Analysis of the prognostic role of an immune checkpoint score in resected non-small cell lung cancer patients

Marta Usó (marta.uso@gmail.com)[1,2†], Eloísa Jantus-Lewintre (jantus_elo@gva.es)[2,3,†], Silvia Calabuig (calabuig_sil@gva.es)[2,4], Ana Blasco (blasco_ana@gva.es)[5], Eva García del Olmo (garcia_evadel@gva.es)[6], Ricardo Guijarro (Ricardo.Guijarro@uv.es)[6], Miguel Martorell (Miguel.Martorell@uv.es)[7], Carlos Camps (camps_car@gva.es)[1,2,5,*], Rafael Sirera (rsirera@btc.upv.es)[3,*]

[1]Department of Medicine, Universitat de València, Valencia, Spain

[2]Molecular Oncology Laboratory, Fundación Investigación, Hospital General Universitario de Valencia, Valencia, Spain

[3]Department of Biotechnology, Universitat Politècnica de València, Valencia, Spain

[4]Department of Pathology, Universitat de València, Valencia, Spain

[5]Medical Oncology Department, Hospital General Universitario de Valencia, Valencia, Spain

[6]Department of Thoracic Surgery, Hospital General Universitario de Valencia, Valencia, Spain;

[7]Centro de Investigación Médica Aplicada (CIMA), Universidad de Navarra, Pamplona, Spain;

[7]Department of Pathology, Hospital General Universitario de Valencia, Valencia, Spain.

†Contributed equally to this work.

*Correspondence: Rafael Sirera, PhD, Associate Professor, Department of Biotechnology, Universitat Politècnica de València, Camino de Vera, s/n, 46022, Valencia, Spain (rsirera@btc.upv.es) or Carlos Camps, MD, PhD, Associate Professor, Department of Medicine, Universitat de València, Av. de Blasco Ibáñez, 15, 46010 València, (

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Abstract

Tumors develop mechanisms to recruit tolerogenic immune cells and to induce the expression of molecules that act as immune checkpoints. This regulation of the immune microenvironment favors immune tolerance to the neoplastic cells. In this study we have investigated the prognostic role of immune-checkpoint expression markers in a cohort of resectable NSCLC patients. RNA was isolated from fresh-frozen lung specimens (tumor and normal lung) (n=178). RTqPCR was performed to analyze the expression to analyze the relative expression of 20 immune-related genes and normalized by the use of endogenous genes selected by GeNorm algorithm. Patients with higher expression levels IL23A, and LGALS2 presented better outcomes. Clustering expression patterns, we observed that patients with higher expression of immunoregulatory genes had better survival rates. Additionally, these data was used to develop a gene expression score. Since CTLA-4 and PD-1 were associated with prognosis based on COX regression analysis (Z-score> 1.5), a multivariate model including these two genes was created. Absolute regression coefficients from this analysis were used in order to calculate the immune-checkpoint score: (PD1 x 0.116) + (CTLA4 x 0.0589) for each case. Kaplan-Meier survival analysis showed that patients with high immune-checkpoint score have longer overall survival (OS) [NR vs 40.4 months, p=0.008] and longer relapse-free survival (RFS) [82.6 vs 23 months, p=0.009]. Multivariate analysis in the entire cohort indicated that the immune-checkpoint score was an independent biomarker of prognosis for OS [HR: 0.308; 95%CI, 0.156-0.609; p=0.001] and RFS [HR: 0.527; 95%CI, 0.298-0.933; p=0.028] in early-stage NSCLC patients. In conclusion, this score provides relevant prognostic information for a better characterization of early-stage NSCLS patients with strikingly different outcomes and who may be candidates for immune-based therapies.

Introduction

Lung cancer is the most common cause of tumor-related death in the world. Approximately 85% are classified as non–small cell lung cancer (NSCLC) and at the time of diagnosis the majority of patients present locally advanced or metastatic disease[1]. Improvements in the understanding of the mechanisms for lung cancer initiation, maintenance, and progression have led to the discovery of a variety of molecularly defined subsets of patients characterized by specific sets of driver mutations[2]. Lung cancers are among the most mutated types of tumors[3], therefore generating new antigens which play a key part in tumor immunity [4] and improved responses to immune-based therapies in NSCLC and other lung tumors[5–7].

Tumor-infiltrating immune cells have been identified in many types of cancers[8] and although some of these cells are potentially capable of eliminating neoplastic cells, ultimately they cannot prevent tumor development and progression[9]. In NSCLC it has been shown that the presence, localization and proportion of helper and specially cytotoxic infiltrating lymphocytes are associated with a favorable prognosis[10–13]. But tumors acquire mechanisms to regulate their immune microenvironment such as the release of a series of factors to subvert normal reaction mechanisms as the modulation of co-stimulatory pathways, also known as immune checkpoints[14], and the induction and attraction of suppressor cells such as myeloid-derived suppressor cells[15,16], tumor-associated macrophages[17], and regulatory T cells[18,19]. The clinical implications of these immunoregulatory elements in tumors are still controversial[20], and their functional or causal relationship between immunosuppressive pathways and immune cells in the tumor microenvironment has not yet been clearly defined in NSCLC[21–24]. Therefore, the study of a great variety of immune-related markers, especially those implicated in immunoregulatory processes, could provide valuable prognostic information that could

help in many applications in future NSCLC clinical practice. Additionally, and regarding laboratory assessments to determine these markers, it is of crucial importance to develop robust methodologies that are not biased by observer subjectivity, are reproducible and allow accurate and affordable large scale determinations. In this study with a cohort of surgically-resected NSCLC patients we have investigated the prognostic value of the quantification of gene expression levels of a large array of immunoregulatory molecules (mRNA by RTqPCR). In this article, we demonstrate for the first time the prognostic relevance of an immune checkpoint score based on the relative expression of two immune checkpoint molecules in resected NSCLC patients.

Results

Patients characteristics

The most relevant demographic and clinicopathological characteristics including age, gender, stage of disease and histology of the 178 NSCLC patients entered in the study are shown in Table 1. The median patient age was 65 years [range: 26-85], 86.5% were male, 47.2% had SCCs, and 59% of the patients were diagnosed at stage I of the disease. Moreover, 80 (45%) relapsed and 76 (42.7%) died during the follow-up. The median follow-up was of 81.23 months [Range: 1-113].

