Immunogenomic Landscape Contributes to Hyperprogressive Disease after Anti-PD-1 Immunotherapy for Cancer

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HIGHLIGHTS
- Mutations/expression changes occur in hyperprogressive tumors after anti-PD-1 therapy
- Immune cell population abundance pattern changed in the hyperprogressive tumors
- ILC3 cells may be enriched in the hyperprogressive tumors after anti-PD-1 therapy
- Post-therapy hyperprogressive tumors were less immunogenic than pre-therapy tumors

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Article

Immunogenomic Landscape Contributes to Hyperprogressive Disease after Anti-PD-1 Immunotherapy for Cancer

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SUMMARY

Although PD-1-blocking immunotherapies demonstrate significant therapeutic promise, a subset of the patients could develop hyperprogressive disease (HPD) with accelerated tumor growth after anti-PD-1 immunotherapy. To elucidate the underlying mechanisms, we compared the mutational and transcriptional landscapes between the pre- and post-therapy tumors of two patients developing HPD after anti-PD-1 immunotherapy. In post-therapy HPD tumors, somatic mutations were found in known cancer genes, including tumor suppressor genes such as TSC2 and VHL, along with transcriptional upregulation of oncogenic pathways, including IGF-1, ERK/MAPK, PI3K/AKT, and TGF-β. We found that post-therapy HPD tumors were less immunogenic than pre-therapy tumors, concurrent with an increased presence of ILC3 cells, a subset of innate lymphoid cells. We also developed a gene expression signature predictive of HPD. In summary, we identified the genomics and immune features associated with HPD, which may help identify patients at risk of adverse clinical outcome after anti-PD-1 immunotherapy.

INTRODUCTION

Immune checkpoint therapies including those targeting PD-1, or its primary ligand PD-L1, have demonstrated therapeutic responses across a broad range of cancer types (Sharma and Allison, 2015). Anti-PD-1 therapy blocks the interaction of PD-1, an inhibitory receptor on tumor-infiltrating T cells, with its ligands PD-L1 and PD-L2 that are predominantly expressed on tumor cells and antigen-presenting cells (APCs), respectively (Topalian et al., 2012). Despite the success of anti-PD-1 immunotherapy in approximately 20%–30% of patients with cancer, the majority of patients do not respond to this treatment (Sharma et al., 2017). In addition, increasing clinical evidence suggests that a significant subset of nonresponsive patients may experience acceleration of disease progression after treatment with anti-PD-1, a phenomenon known as hyperprogressive disease (HPD). Although accurate identification of the frequency of patients developing HPD has been limited by variability in diagnostic criteria, conservative estimates suggest that HPD may occur in as many as 10% of patients treated with anti-PD-1 (Champiat et al., 2017; Kato et al., 2017; Saada-Bouzid et al., 2017).

In contrast to identifying factors that predict responsiveness to PD-1-blocking therapies such as tumor expression of PD-L1, high tumor mutational burden, and the presence of tumor-infiltrating CD8+ T cells, little is known about the mechanisms underlying HPD. Although a pilot study suggested that some patients with MDM2 family amplification or EGFR aberrations developed HPD after treatment with PD-1 or PD-L1 inhibitors (Kato et al., 2017), it is likely that alterations beyond those identified in that study are important in facilitating accelerated disease progression.

To comprehensively examine the mechanisms of HPD, we performed whole-exome sequencing (WES) and RNA sequencing (RNA-seq) analyses of formalin-fixed paraffin-embedded (FFPE) samples of tumors before and after anti-PD-1 therapy in patients with clinical evidence of HPD. We identified individual somatic mutations and mutation clusters associated with clonal evolution that may contribute to the accelerated tumor growth observed in HPD. We also identified characteristic decreases in HPD tumor immunogenicity. Finally, we identified a gene signature that may be predictive of HPD development. These changes were HPD patient specific, and were not found in the tumors of anti-PD-1-treated patients without HPD phenotypes from previous studies. Overall, our study identified the genomics and immune features associated with HPD tumors after anti-PD-1 immunotherapy.
RESULTS

Mutation Patterns Are Altered in HPD Tumors after Anti-PD-1 Treatment

This study included two patients who received anti-PD-1 blockade immunotherapy. Relevant characteristics of the four FFPE tumor samples are summarized in Table 1. Paired tumor samples before and after anti-PD-1 treatment were obtained from a male patient with esophageal squamous cell carcinoma metastatic to lymph nodes (Patient 1) and from a female patient with clear cell renal cell cancer (ccRCC) that had metastasized to the bone (shoulder) and pleura (Patient 2). Following anti-PD-1 treatment that consisted of pembrolizumab (Merck), these two patients demonstrated HPD, as defined by the accelerated tumor growth rate and clinical deterioration using existing criteria (Kato et al., 2017). Each patient demonstrated progression at first radiologic evaluation (less than 2 months after anti-PD-1 therapy initiation). Before enrollment, written informed consent was obtained from all patients to use their tumor samples for research purposes. The study was approved by the Medical College of Wisconsin Institutional Review Board in accordance with federal regulations.

To understand the global changes that take place in HPD tumors after treatment with anti-PD-1, we performed mutational analysis on tumors obtained before and after treatment with pembrolizumab. We observed that Patient 1 had 195 somatic mutations before anti-PD-1 treatment and 338 somatic mutations after treatment (Table S1). There were 154 and 124 common somatic mutations shared by the HPD and pre-therapy tumors for Patients 1 and 2, respectively (Figure S1). Our results were in line with another group’s results showing increased tumor mutation load from baseline in PD (progressive disease) in patients with melanoma after anti-PD-1 therapy (nivolumab) initiation (Riaz et al., 2017). In the latter, the tumor mutation load was decreased in the responding patients (complete response/partial response) from baseline since nivolumab initiation, consistent with immunoediting (Riaz et al., 2017). We also analyzed the mutation profiles of these two patients in the context of known cancer genes based on a comprehensive list of cancer-related genes (downloaded from http://www.bushmanlab.org/links/genelists). There were 47 cancer genes mutated in at least one of the tumors from Patient 1 and 40 cancer genes mutated in at least one of the tumors from Patient 2 (Figure 1, Table S2). Four cancer genes (APH1A, ARHGEF12, GPER1, and KIF14) mutated in the pre-therapy tumor of Patient 1 were not mutated in the HPD tumors, suggesting that the tumor clones containing these four cancer genes were eliminated by anti-PD-1 treatment. However, the HPD tumor of Patient 1 had somatic mutations in 20 cancer genes, including IGFBP2, KMT2C, MAP3K4, MUC16, MUC2, NCOA2, and NOTCH4, which were not present in the pre-therapy tumors. Similar patterns were also observed for Patient 2. Four cancer genes (APC2, OBSCN, PHLP1, and SATB1) that were mutated in the pre-therapy tumor of Patient 2 were not mutated in the HPD tumors, whereas the HPD tumor of Patient 2 had somatic mutations in 21 cancer genes, including IGFBP2, MUC4, NCOA2, NFE2L2, TSC2, and VHL, which were not present in the pre-therapy tumors. The identified mutations in these genes were not present in the tumors of non-HPD patients after anti-PD-1 treatment when compared with previous studies (Biton et al., 2018; Gong et al., 2017; Hanna et al., 2018; Hugo et al., 2016; Miao et al., 2018; Riaz et al., 2017; Rizvi et al., 2015; Teo et al., 2018; Yoshikawa et al., 2017; Zaretsky et al., 2016). These data indicate that the mutational landscape of tumors was significantly altered after anti-PD-1 therapy in patients who demonstrated hyperprogression after anti-PD-1 treatment.

| Patient | Gender | Specimen | Cancer | Treatment | Other Clinical Phenotype | % Tumor |
|---------|--------|----------|--------|-----------|--------------------------|---------|
| #1      | Male   | S1624794 | Esophageal squamous cell carcinoma | Pre-anti-PD-1 | Metastatic to lymph node | 75      |
|         |        | S1707359 | Esophageal squamous cell carcinoma | Post-anti-PD-1 | Metastatic to lymph node | 75      |
| #2      | Female | M16248   | Clear cell renal cell carcinoma | Pre-anti-PD-1 | Metastatic to the pleura and shoulder | 50      |
|         |        | S1701860 | Clear cell renal cell carcinoma | Post-anti-PD-1 | Metastatic to the pleura and shoulder | 75      |

Table 1. Characteristics of the Four FFPE Specimens from Two Patients, Consisting of Paired Pre- and Post-anti-PD-1 (Pembrolizumab) Treatment Samples
For comparison in the context of corresponding cancer populations, we analyzed the numbers of somatic mutations of the esophageal carcinoma (ESCA, n = 184) and kidney renal clear cell carcinoma (KIRC, n = 384) samples from The Cancer Genome Atlas (TCGA). The numbers of nonsilent somatic mutations were in the range of 4–1,763 for ESCA and 15–1,349 for KIRC. The lower quartile, median, and upper quartile were 85, 110, and 168 for ESCA and 54, 77, and 109 for KIRC, respectively (Figure S2). The numbers of nonsilent somatic mutations in the before and after anti-PD-1 therapy tumors of the two HPD patients in this study were 195 and 338 for the patient with ESCA and 156 and 251 for the patient with KIRC. Therefore, they were all above the upper quartiles of TCGA ESCA and KIRC datasets, which suggested that these two patients have an exceptionally high number of somatic mutations compared with the TCGA esophageal cancer (ESCA) and ccRCC (KIRC).

**HPD Tumors Contain Deleterious Mutations and Significantly Activated Oncogenic Signaling Pathways**

To determine if certain genes were altered in both patients with HPD tumors, we searched for gene mutations that were common for the HPD tumors of both patients. Four genes were mutated in the
post-treatment tumors of both patients: NCOR2, GXYLT1, ZFPM1, and IGFBP2 (Figure 2A). There were 96
and 64 subject-specific nonsilent somatic mutations from 154 genes in post-treatment tumors of Patients 1
and 2, respectively (Figures 2B and 2C). The detailed information of these mutations are given in Table S3.

