

APOBEC3G Expression Correlates with T-Cell Infiltration and Improved Clinical Outcomes in High-grade Serous Ovarian Carcinoma

Brandon Leonard^{1,2}, Gabriel J. Starrett^{1,2}, Matthew J. Maurer³, Ann L. Oberg³, Mieke Van Bockstal⁴, Jo Van Dorpe⁴, Olivier De Wever⁵, Jozien Helleman⁶, Anieta M. Sieuwerts⁶, Els M.J.J. Berns⁶, John W.M. Martens⁶, Brett D. Anderson^{1,2}, William L. Brown^{1,2}, Kimberly R. Kalli⁷, Scott H. Kaufmann^{8,9}, and Reuben S. Harris^{1,2,10}

Abstract

Purpose: APOBEC3 DNA cytosine deaminase family members normally defend against viruses and transposons. However, deregulated APOBEC3 activity causes mutations in cancer. Because of broad expression profiles and varying mixtures of normal and cancer cells in tumors, including immune cell infiltration, it is difficult to determine where different APOBEC3s are expressed. Here, we ask whether correlations exist between APOBEC3 expression and T-cell infiltration in high-grade serous ovarian cancer (HGSOC), and assess whether these correlations have prognostic value.

Experimental Design: Transcripts for APOBEC3G, APOBEC3B, and the T-cell markers, CD3D, CD4, CD8A, GZMB, PRF1, and RNF128 were quantified by RT-qPCR for a cohort of 354 HGSOC patients. Expression values were correlated with each other and clinical parameters. Two additional cohorts were used to extend HGSOC clinical results. Immunoimaging was used to colocalize

APOBEC3G and the T-cell marker CD3. TCGA data extended expression analyses to additional cancer types.

Results: A surprising positive correlation was found for expression of APOBEC3G and several T cell genes in HGSOC. Immunohistochemistry and immunofluorescent imaging showed protein colocalization in tumor-infiltrating T lymphocytes. High APOBEC3G expression correlated with improved outcomes in multiple HGSOC cohorts. TCGA data analyses revealed that expression of APOBEC3D and APOBEC3H also correlates with CD3D across multiple cancer types.

Conclusions: Our results identify APOBEC3G as a new candidate biomarker for tumor-infiltrating T lymphocytes and favorable prognoses for HGSOC. Our data also highlight the complexity of the tumor environment with respect to differential APOBEC family gene expression in both tumor and surrounding normal cell types. *Clin Cancer Res*; 22(18): 4746–55. ©2016 AACR.

Introduction

Ovarian cancer is the deadliest gynecologic malignancy worldwide (1). The most common type of ovarian cancer, high-grade

serous ovarian carcinoma (HGSOC), accounts for over 60% of cases, and is the most aggressive reproductive tract malignancy (2). Because of the lack of efficient detection methods, HGSOC generally presents at advanced stages and is associated with high rates of recurrence and mortality. Interestingly, several studies have identified T-cell infiltration as a favorable prognostic factor for HGSOC (3–7). Additional markers of T-cell infiltration and particularly those reflecting high-quality antitumor responses are needed to fully realize the clinical impact of this finding.

The APOBEC enzymes are an 11-member family of zinc-coordinating enzymes that convert cytosines to uracils (C-to-U) in ssDNA (8). The enzymatic activity of specific family members is essential for both adaptive (AID) and innate immune responses [APOBEC3 subfamily members; ref. 9 and references therein]. AID is well studied because of its known roles in antibody diversification through somatic hypermutation and class switch recombination in B cells (10). Other well-studied family members include APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H because of demonstrated anti-HIV-1 activity (11). These enzymes are capable of introducing C-to-U lesions in viral cDNA intermediates that manifest as G-to-A mutations in proviral genomes (12–14). APOBEC3 subfamily members have also been implicated in restricting the replication of many other DNA-based parasites including transposable elements (ref. 9; and references therein). It is important to note that most APOBEC family members are expressed broadly and constitutively (12, 15–17),

¹Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota. ²Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota. ³Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, Mayo Clinic, Rochester, Minnesota. ⁴Department of Pathology, Ghent University Hospital, Ghent, Belgium. ⁵Department of Radiation Oncology and Experimental Cancer Research, Ghent University and Ghent University Hospital, Ghent, Belgium. ⁶Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, the Netherlands. ⁷Women's Cancer Program, Mayo Clinic Cancer Center, Rochester, Minnesota. ⁸Division of Oncology Research, Department of Oncology, Mayo Clinic, Rochester, Minnesota. ⁹Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota. ¹⁰Howard Hughes Medical Institute, University of Minnesota, Minneapolis, Minnesota.

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B. Leonard and G.J. Starrett contributed equally to this article.

Corresponding Author: Reuben S. Harris, Howard Hughes Medical Institute, University of Minnesota, 2231 6th Street South East, Minneapolis, MN 55455. Phone: 612-624-0457; Fax: 612-625-2163; E-mail: rsh@umn.edu

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Translational Relevance

Ovarian cancer is the deadliest cancer of the female reproductive tract, partly due to a lack of biomarkers for early clinical detection and assessment of prognosis. Several members of the APOBEC family of antiviral DNA cytosine deaminases, including *APOBEC3G*, are expressed broadly in human tissues, and *APOBEC3B* is overexpressed in many tumor types, including ovarian cancer. Here, we show that *APOBEC3G* is expressed at surprisingly high levels in T lymphocytes within high-grade serous ovarian tumors. *APOBEC3G* expression correlates positively with improved patient outcomes. The latter result is consistent with prior work demonstrating that T-cell infiltration correlates with improved outcomes. Our results are the first to demonstrate that high *APOBEC3G* levels in ovarian tumors are attributable to T-cell infiltration and not, for instance, expression in other normal cells or tumor cells. Thus, *APOBEC3G* is a new candidate biomarker for T-cell infiltration and positive antitumor immune responses.

but several also can be further upregulated by specific conditions such as *APOBEC3A* by interferon- α (18, 19), *APOBEC3B* by non-canonical NF- κ B activation and HPV infection (20–22), and *APOBEC3C*, *APOBEC3D*, *APOBEC3F*, *APOBEC3G*, and *APOBEC3H* by the combination of T-cell activation and HIV-1 infection (13, 14).

