Mismatch Repair Deficiency Drives Durable Complete Remission by Targeting Programmed Death Receptor 1 in a Metastatic Luminal Breast Cancer Patient

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Established Facts

- The blockade of programmed death receptor 1 (PD-1) or its ligand PD-L1 in advanced breast cancer failed in the majority of patients, with modest response rates reported in luminal breast cancer patients.
- As known from colorectal cancer, mismatch repair deficiency is linked to exceptionally high response rates to immune checkpoint blockade.

Novel Insights

- For the first time, we report on a durable complete remission in a woman with luminal breast cancer after treatment with pembrolizumab, associated with mismatch repair deficiency.
- As a consequence of microsatellite instability, we demonstrated high frequencies of somatic mutations by whole-exome sequencing and identified potential T-cell epitopes by prediction of neoantigens with high affinity binding to one of the patient's HLA.

Keywords

Programmed death receptor 1, PD-1 · Immune checkpoint blockade · Luminal breast cancer · Pembrolizumab · Mismatch repair deficiency · Mutational load · Microsatellite instability

Abstract

Background: In the field of breast cancer tumor biology, triple-negative breast cancer patients are the main focus of current clinical trials exploring the use of immune checkpoint inhibitors due to higher frequencies of somatic mutations, neoantigens, and resulting tumor-spe-

cific T-cell reactivity. **Case Report:** Here, we present the case of a 66-year-old woman with metastatic luminal breast cancer that rapidly responded to monotherapy with pembrolizumab, a monoclonal anti-PD-1 antibody. This patient obtained a partial clinical response within the first cycle of treatment and an ongoing durable complete remission after 12 weeks. Except for a transient immune-related thyroiditis, there were no side effects observed offering remarkable quality of life to the patient. To evaluate the underlying mechanisms, we performed immunohistochemistry, explored the mutational landscape by whole-exome sequencing, and identified

potential T-cell epitopes by prediction of neoantigens with high affinity binding to one of the patient's HLA. Briefly, we found a strong infiltration of CD8+ T cells without staining for PD-L1 in the tumor stroma. Exome sequencing revealed an enormous frequency of somatic and tumor-specific alterations, mainly C>T/G>A transitions. The mutational pattern was further linked to genome instability and deficient mismatch repair supported by the loss of MSH6 protein expression and therefore leading to susceptibility to immune checkpoint blockade. **Conclusion:** Within the overall goal to establish operating procedures for breast cancer immunotherapy, we propose to re-evaluate testing for deficient mismatch repair and to further intensify the search for biomarkers predictive for the success of immune checkpoint modulation including all tumor biologic subtypes of breast cancer.

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Introduction

Monoclonal antibodies targeting programmed death receptor 1 (PD-1) and programmed death-ligand 1 (PD-L1) have proven clinical activity in several cancers such as melanoma, lung cancer, and renal cell cancers, and many more trials are underway [1–3]. So far, blockade of PD-1 or PD-L1 in advanced breast cancer failed in the majority of patients, with objective response rates of 5–19% [4–6]; however, the responses achieved seem to be durable [6]. Best responses have been observed in patients with triple-negative breast cancer (TNBC), who are the main focus of current clinical trials [5]. For luminal type breast cancer, less data is available with limited overall response rates up to 10% [7, 8]. This may be explained based on findings that TNBC are generally more immunogenic and therefore more frequently infiltrated with lymphocytic infiltrates as a consequence of more frequent tumor-specific mutations, thus leading to a higher neoantigen load. Moreover, those tumor-infiltrating lymphocytes are linked to a favorable prognosis and predict for response to chemotherapy in early and advanced TNBC and HER2-positive breast cancers [9–11]. As known from numerous clinical trials, upregulation of PD-L1 is associated with higher benefit of therapy and already part of the clinical decision-making in melanoma, lung cancer, and renal cell cancer. In breast cancer patients, a heterogeneous proportion expresses PD-L1 on either tumor or immune cells [12]. However, the mechanistic basis remains inconclusive since many patients with strong staining for PD-L1 do not respond well, while PD-L1-negative patients frequently respond. This is supported by inconsistent data for the prognostic value of PD-L1 in breast cancer [13].

