Article

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Clinical and Molecular Characteristics Associated With Pleural Metastasis in Advanced Non-Small Cell Lung Cancer

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Background: Pleural metastasis (PM), a driver of malignant pleural effusion (MPE) in lung cancer, remains an unmet clinical need due to the absence of PM/MPE-specific targeted therapies. Therefore, this study aims to explore the clinical and molecular characteristics associated with PM in advanced non-small cell lung cancer (NSCLC).
Methods: This retrospective study analyzed 195 advanced NSCLC patients. Study participants were classified into the PM and non-PM groups based on the occurrence of PM within three years of the initial diagnosis of advanced-stage disease. Clinical and baseline data, including age, gender, smoking history, tumor location, histological classification, tumor-node-metastasis staging (TNM) staging, metastasis profile, and lesion count, were obtained from the hospital's electronic medical records. Genomic DNA was extracted from tumor or liquid biopsy samples, and next-generation sequencing (NGS) was performed using a 139-gene lung cancer panel.
Results: The PM group tended to be older, smoked less, and presented with higher T stages, experiencing less bone metastasis ($p < 0.05$). Mutation frequencies of anaplastic lymphoma kinase (*ALK*), Erb-B2 receptor tyrosine kinase 4 (*ERBB4*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and RB transcriptional corepressor 1 (*RB1*) were significantly lower in the PM group ($p < 0.05$) than those in the non-PM group. However, epidermal growth factor receptor (*EGFR*) driver mutations were significantly higher in the PM group than in the non-PM group ($p < 0.05$). The incidence of PM was highest (73.5%) in patients carrying only *EGFR* driver mutations, without *ALK*, *ERBB4*, *KRAS*, or *RB1* mutations.
Conclusion: This study identified potential clinical and molecular factors involved in the development of PM in advanced NSCLC, providing valuable insights for future mechanistic research.

Keywords: non-small cell lung cancer; pleural metastasis; clinical factors; molecular characteristics; *EGFR*

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, accounting for an estimated 2.2 million new cases and 1.8 million mortalities in 2020 [1,2]. Pleural metastasis (PM), either pleural nodules or effusion, is classified as stage IV disease, indicating an incurable condition [3]. The pleural membranes consist of two layers, the visceral pleura covering the lungs and the parietal layers lining the thoracic cavity. These layers together enclose the pleural space, a sterile and protected environment with lymphatic channels that recycle normal pleural fluid. PM commonly leads to malignant pleural effusion (MPE), a common, clinically significant manifestation characterized by the accumulation of malignant fluid within the pleura. While not all cases of PM present with MPE, its presence typically suggests underlying pleural dissemination in advanced malignancies. MPE significantly contributes to mortality in patients with metastatic pleural tumors, with nearly two million people affected annually [4].

Epidemiological evidence reveals that about 15% of lung cancer patients present with MPE at the time of initial diagnosis, and approximately 50% of those without pleural effusion (PE) develop MPE during advanced stages of the disease [5]. Although nearly all tumors can lead to MPE, over 75% of cases originate from lung, breast, and ovarian cancers, with lung cancer-associated MPE exhibiting the worst survival outcomes [6]. Furthermore, the presence of MPE not only impacts the prognosis but also significantly limits the treatment options [7]. Currently, treatment strategies for patients with PM and MPE primarily involve aggressive management of the primary tumor, pleural fluid

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drainage, and antibiotic administration. However, there are no targeted therapeutic agents specifically for PM or MPE, which significantly impairs both the quality of life and overall survival of lung cancer patients [8].

