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The incidence of lymph node metastasis in patients with different oncogenic driver mutations among T1 non-small-cell lung cancer

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ABSTRACT

Objective: To investigate the incidence and distribution of lymph node metastasis in patients with different gene mutations among pathological T1 non-small-cell lung cancers (NSCLC).
Methods: NSCLC cases resected in our institution between 2016 and 2018 were included. Driver mutation testing was performed in all resected tumor tissues. These patients were grouped by the type of gene mutations. On the basis of protein that mutant-genes encoded involved in the molecular pathway, the genotypes were further classified into four distinct groups: upstream receptor mutant protein (EGFR, HER2 and MET); downstream regulator mutant protein (KRAS and BRAF); fusion mutant protein (ROS1, ALK and RET) and the wild type group. The incidence of lymph node metastasis was compared among different groups.
Results: Of the 1052 patients enrolled, the frequency of positive mutations was 68.0%. The incidence of lymph node metastasis were as follows: wild type (19.3%), ROS1 (72.8%), BRAF (55.5%), ALK (44.7%), HER2 (40%), RET (23.1%), KRAS (15.3%), EGFR (15.3%) and MET mutation (0%) ($P < 0.001$). The incidence of lymph node metastasis was significantly higher in fusion mutant protein group (45.1%) compared with others (wild type 19.3%, downstream regulator mutant protein 19.1%, upstream receptor mutant protein 15.3%, all $P < 0.001$). Patients with fusion genes also showed higher proportion of vascular invasion and positive lymph node ratio of greater than 0.33 compared to others.
Conclusion: Different genotypes of NSCLC have different propensity to develop lymph node metastasis. Cases of fusion gene mutations had a higher risk and burden of lymph node metastasis than other genotypes, which may indicate that more intensive treatment or surveillance strategies should be applied for these patients.

1. Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for the highest percentage. One of the new groups of molecular divisions of NSCLC: driver mutation, has shifted the treatment paradigm to targeted therapy [1]. Drugs designed specifically as inhibitors of molecularly selected targets have dramatically extended the survival times for patients whose tumors harbor actionable oncogenes such as EGFR mutation [2] or translocated ALK, RET, or ROS1 [3–5]. Given the intratumor heterogeneity of NSCLC at the molecular level [6,7], the biological behaviors of NSCLC may differ with respect to different oncogenes. An increasing number of studies have been made to investigate the biologic mechanism of gene mutated NSCLC, such as the associations between metastasis and gene alterations [8,9]. Comprehensive analysis of driver mutation genes and key cancer signaling pathways have become necessary to understand the genetic basis of diseases, biological behaviors, drug resistance, and to modify therapy options accordingly [10,11].

Increased adoption of low-dose spiral computed tomography has resulted in the increase of early-stage lung cancers being diagnosed [12]. Approximately 14.6–16.5% of patients with stage T1 NSCLC have subsequently been shown to have lymph node metastasis [13,14].

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Patients with positive lymph node metastasis have a higher risk of disease recurrence [15]; thus, nodal status is one of the most predominant factors in the accurate staging of patients with resectable NSCLC. Precise status of nodal metastases is the key to deciding whether adjuvant therapy (chemotherapy or radiotherapy) should be delivered [16,17]. Furthermore, identification of associated predictors for lymph node metastasis, would enable the improvement in the strategy of the appropriate extent of lymphadenectomy in early stage NSCLC [18,19].

In NSCLCs, increasing numbers of studies have focused on evaluating the association between lymph node metastasis, impact factors and drainage pattern [20,21]. There have been no studies specifically designed to evaluate which genotype of NSCLC is more likely to have lymph node metastasis. Therefore, an emerging issue concerning the lymph node metastasis in NSCLC is the identification of driver mutation markers for predicting patients who will most likely develop lymph node metastasis. This study aimed to determine the incidence and distribution of lymph node metastasis according to different driver mutations in stage T1 NSCLC.

