TUMOR-ASSOCIATED METABOLIC AND INFLAMMATORY RESPONSES IN EARLY STAGE NON-SMALL CELL LUNG CANCER: LOCAL PATTERNS AND PROGNOSTIC SIGNIFICANCE.

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ABSTRACT

Introduction

Non-small cell lung cancer (NSCLC) patients diagnosed in early stage and surgically-treated have five-year mortality rate >20%. The identification of biomarkers able to predict progression and death may help to identify patients needing closer follow-up.

Methods

A retrospective cohort of early-stage surgically-treated NSCLC patients enrolled in the International Association for the Study of Lung Cancer (IASLC) Staging Project was created, and tissue Microarrays (TMAs) were constructed with tumor and non-tumor lung tissue. Pentose phosphate pathway (PPP) proteins (transketolase [TKT] and transketolase-like 1 [TKTL1]), inflammatory markers (cyclooxygenase-2 [COX-2], tumor necrosis factor alpha [TNF-α], interleukin 1 beta [IL1β], nuclear factor kappa-light-chain-enhancer of activated B cells [NFκB]-p65 and antigen Ki-67), and programmed death-ligand 1 (PDL1) were measured by immunohistochemistry.

Results

NSCLC patients with adenocarcinoma (ADC) or squamous cell carcinoma (SCC) were included in the study (n=199). TKT and TKTL1 were significantly higher in ADC than in non-tumor tissue ($p < 0.001$). Higher values were also observed in NSCLC for all the inflammatory markers, with figures >30% above those of non-tumor tissue ($p < 0.001$). PDL1 analysis showed a higher percentage of positivity in ADC than in non-tumor tissue ($p<0.001$). Multivariate

Cox proportional hazards modeling confirmed that high IL1β level in tumor tissue was independently associated with 3-year mortality in NSCLC [HR= 2.05, 95% CI (1.1-3.7), p= 0.019], a relationship driven by ADC subtype.

Conclusion

This study confirms an increase in metabolic activity and an inflammatory response in tumor tissue of early stage NSCLC, and a significant relationship between high levels of IL1β in the tumor and poor prognosis in ADC.

HIGHLIGHTS

- High metabolic and inflammatory activity characterizes early stage NSCLC.
- Pentose phosphate pathway proteins are overexpressed in adenocarcinoma.
- PDL1 is identified in >15% of early stage lung adenocarcinomas.
- High levels of IL-1β in NSCLC tissue are associated with 3-year mortality in ADC.

KEYWORDS

IL1β, pentose phosphate pathway, PDL1, early-stage, NSCLC, prognosis, inflammation.

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1. INTRODUCTION

Lung cancer (LC) is the leading cause of cancer-related mortality worldwide, accounting for 1.69 million out of the total of 8.8 million cancer-related deaths in 2015 [1]. Non-small cell lung carcinoma (NSCLC) is involved in up to 90% of LC cases, with adenocarcinoma (ADC) and squamous cell carcinoma (SCC) being the major subtypes [2]. Unfortunately, almost 85% of patients with LC remain undiagnosed until the disease is symptomatic and has reached an advanced stage [3], resulting in poor prognosis and an overall 5‑year survival rate of less than 15% [4,5]. LC patients diagnosed in early stages and treated surgically have better prognosis, but their 5-year mortality is still above 20% [6]. Therefore, the identification of biomarkers able to predict which patients submitted to therapeutic surgery present a higher risk of progression and death in the following years may help to improve survival, through the introduction of adjuvant therapies and closer follow-up.

