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Original Article

Clinical features of patients with *MTAP*-deleted bladder cancer

Andre L De Souza[1], Anthony E Mega[1], John Douglass[1], Adam J Olszewski[1], Ece D Gamsiz Uzun[2], Alper Uzun[3,4], Charissa Chou[2], Fenghai Duan[5], Jinyu Wang[6], Amin Ali[2], Dragan J Golijanin[7], Sheldon L Holder[1,2], Galina G Lagos[1], Howard Safran[1], Wafik S El-Deiry[1,2], Benedito A Carneiro[1]

[1]Division of Hematology Oncology, Legorreta Cancer Center at Brown University, Lifespan Cancer Institute, Providence RI, United States; [2]Department of Pathology and Laboratory Medicine, Rhode Island Hospital and Lifespan Medical Center, Providence, RI, United States; [3]Center for Computational Molecular Biology, Brown University, Providence RI, United States; [4]Department of Pediatrics, The Warren Alpert Medical School, Brown University, Providence, RI, United States; [5]Department of Biostatistics and Center for Statistical Sciences, Brown University School of Public Health, Providence, RI, United States; [6]Data Science Initiative, Brown University, Providence, RI, United States; [7]Urology Department, Minimally Invasive Urology Institute, The Miriam Hospital, The Warren Alpert Medical School of Brown University, Providence, RI, United States

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Abstract: Advanced urothelial carcinoma continues to have a dismal prognosis despite several new therapies in the last 5 years. *FGFR2* and *FGFR3* mutations and fusions, PD-L1 expression, tumor mutational burden, and microsatellite instability are established predictive biomarkers in advanced urothelial carcinoma. Novel biomarkers can optimize the sequencing of available treatments and improve outcomes. We describe herein the clinical and pathologic features of patients with an emerging subtype of bladder cancer characterized by deletion of the gene *MTAP* encoding the enzyme S-Methyl-5'-thioadenosine phosphatase, a potential biomarker of response to pemetrexed. We performed a retrospective analysis of 61 patients with advanced urothelial carcinoma for whom demographics, pathologic specimens, next generation sequencing, and clinical outcomes were available. We compared the frequency of histology variants, upper tract location, pathogenic gene variants, tumor response, progression free survival (PFS) and overall survival (OS) between patients with tumors harboring *MTAP* deletion (*MTAP*-del) and wild type tumors (*MTAP*-WT). A propensity score matching of 5 covariates (age, gender, presence of variant histology, prior surgery, and prior non-muscle invasive bladder cancer) was calculated to compensate for disparity when comparing survival in these subgroups. Non-supervised clustering analysis of differentially expressed genes between *MTAP*-del and *MTAP*-WT urothelial carcinomas was performed. *MTAP*-del occurred in 19 patients (31%). Tumors with *MTAP*-del were characterized by higher prevalence of squamous differentiation (47.4 vs 11.9%), bone metastases (52.6 vs 23.5%) and lower frequency of upper urinary tract location (5.2% vs 26.1%). Pathway gene set enrichment analysis showed that among the genes upregulated in the *MTAP*-del cohort, at least 5 were linked to keratinization (FOXN1, KRT33A/B, KRT84, RPTN) possibly contributing to the higher prevalence of squamous differentiation. Alterations in the PIK3 and MAPK pathways were more frequent when *MTAP* was deleted. There was a trend to inferior response to chemotherapy among *MTAP*-del tumors, but no difference in the response to immune checkpoint inhibitors or enfortumab. Median progression free survival after first line therapy (PFS1) was 5.5 months for patients with *MTAP*-WT and 4.5 months for patients with *MTAP*-del (HR = 1.30; 95% CI, 0.64-2.63; P = 0.471). There was no difference in the time from metastatic diagnosis to death (P = 0.6346). Median OS from diagnosis of localized or de novo metastatic disease was 16 months (range 1.5-60, IQR 8-26) for patients with MTAP-del and 24.5 months (range 3-156, IQR 16-48) for patients with MTAP-WT (P = 0.0218), suggesting that time to progression to metastatic disease is shorter in MTAP-del patients. Covariates did not impact significantly overall survival on propensity score matching. In conclusion, MTAP -del occurs in approximately 30% of patients with advanced urothelial carcinoma and defines a subgroup of patients with aggressive features, such as squamous differentiation, frequent bone metastases, poor response to chemotherapy, and shorter time to progression to metastatic disease.

Keywords: Urothelial carcinoma, MTAP, chemotherapy, immunotherapy

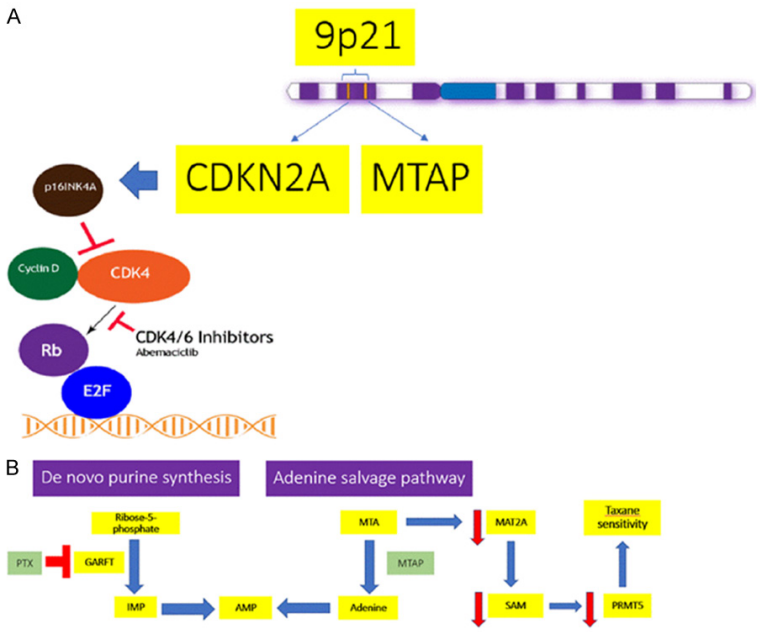

Figure 1. A. In the short arm of chromosome 9 (9p) is segment 21.3 (9p21.3), which contains the genes *MTAP*, *CDKN2A/B* and a series of innate immune response cytokine genes (Type-1 interferons). *CDKN2A* encodes for a suppressor of the cyclin D/CDK4, upregulated in 9p21.3 deletion, which can potentially be inhibited by CDK4/6 inhibitors. B. The MTAP enzyme promotes adenine rescue when the GARFT enzyme in the de novo purine synthesis pathway is inhibited by pemetrexed. Therefore, *MTAP* deletion renders cells sensitive to pemetrexed. MTAP deletion also leads to accumulation of MTA which in turn inhibits MAT2A activity suppressing the levels of SAM, a substrate for PRMT5. AMP = adenosine monophosphate; GARFT = Glycinamide Ribonucleotide Formyltransferase; IMP = inosine monophosphate; MAT2A = Methionine adenosyltransferase 2A; MTA = methyladenosine; MTAP = Methyladenosine phosphorylase; PRMT5 = protein arginine methyltransferase 5; PTX = pemetrexed; SAM = S-Adenosylmethionine.

