Case Report

JOURNAL OF HEPATOLOGY

Personalized peptide vaccine-induced immune response associated with long-term survival of a metastatic cholangiocarcinoma patient

Markus W. Löffler[1,2,13,*], P. Anoop Chandran[2], Karoline Laske[2,3], Christopher Schroeder[4], Irina Bonzheim[5,13], Mathias Walzer[2,6,7], Franz J. Hilke[4], Nico Trautwein[2], Daniel J. Kowalewski[2,3], Heiko Schuster[2], Marc Günder[2], Viviana A. Carcamo Yañez[8], Christopher Mohr[6,7], Marc Sturm[4], Huu-Phuc Nguyen[4], Olaf Riess[4], Peter Bauer[4,13], Sven Nahnsen[6,9], Silvio Nadalin[1], Derek Zieker[1], Jörg Glatzle[1,10], Karolin Thiel[1], Nicole Schneiderhan-Marra[8], Stephan Clasen[11], Hans Bösmüller[5], Falko Fend[5,13], Oliver Kohlbacher[6,7,9,12,13], Cécile Gouttefangeas[2], Stefan Stevanović[2,13], Alfred Königsrainer[1,13], Hans-Georg Rammensee[2,13]

[1]*University Hospital Tübingen, Department of General, Visceral and Transplant Surgery, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany;* [2]*University of Tübingen, Interfaculty Institute for Cell Biology, Department of Immunology, Auf der Morgenstelle 15, 72076 Tübingen, Germany;* [3]*Current address: Immatics Biotechnologies GmbH, Paul Ehrlich Str. 15, 72076 Tübingen, Germany;* [4]*University Hospital Tübingen, Institute of Medical Genetics and Applied Genomics, Calwerstr. 7, 72076 Tübingen, Germany;* [5]*University Hospital Tübingen, Institute of Pathology, Liebermeisterstr. 8, 72076 Tübingen, Germany;* [6]*University of Tübingen, Center for Bioinformatics, Sand 14, 72076 Tübingen, Germany;* [7]*University of Tübingen, Dept. of Computer Science, Sand 14, 72076 Tübingen, Germany;* [8]*NMI Natural and Medical Sciences Institute at the University of Tübingen, Markwiesenstrasse 55, 72770 Reutlingen, Germany;* [9]*University of Tübingen, Quantitative Biology Center (QBiC), Auf der Morgenstelle 10, 72076 Tübingen, Germany;* [10]*Current address: Klinikum Konstanz, Luisenstr. 7, 78464 Konstanz, Germany;* [11]*University Hospital Tübingen, Department of Diagnostic and Interventional Radiology, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany;* [12]*Max Planck Institute for Developmental Biology, Spemannstr. 35, 72076 Tübingen, Germany;* [13]*German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ) partner site Tübingen, Germany*

Background & Aims: We report a novel experimental immunotherapeutic approach in a patient with metastatic intrahepatic cholangiocarcinoma. In the 5 year course of the disease, the initial tumor mass, two local recurrences and a lung metastasis were surgically removed. Lacking alternative treatment options, aiming at the induction of anti-tumor T cells responses, we initiated a personalized multi-peptide vaccination, based on in-depth analysis of tumor antigens (immunopeptidome) and sequencing.

Methods: Tumors were characterized by immunohistochemistry, next-generation sequencing and mass spectrometry of HLA ligands.
Results: Although several tumor-specific neo-epitopes were predicted *in silico*, none could be validated by mass spectrometry. Instead, a personalized multi-peptide vaccine containing non-mutated tumor-associated epitopes was designed and applied. Immunomonitoring showed vaccine-induced T cell responses to three out of seven peptides administered. The pulmonary metastasis resected after start of vaccination showed strong immune cell infiltration and perforin positivity, in contrast to the previous lesions. The patient remains clinically healthy, without any radiologically detectable tumors since March 2013 and the vaccination is continued.
Conclusions: This remarkable clinical course encourages formal clinical studies on adjuvant personalized peptide vaccination in cholangiocarcinoma.
Lay Summary: Metastatic cholangiocarcinomas, cancers that originate from the liver bile ducts, have very limited treatment options and a fatal prognosis. We describe a novel therapeutic approach in such a patient using a personalized multi-peptide vaccine. This vaccine, developed based on the characterization of the patient's tumor, evoked detectable anti-tumor immune responses, associating with long-term tumor-free survival.