In other organ systems, it was shown that tumors with a higher mutational load as a consequence of DNA mismatch repair deficiency leading to microsatellite instability (MSI) turned out to be highly responsive to immune checkpoint blockade [14]. Unlike

Fig. 1. Immunohistochemistry before the start of anti-PD-1 treatment shows **A** invasive ductal carcinoma with intermediate differentiation (HE staining) and **B, C** infiltration of CD3+ and CD8+ lymphocytes as well as **D** negative staining for PD-L1.

with chromosome instability, MSI-high (MSI-H) tumors reflect a different pathway of carcinogenesis. The 4 genes coding for mismatch repair proteins responsible for DNA repair after recombination or exogenic damage are mutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*), mutL homologue 1 (*MLH1*), and postmeiotic segregation increased 2 (*PMS2*). A defect mismatch repair protein leads to the insertion or deletion of short tandem repeats termed microsatellites and facilitates high frequency somatic mutations. In the case of germline mutations of mismatch repair genes, the resulting hereditary syndrome is known as Lynch syndrome I (colon cancer) or Lynch syndrome II (endometrial, ovarian, and other gastrointestinal cancers), named after Henry Lynch [15, 16]. In their landmark phase II trial reporting patients with confirmed mismatch repair deficiency (predominantly colorectal cancer patients), Le et al. [17] found 40–78% response rates to immune checkpoint inhibitors depending on the tumor entity. However, data for breast cancer is heterogeneous and implicates a low incidence of mismatch repair deficiency or microsatellite instability [18–25]. Integrating whole-genome sequencing, recent findings report microsatellite instability of less than 2% [23]. Here, we present the first case of a woman with metastatic, hormone receptor-positive, HER2-negative breast cancer, who achieved a durable complete remission after treatment with pembrolizumab. To the best of our knowledge, this is also the first case of deficient mismatch repair-associated complete remission in breast cancer. Moreover, we discuss implications for future immunotherapy of breast cancer patients with a focus on genomic instability.

Fig. 2. Computed tomography scans **A** before and **B** after 6 months of anti-PD-1 treatment demonstrating complete remission of soft tissue metastatic lesions in the left thoracic wall according to immune-related (ir)RECIST criteria.

Fig. 3. Whole-exome sequencing of a pretherapeutic lesion shows **A** hypermutation, predominantly C>T/G>A transitions, with overall high numbers of single nucleotide variants ($n = 7,419$) and **B** distribution of mutational signatures in accordance with the catalogue of somatic mutations in cancer (COSMIC).

Case Report

A 66-year-old female patient was initially diagnosed in June 2009 with a pT2 pN1 invasive breast carcinoma of no special type (NST) on the left side. The tumor was found to be estrogen receptor(ER)- and progesterone receptor(PR)-positive (ER immunoreactive score 12/12, PR immunoreactive score 3/12). HER2 revealed equivocal expression (2+), but HER2 fluorescent in situ hybridization demonstrated no HER2 copy number gain. Proliferation indices were intermediate (grading 2, Ki-67 15%). Aiming for curative treatment and with test results being suggestive of an excellent prognosis, the patient underwent breast-conserving surgery and adjuvant chemotherapy with 3 cycles of 5-fluoropyrimidine, epirubicin, and cyclophosphamide (FEC) followed by 3 cycles of docetaxel. Thereafter, primary treatment was completed by radiotherapy of the left breast along with initiation of adjuvant endocrine treatment using an aromatase inhibitor (letrozol) and biannual bisphosphonate. After 3 years, ipsilateral axillary lymph node recurrence was diagnosed (ER immunoreactive score 8/12, PR immunoreactive score 5/12). After surgery, adjuvant chemotherapy was performed with capecitabine and vinorelbine followed by radiation of the left axilla. Only 1 month later, the tumor recurred locally in the same area. Due to rapid relapse after first-line endocrine therapy with fulvestrant as a result of

secondary endocrine resistance, the treatment was switched to the steroidal aromatase inhibitor exemestane in combination with the mTOR pathway inhibitor everolimus. Combinatorial therapy had to be terminated due to severe mucositis grade 3–4 according to common terminology criteria of adverse events (CTCAE). Proliferation indices, however, did not change during the course of disease and remained within an intermediate stage. In June 2014, the patient was enrolled in a phase I trial evaluating the safety and efficacy of a HER3-directed monoclonal antibody (RO 5479599) along with the application of pertuzumab and docetaxel. However, treatment had to be discontinued due to sustained diarrhea grade 3–4 according to CTCAE. Moreover, imaging studies confirmed progressive disease with involvement of the contralateral thoracic wall in October 2014. Repeated biopsies of the thoracic wall revealed metastasis of ductal invasive carcinoma of the breast. As shown in figure 1, we found a dense infiltration of CD-8-positive T cells without staining for PD-L1 in the metastatic tumor specimens. Further, a weak positive staining signal for PD-1 was assessed, geographically associated with infiltrating lymphocytes (data not shown). When comparing primary tumor with metastatic lesions, no change, neither in PD-1 nor in PD-L1, was observed (data not shown). Nevertheless, immune checkpoint blockade, targeting PD-1 with pembrolizumab 200 mg