Introduction

Bladder cancer is the 10th most common cancer worldwide with over 570,000 patients diagnosed in 2020 [1]. It is a lethal disease when distant metastases are detected with a 5-year survival rate of 6% [2]. Unresectable locally advanced and metastatic urothelial carcinoma are managed with first line platinum-based chemotherapy (PBC) in platinum eligible patients, which represents 50% of the newly diagnosed patient population [3-7]. Platinum-ineligible patients or those with disease progression within 12 months of platinum-based neo- or adjuvant chemotherapy, are candidates for first-line immune checkpoint inhibitors (ICI) [8, 9]. ICIs have also shown superior overall survival when given as switch maintenance after first line PBC and for patients who are refractory to first line chemotherapy [10-12]. Three other drugs are available for patients who are refractory or ineligible to PBC or ICIs. Two of them are antibody drug conjugates, enfortumab vedotin and sacituzumab govitecan. Enfortumab vedotin targets the ubiquitously expressed surface protein Nectin-4 and delivers the microtubule targeted payload Monomethyl Auristatin E (MMAE); it demonstrated the highest overall and complete response rates among approved therapies for platinum-refractory patients [13, 14]. Sacituzumab govitecan targets trophoblast cell surface antigen 2 (Trop-2), delivers a SN-38 (the active metabolite of irinotecan) payload, and has activity after PBC and ICIs without biomarker selection by Trop2 [15]. The tyrosine kinase inhibitor erdafinitib is used for treatment of bladder cancer harboring *FGFR3* or *FGFR2* molecular alterations refractory to PBC and ICIs [16]. Despite this expanding number of therapeutic agents, it is paramount to identify new biomarkers and therapeutic targets to improve the outcomes of advanced urothelial carcinoma.

One of these biomarkers is the deletion of a gene involved in purine synthesis, *MTAP*, originally described in the context of chromosomal 9p21.3 deletion [17]. Homozygous deletion of 9p21 occurs in 25% of bladder cancers and other tumors such as glioblastoma (41%), pancreatic adenocarcinomas (22%) and lung cancers (15%). The enzyme S-Methyl-5'-thioadenosine phosphatase (MTAP) rescues adenosine production when purine synthesis is inhibited by the anti-metabolite pemetrexed (**Figure 1**). Based on the TCGA dataset, 26.8% of patients with bladder cancer have homozygous *MTAP* gene deletion. Bladder cancer cell lines with *MTAP* deletion (MTAP deficient) have marked sensitivity to pemetrexed compared to *MTAP* wild type (MTAP proficient) cells as exhibited by

the IC50 40 times lower in MTAP deficient cells [18]. When pemetrexed was given to patients with MTAP deficiency, tumor response rates were also superior when compared to patients with MTAP proficient tumors (80% vs 10%) [18]. Retrospective series also showed a 5-28% response rate in this group [19-22]. Therefore, *MTAP* deletion creates a potential therapeutic vulnerability to pemetrexed. Other genes co-deleted with *MTAP* in tumors with 9p21 deletion include *CDKN2A* encoding p16INK4A which regulates cell cycle; and Type 1 interferon genes involved in the innate immune response. In fact, *MTAP* deleted tumors are cold and poorly responsive to immune checkpoint inhibitors [23], possibly through inhibition of methylation of the transcription factor STAT1, involved in the interferon pathway and PD-L1 expression [24]. These findings suggest that 9p21 deletion can impact the tumor sensitivity to ICI and define a clinically relevant molecularly defined subgroup of bladder cancer.

We describe a single-center retrospective analysis of patients with metastatic urothelial carcinoma harboring *MTAP* deletion and its associated clinical, pathological, and genomic features as well as treatment outcomes. These results can inform future trial design of novel therapeutics for this subgroup of patients with advanced urothelial carcinoma and define a novel prognostic and predictive biomarker.

Methods

Aim

The study aimed to describe the clinical, pathologic, and genomic characteristics of patients with advanced urothelial carcinoma harboring *MTAP*-del compared to a cohort of patients with *MTAP*-WT.

Study population and design

Patients with advanced urothelial carcinoma treated at the Lifespan Cancer Institute (period of 2020-2022) who had signed informed consent and had their tumor specimen processed by a Clinical Laboratory Improvement Amendments (CLIA)-approved next generation sequencing platform were included. This study was approved by the Institutional Review Board (IRB) of Rhode Island Hospital under a unified research protocol aimed to investigate molecular features of tumors and biomarkers (IRB# 449060-38, 39). All methods were performed in accordance with the Declaration of Helsinki and our local IRB guidelines and regulations. Informed consent was obtained from all participants and/or their legal guardians. Given the retrospective nature of this research study, formal sample size or power calculations were not performed. The sample size was defined by the number of patients treated at our cancer center with available and adequate results of NGS profiling.

Definition of variables and metrics

Descriptive statistics depicted median and range for continuous demographic variables (age, BMI) and frequency statistics (Chi-square) calculated the odds of chance occurrence of a difference in discrete variables (gender, race/ethnicity, smoking) between the *MTAP*-WT and *MTAP*-del patients. Histology was described as predominantly urothelial, micropapillary, plasmacytoid and others. Carcinoma was predominantly urothelial when over 50% of the specimen was assigned to this histology. Surgical and/or clinical staging was recorded as per AJCC 8th edition. Among important surgical variables, prior non-muscle invasive bladder cancer, intravesical Bacillus Calmette-Guerin (BCG), transurethral resection of bladder tumor (TURBT), cystectomy or nephroureterectomy were reported. Clinical characteristics of importance included *de novo* metastatic disease, number of lines of systemic therapy, prior curative surgery, or palliative radiation.

