Published in final edited form as:

Nat Genet. 2016 April ; 48(4): 356–358. doi:10.1038/ng.3503.

Frequent somatic *CDH1* loss-of-function mutations in plasmacytoid-variant bladder cancer

Hikmat A. Al-Ahmadie^{1,*}, Gopa Iyer^{2,3,*}, Byron H. Lee^{4,*}, Sasinya N. Scott¹, Rohit Mehra⁵, Aditya Bagrodia⁴, Emmet J. Jordan³, Sizhi Paul Gao⁶, Ricardo Ramirez^{6,7}, Eugene K. Cha⁴, Neil B. Desai⁸, Emily C. Zabor⁹, Irina Ostrovnya⁹, Anuradha Gopalan¹, Ying-Bei Chen¹, Samson W. Fine¹, Satish K. Tickoo¹, Anupama Gandhi¹, Joseph Hreiki¹⁰, Agnès Viale¹⁰, Maria E. Arcila^{1,10}, Guido Dalbagni^{2,4}, Jonathan E. Rosenberg^{2,3}, Bernard H. Bochner^{2,4}, Dean F. Bajorin^{2,3}, Michael F. Berger^{1,10}, Victor E. Reuter^{1,2}, Barry S. Taylor^{6,9,10}, and David B. Solit^{2,3,6,10}

¹Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, USA

²Weill Cornell Medical College, Cornell University, New York, New York, USA

³Genitourinary Oncology Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York, USA

⁴Urology Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, New York, USA

⁵Department of Pathology, University of Michigan Health System, Ann Arbor, Michigan, USA

⁶Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York, USA

⁷Weill Cornell Graduate School of Medical Sciences, Cornell University, New York, New York, USA

⁸Department of Radiation Oncology, Memorial Sloan Kettering Cancer Center, New York, New York, USA

Corresponding author: David B. Solit, Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. P: 646-888-2641. solitd@mskcc.org.

*These authors contributed equally to this manuscript

Author Contributions

S.N.S., R.R., M.F.B., A.V., M.E.A., and B.S.T. performed sequencing and analyzed the data. A.B., E.C.Z., and I.O. acquired clinical data and performed statistical analyses. B.H.L., E.J.J., and S.P.G. performed the in vitro experiments. H.A.A., R.M., A.G., Y.B.C., S.W.F., S.K.T., A.G., J.H., and V.E.R. reviewed the pathology and identified tumor specimens for analysis. G.I., E.K.C., N.B.D., and A.G. performed specimen collection and DNA extraction. H.A.A., G.I., B.H.L., G.D., J.E.R., B.H.B., D.F.B., M.F.B., V.E.R., B.S.T., and D.B.S. were involved with the conception and design of the study. All authors assisted with drafting and critically revising the manuscript.

Competing Financial Interests

The authors declare no competing interests as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Accession Codes

dbGaP accession code pending

URLs

www.cbiportal.org

⁹Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York, USA

¹⁰Marie-Josée and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, New York, New York, USA

Abstract

Plasmacytoid bladder cancer is an aggressive histologic variant with a high risk of disease-specific mortality. Using whole exome and targeted sequencing, we find that truncating somatic alterations in the *CDH1* gene occur in 84% of plasmacytoid carcinomas and are specific to this histologic variant. Consistent with the aggressive clinical behavior of plasmacytoid carcinomas, which frequently recur locally, CRISPR/Cas9-mediated knockout of *CDH1* in bladder cancer cells enhanced cell migration.

Keywords

E cadherin; plasmacytoid; bladder cancer; *CDH1*

Bladder cancer is the ninth most common cancer worldwide and the fourth most common cancer in men¹. Cancers arising in the urothelial tract display a wide spectrum of variant morphologies and stage for stage, tumors composed of purely variant histologies tend to have a worse survival. All variant bladder cancer histologies were excluded from The Cancer Genome Atlas (TCGA)^{2,3} and the molecular basis for the morphologic and prognostic heterogeneity of bladder cancers remains ill-defined. To explore whether the morphologic heterogeneity of urothelial tumors has an underlying molecular basis, we characterized the mutational landscape of plasmacytoid urothelial carcinoma, a histologic variant with an aggressive clinical course marked by advanced stage at presentation, chemotherapy resistance, frequent local recurrence, and a peritoneal pattern of spread^{4–7}.

