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Short Report

Pathogenic and targetable genetic alterations in 70 urachal adenocarcinomas

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Key words: urachal cancer, molecular genetics, targeted therapy, colorectal cancer, urothelial carcinoma

Abbreviations: 5-FU: 5-Fluorouracil; BRAF: v-Raf murine sarcoma viral oncogene homolog B; CISH: chromogen ISH; CRC: colorectal cancer (colorectal adenocarcinoma); FFPE: formalin-fixed paraffin embedded; EGFR: epidermal growth factor receptor; ERBB2: erb-B2 receptor tyrosine kinase 2; FGFR: fibroblast growth factor receptor; FISH: fluorescence ISH; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; ISH: in situ hybridization; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MAPK: mitogen-activated protein kinase; MET: tyrosine-protein kinase met/hepatocyte growth factor receptor; MLH1: mutL homolog 1; MMR(d): DNA mismatch repair (deficiency); MSH2: mutS protein homolog 2; MSH6: mutS homolog 6; MSI (-h): microsatellite instability (-high); NGS: next-generation sequencing; NKI: Netherlands Cancer Institute; NRAS: neuroblastoma RAS viral oncogene homolog; OS: overall survival; PD-L1: programmed death-ligand 1; PDGFRA: platelet derived growth factor receptor alpha; PI3K: phosphoinositide 3-kinase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PMS2: postmeiotic segregation increased 2; RAF: rapidly accelerated fibrosarcoma; RFS: recurrence-free survival; SCC: squamous cell carcinoma; SNP: single nucleotide polymorphism; TCGA: the cancer genome atlas; TERT: telomerase reverse transcriptase; TP53: tumor protein p53; TPS: tumor proportion score; UC: urothelial carcinoma; UrC: urachal cancer; WHO: World Health Organization

Additional Supporting Information may be found in the online version of this article.

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Urachal cancer (UrC) is a rare but aggressive malignancy often diagnosed in advanced stages requiring systemic treatment. Although cytotoxic chemotherapy is of limited effectiveness, prospective clinical studies can hardly be conducted. Targeted therapeutic treatment approaches and potentially immunotherapy based on a biological rationale may provide an alternative strategy. We therefore subjected 70 urachal adenocarcinomas to targeted next-generation sequencing, conducted *in situ* and immunohistochemical analyses (including PD-L1 and DNA mismatch repair proteins [MMR]) and evaluated the microsatellite instability (MSI) status. The analytical findings were correlated with clinicopathological and outcome data and Kaplan-Meier and univariable/multivariable Cox regression analyses were performed. The patients had a mean age of 50 years, 66% were male and a 5-year overall survival (OS) of 58% and recurrence-free survival (RFS) of 45% was detected. Sequence variations were observed in *TP53* (66%), *KRAS* (21%), *BRAF* (4%), *PIK3CA* (4%), *FGFR1* (1%), *MET* (1%), *NRAS* (1%), and *PDGFRA* (1%). Gene amplifications were found in *EGFR* (5%), *ERBB2* (2%), and *MET* (2%). We detected no evidence of MMR-deficiency (MMR-d)/MSI-high (MSI-h), whereas 10 of 63 cases (16%) expressed PD-L1. Therefore, anti-PD-1/PD-L1 immunotherapy approaches might be tested in UrC. Importantly, we found aberrations in intracellular signal transduction pathways (RAS/RAF/PI3K) in 31% of UrCs with potential implications for anti-EGFR therapy. Less frequent potentially actionable genetic alterations were additionally detected in *ERBB2* (HER2), *MET*, *FGFR1*, and *PDGFRA*. The molecular profile strengthens the notion that UrC is a distinct entity on the genomic level with closer resemblance to colorectal than to bladder cancer.

What's new?

Urachal cancer (UrC) is a rare but aggressive cancer. In this study, the authors analyzed a number of genes and proteins that might be altered in UrC. They found no evidence of unusual mismatch repair (MMR) or microsatellite instability (MSI) status. However, they did observe aberrations in PD-L1-status, and in intracellular signal-transduction pathways (RAS/RAF/PI3K), as well as alterations in ERBB2 (HER2), MET, FGFR1, and PDGFRA. These results might provide a basis for targeted and/or immunotherapeutic approaches.

