High expression of PD-1 ligands is associated with \textit{kataegis} mutational signature and APOBEC3 alterations

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\textbf{ABSTRACT}

Immunotherapy with checkpoint inhibitors, such as antibodies blocking the programmed cell-death receptor-\textsuperscript{1} (PD-1), has resulted in remarkable responses in patients having traditionally refractory cancers. Although response to PD-1 inhibitors correlates with PD-1 ligand (PD-L1 or PD-L2) expression, PD-1 ligand positivity represents only a part of the predictive model necessary for selecting patients predisposed to respond to immunotherapy. We used all genomic, transcriptomic, proteomic and phenotypic data related to 8,475 pan-cancer samples available in The Cancer Genome Atlas (TCGA) and conducted a logistic regression analysis based on a large set of variables, such as microsatellite instability (MSI-H), mismatch repair (MMR) alterations, polymerase \textdelta{} (POLD1) and polymerase \textepsilon{} (POLE) mutations, activation-induced/apolipoprotein-B editing cytidine deaminases (AID/APOBEC) alterations, lymphocyte markers and mutation burden estimates to determine independent factors that associate with PD-1 ligand overexpression. PD-1 ligand overexpression was independently and significantly correlated with overexpression of PD-L1 and PD-L2. These observations suggest that APOBEC3 alterations, APOBEC3 overexpression and \textit{kataegis} play an important role in the regulation of PD-1 ligand overexpression, and thus, their relationship with immune checkpoint inhibitor response warrants exploration.

\textbf{Introduction}

During the last decade, various immunotherapy approaches have shown promising results in patients having cancer. By modulation of self-reactive cellular immunity (mediated by cytotoxic T-lymphocytes), these therapies permit substantial tumor regression, sometimes leading to durable clinical responses.\textsuperscript{1} One of the most successful strategies in this regard consists of impeding the immune checkpoints located on the surface of T-cells, which act as "off"-switches when bound to their ligands found on antigen-presenting and tumor cells and, thus, are considered protective factors for the cancer cell.\textsuperscript{2} Recently, 3 immune checkpoint inhibitors blocking the programmed cell-death receptor-\textsuperscript{1} (PD-1 or PDCD1) or its ligands—namely PD-L1 (CD274 or B7-H1) and PD-L2 (CD273 or PDCD1LG2) —have been approved by the Food and Drug Administration (FDA): nivolumab, pembrolizumab and atezolizumab; for the treatment of melanoma, Hodgkin’s lymphoma, renal cell cancer, non-small cell lung cancer (NSCLC) and bladder cancer.\textsuperscript{3–8}

PD-1 ligands are constitutively expressed in a variety of normal tissues, such as placenta, heart, lung, liver, spleen, lymph nodes and thymus.\textsuperscript{8} Expression of PD-L1 can be induced in lymphocytes, monocytes, macrophages and epithelial cells after infection by pathogens or inflammatory processes, through the bacterial lipopolysaccharides (LPS), the pro-inflammatory cytokine interferon gamma (IFN$\gamma$), and to a lesser extent by the tumor necrosis factor alpha (TNF$\alpha$).\textsuperscript{8} Aberrant PD-L1 expression may also be observed as a result of various genomic and post-transcriptional alterations, such as after introduction of an ectopic PD-L1 gene promoter by a translocation phenomenon\textsuperscript{10} or after stabilization by truncation of the 3' untranslated region (3'-UTR) of the PD-L1 transcript.\textsuperscript{11} PD-L2 induced expression is less frequent and restricted to limited cell types.\textsuperscript{8} Both PD-L1 and PD-L2 protein overexpression have been described as relevant, albeit imperfect, predictive biomarkers for the response to anti-PD-1 and/or anti-PD-L1 agents.\textsuperscript{12,13} Additionally, PD-L1 and PD-L2 amplification (both genes are located on the same amplicon on the short arm of chromosome 9) has been associated with high response rates to anti-PD-1 agents in Hodgkin’s lymphoma.\textsuperscript{8,14}

Recent evidence has established a link between the genomic instability of cancer and the response to checkpoint blockade in
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The high clinical response rates observed in various colonic and extra-colonic MSI-H tumors and this association may explain, at least in part, the high clinical response rates observed in various colonic and extra-colonic MSI-H tumors after pembrolizumab treatment.\textsuperscript{15,16} PD-L1 expression has also been associated with high tumor mutation burden in melanoma,\textsuperscript{17} NSCLC,\textsuperscript{18} and with additional mechanisms leading to hyper-mutativity, such as POLE and POLD1 aberrations in endometrial carcinoma\textsuperscript{19} and APOBEC3 overexpression in urothelial carcinoma.\textsuperscript{20} However, the molecular mechanisms underlying the association between PD-L1/2 overexpression, the salutary effects of immune checkpoint inhibition and the tumor mutation burden remain largely elusive.