Bioinformatics analyses of these 161 mutations led to the identification of 11 potentially deleterious so-
matic variants in the HPD tumors, which were predicted to be “deleterious” by SIFT, “probably damaging”
by PolyPhen-2, and “potentially associated with cancer” by FATHMM (Table 2). The 11 genes having these
deleterious mutations were TRPC4, POTE, FBN2, KMT2C, FUT10, PQBP1, TSC2, MFSD6, CYP2D6, VHL,
and RAD54B. Of the 11 mutations, 10 were located at evolutionarily conserved sites, as predicted by
GERP++ (scores >2; Table 2). IPA (Ingenuity Pathway Analysis, Qiagen Inc., MD, USA), based on the 11
genes with the deleterious somatic mutations, identified a network involving these mutated genes that
contributes to suppression of the TP53 tumor suppressor and activation of MYC, CCND1, and VEGF onco-
genes (Figure S3). The mutated TSC2 gene carrying a missense mutation, p.Y1611S, was in the center of
this network and is linked to inhibition of the TP53 pathway and activation of the MYC, CCND1, and VEGF path-
ways (Figure S3). TSC2 (also known as TUBERIN) is a tumor suppressor that negatively regulates cellular
signaling networks that control cellular growth and proliferation (Dang et al., 2017). The MuPIT interactive
protein mutation analysis (Niknafs et al., 2013) showed that the pY1611S mutation is located in the Rap/ran-
GAP domain of the TSC2 protein, which is critical for the biological function of TSC2 (Figure S4). Previous
studies showed that TSC2 knockdown transforms mouse and human renal epithelial cells into neoplastic
stem cells that can serially propagate upon re-inoculation in mice (Dang et al., 2017). Together, it is reason-
able to hypothesize that the deleterious p.Y1611S mutation could result in the loss of function of the TSC2
protein, which in turn will lead to uncontrolled proliferation of cancer cells in the HPD tumors that survive
anti-PD-1 treatment.

Based on the differentially expressed genes, IPA identified four significantly activated oncogenic signaling
pathways in the HPD tumors after anti-PD-1 therapy compared with the pre-therapy tumors (p value <0.01,
Z score >2, Figure 3A). They were the insulin growth factor (IGF)-1, extracellular signal-regulated kinase
(ERK)/mitogen-activated protein kinase (MAPK), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/
AKT, and transforming growth factor (TGF)-β signaling pathways. A large number of genes in these onco-
genic pathways were upregulated in the HPD tumors (Figure 3B). Such concerted gene expression changes
can synergistically contribute to the generation of the HPD tumors after anti-PD-1 immunotherapy.

Clonal Evolution Was Detected in HPD Tumors after Anti-PD-1 Therapy

The generation of WES data allowed us to quantify the mutant allele frequencies in all cases. Based on mu-
tation clustering results, we inferred the identity of three clones having distinct sets of mutations (clusters)
in pre-therapy tumors when compared with post-therapy HPD tumors of the two patients. Multiple muta-
tion clusters (n = 3) were present in each of the pre-therapy tumors of the two HPD patients. In Patient 1, the
post-anti-PD-1 treatment HPD tumor was associated with the outgrowth of new clone(s) represented by
mutations in cancer-associated genes including KMT2C, NCOR2, COL28A1, ING3, CAMKK2, and
CARD8 (Figures 4A and 4C). The pre-therapy tumor clone(s) characterized by mutations in APH1A,
ARHGEF12, GPER1, and Kif14 genes was eliminated by anti-PD-1 treatment (Figures 4A and 4C). The
clon(es) represented by mutations in the cancer genes EP400, CUBN, SPP1, PHLPP2, PALB2, ERCC1,
TFRC, MARK4, and MDM4 remained stable under the selection pressure of anti-PD-1 treatment (Figures
4A and 4C). In Patient 2, the post-anti-PD-1 treatment HPD tumor was associated with the evolution of
new clone(s) represented by mutations in the cancer genes BAP1, CARD11, CBFAP2T3, CYP2D6, PRM1,
TSC2, and VHL (Figures 4B and 4D), whereas the pre-therapy tumor clone with mutations in
APC2, CDC27, OBSCN, PHLPP1, and SATB1 was not detectable after anti-PD-1 treatment (Figures 4B
and 4D). Other clones, including those represented by mutations in COL4A3, TTC40, NPHS1, UGT2A3,
RYR1, AGGF1, and LANCL1, remained stable before and after anti-PD-1 treatment (Figures 4B and 4D).

The tumor clonal evolution pattern associated with anti-PD-1 treatment was further validated by analyzing
an independent dataset from a previous study, which conducted WES of paired baseline and relapsed tu-
mors (before and after anti-PD-1 treatment) of four patients with melanoma (Zaretsky et al., 2016). As can be
seen from Figures S5 and S6, all four melanoma cases demonstrated allele clusters after anti-PD-1 therapy. Variant allele frequencies (VAFs) of the Cluster 1 mutations were not significantly changed by PD-1 blockade; Cluster 2 mutations had reduced VAFs but were still prevalent in the relapsing tumor after PD-1 blockade; Cluster 3 mutations represented the newly evolved tumor clone(s) in the relapsing tumor after PD-1 blockade; Cluster 4 mutated genes represented the tumor clone(s) that diminished to

| Gene | Genomic Position | Genomic Mutation | Exon | Protein Alteration | Predicted Effect of Somatic Mutation | SNP137 | ESP MAF |
|------|------------------|------------------|------|--------------------|-------------------------------------|--------|---------|
|      |                  |                  |      |                    | SIFTb | PolyPhen-2 | FATHMMd | GERP++e |
|      |                  |                  |      |                    |       |            |         |         |
| TRPC4 | chr13: 38211734 | c.G2045A         | 10   | p.R682H            | Deleterious (0.00) | Probably damaging (0.999) | Potentially associated with cancer (–2.83) | 6.06   | NA     | NA    |
| POTEE | chr2: 132021334 | c.A2306T         | 15   | p.Y769F            | Deleterious (0.00) | Probably damaging (0.997) | Potentially associated with cancer (–4.69) | NA     | NA     | NA    |
| FBN2  | chr5: 127666313 | c.C4297T         | 33   | p.R1433C           | Deleterious (0.00) | Probably damaging (0.983) | Potentially associated with cancer (–2.9) | 4.21   | NA     | 7.70 x 10^-3 |
| KMT2C | chr7: 151932981 | c.G2690C         | 16   | p.R897P            | Deleterious (0.00) | Probably damaging (0.995) | Potentially associated with cancer (–2.21) | 5.1    | NA     | NA    |
| FUT10 | chr8: 33246817  | c.G876T          | 4    | p.K292N            | Deleterious (0.00) | Probably damaging (1.00) | Potentially associated with cancer (–4.75) | 3.42   | NA     | NA    |
| PQBP1 | chrX: 48759773  | c.C256T          | 4    | p.P86S             | Deleterious (0.00) | Probably damaging (0.996) | Potentially associated with cancer (–1.13) | 5.02   | NA     | NA    |
| TSC2  | chr16: 2137907  | c.A4832C         | 37   | p.Y1611S           | Deleterious (0.02) | Probably damaging (0.997) | Potentially associated with cancer (–3.16) | 4.59   | NA     | NA    |
| MFSD6 | chr2: 191301728 | c.G973A          | 3    | p.G325R            | Deleterious (0.00) | Probably damaging (0.998) | Potentially associated with cancer (–2.42) | 6.07   | NA     | NA    |
| CYP2D6 | chr22: 42522990 | c.C102ST         | 7    | p.T342M            | Deleterious (0.00) | Probably damaging (0.996) | Potentially associated with cancer (–2.26) | 4.06   | NA     | NA    |
| VHL   | chr3: 10191479  | c.C349G          | 2    | p.L117V            | Deleterious (0.00) | Probably damaging (0.994) | Potentially associated with cancer (–6.95) | 3.07   | NA     | NA    |
| RAD54B | chr8: 95411747  | c.T721G          | 6    | p.F241V            | Deleterious (0.01) | Probably damaging (0.996) | Potentially associated with cancer (–3.01) | 5.55   | NA     | NA    |

Table 2. Characteristics of the 11 Deleterious Somatic Mutations in the HPD Tumors after Anti-PD-1 Treatment

ESP, NHLBI Exome Sequencing Project; NA, not available. See also Figure S3.

aGenomic positions are given according to the UCSC Genome Browser hg19 reference assembly.

bSIFT scores range from 0 to 1. The amino acid substitution is predicted to be damaging if the score is ≤0.05 and tolerated if the score is >0.05.

cPolyPhen-2 scores 0.85–1 are interpreted as probably damaging, scores 0.2–0.85 are possibly damaging, and scores 0–0.2 are benign.

dPredictions with FATHMM scores less than 0.75 indicate that the mutation is potentially associated with cancer; otherwise the mutation is not associated with cancer.

eThere is an indication of evolutionary conservation if a given site shows a GERP++ score >2.

fMAFs are according to the NHLBI GO Exome Sequencing Project (ESP65000S-V2 release) Exome Variant Server v.0.0.21 (August 2013).
Figure 3. Activation of Oncogenic Pathways in HP Tumors after Anti-PD-1 Therapy

(A and B) (A) Four oncogenic pathways were activated in the HP tumors. (B) The differentially expressed genes in these oncogenic pathways. Most of the genes were upregulated in the HP tumors after anti-PD-1 therapy. HP, hyperprogressive.
undetectable levels after PD-1 blockade. These data are consistent with our own analysis of tumors from HPD patients before and after anti-PD-1 therapy.

**HPD Tumors Demonstrate Decreased Immunogenicity Relative to Pre-therapy Tumors**

Since anti-PD-1 treatment renders its effects on tumors in a manner completely dependent on immunity, we investigated whether HPD tumors demonstrated changes in their capacity to elicit productive immune reactions using an *in silico* immunophenogram approach (Charoentong et al., 2017). The results showed that HPD tumors had much smaller immunophenoscores compared with the pre-therapy tumors for both patients (Figure 5). Expression of HLAs (human leukocyte antigens) was downregulated in the post-therapy HPD tumors compared with the pre-therapy tumors, whereas checkpoint genes were upregulated in the HPD tumors (Figure 5). These changes resulted in the overall reduction of immunophenoscores in HPD tumors. Consistent with results from the immunophenogram analysis, the differential expression analysis showed that seven genes involved in antigen processing were downregulated in the HPD tumors, i.e., B2M, HLA-B, HLA-DPA1, HLA-DPB1, HLA-DRA, HLA-E, and HLA-F (Figure 6A). In addition, eight genes
encoding immune checkpoints or modulators were upregulated in the HPD tumors, i.e., CTLA4, KDR, CD96, CD70, TNFRSF18, TNFRSF25, BTNL2, and TNFRSF8 (Figure 6A). Changes in expression of these immune-related genes were likely contributors to the weakened immunogenicity of the HPD tumors.

Immune Cell Signatures in HPD Tumors Are Predominately Immunosuppressive

Previous studies have characterized the signature genes of 28 immune cell populations critical to immune responses across multiple cancers (Angelova et al., 2015; Charoentong et al., 2017). Using GSVA (Gene Set Variation Analysis) (Hanzelmann et al., 2013), we evaluated the immune cell landscape in the HPD tumors from our two patients. We identified that the activities of eight immune cell populations were significantly decreased in the HPD tumors after anti-PD-1 treatment (Figure 6B). These populations were monocytes, central memory CD4 T cells, immature dendritic cells, CD56dim NK (natural killer) cells, NK cells, gamma-delta (γδ) T cells, activated dendritic cells, and follicular helper T cells, most of which are linked to functional tumor clearance. In addition, the activities of three immune cell populations, i.e., neutrophils, activated B cells, and neutrophil-like myeloid-derived suppressor cells (MDSC), were upregulated in the hyperprogressors (Figure 6B). These data suggest that the depletion of monocytes, certain types of T cells, NK cells, and dendritic cells may contribute to the ability of HPD tumors to escape immune surveillance. Furthermore, the upregulated neutrophil population as well as the neutrophil-like MDSC (i.e., the MDSC subpopulation with neutrophil signature gene expression) (Zhang et al., 2017) may also contribute to the immune evasion of HPD tumors since these cell populations have been implicated in generating a milieu that attenuates immune responses in the tumor microenvironment (Galdiero et al., 2013; Mishalian et al., 2013; Sagiv et al., 2015; Tuting and de Visser, 2016; Zhang et al., 2017).

