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Summary

Background. Anti-programmed death-1 (PD-1) treatment for advanced non-small-cell lung cancer (NSCLC) has improved the survival of patients. However, a significant percentage of patients do not respond. We examined the use of DNA methylation profiles to determine the efficacy of anti-PD-1 treatment in stage IV NSCLC patients.

Methods. We first established an epigenomic profile based on a microarray DNA methylation signature (EPIMMUNE) in a discovery set of 34 NSCLC tumor samples treated with nivolumab or pembrolizumab. The EPIMMUNE signature was validated in an independent set of 47 NSCLC cases. A derived DNA methylation marker was validated by a single-methylation assay in a validation cohort of 61 NSCLC patients.

Findings. The EPIMMUNE signature in stage IV NSCLC patients treated with anti-PD-1 agents was associated with improved progression-free survival (PFS) (hazard ratio [HR]=0.010, P=0.007; 95% confidence interval [CI]=$3.29x10^{-4}$-0.0282) and overall survival (OS) (HR=0.080, P=0.001; 95% CI=0.017-0.373). The EPIMMUNE-positive signature was not associated with PD-L1 expression, the presence of CD8+ cells, or mutational load. EPIMMUNE-negative tumors were enriched in tumor-associated macrophages (TAMs), neutrophils (TANs), cancer-associated fibroblasts (CAFs) and senescent endothelial cells. The EPIMMUNE-positive signature was associated with improved PFS in a validation cohort (HR=0.330, P=0.006; 95% CI=0.149-0.727). The unmethylated status of T-cell differentiation factor forkhead box P1 (FOXP1) was associated with improved PFS (HR=0.415, P=0.006; 95% CI=0.209-0.802) and OS (HR=0.409, P=0.009; 95% CI=0.220-0.780) in a validation cohort. The EPIMMUNE signature and unmethylated FOXP1 were not associated with clinical benefit in lung tumors that did not receive immunotherapy.

Interpretation. The need to discover predictive biomarkers of response to immune checkpoint inhibition is now pressing. Our study shows that the epigenetic milieu of NSCLC tumors indicate which patients are most likely to benefit from nivolumab or pembrolizumab treatments.

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Research in context

Evidence before this study

There have been significant advances in the clinical care of patients with advanced non-small-cell lung cancer (NSCLC) in recent years arising from the introduction of immunotherapies that target the programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) pathway. Several cellular, immunohistochemical, mutational and expression-based approaches have been proposed to predict response to immune checkpoint inhibition. However, our search of PubMed on April 24th 2018, limited to articles in English, but not by date, using the terms “non-small-cell lung cancer”, “immunotherapy”, “PD-1”, “PD-L1”, “nivolumab”, “pembrolizumab”, “epigenetic”, “DNA methylation, “response”, “biomarker” and “prediction”, yielded no studies addressing whether the presence of epigenetic signatures can identify responders to anti-PD-1 therapy.

Added value of this study

Our findings show that a particular epigenetic profile, termed EPIMMUNE, based on DNA methylation microarrays, is associated with improved progression-free survival and overall survival in stage IV NSCLC patients who received the anti-PD-1 antibodies nivolumab or pembrolizumab. The DNA methylation signature described reflects that the response to PD-1 blockade occurs mainly in immunocompetent primary tumors characterized by particular intrinsic (cancer cell) and extrinsic (microenvironment) settings. The EPIMMUNE signature did not confer any clinical benefit on patients who were not treated with immunotherapy. Among the targets identified in the epigenomic profile, we confirmed that the determination of the unmethylated status of the regulatory T-cell transcription factor FOXP1 is a user-friendly predictor of clinical benefit for anti-PD-1 therapies in advanced NSCLC.

Implications of all the available evidence

This study has identified new biomarkers of clinical response to anti-PD-1 antibodies in NSCLC that can be determined at a global epigenomic level, or simplified at a single methylation locus. The approach can be easily complemented with other strategies, such as PD-L1 or CD8 immunostaining, or mutational load, to identify more accurately those patients who will experience an improved outcome upon PD-1 blockade. The study also identifies cellular components and signaling pathways that, were they accurately targeted, could strengthen the response of resistant patients to immune checkpoint inhibition.

Introduction

Non-small-cell lung cancer (NSCLC) is the primary cause of cancer-associated deaths worldwide.[1] Most patients are diagnosed with metastatic disease, and although systematic therapy for NSCLC has improved with the introduction of drugs targeted against actionable mutations, only a minority of patients carries these amenable genetic targets, and their tumors eventually become resistant to such treatments.[2] Thus, the development of new treatments to improve the outcome of patients with advanced NSCLC is an unmet medical need. In this context, immunotherapy represents an important advance in the management of metastatic NSCLC patients. The use of drugs targeting the programmed death 1 (PD-1) receptor or its ligand, PD-L1, as monotherapy has given rise to a manageable safety profile and notable clinical efficacy, with an unprecedented increase in OS, even in NSCLC patients whose disease progressed while receiving platinum-based chemotherapy.[3-6] PD-1 blockade have also shown to provide clinical benefit as first line therapy in NSCLC.[7,8] These findings have led to the approval of three drugs for patients with advanced NSCLC, two of which target PD-1 (nivolumab and pembrolizumab) and one of which target PD-L1 (atezolizumab).[3-8]

PD-1 is an immune checkpoint receptor expressed in activated B- and T-cells that, through binding to its PD-L1 ligand on cancer cells, inhibits T-cell activation, leading to immune suppression.[9] Thus, activation of the PD-1 / PD-L1 pathway is one mechanism exploited by human malignancies, including NSCLC tumors, to evade immune system control.[9] However, we know very little about the molecular steps involved in avoiding immune surveillance in cancer cells and the repertoire of immunocompetent and immunocompromised tumor types. For example, only 10-30% of unselected NSCLC patients respond to PD-1 blockade by nivolumab.[3,4] Studies suggest that the genetic setting of the tumor, such as one in which a high mutational burden generates numerous neoantigens and the expression of PD-L1 increase the likelihood that a patient will respond to anti-PD-1 / PD-L1.[10,11] However, although PD-L1 expression is enriched in immunotherapy responders,[10-13] the predictive power of PD-L1 is far from perfect. For example, only 44.8% of PD-L1-positive NSCLCs are responsive to pembrolizumab in a first-line setting.[12] Thus, new predictive biomarkers are clearly necessary if we are to be able to select those PD-1 / PD-L1 blockade-responsive NSCLC patients who should receive immunotherapy, and to distinguish them from patients for whom an expensive and, to some degree, toxic drug will not produce any clinical benefit.

For these reasons, we set out to determine whether a profile of DNA methylation, a stable epigenetic chemical mark critical for many cellular activities and disrupted in human

disease,[15,16] and one that is used in the clinical management of gliomas[15] and cancers of unknown primary,[17] is able to predict the clinical response to PD-1 checkpoint blockade in NSCLC patients.

METHODS

Patients and samples

Patients were eligible to enter the study as a member of either the discovery or validation cohort if they had a histologically proven NSCLC and had undergone tumor sampling in any of the participating institutions. The patients had to have been exposed to PD-1 blockade during the course of their disease. The samples were assembled according to the time shedule, first samples in the discovery cohort, later samples for the validation cohorts. The clinical characteristics of the NSCLC samples obtained are summarized in **Table 1**. Patients gave their informed consent to participate in the research, which had received ethical approval from the review board of each institution. The samples were collected after approval outside of any clinical trial. Tumors were collected from patients by surgical resection, CT-guided biopsy or bronchial biopsy. The sampling had to take place when the patient was naïve to any anti-neoplastic treatment for advanced disease, including immunotherapy. Only adjuvant chemotherapy or chemotherapy associated with radiotherapy was accepted, in case of relapse after curative surgery or chemo-radiotherapy. Follow-up involved clinical examination, computed tomography scans or brain magnetic resonance imaging. Radiological assessments for response or progression to immunotherapy were performed according to institution standards, every 3 or 4 cycles. NSCLC tumor samples were studied in order of arrival at the centralized DNA methylation facility, once they had passed the checks for technical quality.