Aggregation of a large number of mutations in a cell can be caused by exposure to exogenous mutagens (such as ultraviolet radiation or tobacco-related carcinogens) or several endogenous mutagenic processes. In particular, tumor hyper-mutation has been associated with different mechanisms impairing the DNA replication fidelity process: (i) loss of DNA damage repair function by mutation, deletion or post-transcriptional regulation of MMR proteins; (ii) alterations of the proof-reading domains of replicative polymerases \( \varepsilon \) and \( \delta \) by mutation of POLE or POLD1 gene; and (iii) unleashed activity of APOBEC (apolipoprotein B mRNA editing cytidine deaminase) enzymes, which leads to a localized hyper-mutation phenomenon called \textit{kataegis}.\textsuperscript{21,22}

APOBEC family members are nucleic acid mutators originally implicated in lipoprotein metabolism (by apolipoprotein-B mRNA editing),\textsuperscript{23} antigen-driven antibody diversification (by somatic hyper-mutation, gene conversion and class-switch recombination of immunoglobulin genes),\textsuperscript{24} and innate anti-retroviral immunity (by inhibition of the replication of retroviruses and retrotransposons).\textsuperscript{25} Although each member of the APOBEC family (AICDA or AID, APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, APOBEC3H and APOBEC4) presents distinct circumstances of expression and specific functions, their activity results almost exclusively in cytosine-to-thymine (C-to-T) transitions in their DNA/RNA substrates. Besides their natural and protective functions, APOBEC enzymes may also cause substantial off-target genotoxic damages (arising mostly during the DNA replication) and thus be implicated in tumor hyper-mutation.\textsuperscript{26}

In this study, we used a large set of pan-cancer tumor samples (TCGA Research Network: \url{http://cancergenome.nih.gov/}) and a series of multivariate regression models to elucidate the overall association between high levels of PD-1 ligand expression and the mutation burden mechanisms mentioned above, especially as it pertains to anomalies correlated with the APOBEC family of enzymes.

**Results**

**Pan-cancer collection description**

A total of 8,475 samples presenting a complete set of data (i.e., with complete annotation of genomic mutations, copy number variations (CNV) and mRNA expression) were included in the regression analysis. The samples corresponded to 32 different cancer types as described in Table S1.

All logistic regression models were built by using single and/or combined predictors gathered by function: MMR alteration (8 markers), polymerases \( \varepsilon \) and \( \delta \) mutation (2 markers), AID/ APOBEC family alteration (36 markers), PD-1 ligand alteration (9 markers), microsatellite phenotype (1 marker), lymphocyte phenotype (11 markers) and mutation burden/genomic instability estimate (5 markers), as defined in Table S2. A minority of samples were evaluated for MSI (1,328 samples—15.7% of the tumors) and only 3,088 (72.9%) and 3,009 (71.0%) tumors were tested by immunohistochemistry to assess lymphocyte and macrophage infiltration. For these particular markers, analysis was done by examining patients with the presence of the marker versus those without, as well as with an unknown status versus those without. For markers corresponding to specific genomic alteration (mutation, copy number variation or mRNA expression), assessment was done as yes versus no for the presence of the marker. Finally, for mutation burden markers, groups-of-interest were delimited at the median: samples with value greater than median compared with samples with value lower or equal to median (Table S2).

When considering the complete collection, median number of mutations per sample was 66.5 (ranging from 0 to 23,950) and minimal number of APOBEC-related alterations was 0 (ranging from 0 to 2,347 minimal load of APOBEC-related alterations per sample). Median number of gains, losses and CNV per sample were 3,228 (ranging from 0 to 14,686), 3,957.5 (ranging from 0 to 14,179) and 8,100 (ranging from 0 to 24,591), respectively.

**Independent predictors of PD-1 ligand overexpression**

A significantly high level of expression of PD-1 ligands was observed in 469 tumors (5.5% of the pan-cancer cohort), and most them \((n = 406, 86.6\%)\) presented at least one molecular alteration within MMR, polymerases \( \varepsilon \) or \( \delta \), APOBEC3 or PD-L1/2 factors (Fig. 1A and B).

In univariate analysis, factors associated with PD-L1 or PD-L2 overexpression were: PD-1 ligand genomic locus amplification, MSI-H phenotype, POLD1 mutation, presence of kataegis, APOBEC3 mutation (APOBEC3G and APOBEC3H single factors were significant), AICDA overexpression, APOBEC3 overexpression (all 7 paralogs were significant), APOBEC4 amplification, monocytes infiltration, overexpression of immune markers (7 single factors were significant), as well as overexpression of IFNy (Table S3).

Interdependent relationships between these factors and PD-1 ligand overexpression were assessed by a logistic regression method adapted to rare events (Firth’s penalized likelihood analysis). The final models, as shown in Table 1, presented a pseudo-\( R^2 \) (likelihood-ratio index of McFadden) of 25.9% and 24.2% (for models using single and combined factors, respectively), demonstrating the percentage of variability of PD-1 ligand overexpression that may be explained by the set of chosen factors.\textsuperscript{27} Particularly, the model obtained with combined factors revealed a strong correlation between the presence of APOBEC alterations and the high level of expression of PD-L1 or PD-L2. APOBEC alterations were represented by the presence of any APOBEC3-
member mRNA overexpression (Odds Ratio OR = 2.7, \( p < 0.0001 \)), the presence of a coding mutation within any of the APOBEC3 paralogs (OR = 2.4, \( p = 0.0027 \)) and the presence of a kataegis signature (OR = 1.3, \( p = 0.0210 \)). Additional positively-related predictors were the presence of a PD-L1/2 amplification (OR = 3.6, \( p < 0.0001 \)); overexpression of IFN\( \gamma \) (OR = 3.1, \( p < 0.0001 \)); overexpression of T-lymphocyte, natural-killer cell, monocyte and macrophage markers (OR ranging from 1.6 to 3.2, \( p < 0.0135 \)); and presence of a POLD1 mutation (OR = 2.1, \( p = 0.0374 \)). All predictors described for the model presented in

