

Use of plasma ctDNA as a potential biomarker for longitudinal monitoring of a patient with metastatic high-risk upper tract urothelial carcinoma receiving pembrolizumab and personalized neoepitope-derived multi-peptide vaccinations: a case report

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ABSTRACT

Upper tract urothelial carcinoma (UTUC) is often diagnosed late and exhibits poor prognosis. Only limited data are available concerning therapeutic regimes and potential biomarkers for disease monitoring. Standard therapies often provide only insufficient treatment options. Hence, immunotherapies and complementary approaches, such as personalized neoepitope-derived multi-peptide vaccine (PNMV), come into focus. In this context, genetic analysis of tumor tissue by whole exome sequencing represents an essential diagnostic step in order to calculate tumor mutational burden (TMB) and to reveal tumor-specific neoantigens. Furthermore, disease progression is essential to be monitored. Longitudinal screening of individually known mutations in plasma circulating tumor DNA (ctDNA) by the use of next-generation sequencing and digital droplet PCR (ddPCR) might be a promising method to fill this gap.

Here, we present the case of a 55-year-old man who was diagnosed with high-risk metastatic UTUC in 2015. After initial surgery and palliative chemotherapy, he developed recurrence of the tumor. Genetic analysis revealed a high TMB of 41.2 mutations per megabase suggesting a potential success of immunotherapy. Therefore, in 2016, off-label treatment with the checkpoint-inhibitor pembrolizumab was started leading to strong regression of the disease. This therapy was then discontinued due to side effects and treatment with a previously produced PNMV was started that induced strong T cell responses. During both treatments, plasma Liquid Biopsies (pLBs) were performed to measure the number of mutated molecules per mL plasma (MM/mL) of a known tumor-specific variant in the *MLH1* gene by ddPCR for longitudinal monitoring. Under treatment, MM/mL was constantly zero. A few months after all therapies had been discontinued, an increase of MM/mL was detected that

persisted in the following pLBs. When MRI scans proved tumor recurrence, treatment with pembrolizumab was started again leading to a rapid decrease of MM/mL in the pLB to again zero. Treatment response was then also confirmed by MRI.

This case shows that use of immunotherapy and PNMV might be a promising treatment option for patients with high-risk metastatic UTUC. Furthermore, measurement of individually known tumor mutations in plasma ctDNA by the use of pLB could be a very sensitive biomarker to longitudinally monitor disease.

CASE REPORT

Background

Upper tract urothelial carcinoma (UTUC) is a rather rare entity of cancer with 60% of invasive carcinoma at the time of diagnosis.¹ The gold standard for high-risk disease is radical nephroureterectomy² and the established first-line therapy for metastasized UTUC is platinum-based adjuvant chemotherapy. However, many patients treated with this first-line therapy suffer from side effects and 26.5% of patients even show disease progression.^{3,4} In the age of next-generation sequencing, it is possible to analyze a tumor comprehensively with respect to its somatic mutations. Thus, one can gain information about tumor-specific mutations to then design individual therapies. Many recent studies have shown that tumor mutational burden (TMB) and microsatellite instability (MSI) are good predictors of response to immunotherapy.^{5,6} This makes UTUC carrying high

TMB a promising target for immunotherapy. Indeed, the immune checkpoint inhibitor (ICI) pembrolizumab as second-line therapy showed significantly longer survival rates, regardless of the PD-L1 (programmed cell death 1 ligand 1) expression.⁷ This approach can be combined with other immune-stimulatory therapies, such as personalized neoepitope-derived multi-peptide vaccines (PNMVs), where genetic analysis of the tumor is used to design peptides mimicking the neoantigens that the tumor is most likely to present on its surface. The synthesized peptides either bind to human leukocyte antigen class I (HLA-I) or are presented via HLA-II by antigen-presenting cells (APCs). The HLA genes are extremely polymorphic making it necessary to haplotype the individual patient's HLA to elicit the best immune response. T cells are then supposed to be primed by interacting with the peptide-presenting APC, so that they recognize the neoantigen-presenting tumor. PNMV is likely most fitting for a patient setting with minimal residual disease to establish a sort of 'cancer surveillance'.⁸ The combination of ICI and PNMV aims at priming the patient's immune system to selectively attack cancer cells and prevent progression. However, up to date, this is largely theory and has not reached clinical practice yet. According to the US National Library of Medicine's Clinical Trials Database, only some studies currently explore the combination treatment, none of them for UTUC.⁹