In recent years, the clinical management of lung cancer has gradually incorporated immunotherapy and targeted therapies based on small-molecule inhibitors, offering new options to improve patient outcomes. However, compared with advances in systemic therapy, research on PM remains limited, particularly in terms of its molecular mechanisms and clinical implications. Investigating the underlying causes and pathogenic mechanisms of PM to identify novel diagnostic biomarkers and therapeutic targets has emerged as a major research priority [9]. During the progression of pleural metastasis, the development of MPE is primarily attributed to cancer cell invasion into the pleura, which, under the influence of immune-inflammatory responses, increases the microvasculature permeability of the visceral and parietal pleura, leading to substantial fluid leakage into the pleural cavity [10]. Additionally, cancer cell infiltration of the lymphatic system disrupts lymphatic drainage, exacerbating fluid accumulation [11]. Pathogenic mechanisms of MPE also involve neovascularization, increased permeability of both existing and newly formed vessels, and tumor cell-induced inflammatory responses within the pleural cavity [12]. Within the pleural microenvironment, tumor cells and host immune cells produce various pro-angiogenesis and pro-inflammatory mediators, such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), which play significant roles in the pathogenesis of MPE [13].

The relationship between genetic alterations and PM largely remains unexplored, with most existing studies investigating this association indirectly through MPE. For example, a retrospective single-center study reported that, among stage IV lung adenocarcinoma (LUAD) patients, the frequency of epidermal growth factor receptor (*EGFR*) driver mutations was higher in those with MPE than in those without MPE [14]. Specifically, the *EGFR* L858R mutation may promote the formation of MPE through the activation of the C-X-C motif chemokine ligand 12-C-X-C chemokine receptor type 4 (CXCL12-CXCR4) signaling pathway [15]. More recently, single-cell RNA sequencing analysis showed that the expression level of the Claudin-4 (*CLDN4*) gene was elevated in non-small-cell lung cancer (NSCLC) patients with recurrent MPE compared to those without recurrence, indicating that *CLDN4* may be involved in MPE pathogenesis [16].

Overall, the potential mechanisms underlying PM in NSCLC largely remain uninvestigated. In this study, we retrospectively enrolled 195 patients with advanced NSCLC. Clinical characteristics and molecular profiles derived from next-generation sequencing (NGS) were compared between PM and non-PM patients, aiming to investigate potential factors associated with PM and to provide new insights into the molecular mechanisms that may contribute to its occurrence.

Materials and Methods

Enrollment of Study Participants

This study was conducted following the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Zhejiang Cancer Hospital (IRB-2025-275 (11T)). Furthermore, written informed consent was obtained from all participants. The study cohort consisted of 195 patients newly diagnosed with stage IV non-small-cell lung cancer (NSCLC) and admitted to Zhejiang Cancer Hospital, China, between July 2018 and July 2021. Clinicopathological information was obtained from the electronic medical records of the hospital. Patients were divided into the PM and non-PM groups based on whether PM developed within 3 years of the initial stage IV NSCLC diagnosis [17]. MPE was confirmed when cancer cells were definitively identified in the pleural fluid by cytopathological examination.

The criteria for patient inclusion were defined as follows: (1) patients with confirmed histopathological diagnosis; (2) those with complete clinical diagnostic data; (3) patients with molecular sequencing results acquired at the time of stage IV lung cancer who received no systemic antitumor therapy before the specimen collection for genetic testing; (4) patients newly diagnosed with stages IV NSCLC, or those with recurrence or metastasis occurring more than 1 year after completion of adjuvant antitumor therapy following surgery and confirmed as stage IV at the time of the first recurrence; (5) patients with identification of PE at initial presentation; and (6) those with a minimum follow-up period of 3 years. The exclusion criteria were as follows: (1) patients with uncontrolled severe infections; (2) those with immune or hematologic conditions unrelated to the tumor; (3) those with congenital diseases or genetic disorders; (4) those with history of severe allergies; and (5) those diagnosed with a second primary tumor.