Introduction

Urachal cancer (UrC) is a rare but aggressive cancer. It is derived from the urachus, an embryological structure extending from the allantois to the fetal bladder. The urachus usually degenerates to form a fibromuscular cord running from the dome of the urinary bladder to the umbilicus, where it forms the median umbilical ligament. Incomplete obliteration occurs in up to 32% of adults but is usually microscopic and asymptomatic.[1]

Approximately 90% of UrC are adenocarcinomas that show remarkable histomorphological similarities with both primary bladder adenocarcinoma and colorectal adenocarcinoma (CRC), which represent the main differential diagnostic entities.[2] The correct differentiation of these entities is important, as therapeutic options differ. While localized UrC is usually treated by partial cystectomy and *en bloc* removal of the umbilical ligament and umbilicus, primary bladder adenocarcinoma usually requires radical cystectomy, and CRC growing into the bladder requires either additional surgical management of the primary tumor or palliative management.

A significant proportion of UrC cases require further therapy due to recurrent and/or metastatic disease in 21–48%.[2] This calls for effective systemic therapy regimes, while radiotherapy has only a limited role.[3] Chemotherapy has traditionally been chosen in analogy to urothelial bladder cancer due to its close anatomical relation. However, non-bladder cancer inspired regimens including 5-Fluorouracil (5-FU) like in CRC show more favorable characteristics in advanced UrC.[2]

In addition, recent molecular data and positive results from the first targeted therapies point towards therapeutic similarities between UrC and CRC. Like in CRC, oncogenic signaling in UrC appears to employ preferably the epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. Moreover, anti-EGFR agents such as cetuximab seem to exert positive effects.[4–6]

To date, genetic data on UrC still is scant or reported only in few cases.[4,5,7–12] In addition, data on non-adenocarcinoma UrC is completely lacking. We therefore analyzed a large cohort of urachal adenocarcinomas in a targeted sequencing approach supplemented with *in situ* and protein expression analyses including evaluation of PD-L1 expression and evaluation of the MMR/MSI status. In addition, a small subset of non-adenocarcinoma UrCs was also analyzed. We aimed to gain more insight in potentially targetable molecular alterations of UrC to eventually facilitate targeted therapy and/or immunotherapy decision-making.

Material and Methods

Cohorts and clinicopathological data

Due to the rarity of UrC, an international, multi-institutional approach was chosen and two cohorts were created.

The first cohort included formalin-fixed paraffin embedded (FFPE) urachal adenocarcinoma samples from 41 patients from nine centers: University Hospital Essen (Germany),[4] Semmelweis University Budapest (Hungary), University of Göttingen (Germany), University Hospital/RWTH Aachen University (Germany), University of Bonn (Germany), Jagiellonian University Cracow (Poland), University Hospital of Rennes (France), Institute of Pathology of Trier (Germany), and the Vancouver Prostate Center (Canada). Additionally, four cases of non-adenocarcinoma histology ($n = 2$: squamous cell carcinoma (SCC); $n = 1$: urothelial carcinoma (UC), $n = 1$: undifferentiated carcinoma) were available and analyzed.

The second, national Dutch cohort included FFPE samples from 29 patients with urachal adenocarcinomas from the Netherlands Cancer Institute (NKI) in Amsterdam (The Netherlands).

Diagnosis of urachal cancer was established after consideration of multi-disciplinary results. Histopathology was evaluated in accordance with (adapted) WHO-criteria[13–15] and review of available material was conducted. Data was requested from cooperating institutions using a standardized 42 parameter datasheet.

The first international multi-institutional cohort was analyzed at the University Hospital Essen (Institute of Pathology) and the second national Dutch cohort at the Netherlands Cancer Institute (NKI) in Amsterdam.

Clinicopathological data was collected retrospectively by chart review and from epidemiological databases. Clinical follow-up of patients varied by tumor stage and individual institutional routines. Overall survival (OS) was defined as the time from diagnosis to death from any cause. Recurrence-free survival (RFS) was defined as the time to occurrence of metastatic disease and/or local recurrence. The study is in accordance with the principles embodied in the Declaration of Helsinki. It was approved before start of experiments by the ethics committee of the Medical Faculty of the University of Duisburg-Essen (16–6902-BO) and included collection of external tumor samples for analyses. The Translational Research Board of the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (CFMPB310) additionally approved analysis of the Dutch cohort.

Targeted next-generation sequencing (NGS)

Customized NGS panels were used to cover important known actionable driver mutations/alterations from CRC, UC and additional main cancer entities (15/13 gene panels; Supporting Information Tables S1–S3).