Fig. 4. Immunohistochemical staining of pretherapeutic lesions for deficient mismatch repair proteins reveals positive nuclear staining signals for MLH1, MSH2, PMS2 and loss of MSH6, indicating deficient mismatch repair.

every 3 weeks was started in August 2015. Clinical observations following the first cycle already demonstrated decreasing cutaneous nodules on the left thoracic wall; after the second cycle, a complete clinical remission was obtained. Computed tomography scans, performed every 3 months beginning 2 months after start of pembrolizumab, confirmed an ongoing complete remission without evidence of disease (fig. 2). Except for a transient immune-related thyroiditis, no side effects were observed offering an excellent quality of life to the patient.

For a deeper understanding, we explored the mutational landscape of one of the metastatic lesions by whole-exome sequencing and found a hypermutated phenotype with an overall high number of 7,419 somatic nucleotide variants (SNV) distributed across all chromosomes (supplementary fig. 1) with a predominance for C>T/G>A transitions (fig. 3). Of note, a high number of short somatic indels was identified as well as an activating mutation in the *ESR1* gene (supplementary table 1). Concerning DNA mismatch repair-associated genes of interest, we identified a somatic frameshift insertion and somatic frameshift deletion in the coding region of *MSH2*. In contrast, the *POLD1* and *POLE* genes, which have recently been tied to increased mutational load, did not harbor mutations in this case [26]. Along that line, the hypermutated phenotype exhibited high numbers of somatic mutations, and the pattern corresponded to distinct signatures (fig. 3) associated with defective DNA mismatch repair (Signatures 6,15,20,21) as defined by the catalogue of somatic mutations in cancer (COSMIC) [27]. Next, mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2) were assessed by nuclear staining as described in the Methods section. This revealed loss of expression in MSH6 while there were regular nuclear staining signals in the other 3 mismatch repair proteins detected (fig. 4). As a consequence, amplification of 7 microsatellite markers, in accordance with the original Bethesda marker panel, confirmed MSI-H. Importantly, the germline determination of coding genes did not indicate mismatch repair deficiency as a result of Lynch syndrome.

Finally, exceptionally high mutational load was linked to corresponding neoantigens by allele-specific HLA-binding prediction to HLA-I as well as HLA-II. Highly suggestive for an antigen-driven T-cell response and indicated by mutational load as well as the presence of tumor-infiltrating lymphocytes, we identified more than 100 high-affinity binding epitopes (supplementary table 2).

Discussion

The historic view of breast cancer as an immunologically silent disease has changed. As demonstrated by the prognostic and predictive value of tumor-infiltrating lymphocytes, breast cancer can be detected by the adaptive immune system and arises within a complex interplay of cancer and host cells in the tumor microenvironment. Blocking of PD-1 by a monoclonal antibody (e.g., pembrolizumab, nivolumab) inhibits the negative regulation of T cells with impressive clinical success in lung cancer, melanoma, and some other solid tumors. However, neoantigens arising from tumor-specific mutations are a prerequisite for the induction of adaptive immunity leading to infiltration of CD8+ T cells and up-regulation of effector pathway activity.

