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Intestinal metaplasia of the urinary tract harbors potentially oncogenic genetic variants

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Abstract

In the urinary tract, there is an uncertain relationship between intestinal metaplasia (IM), primary adenocarcinoma, and urothelial carcinoma. Although IM is usually found adjacent to concurrent urothelial carcinoma or adenocarcinoma, small retrospective series have shown that most bladder biopsies with only IM do not subsequently develop cancer. However, IM with dysplasia does seem to be associated with a higher risk of concurrent malignancy or progressing to cancer. Since the molecular landscape of these lesions has remained largely unexplored, there are significant uncertainties about the oncogenic potential of IM in the bladder and urethra. This study investigated the presence of potentially oncogenic genetic variants in cases of IM with and without dysplasia. Twenty-three (23) cases of IM (3 urethra, 20 bladder) were sequenced using a solid tumor next-generation sequencing panel. Of these, five contained IM with high-grade dysplasia (including a case with paired IM-adenocarcinoma and another with paired IM-urothelial carcinoma) and 18 lacked dysplasia. Oncogenic genetic variants were found in all cases of IM with high-grade dysplasia and in five non-dysplastic IM cases, including mutations and copy number variants commonly seen in primary adenocarcinoma of the bladder and urothelial carcinoma. This study demonstrates that IM can harbor potentially oncogenic genetic variants, suggesting that it might represent a cancer precursor or a marker of increased cancer risk in a subset of cases.

Introduction

Intestinal metaplasia (IM) is relatively uncommon in endoscopic bladder and urethral biopsies, and can be considered a subtype of cystitis/urethritis glandularis [1, 2]. The most common form of cystitis glandularis shows glandular spaces with an inner lining of cuboidal or columnar cells surrounded by benign urothelium [2]. The so called "intestinal type" of cystitis glandularis, which is much less frequent than the usual type, can be regarded as equivalent to IM [2]. In the esophagus and stomach, IM is considered a risk factor for intestinal-type adenocarcinoma as part of a metaplasia-dysplasia-carcinoma sequence [3–5]. However, the association between IM and cancer risk in the urinary tract is still unclear and somewhat debated. In the bladder, IM can be found adjacent to urothelial carcinoma, adenocarcinoma, and squamous cell carcinoma [1, 6]. Nonetheless, seemingly few patients with only IM on biopsy show progression to carcinoma [1, 7, 8]. In contrast, IM with dysplasia seems to have a higher association with concurrent or subsequent carcinoma and likely represents a stronger risk factor for progression when it is the only finding in bladder biopsies [6].

Because the molecular landscape of IM of the bladder and urethra remains largely unexplored, it is uncertain whether urinary tract IM has oncogenic potential from a genetic perspective. Therefore, the objective of this study was to investigate the presence of potentially oncogenic genetic variants in IM of the bladder and urethra.

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Materials and methods

This research was performed with the approval of the Institutional Review Board of Brigham and Women's Hospital.

Cases

A total of 23 cases from 23 different patients were included; 20 from the bladder and 3 from the urethra. The series comprised cases of IM without dysplasia ($n = 18$) and with dysplasia ($n = 5$); the latter group included dysplasia only ($n = 1$), dysplasia adjacent to adenocarcinoma ($n = 1$), dysplasia with concurrent urothelial carcinoma present in a separate sample ($n = 1$), and dysplasia vs superficial fragments of adenocarcinoma (no evidence of invasion, $n = 2$). IM with dysplasia was present in all three urethral cases.

Collection of tissue

The study workflow and a breakdown of the cases is illustrated in Fig. 1. Formalin-fixed paraffin-embedded (FFPE) tissue was collected by coring specific areas from histology tissue blocks ($n = 16$) or manual dissection from 5 μm tissue sections on glass slides ($n = 7$), depending on the abundance and distribution of the lesions of interest. In cases where IM was focal or intermingled with normal urothelium or inflammation, the areas of interest were cored to ensure adequate cellularity and to minimize contamination from other tissues/cells. When there was abundant lesional tissue

present, and the different lesions of interest were clearly separated and amenable to manual dissection, FFPE tissue was scraped off glass slides. Depending on the size of the lesion, coring was performed using disposable 750 μm or 1 mm punches with plungers (Robbins Instruments, Chatham, NJ). H&E stained sections were performed before and after coring to map the areas of interest on the block and to confirm adequate sampling, respectively. Manual dissection of FFPE sections was performed using H&E slides marked by a pathologist for reference.

Normal urothelium was obtained in 11 cases to compare with the corresponding (paired) IM samples. In all of these cases, both the IM and normal urothelium were cored from tissue blocks (Fig. 1). One case showed both IM with dysplasia and adenocarcinoma present on separate tissue fragments, which were manually dissected and differentially sequenced for comparison. Another case showed IM with dysplasia and concurrent high-grade urothelial carcinoma in different samples; both lesions were manually dissected and independently sequenced. For the remaining cases, only IM was collected.

Nucleic acid extraction, sequencing and informatics analysis (oncopanel)

DNA extraction and sequencing were performed as previously described by Garcia et al. and Sholl et al. [9, 10]. Briefly, cellularity was estimated on H&E-stained sections by a pathologist using a threshold of 20% for sample acceptance. DNA was extracted from FFPE scrapings and
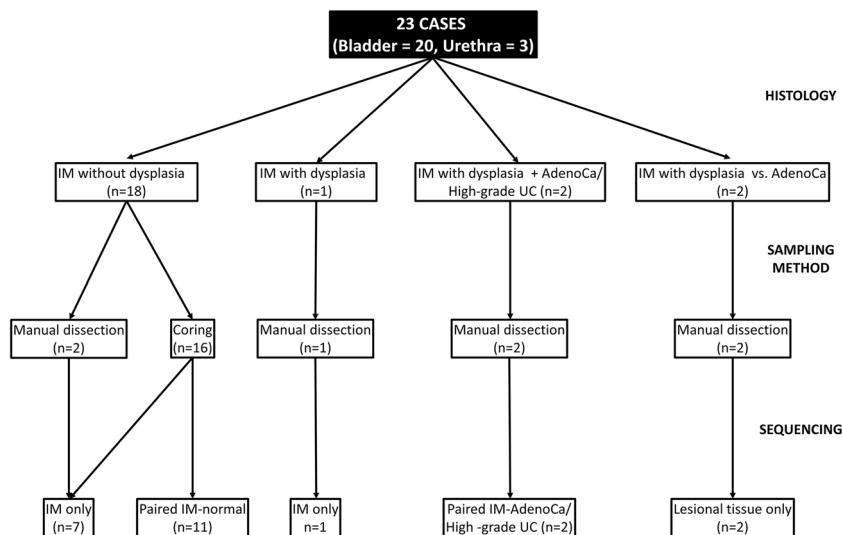
Fig. 1 Study Workflow. IM without dysplasia ($n = 18$), IM with dysplasia ($n = 1$), IM with dysplasia with concurrent adenocarcinoma or urothelial carcinoma ($n = 2$) and IM with dysplasia vs. adenocarcinoma in-situ (no evidence of invasion, $n = 2$). The two cases in the fourth column showed superficial fragments of atypical intestinal epithelium without evidence of invasion. Because both patients had a history of intestinal-type adenocarcinoma status post treatment, the distinction between IM with high-grade dysplasia and superficial fragments of adenocarcinoma or adenocarcinoma in-situ was difficult. AdenoCa adenocarcinoma, IM intestinal metaplasia, UC urothelial carcinoma.