Although the APOBEC3 enzymes have been shown to have important physiologic roles in protecting cells from endogenous and exogenous DNA-based pathogens, their dysregulation has also been linked to cancer. For instance, AID has been linked to various B-cell malignancies (reviewed in ref. 23). A second prominent example is the recent finding that *APOBEC3B* is overexpressed and a significant source of mutation in breast, ovarian, and several other cancers (refs. 17, 24–27; and reviewed in refs. 28–30). *APOBEC3B* deaminates cytosines in genomic DNA to produce promutagenic uracil lesions, which can result in mutations if they are not repaired faithfully. *APOBEC3B* is overexpressed and its mutation signature is overrepresented in genomic sequences of HGSOC and other cancers. Elevated *APOBEC3B* expression levels have also been linked to poor prognosis in multiple cancer types (31–36).

In addition to *APOBEC3B*, related subfamily members have also been implicated in cancer mutagenesis to varying degrees (37–40). Because most APOBEC family members are expressed in many normal cell types and tissues, a major confounding factor in quantification of APOBEC expression levels in tumors is cellular heterogeneity due to surrounding normal tissue and immune cell infiltration. To address these issues, we quantified APOBEC expression in a large cohort of HGSOC patients and asked whether expression levels correlated with immune cell infiltration and could be distinguished from surrounding normal tissues. A strong positive correlation was identified between APOBEC3G expression and markers of T-cell infiltration, and co-expression was confirmed by immunohistochemical and immunofluorescent staining of primary HGSOC specimens. Moreover, high APOBEC3G expression levels correlated significantly with improved prognosis. These findings were extended to multiple cancer types through an analysis of publically available RNA sequencing (RNAseq) data from The Cancer Genome Atlas

(TCGA). Collectively, these results highlight the complexity of APOBEC3 family member expression in HGSOC specimens comprised of tumor cells, surrounding normal tissues, and in many instances infiltrating immune cells. Our studies are the first to identify APOBEC3G as a new candidate biomarker for effective T-cell responses and potentially for immunotherapies against HGSOC.

Materials and Methods

Analysis of expression correlations in Mayo Clinic cohort

Primary tumor samples from 354 HGSOC cases at the Mayo Clinic were selected on the basis of histology, grade, stage, and availability of clinical outcome data (IRB #13-002487). Following cryosectioning of each snap-frozen specimen, TRIzol-based RNA extractions were performed. cDNA was synthesized in triplicate using Transcriptor Reverse Transcriptase (Roche) and reverse transcription-quantitative PCR (RT-qPCR) for APOBEC3G, APOBEC3B, CD3D, CD4, CD8A, GZMB, PRF1, RNF128, and TBP was performed using the primer and probe combinations listed in Supplementary Table S1 (validation in Supplementary Fig. S1). Correlations between APOBEC3G, APOBEC3B, and the various T-cell markers were determined using Spearman's correlation. Spearman's correlation coefficient (r_s) and P values are reported.

Immunohistochemistry

IHC for CD3, CD4, CD8, and APOBEC3G was performed on 7 paraffin-embedded primary HGSOC specimens obtained from patients who underwent debulking surgery at Ghent University Hospital. This study was approved by the ethics committee of the Ghent University Hospital. The tissues were fixed in a 4% formaldehyde solution for 12 to 48 hours and embedded in paraffin. IHC was performed on 3.5- μ m tissue sections on Superfrost slides (Menzel-Gläser) using a Benchmark XT automated slide stainer (Roche), according to the manufacturer's instructions. The following antibodies were used: mouse monoclonal anti-CD3 (clone F7.2.38, dilution 1/10, Dako), rabbit monoclonal anti-CD4 (clone SP35, dilution 1/25, Cell Marque), mouse monoclonal anti-CD8 (clone C8/144B, no dilution, NeoMarkers), and a locally developed rabbit monoclonal anti-APOBEC3G (clone 5211-110-19, dilution 1/50). Although this rabbit monoclonal antibody recognizes APOBEC3G, APOBEC3A, and APOBEC3B due to unavoidable homology, we are confident that it is only detecting APOBEC3G in the immunohistochemistry studies described here because APOBEC3A is not expressed in T lymphocytes and, for reasons still under investigation, this monoclonal antibody does not recognize endogenous APOBEC3B by these procedures. It is important to emphasize that APOBEC3A is not expressed in most normal cell types, and it is only induced to detectable levels in interferon-activated myeloid lineage cells (12, 15, 17, 18). Moreover, comparatively few CD68-positive macrophages were detected in the HGSOC specimens described here and positive signals from APOBEC3A or APOBEC3G expression in this cell type are minor (data not shown). Heat-induced epitope retrieval was done using Cell Conditioning 1 (Roche) for CD3, CD8, and APOBEC3G, and using Cell Conditioning 2 (Roche) for CD4. Visualization of all primary antibodies was achieved with the ultraViewTM Universal DAB Detection Kit (Roche). Counterstaining with hematoxylin, dehydration of the tissue sections, and application of coverslips were carried out using an automated coverslipper (Tissue-Tek).

Immunofluorescent imaging

Fluorescence-based colocalization experiments were done using a subset of the same HGSOC specimens used above for IHC following published procedures (41). After sample preparation, permeabilization, and blocking with 10% goat serum in PBS at room temperature (DAKO X0907), each slide was stained first with rabbit polyclonal anti-APOBEC3G (Sigma Atlas Antibody HPA001812) diluted 1/50 in PBS (RT, 1 hour), second with mouse monoclonal anti-CD3 (described above) diluted 1/10 in PBS (4°C, overnight), and finally with a combination of secondary antibodies diluted 1/500 in PBS [RT, 1 hour; anti-rabbit IgG-AlexaFluor 594 (Invitrogen A11012) and anti-mouse IgG1-AlexaFluor 488 (Invitrogen A21121)]. Finally, slides were stained with DAPI diluted 1/5,000 in methanol (RT, 5 minutes), mounted with a cover slip, and imaged using a fluorescence microscope equipped with appropriate filters (Olympus BX40). Multiple PBS washes were done between each step of the procedures.