Histopathological evaluation and exome sequencing

Histology-guided tumor-type classification of NSCLCs involved review by a pathologist of the tumor's morphological appearance under light microscopy, and of the immunohistochemical (IHC) findings. PD-L1 staining and CD8 content was evaluated as previously described.[18] Whole-exome sequencing was performed on FFPE genomic DNA using Roche's SeqCap EX MedExome Enrichment Kit, following the manufacturer's recommendations. Prepared libraries were sequenced in a HiSeq 2000 instrument (Illumina) with paired-end 100-base reads.

Samples were multiplexed to obtain a raw minimum coverage of 70X. Extended information can be found at **Supplementary Methods**.

DNA methylation procedures and analysis

The DNA methylation status of the discovery cohort was established using bisulfite-converted DNA processed by the Infinium FFPE restoration process and then hybridized on an Infinium MethylationEPIC Array (~850,000 CpG sites) following the manufacturer's instructions for the automated processing of arrays with a liquid handler (Illumina Infinium HD Methylation Assay Experienced User Card, Automated Protocol 15019521 v01).[19] The status of CpG methylation at the FOXP1 CpG site derived from the Infinium MethylationEPIC Array was determined by pyrosequencing analyses. Extended information can be found at **Supplementary Methods**.

Cell enrichment quantification analysis

The beta values for the 301 CpGs of the identified EPIMMUNE signature were used to evaluate its possible individual enrichment among different immune and stroma cell populations where DNA methylation data are available. After determining the methylation status for each CpG based on the responder threshold, the non-informative CpGs were removed, including the positions with the same methylation pattern for all the cell populations considered. The differences present in the methylation profile for each cell type compared with the responders profile were calculated. Finally a similar enrichment analysis were performed by grouping the cell populations by their specific lineage, including myeloid, lymphoid, endothelial or mesenchymal cell lineages and determined their significance through the use of a Wilcoxon signed rank test (pvalue < 0.05). Extended information can be found at **Supplementary Methods**.

Clinical statistical analysis

Assay results were compared with patient outcomes in a double-blind manner. Median follow-up duration was calculated by the inverse Kaplan–Meier method. The significance of the differences between distributions of the groups was estimated with a X^2 test. Progression-free survival (PFS) was defined as the time from the start of anti-PD-1 treatment to the first occurrence of a progression event according to RECIST v1.1. or death. OS was defined as the time from the start of anti-PD1 treatment to death. The Kaplan–Meier method was also used to estimate the PFS and OS, the differences between the groups being calculated with the log-rank test. Hazard ratios (HRs) from univariate Cox regressions were used to determine the association between clinicopathological features with survival. Multivariate Cox

proportional hazards regression was used to identify the independent variables associated with PFS and OS. Extended information can be found at **Supplementary Methods**.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. MD, AMC, MECC, SM, MCdM, DP and ME had access to raw data. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

DISCOVERY COHORT

To define an epigenomic profile associated with those NSCLC patients who would gain clinical benefit from anti-PD-1 treatment, we studied 142 tumoral samples before these cases received immunotherapy. The clinicopathological characteristics of these patients are summarized in **Table 1**. In our initial cohort (discovery cohort) we analyzed 34 NSCLC tumors from patients who underwent anti-PD-1 therapy by using a comprehensive microarray that interrogated the methylation status of around 850,000 CpG sites in the human genome.[19] The clinicopathological characteristics of the 34 patients in the discovery cohort are summarized in **Table 1**. Patients with durable clinical benefit with PD-1 blockade (no progression event or death within the first six months of PD-1 blockade) were classified as “responders” (n=10) and patients with no durable clinical benefit with PD-1 blockade (progression event or death within the first six months of PD-1 blockade) were classified as “non-responders” (n=24). The anti-PD-1-treated NSCLC patients classified as responders also showed fewer progression events (Fisher’s exact test; P=0.005) and deaths from the disease (Fisher’s exact test; P = 0.015) than non-responder patients. An ANOVA was used to rank the greatest methylation differences at the CpG sites (according to differences in the β values) between responders and non-responders. Among the 863,904 CpG sites studied, we found 301 CpG sites (0.03%) at which the methylation levels were significantly associated with clinical response to PD-1 blockade in NSCLC (FDR adjusted p-value P<0.05). **Table S1** describes in the detail the characteristics of the 301 CpG sites. The methylation status of the 301 identified CpG sites, hereafter referred to as the EPIMMUNE epigenetic signature, was significantly associated with PFS (HR=0.010, P=0.007; 95% CI=$3.29\text{x}10^{-4}$-0.0282); log-rank P<10^{-6}) and OS (HR=0.080, P=0.001; 95% CI=0.017-0.373; log-rank P<10^{-3}) (**Fig. 1A**). The EPIMMUNE

negative signature was not an indicator of general poor health, but a specific biomarker of disease specific death in these patients (HR=0.072; P=0.001; 95% CI=0.015-0.334; log-rank P=0.001). The multivariate Cox regression analysis showed that the EPIMMUNE signature was an independent predictor for PFS (HR=0.011, P=0.009; 95% CI=$3.4x10^{-4}$-0.315) and OS (HR=0.081; P=0.001; 95% CI=0.017-0.377) in NSCLC patients receiving PD-1 blockade therapy (**Fig. 1B**). We did not observe any association between the EPIMMUNE signature and any clinicopathological variable (**Table S2**).

In our discovery set of NSCLC cases receiving PD-1 blockade therapy, neither PD-L1 tumoral and stromal expression (**Figure S1**), nor the presence of CD8 cells in tumor or stroma (**Figure S2**) were significantly associated with PFS or OS. However, a high level of expression of tumoral PD-L1 was marginally associated with response according to RECIST criteria (Fisher's exact test, P=0.048). Higher mutational load and its possible association with increased neoantigen burden has also been linked to increased sensitivity to PD-1 blockade in NSCLC.[20] To study this event in our discovery set, we exome-sequenced 22 cases for which we had sufficient DNA left after the DNA methylation microarray hybridization. We identified a median of 183.5 nonsynonymous mutations per sample (range 74 to 970), values that are similar to TCGA NSCLC tumors and other published cohorts.[20] We found that the high *vs.* low mutation burden groups did not differ with respect to PFS and OS (**Figure S3**). The combination of the different patterns of PD-L1 expression described here, the presence of CD8+ in tumor or stroma or the tumor mutational burden with the EPIMMUNE signature did not add extra clinical value: EPIMMUNE-positive patients generally had longer PFS and OS than EPIMMUNE-negative patients, irrespective of their PD-L1, CD8 status and tumor mutational burden (**Figure S4 and Table S2**).

The EPIMMUNE signature identified here for predicting PD-1 blockade response in NSCLC involves factors that are intrinsic and extrinsic to the tumor cells.[10] Tumor cell-intrinsic factors involved in immunotherapy sensitivity include cancer cell-specific changes associated with a variety of oncogenic, tumor suppressor and DNA repair pathways,[10] that affect how the immune system reacts to a given tumor. Here we have used the available DNA methylation patterns in NSCLC cell lines[21] to identify the epigenetic events associated with pure transformed lung cells. In our cases, the EPIMMUNE profile characteristic of NSCLC patients who respond to anti-PD-1 treatment showed: inhibition of β-catenin signaling, targeting genes such as the serine/threonine kinase SGK2 and the cyclic nucleotide phosphodiesterase PDE10A; deficient DNA repair, exemplified by oxidative DNA damage repair glycosylase

MUTYH; and activation of the interferon-gamma (IFN-γ) response, affecting the leucine-rich repeat-containing protein NLRC3, among others (**Table S3**).

