Prognostic Impact of the Number of Peritumoral Alveolar Macrophages in Patients With Stage I Lung Adenocarcinoma

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Abstract

Purpose:

Intratumoral macrophages are reportedly involved in tumor progression in non-small cell lung cancer; however, little is known about the prognostic impact and function of alveolar macrophages (AMs). This study aims to investigate the prognostic impact of the number of peritumoral AMs in patients with stage I lung adenocarcinoma.

Methods:

We investigated 514 patients with pathological stage I lung adenocarcinoma who underwent complete resection with lobectomy or pneumonectomy. The number of peritumoral AMs were counted, and patients were classified into two groups based on the number of peritumoral AMs. Using the Cancer Genome Atlas (TCGA) database of stage I lung adenocarcinoma, we compared gene expression profiles of high and low peritumoral AM contents.

Results:

The median number of peritumoral AMs per alveolar space was 15.5. Patients with a high peritumoral AM content had significantly shorter disease-free survival and overall survival than patients with a low peritumoral AM content (both $p < 0.01$). In the multivariate analyses, a higher number of peritumoral AMs was an independent prognostic factor ($p = 0.02$). The analysis of TCGA database revealed that patients with a high peritumoral AM content had shorter disease-free survival than those with a low peritumoral AM content ($p = 0.04$). Gene expression analysis of TCGA stage I lung adenocarcinoma revealed enrichment of biological processes, such as chemotaxis and epithelial proliferation, in patients with a high peritumoral AM content.

Conclusion:

The number of peritumoral AMs had a strong impact on disease-free survival in patients with stage I lung adenocarcinoma.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (Siegal et al. 2020). Non-small lung cancer (NSCLC) accounts for approximately 80 % of all lung malignancies (Lewis et al. 2014), and adenocarcinomas are the most common type of NSCLC. Early-stage NSCLC patients are generally treated with complete surgical resection of the tumor. However, even after the complete resection of stage I NSCLC patients, the 5-year survival rate was 81–92% (Rami-Porta et al. 2014). Therefore, it is important to identify risk factors for recurrences that are different from conventional viewpoints, such as invasive tumor size, lymphovascular invasion, and pleural invasion.

In addition to tumor cells, tumor tissues are composed of lymphocytes, monocytes, macrophages, and fibroblasts, establishing a complex tumor microenvironment (TME) (Comit et al. 2020; Kalluri. 2016; Shinghal et al. 2019). Not only tumor cells but also non-tumor cells influence tumor progression (Ishii et al. 2016; Hwang et al. 2020). Macrophages are one of the cells that make up TME, and are known to be closely involved in tumorigenesis and progression of lung cancer (Lewis et al. 2006; Bingle et al. 2002; Mantovani et al. 2017; Conway et al. 2016). Intratumoral CD204-positive tumor associated macrophages (TAMs) have been reported to be associated with tumor aggressiveness in lung adenocarcinoma, and squamous cell carcinoma (Ohtaki et al. 2010; Hirayama et al. 2012).

Additionally, some studies have reported that the peritumoral microenvironment influences tumor progression. In pancreatic cancer, the expression of secreted protein acidic and rich in cysteine (SPARC) by peritumoral fibroblasts indicates a poorer prognosis for patients (Infante et al. 2007). Moreover, hyaluronan in peritumoral stroma is associated with cancer cell spreading in breast cancer (Auvinen et al. 2000). Peritumoral macrophages promote tissue remodeling and proangiogenic pathways in hepatocellular carcinoma, by inducing Th17-mediated inflammation in peritumoral stroma (Kuang et al. 2010). For small cell lung cancer, Iriki reported that the macrophages that accumulated near the peripheral areas of tumor nests are important for tumor progression via STAT3 activation, suggesting that peritumoral macrophages may be deeply involved in tumor progression (Iriki et al. 2017).

