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Epigenetic prediction of response to anti-PD-1 treatment in non-small-cell lung cancer: a multicentre, retrospective analysis

Michäel Duruisseaux, Anna Martínez-Cardús*, Maria E Calleja-Cervantes, Sebastian Moran, Manuel Castro de Moura, Veronica Davalos, David Piñeyro, Montse Sanchez-Cespedes, Nicolas Girard, Marie Brevet, Etienne Giroux-Leprieur, Coraline Dumenil, Monica Pradotto, Paolo Bironzo, Enrica Capelletto, Silvia Novello, Alexis Cortot, Marie-Christine Copin, Niki Karachaliou, Maria Gonzalez-Cao, Sergio Peralta, Luis M Montuenga, Ignacio Gil-Bazo, Iosune Baraibar, Maria D Lozano, Mar Varela, Jose C Ruffinelli, Ramon Palmero, Ernest Nadal, Teresa Moran, Lidia Perez, Immaculada Ramos, Qingyang Xiao, Agustin F Fernandez, Mario F Fraga, Marta Gut, Ivo Gut, Cristina Teixidó, Noelia Vilariño, Aleix Prat, Noemi Reguart, Amparo Benito, Pilar Garrido, Isabel Barragan, Jean-François Emile, Rafael Rosell, Elisabeth Brambilla, Manel Esteller*

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 substantial percentage of patients do not respond to this treatment. We examined the use of DNA methylation profiles to determine the efficacy of anti-PD-1 treatment in patients recruited with current stage IV NSCLC.

Methods In this multicentre study, we recruited adult patients from 15 hospitals in France, Spain, and Italy who had histologically proven stage IV NSCLC and had been exposed to PD-1 blockade during the course of the disease. The study structure comprised a discovery cohort to assess the correlation between epigenetic features and clinical benefit with PD-1 blockade and two validation cohorts to assess the validity of our assumptions. We first established an epigenomic profile based on a microarray DNA methylation signature (EPIMMUNE) in a discovery set of tumour samples from patients treated with nivolumab or pembrolizumab. The EPIMMUNE signature was validated in an independent set of patients. A derived DNA methylation marker was validated by a single-methylation assay in a validation cohort of patients. The main study outcomes were progression-free survival and overall survival. We used the Kaplan-Meier method to estimate progression-free and overall survival, and calculated the differences between the groups with the log-rank test. We constructed a multivariate Cox model to identify the variables independently associated with progression-free and overall survival.

Findings Between June 23, 2014, and May 18, 2017, we obtained samples from 142 patients: 34 in the discovery cohort, 47 in the EPIMMUNE validation cohort, and 61 in the derived methylation marker cohort (the T-cell differentiation factor forkhead box P1 [FOXP1]). The EPIMMUNE signature in patients with stage IV NSCLC treated with anti-PD-1 agents was associated with improved progression-free survival (hazard ratio [HR] 0·010, 95% CI $3·29\times10^{-4}$–0·0282; p=0·0067) and overall survival (0·080, 0·017–0·373; p=0·0012). The EPIMMUNE-positive signature was not associated with PD-L1 expression, the presence of CD8+ cells, or mutational load. EPIMMUNE-negative tumours were enriched in tumour-associated macrophages and neutrophils, cancer-associated fibroblasts, and senescent endothelial cells. The EPIMMUNE-positive signature was associated with improved progression-free survival in the EPIMMUNE validation cohort (0·330, 0·149–0·727; p=0·0064). The unmethylated status of FOXP1 was associated with improved progression-free survival (0·415, 0·209–0·802; p=0·0063) and overall survival (0·409, 0·220–0·780; p=0·0094) in the FOXP1 validation cohort. The EPIMMUNE signature and unmethylated FOXP1 were not associated with clinical benefit in lung tumours that did not receive immunotherapy.

Interpretation Our study shows that the epigenetic milieu of NSCLC tumours indicates which patients are most likely to benefit from nivolumab or pembrolizumab treatments. The methylation status of FOXP1 could be associated with validated predictive biomarkers such as PD-L1 staining and mutational load to better select patients who will experience clinical benefit with PD-1 blockade, and its predictive value should be evaluated in prospective studies.

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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 systemic therapy for NSCLC has improved with the introduction of drugs targeted against actionable mutations, only a

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*Contributed equally

Cancer Epigenetics and Biology Program, Bellvitge Biomedical Research Institute, Barcelona, Spain (M Duruisseaux MD, A Martinez-Cardús PhD, M E Calleja-Cervantes PhD, S Moran PhD, M Castro de Moura MSc, V Davalos PhD, D Piñeyro PhD, M Sanchez-Cespedes PhD, Prof M Esteller MD); **Department of Respiratory Medicine, Groupement Hospitalier Est, Hôpital Louis-Pradel, Hospices Civils de Lyon, Lyon, France** (M Duruisseaux, Prof N Girard PhD); **Department of Pathology, Centre de biologie et pathologie Est, Institut de Cancérologie des Hospices Civils de Lyon, Université Lyon 1, Lyon, France** (Prof M Brevet MD); **Department of Respiratory Diseases and Thoracic Oncology, APHP-Hôpital Ambroise Pare, Boulogne-Billancourt, France** (E Giroux-Leprieur MD, C Dumenil MD); **EA4340, UVSQ, University Paris-Saclay, Boulogne-Billancourt, France** (E Giroux-Leprieur); **Department of Oncology, University of Turin, AOU San Luigi, Turin, Italy** (M Pradotto MSc, P Bironzo MD, E Capelletto MD, Prof S Novello MD); **Thoracic Oncology Department, CHU Lille, University of Lille, Lille, France** (Prof A Cortot MD); **UMR 8161—M3T—Mechanisms of Tumorigenesis and Targeted Therapies, CNRS, Institut Pasteur de Lille** (Prof A Cortot) **and Department of Pathology, CHU Lille** (Prof M-C Copin MD), **University of Lille, Lille, France**;