A detailed description of sample processing, DNA extraction, sequencing and data analysis can be found in Supporting Information Document S1. In brief, in both cohorts representative FFPE tumor tissue blocks were selected and DNA was extracted. After target enrichment, libraries were sequenced on an Illumina MiSeq platform (first cohort, Essen) and Illumina Hiseq 2500 platform (second cohort, Amsterdam) followed by mapping to the human genome (version hg19), data filtering and reporting of all non-benign, non-SNP variants found in Cosmic and/or Clinvar (selection of pathogenic alterations).

In situ hybridization (ISH) assays

In cases with evidence for gene amplification detected by NGS (fold change: ≥ 3.5) in the first cohort, the findings were validated by ISH (*ERBB2*: chromogen ISH (CISH), *EGFR*/*MET*: fluorescence ISH [FISH]) followed by immunohistochemistry (IHC). Further details can be found in Supporting Information Document S1.

Immunohistochemistry

p53-IHC analyses were done from available FFPE material from the cohort of Essen ($n = 12$). PD-L1 analyses were performed using clone 22C3 and MMR-status was determined by MLH1-, MSH2-, MSH6, and PMS2-IHC. MMR-IHC results of a subset of cases ($n = 12$) has been published.[16] Due to the limited amount of tissue available, further IHC-assays (HER2, EGFR, and c-MET) were restricted to cases with signs of gene amplification in NGS- and ISH-analyses. Further details can be found in Supporting Information Document S1 and Table S4.

Microsatellite instability (MSI) analyses

In brief, multiplexed PCRs were performed using five primers. MSI-h was defined as the presence of two or more unstable microsatellite markers. Further details can be found in Supporting Information Table S5.

Statistical analyses

Statistical analyses were conducted with SPSS (v23; IBM, Armonk, NY). The Chi square-test, *t* test or Spearman correlation was used when appropriate. Kaplan-Meier survival analyses with the log-rank test were performed to assess the impact of selected variables on OS and RFS. In addition, univariable/multivariable Cox regression analyses were conducted (inclusion criteria for multivariable analysis: $p \leq 0.05$ in univariable analysis). *p*-values ≤ 0.05 were assumed statistically significant, while *p*-values ≤ 0.1 were designated as trends. Genetic alterations were visualized using the OncoPrinter (v.1.0.1) and MutationMapper (v1.0.1) tools.[17,18]

Results

Clinicopathological and prognostic characteristics of urachal adenocarcinomas

Detailed clinicopathological and survival data of the 70 analyzed urachal adenocarcinomas are shown in Tables 1 and 2. Both cohorts were well-balanced in terms of clinicopathological characteristics.

Data on follow-up was available in 64 of the 70 patients (91.4%) with urachal adenocarcinomas. The median follow-up was 49 months (range: 2–212 months). The 5-year OS and RFS rates were 58% and 45% respectively, with a median OS of 109 months (SE: 29.9) and median RFS of 50 months (SE: 13.0).

Table 1. Clinicopathological data in relation to mutational data

			Any mutation			MAPK/PI3K altered		
Table 1		***n* (%)**	**Yes (*n*)**	**Yes %**	***p***	**Yes (*n*)**	**Yes %**	***p***
Sex	Female	24 (34)	18	75	0.920	9	38	0.433
	Male	46 (66)	35	76		13	28	
	Total	70	53	76		22	31	
Age median (min, max)		50 (24, 78)	–		–			–
UrC type	Intestinal	30 (43)	24	80	0.499	10	33	0.846
	Mucinous	26 (37)	18	69		8	31	
	NOS	5 (7)	4	80		1	20	
	Signet ring	4 (6)	4	100		1	25	
	Mixed	5 (7)	3	60		2	40	
	Total	70	53	76		22	31	
Signet ring cells	Yes	11 (17)	10	91	0.202	3	27	0.725
	No	55 (83)	40	73		18	33	
	Total	66	50	76		21	32	
Sheldon	I	0 (0)	0	0	0.165	0	0	0.609
	II	8 (12)	5	63		2	25	
	IIIA	28 (41)	20	71		10	36	
	IIIB	5 (7)	3	60		1	20	
	IIIC	1 (2)	1	100		1	100	
	IIID	1 (2)	1	100		0	0	
	IVA	5 (7)	4	80		1	20	
	IVB	20 (29)	17	85		5	25	
	Total	68	51	75		20	29	
Mayo	I	17 (28)	10	59	0.073	4	24	0.877
	II	19 (31)	14	74		8	42	
	III	5 (8)	4	80		1	20	
	IV	20 (33)	17	85		5	25	
	Total	61	45	74		18	30	
LN status	N0	28 (48)	19	68	0.321	9	32	0.942
	N+	12 (21)	10	83		4	33	
	No LND	18 (31)	15	83		5	28	
	Total	58	44	76		18	31	
Dist. met.	M0	42 (68)	31	74	0.355	13	31	0.795
	M+	20 (32)	17	85		5	25	
	Total	62	48	77		18	29	