Six plasmacytoid-variant bladder tumors were initially analyzed by whole exome sequencing and all 6 harbored nonsense mutations in *CDH1*, the gene encoding E-cadherin (Supplementary Figure 1). In contrast, no *CDH1* truncating mutations were detected within 127 bladder tumors in the TCGA cohort comprised of urothelial carcinoma, not otherwise specified (NOS). To further confirm the association between *CDH1* mutation and plasmacytoid-variant bladder cancer, we performed targeted exon capture and sequencing of 19 additional plasmacytoid-variant bladder tumors (Supplementary Table 1 and Figure 1a), 14 (74%) of which harbored *CDH1* mutations. We also performed a centralized histologic review of the first 62 patients with invasive bladder cancer prospectively sequenced as part of their clinical care. In this prospective cohort, *CDH1* mutations were identified in 6 patients, all of whose tumors exhibited the plasmacytoid-variant histology, whereas no *CDH1* alterations were observed in the 56 non-plasmacytoid-variant samples (Figure 1a). With the exception of *CDH1* alterations, the genomic profile of plasmacytoid-variant tumors was not substantially different from the 183 urothelial carcinoma, NOS tumors in the TCGA or Memorial Sloan Kettering prospective cohorts, with frequent mutations in the tumor

suppressors *TP53* and *RBI*, the chromatin remodeler *ARID1A*, and the targetable kinases *ERBB2* and *PIK3CA* (Figures 1a and 1b).

Plasmacytoid-variant tumors consist of malignant epithelial cells with eccentrically located nuclei growing in a diffuse, discohesive pattern with minimal stromal reaction. These tumors have also been referred to as diffuse or signet ring cell carcinomas. Notably, this morphologic appearance shares similarities with lobular breast and diffuse gastric carcinomas, both of which frequently harbor *CDH1* mutations^{8,9} (Figure 1c). In contrast to the germline point mutations in *CDH1* that typify diffuse hereditary gastric cancers, we identified no germline *CDH1* alterations in the plasmacytoid-variant bladder cancers. The co-mutation pattern of lobular breast and diffuse gastric cancers was also distinct from plasmacytoid-variant bladder carcinoma with the exception of *CDH1* alterations (Supplementary Figure 2).

Variant morphologies often co-exist with urothelial carcinoma, NOS in patients with invasive bladder cancer. To explore whether the plasmacytoid-variant and urothelial carcinoma, NOS components in such tumors evolve from a shared cell of origin, we performed exon capture and deep sequencing of 2 adjacent portions of a bladder tumor that contained distinct regions of plasmacytoid-variant and urothelial carcinoma, NOS histology (Supplementary Figure 3). Both histologic regions shared mutations in *CDKN1A* (A45fs) and *PIK3C2G* (S48R), implying that these were truncal alterations occurring within a common precursor cell. A *CDH1* Y68fs mutation alongside *PTEN*, *NOTCH2*, *FAT4*, and other gene mutations were, however, unique to the plasmacytoid component.

To confirm that the *CDH1* alterations identified in the plasmacytoid-variant tumors resulted in loss of protein expression, we performed immunohistochemistry for E-cadherin. All 31 plasmacytoid tumors from the whole exome, validation, and prospective cohorts exhibited loss of E-cadherin expression, including 5 *CDH1* wild-type tumors. As *CDH1* promoter hypermethylation is an alternative mechanism of E-cadherin loss in other cancer types^{10–12}, we performed bisulfite sequencing of the *CDH1* promoter CpG island. *CDH1* promoter hypermethylation was present in 4 of 5 *CDH1* wild-type plasmacytoid tumors but in none of the *CDH1* mutant or urothelial carcinoma, NOS specimens examined (Supplementary Figure 4). Notably, E-cadherin staining was absent in the invasive component of plasmacytoid-variant tumors but retained within *in situ* regions (Supplementary Figure 5). In sum, the results indicate that loss of E-cadherin expression, most commonly as a result of somatic *CDH1* mutation, is the defining molecular event in plasmacytoid-variant bladder cancers.

Patients with plasmacytoid-variant bladder cancers display a higher cumulative incidence of local recurrence and cancer-specific mortality (Figure 2a–b) and more often exhibit a pattern of peritoneal spread than bladder tumors with pure urothelial carcinoma, NOS histology. To explore whether E-cadherin loss is the molecular basis for the distinct pattern of local invasion observed in patients with plasmacytoid-variant bladder cancers, we performed Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9-mediated knockout of *CDH1* in two urothelial carcinoma cell lines (RT4 and MGHU4) (**On-line Methods** and Figure 2c). Loss of E-cadherin expression resulted in increased migratory

capability of MGHU4 cells (Figure 2d–e) and both RT4 and MGHU4 CDH1 knockouts displayed enhanced migration across a Boyden chamber membrane (Figure 2f) as compared to the parental lines. These results suggest that somatic loss-of-function mutations in *CDH1*, with consequent E-cadherin loss, leads to the enhanced cellular migration and invasive properties characteristic of plasmacytoid-variant tumors.