Survival analysis in Mayo Clinic cohort

Kaplan–Meier curves were constructed by dividing specimens at the median expression level for each gene. Overall survival (OS) data were available for all 354 patients, whereas progression-free survival (PFS) data were only available for 348 patients. *P* values, HRs, and 95% confidence intervals (CI) were determined using Cox regression models on the continuous log₂-transformed expression, adjusting for stage, and debulking status.

Survival analysis in additional cohorts

Ovarian cancer data from TCGA and Gene Expression Omnibus (GEO) were compiled by the 2015 version of the Kaplan–Meier plotter database on a PostgreSQL server (42). GEO accession numbers were GSE14764, GSE15622, GSE18520, GSE19829, GSE23554, GSE26193, GSE26712, GSE27651, GSE30161, GSE3149, GSE51373, and GSE9891. All gene expression data were determined using only the HG-U133A, HG-U133 Plus 2.0, and HG-U133A 2.0 Affymetrix microarray platforms so that comparisons could be made between datasets. Specifically, *APOBEC3G* expression was determined using the 204205_at probe. Grade 3 serous ovarian cancers were the only samples used in this analysis. OS data were available for 856 patients, whereas PFS data were available for 753 patients. Kaplan–Meier plots were constructed and *P* values, HRs, and 95% CIs were calculated using the Mantel–Cox log-rank test.

Medical ethics approval for the Dutch cohort was obtained in part previously ($n = 37$; ref. 43) and in part more recently ($n = 36$ and $n = 15$; MEC-2008-183). *APOBEC3G* mRNA levels were measured by RT-qPCR using an assay on demand (Hs00222415_m1, Applied Biosystems) and three reference genes were measured with the primers listed in Supplementary Table S1 and quantification using SYBR green. Relative *APOBEC3G* expression levels were determined by normalization to the average of 3 reference genes as described previously (31). Kaplan–Meier curves were constructed by dividing specimens at the median expression level for each gene. *P* values, HRs, and 95% CIs were determined using the Mantel–Cox log-rank test.

TCGA analysis

Normalized RNAseqV2 data were downloaded from TCGA in July 2015. *APOBEC3*, *CD3D*, and *CD20* mRNA levels were quan-

tified using normalized read counts. r_s and *P* values for linear models of *APOBEC3G* versus immune-marker genes were calculated using Spearman's rank correlation coefficient with the R statistical environment. Cancer types were grouped by hierarchical clustering (hclust) of the r_s values for each *APOBEC3G* family member in the R statistical environment, and these results were used to generate a dendrogram of these relationships. All data were graphed using the ggplot2 R package (44). *P* values were calculated from the r_s values and adjusted for multiple comparisons using the Benjamini–Hochberg method and significance was defined as a *P* value less than 0.05.

Results

APOBEC3G expression correlates with activated T lymphocyte infiltration in HGSOC

APOBEC3G is expressed constitutively in many different cell lines and tissue types and is also known to be upregulated by HIV-1 infection of primary T lymphocytes, which is one of many distinct mechanisms of immune cell activation (e.g., refs. 12–14). Although virus infection is unlikely to be part of the etiology of ovarian cancer (45), the presence of activated immune cells (infiltrate) in HGSOC is known to correlate with better overall outcomes, most likely due to strong anti-tumor immune responses (3–7). We therefore asked whether *APOBEC3G* expression correlated with T-cell infiltration and clinical outcomes using a cohort of 354 primary HGSOC samples procured at the Mayo Clinic (clinical characteristics in Table 1).

RNA was prepared from fresh-frozen HGSOC tissues, and a previously validated, highly specific RT-qPCR assay was used to assess *APOBEC3G* mRNA levels (12). In parallel, expression of the related gene *APOBEC3B*, which has been implicated in ovarian cancer genome mutagenesis (24), was assayed. In addition, the mRNA levels of several established T-cell markers were quantified, including *CD3D* (total T cells), *CD4* (helper T cells), *CD8A* (cytotoxic T cells), *GZMB* (activated cytotoxic T cells), *PRF1* (activated cytotoxic T cells), and *RNF128* (anergic T cells; primers and probes in Supplementary Table S1; refs. 46, 47).

Strong positive correlations were observed between *APOBEC3G* mRNA expression levels and *CD3D* ($P < 0.0001$, $r_s = 0.6159$), *CD4* ($P < 0.0001$, $r_s = 0.5825$), *CD8A* ($P < 0.0001$, $r_s = 0.6168$), *GZMB* ($P < 0.0001$, $r_s = 0.6591$), and *PRF1* ($P < 0.0001$, $r_s = 0.6422$; Fig. 1A–E). The lone exception was *RNF128* ($P = 0.7665$, $r_s = 0.0161$), which is a marker for T-cell anergy (48), suggesting that the positive signals emanate from *bona fide* activated T lymphocytes (Fig. 1F). Interestingly, *APOBEC3G*

Table 1. Clinical information for Mayo cohort ($n = 354$)

Tumor characteristic	Number
Morphology	
Serous	354 (100%)
Grade	
2	11 (3.1%)
3	343 (96.9%)
Stage	
1	14 (3.9%)
2	8 (2.3%)
3	253 (71.5%)
4	79 (22.3%)
Debulking status	
No residual disease	162 (45.8%)
≤1 cm remaining	144 (40.7%)
<1 cm remaining, possibly 0	48 (13.5%)

