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Neutrophil-rich Pulmonary Carcinoma : Clinicopathological Characteristics and Cytokine Expression and Their Relationship with Lymph Node Metastasis

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Abstract : A small subset of carcinomas of various origins are associated with high numbers of tumor-infiltrating neutrophils (TINs). Here, we examined the characteristics of non-small-cell pulmonary carcinomas with high numbers of TINs, and their relationship with lymph node (LN) metastasis. The study included 100 patients diagnosed and treated for primary pulmonary carcinoma at Showa University Northern Yokohama Hospital from 2011 to 2012. We histopathologically defined tumors with ＞10 neutrophils per high-power field as neutrophilrich. Among the 100 patients, 40 were classed as having neutrophilrich pulmonary cancer (NRPC), and tissue samples from these patients were prepared for further examination. Comparison of the clinicopathological factors (age, gender, tumor size, histological type, and grade) in NRPC cases with or without LN metastasis showed that none of the above factors was significantly correlated with LN metastasis. Immunohistochemical analysis of two cytokines that play a major role in granulopoiesis, granulocyte-colony stimulating factor (G-CSF) and macrophage-CSF (M-CSF), revealed that the expression of M-CSF, but not G-CSF, was significantly correlated with LN metastasis. Furthermore, coexpression of M-CSF and the M-CSF receptor was significantly correlated with LN metastasis, but coexpression of G-CSF and the G-CSF receptor did not show such a correlation. These findings indicate that M-CSF-producing NRPCs show a significantly high lymph node metastasis potential.

Key words : pulmonary carcinoma, neutrophil, lymph node, macrophage colony stimulating factor, granulocyte colony stimulating factor

Introduction

High numbers of tumor-infiltrating neutrophils (TINs) are present in a small subset of carcinomas of various types ; however, there is surprisingly little data about the presence of neutrophils within human tumors[1)]. The number of TINs has been shown to be a strong, independent

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prognostic factor for recurrence-free and overall survival in metastatic[2] and localized clear cell renal cell carcinoma[3], and in head and neck squamous cell carcinoma[4], and a high neutrophil count has been found to be associated with a favorable prognosis in gastric cancer[5]. In contrast, the number of TINs was found to correlate with a high tumor grade in human gliomas[6] and to be related to more aggressive types of pancreatic tumors[7]. Therefore, it is still controversial whether the presence of TINs is indicative of a better or worse host-antitumoral response. Here, we examined the clinicopathological characteristics of pulmonary carcinoma with a high number of TINs, and their relationship with lymph node (LN) metastasis. In the same cohort, we explored the relationship between the principal cytokines in granulopoiesis[8], granulocyte-colony stimulating factor (G-CSF) and macrophage-CSF (M-CSF), and LN metastasis.

Materials and Methods

Patients and samples

This study included 100 patients diagnosed and treated for primary pulmonary carcinoma at Showa University Northern Yokohama Hospital from 2011 to 2012. Clinical information was obtained from hospital records. Tissue samples were obtained by surgical resection, and then fixed in 20% formalin, routinely processed, embedded in paraffin wax, cut into 3-μm-thick sections, and stained with hematoxylin and eosin (H&E). All tissue samples were histopathologically examined by two independent pathologists (T.M. and T.K.).

Neutrophil Counting

Two pathologists (T.M. and T.K.) independently analyzed the H&E-stained sections from each patient, and the numbers of TINs were determined by the following procedure[9]. For each tissue block, the sections were examined under low power magnification to identify areas showing neutrophilic aggregation within the tumor tissue. Then, the numbers of stromal neutrophils were assessed semiquantitatively in 20 non-overlapping high-power fields (HPFs; magnification, 400; 0.08 mm^2) in representative areas of a given tumor on two slides (i.e., 10 fields per slide). Tumors with an average of greater than 10 neutrophils per HPF were considered to be neutrophil-rich carcinomas[9]. Among the 100 patients, 40 had neutrophil-rich pulmonary cancer (NRPC) by the above criterion, and tissue samples from these patients were prepared for further examination.