Medical Oncology Service, Institute of Oncology Rosell, University Hospital Sagrat Cor, QuironSalud Group, Barcelona, Spain, (N Karachaliou MD); **Medical Oncology Service, Quirón Dexeus University Hospital, Barcelona, Catalonia, Spain** (M Gonzalez-Cao MD); **Medical Oncology Service, University Hospital Sant Joan de Reus, Reus, Spain** (S Peralta MD); **Program in Solid Tumors and Biomarkers, Center for Applied Medical Research, Pamplona, Spain** (L M Montuenga MD); **Department of Oncology, Clínica Universidad de Navarra, Pamplona, Spain** (I Gil-Bazo MD, I Baraibar MD); **Department of Pathology, Clínica Universidad de Navarra, Pamplona, Spain** (M D Lozano MD); **Navarra Health Research Institute, Pamplona, Spain** (L M Montuenga, I Gil-Bazo, I Baraibar, M D Lozano); **Pathology Department, University Hospital Bellvitge, Barcelona, Spain** (M Varela MD); **Medical Oncology Department, Catalan Institute of Oncology, Hospital Duran I Reynals, Barcelona, Spain** (J C Ruffinelli MD, R Palmero MD, E Nadal MD); **Medical Oncology Department, Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Barcelona, Spain** (T Moran MD); **Department of Pathology** (L Perez MD) **and Medical Oncology Service** (I Ramos MD, I Barragan PhD), **Hospitales Universitarios Regional y Virgen de la Victoria, Institute of Biomedical Research in Malaga, University of Málaga, Málaga, Spain; Pharmacoepigenetics Group, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden** (Q Xiao MSc, I Barragan); **Cancer Epigenetics Laboratory, Institute of Oncology of Asturias, Hospital Universitario Central de Asturias** (A F Fernandez PhD) **and Nanomaterials & Nanotechnology Research Center** (M F Fraga PhD), **Universidad de Oviedo, Oviedo, Spain; Fundación para la Investigación Biosanitaria de Asturias, Instituto de Investigación Sanitaria del Principado de Asturias, Oviedo, Spain** (A F Fernandez); **CNAG-CRG, Centre for Genomic Regulation, Barcelona Institute**

Research in context

Evidence before this study

Important advances have been made in the clinical care of patients with advanced non-small-cell lung cancer (NSCLC) 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 articles published in English from inception up to April 24, 2018, 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 specific epigenetic profile, termed EPIMMUNE, based on DNA methylation microarrays, is associated with improved progression-free survival and overall survival in patients with stage IV NSCLC who received the anti-PD-1 antibodies nivolumab or pembrolizumab. The EPIMMUNE signature described shows that the response to PD-1 blockade occurs mainly in immunocompetent primary tumours characterised by specific intrinsic (cancer cell) and extrinsic (microenvironment) settings. This 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 forkhead box P1 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 signalling pathways that, were they accurately targeted, could strengthen the response of resistant patients to immune checkpoint inhibition.

minority of patients carry these amenable genetic targets, and their tumours 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 patients with metastatic disease. 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 overall survival, even in patients with NSCLC whose disease progressed while receiving platinum-based chemotherapy.[3–6] PD-1 blockade has also been 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 targets PD-L1 (atezolizumab).[3–8]

PD-1 is an immune checkpoint receptor expressed in activated B cells 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 or PD-L1 pathway is a mechanism by which human malignancies, including NSCLC tumours, evade immune system control.[9] However, very little is known about the molecular steps involved in avoiding immune surveillance in cancer cells and the repertoire of immunocompetent and immunocompromised tumour types. For example, only 10–30% of unselected patients with NSCLC respond to PD-1 blockade by nivolumab.[3,4] Studies suggest that the genetic setting of the tumour, 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 or anti-PD-L1.[10,11] However, although PD-L1 expression is enriched in immunotherapy responders,[10–14] 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 to be able to select those patients with PD-1 and PD-L1 blockade-responsive NSCLC 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 marker crucial 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 origin,[17] can predict the clinical response to PD-1 checkpoint blockade in patients with NSCLC.

Methods

Study design and participants

This multicentre study took place in 15 hospitals in three countries: France (four hospitals), Spain (ten hospitals), and Italy (one hospital). Analyses of tumour samples were done in a centralised DNA methylation facility at the Bellvitge Biomedical Research Institute (Barcelona, Spain).

Patients were eligible to enter the study if they had a histologically proven stage IV NSCLC and had undergone tumour sampling before any antineoplastic treatment for stage IV disease in any of the participating institutions. The patients had to have been exposed to PD-1 blockade during the course of their disease. There were no exclusion criteria. We first established an epigenomic profile based on a microarray DNA methylation signature (EPIMMUNE) in a discovery set of NSCLC tumour samples treated with nivolumab or pembrolizumab, which was then validated in an independent cohort of patients with NSCLC. We also sought the best DNA methylation marker to predict response to PD-1 blockade and went on to validate this derived marker in a further validation cohort. Patients gave their written informed consent to participate in the research, which had received ethics approval from the review board of each institution.

Procedures

Samples were first obtained for the discovery cohort, and later obtained for the validation cohorts. The samples were collected after approval outside of any clinical trial. Tumours were collected from patients by surgical resection, CT-guided biopsy, or bronchial biopsy. Sampling had to take place when the patient was naive to any antineoplastic treatment for advanced disease, including immunotherapy. Only adjuvant chemotherapy or chemotherapy associated with radiotherapy was accepted, in case of relapse after curative surgery or chemoradiotherapy. Follow-up involved clinical examination, CT scans, or brain MRI. Radiological assessments for response or progression to immunotherapy were done according to institution standards every 3–4 cycles. NSCLC tumour samples were studied in order of arrival at the centralised DNA methylation facility, once they had passed the checks for technical quality.

Histology-guided tumour-type classification of NSCLCs involved review by a pathologist (EB) of the tumour's morphological appearance under light microscopy and of the immunohistochemical findings. PD-L1 staining and CD8 content were evaluated as previously described.[18] Whole-exome sequencing was performed on formalin-fixed paraffin-embedded (FFPE) genomic DNA using the SeqCap EX MedExome Enrichment Kit (Roche; Pleasanton, CA, USA), following the manufacturer's recommendations. Prepared libraries were sequenced in a HiSeq 2000 instrument (Illumina; San Diego, CA, USA) with paired-end 100-base reads. Samples were multiplexed to obtain a raw minimum coverage of 70×.

The DNA methylation status of the discovery cohort was established using bisulfite-converted DNA processed by the Infinium FFPE restoration process and then hybridised on an Infinium MethylationEPIC Array (around 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.