Follow-up was defined as time from diagnosis to death or censoring. Censoring was described as last visit in clinic or last telephone encounter with the patient. Median overall survival was designated as time from diagnosis from metastatic disease to death. Median progression free survival was delineated as time from diagnosis to progression or death. All scans were reviewed by the investigator and RECIST 1.1 criteria were used to assess overall response rate to therapy. Disease control rate comprised stable disease for at least 6 months and overall response rate. The best response was assigned to each of the lines of therapy received in the metastatic setting. We documented the few cases of complete pathologic response. *De novo* metastatic disease was defined as metastases demonstrated in scans up to 3 months from date of diagnosis.

Tumor genomic sequencing

Next generation sequencing was performed at a CLIA-certified pathology laboratory (Tempus, Chicago, IL). Formalin-fixed paraffin-embedded tissues underwent hybrid-capture based complete genomic profile, which interrogates the coding exons of up to 700 cancer-related genes and up to 21 gene fusions commonly rearranged in cancer. Tumor mutational burden (TMB), PD-L1 expression, and microsatellite instability are also reported in a subset of tumor specimens, as well as RNA overexpression.

Statistical analyses

Comparison between patient subgroups were made via Chi-square or Fisher's exact tests for categorical variables and unpaired *t* test for continuous variables. Descriptive variables were documented as median, range and interquartile range. Overall survival and progression free survival were estimated by the Kaplan-Meier method with hazard ratio, confidence intervals and P provided by Cox model. Progression free survival was defined as the time started from C1D1 of cancer therapy and lasted until progression on scans or to death from any cause, whichever occurs first. Patients who died prior to therapy were not considered for progression free survival analysis. Overall survival was defined as the time from diagnosis of localized or de novo disease to death from any cause. The technique of nearest neighbor propensity score matching (PSM) was applied to overall survival analysis by accounting for covariate balance between MTAP-del and MTAP-WT groups.

We evaluated relevant gene alterations in urothelial cancer, due to its frequency, prognostic value, or therapeutic relevance (*TP53*, *TERT, ERBB2*, FGFR2, and *FGFR3*). Besides, *RB1* was evaluated due to its importance as a negative predictor for CDK4/6 inhibitors. We also compared the frequency statistics by grouping pathways frequently mutated in urothelial carcinoma that was derived from the list of gene alterations reported in our cohort of patients. These pathways included chromatin remodeling (*ARID1A*, *ARID2A*, *KAT6A*, *KDM6A*, *KMT2C*, *KMT2D*, *NSD1*, *PBRM1*, *SETD2*, *SMARCA4*), homologous repair deficiency (*ATR*, *BAP1*, *BARD1*, *CDK12*, *NBN*, *RAD51*), cell Cycle (*CCND1*, *CDKN1A*, *FBXW7*), PIK3 (*AKT2*, *PHLPP1*, *PI3KCA, PI3KR1*, *PTEN*, *TSC1*, *TSC2*), and MAPK (*BRAF*, *HRAS*, *KRAS*, *MAPK1*, *RAF1*).

Results

Demographics

We included 61 patients with advanced urothelial carcinoma (metastatic or unresectable locally advanced) evaluated by next generation sequencing. Median age was 68 years old, 18 (29%) of patients were female, and 8 (13%) were non-Caucasians. Patients had received 1-4 lines of treatment. Of 61 patients, 19 were *MTAP*-del (31.1%) and 42 were *MTAP*-WT (68.9%). Among the 11 patients with unresectable Stage I-III urothelial carcinoma, 7 patients were *MTAP*-WT, and 4 were *MTAP*--del. Median age at diagnosis in patients who were *MTAP*-WT was 71 years-old (range 49-85, IQR 64-77), while it was 63 years-old (range 52-84, IQR 61-73) for patients who were *MTAP*-del, but the difference between the medians and distribution was not statistically significant (P = 0.2337).

Regarding demographics, the frequency statistics between the *MTAP*-del and *MTAP*-WT populations for gender, ethnicity, or race did not achieve significance. Smoking status (current or former smokers versus never smoker) frequency statistics in patients with deleted versus wild-type *MTAP* was non-significant (P = 0.175584) (**Table 1**).

Clinical and pathologic characteristics

There was a statistically significant association between the presence of squamous differentiation and *MTAP*-del. Nine of the 19 (47.3%) patients with *MTAP*-del had squamous differentiation, as opposed to 11.9% (5/42) patients with *MTAP*-WT (P = 0.006498) (**Table 1**). There was no significant difference in the frequency of other histological variants between the MTAP-del (2 patients with glandular differentiation) and MTAP-WT (plasmacytoid-3, sarcomatoid-2, small cell-1, micropapillary-1, mucinous, clear cell-1).

Bone metastases were present more frequently in patients with *MTAP*-del and tumors were predominantly in the bladder as opposed to the upper urinary tract compared with *MTAP*-WT. Among *MTAP*-del patients with metastases, 10 out of 19 (52.6%) had bone metastases, as

Table 1. Patient demographics and tumor characteristics

	MTAP-del (N = 19)	MTAP-WT (N = 42)	Statistical significance
Age, median (range) (years)	71 (49-85)	63 (52-84)	P = 0.2337
Gender, n (%)			P = 0.1755
Male	16 (84.2)	27 (64.2)	
Female	3 (15.8)	15 (35.8)	
Smoking, n (%)	15 (78.9)	24 (57.1)	P = 0.1755
Squamous differentiation, n (%)	9 (47.4)	5 (11.9)	P = 0.006498
Upper Tract, n (%)	1 (5.2)	11 (26.1)	P = 0.087669
Stage at presentation, n (%)			
T3/4	8 (42.0)	17 (40.2)	P = 0.8718
N1	7 (36.8)	14 (33.3)	P = 0.9809
M1	7 (36.8)	12 (28.6)	P = 0.728254
Metastatic sites, n (%)			
Bone	10 (52.63)	8 (23.5)	P = 0.018
Liver	4 (21.05)	11 (26.1)	P = 0.912

