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Review

The prognostic impact of tumor-associated macrophages and intra-tumoral apoptosis in non-small cell lung cancer

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Summary. Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung malignancies. Tumor-associated macrophages (TAM) are abundant components of NSCLC. Although under certain conditions TAM can kill tumor cells, they can also act as tumor promoters secreting a variety of factors that directly stimulate tumor invasion and metastasis. TAM presents two distinct phenotypes: the classically activated (or M1) phenotype, which is highly pro-inflammatory (phagocytic and cytotoxic), and the alternatively activated (or M2) phenotype, which has anti-inflammatory and pro-tumoral properties. The polarization status of TAM depends on stimulating factors from the tumor microenvironment, and some *in vitro* evidence implies that the phagocytosis of apoptotic bodies derived from tumoral cells is a key factor in M1/M2 modulation, raising the question of whether the evaluation of the apoptotic index (AI) and macrophage polarization have a prognostic role in NSCLC patient survival. The present article systematically reviewed the published series of clinical data that correlated the AI and/or macrophage densities and polarization status (M1/M2) with the outcome of non-small cell lung cancer patients. Even though an overwhelming body of clinical data support that TAM's density, micro-anatomical localization, phenotype and intra-tumoral AI are independent predictors of survival time, no study to date has been conducted to evaluate the impact of these parameters altogether in NSCLC patient outcome. Joint analysis of these biologic factors in future studies might reveal their prognostic value in the management of NSCLC cases.

Key works: Tumor-associated macrophages, Non-small cell lung cancer, Apoptotic Index, Clinical outcome, Prognosis

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Introduction

Lung cancer is the leading cause of cancer deaths throughout the world and is divided into 2 distinct clinical categories, small cell and non-small cell lung carcinoma (NSCLC). NSCLC account for approximately 80% of all diagnosed lung cancer cases (Kawai et al., 2008; Sulpher et al., 2013). Despite numerous clinical trials of promising drugs, no major breakthrough has been made in NSCLC management in the last decades (Passaro et al., 2012; Tong and Taira, 2012). Reflecting that, the prognosis of NSCLC is still poor, with a 5-year survival probability of 49% for early stages, and less than 1% for advanced stages (Siegel et al., 2012). Unfortunately, since most cases are diagnosed with advanced pathologic (p)-stages of disease, curative pulmonary resection is no longer a therapeutic option and multimodality treatment became the indicative management of disease (Al-Shibli et al., 2009). To improve patients' prognosis, it is important to establish biological markers and processes that determine tumors' aggressiveness and predict response toward a particular therapeutic treatment (Tanaka et al., 1999; Muller et al., 2011). Ongoing studies are searching for NSCLC biomarkers that could provide the potential benefits of

prognosis, and could lead to important applications, such as drug targeting (Castro et al., 2010; Muller et al., 2011). For example, the treatment and diagnosis of NSCLC has been revolutionized by the development of targeted agents (e.g.: the FDA-approved targeted drugs *Erlotinib* and *Gefitinib* for patients harboring specific *EGFR* mutations) (Heist and Engelman, 2012). Following these new advances in therapies, a better understanding of the role of local microenvironment in tumor promotion and progression might be helpful to establish new strategies against NSCLC.

The tumor microenvironment is composed of proliferating neoplastic cells, a vascular network, the extracellular matrix produced by fibroblasts and infiltrated immune cells (Schmieder et al., 2012). Solid tumors are composed by a large mass of immune cells that could reach approximately 60% of total cells, contributing to a unique chronic inflammatory microenvironment that influences both negatively and positively the biological properties of tumor tissue (Fig. 1) (Pollard, 2004; Grivennikov et al., 2010). One of these inflammatory cells presented in high amounts in tumors are the macrophages (MΦ). Tumor-associated macrophages (TAM) have complex functions in their interaction with neoplastic cells because of their capacity to polarize into two different phenotypes (M1 or M2) (Mantovani et al., 2002). The M1 (or classically activated) phenotype of macrophages is thought to be induced *in vitro* by interferon-γ, in combination with lipopolysaccharide (LPS) and/or tumor necrosis factor (TNF)-α. M1 macrophages are associated with the expression of TNF-α, interleukin (IL)-12, IL-1,

