Perspective

Tumor intrinsic resistance to anti-programmed death 1

Jacqueline Liu, Ting Fang, Adil I. Daud

Helen Diller Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA

Correspondence to: Adil I. Daud. Helen Diller Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA. Email: adaud@medicine.ucsf.edu.

Comment on: Zaretsky JM, Garcia-Diaz A, Shin DS, *et al*. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. N Engl J Med 2016;375:819-29.

Abstract: Immunotherapy has risen to the forefront of systemic treatment options against advanced melanoma, demonstrating both increased efficacy and decreased toxicity. Specifically, immune checkpoint blockades with agents that inhibit the interaction between programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) have achieved significant success in the clinical setting. However, patients can experience progression following an initial response to anti-PD-1 therapy, and this has fueled an interest in elucidating the potential mechanisms underlying adaptive immune resistance. In this study, Zaretsky *et al*. presents hypothesis-generating data that mutations within pathways involving interferon signaling and antigen presentation contribute to relapse.

Keywords: Melanoma; immune checkpoint blockade; programmed death 1 (PD-1), Janus kinase (JAK); interferon-gamma

Submitted Nov 12, 2016. Accepted for publication Nov 29, 2016.
doi: 10.21037/tcr.2016.12.55
View this article at: http://dx.doi.org/10.21037/tcr.2016.12.55

Immune checkpoint inhibition has revolutionized therapy for patients with advanced melanoma in the last decade, particularly the use of antibodies against programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) proteins. Despite the unprecedented success of PD-1 blockade therapy in melanoma, progression of disease occurs after 21 months in approximately 25% of patients who initially achieved an objective response. When PD-1 interacts with its two ligands, PD-L1 and PD-L2, it inhibits TCR downstream signaling and inhibits T-cell activity (1). During an active response with PD-1 blockade, tumor-infiltrating CD8+ T cells are the effectors in tumor regression by mounting an interferon-gamma/granzyme mediated attack on tumor cells. First, naive CD8+ T-cells undergo genetic differentiation to function as CD8+ effector T-cells. Subsequently, genes encoding cytokines, chemokines, and other soluble factors associated with cytolysis are expressed (2). IFN-gamma, a central cytokine secreted by effector CD8+ T-cells, induces macrophage activation and promotes anti-tumor responses (3,4). CD8+ T cells can also mediate cytolytic cell death via the perforin-granzyme effector pathway (5). Calcium-dependent release of perforin and granzymes from lytic granules occurs upon antigen recognition on the surface of target cells. Perforin polymerizes to form transmembrane pores within target cell membranes. Granzymes activate enzyme cascades that trigger apoptosis (6). Studies have revealed that both perforin and granzymes are necessary for effective cell killing. Mast-cell lines transfected with genes encoding perforin alone and granzyme B alone experience profound defects in cytotoxic T cell activity and complete loss in cytolysis, respectively. However, mast-cell lines transfected with both genes induce DNA fragmentation as effectively as granules from cytotoxic cells (5). This suggests that perforin compromises the integrity of the target cell membrane, allowing granzymes to enter the cell. Furthermore, recent findings have shown that increased expression levels of IFN-γ and granzyme B in T cells correlate with favorable outcomes (7,8). Moreover, it is thought that the success of immune checkpoint blockade therapies can be predicted by a presence of pre-existing antigen-specific CD8+ T cells that are negatively regulated through PD-1/PD-L1

mediated adaptive immune resistance (9,10). In this study, Zaretsky *et al.* determined that CD8+ T cells were also abundant in the tumor margin at the time of recurrence. They proposed that the reasons for loss of T-cell cytotoxic activity were attributed to lack of sensitivity to effector molecules by cancer cells and diminished tumor antigen recognition.

To explore the biology underlying acquired resistance, Zaretsky *et al.* commenced by studying 78 patients from the University of California, Los Angeles with metastatic melanoma who were treated with pembrolizumab, an anti-PD-1 antibody. Of this cohort, 4 patients were found to have exhibited promising initial response, followed by a period of dormancy and abrupt delayed progression. This clinical pattern of relapse fueled a hypothesis that there were tumor mutations contributing to the immune-mediated clonal selection underlying tumor growth. To investigate this theory, whole-exome sequencing analysis was performed on the DNA extracted from baseline and relapsed solid tumors of the four patients or early-passage primary cell lines. As expected, there were a number of genetic similarities between the tissues extracted from the two time points.