The β values for the 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 were available. After determining the methylation status for each CpG on the basis of 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 was performed by grouping the cell populations by their specific lineage, including myeloid, lymphoid, endothelial, or mesenchymal cell lineages, and their significance was determined with a Wilcoxon signed rank test ($p<0·05$). Extended information on these procedures can be found in the appendix.

Outcomes

The study outcomes were progression-free survival, overall survival, and disease-specific survival. Progression-free survival was defined as the time from the start of anti-PD-1 treatment to the first occurrence of a progression event according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 or death. Overall survival was defined as the time from the start of anti-PD-1 treatment to death. Disease-specific survival was defined as the time from the start of anti-PD-1 treatment to death from lung cancer.

Statistical analysis

We included as many patients as were eligible between the study opening and closing dates in our analyses. Assay results were compared with patient outcomes in a double-blind manner. An ANOVA test was performed to rank the greatest methylation differences between responders and non-responders. A supervised classification model based on elastic-net regularised logistic regression was used to predict responder (EPIMMUNE positive) and non-responder (EPIMMUNE negative) status. 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 χ^2 test. The Kaplan-Meier method was also used to estimate progression-free and overall survival, with the differences between the groups calculated with the log-rank test. Hazard ratios (HRs) from univariate Cox regressions were used to determine the association between clinicopathological features and survival. Multivariate Cox proportional hazards regression was used to identify the independent variables associated with progression-free and overall survival. Further details can be found in the appendix.

of Science and Technology, Barcelona, Spain (M Gut PhD, I Gut PhD)**; Pathology Department** (C Teixidó PhD) **and Medical Oncology Department** (N Vilariño MD, A Prat MD, N Reguart MD)**, Hospital Clinic, Institut d'Investigacions Biomédiques August Pi i Sunyer, Barcelona, Spain; Pathology Department** (A Benito MD) **and Medical Oncology Service** (P Garrido MD)**, University Hospital Ramon y Cajal, Madrid, Spain; Department of Pathology Department, APHP-Hôpital Ambroise Pare, Boulogne-Billancourt, France** (J-F Emile MD)**; Catalan Institute of Oncology, Germans Trias i Pujol Health Sciences Institute and Hospital, Barcelona, Spain** (Prof R Rosell MD)**; Centre Hospitalier Grenoble, INSERM U1219, University UGA, Grenoble, France** (Prof E Brambilla MD)**; Centro de Investigación Biomédica en Red de Cancer, Madrid, Spain** (L M Montuenga, I Gil-Bazo, M D Lozano, P Garrido, Prof M Esteller)**; Physiological Sciences Department, School of Medicine and Health Sciences, University of Barcelona, Spain** (Prof M Esteller)**; and Institucio Catalana de Recerca i Estudis Avançats, Barcelona, Spain** (Prof M Esteller)

Correspondence to:
Prof Manel Esteller, Cancer Epigenetics and Biology Program, Bellvitge Biomedical Research Institute, 08908 L'Hospitalet, Barcelona, Spain
mesteller@idibell.cat

See Online for appendix

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, AM-C, MEC-C, 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.

	EPIMMUNE discovery cohort (n=34)	EPIMMUNE validation cohort (n=47)	FOXP1 methylation status validation cohort (n=61)
Age, years	61 (40–80)	62 (38–99)	63 (43–79)
Sex			
Male	29 (85%)	34 (72%)	44 (72%)
Female	5 (15%)	13 (28%)	17 (28%)
ECOG performance status score			
0–1	25 (74%)	40 (85%)	49 (80%)
≥2	9 (26%)	5 (11%)	9 (15%)
Unknown	0	2 (4%)	3 (5%)
Smoking history			
Current or former smoker	31 (91%)	37 (79%)	57 (93%)
Never smoker	3 (9%)	6 (13%)	4 (7%)
Unknown	0	4 (9%)	0
Disease stage at diagnosis			
II or III	10 (29%)	10 (21%)	11 (18%)
IV	24 (71%)	36 (77%)	48 (79%)
Unknown	0	1 (2%)	2 (3%)
Histology at diagnosis			
Adenocarcinoma	28 (82%)	33 (70%)	45 (74%)
Squamous cell carcinoma	6 (18%)	7 (15%)	15 (25%)
Others	0	3 (6%)	1 (2%)
Unknown	0	4 (9%)	0
Brain metastases			
Yes	14 (41%)	8 (17%)	8 (13%)
No	20 (59%)	36 (77%)	43 (70%)
Unknown	0	3 (6%)	10 (16%)
Type of sample			
Biopsy or cytology	16 (47%)	37 (79%)	47 (77%)
Surgical resection	18 (53%)	10 (21%)	14 (23%)
Previous chemotherapy treatment			
Cisplatin based	15 (44%)	28 (60%)	17 (28%)
Carboplatin based	17 (50%)	9 (19%)	40 (66%)
Others	0	6 (13%)	0
No previous treatment	0	1 (2%)	2 (3%)
Unknown	2 (6%)	3 (6%)	2 (3%)
Anti-PD-1 agent			
Nivolumab	33 (97%)	44 (94%)	57 (93%)
Pembrolizumab	1 (3%)	3 (6%)	4 (7%)
Line of PD-1 blockade			
First	0	7 (15%)	2 (3%)
Second	24 (71%)	30 (64%)	35 (57%)
Third	7 (21%)	6 (13%)	16 (26%)
Further lines	3 (9%)	4 (9%)	8 (13%)
Clinical benefit to PD-1 blockade			
Durable clinical benefit	10 (29%)	16 (34%)	25 (41%)
No clinical benefit	24 (71%)	31 (66%)	36 (59%)

(Table continues on next page)

Results

Patients were enrolled between June 23, 2014, and May 18, 2017. To define an epigenomic profile associated with patients who would gain clinical benefit from anti-PD-1 treatment, we studied 142 tumoral samples before these patients received immunotherapy (table). In our initial cohort (discovery cohort), we analysed 34 NSCLC tumours from patients who underwent anti-PD-1 therapy using a comprehensive microarray that interrogated the methylation status of 863 904 CpG sites in the human genome (table).[19] Patients with durable clinical benefit with PD-1 blockade (defined as no progression event or death within the first 6 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 6 months of PD-1 blockade) were classified as non-responders (n=24). Those patients classified as responders showed fewer progression events (six [60%] *vs* 24 [100%], Fisher's exact test p=0·0045) and deaths from the disease (three [30%] *vs* 19 [79%] Fisher's exact test p=0·015) than did non-responder patients.