Immune-related gene expression patterns and their association with survival

We measured the expression of 20 immune-related genes in primary lung tumor and paired noncancerous tissues (adjacent healthy lung tissue) using RTqPCR. Using this criteria, we found that FOXP3 (3.87X) and CD25 (2.66X) were overexpressed, whilst CD1C (0.42X), CD127 (0.40X), PD1 (0.38X), and CCL2 (0.25X) were downregulated in the tumor compared to normal-paired tissue. Unsupervised hierarchical clustering analysis was used to group patients based on the similarity of their expression patterns. Patients were classified into a cluster tree with two major subgroups according to the expression of genes related to conventional and regulatory T cells (CD4, CD8, CD127, FOXP3, CD25, CTLA4, PD1 and PDL1) and genes involved in different immunoregulatory processes (IL10, IL23A, CCL2, NRP1, LGALS1, LGALS2, CD1C, and CD209). Patients in Cluster I had lower expression levels of most of the genes analyzed, whilst Cluster II comprised patients with higher gene expression levels (Figure 1).

Kaplan-Meier survival analysis using the two major clusters showed that patients in Cluster II had longer relapse free survival (RFS) (81.2 vs. 26.2 months, $p = 0.027$) and overall survival

(OS) (not reached (NR) vs. 46.6 months, p = 0.040) than patients in Cluster I (Figure 2). We also analyzed their prognostic value according to histology, and observed that ADC patients classified in Cluster II had a significantly better RFS (81.2 vs. 17.8 months, p = 0.005) and OS (NR vs. 42.9 months, p = 0.034) than patients in Cluster I. Although it is of interest that the unsupervised cluster analysis of immune-related genes was able to identify a group of patients with a better prognosis, hierarchical clustering can only be applied retrospectively and cannot be used to predict a patient's future outcome. Therefore, next we investigated the prognostic value of genes analyzed individually or as small groups.

Individual immune-related genes associated with survival

Kaplan-Meier analysis was carried out indicated that patients with high levels of IL23A presented better RFS (81.2 vs. 23.4 months, p = 0.003; Supplementary Figure 1a-b) and better OS (NR vs. 43.4 months, p = 0.001). Another gene that correlated with better RFS (NR vs. 26.2 months, p=0.002) and OS (NR vs. 46.6, p=0.007) was LGALS2, which encodes galectin-2 (Supplementary Figure 1c-d).

Survival analysis was also performed according to the patient histology. Kaplan-Meier test performed with ADC patients (including ADCs and adenosquamous histology), showed the same association between high IL23A and LGALS2 and better prognosis that were found in the entire cohort. Furthermore, the group of patients with high CTLA4 expression levels had better RFS (81.2 vs. 18.2 months, p = 0.002, Supplementary Figure 2a-b) and OS (NR vs. 37 months). We also found that high levels of IL10 correlated with a higher RFS (49.3 vs. 18.8 months, p = 0.029, Supplementary Figure 2c-d) and OS (81.2 vs. 37 months, p = 0.030).

Immune checkpoint score (ICS) is a prognostic biomarker for RFS and OS in NSCLC

We also intended to create a gene expression score based on a multi-gene signature, which can provide more accurate predictions than a model using single genes[25,26]. Univariate Cox regression analysis was performed considering overall survival as a dependent variable. Genes were ordered on the basis of their prognostic power (univariate Z-score, Supplementary Figure 3) and according to this ranking, the expression of two genes, PD1 and CTLA4 (both considered immune checkpoint molecules), were found to be associated with survival (Z-score >2), and therefore, were selected to construct a risk signature. We constructed a model based on the relative contribution of these two genes in the multivariate analysis (considering the absolute regression coefficients, see table 2), and the resulting score was named ICS (Immune Checkpoint Score), with the following equation: **(PD1 x 0.116) + (CTLA4 x 0.058)**.

Kaplan-Meier analysis showed that patients with a high ICS (> median) had longer RFS (82.6 vs. 23 months, $p = 0.009$; Figure 3) and OS (NR vs. 40.4 months, $p = 0.008$). We also performed a stratified analysis by TNM staging and histology. We found that for ADC patients, the association between high ICS and prognosis was stronger than for the entire cohort of patients (RFS: NR vs. 16.2 months, $p < 0.001$ and OS: NR vs. 34.4 months, $p = 0.002$). To evaluate the potential use of the ICS as an independent prognostic biomarker, a multivariate analysis was performed, where significant analytical and clinicopathological variables from the univariate analysis were entered in the study. The variables included were: Lymph node involvement, KRAS status, cluster classification, FOXP3, CD4, CD127, IL23, LGALS2, the expression score, and the immune checkpoint score. Results obtained from this multivariate analysis indicated that KRAS status and the immune checkpoint score were independent biomarkers for both OS and RFS, and in the later, CD127 expression was also identified as an independent biomarker (see table 3).

Discussion

Although most of the patients in early stage NSCLC (stages I-IIIA) are treated surgically with curative intent, the associated survival is less than optimal, with a 5-year survival rate ranging from 50% for stage IA to 15% for stage IIIA[27]. Currently, there are still gaps in the approach used for selecting patient's adjuvant therapies based on the surgical or TNM stage alone. So, a great challenge in the management of patients with resected NSCLC is to develop new biomarkers that could help in identifying subjects at the greatest risk of recurrence and their potential response to specific treatments. Over the last decade, the field of tumor immunology has changed, and it is now accepted that the immune system plays a pivotal role in cancer. We are in the immunotherapy era not only for the efficacy of the new therapeutic armamentarium based in the blockage of immune checkpoint inhibitors but also for the relevance of the immune-derived prognostic and predictive biomarkers related to immunoregulatory processes. Thus, the studies of immune-related markers, especially those implicated in immunoregulatory processes, like our paper, could provide valuable prognostic information in resected NSCLCs that could help in future clinical practice.