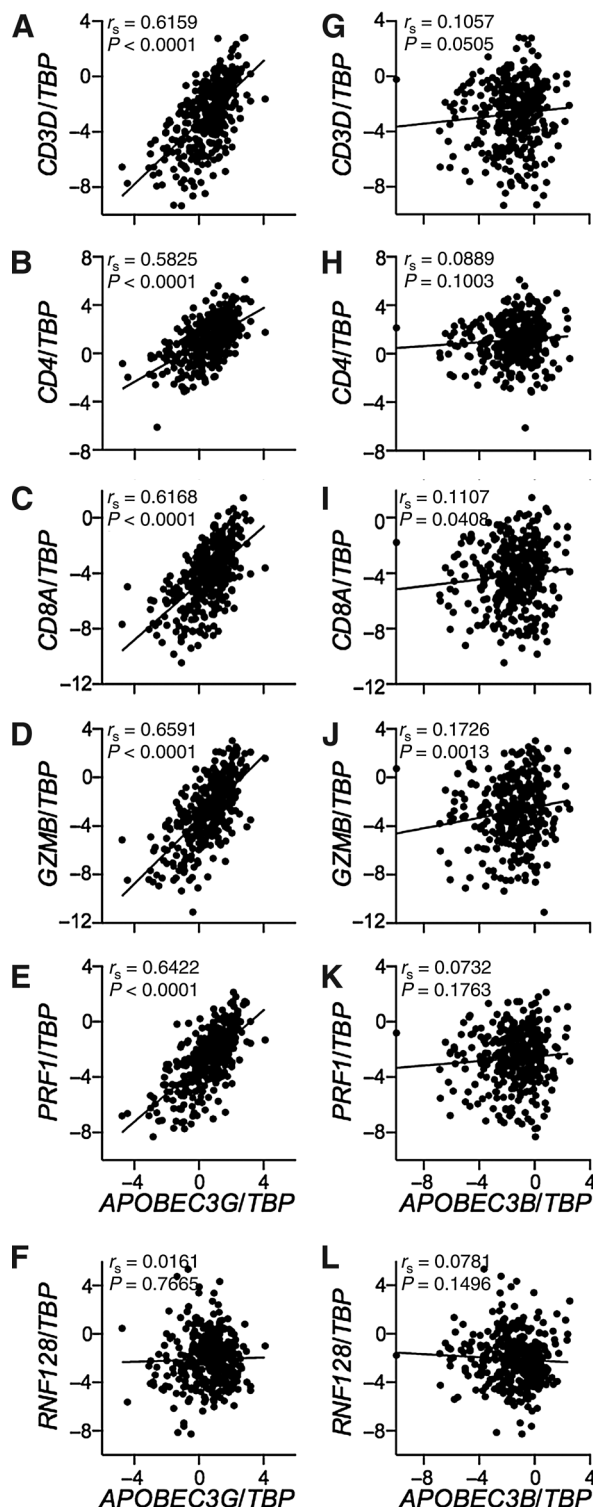


Figure 1. Correlations between *APOBEC3* expression and T-cell markers in HGSOc. Dot plots illustrating correlations between *APOBEC3G* (A–F) or *APOBEC3B* (G–L) expression and the indicated T-cell marker ($n = 354$). mRNA expression was determined using RT-qPCR and normalized to the housekeeping gene *TBP*. Spearman's correlation coefficients (r_s) and P values are shown. Best-fit lines are shown for qualitative comparison, and were calculated using linear regression models.

expression showed a similar positive correlation with *CD8A* and *CD4*, suggesting that *APOBEC3G* is expressed in both the cytotoxic and helper T-cell subsets (Fig. 1B vs. 1C). This result was corroborated by similarly strong positive correlations between *APOBEC3G* and two markers of CTL activation, *GZMB* and *PRF1* (Fig. 1D and E). In contrast, weaker and less significant correlations were found between *APOBEC3B* and the expression of any of these T-cell genes (Fig. 1G–L). This result was expected, however, because prior studies have indicated that *APOBEC3B* is only expressed at very low levels in normal tissues and upregulated in tumor cells (12, 17, 24, 25). Taken together, our data confirm prior studies documenting T-cell infiltration in HGSOc (3–7) and, importantly, reveal an unanticipated association between high levels of *APOBEC3G* expression and CTL activation in HGSOc. This result for *APOBEC3G* was especially unexpected given its broad expression profile documented previously by several groups including our own (12, 15–17).

APOBEC3G protein visualization in infiltrating T lymphocytes in HGSOc

To determine whether *APOBEC3G* protein expression colocalizes with the same T-cell markers analyzed above at the mRNA level by RT-qPCR, we performed hematoxylin staining and immunohistochemistry for CD3, CD4, CD8, and *APOBEC3G* on seven unrelated HGSOc samples (representative image sets in Fig. 2A–T with hematoxylin-eosin staining in Fig. 2A, F, K, and P). As expected from the RT-qPCR analysis above, several of these additional tumor specimens showed clear evidence for T lymphocyte infiltration. Two HGSOc lesions contained low expression of all examined T-cell markers (representative images for CD3, CD4, and CD8 in Fig. 2B–D) and correspondingly low levels of *APOBEC3G* expression (representative image in Fig. 2E). In contrast, two tumors showed extensive T lymphocyte infiltration (representative images for CD3, CD4 and CD8 in Fig. 2G–I, L–N, and Q–S). Interestingly, there was a strong colocalization between these markers and the expression of *APOBEC3G* in these tumors (representative image in Fig. 2J, O, and T). Moreover, different regions of a single HGSOc can be heterogenous, with one region showing dense clusters of *APOBEC3G*-high infiltrating T cells and another showing a more dispersed distribution (compare Fig. 2F–J and K–O). The remaining three samples showed moderate expression of both infiltrating T-cell markers and *APOBEC3G*, and colocalization was still observed (data not shown). These immunohistochemical experiments confirm the above correlations and show that *APOBEC3G* is indeed expressed at the protein level within tumor infiltrating T lymphocytes.

To directly test whether *APOBEC3G* is expressed within tumor infiltrating T lymphocytes, we performed a series of fluorescence-based colocalization experiments with CD3 and *APOBEC3G*. Here, we were only able to image well-isolated infiltrating T lymphocytes because groups of cells caused excessive background fluorescence. Nevertheless, these experiments enabled us to demonstrate for the first time that CD3 and *APOBEC3G* are indeed co-expressed in the same T lymphocyte (representative image in Fig. 2U–X). Moreover, the DAPI co-stain and $\times 1,000$ total magnification combine to show that both proteins are excluded from the nuclear compartment and predominantly cytoplasmic (the additional cell surface localization of CD3 is more difficult to visualize in tissue cross sections).