A comparison between the genomic compositions of baseline and relapsed tumors from Patient 1 showed an abundance of paralleled nonsynonymous mutations and loss of heterozygosity events. Surprisingly, 92.5% of the 1,173 nonsynonymous mutations originally in the baseline samples were also present in tumors at the time of relapse. Moreover, there was close similarity in loss-of-heterozygosity (LOH) events between baseline and relapsed tumors. Since LOH is known to be involved in the process of exposing a mutated suppressor gene following the deletion of the wild type allele at a particular locus (11), it was unsurprising that this genetic event was so prominent in patients who progressed. Zaretsky *et al.* reasoned that genetic differences in the relapsed tumors might have also been attributed to additive effects of the LOH events. Moreover, new mutations were discovered in tumor samples procured from Patient 1 after progression.

Of the 53 new mutations in the relapsed samples of Patient 1, there were 3 homozygous mutations identified. The Q503* nonsense mutation in the gene encoding Janus kinase (*JAK1*) was of particular interest in this study because defects in the JAK/STAT signal transduction pathway have been previously associated with loss of growth-restraining functions in human melanoma cell lines (12,13). Since the mutation in Patient 1 was upstream of the kinase domain, Zaretsky *et al.* hypothesized that it likely truncated the protein and disrupted its functional capacity. In Patient 1, the chromosome that contained *JAK1* (chromosome 1p) housed 36 fewer heterozygosity mutations and 20 more homozygous mutations at relapse than at baseline.

Findings from Patient 2 show that relapsed tumors had 95.8% of the 240 nonsynonymous mutations seen in baseline tumors. Of the 76 new nonsynonymous mutations detected at relapse, *JAK 2* was the only homozygous mutation. This mutation, contained in chromosome 9, likely underwent a LOH event comparable to that seen in *JAK 1*. Together, the similar genetic makeup of baseline and relapsed tumors seen in Patient 1 and 2 allow for the hypothesis that anti-PD-1 resistant cancers are derived directly from baseline tumors and precede clonal selection.

After identifying the *JAK* mutations, Zaretsky *et al.* proceeded to assess how the disruption of the JAK/STAT pathway affected cell function. Cell lines M420 (wild-type *JAK2*) and M464 (*JAK2* F547 splice site mutation) were developed to represent baseline and relapsed tumors, respectively. In order to confirm that the M464 cell line was a genuine and comprehensive representation of the original tumor, whole-exome genome analysis was used for comparison between the two. Western blots were subsequently employed to investigate whether the cell lines responded differently to signaling molecules. Upon stimulation from interferon alfa, beta, and gamma in the M420 cell line, there was expected increase in the signal transducer and activator of both transcription 1 (STAT1) and interferon regulatory factor (IRF), phosphorylation of STAT1, and downstream production of PD-L1, antigen processing 1 (TAP1), and major histocompatibility complex (MHC) class I. In other words, the interferon alfa, beta, and gamma signal transduction pathways were functioning as expected in the wild-type M420 cell line. In M464, however, there was only a noted response to interferon alfa and beta stimuli, but a clear loss of sensitivity to interferon gamma. The Western blot analysis revealed significantly decreased expression of pSTAT1, PD-L1, and MHC class I after exposure to interferon gamma. Moreover, there was a decrease in expression of several interferon-induced genes in M464. These results were aligned with the well-studied JAK/STAT pathway, where dimerization of interferon gamma receptors leads to activation of JAK1 and JAK2, followed by subsequent phosphorylation of STAT (14). With the data from Zaretsky *et al.*, it is feasible that the disruption of the JAK/STAT pathway facilitated tumor outgrowth by reducing the antitumor effects of interferon

signaling.

Interferon gamma has been recognized as a critical cytokine associated with host defense and antitumor mechanisms. The interferon gamma induced JAK/STAT signal transduction pathway participates in processes of antiviral and bacterial defense, innate immunity, acquired immunity, and cell growth inhibition (15,16). As a result of the diverse immunomodulatory effects mediated by interferon gamma, particularly cell growth arrest, a loss of response to this cytokine can render the host immune system vulnerable to neoplastic growth (17). Zaretsky *et al.* presented provocative evidence that *JAK1* and *JAK2* mutations prevent the cytotoxicity of interferon gamma and contribute to tumor escape.