Alternatively, tumor cell-extrinsic mechanisms that lead to immunoresponse include elements other than the cancer cells, including other cellular lineages that are present in the primary tumor.[10] Taking advantage of the available DNA methylation profiles of particular cell populations (**Table S4**), we have identified cell lineages enriched in our EPIMMUNE signature (**Table S5**)**.** The carefully dissected DNA methylation patterns of the T-cell, B-cell and myeloid lineages available from the International Human Epigenome Consortium (IHEC)[22] and other databases (https://www.ncbi.nlm.nih.gov/geo/ and https://www.ncbi.nlm.nih.gov/sra) allow the molecular dissection of various immune classes in our set of NSCLCs. We observed that the EPIMMUNE-negative signature that characterizes the lack of response to PD-1 blockade identifies NSCLC tumors with an enrichment of cell populations derived from the myeloid lineage in comparison with the over-representation of the lymphoid lineage in the EPIMMUNE-positive group (Wilcoxon test, P<0.001). EPIMMUNE-negative tumors were particularly enriched in tumor-associated macrophages (TAMs) (Wilcoxon test, P<0.001) and tumor-associated neutrophils (TANs) (Wilcoxon test, P<0.001). These observations are consistent with the proposed role of both cell populations as key contributors to resistance to anti-tumor immunotherapeutic approaches.[9] Conversely, the tumors of EPIMMUNE-positive NSCLC patients were enriched in the lymphoid lineage, particularly CD4+ α/ß T-cells with the capacity to produce IFN-γ [23] (Wilcoxon test, P<0.001), CD8+ α/ß central memory T-cells that represent an important fraction of the tumor-reactive T-cells[24] (Wilcoxon test, P<0.001), and natural killer (NK) cells (Wilcoxon test, P<0.001) that mediate the anti-tumor responses without prior sensitization or recognition of specific tumor antigens.[25] As part of this extrinsic epigenetic signature, we also assessed the presence of two cellular types that are present in the tumoral microenvironment: cancer-associated fibroblasts (CAFs) and endothelial cells.[9] For CAFs, taking advantage of our recent dissection of the DNA methylation profile of these cells in NSCLCs,[26] we were able to determine that the EPIMMUNE-negative signature also identifies NSCLC tumors that are enriched in CAFs (Wilcoxon test, P<0.001), an observation that is consistent with the proposed role of these cells as contributors to immunotherapy resistance.[27] Using the available DNA methylation profiles for the various subclasses of endothelial cells,[22,28] we observed that EPIMMUNE-negative tumors were enriched in endothelial cells with a senescence phenotype characterized by diminished proliferation, migration and spreading capacity, and a large number of progenitor endothelial cells (Wilcoxon test, P<0.001).[29] This profile describes a hypoxic microenvironment associated with

the overexpression of immunosuppressive cytokines such as vascular endothelial growth factor (VEGF).[30] Conversely, the EPIMMUNE-positive tumors presented more normally activated endothelial cells, which can contribute to the successful recruitment of immune effector cells.

VALIDATION COHORTS

Once we had identified the EPIMMUNE signature as being a predictor of response to PD-1 blockade in the discovery set of stage IV NSCLCs, we examined whether the characterized DNA methylation profile was also able to discriminate clinical outcome in an independent validation set of advanced NSCLC cases treated with anti-PD-1 therapies. Thus, using the same DNA methylation microarray platform, we interrogated 47 additional NSCLC specimens from patients who received nivolumab or pembrolizumab. The clinicopathological characteristics of the patients in this validation cohort are listed in **Table 1**. We found that the EPIMMUNE-positive signature was also associated with improved PFS in the studied validation cohort of NSCLC cases receiving anti-PD-1 therapy (HR=0.330, P=0.006; 95% CI=0.149-0.727; log-rank P=0.004) (**Fig. 2A**). A trend between the EPIMMUNE-positive signature and improved OS was also observed (HR=0.458, P=0.068; 95% CI=0.197-1.061; log-rank P=0.060) (**Fig. S5**); a trait that it was also observed for the EPIMMUNE negative signature and disease specific death (HR=0.465; P=0.079; 95% CI=0.198-1.092; log-rank P=0.048) (**Fig. S5**)**.** The presence of the EPIMMUNE-positive signature in the validation cohort was enriched in those patients without tumor progression (5 of 7, 71%), whereas EPIMMUNE-negative cases frequently experienced tumor progression (31 of 40, 78%) (Fisher's exact test; P=0.018). Multivariate Cox regression analysis showed that the EPIMMUNE signature was an independent predictor of PFS (HR=0.336, P=0.007; 95% CI=0.151-0.747) in the interrogated validation cohort of NSCLC cases treated with anti-PD-1 therapy (**Fig. 2B**). We did not observe any association between the EPIMMUNE signature and any clinicopathological variable (**Table S2**).

Having identified the EPIMMUNE signature as being a predictor of response to PD-1 blockade in the discovery and validation sets of NSCLCs, we investigated whether the epigenomic profile obtained was also present in other NSCLC cohorts, such as those contributed by TCGA. Most of the available DNA methylation data from this malignancy are derived from a previous DNA methylation microarray of lower resolution, which interrogates approximately 450,000 CpG sites[31] and contains 146 of the 301 CpGs (48%) of the EPIMMUNE signature. Importantly, this reduced epigenetic profile, which we call EPIMMUNE-

TCGA, was still able to predict clinical response, PFS (HR=0.124, P<0.001; 95% CI=0.043-0.356; log-rank P<10^{-5}) and OS (HR=0.293, P=0.026; 95% CI=0.100-0.863; log-rank P=0.019) in our discovery set of NSCLCs treated with anti-PD-1 therapies (**Fig. 3A**). The EPIMMUNE-TCGA negative signature did not indicate overall poor health, but particularly disease specific death in the discovery cohort (HR=0.210; P=0.009; 95% CI=0.065-0.682; log-rank P=0.005). The EPIMMUNE-TCGA signature was not associated with clinical outcome in the validation cohort (**Fig. S6**)**.** Remarkably, for NSCLC cancer patients who did not receive immunotherapy, such as those included in TCGA projects (https://cancergenome.nih.gov/), we found that the EPIMMUNE-TCGA signature was not associated with OS (HR=0.989, P=0.967; 95% CI=0.587-1.665; log-rank P=0.927) (**Fig. 3B**). These results reinforce the role of the observed epigenomic profile, not as a general factor of improved outcome, but as a particular predictive biomarker of response to anti-PD-1 therapies.

Having selected the sites that were associated with known genes from the 301 CpGs in the EPIMMUNE signature (191 CpGs, 63%), we sought to validate the best single DNA methylation marker that could predict response to PD-1 blockade therapy in NSCLC patients. The top gene according to a CpG of the EPIMMUNE signature located in a regulatory region with the highest ANOVA value and greatest CpG methylation difference between PD-1 blockade responders and non-responders in the discovery cohort (**Table S1**), with additional biological plausibility, was the T-cell-related forkhead box P1 (FOXP1) transcription factor.[32,33] The unmethylated status of FOXP1 has been associated with quiescent naïve CD4+ T-cells,[32,33] so it is reasonable to speculate that the release of the PD-1/PD-L1 immunosuppression axis through the use of the anti-PD-1 antibody will allow the activation of these naïve T-cells, an event that has already been linked to FOXP1 hypermethylation.[32,33] FOXP1 unmethylated status was associated with extended PFS in the studied discovery cohort (HR=0.423, P=0.032; 95% CI=0.192-0.928; log-rank P=0.027) (**Fig. 4A**), but not with OS (**Fig. S7**). The Cox multivariate regression model showed that FOXP1 methylation status was an independent prognostic factor of PFS in the discovery cohort (HR=0.364; P=0.015; 95% CI=0.161 to 0.822) (**Fig. 4B**). The combination of the PD-L1 and CD8 status or tumor mutational burden did not improve the prediction of anti-PD-1 response provided by FOXP1 unmethylated status alone (**Figure S8**). FOXP1 unmethylated status also was associated with extended OS in the microarray validation cohort (HR=0.486, P=0.045; 95% CI=0.239-0.987; log-rank P=0.041) (**Fig. 4C**), but not with PFS (**Fig. S7**). The Cox multivariate regression model showed that FOXP1 methylation status was an independent prognostic factor of PFS in the discovery cohort (HR=.; P=.; 95% CI=. to .) (**Fig. 4D**).