cores using standard commercial kits (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Sequencing libraries were prepared using TruSeq LT library preparation kit (Illumina, San Diego, California) with a DNA input of 200 ng (threshold of 100 ng). Sequences of interest were selected by hybridization to a set of custom-designed capture probes (Agilent SureSelect; Agilent Technologies, Santa Clara, CA). Sequencing was performed on an Illumina HiSeq 2500 System (Illumina, San Diego, CA). Deconvolution of pooled samples, read alignment, variant calling (single nucleotide variants, insertion-deletions, copy number variants (CNVs) and structural variants) and annotation was carried out using an institutional bioinformatic pipeline [9–11]. To remove contaminating germline variants in this tumor-only sequencing assay, variants present at a population frequency of >0.1% in the gnomAD database (Broad Institute) were filtered out. Additionally, the informatics analysis of Oncopanel includes in-house developed algorithms for detection of mismatch repair deficiency and several mutational signatures (smoking, UV, and APOBEC) [12]. All variants were reviewed and approved by a molecular genetic pathologist (LMS) and evaluated for pathogenicity.

Immunohistochemistry

Immunohistochemistry for MTAP and IDH2 R172 mutant proteins was performed using primary anti-MTAP (Cat# sc-100782, clone 42-T, Santa Cruz Biotechnology, TX) and mutation-specific anti-IDH1 R132/IDH2 R172 (Cat# MABC1103, clone R132/172, Millipore Sigma, Burlington, MA) mouse monoclonal antibodies. Paraffin-embedded sections were incubated in hydrogen peroxide and absolute alcohol for 30 min to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed in a pressure cooker with sodium citrate buffer (10 mM, pH6). After blocking of non-specific binding sites, tissue sections were incubated with anti-MTAP (dilution 1:75, overnight incubation) and anti-IDH1 R132/IDH R172 (dilution 1:350) primary antibodies. A Novolink Polymer Detection System (Leica Biosystems, Buffalo Groove, IL) was used for detection. Staining was performed in a Link48 automated platform (Agilent, Santa Clara, CA, USA).

Cohort of primary bladder adenocarcinomas for comparison

An institutional cohort of 30 primary bladder adenocarcinomas previously sequenced with Oncopanel was analyzed. These patients were part of consented institutional protocols (DFCI protocols 11-104/17-000). This cohort was evaluated as a comparator; relevant genes affected in >20% of cases

were extracted, and their frequencies were plotted as presented in the results section.

Clinical and pathologic follow-up data

Electronic medical records, laboratory information systems and available scanned outside pathology reports were reviewed to obtain follow-up information.
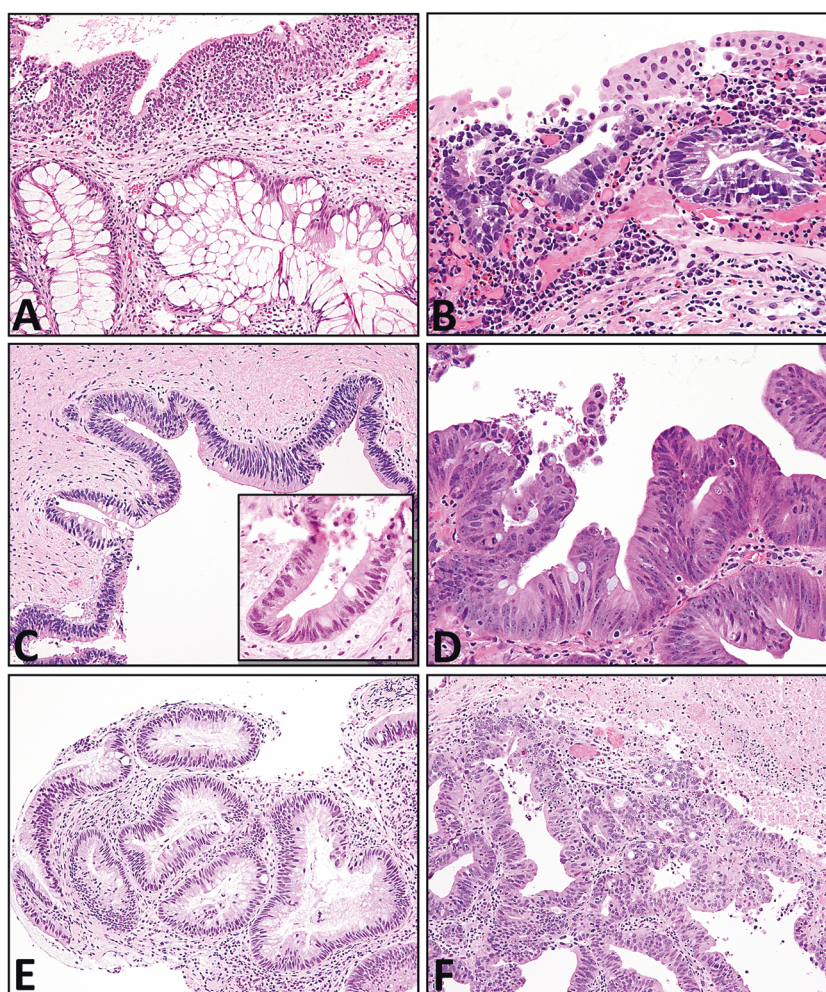
Results

General histopathologic and clinical characteristics of the cases

All cases of IM without dysplasia ($n = 18$) arose in the bladder, with the extent of IM ranging from focal (up to three discrete foci and <50% of the tissue) to diffuse (>3 discrete foci or >50% of the tissue) (Fig. 2a). Most cases demonstrated tall columnar epithelium with interspersed goblet cells, but a subset showed predominantly columnar mucinous cells. Nuclei were elongated, containing delicately dispersed chromatin and variably sized basophilic nucleoli. A mixed inflammatory infiltrate including plasma cells, lymphocytes, eosinophils, and neutrophils was almost invariably present. Mitoses and morphologic features suggestive of malignancy (e.g., enlarged hyperchromatic nuclei or atypical mitotic figures) were uniformly absent in IM without dysplasia. A single focus of IM with scattered Paneth cells was seen in only one sample. One case had a somewhat polypoid appearance on cystoscopy and was diagnosed as polypoid cystitis glandularis with IM.