Identification of MPE

MPE identification was confirmed through cytopathological examination. Briefly, pleural effusion samples were collected via thoracentesis using sterile aspiration kits, followed by centrifugation at 1500 rpm for 10 minutes. The resulting cell pellet was smeared onto slides, fixed, and underwent cytological staining using the Papanicolaou method. Furthermore, morphological assessment was independently conducted by two board-certified pathologists, and MPE diagnosis was confirmed based on cytological features. Clinical data and radiological findings were also reviewed to support the cytological diagnosis.

Collection of Clinical Information

Clinical data for all 195 patients were collected from electronic medical records and included age, gender, smoking history, tumor location, histological classification, TNM staging, metastasis profile, and lesion count. The number of lesions was defined as the sum of the primary tumor and all metastatic lesions found in both lungs.

Targeted DNA Sequencing and Bioinformatics Analysis

Out of the 195 patients, 192 provided tumor tissue or body fluid samples for molecular sequencing analysis. Sample sources included lung or pleural tumor tissue in 51 cases, pleural fluid in 84 cases, and other tissues or fluids in 57 cases, comprising 28 liver metastases, 16 bone metastases, 11 adrenal metastases, one pericardial effusion, and one cerebrospinal fluid specimen. NGS was performed at a Clinical Laboratory Improvement Amendments (CLIA)- and College of American Pathologists (CAP)-certified laboratory (Nanjing Geneseeq Technology Inc., Nanjing, China). Genomic DNA was extracted from tumor or liquid biopsy samples and genetically analyzed using a 139-gene lung cancer panel (Pulmocan®, Nanjing Geneseeq Technology Inc., Nanjing, China). Sequencing was performed on the Illumina HiSeq 4000 NGS platforms (Illumina, Inc., San Diego, CA, USA), achieving a targeted mean coverage depth of $\geq$500× for tumor tissue DNA and $\geq$5000× for cfDNA from liquid biopsy samples.

Sequencing data were demultiplexed using bcl2fastq (v2.19, Illumina, Inc., San Diego, CA, USA) and analyzed with Trimmomatic to remove low-quality bases (quality score <15) or N bases. Then, the data were aligned to the human reference genome (hg19) employing the Burrows-Wheeler Aligner (bwa-mem) and further processed using the Picard suite and the Genome Analysis Toolkit (GATK). Single-nucleotide variants (SNVs) and insertion/deletions (indels) were detected using VarScan2 and HaplotypeCaller/UnifiedGenotyper in GATK. Common SNPs were excluded by referencing the dbSNP and 1000 Genome data sets.

A mutation was called when the mutant allele frequency (MAF) cutoff was $\geq$0.5% for tissue samples, 0.1% for liquid biopsy samples, with a minimum of at least three unique mutant reads on different strands, high-quality scores, and manual verification in Integrative Genomics Viewer Software (IGV, Broad Institute, Cambridge, MA, USA). Gene fusions were identified using FACTERA, and copy number variation (CNV) was analyzed with ADTEx. The log2 ratio cut-off for copy number gain was defined as 2.0 for tissue samples and 1.6 for cfDNA samples. However, a log2 ratio cut-off of 0.6 was used for copy number loss in all sample types. Following quality control, six patients either experienced sequencing failures or did not meet the analysis criteria, leaving 186 patients for further molecular analysis.

Statistical Analysis

Statistical analysis was conducted using SPSS 26.0 (IBM Corp., Armonk, NY, USA). All variables were presented as categorical data, expressed as the number of patients (percentage). Group comparisons were performed using the *Chi-square* test or the Continuity Correction *Chi-square* test, as appropriate. Moreover, trends in the incidence rates between different groups were evaluated using a two-sided Cochran-Armitage test. A p-value < 0.05 was considered statistically significant.