For the purpose of staging, clinicians mainly rely on imaging techniques. But these struggle to detect early progression of disease. As a result, UTUC and disease progression are often detected late in an advanced state, so biomarkers are urgently needed in the treatment of UTUC.¹ Easy to obtain are blood samples, but there is very limited data available on blood-based biomarkers in UTUC.¹⁰ However, there are some candidates for biomarkers at stake. One promising candidate presents circulating tumor DNA (ctDNA) that can be sampled from the patient's peripheral blood by a method called plasma Liquid Biopsy (pLB). The amount of ctDNA in the peripheral blood is thought to correlate with tumor mass. A positive correlation has been shown for various cancer types.^{11,12} Measurement of individually known variants in plasma ctDNA by digital droplet PCR (ddPCR) represents a sensitive method to monitor disease activity longitudinally, that may match the need for a clinical biomarker.¹³

Here, we introduce the case of a patient with high-risk, metastatic UTUC treated with immunotherapy as well as PNMV whose course of disease has been monitored by pLB-based ddPCR.

Case presentation

A 55-year-old man was diagnosed with high-risk UTUC of the right ureter in April 2015. Staging and grading revealed stage pT2 N1 M0 (1/1) G2 with a paraaortal lymph node metastasis. The patient underwent radical nephroureterectomy in May 2015 followed by two cycles of palliative chemotherapy (gemcitabine/carboplatin).

In July 2015, the chemotherapy was discontinued due to side effects, especially urticaria.

Two months later, in September 2015, the patient developed tumor recurrence in the bladder and hence underwent transurethral resection of the bladder (TUR-B). Four days later, a CT scan revealed extended infestation of cervical, supraclavicular, retroperitoneal, and retrocrural lymph nodes and a second CT scan in March 2016 showed strong progression of the lymph node metastases (figure 1A). Later in March, a bone metastasis of the right seventh rib was discovered.

The patient's tumor tissue was genetically analyzed by whole exome sequencing (WES) in order to potentially reveal new treatment targets. For the analysis, tissue from the TUR-B procedure from September 2015 was used. Results are displayed in online supplemental table S1. The genetic analysis showed a very high TMB of 41.2 mutations per megabase. One important tumor-specific genetic variant detected by WES analysis was the one of the *MLH1* gene (*MLH1* c.883A>T; p.Ser295Cys). The *MLH1* gene encodes for the MutL homolog protein 1, which plays an important role in the mismatch repair (MMR). Defects in *MLH1* are associated with MSI, which was also predicted in our patient. For more details of the technical methods, please see the online supplemental material.

Even though PD-L1 testing was not established at this time, immunotherapy was recommended based on DNA MMR deficiency and high TMB in the tumor genome, and in April 2016 off-label therapy with the ICI pembrolizumab (2 mg/kg body weight every 3 weeks) was started. The patient generally tolerated the immunotherapy well, but his fifth cycle of pembrolizumab in July 2016 was delayed for 10 days due to subclinical elevations in liver transaminases.

Furthermore, because of the high TMB, the tumor was considered likely to have many mutated antigens present on its surface providing a rationale for a treatment attempt with PNMVs. In order to produce such vaccines, the tumor specimen was analyzed by WES, followed by prediction of neoepitopes and selection of peptides (see online supplemental material). Seven major histocompatibility complex (MHC) class I peptides and three longer MHC class II peptides were chosen that were then used for the production of the personalized vaccine (table 1). The vaccine was produced as an individual healing attempt in Tuebingen, Germany, in May 2016, but following the patient's wish was not yet applied at this time.

Instead, treatment with pembrolizumab was continued and a CT scan was performed in June 2016. This scan showed strong regression of the metastases and great response to the immunotherapy (figure 1B). Additional CT scans in October 2016 and January 2017 revealed further regression of the disease and no new lesions were found at any time point.