Among the CpG sites studied, we found 301 CpG sites (<1%) at which the methylation levels were significantly associated with clinical response to PD-1 blockade in NSCLC (false discovery rate adjusted p<0·05 for all sites). The characteristics of these 301 CpG sites are described in detail in the appendix. The methylation status of these sites, hereafter referred to as the EPIMMUNE epigenetic signature, was significantly associated with progression-free survival and overall survival (figure 1). The EPIMMUNE negative signature was not an indicator of general poor health but a biomarker of disease-specific death in these patients (HR 0·072, 95% CI 0·015–0·334, p=0·0012; log-rank p=0·0010). The multivariate Cox regression analysis showed that the EPIMMUNE signature was an independent predictor for progression-free survival and overall survival in patients with NSCLC receiving PD-1 blockade therapy (appendix). We did not observe any association between the EPIMMUNE signature and any clinicopathological variable (appendix).

In our discovery set, neither PD-L1 tumoral and stromal expression nor the presence of CD8 cells in tumour or stroma were significantly associated with progression-free or overall survival (appendix). 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 samples for which we had sufficient

DNA left after the DNA methylation microarray hybridisation. We identified a median of 183·5 non-synonymous mutations per sample (IQR 128·0–436·5), values that are similar to those for The Cancer Genome Atlas (TCGA) NSCLC tumours and other published cohorts.[20] We found that the high mutation burden groups did not differ from the low mutation burden groups with respect to progression-free and overall survival (appendix). The combination of the different patterns of PD-L1 expression described here, the presence of CD8+ in tumour or stroma, or the tumour mutational burden with the EPIMMUNE signature did not add extra clinical value: EPIMMUNE-positive patients generally had longer progression-free and overall survival than did EPIMMUNE-negative patients, irrespective of their PD-L1 status, CD8 status, and tumour mutational burden (appendix).

The EPIMMUNE signature we identified for predicting PD-1 blockade response in NSCLC involves factors that are intrinsic and extrinsic to the tumour cells.[10] Tumour cell-intrinsic factors involved in immunotherapy sensitivity include cancer cell-specific changes associated with a variety of oncogenic, tumour suppressor, and DNA repair pathways[10] that affect how the immune system reacts to a given tumour. 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 patients with NSCLC who respond to anti-PD-1 treatment showed inhibition of β-catenin signalling, 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-γ response, affecting the leucine-rich repeat-containing *NLRC3*, among others (appendix).

Alternatively, tumour cell-extrinsic mechanisms that lead to immunoresponse include elements other than the cancer cells, including other cellular lineages that are present in the primary tumour.[10] Taking advantage of the available DNA methylation profiles of particular cell populations, we identified cell lineages enriched in our EPIMMUNE signature (appendix). The carefully dissected DNA methylation patterns of the T-cell, B-cell, and myeloid lineages available from the International Human Epigenome Consortium[22] and other databases such as Gene Expression Omnibus and Sequence Read Archive allow the molecular dissection of various immune classes in our set of NSCLCs. We observed that the EPIMMUNE-negative signature that characterises the little response to PD-1 blockade identifies NSCLC tumours 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<0·0001). EPIMMUNE-negative tumours were particularly enriched in tumour-associated macrophages (Wilcoxon test p<0·0001) and tumour-associated neutrophils (Wilcoxon test p<0·0001). These observations

	EPIMMUNE discovery cohort (n=34)	EPIMMUNE validation cohort (n=47)	FOXP1 methylation status validation cohort (n=61)
(Continued from previous page)			
RECIST response to PD-1 blockade			
Complete response	0	1 (2%)	0
Partial response	8 (24%)	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%)
Progression-free survival since PD-1 blockade, months	2·13 (0·43–24·16)	2·70 (0·03–41·39)	3·80 (0·20–22·67)
Overall survival since PD-1 blockade, months	8·46 (0·92–24·59)	6·43 (0·03–48·16)	7·77 (0·85–30·00)

Data are n (%) or median (range). FOXP1=forkhead box P1. ECOG=Eastern Cooperative Oncology Group. PD-1=programmed death-1. RECIST=Response Evaluation Criteria In Solid Tumors. NSCLC=non-small-cell lung cancer.

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

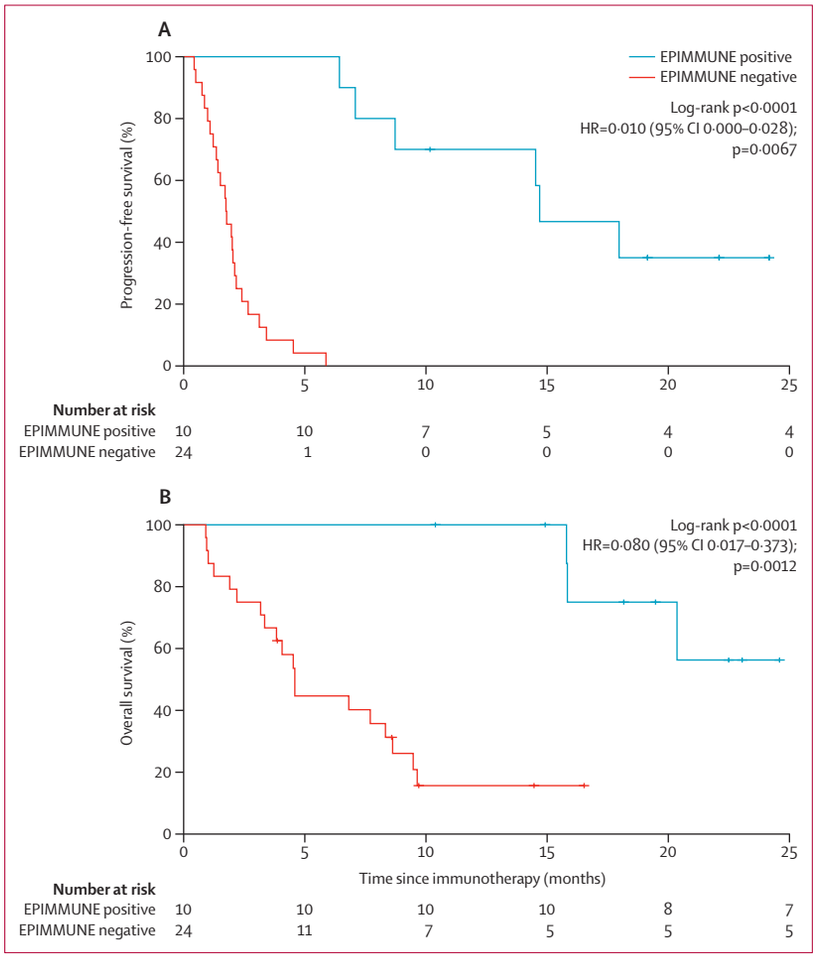

Figure 1: **EPIMMUNE association with progression-free survival and overall survival in the discovery cohort**
Kaplan-Meier analysis of progression-free survival (A) and overall survival (B) in 34 patients with NSCLC by EPIMMUNE signature. NSCLC=non-small-cell lung cancer. HR=hazard ratio.