Alveolar macrophages (AMs) reside in the alveolar space, and playing a crucial role in lung homeostasis (Lee et al. 2015; Allard et al. 2018). Additionally, AMs are major cells that constitute the peritumoral microenvironment. In the mouse model, AMs perform self-renewal throughout their life cycle (Guilliams et al. 2013, unlike stromal macrophages that are recruited from the circulation (Pollard et al. 2004; Sica et al. 2012). In breast cancer, AMs secrete transforming growth factor-beta in a premetastatic niche, promoting lung metastases (Sharma et al. 2015). Moreover, AMs mainly advance lung metastasis by generating leukotriene B4 in hepatocellular carcinoma (Nosaka et al. 2018). Speth et al found SOCS3 secreted by AMs inhibits tumor progression in lung cancer (Speth et al. 2019).

Based on these findings, we hypothesized that peritumoral AMs are associated with the prognosis of lung cancer. Thus, this study aims to investigate the prognostic impact of the number of peritumoral AMs in patients with stage I lung adenocarcinoma.

Materials And Methods

Patients

Patients with pathological stage I adenocarcinoma who underwent lobectomy or pneumonectomy with systemic lymph node dissection at the National Cancer Center East between January 2011 and December 2015 were enrolled in this study (Supplementary Fig. 1). We excluded patients who received preoperative therapy and those with adenocarcinoma in situ, undetectable tumors in surgical specimens, and synchronous lung cancer. Finally, 514 patients were enrolled in this study. The study was approved by the

institutional review board of the National Cancer Center Hospital East (IRB approval number 2020 – 239). The clinical characteristics of patients were retrospectively retrieved from the Thoracic Surgical Database of the National Cancer Center Hospital East.

Pathologic evaluation

All surgical specimens were fixed in 10% formalin, and embedded in paraffin. All tumors were cut at 5-mm intervals, and 4µm thick-sections were stained using the hematoxylin and eosin (HE) method. The Verhoeff-van-Gieson (VVG) method was used to visualize elastic fibers to identify lymphovascular and pleural invasion. Histological diagnosis was based on the fourth edition of the World Health Organization series, and the disease stages were categorized according to the guidelines of the 8th edition of the TNM Classification of Malignant Tumors.

Calculation of the number of alveolar macrophages

We selected the largest cross-sectional slide for each patient to calculate the number of AMs (Figs. 1A-D). If the tumor was on multiple slides in the largest cross-section, we counted all these slides. The HE slides were scanned and captured using a digital slide scanner (Aperio VERSA SL200; Leica, Biosystems, Nußloch, Germany) and were reviewed by two pathologists (O.N and G.I) who were blinded to the clinicopathologic information of each slide. The peritumoral alveolar space was defined as the air space outside the tumor within three alveoli, and the number of macrophages per alveolar space was counted. AMs were distinguished from spread through air spaces (STAS) of tumor cells using the following methods. Macrophages in smokers typically have cytoplasm containing faint brown pigment and black carbon granules, whereas in nonsmokers the pigment is lacking and the cytoplasm is sometimes foamy. Nuclei are small, uniform, and regular, without atypia. Nuclear folds are frequent, and nucleoli are inconspicuous or absent. Contrastingly, STAS generally lack cytoplasmic pigment or foamy cytoplasm. They often grow in cohesive clusters and nuclei are atypical with hyperchromasia and frequent nucleoli. The number of peritumoral AMs was determined as the average of the top five AMs in each patient.

We investigated the correlation between the number of peritumoral AMs in HE-stained and peritumoral CD68-positive AMs. Forty patients were examined for CD68 expression in the peritumoral alveolar space (Supplementary Figs. 2A, B). The distribution of peritumoral AMs on HE slides and CD68-positive macrophages in each patient strongly correlated ($p < 0.01$, $r^2 = 0.91$) (Fig. 1E). Therefore, we assessed that HE slides were efficient for counting the number of peritumoral AMs, and later the number of peritumoral AMs was evaluated using HE slides only.