Infiltrating immune cells of the acquired and innate immune response are organized in the lung tissue[20,28] and their scoring of the type, density and localization has demonstrated to be a prognostic factor in cancer, even as useful as the pathological characteristics[9,10]. Additionally, in the tumor, cancer cells and other components of the microenvironment release chemokines and chemokine receptors[29,30] that on one side regulate the migration of immune cells[31,32] and on the other side act as molecules that promote the proliferation and migration of the neoplastic cells[33,34]. A favorable environment for the growth of tumor cells implies the specific attraction of cells with known immunosuppressive properties as regulatory T cells, Tregs[35–37] and myeloid-derived suppressor cells (MDSCs)[38–41]. Moreover, cancer cells can produce cytokines with immunosuppressive functions as IL-10[42] and TGF-beta[43] together

with enzymes such as indoleamine 2,3-dioxygenase (IDO)[44] that can impair metabolites needed for immune cells to thrive in the environment. But of special interest is the capability of the tumoral cells to express certain immune checkpoint receptors that promote tolerance of the immune system to the tumoral cells and lead the escape from immune surveillance. The maximum exponents of the checkpoint receptor are the cytotoxic T-lymphocyte–associated protein-4 (CTLA-4)[45], programmed cell death protein-1 (PD-1) and its ligand PD-L1[46,47]. In our work we have analyzed the expression levels by RTqPCR of a large variety of cellular and soluble immuneregulation-related biomarkers associated with components of the physiologic or pathophysiologic immune response.

In this study gene expression immune-related biomarkers were assessed in fresh-frozen tumor and normal lung tissue samples from resected-NSCLC patients. An introductory analysis based on unsupervised clustering of the immune-related genes indicated that the group of patients with the highest expression levels of immune-related genes had better outcomes than the other group, although most of these genes were involved in immunoregulatory processes. This is not the first study to observe that patients with higher expression of genes related to immunoregulation have better survival rates[48]. In fact, in breast cancer, a molecular signature obtained from microarray data analysis, which was associated with relapse-free patients had a higher representation of genes involved in B cell development and antigen presentation, but also of genes involved in T-cell apoptosis, CTLA4 signaling, or activation of IL23R, which are all pathways involved in the negative regulation of effector T cells[49]. In a recent study, the expression of immunosuppressive factors such as PD1, PDL1, CTLA4, and FOXP3 measured in 481 breast tumors, were highly significant predictors of therapy response and improved outcome[50]. Our observations are consistent with the present idea that there are two different phenotypes regarding the tumoral infiltration of immune cells, in the sense that the most immune components present in the tumoral microenvironment, even if they are

immunoregulatory, the better prognosis of the disease[51]. By contrast those tumors that are not able to recruit immune cells had worst prognosis[48,52].

Since hierarchical clustering can only be applied retrospectively and cannot be used to predict a patient's future outcome, we investigated the prognostic value of genes analyzed individually. This individual survival analysis revealed associations between markers like IL23A, and LGALS2 with better outcomes. IL23 is considered the master switch in several T cell-mediated inflammatory disorders, but the antitumor activity of IL23 is controversial. On the one hand, it has been shown that pro-inflammatory cytokines, including IL17A, IL6, and IL23 can impair $CD8^+$ T cell-mediated immune surveillance and promote tumor neovascularization[53]. But on the other hand, other groups have reported that IL23 exerts antitumor activity by stimulating T cells and natural killer (NK) cells[54]. Its prognostic value was studied in ovarian cancer, and an improved OS was observed in patients with high p19 mRNA expression (expressed by the IL23A gene)[55]. In lung cancer, a recent study in NSCLC tumor samples and cell lines reported that gemcitabine, a chemotherapy drug indicated for first-line treatment of NSCLC, induced IL23A expression and that it was found to induce NSCLC cell line proliferation. However, they failed to correlate IL23A expression with NSCLC patient prognosis[56]. A for LGALS2, in contrast to galectin-1 and galectin-3, relatively few studies have examined the expression of galectin-2 in animals and human tumors. Galectins are members of a highly conserved family of β-galactoside-binding lectins, which have a broad variety of functions including immune function regulation. The most extensively-studied galectin function is their regulation of apoptosis. Furthermore, galectin-1 functions as a soluble mediator used by tumor cells to evade the immune response[57]. Similar to our findings that lower LGALS2 expression is associated with a worse outcome, in gastric cancer, it has been reported that decreased galectin-2 expression is associated with LN involvement and advanced clinical stage[58].

In other to get a deeper insight into the immune regulatory process, a score composed with the expression of immune checkpoint related genes (ICS, immune checkpoint score) was constructed following a mathematical model[25,26]. Expression scores are well established methods for separating patients into prognostic group and our score includes the expression levels and regression coefficients from the cox analysis of CTLA4 and PD1, as follows, ICS = (PD1 x 0.116) + (CTLA4 x 0.058). When dichotomizing the cohort attending to the median level of the ICS, the Kaplan Meier analysis revealed that a high ICS (ICS above the median) was associated with longer OS and RFS. The ICS in the Cox regression model, including all the significant variable, demonstrated to be an independent prognostic biomarker, along with *KRAS* status. The ICS encompasses the expression of two genes, CTLA4 and PD1, which have become of great interest in the last few years. CTLA4 overexpression is more common in ADC and appears to be an independent prognostic factor in NSCLC[45]. This is because researchers have demonstrated the importance of how the immune checkpoint blockade leads to robust antitumor effects in patients with metastatic melanoma, NSCLC, and other tumor types. In fact, targeted therapy with blocking antibodies to this immune checkpoint are one of the more promising therapeutics in many different tumors. So regarding the prognostic implication of CTLA-4 expression in NSCLC there are contradictory and opposite results in the literature. It has been described that high expression of CTLA-4, but not PDCD1 predicts worse survival in NSCLC[46] and other malignancies like nasopharyngeal[59] or esophageal carcinoma[60]. Contrarily, other authors have found a reduced death rate in radically resected NSCLC overexpressing CTLA-4[45].