Once we had determined that FOXP1 unmethylation was associated with improved PFS in the discovery cohort, we sought to validate the DNA methylation marker in an additional independent validation cohort of 61 NSCLC patients who received PD-1 blockade therapy. The clinicopathological characteristics of the 61 patients in this validation cohort are listed in **Table 1**. The CpG methylation levels at the described sites were analyzed by pyrosequencing to test a more affordable and large-scale, user-friendly approach. We found that FOXP1 unmethylated status was also significantly associated with PFS following anti-PD-1 treatment in the validation cohort of NSCLC samples analyzed by pyrosequencing (HR=0.415, P=0.006; 95% CI=0.209-0.802; log-rank P=0.005) (**Fig. 5A**). The presence of FOXP1 hypomethylation was also significantly associated with OS (HR=0.409, P=0.009; 95% CI=0.220-0.780; log-rank P=0.007) (**Fig. 5A**). FOXP1 hypomethylation was also associated with disease specific death (HR=0.322; P=0.003; 95% CI=0.152-0.0683; log-rank P=0.002). According to the multivariate Cox regression analysis, FOXP1 unmethylated status was an independent predictor of PFS (HR=0.442, P=0.016; 95% CI=0.228-0.858) and OS (HR=0.457, P=0.028; 95% CI=0.227-0.918) (**Fig. 5B**). Finally, we also established that FOXP1 methylation status was not associated with OS (HR=0.980, P=0.889; 95% CI=0.736-1.305; log-rank P=0.889) in the TCGA NSCLC cancer patients who have not received immunotherapy (**Fig. 5C**). Interestingly, because RNA sequencing data are available for the TCGA samples, we were able to show that FOXP1 hypermethylation was associated with transcript downregulation (**Fig. S9**), suggesting the possible use in future studies of immunohistochemistry approaches to evaluate the role of FOXP1 in anti-PD1 response prediction. These findings imply that FOXP1 epigenetic status is not an overall prognostic factor in NSCLC, but a specific biomarker predicting PD-1 blockade response.

DISCUSSION

The widespread use of monoclonal antibodies to target immune checkpoints, such as PD-1, PD-L1, and the cytotoxic T-lymphocyte antigen 4 (CTLA-4), has markedly improved the outcome of patients with advanced cancer.[9,10] However, despite the overall significant impact on their prognosis, a substantial percentage of patients do not receive any clinical benefit. This means that, from the patients' point of view, they may suffer adverse reactions but no positive response, while from a healthcare policy perspective, expensive drugs are administered that are not sufficiently cost-effective. Thus, it is essential for medical oncology to find biomarkers that will predict the response to immune checkpoint molecules. Current proposed biomarkers of response to PD-1 blockade include expression of particular proteins,

RNA transcription profiles, characteristic mutational landscapes, intratumoral cell type composition and immunoscores based on the expression of relevant genes.[9,10] However, even for the most commonly used biomarker of response, PD-L1 staining, there are significant exceptions for responders and non-responders.[13] In our case, the small size of our discovery cohort, although afforded the ability to identify a classifier, could explain having not observed an association of PD-L1 and clinical outcome. Interestingly, a survival benefit associated with the combination of pembrolizumab and chemotherapy have been observed in all subgroups of PD-L1 tumor proportion scores, however, the greatest relative benefit was found in those tumors with a PD-L1 tumor proportion score of 50% or greater.[34] The EPIMMUNE signature described here provides another step towards filling the gap in our knowledge, as is demanded by the more modern and precise approaches of today's cancer medicine. Importantly for clinical praxis, the demonstrable feasibility of moving from the "omic" approach to a sensitive pyrosequencing PCR-based assay for the FOXP1 biomarker could simplify the process and reduce the costs of the analysis allowing a more affordable large-scale approaches. Furthermore, the methylation microarray technique requires double amount of DNA than the pyrosequencing strategy, further facilitating the use of the latest in the scarce biological material of these patients, which is also in great demand for other tests.

The EPIMMUNE signature identified here has a value beyond its ability to predict the response to PD-1 blockade, whereby it also provides biological explanations of the intimate cellular networks involved in determining immune-checkpoint inhibition. The epigenetic profile observed in NSCLC patients who, following anti-PD-1 therapy, have no substantive clinical response, is characteristic of tumors that are enriched in a particular immune microenvironment characterized by an enrichment in myeloid lineage-derived cells, such as tumor-associated macrophages (TAMs) and neutrophils. These PD-1 blockade-resistant tumors are also enriched in cancer-associated fibroblasts (CAFs) and senescent endothelial cells. Interestingly from a clinical practice standpoint, the presence of cell populations associated with the EPIMMUNE negative signature, such as of TAMs, TANs, CAFs and senescent endothelial cells, can also be assessed by immunohistochemistry.[10] The epigenetic setting of NSCLC patients who do not respond to the PD-1 blockade described here is therefore compatible with the so-called immunologically "cold" or immuno-ignorant tumors, and identifies cases in which interventions to transform them into "hot" or more immunosensitive tumors can be examined.[10] There is a wide spectrum of drugs that have the potential to elicit a stronger immune response, particularly in the scenario described here, in which NSCLC patients who do not respond to PD-1 blockade exhibit exaggerated TAM and

CAF activity and an immunosuppressive endothelial milieu. For example, the specific inhibition of the macrophage-associated phosphoinositide 3-kinase γ (PI3K-γ); or the blockade of the receptor of colony-stimulating factor 1 (CSF-1R), that it is under clinical trials to test its combination with immune checkpoint blockade. In addition, CAFs can be targeted to increase the response of the EPIMMUNE-negative tumors to PD-1 blockade, inhibiting the CAF recruitment-associated chemokine (C-X-C motif) ligand 12 (CXCL12) or the focal adhesion kinase (FAK).[27] Finally, targeting endothelial cells, the normalization of the tumor vasculature by the use of antiangiogenic agents, which enhances the infiltration of CD4+ T and CD8+ cells and blocks myeloid-derived suppressor cell function,[30] is a very attractive therapeutic approach. Preliminary findings suggest that blocking angiogenesis, for example, by using bevacizumab or ramucirumab, increases the efficacy of immune checkpoint inhibitors.[35]

Finally, the epigenetic landscape of the NSCLC patients determined in this study could, by itself, also be a target for therapies. The DNA methylation marks studied here and the histone modifications can be reverted by the use of epigenetic drugs that facilitate the conversion from a “cold” tumor microenvironment to an immunoresponsive one. In this regard, demethylating agents enhance chemokine production by T helper 1 (T_H1)-type T-cells and T-cell trafficking into the tumor, providing better responses to immunotherapies in preclinical models.[36,37] Most importantly, DNA demethylating agents and other epigenetic drugs, such as histone deacetylase inhibitors, have been clinically approved for use in the treatment of some subtypes of leukemias and lymphomas.[15] This has facilitated the inclusion of these agents, in combination with immune checkpoint inhibitors, in several Phase II and III clinical trials in lung cancer (NCT02638090 and NCT01928576) and other solid tumor types (NCT03264404, NCT03182894 and NCT02816021).

In conclusion, we report that the establishment of DNA methylation profiles in NSCLC tumor samples constitutes a predictive tool for selecting patients who stand to gain clinical benefit from anti-PD-1 therapy. The EPIMMUNE signature identified here, at the single-locus level and at the more comprehensive genomic level, provides insight into the immune molecular and cellular milieu of primary tumor specimens, which is a critical microenvironment for determining the response to immune checkpoint inhibitors. Our findings also warrant follow-up studies to check their ability to predict tumoral response to drugs targeting other immune-related proteins, such as PD-L1 and CTLA-4. Although further prospective clinical studies are needed to establish its true value, the epigenetic biomarkers identified herein could be helpful for selecting those patients for whom immunotherapy or strategies acting on

specific intratumoral cell subpopulations could be assessed in cancer-type-specific studies and basket clinical trials.