Among the breast cancer subtypes, this is mainly attributed to TNBC with higher rates of somatic mutations and tumor-infiltrating lymphocytes [28]. However, all tumor biologic subgroups comprise tumors with high mutational burden [29]. This is demonstrated by the present case, who is, unexpectedly, a hormone receptor-positive luminal B breast cancer patient. However, whole-exome sequencing revealed high numbers of somatic mutations, especially if compared to other ER-positive breast cancers [29]. For the reasons described above and in contrast to TNBC, clinical trials exploring the clinical activity of checkpoint inhibitors in ER-positive breast cancer patients are rare. An expansion of the phase Ib study KEYNOTE 028 enrolled 25 heavily pretreated ER/PR+, HER2-, PD-L1+ patients and reported an overall response rate of 12%, including 3 partial responses. Similarly, our patient was heavily pretreated with several lines of chemotherapy and anti-hormonal treatments, exhibiting secondary endocrine resistance before pembrolizumab was started. Whether distinct therapies in the former history of the patient paved the way for the clinical success of immune checkpoint blockade remains elusive. However, higher response rates have been linked to patients with less previous lines of therapy [30]. Along that line, pembrolizumab as an adjunct to the neoadjuvant treatment of choice in a cohort of early breast cancer patients substantially increased the rate of pathologic complete responses [31]. Another study explored the combination of anti-PD-L1 treatment in combination with nab-paclitaxel in metastatic breast cancer demonstrating overall response rates of 41.7–77.8% in the PD-L1-positive subgroup. Based on these results, the combination of the anti-PD-L1 monoclonal antibody atezolizumab and nab-paclitaxel is under evaluation in frontline treatment, and results are eagerly awaited [30]. Surprisingly, in the tumor specimens of the patient presented here, no staining for PD-L1 was observed in serial immunohistochemical analysis during the course of metastatic disease. Therefore, upregulation of PD-L1 seems not to be the critical immune escape mechanism to overcome immune surveillance. It is important to note that the absence of PD-L1 does not preclude the possibility that immune checkpoint inhibition will lead to disease control as demonstrated in our report. Indeed, even in cancers suggesting a clear predictive value of PD-L1 (e.g., melanoma, lung cancer), clinical and pathologic standards are an ongoing matter of debate [32]. More importantly, not all metastatic lesions could be

monitored for PD-L1 expression in this case. This represents an open question since site-specific regulation of PD-L1 may have an important impact and remains to be further explored.

In addition to inherited autosomal dominant syndromes, deficiency of DNA mismatch repair proteins (MLH1, MSH2, MSH6, PMS2) as a result of sporadic mutations or promoter methylation (termed microsatellite instable, MSI-H) is the most common cause of increased tumor mutational burden. Here, somatic mismatch repair deficiency was diagnosed by immunohistochemical loss of MSH6 protein expression and corresponding amplification of 7 out of 7 microsatellite markers (MSI-H). In accordance with the literature, not implicating a breast cancer involvement in Lynch syndrome, no germline mutations were found in our case. However, the frequency and impact of other genetic alterations identified in this breast cancer case, especially somatic frameshift insertion and deletion in the coding region of the *MSH2* gene, warrant further research [33]. For the identification of predictive markers, comprehensive genomic and transcriptomic profiling may be of particular interest in large clinical trials investigating immune checkpoint blockade in breast cancer.

From a clinical perspective, Food and Drug Administration approval of pembrolizumab for MSI-H or dMMR cancers, irrespective of the tumor entity, was given in May 2017. 5 uncontrolled, multi-cohort, multi-center, single-arm clinical trials [17, 34] reported on 149 patients with MSI-H or dMMR cancers. In addition to 59 patients diagnosed with colorectal cancer, 14 other cancer entities were included. There were 11 complete responses and 48 partial responses observed with an overall response rate of 39.6%. Since only 2 breast cancer patients were part of these studies, our report is also the first case of dMMR breast cancer demonstrating a durable complete response to immune checkpoint blockade.

In order to improve patient outcomes in breast cancer, we propose to re-evaluate testing for dMMR and to further intensify the search for biomarkers predictive for the success of immune checkpoint modulation including all tumor biologic subtypes of breast cancer. With respect to the rapidly increasing number of immunologically active therapies (e.g., mutanome-based vaccines, CAR T cells, other checkpoint inhibitors, immunomodulatory drugs), this is in line with the overall goal to establish operating procedures for the stratification of breast cancer immunotherapy.

Methods

Serial tissue samples were provided by the NCT Heidelberg Tissue Bank in accordance with its regulations and after approval by the Ethics Committee of Heidelberg University.

Immunohistochemistry and Typing of Microsatellite Instability

As part of clinical routine, histopathological and tumor biologic parameters were assessed according to protocols of the Department of Pathology at Heidelberg University Hospital by determination of tumor size, nodal involvement, tumor grading, proliferation index (Ki-67), and expression levels of ER, PR, and HER2.