Keywords: Primary liver cancer; Cholangiocarcinoma; Immunotherapy; Peptides; HLA; Immunopeptidome; Anti-tumor T cell response.
Received 19 February 2016; received in revised form 16 June 2016; accepted 29 June 2016
* Corresponding author. Address: University of Tübingen, Interfaculty Institute for Cell Biology, Department of Immunology, Auf der Morgenstelle 15, 72076 Tübingen, Germany. Tel.: +49 7071 29 80992; fax: +49 7071 29 5653.
E-mail address: markus.loeffler@med.uni-tuebingen.de (M.W. Löffler).
Abbreviations: CCA, cholangiocarcinoma; CT, computerized axial tomography; CCND, cyclin D; DMSO, dimethyl sulfoxide; ELISPOT, enzyme linked immune-spot assay; FFPE, formaldehyde fixed-paraffin embedded tissue; FPKM, fragments per kilobase mapped; ^{18}FDG, fluorodeoxyglucose (^{18}F); HLA, human leucocyte antigen; iCCA, intrahepatic cholangiocarcinoma; ICS, intracellular cytokine staining; IDH, isocitrate dehydrogenase; KMT2C, lysine N-methyltransferase 2C; LLoQ, lower limit of quantification; Mbp, megabase pairs; MMP, matrix metalloproteinase, PBRM, protein polybromo-1; PBMC, peripheral blood mononuclear cells, PET, positron emission tomography; RGS, regulator of G-protein signaling, SIMOA, single molecule array; SSP-PCR, single specific primer polymerase chain reaction; WES, whole exome sequencing; WTS, whole transcriptome sequencing.

Case Report

Introduction

Cholangiocarcinomas (CCA), heterogeneous malignant epithelial tumors originating from hepatic ductal cells, are typically diagnosed in a late stage and have a particularly poor prognosis [1]. CCA are rare in the Western world (0.5–1.5/100,000 persons), while they are the second most frequent primary liver cancer worldwide [2]. Risk factors include liver cirrhosis, viral hepatitis, hepatobiliary flukes and primary sclerosing cholangitis [1]. A recent systematic meta-analysis of 4,756 patients with intrahepatic cholangiocarcinoma (iCCA) reported 5-year survival rates between 5 and 56% and a median survival of 28 months [3]. Surgical resection of tumors is so far the only effective therapeutic strategy and neither adjuvant chemotherapy nor radiotherapy has shown any clear benefit [3]. Prognosis of lymph node metastasis in iCCA is usually fatal and the reported 5-year survival rates are 0–8% [4,5].

Novel treatment options using targeted agents off-label or *loco*-regional therapies have only shown limited success in CCA [6]. Intra and inter tumor genetic heterogeneity among iCCA, evidenced by whole exome sequencing (WES), calls for personalized therapies [7]. An encouraging new immunotherapy, using adoptive transfer of *ex vivo* expanded autologous $CD4^+$ T cells targeting a mutated antigen, yielded spectacular clinical results in a patient with non-resected metastatic CCA [8]. This success, previously unheard of in CCA, points towards the possible effectiveness of immunotherapies. Furthermore, multi-peptide vaccine-induced immune responses correlate positively with increased survival in cancer [9,10]. In line with such promising immunotherapeutic developments, we report our findings in a patient with metastatic iCCA, treated with a personalized multi-peptide vaccination. Since initial diagnosis and first tumor resection, the patient had tumor recurrences twice in the liver, as well as a lung metastasis, underlining the aggressiveness of the disease. The vaccine was designed based on WES, whole transcriptome sequencing (WTS) and HLA ligandome analysis of the tumors and induced long-term functional vaccine specific T cells. Remarkably, the tumor did not metastasize further and the patient is currently tumor-free, five years after initial diagnosis and 41 months after initiation of vaccination, suggesting therapeutic effectiveness.

Materials and methods

Next-generation-sequencing

As part of a research project (IndividuaLIVER, approved by the Institutional Review Board at the University Hospital of Tübingen), WES of tumor and normal tissue was performed for L06/10, L04/12 and P03/13 (Fig. 1). In addition, the transcriptome of tumor tissue of L06/12 and P03/13 was sequenced. Further details are provided in Supplementary Tables 6–8; and Supplementary Tables 11 and 14.