Immunohistochemical staining

Two hematopoietic growth factors, G-CSF and M-CSF, and their receptors, G-CSFR and M-CSFR, were examined immunohistochemically; the details of the antibodies used and the antigen retrieval and incubation steps are listed in Table 1. After endogenous peroxidase activity was inhibited by using hydrogen peroxide solution, the sections were incubated with primary antibody. A secondary antibody was raised against biotinylated immunoglobulin and conjugated with avidin-horseradish peroxidase. The staining of each sample was visualized by using a Ventana I-View DAB universal kit (Roche, Tokyo, Japan). After nuclear staining with Mayer's

Table 1. Antibodies for immunohistochemistry

Antibody	Clone	Source	Dilution	Antigen retrieval	Incubation (37°C) (min)
G-CSF	5.24	Calbiochem	1 : 50	Heat-treatment (100°C) with saponin (0.1%) for 30 min	32
M-CSF	EP1179Y	GeneTeX	1 : 100	Heat-treatment (100°C) with EDTA (ph 8.5) for 60 min	32
G-CSFR	S-1284	Abcam	1 : 80	Heat-treatment (100°C) with EDTA (ph 8.5) for 8 min	16
M-CSFR	EP1037Y	GeneTeX	1 : 100	Heat-treatment (100°C) with EDTA (ph 8.5) for 60 min	10

G-CSF, granulocyte-colony stimulating factor ; M-CSF, macrophage-colony stimulating factor ; G-CSFR, granulocyte-colony stimulating factor receptor ; M-CSFR, macrophage-colony stimulating factor receptor

hematoxylin, the antibody-antigen reaction was enhanced by using 0.5% copper sulfate. Positive and negative controls for the antibodies were prepared in accordance with their data sheets.

Two pathologists (T.M. and T.K.), who were blinded to the patients' clinical data, examined the immunostained tissues. Because there is no standard cut-off for immunopositivity for G-CSF, G-CSFR, M-CSF, or M-CSFR, we determined the cases to be immunopositive when more than 10% of the tumor cells showed distinct membranous and cytoplasmic staining.

Statistical analysis

The chi-square test was used to compare characteristics between groups. *P* values less than 0.05 were considered to be statistically significant.

Results

Clinicopathological findings

The clinicopathological characteristics of the 40 cases of NRPC included in this study are listed in Table 2. The proportion of patients with LN metastasis (LN(+)) in these NRPC cases (11/40 ; Table 2) was not statistically different to that in the non-NRPC cases (12/60), which were not examined further in this study.

The median age of the patients with NRPC was 66.0 years (ranging from 39–83 years), with 21/40 patients aged ≤ 65 years. There was no significant difference between the proportion of patients aged ≤ 65 years in the LN(+) and LN(−) subgroups (7/11 [64%] and 14/29 [48%], respectively ; Table 2).

Twenty-six of the patients were male, and 14 were female. The gender distributions in the LN (+) and LN(−) cases were not significantly different from each other (8/11 [72%] males versus 18/29 [62%] males, respectively ; Table 2).

Eighteen patients had tumors classified as T1 (tumor size ≤ 3 cm) and 22 had tumors classified as T2 (tumor size > 3 cm). The tumor sizes in the LN(+) cases (5/11 [45%] T1) were not significantly different from those in the LN(−) cases (13/29 [45%], respectively ; Table 2).

Table 2. Clinicopathological findings for neutrophil-rich pulmonary carcinoma

Clinicopathological findings	LN(+)	LN(−)	*P*-value
Age (years) ≤ 65 : > 65	7 : 4	14 : 15	N.S
Gender M : F	8 : 3	18 : 11	N.S.
Size T1 : T2	5 : 6	13 : 16	N.S.
Histology Ad : Sq : AqSq : L	3 : 6 : 0 : 2	16 : 9 : 3 : 1	N.S.
Grade G1, 2 : G3, 4	8 : 3	23 : 6	N.S.