Since February 2017, pLBs were performed regularly as a potential longitudinal monitoring of the patient's disease. From pLB-derived ctDNA, the amount of MM/

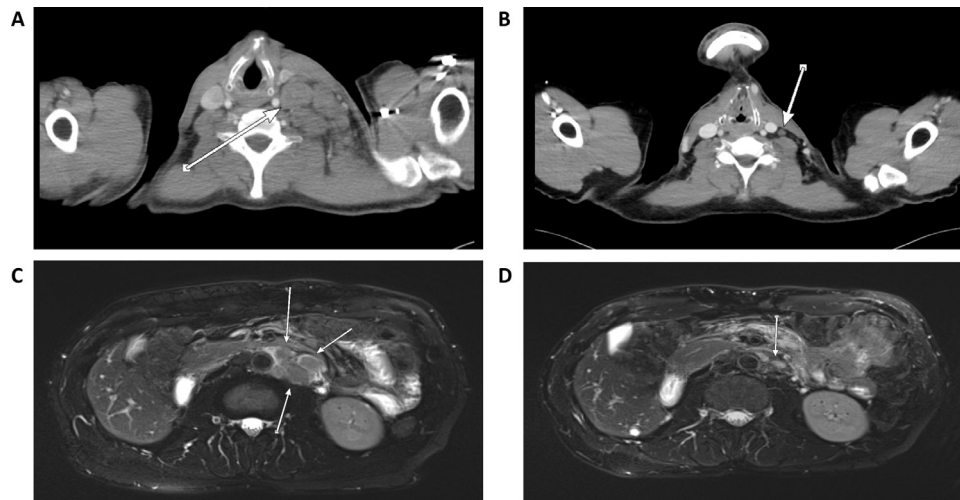


Figure 1 CT/MRI scans at different time points over the course of the disease. (A) CT scan from March 2016 before start of initial treatment with pembrolizumab showing strong lymph node metastases (arrow). (B) CT scan from April 2018 under initial therapy with pembrolizumab showing shrinkage of the metastases (arrow) indicating great treatment response supported by mutated molecules per mL plasma (MM/mL) constantly at zero. (C) MRI scan from November 2019, 6 months after discontinuation of treatment with personalized neoepitope-derived multi-peptide vaccines showing reappearance of enlarged paraaortal lymph nodes (arrows) corresponding with increasing MM/mL between 44.49 and 188.01. (D) MRI scan from February 2020, 3 months after re-initiation of treatment with pembrolizumab showing shrinkage of the enlarged lymph nodes (arrow) indicating a new treatment response corresponding with MM/mL returning to zero.

mL of the tumor-specific genetic variant of the *MLH1* gene was measured by ddPCR. The *MLH1* variant was chosen to be measured in pLB as it is considered one of the tumor's driver mutations and was not part of the 10 peptides used in the vaccine. Under the first course of treatment with pembrolizumab, the amount of MM/mL observed in the pLB was always zero (figure 2).

Further CT scans from April 2017 until April 2018 showed stable disease. In October 2017, after the 26th cycle of pembrolizumab in total, the immune therapy was discontinued due to the development of unclear urticaria.

In February 2018, under stable disease conditions and after long discussions with the patient and his physicians, the patient decided to receive his first PNMV. Some concerns existed regarding the fact that the PNMV had been produced based on 2 year old WES data. Nonetheless, having in mind the possibility of a shifted tumor mutational profile, the PNMV was applied after long consideration as it still presented a valuable treatment option at that time. The vaccine was always injected intracutaneously in the left thigh followed by a subcutaneous injection of an adjuvant (granulocyte-macrophage colony-stimulating factor/Leukine). Over the course of 16 days, he received three more vaccinations (priming phase), followed by one vaccination every 4 weeks to 6 weeks (boosting phase).