In summary, we report *CDH1* alteration as the pathognomonic feature of plasmacytoid-variant bladder cancer, a disease subtype with an aggressive clinical behavior and poor prognosis. Loss of E-cadherin expression, as a result of *CDH1* somatic mutation or promoter hypermethylation, is associated with enhanced cellular migration, likely explaining the unique peritoneal pattern of disease dissemination and poor clinical outcome of patients with this disease. While inactivating mutations in *CDH1* were found exclusively in plasmacytoid-variant tumors, the pattern of co-altered genes was similar to bladder cancers with uniformly urothelial carcinoma, NOS histology. This suggests that both histologic subtypes likely evolve from a common cell of origin, with *CDH1* alterations demarcating a distinct evolutionary path. The frequent presence of clinically actionable alterations in genes such as *ERBB2*, *PIK3CA*, and *TSC1* and the poor prognosis of patients with this disease imply that early use of targeted agents, as part of a multi-modality treatment approach, should be considered for patients with plasmacytoid-variant bladder cancers.

Online Methods

Sample Acquisition

Following Institutional Review Board approval, we identified tissue samples from all patients with a diagnosis of plasmacytoid or signet ring cell bladder carcinoma evaluated at Memorial Sloan Kettering Cancer Center between 1993 and 2014. All patients included in this analysis had written informed consent. Each specimen underwent detailed histopathologic examination initially by a single genitourinary pathologist (HAA) to confirm plasmacytoid morphology. Hematoxylin & eosin (H&E) slides were subsequently reviewed by a panel of six genitourinary pathologists (AG, YC, SWF, SKT, AG, and VER). Representative formalin-fixed, paraffin-embedded (FFPE) sections from each sample were selected for analysis. In a subset of cases, macro-dissection was performed to enrich for tumor content and minimize stromal tissue contamination. Matching normal tissue for germline DNA was obtained from uninvolved FFPE tissue sections of benign lymph nodes collected as part of surgical resection. Immunohistochemistry for E-cadherin was performed from slides taken from the same tissue block which was utilized for Next Generation sequencing analysis.

Clinical Data Analysis

Clinical data was accessioned for 53 patients treated at Memorial Sloan Kettering Cancer Center between 7/1994–4/2014 who had predominantly plasmacytoid histology. Among this group, 16 patients had metastases at presentation and were excluded from recurrence and survival analyses. Two patients had treatment and follow up at outside hospitals and also were excluded, leaving 37 patients that were included in the survival analyses. Patient demographics, pathologic stage, surgical margin status, clinical follow-up, and the use of

preoperative chemotherapy in the neoadjuvant or consolidative setting were obtained. The same clinicopathologic data was collected for 978 patients who received definitive treatment for pure bladder urothelial carcinoma between 5/2001–3/2010. Median follow-up time among survivors was 71.8 months (min = 0.4 months, max = 150 months). During follow-up, 437 patients died. Of those deaths, 270 were due to cancer. There were 348 recurrences during follow-up, of which 170 were local recurrences.

Baseline characteristics were compared by histology using the Fisher's exact test for categorical factors and the Wilcoxon test for continuous factors. Follow-up time started at date of radical cystectomy or definitive treatment. Because many patients died of causes other than cancer and because many patients died without recurrence, we used competing events methods when calculating cancer-specific mortality (CSM) and local recurrence-free survival. Cumulative incidence estimated CSM and local recurrence-free survival, and Gray's test was used to compare groups. Multivariable survival analyses were conducted using Cox regression or competing risks regression.

A p-value <0.05 was considered statistically significant. All analyses were conducted in R software version 3.1.1 (R Core Development Team, Vienna, Austria).

Whole Exome Sequencing

Four FFPE and two frozen tumors with matched normal tissue underwent DNA extraction with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following manufacturer's instructions. These six samples were subjected to whole exome sequencing. Between 1.5 and 2 µg of genomic DNA was captured by hybridization using the SureSelect XT Human All Exon V4 (Agilent Technologies). Samples were prepared according to the manufacturer's instructions.