### Table 1. Multivariate analysis of association\(^a\) between all factors and PD-1 ligand mRNA overexpression, using single factors (model A) or relevant combined factors (model B).

| Subgroups and factors | A. Model using only single factors \( R^2 = 25.9\% \) | B. Model using single and combined factors\(^b\) \( R^2 = 24.2\% \) |
|-----------------------|--------------------------------------------------|--------------------------------------------------|
|                       | \( p \) value | OR | CI 95\% | \( p \) value | OR | CI 95\% |
| POLYMERASES \( \delta \) and \( \epsilon \) | | | | | | |
| POLD1 mutated         | 0.0172       | 2.2 | [1.2–4.3] | | | |
| MUTATION BURDEN       | | | | | | |
| Kataegis estimate     | 0.0210       | 1.3 | [1.0–1.7] | | | |
| PD-L1 LIGAND           | | | | | | |
| PD-L1 amplified\(^c\) | <0.0001       | 3.8 | [2.9–4.9] | 0.0001 | 3.6 | [2.8–4.6] |
| PD-L2 amplified\(^c\) | <0.0001       | 3.8 | [2.9–4.9] | | | |
| Any PD-1 ligand amplified | | | | | | |
| AID/APOBEC FAMILY     | | | | | | |
| APOBEC3B mutated       | 0.0267       | 3.4 | [1.2–10.2] | | | |
| APOBEC3D mutated       | 0.0224       | 3.8 | [1.2–11.7] | | | |
| APOBEC3H mutated       | 0.0257       | 4.0 | [1.2–13.4] | | | |
| Any APOBEC3 mutated    | | | | | | |
| APOBEC3A overexpressed | <0.0001       | 4.2 | [2.9–6.0] | | | |
| APOBEC3C overexpressed | <0.0001       | 2.3 | [1.5–3.4] | | | |
| APOBEC3G overexpressed | <0.0001       | 2.1 | [1.4–3.2] | | | |
| Any APOBEC3 overexpressed | | | | | | |
| LYMPHOCYTE FACTORS    | | | | | | |
| CD3\( \gamma \) overexpressed | <0.0001    | 2.7 | [1.8–4.0] | | | |
| CD4\( \gamma \) overexpressed | 0.0005       | 2.0 | [1.4–2.9] | | | |
| CD8A overexpressed     | 0.0004       | 2.1 | [1.4–3.2] | | | |
| NCAM1 overexpressed    | 0.0149       | 1.9 | [1.1–3.3] | | | |
| CD14 overexpressed     | <0.0001       | 3.0 | [2.0–4.4] | | | |
| IFN\( \gamma \) overexpressed | <0.0001       | 2.7 | [1.8–4.0] | | | |

\(^a\)Alterations with a \( p \) value \( <0.05 \) in univariate analysis were selected for multivariate analysis, respecting each model (i.e., using only single factors or single and relevant combined factors). A Fifth-corrected logistic regression with backward elimination was performed until all factors influencing the model present a \( p \) value \( \leq 0.05 \). Only factors with \( p \) value \( <0.05 \) for a positive association in multivariate analysis are shown.

\(^b\)Combined factors as outlined in Table S2. For instance, any MMR member altered, any APOBEC3 member overexpressed, any PD-1 ligand mutated and total number of CNV represent combined factors.

\(^c\)PD-L1 amplification and PD-L2 amplification are co-linear variables.

Abbreviations: AID = activation-induced deaminase; APOBEC = apolipoprotein B editing complex; CI 95\% = 95\% confidence interval; CNV = copy number variation; MMR = mismatch repair; OR = odds ratio.
Table 1B remained significant after application of the re-sampling method (1,000 replicates, \( p < 0.05 \)).

These data confirmed the association previously observed between the PD-1 ligand expression and the presence of tumor mutagenesis processes, and to a lesser extent \( POLD1 \) alterations; but above all, allowed to highlight the critical role played by APOBEC enzymes and their dysfunction. Interestingly, kataegis-signature and APOBEC alterations appeared to be related to PD-L1/2 overexpression independently of the immune cell infiltrate and IFN\( \gamma \) release (Fig. 2).

**Independent predictors of tumor mutation burden**

In univariate analysis, the factors positively associated with a high tumor mutation burden (i.e., total number of mutations per tumor greater than 66.5) were: MSI-H phenotype, MMR alteration (all MMR proteins were significant), \( POLD1 \) or \( POLE \) mutation, high number of CNV (gains, losses or combined factor), kataegis-signature, APOBEC3 mutation or amplification (all paralogs were significant), APOBEC3 overexpression (more specifically APOBEC3A/B/F/G overexpression), PD-L1 downregulated genomic mutation or amplification, PD-L1 overexpression (but not PD-L2), presence of a lymphocyte or monocyte infiltrate and IFN\( \gamma \) overexpression (Table S4).