LN(+), positive for lymph node metastasis; LN(−), negative for lymph node metastasis; T1 and T2, tumor size of ≤3 cm and >3 cm, respectively; Ad, adenocarcinoma; Sq, squamous cell carcinoma; AdSq, adenosquamous carcinoma; L, large cell neuroendocrine carcinoma; G1, 2, well or moderately differentiated; G3, 4, poorly differentiated or undifferentiated; N.S, not significant

The tumors of 19 patients were classed as adenocarcinoma, 15 were squamous cell carcinoma, 3 were adenosquamous carcinoma, and 3 were large neuroendocrine cell carcinoma. Among the 11 LN(+) cases, there were 3 cases of adenocarcinoma (27%), 6 cases of squamous cell carcinoma (55%), and 2 cases of large neuroendocrine cell carcinoma (18%), whereas among the 29 LN(−) cases, there were 16 cases of adenocarcinoma (55%), 9 cases of squamous cell carcinoma (31%), 3 cases of adenosquamous carcinoma (10%), and 1 case of large neuroendocrine cell carcinoma (3%). The differences in proportions of histopathological types between LN(+) and LN(−) cases were not statistically significant.

The tumors of 21 patients were classified as grade 1 or 2 (G1, 2; well or moderately differentiated) and 19 were grade 3 or 4 (G3, 4; poorly differentiated or undifferentiated). Among the 11 LN(+) cases, 8 were G1, 2 (72%) and 3 were G3, 4, whereas among the 29 LN(−) cases, 23 were G1, 2 (79%) and 6 were G3, 4. This difference was not statistically significant.

Immunohistochemical findings

To examine the protein expression of hematopoietic growth factors and receptors in NRPC, we performed immunohistochemical staining for G-CSF, M-CSF, and their respective receptors (Table 3; Fig. 1).

Of the 40 patients with NRPC, 19 were G-CSF-immunopositive and 20 were M-CSF-immunopositive. The proportion of LN(+) cases that were G-CSF-immunopositive (5/11 [45%]) was not significantly different from the proportion of LN(−) cases that were G-CSF-immunopositive (14/29 [48%]). In contrast, the proportion of LN(+) cases that were M-CSF-immunopositive (9/11 [82%]) was significantly higher than the proportion of LN(−) cases that were M-CSF-immunopositive (11/29 [38%]; $P = 0.015$).

Table 3. Immunohistochemical analysis of neutrophil-rich pulmonary carcinoma

Markers	LN(+) % (n)	LN(−) % (n)	*P*-value
G-CSF	45% (5/11)	48% (14/29)	N.S
M-CSF	82% (9/11)	38% (11/29)	0.015
G-CSFR	55% (6/11)	62% (18/29)	N.S.
M-CSFR	36% (4/11)	24% (7/29)	N.S.
G-CSF + G-CSFR	18% (2/11)	34% (10/29)	N.S.
M-CSF + M-CSFR	45% (5/11)	14% (4/29)	0.046

LN(+), positive for lymph node metastasis; LN(−), negative for lymph node metastasis; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; G-CSFR, granulocyte-colony stimulating factor receptor; M-CSFR, macrophage-colony stimulating factor receptor; N.S, not significant

Twenty-four patients were G-CSFR-immunopositive and 11 were M-CSFR-immunopositive. Neither receptor was expressed at a significantly different level between the LN(+) and LN(−) cases (G-CSFR, 6/11 [55%] versus 18/29 [62%], respectively; and M-CSFR, 4/11 [36%] versus 7/29 [24%], respectively).

Twelve patients coexpressed G-CSF and G-CSFR and 9 coexpressed M-CSF and M-CSFR. Coexpression of G-CSF and G-CSFR was observed in 2 of the 11 LN(+) cases (18%) and in 10 of the 29 LN(−) cases (34%). This difference was not statistically significant. In contrast, 5 of the 11 LN(+) cases coexpressed M-CSF and M-CSFR (45%), but only 4 of the 29 LN(−) cases coexpressed M-CSF and M-CSFR (14%), a statistically significant difference ($P = 0.046$).