Dysplastic IM ($n = 5$) was found in the bladder ($n = 2$) and urethra ($n = 3$), and showed mucin depletion, pseudostratification, nuclear enlargement and hyperchromasia (Fig. 2b–f). Prominent basophilic nucleoli and numerous mitotic figures were readily identified. A single gland with focal architectural complexity somewhat suspicious for, but not diagnostic of adenocarcinoma was present in one case. This sample was considered dysplastic IM for the purposes of the study. One case of dysplastic IM (case 20) contained IM with high-grade dysplasia and invasive adenocarcinoma present in separate tissue fragments, which were differentially dissected and sequenced for comparison (Fig. 2e, f). Another case (case 21) also showed concurrent high-grade papillary urothelial carcinoma in separate biopsies, which was sequenced in parallel for comparison. Two additional cases, one in the bladder and one in the urethra, showed superficial fragments of atypical intestinal epithelium, without clear evidence of invasion. Both patients had a history of intestinal-type adenocarcinoma status post treatment, making the distinction between IM with high-grade

Fig. 2 Histology of intestinal metaplasia (IM), IM with dysplasia and primary adenocarcinoma of the bladder and urethra. a Prototypical case of bladder IM without dysplasia showing abundant goblet cells adjacent to normal urothelium. **b** IM with dysplasia below the urothelial surface. Unlike non-dysplastic IM, these cases show marked mucin depletion, nuclear enlargement and hyperchromasia. **c** IM with dysplasia in urethra of a patient with a history of primary urethral adenocarcinoma. Foci of non-dysplastic IM were also present adjacent to the dysplastic foci (inset). **d** Urethral lesion showing highly atypical intestinal epithelium without evidence of invasion. However, this patient also had a history of a primary adenocarcinoma of the bladder, suggesting that the lesion might represent superficial fragments of adenocarcinoma. Paired IM with dysplasia (**e**) and primary adenocarcinoma of the urethra (**f**) were present in separate tissue fragments in the biopsy of case 20.

dysplasia and superficial fragments of adenocarcinoma or adenocarcinoma in-situ very difficult.

Next-generation sequencing (NGS) results

A total of 36 samples from 23 individual patients were sequenced. The breakdown of the cases and samples is as follows: 11 patients with paired non-dysplastic IM-normal urothelium (22 samples), 10 patients with only IM (7 non-dysplastic and 3 dysplastic, 10 samples), 1 case with paired dysplastic IM-adenocarcinoma (2 samples) and 1 case with paired dysplastic IM-high-grade urothelial carcinoma (2 samples). Potentially oncogenic genetic variants were detected in a subset of 10/23 IM cases (43%), comprising 5 cases of non-dysplastic IM and all 5 cases of IM with dysplasia (including the cases with paired adenocarcinoma and high-grade urothelial carcinoma) (Fig. 3). All 23 cases were mismatch-repair proficient. Overall, the mutational burden and number of CNVs was higher in IM with dysplasia and adenocarcinoma than in IM without dysplasia and normal urothelium (Table 1 and Fig. 3). No oncogenic

genetic variants were seen in normal urothelium. Of note, our assay only considers variants present at allele frequencies higher than 3% to reduce the risk of false positives, which might increase the rate of false negatives for real low-level variants corresponding to small clones within the histologically normal urothelium. A summary of the specific single nucleotide variants (SNVs) and indels present in each case is presented in Table 2.

IM without dysplasia

Of the 18 cases of IM without dysplasia, 5 showed potentially oncogenic somatic variants (Fig. 3, cases 1–5). Of note, there were no significant morphologic differences between non-dysplastic IM cases with and without potentially oncogenic variants. Two of the five cases (cases 1 and 2) had paired morphologically normal urothelium for comparison, whereas cases 3, 4, and 5 did not. IM from cases 1 and 2 showed *Lysine Methyltransferase 2D* (*KMT2D*) and *Stromal Antigen 2* (*STAG2*) mutations, respectively. These variants were not seen in the paired
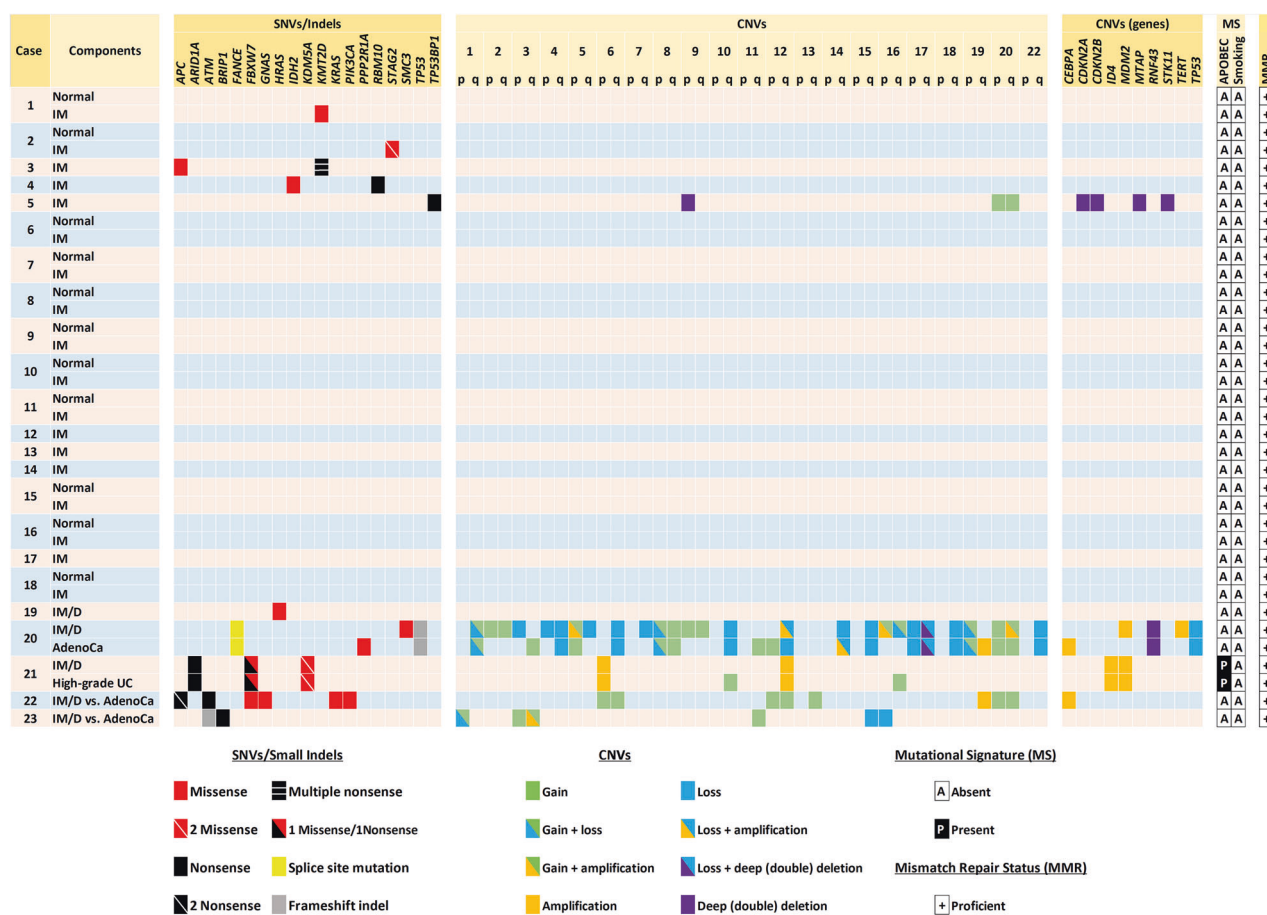
Fig. 3 Summary of next-generation sequencing results. AdenoCa adenocarcinoma, IM intestinal metaplasia, IM/D intestinal metaplasia with dysplasia. Only 10/23 (43%) tested cases have positive findings as shown.