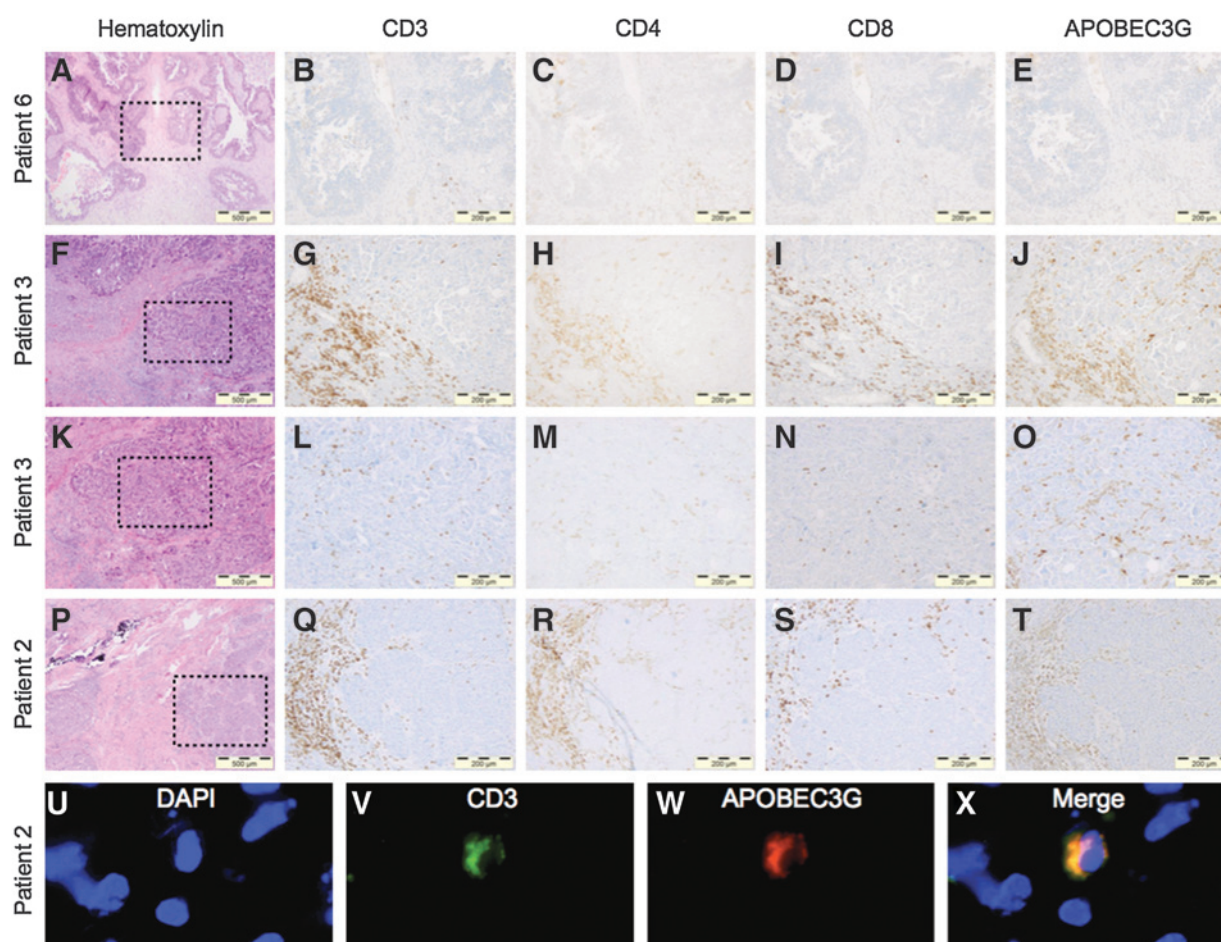


Figure 2.

Immunohistochemistry and immunofluorescence of T-cell markers in HGSOc. Photomicrographs of immunohistochemistry and immunofluorescence staining performed on HGSOc specimens illustrating the association between levels of T lymphocyte infiltration and the intensity of APOBEC3G expression. Representative staining of one HGSOc specimen with low (patient 6) and three staining sets from two HGSOc specimens with high (patients 3 and 2) levels of T-cell infiltration are shown. The images depict hematoxylin-eosin (A, F, K, and P), CD3 (B, G, L, and Q), CD4 (C, H, M, and R), CD8 (D, I, N, and S), and APOBEC3G (E, J, O, and T). The dotted box in the $\times 40$ hematoxylin images indicates the approximate location of the subsequent panels at $\times 100$ magnification. The bottom row shows representative $\times 1,000$ magnification images of colocalization of CD3 and APOBEC3G by immunofluorescent staining. DAPI-stained nuclei are blue.

***APOBEC3G* is a candidate biomarker for improved HGSOc patient outcomes**

Long-term clinical follow-up data were available for all of the Mayo Clinic HGSOc patients. The aforementioned gene expression data were, therefore, correlated with clinical information to determine whether *APOBEC3G* expression levels predict the length of PFS and/or OS in HGSOc. As a positive control, the T-cell markers above were also analyzed with respect to clinical information. Kaplan–Meier plots were constructed by splitting each gene-expression dataset at the median to create high- and low-expression groups (Fig. 3A and B). *P* values, HRs, and 95% CIs were determined using Cox regression models on the $\log_2$ -transformed expression that were corrected for stage and debulking status (Table 2).

Higher expression levels of *CD3D* ($P = 0.020$; HR, 0.94; 95% CI, 0.90–0.99), *CD4* ($P = 0.0046$; HR, 0.90; 95% CI, 0.84–0.97), *CD8A* ($P = 0.0053$; HR, 0.93; 95% CI, 0.88–0.98), *GZMB* ($P = 0.011$; HR, 0.94; 95% CI, 0.90–0.99), and *PRF1* ($P = 0.0049$; HR, 0.91; 95% CI, 0.86–0.97) were all associated with

improved PFS (Fig. 3A and Table 2). As expected, *RNF128* ($P = 0.43$; HR, 0.97; 95% CI, 0.91–1.04) did not correlate with PFS (Fig. 3A and Table 2). Interestingly, *APOBEC3G* ($P < 0.0001$; HR, 0.81; 95% CI, 0.73–0.89) surpassed all of these genes as the most indicative marker of improved PFS in HGSOc (Fig. 3A). The results compiled from an analysis of OS largely mirrored those of PFS (Fig. 3B and Table 2).

Next, we used Kaplan–Meier plotter (kmplot.com) to generate a validation cohort using HGSOc data from TCGA and GEO. In this composite analysis of HGSOc patients, high levels of *APOBEC3G* correlated with improved durations of PFS ($P = 0.0057$; HR, 0.78; 95% CI, 0.65–0.93) and OS ($P = 0.063$; HR, 0.84; 95% CI, 0.70–1.01; Supplementary Fig. S2). Although only the former correlation reached statistical significance, the latter trended toward significance and also supported the observations above with the HGSOc Mayo Clinic cohort. A larger degree of variation is to be expected in this composite cohort because clinical variables are more difficult to take into account.