ILC3 Innate Lymphocytes Are Upregulated in HPD Tumors

Recent studies have revealed the importance of innate lymphoid cells (ILCs) in homeostasis and inflammation of tumors (Bjorklund et al., 2016; Wallrapp et al., 2017). Although three main populations of ILCs, ILC1, ILC2, and ILC3, have been categorized based on their transcription factor profiles and secreted cytokines (Spits et al., 2013), little is known about their roles in carcinogenesis and immunotherapy resistance. To evaluate ILCs in HPD tumors, we analyzed the transcriptional levels of the marker genes characteristic of the ILC1, ILC2, and ILC3 populations (Bjorklund et al., 2016; Wallrapp et al., 2017). GSEA (Subramanian et al., 2005) showed that the ILC3 marker genes were significantly enriched among the top upregulated genes in the HPD tumors after anti-PD-1 treatment (Figures 7A and 7B). In contrast, the ILC1 and ILC2 marker genes were not enriched in either the up- or downregulated genes in the HPD tumors (Figure S7). These data suggest that the ILC3 population is activated in HPD tumors. To validate this finding, we analyzed the RNA-seq data from other studies that evaluated tumor changes in response to anti-PD-1 therapies. Analysis of the transcriptomes of responding (n = 15) and nonresponding (n = 13) pre-treatment melanoma tumors from the patients subject to PD-1 blockade (Hugo et al., 2016) showed that ILC3 marker genes were commonly upregulated in the melanoma tumors resistant to anti-PD-1 therapy (Figure 7C). Based on the RNA-seq data of the Kras<sup>G12D</sup> mouse model, we also found that there were a large number of ILC3 marker genes significantly upregulated in murine lung adenocarcinoma tumors that were resistant to anti-PD-1 therapy when compared with untreated tumors (Koyama et al., 2016) (Figure 7D). These results are concordant with our HPD RNA-seq data, suggesting that enrichment of the ILC3 population in the HPD tumors may be a characteristic feature of tumors that are insensitive to anti-PD-1. This finding is consistent with the previous report that ILC3 lymphocytes contribute to the initiation and progression of cancers (Fung et al., 2017). The mechanistic connection between ILC3 population and anti-PD-1 therapy effect is unknown. However, it was reported that ILC3 may promote the growth of mutant tumor cells that express the receptors needed for oncogenic pathways (Fung et al., 2017; Kirchberger et al., 2013). Our and others’ data (Riaz et al., 2017) suggested that anti-PD-1 therapy increased tumor mutation burden in patients with cancer with hyperprogressive or progressive tumor phenotype. Therefore, activated ILC3 cell population may be required for the promotion of the growth of more mutant cells in the patients with cancer with HPD or PD subjected to anti-PD-1 therapy.

Pro-inflammatory Pathways Were Activated in the Pre-therapy Tumors of Patients with HPD and Further Activated by Anti-PD-1 Therapy

PD-1 has been demonstrated to inhibit excessive inflammatory responses during infection in mouse models (Lazar-Molnar et al., 2010). To identify the inflammatory changes in HPD tumors, we evaluated changes in inflammatory-related genes included in the “hallmark inflammatory” gene set (Liberzon et al., 2011, 2015). To characterize the inflammation activity in post-anti-PD-1 treatment HPD tumors versus...
Patient 1

Pre-therapy tumors

Immunophenoscore: 4

Post-αPD-1 HPD tumors

Immunophenoscore: 0

Patient 2

Immunophenoscore: 6

Post-αPD-1 HPD tumors

Immunophenoscore: 3

MHC: Antigen Processing

CP: Checkpoints | Immunomodulators

EC: Effector Cells

SC: Suppressor Cells

Sample-wise (averaged) z-score

Weighted z-score
pre-treatment tumors, we again utilized GSVA, which identified four founder datasets of inflammation pathways that were significantly enhanced in the HPD tumors after anti-PD-1 treatment (Figure 8A). In each of these four pro-inflammatory datasets, many more genes were up- than downregulated (Figures 8B–8E), suggesting an overall pro-inflammatory trend after anti-PD-1 treatment.

For comparison, we analyzed the gene expression data of tumor samples from the GSE52562 dataset before anti-PD-1 treatment (Westin et al., 2014). This dataset included two potential HPD patients whose progression-free survival (PFS) was less than 2 months post-pidilizumab treatment (SAMPLE.25 and SAMPLE.5 in Table S4) and four responsive patients whose PFS was more than 2 years (24 months) after treatment (SAMPLE.23, SAMPLE.19, SAMPLE.13, and SAMPLE.17 in Table S4). This analysis showed that the tumors of HPD patients have elevated inflammation pathway activity (mainly chemokine activity) even before anti-PD-1 therapy when compared with tumors from non-HPD patients (Figure S8). These and our data collectively suggested that anti-PD-1 therapy further boosts the pre-existing high levels of inflammation in patients who subsequently develop HPD in ways that are not conducive to promoting tumor rejection.

HPD-Associated Gene Expression Signature

Based on the pre-therapy tumor expression data of Dataset_1 (See Transparent Methods), we developed a 121-gene set to differentiate HPD patients from non-HPD patients (Figure S9, Table S5). The effectiveness of this 121-gene classifier in the identification of HPD patients was tested using the pre-therapy tumor expression data from Dataset_2 (See Transparent Methods). This classifier had an area under curve (AUC) value of 0.91 (95% confidence interval [CI], 0.87–0.96), a sensitivity of 71% (95% CI, 51%–87%), and a specificity of 93% (95% CI, 80%–99%) in predicting HPD patients in Dataset_2 (Figure 9A). Kaplan-Meier analysis of TCGA data showed that the 121-gene expression signature can significantly separate low-risk group from high-risk group in the 13 major types of cancers including melanoma (SKCM), glioma, and carcinomas of the esophagus (ESCA), stomach (STAD), breast (BRCA), kidney (KIRC), bladder (BLCA), liver (LIHC), head and neck (HNSC), lung (LUAD and LUSC), colon (COAD), and pancreas (PAAD) (Figures 9B–9D and S10–S12). This panel was able to identify extremely high-risk groups in ESCA, COAD, and PAAD (Figures 9B–9D).

DISCUSSION

Checkpoint blockade with anti-PD-1 antibodies has resulted in excellent responses in a subset of patients with cancer. However, there is a sizable proportion of patients with cancer who do not respond to anti-PD-1 treatment, with a subset of these patients developing hyperprogression with accelerated tumor growth after anti-PD-1 immunotherapy (Champiat et al., 2017; Kato et al., 2017). Currently, there is a lack of systematic studies to identify the genes or immune factors that predict resistance to immune checkpoint inhibition or HPD in response to anti-PD-1 treatment. In this study, we utilized WES and RNA-seq approaches to identify the mutation spectrum and gene expression profiling changes in HPD tumors when compared with pre-therapy tumors. We also performed pathway and tumor immunogenicity analyses based on the RNA-seq data. Finally, we combined our data with publicly available datasets and developed an HPD gene expression signature capable of predicting patients unlikely to respond to anti-PD-1.

The mutation analysis highlighted 11 genes with deleterious mutations in the HPD tumors after anti-PD-1 therapy (Table 2). Most of these genes have not been adequately studied in the context of cancer before. However, a query of this 11 mutated gene set in the cBioPortal website (http://www.cbioportal.org/) (Cerami et al., 2012; Gao et al., 2013) showed that this gene set has somatic mutations or copy number aberrations (CNAs) in 8,887 (22%) of the 41,320 sequenced patients. The alterations of these 11 genes were most frequent in the six major cancer types with an alteration frequency >30% (Figure S13), i.e., prostate cancer (70.8% tumor samples had mutations or CNAs in at least one of the 11 genes), melanoma (50.2% altered), renal cell carcinoma (45.3% altered), brain cancer (33.3% altered), breast cancer (31.1% altered), and colorectal adenocarcinoma (31.0% altered). These data support the cancer linkage to these 11 genes, the mutations of which could contribute to the tumor hyperprogressive phenotype.
Among the 11 genes, some have tumor suppressive properties, good examples being TSC2 and VHL. Inactivating mutations in TSC2 that encode the protein tuberin lead to constitutive activation of mTOR kinase through the Rheb-GTP signaling axis (Menon et al., 2014; Zoncu et al., 2011), which in turn induces cell growth, motility, invasion, and development of tumors (Goncharova et al., 2004, 2006). These outcomes were consistent with our observation that the deleterious pY1611S mutation in the key Rap/ran-GAP domain of the TSC2 protein (Table 2, Figure S4) occurred in the hyperprogressive tumors after anti-PD-1 therapy. We also found that the VHL gene had a deleterious mutation—pL117V—in the ccRCC hyperprogressive tumors after anti-PD-1 treatment (Table 2). VHL, located on chromosome 3p25, is a major tumor suppressor gene involved in ccRCC oncogenesis (Gossage et al., 2015). Interestingly, a recent study found that PD-L1 expression was associated with dense PD-1 expression and wild-type VHL ccRCC, but not with mutated/inactivated VHL ccRCC (Kammerer-Jacquet et al., 2017). Therefore, only the patients with ccRCC with wild-type VHL may benefit from immunotherapies inhibiting PD-L1/PD-1 (Kammerer-Jacquet et al., 2017). In our case, we found that only the post-anti-PD-1 therapy hyperprogressive ccRCC tumor had detectable deleterious VHL mutation, but the pre-therapy ccRCC tumor did not. This suggested that the selection pressure of anti-PD-1 therapy eliminated most of the wild-type VHL ccRCC cells but had little effect on cells with mutated VHL ccRCC, such that these mutated cells were highly enriched in the post-therapy HPD tumors. This has significant implications in that it suggests that ccRCC cells with an altered/mutated VHL gene may be a key factor leading to HPD after anti-PD-1 therapy.

The pre- and post-treatment tumors in this study were acquired through biopsy from the primary lesion. After anti-PD-1 therapy, the initial minor subclones of somatic mutations could be boosted by the treatment and expanded in the tumor samples of the two HPD patients as shown in Figure 4, which contributed to the tumor heterogeneity that may account for changes in the mutational and/or expression landscape. Clonal evolution analysis (Figure 4) indicates that HPD tumor-specific mutations in TSC2 and VHL along with mutations in a number of other cancer genes including KMT2C, NCOR2, COL28A1, ING3, CAMKK2, CARD8, BAP1, CARD11, CBFA2T3, CYP2D6, and PBRM1 could be significant to the progression of nonaggressive pre-therapy tumors to the hyperprogressive state after anti-PD-1 treatment. Figure S3 showed that the mutated KMT2C, TSC2, VHL, and CYP2D6 genes were involved in the gene-gene interaction at the molecular level.