Before starting the vaccine treatment, an initial immune monitoring had been performed in February 2017, showing no specific T cell response against any of the used peptides. After having received the ninth vaccination, a second immune monitoring was performed to gain knowledge about a first immunological response. This monitoring revealed strong CD4⁺ T cell responses

against 4 of the 10 chosen peptides. A third and fourth immune monitoring were performed at the time of the 17th vaccination and 2 months after the 18th vaccination in March and July 2019. These revealed strong CD4⁺ and CD8⁺ T cell responses against many of the chosen peptides indicating a successful vaccination approach (table 1). Throughout the whole PNMV treatment, 10 pLBs were performed by the use of ddPCR at an average interval of 4–6 weeks to longitudinally monitor tumor burden and the amount of MM/mL was constantly zero (figure 2).

After the 18th vaccination in May 2019, the patient developed an allergic reaction with local swelling, itching, nausea and strong urge to urinate, leading to discontinuation of the vaccination therapy. At this time point, the amount of MM/mL measured in the pLB was zero and MRI scans showed no signs of tumor progression. After cessation of the vaccination therapy, the patient received no new treatment immediately. Nevertheless, pLBs (figure 2) and MRI scans for monitoring were performed regularly.

In August 2019, an MRI scan revealed unclear enlargement of a left paraaortal infrarenal lymph node. Therefore, a control MRI was scheduled for 3 months later. Meanwhile, two pLBs were performed on first and 23rd of October 2019 which showed increment of the amount of MM/mL to first 3.63 and then 44.49. In November 2019, the control MRI revealed further enlargement of the left paraaortal lymph node as well as new enlargement of a left iliacal commune lymph node (figure 1C). Therefore, the treating oncologists decided to start a new cycle of treatment with pembrolizumab. Unfortunately, before re-initiation of pembrolizumab, a new analysis of the genomic profile of the tumor tissue could not be performed

Table 1 Overview of the immune monitoring results

Peptide-specific immune responses		September 13, 2018 (V9)			March 14, 2019 (V17)			July 08, 2019 (post V18)		
No.	Peptide sequence	Gene and somatic variant	HLA	VAF (%)	CD4	CD8	CD4	CD8	CD4	CD8
1	HSYRGTGGIFK	TBL1XR1 NM_024665 c.G1462A;p.E488K	HLA-A*03:01	36	-	-	++ SI: 4.3 (1.7%)	+ SI: 2.1 (0.6%)	Pool peptide 1+5 +++ SI: 7.3 (0.4%)	Pool peptide 1+5 ++++ SI: 15.9 (6.5%)
2	MMFRNYQRK	CHD4 NM_001273 c.G1705A;p.V569M	HLA-A*03:01	38	-	-	-	++ SI: 3.8 (1.8%)	-	+ SI: 3.2 (0.6%)
3	EVSAAHRAHYF	DNMT3A NM_022552.4 c.G2375A;p.R792H	HLA-A*26:01	36	+++ SI: 7.7 (1.1%)	(+) positive tendency	+++ SI: 2.2 (0.6%)	-	Pool peptide 3+7 ++++ SI: 21.6 (1.8%)	Pool peptide 3+7 -
4	DPTASVPSM	KIAA1549 NM_001164665 c.G4753A;p.V1585M	HLA-B*35:01	33	+++ SI: 5.6 (4.3%)	-	++ SI: 3.7 (1.6%)	-	Pool peptide 4+6 ++++ SI: 11.6 (0.5%)	Pool peptide 4+6 -
5	HIPIIWATS	PIK3C2B NM_002646 c.G1919A;p.R640H	HLA-A*26:01	45	-	-	++++ SI: 13.1 (6.6%)	+++ SI: 6.2 (2.9%)	Pool peptide 1+5 +++ SI: 7.3 (0.4%)	Pool peptide 1+5 ++++ SI: 15.9 (6.5%)
6	VORRAQKLF	KEL NM_000420 c.A869T;p.E290V	HLA-B*15:01	37	-	-	-	+ SI: 2.1 (1.0%)	Pool peptide 4+6 ++++ SI: 11.6 (0.5)	Pool peptide 4+6 -
7	AFDDKTRLV	WWC3 NM_015691 c.T662C;p.V221A	HLA-C*04:01	33	-	-	-	-	Pool peptide 3+7 ++++ SI: 21.6 (1.8%)	Pool peptide 3+7 -
8	TGVPQSRPHIPRTQPQP	RNF43 NM_017763.4 c.C1742A;p.P581H	Class II	36	++++ SI: 39.3 (1.7%)	-	+ SI: 2.5 (0.7%)	-	++ SI: 4.1 (0.3%)	-
9	MFKGVASSQLPKGTKT	SETD2 NM_014159.6 c.G158A;p.R53Q	Class II	38	++++ SI: 37.5 (0.9%)	-	+++ SI: 9.5 (2.0%)	-	++++ SI: 35.9 (2.4%)	-
10	DWNPHQDLHAQDRAHRI	SMARCA4 NM_001128844.1 c.A3555T;p.Q1185H	Class II	33	-	-	-	-	-	-