2. Methods

2.1. Patients

From 2016 to 2018, we consecutively collected the patients with pathological stage T1 NSCLC tumors who underwent lobectomy/sublobectomy and lymph node dissection (including systematic lymph node dissection and lymph node sampling) at the First Affiliated Hospital of Guangzhou Medical University. All procedures performed were in accordance with the ethical standards of the Helsinki Declaration and applicable regulatory requirements. Written informed consent was obtained from all patients to permit genetic analysis of biological samples.

Inclusion criteria were: (1) single primary NSCLC; (2) the pathologic T stage of T1; (3) underwent anatomical resection combined with lymphadenectomy (systematic lymph node dissection or systematic lymph node sampling) compliant with the National Comprehensive Cancer Network (NCCN) criteria [22,23]; (4) all resected-tissues and lymph nodes were proven by final pathology with paraffin blocks; and (5) sufficient resected-tissue for mutational analyses. Patients were excluded for: (1) multiple lung cancer, (2) non-invasive cancer (e.g. adenocarcinoma in situ, minimally invasive adenocarcinoma) or small cell lung cancer, (3) preoperative neoadjuvant therapy, (4) preoperative diagnostic biopsy; and (5) local or distant metastasis.

2.2. Driver mutation analysis and grouping

Genomic driver gene mutation testing was performed in all included patients and only in resected tumor tissues. Gene alterations such as EGFR, HER2, MET, KRAS, BRAF, ALK, ROS1 and RET were tested by a targeted next-generation sequencing method [24,25]. For the purpose of better analyses and to assess the impact of mutant genes, based on the protein that mutant-genes encoded involved in the signaling pathway, we classified the genotypes into four distinct groups: upstream receptor mutant protein (EGFR, HER2 and MET), downstream regulator mutant protein (KRAS and BRAF), fusion mutant protein (ROS1, ALK and RET) and the wild type group.

2.3. Evaluation of node metastasis and further analysis

The incidence of lymph node metastasis (or lymph node metastatic rate) was defined as the number of patients with lymph node metastasis divided by the number of patients with resected lymph nodes. The incidence of lymph node metastasis was analyzed for each pathologic N stage (N1, skip N2 and N1 + N2) based on different genotype and mutation groups. Vascular invasion and lymph node ratio (LNR) are both adverse prognostic factors in NSCLC, therefore, the incidence of vascular invasion and LNR greater than 0.33 [26] were calculated according to different mutations. Subgroup analyses explored the incidence of lymph node metastasis in different mutation groups by gender, age and smoking history. The associations between lymph node metastasis and clinicopathological characteristics were also analyzed.

2.4. Statistical analysis

Statistical analysis was performed using SPSS statistics version 23.0 (IBM Corp, Armonk, NY). The enumeration data was described as number (percentage), and the measurement data was described as mean ± standard deviation (SD). Comparisons between categorical variables were performed using Pearson Chi-square test or Fisher's exact test, as appropriate. Non-parametric analysis (Mann-Whitney U test or Kruskal-Wallis test) was used for the ordinal / ranked variables and data of non-normal distribution. Variables that were statistically significant factors for lymph node metastasis by univariate analysis were used to for the multivariate logistic regression analysis. All statistical tests were two-sided and all tests were considered significant for P values below 0.05. The Bonferroni correction was also used for adjustment during multiple comparisons.

3. Results

3.1. Patients' characteristics

A total of 1052 patients met the inclusion criteria and were enrolled in this study. The baseline characteristics of the included cases are summarized in Table 1. Of the enrolled patients, 502 (47.7%) were female, 732 (69.6%) were never-smokers, and 526 (50.0%) were older than 60 years. The median tumor diameter was 2.0 cm (range of 0.3–3.0 cm). Adenocarcinoma (949, 90.2%) was the predominant pathological type.