Mutational data is separately displayed for cases with any detected mutation and for cases with MAPK/PI3K-pathway activation (mutations in intracellular signal transduction pathways). Detailed clinicopathological data separately displayed for both cohorts can be found in Supporting Information Table S3.
Abbreviations: UrC, urachal cancer, LN, lymph node, Dist. met., distant metastasis, NOS, not otherwise specified, LND, lymph node dissection.

Genomic alterations in urachal adenocarcinomas

Detailed information is shown in Figure 1, Supporting Information Document S1, Figure S1, and Table S2.

Overall, 73 pathogenic mutations and four gene amplifications were detected in the 70 analyzed urachal adenocarcinomas. At least one genomic alteration was found in 55 of 70 patients (79%). All reported mutations were predicted to be pathogenic.

Missense mutations were the most common type of alteration ($n = 64$), followed by truncating mutations ($n = 4$), and nonsense mutations ($n = 2$). There was one deletion, one insertion and one splice-site mutation.

Two of the four patients with gene amplifications were female (50%), three were intestinal type UrC (*EGFR*-amplified: $n = 2$, *ERBB2*-amplified: $n = 1$), one was a signet ring

Table 2. Univariable (a) and multivariable (b) Cox-analyses in the total cohort of urachal adenocarcinomas

		Overall survival (OS)			Recurrence-free survival (RFS)		
Table 2a		**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Sex	Female	*ref.*			*ref.*		
	Male	0.801	0.327–1.958	0.626	1.462	0.591–3.614	*0.411*
Age	≤50 years	*ref.*			*ref.*		
	>50 years	0.725	0.319–1.647	0.443	0.607	0.282–1.304	0.201
UrC type: intestinal	No	*ref.*			*ref.*		
	Yes	0.589	0.253–1.369	0.218	0.488	0.222–1.074	0.075
UrC type: mucinous	No	*ref.*			*ref.*		
	Yes	1.649	0.740–3.677	0.222	1.189	0.566–2.497	0.648
Signet ring cells	No	*ref.*			*ref.*		
	Yes	2.148	0.832–5.548	0.114	1.725	0.685–4.345	0.247
Sheldon stage	≤IIIa	*ref.*			*ref.*		
	>IIIb	2.405	1.060–5.456	0.036	1.801	0.858–3.782	0.120
Mayo stage	I	*ref.*			*ref.*		
	≥II	4.497	1.320–15.313	0.016	2.513	0.943–6.696	0.065
LN status	N0	*ref.*			*ref.*		
	N+	2.888	1.065–7.828	0.037	4.565	1.615–12.902	0.004
Dist. met.	M0	ref.			*ref.*		
	M+	2.43	1.085–5.441	0.031	1.564	0.701–3.493	0.275
Any mutation	No	*ref.*			*ref.*		
	Yes	2.56	0.762–8.596	0.128	0.922	0.390–2.180	0.854
MAPK/PI3K alterated	No	*ref.*			*ref.*		
	Yes	0.812	0.320–2.061	0.661	0.897	0.396–2.033	0.795

	Overall survival (OS)				Recurrence-free survival (RFS)		
Table 2b	**HR**	**95% CI**	***p***		**HR**	**95% CI**	***p***
Sheldon stage (≤IIIa/>IIIb)	2.203	0.965–5.026	0.061	**LN Stage (N0/N+)**	2.462	0.757–8.013	0.134
Any mutation (no/yes)	2.216	0.653–7.517	0.202	**Any mutation (no/yes)**	1.343	0.279–6.471	0.713
Sheldon stage (≤IIIa/>IIIb)	2.384	1.043–5.450	0.039	**LN Stage (N0/N+)**	2.841	0.836–9.663	0.094
MAPK/PI3K altered (no/yes)	0.931	0.363–2.391	0.882	**MAPK/PI3K altered (no/yes)**	0.362	0.067–1.945	0.236
Mayo stage (I/≥II)	4.035	1.171–13.909	0.027	–			
Any mutation (no/yes)	1.81	0.525–6.243	0.348	–			
Mayo stage (I/≥II)	4.563	1.337–15.577	0.015	–			
MAPK/PI3K altered (no/yes)	0.846	0.326–2.196	0.732	–			

OS-related multivariable analysis models were calculated for Sheldon and Mayo stages separately (both $p < 0.05$ in univariable analysis). As both staging systems include information on status of lymph nodes and distant metastasis, these factors were excluded from OS-related models. For RFS, only lymph node status exhibited a p values < 0.05 in univariable analyses. Therefore, RFS models have been calculated separately including lymph node status.
Abbreviations: HR, hazard ratio, CI, confidence interval, LN, lymph node, Dist. met., distant metastasis.

cell type UrC (*MET*-amplified; Supporting Information Fig. S2) and all were alive at last follow-up.