Table 2. Mutational landscape of MTAP-del and MTAP-WT urothelial carcinomas

Genomics (n, %)	MTAP-del (N = 19)	MTAP-WT (N = 42)	*P* value
PD-L1 > 10%	8 (42)	10 (24)	P = 0.251042
TMB > 10 m/MB	2 (10)	6 (14)	P = 0.994643
Gene alteration			
FGFR2/3	3 (16)	10 (24)	P = 0.7108
TERT	17 (89)	20 (48)	P = 0.004
TP53	10 (53)	29 (69)	P = 0.3428
RB1	1 (5)	9 (21)	P = 0.2278
ERBB2	1 (5)	5 (12)	P = 0.7320

Table 3. Alterations in gene pathways in MTAP-del and MTAP-WT urothelial carcinomas

Gene pathway, n (%)	MTAP-del	MTAP-WT	Statistical significance
PIK3	9 (47)	7 (17)	P = 0.027
MAPK	2 (10.5)	8 (19)	P = 0.646
COMPASS	13 (68)	26 (62)	P = 0.839
Beta-catenin	5 (26)	6 (14)	P = 0.4400
Circadian Rhythm	5 (26)	7 (17)	P = 0.4400
Cell Cycle	4 (21)	5 (12)	P = 0.5870
Apoptosis	4 (21)	4 (9)	P = 0.4089
Other FGF pathway	4 (21)	3 (7)	P = 0.5579
HRD	3 (16)	12 (28)	P = 0.4517
Androgen receptor	3 (16)	3 (7)	P = 0.557903
TP53 regulators	3 (16)	0	N/A

opposed to 8 out of 42 (23.5%) who were *MTAP*-WT (P = 0.0182). Among patients with *MTAP*-del, 4 out of 19 (21%) had liver metastases, while 11 out of 42 (26.1%) *MTAP*-WT patients had liver metastases (P = 0.912) (**Table 1**). For the whole population, 12 patients (19.7%) had upper tract disease and 49 had bladder tumors. Only one patient (5.2%) with *MTAP*-del had upper tract disease as opposed to 11 (26.1%) of the patients with *MTAP*-WT (P = 0.087), a nonsignificant trend. Therefore, 94.8% of patients with *MTAP*-del had bladder cancer; conversely, 73.9% of patients with *MTAP*-WT had bladder cancer. There was no correlation between *de novo* metastatic presentation and *MTAP* status (P = 0.688). Seventeen of the 42 (40.4%) *MTAP*-WT, and 8 of 19 (42%) *MTAP*-del patients had T3 or T4 staging (P = 0.871873). N1 disease was found in 14 of 42 (33.3%) patients with *MTAP*-WT and 7 of 19 patients (36.8%) with *MTAP*-del (P = 0.980).

Genomic analysis

The prevalence of *TERT* and *PIK3* mutations was higher among MTAP-del tumors, as well as overexpression of MAPK pathway (**Tables 2** and **3**). **Table 2** shows the genetic landscape in patients with advanced urothelial carcinoma between

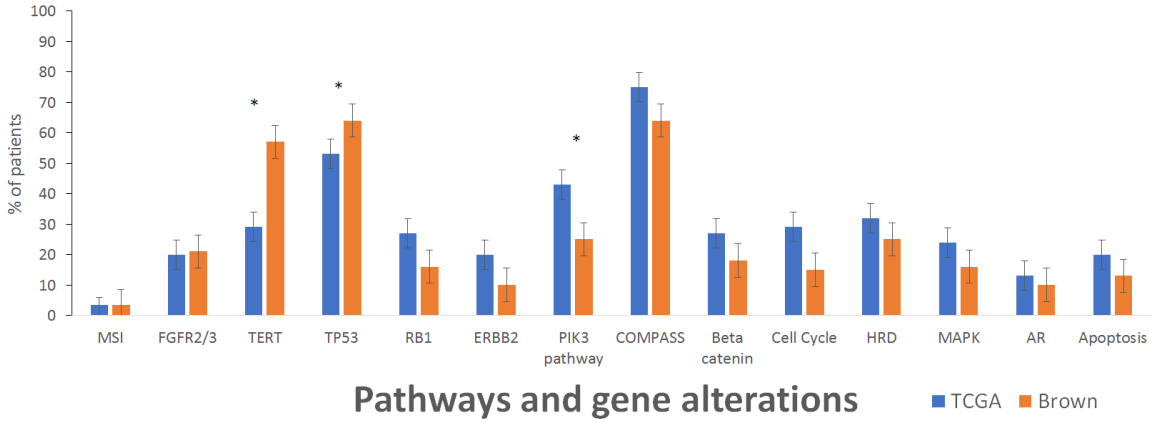

Figure 2. Comparison of frequency of the most prevalent gene and pathway alterations between the TCGA/MSK 2020 cohort (N = 476) and the present study cohort (N = 61). Notice the statistically significant difference in the prevalence of *TERT* promoter alterations (P < 0.00001), *TP53*, and PIK3 (*AKT2*, *PHLPP1*, *PIK3CA*, *PIK3R1*, *TSC1*, *TSC2*, *PTEN*) pathway alterations. The *TERT* alteration difference is attributed to the fact that the TCGA does not sequence promoter mutations, responsible for the majority of *TERT* alterations. COMPASS: COMplex of Proteins Associated with Set-1. (*) = P < 0.05.

MTAP-del and *MTAP*-WT. Primary tumor specimens were utilized for next generation sequencing in 14 patients with *MTAP*-del and 26 patients with *MTAP*-WT (P = 0.5446).

There was no significant difference in tumor mutational burden and PD-L1 expression (defined as CPS > 10%) between *MTAP*-del and *MTAP*-WT (P = 0.251042). *TERT* promoter missense mutations and PIK3 pathway gene alterations were more prevalent in *MTAP*-del patients (79% and 42%, respectively) than *MTAP*-WT (32.0% and 11.4%, respectively (P = 0.004864 for TERT; and P = 0.027094 for PIK3 pathway) (**Tables 2** and **3**).

