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Expression of immune checkpoints genes in tumour samples from resectable NSCLC patients. Role as prognostic biomarkers.

Trabajo de fin de grado

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Title:

Expression of immune checkpoints genes in tumour samples from resectable NSCLC patients. Role as prognostic biomarkers.

Summary:

Lung cancer is the leading cause of cancer-related death worldwide, with a 5-year survival lower than 5% in advanced stages of the disease. Non-Small Cell Lung Cancer (NSCLC) represents approximately 80% of lung cancer cases. Despite the advances achieved in the last years, there is still an urgent need to develop new and more efficient therapeutic strategies in this type of cancer and a lack of knowledge concerning the tumour microenvironment.

Although it is widely known that the immune system is capable of preventing cancer initiation and its progression, it is also known that one of the hallmarks of cancer is the evasion of the immune surveillance through different mechanisms, one of which is the inhibition of antitumour T cell response. This research focuses on the fact that cancer cells induce inhibitory signals to evade the immune response.

The expression of 8 genes involved in immune-regulation (*PD-L1, PD-L2, IDO-1, IDO-2, ICOS-LG, CD5, CD6 and CD200*) was analysed by RTqPCR in 201 paired fresh frozen tumour and normal tissue samples of resected NSCLC. Relative expression was calculated by Pfaffl formulae using *ACTB, CDKN1B* and *GUSB* as endogenous controls. Non-parametric tests were used for correlations between clinico-pathological and analytical variables and survival was assessed by Cox regression analysis. For those statistically significant analysis, Kaplan-Meier curves (log-rank test), were represented, considering significant $p<0.05$.

Patients with higher expression of *CD5* and *IDO-2* had a significant increase in overall survival (OS, 53.3 months vs NR, p=0.011 and 51.9 months vs NR; p=0.050, respectively). Regarding the analysis performed in the adenocarcinoma (ADC) subgroup, it was observed a tendency of longer OS and relapse-free survival (RFS) in those patients with high expression levels of *PD-L1, IDO-1* and *IDO-2*. A score including three genes: *PD-L1, IDO-1* and *IDO-2* was generated (*PDIDO* score). Patients with high expression levels of the *PDIDO* score show better RFS (17.9 months vs NR; p=0.001) and OS (29.8 vs NR months; p=0.0002). Multivariate analysis established that *PDIDO* score was an independent prognostic factor for RFS [HR, 0.274; 95%CI, 0.126-0.593; p=0.001] and OS [HR=0,267; 95% CI, 0.113-0.630; p=0.003].

Altogether, the study of the immune profile in resected NSCLC has allowed the establishment of *PDIDO* score as an independent biomarker for RFS and OS in the ADC group of patients. Moreover, *CD5* would be a feasible prognostic biomarker for OS regardless of histology in resectable NSCLC.

Key words: Non-Small Cell Lung Cancer, Relative gene expression, Real-Time quantitative PCR, immune-regulation.

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Título:

Expresión de genes de inmuno *checkpoints* en muestras tumorales de pacientes con cáncer de pulmón no microcítico en estadios resecables. Posible rol como biomarcadores pronósticos.

Resumen:

El cáncer de pulmón es la principal causa de muerte debida a cáncer a nivel mundial, con una supervivencia a los 5 años menor del 5% en estadios avanzados de la enfermedad. El cáncer de pulmón no microcítico representa aproximadamente un 80% de los casos de cáncer de pulmón. A pesar de los avances logrados en los últimos años, hay una gran necesidad de desarrollo de nuevas estrategias terapéuticas más eficientes contra este tipo de cáncer y una falta de comprensión del microambiente tumoral.

Por un lado, se sabe que el sistema inmune es capaz de evitar el cáncer, así como su progresión, pero también se sabe que una de las *características distintivas* del cáncer es la capacidad de evadir la vigilancia inmunológica a través de distintos mecanismos, siendo uno de ellos la inhibición de la respuesta antitumoral mediada por las células T. Este trabajo se basa en el hecho de que las células tumorales inducen señales inhibitorias que les permiten evadir la respuesta inmune.

Se analizó la expresión de 8 genes relacionados con la inmunoregulación (*PD-L1, PD-L2, IDO-1, IDO-2, ICOS-LG, CD5, CD6* y *CD200*) mediante RTqPCR en 201 muestras pares de tejido tumoral fresco congelado y del correspondiente tejido normal. Se calculó la expresión génica relativa mediante la fórmula de Pfaffl utilizando *ACTB, CDKN1B* y *GUSB* como controles endógenos. Con el fin de establecer correlaciones entre las variables analíticas y clinicopatológicas, se realizaron tests no paramétricos. Se analizó la supervivencia por análisis de regresión de Cox, y se representaron las curvas Kaplan-Meier (test log-rank) de aquellos análisis estadísticamente significativos ($p<0.05$).

Aquellos pacientes con altos niveles de *CD5* e *IDO-2* presentaron mayor supervivencia global (SG, 53.3 meses vs NA, p=0.011 y 51.9 meses vs NA; p=0.050, respectivamente). En cuanto al análisis realizado en el subgrupo de adenocarcinoma (ADC), se observó una tendencia de mayor supervivencia libre de recaída (SLR) y SG en aquellos pacientes con altos niveles de expresión de *PD-L1, IDO-1* e *IDO-2.* Se generó una firma génica (firma *PDIDO*) basada en la expresión de los genes *PD-L1, IDO-1* e *IDO-2*. Los pacientes con altos niveles de expresión de la firma tuvieron mayor SLR (17.867 vs NA meses; p=0.001) y SG (29.83 vs NA meses; p=0.0002). El análisis multivariante estableció la firma *PDIDO* como factor pronóstico independiente para SLR [HR, 0.274; 95%CI, 0.126-0.593; p=0.001] y SG [HR, 0.267; 95% CI, 0.113-0.630; p=0.003].

Por tanto, el estudio del perfil inmunológico en muestras resecadas de CPNM ha permitido establecer la firma *PDIDO* como biomarcador independiente de SLR y SG en el grupo de pacientes con ADC. Además, *CD5* podría establecerse como biomarcador pronóstico de SG independientemente del tipo histológico en estadios tempranos de CPNM.

Palabras clave: Cáncer de pulmón no microcítico, PCR cuantitativa a tiempo real, Expresión génica relativa, inmunoregulación.

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Títol:

Expressió de gens d'immuno *checkpoints* en mostres tumorals de pacients amb càncer de pulmó no microcític en estadis ressecables. Possible rol com a biomarcadors pronòstics.

RESUM

El càncer de pulmó es la principal causa de mort deguda al càncer a nivell mundial, amb una supervivència als 5 anys menor al 5% en estadis avançats. El càncer de pulmó no microcític representa aproximadament un 80% dels casos de càncer de pulmó. Malgrat els avanços obtinguts en els últims anys, hi ha una gran necessitat de desenvolupament de noves estratègies terapèutiques més eficients contra aquest tipus de càncer i la falta de coneixement del microambient tumoral.

D'una banda, és conegut que el sistema immunològic es capaç d'evitar el càncer i la seua progressió però també es sap que una de les *característiques distintives* del càncer es la capacitat d'evadir la vigilància immunològica mitjançant diversos mecanismes, com la inhibició de la resposta antitumoral depenent de cèl·lules T. Aquest treball es basa en el fet de que les cèl·lules tumorals indueïxen senyals inhibitòries que els permeten evadir la resposta immune.

Es va analitzar l'expressió de 8 gens relacionats amb la immunoregulació (*PD-L1, PD-L2, IDO-1, IDO-2, ICOS-LG, CD5, CD6* i *CD200*) mitjançant RTqPCR en 201 mostres parelles de teixit tumoral fresc congelat i del corresponent teixit normal. Es va calcular l'expressió gènica relativa mitjançant la fórmula de Pfaffl utilitzant *ACTB, CDKN1B* i *GUSB* com controls endògens. Es van realitzar tests no paramètrics amb la fi d'establir correlacions entre les variables analítiques i clinicopatològiques. La supervivència es va analitzar amb anàlisis de regressió de Cox i en aquells casos en què els resultats foren significatius ($p<0.05$), es van representar les corbes Kaplan-Meier (test log-rank).

Aquells pacients amb alts nivells de *CD5* e *IDO-2* presentaren major supervivència global (SG, 53.3 mesos vs. NA, p=0.011 i 51.9 mesos vs. NA; p=0.050, respectivament). En l'anàlisi realitzat en el subgrup d'adenocarcinoma, es va observar una tendència de major supervivència lliure de recaiguda (SLR) i SG en aquells pacients amb alts nivells d'expressió de *PD-L1, IDO-1* e *IDO-2.* Es va generar una firma genètica (*PDIDO*) basada en l'expressió dels gens *PD-L1, IDO-1* e *IDO-2*. Els pacients amb alts nivells d'expressió de la firma presentaren major SLR (17.867 mesos vs. NA; p=0.001) i SG (29.83 mesos vs. NA; p=0.0002). L'anàlisi multivariant va establir la firma *PDIDO* com un factor pronòstic independent de SLR [HR, 0.274; 95%CI, 0.126-0.593; p=0.001] i SG [HR, 0.267; 95%CI, 0.113-0.630; p=0.003).

Per tant, l'estudi del perfil immunològic en mostres ressecades de CPNM ha permés establir la firma *PDIDO* com biomarcador independent de SLR i SG en el grup de pacients amb ADC. A més, CD5 podria establir-se com a biomarcador pronòstic de SG independentment del tipus histològic en estadis inicials de CPNM.

Paraules clau: Càncer de pulmó no microcític, PCR quantitativa a temps real, expressió gènica relativa, immunoregulació.

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ABBREVIATIONS

ACTB: Actin beta
ADC: Adenocarcinoma
ALCAM: Activated leukocyte cell adhesion molecule
ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase
APC: Antigen presenting cell
CAF: Cancer-associated fibroblasts
CD166: Cluster of differentiation 166
CD200: Cluster of differentiation 200
CD273: Cluster of differentiation 273
CD274: Cluster of differentiation 274
CD275: Cluster of differentiation275
CD279: Cluster of differentiation 279
CD28: Cluster of differentiation 28
CD4: Cluster of differentiation 4
CD40: Cluster of differentiation 40
CD40L: Cluster of differentiation 40 ligand
CD5: Cluster of differentiation 5
CD6: Cluster of differentiation 6
CD72: Cluster of differentiation 72
CD8: Cluster of differentiation 8
CD80: Cluster of differentiation 80
CD86: Cluster of differentiation 86
CDKN1B: Cyclin-dependent kinase inhibitor
cDNA: complementary DNA
CI: Confidence interval
Cp: Crossing point
Cq: Quantitative cycle
CTL: Cytotoxic T lymphocytes
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4
DC: Dendritic cells
DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotides
E: Efficiency
EGFR: Epidermal growth factor
GUSB: Glucuronidase beta
HER2: Human Epidermal receptor growth factor 2
ICOS: Inducible co-stimulator
ICOS-LG: Inducible co-stimulator ligand
IDO: Indoleamine 2,3-dioxygenase
IFNγ: Interferon γ
IL1: Interleukin 1
IL10: Interleukin 10
IL2: Interleukin 2

LAG-3: Lymphocyte activation gene 3
LCC: Large cell carcinoma
LN: Lymph nodes
MDSC: Myeloid-derived suppressor cell
MHC: Major histocompatibility complex
NA: Not available
NF: Nuclease-free
NFQ: Non-Fluorescent Quencher
NGS: Next generation sequencing
NK: Naturall killer
NR: Not reached
NS: Not specified
NSCLC: Non-small cell lung cancer
OS: Overall survival
PD-1: Programmed cell death 1
PD-L1: Programmed cell death 1 ligand
PD-L2: Programmed cell death 2 ligand
PS: Performance status
RECIST: Response evaluation criteria for solid tumours
RFS: Relapse free survival
RNA: Ribonucleic acid
RTqPCR: Quantitative real-time polymerase chain reaction
SCC: Squamous cell carcinoma
UNG: Uracil-DNA N-glycosylase
TAM: Tumour-associated macrophages
Th: Helper T cells
TCR: T cell receptor
TIL: Tumour-infiltrating lymphocyte
TIM-3: T cell immunoglobulin and mucin domain
TNF: Tumour necrosis factor
TNM: Tumour/Nodules/Metastasis
Treg: Regulatory T cells
TTR: time to relapse
WT: Wild type

1. INTRODUCTION

1.1. THE CONCEPT OF CANCER

Cancer is a group of pathologies that can affect almost any part of the body, classified in more than 100 different types depending on the affected organ, each type divided as well into different subtypes of tumours (National Cancer Institute; NCI, 2017).

Cancer is a disease involving dynamic changes in the genome (Ye *et al.*, 2007) and in the microenvironment (Quail and Joyce, 2013); characterised by abnormal cells dividing without control, which can acquire the ability of invasion and as a consequence, metastasize different tissues (Weinberg, 2007).

In 2000, Hanahan and Weinberg defined six essential characteristics of cancer cells: self-sufficiency in growth signals, evasion to antigrowth signals, limitless of replicative potential, apoptosis evasion, sustained angiogenesis and tissue invasion and metastasis (Hanahan *et al.*, 2000). In 2011, the same authors stated that the existence of tumours relies as well on normal cells recruited conforming the tumour microenvironment and added four new hallmarks: cellular energetics deregulation, genome instability and mutation, immune destruction evasion, tumour-promoting inflammation (Figure 1) (Hanahan and Weinberg, 2011).