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Figure Legends

Figure 1. DNA methylation predictive signature (EPIMMUNE) association with progression-free survival (PFS) and overall survival (OS) in a discovery cohort of NSCLC patients treated with anti-PD-1 therapy. (A) Kaplan–Meier analysis of PFS (left) and OS (right) in the 34 NSCLC patients according to the EPIMMUNE signature, defined by the methylation status of the 301 CpG sites [EPIMMUNE-positive (+) signature]. P is calculated using the log-rank function. Univariate Cox regression analysis is represented as the hazard ratio (HR) with a 95% confidence interval (95% CI). Values of $P<0.05$ are considered to be statistically significant. The number of events is shown from 5 to 25 months for both groups. (B) PFS and OS multivariate Cox regression analysis, illustrated as a forest plot, taking into account various clinical parameters in the discovery cohort of NSCLC patients treated with anti-PD-1 agents. Values of P are those corresponding to HRs, with a 95% CI, associated with anti-PD-1 treatment. Covariates with an associated value of $P<0.05$ were considered to be independent prognostic factors of PD-1 blockade outcome.

Figure 2. EPIMMUNE signature analysis in validation cohort of NSCLC patients treated with anti-PD-1 agents. (A) Kaplan–Meier analysis of PFS in the 47 NSCLC patients by EPIMMUNE signature, defined by the methylation status of the 301 CpG sites [EPIMMUNE-positive (+) signature]. P is calculated using the log-rank function. Univariate Cox regression analysis is represented by the hazard ratio (HR) with a 95% confidence interval (95% CI). Values of $P<0.05$ are considered statistically significant. The number of events from 10 to 40 months is shown for both groups. (B) PFS multivariate Cox regression analysis, represented as a forest plot, taking into account clinical characteristics in the validation cohort of NSCLC patients treated with anti-PD-1 agents. Values of P correspond to HRs, with 95% CIs, associated with anti-PD-1 treatment. Covariates with an associated value of $P<0.05$ are considered as independent prognostic factors of PD-1 blockade outcome.

Figure 3. Kaplan–Meier estimates of clinical outcome with respect to the presence of the EPIMMUNE-TCGA signature in patients with NSCLC. (A) Kaplan–Meier estimates of PFS (left) and OS (right) with respect to the presence of the EPIMMUNE-TCGA signature in the discovery cohort of NSCLC patients who received anti-PD-1 treatment. (B) Kaplan–Meier estimates of OS with respect to the presence of the EPIMMUNE-TCGA signature in NSCLC

patients available from the TCGA database. None of these patients received anti-PD-1 treatment.

Figure 4. Survival analysis estimates of clinical outcome with respect to FOXP1 methylation status in NSCLC microarray discovery and validation cohorts. (A) Kaplan–Meier estimates of PFS with respect to FOXP1 methylation status in the discovery cohort of NSCLC patients who received anti-PD-1 treatment. (B) PFS multivariate Cox regression analysis, represented by a forest plot, taking into account the various clinical characteristics of the discovery cohort of NSCLC patients treated with anti-PD-1 agents. (C) Kaplan–Meier estimates of OS with respect to FOXP1 methylation status in the microarray validation cohort of NSCLC patients who received anti-PD-1 treatment. (D) OS multivariate Cox regression analysis, represented by a forest plot, taking into account the various clinical characteristics of the microarray validation cohort of NSCLC patients treated with anti-PD-1 agents.

Figure 5. Survival analysis estimates of clinical outcome with respect to FOXP1 methylation status in NSCLC pyrosequencing validation cohort and TCGA samples. (A) Kaplan–Meier estimates for PFS (left) and OS (right) with respect to FOXP1 methylation status by pyrosequencing analysis in the validation cohort of NSCLC patients who underwent PD-1 blockade therapy. (B) PFS and OS multivariate Cox regression analysis, represented by a forest plot, taking into account the clinical characteristics of the validation cohort of NSCLC patients treated with anti-PD-1 agents. Values of $P<0.05$ are considered to be statistically significant. In multivariate analyses, significant covariates are considered independent prognostic factors of PD-1 blockade outcome. (C) Kaplan–Meier estimates of OS with respect to FOXP1 methylation status in the NSCLC cohort from TCGA database.

Tables

Table 1. Clinical characteristics of discovery and validation NSCLC cohorts, and efficacy of anti-PD-1 therapy.

Supplemental Appendix

Supplementary Methods

Supplementary Figure 1. Kaplan–Meier estimates of PFS and OS with respect to tumoral and stromal PD-L1 expression in the discovery cohort of NSCLC patients who received anti-PD-1 treatment.

Supplementary Figure 2. Kaplan–Meier estimates of PFS and OS with respect to tumoral and stromal CD8+ presence in the discovery cohort of NSCLC patients who received anti-PD-1 treatment.

Supplementary Figure 3. Kaplan–Meier estimates of PFS and OS with respect to tumor mutation burden (TMB) in the discovery cohort of NSCLC patients who received anti-PD-1 treatment.

Supplementary Figure 4. Combination of the EPIMMUNE signature with PD-L1, CD8+ and tumor mutation burden (TMB) assessments in the discovery cohort.

Supplementary Figure 5. Kaplan–Meier estimates of OS and disease specific survival with respect to the EPIMMUNE signature in the validation cohort of NSCLC patients who received anti-PD-1 treatment.

Supplementary Figure 6. Kaplan–Meier estimates of PFS and OS with respect to the EPIMMUNE-TCGA signature in the microarray validation cohort of NSCLC patients who received anti-PD-1 treatment.

Supplementary Figure 7. Kaplan–Meier estimates of OS and PFS with respect to FOXP1 methylation status in the discovery and validation microarray cohorts, respectively.

Supplementary Figure 8. Combination of the FOXP1 methylation status with PD-L1, CD8+ and tumor mutation burden (TMB) assessments in the discovery cohort.

Supplementary Figure 9. Correlation between methylation beta values and expression for FOXP1 in primary lung tumors from TCGA.

Table S1. Description of the 301 CpG sites of the EPIMMUNE signature.

Table S2. Association between EPIMMUNE signature and clinical covariables in discovery and validation cohorts.

Table S3. Enrichment for the identified cancer pathways of the EPIMMUNE 301 CpG sites.

Table S4. DNA methylation sources used for cell lineage analyses.

Table S5. Enrichment for different cell populations of the EPIMMUNE 301 CpG sites

Contributors

MD, AMC and ME designed the study, contributed to the analysis, and wrote the first draft of the manuscript. AMC, MECC, SM, MCdM, VD, DP, MSC, AFF and MFF did further data analysis. MG and IG developed the exome sequencing. In-depth clinical and pathological characterization and recruitment of patients were carried out by MD, AMC, NG, MB, EGL, CD, MP, PB, EC, SN, AC, MCC, NK, MGC, SP, LM, IGB, IB, MDL, MV, JCR, RP, EN, TM, LP, IR, QX, CT, NV, AP, NR, AB, PG, IB, JFE, RR and EB. All authors helped draft the manuscript or revise it critically for significant intellectual content, and made substantial contributions to the concept and design of the study and acquisition, analysis, and interpretation of data.

Declaration of interests

MD has received research funding for institutional research project from Novartis and Pfizer unrelated to the present work. MD has served as a consultant (advisory board) for Pfizer, Boehringer Ingelheim and Bristol-Myers Squibb. ME and AMC report receiving personal fees from Ferrer International S.A. that are unrelated to the submitted work. EN received consultancy fees from Merck Sharp and Dohme MSD and Bristol-Myers Squibb. The other authors declare no conflicts of interest.