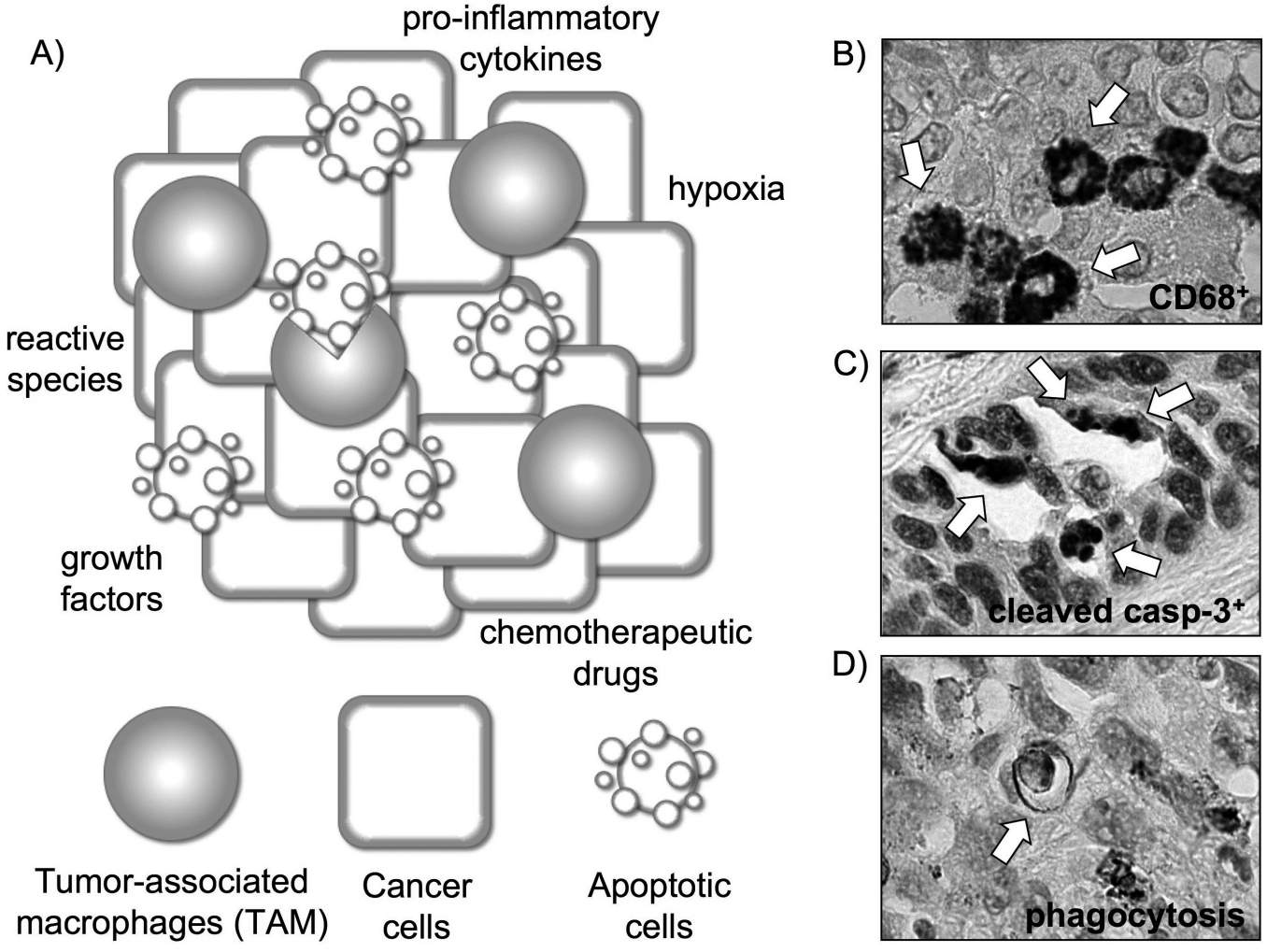

Fig. 1. Interactions between TAMs and viable/apoptotic NSCLC cells in tumor islets **(A).** The abundance of CD68-positive TAM cells (**B**, arrows), intratumoral apoptosis (identified with cleaved/active caspase-3 antibody) (**C**, arrows) and the phagocytosis of apoptotic bodies by CD68-positive TAM cells in tumor islets (**D**, arrow) are shown. Representative images were obtained by immunohistochemistry of formalin-fixed paraffin-embedded NSCLC tissues.

inducible nitric oxide synthase (iNOS), and are responsible for a pro-inflammatory and cytotoxic response against tumoral cells (Mantovani et al., 2004). On the other hand, M2 (also known as alternatively activated) macrophages are known to be modulated by IL-4, IL-13 and IL-10, and are associated with tumor progression by secreting molecules like vascular endothelial growth factor (VEGF) and transforming growth factor (TFG-ß) (Biswas et al., 2008; Ohri et al., 2009; Qian and Pollard, 2010; Ruffell et al., 2012). In addition, M2 macrophages are described to be the predominant phenotype of TAM in solid tumors (Anderson and Mosser, 2002).

Even though the role of different macrophage phenotypes in tumor progression has been extensively reviewed (Anderson and Mosser, 2002; Gordon, 2003; Sica et al., 2006; Biswas et al., 2008; Mosser and Edwards, 2008; Gordon and Martinez, 2010) and most studies suggest that TAM are associated with poor clinical prognosis, some contradictory data can be found in the literature even for the same histological type of tumors. Specifically, in lung cancer, studies demonstrated a positive, negative and inconclusive prognostic significance of TAM densities (Zhang et al., 2012). Therefore, the prognostic value of TAM for patients with lung tumors remains controversial. Indeed, several works support a dual role for macrophages in the regulation of tumor proliferation and immune control, which indicates that more studies are necessary to address the role that the local tissue microenvironment plays in determining the macrophage phenotype (Kataki et al., 2002).

Multiple characteristics of solid tumors, including hypoxia and abundant cell death, such as apoptosis or necrosis, influence macrophage functions (Fig. 1) (Ruffell et al., 2012). Immune response and chemotherapeutic agents used regularly in NSCLC management, such as cisplatin and carboplatin, induce cell death in different ways. In immune response, production of TNF-α and other pro-inflammatory cytokines induce cell death by stimulation of death receptors in the tumoral cell membrane (involved in the so-called extrinsic apoptotic pathway), and nitric oxide ($NO^{\bullet}$) and other reactive species (RS) induce programmed cell death by increasing the oxidative stress inside the cell (Weigert and Brüne, 2008). On the other hand, cisplatin and carboplatin induce DNA damage, leading the target cells to commit apoptosis by activating the mitochondrial (intrinsic) pathway (Siddik, 2003). In addition, hypoxic cores inside the tumor also elicit tumor destructive reactions, leading mainly to a necrotic type of cell death.