There was no significant difference in the frequency of mutations in *RB1, TP53, FGFR2, FGFR3, ERBB2* genes between *MTAP*-del and *MTAP*-WT patients (**Table 3**). The pathways investigated encompassed chromatin remodeling (*KDM6A, KMT2C, KMT2D, ARID1A, ARID2, PBRM1, KAT6A, NSD1, SETD2, SMARCA4*), FGF pathway (*FGF3, FGF4, FGF19, FRS2*), homologous repair deficiency (*ATR, BAP1, BARD 1, RAD51, CDK12, NBN*), cell cycle (*CCND1, CDKN1A, FBXW7*), mitogen-activated protein kinase (MAPK- *BRAF, HRAS, KRAS, MAPK1, RAF1*), PIK3 (*AKT2, PHLPP1, PIK3CA, PIK3R1, TSC1, TSC2, PTEN*), androgen receptor (*AR, NCOR1, SPOP*), apoptosis (*RBM10, CAS8, MCL1*), beta-catenin (*APC, FAT1, LPR1, TCF7L2*), and circadian rhythm genes (*ELF3, ZFHX3*). Only two patients with *MTAP*-WT had microsatellite instability (MSI), and no patient with *MTAP*-del had MSI. **Figure 2** describes the frequency of these genes and pathways between the TCGA/MSK 2020 cohort and the present study cohort. Notice that *TERT* alterations differ between the two cohorts because TCGA and MSK do not sequence *TERT* promoters, which represents most of the alterations in this gene.

We assessed the distribution of mRNA overexpression between patients with *MTAP*-del and *MTAP*-WT. A total of 23 out of 42 patients with *MTAP*-WT and 15 out of 19 patients with *MTAP*-del had available mRNA sequencing data. The mRNA overexpression from genes related to the MAPK pathway (BRAF, HRAS, MAP2K2, NRAS) was more frequent among *MTAP*-del patients (63%) than *MTAP*-WT patients (33.3%) (P = 0.0572). RNA overexpression of genes related to the PIK3, or cell cycle pathways were not correlated with *MTAP*-del. Overexpression of FGFR, MYC and AR was identified only among 10 patients with *MTAP*-WT tumors.

We also performed unsupervised hierarchical cluster analysis of gene expression to compare differentially expressed genes in *MTAP*-del and *MTAP*-WT tumors. Among the 50 most downregulated genes in the *MTAP*-del group, 17 have not been previously described in urothelial carcinoma (*ACSM2A, ACSM2B, APOH, ARMC3, DDC, GP2, HGD, KCNG3, KNCV2, NKX2-2, PAPC1L2A, PKHD1, SYT13, TGM3*)

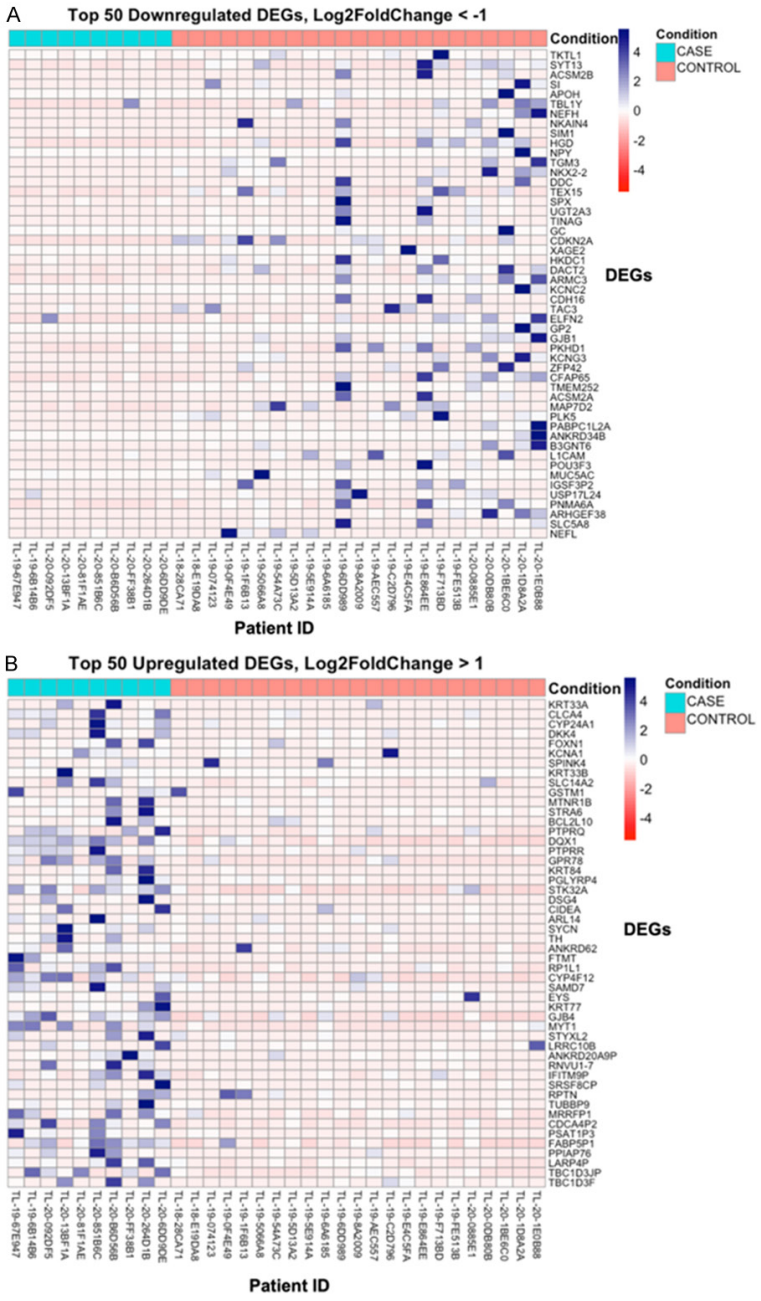

Figure 3. Pathway enrichment analysis of most downregulated (A) and most upregulated genes (B) in *MTAP*-del (cases) and *MTAP*-WT urothelial carcinoma (controls).

(**Figure 3A** and **3B**). Among the 50 most upregulated genes in the *MTAP*-del group, 8 have not been described previously in bladder cancer (*DKK4, FTMT, GPR78, KRT33A, KRT33B, LARP4P, MTNR1B, SYCN*). Among the genes that were previously linked to bladder cancer, some under- or over-expressed genes are linked to aberrant methylation. Some of the genes in this cohort regulate circadian rhythm, cell adhesion, ferroptosis/apoptosis, channels and transporters, and vesicular trafficking. We observed a higher frequency of squamous differentiation in *MTAP*-del-urothelial carcinoma. Keratinization may explain this squamous differentiation (for example genes for keratin-*KRT32A*, *KRT32B*, *KRT77* and *KRT84*-are upregulated in MTAP-deleted tumors, **Figure 3B**). Among the genes upregulated in our *MTAP*-del cohort, at least 5 trigger keratinization (such as FOXN1, KRT33A/B, KRT84, RPTN).