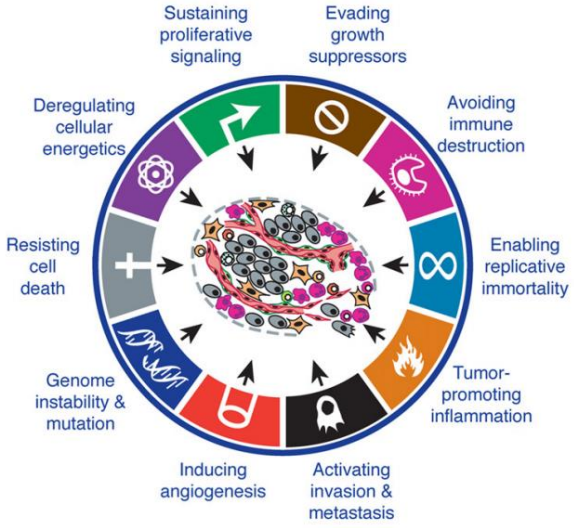

Figure 1. The ten hallmarks of cancer. *(Hanahan and Weinberg, 2011).*

Therefore, tumours are complex masses which have lost the normal structure and function of the tissue, composed not only by cancer cells but also by recruited cells that enhance the previously mentioned characteristics.

1.2. LUNG CANCER

1.2.1. Epidemiology

Nowadays the incidence of cancer in Spain is around 250.000 new cases every year, being 28.347 the number of new lung cancer cases diagnosed. In addition, cancer is the second cause of death in Spain, being lung cancer responsible of the largest number of deaths related to cancer in

Spain, causing a total of 21.220 deaths in 2014, and one and a half million deaths worldwide (National Statistics Institute; INE, 2016 and Spanish Lung Cancer Group; SEOM, 2017).

The incidence of cancer keeps increasing both in number of cases and deaths (Fitzmaurice *et al.*, 2017), as well as female lung cancer incidence and mortality rates as a consequence of the smoking patterns acquired (Hashim *et al.*, 2016; Malvezzi *et al.*, 2017).

1.2.2. Risk Factors

There is a lot of scientific evidence supporting the fact that smoking is responsible of approximately 80% of lung cancer cases worldwide (Agudo *et al.*, 2012). In addition, there are other factors associated with increased risk of lung cancer, such as outdoor air pollution and dietary habits (Tanvetyanon and Bepler, 2008; Bagnardi *et al.*, 2010). Furthermore, approximately a 15% of lung cancer in the UK have been linked to occupational exposures due to asbestos, silica, radon, diesel engine exhausts and mineral oils among others (Parkin, 2011). Nevertheless, as the effect of these factors in non-smoker population remains unknown, more research should be done in order determine the genetic effect on lung cancer incidence, thus understanding its epidemiology (Wakelee *et al.*, 2007).

1.2.3. Diagnosis and prognosis

At present, the diagnosis of lung cancer is primarily based on symptoms (e.g., cough, chest pain, hemoptysis, shortness of breath, weakness), and detection often occurs at advanced stages of disease when curative intervention (e.g., surgery) is no longer possible (Jantus-Lewintre *et al.*, 2012). Diagnosis is based on imaging techniques and a confirmatory biopsy, in order to obtain tissue samples for pathological analysis (Gridelli *et al.*, 2015).

Prior to prognosis and treatment determination, accurate staging is needed (Gridelli *et al.*, 2015). Nowadays it is followed the 7th edition of the TNM classification (Supplementary Table 1, Appendix I), based on the size and degree of locoregional invasion by the primary tumour (T), the extent of regional lymph node involvement (N) and the presence or absence of intrathoracic or distant metastases (M) (Shepherd *et al.*, 2007; Mirsadraee *et al.*, 2012).

1.2.4. Pathology and classification

Lung cancer is generally originated in bronchial epithelium and characterised by a slow growth rate. Non-small cell lung cancer (NSCLC) major histological subtypes are adenocarcinoma (ADC) and squamous cell carcinoma (SCC), representing 50 and 40% of the cases, respectively. Among other histological subtypes, the most important is known as large cell carcinoma (Chen *et al.*, 2014).

From the genomic point of view, lung cancer is one of the most mutated solid tumours, accumulating a mean of 200 mutations per tumour (Figure 2) (Alexandrov *et al.*, 2013). Interestingly, tumours from smokers accumulate ten times more mutations than tumours from never-smokers (Gridelli *et al.*, 2015).

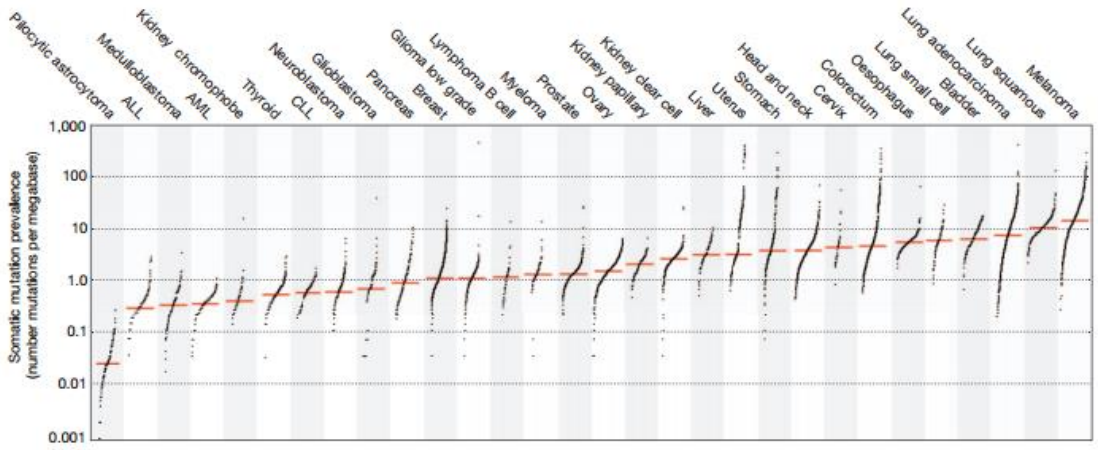

Figure 2. Representation of somatic mutations accumulated in different types of cancer *(Alexandrov et al., 2013).*

Nowadays it is known that there are driver oncogene mutations that lead to different types of oncogenic transformations. This has enhanced the molecular classification of NSCLC, based on activating mutations on the tyrosine kinase known as Epidermal Growth Factor (*EGFR*), as well as anaplastic lymphoma kinase (*ALK*) gene rearrangements. *KRAS*, *BRAF* and *ROS* mutations are also frequently analysed as well as *HER2* amplifications (Gridelli *et al.*, 2015).

1.2.5. Treatment

The treatment options for NSCLC are based mainly on the stage of the cancer, but other factors such as anatomopathological classification and performance status (PS) are also important. Surgery is the primary option for treating early-stage NSCLC. However, a proportion of lung cancer patients develop recurrence, even after curative resection (García-Campelo *et al.*, 2015).

In addition, most lung cancer patients are diagnosed in advanced stages of the disease, when surgery, chemotherapy or radiotherapy are not very effective. In those cases, different types of chemotherapy are used depending not only on histology or patient's PS, but also on its mutational status due to the availability of targeted therapies. Furthermore, a potential alternative is immunotherapy, which consists of treatments that stimulate the patients' immune system in order to detect and attack cancer cells more efficiently (Novello *et al.*, 2016; García-Campelo *et al.*, 2015; Gridelli *et al.*, 2015; Jiang and Zhou, 2015).

1.3. IMMUNE SYSTEM AND CANCER

The immune system plays an important role in the maintenance of the integrity of the organism; it is not only involved in protection against pathogens, but also in cancer prevention, development and defence. First, it can protect the host from virus-induced tumours by eliminating or suppressing viral infections. Second, it prevents the inflammatory environment that leads to tumourigenesis by eliminating pathogens and inflammation. Third, tumour cells present genetic and cellular alterations that allow their identification and destruction by means of tumour-specific antigens or molecules induced by cellular stress. This last function is called immunosurveillance (Vesely *et al.*, 2011; Candeias and Gaipl, 2016).

There are two different types of immune responses, innate (non-specific immunity) and adaptive (specific immunity), which are interconnected by cytokines and antibodies production (Vesely *et al.*, 2011).

The innate immune system, which represents the first line of defence against foreign pathogens and transformed cells, is composed of macrophages, neutrophils, dendritic cells (DCs), mast cells, eosinophils, basophils, natural killer (NK) cells, and NK T cells (Bremnes *et al.*, 2011; Vesely *et al.*, 2011).

The adaptive immune system comprises B and T cells that further promote activation of innate immunity and support the expansion and production of tumour-specific T cells and antibodies. There are two main T cell subtypes: cytotoxic T cells (CTL, CD8+) and T helper cells (Th,CD4+), which is formed by different subtypes, being Th1, Th2, and Th17 the most important (Bremnes *et al.*, 2011; Vesely *et al.*, 2011). These cells express antigen-specific receptors that allow a flexible and broad number of responses.

In T-cell mediated responses, three main steps are required. First, antigen should be recognised by Major Histocompatibility Complex (MHC) by means of antigen presenting cells (APC) through CD3 T cell receptor (TCR). The second step consists of triggering activation signals by binding of B7 and CD28. The third step is cytokine secretion, which allows T cell proliferation. Afterwards, B7 molecules bind to CTLA-4 stopping the immune response, in order to avoid excessive immune responses. An equilibrium between activating and inhibitory signals is required to maintain homeostasis and self-tolerance (Zang and Allison, 2007; Pardoll, 2012).

The generation of cancer immunity is a cyclic process that is shown in Figure 3, divided in different steps: in the first step, neo-antigens created by oncogenesis are released and captured by dendritic cells (DCs). In the second step, DCs present the captured antigens on MHCI and MHCII molecules to T cells, resulting in the activation of effector T cell responses against the cancer-specific antigens (step 3), which move to the tumour bed (step 4), and infiltrate it (step 5). The sixth step shows recognition of cancer cells by T cells through interaction between its T cell receptor (TCR) and its specific antigen bound to MHCI, leading to target cancer cell destruction (step 7) (Chen and Mellman, 2013).

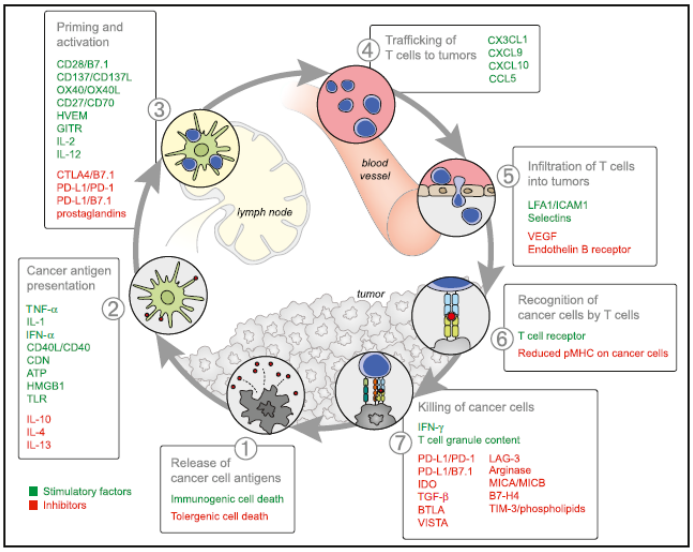

Figure 3. The cancer-immunity cycle *(Chen and Mellman, 2013).*

The immune system plays an important role in the case of NSCLC, which is characterised by a considerable immune infiltration caused by the amount of alterations provoked by the carcinogens present in tobacco smoke (Vogelstein *et al.*, 2013).

1.3.1. Tumour microenvironment

Cancer cells develop in a complex microenvironment, which is required for sustained growth, invasion and spread. Tumour microenvironment consists of diverse cell types such as cancer stem cells, pericytes, endothelial cells, cancer-associated fibroblasts (CAF), and immune cells that are attracted by tumour-cell-derived factors and embedded in an extracellular matrix (Figure 4). The regulatory factors are soluble mediators such as cytokines, chemokines and growth factors among others (Bremnes *et al.*, 2011; Fridman *et al.*, 2012; Becht *et al.*, 2015).

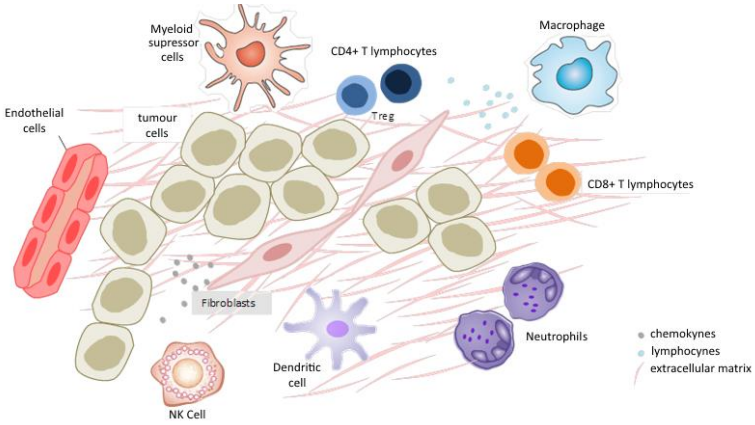

Figure 4. Cancer cells and its microenvironment *(Jantus-Lewintre and Usó, 2015).*

Tumour microenvironment plays an important role avoiding immune surveillance, which consists of detection and destruction of nascent tumour cells. As it can be seen in Figure 5, at the beginning of oncogenesis, transformed cells are killed due to the recognition of neo-antigens by immune cells, but as tumours are comprised of different immunogenic clones, there are some tumour cells that present immune evasion or suppression strategies and scape the immune system; hence, these clones will remain alive and allow tumour development. This selection process is known as immunoedition (Prendergast, 2008).