For the **Gene Expression Omnibus database** see https://www.ncbi.nlm.nih.gov/geo

For the **Sequence Read Archive** see https://www.ncbi.nlm.nih.gov/sra

are consistent with the proposed role of both cell populations as key contributors to resistance to antitumour immunotherapeutic approaches.[9] Conversely, the tumours of EPIMMUNE-positive patients with NSCLC were enriched in the lymphoid lineage, particularly CD4+ α/β T cells with the capacity to produce interferon-γ[23] (Wilcoxon test p<0·0001), CD8+ α/β central memory T cells that represent an important fraction of the tumour-reactive T cells[24] (Wilcoxon test p<0·0001), and natural killer cells (Wilcoxon test p<0·0001) that mediate the antitumour responses without prior sensitisation or recognition of specific tumour 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 and endothelial cells.[9] For cancer-associated fibroblasts, 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 tumours that are enriched in cancer-associated fibroblasts (Wilcoxon test p<0·0001), 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 tumours were enriched in endothelial cells with a senescence phenotype characterised by diminished proliferation, migration, and spreading capacity and a large number of progenitor endothelial cells (Wilcoxon test p<0·0001).[29] This profile describes a hypoxic microenvironment associated with the overexpression of immunosuppressive cytokines such as vascular endothelial growth factor.[30] Conversely, the EPIMMUNE-positive tumours presented more normally activated endothelial cells, which can contribute to the successful recruitment of immune effector cells.

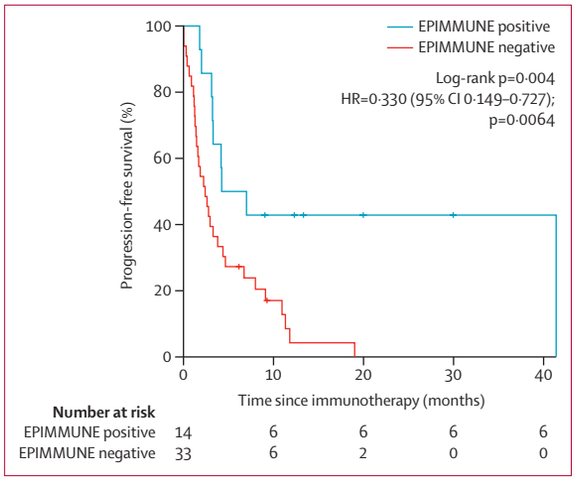

Figure 2: **EPIMMUNE signature analysis in validation cohort of patients with NSCLC treated with anti-PD-1 agents**
Kaplan-Meier analysis of progression-free survival in 47 patients with NSCLC by EPIMMUNE signature. HR=hazard ratio. NSCLC=non-small-cell lung cancer.

For more on **TCGA projects** see https://cancergenome.nih.gov

Having identified the EPIMMUNE signature as being a predictor of response to PD-1 blockade in the discovery set, we examined whether the characterised DNA methylation profile could also discriminate clinical outcome in an independent validation set of patients with advanced NSCLC 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 (table). We found that the EPIMMUNE-positive signature was associated with improved progression-free survival in the studied validation cohort of patients with NSCLC receiving anti-PD-1 therapy (figure 2). We also observed a non-significant association between the EPIMMUNE-positive signature and improved overall survival (HR 0·458, 95% CI 0·197–1·061, p=0·068; log-rank p=0·060; appendix); this result was reflected in an association between the EPIMMUNE-negative signature and disease-specific death (0·465, 0·198–1·092, p=0·079; log-rank p=0·048; appendix). In the validation cohort, the EPIMMUNE-positive signature was enriched in five (71%) of the seven patients who did not experience tumour progression, whereas EPIMMUNE-negative patients frequently experienced tumour progression (31 [78%] of 40 patients; Fisher's exact test p=0·018). Multivariate Cox regression analysis showed that the EPIMMUNE signature was an independent predictor of progression-free survival in the interrogated validation cohort of NSCLC cases treated with anti-PD-1 therapy (appendix). We did not observe any association between the EPIMMUNE signature and any clinicopathological variable (appendix).

We then 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 (48%) of the 301 CpGs of the EPIMMUNE signature. Importantly, this reduced epigenetic profile, which we call EPIMMUNE-TCGA, was still able to predict clinical response, progression-free survival, and overall survival in our discovery set of NSCLCs treated with anti-PD-1 therapies (figure 3A). The EPIMMUNE-TCGA negative signature did not indicate overall poor health but particularly disease-specific death in the discovery cohort (HR 0·210, 95% CI 0·065–0·682, p=0·0092; log-rank p=0·0053). The EPIMMUNE-TCGA signature was not associated with clinical outcome in the validation cohort (appendix). Remarkably, for patients with NSCLC who did not receive immunotherapy, such as those included in TCGA projects, we found that the EPIMMUNE-TCGA signature was not associated with overall survival (figure 3B). These results reinforce the role of the observed epigenomic profile, not as a general factor of improved outcome but as a specific predictive biomarker of response to anti-PD-1 therapies.