Typically, cells dying by apoptosis are thought to be promptly phagocytized by mechanisms that fail to incite pro-inflammatory or immune reaction (Fig. 1) (Gregory and Devitt, 2004). Indeed, signaling molecules and membrane receptor in apoptotic cells (e.g.: externalized phospholipid phosphatidylserine) are recognized by macrophages, generating a cascade of cell signals leading to the phagocytosis of apoptotic bodies. This process is believed to be responsible for a M1-to-M2 shift in macrophage phenotype, which will then secrete anti-inflammatory mediators, most notably TGF-ß but also IL-10, prostaglandin E2 (PGE2), lactoferin and VEGF (Gregory and Pound, 2011). Thus, many lines of *in vitro* data have suggested that intratumoral phagocytosis of apoptotic cells by macrophages could be to a large extent responsible for the modulation of immune response, especially the M2 polarization of TAM (Reiter et al., 1999; Weigert et al., 2007; Weigert and Brüne, 2008) . This critical event has been associated with dampening of the immune responses, leading to tumor promotion, progression, and metastasis (Sica et al., 2008). So, the macrophage polarization to the M2 phenotype appears to be a key event in tumoral progression, and it seems that phagocytosis of apoptotic cells is a very important element of this polarization.

For these reasons, the apoptotic index (AI) and TAM polarization in solid tumors could have an intrinsic relationship with tumor progression, influencing patient outcome and overall survival (Törmänen et al., 1995; Tanaka et al., 1999; Kim et al., 2008; Ohri et al., 2009; Dworakowska et al., 2009). Therefore, the major goal of this work is to review and compile the available clinical data in the literature that correlates the apoptotic index and/or macrophage densities and the polarization status (M1 or M2) with the outcome of non-small cell lung cancer patients. We aimed to evaluate the clinical relevance of these important biological processes, highlighting the clinical use of these parameters for future improvement in the management of NSCLC patients.

Macrophage polarization in non-small cell lung cancer patient prognosis: a clinical update

Many studies have been conducted trying to elucidate the role of macrophages in tumor growth and their prognostic value (Leek et al., 1996; Shimura et al., 2000). One recent study concluded that high TAM density seems to be associated with a worse overall survival in patients with gastric, urogenital and head and neck cancers, and with better overall survival in patients with colorectal cancer (Zhang et al., 2012). In lung cancer, their analysis showed conflicting results about the prognostic significance of counting TAM in tumor tissues (Zhang et al., 2012). Thus, the prognostic value of TAM quantification for patients with lung tumors remains controversial.

For this reasons, we reviewed the medical literature searching for prospective/retrospective clinical studies that evaluated not just the impact of macrophage densities (cell number), but also their stromal and/or parenchymal (tumor islets) micro-anatomical localization and polarization (M1/M2) status, with the NSCLC patient survival rate (overall survival). The characteristics of these studies, performed in formalin-fixed paraffin-embedded tissue, are summarized in Table

1 and discussed below.

Macrophage densities

Trying to elucidate the importance of macrophage density on patient survival/outcome, Arenberg and collaborators (Arenberg et al., 2000), using immunohistochemistry (IHC) detection of HAM-56+ cells as macrophage marker, performed a prospective study with 15 consecutive patients who had undergone thoracotomy for suspected primary bronchogenic carcinoma. The authors followed the patients for an average of 76 months and found that those patients who died (n=7) had significantly higher numbers of macrophages than did those who remained free of recurrence (n=8) (92.3±19.8 vs. 49.2±6.6 macrophages/x400 magnification field, respectively) (P<0.05). Corroborating these findings, Chen and collaborators (Chen et al., 2003) demonstrated that the median survival for patients with high density of TAM (≥162 macrophages/x200 magnification field, 16 months, n=18) was significantly shorter when compared to patients with low density (<162 macrophages/X200 magnification field, 45 months, n=17) (log-rank test, P=0.025). Their study was performed in a retrospective cohort of 35 patients who had undergone curative resection of early p-stage (I, II or IIIa) of NSCLC cases, using IHC of CD68+ cells as macrophage marker. The same group, in a follow-up study (Chen et al., 2005)

Table 1. Characteristics of the eligible studies that evaluated the clinical significance of macrophage (MΦ) densities, micro-anatomical localization and M1/M2 phenotypes as prognostic biomarkers in NSCLC.