HLA: human leukocyte antigen type, for which binding was predicted. VAF: variant allele frequency, detection frequency of the mutated allele. The observed frequencies could be influenced by tumor content and copy number variations and do not directly equal the proportion of the mutation in the tumor. SI: Stimulation Index, x-fold increase of polyfunctional reactive T cells (at least two of the markers CD154, IFN- γ , TNF and/or IL-2 positive) in the stimulated specimen compared to the according negative control specimen. Additional information: proportion (%) of reactive T cells (IFN- γ , TNF, IL-2 and/or CD154 positive) of the CD4⁺ or CD8⁺ T cell population after in vitro amplification. Does not directly equal the frequency of specific cells in vivo. +, lightly positive; ++, positive; +++, strongly positive; ++++, very strongly positive; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin.

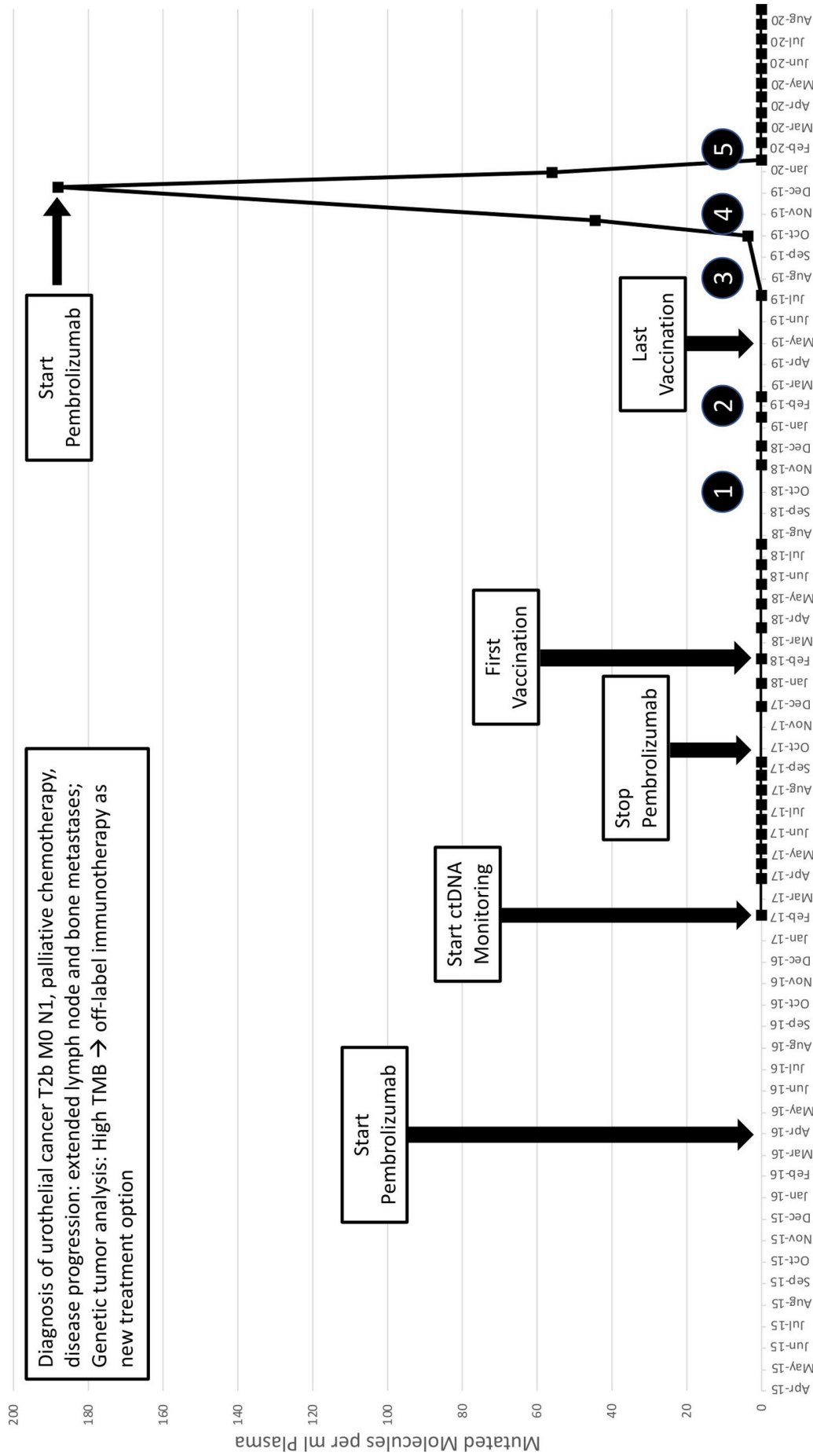


Figure 2 Timeline of the patient's treatment course. Starting in February 2017, MM/mL were measured via pLB and remained at undetectable levels until July 2019. Circled numbers indicated successive MRI scans: (1) and (2) no detected changes; (3) unspecified lymph node enlargement; (4) assured tumor progression and (5) tumor regression. ctDNA, circulating tumor DNA; MM/mL, mutated molecules per ml plasma; pLB, plasma Liquid Biopsy; TMB, tumor mutational burden.

because neither surgery nor another biopsy was carried out, and the amount of cell free DNA (cfDNA) in the blood, which at this time point ranged between 3.51 ng/mL plasma and 4.78 ng/mL plasma, as well as the tumor content in the cfDNA indicated by ddPCR were too low for a comprehensive sequencing approach.

When the patient received his first dose of pembrolizumab at the beginning of December 2019, the amount of MM/mL measured in the pLB was 188.01. Three weeks later, the patient received his second dose of pembrolizumab. Interestingly, at this time, the amount of MM/mL had already decreased to 56.01 suggesting a strong therapy response. On January 17, 2020, another pLB was performed revealing a further decrease of the amount of MM/mL to again zero under immune therapy and a control MRI scan performed on February 28, 2020, showed significant regression of the lymph nodes confirming a treatment response (figure 1D). Up until today, the patient remains in remission. Ten more pLBs were conducted between February 2020 and August 2020 and the amount of MM/mL measured was constantly zero (see figure 2). The latest CT and MRI scans performed on September 18, 2020, showed no signs of tumor recurrence.