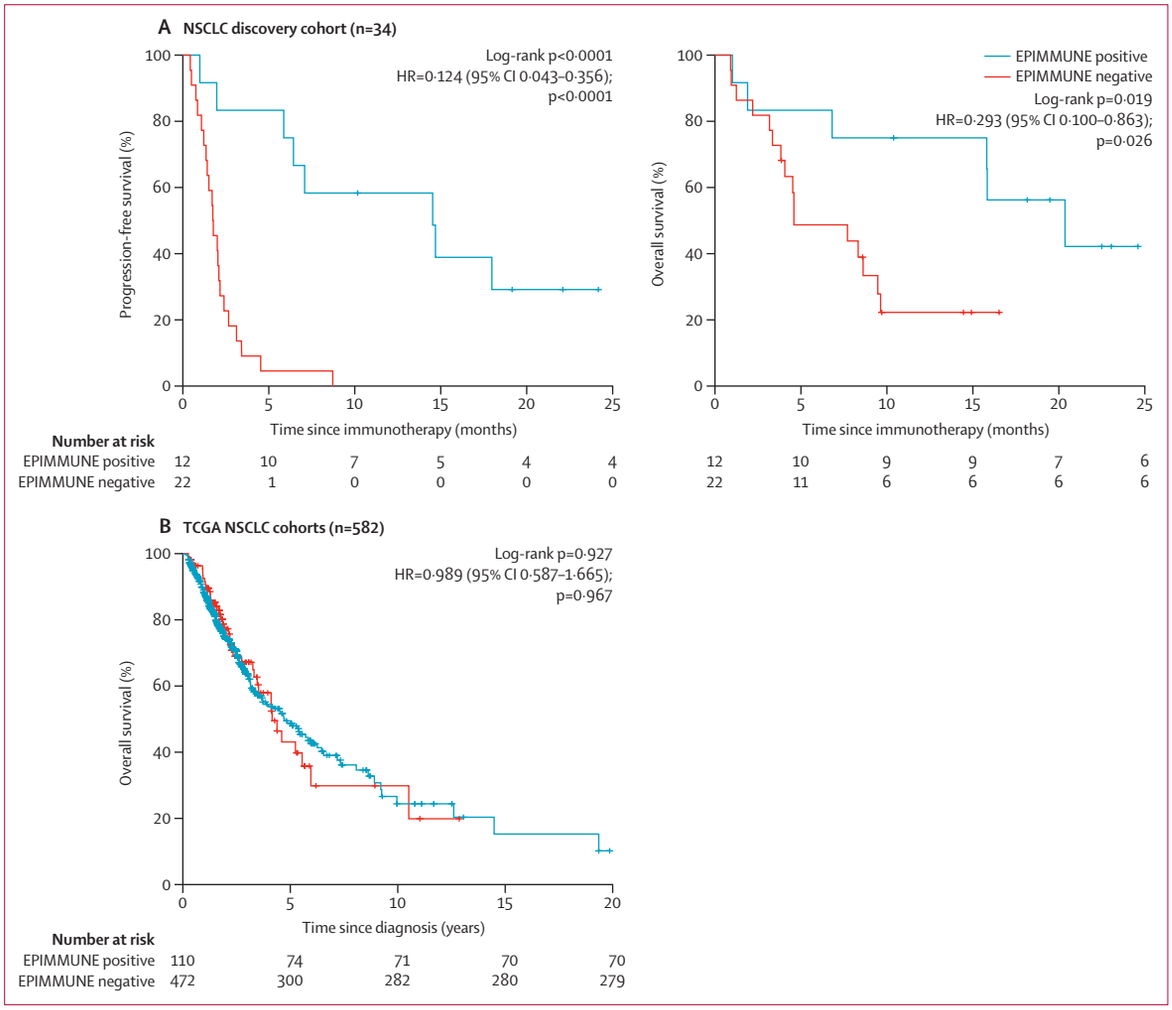

Figure 3: **Kaplan-Meier estimates of clinical outcome by presence of the EPIMMUNE-TCGA signature in patients with NSCLC**
(A) Kaplan-Meier estimates of progression-free survival and overall survival by EPIMMUNE-TCGA signature in the discovery cohort. (B) Kaplan-Meier estimates of overall survival by EPIMMUNE-TCGA signature in patients with NSCLC available from the TCGA database, none of whom received anti-PD-1 treatment. NSCLC=non-small-cell lung cancer. TCGA=The Cancer Genome Atlas.

191 CpG sites (63%) from the 301 CpGs in the EPIMMUNE signature were associated with known genes (appendix), and we sought to validate the best single DNA methylation marker that could predict response to PD-1 blockade therapy in patients with NSCLC. 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 (appendix), 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 naive 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 naive T cells—an event that has already been linked to FOXP1 hypermethylation.[32,33] FOXP1 unmethylated status was associated with extended progression-free survival in the studied discovery cohort (figure 4A), but not with overall survival (appendix). The Cox multivariate regression model showed that FOXP1 methylation status was an independent prognostic factor of progression-free survival in the discovery cohort (appendix). The combination of the PD-L1 and CD8 status or tumour mutational burden did not improve the prediction of anti-PD-1 response provided by FOXP1 unmethylated status alone (appendix). FOXP1 unmethylated status also was associated with extended overall survival in the microarray validation cohort (figure 4B), but not with progression-free survival (appendix). The Cox multivariate regression model showed that FOXP1 methylation status was an independent prognostic factor of progression-free survival in the discovery cohort (appendix).