Description	Population	Size	Diagnostic technique	Analytic result	P	Reference
Prospective association of MΦ density with patient outcome	Patients undergoing thoracotomy	15	IHC of HAM56$^+$	High MΦ density is negatively correlated with survival	<0.05	Arenberg et al., 2000
Retrospective association of MΦ density with patient outcome with 3 year follow-up	Patients with primary NSCLC who underwent surgery	117	IHC of CD68$^+$	No correlation on 3-years survival	NS	Toomey et al., 2003
Retrospective association of MΦ density with patient outcome	Surgically resected (Stage I-IIIa)	35	IHC of CD68$^+$	High MΦ density is negatively correlated with probability of survival	=0.024	Chen et al., 2003
Retrospective association of MΦ densities with 5-years patient survival (stromal vs. islets)	Stage I-IV	175	IHC of CD68$^+$	High MΦ in tumor islet and stromal are a predictor of survival	<0.001	Welsh et al., 2005
Retrospective association of MΦ density with patient outcome	Stage I-IIIb	41	IHC of CD68$^+$	MΦ negatively correlated with survival	<0.05	Chen et al., 2005
Retrospective association of MΦ with the clinical outcome (stromal vs. islets)	Stage IV	199	IHC of CD68$^+$	High MΦ in islets is a predictor of survival	<0.001	Kawai et al., 2008
Retrospective association of MΦ density with patient survival (stromal vs. islets)	(Stage I-IV)	144	IHC of CD68$^+$	High MΦ density in islets is a predictor of survival	<0.001	Kim et al., 2008
Retrospective association of MΦ (M1 or M2) densities in islet or stroma with patient survival	Poor (7-months) vs. extended (92-months) survival (Stage I-IV)	40	IHC of CD68$^+$/HLA-DR$^+$, iNOS$^+$, MRP8/14$^+$, TNF-α^+ for M1 and CD68$^+$/CD163$^+$ for M2	High MΦ (M1) density in islets is a predictor of survival	<0.001	Ohri et al., 2009
Retrospective association of MΦ density in islet or stroma with patient survival	Surgically resected (Stage I-IIIa)	335	IHC of CD68$^+$	No correlation on 16-years patients' survival	NS	Al-Shibli et al., 2009
Retrospective association of MΦ density in islet or stroma with patient survival	(Stage I-IV)	99	IHC of CD68$^+$	High MΦ density in islets and low in stroma is a predictor of patients' survival	<0.001	Dai et al., 2010
Retrospective association of MΦ (M1 or M2) densities in islet or stroma with patient survival	Short (1-year) vs. long (5-year) survival (Stage I-IV)	100	IHC of CD68$^+$/HLA-DR$^+$ (M1) and CD68$^+$/CD163$^+$ (M2)	High MΦ M1 density in islets is a predictor of survival	<0.001	Ma et al., 2010
Retrospective association of M2 phenotype and patient outcome	Stage Iab-IIIa	170	IHC of CD68$^+$/CD204$^+$ for M2	High MΦ M2 density phenotype is a predictor of survival	=0.007	Ohtaki et al., 2010
Retrospective association of M1/M2 phenotypes and patient outcome	Patients undergoing lobectomy or pneumonectomy	65	IMF of CD68$^+$/iNOS$^+$ for M1 and CD68$^+$/CDMRR$^+$ for M2	High TAMs and M2 density phenotype is a predictor of survival	<0.001	Zhang et al., 2011
Prospective association of abundance and distribution of immune cells with patient outcome	Surgically resected ($T_{1\text{-}3}N_{0\text{-}2}M_0$)	65	IHC of CD68$^+$, CD3$^+$, CD4$^+$, CD8$^+$, CD20$^+$, S100$^+$, CD1a$^+$	High MΦ density is a predictor of survival	=0.03	Da Costa Souza et al., 2012

MΦ, macrophages; M1, classically-activated macrophages; M2, alternatively-activated macrophages; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; IMF, immunofluorescence; NS, no significant.

confirmed that the median relapse-free survival for patients with high density of TAMs (≥163 macrophages/x200 magnification field, 7 months, n=21) was also significantly shorter than for patients with a low density of TAMs (<163 macrophages/x200 magnification field, 26 months, n=20) (log-rank test, P=0.018). Despite the small size of the cohorts (the median sample size was 31 patients), all these studies found a significantly shorter relapse-free survival of patients with high TAM density.

In contrast to these findings, the recent study of da Costa Souza (2012) showed that the 5-year survival rate in patients with high macrophage density is correlated with high probability of survival (median of 4.5% of cells as cut-off point, estimated median survival of 76 vs. 30 months for the high-risk group) (log-rank test, P=0.02). They performed a prospective study with 65 patients using IHC of CD68+ cells. Moreover, Toomey and collaborators (Toomey et al., 2002) used a retrospective cohort of 117 patients who had undergone curative surgery and showed that the density of CD68+ cells was not associated with a 3-year survival time (Cox-regression analysis, P=0.24). They found an average of 10-50 macrophages/x400 magnification field (of around 500 tumor cells in total). These studies reinforced previous inconsistent findings (Zhang et al., 2012), and suggested that other biological/clinical variables should be included to strengthen the prognostic role of TAM in NSCLC.

Stromal vs. tumor islets macrophages' densities

One important biological feature that should be considered is the micro-anatomical localization of macrophages. Solid tumors are known to have distinct but interdependent compartments: the parenchyma of neoplastic cells (tumor islets, or nests) and the stroma that, in many tumors, is separated by a basal lamina (Tlsty and Coussens, 2006). The stroma comprises nonmalignant supporting tissue, including connective tissue, blood vessels, and inflammatory cells. Solid tumors require the stroma to grow beyond a minimal size of 1 to 2 mm, since it provides the vascular supply that tumors require for obtaining nutrients, gas exchange and waste disposal. This compartment may also limit the influx of inflammatory cells or may limit the egress of tumor cells (invasion) (Elgert et al., 1998; Welsh et al., 2005). Hence, the evaluation of TAM distribution between compartments could have important prognostic implications.