A total of 1052 pathological stage T1 NSCLC cases were sequenced for driver mutations. The frequency of oncogenic driver mutations in NSCLC and in lung adenocarcinoma are presented in Supplementary Figure S1. EGFR mutations were detected in 550 patients (52.3%), KRAS mutation in 85 (8.1%), ALK rearrangement in 38 (3.6%), RET rearrangement in 13 (1.2%), ROS1 rearrangement in 11 (1.0%), while patients with alterations in BRAF, HER2 and MET accounted for less than 1.0%. These key mutations were mutually exclusive. The mutation frequency in lymph node negative and lymph node positive patient subgroups are provided in Supplementary Figure S2.

The mean number of lymph nodes examined of the entire cohort was 15.9 and the median number was 14.0. No significant difference in the number of examined lymph nodes was observed in patients who were lymph node negative and lymph node positive (mean number: 15.9 vs. 16.4, $P = 0.273$) (Supplementary Table S1). Likewise, the number of examined lymph nodes was comparable in different mutation groups ($P > 0.05$).

3.2. General incidence of lymph node metastasis

Lymph node involvement was confirmed in 197 (18.7%) patients: sixty cases (5.7%) were N1 positive only, 44 (4.2%) were skip N2 positive (N2 positive only), and 93 (8.8%) were N1 and N2 positive.

The incidence of lymph node status for each genotype is presented in Table 2 and Fig. 1A. The top three genotypes with the highest lymph node metastatic rate were ROS1 rearrangement (72.8%), BRAF mutations (55.5%) and ALK rearrangement (44.7%), respectively. Next were HER2 mutations (40.0%) and RET rearrangement (23.1%). The lymph node metastatic rate in EGFR mutations (15.3%) and KRAS mutations (15.3%) were relatively low and comparable. In patients with MET mutations, no lymph node metastasis was observed.

Table 1
Clinicopathologic characteristics of the included patients.

Variables	Number	%
All patients	1052	100
Age (Mean ± SD)	59.6 ± 10.4	
< = 60y	526	50.0
> 60y	526	50.0
Gender		
Male	550	52.3
Female	502	47.7
Smoking history		
Never	732	69.6
Smoker	320	30.4
Laterality		
Left	425	40.4
Right	627	59.6
Tumor size		
≤1cm	97	9.2
1-2cm	535	50.9
2-3cm	420	39.9
N stage		
N0	855	81.3
N1	60	5.7
Skip N2	44	4.2
N1 + N2	93	8.8
Histology		
Adenocarcinoma	949	90.2
Squamous cell carcinoma	58	5.5
Others	45	4.3
Vascular Invasion		
Absent	913	86.8
Present	139	13.2
Genotype		
Wild type	337	32.0
EGFR 19del	242	23.0
EGFR L858R	270	25.7
EGFR rare mutation	38	3.6
HER2 mutation	5	0.5
MET mutation	4	0.4
KRAS mutation	85	8.1
BRAF mutation	9	0.9
ALK rearrangement	38	3.6
ROS1 rearrangement	11	1.0
RET rearrangement	13	1.2

3.3. Lymph node metastasis for different mutation groups

The incidence of lymph node involvement stratified by different mutation groups was summarized in Table 3 and Fig. 1B.

Wild type group (n = 337): There were 65 (19.3%) lymph node positive patients. The incidence of N1, skip N2 and N1 + N2 positive were 5.0%, 3.6% and 10.7%, respectively.

Upstream receptor mutant protein group (n = 559): There were 86 (15.3%) lymph node positive patients. The incidence of N1, skip N2 and N1 + N2 positive were 4.8%, 3.0% and 7.5%, respectively.

Downstream regulator mutant protein group (n = 94): There were 18 (19.1%) lymph node positive patients. The incidence of N1, skip N2 and N1 + N2 positive were 7.4%, 6.4% and 5.3%, respectively.

Fusion mutant protein group (n = 62): There were 28 (45.1%) lymph node positive patients. The incidence of N1, skip N2 and N1 + N2 positive were 14.5%, 14.5% and 16.1%, respectively.

