduce background signals, we applied stringent criteria considering genes with an adjusted p value by false discovery rate ≤ 0.05, a mean readcount > 5 and a log2 fold change ≤ -2 or ≥ 2 as being significantly differentially regulated between two conditions.

In order to attribute the differential gene expression of OD patients to neuronal patterns, data were analyzed using the following bioinformatics resources: gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Online Mendelian Inheritance in Man (OMIM).

Sequencing data were up-loaded to NCBI GEO database (GSE101968; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101968).

Results

Transcriptome profiling: quality analysis and gene expression

Quality control of RNA from three representative tissue samples per group revealed sufficient integrity and amount in order to prepare high-quality cDNA libraries. On average, each sample was covered by about 53 million reads of which 81% were unique hits giving a very robust database for the downstream evaluation of both transcriptomes.

Hierarchical clustering of the expression patterns showed significant differences between controls and OD patients, as demonstrated by a striking decrease of the correlation coefficient down to an average of 0.62 across the two groups (Fig. 1a, suppl. Table 1). Thus, significant differences between controls and OD group, as well as genetic homogeneity within each group allowed further comparison and analyses, in spite of the small sample size.

One hundred eighty-three genes were found to be significantly upregulated in the OD group compared with controls and 762 genes showed significant downregulation (Fig. 1b, suppl. Table 1B-D).

Transcriptome profiling: alignment with biological databases

In order to evaluate the significant differences in gene expression profiles with regard to neuronal patterns, only the coding 560 downregulated genes were further analyzed (suppl. Table 1D) using common bioinformatic databases for in silico analysis.

In order to attribute the differential gene expression of OD patients to neuronal patterns, data were analyzed using the following bioinformatics resources: gene ontology, Kyoto

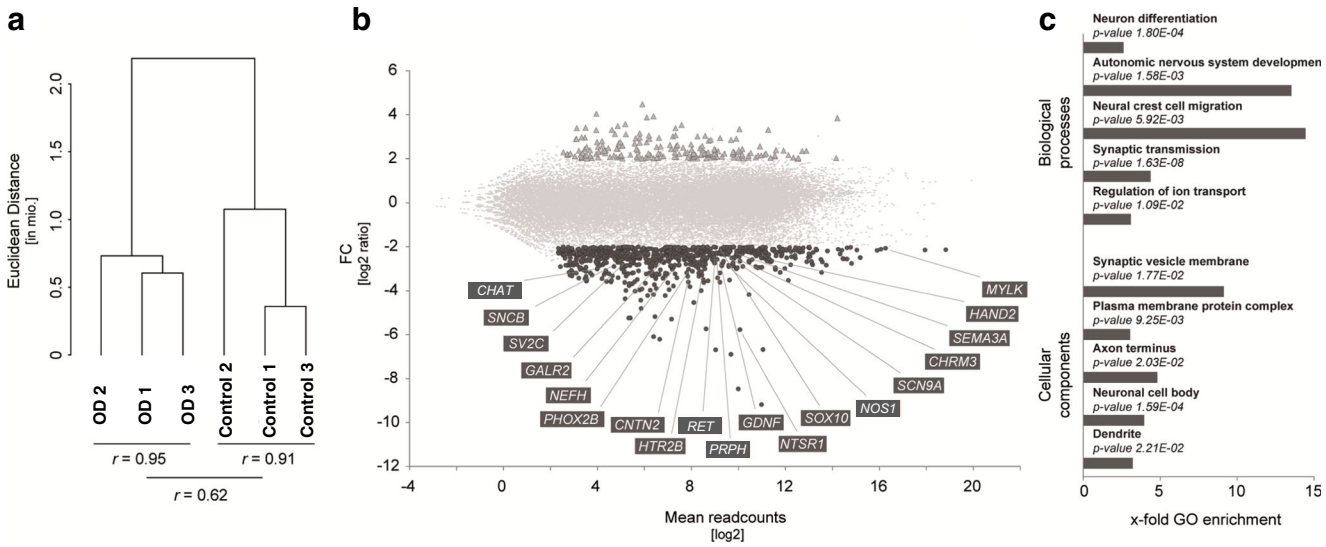

Fig. 1 Transcriptomic alterations in rectal tissue of patients with obstructed defecation. **a** Dendrogram displaying the hierarchical clustering (Euclidean distance) of the six samples investigated. Analysis is based on readcounts, showing genetic homogeneity within the groups and allowing further comparison; OD, obstructed defecation. **b** Scatter plot of mean readcounts versus fold change for all genes analyzed. Light gray triangles represent genes being significantly upregulated in OD while downregulated genes are marked with dark gray circles. Individual genes known to be associated with the enteric nervous system are highlighted. **c** Gene ontology (GO) analysis shows that genes downregulated in OD samples are predominantly associated to biological processes and cellular compartments linked to neuronal-associated functions. The x-fold enrichment is calculated as the ratio of genes in the dataset associated with the respective term relative to the genomic background

Encyclopedia of Genes and Genomes, and Online Mendelian Inheritance in Man.