In an attempt to validate the initial results, additional cell lines were developed and modified for *in vitro* cultures. Human melanoma cell line M407 was established using CRISPR Cas9, which was in turn used to engineer *JAK1*-knockout and *JAK2*-knockout sublines. M407 and its sublines expressed NY-ESO-1, the cancer-testis antigen, which allowed them to interact with T cells expressing NY-ESO-1-specific T-cell receptors. Together, these cell lines provided a means to study T-cell cytotoxic activity in patients with *JAK1* and *JAK2* mutations. After 24 hours of *in vitro* culture, the high levels of interferon gamma production confirmed that the NY-ESO-1-specific T-cell receptors recognized the M407 parental line and its two sublines equally. Next, the M420, M464, M407, *JAK1*-knockout, and *JAK2*-knockout cell lines were cultured with recombinant interferon alfa, beta, and gamma. M420 and M407 served as negative controls; the two cell lines responded to all three interferon cytokines with expected dose-dependent growth arrest. However, the M464 and *JAK2*-knockout subline only experienced interferon alfa and beta induced growth inhibition, but lacked a response to interferon gamma signaling. Alternatively, the *JAK1*-knockout subline was unresponsive to all three cytokines. Since JAK1 is downstream of interferon alfa, beta, and gamma, it is reasonable that the mutation in *JAK1* disrupted all associated signaling transduction pathways. In order to ensure that these effects were not a result of a general decrease in T-cell recognition, the cell lines were cultured with 2'3'-cGAMP, a known cytosolic agonist of the stimulator of interferon genes (STING), which upregulates interferon beta. As expected, treatment with 2'3'-cGAMP induced growth inhibition of all cell lines except the *JAK1*-knockout subline of M407. Collectively, these results again stress the importance of *JAK1* and *JAK2* in the interferon gamma signaling pathway, which consequently plays a role in preventing tumor progression.

Aside from the detrimental effects of tumor cell insensitivity to effector molecules, as shown through Patient 1 and Patient 2, loss of antigen presentation as a result of mutations in MHC class I components also disrupts cytotoxic T-cell activity. Following whole-genome sequencing of both pre-treatment and post-treatment progressive lesions of Patient 3, a 4-bp S14 frame-shift deletion in exon 1 of beta-2-microglobulin was identified as a relapse-specific homozygous mutation. Beta-2-microglublin is a soluble protein that plays a central role in MHC class I molecule folding and cell surface localization (18). Despite the continued production of MHC class I molecules in Patient 3, there was a loss of outer-membrane localization of this heterodimer. Additionally, there were no observed MHC compensatory mechanisms, such as an upregulation of MHC class II molecules. These results support previous discoveries that loss of beta-2-microglublin allows antigens to evade recognition by antitumor CD8+ T cells, ultimately leading to acquired resistance to immunotherapy (18).

Though loss-of-function mutations were found in Patients 1, 2, and 3, there was no clear genetic explanation for the immune-resistant progression observed in Patient 4. Immunohistochemical staining analyses of baseline tumor samples from the first three patients yielded preexisting CD8 T-cell infiltrates and PD-L1 expression in the tumor margin, which are acknowledged predictors of response to PD-1 blockade therapy (9). The tumor biopsy sample from Patient 4 at time of response revealed a significant increase in intratumoral CD8 T-cell infiltrates and PD-L1 expression, but PD-L1 expression was not present at baseline or relapse. This lack of PD-L1 expression at either time points was interesting because of the close proximity of cancer cells to T cells and PD-L1-expressing stroma. These observations fed the notion that there are nongenetic factors influencing expression of interferon-inducible genes. This presents alluring opportunities for future research efforts on alternative routes of gene induction, presumably through epigenetic mechanisms (16).

While Zaretsky *et al.* drew a relationship between inactivation of interferon induced *JAK1* or *JAK2* signaling and adaptive immune resistance, there has been evidence suggesting that activation of JAK/STAT signaling can also drive malignancies to some degree (19). Though STAT1 has been shown to be a primary transactivator downstream of interferon gamma (20,21), other transactivators are also subject to interferon gamma signaling, such as STAT3 (22,23).

A number of clinical investigations have confirmed the role of activated STAT3 as an oncogene, mediating proteins that are involved in cancer cell proliferation and angiogenesis (24-26). Such findings even inspired a Phase I study that administered AZD1480, an inhibitor of JAK1 and JAK2, as a monotherapy to treat 38 patients with solid tumors. While there was a reduction in PSTAT3 within granulocytes of one patient, indicated by an analysis of pre- and post-treatment tumor biopsies, there was an overall lack of significant antitumor effects with the use of AZD1480 (27).