Embracing this idea, Welsh and collaborators (Welsh et al., 2005) evaluated in a retrospective cohort of 175 patients with NSCLC who had undergone resection with curative intent (p-stages I, II and IIIa), the parenchymal (tumor islets) and stromal densities of macrophages (Table 1). Stromal $CD68^+$ macrophages presented a median of 174 cells/mm^2 (ranging from 5 to 3,310) and tumor islets a median of 131 cells/mm^2 (ranging from 1 to 891). They also determined the islet/stromal ratio of MΦ number in tumor samples. Kaplan-Meier survival curve and Cox-regression analysis showed that high macrophage numbers in islets (>174 cells/mm^2, 2,244 days vs. <174 cells/mm^2, 334 days, P<0.001) and high islet/stroma ratio (2,147 vs. 312 days, P<0.001) emerged as independent favorable prognostic indicators, whereas, in IIIa p-stage, high macrophage density in stroma reflected a poor prognosis (224 vs. 936 days, P=0.022). In accordance with these results, Kim and collaborators, using a retrospective study of NSCLC 144 patients who had undergone curative intended surgery, found that patients with high tumor islet macrophage density survived longer compared to patients with low tumor islet macrophage density (P=0.0002), concluding that a high number of tumor islet macrophages was an independent favorable prognostic factor for patients with resected NSCLC (Kim et al., 2008). The same results were obtained in a retrospective study performed by Dai and collaborators, with IHC detection of CD68+ cells in 99 NSCLC patients with different p-stages (I-IV). They confirmed the favorable prognosis represented by high islet/stromal macrophage ratio and the unfavorable prognosis of patient with a high density of macrophages in the stroma (Dai et al., 2010). Furthermore, the study of Kawai and collaborators performed with a retrospective cohort of 199 NSCLC patients in advanced stage IV of disease, also showed that a high islets/stromal macrophage ratio was associated with better prognosis (Kawai et al., 2008). They also found that high stromal macrophage density was associated with poor prognosis. In contrast to these previous studies, Al-Shibli, using IHC of CD68+ in a retrospective cohort of 335 patients, showed that macrophage density/micro-anatomical localization did not have any statistical correlations with patient disease specific survival (Al-Shibli et al., 2009). This was the first work that showed no relationship between macrophage density/micro-anatomical localization and patient outcome/survival in NSCLC.

The studies addressed until now have some contradictory results about macrophage micro-anatomical localization and NSCLC patient outcome/survival. Nevertheless, they overall suggest that high macrophage densities in islets and stroma could be a prognostic marker in NSCLC. Therefore, the micro-anatomical localization analysis of TAMs could be a new approach in clinical predicting studies.

Macrophage phenotypes

The previous works mentioned showed that TAMs have an intrinsic relationship with NSCLC patient overall survival time in according with their density *per se* or their micro-anatomical localization. Beyond these features, macrophage polarization is suggested as a key factor in tumor progression and metastasis (Mantovani et al., 2002; Lewis and Pollard, 2006; Sica et al., 2006; Allavena et al., 2008; Qian and Pollard, 2010). It is well known that macrophages can polarize to a M1 (classic)

or M2 (alternative) phenotype. Then, the puzzle of how macrophage density in different micro-anatomical localization could be associated with better or worse prognosis in NSCLC may be solved by the investigation of the M1/M2 polarization status of TAMs.

In 2009, Ohri and collaborators, based on the marked survival advantage of NSCLC patients presenting a high number of macrophages in their tumor islets, identified CD68+ macrophages expressing M1 (iNOS, MRP8/14, TNF-α, and HLA-DR) or M2 (CD163 and VEGF) double-staining markers in islets and stroma of surgically resected tumors from 20 patients with extended survival (ES) (median 92.7 months) and 20 with poor survival (PS) (median 7.7 months) (Ohri et al., 2009). Their study also confirmed that a high macrophage count in tumor islets was correlated with a better overall survival (median of 33.95 cells/mm^2 in ES group vs. 4.02 cells/mm^2 in PS group, P<0.001) but, more than that, the data demonstrated the relevance of macrophage phenotype in NSCLC patients' overall survival. Interestingly, more than 70% of islet TAMs were positive for M1 markers in the ES group. They concluded that the survival advantage conferred by macrophages infiltrated in tumor islets was related to their cytotoxic (M1) antitumor activity. Moreover, Ma and collaborators conducted a study to determine whether the micro-localization of M1/M2 macrophages densities are associated with NSCLC patient overall survival time (Ma et al., 2010). Their retrospective cohort was stratified into patients with an average of 1-year survival (short survival group) (n=50) and patients with an average of 5-year survival (long survival group) (n=50), and tumor biopsies were double-stained IHC for M1 (CD68+/HLA-DR+ cells) and M2 (CD68+/CD163+ cells). Even though the overall analysis of Ma's study indicates that approximately 70% of TAMs are M2 macrophages, the long survival group also presented a significantly higher M1 macrophage count in the NSCLC tumor islets and stroma as compared to short survival group (approximately 70 cells/mm^2 vs. 17 cells/mm^2, P<0.001), thus establishing it as an independent predictor of patient survival time. More interestingly, the M1/M2 macrophage ratio in the tumor islets and in stroma from long survivors was 9- and 2-fold higher when compared to short survival patients, respectively. In this context, evaluating specifically the prognostic role of stromal M2 macrophage density, Ohtaki and collaborators found a significant association between the number of CD204+ macrophages and poor outcome (Ohtaki et al., 2010). Their study was based on recent evidence that demonstrated high expression of CD204 in M2 macrophages (Komohara et al., 2008; Kawamura et al., 2009; Ohtaki et al., 2010). They used a retrospective cohort of 170 patients with lung adenocarcinoma who had undergone surgery with curative intent (I-IIIa stages). Moreover, they also evaluated the expression level of several cytokines and found an association between IL-10 and monocyte chemoattractant protein-1 (MCP-1/CCL2), both molecules involved with the tumor-promoting phenotype of TAMs, with the number of CD204+ cells. The authors suggested that MCP-1 and IL-10 derived from tumor cells or stromal cells induce differentiation, accumulation, and migration of M2 macrophages into lung cancer tissue, outlining the macrophages that promote tumor progression (Ohtaki et al., 2010).