Activating MAPK/PI3K-alterations were common events in urachal adenocarcinomas with 22 mutations in *K-/NRAS*, *BRAF* or *PIK3CA* affecting 22 of 70 patients (31%; Fig. 1, Supporting Information Fig. S1). These alterations were mutually exclusive, whereas in 16 of 22 cases (73%) a concomitant TP53 mutation was detected. All mutations affected functional regions with predicted pathogenicity (Supporting Information Fig. S1).

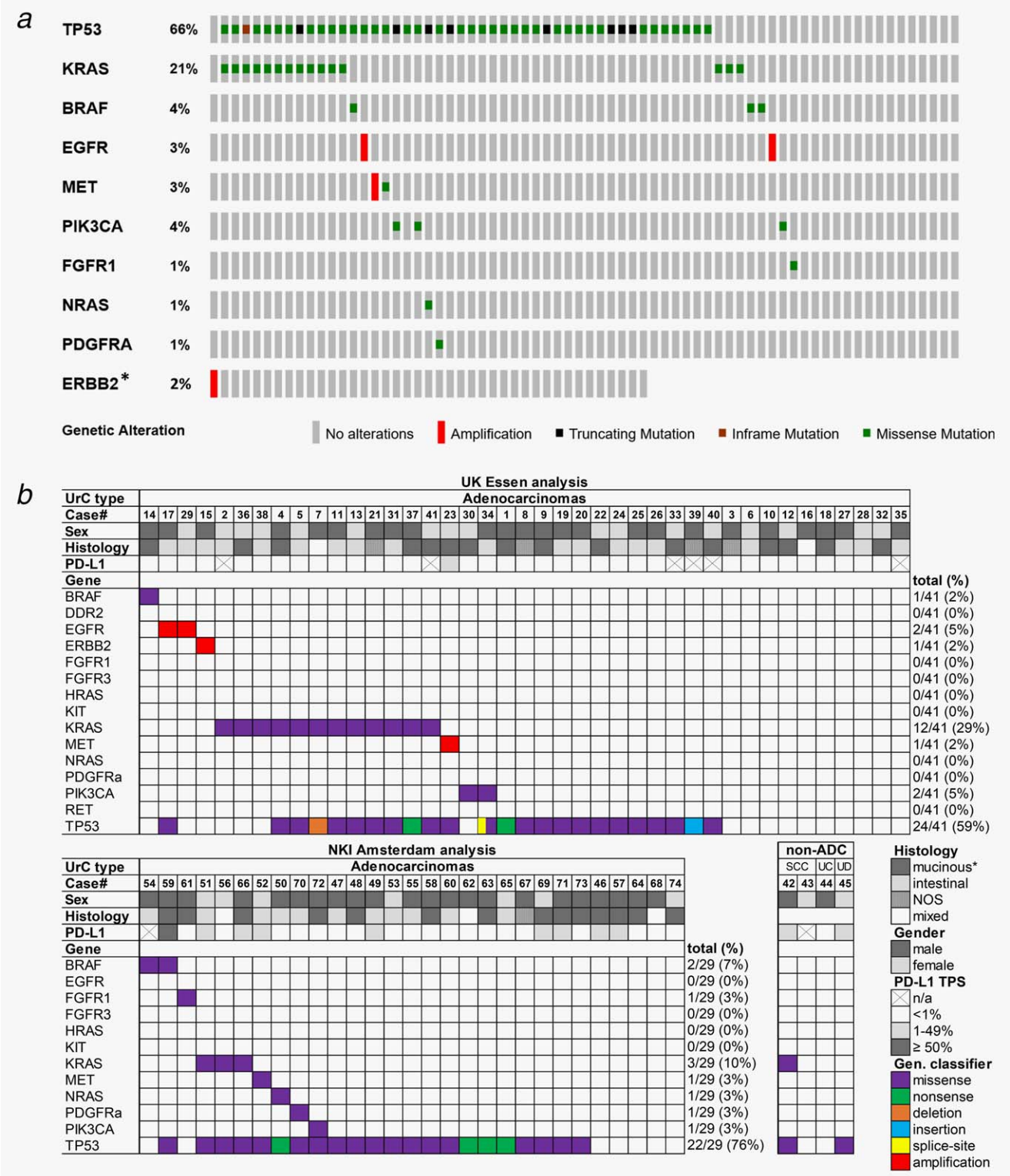

Figure 1. Oncoprints of the genetic alterations in UrC. (*A*) Overview of the genetic alterations detected in the total cohort urachal adenocarcinomas. (*B*) A more detailed version including information on sex, histology, PD-L1 expression and genetic alteration type also in the non-adenocarcinoma UrC cases. Gene amplifications (including *ERBB2**, $n = 41/70$) were evaluated in the first cohort only. Details on the genetic events can be found in Supporting Information Table S2. Abbreviations: NOS, not otherwise specified; SCC, squamous cell carcinoma; TPS, PD-L1 tumor proportion score; UC, urothelial carcinoma; UD, undifferentiated carcinoma. mucinous*: cases with predominant signet ring cell morphology included. [Color figure can be viewed at wileyonlinelibrary.com]

The first, multi-institutional cohort showed more *KRAS* mutations ($p = 0.059$). No other significant differences regarding molecular alterations between both cohorts were noted. In particular, there were no statistically significant differences in clinico-pathological or in any molecular parameters between mucinous and intestinal type urachal carcinomas.

In 66 of 70 cases (94.3%) tissue for MMR-IHC was available with technical feasibility of IHC of all four proteins (MLH1, MSH2, MSH6, and PMS2) in 61 of 70 cases (87.1%). The molecular MSI-analyses were feasible in 56 of 70 cases (80%). A negative MMR expression was detected in one case (MSH2). In this and all other cases, all remaining MMR-markers were positive and no MSI-h was detected.