Gene expression signature analysis on the Cancer Genome Atlas data sets

We analyzed the difference in gene signatures between the number of AMs in lung adenocarcinoma, using the Cancer Genome Atlas-Lung Adenocarcinoma (TCGA- LUAD) datasets. All analyses were performed using R software (version 4.0.2) (<https://www.r-project.org/>; The R Foundation for Statistical Computing, Vienna, Austria). We downloaded the data of 155 patients with stage I lung adenocarcinoma

in June 2020 from the Genomic Data Common (GDC) Data Portal (<https://portal.gdc.cancer.gov>) using the R/Bioconductor package TCGA biolinks. Using representative digital slides of each patient that can be viewed on the site, we counted and calculated peritumoral AMs and divided them into two groups in the manner described above. Twenty-five patients were excluded because of difficulty in evaluating peritumoral AMs. Differentially expressed genes between the high and low AM groups were identified using the DESeq2 package (version 1.28.1) [Love MI et al]. Genes were extracted using adjusted p-values (padj) of < 0.05 and $|\log_2 \text{fold changes} (\log_2\text{FC})|$ of > 1 . We performed hierarchical clustering of genes that differed in expression between the high and low AM groups ($|\log_2\text{FC}| > 1$), and heatmap analysis was performed using gplots (version 3.0.4) [Warnes GR et al.] and genefilter (version 1.70.0) [Gentleman R et al.] packages. Additionally, the differentially expressed genes were assessed for enrichment analysis using Metascape (<http://metascape.org/gp/index.html#/main/step1>) [Zhou Y et al]. The Metascape pathway enrichment analysis used terms across different ontology sources, such as Gene Ontology (GO).

Statistical analysis

Associations between the clinicopathologic variables and the number of peritumoral AMs were analyzed using Fisher's exact test (for categorical variables) and the Mann-Whitney U test (for continuous variables). The number of peritumoral AMs in each predominant subtype group was compared using the Mann-Whitney U test. Pearson correlation coefficient was used to evaluate the correlation between the number of peritumoral AMs on HE slides and the number of CD68-positive peritumoral AMs. To evaluate prognosis, receiver operating characteristic curves of the number of peritumoral AMs were used. Overall survival (OS) was defined as the interval between the date of surgery and the date of death from any cause or the last follow-up. Disease-free survival (DFS) was defined as the time between the date of surgery and the date of recurrence detection, death from any cause, or the last follow-up. Survival curves were estimated using the Kaplan-Meier method, and differences in OS and DFS were compared by using the log-rank test. Hazard ratios (HRs) were estimated using the Cox proportional hazards model. The date of data cut-off was May 2019 at our institution. These data were analyzed using EZR version 1.51, a graphical user interface for R program.

Results

Correlation between clinicopathological factors and the number of peritumoral AMs

The median number of peritumoral AMs was 15.5 per alveolar space, and we divided patients into two groups based on this value. Patients with a higher number of peritumoral AMs were men, with smoking history; high-grade pathologic T stage; larger pathologic total and invasive tumor sizes; the presence of pleural invasion, lymph vessel invasion and vascular invasion; and a higher proportion of invasive predominant subtypes (Table 1).

Table 1
Clinicopathologic characteristics of patients with high- vs low-peritumoral AMs

	Overall	peritumoral	peritumoral	
	Cohort	AMs < 15.5	AMs ≥ 15.5	
Variables	(n = 514)	(n = 257)	(n = 257)	<i>p</i>
Age(y)				
median	69 (33–93)	68 (35–87)	69 (33–93)	
< 65	152	75	77	0.92
≥65	362	182	180	
Sex				
female	250	149	101	< 0.01
male	264	108	156	
Smoking history				
never	232	136	96	< 0.01
ever/current	282	121	161	
Pathologic T stage				
1mi/1a/1b/1c	51/122/153/70	39/82/82/23	12/40/71/47	< 0.01
2a	118	31	87	
Total tumor size(mm)				< 0.01
median(range)	24 (7–75)	22 (7–62)	25 (8–75)	
Invasive tumor size(mm)				< 0.01
median(range)	14 (1.5–40)	11 (1.5–40)	18 (2–40)	
Pleural invasion				< 0.01
absent	411	230	181	
present	103	27	76	
Lymphatic permeation				< 0.01
absent	480	250	230	
present	34	7	27	
Vascular invasion				< 0.01