Figure 6. Changes in the Expression of Critical Immune-Related Genes and the Activity of Immune Cell Populations Contribute to Decreased Immunogenicity in the Post-a-PD-1 HPD Tumors

(A) Seven genes involved in antigen processing were downregulated, whereas eight genes encoding immune checkpoints or modulators were upregulated in hyperprogressor tumors.

(B) The activity of eight immune cell populations were weakened and three were strengthened, as detected by GSVA method.

See also Figure S14.
Figure 7. The ILC3 Population Was Activated in the HPD Tumors after Anti-PD-1 Therapy

(A–D) (A) GSEA showed that ILC3 marker genes were significantly enriched in the top upregulated genes in HPD tumors resistant to anti-PD-1 therapy. (B) Most of the differentially expressed ILC3 marker genes in the HPD tumors resistant to anti-PD-1 treatment were upregulated. (C) A higher percentage of ILC3 marker genes were upregulated in the nonresponding melanoma tumors resistant to anti-PD-1 therapy based on the analysis of data from an independent study in humans. (D) Upregulation of ILC3 marker genes comparing anti-PD-1-treatment-resistant mouse tumors with untreated tumors in the KrasG12D mouse model.
Figure 8. Activation of Inflammatory Pathways in the HPD Tumors after Anti-PD-1 Treatment

(A) GSVA identified the activation of four founder datasets of inflammation pathways.

(B) Differentially expressed genes in the inflammatory signature of RESPONSE_TO_CHEMICAL_STIMULUS;

(C) Differentially expressed genes in the inflammatory signature of KEGG_CHEMOKINE_SIGNALING_PATHWAY;

(D) Differentially expressed genes in the inflammatory signature of INFLAMMATORY_RESPONSE; (E) Differentially expressed genes in the inflammatory signature of CHEMOKINE_ACTIVITY. In each of the four pro-inflammatory datasets from (B–E), there were much more upregulated than downregulated genes. See also Figure S8.
network leading to suppression of the TP53 pathway activity. Previous studies showed that KMT2C (MLL3) co-activates TP53, whereas KMT2C levels decrease during cancer progression, which correlates with distinct clinical stages (Ford and Dingwall, 2015; Lee et al., 2009; Rabello et al., 2018). These results are consistent with our observations in HPD tumors after anti-PD-1 treatment.

Our RNA-seq data revealed that the IGF-1, ERK/MAPK, PI3K/AKT, and TGF-β signaling pathways were activated in the HPD tumors after anti-PD-1 therapy (Figure 3). Recent studies have found that TGF-β signaling may play an important role in resistance to immunotherapy. For example, Mariathasan et al. reported that lack of response to anti-PD-L1 antibody was associated with TGF-β signaling in fibroblasts and the exclusion of CD8+ T cells, indicating that TGF-β-mediated stromal remodeling restricts T cell infiltration to
suppress antitumor immunity and that TGF-β inhibition may enhance the efficacy of immune checkpoint blockade (Mariathasan et al., 2018). In parallel, Tauriello et al. found that single-agent PD-1/PD-L1 inhibition had little effect, but co-targeting TGF-β produced a robust antitumor immune response that could prevent the development of metastasis and eliminate established metastases in a mouse model (Tauriello et al., 2018). Collectively, these studies indicate that inhibiting TGF-β could significantly improve the efficacy of anti-PD-1/anti-PD-L1 treatment (Mariathasan et al., 2018; Tauriello et al., 2018). Herein, our data suggest that enhanced TGF-β signaling could also contribute to the development of HPD after anti-PD-1 therapy. Therefore, inhibiting TGF-β signaling may also help prevent the development of HPD in response to anti-PD-1 treatment. Another interesting finding is the activation of PI3K/AKT in HPD tumors. A recent study demonstrated that the activity of PI3K/AKT signaling was crucial for lymphomas with PD-1 deletion (Wartewig et al., 2017). Therefore, when the tumors are exposed to anti-PD-1 therapy, elevated PI3K/AKT signaling may be another important mechanism for the survival, progression, or even hyperprogression of the tumor cells.

The HPD tumors had reduced tumor immunogenicity when compared with the pre-therapy tumors. Such reduction may be caused by downregulation of antigen-processing genes, including several HLA genes and B2M, and upregulation of certain immune checkpoint or modulator genes other than PD-1/PD-L1 (Figures S5 and 6). In the context of studying 28 immune cell populations critical to pan-cancer immunogenomics (Angelova et al., 2015; Charoentong et al., 2017), we found that the activity of eight immune cell populations were weakened and two were strengthened in the HPD tumors. The weakened immune cell populations including monocytes, CD4 helper T cells, dendritic cells, and NK cells may contribute to the ability of HPD tumors to escape immune surveillance. The enhanced cell populations such as neutrophils are known to have a number of pro-tumor properties (Galdivier et al., 2013; Mishalian et al., 2013; Sagiv et al., 2015; Tuting and de Visser, 2016), thus the increase in neutrophil activity in HPD tumors was not surprising.

The two patients developed HPD after anti-PD-1 therapy, indicating the adverse immunity changes that may result in an immunosuppressive environment. The decreased portion of immune cell phenotypes after anti-PD-1 therapy led us to speculate whether anti-PD-1 therapy contributed to accelerated AICD (activation-induced cell death) in these two patients. To test this hypothesis, we applied the GSVA approach to the apoptosis gene sets collected in the MSigDB database (Libervon et al., 2015). It can be seen that five apoptosis gene sets were activated in the two patients after anti-PD-1 therapy (Figure S14A), of which 27 apoptotic genes including marker genes in caspase/bcl2 pathways (CASP3, CASP7, BNIP2, and BNIP3L) were significantly upregulated (Figure S14B). This indicated that the accelerated AICD may occur in the anti-tumor activating lymphocytes, which accounted for the decreased portion of immune cell phenotypes and enhanced immunosuppressive environment after anti-PD-1 therapy.

So far, cancer immunotherapies have largely focused on T lymphocytes. However, ILCs could also play important roles in the immune response. ILCs were classified into cytotoxic ILCs, such as NK cells, and helper-like ILCs, such as the ILC1, ILC2, and ILC3 subsets. Much of the role of ILCs other than NK cells in cancer and immunotherapy remain elusive. ILCs might represent promising targets in the context of cancer therapy because they are endowed with potent immunomodulatory properties. In the present study, we analyzed the dynamic changes in the activity of ILC populations associated with anti-PD-1 therapy. This represents the first study analyzing the ILC populations in hyperprogressive tumors after anti-PD-1 therapy. Although ILC1 and ILC2 subsets did not show significant changes according to GSEA (Figure S7), the ILC3 population was activated in HPD tumors compared with pre-therapy tumors (Figure 7). Among the three subsets of ILCs, the role of ILC3 is gaining increased interest for its potential tumor-promoting activities. ILC3 that produces interleukin (IL)-22 has also been shown to promote tumor growth mediated via STAT3 activation (Kirchberger et al., 2013). Another study showed that ILC3 promoted lymphatic metastasis by modulating the local chemokine milieu of cancer cells (Irshad et al., 2017). ILC3 may also promote tumor formation and progression by suppressing T cell responses (van Beek et al., 2016). It had been shown that intestinal ILC3 cells limit T cell responses and induce T cell death via outcompeting T cells for IL-2 (Hepworth et al., 2015). We observed upregulated expression of ILC3 marker genes by anti-PD-1 immunotherapy in the two HPD patients, which may contribute to the suppression of T cell responses or the induction of T cell death. Our findings were in line with those of previous studies, indicating that inhibiting ILC3 may complement anti-PD-1 treatment to reduce the likelihood of developing hyperprogressive tumors after the therapy.
It is worth mentioning that IL-22 expression was not detected in the before and after anti-PD-1 treatment FFPE samples of the two patients, which may be due to the influence of the degradation of the RNA samples from the FFPE specimens on gene expression study. However, previous studies have defined a large group of marker genes whose expressions were characteristic of the ILC3 cell population (Bjorklund et al., 2016; Wallrapp et al., 2017). For example, the ILC3 cells were defined by using a repertoire of around 400 genes (Bjorklund et al., 2016; Wallrapp et al., 2017), which became the basis of our analyses on ILC3 cells. Therefore, we analyzed the expression pattern changes of these marker genes to study the dynamic changes of ILC cell populations in response to the anti-PD-1 immunotherapy in the tumors of the HPD patients (Figures 7 and S7).

Previous research showed that PD-1-deficient mice were extraordinarily sensitive to tuberculosis and had much shorter survival times compared with wild-type mice (Lazar-Molnar et al., 2010). This sensitivity results from the need for the PD-1 pathway to control excessive inflammatory responses to tuberculosis infection in the lungs of mice (Lazar-Molnar et al., 2010). This led us to hypothesize that the PD-1 pathway may also be required to control excessive inflammatory responses in patients susceptible to HPD. If anti-PD-1 therapy is administered to HPD patients, it may contribute to tumor growth by further upregulating inflammatory pathway activities. The analyses of our data and those of others (Westin et al., 2014) confirmed this hypothesis by showing that anti-PD-1 therapy can further boost the pre-existing high levels of inflammation in HPD patients, and thus contribute to the hyperprogressive phenotype (Figures 8 and S8).

On the basis of genome-wide expression data of tumors from our study, and two publicly available datasets (before anti-PD-1 therapy) (Riaz et al., 2017; Westin et al., 2014), we identified and validated a 121-gene expression signature that can distinguish HPD patients from non-HPD patients. This may have significant clinical predictive value to identify patients who are suitable for anti-PD-1/anti-PD-L1 immunotherapy. Having validated this gene set, we examined whether there exists any mechanism that might explain its association with HPD. Interestingly, most of these genes (70 of 121) belonged to gene sets that we identified as significant to different aspects of the HPD tumors in our samples. Specifically, these genes could be classified into the following six categories that were described above as important contributors to the HPD phenotype (Figure S9): (1) somatic mutated gene sets; (2) oncogenic pathways of IGF-1, ERK/MAPK, PI3K/AKT, and TGF-β; (3) immune checkpoint genes; (4) ILC3 population marker genes; (5) marker genes for other immune populations like monocytes, CD4 T cells, and dendritic cells; and (6) differentially expressed genes in post-anti-PD-1 HPD tumors versus pre-anti-PD-1 non-HPD tumors. Thus, a significant portion of these HPD signature genes could be involved in the critical biological processes important to tumor evolution, infiltrated immune cells, and tumor-microenvironment interactions. However, although we validated the 121-gene set, more patient cohorts subjected to anti-PD-1 therapy that contain HPD patients and non-HPD patients are needed for prospective validation.