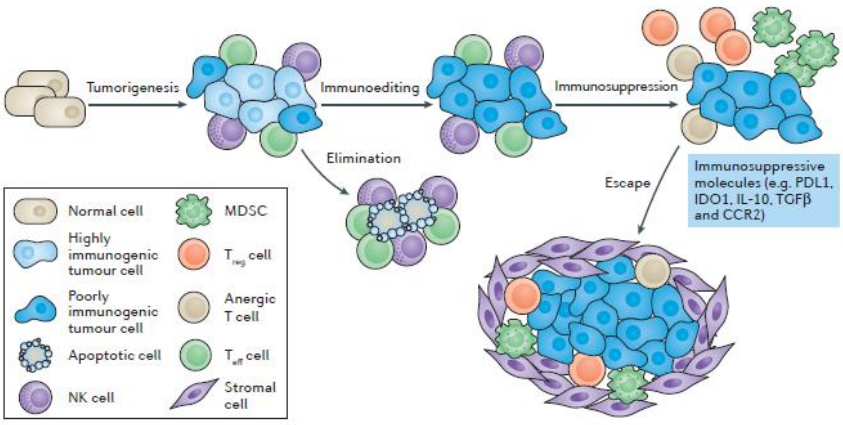

Figure 5. Role of immune system during oncogenesis *(Yarchoan et al., 2017).*

This has demonstrated that cancer cells can not only avoid immunosurveillance, but also exploit native immune mechanisms to their own benefit ensuring cancer initiation, promotion and progression (Vesely et al., 2011).

Therefore, tumour microenvironment can be associated with patient prognosis, and depending on its composition it can be a therapeutic target, although it often depends on the tissue context and cellular stimuli (Bremnes *et al.*, 2011).

1.3.2. Immune-system related biomarkers in lung cancer

Immune evasion is also achieved by regulation of the tumour-infiltrating immune cells. This can be mediated by soluble factors such as cytokines, prostaglandins, polyamines or enzymes, released by tumour cells, stromal cells or infiltrating myeloid cells. Another strategy consists of the increased expression of shed antigens or inhibitory molecules by tumour or dysfunctional immune cells of the tumour microenvironment, which leads to deregulation of T cell activity (Moss *et al.*, 2006; Fauci *et al.*, 2012). The expression of these molecules is frequently used as cancer biomarkers and also as therapeutic targets.

1.3.2.1. Immune checkpoint molecules

The most frequent strategy followed by tumours to escape from the immune system consists of altering the immune checkpoints, which are control points in charge of regulating specific immune response. Some molecules that act as immune checkpoints are CTLA-4, PD-1, TCR, LAG-3, TIM-3, among others. It has to be remarked that B7 family molecules play a key role in checkpoint regulation in cancer (Figure 6) (Pardoll, 2012; Ceeraz and Nowak, 2014; Topalian *et al.*, 2016).

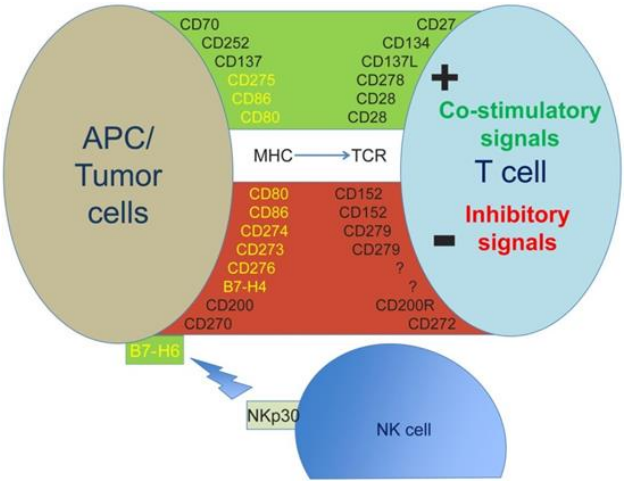

Figure 6. Antigens expressed on the cell surface of tumour cells or APC and their corresponding receptors or ligands on the surface of T cells or NK cells. *B7 family members are shown in yellow.*
APC: Antigen presenting cell; MHC: Major Histocompatibility Complex; NK: Natural killer cell; TCR: T cell receptor (Greaves and Gribben, 2013).

1.3.2.1.1. PD-L1 and PD-L2

The two ligands for Programmed-cell death receptor 1 (PD-1, also known as CD279) are PD-L1 (also known as B7-H1 and CD274) and PD-L2 (also known as B7-DC and CD273), being cell-surface glycoproteins from B7 family. They are co-inhibitory molecules (Figure 7), thus they limit T cell

proliferation and cytokine secretion activity when bound to its receptor (PD-1), which is expressed on activated T cells or B cells. Therefore, they regulate peripheral CD4 and CD8 T cell tolerance, preventing autoimmunity and maintaining T cell homeostasis (Freeman *et al.*, 2000; Francisco *et al.*, 2009; Obeid *et al.*, 2016).

PD ligands and PD-1 supress anti-tumour immunity and promote tumour progression by inactivating T cells, and activating tumour-suppressive cell populations. It has been demonstrated that some tumours use this pathway to obtain immune resistance. PD-L1 and PD-L2 are expressed by tumour cells, tumour infiltrating immune cells and also tumour-associated fibroblasts (Nazareth *et al.*, 2007; Lesterhuis *et al.*, 2011; Obeid *et al.*, 2016). Nevertheless, their expression depends on different stimuli, and it has been stated that they could have different functions in type 1 and type 2 responses regulations, as Th1 cells enhance PD-L1 expression whilst Th2 cells up-regulate PD-L2 (Loke and Allison, 2003; Ghiotto *et al.*, 2010).

1.3.2.1.2. CD200

Cluster of Differentiation 200 (CD200, also known as OX-2) is a membrane glycoprotein that suppresses immune activity. CD200 is consistently expressed on thymocytes, T and B lymphocytes, neurons and endothelial cells. It is related to the B7 family of co-inhibitory receptors (Figure 7) and interacts with CD200R triggering a signal to supress T-cell mediated immune responses, concretely by modulating macrophage or dendritic cell activity and inducing regulatory T cells. CD200R is expressed mostly by myeloid cells on macrophages and monocytes and some T cells (Moreaux *et al.*, 2008; Snelgrove *et al.*, 2008). As it has an immunosuppressive role, its overexpression could enhance immune scape (Kawasaki *et al.*, 2007). Nevertheless, it plays a bidirectional role in cancer, by exerting not only immunosuppressive but also anti-inflammatory effects (Erin *et al.*, 2015).

1.3.2.1.3. ICOS-LG

Inducible T-cell co-stimulator ligand (ICOS-LG, also known as CD275, B7-H2, B7RP-1, LICOS and GL5) is a B7 homolog protein. ICOS-LG is constitutively expressed on B cells, dendritic cells and macrophages and can be induced in non-hematopoietic cells, whilst its receptor is ICOS (CD274), expressed on activated T cells and resting memory T cells. ICOS-LG is upregulated on activated T cells and acts as a co-stimulatory signal for T-cell proliferation and cytokine secretion (Figure 7); it also induces B-cell proliferation and differentiation. Their interaction plays an important role in mediating local tissue responses to inflammatory conditions, as well as in modulating the secondary immune response by co-stimulating memory T-cell function. ICOS-LG can also bind CD28 and CTLA-4 (Yoshinaga *et al.*, 1999; Paulos *et al.*, 2010; Yao *et al.*, 2011).

Regarding tumour expression of ICOS-LG, it has been described that it is mainly expressed on tumour-associated macrophages and tumour cells. It can increase T cell-mediated tumour immunity by co-stimulating Tregs (Martin-Orozco *et al.*, 2010; Zhang *et al.*, 2016).

1.3.2.2. Immunosuppresive factors

Some tumours present alterations in their metabolism which lead to an accumulation of immune-inhibitory molecules. This is supported by the fact that T cells are sensitive to low tryptophan levels, which leads to proliferative arrest. In addition, as a consequence of essential

nutrient depletion, immune-suppressive metabolites are accumulated (Munn *et al.*, 2005; Pardoll, 2012).

1.3.2.2.1. IDO-1 and IDO-2

IDO (indoleamine 2,3 dioxygenase) is an intracellular enzyme that degrades the essential amino acid tryptophan (Trp) along the kynurenine pathway. IDO comprises 2 isozymes (IDO-1 and IDO-2) and is expressed by both, tumour and infiltrating immune cells, such as eosinophils, granulocytes, dendritic cells and macrophages (Astigiano *et al.*, 2005; Metz *et al.*, 2007; Löb *et al.*, 2009). There are different opinions regarding IDO expression in tumours, while some authors state IDO is constitutively expressed on tumour cells (Uyttenhove *et al.*, 2003), others state that IDO is not constitutively expressed in tumour cells, APCs, epithelial cells or fibroblasts, but it is induced by inflammatory stimulus, by means of soluble factors such as IFN-ɣ, TNF-α or IL-1 (Jurgens *et al.*, 2009; Zhai *et al.*, 2016). Tryptophan degradation is also triggered by CTLA-4-CD80/86 or CD40-CD40L ligation on activated T Cells (Grohmann *et al.*, 2002).

These enzymes inhibit immune responses through the local depletion of amino acids that are essential for anabolic functions in lymphocytes (particularly T cells) or through the synthesis of specific natural ligands for cytosolic receptors. As it can be seen in Figure 7, IDO is the limitant reactant of tryptophan catabolism (Löb *et al.*, 2009; Zhai *et al.*, 2015). Its activity causes a decrease in tryptophan availability and as a consequence an accumulation of kynurenines, active catabolites that that induce apoptosis in CD4+ T cells (Löb *et al.*, 2009; Von Bubnoff *et al.*, 2011).

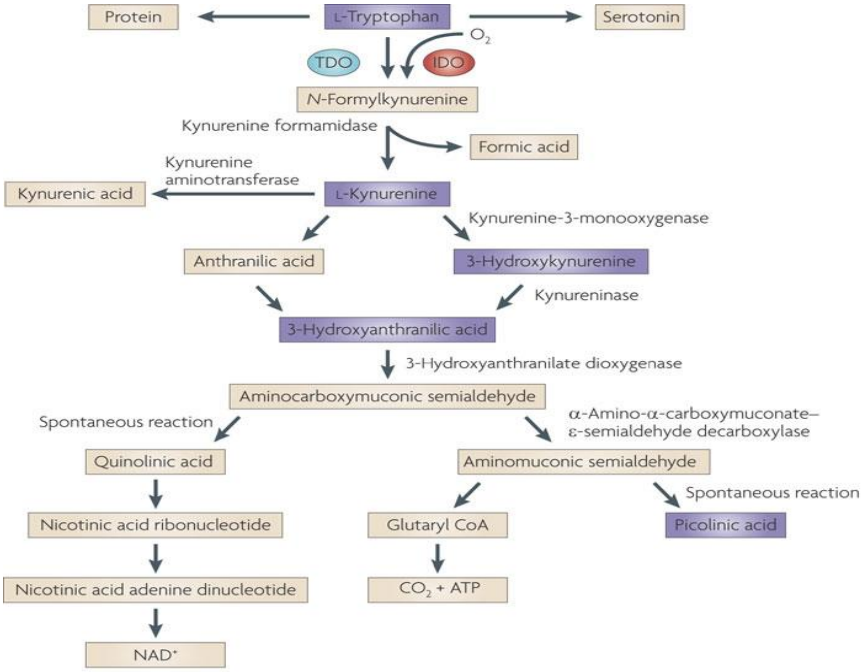

Figure 7. IDO enzyme and kynurenine in cancer cells. *Indoleamine-2,3-dioxygenase (IDO)-induced tryptophan catabolism along the kynurenine pathway; TDO, tryptophan dioxyenase ; IDO indoleamine 2,3 dioxygenase (Löb et al., 2009).*

Regarding tumour activity, IDO enhances tumour immune scape as it has been established that secreted kynurenine binds effector T cell receptors, thus tumour-infiltrating lymphocytes are anergic and do not proliferate (Godin-Ethier *et al.*, 2011).

1.3.2.3. T lymphocyte receptors

1.3.2.3.1. CD5 and CD6

CD5 and CD6 are closely related lymphocyte surface receptors of the scavenger receptor cysteine-rich superfamily, both expressed on the same lymphocyte populations (thymocytes, mature T cells and B cells) (Gimferrer *et al.*, 2003). Cluster of differentiation 5 (CD5, also known as T1, Leu1, and Tp67) ligands are CD72 and CD5 itself (Biancone *et al.*, 1996; Bikah *et al.*, 1998). Cluster of differentiation 6 (CD6, also known as Tp120) ligand is ALCAM (also known as CD166), an adhesion molecule that belongs to the Ig superfamily (Gimferrer *et al.*, 2003). Both, CD5 and CD6 are T cell receptor inhibitory molecules involved in anti-tumour immune responses, as they co-localize with the TCR/CD3 complex at the immune synapse (Figure 8).

It has been discovered that CD5 down-regulation on cytotoxic T lymphocytes within the tumour microenvironment improves their cytotoxic activity as well as cytokine secretion (Tabbekh *et al.*, 2013; Dirican *et al.*, 2015).

Although little is known about CD6, it also inhibits T cell activation and proliferation, by interfering with early cell-cell interactions needed for immune synapse maturation (Osorio *et al.*, 1998), and CD6/CD166 interaction mediates thymocyte-thymic epithelial cell adhesion (Gimferrer *et al.*, 2004).