Efficacy of systemic therapies

Among the patients with *MTAP*-WT who received systemic therapy in the metastatic setting, median number of lines was 2 (range 1-4), and for patients with *MTAP*-del who received systemic antineoplastic treatment, median was also 2 prior lines of therapy (range 1-3).

Among patients with *MTAP*-WT, 35.7% (15/42) received chemotherapy in the metastatic setting. There were two complete responses (CRs), six partial responses (PRs), and 1 stable disease (SD) leading to an overall response rate (ORR) of 53%, and a disease control rate (DCR) of 60% (9/15). Among *MTAP*-del patients with metastatic disease, 47.3% (9/19) received chemotherapy in the metastatic setting with no responses to chemotherapy and only one stable disease as best response (DCR of 11%). This comparison of DCR rates between MTAP-del and MTAP-WT suggested a trend towards correlation of MTAP-del and poor response to chemotherapy (P = 0.0543), although the frequency statistics for overall response rate (ORR) was non-significant (P = 0.1024) (**Table 4**).

Table 4. Disease control rate and response rate

	MTAP-del	MTAP-WT	Statistical Significance
Disease control rate (%)			
Platinum-based chemotherapy	11	60	P = 0.0543
Immune checkpoint inhibitor	33	42	P = 0.8651
Enfortumab	57	50	P = 0.7638
Response rate			
Platinum-based chemotherapy	0	53	P = 0.1024
Immune checkpoint inhibitor	8	19	P = 0.6762
Enfortumab	43	33	P = 0.3420

Among patients with *MTAP*-WT treated in the metastatic setting, 73.8% (31/42) received immune checkpoint inhibitors. There were 5 CRs, 1 PR, and 7 SD to immune checkpoint inhibitors, for an ORR of 19%, and a DCR of 41.93% (13/31). Median duration of response among the 6 responders to immune checkpoint inhibitors was 20.5 months (range 6-65, IQR 11-36). Among *MTAP*-del patients with metastatic disease, 63.15% (12/19) had immune checkpoint inhibitors, with 1 CR and 3 SDs, for an ORR of 8% and a DCR of 33.3% (4/12). The DCR and ORR difference between the groups were not statistically significant (DCR: P = 0.8651; ORR: P = 0.6762; **Table 4**). The patient with *MTAP*-del who had CR continues to show no evidence of progression, with a duration of response of 12 months.

Among patients with *MTAP*-WT treated in the metastatic setting, 14.2% (6/42) had enfortumab. There were 2 PRs and 1 SD to enfortumab, for an ORR of 33.3%, and a DCR of 50% (3/6). Among *MTAP*-del patients with metastatic disease, 36.8% (7/19) had enfortumab, with 3 PRs and 1 SD for an ORR of 42.8% and a DCR of 57.14% (P = 0.7638). Only 3 out of 42 patients with *MTAP*-WT were given erdafitinib, and there was 1 PR to erdafitinib. No patient with *MTAP*-del were treated with erdafitinib. No patient in our cohort received sacituzumab govitecan at the time of data collection.

Progression free survival (PFS) analysis

Among the 32 patients who were *MTAP*-WT, 2 had no progression event, 2 were lost to follow-up and 28 had progression event (87.50%). Among the 15 patients with *MTAP*-del, 1 had no progression event and 14 had progression event (93.33%). Median progression free survival after first line therapy was 4 months (range 1.00-17.00, IQR 1.00-5.00) for patients with *MTAP*-del and 6.00 months (range 1.00-36.00, IQR 2.50-11.00) for patients with *MTAP*-WT (based on the Cox regression model: HR: 0.50 (95% CI, 0.26-0.97, *P*-value =0.0411, reference = 'MTAP-del'). Thus, PFS in patients with *MTAP*-WT is significantly better than that in patients *MTAP*-del, as shown in the Kaplan-Meier estimation of progression free survival (**Figure 4A**).

Propensity score matching (PSM) for PFS

We utilized the PSM matched data and deleted the patients who did not receive first line therapy (30 *MTAP*-WT patients, 2 had no progression event, 2 were lost to follow-up and 26 had progression event; 15 *MTAP*-del patients, 1 had no progression event and 14 had progression event). **Table 5** shows that we have attained a relatively high degree of balance on the five covariates included in the model (i.e., all *P* values < 0.05). Median progression free survival after first line therapy was 4.00 months (range 1.00-17.00, IQR 1.00-5.00) for patients with *MTAP*-del and 6.00 months (range 1.00-36.00, IQR 2.00-11.00) for patients with *MTAP*-WT (based on the Cox regression model: HR: 0.539 (95% CI, 0.28-1.05, *P*-value = 0.0682, reference = 'MTAP-del'). This indicates that after adjusting for the effects of covariates via PSM, there was a trend for a better PFS in patients with *MTAP*-WT compared to *MTAP*-del, as shown in the Kaplan-Meier estimation of progression free survival (**Figure 4B**).

Survival

Patients with *MTAP*-del have more aggressive disease, as seen by a shorter time to progression to metastatic disease. Among the 42 patients who were *MTAP*-WT, 12 are alive, 4 were lost to follow-up and 26 have deceased (59%). Among the 19 patients with *MTAP*-del, 4 are alive and 15 passed (78.94%), with no loss of follow-up. Median overall survival was 26 months (range 2.50-60.00, IQR 11-46) for patients with *MTAP*-del and 43 months (range 2.00-283.00, IQR 18-110) for patients with *MTAP*-WT (based on the Cox regression model: HR: 0.46; 95% CI: 0.23-0.91; *P*-value: 0.0266; reference: 'MTAP-del'). Thus, OS in patients with *MTAP*-WT is significantly better than that in patients with *MTAP*-del, as shown by the