Carcinogenesis evolves through genetic and epigenetic changes which allow cells to acquire the specific characteristics of malignancy [7,8], but tumor progression also depends on complex interactions between host genetic susceptibility and the local environment [9]. The chronic and uncontrolled cell proliferation that characterizes carcinogenesis involves not only a deregulated control of cell proliferation, but also the adjustments of energy metabolism necessary to increase cell growth and division [8].The activity of the pentose phosphate pathway (PPP) enables tumor cell proliferation by generating pentose phosphates and ribonucleotides which favor the high rate of nucleic acid synthesis of cancer cells. This pathway is also a major source of nicotinamide adenide dinucleotide phosphate (NADPH), which is required for

cell survival under stress conditions [10–12]. PPP contains two distinct metabolic branches, the oxidative and the non-oxidative branches. Flux through the oxidative branch is mainly regulated through enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate-dehydrogenase (6PGD). The enzyme transketolase (TKT) is one of the main regulators of the non-oxidative branch, while transketolase-like 1 (TKTL1) is an isoenzyme of TKT which is also thought to participate in the regulation of the PPP [13], although its precise role is still a matter of debate. TKTL1 has been shown to be upregulated in various cancer tissues, and its overexpression is correlated with many relevant cancer-related mechanisms such as invasiveness, therapeutic resistance, and poor prognosis [10,14,15].

The communication between tumor cells and their microenvironment (TME), which is composed of different cell subpopulations and an extracellular matrix (ECM), is also critical for tumor growth and progression [16,17]. Tumor stroma consists of fibroblasts, macrophage-lineage cells and vascular endothelial cells, with variable amounts of extracellular matrix, all of which contribute a support structure for tumor growth [18]. Inflammatory cells are a key component of the microenvironment of carcinomas and influence cancer initiation and promotion by secreting cytokines, growth factors and chemokines, which stimulate proliferation of epithelia as well as the generation of reactive oxygen species that can cause DNA damage [19]. Inflammation is involved in all stages of tumorigenesis, from malignant transformation and tumor initiation to the invasion and metastasis of established tumors [20].

Understanding the metabolic changes of tumor cells and the nature of their microenvironment is important for identifying prognostic markers in early stage

LC, and may allow the development of LC therapies targeting aspects of TME which influence the disease's progression. The aim of the present study was to identify molecular biomarkers related to cell metabolism and local inflammation in cancer tissue from early stage surgically-treated NSCLC patients that may be a potential target for specific therapies, and its relationship to prognosis.

2. METHODS

2.1 Design and population

The present study was nested in the International Association for the Study of Lung Cancer (IASLC) Staging Project, which has the aim of improving staging accuracy in LC. It was performed on the cases included by the Bronchogenic Carcinoma Cooperative Group of the Spanish Society of Pneumology and Thoracic Surgery (GCCB-II) in the Project between 2009 and 2012. The eighth edition of the tumor, node and metastasis (TNM) classification for LC was published in 2016 [21,22], and the innovations introduced in this edition were based on the data-driven recommendations of the IASLC [23,24]. The Project analyzed 77,154 evaluable patients recorded by 35 centers from 16 countries around the world [25]. Data entry and analysis were performed by Cancer Research and Biostatistics (CRAB), a non-profit organization based in Seattle, Washington. The inclusion criterion was a pathologic diagnosis of LC in patients without any associated severe renal or hepatic disease that might compromise survival in the following three years. The Spanish GCCB-II contributed 2,362 prospectively registered cases to the IASLC International Database to inform the eighth edition of the TNM classification [26]. Eighteen of the hospitals

participating in the GCCB-II included 1,035 surgically resected LC patients, representing 42.8% of the Spanish cohort. In this study, we retrospectively analyzed tissue samples from these surgical patients enrolled in the IASLC Staging Project and recorded at the participating Spanish hospitals [27]. The research protocol was approved by the reference regional research and ethics committee for the study (Fundació Parc Taulí reference PI12/02040) and by the local research and ethics committees of all participating centers. Written informed consent was obtained from all participating patients in accordance with the current legal regulations (RD 1716/2011) in Spain.

2.2 Clinical variables

The baseline clinical variables included in the IASLC database have been described elsewhere [25,28]. In brief, baseline clinical information included demographic data, smoking status, comorbidities, tumor location, blood analyses, results from staging tests, lung function, details on surgical treatment, pathological diagnosis and TNM descriptors. Survival was assessed annually, and overall mortality three years after surgical treatment was considered the main outcome for the present study.