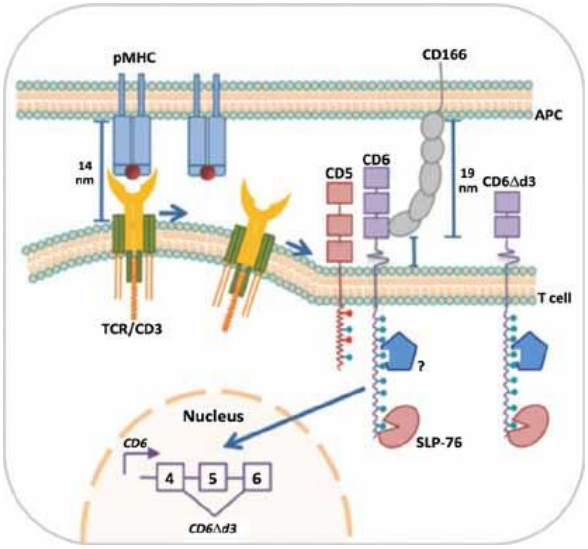

Figure 8. Immune synapse representation involving CD5 and CD6 *(Santos et al., 2016).*

By contrast, CD5-CD5L interaction during T cell-dependent immune responses co-stimulates T and B cell activation and proliferation in a pathway similar to CD28/CTLA-4-B7.1/B7.2 and CD40-CD40L (Biancone *et al.*, 1996; de Wit *et al.*, 2011). Furthermore, CD6 also acts as a co-stimulatory molecule synergizing with TCR to enhance T cell proliferation (Gimferrer *et al.*, 2004). Thus, CD5 and CD6 have not only an inhibitory but also a co-stimulatory role.

Tumour microenvironment can be associated with patient's prognosis in cancer. As an example, a strong cytotoxic lymphocyte infiltration correlated with good prognosis in different tumour types, including lung cancer. By contrast, regulatory T cell infiltration is associated to a worse

prognosis as it induced anti-tumour effector T cells blockade or chronic inflammation disease (Bremnes *et al.*, 2011; Fridman *et al.*, 2012; Becht *et al.* 2015).

Therefore, analysing the gene expression of *PD-L1, PD-L2, CD200, CD5, CD6, ICOS-LG, IDO-1* and *IDO-2*, could help to determine their potential role in tumour immune escape. The validation of these immunoregulatory genes as biomarkers in NSCLC would not only facilitate information about disease prognosis but also about potential therapeutic targets.

2. OBJECTIVES

NSCLC is a heterogeneous and ethiopathologically complex disease. The knowledge of lung tumour microenvironment is leading to a better understanding of the evasion of immune surveillance and the development of new therapies, but in NSCLC remains largely unknown. The main objective of this Degree's thesis is to analyse immunoregulatory gene expression that could provide relevant information as potential prognostic biomarkers in resectable NSCLC.

To do so, the specific aims of this study are:

1. To select a cohort of resectable NSCLC patients with paired normal/tumour samples and complete electronic medical records in order to analyse demographic and clinico-pathological characteristics of this specific patient's population.

2. To analyse the relative gene expression levels of the immunoregulatory genes *PD-L1, PD-L2, CD200, CD5, CD6, ICOS-LG, IDO-1 and IDO-2* by RTqPCR on tumour samples from resected NSCLC.

3. To study correlations between expression levels of the analysed genes and some relevant clinico-pathological features.

4. To evaluate the relative gene expression of the analysed genes alone or in combination as prognostic biomarkers for resectable NSCLC and in different histology subsets of patients.

3. MATERIALS AND METHODS

3.1. PATIENT COHORT AND SAMPLE COLLECTION

In this study 201 patients from *Consorcio Hospital General Universitario de Valencia* with NSCLC were included between 2004 and 2016. These patients had resected, non-pre-treated stage I to IIIA cancer according to the American Joint Committee on Cancer staging manual with a confirmed histological diagnosis of NSCLC. The study was conducted in accordance with the Declaration of Helsinki and the institutional ethical review board approved the protocol. All patients had signed the informed consent prior to the collection of their biological samples.

Specimens collected consist of 201 fresh tissue samples obtained from surgical resection of patient's tumour, after immediate separation of tumour and adjacent normal lung tissue by a pathologist. Tissue samples were preserved in RNAlater® (Applied Biosystems, USA) to prevent RNA degradation, and fresh-frozen at -80ºC until their analysis.

3.2. RNA ISOLATION

RNA and DNA were isolated from fresh-frozen tissue samples by using the TRIzol method (TRI Reagent®, Sigma, USA), following the manufacturer's instructions. Briefly, a piece of 20-30mg of tissue was dissected and 1 mL of TriReagent® was added, and homogenized in a TissueLyser (Qiagen, Germany) by using tungsten beads. Chloroform was added to solubilize lipid content and after sequential centrifugations and incubations three phases could be distinguished: RNA, DNA and proteins. The aqueous phase containing the RNA was separated and subsequently precipitated with isopropanol and washed with ethanol. Afterwards, samples were re-suspended in Nuclease-free (NF) water (Qiagen, Germany) and treated with DNAse (Sigma, USA) to eliminate traces of genomic DNA.

The DNA interphase was recollected in absolute ethanol and was washed first with buffer (10% ethanol/0.1M sodium citrate) and then with 75% ethanol. It was re-dissolved in NF water and stored at -80ºC until further analysis.

RNA quantification was performed using a nano-spectrophotometer (Nano Drop 2000C, Thermo Fisher Scientific, USA), and its quality was also assessed by considering the absorbance ratios 260/280 and 260/230. Samples with suboptimal concentration (<76ng/µl) or quality were excluded from the study. Samples were stored at -80ºC until further analysis.

3.3. REVERSE TRANSCRIPTION

The RNA samples were retrotranscribed using the High Capacity cDNA Reverse Transcription (RT) Kit® (Applied Biosystems, USA). The reactions contained 2 µl of RT buffer, 0.8 µl of dNTPs, 2 µl of random RT primers, 1 µl of RNAse inhibitor, 1 µl of MultiScribe Reverse Transcriptase®, varying sample volumes to obtain a final RNA concentration of 1000ng, and NF water (Qiagen, Germany) up to a final volume of 20µl. The reaction took place in a MasterCycler® thermocycler (Eppendorf, Germany), and the cycling program consisted of of a pre-incubation (10 minutes at 25ºC) to activate the enzyme, the retrotranscription of RNA to cDNA (2 hours at 37ºC) and a final step to denature the

enzyme (5 minutes at 85ºC). Resulting cDNA was diluted in NF water to a final concentration of 250ng/µl and stored at -20ºC until further analyses.

3.4. QUANTITATIVE REAL TIME PCR (RTqPCR)

Gene expression of the cDNA previously obtained was analysed by RTqPCR using a cocktail of primers and hydrolysis probes (TaqMan®, Applied Biosystems, USA), designed to bind inter-exon regions of target genes and therefore preventing genomic DNA amplification. The structure of the hydrolysis probes includes a reporter dye linked to the 5' end and a non-fluorescent quencher (NFQ) at the 3' end as well as a Minor Groove Binder (MGB) attached to the NFQ in order to increase the melting temperature (T_m) without increasing the length of the probe (Figure 9) (VanGuilder *et al.*, 2008).

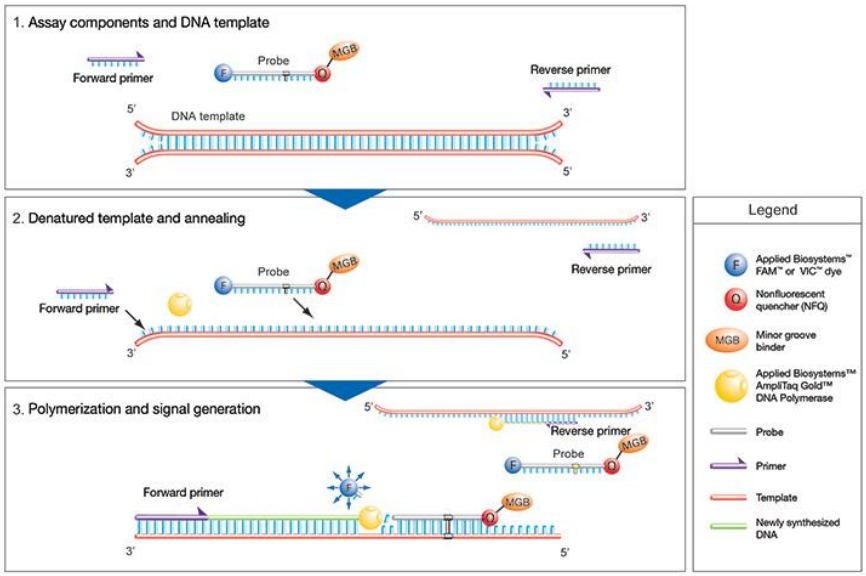

Figure 9. TaqMan® qPCR reaction steps *(Figure from Life Technologies).*

Each reaction was performed in a 384-Well Plate with a final volume of 5µl comprising: 2.5 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1.25 µl of NF water, 0.25 µl of TaqMan® Gene Expression Assay mix (Applied Biosystems)(Table 2) and 1 µl of sample cDNA. The TaqMan ® Gene Expression Master Mix contains DNA polymerase, dNTPs and a uracil N-glycolase enzyme that prevents reamplification of possible contaminants' amplicons.

The reactions took place in a thermocycler (LightCycler 480, Roche, Switzerland), following the cycling conditions shown in Table 1. The pre-PCR phase is required to ensure optimal UNG enzyme activity and activation of the AmpliTaq Gold enzyme.

Table 1. Temperature program of RT-qPCR.

	Temperature (ºC)	Duration (min:sec)	Phase
Pre-PCR	50	2:00	Amperase UNG
	95	10:00	Hot Start
PCR	95	00:15	Denaturation
(45 ciclos)	60	01:00	Merging-Extension

UNG: Uracil N-glycolase

In order to confirm the absence of contamination, no-template controls (NTC) were included in each run, as well as human reference cDNA (Clontech, USA) which was used as a positive control and interplate normalizer.

3.4.1. Gene expression relative quantification

The efficiency of each TaqMan® assay was evaluated by carrying out serial dilutions (50ng/µL, 5 ng/µL, 0.05 ng/µL, 0.005 ng/µL and 0.0005 ng/µL) of the commercial human cDNA above mentioned. The efficiency (E) of one cycle in the exponential phase was calculated by using the following equation: $E=10^{-1/slope}$.

To correct sample deviations due to varying sample concentration, the endogenous genes *ACTB*, *CDKN1B* and *GUSB* which have constant expression were used to calculate the normalization factor following Pfaffl formula. These endogenous genes have previously been evaluated using GeNorm software, which calculates the gene-stability for different control genes, allowing selection of the best internal controls (Vandesompele *et al.*, 2002; Bustin *et al.*, 2009).

After N amplification cycles, the fluorescent signal will overcome the background fluorescence establishing the quantification cycle (Cq), which will allow the determination of the relative quantity of each sample (VanGuilder *et al.*, 2008). All samples were analysed by duplication, and the mean Cq value was used for further analysis. Certain samples had gene expression levels below the limit of detection, so their relative gene expression was calculated with the maximum Cq value, which corresponds to the minimum detectable expression.

In this study 8 genes (shown in Table 2) were analysed, selected for their potential role in immune surveillance, described in other pathologies according to a PubMed database search, which suggest their role in in cancer immunity.

Table 2. Genes analysed in this study and their Taqman® assays used for RTqPCR.

Gene	Full Name	Assay	Amplicon Length
ACTB	Actin, Beta	Hs99999903_m1	171
CDKN1B	Cyclin-dependent kinase inhibitor	Hs00153277_m1	71
GUSB	Glucuronidase, beta	Hs01558067_m1	71
CD274	CD274 molecule	Hs01125301_m1	89
PDCD1LG2	Programmed cell death 1 ligand 2	Hs01057777_m1	61
IDO-1	Indoleamine 2,3-dioxygenase 1	Hs00984148_m1	66
IDO-2	Indoleamine 2,3-dioxygenase 2	Hs01589373_m1	101
CD5	CD5 molecule	Hs00204397_m1	114
CD6	CD6 molecule	Hs00198752_m1	88
CD200	CD200 molecule	Hs01033303_m1	64
ICOS-LG	Inducible T-cell co-stimulator ligand	Hs00323621_m1	59

Relative gene expression levels were determined based on the ratio of the target gene expression to the reference gene expression by using the Pfaffl formula (Equation 1). The relative quantification is calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control (Pfaffl, 2001). The geometric mean of the expression of the three

endogenous genes above mentioned was considered as the expression of the reference gene (Vandesompele *et al.*, 2002).

$$Ratio = \frac{E_{target}^{\Delta Cp\ target\ (Control-Sample)}}{E_{ref}^{\Delta Cp\ ref\ (Control-Sample)}}$$

Equation 1. Pfaffl formula for relative gene expression ratio. *Target: gene analysed; Ref: endogenous gene; E: Gene efficiency; Cp: Crossing point; ΔCp: Expression difference between normal and tumour tissue.*

3.5. DATA ANALYSIS

Prior to statistical analyses, expression data were carefully reviewed and those values considered as outliers were excluded.

First of all, descriptive analyses were conducted to determine the clinico-pathological and demographic characteristics of the patient's cohort, as well as the expression of the target genes.

The next statistical analysis performed consisted of a Kolmogorow-Smirnov test, to determine whether or not the data followed a normal distribution.

Relative gene expression was assessed taking into account the median values as median is less affected by data variability than mean, ensuring statistical robustness. If the value of the target gene varies from 0.5 to 2.0, its expression does not change between tumour tissue and the normal pulmonary parenchyma. Therefore, it will be considered that there is differential expression between tumour and normal tissue if the ratio value is higher than 2, indicating that the target gene is overexpressed in tumour, or if ratio value is lower than 0.5, which indicates that the target gene is underexpressed (Usó *et al.*, 2016).

Gene correlations were analysed by using Spearman's rank test, and gene expression was correlated with clinico-pathological variables using the non-parametric tests Mann Whitney U to compare two independent groups and Kruskall-Wallis to compare more than two independent groups.