With similar intent, Zhang and collaborators (2011) used immunofluorescence staining determination of CD68+ cells, macrophage mannose receptor-positive (MRR+, as a specific M2 marker) and inducible nitric oxide synthase-positive (iNOS+, as a specific M1 marker) cells in tumor biopsies from a retrospective cohort of 65 patients with lung adenocarcinoma who had undergone either lobectomy or pneumoctomy. Kaplan-Meier curves showed that high TAM counts (>102 vs. ≤102 cells per high magnification field, P<0.001) and high M2 phenotype (>82 vs. ≤82 cells per high magnification field, P<0.001) were associated with poor survival. In the lung adeno-carcinomas sample, they showed that an overall percentage of 79% (±16.27%) were of M2 TAMs (representing a hazard ratio of 4.28, P=0.038). Moreover, total macrophage count and M2 polarized TAM numbers were significantly associated with p-TNM staging: high number and M2 TAMs were associated with advanced stages (III and IV) when compared with initial stages (I and II). Furthermore, they analyzed the cytokine profile of the lung adenocarcinoma compared to a benign lung lesion and found that lung adenocarcinoma had a high expression of IL-4 and IL-10 (anti-inflammatory cytokines), and low expression of interferon-gamma and IL-12 (pro-inflammatory cytokines), suggesting that during tumor progression the M1 macrophages could be shifting to an alternative phenotype in response to micro-environmental changes.

Taking into account all the clinical-pathological information available in the abovementioned studies (Table 1), it became clear that tumor-associated macrophages have pro-tumorigenic as well as anti-tumorigenic contributions in lung tumors. Furthermore, our meta-data analysis suggests that the joint analysis, including the discrimination between macrophage count in tumor islets and/or stroma, in combination with the M1/M2 polarization status, is a more useful clinical information for the oncologist and an important step towards predicting NSCLC patient outcome. Differences in these biological parameters could be responsible for the conflicting results described in previous reports. Altogether, these data suggest that TAM influence on patients' prognosis depends on the balance between M1/M2 phenotypes in tumor islets (e.g.: high M1 density in tumor islets in early clinical stages I-IIIa represents favorable prognosis, in spite of high M2 density in tumor islets in advanced stages IIIb-IV, which reflects an unfavorable outcome) (Table 1), implying that several components and factors of the micro-environment of the tumor tissue (cytokines, growth factors, pO_2, oxidants levels, chemotherapeutic agents,

intracellular components and/or apoptotic bodies) could be key players in this modulation. Therefore, due to their intrinsic relationship with NSCLC progression and patient outcome/survival, the evaluation of TAMs has the potential to be incorporated in clinics as a prognostic biomarker in non-small cell lung cancer cases. At this point, we are going to explore the clinical available data addressing the role of intra-tumoral apoptosis (as percentages of apoptotic cells and bodies among tumor cells) in TAM polarization and/or in the prediction of NSCLC patient survival time.

Apoptosis in the tumor milieu: A major factor for TAM polarization and unfavorable outcome in NSCLC?

In the human lung, due to the intense cell death caused by acute injury (such as cigarette smoking), or within the pro-inflammatory micro-environment where solid lung tumors have developed (with chemotherapeutic agents and hypoxic cores), the initial responses to unwanted/damaged cells involves the recruitment of professional phagocytes such as macrophage cells (Savill, 1997; Serhan and Savill, 2005). Macrophages are pivotal players in the removal of dead cells (Savill, 1997; Serhan and Savill, 2005), preventing the secondary inflammatory responses mediated by the release of intracellular components (Hodge et al., 2002). The histologic determination of the amount of apoptosis in tumor islets has been established as a key event in the balance between cell proliferation/cell death processes during tumor progression (Gonzalez et al., 2001; Mantovani et al., 2002; Siddik, 2003), being useful information for predicting response to chemotherapy (Lankelma et al., 1999; Moertel et al., 1992). More importantly, as explored in the introductory section, several in vitro experiments suggest that secreted compounds or integral receptors and molecules in the surface of apoptotic cells can trigger the modulation from anti- to a pro-tumoral phenotype of innate immune cells (Gregory and Devitt, 2004; Weigert et al., 2007; Biswas et al., 2008; Gregory and Pound, 2011). Moreover, the molecular pathways of apoptosis are controlled by genes that either promote (e.g.: Bax, Fas, Bid) or inhibit (e.g.: Survivin, Bcl-xL, Bcl-2) the activation of the caspase cascade, whose levels have already been investigated and reported to be correlated with better or worse patient outcome (Estrov et al., 1998; Volm and Koomägi, 2000; Singhal et al., 2005). Despite all these implications of apoptosis and cancer described above, the significance of apoptosis in NSCLC as a biologic marker, especially as a prognostic factor, remains controversial. In this section, we search the literature focusing specifically on the available medical data that has evaluated the role of apoptotic index (percentage of apoptotic cells or apoptotic bodies per 100 viable tumor cells) in patient outcome (Table 2). We did not aim to evaluate the prognostic role of pro- or anti-apoptotic genes/protein levels in NSCLC tumor tissues, since this biological information could mainly reflect the resistance/sensitivity of tumor cells to undergo apoptosis.

Trying to associate whether apoptosis has a relationship with patient outcome in non-small cell lung

Table 2. Characteristics of the eligible studies that evaluated the clinical significance of the Apoptotic Index (AI) as a prognostic biomarker in NSCLC.