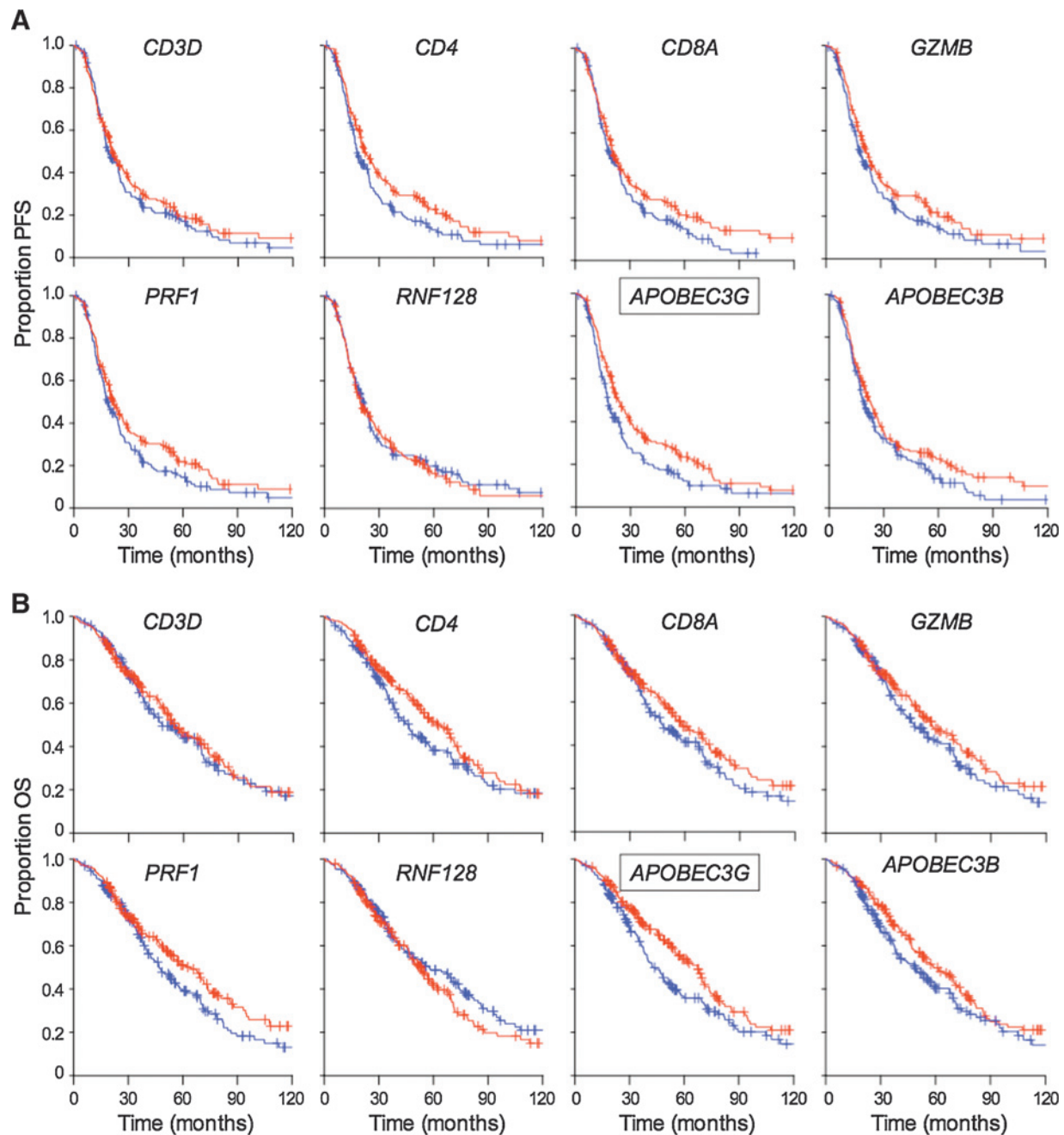


Figure 3. Clinical correlates of T-cell marker expression in HGSOC. Kaplan-Meier plots illustrating associations between PFS (**A**, $n = 354$) or OS (**B**, $n = 348$) and either one of the conventional T-cell markers or *APOBEC3G* or *APOBEC3B* expression in the Mayo cohort of patients. Samples were split at the median expression level for each gene with red representing tumors with high and blue representing tumors with low mRNA levels.

Finally, we analyzed HGSOC specimens from a Dutch cohort consisting of 88 patient samples (clinical information in Supplementary Table S2). *APOBEC3G* expression was quantified using an independent RT-qPCR strategy (housekeeping gene primer sequences in Supplementary Table S1 and see Materials and Methods for details). Again, *APOBEC3G* expression levels associated with improved OS and more weakly with PFS (Supplementary Fig. S2).

APOBEC3B expression levels do not associate with HGSOC outcomes

APOBEC3B has been implicated recently as an endogenous mutagen in several cancers (refs. 28–30; and references therein), including ovarian cancer (24). Moreover, its overexpression has been linked to poor patient outcomes in multiple cancer types (31–36). Using the Mayo cohort gene-expression data and clinical information from above, we asked whether *APOBEC3B* affects

Table 2. Mayo cohort Cox regression analysis

Gene	Parameter ^a	P value	HR ^b (95% CI) ^c
CD3D	PFS	0.020	0.94 (0.90–0.99)
CD3D	OS	0.087	0.95 (0.90–1.01)
CD4	PFS	0.0046	0.90 (0.84–0.97)
CD4	OS	0.018	0.91 (0.84–0.98)
CD8A	PFS	0.0053	0.93 (0.88–0.98)
CD8A	OS	0.015	0.93 (0.87–0.99)
GZMB	PFS	0.011	0.94 (0.90–0.99)
GZMB	OS	0.047	0.95 (0.90–1.00)
PRF1	PFS	0.0049	0.91 (0.86–0.97)
PRF1	OS	0.018	0.92 (0.86–0.99)
RNF128	PFS	0.43	0.97 (0.91–1.04)
RNF128	OS	0.44	0.97 (0.91–1.04)
APOBEC3G	PFS	<0.0001	0.81 (0.73–0.89)
APOBEC3G	OS	0.0003	0.82 (0.73–0.91)
APOBEC3B	PFS	0.034	0.92 (0.85–0.99)
APOBEC3B	OS	0.06	0.93 (0.87–1.00)

^aPFS and OS (*n* = 354 and *n* = 348, respectively).
^bHR, hazard ratio.
^cCI, confidence interval.

patient outcomes in HGSOc. These analyses revealed a trend toward high *APOBEC3B* and improved, rather than worsened, PFS and OS outcomes, although these relationships were less significant statistically than those for *APOBEC3G* (Fig. 3).