PCR amplification of the libraries was carried out for 6 cycles in the pre-capture step and for 10 cycles post-capture. Samples were barcoded and run on a HiSeq 2500 in 100 bp paired-end runs using the TruSeq SBS Kit v5 (Illumina). The average number of read pairs per sample was 63 million, the average duplication rate was 12%, and 94.4% of the targeted regions were sequenced at 30X or higher coverage. Sequencing data is available on the publicly accessible cBio Portal for Cancer Genomics.¹³

Sequence Alignment and mutation identification

Sequence alignment and mutation identification were performed as previously described.¹⁴ Briefly, paired-end sequencing data from exome capture libraries were aligned to the reference human genome (hg19) using the Burrows-Wheeler Aligner.¹⁵ De-duplication, base quality recalibration, and multiple-sequence realignment were performed using Picard suite and the Genome Analysis Toolkit prior to mutation detection.^{16,17} BAM files were coordinate-sorted and processed for both point mutations and small insertions and deletions (indels) less than 50bp in length. Single-nucleotide variants were detected with MuTect, a Bayesian framework for the detection of somatic mutations, and indels were detected using Pindel.^{18,19}

Targeted exon and capture sequencing

A solution phase hybridization-based exon capture and massively parallel DNA sequencing assay was used to profile the mutation and copy number alteration profile of 300 cancer-associated genes in 25 plasmacytoid-variant tumor samples as previously described.²⁰ Six additional plasmacytoid-variant tumors were subjected to a CLIA-certified assay (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets, or MSK-IMPACT) that screens 341 cancer-associated genes using the same technology.²¹ Sequencing data was deposited into the cBio Portal (see above).

Bisulfite sequencing

Five hundred ng of genomic DNA was modified with sodium bisulfite using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA). PCR primers were used to amplify a 259 bp fragment of a CpG island beginning 311 bp upstream of the *CDH1* translation start site (Supplemental Table 2). The PCR product was then subcloned into the pSC-A-amp PCR Cloning Vector (Agilent Technologies, La Jolla, CA) according to the manufacturer's instructions for Sanger sequencing. Bisulfite conversion efficiency was calculated by dividing the number of non-CpG cytosines converted to thymines by the total number of non-CpG cytosines, and clones with conversion efficiencies less than 95% were not analyzed. Data analysis was performed using BISMA.²²

CRISPR/Cas9-mediated knockout of *CDH1*

LentiCRISPR v2 plasmid deposited by Sanjana et al²³ was obtained from Addgene (Cambridge, MA), and a D10A mutation was introduced in the Cas9 coding sequence to improve the specificity of genome editing. Oligonucleotides targeting exon 3 of *CDH1* were subcloned into LentiCRISPR v2 nickase (Supplemental Table 2). Lentivirus was produced by transfecting these two constructs together with psPAX2 and pMD2.G (Addgene, Cambridge, MA) in 293T cells, and RT4 and MGHU4 (gift from Margaret Knowles, University of Leeds) bladder cancer cells were infected. Single clones of transduced cells were screened for CDH1 expression by immunoblot (E-cadherin antibody from Cell Signaling, Cat. No. 3195), and those without detectable CDH1 protein were used to perform further experiments.

Wound Healing Assay

MGHU4 and MGHU4 CDH1 knockout clones were plated on a 60 mm dish in minimal essential media (MEM) with 10% FBS and allowed to divide to create a confluent monolayer. Media was changed to MEM with 2% FBS 8 hours before the wound assay was performed. The monolayer was scratched with a P200 pipet tip, cell debris was removed by washing once with MEM with 2% FBS, and the cells were cultured in MEM with 2% FBS. Image acquisition was performed immediately after scratching and at 16 and 20 hours after scratching. Images were analyzed using the Microscope Image Analysis Toolbox (MiToBo) extension for ImageJ.

Boyden Chamber Cell Migration Assay

Each assay experiment was conducted within a 24-well plate. Cells (5×10^4) were seeded onto the upper chamber of a transwell chamber insert (8.0 μm), and media containing 10% Fetal Bovine Serum (FBS) and 20 ng/ml Epidermal Growth Factor (EGF) was placed outside the insert to serve as a chemoattractant. After a 24 hour incubation period, migrated cells were fixed and stained by crystal violet (10%) / formaldehyde (37%) solution, followed by microscopic examination. Five random views were selected to count the migrated cells. Each experiment was repeated independently three times. P-values were generated using t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Translational and Integrative Medicine Research Fund award (H.A.A.), Cycle for Survival (H.A.A. and B.S.T.), the Josie Robertson Foundation (B.S.T.), and the Marie-Josée and Henry R. Kravis Center for Molecular Oncology. This study was also funded in part by the Sloan Kettering Institute for Cancer Research Cancer Center Support Grant (P30CA008748).