In our cohort, we have found that for ADC patients, the association between high ICS and prognosis was stronger than for the entire group of patients. This results are in concordance with recent reports that indicate that in squamous NSCLC treated with nivolumab, an anti-PD1 monoclonal antibody, the improvement in overall survival was independent of PD-L1

expression[61] while in contrast for nonsquamous NSCLC this benefit was only observed in those patients with PD-L1 expression[62]. In a similar way, PD1 is also an immune checkpoint receptor with immunosuppressive properties. However, in contrast to CTLA4, PD1 is activated during the effector stages of T cell activation, interaction with its ligand (PDL1) occurs primarily in peripheral tissues instead of lymph nodes, and importantly, it can be expressed in tumor tissue as well as in immune cells[47,63]. The expression of immune checkpoints, PD1 and PDL1, in infiltrating immune cells by IHC was correlated with better responses to immune checkpoint blockade treatment, suggesting that the presence of these biomarkers might indicate that these tumors have already been recognized by the immune system, and therefore they are key predictors of clinical treatment responses[52,64]. Additionally, in one publication the expression of PD1 and PDL1 in a cohort of 125 NSCLC has been assessed in tissues in order to evaluate if they were differently expressed according to the presence or absence of EGFR mutations, ALK translocation, or KRAS mutations. Although they observed that the sensitivity to treatment was higher and the OS was longer in patients treated with EGFT TKIs when PDL1 expression was higher, no differences were observed for PD1[47]. As for PDL1, its expression in two large NSCLC patient cohorts has been analyzed, observing that high expression of PDL1 protein or mRNA was associated with a better outcome[24]. Furthermore, tumor PD-L1 expression was associated with improved overall survival in NSCLC with adjuvant therapy[65]. Also, in NSCLC patients treated with EGFR inhibitors the response rate, time to progression and survival was higher in PD-L1 positive vs PD-L1 negative patients, but with no difference in PD1 positive vs PD-1 negative groups[47] However, we failed to obtain this correlation in our data when the prognostic value of the markers was analyzed individually. This discrepancy could be explained by methodological differences as we performed quantitative PCR whereas Velcheti et al. used IHC and in situ hybridization. But the IHC determinations of PD1 and PDL1 at the protein levels in NSCLC

is heterogeneous even with interassay discordance. This differences are caused by the antibodies used, with a range in specificity and affinity[66] and tests that harmonize immune checkpoint determinations in NSCLC and other cancers are indeed needed[67]. For this reason we selected for our study the use of RTqPCR, the gold standard method for gene expression quantification with high sensitivity and specificity and clinically applicable for detecting patient subgroups with specific prognostic characteristics. Other advantages of this technology are that it requires a low RNA input, it is less time consuming than other methods, and it is robust and flexible. This is of great importance because current clinicopathological staging methods have limited success in predicting patient survival and great outcome uncertainty for same-stage NSCLC cancers remains and today, we still cannot predict which patients will be cured and which ones will suffer recurrence or death after surgical resection. Very recently, an in silico study using mRNA data from The Cancer Genome Atlas (TCGA) from 11 tumor types has demonstrated heterogeneous immune infiltrates are present and in general are linked to improved prognosis[68]. If these predictive markers are a reflection of a pre-existing immune recognition, and taking the theory that predictive markers are also likely to be of prognostic value into account[69], our results suggest that immune checkpoint marker expression may also be of future value as a new prognostic NSCLC biomarker. Thus, the immune checkpoint score may reflect a favorable immune context, in which the immune system recognizes the tumor. We propose that these results may also have some therapeutic value for managing NSCLC via emerging targeted immunotherapies, especially immune checkpoint blockade-based therapies, and so further studies to asses both of these uses should be conducted in order to better understand these processes.

Taken together, our results indicate the existence of two possible immune-scenarios in NSCLCs. In the first, the tumor is recognized by the immune system and a T-cell response is activated, which in turn activates also immunoregulatory pathways. In this case patients had

better outcomes. In the second scenario, which is associated with worse outcomes, the immune system does not recognize the tumor and there is no immune response activation, therefore immunoregulatory pathway activation is not required. These results provide new insight into the tumor immunity field in NSCLC, and could be useful in the future development of prognostic and therapeutic tools.

Patients and Methods

Patients and tissue samples

This retrospective study included 178 patients with resected NSCLC from the General University Hospital of Valencia who underwent surgery between 2004 and 2013 and who fit the eligibility criteria: resected, non-pretreated stage I to IIIA patients (according to the American Joint Committee on Cancer Staging manual) with a histological diagnosis of NSCLC. The study was conducted in accordance with the Declaration of Helsinki, and the institutional ethical review board approved the protocol. The most relevant demographic and clinicopathological characteristics of the cohort are shown in Table 1. Patient tumor and adjacent normal lung specimens were obtained at the time of surgery and were preserved in RNAlater (Applied Biosystems, USA) to avoid degradation of RNA. The samples were frozen at -80° C until the analysis. REMARK recommendations on the studies of prognostic tumor markers found in tissues, blood and other body fluids were followed[70]. Relapse-free survival (RFS) was estimated as the time from surgery to recurrence or death from the disease, whereas overall survival (OS) was defined as the time from diagnosis to the date of death or last follow up. Additionally, *K-RAS* mutation status was assessed for the whole cohort using the theraScreen® KRAS Pyro® kit (Qiagen). This kit is used for quantitative detection of mutations in codons 12, 13, and 61 of the human *KRAS* gene by pyrosequencing.

Quantitative real time PCR of immune-related genes

RNA from frozen tissue samples was extracted using standard TRIZOL (Invitrogen) methods. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions with 1.0 μg of total RNA using random hexanucleotides. The thermal cycling conditions were as follows: 10 min at 25° C, 120 min at 37° C and 5 s at 85 °C. RTqPCR was performed using Universal Master