APOBEC expression correlates with immune cell markers in multiple human cancers

To extend our findings from HGSOc to additional human cancers, publicly available RNAseq data from TCGA were analyzed to determine whether correlations exist between expression of *APOBEC* genes and immune cell markers. At the time of these analyses, the TCGA had RNAseq data available for 7,861 samples spanning 22 different tumor types (details in Supplementary

Table S3). For each tumor type, expression of each *APOBEC* family member was quantified and correlated with the T-cell marker *CD3D* (Fig. 4; top heatmap). Hierarchical clustering was also performed to elucidate similar correlation patterns between cancer types (Fig. 4A top). These analyses revealed that, in addition to *APOBEC3G*, *APOBEC3D*, and *APOBEC3H* also correlated significantly with *CD3D* across multiple cancer types. *APOBEC3F*, also known to be expressed broadly and in T cells (12, 13, 15–17), did not correlate as strongly. The same analysis was also performed with *CD20*, a well-known marker for B cells (Fig. 4 bottom heatmap). The expression of the antibody diversification gene, *AID*, was the only *APOBEC* family member that significantly correlated with *CD20* in a majority of cancer types (Fig. 4B). These analyses indicate that much of the expression of several *APOBEC* family members in cancer is likely due to T- and B-cell infiltration.

Discussion

Our studies have led to the surprising identification of *APOBEC3G* as a new candidate biomarker for activated T lymphocyte infiltration in HGSOc and improved patient outcomes. This result was unexpected because prior work has shown that *APOBEC3G* is expressed broadly, constitutively, and in some instances inducibly (12, 13, 15–17). The analysis of a cohort of 354 HGSOc patients also identified a strong correlation between *APOBEC3G* and several markers of T-cell infiltration (Fig. 1A–E). These results were validated at the protein level by immunohistochemistry and immunofluorescent staining of independent HGSOc tumor samples (representative image sets in Fig. 2). Clinical data revealed that *APOBEC3G* also associates with improved outcomes in a large HGSOc cohort as well as in two additional independent ovarian cancer cohorts (Table 2, Fig. 3, and Supplementary Fig.

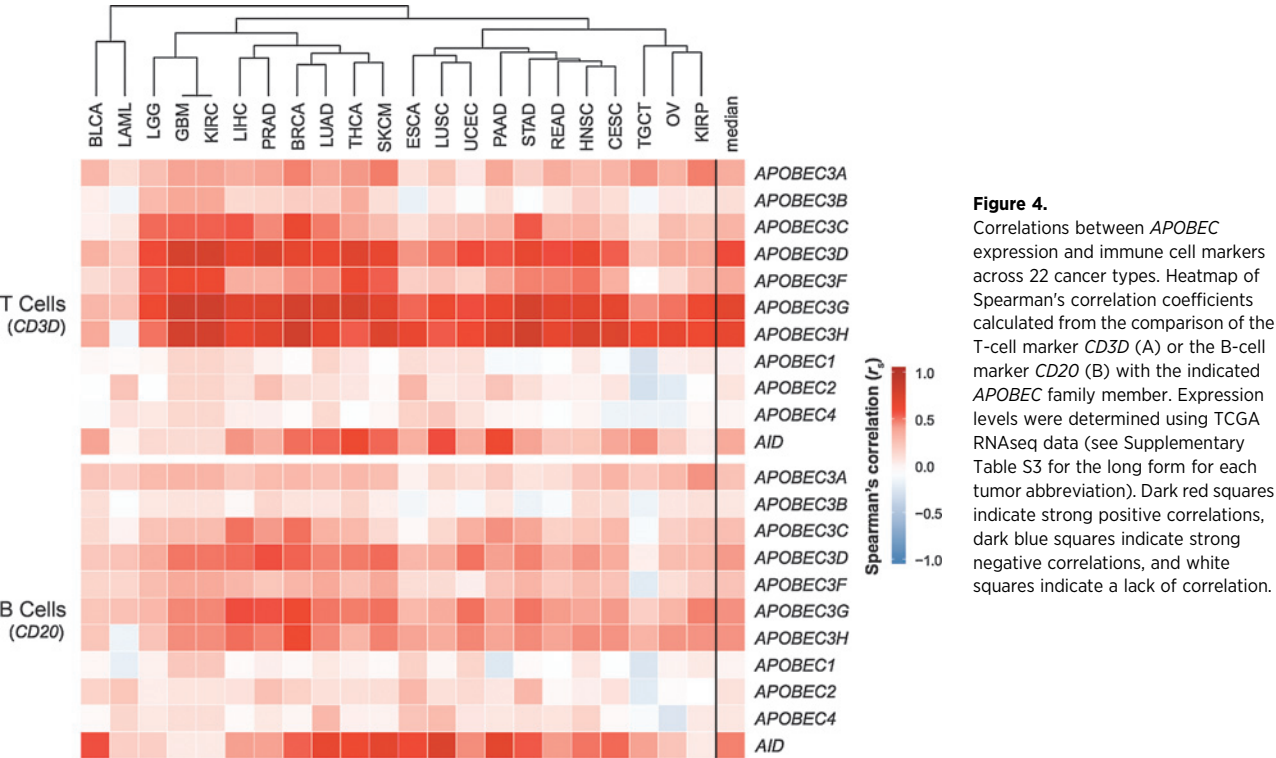


Figure 4. Correlations between *APOBEC* expression and immune cell markers across 22 cancer types. Heatmap of Spearman's correlation coefficients calculated from the comparison of the T-cell marker *CD3D* (A) or the B-cell marker *CD20* (B) with the indicated *APOBEC* family member. Expression levels were determined using TCGA RNAseq data (see Supplementary Table S3 for the long form for each tumor abbreviation). Dark red squares indicate strong positive correlations, dark blue squares indicate strong negative correlations, and white squares indicate a lack of correlation.