In multivariate analysis, the overall tumor mutation burden was modeled with a pseudo-R\(^2 \) of 30.3% (Table 2A). Factors considered independent predictors for the presence of a high number of point mutations in a tumor included MSI-H status.
(OR = 54.7, \( p < 0.0001 \)), MMR alteration (OR = 1.6, \( p < 0.0001 \)), polymerases \( \delta \) or \( \epsilon \) catalytic subunits mutation (OR = 4.0, \( p = 0.0002 \) and OR = 5.7, \( p < 0.0001 \), respectively), as well as kataegis-signature (OR = 14.1, \( p < 0.0001 \)), AID/APOBEC family members mutation or amplification (OR ranging from 1.2 to 13.3, \( p < 0.0129 \)) and a high number of CNV (more than 8,100 CNV per sample, OR = 2.0, \( p < 0.0001 \)). Factors related to the immune modulation, like PD-1 ligand mutation (OR = 5.9, \( p = 0.0222 \)), lymphocyte infiltration (OR = 1.4, \( p = 0.0005 \)) or T-lymphocyte CD8A marker overexpression (OR = 1.4, \( p = 0.0214 \)), were also linked to the tumor mutation burden (Table 2A).

Remarkably, neither APOBEC3 nor PD-L1 overexpression were negatively correlated with the presence of (natural-killer cell marker) and IL3RA (dendritic cell marker) in 1,604 tumors (18.9%). In this pan-cancer collection, the kataegis-signature was positively associated with MMR alteration, POLE mutation, overall mutation burden, total number of CNV, APOBEC3 or APOBEC4 mutation, AID/APOBEC amplification (of all 11 paralogs), APOBEC3 overexpression, PD-1 ligand alteration (mutation, amplification or overexpression), lymphocyte and monocyte infiltrate and overexpression of CD3G and MS4A1. In this case, MSI-H, APOBEC1/2/4 overexpression as well as NCAM1 (natural-killer cell marker) and IL3RA (dendritic cell marker) overexpression were negatively correlated with the presence of a kataegis-signature (Table S5).

Similarly to the tumor mutation burden, the presence of kataegis was modeled by multivariate analysis using a set of pre-defined combined factors, with a pseudo-\( R^2 \) of 30.0%. The predictors of kataegis-signature were the presence of a MMR alteration (OR = 1.2, \( p = 0.0133 \)), the overall mutation burden (OR = 14.1, \( p < 0.0001 \)), a high number of CNV (OR = 1.4, \( p = 0.0005 \)), APOBEC3 or APOBEC4 amplification (OR = 1.6, \( p < 0.0001 \) and OR = 1.4, \( p < 0.0001 \)), APOBEC3 overexpression (OR = 1.6, \( p = 0.0016 \)), the presence of a PD-1 ligand altered by mutation (OR = 2.8, \( p = 0.0124 \)), amplification (OR = 1.3, \( p = 0.0070 \)) or overexpression (OR = 1.4, \( p = 0.0099 \)), and the presence of a lymphocyte infiltrate (OR = 1.2, \( p < 0.0001 \)) (Table 2B). All factors, except the correlation between the lymphocyte infiltrate and the kataegis-signature, were confirmed by the internal validation procedure (1,000 replicates, \( p < 0.05 \)).

Such analysis allowed to confirm the existence of a bi-lateral relationship between PD-L1/2 expression and the presence of kataegis, along with a bi-directional relationship between kataegis and the overall tumor burden, as represented in the probabilistic graphical model in Fig. 2.

Independent predictors of APOBEC3 mRNA overexpression

A significant overexpression of APOBEC3 (including APOBEC3 isoforms A, B, C, D, F, G and H) was observed in 1,394 (16.4%) of the tumors. The factors associated with APOBEC3 overexpression in univariate analysis were: MMR altered (particularly MLH3 and MSH3), the number of genes lost and/or gained, the number of mutations as well as the number of kataegis-related mutations, APOBEC3C mutation, AICDA, APOBEC1 and APOBEC3 amplification, AICDA overexpression, PD-L1/2 amplification and overexpression, presence of a lymphocyte infiltrate with overexpression of lymphocyte markers CD3G, CD4+, CD8A, MS4A1, IL3RA, CD14 and CD33 and IFN\( \gamma \) overexpression (Table S6).

Independent factors significantly associated with APOBEC3 overexpression in multivariate analysis (pseudo-\( R^2 = 14.0% \)) were the total number of APOBEC-related alterations (OR = 1.2, \( p = 0.0048 \)), the overexpression of PD-1 ligand (OR = 2.6, \( p < 0.0001 \)), amplification of APOBEC3 genes (OR = 2.3, \( p < 0.0001 \)) or overexpression of AICDA (OR = 2.2, \( p < 0.0001 \)), as well as expression of IFN\( \gamma \) (OR = 3.7, \( p < 0.0001 \)) and lymphocyte markers (all excepted NCAM1 and CD14, OR = 1.5–3.5, \( p < 0.0131 \)) (Table 3A). All predictors for the model were confirmed by internal validation (1,000 replicates, \( p < 0.05 \)), and bidirectional dependences between APOBEC3 overexpression and kataegis, plus APOBEC3 overexpression and PD-1 ligand overexpression, were inferred in the graphical model in Fig. 2.