Lymph node status had remarkably higher positive results in the fusion mutant protein group compared with others (all $P < 0.001$). However, the incidence of lymph node metastasis in the upstream receptor mutant protein, downstream regulator mutant protein and wild type group were similarly comparable. In addition, the lymph node metastatic rate of the fusion mutant protein group remained the highest, regardless of lymph node status (N1, skip N2 or N1 + N2). Further investigation of lymph node metastatic rate by lymph node stations for different mutation groups are shown in Supplementary Table S2. In most stations, high incidence of lymph node metastasis was observed in the fusion mutant protein group.

3.4. Further analysis

The incidence of vascular invasion and LNR > 0.33 can be seen in Fig. 2A and B. Vascular invasion occurred most frequently in patients with fusion mutations compared with other three groups ($P < 0.002$). When further stratified by lymph node status, fusion mutations were observed with the highest rate of vascular invasion in both the lymph node positive and negative group (Supplementary Figure S3). Similarly, patients with fusion mutant protein also showed a higher percentage of LNR > 0.33 compared to others ($P < 0.004$). In the entire cohort, ROS1-positive patients showed the highest proportion of vascular invasion as well as LNR > 0.33.

In subgroup based on gender, age and smoking history, the fusion mutant protein group was still observed with the highest proportion of lymph node metastasis (Supplementary Figure S4).

There was a significant association between lymph node involvement and age, smoking history, tumor size, vascular involvement, genotype and mutant protein on the univariate analysis (Supplementary Table S3). The following genes were associated with a significantly increased risk of lymph node involvement in multivariate analyses (Supplementary Table S4): ROS1 rearrangement ($P < 0.001$), BRAF mutation ($P = 0.005$), and ALK rearrangement ($P = 0.023$). When grouped by mutant protein, a significantly higher risk of nodal involvement was observed in fusion mutant protein group in the multivariate analysis ($P = 0.002$).

4. Discussion

In this study, we collected real-world data and performed an

Table 2
The incidence of different lymph node status for specific genotype (n = 1052).

	N status					
	N0 (%)	N1 (%)	Skip N2 (%)	N1 + N2 (%)	Total (%)	P-value
Genotype						< 0.001*
Wild type	272 (80.7%)	17 (5.0%)	12 (3.6%)	36 (10.7%)	337 (100%)	
EGFR	466 (84.7%)	27 (4.9%)	16 (2.9%)	41 (7.5%)	550 (100%)	
HER2	3 (60.0%)	0	1 (20.0%)	1 (20.0%)	5 (100%)	
MET	4 (100.0%)	0	0	0	4 (100%)	
KRAS	72 (84.7%)	5 (5.9%)	5 (5.9%)	3 (3.5%)	85 (100%)	
BRAF	4 (44.4%)	2 (22.2%)	1 (11.1%)	2 (22.2%)	9 (100%)	
ALK	21 (55.3%)	6 (15.8%)	7 (18.4%)	4 (10.5%)	38 (100%)	
ROS1	3 (27.3%)	2 (18.2%)	2 (18.2%)	4 (36.4%)	11 (100%)	
RET	10 (76.9%)	1 (7.7%)	0	2 (15.4%)	13 (100%)	

Incidence as the number of patients with accordingly lymph node status divided by the number of patients with specific genotype.
* Statistical analysis performed by Kruskal-Wallis test.