Description	Population	Size	Diagnostic technique	Analytic result	P	Reference
Retrospective association of basal AI (≤1.5% vs. >1.5%) with patient outcome	Surgically resected (Stage I-IIIa)	75	TUNEL$^+$ cells	High apoptotic index correlated with shortened survival	<0.01	Törmänen et al., 1995
Retrospective association of AI and patient survival	Surgically resected (Stage I-III)	178	TUNEL$^+$ cells	No correlation with patient survival	NS	Stammler and Volm, 1996
Retrospective association of basal AI with patient outcome (<1.0 vs. >1.0% of median)	Surgically resected (Stage I-IIIa)	173	Morphological analysis of H&E slides	No correlation on 5-years overall survival	NS	Komaki et al., 1996
Retrospective association of AI ranges (<0.5%; 0.5-1.1%; 1.1-2.5%; ≥2.5%) with patient outcome	Surgically resected (Stage I-IIIa)	236	TUNEL$^+$ cells with H&E confirmation	High apoptotic index is a predictor of survival	=0.003	Tanaka et al., 1999
Retrospective association of AI in squamous cell carcinoma with patient survival	Patients with squamous cell carcinoma	134	Morphological analysis of H&E or anti-ASP	High apoptotic index correlated with shortened survival	=0.036	Gosh et al., 2001
Retrospective association of basal AI with patient outcome (<1.4 vs. ≥1.4%)	Surgically resected (Stage I-IIIa)	50	TUNEL$^+$ cells with H&E confirmation	High apoptotic index correlated with shortened survival	=0.03	Dworakowska et al., 2005
Retrospective association of basal AI with patient outcome (median of 0.8% vs. 25th and 75th percentile)	Surgically resected (Stage I-IV)	170	TUNEL$^+$ cells with H&E confirmation	No correlation on 5-years survival	NS	Dworakowska et al., 2009

AI, apoptosis index; NSCLC, non-small cell lung cancer; H&E, Hematoxylin and eosin-stain; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling assay; anti-ASP, antibody against apoptosis-specific protein; SQC, squamous cell carcinoma; AdC, adenocarcinoma; LCC, large cell carcinoma; NS, no significance.

carcinomas, Törmänen and collaborators evaluated, in a retrospective cohort of 75 patients, the apoptotic index (AI) using terminal deoxynucleotidyl transferase dUTP nick end labeling assay-positive (TUNEL+) cells (Törmänen et al., 1995). Their data showed that patients with high AI had a shortened survival as compared to patients with low AI (>1.5% cells vs. ≤1.5% cells; log rank test of P<0.01). Furthermore, in a multivariate analysis, enhanced apoptosis showed a 1.9-fold risk for shortened survival (95% CI, 1.04-3.60; P<0.05). Interestingly, the percentage of apoptotic cells in lung carcinoma was significantly higher in poorly differentiated (grade III) carcinomas than in low-grade carcinomas (grades I-II) (P<0.02), as described for others types of tumors (Hirvikoski et al., 1999; Yamasaki et al., 1997; Lipponen et al., 1994), although no association with the p-TNM status was observed. In contrast, the study from Stammler and Volm found no relationship between apoptotic index (according with a median value) and patient survival in NSCLC (P=0.22) (Stammler and Volm, 1996). Their study was performed with a retrospective cohort of 176 patients, using TUNEL+ cells to evaluate apoptotic index (AI=apoptotic cells/viable tumor cells x 100). The apoptotic index was in the range of 0.0 to 1.7% for all cases (mean value ± standard deviation: 0.37±0.29; median: 0.25) (Stammler and Volm, 1996).

Komaki et al. (1996), in a retrospective cohort of 173 patients, evaluated the apoptotic index (AI ranged from 0.2% to 2.8%, with a median of 1.0%), using haematoxylin and eosin (H&E) staining to score apoptotic cells as having characteristic heavily condensed aberrant nuclei with homogeneous dark basophilia. Even though the overall 5-year survival was not statistically different between patients in regard to high or low apoptotic index (Table 2), a high level of apoptosis was associated with the worst survival of patients with adenocarcinomas (AdC) and large cell carcinomas (LCC) (P<0.001). Additionally, patients with high apoptosis showed significantly better 5-year overall (P=0.008) survival in the squamous cell carcinomas (SQC) group. Multivariate analysis showed that apoptosis was a significant predictor of 5-year distant metastasis (P=0.01). Based on these result, the authors suggested that this observation may have an effect on the treatment selection for the subset of Nl NSCLC patients.

In the article of Tanaka and collaborators, detailed examination of AI in lung cancer tissue identified two borderline values that determined postoperative prognosis, establishing the biologic and clinical significance of AI in NSCLC (Tanaka et al., 1999). In a retrospective cohort of 236 consecutive patients with pathologic (p)-stage I to IIIa NSCLC, who underwent complete tumor resection and mediastinal lymph node dissection without any preoperative therapy, AI was evaluated by TUNEL+ cells (with haematoxylin counterstaining) and expressed as number of apoptotic cells/1000 cells. The mean AI found for all 236 patients studied (adjusted for % of viable tumor cells) was 1.88±0.14%, with a median of 1.1%. No significant correlation was observed between AI and sex, performance status (PS), histologic type, or p-stage. All patients were divided into four AI groups using the 25th, 50th, and 75th percentile values (0.5, 1.1, and 2.5%, respectively) (AI<0.5%, 0.5≤AI<1.1%, 1.1≤AI<2.5%, AI≥2.5%), and the 5-year survival rate was 74.7%, 51.6%, 57.8% and 83.2%, respectively. Their findings demonstrated that the prognosis of these moderate AI groups (0.5≤AI<1.1%, 1.1≤AI<2.5%) was significantly worse compared with low AI group (<0.5%). Interestingly, patients with the highest AI (≥2.5%) had the most favorable prognosis. AI proved to be an independent prognostic factor in NSCLC.