A total of four tumor samples (Formalin-Fixed, Paraffin Embedded tissue (FFPE) shavings; L06/10; L03/11; L04/12; P03/13) and one reference sample (blood) were sequenced with a custom cancer panel (SureSelect XT; Agilent, Waldbronn, Germany) covering 1.566 Mbp of coding sequence (Supplementary Table 3). On average a two-fold higher read count was generated for the tumor samples compared to the reference sample (39,900,242 reads *vs.* 17,748,620 reads). This yielded a mean coverage depth of 1,065x for the tumor samples with 98% of regions of interest covered with at least 100x and 90% of the regions covered with at least 500x (For further details see Supplementary Tables 4 and 5). The reference sample was sequenced to a mean coverage depth of 705x and a 98% 100x-coverage of the target region. Bioinformatic analysis for WES, WTS and panel sequencing followed established in-house pipelines. Further details including the bioinformatics analysis pipeline can be found in the Supplementary materials and methods.

HLA typing

Two-digit HLA class I and II typing was performed by SSP-PCR at the Department of Transfusion Medicine, University of Tübingen, following clinical routines. Typing at four-digit resolution using the WES data was performed by OptiType [11] for HLA class I and further manual curation for HLA class II alleles, determining the patient's HLA allelotype to be: HLA-A*03:01/A*29:01, B*07:05/B*35:01, C*04:01/C*15:05 and HLA-DRB1*01:01/DRB1*11:01/DRB3*02:02, DQB1*03:01/DQB1*05:01. The results of OptiType are in accordance with the PCR-based two-digit HLA typing.

Mass spectrometry

HLA ligands were immunoprecipitated from cryopreserved tumor tissues and liver tissue (L03/10) as previously described [12] using the pan-HLA class I antibody, W6/32 (manufactured in-house). HLA ligands were purified using 3 kDa cut-off centrifugal filters (Amicon, Maerck Millipore, Carrightwohill, Ireland), desalted (C18 ZipTip; Merck Millipore, Darmstadt, Germany), concentrated (vacuum centrifuge; Bachofer, München, Germany) and analyzed by LC-MS/MS as previously described [13]. Further details are provided in Supplementary materials and methods and Supplementary Table 17. An individualized protein database containing tumor-specific somatic mutations identified by WES/WTS was generated in FASTA format and used for spectral annotation using the Mascot search engine (v2.2.0.4, Matrix Science, Boston, MA).

T cell in vitro immunomonitoring

The patient received 32 vaccinations in total between September 2012 and February 2016 (ongoing). EDTA-anticoagulated blood (45 ml) and serum (5.5 ml) was drawn at two time points before vaccination and at regular intervals during the course of vaccination. T cell responses to all vaccinated peptides (Table 1) were monitored in peripheral blood mononuclear cells (PBMCs) isolated from blood drawn before vaccination (either during the pre-vaccination screening (scr) or at the first vaccination appointment (1V)) as well as during vaccinations 5V, 6V, 8V, 10V, 12V, 15V, 22V and 25V. PBMCs were pre-stimulated *in vitro* using the vaccinated peptides and relevant negative control peptides and expanded using IL-2 for 12 days (see details in Supplementary materials and methods) [14,15]. Responses to HLA class I peptides were monitored by IFN-γ ELISPOT and HLA-peptide multimer staining, while responses to HLA class II peptides and RGS-5 peptide were determined by IFN-γ ELISPOT and ICS (for IFN-γ, TNF-β, IL-2 and IL-10) (all techniques are elaborated in Supplementary materials and methods).

Histopathology

Tissue sections from paraffin embedded diagnostic tumor material obtained after surgical resections (L06/10; L03/11; L04/12; P03/13) were assessed by a consultant pathologist and FFPE shavings (>90% tumor fraction) used to isolate DNA for panel sequencing (above) and *IDH1* PCR. Further sections were used for additional immunohistochemical (IHC) analyses to complement CK7, Hep Par 1, Napsin A and TTF1 stainings, which had already been assessed during routine histopathology.

IHC staining was performed on an automated immunostainer, following the manufacturer's protocol (Benchmark, Ventana Medical Systems, Tucson, AZ). Respective commercially available antibodies used, sources and dilutions are summarized in Supplementary Table 1. Sections were assessed by a consultant pathologist as negative (−), positive with low (+), intermediate (++) and strong (+++) staining in immunohistochemistry or by evaluating positive cells in 10 high power fields counted and cell densities for the epithelial (E) and stromal (S) compartment respectively.

Results

Clinical course

A 56-year-old female patient, previously asymptomatic and healthy without any established risk factors for primary liver cancers, was sonographically diagnosed with a big unilocular mass in the right hepatic lobe (Ø 11.5 cm) in June 2010. Following atypical liver tri-segment resection (segments IVb, V, VI) and radical