Survival analyses were performed considering Relapse Free Survival (RFS) and Overall Survival (OS); RFS spans from the date of surgery to the date of relapse or exitus and OS is calculated from the date of surgery to the date of exitus, following the Response Evaluation Criteria in Solid Tumours (RECIST) criteria. For those patients who had not relapsed or passed out, the last recorded follow-up was considered the end of the study (Therasse *et al.*, 2000).

In order to assess whether the analysed genes had prognostic value, univariate Cox regression was performed for each target gene. For those genes with significant prognostic value, survival was assessed by Kaplan-Meier curves using the log-rank test. Prior to that, continuous variables were dichotomized using the median as a cut-off value.

In order to obtain more significant results, expression prognostic scores were calculated by combining different markers following the Z-score method. First, univariate Cox analysis was performed with each target gene expression and Z-score was calculated dividing its regression

coefficient by its error. Those genes whose |Z-score| was higher than 1.5, were introduced in a multivariate Cox analysis to obtain the coefficient regression which will be used to calculate the score. The prognostic value of the gene score was calculated as previously described (Lossos *et al.*, 2004; Schetter *et al.*, 2009; Usó *et al.*, 2017).

To establish independent prognostic biomarkers, a multivariate Cox regression was performed using clinico-pathological variables and dichotomized gene expression potential markers. The Hazard ratio determines its significance for a 95% confidence Interval (Bradburn *et al.*, 2003).

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 15.0 software (Chicago, IL), considering statistically significant $p<0.05$.

4. RESULTS AND DISCUSSION

4.1. COHORT DESCRIPTIVE ANALYSIS

This study included 201 patients with NSCLC in resectable stages (I-IIIA), who underwent resection at *Consorcio Hospital General Universitario de Valencia.* The patient cohort was characterised by a median age of 65 years, 86% of the patients were males and 88% were current or former smokers. Regarding histology, 46% (93/201) of the cases were SCC and 44% (88/201) ADC. 73 patients (37.2%) received adjuvant chemotherapy post-surgery. The most relevant demographic and clinico-pathological characteristics are shown in Supplementary Table 2 (Appendix I).

4.2. GENE EXPRESSION ANALYSIS

For relative gene expression determination, 201 paired samples (tumour and adjacent normal lung) tissues were analysed. RNA was isolated from small pieces of tissue. An optimal RNA concentration was obtained from all the samples; the median for normal adjacent tissues was 325ng/µl and 1063.1ng/µl [83.2-2616.7] for tumour tissues.

4.2.1. Rna quality assessment

The quality of the extracted RNA was assessed using a nanospectrophotometer. The ratios A260/280 and A260/230 ranged from 1.8 to 2.0 and from 2.0 to 2.2, respectively in 80% of the samples, indicating the extraction protocol is appropriated to obtain good quality RNA for gene expression analysis.

4.2.2. Efficiencies and linearity

The efficiency for each Taqman® assay used in this study was determined using the Cq slope method. Cq values for serial dilutions of the target template were obtained and plotted against log values of target DNA concentration in order to calculate the slope of each standard curve. Results are shown in Table 3.

Table 3. Efficiencies for the Taqman® assays used in this study.

Gene	Code	Slope	Efficiency	% Efficiency
ACTB	Hs99999903_m1	-3.52	1.92	96.10
CD200	Hs01033303_m1	-3.78	1.85	92.35
CD5	Hs00204397_m1	-3.66	1.88	93.75
CD6	Hs00198752_m1	-3.46	1.95	97.25
CDKN1B	Hs00153277_m1	-3.71	1.86	93.00
GUSB	Hs01558067_m1	-3.22	2.00	100.00
ICOS-LG	Hs00323621_m1	-3.73	1.85	92.65
IDO-1	Hs00984148_m1	-3.56	1.91	95.00
IDO-2	Hs01589373_m1	-3.69	1.87	93.40
PD-L1	Hs01125301_m1	-3.22	2.00	100.00
PD-L2	Hs01057777_m1	-3.41	1.97	98.25

4.2.3. Relative gene expression quantification

In order to determine whether the analysed genes were overexpressed or underexpressed in tumours, the fold-change was used, which corresponds to the ratio of the expression in the tumour and the normal tissue. Relative gene expression data are shown in Table 4.

Table 4. Gene expression data of the analysed genes.

	PD-L1	PD-L2	CD200	CD5	CD6	ICOS-LG	IDO-1	IDO-2
N	200	201	200	200	201	201	193	200
Mean	2.63	1.27	1.52	1.85	2.24	2.35	2.94	2.04
Median	1.03	0.63	0.85	1.09	1.20	1.20	1.04	0.84
Minimum	0.00	0.01	0.02	0.01	0.01	0.02	0.00	0.01
Maximum	56.14	14.27	21.69	19.00	23.72	128.53	30.21	27.26

Relative gene expression was assessed taking into account the median values as it is less affected by data variability than mean value, ensuring statistical robustness. Thus, relative gene expression median values were represented (Figure 10).

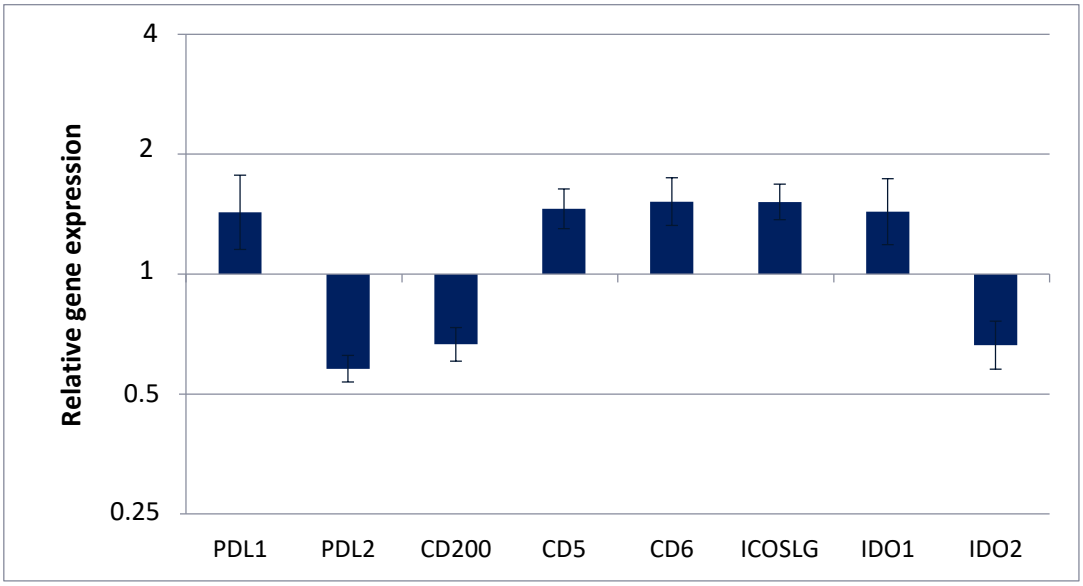

Figure 10. Relative mRNA expression levels of the 8 genes analysed. *Relative gene expression median is represented for each gene. The results represented are median ± intercuartile range. Down-regulated genes were represented after transformation by -1/(median).*

It was considered that a gene was overexpressed when the median of its relative gene expression was above 2, and it was considered to be underexpressed when it was below 0.5. Using this criteria, we found a tendency of *PD-L2* (0.63X) to be down-regulated in tumour, whereas no significant changes were obtained in the rest of analysed immune markers.

Previous studies have reported that *PD-L2* expression is lower than *PD-L1* in several tissues, including human respiratory tract epithelial cells (Pinchuk et al., 2008; Rozali *et al.*, 2012). This could be explained by the statement that *PD-L1* is expressed by both tumour cells and tumour infiltrating immune cells, whilst *PD-L2* expression is mainly detected only in tumour cells (Obeid *et al.*, 2016).

Regarding the expected expression of *ICOS-LG* and *CD200* in lung cancer, there are scarce and controversial published data analysing their role in carcinogenesis. Moreover, *CD200* has been implicated in anti-tumour T cells suppression and also as an inhibitor of tumour growth, demonstrating a possible dual function in cancer development (Stanciu *et al.*, 2006; Siva *et al.*, 2008; Talebian and Bai, 2012).

CD5 and *CD6* expression almost did not vary between tumour and normal tissue in our cohort, although it has been stated that infiltrating T lymphocytes in thoracic tumours express *CD5* (Dirican *et al.*, 2015). Even though, it is important to remark that more than 1/3 NSCLC show no lymphocyte infiltration pattern in their tumours (immune desert) as was recently published by our group (Usó *et al.*, 2017).

Regarding *IDO-1* and *IDO-2,* both genes are reported as up-regulated in almost all human cancers (Sorensen *et al.*, 2011). In lung cancer, Karanikas et al. found that *IDO-1* was more expressed in lung tumour than in normal tissue and in lung cancer cell lines, but the number of samples included in this study was very low (N=28) (Karanikas *et al.*, 2007).

4.2.4. Gene correlation analysis

Non-parametric Spearman's rank test was performed in order to investigate the association between the analysed immunoregulatory markers, considering the relative expression values as continuous variables. Results are shown in Supplementary Table II (Appendix I).

As all the analysed genes are related to modulation of immune-surveillance in tumour microenvironment, they were expected to be correlated. The Spearman's correlation coefficient obtained showed a strong positive correlation among the studied genes ($p<0.0001$) in all cases except on the analysis between *IDO-1* and *CD200* or *ICOS-LG*.

These correlations can be related to their function, as PD-L1, PD-L2, ICOS-LG and CD200 are immune checkpoint molecules, which mediate second signals that modulate T cell responses, and CD5 and CD6 are involved in T cell proliferation, as previously mentioned. In addition, it is known that these functions are enhanced by metabolic enzymes such as IDO. Herein, these molecules are co-expressed in the tumour microenvironment because all of them play a role in tumour immune regulation.

4.2.5. Gene expression correlation with clinico-pathological variables

In order to establish a relationship between patient's clinico-pathological variables and gene expression levels, non-parametric tests such as Mann-Whitney U, Kruskall-Wallis and Chi-square tests were performed (significant results are shown in Table 5). Demographic and clinico-pathological variables analysed were sex, age (considering 65 years as a cut-off value), smoking status, performance status, tumour size (considering a cut-off of 3.5cm), lymph node involvement, stage, histology, differentiation grade and mutational status of *EGFR* and *KRAS*.

Table 5. Relative gene expression differences depending on clinico-pathological variables.

Variable	Gene		N	Median	Range[min-max]	p-value
Sex	IDO-2	Female	29	1.249	0.08-9.66	0.038
		Male	170	0.788	0.01-27.26	
Age	PD-L2	=< 65	95	0.9002	0.04-14.11	0.010
		> 65	93	0.532	0.01-14.27	
	CD5	=< 65	94	1.242	0.04-13.00	0.030
		> 65	93	0.872	0.01-19.00	
Tumour size	PD-L2	<=3,5	102	0.551	0.01-9.46	0.022
		>3,5	98	0.763	0.05-14.27	
Differentiation grade	PD-L2	Well/Moderate	117	0.583	0.01-14.27	0.007
		Poor	48	1.114	0.03-6.49	
	CD5	Well/Moderate	117	1.017	0.01-13.31	0.016
		Poor	47	1.695	0.02-10.77	
	CD6	Well/Moderate	117	0.947	0.01-18.32	0.013
		Poor	48	1.551	0.01-12.90	

No significant correlations were obtained for clinico-pathological variables such as smoking habits, lymph node involvement, PS, histology, stage, or *EGFR/KRAS* mutational status. However, some studies have reported correlation between lymph node involvement and expression of *IDO* and *PD-L2* in melanoma (Obeid *et al.*, 2016).

Although Mann-Whitney U results state that there is a significant differential expression of *IDO-2* among different sexes, this could be due to the lower amount of women in the patient's cohort as a consequence of the epidemiology of the disease; hence more analysis should be done in order to validate this result.

Interestingly, higher levels of *PD-L2* correlate with bigger tumours (>3.5cm) as shown in Figure 11a, which is line with a previous study reporting that *PD-L2* expression stabilises with tumour size (Obeid *et al.*, 2016). By contrast, in another study in lung adenocarcinoma, *PD-L1* expression correlated with tumour size and lymph node involvement, but such correlation was not found in the case of *PD-L2* expression (Zhang *et al.*, 2014).

The most remarkable correlation is established between tumour's differentiation grade and *PD-L2, CD5* and *CD6* expression (Figure 11b-d). It has been found higher expression of these immunoregulatory markers in poor differentiated tumours. These results are in concordance with previous studies that found higher *PD-L2* expression in poor and moderately differentiated tumour than in well-differentiated (Mo *et al.*, 2016). Another study revealed higher *CD5* expression in differentiating thymic carcinomas than in poorly differentiated carcinomas of lung origin, as opposite to our results. (Asirvatham *et al.*, 2014). Therefore, further research is required to clear the relationship between tumour differentiation grade and immunoregulatory gene expression.