Mix and TaqMan Gene Expression Assay (Applied Biosystems, USA), to analyze the relative expression of 20 immune-related genes: Chemokine ligand 2 (CCL2; assay ID Hs00234140_m1), CCL22 (Hs99999075_m1), CD1C (Hs00233509_m1), CD127 (Hs00233682_m1), CD209 (Hs00253550_m1), CD25 (Hs00166229_m1), CD4 (Hs00181217_m1), CD8 (Hs00233520_m1), C-type lectin domain family 4, member C (CLEC4; Hs01092462_m1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4; Hs01011591), forkhead box P3 (FOXP3; Hs00203958_m1); indolamine-1 (IDO1; Hs00984148_m1) interleukin 10 (IL10; Hs00961622_m1), IL23A (Hs00413259_m1), lectin galactoside-binding soluble 1 (LGALS1; Hs00355202_m1), LGALS2 (Hs00197810_m1), neuropilin 1 (NRP1; Hs00826125_m1), programmed cell death 1 (PD1; Hs01550088_m1), PDL1 (Hs01125301_m1), and transforming growth factor beta 1 (TGFB1; Hs00171257_m1). Using GeNorm software, actin beta (ACTB; Hs01060665_g1), glucuronidase beta (GUSB; Hs01558067_m1), and cyclin-dependent kinase inhibitor (CDKN1B; Hs00153277_m1) were use as endogenous controls. The thermal cycling parameters were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For efficiency calculations of each gene, we used the random-primed qPCR Human Reference cDNA (Clontech, USA). Relative gene expression levels were expressed as the ratio of target gene expression to reference gene (GUSB) expression by using the Pfaffl formula[71]. It was considered a gene to be overexpressed when the median of the relative gene expression of the pathological area referred to the adjacent healthy tissue was higher than 2 and underexpressed when it was less than 0.5. Gene expression levels were dichotomized as “high” and “low” according to the median of each case.

Statistical analysis

Non-supervised hierarchical analysis was carried out with Cluster software (version 3.0) and visualized with Tree View software version 1.0.6 which can be found at http://rana.lbl.gov/EisenSoftware.htm [72]. All analyses were carried out on normalized and log2-transformed dataset values. Uncentered correlation was used as the similarity metric and average linkage was used as the clustering method. Continuous variables were compared by non-parametric Mann–Whitney U and Kruskall-Wallis tests. A Spearman rank test was used to test for correlations between continuous variables, and the association between dichotomized variables was evaluated by the Chi-square test. Survival analysis was performed using a univariate Kaplan-Meier (log-rank) test method with clinicopathological variables, dichotomized gene expression marker levels, and immune cell infiltration levels. Finally, to assess the independent value of the tested biomarkers, a Cox proportional hazard model for multivariate analyses was used. All significant variables from the univariate analyses were entered into the multivariate analyses in a forward stepwise Cox regression analysis. Furthermore, we also calculated gene expression scores based on multi-gene signatures using a method previously reported[25,26]. Univariate Cox regression analysis on the training cohort was used to select genes associated with mortality (Z-score > 2) which were afterwards included in a multivariate risk model. All genes were included for these purposes, and expression values for all analyses are continuous variables. For multivariate Cox regression models, missing values for genes were replaced with the average value. A probability of 95% ($p < 0.05$) was considered statistically significant for all analyses. The statistical analyses were done using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 15.0.

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Table 1. Clinicopathological characteristics of the patients included in the study.

Characteristics	N	%
Age at surgery (median, range):	**65 [26-85]**	
Gender		
Male	154	86.5
Female	24	13.5
Stage		
I	105	59
II	35	19.7
IIIA	38	21.3
Histology		
SCC	84	47.2
ADC	74	41.6
Others	20	11.2
Performance Status		
0	118	66.3
1-2	60	33.7
Differentiation grade		
Poor	43	24.2
Moderate	77	43.3
Well	31	17.4
NS	27	15.2
Smoking Status		
Current	86	48.3
Former	72	40.4
Never	20	11.3

ADC, adenocarcinoma; SCC, squamous cell carcinoma; NS, not specified.

Table 2. Results from the multivariate Cox regression model for OS.

Variable	Regression Coefficient	SE	*p*-value	HR	95% CI
PD1 expression	-0.116	0.075	0.121	0.890	0.769-1.031
CTLA4 expression	-0.058	0.035	0.102	0.944	0.881-1.012

CI, confidence interval; HR, hazard ratio; OS, overall survival; SE, standard error.

Table 3. Multivariate Cox regression model results, including all the significant variables**.**

	OS			RFS		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
KRAS status						
Mutated vs. WT	2.984	1.338-6.659	0.008	3.807	1.764-8.214	0.001
Immune checkpoint score						
High vs. Low	0.308	0.156-0.609	0.001	0.527	0.298-0.933	0.028

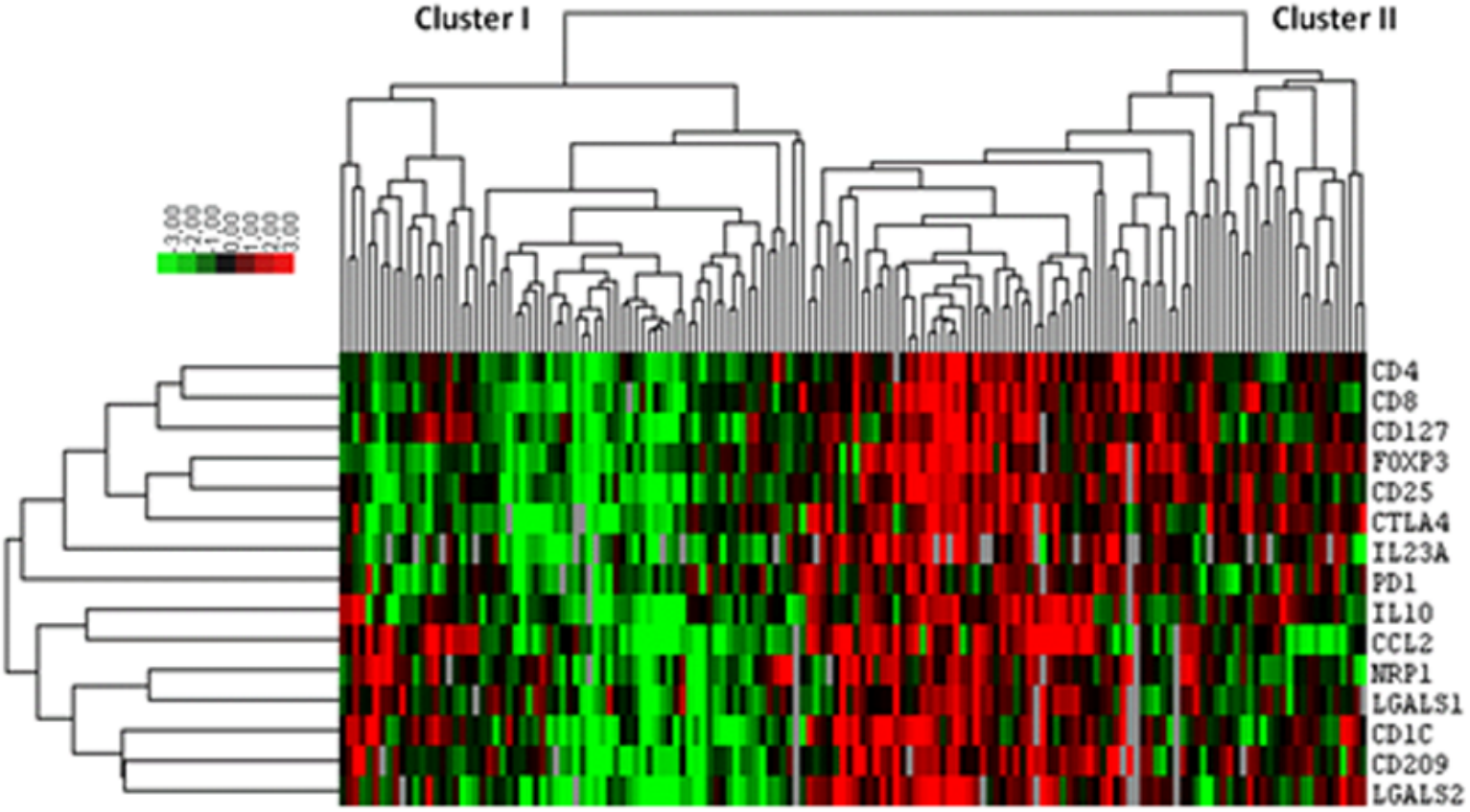
CI, confidence interval; HR, hazard ratio; OS, overall survival; RFS, relapse-free survival; WT, wild type.