In 63 of 70 cases (90%) sufficient material was available for PD-L1-IHC. In 10 of these 63 cases (15.9%) a specific PD-L1-expression in the tumor cells was detectable (Fig. 1) with a tumor proportion score (TPS) of 1–49% in 9 of 10 cases (90%) and a TPS $\geq$ 50% in one Case (10%). PD-L1-expression was not restricted to intestinal type UrC (3/10; 30%). The majority of PD-L1 expressing cases were found in mucinous type UrC (7/10; 70%) including the only case with high PD-L1-expression (TPS $\geq$ 50%). No obvious clustering of cases with PD-L1-expression and other molecular events was obvious. However, both cases with *MET*-alterations exhibited PD-L1 expression.

Correlation between genomic alterations and clinical outcomes

Clinicopathological factors were not associated with any genetic event, except for a trend to higher Mayo tumor stage in UrC with MAPK/PI3K-alterations ($p = 0.073$). Organ confined disease, that is Sheldon[19] Stage $\leq$IIIA and Mayo[20] Stage I, and absence of metastatic disease were univariable predictors of more favorable OS ($p = 0.036$, $p = 0.016$, $p = 0.037/p = 0.031$) while only absence of lymph node metastasis predicted better RFS ($p = 0.004$) in univariable analysis. A trend to more favorable RFS was found in intestinal type UrC ($p = 0.075$; log-rank test: $p = 0.068$, Supporting Information Fig. S3) and in organ confined disease in the Mayo system ($p = 0.056$).

Presence of PD-L1 expression was associated with adverse PFS ($p = 0.002$; log-rank test), however, there were only three events in the PD-L1 positive group, thus limiting its significance. No such association was detected regarding OS.

In multivariable Cox-analyses for OS and RFS, presence of any mutation or MAPK/PI3K-alteration did not exhibit prognostic influence (Table 2).

Additionally, no correlation was observed between the mutational status of *TP53* and its IHC staining pattern. A higher rate of *TP53* mutation was observed in UrC with signet ring cells ($p = 0.05$). No other clinicopathological or prognostic associations were noted for *TP53* and/or *KRAS* mutational status.

Results in non-adenocarcinoma UrC

The results of the four cases of non-adenocarcinoma UrC are included in Figure 1 and Supporting Information Table S2. Due to the low number of cases, no correlation analyses with clinicopathological data were performed.