2.3 Sample processing

Formalin-fixed paraffin embedded tissue samples were obtained from participating hospitals and stored in the Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES) Pulmonary Biobank Platform

(PBP), part of the Spanish Biobank Network [29]. A pathology panel formed by three experts evaluated the samples, confirmed the histology and selected the appropriate area to perform Tissue Microarrays (TMAs), identifying an area with abundant malignant cells and a second area with preserved lung tissue distant from the tumor when possible. TMAs were prepared at the Centro de Investigación Médica Aplicada (CIMA) of the Universidad de Navarra. From each block, three cylinders of 0.1 cm in diameter from a tumor zone and two cylinders from the non-tumor area were obtained. Independent TMAs were created for each histological subtype.

2.4 Biological marker analysis

2.4.1 Cell metabolism

The pentose phosphate pathway (PPP) proteins transketolase (TKT) and its isoform transketolase-like 1(TKTL1) were measured by immunohistochemical (IHC) staining with TKT (clone1925-250, isotype IgG1) and TKTL1 (clone 7D10, isotype IgG1) antibodies (ABnostics, Germany) in tumor and non-tumor TMAs. Details on the experimental procedures are described in the Supplementary Material.

2.4.2 Inflammatory response

Cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL1β), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-p65 and antigen Ki-67 were measured by IHC in the tumor and non-

tumor TMAs with the following antibodies: COX-2 (anti-COX-2 antibody, Santa Cruz Biotechnology, Dallas, TX, USA), NF-κB (p65) (anti-NF-κB (p65), Santa Cruz), ki-67 (anti-ki67 antibody, Millipore Iberica, CA, USA), TNF-α and IL1β (Santa Cruz). Details on the experimental procedures are described in the Supplementary Material.

2.4.3 Transmembrane proteins

Programmed death-ligand 1 (PDL1) was measured by IHC with the commercially available 22C3 pharmDx Autostainer 48.8 (DAKO Agilent Pathology Solutions, Santa Clara, CA) in tumor and non-tumor TMAs. The samples were considered positive for PDL1 when the stain was positive in ≥1% of the cells independently of the percentage of membrane stained. The results were expressed as percentages of positivity in accordance with a predefined scale: <1%; 1-5%, 5-50% and >50% [30].

2.5 Statistical analysis

Clinical data on the patients participating in the IASLC Staging Project were obtained from the CRAB and entered into a database with the results of the molecular biomarkers assessed. SPSS software version 15.0 (Chicago, IL, USA) and R package (http://www.r-project.org) were used for analyses. Categorical variables were expressed as absolute and relative frequencies, continuous variables as means and standard deviations (SD), with confidence intervals (CI) of the mean when required, and non-normally distributed data as

medians and interquartile ranges (IQR). Results for PPP variables were expressed as OD units, after their normalization with the mean value of non-tumor tissue. Inflammatory markers and transmembrane proteins were expressed as scale units from a semiquantitative score.

Three-year mortality after resection was considered the main outcome for the present study, according to the criteria used by the IASLC for the staging classification for its eighth edition. First, over and under-expression of the molecular markers assessed in tumor tissue was determined, using the non-tumor lung tissue as the reference. Second, the relationship between clinical characteristics and the main outcome was examined in order to define a clinical model for the cohort. And finally, the prognostic capacity of the molecular biomarkers was assessed, with adjustment for the statistically significant variables predefined in the clinical model.