histologically normal urothelium. Case 3 had multiple *KMT2D* mutations, suggesting biallelic loss of the gene. Additionally, this case harbored an *Adenomatous Polyposis Coli* (*APC*) *p.I1307K* variant that was most likely germline (VAF ~50%). Germline *APC p.I1307K* is a known genetic risk factor for development of colorectal adenocarcinoma in Ashkenazi Jewish populations [13]. This patient had a remote history of low-grade papillary urothelial carcinoma (pTa) status-post treatment, but there was no morphologic evidence of residual urothelial carcinoma in the current specimen and IM was collected by coring, minimizing the chance of contamination. Case 4 had a well-characterized hotspot *Isocitrate Dehydrogenase 2* (*IDH2*) variant. Mutation-specific antibodies for *IDH1 R132X/IDH2 R172X* variants demonstrated expression of mutant IDH2 protein in IM, consistent with the NGS findings (Fig. 4a, b). Expression of the mutant IDH2 protein was not seen in adjacent stromal or inflammatory cells. In case 5, there was a deep (double copy) 9p21.3 deletion encompassing *Cyclin-Dependent Kinase Inhibitor 2A* (*CDKN2A*), *Cyclin-Dependent Kinase Inhibitor 2B* (*CDKN2B*) and *Methyl-thioadenosine Phosphorylase* (*MTAP*), in addition to

chromosomal-level gains of chromosome 20 and a *Tumor Protein P53 Binding Protein 1* (*TP53BP1*) mutation. Immunohistochemistry showed loss of MTAP protein expression in IM, which contrasted with the positivity seen in adjacent stromal and inflammatory cells (Fig. 4c, d). Interestingly, per clinical notes, this patient had a history of Peutz–Jeghers syndrome, consistent with the double copy loss of *Serine/Threonine Kinase 11* (*STK11*) exon 3 detected by NGS (Fig. 5).

Dysplastic IM

Case 19 harbored a well-known hotspot *Human Homologue of Harvey Rat Sarcoma Viral Oncogene* (*HRAS*) mutation. No additional genetic variants were identified.

Paired dysplastic IM-adenocarcinoma and dysplastic IM-papillary urothelial carcinoma

The one case (Fig. 3, case 20) with paired dysplastic IM-adenocarcinoma showed multiple somatic variants and numerous CNVs in both samples, including chromosomal-

Table 1 General next-generation sequencing metrics and mutational burden.

CN	Comp	Cell (%)	MR	Q30%	TMB
1	Normal	100	157	97	6.844
	IM	~50	149	97	5.323
2	Normal	100	200	96	5.323
	IM	~50	286	98	6.083
3	IM	~20	260	98	4.562
4	IM	~30	241	97	4.562
5	IM	~80	263	98	5.323
6	Normal	100	298	97	3.042
	IM	~80	248	98	3.802
7	Normal	100	102	96	0.76
	IM	~80	129	95	0.76
8	Normal	100	341	98	5.323
	IM	~80	264	97	3.042
9	Normal	100	289	98	3.802
	IM	~80	260	98	3.802
10	Normal	100	306	98	3.042
	IM	~80	279	98	3.802
11	Normal	100	285	98	0.76
	IM	~80	244	98	0.76
12	IM	~50	203	97	1.521
13	IM	~80	196	98	4.562
14	IM	~80	222	98	2.281
15	Normal	100	118	96	0.76
	IM	~80	126	96	1.521
16	Normal	100	100	95	4.562
	IM	~80	90	93	4.562
17	IM	~80	247	98	3.802
18	Normal	100	200	97	4.562
	IM	~50	250	98	4.562
19	IM/D	~70	174	96	1.521
20	IM/D	~40	249	98	6.083
	AdenoCa	~40	346	98	5.323
21	IM/D	~20	202	97	20.531
	High-grade UC	~50	209	98	25.094
22	IM/D[a]	~50	243	97	7.604
23	IM/D[a]	~20	202	97	3.802

AdenoCa adenocarcinoma, *Cell* estimated cellularity, *CN* case number, *Comp* histologic component, *IM* intestinal metaplasia, *IM/D* intestinal metaplasia with dysplasia, *MR* mean number of aligned high-quality reads, *Q30%* Percentage of exons having more than 30 aligned reads, *TMB* mutational burden (in mutations per Mb), *UC* urothelial carcinoma.