Discussion

Systemic therapy is often required in UrC because patients present with advanced stage disease or they progress after initial locoregional intervention. Investigation of the efficacy of chemotherapy has been limited to retrospective series. Due to the rarity of UrC, it is not feasible to perform large prospective clinical studies to evaluate the clinical benefit of various systemic therapies. Molecular alterations, however, may provide the rationale for targeted therapeutic approaches and/or immunotherapy.

The most common genetic alterations in urachal adenocarcinomas we detected were *TP53* mutations (66%). This high number indicates the common role of inactivation of this tumor suppressor gene also in UrC. Although *TP53* has in the past been considered mostly undruggable, recently multiple efforts of targeting *TP53* altered tumors are in development.[21]

In addition, we found a subset of UrC characterized by dysfunctional and activated oncogenic MAPK- and PI3K-pathways.[4,5] As EGFR signaling mainly occurs through these pathways,[6] we grouped together all UrC with any pathogenic mutational event in *KRAS, NRAS, BRAF*, or *PIK3CA* ($n = 22$; 31%). This subset is additionally equivalent to all UrCs with mutations in intracellular signal transduction pathways and was termed 'MAPK/PI3K-altered' subset. All these alterations were mutually exclusive.

These findings together with the results of the MMR-/MSI- and PD-L1-analyses have important therapeutic and ontogenetic implications.

Due to histopathological and certain clinical similarities of UrC and CRC, chemotherapy in advanced UrC has often been adapted from CRC with 5-FU containing approaches. This has been shown to be more effective compared to regimes used for UC.[2,22] In accordance with this observation, we did not find evidence of MMR-d/MSI-h in UrC, which leads to a hypermutated phenotype and in CRC has been implicated in decreased 5-FU-efficacy.[23] Our single UrC-case with immunohistochemical MSH2-loss seems to represent a false negative result, as MSH6-protein expression was retained which should not be the case in MSH2-deficient tumors.[7] Additionally, no evidence of MSI-h was detected on the DNA level by microsatellite PCR analyses neither in this nor in any other case.

Another recent and efficient anti-cancer strategy is targeting tumoral immune-escape mechanisms, in which inhibitors of the PD-1/PD-L1-axis are of particular therapeutic interest. PD-1/PD-L1 inhibitors have proven efficacy in several types of advanced cancer, including melanoma, non-small-cell lung cancer, renal cell carcinoma and bladder cancer.[24] In most studies, cases with immunohistochemical PD-L1-expression on tumor cells and/or tumor-related immune-cells exhibited better responsiveness to PD-1/PD-L1-inhibitors. However, as

also in tumors without PD-L1-expression positive anti-tumoral effects can be detected, PD-L1-IHC testing is considered complementary with the exception of the companion diagnostic 22C3-test in NSCLC.[24] With regard to UrC, these results implicate that PD-1/PD-L1-inhibitors might be effective in advanced disease despite its modest rate of PD-L1-expression (16%). In fact, others have recently reported on a case in UrC with atezolizumab-therapy, an anti-PD-L1-antibody, resulting in stable disease.[25] However, as MMR-d/MSI-h does not seem to be a hallmark of UrC at least in our cohort, immune escape mechanisms employing the PD-1/PD-L1 axis do not seem to be the defining molecular characteristic of UrC. This also has implications for the use of pembrolizumab in UrC, an anti-PD-1-antibody, which recently gained FDA-approval in MMR-d/MSI-h tumors irrespective of site of origin.

Therefore, targeted therapy approaches are of prominent therapeutic interest in advanced UrC.

Recently, *Collazo-Lorduy* and colleagues reported a successful application of cetuximab, an anti-EGFR monoclonal antibody which is used in metastasized CRC, in one UrC patient who was pretreated with chemotherapy. This treatment was justified by the presence of an *EGFR* amplification and absence of a *KRAS* mutation.[5]

In CRC, it has been shown that MAPK-/PI3K-activation downstream of the EGF receptor is associated with anti-EGFR therapy resistance.[26] We detected two cases in our cohort with coexisting *EGFR* gene amplification and without activating MAPK-/PI3K-mutations thus *EGFR* amplification is a rare event in UrC. Therefore, it seems reasonable to test for *EGFR* gene amplifications and activating MAPK/PI3K-pathway mutations in advanced UrC when an anti-EGFR therapy is considered. This approach would be similar to the concept of complementary/companion diagnostics in CRC.[27]

In addition to these therapeutic considerations, the mutational profile of UrC shows similarities to that of CRC. Most of the detected activating MAPK/PI3K-pathway mutations were also common in CRC when compared to The Cancer Genome Atlas (TCGA) data. [28] This is especially prominent in the clustering of codon G12/G13 *KRAS* alterations but also for the remaining *KRAS*, *NRAS*, and *PIK3CA* mutations (Supporting Information Fig. S1).[28] Interestingly, both *EGFR* amplified cases we detected were intestinal type UrCs.