Discussion

High-risk UTUC represents a type of cancer that is associated with a high morbidity and bad clinical outcomes. As of today, standard treatment options include surgery and chemotherapy, but mainly lack successful personalized targeted therapies.¹⁴

Here, we present the case of a 55-year-old man diagnosed with high-risk metastatic UTUC who developed recurrence of the disease after having undergone standard treatment. We put emphasis on the importance of genetic analysis of tumor tissue by WES in order to reveal new personalized treatment targets. In the specific case presented here, WES showed the presence of MSI and a very high TMB (41.2 mutations per megabase) of over 10 fold the mean TMB found in UTUC (2.91 mutations per megabase),¹⁵ placing it into the highest quartile of TMB in metastatic urothelial carcinoma^{16 17} and thereby indicating a potential treatment success of ICI.⁵ Therefore, in 2016, the decision was made to treat the patient with pembrolizumab. At this time point, the use of immunotherapy for the treatment of UTUC was still declared off-label, but since then several studies have shed light on the success of this type of treatment leading to FDA approval for pembrolizumab as second-line therapy for UTUC in May 2017.¹⁸ In the same year, the FDA even approved the use of immunotherapy for the treatment of MSI-high solid tumors in general, independent of their entity.¹⁹

With the goal to further enhance the immunotherapy, we also produced a PNMV based on the genetic analysis of the tumor that induced very strong CD4⁺ as well as CD8⁺ T cell responses in the patient. This type of therapeutic anti-cancer peptide vaccine is a very new approach to target tumor cells and is still considered an individual

healing attempt. In Germany, there is a unique situation for individual healing attempts, which are outside of otherwise required permission, and are possible even without obtaining an ethical approval. Nevertheless, it is important to document an interdisciplinary team decision together with the patient.