Results

Comparison of Demographics Between the PM and Non-PM Groups

Based on whether PM developed within three years of the initial diagnosis of advanced lung cancer, patients were divided into two groups: the PM group (n = 117) and the non-PM group (n = 78). The median age was 64 years in the PM group and 62 years in the non-PM group. In the PM group, 58 patients (49.6%) were aged $\geq$65 years, 73 (62.4%) were females, and 30 (25.6%) had a history of smoking. In the non-PM group, 25 cases (32.1%) were aged $\geq$65 years, 41 (52.6%) were females, and 35 (44.9%) had a smoking history (Table 1). Furthermore, there was no statistically significant difference in gender distribution between the two groups ($p > 0.05$). However, a substantial difference was observed for age $\geq$65 years and smoking history, with the PM group having a higher proportion of older patients and a lower rate of smoking history ($p <$ 0.05, Table 1).

Comparison of Clinical Characteristics Between the PM and Non-PM Groups

No statistically significant differences were observed between the PM and non-PM groups regarding histological subtypes, N staging, liver and brain metastasis, tumor location, number of tumors, and presence of bilateral lung tumors ($p > 0.05$). However, significant differences were found in T staging and the rate of bone metastasis, with the PM group exhibiting a higher T stage but a lower rate of bone metastasis ($p < 0.05$, Table 1). These findings suggest that a larger tumor size may be more likely to lead to PM. Throughout the entire disease course, the incidence of bone metastasis was 28.2% in patients with PM compared to 62.8% in those without PM ($p < 0.05$).

Comparison of Gene Alteration Characteristics Between the PM and Non-PM Groups

Of the total of 195 patients, 192 provided tumor tissues or body fluid samples for NGS analysis. After NGS and bioinformatics quality control analysis, 186 cases were included in the subsequent mutation analysis, consisting of 112 patients in the PM group and 74 in the non-PM group. The genomic landscape of these 186 patients is depicted in Fig. 1.

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Table 1. Comparison of demographics and clinical characteristics between the PM and non-PM groups.

Characteristics		PM group (n = 117)	Non-PM group (n = 78)	χ^2	*p*-value
Age				5.877	0.015
<65 y		59 (50.4%)	53 (67.9%)		
≥65 y		58 (49.6%)	25 (32.1%)		
Gender				1.862	0.172
Female		73 (62.4%)	41 (52.6%)		
Male		44 (37.6%)	37 (47.4%)		
Smoking history				8.248	0.016
Smoker		30 (25.6%)	35 (44.9%)		
Non-smoker		86 (73.5%)	43 (55.1%)		
Unknown		1 (0.9%)	0 (0.0%)		
Histological classification				0.949	0.622
LUAD		114 (97.4%)	74 (94.9%)		
LUSC		2 (1.7%)	3 (3.8%)		
Unknown		1 (0.9%)	1 (1.3%)		
T staging				10.837	0.028
x		14 (12.0%)	16 (20.5%)		
1		23 (19.7%)	26 (33.3%)		
2		46 (39.3%)	21 (26.9%)		
3		12 (10.3%)	8 (10.3%)		
4		22 (18.8%)	7 (9.0%)		
Lymph node (N staging)				0.994	0.608
x		1 (0.9%)	2 (2.6%)		
0		25 (21.4%)	15 (19.2%)		
≥1		91 (77.8%)	61 (78.2%)		
Metastatic site					
Bone	Yes	33 (28.2%)	49 (62.8%)	23.012	<0.001
	No	84 (71.8%)	29 (37.2%)		
Liver	Yes	16 (13.7%)	17 (21.8%)	2.195	0.138
	No	101 (86.3%)	61 (78.2%)		
Brain	Yes	17 (14.5%)	18 (23.1%)	2.321	0.128
	No	100 (85.5%)	60 (76.9%)		
Adjacent to pleura				0.716	0.699
Yes		18 (15.4%)	13 (16.7%)		
No		98 (83.8%)	65 (83.3%)		
Unknown		1 (0.9%)	0 (0.0%)		
Number of Lung Tumor				6.369	0.095
0		0 (0.00%)	3 (3.8%)		
1		68 (58.1%)	38 (48.7%)		
≥2		48 (41.0%)	37 (47.4%)		
Unknown		1 (0.9%)	0 (0.0%)		
Distribution of tumors in lungs				4.004	0.135
Single		79 (67.5%)	42 (53.8%)		
Bilateral		36 (30.8%)	33 (42.3%)		
Unknown		2 (1.7%)	3 (3.8%)		