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Table 1

Table 1. Clinical characteristics of discovery and validation NSCLC cohorts and efficacy of anti-PD-1 therapy

Characteristics	NSCLC cohorts		
	Discovery cohort EPIMMUNE (N = 34)	**Validation cohort** EPIMMUNE (N = 47)	**Validation cohort** FOXP1 methylation status (N = 61)
Age (years) - Median [range]	61 [40 - 80]	62 [38 - 99]	63 [43 - 79]
Gender - Frequency (%)			
Male	29 (85%)	34 (72%)	44 (72%)
Female	5 (15%)	13 (28%)	17 (28%)
ECOG performance status score - Frequency (%)			
ECOG 0-1	25 (74%)	40 (87%)	49 (80%)
ECOG 2 or more	9 (26%)	5 (11%)	9 (15%)
Unknown	0 (0%)	2 (2%)	3 (5%)
Smoking history - Frequency (%)			
Current or Former smoker	31 (91%)	37 (79%)	57 (93%)
Never smoker	3 (9%)	6 (13%)	4 (7%)
Unknown	0 (0%)	4 (8%)	0 (0%)
Disease stage at diagnosis - Frequency (%)			
II or III	10 (30%)	10 (21%)	11 (18%)
IV	24 (70%)	36 (77%)	59 (79%)
Unknown	0 (0%)	1 (2%)	2 (3%)
Histology at diagnosis - Frequency (%)			
Adenocarcinoma	28 (82%)	33 (70%)	45 (74%)
Squamous cell carcinoma	6 (18%)	7 (15%)	15 (25%)
Others	0 (0%)	3 (6%)	1 (1%)
Unknown	0 (0%)	4 (9%)	0 (0%)
Brain metastases - Frequency (%)			
Yes	14 (41%)	8 (17%)	8 (13%)
No	20 (59%)	36 (77%)	43 (71%)
Unknown	-	3 (6%)	10 (16%)
Type of sample - Frequency (%)			
Biopsy or cytology	16 (47%)	37 (79%)	47 (77%)
Surgical resection	18 (53%)	10 (21%)	14 (23%)
Previous chemotherapy treatment - Frequency (%)			
Cisplatin-based	15 (44%)	28 (60%)	17 (28%)
Carboplatin-based	17 (50%)	9 (19%)	40 (66%)
Others	0 (0%)	6 (13%)	0 (0%)
No previous treatment	0 (0%)	1 (2%)	2 (3%)
Unknown	2 (6%)	3 (6%)	2 (3%)
Type of anti-PD-1 agent - Frequency (%)			
Nivolumab	33 (97%)	44 (94%)	57 (93%)
Pembrolizumab	1 (3%)	3 (6%)	4 (7%)
Line of PD-1 blockade - Frequency (%)			
First	0 (0%)	7 (15%)	2 (3%)
Second	24 (70%)	30 (64%)	35 (57%)
Third	7 (21%)	6 (13%)	16 (26%)
Further lines	3 (9%)	4 (8%)	8 (14%)
Clinical benefit to PD-1 blockade - Frequency (%)			
Durable clinical benefit	10 (29%)	16 (34%)	25 (41%)
No clinical benefit	24 (71%)	31 (66%)	36 (59%)
Response to PD-1 blockade (RECIST)- Frequency (%)			
Complete response	0 (0%)	1 (2%)	0 (0%)
Partial response	8 (23%)	8 (17%)	15 (25%)
Stable disease	3 (9%)	10 (21%)	16 (26%)
Progressive disease	22 (65%)	22 (47%)	28 (46%)
Not evaluable	1 (3%)	6 (13%)	2 (3%)
PFS since PD-1 blockade (months) - Median [range]	2.131 [0.426 - 24.163]	2.704 [0.030 – 41.39]	3.800 [0.200 - 22.670]
OS since PD-1 blockade (months) - Median [range]	8.459 [0.918 - 24.590]	6.429 [0.030 – 48.16]	7.770 [0.850 – 30.00]

Abbreviations: ECOG = Eastern Cooperative Oncology Group; OS= Overall survival; PFS = Progression-free survival

Figure 1

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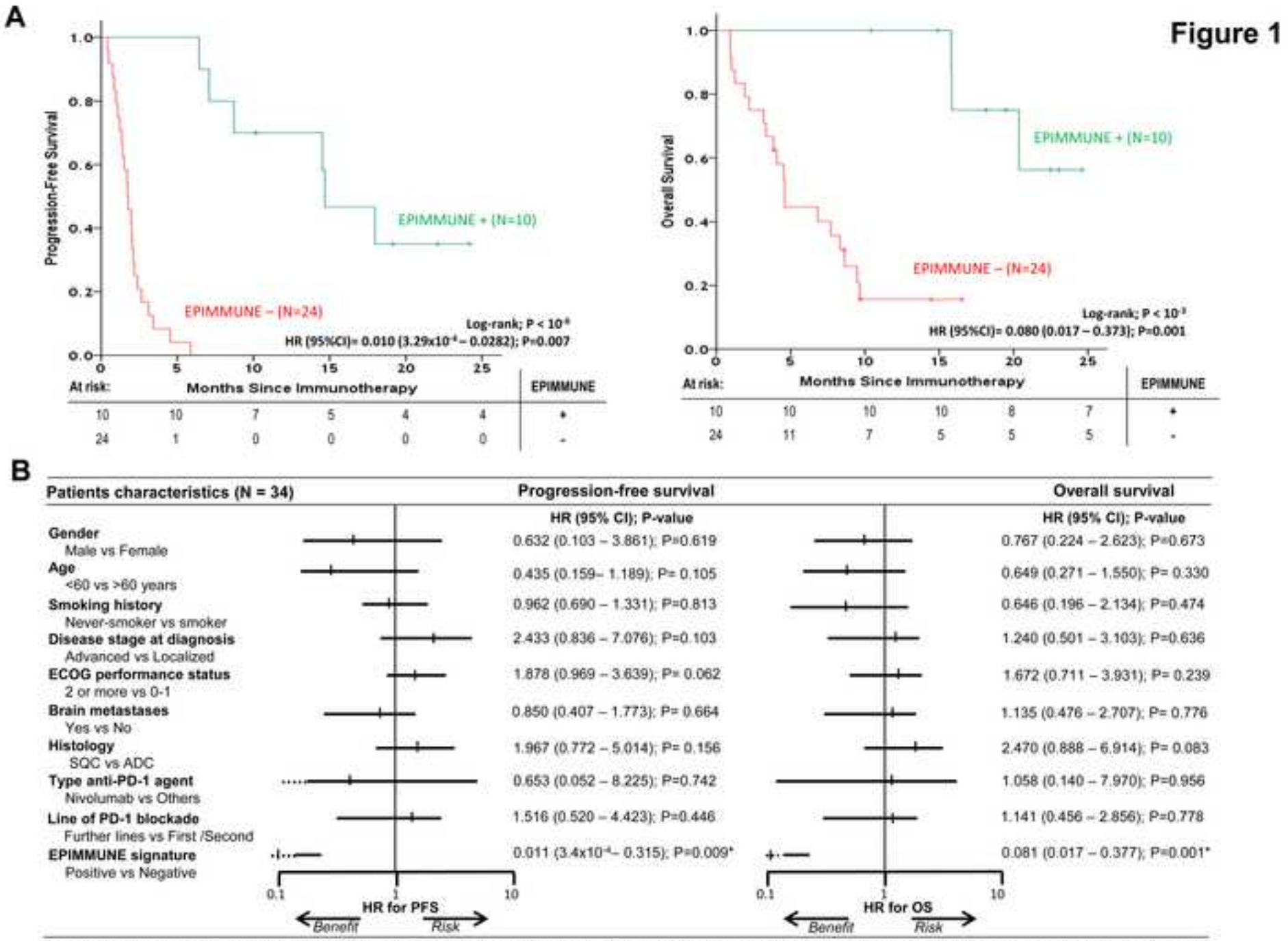