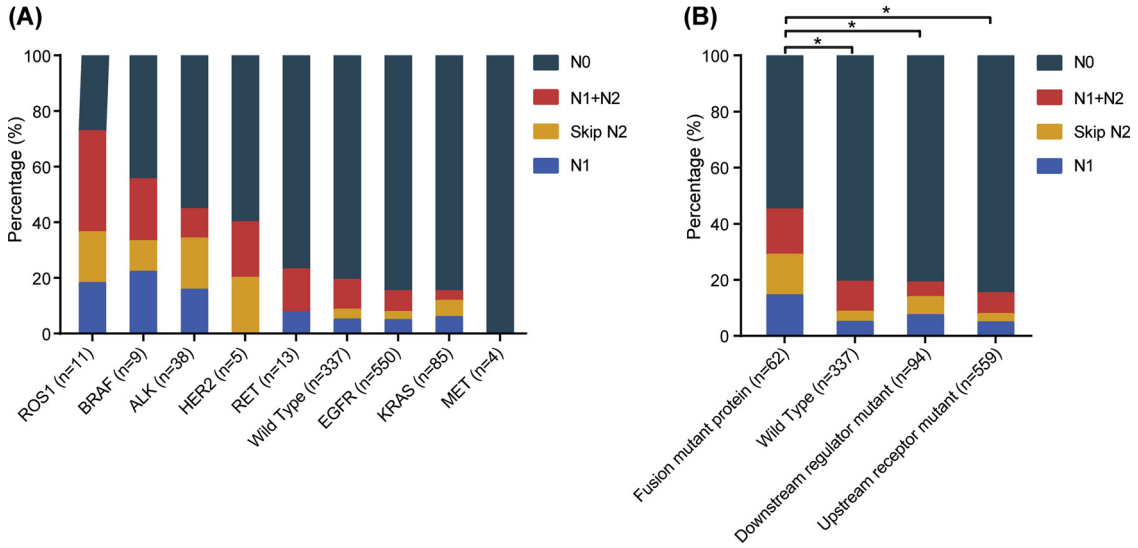

Fig. 1. The distribution of lymph node status according to **(A)** different genotypes and **(B)** different mutant-protein groups. The N0, N1, skip N2 and N1 + N2 patients are shown in different colors. *Denotes significant difference ($P < 0.001$, Statistical analysis performed by Kruskal-Wallis test).

analysis of the incidence and distribution of lymph node metastasis between different well-identified driver mutations among stage T1 NSCLC. The incidence of lymph node metastasis was significantly higher in the fusion mutant protein group compared with others. Further analysis also showed fusion genes have a higher rate of vascular invasion and LNR > 0.33. Additionally, fusion mutation was a significant risk factor for lymph node metastasis. To our knowledge, this is the first and largest study to comprehensively investigate which genotype of NSCLC is more likely to have lymph node metastasis. The results showed discrepant lymph node status in different genotypes of NSCLC, suggesting that the genomic features might play an important role in heterogeneous lymph node metastasis.

Mutations of driver genes contribute to oncogenesis and progression of tumor. In particular, mutation status of early stage NSCLC may help identify groups of patients with small size tumors at high risk for lymph node metastasis that may receive the most benefit from adjuvant therapy. Genetic factors associated with an increased risk of lymph node metastasis are not certain. Consistent with our results, Li et al. [27] performed a retrospective study including 675 patients with early stage lung adenocarcinomas, the results suggested that ALK rearrangement was more common in those with lymph node metastasis compared with EGFR mutations, while no significance difference was observed between EGFR, KRAS and wild type mutations in terms of node metastasis. Another study of patients with completely resected stage IA lung adenocarcinoma by Shin et al. [28] reported that ALK rearrangement was associated with more regional lymph node metastasis and unfavorable disease-free survival compared to ALK-negative patients. In a prevalence analysis [29] of ROS1 fusion, ROS1 status was significantly correlated with lymph node metastasis and ROS1 positive was found higher in patients at advanced node stages. Similarly, another study [30] reported that patients harboring RET fusion gene tended to present with more N2 stage (54.5%) in small tumors (≤3 cm), significantly higher than the other lung adenocarcinomas without RET fusion (22.6%).

In our study, we included more patients and have more target genes involved. Only patients with pathological T1 NSCLC were included, because a larger tumor is correlated with a higher risk for the development of lymph node metastasis. A targeted next-generation sequencing method was used for this mutational analysis in 1052 NSCLC patients. The frequencies of driver mutations in our study were similar to those reported by multinational epidemiologic studies [24,31–34] in NSCLC among East Asian populations, which ensured the reliability of our study. The change in the frequency of driver mutations from node negative to node positive disease indicated that some oncogenic mutations occur during the carcinogenetic process from less aggressive to more aggressive tumors. The molecular characteristics reported by other studies [35–38] also support the multistep tumorigenesis and progression in lung cancers.