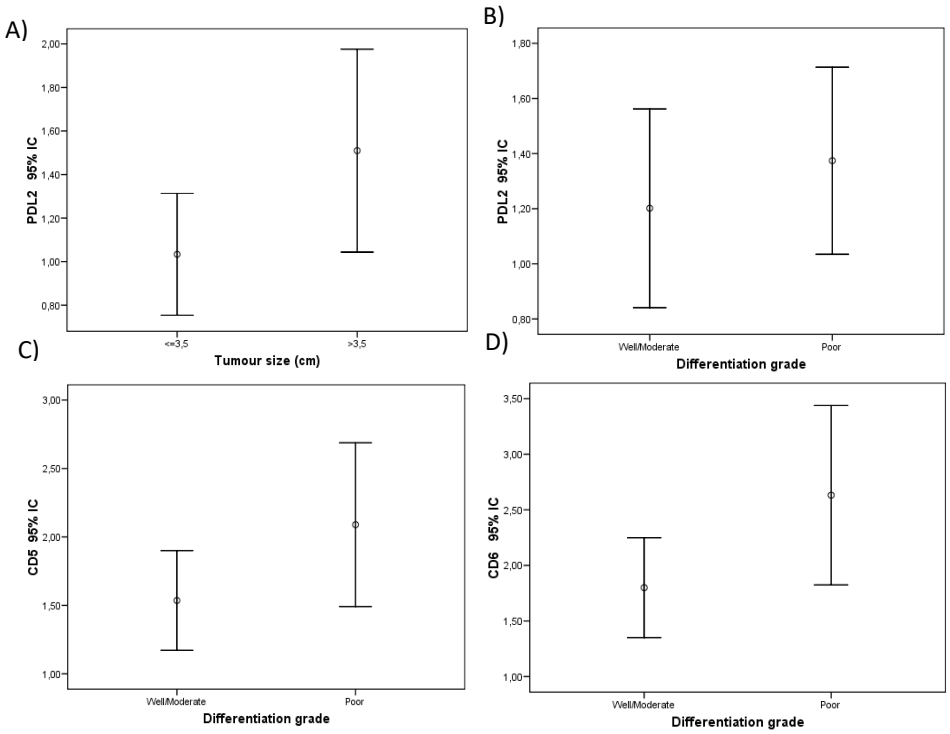

Figure 11. Representation of correlation between clinico-pathological variables and gene expression markers *based on error bars for 95% confidence interval (CI) of the mean. A) Representation of PD-L2 according to tumour size, B) representation of PD-L2 according to differentiation grade, C) representation of CD5 according to differentiation grade and D) representation of CD6 according to differentiation grade.*

4.2.6. Immune-related gene expression patterns association with survival analysis

Of the 201 resected NSCLC patients included in the study, 84 (41.8%) relapsed and 114 (56.8%) died during the follow-up. The median follow-up was of 34.2 months (0.6-133.8).

4.2.6.1. Clinico-pathological variables

The prognostic value of the different clinico-pathological variables was assessed using the univariate Cox regression method for RFS and OS (Table 6).

Table 6. Entire cohort: Results from univariate survival analysis based on clinico-pathological variables.

Varables	RFS			OS		
	HR	95,0% CI	p-value	HR	95,0% CI	p-value
Sex *Male vs. Female*	1.822	0.883-3.761	0.104	2.313	0.934-5.730	0.070
Age *>65 vs. ≤65*	1.287	0.852-1.944	0.231	1.364	0.861-2.161	0.186
Smoking habit *Current vs. Former vs. Never*	1.082	0.800-1.464	0.608	0.913	0.657-1.269	0.589
PS *1/2 vs 0*	1.637	1.080-2.482	**0.020**	1.833	1.185-2.835	**0.007**
Tumour size *>3.5 cm vs. ≤ 3.5 cm*	1.421	0.947-2.132	0.089	1.353	0.859-2.132	0.192
Stage *II/IIIA vs. I*	1.273	0.995-1.628	0.055	1.302	0.989-1.715	0.060
Histology *ADC vs. SCC vs. Others*	1.086	0.812-1.453	0.579	0.975	0.699-1.358	0.879
Lymph node involvement *Yes vs. No*	2.046	1.340-3.123	**0.001**	1.622	0.998-2.363	**0.051**
Differentiation grade *Poor vs. Well/Moderate*	1.106	0.815-1.499	0.518	0.975	0.697-1.365	0.884
***KRAS* Mutational Status** *Mutated vs. Wild type*	1.978	1.124-3.481	**0.018**	2.038	1.107-3.751	**0.022**
***EGFR* Mutational Status** *Wild type vs. Mutated*	0.991	0.392-2.507	0.984	1.036	0.366-2.932	0.947
Chemotherapy *Yes vs. No*	1.864	1.240-2.800	**0.003**	1.446	0.918-2.278	0.112

HR: Hazard ratio; CI: 95% Confidence interval, OS: Overall survival; RFS: relapse free survival; PS: performance status; EGFR: epidermal growth factor receptor.

Significant results obtained from the univariate analysis were also evaluated using the Kaplan-Meier method (log-rank test) in order to obtain the survival plots (RFS: Figure 12; OS: Figure 13).

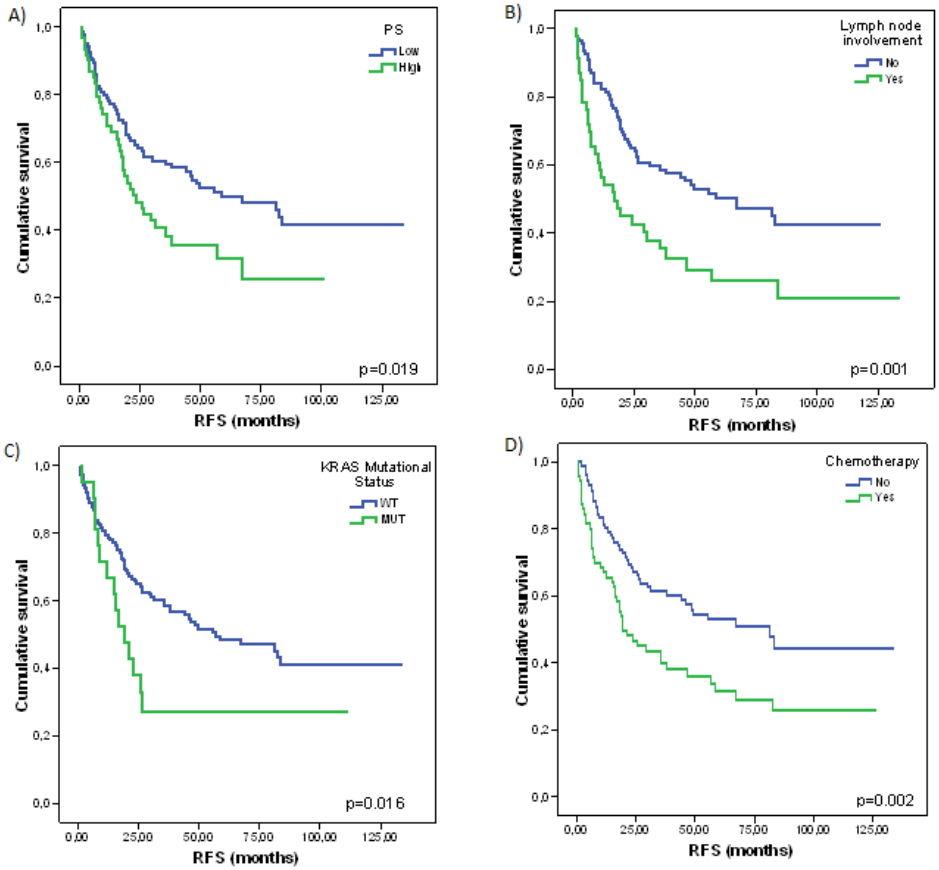

Figure 12. Kaplan-Meier curves for clinico-pathological variables significantly associated to RFS: *A) Performance status (PS), B) lymph node involvement, C) KRAS mutational status and d) chemotherapy.*

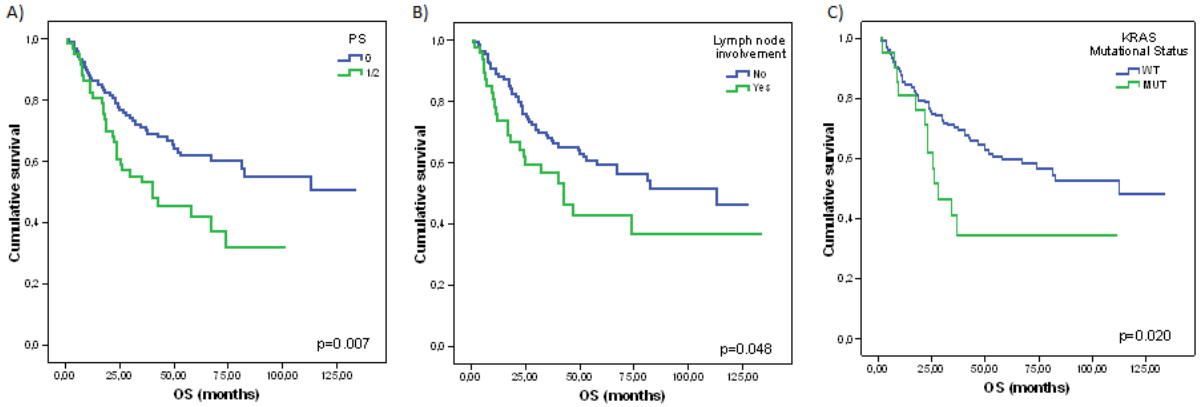

Figure 13. Kaplan-Meier curves for clinico-pathological variables significantly associated to OS: *A) Performance status (PS), B) lymph node involvement and C) KRAS mutational status*

The survival analysis showed that patients with good performance status (PS=0) had better RFS and OS (p=0.019 and p=0.007, respectively), in concordance with previous results (Peters *et al.*, 2014). Lymph node involvement and *KRAS* mutations define a group of patients associated with worse clinical outcomes, as previously reported (Meng *et al.*, 2013; Suzuki *et al.*, 2013; Qiang *et al.*, 2015; Yagishita *et al.*, 2015).

Those patients who had received adjuvant chemotherapy had better RFS (p=0.002), but it did not have any association with OS, as previously reported (Peters *et al.*, 2014).

4.2.6.2. Immune-related biomarkers

The prognostic value of the expression of immunoregulatory genes was assessed using the univariate Cox regression method for RFS and OS (Table 7). For those cases in which significant associations were found, Kaplan-Meier curves were represented (RFS: Figure 14A and OS: Figure 14B) and C). Prior to that, gene expression data were dichotomised according to the median of each gene, splitting the data into two groups: i)"high" corresponding to those samples whose expression values were > median, and ii) "low" for those samples whose expression values were ≤ median.

Table 7. Entire cohort: Univariate survival analysis based on immunoregulatory genes expression.

		RFS			OS		
		HR	**95,0% CI**	**p-value**	**HR**	**95,0% CI**	**p-value**
PD-L1	*High vs. Low*	0.949	0.627-1.436	0.803	0.763	0.483-1.206	0.247
PD-L2	*High vs. Low*	0.833	0.556-1.248	0.376	0.830	0.528-1.305	0.420
CD200	*High vs. Low*	1.204	0.802-1.806	0.370	1.076	0.685-1.692	0.750
CD5	*High vs. Low*	0.759	0.507-1.138	0.182	0.555	0.350-0.879	**0.012**
CD6	*High vs. Low*	1.005	0.671-1.503	0.982	0.968	0.617-1.520	0.889
ICOS-LG	*High vs. Low*	0.962	0.642-1.443	0.852	1.035	0.658-1.628	0.883
IDO-1	*High vs. Low*	0.873	0.579-1.316	0.516	0.809	0.510-1.283	0.367
IDO-2	*High vs. Low*	0.640	0.425-0.965	**0.033**	0.635	0.401-1.004	**0.052**

HR: Hazard ratio; CI: 95% Confidence interval, OS: Overall survival; RFS: relapse free survival; PS: performance status; EGFR: epidermal growth factor receptor

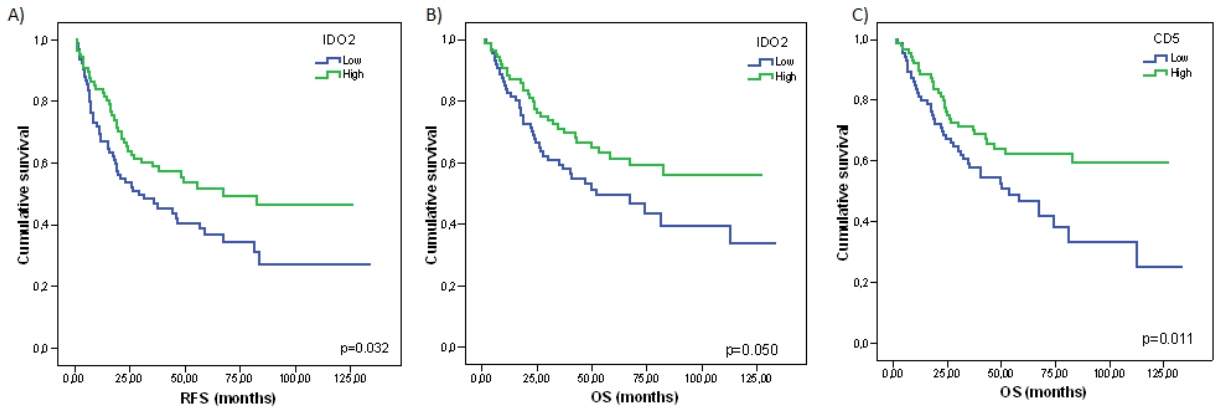

Figure 14. Kaplan-Meier curves for immunoregulatory genes expression in the entire cohort.
A) IDO-2 expression associated to RFS, B) IDO-2 expression associated to OS C)CD5 expression associated to OS

Univariate Cox analysis found that high expression levels of *IDO-2* were associated with RFS and *CD5* and *IDO-2* were associated with OS. Kaplan-Meier analysis was performed to obtain the survival plots and showed that patients with high expression levels of *IDO-2* had better RFS (29.300 vs. 66.97 months; p=0.032) and high expression levels of *CD5* and *IDO-2* had better OS (53.3 months vs. NR; p=0.011 and 51.9 months vs. NR; p=0.050, respectively).