Though this study presents the novel insight into the mechanism of adaptive resistance, there remain areas of possible contention. Firstly, the number of tumors and patients profiled is very small and the nature of the analyses indicates the possibility that these mutations are not causal. Additionally, there was a treatment history inconsistency in the study cohort because one patient was not treatment naïve at the time of starting pembrolizumab. Patient 1 had been administered a course of vemurafenib, a BRAF inhibitor, after baseline biopsy procurement and prior to initiation of pembrolizumab. The effects of using targeted therapy prior to anti-PD1 treatment are not yet completely understood. Next, the study failed to thoroughly explain the acquired resistance observed in Patient 4. It was merely proposed that unknown nongenetic factors contributed to tumor outgrowth. Furthermore, whole exome sequencing analysis of samples acquired at time points before anti-PD1 treatment and after relapse may suggest a correlation between *JAK* mutations and tumor progression, but it does not exclude the possibility of other factors influencing immune evasion. The argument for direct causation would have been more compelling if there was evidence that the onset of progression occurred momentarily after and as a result of *JAK* dysregulation. However, the tumor responses were evaluated periodically every 12 weeks, so the precise start of progression could not be determined, especially during the period between imaging assessments. Sequencing analysis was only performed on relapsed tissue after its detection, so it was ambiguous whether progression occurred prior to or after the emergence of the *JAK* mutations.

To study the *JAK* mutations and their role in the interferon gamma signaling pathway, human melanoma cell lines were engineered for *in vitro* analysis. While the application of *in vitro* co-culture assays facilitated the understanding of interferon signaling pathways, differences between *in vitro* and *in vivo* processes must be taken into account. Historically, *in vitro* tumor cell lines have provided efficient platforms to study therapy resistance (28). However, there are biological differences between *in vitro* tumors and *in vivo* human neoplasms, ranging from dissimilarities in immunogenicity to the tumor-host interaction (29). As a result, the *in vitro* cell lines and assays used to explain the functional effects of the *JAK1* and *JAK2* mutations were likely not comprehensively representative of melanomas within the human body. As a consequence, there may be other genetic and epigenetic mechanisms inducing the acquired resistance to anti-PD1 therapy within the body, aside from the noted *JAK* mutations studied *in vitro*.

Zaretsky *et al.* nevertheless contributed considerably to the understanding that the JAK/STAT pathway mediates cell-growth arrest. The study was certainly hypothesis-generating, focusing on the roles of *JAK1* and *JAK2* mutations in interferon gamma signaling insensitivity and tumor progression. Further investigation is required to corroborate these findings. It may also be noteworthy to explore the molecular processes behind relapses from metastatic melanoma patients who received other treatment options, such as anti-CTLA4 therapy, combination immunotherapies, and targeted therapies. Additionally, while this study investigated a patient population with acquired response to PD-1 inhibition, there are many cancer patients who lack an initial objective response to checkpoint blockade therapy (30). Research endeavors have already been initiated towards identifying predictive biomarkers for response to treatment. Understanding the molecular pathways involved in both initial response and delayed relapse for anti-PD1 treatment is essential for clinical efficacy.