The highest-ranking node metastatic rate of different genotypes was ROS1 rearrangement (72.8%) in our study. Our study showed that the lymph node metastasis exhibited mainly in fusion mutations. When further stratified by age, gender and smoking history, patients with fusion mutations still showed a higher node-metastatic rate compared with other mutations. The presence of fusion genes was a statistically significant predictor of lymph node metastasis. Tumor-associated lymph node involvement is a complicated process, different genotypes present with different genomic features, lymph node metastasis may be driven by gene mutations as well as signaling pathways activated by

Table 3
The incidence of different lymph node status for different mutation types (n = 1052).

	N status					
	N0 (%)	N1 (%)	Skip N2 (%)	N1 + N2 (%)	Total (%)	P-value
Mutation Type						< 0.001*
Wild type	272 (80.7%)	17 (5.0%)	12 (3.6%)	36 (10.7%)	337 (100%)	
Upstream receptor mutant	473 (84.6%)	27 (4.8%)	17 (3.0%)	42 (7.5%)	559 (100%)	
Downstream regulator mutant	76 (80.9%)	7 (7.4%)	6 (6.4%)	5 (5.3%)	94 (100%)	
Fusion mutant protein	34 (54.8%)	9 (14.5%)	9 (14.5%)	10 (16.1%)	62 (100%)	

Incidence as the number of patients with accordingly lymph node status divided by the number of patients with specific mutation type.
* Statistical analysis performed by Kruskal-Wallis test.

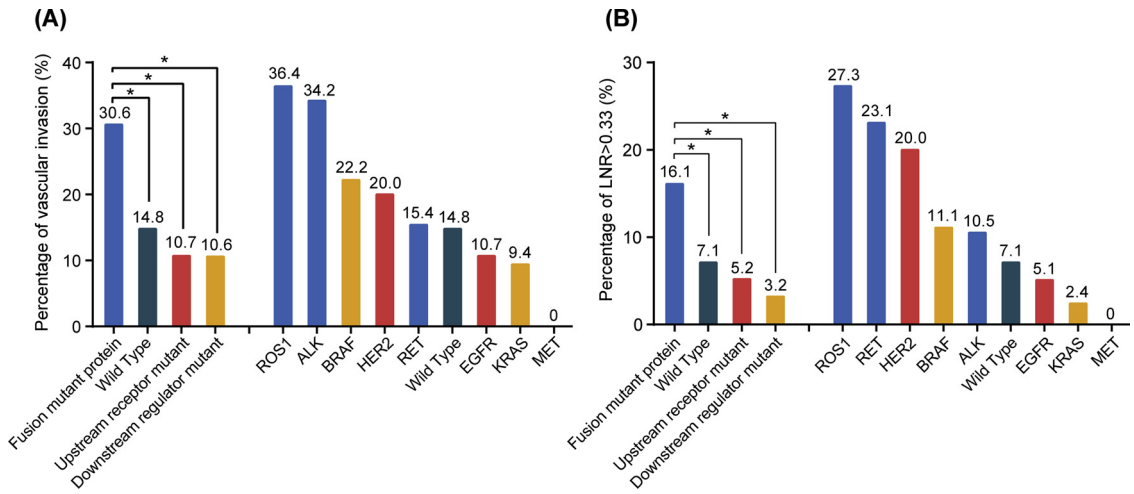

Fig. 2. The incidence of **(A)** vascular invasion and **(B)** lymph node ratio (LNR) > 0.33 by different mutant-protein groups and different genotypes. Patients with different mutation types are shown in different colors. *Denotes significant difference.

gene alterations. Previous studies [30,39–42] also have reported that NSCLC harboring ROS1, ALK or RET fusions share some clinicopathological characteristics, such as young age of onset and light / never smoking history, suggesting that similar routes of oncogenesis might exist in these fusion subtypes of NSCLC. Tumors activated by fusion mutations tend to have lower mutational burden and less neoantigens [43], which may lead to the disorders of immune recognition against cancer and promote the metastasis of lymphatic drainage through immune escape.