[a]In these cases, the differential diagnosis was between intestinal metaplasia with high-grade dysplasia and superficial fragments of adenocarcinoma (no clear evidence of invasion in the sample) or adenocarcinoma in situ.

level loss of chromosomes 4 and 17, chromosomal-level gain of chromosome 20 and multiple arm-level and regional

events (Fig. 3). Of note, both the IM and adenocarcinoma showed biallelic loss of *Tumor Protein P53* (*TP53*), whereas *Structural Maintenance Of Chromosomes 3* (*SMC3*) and *Protein Phosphatase 2 Scaffold Subunit Alpha* (*PPP2R1A*) mutations were exclusive to the dysplastic IM and adenocarcinoma, respectively. The IM also showed amplification of a region of chromosome 12 encompassing *Human Homologue of Mouse Double Minute 2* (*MDM2*).

Case 21 was more complex in that it harbored multiple somatic SNVs, including both cancer-relevant mutations (see Table 2) and numerous variants of uncertain significance. Case 20 showed an APOBEC mutational signature, which is associated with upregulation of APOBEC enzymes and might predict somewhat favorable clinical outcomes in urothelial carcinoma [14, 15]. Regional amplifications of 6p and 12q were also identified, the latter involving the *MDM2* locus. Paired urothelial carcinoma also showed an APOBEC signature and a very similar mutational profile, with identical disease-relevant mutations and a few different variants of uncertain significance (not shown), as well as a slightly higher mutational burden. Akin to dysplastic IM, the CNV profile of the urothelial carcinoma also showed 6p and 12q amplifications, with additional gains of 10q and 16q.

Dysplastic IM versus adenocarcinoma

The two cases (Fig. 3, cases 22 and 23) with a differential diagnosis of IM with dysplasia vs. adenocarcinoma showed multiple somatic genetic variants. Case 1 demonstrated biallelic loss of *APC* with multiple concurrent cancer-relevant mutations (Fig. 3 and Table 1). Chromosomal-level gains of chromosomes 6, 12, and 20, arm-level gain of 13q and 19q13.11 amplification (locus encompassing *CCAAT Enhancer Binding Protein Alpha*, *CEBPA*) were also present. Case 2 had *Ataxia Telangiectasia Mutated* (*ATM*) and *BRCA1 Interacting Protein C-Terminal Helicase 1* (*BRIP1*) variants, and multiple CNVs including a focal 3q amplification (Fig. 3).

Frequency of genetic variants in a cohort of primary bladder adenocarcinomas

In general, the genes with the highest frequency of variants were *TP53*, *AT-Rich Interaction Domain 1A* (*ARID1A*), *KMT2D*, *Lysine Demethylase 6A* (*KDM6A*) and *Fibroblast Growth Factor Receptor 3* (*FGFR3*). All these genes showed variants in 20% or more of the cases (Fig. 6).

Clinical follow-up

Demographic and relevant clinical follow-up data for the cases with positive genetic findings are summarized in Table 3.

Table 2 Single nucleotide variants and indels.

Case	Component	Gene	Nucleotide change	Amino-acid change
1	Normal	–	–	–
	IM	KMT2D	c.16295G>A	p.R5432Q
2	Normal	–	–	–
	IM	STAG2	c.1225G>A	p.D409N
		STAG2	c.1231G>A	p.E411K
3	IM	APC	c.3920T>A	p.I1307K
		KMT2D	c.2398C>T	p.Q800*
		KMT2D	c.11674C>T	p.Q3892*
		KMT2D	c.2782C>T	p.Q928*
		KMT2D	c.1925C>A	p.S642*
4	IM	IDH2	c.516G>T	p.R172S
		RBM10	c.94C>T	p.R32*
5	IM	TP53BP1	c.5431C>T	p.R1811*
6	Normal	ND/NA	–	–
	IM	ND/NA	–	–
7	Normal	ND/NA	–	–
	IM	ND/NA	–	–
8	Normal	ND/NA	–	–
	IM	ND/NA	–	–
9	Normal	ND/NA	–	–
	IM	ND/NA	–	–
10	Normal	ND/NA	–	–
	IM	ND/NA	–	–
11	IM	ND/NA	–	–
12	IM	ND/NA	–	–
13	IM	ND/NA	–	–
14	IM	ND/NA	–	–
15	Normal	ND/NA	–	–
	IM	ND/NA	–	–
16	Normal	ND/NA	–	–
	IM	ND/NA	–	–
17	IM	ND/NA	–	–
18	Normal	ND/NA	–	–
	IM	ND/NA	–	–
19	IM/D	HRAS	c.182A>G	p.Q61R
20	IM/D	FANCE	c.1383+1G>A	–
		SMC3	c.3353G>T	p.G1118V
		TP53	c.181_182delGA	p.D61*
	AdenoCa	FANCE	c.1383+1G>A	–
		PPP2R1A	c.770G>T	p.W257L
		TP53	c.181_182delGA	p.D61*
21	IM/D	ARID1A	c.6817C>T	p.Q2273*
		FBXW7	c.1637C>T	p.S546L
		FBXW7	c.907C>T	p.Q303*
		KDM5A	c.3532G>A	p.E1178K
		KDM5A	c.2113G>A	p.D705N

Table 2 (continued)

Case	Component	Gene	Nucleotide change	Amino-acid change
	High-grade UC	ARID1A	c.6817C>T	p.Q2273*
		FBXW7	c.1637C>T	p.S546L
		FBXW7	c.907C>T	p.Q303*
		KDM5A	c.3532G>A	p.E1178K
		KDM5A	c.2113G>A	p.D705N
22	IM/D vs. AdenoCa	APC	c.4348C>T	p.R1450*
		APC	c.2097G>A	p.W699*
		ATM	c.8539G>T	p.E2847*
		FBXW7	c.1393C>T	p.R465C
		GNAS	c.992C>T	p.A331V
		KRAS	c.35G>A	p.G12D
		PIK3CA	c.1633G>A	p.E545K
23	IM/D vs. AdenoCa	ATM	c.8425_8426insA	p.S2812Vfs*3
		BRIP	c.2392C>T	p.R798*

10/23 (43%) tested cases had positive findings.

AdenoCa adenocarcinoma, *IM* intestinal metaplasia, *IM/D* intestinal metaplasia with dysplasia, *ND/NA* no mutations detected/not applicable, *UC* urothelial carcinoma.

Discussion

In the stomach and esophagus, it has been shown that IM may harbor oncogenic genetic variants that make it prone to malignant transformation as further genetic changes accumulate in susceptible IM subclones [16, 17]. Conversely, in the urinary tract, the relationship between IM and cancer is unclear and somewhat debated. Although IM can recur locally, most series have shown that only a small number of patients develop adenocarcinoma during follow-up [6, 7]. However, IM with and without dysplasia is often found in association with both gland-forming and non-gland-forming malignancies of the urinary tract [1, 6]. In fact, one of the largest available series of cystitis glandularis, which includes 19 cases of IM, shows that this lesion is more commonly associated with concurrent urothelial carcinoma than with primary adenocarcinoma of the bladder [1]. However, because of the retrospective design and relatively small size of most studies, the true significance of urinary tract IM as a cancer risk factor is not well-established.