	Overall	peritumoral	peritumoral
absent	411	238	173
present	103	19	84
Predominant subtype			< 0.01
Lepidic	260	180	80
Papillary	115	42	73
Micropapillary	1	1	0
Acinar	81	27	54
Solid	57	7	50
EGFR mutation (n = 244)			0.36
absent	120	45	75
present	124	54	70

Correlation between the numbers of peritumoral AMs and the predominant subtype

The relationships between the number of peritumoral AMs and predominant subtypes are presented in Fig. 2, wherein the micropapillary-predominant subtype was excluded because of one case. The median number of peritumoral AMs for the predominant subtype was 9.6 in lepidic, 20.4 in papillary, 20.2 in acinar, 27.2 in solid subtypes. The number of peritumoral AMs in the predominantly solid subtype was significantly higher than that in the acinar subtype ($p < 0.01$), papillary subtype ($p < 0.01$), and lepidic subtype ($p < 0.01$). There were no significant differences between the number of peritumoral AMs in the predominantly acinar and papillary subtypes ($p = 0.78$), and the number of peritumoral AMs in these two subtypes was significantly higher than that in the lepidic subtype (both $p < 0.01$).

Survival by the number of peritumoral AMs

The median follow-up time of patients was 61.8 months. Patients with a high peritumoral AM content showed significantly shorter OS ($p < 0.01$, 5-year OS rate: 95% vs. 83.4 %) (Fig. 3A) and DFS ($p < 0.01$, 5-year DFS rate: 91.3% vs. 73.5%) (Fig. 3B) than patients with a low peritumoral AM content in stage I adenocarcinoma. In stage IA, patients with a high AM content showed significantly shorter OS ($p = 0.02$, 5-year OS rate: 95.3% vs. 88.7%) (Fig. 3C) and DFS ($p < 0.01$, 5-year DFS rate: 93.2% vs. 81.4%). In stage IB, patients with a high peritumoral AM content showed significantly shorter OS ($p = 0.03$, 5-year OS rate: 92.4 % vs. 72.8 %) (Fig. 3D) and DFS ($p = 0.03$, 5-year OS rate: 77.2% vs. 57.8%) (Fig. 3F).

Prognostic significance of the number of peritumoral AMs

In the univariate analysis, a larger invasive tumor size; the presence of pleural invasion, lymphatic permeation, presence of vascular invasion; and a higher number of peritumoral AMs were significantly associated with shorter DFS (Table 2).

Table 2
Univariate and multivariate analysis for disease-free survival based on the number of peritumoral AMs

Variable	Univariate			Multivariate		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Age(y)						
<65	ref.					
≥65	1.31	0.83–2.08	0.24			
Sex						
Female	ref.					
Male	1.32	0.88–1.98	0.18			
Smoking history						
Never	ref.					
Ever/Current	1.29	0.85–1.94	0.23			
Invasive tumor size(cm)						
(+ 1.0cm)	2.25	1.83–2.78	< 0.01	1.60	1.23–2.07	< 0.01
Pleural invasion						
absent	ref.			ref.		
present	3.12	2.11–4.81	< 0.01	1.14	0.69–1.86	0.62
Lymph vessel invasion						
absent	ref.			ref.		
present	3.02	1.77–5.18	< 0.01	1.37	0.79–2.40	0.26
Vascular invasion						
absent	ref.			ref.		
present	5.07	3.39–7.58	< 0.01	2.55	1.56–4.24	< 0.01
Number of peritumoral AMs						
low (< 15.5)	ref.			ref.		
high (≥ 15.5)	3.44	1.93–4.80	< 0.01	1.85	1.11–3.09	0.02

In the multivariate analysis, a higher number of peritumoral AMs was an independent prognostic factor for DFS (HR 1.85, 95% confidence interval (CI): 1.11–3.09, $p = 0.02$), in addition to invasive tumor size and vascular invasion.