S2). Finally, a global analysis of gene expression in 22 cancer types identified a similar correlation for two additional *APOBEC3* genes, *APOBEC3D* and *APOBEC3H*, and a marker of T cells, *CD3D* (Fig. 4). Together, these data suggest that *APOBEC3G* expression levels in tumor infiltrating T lymphocytes may be a predictive biomarker for strong anticancer T-cell responses and improved HGSOC outcomes.

Over the past several years, substantial evidence has accumulated to indicate that *APOBEC3B* and *AID* contribute to cancer genome mutagenesis. *APOBEC3B* is thought to mutate the genomes of several different cancer types, including breast, lung, bladder, cervical, head/neck, and ovarian (17, 24–27). The carcinogenic effect of *AID* appears more limited, as its characteristic deamination signature has only been found in certain types of B-cell leukemias and lymphomas (23, 27). The idea that *AID* expression is constrained to B lineage cell types is consistent with our data showing that *AID* mRNA expression correlates with *CD20* mRNA expression in several solid tumor types (Fig. 4 bottom heatmap). In addition to *APOBEC3B* and *AID*, several other *APOBEC* family members have also been implicated in carcinogenesis (37–40). For example, it has been proposed that *APOBEC3G* drives hepatocellular carcinoma tumorigenesis (37). This idea, however, is difficult to reconcile with our observation that *APOBEC3G* mRNA expression in HGSOC and other tumor types correlates with the expression of activated T lymphocyte markers and, at least for HGSOC, this strong correlation could be validated visually at the protein level (Figs. 1, 2, and 4). Additional studies are clearly warranted to extend the *APOBEC3G* immunohistochemistry results described here to other tumor types, including hepatocellular carcinoma.

We were also surprised by the analysis of *APOBEC3B* in HGSOC, which revealed no significant clinical correlations and even a slight trend in the Mayo Clinic cohort toward better OS (Fig. 3). This finding differs significantly from estrogen receptor (ER)-positive breast cancer, where high *APOBEC3B* expression associates with shorter periods of disease-free survival and poorer rates of OS (31–34). A previous deep-sequencing study highlighted the similarities between HGSOC and another type of breast cancer, triple negative breast cancer (TNBC; ref. 49), and, also found no correlation between *APOBEC3B* expression and survival in TNBC (32). One major difference between these two cancer types and ER-positive breast cancer is therapeutic options. There are multiple targeted therapeutics available for the treatment of ER-positive breast cancer that are administered on the basis of molecular markers. In contrast, nearly all HGSOC and many TNBC patients are treated with platinum-based therapies. Because platinum-based therapies induce DNA damage, it is possible that these drugs become synergistic with *APOBEC3B*-catalyzed cytosine deamination and create a synthetic lethal state in cancer cells. This speculation is plausible because increased mutation loads have been shown to correlate with improved clinical outcomes in HGSOC patients treated with cisplatin (50). Furthermore, a synergistic effect created by these two forms of DNA damage could explain the slight trend found here toward a positive correlation between increased *APOBEC3B* expression and improved outcomes. Another possible explanation is that the levels of *APOBEC3B* mutagenesis in ovarian cancer may be not high enough to manifest clinically. Indeed, the *APOBEC3B* mutation signature is not as strong in ovarian cancer as it is in many other cancer types despite similar mRNA and protein expression

levels (17, 21, 24–27). The underlying causes for this apparent discordancy are unknown, but several factors could be involved, including altered DNA repair capacities, differential protein regulation, and mutational contributions from other sources. More work is needed to determine the threshold of *APOBEC3B* mutagenesis required for a clinical impact.

Our global analysis of TCGA data revealed that the correlation between *APOBEC3F* and *CD3D* was substantially diminished as compared with *APOBEC3D*, *APOBEC3G*, and *APOBEC3H* (Fig. 4). This is surprising because *APOBEC3F* is thought to be broadly expressed and play an equally important role as *APOBEC3D*, *APOBEC3G*, and *APOBEC3H* in HIV-1 restriction in CD4-positive T lymphocytes, and prior studies have found that *APOBEC3F* is expressed at comparable levels to *APOBEC3D* and higher than *APOBEC3H* in human primary CD4⁺ T cells (12–14). Our data suggest that *APOBEC3F* may be under- and/or heterogeneously expressed in T cells associated with the tumor microenvironment. These differences are potentially important, as the overall *APOBEC* family member expression profile may be a useful property for distinguishing antiviral from antitumor immune responses. It will be interesting in future studies to determine whether *APOBEC3G*, the overall *APOBEC* family expression profile, *APOBEC* mutation signature, and/or classical markers for T-cell activation are the best predictors of successful clinical responses to immunotherapies for HGSOC as well as for other tumor types.

Disclosure of Potential Conflicts of Interest

J.W.M. Martens reports receiving a commercial research grant from Philips Research. R.S. Harris has ownership interest (including patents) in and is a consultant/advisory board member for ApoGen Biotechnologies Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: B. Leonard, G.J. Starrett, A.L. Oberg, S.H. Kaufmann, R.S. Harris

Development of methodology: B. Leonard, M. Van Bockstal, J. Van Dorpe, O. De Wever, A.M. Sieuwerts, R.S. Harris

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Leonard, G.J. Starrett, M. Van Bockstal, J. Van Dorpe, J. Helleman, A.M. Sieuwerts, E.M.J.J. Berns, J.W.M. Martens, B.D. Anderson, W.L. Brown, K.R. Kalli

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Leonard, G.J. Starrett, M.J. Maurer, A.L. Oberg, A.M. Sieuwerts, E.M.J.J. Berns, B.D. Anderson, R.S. Harris

Writing, review, and/or revision of the manuscript: B. Leonard, G.J. Starrett, M.J. Maurer, A.L. Oberg, M. Van Bockstal, J. Van Dorpe, A.M. Sieuwerts, J.W.M. Martens, K.R. Kalli, S.H. Kaufmann, R.S. Harris

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Leonard, M. Van Bockstal, W.L. Brown, K.R. Kalli

Study supervision: B. Leonard, R.S. Harris

Other (connected the research teams): J.W.M. Martens

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