Independent predictors of APOBEC3 mutation

Non-silent mutations of APOBEC3 were observed in 171 (2%) of the tumors. At the univariate level, the presence of a mutation in one of the 7 APOBEC3 paralogs was related to MSI-H phenotype, the presence of MMR alteration (MLH3, MSH2, MSH6 or PMS2 proteins), presence of mutagenesis process (overall mutation burden and kataegis), the presence of a non-silent mutation within POLD1, POLE, AICDA, APOBEC1, APOBEC2, APOBEC4 and PD-1 ligand genes, APOBEC4 amplification, PD-L1/2 overexpression and CD14 overexpression (Table S7).

The presence of a mutation in any of the APOBEC3 genes was modeled with a correlational pseudo-\( R^2 \) of 20.1% by several positive independent predictors including MSI-H (OR = 5.4, \( p < 0.0001 \)), high number of non-silent mutations (total number of mutations greater than 66.5, OR = 6.7, \( p < 0.0246 \)), mutations within POLE, APOBEC1, APOBEC2 and PD-L1/2 (OR ranging from 3.0 to 6.4, \( p < 0.0001 \)), and overexpression of PD-1 ligands (OR = 2.0, \( p = 0.0067 \)) (Table 3B). These predictors, with the exception of MSI-H status, were confirmed by the bootstrapping method (1,000 replicates, \( p < 0.05 \)) and added to the graphical model in Fig. 2.
Table 3. Multivariate analysis of association\textsuperscript{a} between all factors and APOBEC3 members (APOBEC3A/B/C/D/F/G/H) mRNA overexpression (model A) or mutation (model B).

| Subgroups and factors | A. APOBEC3 Overexpression model\textsuperscript{b} $R^2 = 14.0\%$ | B. APOBEC3 Mutation model\textsuperscript{b} $R^2 = 20.1\%$ |
|-----------------------|---------------------------------|--------------------------------|
|                       | $p$ value | OR | CI 95% | $p$ value | OR | CI 95% |
| MICROSATellite STATUS |          |    |        |          |    |        |
| MSI-high\textsuperscript{i} | ns | — | — | <0.0001 | 5.4 | [3–9.4] |
| POLYMERASES $\beta$ and $\gamma$ | ns | — | — | <0.0001 | 6.4 | [4.2–9.8] |
| MUTATION BURDEN |          |    |        |          |    |        |
| Number of mutations | 0.0048 | 1.2 | [1.1–1.5] | ns | — | — |
| Koteogics estimate |          |    |        |          |    |        |
| Any PD-1 ligand mutated | ns | — | — | 0.0246 | 3.0 | [1.2–7.7] |
| Any PD-1 ligand overexpressed | <0.0001 | 2.6 | [2–3.2] | 0.0067 | 2.0 | [1.2–3.4] |
| AID/APOBEC FAMILY |          |    |        |          |    |        |
| APOBEC7 mutated | ns | — | — | 0.0009 | 4.0 | [1.8–9.1] |
| APOBEC2 mutated | ns | — | — | 0.0057 | 4.7 | [1.6–14.1] |
| APOBEC1 amplified | 0.0461 | 1.2 | [1–1.3] | ns | — | — |
| APOBEC3 amplified | <0.0001 | 2.3 | [1.9–2.7] | ns | — | — |
| AICDA overexpressed | <0.0001 | 2.2 | [1.5–3.3] | ns | — | — |
| LymphocYTE MARKERS |          |    |        |          |    |        |
| CD3G overexpressed | <0.0001 | 2.0 | [1.5–2.8] | ns | — | — |
| CD4\textsuperscript{c} overexpressed | 0.0131 | 1.5 | [1.1–2] | ns | — | — |
| CD8A overexpressed | <0.0001 | 3.5 | [2.6–4.8] | ns | — | — |
| MS4A1 overexpressed | 0.0040 | 1.8 | [1.2–2.8] | ns | — | — |
| IL3RA overexpressed | 0.0025 | 1.6 | [1.2–2.1] | ns | — | — |
| CD3D overexpressed | <0.0001 | 2.1 | [1.5–2.9] | ns | — | — |
| IFN$\gamma$ overexpressed | <0.0001 | 3.7 | [2.7–5] | ns | — | — |

\textsuperscript{a}Alterations with a $p$ value $\leq 0.25$ in univariate analysis were selected for multivariate analysis, respecting each model (i.e., using only single factors or single and relevant combined factors). A Fifth-corrected logistic regression with backward elimination was performed until all factors influencing the model present a $p$ value $\leq 0.05$. Only factors with $p$ value $\leq 0.05$ for a positive association in multivariate analysis using combined models are shown.

\textsuperscript{b}Combined factors as outlined in Table S2. For instance, any MMR member altered, any APOBEC3 member overexpressed, any PD-1 ligand mutated and total number of CNV represent combined factors.

\textsuperscript{i}MSI-status was dichotomized as MSI-high versus MSI not-high (MSH or MSI-low) and unknown versus MSI not-high (MSH or MSI-low).

\textsuperscript{c}Abbreviations: AID (also called tumor mutation burden) and the expression of PD-1 ligand, which is often considered to be a predictive feature for response to immune checkpoint blockade mediated by agents, such as nivolumab, pembrolizumab or atezolizumab.\textsuperscript{12,13,19} Recently, several publications have reported a correlation between the presence of a large number of mutations (also called tumor mutation burden) and the expression of PD-L1.\textsuperscript{17,19} The aim of this study was to clarify, using a large collection of samples, the molecular and cellular factors associated with the overexpression of PD-1 ligands in tumor cells, especially as it pertains to the mechanisms leading to an accumulation of mutations in such cells, and this, independently of the cancer subtype.