Relationships between independent variables, both clinical and molecular, and the outcome were evaluated using Student's t test, one-way ANOVA, Mann-Whitney U, and Chi-square (χ^2) tests, as required. Sensitivity and specificity values from ROC curves and the Youden index were used to establish optimal cut-off levels for the molecular biomarkers assessed to discriminate between patients who did and did not survive after three years. Kaplan-Meier analysis was used for survival analysis and multivariate Cox proportional hazards regression to evaluate survival after adjusting for covariates. Sociodemographic, clinical and molecular variables showing a relationship with the outcome in first-step univariate models ($p<0.10$) were included in survival models as covariates. All the tests used were two-sided, and a p value of 0.05 or less was reported as statistically significant.

3. RESULTS

3.1 Patients and material

Seven of the 18 Spanish hospitals (38.9%) which included surgically-treated patients in the eighth edition of IASLC staging project agreed to participate in the present study. We collected 253 early stage lung cancer samples from the 1,035 surgically-treated patients included in the IASLC database in Spain (34.4%). The pathology panel identified 233 samples suitable for the preparation of TMAs. After integrating complete baseline clinical and 3-year follow-up information, 222 samples comprised the final cohort, which included patients with NSCLC and other histology subtypes. Patients with final diagnosis of NSCLC (ADC and SCC) (n=199) formed the cohort for the present study (Table 1). Seventy-nine patients from this cohort died in the three years following their therapeutic surgery (39.7%).

3.2 Local metabolic activity and inflammation in tumor and non-tumor tissue

Local metabolic activity and inflammatory response were measured in both tumor and adjacent non-tumor tissue, to identify specific hypermetabolic and inflammation patterns morphologically related to malignant tissue.

Regarding PPP markers, TKT and TKTL1 levels were measured in ADC samples (n=89), and their values in tumor tissue were expressed as a ratio against the levels in non-tumor tissue. TKT levels were measured in 85 samples from tumor and in 55 samples from non-tumor tissue. The mean expression of TKT in tumor tissue was twice the value obtained in healthy tissue (mean 2.70

[SD 0.88] 95%CI 2.5-2.9, p<0.001). TKTL1 levels were also measured in 88 samples from tumor and in 53 samples from non-tumor tissue, and the mean expression of TKTL1 in tumor tissue was also significantly increased (mean 1.29 [SD 0.29] 95%CI 1.23-1.35, p<0.001). These results confirmed the role of TKT and TKTL1 in early stage LC.

The local inflammatory response was measured in the full population sample of NSCLC. Levels of all biomarkers were significantly higher in malignant tissue and more than 30% above the non-tumor tissue reference values, a finding that confirms the local inflammatory activity in NSCLC tissue (Table 2).

PDL1 analysis in ADC samples (n=89) showed a significantly higher percentage of positivity in tumor samples than in adjacent non-tumor tissue (p<0.001, χ^2 test). PDL1 was found in 11 cases of tumor tissue (1-5%: 1 case; 5-50%: 6 cases and >50%: 4 cases) and in only 1 case in adjacent non-tumor tissue (5-50%). These results confirm the differential expression pattern of this oncogenic driver in early stage lung ADC.

3.3 Relationship between biological and clinical markers

Overall, baseline clinical variables were not associated with metabolic and inflammatory biomarkers and transmembrane proteins in tumor tissue in the cohort. However, higher staging was associated with increased levels of inflammatory marker NFκB-p65 in ADC (p= 0.022, ANOVA).

3.4 Association between biological markers and survival

Regarding baseline clinical characteristics, only pathological staging was related to 3-year mortality (Table 3). PPP biomarkers did not show any association with 3-year mortality after surgical treatment. Similar results were found for inflammatory markers, although a clear trend was found for IL1β, which was higher in the tumor tissue of patients who died in the three years immediately after tumor resection. Among membrane receptors, PDL1 was not related to 3-year mortality (Table 4).