GO analysis for the domains "biological process" and "cellular compartments" showed a significantly enriched association of these genes to neuromuscular-associated processes and compartments, e.g., ENS development (enrichment, 16-fold; *p* value, 1,58E-03) or synaptic transmission (enrichment, 4-fold; *p* value, 1,63E-08) (Fig. 1c). Genes included those well-described for enteric neuronal (subtype) cells, such as RET, PRPH, NOS1, and CHAT; those for enteric glial cells such as SOX10 and GDNF; and those for synaptic transmission (e.g., SNCB, SV2C), and neuronal receptors or ion channels (e.g., GALR2, HTR2B, NTSR1, CHRM3, SCN9A) (representatively highlighted in Fig. 1b) (abbreviations in suppl. Table 1). KEGG pathway analysis identified a number of relevant genes associated with neuroactive ligand-receptor interactions (e.g., GALR2, NTSR1, CHRM3) and axon guidance (SEMA3A). OMIM analysis further confirmed that downregulated gene patterns in OD were associated with Hirschsprung disease (RET, GDNF) [7].

Discussion

In our study, transcriptome profiling revealed significant alterations in ENS-associated gene expression in patients with OD. Most strikingly, expression levels of key ENS genes were significantly downregulated and linked to numerous major neuronal-associated processes, e.g., ENS development and synaptic transmission. These findings are suggestive for an impairment of the ENS in OD. Further, the genetic alterations observed in OD resemble those described in aganglionic Hirschsprung disease.

To date, little descriptive data exist about the pathophysiology of OD, revealing a link between ENS and OD. In an immunohistochemical analysis, Bassotti et al. observed reduced glia and an increased number of interstitial cells of Cajal in obstructed patients [4]. For STC, more descriptive data exist, emphasizing the involvement of ENS, characterized by reduced ganglia, neurons [3, 8], interstitial cells of Cajal, and altered neurochemical code with a decrease in ChAT subpopulation [8–10]. Consequently, STC is categorized as hypoganglionosis in the London Classification of gastrointestinal neuromuscular pathology.

To our knowledge, high-throughput transcriptome analysis has never been published before for constipated patients. Recently, one study performed next-generation sequencing in $n = 11$ patients suffering from aganglionosis in Hirschsprung disease in order to detect new coding variants. Most of the reported genes were also significantly downregulated in our patients' cohort, such as RET, SOX10, PROKR1, PHOX2B, GDNF, and L1CAM, and GFRA1, one of the new coding variants proposed by the authors (suppl. Table 1D) [11].

As there is a significant overlap of patients, who suffer from OD and STC with 49% of OD patients presenting with delayed colonic transit [1, 12], the clinically practiced strict distinction between STC and OD may not correctly reflect the

underlying pathophysiology. Changes in the ENS might be the joint component of constipation subtype, which may also explain why treating OD leads to an improvement of STC and vice versa, and might also explain recurrences or persistence of constipation after treatment in some patients. For example, application of the selective 5-HT4 serotonin receptor agonist prucalopride that promotes colonic motility improves function not only in STC but also in OD [13]. In addition, surgical treatment of OD by laparoscopic ventral rectopexy leads to a lessening of constipation in patients accompanied with STC the same way as it does in patients with normal-transit constipation [14].

Our study clearly shows transcriptomic alterations in rectal tissue of OD patients that affect ENS functions. However, there are several limitations to the study. First, the number of patients' samples for transcriptome profiling is relatively small due to the costly analysis. Nonetheless, hierarchical clustering demonstrated significant genetic differences between controls and OD, as well as genetic homogeneity within the control and the OD group, allowing further comparison in spite of the small sample size. Second, due to operatively altered tissue samples and decreasing numbers of STARR procedures, additional analysis on protein level could not be evaluated in this study. Lastly, our data describe the association of downregulated ENS-related gene expression and OD for the first time, but whether the alterations of the ENS are causative for OD cannot be answered by this analysis. Further studies illuminating the etiology of chronic constipation and STC as well as OD are much awaited, underlining the exploratory character of the present pilot study.

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Compliance with ethical standards

The study was approved by an institutional review board (Ethics committee, University Würzburg, Germany, approval number 23/12). All patients gave their informed, written consent.

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

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