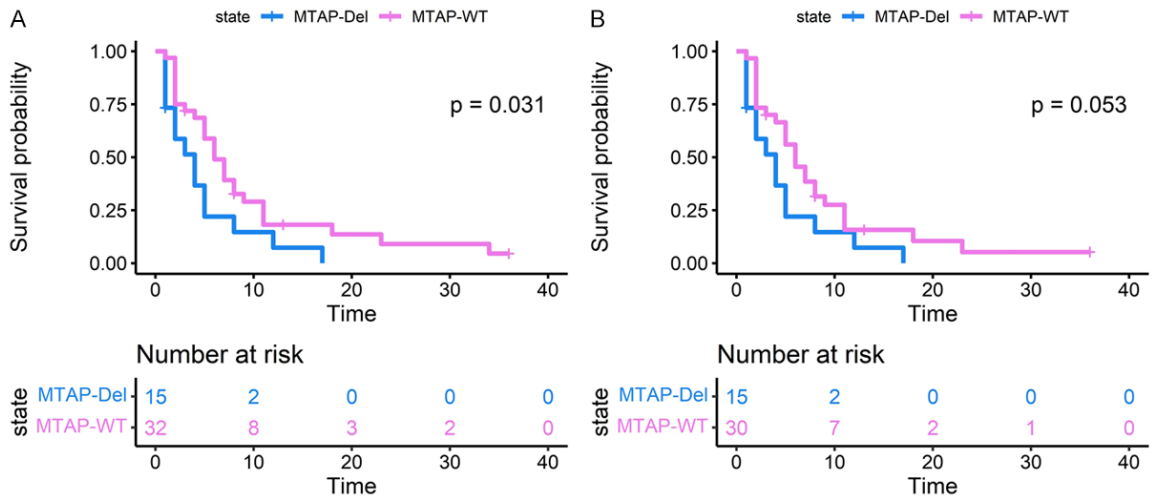

Figure 4. Kaplan-Meier estimation of Progression-free survival after first line therapy, which could be chemotherapy or immune checkpoint inhibitor prior to propensity score matching (A), and after propensity score matching (B).

Table 5. Summary of covariate distribution between MTAP groups after propensity score matching for progression-free survival analysis

	MTAP-del	MTAP-WT	*P*-value
Age (mean (SD))	64.48 (9.54)	69.04 (10.60)	0.168
Gender = 1 (%)	2 (13.3)	10 (33.3)	0.283
Histology = 1 (%)	8 (53.3)	9 (30.0)	0.232
Prior NMIBC = 1 (%)	11 (73.3)	18 (60.0)	0.582
Age (mean (SD))	64.48 (9.54)	69.04 (10.60)	0.168
Gender = 1 (%)	2 (13.3)	10 (33.3)	0.283
Prior surgery (%)			0.214
0	7 (46.7)	17 (56.7)	
1	7 (46.7)	7 (23.3)	
2	1 (6.7)	6 (20.0)	

Kaplan-Meier estimation of overall survival between two groups (**Figure 5A**).

Propensity score matching with cox regression for overall survival

We utilized the propensity score method to balance in the distribution of the baseline covariates, then ran cox regression for the matched data set. First, we conducted the propensity score method to the data set which was selected randomly (38 *MTAP*-WT patients, 10 are alive, and 28 have deceased; 19 *MTAP*-del patients, 4 are alive, and 15 passed). **Table 6** below indicates that we have attained a relatively high degree of balance on the five covariates included in the model (i.e., all *P* values > 0.05).

After Propensity Score Matching, median overall survival was 26 months (range 2.50-60.00, IQR 11-46) for patients with *MTAP*-del and 42 months (range 2.00-283, IQR 18-105) for patients with *MTAP*-WT (Based on the Cox regression model: HR: 0.49 (95% CI, 0.25-0.98, *P*-value =0.0447, reference = '*MTAP*-del'). So we can see that after adjusting for the effects of covariates via Propensity Score Matching, overall survival in patients with *MTAP*-WT is still significantly better than that in patients with *MTAP*-del, as shown by the Kaplan-Meier estimation of overall survival between two groups (**Figure 5B**).

We did not find a statistically significant impact of gender on overall survival time on propensity score matching (HR: 0.65, 95% CI, 0.30-1.40, P = 0.287). Also, patients with pure urothelial carcinoma or poorly differentiated without variants had similar survival to patients with variant histology (HR: 0.98, 95% CI, 0.48-2.00, P = 0.949). Furthermore, patients survived longer if they underwent curative cancer surgery compared to patients who did not (if prior radical cystectomy or cystoprostatectomy: HR: 0.57. 95% CI, 0.28-1.20, P = 0.125; if prior nephroureterectomy or urethrectomy: HR 0.82, 95% CI, 0.31-2.20, P = 0.693). Patients without prior non-muscle invasive bladder cancer had a shorter overall survival than patients with prior non-muscle invasive bladder cancer (HR: 1.75, 95% CI, 0.77-4.00, P = 0.182).

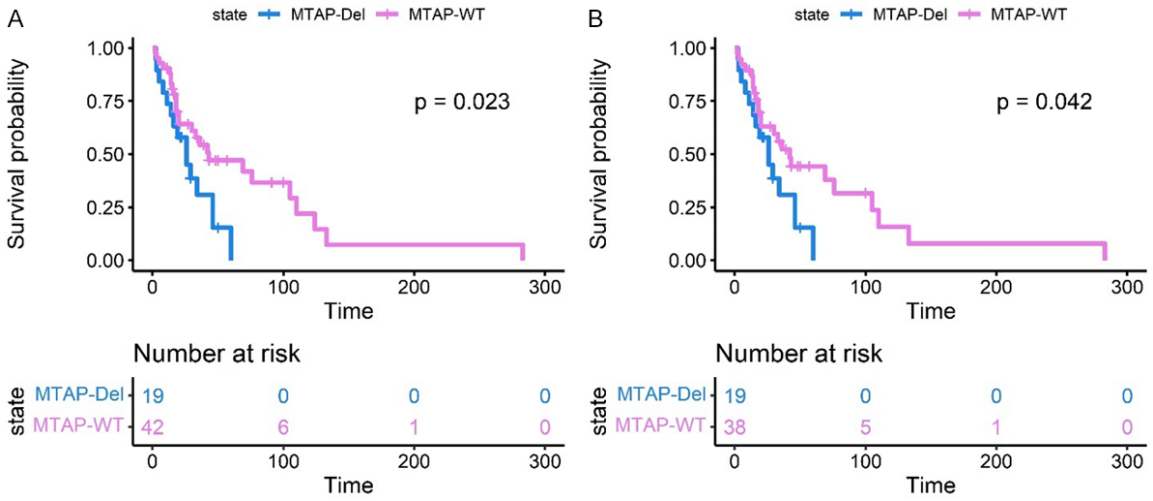

Figure 5. Overall survival from time of diagnosis prior to propensity score matching (A), and after propensity score matching (B).