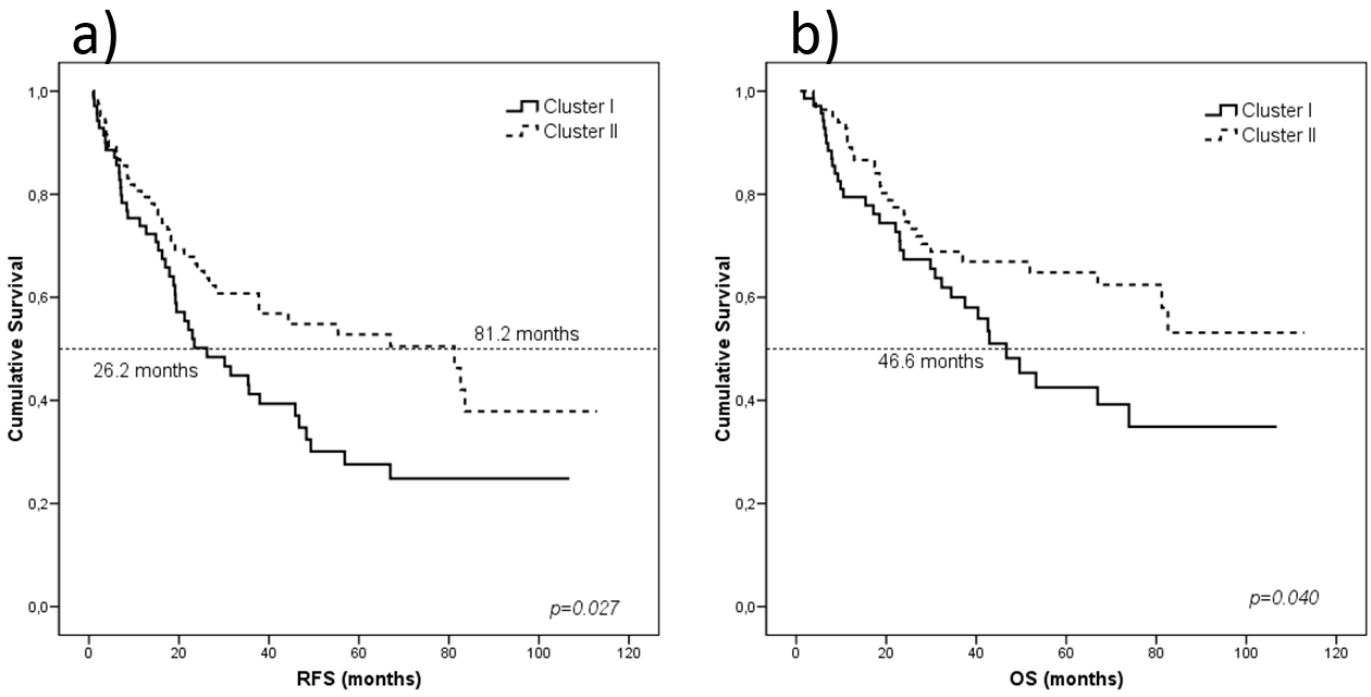
Legend to the figures

Figure 1. Hierarchical cluster based on selected gene expression. Patients in the original cohort were clustered into a hierarchical tree based on the expression of immune related genes. The clustering separated the patients into two distinct groups. Red indicates high expression and green indicates low expression levels.

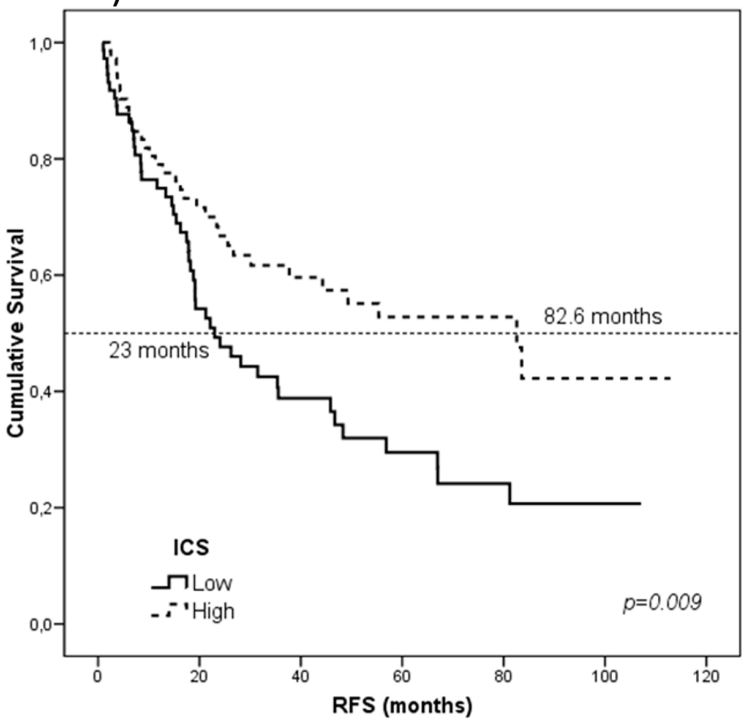
Figure 2. Kaplan-Meier plots for OS and RFS according to the clustering classification of patients. a) RFS and b) OS. Solid line represents patients classified in Cluster I, whilst dashed line represents patients in Cluster II. P-values were calculated using the Kaplan-Meier test.