Given the difficulties inherent to collecting data prospectively and gathering a large number of cases of a relatively rare entity, alternative strategies are required to explore the oncogenic potential of IM and the relationship between IM and urinary tract malignancies. A reasonable approach is to perform comparative immunophenotypic and molecular studies at the tissue level. Immunohistochemistry

Fig. 4 Histology and Immunohistochemistry of Cases 4 and 5. a In case 4, IM showed a predominance of mucinous cells and absence of architectural and/or cytologic atypia. b IDH1 R132X/IDH2 R172X mutation-specific immunohistochemistry demonstrates cytoplasmic expression of mutant IDH2 protein in the cytoplasm of IM cells, consistent with the *IDH2 R172S* mutation identified by NGS (Oncopanel). c Lesion from the posterior bladder wall of case 5 showing non-dysplastic IM. d MTAP expression is lost in the intestinal-type epithelium but retained in the stromal and inflammatory cells. e The copy number plot corresponding to chromosome 9 shows deep (double copy) loss of a region within 9p21.3 encompassing the sequences of *CDKN2A*, *CDKN2B* and *MTAP*. Copy number changes are presented as log2 ratios.
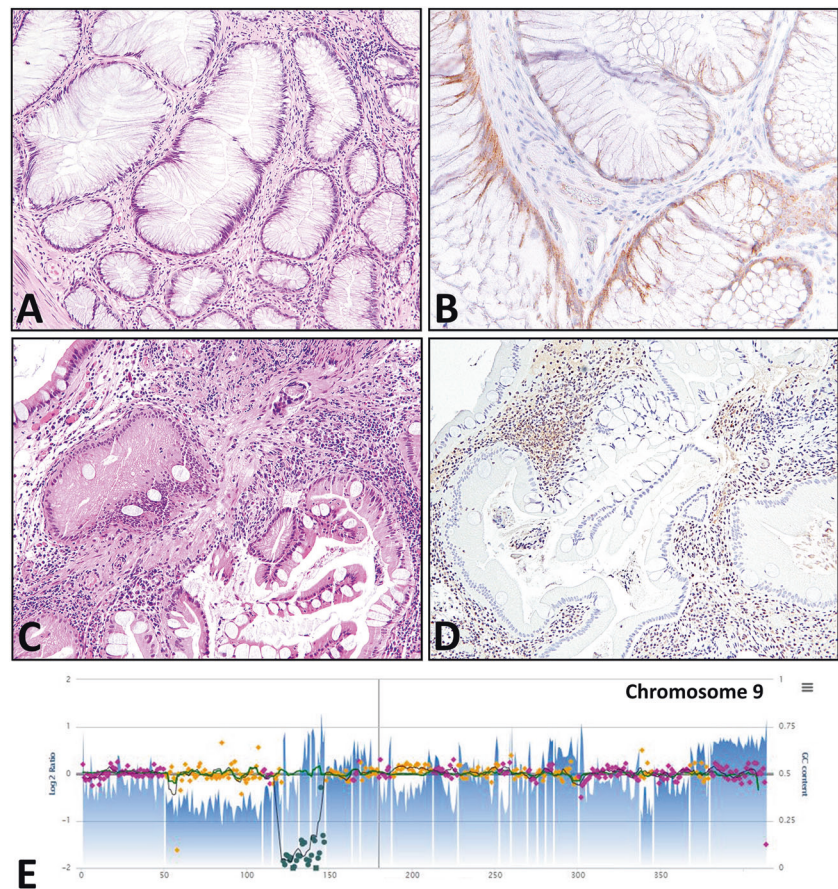
has shown that, unlike usual cystitis cystica/glandularis and normal urothelium, IM expresses keratin 20 and CDX2, and loses expression of keratin 7 [18]. However, very little is known about the molecular characteristics of IM of the urinary tract. Prior studies of single cases have shown evidence of loss of heterozygosity of the *D2S123* microsatellite sequence, as well as *Telomerase Reverse Transcriptase* (*TERT*) promoter and *F-Box And WD Repeat Domain Containing 7* (*FBXW7*) mutations [19, 20]. Additionally, loss of heterozygosity of *TP53* can be seen in dysplastic glandular foci within IM [19]. IM of the bladder has also been associated with telomere shortening and chromosomal gains [21]. More recently, Amin *et al.* performed mutation analysis on a limited number of colon cancer-related genes to compare IM of the bladder, tubular adenomas, and papillary urothelial carcinomas [22]. Their study found *APC* and *Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog* (*NRAS*) mutations in 2 of 9 IM cases.

In the present study, 2 of 23 patients had a suspected or known germline event (~10%) which is roughly in keeping with the rates of significant germline events detected by routine profiling in cancer cohorts [23]. Interestingly, case 3 (from a patient who had no history of colon carcinoma) demonstrated a likely germline *APC p.I1307K* variant,