While an association between mutation burden and increased PD-L1 or PD-L2 expression was confirmed in our pan-cancer dataset in univariate analysis, we show that, in multivariate analysis, PD-L1 and PD-L2 overexpression appears to be rather highly and independently correlated with Kataegis—a specific localized hyper-mutational process caused by the AID/APOBEC family of enzymes—as well as with overexpression of APOBEC3 members (Table 1 and Fig. 2). These results are consistent with the observation recently described in urothelial carcinoma, where the expression of APOBEC3F was associated with the expression of PD-L1 in tumor cells, while the expression of APOBEC3A, APOBEC3D and APOBEC3H was associated with the expression of PD-L1 in tumor infiltrating mononuclear cells.\textsuperscript{20}

The mechanisms by which APOBEC3 and PD-L1/2 overexpression are correlated may be explainable by several hypothesises (Fig. 3A–C). First, a viral infection may result in both overexpression of APOBEC3 and PD-1 ligands (Fig. 3A). The pathogen entry induces the secretion of cytokines (such as IFN$\gamma$) by immune cells in virally infected hosts, which in turn can increase both APOBEC3 and PD-L1 expression via...
transcriptional regulation. \textsuperscript{30} De facto, in our study, a strong positive association was found between IFN\(\gamma\) and PD-1 ligand expression (OR = 2.6, \(p < 0.0001\)), as well as between IFN\(\gamma\) and APOBEC3 expression (OR = 3.7, \(p < 0.0001\)). However, if the induction of APOBEC3 enzymes in response to a pathogen appears to be a mechanism of host defense (by interfering with proper replication of retroviruses), the induction of PD-1 ligand seems rather counter-intuitive as favoring immune tolerance and viral persistence. Indeed, the paradoxical overexpression of immune checkpoints has been described as a trigger of T-lymphocyte exhaustion after chronic pathogen exposure\textsuperscript{31} and a mechanism of tissue protection against early T-cell mediated damaging inflammatory events in various human non-infectious diseases and animal models.\textsuperscript{32,33} Also, one may think that upregulation of PD-L1 represents a second-step in the viral invasion mechanism, producing conditions that allow the virus natural reservoir’s persistence and continuous replication, and therefore, evading the host immune response. Indeed, it has been recently shown that viruses themselves can disrupt the 3’ region of the PD-L1 gene resulting in a marked elevation of PD-L1 transcripts.\textsuperscript{11} Another hypothesis to explain the concurrent overexpression of PD-1 ligand and APOBEC3 enzymes could relate to the existence of a common signaling cascade distinct from infectious processes (Fig. 3B). Initially, APOBEC3 members were called “phorbolins,” as their expression could be induced by the plant-derived compounds called phorbols.\textsuperscript{34} Interestingly, phorbol esters (e.g., phorbol-12-myristate-13-acetate (PMA), 12-O-Tetradecanoylphorbol-13-acetate (TPA)), are potent activators of the protein kinase C (PKC) used in biomedical research to promote carcinogenesis or stimulate T-cell activation, and have also been described as inducers of PD-1 ligand expression.\textsuperscript{35} Phorbols are tumor promoters that induce malignant transformation when associated with other carcinogens. They are considered as indirect DNA damaging agents by formation of superoxide radicals\textsuperscript{36} and thus may amplify the hyper-mutation signature observed in certain tumors. A third hypothesis that would explain the association observed between overexpression of APOBEC3 and PD-1 ligands could be based on a selection concept (Fig. 3C). If cells exhibiting high levels of mutation burden fail to upregulate PD-L1, they may be eliminated and hence not observed. As APOBEC enzymes and PD-1 ligands share the same pattern of expression, the latter offers the possibility for tumor cells harboring localized hyper-mutation patterns to escape the immune system. Of interest in this regard, recent data suggests that the relationship between prognosis and genomic instability as reflected by copy number alterations and mutation clonality in tumor cells is an inverted U shape—those individuals harboring tumors with the lowest and highest genomic instability do better than those with intermediate levels.\textsuperscript{37} The latter could be due to the fact that, when a cell presents a high genomic instability—regardless of the mechanism of mutagenesis—this highly modified genotype generates multiple neo-antigens, which leads to the activation of the immune system and the subsequent elimination of the cell. The only cells persisting in this setting are those producing factors such as PD-L1, allowing them to successfully evade immune elimination and thus form a tumor.\textsuperscript{38,39}

Interestingly, mutations within APOBEC3 genes appeared to be independently associated with PD-1 ligand overexpression along with, but not directly correlated with, APOBEC3 overexpression (Table 1). The exact mechanism linking the presence of mutations within APOBEC genes and the high expression
level of PD-L1 ligand remains to be elucidated, but suggests either a cause-and-effect relationship between the APOBEC enzyme functions and the expression of PD-L1 ligands, and/or supports the hypothesis that the observation of tumor cells concurrently harboring DNA damaging mutations and immune tolerance markers may reflect positive selection of tumor cells, as proposed by the clonal-evolution model (Fig. 3C). While a recent study reported an enrichment of kataegis-related mutation pattern in tumors presenting common APOBEC3 variants,40 our analysis shown that APOBEC3 mutations were linked to the total number of mutations in the tumor (Table 3B), and the latter and kataegis were associated with each other (Table 2). APOBEC3 mutations were also independently correlated with MSI, MMR alterations and POLE mutations (Table 3B). All these independent mutagenesis mechanisms contribute to the accumulation of genomic alterations in tolerated tumor cells and may explain, albeit perhaps not independently, the association observed between the high mutation burden and PD-L1 positive tumors.