Most current clinical trials investigating anti-tumor peptide vaccines focus on the use of tumor-associated antigens,²⁰ but some very recent publications show that PNMVs might be an even more promising therapy option for many tumor entities, including those with limited other available therapies, for example, glioblastoma.^{21 22} Recently, some studies also reported promising results in designing neoantigen-based off-the-shelf vaccines for MMR-deficient colorectal cancer,^{23 24} but these vaccines are less personalized as they are based on the same neoantigens that are considered most likely to be present in MMR deficient cancers for all patients.

In our patient, the high TMB possibly led to an abundance of tumor neoantigens on the tumor cell surface that could be targeted by the vaccine. Due to the big pool of potentially eligible peptide sites, we were able to select some of very high predicted HLA haplotype affinity making this type of treatment more likely to be successful. Still, other patients treated with PNMV who had only an intermediate or even low TMB also showed response to the therapy (unpublished data) suggesting that not only the quantity of targetable tumor neoantigens seems to be important for treatment response, but also their quality. Nevertheless, more research is needed to fully investigate the effects of such vaccines in larger patient cohorts.

Unfortunately, our patient experienced tumor recurrence. We think this might be explained by an immunologic escape mechanism the tumor acquired during the vaccine therapy. This mechanism has been described for other types of anti-tumor vaccinations before, such as an EGFRvIII-targeted (epidermal growth factor receptor transcript variant 3) peptide vaccine for the treatment of glioblastoma.²⁵ Nevertheless, to fully investigate this theory, sequencing of the current tumor tissue in comparison to the data from the previous analysis in 2015 would be crucial, which was not possible in our patient due to two main reasons: First, the amount of cfDNA in the blood was low (see lines 145 f), and second, the frequency of the MLH1 driver mutation in the pLBs indicated a tumor content of maximum ~20% in the cfDNA in one sample, which represents the lower border for tumor sequencing. Thus, a characterization of the tumor based on panel sequencing or WES of cfDNA would have only been possible with extremely limited sensitivity. These two limitations (cfDNA amount and tumor content) did not suggest a panel sequencing or WES approach to potentially bring up new relevant insights.

Even though our patient did not receive ICI and PNMV at the same time, recent studies show that combining the two types of therapies can lead to mutual reinforcement.^{26 27}

Furthermore, monitoring of the disease progress is a very important aspect in the treatment of UTUC. To the best of our knowledge, there are currently no valid biomarkers available to longitudinally monitor a patient with this tumor entity.¹⁰ Physicians mainly rely on imaging techniques, which tend not to be very sensitive, making it hard to detect potential recurrence of the tumor early, as a recent study showed for the closely related entity of bladder cancer.²⁸ Here, we propose the use of pLB-based measurements of individually known mutations in ctDNA as a potential biomarker for longitudinal monitoring of the disease, which goes in line with the findings of Chalfin *et al* from 2019.²⁹ In our patient, these measurements have proven to be a sensitive method to detect potential tumor recurrence in a very early stage, provided that the chosen mutations are not subject to some selection pressure caused by the therapy. Most interestingly, constant increases of the amount of MM/mL were observed earlier than specific changes in MRI scans, giving first hints about an ongoing tumor recurrence 1 month before imaging techniques specifically detected it. Furthermore, after new induction of treatment with immunotherapy, MM/mL decreased again rapidly indicating a treatment success. Nevertheless, more in-depth research with a larger patient cohort needs to be conducted to fully investigate the potential of such measurements as a biomarker for UTUC and other types of cancer.

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