Cancer Genetics 235–236 (2019) 18–20

Cancer Genetics

SHORT COMMUNICATION

Classic bladder exstrophy and adenocarcinoma of the bladder: Methylome analysis provide no evidence for underlying disease-mechanisms of this association

Amit Sharma[a,b,*], Holger Fröhlich[c], Rong Zhang[d,e], Anne-Karoline Ebert[f], Wolfgang Rösch[g], Henning Reis[h], Glen Kristiansen[i], Jörg Ellinger[j], Heiko Reutter[d,e,k]

[a] *Department of Neurology, University Clinic Bonn, Sigmund-Freud Str. 25, D-53127 Bonn, Germany;* [b] *Department of Ophthalmology, University Clinic Bonn, Bonn, Germany;* [c] *Bonn-Aachen International Center for IT, University of Bonn, Germany;* [d] *Institute of Human Genetics, University Hospital of Bonn, Bonn, Germany;* [e] *Department of Genomics, Life & Brain Center, Bonn, Germany;* [f] *Department of Urology and Paediatric Urology, Ulm University, Ulm, Germany;* [g] *Department of Paediatric Urology, Clinic St. Hedwig, University Medical Center Regensburg, Regensburg, Germany;* [h] *Faculty of Medicine, Institute of Pathology, University Duisburg-Essen, Essen, Germany;* [i] *Institute of Pathology, University Hospital Bonn, Bonn, Germany;* [j] *Department of Urology, University Hospital Bonn, Bonn, Germany;* [k] *Department of Neonatology and Pediatric Intensive Care, Children's Hospital, University of Bonn, Bonn, Germany*

Abstract

The bladder exstrophy-epispadias complex (BEEC) represents the severe end of uro-rectal malformation spectrum involving aberrant embryonic morphogenesis of the cloacal membrane and the urorectal septum. The most common form of BEEC is isolated classic bladder exstrophy (CBE). Long-term complications in CBE are malignancies of the bladder with 95% of them being adenocarcinomas. Since CBE and adenocarcinoma of the bladder are rare entities, their frequent co-occurrence suggests a common etiology. Recent studies suggest that promoter methylation of various genes play a crucial role during the phenotypical morphogenesis of adenocarcinomas of urinary bladder. To examine, whether epigenetic processes such as DNA methylation patterns are potentially associated with CBE, we performed Illumina 450 K methylation arrays in blood ($n=10$) and tissue samples ($n=2$) of CBE patients and healthy matched controls ($n=12$). In our analysis, we found total lack of methylation in the blood and methylation differences were restricted to 10 CpG sites in the tissue samples. In comparison to other bladder anomalies, CBE tissue methylation profiles differ from those of adenocarcinoma, adenocarcinoma with CBE, urothelial carcinoma and urachal carcinoma. In this preliminary study, we did not provide any strong evidence of major DNA methylation alterations which would be suggestive for strong underlying epigenetic mechanism. However, larger studies are required to provide more robust statistical evidence to exclude smaller effects in the tissues.

Keywords Classic bladder exstrophy, Adenocarcinoma of the urinary bladder, DNA methylation.

Introduction

The bladder exstrophy-epispadias complex represents the severe end of human congenital anomalies of the kidney and urinary tract (CAKUT) that also involves defect in abdominal wall, pelvis, genitalia, occasionally the spine and anus [1,2].

Received May 22, 2018; received in revised form March 27, 2019; accepted May 29, 2019

*Corresponding author at: Department of Neurology, University Clinic Bonn, Sigmund-Freud Str. 25, D-53127 Bonn, Germany.

E-mail address: amit.sharma@ukbonn.de

2210-7762/$ - see front matter
https://doi.org/10.1016/j.cancergen.2019.05.004

The severity-spectrum of the BEEC comprises the mild form (epispadias/E), intermediate form (classic bladder exstrophy/CBE), and the severe form (exstrophy of the cloaca/CE). Long-term complications of CBE are malignancies of the bladder with 95% of these being adenocarcinomas [3]. Since CBE and adenocarcinoma of the bladder are rare entities, their frequent co-occurrence suggests a common etiology [4]. Although, the genetic determinants related to BEEC and their potential molecular mechanisms have already been discussed [5]. Still, the non-genetic risk factors contributing to its etiology remain largely unknown. Recently, Kolarova et al. [6] described a CBE patient with hypomethylation of the imprinted PLAGL1 locus in chromosome 6q24. The hypomethylation was verified by MS-MLPA and the authors proposed that the methylation loss was probably caused by a mosaic epimutation thus suggesting a link between CBE and imprinting disorders [6]. In the present study, we aim to examine whether aberrant DNA methylation patterns are potentially associated with CBE of the urinary bladder. We therefore analyzed whole blood and tissue samples from CBE patients and healthy matched controls. Furthermore, we analyzed tissue samples of the urinary bladder from one CBE patient with adenocarcinoma, one patient with primary adenocarcinoma of the urinary bladder, one tissue sample of the urinary bladder from CBE patient without adenocarcinoma, one urachal carcinoma sample and two urothelial carcinoma samples. These samples were compared to the tissue samples of the urinary bladder which were neither affected by adenocarcinoma nor derived from the CBE patients or any other patient with congenital urinary tract anomaly.