Figure 3. Kaplan-Meier plots for OS and RFS according to the immune checkpoint expression score (ICS). The score was divided as low and high according to its median. Solid line represents patients with low levels of expression, whilst dashed line represents patients with high scores. P-values were calculated using the Kaplan-Meier test.

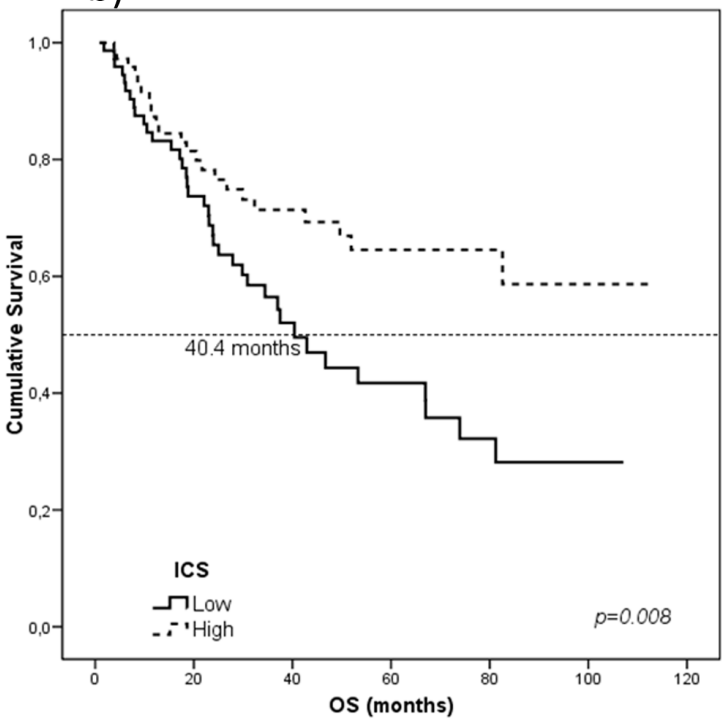

Cluster I
Cluster II
CD4
CD8
CD127
FOXP3
CD25
CTLA4
IL23A
PD1
IL10
CCL2
NRP1
LGALS1
CD1C
CD209
LGALS2


a)
Cluster I
Cluster II
Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
81.2 months
26.2 months
p=0.027
0
20
40
60
80
100
120
RFS (months)
b)
Cluster I
Cluster II
Cumulative Survival
46.6 months
p=0.040
OS (months)

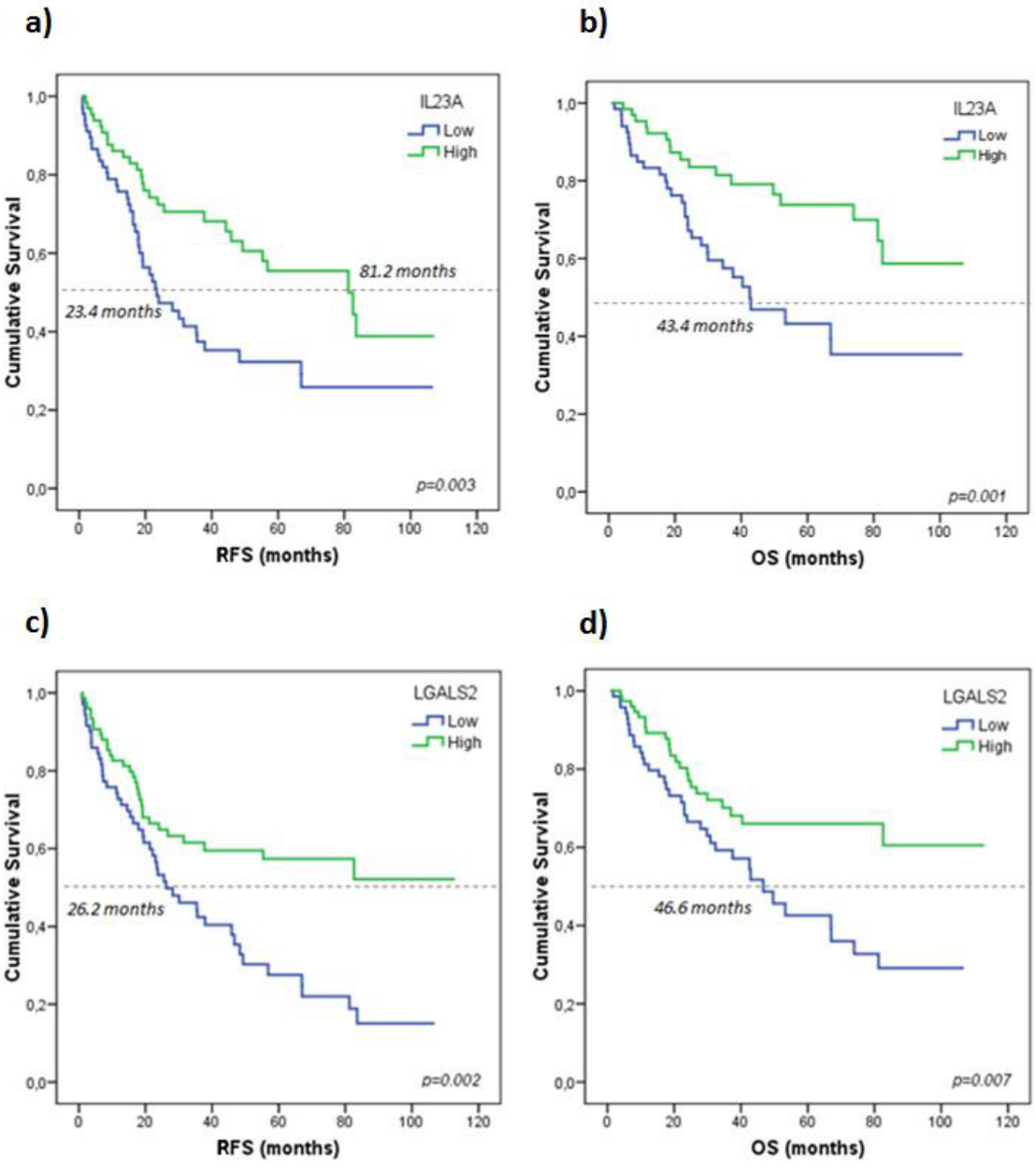
a)