In conclusion, PD-1 therapy is one of several therapeutic agents championing tumor immunosurveillance in patients with advanced melanoma. The success of treatment heavily relies on the ability of cytotoxic T cells to recognize tumor associated antigens. There is evidence suggesting that dysfunction of the JAK/STAT signal cascade pathway is key in allowing tumor escape and progression. Similarly, the role of STAT family transcription factors in MHC class I and MHC class II expression on the surface of neoplastic cells further emphasizes the functional significance of interferon gamma in stimulating the JAK/STAT pathway. Monitoring changes in the tumor profile through whole genome sequencing provides a comprehensive index of therapeutic targets and paves the way to improving patient prognosis with immunotherapeutic intervention.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Xia Fang (Department of Hematology, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai, China).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2016.12.55). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Postow MA, Callahan MK, Wolchok JD. Immune Checkpoint Blockade in Cancer Therapy. J Clin Oncol 2015;33:1974-82.
2. Nolz JC, Starbeck-Miller GR, Harty JT. Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions. Immunotherapy 2011;3:1223-33.
3. Boehm U, Klamp T, Groot M, et al. Cellular responses to interferon-gamma. Annu Rev Immunol 1997;15:749-95.
4. Vaseghi H, Sanati MH, Jadali Z. T-helper Cell Type-1 Transcription Factor T-Bet Is Down-regulated in Type 1 Diabetes. Iran J Allergy Asthma Immunol 2016;15:386-93.
5. Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science, 2001.
6. Trapani JA, Smyth MJ, Apostolidis VA, et al. Granule serine proteases are normal nuclear constituents of natural killer cells. J Biol Chem 1994;269:18359-65.
7. van Houdt IS, Sluijter BJ, Moesbergen LM, et al. Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression. Int J Cancer 2008;123:609-15.
8. Anegawa H, Otsuka A, Kaku Y, et al. Upregulation of granzyme B and interferon-γ mRNA in responding lesions by treatment with nivolumab for metastatic melanoma: a case report. J Eur Acad Dermatol Venereol 2016;30:e231-e232.
9. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568-71.
10. Daud AI, Loo K, Pauli ML, et al. Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. J Clin Invest 2016;126:3447-52.
11. Ryland GL, Doyle MA, Goode D, et al. Loss of heterozygosity: what is it good for? BMC Med Genomics 2015;8:45.
12. Pansky A, Hildebrand P, Fasler-Kan E, et al. Defective Jak-STAT signal transduction pathway in melanoma cells resistant to growth inhibition by interferon-alpha. Int J Cancer 2000;85:720-5.
13. Wilks AF, Harpur AG, Kurban RR, et al. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol Cell Biol 1991;11:2057-65.
14. Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci 2004;117:1281-3.
15. Shuai K, Liao J, Song MM. Enhancement of antiproliferative activity of gamma interferon by the specific inhibition of tyrosine dephosphorylation of Stat1. Mol Cell Biol 1996;16:4932-41.
16. Dunn GP, Sheehan KC, Old LJ, et al. IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. Cancer Res 2005;65:3447-53.
17. Berner V, Liu H, Zhou Q, et al. IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. Nat Med 2007;13:354-60.
18. Restifo NP, Marincola FM, Kawakami Y, et al. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. J Natl Cancer Inst 1996;88:100-8.
19. Thomas SJ, Snowden JA, Zeidler MP, et al. The role of JAK/STAT signalling in the pathogenesis, prognosis and

treatment of solid tumours. Br J Cancer 2015;113:365-71.
20. Bromberg JF, Horvath CM, Wen Z, et al. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. Proc Natl Acad Sci U S A 1996;93:7673-8.
21. Wong LH, Krauer KG, Hatzinisiriou I, et al. Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. J Biol Chem 1997;272:28779-85.
22. Schindler C, Plumlee C. Inteferons pen the JAK-STAT pathway. Semin Cell Dev Biol 2008;19:311-8.
23. Zaidi MR, Merlino G. The two faces of interferon-γ in cancer. Clin Cancer Res 2011;17:6118-24.
24. Watson CJ, Miller WR. Elevated levels of members of the STAT family of transcription factors in breast carcinoma nuclear extracts. Br J Cancer 1995;71:840-4.
25. Barbieri I, Pensa S, Pannellini T, et al. Constitutively active Stat3 enhances neu-mediated migration and metastasis in mammary tumors via upregulation of Cten. Cancer Res 2010;70:2558-67.
26. Kasprzycka M, Marzec M, Liu X, et al. Nucleophosmin/anaplastic lymphoma kinase (NPM/ALK) oncoprotein induces the T regulatory cell phenotype by activating STAT3. Proc Natl Acad Sci U S A 2006;103:9964-9.
27. Plimack ER, Lorusso PM, McCoon P, et al. AZD1480: a phase I study of a novel JAK2 inhibitor in solid tumors. Oncologist 2013;18:819-20.
28. Voskoglou-Nomikos T, Pater JL, Seymour L. Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. Clin Cancer Res 2003;9:4227-39.
29. Malling HV, Frantz CN. In vitro versus in vivo metabolic activation of mutagens. Environ Health Perspect 1973;6:71-82.
30. Meng X, Huang Z, Teng F, et al. Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy. Cancer Treat Rev 2015;41:868-76.

Cite this article as: Liu J, Fang T, Daud AI. Tumor intrinsic resistance to anti-programmed death 1. Transl Cancer Res 2016;5(Suppl 7):S1515-S1520. doi: 10.21037/tcr.2016.12.55