PM, pleural metastasis; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

In the PM group, the most frequent alterations included mutations in *EGFR* (69.6%), tumor protein p53 (*TP53*) (60.7%), and catenin beta-1 (*CTNNB1*) (9.8%), along with copy number variation (CNV) in *EGFR* (17.0%) and *MYC* (10.7%). The non-PM group displayed high-frequency mutations in *TP53* (73.0%), *EGFR* (56.8%), anaplastic lymphoma kinase (*ALK*) (12.2%), RB transcriptional corepressor 1 (*RB1*) (12.2%), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (10.8%), and ROS proto-oncogene 1 (*ROS1*) (10.8%), as well as CNV in *EGFR* (12.2%) and *MYC* (9.5%). Comparative details of gene variations between the PM and non-PM groups are presented in Table 2, which shows the genes altered in at least three patients in either the PM or non-PM group.

Furthermore, the PM group exhibited significantly lower mutation frequencies in *ALK,* Erb-B2 receptor tyro-

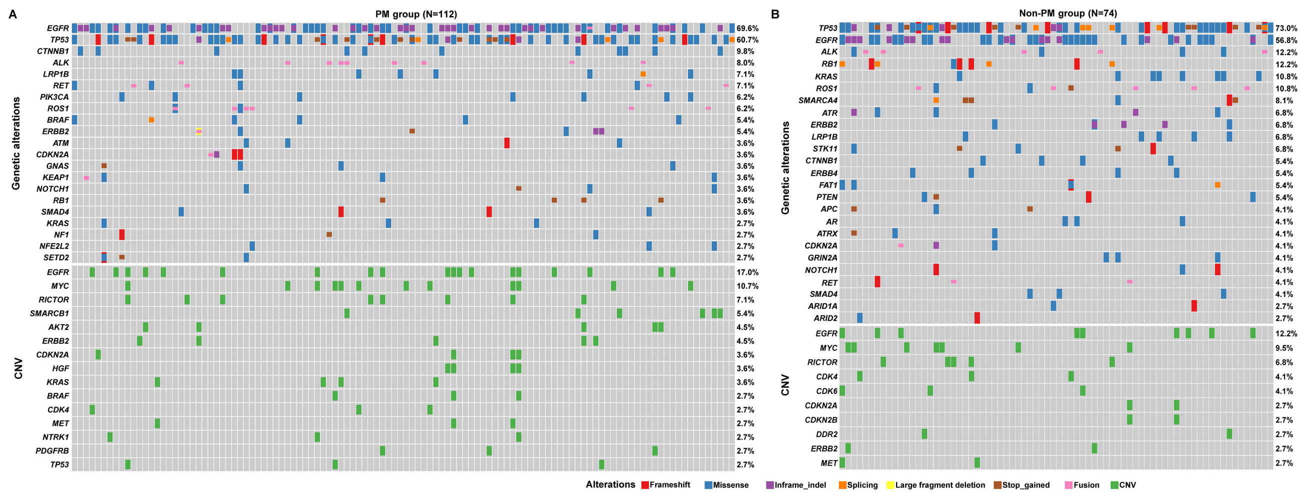

Fig. 1. Genomic landscape of 186 patients who underwent NGS analysis on tumor tissue or body fluid samples. Genetic profile of (A) the genes altered in at least three patients in the PM group (n = 112), and (B) the genes altered in at least two patients in the non-PM group (n=74). PM, pleural metastasis; CNV, copy number variation; indel, insertion and deletion; NGS, next-generation sequencing.

sine kinase 4 (*ERBB4), and KRAS, RB1* compared to the non-PM group ($p < 0.05$). Conversely, the frequency of *EGFR* mutations was higher in the PM group, although this trend did not reach statistical significance ($p = 0.072$). Based on its clinical relevance, *EGFR* was still considered a crucial gene despite the lack of statistical significance.