As mentioned above, although tumor mutation burden correlated with PD-L1/2 ligand overexpression in univariate analysis, the overall mutation burden factor was not retained in our final multivariate model. Previous reports have described an independent relationship between PD-L1 expression and the number of mutations presented by a tumor,15,16,19 but APOBEC expression was not evaluated in these studies. Our results suggest that the factors that most significantly and independently correlated with PD-L1/2 overexpression relate to APOBEC dysregulation and the resulting kataegis mutational signature; and these factors are in turn independently linked to the overall mutation burden (Fig. 2). In this study, an additional factor independently related to PD-1 ligand overexpression was the presence of a mutation within the POLD1 gene, encoding the catalytic subunit of polymerase δ and likewise involved in tumor genomic instability.41 However, POLD1 mutations represent a rare tumor event (1.2% of all tumors) and the related POLE mutations were not associated with PD-L1 and/or PD-L2 overexpression in the multivariate analysis.

All predictive factors defined in our study have been extracted from a large pan-cancer cohort, regardless of the tumor histology. One might suggest evaluation of those markers among different diagnoses, as the genomic backgrounds and mutagenesis process exposures may differ between them (e.g., effect of UV radiation in melanoma, tobacco-related carcinogens in lung cancer, human papillomavirus in cervical cancer). Also, the rather low frequency of some of our variables (e.g., PD-1 ligand overexpression and APOBEC3 mutation are present in 5.5% and 2% of the tumors, respectively) may legitimately raise concerns regarding the goodness-of-fit of the described regression models. We specifically used statistical methods adapted to rare events, such as the Firth’s penalized-likelihood regression and internally evaluated the performance of our predictive models by using a re-sampling method, generating regression estimates for 1,000 replicates of n = 8,475 sample-size sets. Although the main conclusions of the study require external validation on an independent cohort, internal validation of our logistic models confirmed the key relationships existing between PD-1 ligands expression and all factors entered. Three factors, however, did not show a significant correlation when assessed by the bootstrapping method, despite their initial description in the primary models. These factors, namely the MSI phenotype, lymphocyte and macrophage infiltrate estimators, were evaluated only in specific tumor types and, therefore, exhibited a high rate of missing values (84.3%, 27.1% and 29%, respectively).

In conclusion, our study confirms the strong association between the expression of immune checkpoints and the presence of a high level of DNA instability, represented not only by the overall number of mutations, but more importantly by the unleashed activity of APOBEC enzymes. The observation of APOBEC3 variants (and not just APOBEC3 mRNA overexpression) independently associated with the overexpression of PD-1 ligands constitutes an argument in favor of a causative relationship. The concurrent observation of APOBEC function dysregulation and immune checkpoint expression may also be evidence for a positive selection process, by which late-stage tumors that have accumulated a high load of kataegis-related mutations are able to evade the immune system. It is already known that high tumor mutation burden, as well as PD-L1 overexpression, can correlate with better response to inhibitors of the PD1/PD-L1 axis.18 But the causative origin of such mutation burden, the relationship between these 2 factors, as well as the evaluation of AID/APOBEC markers by assessment of activity, genomic mutation and/or mRNA expression in human tumors merit a deeper investigation. Finally, the search for biomarkers that predict response is a central focus of the checkpoint inhibitor field.42 Once activated, it is crucial to know precisely, which neo-antigens are recognized by the immune system. Tumor mutational burden has emerged as an important predictor of response to immunotherapy, but mainly because a high number of mutations increases the likelihood of generating a neo-antigen which elicits immune reaction. However, it is clear that not all patients with high tumor mutational burden respond, and it is, therefore, crucial to determine the specific subset of mutations (and hence specific subset of neo-antigens) that elicit an immune reaction. As a kataegis-signature is found in more than 18.9% of the TCGA pan-cancer cohort, APOBEC alterations and the related kataegis-specific mutational load merits investigation in this regard, especially because of their association with high PD-L1 expression and viral infections.

Material and methods

Data retrieval and processing

Mutation, copy number variation, mRNA expression, protein expression, molecular phenotype (MSI and mutation burden estimates) data were downloaded from the community resource project TCGA, using the Broad GDAC Firehose website (https://gdac.broadinstitute.org/—standardized data run release 2016_01_28). A total of 11,245 samples corresponding to unique patients and related to 33 distinct tumor types were curated. All samples were published and available without restriction of use on the date of May, 1st 2016. All data used in this study respected the TCGA’s Human Subjects Protection and Data Access Policies (https://cancergenome.nih.gov/abouttcga/policies/tcga-human-subjects-data-policies).
Whole-genome level 2 somatic mutation data were available for 9,103 tumor samples (all cohorts except mesothelioma), and lists of significant variants were generated using the MutSig2CV algorithm (http://www.broadinstitute.org/cancer/tcga/mutsig), taking into account the somatic background mutation rate for each gene as well as neighbor genes.43 All non-synonymous missense, nonsense, non-stop, del/insertions, frameshift or splicing site mutations within the genes of interest (described in Table S2) were kept for the analysis.