Evaluation of the survival impact of the number of peritumoral AMs

Patients were stratified into two groups according to the different cut-off values of the number of peritumoral AMs. Multivariate analyses showed that although the cut-off value was 10, there was no significant difference between patients with high and low peritumoral AMs. Both the cut-off values of 20 and 30 were independent factors for DFS (HR 2.01, 95% confidence interval: 1.28–3.15, $p < 0.01$; HR 3.27, 95% CI: 2.10–5.08, $p < 0.01$, respectively), and the hazard ratio gradually increased as the cut-off value increased (Table 3). This result suggested the possibility that the higher the number of peritumoral AMs, the more likely the tumor would recur.

Table 3
Summary of the results of multivariate analyses for disease-free survival about the number of peritumoral AMs

Number of peritumoral AMs	n	HR	95% CI	<i>p</i>
> 10 vs. ≤10				
> 10	182	ref.		
≤10	332	1.32	0.75–2.34	0.33
> 15.5 vs. ≤15.5 (Median)				
> 15.5	257	ref.		
≤15.5	257	1.85	1.11–3.09	0.02
> 20 vs. ≤20				
> 20	321	ref.		
≤20	193	2.01	1.28–3.15	< 0.01
> 30 vs. ≤30				
> 30	413	ref.		
≤30	101	3.27	2.10–5.08	< 0.01

Survival and gene ontology analysis by the number of peritumoral AMs in TCGA database

Of the 130 patients, the median number of peritumoral AMs that we calculated on TCGA digital slides was 11.3, and we divided them into two groups using this value. Patients with a higher number of peritumoral AMs showed significantly shorter DFS ($p = 0.04$) (Fig. 4A), whereas there was no significant difference in OS (data not shown).

We extracted 212 differentially expressed genes (DEGs) between patients with high and low peritumoral AMs. A heatmap of 212 DEGs is presented in Fig. 4B. The Metascape pathway enrichment analysis was performed, which identified the top 20 biological processes in the DEGs, including chemotaxis (GO: 0006935) and epithelial proliferation (GO: 0050673) (Fig. 4C).

Discussion

In our study, clinicopathological factors showed that patients with a high peritumoral AM content had more aggressive tumors, such as with larger pathologic invasive tumor size; the presence of pleural invasion, lymphatic permeation, and a higher proportion of more invasive predominant subtype, than patients with a low peritumoral AM content. The multivariate analysis revealed that the number of peritumoral AMs was an independent prognostic factor. These results suggest that the number of peritumoral AMs is a strong factor for poor prognosis in patients with stage I lung adenocarcinoma. To the best of our knowledge, this is the first report showing that the number of AMs is associated with the prognosis of stage I lung adenocarcinoma.

In the analysis using the TCGA dataset, patients with a high peritumoral AM content showed shorter DFS than those with a low peritumoral AM content, indicating that the results using our hospital cohort are reproducible. The significant difference in some GO pathways, such as chemotaxis and epithelial proliferation, might be one of the reasons why the prognosis was poorer in the group with a high peritumoral AM content.

Previous reports have described the relationship between the tumor and the microenvironment around the tumor. In some types of cancer, peritumoral macrophages facilitate the progression in tumor cells (Kuang et al. 2010; Iriki et al. 2017). These reports indicate that the microenvironment around the tumor, not only within the tumor, is involved in tumor progression. Alternatively, Sharma et al. reported that AMs provide an environment where lung metastases are likely to occur by suppressing antitumor T cell responses in breast cancer (Sharma et al. 2015). Future studies are needed to examine the gene expression profiles of AMs around tumors.

We suggest two possible reasons why the number of peritumoral AMs differs depending on the patient: (1) Cancer cells themselves mobilize the peritumoral AMs, and the ability to attract AMs varies depending on the characteristics of cancer cells in each case. (2) AMs that are originally present in alveoli before tumorigenesis and the characteristics of AMs in each case may influence the development of adenocarcinoma. To verify our theory, we made the following considerations: We defined nonadjacent alveolar space as the air space 2 mm away from the tumor edge on the largest cross-sectional slide, to avoid overlapping with the peritumoral alveolar space, and counted macrophages per alveolar space