Methods

Patients, controls, and DNA isolation

The present study was part of an ongoing multicenter investigation of the molecular genetic causes of BEEC. The study was approved by the ethics committee of the Medical Faculty of the University of Bonn (No. 146/12). Written informed consent was obtained from all participants prior to inclusion. Blood samples were obtained from patients and a population-based control group. Tissue samples were obtained from Institute of Pathology at the University Hospital Bonn and Institute of Pathology at the University Duisburg-Essen, Germany. The description of samples used in this study is shown in supplementary Table 1. Genomic DNA from blood and tissue samples was isolated by using standard protocol and was chemically modified using sodium bisulfite to converts all unmethylated cytosine to uracil using EZ DNA methylation kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions.

Methylation analysis

Comparison of peripheral blood samples was carried out between CBE patients ($n=10$) and age and gender matched healthy controls ($n=10$). In addition, three tissue samples of the urinary bladder (CBE patient with adenocarcinoma, patient with primary adenocarcinoma, CBE patient without adenocarcinoma), healthy tissue ($n=4$) samples, urothelial carcinoma tissue samples ($n=4$), and urachal carcinoma samples ($n=2$) were included. Analysis of the Illumina 450 K chips was carried out using R-package "missMethyl" [7]: Initial quality control revealed five patient tissue samples (2 controls, 2 urothelial carcinoma tissue samples and 1 urachal carcinoma samples) with more than 1% undetectable probes, where "undetectable" was defined by a detection *p*-value above the typical 1% threshold. These five samples were omitted and functional normalization was applied to the remaining samples [8]. Probes containing SNPs as well as cross-reactive probes were omitted. A collection of cross-reactive probes was obtained from [9]. Beta values of the remaining 393,639 CpGs were logit transformed to *M*-values to allow for application of limma [10]. The corresponding design matrix included the patient phenotype (cell type plus diagnosis) as well as age and gender to account for confounding effects. Moreover, we fitted array quality weights [11] for differential CpG detection. We mainly performed two types of differential CpG analysis: (1) CBE vs. healthy controls using only blood samples; (2) CBE vs. healthy controls using only tissue samples. In addition, we performed hierarchical clustering (average linkage) of patient samples while focusing on those 1280 CpGs which were nominally significant ($p< 0.001$) according to limma *F*-test, i.e., to check across different samples. We used a robust empirical Bayes procedure for limma for moderation of statistical power [12].

Results

CBE methylome analysis in whole blood and tissue samples

After correction of age and gender effects we did not observed any statistically significant CpGs in the blood samples of CBE patients and healthy controls. While in case of CBE tissues, we observed 10 statistically significant CpGs (adjusted *p*-value $< 5\%$) with at least 25% increase or decrease of DNA methylation (supplementary Fig. 3, supplementary Table 2). Eight of these CpGs were hyper-methylated in CBE patient tissue and two were found to be hypo-methylated (cg17209507, cg09687907). However, none of these CpG sites have been previously linked to bladder related phenotypes or pathways.

Focusing on *ISL1* and *PLAGL1* we found no statistically significant differences in methylation status at these loci between patients and control samples. However, 20 CpGs in *PLAGL1* and 19 CpGs in *ISL1* showed at least 25% increase or decrease of DNA methylation. Here again, the lack of statistical significance might be explainable by the low number of samples.

Comparison of CBE methylome with other bladder cancer tissue types

To obtain a better understanding of the similarity of CBE methylome from different cancer types (2 urothelial carcinoma, 1 adenocarcinoma, 1 adenocarcinoma with CBE, 1 urachal carcinoma), we performed hierarchical clustering (average linkage) while focusing on 1280 highly variable CpGs (see methods). Supplementary Fig. 1 confirmed the high similarity of CBE blood and healthy blood samples. Moreover,

a more pronounced difference of the two CBE tissues from healthy and cancer tissues was visible. The impression was further confirmed by a UMAP projection of the data (Supplementary Fig. 2), which is a modern non-linear manifold embedding technique to visualize higher dimensional data in 2 dimensions.

Discussion

Since CBE and adenocarcinoma of the urinary bladder are rare entities, their frequent co-occurrence suggests a common etiology. Previous studies suggest epigenetic alterations to be involved in the etiology of both entities. Therefore, herein, we investigated the possible involvement of epigenetic alterations particularly in CBE of the urinary bladder. In the present study, genome wide methylation levels in whole blood did not reveal any significant alterations in patients compared to the healthy controls. We have observed few CpG sites in two tissues samples with minor differences in their methylation status between patient and control groups, which might be a stochastic effects and requires bigger set of samples to prove the significance effect with strong statistical power. In specific focus to *ISL1,* a major susceptibility gene [13,14] for CBE methylation appears to have very restricted effect. Similarly, in case of *PLAGL1*, which previously showed hypomethylation in only one patient might have stochastic or polymorphism related methylation effect. However, large data sets are required to obtain any statistical significance. Considering our current data and in addition to previous reports there is no evidence that the epigenome, in particular DNA methylation plays any major role in the formation of CBE of the urinary bladder.