Staging of lymph node metastasis is essential and a prognostic factor to determine the extent of disease and consequently the patient's prognosis. With advances in lung cancer screening, more pulmonary nodules and mutations were detected at early stage. The necessary extent of intraoperative lymph node dissection in small size tumors (stage T1) is a controversial issue. Therefore, identification of associated mutation prediction factors regarding the risk of lymph node metastasis would be helpful.

In addition, this study showed a discrepancy of vascular invasion between different gene mutations. Patients with fusion mutant genes showed a higher propensity of lymph node metastasis and higher incidence of vascular invasion. Furthermore, fusion mutations were observed with a significantly higher rate of vascular invasion in the lymph node positive group compared with the node negative group, which indicates that fusion mutations may promote the metastasis of lymph node through more vascularization in tumors. Overall, these results suggested that lymph node status and the vascular invasion were heterogeneous in different gene mutated NSCLC, which illustrated that the existence of different genomic characteristics may potentially predict risk of node or vascular metastases. Our findings may be useful by providing information of the risk of lymph node metastasis and thus allow recommendations for further therapeutic approaches in patients found harboring driver gene mutations, especially those that have undergone limited resection without complete dissection of the regional and mediastinal lymph nodes. According to our results, more caution should be put on patients with ROS1 rearrangement, BRAF mutations and ALK rearrangement which presented with the highest incidence of node metastasis (72.8%, 55.5% and 44.7%, respectively). Therefore, enhancement of subsequent treatments (e.g. adjuvant targeted therapy / chemotherapy / radiotherapy) may be advised for these patients. Moreover, when patients with early stage NSCLC were identified with fusion mutations by biopsy, more radical and complete nodal dissection would be advisable according to our findings.

To date, data in the literature is not sufficient to conclude which genotype of NSCLC is more likely to have lymph node metastases, we believe that information on this essential topic is scarce and our study contributes important data to this issue. Furthermore, our findings may facilitate the understanding of the mechanisms of lymph node metastasis. Further studies with larger cohorts are warranted, as well as research into additional molecular factors that might be responsible for more or less aggressive lymph node metastasis and vascular spreading.

Some limitations of this study exist that must be taken into account: first, it is a monocentric study; second, the sample size may limit the statistical power, though, it should be noted that, this is the largest study to describe detailed lymph node metastasis status in driver mutant NSCLC; third, it is a retrospective study, however, to ensure the accuracy and comparability of data, we tried to limit confounding factors: the patients were consecutively enrolled and no mutational status was known prior to the resection. All included patients underwent uniform and standard anatomical resection with lymphadenectomy fulfilling the NCCN criteria [22]. Based on our previous research [44], a greater number of lymph nodes sampled is associated with more-precise node staging and the examination of more lymph nodes can reduce risk of undetected positive lymph nodes. The mean number of examined lymph nodes was high (15.9 in our study), which confirmed the accuracy of the staging. In addition, no significant difference in the examined lymph node count was found between lymph node negative and lymph node positive groups, or between different mutation groups. Additionally, we only included pathological T1 NSCLC because small tumors (3 cm or less) in early stage disease may provide a more realistic reflection of the actual functions of gene alternations. Finally, we believe that standard surgery and careful data collection in this study permit a strong and valid statistical analysis.

5. Conclusion

Different gene mutation features might contribute to the heterogeneous propensity of lymph node metastasis in patients with NSCLC. Cases of fusion mutations had a higher risk of lymph node metastasis than other mutations, which suggested that careful surveillance for recurrence and enhanced treatment should be applied for this subset of patients.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.06.026.

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