which constitutes a well-recognized genetic risk factor for colorectal cancer [13]. This raises the possibility that *APC p.I1307K* might also have an association with glandular lesions of the bladder, although this is speculative. The second patient (case 5) had a history of Peutz-Jeghers Syndrome, which is consistent with the identification of biallelic deletion of exon 3 of *STK11*. Deletion of exon 3 of *STK11* has been previously reported in patients with Peutz-Jeghers and had been identified in a germline sample of case 5 according to clinical notes [24, 25].
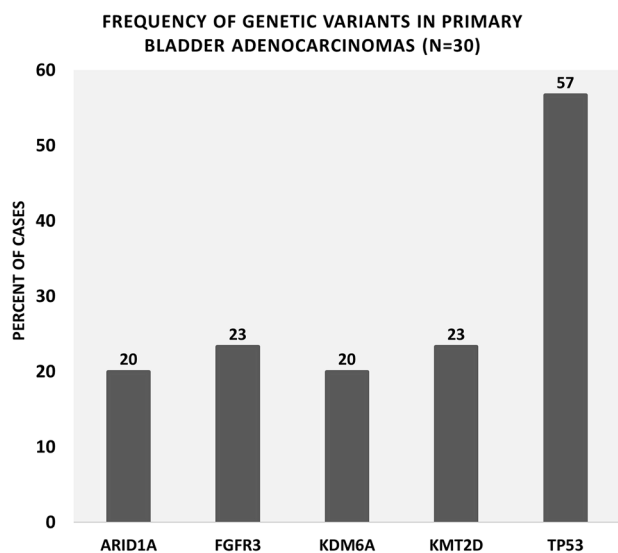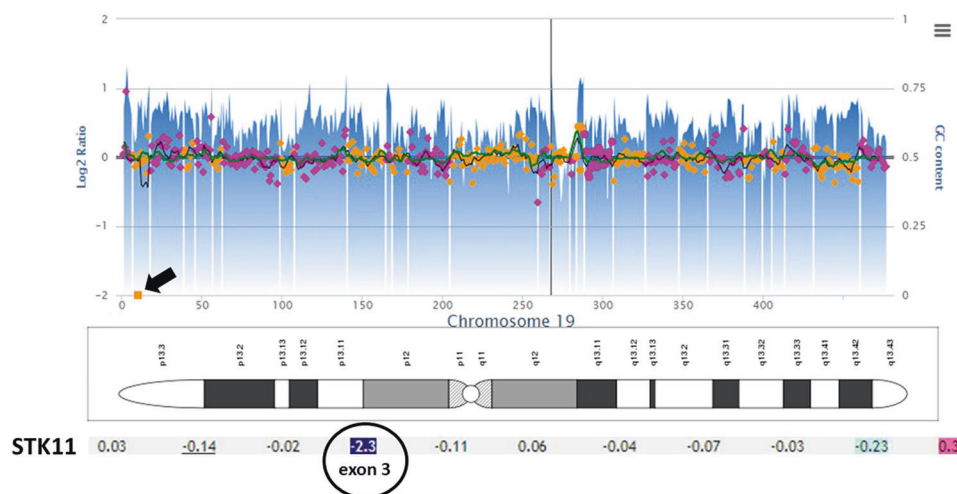
In the present study, 5/18 cases (28%) with non-dysplastic IM harbored potentially oncogenic somatic genetic variants in the metaplastic epithelium (Fig. 3). Of these five cases, only one had a remote history of low-grade papillary urothelial carcinoma (pTa). Some of the variants identified in IM without dysplasia, such as *KMT2D* (cases 1 and 3), *STAG2* (case 2) and *RNA Binding Motif Protein 10* (*RBM10*, case 4) mutations, have been described both in non-nvasive and invasive urothelial carcinoma [26, 27].

Cases 4 and 5 show additional interesting results and merit individual discussion. Case 4 harbored an activating hotspot *IDH2 p.R172S* mutation confirmed by mutation-specific immunohistochemistry (Fig. 5). This particular

Fig. 5 STK11 exon3 deletion in a patient with history of Peutz–Jeghers Syndrome (case 5). Chromosome 19 copy number plot demonstrating focal double copy loss of exon 3 of *STK11* (black arrow and black circle). Copy number changes are presented as log2 ratio values.

Fig. 6 Frequency of genetic variants of disease-relevant genes present in primary adenocarcinomas of the bladder. Data was extracted from an institutional cohort of primary bladder adenocarcinomas sequenced by Oncopanel (*n* = 30, including two possible urachal tumors).

usually co-deleted and loss of MTAP expression by immunohistochemistry is therefore a a useful surrogate marker of homozygous *CDKN2A/P16* loss in multiple lesions, including non-dysplastic IM [29]. Homozygous deletion of this chromosomal region (9p21.3) in case 5 was confirmed by MTAP immunohistochemistry (Fig. 4).

The data presented herein shows that IM with high-grade dysplasia always harbored at least one, but usually multiple, genetic variants (Fig. 3), including several mutations that have been described in primary adenocarcinomas of the bladder [20, 28]. These included, but were not restricted to, *APC* (case 22), *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha* (*PIK3CA*, case 22), *FBXW7* (cases 21 and 22) and *Kirsten Rat Sarcoma Viral Oncogene Homolog* (*KRAS*, case 22) mutations. Interestingly, some cases of IM with dysplasia showed variants that are also commonly seen in urothelial carcinoma of the bladder, such as *ARID1A* (case 21), *HRAS* (case 19) and *Lysine Demethylase 5A* (*KDM5A*, case 21) mutations [26]. Moreover, IM of case 21 showed an APOBEC mutational signature, which can be seen in multiple tumors including urothelial carcinoma [14, 15], and the mutational and CNV profile of this lesion was markedly similar to that seen in separate foci of concurrent urothelial carcinoma. In case 20, paired dysplastic IM-primary bladder adenocarcinoma samples showed very similar mutational profiles with biallelic inactivation of *TP53*. Despite the absence of overtly invasive tumor in cases 22 and 23, both patients had a history of adenocarcinoma. Therefore, it is possible that the lesion represented in the samples was recurrent adenocarcinoma without evidence of invasion. Four of 5 (80%) IMs with dysplasia harbored multiple CNVs, which were particularly complex in the case with paired dysplastic IM-adenocarcinoma. *TERT* promoter mutations were not detected in any case.

This study has limitations that need to be discussed. First, there was no normal urothelium for comparison in a

variant, *IDH2 p.R172S*, has been described as a driver event in primary bladder adenocarcinoma [28]. Case 5 is the only non-dysplastic IM case with CNVs, which included biallelic loss of tumor suppressors *CDKN2A* (p16) and *CDKN2B*. In urothelial carcinoma, *CDKN2A (*p16*)* inactivation/deletion characterizes a specific subset of tumors and is mutually exclusive with loss of *Retinoblastoma-Associated Protein* (*RB1*) [26]. Homozygous deletion of *CDKN2A* was also observed in a small number of cases (2/30) in our series of primary bladder adenocarcinomas. The *MTAP* gene, which encodes an enzyme that plays a major role in the salvage of methionine and adenine and is therefore considered a ubiquitously expressed housekeeping gene, is located in close proximity to *CDKN2A*. Consequently, these genes are

Table 3 Demographic and follow up data of patients with positive molecular findings.