To better define HPD, especially to differentiate HPD from intermediate and/or late tumor progression, we compared the mutational and gene expression of the two original samples in our study with the pre-treatment tumor samples of the four patients (#28, #9, #26, #38) who developed intermediate and/or late tumor progression (Table S6). Mutation analysis showed that 40 cancer genes had nonsilent somatic mutations in the original tumors of the HPD patients but no mutations in the tumors of the patients whose tumor progression was intermediate and/or late (Figure S15). These genes include, for example, *MUC13, MUC6, APC2, ARID2, CDK4, EP400, MARK4, MDM4, MUC2, NOTCH1, and SLIT2*. Previous research demonstrated that *MDM4* alteration was significantly associated with hyperprogression in patients subjected to immunotherapy (Kato et al., 2017), which was consistent with our results. We tabulated the information of these 40 HPD-associated cancer genes in Table S7. At the transcriptome level, GSEA identified four gene sets from the MsigDB database that were significantly altered in the tumors of HPD patients compared with the patients with intermediate and/or late tumor progression. These gene sets were: *HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY, HALLMARK_DNA_REPAIR, HALLMARK_ADIPONECINOSIS*, and *SINGH_KRAS_DEPENDENCY_SIGNATURE*. The first three pathways, i.e., the reactive oxygen species pathway, the DNA repair pathway, and the adipogenesis pathway, were significantly inhibited, whereas the KRAS signaling pathway was significantly activated in the tumors of HPD patients relative to the patients with intermediate and/or late tumor progression (Figure S16A). The corresponding gene expression changes of the above significantly altered pathways were also shown (Figure S16B). Together, these mutational and transcriptional changes of the tumors between the HPD and the intermediate/late tumor progression patients may contribute to the better characterization of the HPD condition.
Overall, our comprehensive analysis of HPD tumors after anti-PD-1 therapy and pre-therapy tumors identified the genomics and immune factors contributing to the hyperprogression phenotypes, such as deleterious somatic mutations in important tumor suppressors such as TSC2 and VHL, downregulated antigen-processing genes, and upregulated immune checkpoints or modulators other than PD-1/PD-L1. We also identified immune cell populations with significant activity changes in the HPD tumors; particularly the ILC subset, ILC3, was found to be activated in the HPD tumors after anti-PD-1 treatment. A gene expression signature for HPD tumors was also identified and validated using our samples and publicly available datasets. Our findings may contribute to understanding the mechanisms of the development of HPD after anti-PD-1 treatment, which is important to identify patients at high risk of developing HPD.

Limitations of Study
In this study, we analyzed the genomics, transcriptomics, and immunogenicity of two patients subjected to anti-PD-1 immunotherapy who developed hyperprogression after the treatment. We acknowledged that the patient sample size was small in this study. This is because the majority of the patients either did not develop hyperprogression or had the pseudo-hyperprogressive phenotype after checkpoint immunotherapy. Further larger patient samples involving more HPD patients treated with anti-PD-1 are needed to validate and extend our findings. Another limitation is that we only profiled HPD tumor sample one time upon hyperprogression after anti-PD-1 immunotherapy and did not collect post-hyperprogression tumor samples at later time points. This design rendered us unable to investigate whether the associated immunosuppressive profiles of HPD tumors remain as such even at later time points. However, our study is innovative in terms of analyzing both the before- and after-immunotherapy DNA/RNA samples of the HPD patients and serves as the starting point for similar studies that are lacking in the field.

Currently, the two outside datasets we used in the manuscript were the only ones that have the transcriptome-level gene expression data available publicly for us to develop a gene expression profile for HPD (Riaz et al., 2017; Westin et al., 2014). Based on the available data, we characterized a 121-gene expression profile to differentiate HPD patients from non-HPD patients in both the datasets (Riaz et al., 2017; Westin et al., 2014) with high AUC values and high sensitivity and specificity as described in the manuscript. More HPD patients with well-profiled transcriptome data and detailed clinical information related to the anti-PD-1 treatment are needed to verify our gene expression signature.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND SOFTWARE AVAILABILITY
The WES and RNA-seq raw sequence reads data from the before and after anti-PD-1 immunotherapy FFPE samples from the two cancer patients (4 FFPE samples) have been deposited in the Sequence Read Archive under accession number of PRJNA503522.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Transparent Methods, 16 figures, and 7 tables and can be found with this article online at https://doi.org/10.1016/j.isci.2018.10.021.

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AUTHOR CONTRIBUTIONS
M.Y. and Y.W. conceived the project and revised the manuscript. D.X. performed the experiment and all the data analyses and writing of the paper. A.K.S and A.C.M collected the samples for this study. B.G. recruited the patients and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
Hyperprogressive disease is a new pattern of tumor growth that is associated with rapid tumor progression and poor prognosis. The cBio cancer genomics portal is an open platform for exploring multidimensional cancer genomics. Single-cell RNA sequencing has emerged as a powerful tool for studying the heterogeneity of human cells. The expanding role of innate lymphoid cells and their T-cell counterparts in gastrointestinal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy.

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Supplemental Information

Immunogenomic Landscape Contributes to Hyperprogressive Disease after Anti-PD-1 Immunotherapy for Cancer

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Figure S1. The number of somatic mutations in the pre- and post- anti-PD-1 treatment tumor samples of the two patients. Related to Figure 1 and Figure 2. A) Patient 1; B) Patient 2.
Figure S2. The distribution of nonsilent somatic mutations in the two TCGA cancer types analyzed in the hyperprogressive tumor context in the present study. Related to Figure 1 and Figure 2. The numbers of nonsilent somatic mutations of the esophageal carcinoma (ESCA, n=184) and kidney renal clear cell carcinoma (KIRC, n=384) samples from TCGA.
Figure S3. Key mutated cancer genes interacting network. Related to Table 2. Based on the eleven genes with the deleterious somatic mutations, a mechanistic network was built by IPA in which ten genes carrying these mutations resulted in the suppression of TP53 tumor suppressor pathway and activation of MYC, CCND1 and VEGF oncogenic pathways.
Figure S4. Key mutation in the TSC2 protein. Related to Table 2. The 3D structure of the TSC2 protein and the location of the amino acid residue harboring the p.Y1611S mutation, which is within the Rap/ran-GAP domain of the TSC2 protein critical to its biological function.
Figure S5. Clonal evolution from the pre-anti-PD1 therapy baseline tumor to post-anti-PD-1 relapsing tumor in the four melanoma patients from a previous study. Related to Figure 4. The graphical representation of clonal evolution in the four melanoma patients: (A) Case #1; (B) Case #2; (C) Case #3; (D) Case #4.
Figure S6. The mutation clusters representing clonal evolution from the pre-anti-PD1 therapy baseline tumor to post-anti-PD-1 relapsing tumor in the four melanoma patients from a previous study. Related to Figure 4. The mutation clusters detected in the pre-anti-PD1 therapy baseline tumor to post-anti-PD-1 relapsing tumor in the patients: (A) Case #1; (B) Case #2; (C) Case #3; (D) Case #4. The relationship between the clusters in the pre-therapy and post-therapy tumors are indicated by lines linking them.
Figure S7. The ILC1 and ILC2 populations activity do not have significant changes in the HPD tumors after anti-PD-1 therapy. Related to Figure 7. (A) The ILC1 and (B) the ILC2 marker genes were not enriched in either the top up- or down-regulated genes in the HPD tumors.
Figure S8. Pre-α-PD-1 therapy tumors of hyperprogressive patients have elevated inflammation pathway activity (mainly chemokine activity) compared to the responsive patients. Related to Figure 8. (A) GSVA identified the activation of two founder data sets of inflammation pathways in the pre-therapy tumors of HPD patients compared to the non-HPD patients; (B) The chemokine encoding genes that were up-regulated in the pre-therapy tumors of HPD patients compared to the non-HPD patients.
Performance of the 121-gene set in the discovery dataset (Dataset_1)

I) Clonal mutated genes:
\textit{AFF1, HIVEP1, NOTCH3, SATB1, TSC2, HSPG2, ARID2, SPP1, NFE2L2, CARO6, CYP2D6, YHL, OBSCN}

II) Oncogenic pathways of IGF-1, ERK/MAPK, PI3K/AKT, TGF-\beta:
\textit{EP300, SMURF1, TLN1, YWHAQ, LAMTOR3, YWHAE, PPM1L}

III) Immune checkpoint:
\textit{TNFRSF25, KDR, CD96, CTLA4}

IV) ILC3 population marker genes:
\textit{CLSTN3, SLC27A1, TSPAN3, TCF4, CORO1C, CD96, SORT1, TRIO, UBTF, GOLIM4, TLN1, CD63, FUCA2, ZFP36L1, SSBP2, OBFC1, GPR18, YWHAQ, ARMC9}

V) Marker genes for other immune populations like monocytes, CD4 T cells and dendritic cells:
\textit{HIVEP2, NOTCH3, CD63, CARD8, GPR18, ATP5L, CCNA1, ANXA5, COL4A1, ARL1}

VI) Differential expressed genes in post-anti-PD-1 HPD tumors vs pre-anti-PD-1 non-HPD tumors:
\textit{DGKD, FAM104B, TRIP12, EP300, CLSTN3, SLC27A1, GBF1, KLFDC8B, CPT1A, SMURF1, CAMSAP1, CSNK1G1, SLC25A34, GALNT10, TNKS2, PTPN3, ADAR}

Figure S9. Results of the 121-gene expression signature in the discovery data set (Dataset_1). Related to Figure 9. ROC curves was shown for separating HPD patients from non-HPD patients in the discovery data set (4 HPD vs 16 non-HPD patients, AUC=1). The majority of these genes (70 of 121) belonged to the gene sets that we identified as significant to different aspects of the HPD tumors in our samples. Specifically, these genes were classified into the following six categories.
Figure S10. Kaplan–Meier analysis showed that the 121-gene set classifier can separate significantly low- and high-risk groups in the 13 major TCGA cancers. Related to Figure 9. The Kaplan–Meier curves of the TCGA cancer types of (A) LUSC, (B) STAD, (C) glioma, (D) BRCA were shown in this figure.
Figure S11. Kaplan–Meier analysis showed that the 121-gene set classifier can separate significantly low- and high-risk groups in the 13 major TCGA cancers. Related to Figure 9. The Kaplan–Meier curves of the TCGA cancer types of (A) KIRC, (B) BLCA, (C) LIHC, (D) LUAD were shown in this figure.
Figure S12. Kaplan–Meier analysis showed that the 121-gene set classifier can separate significantly low- and high-risk groups in the 13 major TCGA cancers. Related to Figure 9. The Kaplan–Meier curves of the TCGA cancer types of (A) HNSC, (B) SKCM were shown in this figure.
Figure S13. The mutation analysis highlighted eleven genes with deleterious mutations in the HPD tumors after anti-PD-1 therapy. Related to Table 2. Most of these genes have not been adequately studied in the cancer context before. Querying the HPD tumors associated 11-mutated-gene set in the cBioPortal website (http://www.cbioportal.org/) showed that this gene set had somatic mutations or copy number aberrations (CNAs) in 8887 (22%) of 41320 sequenced patients. The frequencies of tumor samples having somatic alterations in at least one of the eleven genes among each type of cancers archived in cBioPortal were shown in the figure.
Figure S14. Changes of the apoptosis pathway activity in the after anti-PD-1 immunotherapy tumors of the HPD patients. Related to Figure 5 and Figure 6. (A) Five apoptosis gene sets were activated in the two patients after anti-PD-1 immunotherapy; (B) 27 apoptotic genes of these five apoptosis gene sets including marker genes in caspase/bcl2 pathways (CASP3, CASP7, BNIP2, BNIP3L) were significantly up-regulated.
Mutation analysis showed that 40 cancer genes had somatic mutations in the original tumors of the HPD patients but no mutations in the tumors of the four patients whose tumor progression was intermediate and/or late.

| 2 HPD Pts | 4 Pts with intermediate and/or late tumor progression |
|-----------|-----------------------------------------------------|
|           |                                                     |

40 cancer genes with somatic mutations in the original tumors of the HPD patients but no mutations in the tumors of the four patients whose tumor progression was intermediate and/or late.