3.5 Survival analysis

Kaplan-Meier analysis showed that higher stage ($p<0.001$) and cardiac comorbidity ($p= 0.033$) were significantly associated with mortality (Figure 1 and 2, Supplementary material). Accordingly, a clinical model for the identification of mortality risk factors was created including all variables that showed an association with the outcome ($p<0.10$) in the survival analyses performed. Multivariate Cox proportional hazards regression confirmed that these two clinical variables showed a statistically significant relationship with the outcome, stage [HR= 1.59, 95% CI (1.32-1.91), $p<0.001$] and cardiac comorbidity [HR= 1.779, 95% CI (1.14-2.77), $p= 0.011$].These variables were included in subsequent survival analyses assessing the prognostic capacity of the molecular biomarkers examined.

The Youden index was used to establish optimal cutoffs of the molecular biomarkers assessed which most accurately identified survivors and non-survivors after three years. Only patients with IL1β levels above 1356 (arbitrary units) showed significantly higher mortality ($p= 0.0074$) (Figure 1). When we

analyzed the effect of IL1β levels in ADC and SCC subtypes separately, the significant differences were found in ADC subtype (p= 0.0062) and not in SCC subtype (p= 0.38) (Figure 2A and 2B).

The multivariate Cox proportional hazards model, after adjustment for staging and cardiac comorbidity, confirmed the prognostic power of high IL1β levels in tumor tissue [HR= 2.05, 95% CI (1.1-3.7), p= 0.019]. When we assessed NSCLC subtypes individually, this prognosis power was statistically significant for ADC [HR= 2.901, 95% CI (1.242-6.776), p= 0.014], but not for SCC [HR= 1.314 (0.956-1.807), p= 0.461].

4. DISCUSSION

The present study confirms the existence of local changes in the tumor and its microenvironment in early stage NSCLC, as shown by the high levels of molecular biomarkers related to local metabolic activity and inflammation in tumor tissue compared to its surrounding lung tissue. Furthermore, this study shows the prognostic capacity of IL1β, a biomarker which is not directly related to the tumor cells but rather to their microenvironment. Higher levels of IL1β in early stage lung cancer patients were associated with lower 3-year-survival, after adjusting for the significant clinical variables. The measurement of this marker in tissue samples of early stage LC patients may favor the identification of those who may benefit from closer follow-up and additional treatments.

In the present study, the PPP proteins TKT and its isoform TKTL1 showed higher levels in tumor tissue than in the surrounding lung tissue of ADC subtype. Activation of oncogenic signaling pathways adapts the tumor cell

metabolism to the dynamic tumor microenvironment (TME), where nutrient and oxygen concentrations are spatially and temporally heterogeneous [11]. Therefore, tumor cells switch their core metabolism to meet the increased requirements of cell growth and division by enhancing key metabolic pathways such as glycolysis and PPP [31]. Cancer cells can accelerate PPP activity by raising the expression of specific enzymes: TKT appears elevated in pancreatic cancer, and TKTL1 is highly expressed in various cancers and has been related to tumor invasiveness, therapeutic resistance, and poor prognosis [10,14,15,32,33]. TKTL1 expression has also been found to be elevated in NSCLC, with ADC being more strongly positive than SCC [34].The capacity to degrade glucose under anaerobic conditions is critical to tumor growth, especially when the cells are carried away from the basement membrane, thus diminishing their oxygen supply. In this situation, TKTL1 overexpression confers an advantage for malignant cells, allowing them to grow faster and metastasize [35,36]. In fact, TKTL1-suppressed cells display significantly decreased growth and proliferation rates [36] and inhibitors of TKT activity or gene expression also suppress tumor growth. Dietary studies have also indicated that the inhibition of the PPP minimizes tumor growth [13,36,37]. Although it has been reported that TKTL1 contributes to total transketolase activity [38], with a similar metabolic role to TKT, this protein also seems to participate in other metabolic processes such as DNA hypomethylation, fatty acid synthesis, and even the one-substrate reaction catalyzing the transformation of xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetate [39,40]. To our knowledge, this is the first time that different patterns of expression in TKT and TKTL1 in NSCLC progression have been shown in the same tissue samples. However, high

levels of these proteins did not show a relationship with 3-year mortality in the present study, a result that does not support the hypothesis of a specific role forTKTL1 with a potential effect in cancer development.