These results are in agreement with the fact that *IDO* expression inhibits proliferation of tumour cells *in vitro* due to the decrease on tryptophan (Aune and Pogue, 1989). However, the role

of IDO has previously been studied by immunohistochemistry in NSCLC, and high expression was associated with shorter survival rates, although the authors stated that the prognostic role of IDO-positive infiltrates should be further studied (Karanikas *et al.*, 2007). Regarding previous analysis in other cancer types, on the one hand, it has been stated that *IDO* expression in hepatocellular carcinoma correlated with lower OS (Pan *et al.*, 2008), but on the other hand high *IDO* expression in breast tumours correlated with increased OS, supporting the dual role that *IDO* activation may play in carcinogenesis and modulation of tumour microenvironment (Soliman *et al.*, 2013).

In our cohort, higher levels of relative expression of *CD*5 define a subset of patients with better outcome. CD5 is an inhibitory molecule involved in anti-tumour immune-responses by reducing cytotoxic T lymphocytes activity and cytokine secretion (Tabbekh *et al.*, 2013), but it has also been discovered a co-stimulatory function during T cell dependent immune responses, which leads to T and B cell activation and proliferation (de Wit *et al.*, 2011). Previous studies have associated low *CD5* expression levels with improved survival in NSCLC (Dirican *et al.*, 2015). Interestingly, a recent study found that different *CD5* haplotypes can be associated either to better or worse survival in melanoma, which could explain these controversial results (Potrony *et al.*, 2016).

According to our results, previous studies found no correlation between *PD-L1* expression and OS in melanoma cells and NSCLC (Konishi *et al.*, 2004; Gadiot *et al.*, 2011). Nevertheless, *PD-L1* and *PD-L2* expression were correlated with poor prognosis in advanced esophageal cancer (Ohigashi *et al.*, 2005). By contrast, Obeid *et al*. stated that *PD-L1* and *PD-L2* correlated with increased amounts of immune cells in the microenvironment and their expression was associated with longer OS in advanced melanoma. These controversial results could be explained by the fact that although it is well established that PD-1/PD-L1 have an inhibitory role on T cell function in the tumour microenvironment, PD-L1 expression can be induced by interferon secretion, and PD-L2 expressed on B cells enhances anti-tumour protection by increasing Th1 and Th17 responses, displaying a dual effect on the modulation of immune response against tumours (Francisco *et al.*, 2009; Tomihara *et al.*, 2012).

Although the rest of genes did not show significant results, they have already been described in the bibliography in other cancer types, such as *CD6*, that correlated with more aggressive disease in breast cancer (Burkhardt *et al.*, 2006). Regarding *CD200* and *ICOS-LG* expression, controversial results have been found, as they have been associated with improved survival as well as with poor prognosis in different cancer types (Tonks *et al.*, 2007; Faget *et al.*, 2013; Erin *et al.*, 2015; Zhang *et al.*, 2016), supporting their dual role in tumour immune-regulation.

4.2.6.3. Subanalysis according to histology

The prognostic value of clinico-pathological and experimental variables was also assessed according to histology.

The ADC subgroup comprised 88 patients: of these, 30 (38.5%) relapsed and 28 (35.9%) died during the follow-up of this study. In the univariate analysis for clinico-pathological variables, PS, lymph node involvement, *KRAS* mutational status and chemotherapy (adjuvant treatment) were associated with RFS, whereas only *KRAS* mutational status was associated with OS (Table 8).

Table 8. ADC subgroup: Results from univariate survival analysis based on clinico-pathological variables.

Variable	RFS			OS		
	HR	95,0% CI	p-value	HR	95,0% CI	p-value
Sex *Male vs. Female*	2.075	0.912-4.721	0.082	2.004	0.761-5.821	0.160
Age *>65 vs. ≤65*	1.069	0.561-2.037	0.840	1.352	0.634-2.880	0.435
Smoking habit *Current vs. Former vs. Never*	1.378	0.909-2.088	0.131	1.262	0.776-2.053	0.348
PS *1/2 vs 0*	1.874	0.980-3.582	0.058	1.501	0.735-3.064	0.265
Tumour size *>3.5 cm vs. ≤ 3.5 cm*	1.491	0.788-2.819	0.219	1.274	0.607-2.676	0.522
Stage *II/IIIA vs. I*	1.132	0.754-1.698	0.550	0.860	0.515-1.434	0.563
Lymph node involvement *Yes vs. No*	3.314	1.634-6.723	**0.001**	1.394	0.561-3.466	0.475
Differentiation degree *Poor vs. Well/Moderate*	0.818	0.514-1.302	0.397	0.780	0.453-1.342	0.369
***KRAS* Mutational Status** *Mutated vs. Wild type*	2.446	1.164-5.140	**0.018**	2.661	1.153-6.144	**0.022**
***EGFR* Mutational Status** *Wild type vs. Mutated*	0.834	0.290-2.403	0.737	0.819	0.242-2.768	0.748
Chemotherapy *Yes vs. No*	2.458	1.290-4.683	**0.006**	1.170	0.552-2.480	0.683

HR: Hazard ratio; CI: Confidence interval, OS: Overall survival; RFS: relapse free survival; PS: performance status; EGFR: epidermal growth factor receptor

The prognostic value of the immune markers was also assessed within the ADC subgroup, using the Cox regression method (Table 9)

Table 9. Survival univariate analysis based on target genes expression in ADC subgroup.

		RFS			OS		
		HR	95,0% CI	p-value	HR	95,0% CI	p-value
PD-L1	*High vs. Low*	0.651	0.342-1.236	0.189	0.556	0.262-1.181	0.126
PD-L2	*High vs. Low*	0.630	0.329-1.207	0.164	0.752	0.357-1.587	0.455
CD200	*High vs. Low*	1.485	0.779-2.831	0.230	1.285	0.607-2.720	0.513
CD5	*High vs. Low*	0.744	0.392-1.413	0.366	0.583	0.274-1.239	0.161
CD6	*High vs. Low*	0.742	0.390-1.415	0.365	0.810	0.384-1.708	0.580
ICOS-LG	*High vs. Low*	0.793	0.415-1.515	0.482	0.920	0.432-1.958	0.828
IDO-1	*High vs. Low*	0.547	0.283-1.058	0.073	0.516	0.241-1.107	0.089
IDO-2	*High vs. Low*	0.343	0.176-0.668	**0.002**	0.381	0.175-0.830	**0.015**

HR: Hazard ratio; CI: Confidence interval, OS: Overall survival; RFS: relapse free survival; PS: performance status; EGFR: epidermal growth factor receptor.

Kaplan-Meier curves for *IDO-2* show that the expression of this gene has prognostic information in ADC patients (Figure 15). Particularly, the group of patients with higher expression

levels of *IDO-2*, presented a significant improve in RFS and also in OS compared with those patients with lower levels of *IDO-2* (RFS: 15.43 months vs. NR; p=0.001 and OS: 37.00 months vs. NR; p=0.012).

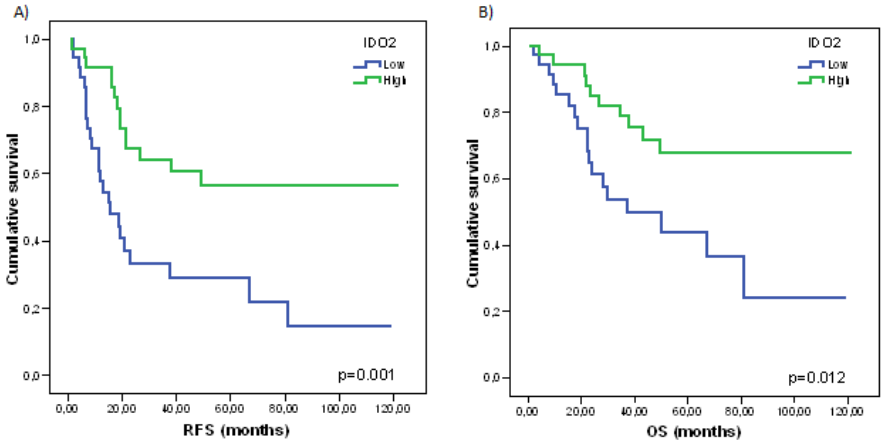

Figure 15. Kaplan-Meier curves for IDO-2 expression associated to A) OS and B) RFS in ADC subgroup

The SCC subgroup comprised 90 patients; 96.6% were current or former smokers. Of these, 40 (44.4%) relapsed and 39 (43.3%) died. In contrast to the findings in ADC patients, no significant association was found between the clinico-pathological or experimental variables and OS or RFS in this group of patients (data not shown).

4.2.7. Expression score analysis

We also decided to create a prognostic expression score, which can provide more accurate prognostic information than models using single genes (Sanmartín *et al.*, 2014; Usó *et al.*, 2017). As explained in materials and methods, a univariate Cox regression analysis with expression data was performed in order to identify which genes were moderately associated with survival (|Zscore>1.5). Results are shown in Table 10.

Table 10. Univariate analysis of the expression of the target genes for OS in the entire cohort.

	Regression coefficient	Error	HR	95,0% CI	p-value	Zscore
PD-L1	-0.009	0.017	0.991	0.958-1.025	0.602	-0.522
PD-L2	0.067	0.056	1.069	0.959-1.192	0.229	1.204
CD200	-0.033	0.045	0.967	0.886-1.056	0.460	-0.740
CD5	-0.034	0.061	0.967	0.858-1.090	0.579	-0.555
CD6	-0.003	0.040	0.997	0.922-1.078	0.941	-0.073
ICOS-LG	0.007	0.057	1.007	0.901-1.125	0.905	0.119
IDO-1	-0.006	0.026	0.994	0.944-1.045	0.803	-0.250
IDO-2	0.023	0.044	1.024	0.939-1.115	0.596	0.531

HR: Hazard ratio; CI: 95% Confidence interval

In the entire cohort, and according to the values of Z-score, none of the analysed genes had enough involvement in order to be introduced in a score with prognostic value.

Then, we conducted the same analysis in the ADC subgroup (Table 11).

Table 11. Univariate analysis of the expression of the immunoregulatory genes for OS in ADC subgroup.

Gene	Regression coefficient	Error	HR	95,0% CI	p-value	Zscore
PD-L1	-0.182	0.101	0.833	0.684-1.015	0.070	**-1.809**
PD-L2	-0.127	0.199	0.881	0.597-1.300	0.523	-0.638
CD200	-0.007	0.036	0.993	0.924-1.066	0.840	-0.201
CD5	0.008	0.110	1.008	0.812-1.251	0.942	0.073
CD6	0.006	0.067	1.006	0.881-1.148	0.933	0.084
ICOS-LG	-0.038	0.106	0.963	0.782-1.185	0.721	-0.357
IDO-1	-0.183	0.105	0.833	0.678-1.024	0.082	**-1.738**
IDO-2	-0.195	0.120	0.823	0.650-1.040	0.103	**-1.629**

HR: Hazard ratio; CI: Confidence interval

PD-L1, *IDO-1* and *IDO-2* were selected due to their association with mortality (|Z-score|>1.5). A multivariate Cox regression analysis was performed to obtain their regression coefficient, which was used to calculate the score and generate the "*PDIDO* score", as indicated in Table 12 and Equation 2.

Table 12. Multivariate Cox regression analysis with the selected genes for the score calculation.

Gene	Regression coefficient	Error	HR	95,0% CI	p-value
PD-L1	-0.179	0.095	0.836	0.694-1.006	0.059
IDO-1	-0.173	0.108	0.841	0.681-1.039	0.108
IDO-2	-0.097	0.123	0.907	0.713-1.154	0.429

ADC: adenocarcinoma; HR: Hazard ratio; CI: 95% Confidence interval

PDIDO score = 0.179 * PD-L1 + 0.173 * IDO-1 + 0.097 * IDO-2

Equation 2. PDIDO score calculation.

The value of PDIDO score for each patient was calculated, and their prognostic value was analysed after dichotomization using the median as cut off. Again, two groups were generated, named "High" (> median) or "Low" (≤ median). Kaplan-Meier analysis showed that patients with high *PDIDO* score had significant better RFS (17.867 months vs. NR; p=0.001) and OS (29.83 months vs. NR; p=0.0002) than those presented by the genes itself (Figure 16).

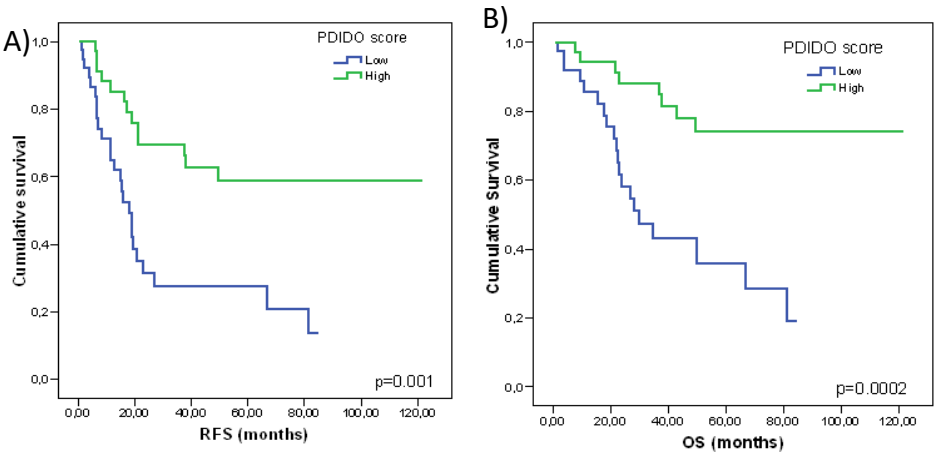

Figure 16. Kaplan-Meier curves of *PDIDO* score for A) OS and B) RFS in ADCs.

Therefore, *PDIDO* score is associated to better prognosis, although its components have been stablished as poor prognostic biomarkers in ADC (Zhang *et al.*, 2014, 2017). The favourable prognostic value of *PDIDO* score can be explained by the fact that although these genes are immunosuppressive factors, their presence contributes to tumour inflammation, which allows tumour-recognition by the immune system, facilitating tumour-cells destruction (Usó *et al.*, 2017).