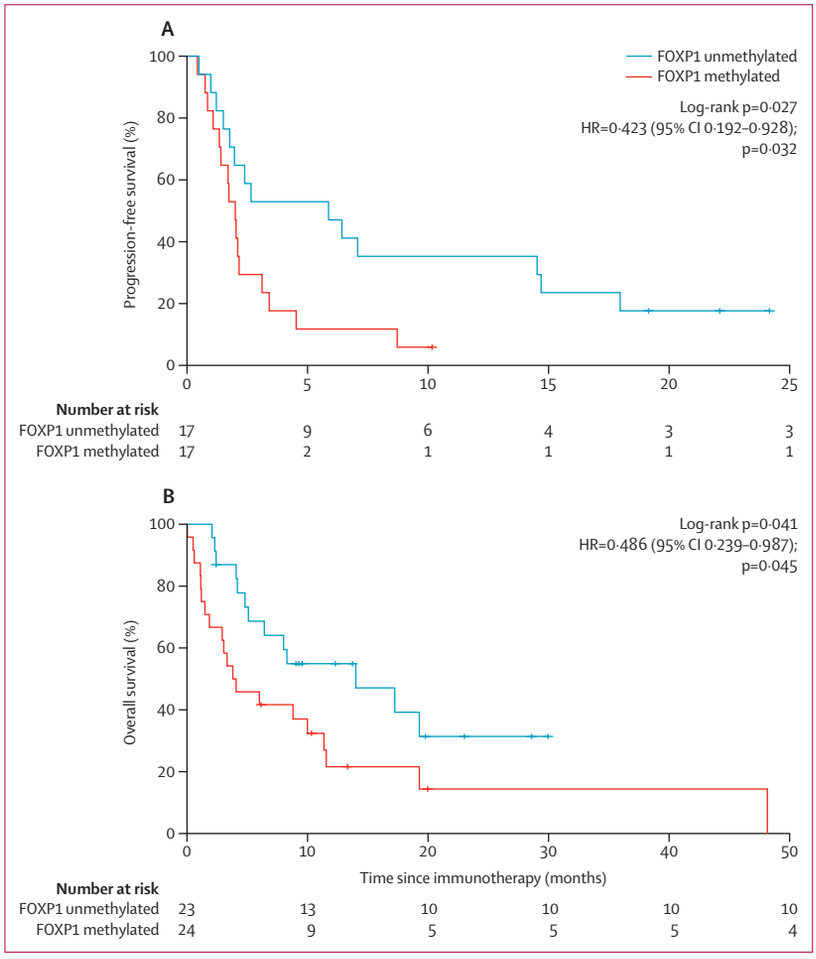

Figure 4: **Survival analysis estimates of clinical outcome by FOXP1 methylation status in NSCLC microarray discovery and validation cohorts**

(A) Kaplan-Meier estimates of progression-free survival by FOXP1 methylation status in the discovery cohort. (B) Kaplan-Meier estimates of overall survival with respect to FOXP1 methylation status in the microarray validation cohort. HR=hazard ratio. FOXP1=forkhead box P1.

We then sought to validate the DNA methylation marker in an additional independent validation cohort of 61 patients with NSCLC who had received PD-1 blockade therapy (table). The CpG methylation levels at the described sites were analysed by pyrosequencing to test a more affordable and large-scale, user-friendly approach. We found that FOXP1 unmethylated status was also associated with progression-free survival following anti-PD-1 treatment in the validation cohort of NSCLC samples analysed by pyrosequencing (figure 5A). The presence of FOXP1 hypomethylation was also associated with overall survival (figure 5B). FOXP1 hypomethylation was also associated with disease-specific death (HR 0·322, 95% CI 0·152–0·068, p=0·0034; log-rank p=0·0020). According to the multivariate Cox regression analysis, FOXP1 unmethylated status was an independent predictor of progression-free survival and overall survival (appendix). Finally, we also established that FOXP1 methylation status was not associated with overall survival in the TCGA patients with NSCLC who had not received immunotherapy (figure 5C). Because RNA sequencing data are available for the TCGA samples, we were able to show that FOXP1 hypermethylation was associated with transcript downregulation (appendix).

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 might experience adverse reactions with no positive response, whereas from a health-care policy perspective, expensive drugs are administered that are not sufficiently cost-effective. Thus, discovery of biomarkers that will predict response to immune checkpoint molecules is essential. 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 important exceptions for responders and non-responders.[13] In our case, although we were able to identify a classifier from our discovery cohort, its small size could explain our having not observed an association of PD-L1 and clinical outcome. Interestingly, a survival benefit associated with the combination of pembrolizumab and chemotherapy has been observed in all subgroups of PD-L1 tumour proportion scores; however, the greatest relative benefit was found in those tumours with a PD-L1 tumour proportion score of 50% or greater.[34] The EPIMMUNE signature described here provides another step towards filling the gap in medical 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 so-called 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 approach. Furthermore, the methylation microarray technique requires double the amount of DNA than does the pyrosequencing strategy, further facilitating the use of the latter in the scarce biological material of these patients, which is also in great demand for other tests. It is worth mentioning in this regard that both EPIMMUNE signature and FOXP1 epigenetic status are not overall prognostic factors in NSCLC, but are specific biomarkers predicting PD-1 blockade response.

The EPIMMUNE signature has a value beyond its ability to predict the response to PD-1 blockade—ie, it also provides biological explanations of the intimate

cellular networks involved in determining immune-checkpoint inhibition. The epigenetic profile observed in patients with NSCLC who, following anti-PD-1 therapy, have no substantive clinical response, is characteristic of tumours that are enriched in a particular immune microenvironment characterised by an enrichment in myeloid lineage-derived cells, such as tumour-associated macrophages and neutrophils. These PD-1 blockade-resistant tumours are also enriched in cancer-associated fibroblasts and senescent endothelial cells. Interestingly from a clinical practice standpoint, the presence of cell populations associated with the EPIMMUNE-negative signature, such as of tumour-associated macrophages and neutrophils, cancer-associated fibroblasts, and senescent endothelial cells, can also be assessed by immunohistochemistry.[10] The epigenetic setting of patients with NSCLC who do not respond to the PD-1 blockade described here is therefore compatible with the so-called immunologically cold or immuno-ignorant tumours, and identifies cases in which interventions to transform them into so-called hot or more immuno-sensitive tumours 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 patients with NSCLC who do not respond to PD-1 blockade exhibit exaggerated tumour-associated-macrophage and cancer-associated-fibroblast activity and an immunosuppressive endothelial milieu—for example, the specific inhibition of the macrophage-associated phosphoinositide 3-kinase γ or the blockade of the receptor of colony-stimulating factor 1, which is under clinical trials to test its combination with immune checkpoint blockade. In addition, cancer-associated fibroblasts can be targeted to increase the response of the EPIMMUNE-negative tumours to PD-1 blockade, inhibiting the cancer-associated-fibroblast recruitment-associated chemokine ligand 12 or the focal adhesion kinase.[27] Finally, targeting endothelial cells, the normalisation of the tumour vasculature by the use of antiangiogenic agents, which enhances the infiltration of CD4+ and CD8+ T 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 patients with NSCLC determined in this study could, by itself, also be a target for therapies. The DNA methylation markers studied here and the histone modifications can be reverted with epigenetic drugs that facilitate the conversion from a cold tumour microenvironment to an immunoresponsive one. In this regard, demethylating agents enhance chemokine production by T helper 1-type T cells and T-cell trafficking into the tumour, providing better responses to immunotherapies in preclinical models.[36,37] Most importantly, DNA demethylating agents and other