ADC: adenocarcinoma; HR: Hazard Ratio; 95%CI: 95% of Confidence Interval; OS: Overall survival since PD-1 blockade; PFS: Progression-Free Survival since PD-1 blockade; SQC: squamous cell carcinoma.*Co-variable with P-value (P) under 0.05 are considered as statistical significant independent prognostic factor for PFS or OS to anti-PD1 blockade

Figure 2

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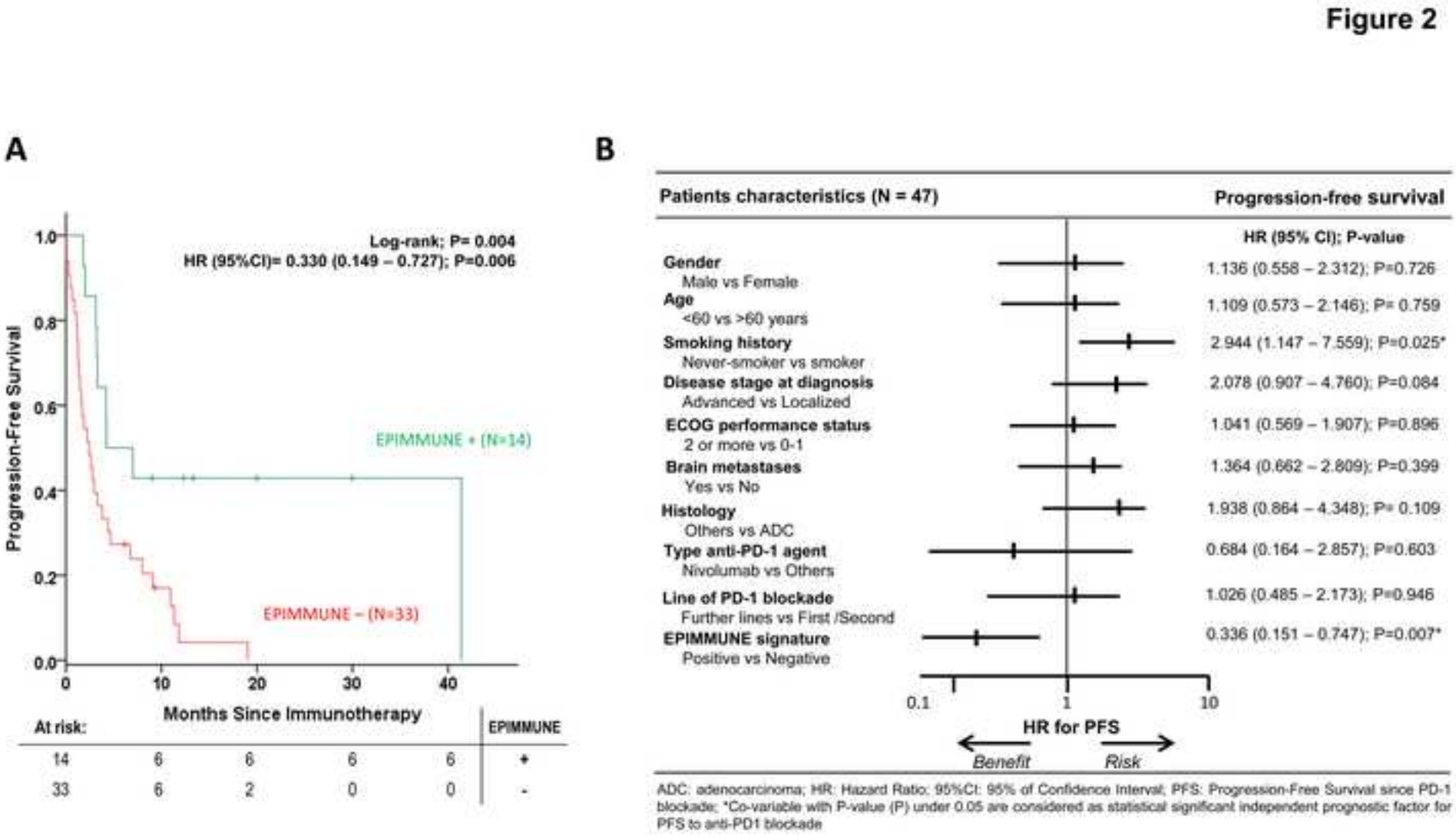

ADC: adenocarcinoma; HR: Hazard Ratio; 95%CI: 95% of Confidence Interval; PFS: Progression-Free Survival since PD-1 blockade; *Co-variable with P-value (P) under 0.05 are considered as statistical significant independent prognostic factor for PFS to anti-PD1 blockade

Figure 3

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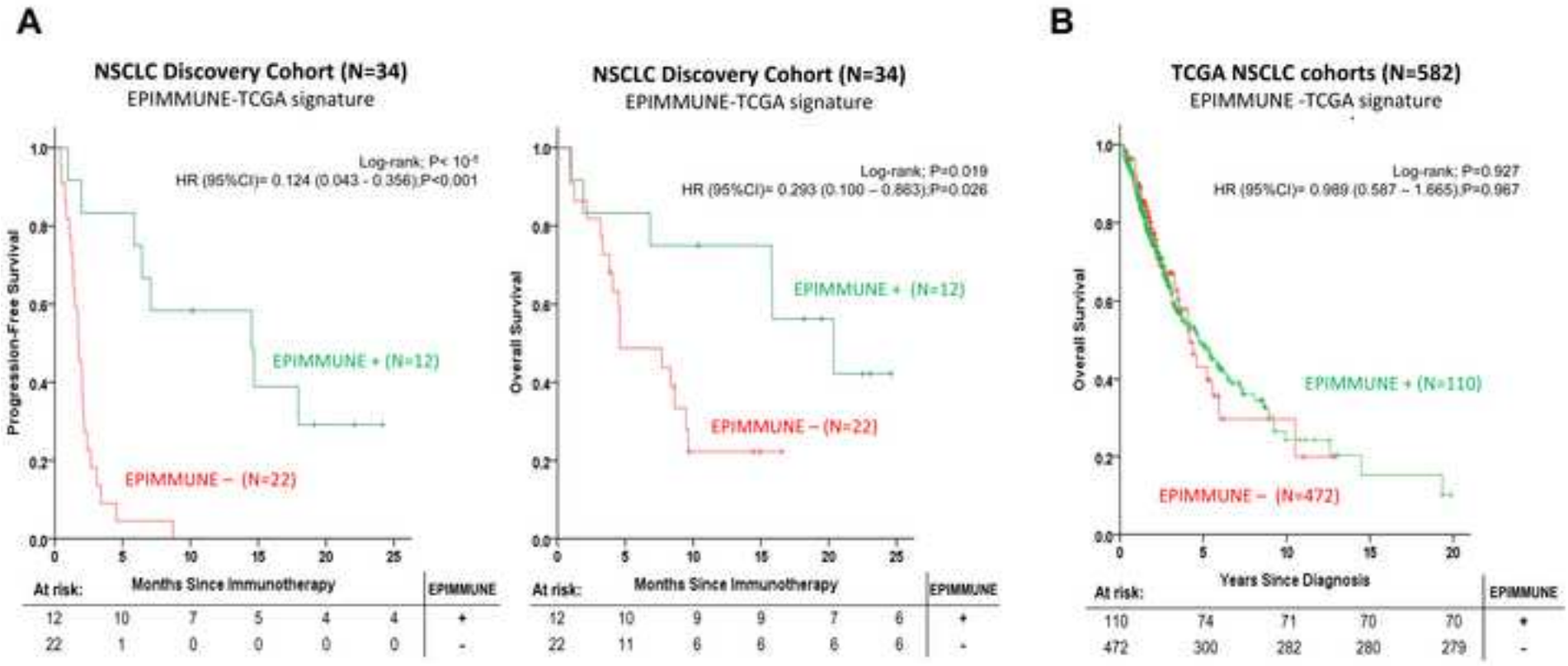