Discussion

Caruso *et al*[9], who were the first to define neutrophil-rich carcinoma in the stomach with the criterion of >10 TINs/HPF, revealed an association between high neutrophil levels and a favorable prognosis in female patients. Here, by using the same criterion, we found that 40% of a series of 100 resected pulmonary carcinoma cases were NRPC. Wei *et al*[10] found a similar percentage (i.e., 38%) of NRPC in a series of 114 pulmonary carcinomas. In pulmonary carcinoma, Lee *et al*[11] found no correlation between the numbers of TINs and prognosis; however, Bellocq *et al*[12] reported that a high number of TINs was associated with a significantly poor outcome. Here, although we did not consider prognosis, we found no significant difference in the proportion of patients with LN metastasis between the NRPC (11/40) and non-NRPC (12/60) cases. Therefore, the NRPC cases were considered to include both high and low LN metastasis potential cases.

We found that no factors among the clinical (age, gender) or histopathologic (tumor size, grade) factors significantly correlated with LN metastasis in the NRPC cases. Histological type also did not significantly correlate with LN metastasis, but further study is necessary because this study includes too few cases of each histological type to be statistically reliable.

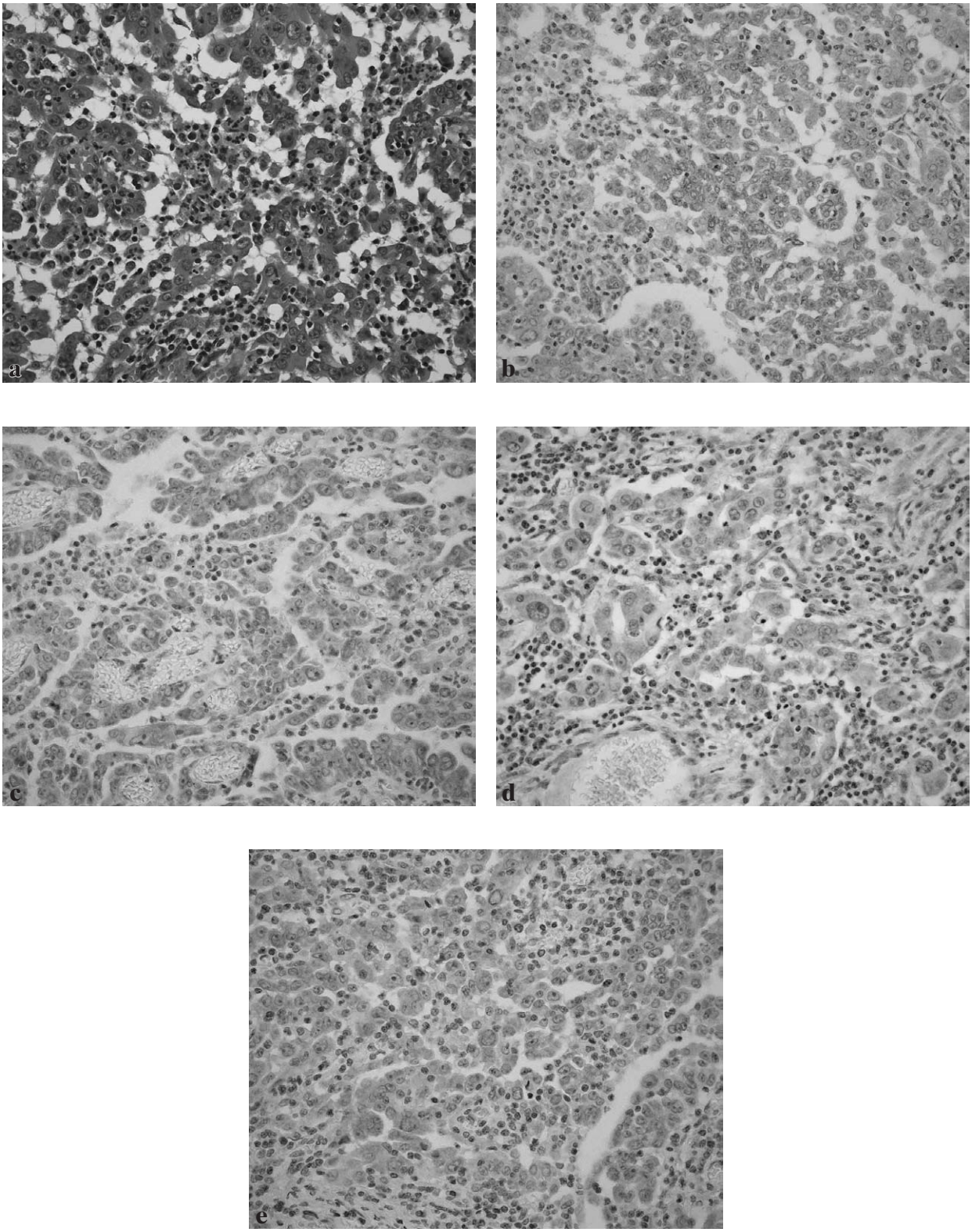

Fig. 1. Production of hematopoietic growth factors and their receptors in neutrophil-rich pulmonary carcinoma. Representative high power (×400 magnification) views of a) H&E staining, b) immunoreactive G-CSF (brown), c) immunoreactive M-CSF (brown), d) immunoreactive G-CSFR (brown), and e) immunoreactive M-CSFR (brown).

Non-small-cell lung carcinomas secrete cytokines involved in granulopoiesis[8], although the reason for this phenomenon is unknown. Here, we explored the expression of M-CSF, G-CSF, and their receptors in NRPC. We found that LN metastasis was significantly correlated with M-CSF immunopositivity but not G-CSF immunopositivity in tumor