References

1. Siegel RL, Miller KD, Jemal A. CA Cancer J Clin. 2015; 65:5–29. [PubMed: 25559415]
2. Amin MB. Mod Pathol. 2009; 22(Suppl 2):S96–S118. [PubMed: 19494856]
3. Chalasani V, Chin JL, Izawa JJ. Can Urol Assoc J. 2009; 3:S193–8. [PubMed: 20019984]
4. Dayyani F, et al. J Urol. 2013; 189:1656–61. [PubMed: 23159581]
5. Kaimakliotis HZ, et al. Urology. 2014; 83:1112–6. [PubMed: 24582117]
6. Keck B, et al. BMC Cancer. 2013; 13:71. [PubMed: 23394492]
7. Nigwekar P, et al. Am J Surg Pathol. 2009; 33:417–24. [PubMed: 19011563]
8. Cancer Genome Atlas, N. Nature. 2012; 490:61–70. [PubMed: 23000897]
9. Cancer Genome Atlas Research, N. Nature. 2014; 513:202–9. [PubMed: 25079317]
10. Graff JR, et al. Cancer Res. 1995; 55:5195–9. [PubMed: 7585573]
11. Grady WM, et al. Nat Genet. 2000; 26:16–7. [PubMed: 10973239]
12. Corso G, et al. J Clin Oncol. 2013; 31:868–75. [PubMed: 23341533]
13. Cerami E, et al. Cancer Discov. 2012; 2:401–4. [PubMed: 22588877]
14. Johnson BE, et al. Science. 2014; 343:189–93. [PubMed: 24336570]
15. Li H, Durbin R. Bioinformatics. 2009; 25:1754–60. [PubMed: 19451168]
16. McKenna A, et al. Genome Res. 2010; 20:1297–303. [PubMed: 20644199]
17. DePristo MA, et al. Nat Genet. 2011; 43:491–8. [PubMed: 21478889]
18. Cibulskis K, et al. Nat Biotechnol. 2013; 31:213–9. [PubMed: 23396013]
19. Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. Bioinformatics. 2009; 25:2865–71. [PubMed: 19561018]
20. Won HH, Scott SN, Brannon AR, Shah RH, Berger MF. J Vis Exp. 2013:e50710. [PubMed: 24192750]
21. Cheng DT, et al. J Mol Diagn. 2015; 17:251–64. [PubMed: 25801821]
22. Rohde C, Zhang Y, Reinhardt R, Jeltsch A. BMC Bioinformatics. 2010; 11:230. [PubMed: 20459626]
23. Sanjana NE, Shalem O, Zhang F. Nat Methods. 2014; 11:783–4. [PubMed: 25075903]

Figure 1. Comparison of the genomic landscape of plasmacytoid-variant bladder cancers to urothelial carcinoma, NOS cancers

a) Heatmap comparing the frequency and distribution of *CDH1* alterations and select co-altered genes within 25 plasmacytoid-variant bladder cancers (including the 6 tumors analyzed by WES), a prospective cohort of 62 urothelial carcinomas (including 6 with plasmacytoid-variant histology), and 121 muscle-invasive urothelial carcinoma, NOS samples (urothelial TCGA). Star: Six *CDH1* mutant plasmacytoid-variant tumors from the prospective clinical cohort. **b)** Comparison of frequencies of select genetic alterations between 31 plasmacytoid-variant bladder cancers, 127 urothelial TCGA samples, and a prospective institutional cohort of 56 urothelial carcinoma, NOS tumors. Asterisk: $p < 0.05$. **c)** Representative H&E images of plasmacytoid-variant bladder, lobular breast, and diffuse gastric carcinomas (top). Distribution of *CDH1* alterations in all 3 tumor types are displayed (bottom). Stars: nonsense, frameshift, splice site mutations; triangles: point mutations; bars: indels. Cad_pro: Cadherin prodomain; Cadherin_C: Cadherin cytoplasmic domain.

Figure 2. Clinical and biologic effects of *CDH1* loss

a–b) Cancer-specific mortality and disease recurrence following radical cystectomy are shown in patients with plasmacytoid-variant or urothelial carcinoma, NOS tumors. **c)** Immunoblot of RT4 and MGHU4 bladder cancer cell lines subjected to CRISPR-mediated genetic deletion of *CDH1*. **d–e)** *In vitro* scratch assay of MGHU4 cells with or without *CDH1* deletion. Error bars represent median and interquartile range. **f)** Boyden chamber assay of RT4 and MGHU4 cells with or without *CDH1* deletion. Asterisk: $p < 0.05$.