1,0
0,8
0,6
0,4
0,2
0,0
Cumulative Survival
0
20
40
60
80
100
120
RFS (months)
82.6 months
23 months
ICS
Low
High
p=0.009

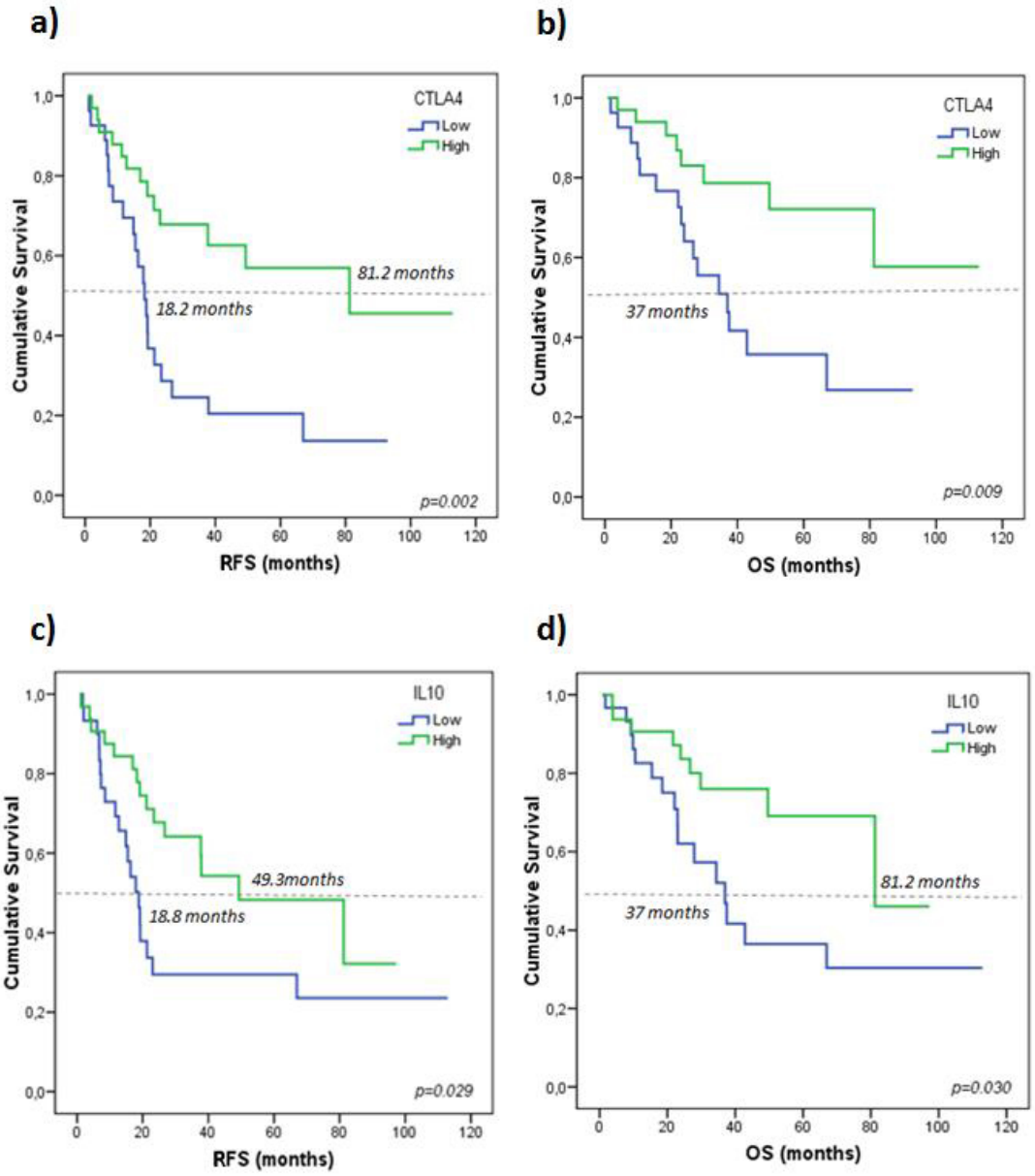
b)


1,0
0,8
0,6
0,4
0,2
0,0
Cumulative Survival
0
20
40
60
80
100
120
OS (months)
40.4 months
ICS
Low
High
p=0.008

Supplementary Figure 1. Kaplan-Meier plots for OS and RFS according to gene expression levels. a-b) IL23A, and c-d) LGALS2. Gene expression levels were dichotomized according to the median. Blue line represents patients with low levels of expression, whilst green line represents patients with high levels. P-values calculated using the Kaplan-Meier test.



Supplementary Figure 2. Kaplan-Meier plots for OS and RFS according to gene expression levels in ADCs. a-b) CTLA4; and c-d) IL10. Gene expression levels were dichotomized according to the median. Blue line represents patients with low levels of expression, whilst green line represents patients with high levels. P-values calculated using the Kaplan-Meier test.



Supplementary Figure 3. Univariate analysis of the expression of 20 immunoregulatory genes for OS. The genes are ranked based on their predictive power (univariate Z-score). Dashed lines indicate |Z-score|= 1.5. This criterion was used to select genes to include in the multivariate Cox regression model used to calculate the expression score