4.2.8. Multivariate Cox analysis

In order to state whether the analysed variables (clinical, analytical, scores) could be established as independent prognostic biomarkers, a multivariate model for RFS and OS was performed including all the variables that were significantly associated with prognosis ($p < 0.05$) in the previous univariate analyses.

In the entire cohort, analysis for RFS included the following variables: lymph node involvement, PS, *KRAS* mutational status, adjuvant chemotherapy and *IDO-2*. For OS, variables included were: lymph node involvement, PS, *KRAS* mutational status, *CD5* and *IDO-2*. Significant results are shown in Table 13.

Table 13. Multivariate Cox regession analysis for RFS and OS in the entire cohort.

Variable	RFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
***KRAS* Mutational Status** *Mutated vs. WT*	2.114	1.173-3.809	0.013	2.007	1.065-3.782	0.031
Lymph node involvement *Yes vs No*	1.947	1.225-3.092	0.005	1.840	1.097-3.087	0.021
PS *1/2 vs 0*	1.561	1.001-2.435	0.050	1.773	1.113-2.824	0.016
CD5 *High vs. Low*				0.539	0.329-0.883	0.014

OS: Overall survival; RFS: Relapse-free survival; HR: Hazard ratio; CI: Confidence interval; PS: Performance Status

Besides *KRAS* mutational status and lymph node involvement, two pathological variables that have previously been associated with prognosis in resectable NSCLC (Meng *et al.*, 2013; Suzuki *et al.*, 2013; Qiang *et al.*, 2015; Yagishita *et al.*, 2015), this is the first time that CD5 is reported as an independent prognostic biomarker for OS in resectable NSCLC [HR,0.536; 95% CI, 0.329-0.883; p=0.013].

Multivariate analysis was also performed with significant results for ADC patients. In this case, multivariate analysis for RFS included PS, lymph node involvement, *KRAS* mutational status, chemotherapy, *IDO-2* and *PDIDO* score. For OS variables included were *KRAS* mutational status, *IDO-2* and *PDIDO* score. Significant results are shown in Table 14.

Table 14. Multivariate Cox regression analysis for RFS and OS in the ADC subgroup.

Variable	RFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
***KRAS* Mutational Status** *Mutated vs. WT*				2.444	1.058-5.645	0.036
***PDIDO* score** *High vs. Low*	0.274	0.126-0.593	0.001	0.267	0.113-0.630	0.003

OS: Overall survival; RFS: Relapse-free survival; HR: Hazard ratio; CI: Confidence interval

Our results indicate that the *PDIDO* score is an independent biomarker for both RFS [HR, 0,274; 95% CI, 0.126-0.593; p=0,001] and OS [HR, 0.267; 95%CI, 0.113-0.630; p=0.003]. Moreover, its prognostic value proved to be stronger for OS than factors such as *KRAS* mutational status in ADC patients, as it comprises molecules from two important immunoregulatory pathways. The role of *PDIDO* score in tumour microenvironment confirms the idea that the presence of immune factors enhances tumour inflammation triggering anti-tumour T cell responses, and is related to a better prognosis that non-inflamed tumours, which are not recognized by the immune system (Usó *et al.*, 2017).

In summary, the analysis of the immune profile in resected samples of NSCLC has allowed the establishment of independent prognostic biomarkers based on the expression of immunoregulatory genes associated with better outcomes. The most remarkable finding is that *PDIDO* score is an independent prognostic biomarker for overall survival and relapse-free survival in early stages of lung ADC that could be introduced to the clinical practice to allow easy identification of patients with good outcome.

5. CONCLUSSIONS

1. A large cohort including 201 NSCLC patients with paired tumour and normal tissue samples was analysed and accomplished the eligibility criteria to be included in the present study. Moreover, demographics and the clinical behaviour of this cohort was the expected for patients in resectable stages.

2. Gene expression levels analysis showed that the studied genes were neither significantly overexpressed nor underexpressed in tumour than in normal tissue samples.

3. Gene correlation analysis showed that the studied genes follow a co-expression pattern as they are all involved in tumour-immune regulation. Statistically significant correlations among gene expression and clinico-pathological variables, such as increased levels of *PD-L2* according to tumour size, and the positive correlations between poorly differentiated tumours and *PD-L2*, *CD5* and *CD6*.

4. a. Survival analyses in the entire cohort associated clinico-pathological variables such as *KRAS* mutational status, lymph node involvement and performance status with poor prognosis, as well as the immunoregulatory gene *CD5*, which was established as an independent biomarker for overall survival.

   b. In lung adenocarcinomas, a three gene score (*PDIDO* score) demonstrated to be independently and significantly associated with prognosis in both, relapse-free survival and overall survival.

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7. APPENDICES

APPENDIX I. SUPPLEMENTARY TABLES

Supplementary table 1. TNM7 classification and staging *(Goldstraw et al., 2007).*

Occult Carcinoma	Tx	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1a,b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T1a,b	N1	M0
	T2a	N1	M0
	T2b	N0	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1, T2	N2	M0
	T3	N1,N2	M0
	T4	N0, N1	M0
Stage IIIB	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1a,b

T: Tumour size; N: lymph node involvement; M: metastasis.

Supplementary Table 2. Patient characteristics description.

Total		N 201	% 100
Age	Median Range	65 26-85	
Sex	Female Male	29 172	14,4 85,6
Smoking Status	Current Former Never	96 83 24	47,3 40,9 11,8
Perfomance Status (PS)	0 1 2	135 68 1	66,2 33,3 0,5
Tumour size	< 3cm 3 - 5cm 5 - 7cm > 7cm	87 67 29 18	43,3 33,3 14,4 9
Lymph node involvement	Yes No	52 149	25,9 74,1
Stage	1-A 1-B 2-A 2-B 3-A	43 61 35 22 40	21,4 30,3 17,4 10,9 19,9
Histology	SCC ADC Others	93 88 20	46,3 43,8 10
Differentiation degree	Poor Moderate Well NS	48 83 34 36	29,1 50,3 20,6
EGFR Mutational Status	Mutated Wild-type NS	12 89 100	11,9 88,1
KRAS Mutational Status	Mutated Wild-type NS	22 146 33	13,1 86,9
Adjuvant chemotherapy (post-surgery)	Yes No NS	73 123 5	37.2 62.8
Relapse	Yes No	84 117	41,8 58,2
Exitus	Yes No	114 87	56,7 43,3

**SCC: Squamous cell carcinoma ADC: Adenocarcinomas, NS: Not specified*

Supplementary Table 3. Gene correlation analysis results.

		PD-L1	PD-L2	CD200	CD5	CD6	ICOS-LG	IDO-1	IDO-2
PD-L1	**Coeficiente de correlación**	1	,502(**)	,330(**)	,496(**)	,482(**)	,372(**)	,150(*)	,312(**)
	Sig. (bilateral)	.	0	0	0	0	0	0,038	0
	N	198	198	198	197	198	197	190	197
PD-L2	**Coeficiente de correlación**	,502(**)	1	,599(**)	,600(**)	,665(**)	,554(**)	,242(**)	,549(**)
	Sig. (bilateral)	0	.	0	0	0	0	0,001	0
	N	198	200	200	199	200	199	192	199
CD200	**Coeficiente de correlación**	,330(**)	,599(**)	1	,531(**)	,560(**)	,617(**)	-0,027	,396(**)
	Sig. (bilateral)	0	0	.	0	0	0	0,708	0
	N	198	200	200	199	200	199	192	199
CD5	**Coeficiente de correlación**	,496(**)	,600(**)	,531(**)	1	,901(**)	,541(**)	,207(**)	,654(**)
	Sig. (bilateral)	0	0	0	.	0	0	0,004	0
	N	197	199	199	199	199	198	191	198
CD6	**Coeficiente de correlación**	,482(**)	,665(**)	,560(**)	,901(**)	1	,621(**)	,184(*)	,636(**)
	Sig. (bilateral)	0	0	0	0	.	0	0,011	0
	N	198	200	200	199	200	199	192	199
ICOS-LG	**Coeficiente de correlación**	,372(**)	,554(**)	,617(**)	,541(**)	,621(**)	1	-0,061	,438(**)
	Sig. (bilateral)	0	0	0	0	0	.	0,404	0
	N	197	199	199	198	199	199	191	198
IDO-1	**Coeficiente de correlación**	,150(*)	,242(**)	-0,027	,207(**)	,184(*)	-0,061	1	,396(**)
	Sig. (bilateral)	0,038	0,001	0,708	0,004	0,011	0,404	.	0
	N	190	192	192	191	192	191	192	191
IDO-2	**Coeficiente de correlación**	,312(**)	,549(**)	,396(**)	,654(**)	,636(**)	,438(**)	,396(**)	1
	Sig. (bilateral)	0	0	0	0	0	0	0	.
	N	197	199	199	198	199	198	191	199

*** Correlation is significant at 0,01 level (bilateral). * Correlation is significant at 0,05 level (bilateral).*

APPENDIX II. COMMUNICATIONS DERIVED FROM THIS STUDY

Abstract SEOM 2017 (Submitted)

ESTABLECIMIENTO DE UNA FIRMA DE GENES INMUNOREGULADORES CON VALOR PRONÓSTICO EN ESTADIOS TEMPRANOS DE ADENOCARCINOMAS DE PULMÓN.

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Introducción y objetivos

El adenocarcinoma (ADC) es uno de los subtipos de cáncer de pulmón más frecuente. El estudio de la evasión de la vigilancia inmune en el microambiente tumoural ha dado lugar al desarrollo de nuevas terapias contra el cáncer. Este trabajo se centra en el análisis de genes inmunoreguladores como posibles biomarcadores pronóstico.

Materiales y métodos

Se utilizó una cohorte de 88 pacientes con ADC en estadios resecables. Se analizó la expresión de 8 genes relacionados con la regulación inmune (*PD-L1, PD-L2, IDO-1, IDO-2, ICOS-LG, CD5, CD6* y *CD200*) mediante RTqPCR en muestras pareadas de tejido normal y tumoural, y se calculó la expresión relativa utilizando la fórmula de Pfaffl. La supervivencia se determinó mediante análisis de regresión de Cox. Se consideraron significativos aquellos análisis en que $p<0,05$, y se representaron mediante curvas Kaplan-Meier (test log-rango) tras dicotomizar los datos tomando la mediana como valor de corte.

Resultados

La cohorte de pacientes se caracterizó por una mediana de edad de 65 años y buen estado funcional (PS=0) en un 77% de los pacientes. Se observó una tendencia de mejor supervivencia global (SG) y mejor supervivencia libre de enfermedad (SLE) en aquellos pacientes con altos niveles de expresión de *PD-L1*, *IDO-1* e *IDO-2*. Se creó una firma de expresión basada en la expresión matemática: 0.179 * *PD-L1* + 0.173 * *IDO-1* + 0.097 * *IDO-2*. Los pacientes con altos niveles de expresión de la firma presentaban mejor SG (29,83 vs NA meses; p=0.0002) y SLE (17,867 vs NA meses; p=0.001). El análisis multivariatee confirmó que la firma es un biomarcador pronóstico independiente para SG (HR=0,267 [0,113-0,630]; p=0.003] y SLE (HR=0,274 [0,126-0,593]; p=0,001).

Conclusión

El análisis del perfil inmunológico de muestras resecadas de ADC ha permitido establecer una firma de genes reguladores como biomarcador pronóstico independiente para SG y SLE en estadios tempranos de ADC de cáncer de pulmón no microcítico.

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Abstract ESMO 2017 (Submitted)

ANALYSIS OF IMMUNOREGULATORY BIOMARKERS IN EARLY STAGES OF NON-SMALL CELL LUNG CANCER

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Introduction: The study of the tumour microenvironment is leading to a better understanding of the evasion of immune surveillance and the development of new therapies. This research focuses on the analysis of immunoregulatory genes as potential prognostic biomarkers in resectable non-small cell lung cancer (NSCLC).

Materials and methods: The expression of 8 genes involved in immune-regulation (*PD-L1, PD-L2, IDO-1, IDO-2, ICOS-LG, CD5, CD6 and CD200*) was analysed by RTqPCR in 257 paired fresh frozen tumour and normal tissue samples of resected NSCLC. Relative expression was calculated by Pfaffl formulae using *ACTB, CDKN1B* and *GUSB* as endogenous controls. Non-parametric tests were used for correlations between clinico-pathological and analytical variables and survival was assessed by Kaplan-Meier curves (long rank-test), considering significant $p<0.05$.

Results: Patient`s median age was 64 years, 82% were males, 88% were former or current smokers, 47% were adenocarcinomas (ADC). Patients with higher expression of *CD5* and *IDO-2* had a significant increase in overall survival (OS, 53.3 vs NR months, p=0.032; 51.9 vs NR months, p=0.049, respectively). A signature combining the expression of *CD5* and *IDO-2* was able to better prognosticate survival (40.4 vs NR months, p=0.028). The multivariate analysis (including clinico-pathological and analytical variables) showed that this signature has independent prognostic information OS (HR=0.553 [0.344-0.887], p=0.016).

Moreover, in the subgroup of ADC increased expression of *CD5* and *IDO-2* was associated with longer OS as well as increased relapse-free survival (RFS, 19.1 vs NR months, p=0.045; 18.8 and 67.0 months, p=0.029, respectively). The multivariate analysis established this gene signature as an independent prognostic biomarker for OS (HR=0.380 [0.166-0.872]; p=0.026) and RFS (HR=0.288 [0.139-0.597]; p=0.002).

Conclusion: The analyses revealed the prognostic value of *CD5* and *IDO-2*, being their combination an independent prognostic marker in resectable NSCLC.

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