Analysis of EGFR Driver Mutations in the PM and Non-PM Groups

We analyzed the distribution of *EGFR* driver mutations between the two groups, including *EGFR* L858R, exon 19 deletion (19del), exon 20 insertion (20 ins), L861Q, G719X, and S768I. The frequency of *EGFR* driver mutations was significantly higher in the PM group than the non-PM group [73.21% (82/112) vs 56.76% (42/74), $p < 0.05$].

Integrated Analysis of the Molecular Factors

Since the positive rates of many gene mutations were 0, the data did not meet the conditions for Logistic regression analysis. Therefore, we conducted an integrated analysis of all molecular alterations concerning the occurrence of PM.

As shown in Table 3, the highest incidence of PM (73.5%) was observed in those harboring only *EGR* driver mutations without coexisting *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations. When both *EGFR* driver mutations and *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations were either concurrently present or absent, the PM incidence rates were similar, at 63.1% and 65.2%, respectively. The lowest incidence of PM (42.2%) was observed in those harboring only *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations without *EGR* driver mutations. Overall, the incidence of PM varied significantly across the four groups ($p < 0.05$, Table 3).

Discussion

To explore the potential factors influencing PM and the underlying molecular mechanisms, we compared the clinicopathological characteristics and molecular features of advanced NSCLC patients with and without PM. Clinically, patients aged $\geq$65 years and non-smokers were more likely to develop PM. Furthermore, larger tumor size was also associated with an increased risk of PM. Older age is often accompanied by reduced systemic immunity [18], while larger tumors may be more prone to direct invasion to adjacent pleural tissue, making PM more likely under these conditions. Of particular interest was the relationship of PM with smoking history and bone metastasis. A study published in *Cell* (2020) suggested that lung cancer in non-smokers represents a different and unique disease entity compared to that in smokers, with distinct pathogenic mechanisms and molecular pathways, potentially leading to different responses to targeted drugs [19]. However, due to the scarcity of research comparing the differences in metastatic organ patterns between smokers and non-smokers, the specific characteristics of distant metastasis in these two groups remain unclear. Our findings indicated that smokers exhibited a lower incidence of PM. The underlying mechanisms of these differences could have significant implications for NSCLC treatment and possess high potential research value.

The biological susceptibility to specific organ metastases may vary across molecular subgroups. Understanding these differences could be meaningful for thorough metastatic screening and for guiding preventive and treatment decision-making. The 2021 NCCN guidelines further emphasize the clinical requirement of evaluating multiple gene statuses before initiating NSCLC treatment, and simultaneous detection of multiple gene mutations has grad-

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Table 2. Comparison of gene alterations between the PM and non-PM groups.