Case[a]	Sex	Age	Lesion-location	Relevant clinical history and follow-up
1	M	22	IM-Bladder	The patient had a history of cystitis cystica/cystitis glandularis. FU is limited to 2 mo post-TURBT. The lesion analyzed in this study caused bladder outlet obstruction. In a post-operative control (2 months), recurrent bladder outlet obstruction was mentioned, but the patient was lost to FU afterwards.
2	M	55	IM-Bladder	The patient presented with recurrent florid cystitis cystica/glandularis and developed bilateral ureteral obstruction requiring placement of ureteral stents. There was no evidence of malignancy after a FU period of 1 yr and 10 mo.
3	F	59	IM-Bladder	According to clinical notes, there was a history of low-grade pTa 9–10 years prior to the diagnosis of IM, with no documented recurrences, and a long-standing history of interstitial cystitis. The patient underwent radical cystectomy for intractable symptoms of interstitial cystitis and overactive bladder, after failing multiple therapies. The cystectomy did not show evidence of malignancy/tumors, and the patient was lost to FU 2 months after the surgery.
4	M	55	IM-Bladder	Recent case, no FU data available.
5	F	30	IM-Bladder	The patient showed a recurrent bladder lesion that was removed at an outside hospital 3 yr and 3 mo later. This lesion was benign according to clinical notes. The patient showed NED after a FU period of 9 yr and 8 mo.
19	M	69	IM/D-Urethra	No FU data available.
20	F	50	IM/D and concurrent AdenoCa-Urethra	No FU data available.
21	M	65	IM/D and concurrent high-grade UC-Bladder	The patient had a history of high-grade papillary UC with focal lamina propria invasion s/p intravesical BCG. The specimen analyzed herein showed concurrent dysplastic IM (posterior wall) and recurrent high-grade papillary UC (dome and right lateral walls) with focal lamina propria invasion. The patient underwent radical cystoprostatectomy that showed pT1 N0 high-grade papillary UC. The patient showed NED after a FU period of 3 yr and 3 mo.
22	M	75	IM/D vs. AdenoCa-Bladder	The patient had an established history of bladder adenocarcinoma. The specimen analyzed herein did not show evidence of invasion (IM with dysplasia vs AIS/superficial fragments of adenocarcinoma). Abdominal wall metastases were diagnosed 1 yr after TURBT, followed by disseminated pelvic and intraabdominal metastases. The patient died of disease after a FU period of 3 yr.
23	M	73	IM/D vs. AdenoCa-Urethra	The patient had a history of prostatic adenocarcinoma s/p brachytherapy and urethral adenocarcinoma. Radical cystoprostatectomy was performed, which showed a pT2 N0 urethral adenocarcinoma. The patient showed NED after a FU period of 2 yr and 2 mo.

AdenoCa adenocarcinoma, *AIS* adenocarcinoma in-situ, *BCG* Bacillus Calmette-Guerin, *FU* follow up, *IM* intestinal metaplasia, *IM/D* intestinal metaplasia with dysplasia, *mo* months, *NED* no evidence of disease, *s/p* status post, *TURBT* transurethral resection of bladder/urethral tumor, *UC* urothelial carcinoma, *yr* years.

[a]Case numbers correspond to the cases with positive molecular findings. Cases 6–18 showed intestinal metaplasia without dysplasia and no positive findings by next-generation sequencing

subset of samples, which is an almost unavoidable problem in studies of archival FFPE tissue. Therefore, the interpretation of variants as germline and/or somatic in cases without paired normal tissue is somewhat speculative. However, the variant allele frequency/fraction (VAF) is a particularly valuable metric that can be used to interpret variants as somatic or potentially germline [30]. In this study, the VAF of the *APC p.I1307K* variant was supportive of a germline origin, in keeping with previously published data [13]. The other potentially germline variant detected in this series (homozygous deletion of *STK11*) had been previously identified as the cause of Peutz-Jeghers syndrome in a germline sample of case 5 according to available clinical notes. All the other variants identified in the study showed sequencing metrics consistent with a somatic origin.

Moreover, mutation-specific IDH1 and MTAP immunohistochemistry demonstrated that *IDH1 p.R172S* mutation and *MTAP* loss were only present in the lesional tissue (IM) of cases 4 and 5, respectively. Despite the lack of paired normal tissue in a subset of cases, this is the first molecular study of urinary tract IM to include non-lesional tissue control for a significant proportion of the samples (11/23 cases). Also, paired dysplastic IM-adenocarcinoma and dysplastic IM-high-grade urothelial carcinoma were analyzed in parallel in two cases. Another potential weakness of our approach is the use of coring and manual dissection instead of laser microdissection. However, multiple measures were taken to avoid contamination and ensure correct sampling, as described above. Finally, our cases have limited clinical follow-up data.

Despite the aforementioned limitations, this series presents the largest and most comprehensive molecular characterization of IM with and without dysplasia to date. Given the short follow-up of most cases, the absence of progression to malignancy in patients with non-dysplastic IM harboring potentially oncogenic mutations should be interpreted cautiously; especially considering that the interval between diagnosis of IM and development of high-grade dysplasia or adenocarcinoma in other organs is 5–10 years, but not uncommonly much longer [31, 32]. In this regard, data from studies of upper gastrointestinal lesions show that while IM significantly increases the risk of adenocarcinoma, only a small fraction of cases eventually develop an overt malignancy [33]. Unlike upper gastrointestinal tract IM, whose prevalence is known thanks to endoscopic screening programs [33] of high-risk populations, the true incidence of urinary tract IM is unknown. This series demonstrated potentially oncogenic genetic variants in 5/18 (28%) cases of non-dysplastic IM, suggesting that biologic progression to dyplasia and carcinoma is theoretically possible in a subset of cases.

Of note, highly recurrent genetic variants were not identified. This suggests that, if genitourinary IM is indeed a precursor, it might progress via diverse oncogenic pathways and give rise to different types of cancer. This contrasts with the somewhat linear molecular evolution of esophageal IM, where progression to dysplasia and adenocarcinoma consistently correlates with sequential biallelic loss of *CDKN2A* and *TP53* [16]. Similarly, urothelial carcinoma in situ, a known precursor of muscle-invasive bladder cancer, usually shows consistent genetic alterations of *TERT*, *TP53*, and *CDKN2A* [34, 35].

The results presented herein show that IM with high-grade dysplasia invariably harbors oncogenic genetic variants associated with both urothelial carcinoma and primary adenocarcinoma of the bladder. Therefore, the presence of IM with dysplasia in the absence of concurrent cancer in bladder and urethral biopsies likely warrants close follow-up. Unfortunately, there were no significant morphologic differences between the subsets of non-dysplastic IM cases with and without potentially oncogenic genetic variants. Two of the non-dysplastic IM cases (cases 4 and 5) with oncogenic genetic variants showed florid IM, but so did multiple cases without molecular findings. Identification of biallelic *CDKN2A* loss in case 5 suggests that the presence of IM – even without dysplasia – might warrant clinical follow-up in the context of Peutz-Jeghers syndrome. Interestingly, some of the variants detected in IM have been also identified in urothelial carcinoma, suggesting that IM might have a relationship to urothelial cancer. In this regard, it is uncertain if IM that harbors genetic variants frequently seen in urothelial carcinoma is a direct cancer precursor or part of a field change. Further studies are required to answer these questions.

Compliance with ethical standards

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