cells. Similar to our results, Kaminska *et al*[13] found a significant correlation between the stage of non-small-cell lung cancer and the serum M-CSF level but not the serum G-CSF level. Furthermore, Katsumata *et al*[14] and Kaminska, *et al*[13] showed the prognostic value of serum M-CSF level but not the serum G-CSF level. Therefore, although both M-CSF and G-CSF are well known to be expressed in pulmonary carcinoma cells with neutrophilia[8, 15–17] and both of them induce TINs, only M-CSF appears to promote a more advanced stage disease.

The exact role of TINs is still controversial. In some experimental tumors, TINs are cytolytic and can eliminate tumor cell populations[18], whereas in others, TINs contribute to the invasive potential of the tumor[19, 20]. Recently, TINs have been functionally subclassified as protumor phenotype (N2) and antitumor phenotype (N1)[1]. Our finding that M-CSF-immunopositive NRPC cases, but not G-CSF-immunopositive NRPC cases, showed a significantly higher occurrence of LN metastasis raises the possibility that TINs chemoattracted by M-CSF can be functionally subclassified as protumor phenotype (N2).

Furthermore, in the current study, LN metastasis showed a significantly high correlation with the coexpression of M-CSF and M-CSFR, but not G-CSF and G-CSFR. Previous studies demonstrated that M-CSF is involved in the autocrine growth mechanism in lung carcinomas, facilitating tumor growth and invasion[21–23]. Although both M-CSF and G-CSF promote tumor cell invasion by increasing production of gelatinases[24], coexpression of M-CSF and M-CSFR is reported to have an advantage for tumor invasion by the autocrine growth mechanism in non-small-cell lung cancer[17], which may explain the high LN metastasis potential in the NRPC cases examined here.

In conclusion, our results indicate that M-CSF-producing NRPC shows a significantly high lymph node metastasis potential.

Conflict of interest

The authors declare no conflict of interest.

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