Focal CNV corresponding to level 3 genome-wide single nucleotide polymorphism (SNP) array data were normalized and assessed at the gene-level using the GISTIC2 protocol (http://www.broadinstitute.org/tcga/about), where a “deep loss” was documented by the value (−2), a “single-copy loss” by the value (−1), a “low-level gain” by the value (+1) and an “amplification” by the value (+2). Both levels of losses or gains were integrated without distinction in the further analysis. A total of 10,845 tumor samples related to 33 cohorts were thereby implemented.

The sequencing-based mRNA expression (RNAseqV2, level 3) was available for 10,261 tumor samples (33 cohorts). Expression signals were integrated and normalized for each gene, per sample, using the protocol RSEM (RNA-Sequencing by Expectation Maximization).45 The standard score (z-score) for each gene per sample was calculated using the mean and standard deviation of values found in all similar tumors (same tumor type) that are diploid for the said gene. A z-score ≥2 standard deviations was used as threshold of overexpression, whereas a threshold ≤(−2) standard deviations was used to qualify underexpressed genes.

Microsatellite stability status was available for 1,909 samples, distributed in 7 cohorts (colon and rectal carcinoma, esophageal carcinoma, pancreatic adenocarcinoma, stomach adenocarcinoma, uterine corpus endometrial carcinoma and uterine carcinosarcoma). Tumors were designated as MSI-H, if there was instability in at least 3 of 7 markers (mononucleotides microsatellites BAT25, BAT26, BAT40, TGFB2R and dinucleotides microsatellites D2S123, D5S346, D17S250), as reflected by fragment analysis of tumor compared with normal tissue.

The total number of CNV (estimated in number of genes lost and number of genes gained), overall number of mutations and number of mutations related to APOBEC (i.e., respecting the CpG promoter sequence) were retrieved from the Broad GDAC Firehose website and correspond to analyses performed in August 2015 with the P-MACD (Pattern of Mutagenesis by APOBEC Cytidine Deaminases) calculation process.21,46

MLH1, MSH1, MSH6 and PMS2 protein immunostaining as well as lymphocyte and macrophage infiltration estimates obtained by hematoxylin and eosin staining (H&E) were downloaded from the NCI TCGA Data Portal interface (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp), and were available for 479; 7,774 and 7,514 samples, respectively.

**Study design**

A pan-cancer cohort including all unique samples presenting complete mutations, CNV and mRNA expression data description as processed above was used for the analysis (n = 8,475).

Missing values for microsatellite status and lymphocyte/macrophage infiltrate variables were categorized as “unknown” and included in the statistics. Factors of interest were sub-grouped by biological function, as shown in Table S2.

**Statistical analysis**

Association of all factors with PD-1 ligand overexpression (modeling the overexpression of either PD-L1 or PD-L2), the overall tumor mutation burden (modeling the presence of a high number of mutations, independently from their mechanism of origin), kataegis estimate (modeling the specific mutational activity of APOBEC members), APOBEC3 paralogs overexpression (modeling the overexpression of members APOBEC3A/B/C/D/F/G/H), or APOBEC3 paralogs mutations (modeling the presence of a mutation within the genes APOBEC3A/B/C/D/F/G/H) was assessed by univariate analysis using a Fisher’s exact test. A logistic regression based on all factors presenting a two-tailed p value ≤0.25 in the univariate analysis was run to assess the best set of predictors for each dependent factor. The Firth’s penalized-likelihood method (addressing issues of quasi-complete separation of data and low frequency events, as observed in this cohort) was used for model fitting, using both single factors and combined sets of co-variates. A backward elimination selection procedure was run until all significant factors were found; a p value ≤0.05 was considered statistically significant for final conclusions. The goodness-of-fit was assessed for each model by the McFadden’s pseudo-R2 (representing the percentage of outcome’s variability that is accounted for by the particular set of variables), re-scaled from 0% to 100% for ease of interpretation.27 Internal validation of the logistic regression models was conducted after bootstrapping of the initial cohort (random re-sampling with replacement method, generating 1,000 replicates of 8,475 sample-size sets).49 A graphical model expressing conditional dependences between variables was built using the series of regression models obtained.28

All statistical analyses were conducted by AB using SAS® University Edition software (http://support.sas.com/software/products/university-edition/).

**Disclosure of potential conflicts of interest**

RK has research funding from Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, and Guardant, as well as consultant fees from Sequenom and Actuate Therapeutics and an ownership interest in Novena, Inc. and CureMatch, Inc. IT has an ownership interest in CureMatch, Inc.

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**Author contributions**

Conception and design: AB, RK, IT. Development of methodology: AB, RK, IT. Acquisition of data: AB. Analysis and interpretation of data: AB, RK. Writing, review and revision of the manuscript: AB, RK. Editing and approval of final version: AB, IT, RK.
Data and materials availability
All the data used in this article were downloaded from the community resource project TCGA (https://gdac.broadinstitute.org/—standardized data run release 2016_01_28) and were available without restriction of use on the date of May 1, 2016. All analysis results are shown in Supplementary Materials.

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