Gene	PM group		Non-PM group		χ^2	*p*-value
	No. of WT	No. of alter.	No. of WT	No. of alter.		
ALK mut.	112	0	70	4	3.885	0.049 [a]
ERBB4 mut.	112	0	70	4	3.885	0.049 [a]
KRAS mut.	109	3	66	8	3.935	0.047 [a]
RB1 mut.	108	4	65	9	5.059	0.025
ATR mut.	111	1	69	5	3.209	0.073 [a]
STK11 mut.	111	1	69	5	3.209	0.073 [a]
SMARCA4 mut.	110	2	68	6	2.928	0.087 [a]
GRIN2A mut.	112	0	71	3	2.414	0.120 [a]
FAT1 mut.	111	1	70	4	1.958	0.162 [a]
EGFR mut.	34	78	32	42	3.232	0.072
TP53 mut.	44	68	20	54	2.967	0.085
ATM mut.	108	4	74	0	1.270	0.260 [a]
PTEN mut.	110	2	70	4	0.890	0.345 [a]
BRAF mut.	106	6	73	1	1.023	0.312 [a]
KEAP1 mut.	109	3	74	0	0.680	0.409 [a]
NFE2L2 mut.	109	3	74	0	0.680	0.409 [a]
APC mut.	111	1	71	3	0.880	0.348 [a]
AR mut.	111	1	71	3	0.880	0.348 [a]
ATRX mut.	111	1	71	3	0.880	0.348 [a]
PIK3CA mut.	105	7	72	2	0.569	0.451 [a]
ROS1 mut.	110	2	71	3	0.224	0.636 [a]
CTNNB1 mut.	101	11	70	4	1.172	0.279
GNAS mut.	108	4	73	1	0.205	0.650 [a]
RET mut.	108	4	73	1	0.205	0.650 [a]
ERBB2 mut.	106	6	69	5	0.006	0.937 [a]
ROS1 fusion	106	6	69	5	0.006	0.937 [a]
ALK fusion	103	9	69	5	0.105	0.746
RET fusion	108	4	72	2	0.000	1.000 [a]
LRP1B mut.	104	8	69	5	0.010	0.919
NOTCH1 mut.	108	4	71	3	0.000	1.000 [a]
SMAD4 mut.	108	4	71	3	0.000	1.000 [a]
ASXL1 mut.	108	4	72	2	0.000	1.000 [a]
CDKN2A mut.	109	3	72	2	0.000	1.000 [a]
NF1 mut.	109	3	72	2	0.000	1.000 [a]
SETD2 mut.	109	3	73	1	0.009	0.925 [a]
SMARCB1 CNV	106	6	74	0	2.560	0.110 [a]
HGF CNV	108	4	74	0	1.270	0.260 [a]
AKT2 CNV	107	5	74	0	1.903	0.168 [a]
BRAF CNV	109	3	74	0	0.680	0.409 [a]
PDGFRB CNV	109	3	74	0	0.680	0.409 [a]
CDK6 CNV	111	1	71	3	0.880	0.348 [a]
EGFR CNV	93	19	65	9	0.804	0.370
KRAS CNV	108	4	73	1	0.205	0.650 [a]
CDK4 CNV	109	3	71	3	0.009	0.924 [a]
ERBB2 CNV	107	5	72	2	0.050	0.823 [a]
MYC CNV	100	12	67	7	0.076	0.782
RICTOR CNV	104	8	69	5	0.010	0.909
CDKN2A CNV	108	4	72	2	0.000	1.000 [a]
MET CNV	109	3	72	2	0.000	1.000 [a]
NTRK1 CNV	109	3	72	2	0.000	1.000 [a]
TP53 CNV	109	3	72	2	0.000	1.000 [a]

PM, pleural metastasis; mut., mutation; WT, wild type; alter., alteration; CNV, copy number variation; No., number. [a], Continuity Correction.

Table 3. Integrated analysis of the molecular factors concerning the occurrence of PM [n (%)].

Categories	n	PM incidence	χ^2	p-value
Category 1	34	25 (73.5)	9.128	0.028
Category 2	84	53 (63.1)		
Category 3	23	15 (65.2)		
Category 4	45	19 (42.2)		

Category 1: Carrying *EGR* driven mutations without mutations in *ALK*, *ERBB4*, *KRAS*, or *RB1*; Category 2: Harboring *EGR* driver mutations, along with at least one mutation in *ALK, ERBB4, KRAS*, or *RB1*; Category 3: Carrying *EGR* driver wild-type, with no mutations in *ALK, ERBB4, KRAS*, or *RB1*; Category 4: EGFR driver wild-type, along with at least one mutation in *ALK, ERBB4, KRAS*, or *RB1*.