Table 6. Summary of covariate distribution between MTAP groups after propensity score matching for overall survival analysis

	MTAP-del	MTAP-WT	*P*-value
Count (n)	19	38	
Age (mean (SD))	66.54 (9.54)	70.46 (10.25)	0.170
Gender = 1 (%)	3 (15.8)	13 (34.2)	0.252
Histology = 1 (%)	12 (63.2)	15 (39.5)	0.159
Prior NMIBC = 1 (%)	15 (78.9)	22 (57.9)	0.202
Prior surgery (%)			0.397
0	10 (52.6)	18 (47.4)	
1	8 (42.1)	13 (34.2)	
2	1 (5.3)	7 (18.4)	

Discussion

We describe here the clinical and pathologic characteristics of a cohort of patients with advanced urothelial carcinoma and *MTAP*-del and compared with a population with *MTAP*-WT tumors. Patients with *MTAP*-deleted tumors had a higher prevalence of the squamous histology variant, bone metastases, and shorter time to progression of metastatic disease.

Our cohort of patients with *MTAP*-del had a 47.3% prevalence of squamous cell differentiation, while 11.9% of our *MTAP*-WT patients had squamous differentiation. Squamous differentiation is found in 16-22% of patients with bladder cancer [25-27] and is associated with loss of function mutations in Ppar-gama, a transcription factor that heterodimerizes with retinoic acid and suppresses NF-kb expression, a central mediator of urothelial inflammation [28]. As NF-kb upregulates type-1 interferon response [29], a set of genes that are absent in 9p21.3 deletion, we hypothesize that the high prevalence of the squamous variant results from the interruption of a feedback loop between type 1-interferon and Nf-kb. MTA accumulation in 9p21.3 deletion also suppresses STAT1, a transcription factor in the interferon pathway [30]. Our gene set enrichment analysis also shows upregulation of genes involved in keratinization, which may play a role on squamous differentiation. To the best of our knowledge this is the first series to document the high prevalence of squamous differentiation in a *MTAP*-del cohort of patients with advanced urothelial carcinoma. This may have implications for the treatment decisions in the neoadjuvant, adjuvant, and first line treatment of patients with urothelial carcinoma and squamous differentiation.

Loss of Type 1 interferon genes in 9p21 chromosomal deletion is a predictor of poor response to immune checkpoint inhibitors in melanoma, urothelial carcinoma, and non-small cell lung cancer [31-33]. It has been shown that *MTAP*-del tumors are cold and poorly responsive to immune checkpoint inhibitors in urothe-

lial carcinoma [23], possibly through inhibition of methylation of the transcription factor STAT1, involved in the interferon pathway and PD-L1 expression [24]. Alhalabi et al showed that *MTAP*-del confers a shorter overall survival in patients treated with immune checkpoint inhibitors after platinum-based therapy. Our results did not show a significant association between *MTAP*-del with overall response or median duration of response to chemotherapy or immune checkpoint inhibitors, possibly due to the small sample size. However, there was a correlation between *MTAP*-del and higher disease control rate to chemotherapy.

The present study describes patients with an aggressive subset of metastatic urothelial carcinoma, including faster progression to metastatic disease and poor response to chemotherapy in agreement with previous studies. A retrospective analysis from the phase 2 trial investigating the efficacy of the immune checkpoint atezolizumab as first line treatment in cisplatin-ineligible patients with locally advanced or metastatic urothelial carcinoma showed poor response of *MTAP*-del urothelial carcinoma to immunotherapy [34]. Considering the recent establishment of standard of care adjuvant nivolumab after radical cystectomy in patients with high-risk muscle invasive bladder cancer [35], we could consider investigating the benefit of adjuvant nivolumab among the subgroup of patients with *MTAP*-del vs *MTAP*-WT that could reveal improved outcomes among patients with *MTAP*-WT tumors. Also, considering the upregulation of MAPK and PI3K pathways in this cohort, the therapeutic potential of targeted agents including SOS1 inhibitors, RAF/MEK stabilizers, PI3K, AKT and mTOR inhibitors could be considered in future studies.

Squamous variant of bladder cancer has poor clinical outcomes and response to chemotherapy. The higher prevalence of squamous differentiation may render *MTAP*-del advanced urothelial carcinomas sensitive to taxanes and may be a factor in the aggressive feature of these tumors. Prior studies have shown *MTAP*-del cell lines and xenografts are sensitive to MAT2 inhibitor combined with taxanes [36]. The higher prevalence of bone metastasis instigates confirmation that *MTAP* is not implicated in the establishment of the metastatic niche. Ongoing efforts in our group include to investigate differential gene expression profile between patients with bone metastases and other sites of disease, including the role of *RPTN* in MTAP-deleted urothelial carcinomas *RPTN* is overexpressed in *MTAP*-del tumors. *RPTN* is upregulated in 15% of a molecular subtype of muscle invasive bladder cancer enriched in PPARG upregulation. The transcription factor PPARG dimerizes with retinoic acid receptor to regulate squamous differentiation. Also, the present study confirms the rarity of *MTAP* deletion observed in the TCGA cohort of upper tract urothelial carcinomas, with only one patient with *MTAP*-del progressing to metastatic disease after prior nephroureterectomy.

The limitations of the present study include the retrospective nature of this work, the small sample of patients, the patient population limited to one cancer center and the fact that therapies like erdafitinib, enfortumab, and sacituzumab govitecan were introduced when the first sequenced patients had already deceased. Our database did not capture known prognostic factors in metastatic urothelial carcinoma such as performance status and hemoglobin. However, we did show that there was no difference in the prevalence of liver metastasis, another important prognostic factor after platinum-based therapy.

Conclusions

MTAP deletion represents a common subtype of advanced urothelial carcinoma and is characterized by aggressive features, such as squamous differentiation, frequent bone metastases, poor response to chemotherapy and shorter time to progression to metastatic disease. Prior studies have documented retrospectively the poor response of *MTAP*-del advanced urothelial to immunotherapy. To the best of our knowledge this is the first study to report a higher prevalence of squamous differentiation and poor response to first line combination chemotherapy in *MTAP*-del advanced urothelial carcinoma.

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This study was approved by the Institutional Review Board of Rhode Island hospital under a unified molecular consent (IRB# 449060-38, 39). Informed consent was obtained from all subjects.

Disclosure of conflict of interest

None.

Address correspondence to: Andre L De Souza, Division of Hematology Oncology, Legorreta Cancer Center at Brown University, Lifespan Cancer Institute, Providence RI, United States. E-mail: andre_desouza@brown.edu

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