HER2 status was assessed in accordance with current guidelines [35]. ER and PR status was analyzed as percentage of positive nuclear staining. Further, tumor specimens were immunostained for CD3 (clone 2GV6), CD8 (clone C8/144B), CD20 (clone L26), PD-1 (clone NAT105), and PD-L1 (clone E1L3N) first. Second, expression of mismatch repair proteins were analyzed using antibodies for MLH1 (clone M1), MSH6 (clone 44), MSH2 (clone G219-1129), and PMS2 (clone EPR3947). Finally, microsatellite instability was determined using the marker panel BAT25, BAT26, D5S346, D2S123, D17S250, complemented by BAT40 and CAT25, as previously described [36, 37].

Whole-Exome Sequencing

Genomic DNA from formalin-fixed and paraffin-embedded tissues was extracted from cell pellets using the QIAGEN DNeasy purification kit (QIAGEN, Hilden, Germany). Libraries of genomic DNA were prepared using the Illumina Paired-End Sample Prep kit (Illumina, Inc., San Diego, SA, USA) according to the manufacturer's guidelines followed by quality control using TapeStation (Agilent, Santa Clara, CA, USA) and fluoroemetric quantification using Qubit (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation for low-input whole-exome sequencing was conducted according to the manufacturer instructions (Agilent Low Input Sure Select v5+UTR) using 200 ng input material. Next, whole-exome sequencing was conducted on the HiSeq 4000 device (Illumina) by pooling 2 samples per lane using 101 bp paired end reads.

Mapping and Analysis of Whole-Exome Sequencing Data

Exome sequencing reads were aligned to the 1000 Genomes Phase 2 assembly of the Genome Reference Consortium human genome (build 37, version hs37d5) using BWA-MEM [38] (v0.7.8). SNV and short indel calling was performed using in-house pipelines based on SAMtools mpileup (v0.1.19), BCFtools (v0.1.19), and Platypus (v0.8.1) as described previously [39] and updated more recently [40], with the exception of setting the mpileup threshold for minimum read mapping quality in the tumor to 30.

Supervised mutational signature analysis of high-confidence somatic single nucleotide variants (SNVs) was performed using non-negative matrix factorization formalism as described previously [41]. A predefined set of 30 canonical mutational signatures was used for decomposition of somatic SNVs (cancer.sanger.ac.uk/cosmic/signatures), which were further re-normalized using the observed trinucleotide frequencies in the human exome to the ones of the human genome.

Neoantigen Prediction

First, the HLA type of the patient was determined using the HLA typing tool Phlat to infer the 4-digit HLA genotype from the corresponding whole exome data. Second, NetMHCpan (version 2.8) and NetMHCIIpan (version 3.0) were used to predict allele-specific HLA-binding for both, HLA class I and HLA class II. To annotate

DNA mutations to protein sequences, the stand-alone software packages of the tools were integrated in a shell-based analysis pipeline and HLA-binding predictions performed for the mutated and corresponding wildtype protein stretches. Neoepitopes were predicted by defining all 9–11mers resulting from mutations and determining whether the predicted binding affinity to one of the patient's HLA alleles was <500 nM, dismissing those with a lower binding affinity. In order to remove neoantigens arising from genes without expression, predicted neoantigens were compared with TCGA RNAseq data for ductal invasive breast carcinoma.

Online Supplemental Material

Suppl. table 1. Identified somatic mutations by whole-exome sequencing

Suppl. table 2. Predicted neoantigens with high-affinity binding

Suppl. fig. 1. Intermutation distances of somatic mutations.

To access the supplemental material please refer to www.karger.com/?DOI=492580.

Patient Consent and Ethical Statement

The patient provided signed written consent for sample and gene analysis according to the German Gene Diagnosis Act.

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Author's Contributions

C. Fremd and A. Schneeweiss designed the treatment and research plan, collected and interpreted the data, and wrote the manuscript. M. Hlevnjak and M. Zapatka analyzed and interpreted exome sequencing data and reviewed the manuscript. H.P. Sinn and M. Kloor carried out immunohistochemistry and microsatellite typing. V. Thewes, P. Lichter, D. Jäger, I. Zörnig, N. Halama, Z. Kosaloglu, F. Schütz, F. Marmé, P. Schirmacher, and N. Fejzibegovic contributed to interpretation of data. All authors read and approved the final version.

Disclosure Statement

The authors declare no potential conflicts of interest.

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