However, not only similarities but also differences were detected. For example, we identified three non-classical, *BRAF* mutations (G469A: $n = 1$, D594G: $n = 2$) adding to the four classical V600E mutations reported previously.[4] While the G469A *BRAF* mutation was relatively common in cancer, the D594G variant has rarely been detected in cancer. Interestingly, although being located in the BRAF-kinase region, the D594G variant has been described in a case of metastatic CRC responding to cetuximab.[27]

In addition, a comparison with TCGA data for CRC revealed lower frequencies of almost all analyzed genetic alterations in UrC with the exception of *TP53* mutations. (Supporting Information Fig. S4).[28] However, although certain differences to CRC are apparent, the disparity to UC is more pronounced (Supporting Information Fig. S4). This notion is further supported by the very low incidence of Telomerase reverse transcriptase (*TERT*) promoter mutations in urachal adenocarcinomas (4%), which is otherwise a very common event in UC but not in CRC.[29–31] Interestingly, our findings regarding MMR-d/MSI-h in UrC are more comparable to figures in UC[32] than in CRC.[23,28] Regarding the rate of PD-L1-expression, UrC (16%) seems to be in the lower range of figures reported for UC (14–28%)[33] and CRC (9–43%).[34,35] Taken together, UrC appears to be a distinct entity on the genomic level with closer resemblance to CRC than to UC. Further analyses are needed to address this issue while the present study was not primarily designed from an ontogenetic point of view.

In addition to alterations related to intracellular signal transduction pathways, we also detected cases with other pathogenic and possibly targetable genetic events. For example, pathogenic *FGFR1*, *MET*, and *PDGFR*A mutations and *MET*- and *ERBB2*-gene amplifications were detected in one case each, possibly identifying therapeutic targets in these patients.

Interestingly, both UrC-cases with *MET*-alterations exhibited PD-L1-expression and first (pre-)clinical evidence of the relationship between PD-L1 upregulation and *MET*-alterations is emerging.[36] Moreover, one case with high level *ERBB2* gene amplification and HER2-protein expression was detected (Supporting Information Fig. S2). HER2 is another member of the EGFR family and a well-known therapeutic target in breast and gastric cancer and under clinical investigation in other cancers such as non-small cell lung cancer.[37–39]

Our analysis has certain limitations. Due to the rarity of the disease, prospective analyses can hardly be conducted. We therefore chose a retrospective multi-institutional approach to maximize our sample size. In addition, we analyzed a first multi-institutional, international cohort and a second national Dutch cohort at different sites. Through this approach we expected to gain a more comprehensive and homogenous estimation of genetic events in UrC. This approach holds, however, the disadvantage of analytical differences between laboratories. This applies to the use of MiSeq and HiSeq-platforms at the different sites. While the enhanced capabilities and sensitivity of the HiSeq-platform are accepted, both sites were in contact to obtain best comparable analytic data. Although our cohort is the largest analyzed in the literature, the case number remains low, which precludes adequate statistical power for some sub-group analyses. This is also important when considering the results of survival analyses as inevitably the samples were collected from patients with a range of different stages and treatments

confounding the possible survival impact of genetic alterations. Additionally, the employed gene panel was of modest size which has to be kept in mind in regard to the patients where no genomic aberration was detectable.

In conclusion, we have analyzed the largest cohort of UrC to date in an international, multi-institutional targeted sequencing approach supplemented with *in situ* hybridization and protein expression analyses including evaluation of PD-L1 expression and MMR/MSI-status. Although MMR-d/MSI-h does not seem to be a hallmark of UrC, anti-PD-1/PD-L1 immune therapy might be a potential therapeutic option given the PD-L1 expression rate in UrC. In particular, our results underline the role of MAPK/PI3K-pathway dysregulation which was detected in 31% of urachal adenocarcinomas. This has important implications for possible anti-EGFR therapy in UrC and suggests that targeted testing of MAPK/PI3K-pathway alterations would be reasonable in patients with advanced UrC before considering anti-EGFR therapy. In addition, further potentially druggable alterations were found in *ERBB2* (HER2), *MET*, *FGFR1*, and *PDGFRA* in a subset of patients. Our data support the notion of UrC being a distinct entity on the genomic level with closer resemblance to CRC than to UC.

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