Conclusion

Herein, we did not observe any statistically significant alterations of DNA methylation levels in the blood of CBE patients. In CBE tissues, we found only 10 differentially methylated locations in whole genome with at least 25% hyper- or hypomethylation. Furthermore, our analysis also did not provide clear evidence for *ISL1* and *PLAGL1* methylation difference related to CBE. However, the low number of tissue samples (two CBE tissues and two healthy tissues) could be the reason for the lack of statistical significance. Using unsupervised cluster analysis, we could show that healthy tissues together with cancer tissues group separately from CBE samples. Altogether this analysis thus provides no clear hint that epigenetic change, in particular DNA methylation probably contributes significantly to the formation of CBE of the urinary bladder.

Funding details

This work was funded by a grant of the German Research Foundation (Deutsche Forschungsgemeinschaft, RE 1723/1-3).

Disclosure statement

The authors declare no conflict of interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.05.004.

References

[1] Ebert AK, Reutter H, Ludwig M, Rosch WH. The exstrophy-epispadias complex. Orphanet J Rare Dis 2009;4:23.
[2] Reutter H, Qi L, Gearhart JP, Boemers T, Ebert AK, Rosch W, Ludwig M, Boyadjiev SA. Concordance analyses of twins with bladder exstrophy-epispadias complex suggest genetic etiology. Am J Med Genet A 2007;143a:2751–6.
[3] Engberg, Magdalena Fossum. and Agneta. Nordenskjöld. Bladder exstrophy and the risk of urinary bladder cancer in Sweden 1952-2012. 29th Congress of the ESPU, Helsinki, Finland, 2018, S18–5 (PP).
[4] Xiong W, Peng R, Zhu L, Zhong Z. Bladder exstrophy-epispadias complex with adenocarcinoma in an adult patient: a case report. Exp Ther Med 2015;10:2194–6.
[5] Reutter H, Keppler-Noreuil K, Catherine EK, Thiele H, Yamada G, Ludwig M. Genetics of bladder-exstrophy-epispadias complex (BEEC): systematic elucidation of mendelian and multifactorial phenotypes. Curr Genom 2016;17:4–13.
[6] Kolarova J, Bens S, Ammerpohl O, Hilger AC, Zhang R, Reutter H, Siebert R. PLAGL1 epimutation and bladder exstrophy: coincidence or concurrent etiology? Birth Defects Res A Clin Mol Teratol 2016;106:724–8.
[7] Phipson B, Maksimovic J, Oshlack A. missMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform. Bioinformatics 2016;32:286–8.
[8] Fortin JP, Labbe A, Lemire M, Zanke BW, Hudson TJ, Fertig EJ, Greenwood CM, Hansen KD. Functional normalization of 450k methylation array data improves replication in large cancer studies. Genome Biol 2014;15:503.
[9] Chen YA, Lemire M, Choufani S, et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics 2013;8:203–9.
[10] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3 Article3.
[11] Ritchie ME, Diyagama D, Neilson J, van Laar R, Dobrovic A, Holloway A, Smyth GK. Empirical array quality weights in the analysis of microarray data. BMC Bioinform 2006;7:261.
[12] Phipson B, Lee S, Majewski IJ, Alexander WS, Smyth GK. Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. Ann Appl Stat 2016;10:946–63.
[13] Zhang R, Knapp M, Suzuki K, Kajioka D, Schmidt JM, Winkler J, Yilmaz O, Pleschka M, Cao J, Kockum CC, Barker G, Holmdahl G, Beaman G, Keene D, Woolf AS, Cervellione RM, Cheng W, Wilkins S, Gearhart JP, Sirchia F, Di Grazia M, Ebert AK, Rosch W, Ellinger J, Jenetzky E, Zwink N, Feitz WF, Marcelis C, Schumacher J, Martinon-Torres F, Hibberd ML, Khor CC, Heilmann-Heimbach S, Barth S, Boyadjiev SA, Brusco A, Ludwig M, Newman W, Nordenskjold A, Yamada G, Odermatt B, Reutter H. ISL1 is a major susceptibility gene for classic bladder exstrophy and a regulator of urinary tract development. Sci Rep 2017;7:42170.
[14] Sharma A, Dakal TC, Ludwig M, Fröhlich H, Mathur R, Reutter H. Towards a central role of ISL1 in the bladder exstrophy-epispadias complex (BEEC): Computational characterization of genetic variants and structural modelling. Genes 2018. doi:10.3390/genes9120609.