Figure S15. Comparison of the somatic mutation profiles of pretreatment tumor samples between HPD patients and a subset of non-HPD patients. Related to Figure 9. Mutation analysis showed that 40 cancer genes had somatic mutations in the original tumors of the HPD patients but no mutations in the tumors of the patients whose tumor progression was intermediate and/or late.
Figure S16. GSVA analysis of the transcriptional profiles of pretreatment tumor samples between HPD patients and a subset of non-HPD patients. Related to Figure 9. (A) Four gene sets were significantly altered in the tumors of HPD patients compared to the patients with intermediate and/or late tumor progression; (B) The corresponding gene expression changes of the above significantly altered pathways were also shown.
Table S1, Table S2, Table S3 were the supplemental Excel files.

Table S4. The clinical information of the eighteen follicular lymphoma patients from the GSE52562 study, among whom two patients had PFS less than two months together with advanced tumor progression phenotypes after anti-PD-1 treatment. Related to Figure 9.

| ExpId     | SampleID | gender | age | pfs.censorship | pfs.time.month | treatment       | tissue        | HPDstatus |
|-----------|-----------|--------|-----|----------------|----------------|-----------------|---------------|-----------|
| GSM1269893| SAMPLE.25  | F      | 67  | 1              | 1.8            | pre-pidilizumab | tumor biopsy  | HPD       |
| GSM1269873| SAMPLE.5   | F      | 79  | 1              | 2.0            | pre-pidilizumab | tumor biopsy  | HPD       |
| GSM1269883| SAMPLE.15  | M      | 46  | 1              | 3.7            | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269886| SAMPLE.18  | M      | 69  | 0              | 4.1            | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269877| SAMPLE.9   | F      | 58  | 1              | 6.5            | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269888| SAMPLE.20  | F      | 56  | 0              | 7.1            | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269875| SAMPLE.7   | M      | 60  | 1              | 10.1           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269889| SAMPLE.21  | F      | 62  | 1              | 12.7           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269871| SAMPLE.3   | M      | 51  | 1              | 13.5           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269894| SAMPLE.26  | M      | 58  | 1              | 15.3           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269890| SAMPLE.22  | M      | 70  | 1              | 18.6           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269892| SAMPLE.24  | M      | 63  | 0              | 18.8           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269879| SAMPLE.11  | M      | 67  | 1              | 19.6           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269869| SAMPLE.1   | F      | 61  | 1              | 21.6           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269891| SAMPLE.23  | F      | 37  | 0              | 26.5           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269887| SAMPLE.19  | F      | 41  | 0              | 30.4           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269881| SAMPLE.13  | M      | 58  | 0              | 30.8           | pre-pidilizumab | tumor biopsy  | nonHPD    |
| GSM1269885| SAMPLE.17  | F      | 45  | 0              | 35.0           | pre-pidilizumab | tumor biopsy  | nonHPD    |

Table S5. The information of the 121 genes in the expression signature of pre-anti-PD-1 treatment tumors that may be predictive of HPD (hyperprogressive disease) patients after anti-PD-1 immunotherapy. Related to Figure 9.

| Gene Symbol | Entrez Gene Name                          | Location                  | Type(s)             |
|-------------|-------------------------------------------|---------------------------|---------------------|
| AAK1        | AP2 associated kinase 1                   | Cytoplasm                 | kinase              |
| ACOT1       | acyl-CoA thioesterase 1                   | Cytoplasm                 | enzyme              |
| ACOT2       | acyl-CoA thioesterase 2                   | Cytoplasm                 | enzyme              |
| ADAR        | adenosine deaminase, RNA specific         | Nucleus                   | enzyme              |
| AFF1        | AF4/FMR2 family member 1                 | Nucleus                   | transcription regulator |
| ANKS6       | ankyrin repeat and sterile alpha motif domain containing 6 | Cytoplasm | other |
| ANXA5       | annexin A5                               | Plasma Membrane           | transporter          |
| ARID2       | AT-rich interaction domain 2             | Nucleus                   | transcription regulator |
| Gene Symbol | Description | Cellular Location | Function |
|-------------|-------------|------------------|----------|
| ARL1        | ADP ribosylation factor like GTPase 1 | Cytoplasm enzyme |          |
| ARMC9       | armadillo repeat containing 9 | Cytoplasm other |          |
| ATF7IP      | activating transcription factor 7 interacting protein | Nucleus transcription regulator |          |
| ATP11C      | ATPase phospholipid transporting 11C | Plasma transporter |          |
| ATP5L       | ATP synthase membrane subunit g | Cytoplasm enzyme |          |
| BAZ1B       | bromodomain adjacent to zinc finger domain 1B | Nucleus transcription regulator |          |
| BAZ2A       | bromodomain adjacent to zinc finger domain 2A | Nucleus transcription regulator |          |
| C17orf97    | chromosome 17 open reading frame 97 | Other other |          |
| CAMSAP1     | calmodulin regulated spectrin associated protein 1 | Cytoplasm other |          |
| CARD8       | caspase recruitment domain family member 8 | Nucleus other |          |
| CCNA1       | cyclin A1 | Nucleus other |          |
| CCNT1       | cyclin T1 | Nucleus transcription regulator |          |
| CD63        | CD63 molecule | Plasma other |          |
| CD96        | CD96 molecule | Plasma other |          |
| CHD4        | chromdomain helicase DNA binding protein 4 | Nucleus enzyme |          |
| CLSTN3      | calsyntenin 3 | Plasma other |          |
| COL4A1      | collagen type IV alpha 1 chain | Extracellular other |          |
| COL4A2      | collagen type IV alpha 2 chain | Extracellular other |          |
| COMMDO9     | COMM domain containing 9 | Cytoplasm other |          |
| CORO1C      | coronin 1C | Cytoplasm other |          |
| CPT1A       | carnitine palmitoyltransferase 1A | Cytoplasm enzyme |          |
| CREB2ZF     | CREB/ATF bZIP transcription factor | Nucleus transcription regulator |          |
| CSNK1G1     | casein kinase 1 gamma 1 | Cytoplasm kinase |          |
| CTLA4       | cytotoxic T-lymphocyte associated protein 4 | Plasma transmembrane receptor |          |
| CYP2D6      | cytochrome P450 family 2 subfamily D member 6 | Cytoplasm enzyme |          |
| DGKD        | diacylglycerol kinase delta | Cytoplasm kinase |          |
| DIAPH1      | diaphanous related formin 1 | Plasma other |          |
| EID2        | EP300 interacting inhibitor of differentiation 2 | Nucleus other |          |
| ELK4        | ELK4, ETS transcription factor | Nucleus transcription regulator |          |
| EP300       | E1A binding protein p300 | Nucleus transcription regulator |          |
| ERN1        | endoplasmic reticulum to nucleus signaling 1 | Cytoplasm kinase |          |
| FAHD1       | fumarate acetoacetate hydrolase domain containing 1 | Cytoplasm enzyme |          |
| FAM104B     | family with sequence similarity 104 member B | Other other |          |
| FBXL17      | F-box and leucine rich repeat protein 17 | Other other |          |
| Gene     | Description                                                                 | Location       | Function                  |
|----------|------------------------------------------------------------------------------|----------------|--------------------------|
| FPGT     | fucose-1-phosphate guanylyltransferase                                        | Cytoplasm      | enzyme                   |
| FUBP3    | far upstream element binding protein 3                                       | Nucleus        | transcription regulator   |
| FUCA2    | alpha-L-fucosidase 2                                                         | Extracellular  | enzyme                   |
| GALNT10  | polypeptide N-acetylgalactosaminyltransferase 10                             | Cytoplasm      | enzyme                   |
| GALNT2   | polypeptide N-acetylgalactosaminyltransferase 2                             | Cytoplasm      | enzyme                   |
| GAPVD1   | GTPase activating protein and VPS9 domains 1                                 | Cytoplasm      | other                    |
| GATAD2B  | GATA zinc finger domain containing 2B                                        | Nucleus        | transcription regulator   |
| GBF1     | golgi brefeldin A resistant guanine nucleotide exchange factor 1             | Cytoplasm      | other                    |
| GOLM4    | golgi integral membrane protein 4                                            | Cytoplasm      | other                    |
| GPR18    | G protein-coupled receptor 18                                                | Plasma Membrane| G-protein coupled receptor |
| HADH     | hydroxyacyl-CoA dehydrogenase                                                 | Cytoplasm      | enzyme                   |
| HHLA3    | HERV-H LTR-associating 3                                                      | Other          | other                    |
| HIVEP1   | human immunodeficiency virus type I enhancer binding protein 1              | Nucleus        | transcription regulator   |
| HIVEP2   | human immunodeficiency virus type I enhancer binding protein 2              | Nucleus        | transcription regulator   |
| HMBS     | hydroxymethylbilane synthase                                                  | Cytoplasm      | enzyme                   |
| HPGDS    | hematopoietic prostaglandin D synthase                                       | Cytoplasm      | enzyme                   |
| HSPG2    | heparan sulfate proteoglycan 2                                               | Extracellular Space | enzyme          |
| KDM6B    | lysine demethylase 6B                                                         | Extracellular Space | enzyme           |
| KDR      | kinase insert domain receptor                                                 | Plasma Membrane| kinase                 |
| KLHDC8B  | kelch domain containing 8B                                                    | Cytoplasm      | other                    |
| LAMTOR3  | late endosomal/lysosomal adaptor, MAPK and MTOR activator 3                 | Cytoplasm      | other                    |
| LGALS12  | galectin 12                                                                  | Extracellular Space | other                  |
| LNPEP    | leucyl and cystinyl aminopeptidase                                            | Cytoplasm      | peptidase                |
| LRP6     | LDL receptor related protein 6                                                | Plasma Membrane| transmembrane receptor  |
| MAGEH1   | MAGE family member H1                                                         | Cytoplasm      | other                    |
| MEF2D    | myocyte enhancer factor 2D                                                    | Nucleus        | transcription regulator   |
| MTIF3    | mitochondrial translational initiation factor 3                               | Cytoplasm      | translation regulator     |
| NFE2L2   | nuclear factor, erythroid 2 like 2                                            | Nucleus        | transcription regulator   |
| NOTCH3   | notch 3                                                                      | Plasma Membrane| transcription regulator   |
| NPLOC4   | NPL4 homolog, ubiquitin recognition factor                                   | Nucleus        | other                    |
| NSD1     | nuclear receptor binding SET domain protein 1                                | Nucleus        | transcription regulator   |
| NUP188   | nucleoporin 188                                                               | Nucleus        | other                    |
| OBSCN    | obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF               | Cytoplasm      | kinase                   |
| OTUD7B   | OTU deubiquitinase 7B                                                         | Cytoplasm      | peptidase                |
PAK2 p21 (RAC1) activated kinase 2
PCDHGB7 protocadherin gamma subfamily B, 7
PHF8 PHD finger protein 8
PPM1L protein phosphatase, Mg2+/Mn2+ dependent 1L
PPP2R3C protein phosphatase 2 regulatory subunit B'gamma
PTPN3 protein tyrosine phosphatase, non-receptor type 3
PTS 6-pyruvoyltetrahydropterin synthase
RANGAP1 Ran GTPase activating protein 1
SATB1 SATB homeobox 1
SERPINF1 serpin family F member 1
SETX senataxin
SLC25A34 solute carrier family 25 member 34
SLC27A1 solute carrier family 27 member 1
SLC38A6 solute carrier family 38 member 6
SLC6A6 solute carrier family 6 member 6
SMURF1 SMAD specific E3 ubiquitin protein ligase 1
SNAPC4 small nuclear RNA activating complex polypeptide 4
SORT1 sortilin 1
SPEN spen family transcriptional repressor
SPIN2A spindlin family member 2A
SPP1 secreted phosphoprotein 1
SSBP2 single stranded DNA binding protein 2
OBFC1 STN1, CST complex subunit
SYTL4 synaptotagmin like 4
TCF4 transcription factor 4
TEX261 testis expressed 261
TGOLN2 trans-golgi network protein 2
TIMM8B translocase of inner mitochondrial membrane 8 homolog B
TLN1 talin 1
TMEM99 transmembrane protein 99
TNFRSF25 TNF receptor superfamily member 25
TNKS2 tankyrase 2
TRIO trio Rho guanine nucleotide exchange factor
TRIP12 thyroid hormone receptor interactor 12
TSC2 TSC complex subunit 2
TSPAN3 tetraspanin 3
| Gene      | Description                                                                 | Location       | Role                |
|-----------|-----------------------------------------------------------------------------|----------------|---------------------|
| UBTF      | upstream binding transcription factor, RNA polymerase I                      | Nucleus        | transcription regulator |
| KIAA2018  | upstream transcription factor family member 3                               | Other          | other               |
| VHL       | von Hippel-Lindau tumor suppressor                                           | Nucleus        | transcription regulator |
| WDR44     | WD repeat domain 44                                                         | Cytoplasm      | other               |
| YWHAE     | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon | Cytoplasm      | other               |
| YWHAQ     | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta  | Cytoplasm      | other               |
| ZFP36L1   | ZFP36 ring finger protein like 1                                             | Nucleus        | transcription regulator |
| ZNF609    | zinc finger protein 609                                                      | Nucleus        | other               |
| ZNF878    | zinc finger protein 878                                                      | Other          | other               |
Table S6. The clinical information of the 51 melanoma patients subjected to nivolumab immunotherapy from the CA209-038 study, among whom 21 patients had PFS less than two months together with post-therapy tumor progression phenotypes. Related to Figure 9.