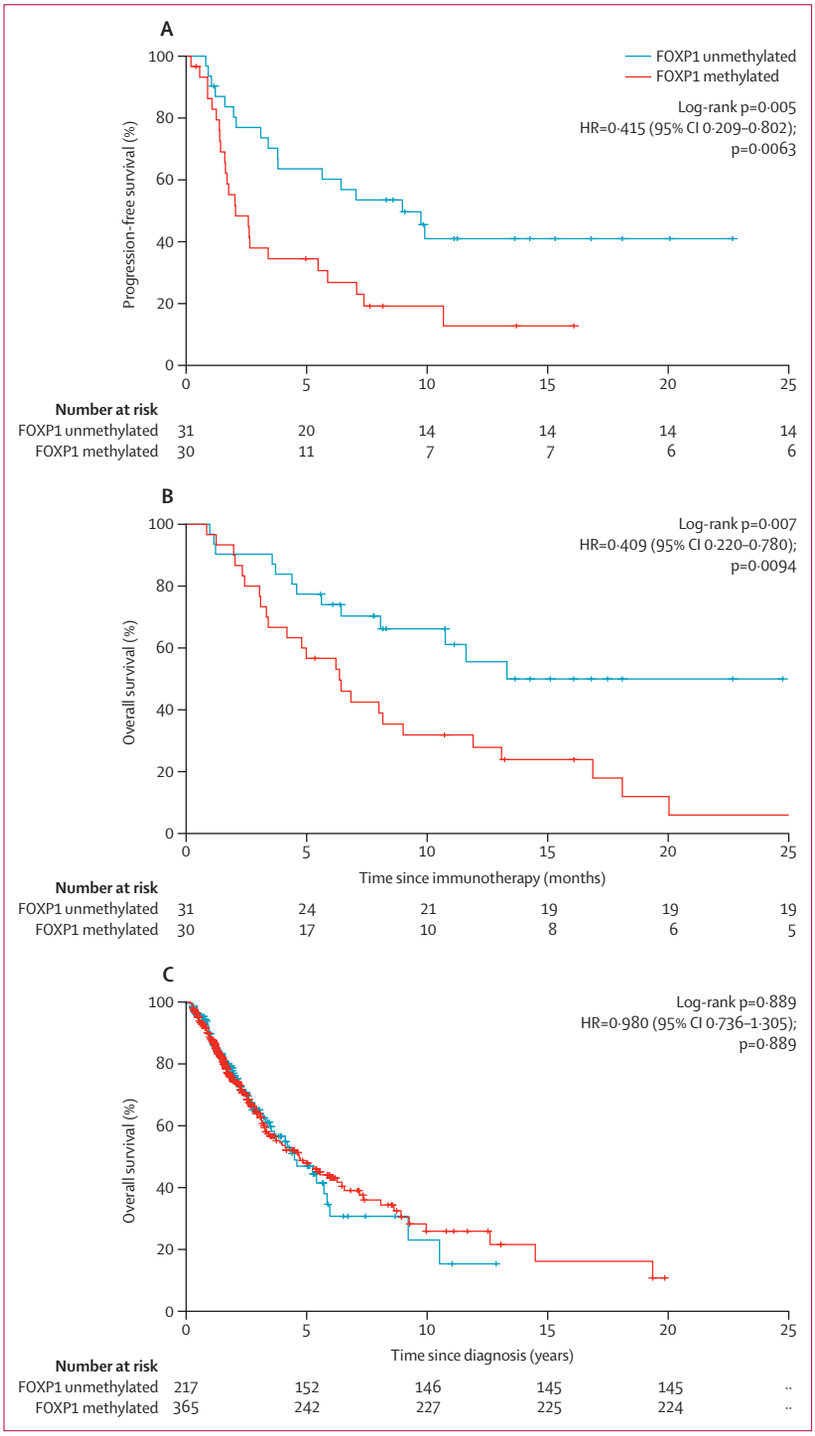

Figure 5: **Survival analysis estimates of clinical outcome by FOXP1 methylation status in NSCLC pyrosequencing validation cohort and TCGA samples**

(A) Kaplan-Meier estimates for progression-free survival (A) and overall survival (B) by FOXP1 methylation status by pyrosequencing analysis in the validation cohort. (C) Kaplan-Meier estimates of overall survival by FOXP1 methylation status in the NSCLC cohort from the TCGA database. HR=hazard ratio. NSCLC=non-small-cell lung cancer. FOXP1=forkhead box P1.

epigenetic drugs, such as histone deacetylase inhibitors, have been clinically approved for use in the treatment of some subtypes of leukaemias and lymphomas.[15] This has facilitated the inclusion of these agents, in combination with immune checkpoint inhibitors, in several phase 2 and 3 clinical trials in lung cancer (NCT02638090 and NCT01928576) and in other solid tumour types (NCT03264404, NCT03182894, and NCT02816021).

In conclusion, we report that the establishment of DNA methylation profiles in NSCLC tumour 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 tumour specimens, which is a crucial microenvironment for determining the response to immune checkpoint inhibitors. Our findings also warrant follow-up studies to check the ability of such profiles 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 the true value of the EPIMMUNE signature, 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.

Contributors

MD, AM-C, and ME designed the study, contributed to the analysis, and wrote the first draft of the manuscript. AM-C, MEC-C, SM, MCdM, VD, DP, MS-C, AFF, and MFF did further data analysis. MG and IG developed the exome sequencing. In-depth clinical and pathological characterisation and recruitment of patients were carried out by MD, AM-C, NG, MB, EG-L, CD, MP, PB, EC, SN, AC, M-CC, NK, MG-C, SP, LMM, IG-B, IBara, MDL, MV, JCR, RP, EN, TM, LP, IR, QX, CT, NV, AP, NR, AB, PG, IBarr, J-FE, RR, and EB. All authors helped to draft the manuscript or revise it critically for 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, outside of the submitted work. MD has served as a consultant (advisory board) for Pfizer, Boehringer Ingelheim, and Bristol-Myers Squibb, outside of the submitted work. ME and AM-C report personal fees from Ferrer International, outside of the submitted work. EN has received consultancy fees from Merck Sharp & Dohme and Bristol-Myers Squibb, outside of the submitted work. All remaining authors declare no competing interests.

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