(Supplementary Figs. 3A, B). There was a strong correlation between peritumoral and nonadjacent AMs ($r^2 = 0.723$; Supplementary Fig. 3C). The median number of nonadjacent AMs was 8.6 per alveolar space. In the multivariate analysis, there were no significant differences between patients with a high and low non-adjacent AM content with the cut-off values of 8.6 and 10 ($p = 0.54$ and 0.50 , respectively) (Supplementary Tables 1, 2). For the cut-off value of 20, patients with a high nonadjacent AM content showed poorer DFS than those with a low nonadjacent AM content (HR 1.57, 95% CI: 1.21–2.04, $p < 0.01$) (Supplementary Table 2). This result may indicate that highly malignant tumors are likely to occur in cases with many AMs where the tumor develops. However, we cannot rule out the possibility that AMs 2mm apart may also be mobilized by cancer cells. Further studies are needed to elucidate why the number of peritumoral AMs varies for each case, further studies will be needed.

There are some limitations to our study. First, this was a retrospective study in a single institution. Second, we did not evaluate the number of AMs on all slides with tumors. Third, the number of AMs is affected by how the lungs are inflated during fixation. Therefore, we should interpret the results of this study with caution.

The prognostic influence of the number of peritumoral AMs in our study suggests that both intratumoral and the peritumoral microenvironments are important. Recent studies have investigated novel therapeutic strategies aimed at depleting TAMs and/or reprogramming their tumor-promoting effects (Mantovani et al. 2017; Anfray et al. 2020; Sarode et al. 2020). Considering the results of our study, there may be a need for therapeutic efforts targeting AMs, not only TAMs.

Abbreviations

NSCLC, non-small cell lung cancer; TME, tumor microenvironment; TAM, tumor associated macrophage; AM, alveolar macrophage; AIS, adenocarcinoma in situ; HE, hematoxylin and eosin; VVG, Verhoeff-van-Gieson; STAS, spread through air space; TCGA, The Cancer Genome Atlas; GO, Gene Ontology; OS, overall survival; DFS, disease-free survival.

Declarations

Funding:

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Conflicts of interest:

The authors declare that they have no conflict of interest.

Availability of data and material:

There are no availability of data and material.

Code availability:

There are no code availability.

Authors' contributions:

ON and GI participated in the study design, data collection, data analysis, manuscript writing and manuscript review. All other authors participated in manuscript review and revision and provided final approval for submission.

Ethics approval:

This retrospective study was approved by the Institutional Review Board (IRB approval number; 2020-239).

Consent to participate:

Informed consent was obtained from all patients.

Consent to publication:

Comprehensive informed consent was obtained from all patients.

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Figures

Figure 1

Figure 1

Microscopic features of high and low peritumoral macrophage contents. (A) Low-power view, and (B) high-power view of a high peritumoral macrophage content. (C) Low-power view, and (D) high-power view of a low peritumoral macrophage content. (E) Correlation between the number of peritumoral alveolar macrophages on HE slides and CD68 immunostaining slides. The x-axis shows the number of

peritumoral AMs on the HE slide, and the y-axis shows the number of peritumoral CD68-positive AMs. AMs, alveolar macrophages; HE, hematoxylin and eosin

Figure 2

Figure 2

Numbers of peritumoral AMs in each predominant subtypes: lepidic, papillary, acinar, and solid. Statistical analysis was conducted in each two groups.

Figure 3

Figure 3

Kaplan-Meier analysis according to the number of peritumoral AMs. (A) Overall survival (OS), (B) Disease-free survival (DFS) in stage I patients. (C) OS, (D) DFS in stage IA patients. (E) OS, (F) DFS in stage IB patients. AMs, alveolar macrophages

Figure 4

Figure 4

Survival and gene ontology analyses by the number of peritumoral AMs in the TCGA database. (A)Kaplan-Meier analysis of disease-free survival in the TCGA database. (B)Heat map compared with higher and lower peritumoral AM content. (C)Gene ontology and pathways were significantly higher in high peritumoral AM content than in low peritumoral AM content.

Supplementary Files

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- [AMpaper.SuppFigure1.pptx](#)
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