| PatientID | Sample | SampleType | PFS Censorship | Clinical Phenotype | PFS (days) | HPD status |
|-----------|--------|------------|----------------|-------------------|------------|------------|
| Pt103     | Pt103_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt106     | Pt106_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 56 | HPD |
| Pt11      | Pt11_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 59 | HPD |
| Pt17      | Pt17_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 48 | HPD |
| Pt1       | Pt1_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 54 | HPD |
| Pt24      | Pt24_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt27      | Pt27_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt29      | Pt29_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt31      | Pt31_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt39      | Pt39_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 57 | HPD |
| Pt46      | Pt46_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 51 | HPD |
| Pt47      | Pt47_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 57 | HPD |
| Pt52      | Pt52_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 57 | HPD |
| Pt5       | Pt5_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 56 | HPD |
| Pt62      | Pt62_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 56 | HPD |
| Pt66      | Pt66_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 59 | HPD |
| Pt78      | Pt78_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt84      | Pt84_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 50 | HPD |
| Pt85      | Pt85_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 49 | HPD |
| Pt8       | Pt8_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 52 | HPD |
| Pt90      | Pt90_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 44 | HPD |
| Pt101     | Pt101_Pre | Pre-anti-PD-1 tumor | 0 | PARTIAL RESPONSE | 612 | nonHPD |
| Pt10      | Pt10_Pre | Pre-anti-PD-1 tumor | 0 | STABLE DISEASE | 119 | nonHPD |
| Pt18      | Pt18_Pre | Pre-anti-PD-1 tumor | 0 | NA | 519 | nonHPD |
| Pt23      | Pt23_Pre | Pre-anti-PD-1 tumor | 0 | DEATH PRIOR TO DISEASE ASSESSMENT | 52 | nonHPD |
| Pt26      | Pt26_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 294 | nonHPD |
| Pt28      | Pt28_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 61 | nonHPD |
| Pt2       | Pt2_Pre | Pre-anti-PD-1 tumor | 1 | STABLE DISEASE | 115 | nonHPD |
| Pt30      | Pt30_Pre | Pre-anti-PD-1 tumor | 0 | PARTIAL RESPONSE | 603 | nonHPD |
| Pt34      | Pt34_Pre | Pre-anti-PD-1 tumor | 1 | NA | 834 | nonHPD |
| Pt36      | Pt36_Pre | Pre-anti-PD-1 tumor | 1 | NA | 737 | nonHPD |
| Pt37      | Pt37_Pre | Pre-anti-PD-1 tumor | 0 | STABLE DISEASE | 176 | nonHPD |
| Pt38      | Pt38_Pre | Pre-anti-PD-1 tumor | 0 | PROGRESSION | 167 | nonHPD |
| Pt3       | Pt3_Pre | Pre-anti-PD-1 tumor | 0 | PARTIAL RESPONSE | 583 | nonHPD |
| Pt44      | Pt44_Pre | Pre-anti-PD-1 tumor | 0 | PARTIAL RESPONSE | 560 | nonHPD |
| Pt48      | Pt48_Pre | Pre-anti-PD-1 tumor | 1 | NA | 1046 | nonHPD |
| Pt49      | Pt49_Pre | Pre-anti-PD-1 tumor | 1 | PARTIAL RESPONSE | 827 | nonHPD |
| Pt4       | Pt4_Pre | Pre-anti-PD-1 tumor | 0 | STABLE DISEASE | 175 | nonHPD |
| Pt59      | Pt59_Pre | Pre-anti-PD-1 tumor | 0 | STABLE DISEASE | 111 | nonHPD |
| Pt65      | Pt65_Pre | Pre-anti-PD-1 tumor | 1 | STABLE DISEASE | 280 | nonHPD |
| Pt67      | Pt67_Pre | Pre-anti-PD-1 tumor | 0 | STABLE DISEASE | 281 | nonHPD |
| Pt72      | Pt72_Pre | Pre-anti-PD-1 tumor | 0 | PARTIAL RESPONSE | 333 | nonHPD |
| Pt76      | Pt76_Pre | Pre-anti-PD-1 tumor | 0 | NA | 10 | nonHPD |
Table S7. The information of the 40 HPD associated cancer genes having nonsilent somatic mutations in the original tumors of the HPD patients but no mutations in the tumors of the patients whose tumor progression was intermediate and/or late. Related to Figure 9.

| Symbol | Entrez Gene Name | Location | Type(s)     |
|--------|------------------|----------|-------------|
| ABCB5  | ATP binding cassette subfamily B member 5 | Plasma Membrane | transporter |
| AFF1   | AF4/FMR2 family member 1 | Nucleus | transcription regulator |
| APC2   | APC2, WNT signaling pathway regulator | Cytoplasm | enzyme |
| APH1A  | aph-1 homolog A, gamma-secretase subunit | Cytoplasm | peptidase |
| ARHGIF12 | Rho guanine nucleotide exchange factor 12 | Cytoplasm | other |
| ARID2  | AT-rich interaction domain 2 | Nucleus | transcription regulator |
| BRIP1  | BRCA1 interacting protein C-terminal helicase 1 | Nucleus | enzyme |
| CDK4   | cyclin dependent kinase 4 | Nucleus | kinase |
| CLTC1L | clathrin heavy chain like 1 | Plasma Membrane | other |
| COL4A3 | collagen type IV alpha 3 chain | Extracellular Space | other |
| CUBN   | cubulin | Nucleus | enzyme |
| ELOVL5 | ELOVL fatty acid elongase 5 | Cytoplasm | enzyme |
| EP400  | E1A binding protein p400 | Nucleus | other |
| ERCC1  | ERCC excision repair 1, endonuclease non-catalytic subunit | Nucleus | enzyme |
| FRYL   | FRY like transcription coactivator | Other | other |
| GPER1  | G protein-coupled estrogen receptor 1 | Plasma Membrane | G-protein coupled receptor |
| HIVEP1 | human immunodeficiency virus type 1 enhancer binding protein 1 | Nucleus | transcription regulator |
| HSPG2  | heparan sulfate proteoglycan 2 | Extracellular Space | enzyme |
| KIF14  | kinesin family member 14 | Cytoplasm | enzyme |
| LIFR   | LIF receptor alpha | Plasma Membrane | transmembrane receptor |
| MARK4  | microtubule affinity regulating kinase 4 | Cytoplasm | kinase |
| MDM4   | MDM4, p53 regulator | Nucleus | enzyme |
| MUC13  | mucin 13, cell surface associated | Extracellular Space | other |
| MUC2   | mucin 2, oligomeric mucus/gel-forming | Extracellular Space | other |
| MUC6   | mucin 6, oligomeric mucus/gel-forming | Extracellular Space | other |
| NCTCH1 | notch 1 | Plasma Membrane | transcription regulator |
| PALB2  | partner and localizer of BRCA2 | Nucleus | other |
| PCM1   | pericentriolar material 1 | Cytoplasm | other |
| PDE11A | phosphodiesterase 11A | Cytoplasm | enzyme |
| PHLP1  | PH domain and leucine rich repeat protein phosphatase 1 | Cytoplasm | enzyme |
| PHLP2  | PH domain and leucine rich repeat protein phosphatase 2 | Cytoplasm | enzyme |
| PPM1E  | protein phosphatase, Mg2+/Mn2+ dependent | Nucleus | phosphatase |
| PRKCI  | protein kinase C iota | Cytoplasm | kinase |
| RANBP17 | RAN binding protein 17 | Nucleus | transporter |
| SATB1  | SATB homeobox 1 | Nucleus | transcription regulator |
| SLIT2  | slit guidance ligand 2 | Extracellular Space | other |
| SPTA1  | spectrin alpha, erythrocytic 1 | Cytoplasm | other |
| SSX1   | SSX family member 1 | Nucleus | transcription regulator |
| TFRC   | transferrin receptor | Plasma Membrane | transporter |
| VAV3   | vav guanine nucleotide exchange factor 3 | Extracellular Space | cytokine |
Transparent Methods:

Whole-exome sequencing (WES) and RNA-seq experimentation and data analyses

For each set of paired tumor samples, a section of formalin-fixed tissue was examined with hematoxylin and eosin (H&E) staining to confirm the presence of tumor and determine the relative tumor burden. At least five 10-mm FFPE slides were used for each tumor specimen, from which DNA and RNA were purified by a commercial vendor (Omega Bio-tek, Inc., Norcross, GA 30071) and subjected to WES and RNA-seq after library purification. The Illumina Nextera Rapid Capture Exome kit was used for the preparation of exome libraries, which were sequenced to the average depth of 150 X coverage in the paired end 150 bp (PE150) mode with a HiSeq 4000 system. The Illumina TruSeq RNA Access kit was used for the preparation of total RNA libraries that were sequenced to the average depth of 75 million reads in the paired end 100 bp (PE100) mode using the HiSeq 2500 system.

The WES short reads were aligned to a reference genome (NCBI human genome assembly hg19) using the BWA (Burrows-Wheeler Aligner) program (Li and Durbin, 2009). Each alignment was assigned a mapping quality score by BWA (Li and Durbin, 2009), which generated a Phred-scaled probability that the alignment is correct. Reads with low mapping quality scores (< 5) were removed to reduce the false positive rate. The PCR duplicates were detected and removed using Picard software. Local realignment of the BWA-aligned reads was performed using the Genome Analysis Toolkit (GATK) (McKenna et al., 2010). VarScan 2 (Koboldt et al., 2012) was used to identify somatic variants based on the local realignment results comparing each tumor with the two reference blood samples. Default parameters in VarScan 2 were used. The lists of shared SNVs/indels were then annotated using ANNOVAR (Wang et al., 2010). Single nucleotide polymorphisms (SNPs) were filtered against dbSNP version 142 (dbSNP 142). Plots of mutations were generated using the “oncoPrint” function provided by the R package – ComplexHeatmap (Gu et al., 2016). To identify somatic mutations with the most significant functional consequences, we predicted the impact of the mutations on
HPD tumors using the bioinformatics programs SIFT, PolyPhen-2, and FATHMM according to our previous approaches (Xiong et al., 2015). Network analysis of the eleven genes having deleterious mutations in HPD tumors was performed and graphically depicted using Ingenuity Pathway Analysis software (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Mapping of the p.Y1611S mutation to the 3D structure of the TSC2 protein was performed using MuPIT software (Niknafs et al., 2013). The bioinformatics tools SciClone (Miller et al., 2014) and Clonevol (Dang et al., 2017) were used to identify the clonal structures of the paired tumors of the two HPD patients. Plots of the clonal mutation clusters were generated using the fishplot software feature (Miller et al., 2016).

RNA-seq sample quality was analyzed using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequence data reads in fasta format were first processed through Perl scripts (Haas et al., 2013). Data were then refined by removing reads containing adapter, poly-N, or low-quality reads (Pei et al., 2016; Wang et al., 2015). All downstream analyses were based on refined data. The “rsem prepare reference” script of the RSEM package was used to generate reference transcript sequences by using the gene annotation file (GTF) format and the full genome sequence (FASTA) format of human GRCh37 assembly. All of the quality reads of different samples were mapped to generated reference transcript sequences using the Bowtie-2 program (Langmead et al., 2009) to determine the identity between cDNA sequences and corresponding genomic exons in regions of exact matches. The “rsem calculate expression” script of RSEM was used to analyze both the alignment of reads against reference transcript sequences and the calculation of relative abundances. Normalized gene expression values in TPM (Transcripts Per Kilobase Million) were used as input of the AltAnalyze software (Olsson et al., 2016) for differential gene expression analysis. FDR (False discovery rate) corrected $P$-values of less than 0.05 were used as criteria for significantly regulated genes.
To perform oncogenic pathway or network analysis, the list of differentially expressed genes between paired pre- and post-anti-PD-1 therapy tumors of the two patients was analyzed through the use of IPA. The GSVA (Gene Set Variation Analysis) (Hanzelmann et al., 2013) and GSEA (Gene Set Enrichment Analysis) (Subramanian et al., 2005) approaches were used to analyze the activity and enrichment of immune cell populations, respectively. GSEA analysis was performed for pre-ranked differentially expressed genes using the option ‘GseaPreranked’. One thousand permutations were used to calculate significance. A gene set was considered to be significantly enriched in one of the two groups when the $P$ value was lower than 0.05 and the FDR was lower than 0.25 for the corresponding gene set. For inflammatory pathway analysis, we performed a focused gene expression study by analyzing the changes of the inflammatory related genes included in the Hallmark gene set for inflammatory response named “HALLMARK_INFLAMMATORY_RESPONSE” downloaded from the MSigDB database (Liberzon et al., 2015; Liberzon et al., 2011). The GSVA approach (Hanzelmann et al., 2013) was used to characterize the activity of inflammation pathways in the post-anti-PD-1 treatment HPD tumors vs pre-treatment tumors. All heatmaps of gene expression were generated using the R package – heatmap3 (https://cran.r-project.org/web/packages/heatmap3/).

**Tumor immunogenicity analysis**

Immunogenicity of the pre-anti-PD-1 treatment tumors and post-treatment HPD tumors was analyzed using published criteria (Charoentong et al., 2017; Hakimi et al., 2016). The immunophenoscore (IPS) was calculated on an arbitrary 0–10 scale based on the sum of the weighted averaged Z score of the four categories shown in Figure 5 in accordance to the previous methods (Charoentong et al., 2017; Tappeiner et al., 2017). Briefly, the four categories include 20 single factors such as the presence of specific immune cell types along with the abundance of MHC molecules, or molecules known to act as immunoinhibitors or immunostimulators. For each determinant, a sample-wise Z score from gene expression data
was calculated. For the six cell types, an average Z score from the corresponding metagenes was calculated. The metagenes were defined previously as non-overlapping sets of genes that are representative for specific immune cell subpopulations and are not expressed in normal tissue (Charoentong et al., 2017). The detailed list of genes included in the metagenes were available from the same literature (Charoentong et al., 2017). The determinants were then divided into four categories—effector cells (activated CD4+ or CD8+ T cells and effector memory CD4+ T cells or CD8+ T cells), and suppressive cells (Tregs and MDSCs [myeloid-derived suppressor cells]), MHC-related molecules, and checkpoints or immunomodulators are color-coded in the outer part of the wheel (red: positive Z score, blue: negative Z score).

**Development and validation of an HPD classifier based on gene expression data**

Previously, no gene expression signature had been identified to predict which patients might develop HPD after receiving anti-PD-1 immunotherapy. To identify such predictors, we analyzed the publicly available gene expression data sets of the anti-PD-1 immunotherapy studies that may contain subsets of patients that acquired HPD. Similar to previous studies (Champiat et al., 2017; Kato et al., 2017; Saada-Bouzid et al., 2017), we defined HPD as (1) progression at first restaging on therapy, (2) increase in tumor size > 50%, and (3) >2-fold increase in tumor growth rate (TGR). Based on these criteria, we identified two cohorts in these datasets that received anti-PD-1 treatment and contained patients that developed putative HPD. The first study (Accession # “GSE52562” in the GEO database) performed gene expression profiling of tumor biopsies before and after pidilizumab (a humanized anti-PD-1 monoclonal antibody, also called “CT-011”) therapy in patients with relapsed follicular lymphoma (Westin et al., 2014). Previously, it was suggested that binding to PD-1 was the main driver for pidilizumab’s activity. Recent analyses show that pidilizumab binds to a hypoglycosylated/nonglycosylated form of PD-1 that is present on a distinct subpopulation of exhausted T cells (Fried et al., 2018). Nevertheless, multiple studies have shown that pidilizumab can affect PD-1 function either through binding or
other mechanisms, so pidilizumab treatment is still considered as anti-PD-1 therapy (Abdin et al., 2018; Benson et al., 2010; Jelinek and Hajek, 2016; Mkrtichyan et al., 2011; Rosenblatt et al., 2011; Westin et al., 2014). Two of eighteen follicular lymphoma patients from this study had PFS less than two months after anti-PD-1 treatment. These two patients were classified as HPD patients, while the other sixteen were non-HPD patients (Table S4). To develop an HPD-associated gene expression signature, the pre-therapy tumor expression data of our two HPD patients were combined with the pre-treatment tumor expression data of the two HPD patients and sixteen non-HPD patients from the GSE52562 study. This was used as the HPD signature discovery dataset (called “Dataset_1”). Another study (quoted as “CA209-038”) assessed transcriptome changes in tumors from the patients with advanced melanoma before and after nivolumab immunotherapy (Riaz et al., 2017). This CA209-038 study had 21 advanced melanoma patients having PFS < 2 months after anti-PD-1 immunotherapy. Therefore, these 21 patients were classified as the HPD patients while the other 31 patients were classified as non-HPD patients (Table S6). These 51 patients had pre-therapy gene expression data available, and this dataset was used as the validation dataset (called “Dataset_2”).

Based on the genome-wide expression data of Dataset_1 and Dataset_2, we developed and validated a 121-gene classifier using the cancerclass R package (Budczies et al., 2014). The performance of the 121-gene set as a classifier was evaluated with the use of receiver-operating-characteristic curves, calculation of AUC (Hanley and McNeil, 1982), and estimates of sensitivity and specificity implemented in the cancerclass R package (Jan et al., 2014). This classification protocol starts with a feature selection step and continues with nearest-centroid classification. Fisher’s exact test was used for categorical variables. All confidence intervals are reported as two-sided binomial 95% confidence intervals. Statistical analysis was performed with R software, version 3.2.3 (R Project for Statistical Computing). We also tested the prognostic performance of the 121-gene signature using gene expression data from the TCGA tumor samples in conjunction with the online biomarker validation tool and database –
SurvExpress (Aguirre-Gamboa et al., 2013). Specifically, Kaplan-Meier survival analyses were implemented to estimate the survival functions after the samples were classified into two risk groups according to their risk scores based on the 121-gene set. Differences in survival risk between the two risk groups were assessed using the Mantel-Haenszel log-rank test.

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