Figure 4

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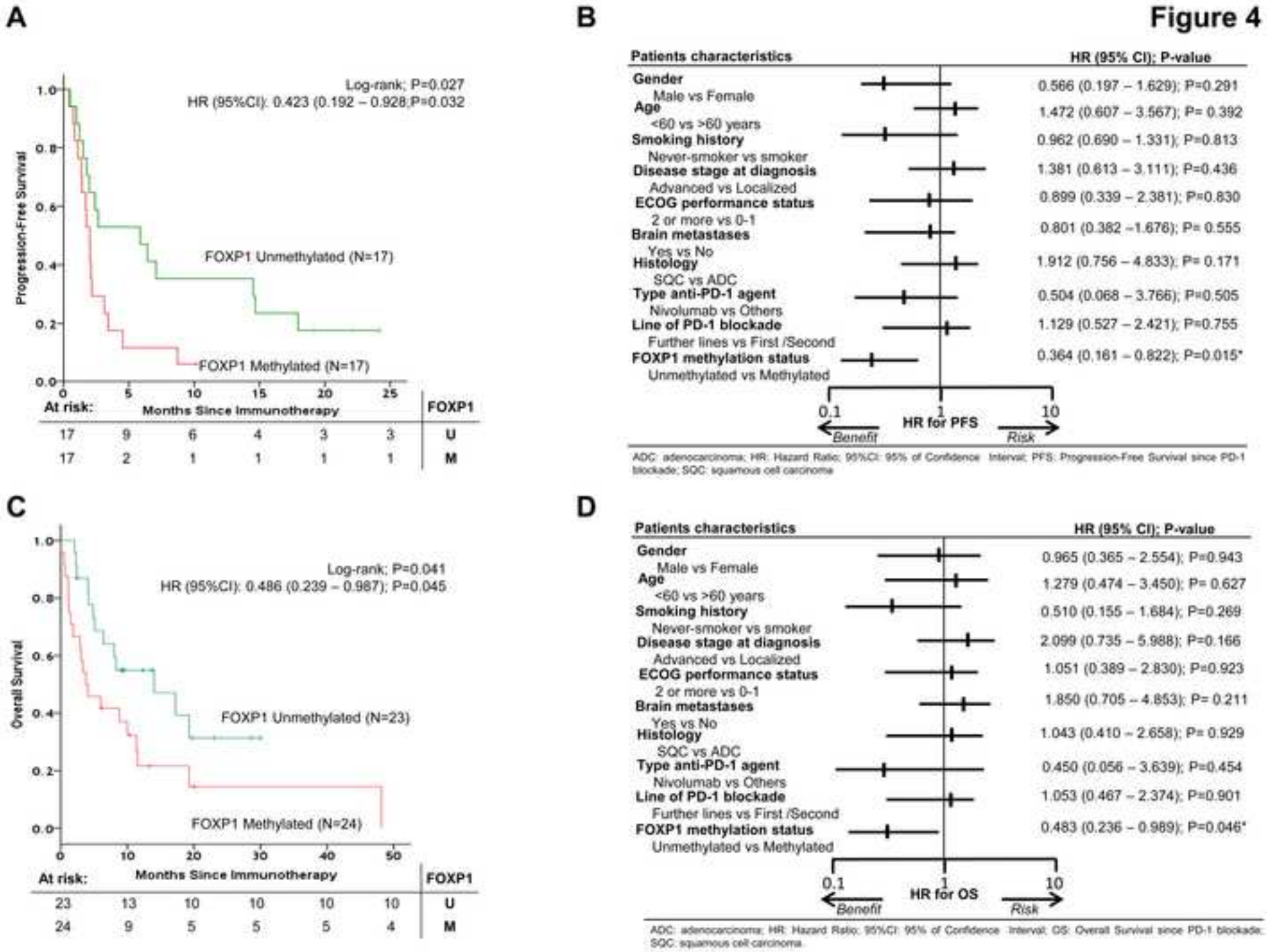

Figure 5

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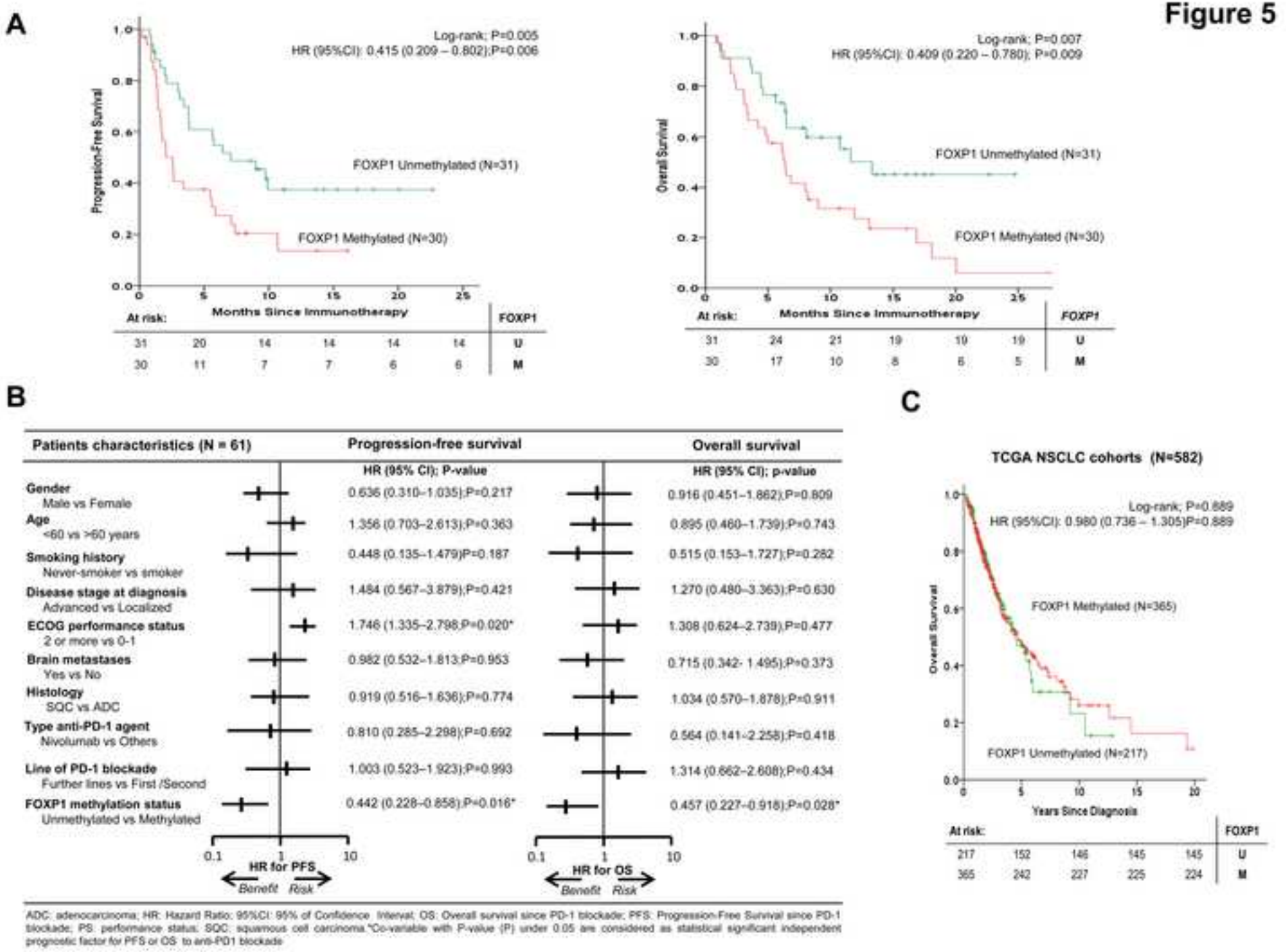

Supplementary Material (appendix)

Click here to download Necessary Additional Data: Supplementary appendix.pdf