Ghosh et al. (2001), in a retrospective cohort of 134 patients with squamous cell carcinoma, used haematoxylin/eosin staining and apoptosis specific protein (ASP) immune quantification to evaluate apoptotic index (AI=number of apoptotic cells/10.000 malignant cells). The value of the AIs obtained by H&E staining for all 134 cases (adjusted for % of viable tumor cells) ranged from 0.024 to 1.455%, with a mean of 0.302 (SD, 0.24; median, 0.22). In all cases the count obtained by anti-ASP staining was higher than that obtained by H&E staining, but the two sets of AIs correlated very strongly (R^2=0.9839; P<0.001). Patients were grouped into high and low apoptosis groups based on the AI of the tumor (>0.5% was regarded as high). Overall analysis of their series of SCCs of the lung showed patients whose tumors had a low AI surviving longer. The mean survival of patients was 109 and 172 weeks, respectively (P=0.036). They concluded that AI, as measured by histological techniques, could be used as a prognostic guide in squamous lung cancer cases. In addition, Dworakowska et al. (2005), in a pilot study performed with a retrospective cohort of 50 patients, also assessed the prognostic relevance of apoptotic index (using TUNEL-stained cells with serial H&E counterstained sections) in non-small cell lung cancer patients. The mean and median AI (adjusted for % of viable tumor cells) calculated for all 50 patients was 1.4% and 0.9%, respectively. Median survival for patients with lower (<1.4%) and higher (≥1.4%) AI was 43 months and 22 months, respectively, with a 5-year survival probability of 60 and 25%, respectively (P=0.03). Multivariate analysis showed that the only variable associated with shortened overall and disease-free survival was AI (P=0.03, HR=2.9, 95% CI 1.95-3.86), concluding that AI is a major influence in NSCLC survival. The most important finding of Dworakowskas' study is the negative prognostic impact of high AI in NSCLC patients.

Finally, attempting to verify the robustness of their previous findings, Dworakoska and collaborators (Dworakowska et al., 2009) re-evaluated the prognostic role of AI in a larger group of 170 NSCLC cases. The apoptotic index (AI) grading was expressed as the number of TUNEL+ cells (with haematoxylin/eosin counterstain), comparing the 25th, 50th and 75th

percentile groups (mean AI in 168 positive cases was 1.2±1.0 SD, with median, 25th and 75th percentile of 0.8, 0.5 and 1.4%, respectively) (AI<0.5%, 0.5≤AI<0.8%, 0.8≤AI<1.4%, AI≥1.4%) (values adjusted for % of viable tumor cells). Different from what was obtained in their pilot study, no association between the apoptotic index and patient 5-year/overall survival in NSCLC was found.

So far, based on the presence of positive (Tanaka et al., 1999), negative (Törmänen et al., 1995; Tanaka et al., 1999; Ghosh et al., 2001; Dworakowska et al., 2005) and non-significant (Komaki et al., 1996; Stammler and Volm, 1996; Dworakowska et al., 2009) studies addressing the prognostic significance of apoptotic index in non-small cell lung cancer, the clinical association of the apoptotic index with patient outcome/survival in NSCLC is still controversial. It is important to emphasize that apoptosis appears to be a rare event in routine histological tissue sections because the prompt phagocytic clearance mechanism effectively narrows the window of observational opportunity (Kerr et al., 1972). However, there is a strong association between high apoptotic index (AI values greater that 1.16±0.6%) and a lower overall survival. Quantitative aggregation of the survival results demonstrated that the mean overall survival of patients with low AI compared to high AI was 35.09±10.0 vs. 23.01±6.3 months (P<0.05) and a 5-year overall survival probability of 49.75% vs. 31.02% (P=0.027), respectively. Median sample size for the abovementioned studies (n=7) was 144±64 patients (range=50-236). These studies reinforce that joint analysis of several factors, such as proliferative index, metastasis, or TAM densities in tumor islets, and AI, might provide additional prognostic information in NSCLC patients. Therefore, the apoptotic index has potential to be a biomarker in NSCLC.

Conclusion

Much effort has been made to try to find and establish useful biomarkers in lung cancer that could guide physicians to decide the best treatment for patients. Taking into account all potential bias that exists between different studies evaluated here, our meta-data analysis showed that macrophage density, their micro-anatomical localization and phenotype, and the apoptotic index are all effective biological parameters to predict patient survival, independently. However, no study has addressed the prognostic role of these biological variables in combination. Several authors (Mantovani et al., 2002, 2004; Biswas et al., 2008; Qian and Pollard, 2010; Ruffell et al., 2012) already showed the intrinsic relationship between macrophages and tumor development and progression, probably because of their capability to shift from a pro-inflammatory and anti-tumoral (M1) to anti-inflammatory and pro-tumoral (M2) phenotype. An interesting observation is that TAMs in tumor islets seem to change their polarization status from M1 to M2 during tumor promotion (early to advanced p-stages). The histologic determination of the amount of apoptosis in tumor islets has already been established as a useful clinical parameter correlated with tumor cell proliferation (Siddik, 2003; Gonzalez et al., 2001; Mantovani et al., 2002), and to predict the response to chemotherapeutic regimen (Moertel et al., 1992; Lankelma et al., 1999). Since the process of phagocytosis of apoptotic bodies derived from dying tumor cells is a key factor in shifting macrophages' phenotype in the tumor milieu, future studies should be designed to address the prognostic impact of TAMs (densities, micro-anatomical localization and phenotype) in combination with the intra-tumoral apoptosis in non-small cell lung cancer.

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