The present study confirms the high inflammatory activity in NSCLC, with levels of all the biomarkers measured being significantly higher in malignant tissue than in non-tumor tissue. Besides, the results obtained show that high IL1β levels are an independent risk factor of poor prognosis in early stage NSCLC, confirming that inflammation plays a key role in its progression. IL1β is a pleiotropic cytokine that promotes tumor proliferation, angiogenesis and metastases [41]. Sustained induction of IL1β enhances the intensity of the inflammatory response and creates an inflammatory microenvironment that is advantageous for tumor initiation and/or progression [42]. Higher levels of IL1β have also been found in other solid tumors such as breast, colon, head and neck and melanomas, and patients with IL1β-producing tumors have worse prognoses [41]. A study of melanoma cells *in vitro* found that IL1β and vascular endothelial growth factor (VEGF) act in a complementary manner in the induction of angiogenesis. In that study, VEGF neutralization resulted in only an initial tumor inhibition, which was followed by tumor recurrence; blocking IL1β was associated with a decreased tumor growth for extended periods of time, a finding that supported a key role for IL1β in tumor progression [43]. An association between COX-2 expression with IL1β–induced angiogenesis and tumor growth *in vitro* and *in vivo* has also been suggested [44]. COX-2 levels were higher in tumor tissue than in non-tumor tissue in our study, although we did not find any clear association between COX-2 levels and 3-year mortality. However, COX-2 and IL1β levels were significantly correlated (data not shown),

suggesting a degree of synergy between these two biomarkers. Similarly, higher levels of TNFα were found in tumor tissue, supporting a role for this inflammatory mediator in the development of LC. TNFα is produced constitutively by many malignant cells and may directly contribute to oncogenic activation, DNA damage and epithelial-to-mesenchymal transition (EMT) [45]. In fact, in another study, high levels of expression of TNFα made a major contribution to an 11-gene signature of poor prognostic significance in stage I lung cancer [46].

The advent of targeted therapies has drawn attention to the predictive value of new molecular biomarkers, which allow the selection of patients who may experience a clinical benefit from adjuvant therapies [47]. Among the immune checkpoint proteins, one of the most studied in NSCLC clinical trials is PD-1 and its two ligands PD-L1 and PD-L2 [48]. PDL1 is present on antigen-presenting cells (APCs), including tumor cells, and interacts with its receptor (PD1) on T cells inhibiting T-cell effector functions [49]. Activation of inhibitory T-cell checkpoint interactions has been demonstrated in NSCLC and suppresses the anti-tumor immune response [48–50]. We did not find an association between PD-L1 and 3-year mortality in our study, but the identification of this ligand in more than 10% of the ADC samples opens up the possibility of adjuvant immunotherapy in early stage NSCLC patients who show an inflammatory response pattern in the tumor microenvironment that puts them at risk of progression.

The present study has some limitations that should be taken into account: NSCLC patients were analyzed as a single group, because statistically significant differences in the biomarkers measured were not found between

ADC and SCC (data not shown). However, we cannot rule out the possibility that differences in some of the biomarkers assessed would be found between these subtypes when assessed in larger cohorts. Besides, this is a retrospective cohort analysis which needs to be validated in larger prospective cohorts to confirm the potential clinical use of the biomarkers shown to be related to prognosis in this study. Future studies should take into account the characteristics of the IHC performed in the present study.

5. CONCLUSION

This study confirms the increased metabolic activity in tumor tissue of early stage NSCLC, which is paralleled by a well-defined inflammatory response in its microenvironment. Furthermore, local inflammation in tumor tissue was associated with poor prognosis, as shown by the relationship between high levels of IL1β in the tumor sample and 3-year mortality. These results were driven mainly for ADC subtype. Accordingly, this inflammation biomarker may contribute to the identification of patients who are at risk of recurrence and may be candidates for adjuvant therapies.

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