ually been applied to guide targeted therapy selection [20]. We performed molecular profiling on 139 genes using NGS in patients with and without PM to delve deeper into their molecular characteristics. Conventional genetic testing commonly relies on surgical resection or biopsy samples, which can be challenging to acquire, particularly in patients with inoperable advanced-stage NSCLC or recurrent metastases, due to the difficulty of obtaining adequate histological samples for sequencing [21–23]. In this study, specimen sources included lung or pleural tissue (n = 51), pleural fluid (n = 84), and other tissues or fluids (n = 57). Notably, pleural fluid samples, which are relatively easy to acquire, provided relatively accurate sequencing results, serving as a viable alternative when tissue samples were unavailable. These results underscore the potential of less invasive sampling methods to obtain critical diagnostic and prognostic information, thereby expanding the possibilities for personalized treatment in NSCLC, especially for patients who are unable to undergo surgery or biopsy.

Molecular sequencing revealed that mutation frequencies of *ALK, ERBB4, KRAS, RB1*, Ataxia Telangiectasia and Rad3 related (*ATR*), and Serine/threonine kinase 11 (*STK11)* were significantly higher in the non-PM group compared to the PM group. Conversely, the overall mutation frequency of *EGFR* was higher in the PM group, although the difference was statistically insignificant; however, the frequency of *EGFR* driver mutation was substantially higher in the MP group. Since the positive rates of many gene mutations were 0, our data did not meet the requirements for Logistic regression analysis. Therefore, we conducted an integrated analysis of all molecular concerning the occurrence of PM. The highest incidence of PM (73.5%) was found in those with *EGR* driver mutations but without *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations. When both *EGR* driver mutations and *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations were either concurrently present or absent, the PM incidence rates were comparable, at 63.1% and 65.2%, respectively. The lowest PM incidence (42.2%) occurred in those with only *ALK*, *ERBB4*, *KRAS*, and *RB1* mutations and no *EGR* driver mutations.

Previous studies have also found a higher rate of PM in LUAD patients carrying *EGFR* mutations [14,15]. Intriguingly, we observed an obvious decrease in PM incidence from scenarios characterized by solely *EGR* driver mutations. These findings indicate that the occurrence of PM in NSCLC may be influenced by a competitive or trade-off effect among different molecular factors.

KRAS mutations have been reported to be associated with the formation of PE, with mutant *KRAS* promoting MPE development [24]. Conversely, our study showed a lower frequency of *KRAS* mutations in the PM group. This finding does not necessarily indicate a protective role of *KRAS* mutations against PM. We speculate that the recent availability of *KRAS*-targeted therapies may have improved clinical outcomes in patients with mutation in *KRAS*, thereby altering disease progression and metastatic patterns, which could in part explain the observed difference. However, given the limited number of *KRAS*-mutant cases in our study, this observation warrants validation in larger and mechanistically designed studies.

The cell cycle checkpoint proteins ATR and its major downstream effector Checkpoint kinase 1 (CHEK1) play a crucial role in preventing cells with damaged or incompletely replicated DNA from entering mitosis, especially when challenged by DNA-damaging agents, such as radiation therapy or chemotherapeutic drugs, that are the primary modalities to treat cancer [25,26]. The Erb-b2 receptor tyrosine kinase (ErbB) family of receptors (*ErbB1, ErbB2, ErbB3,* and *ErbB4*) have been recognized to play important roles in tumor development. Notably, ErbB4 appears to be unique among these receptors in exhibiting growth-inhibitory properties and has well-defined roles in normal tissue development [27]. Serine/threonine kinase 11/liver kinase B1 (*STK11/LKB1)* mutations disrupt AMP-activated protein kinase (AMPK) regulation, resulting in abnormal mechanistic target of rapamycin kinase (mTOR) and hypoxia-inducible factor 1-alpha (HIF-1α) expression. *LKB1*-deficient cancer cells contain an overactive AMPK "energy sensor", which inhibits cellular death and promotes glucose, lipid, and protein synthesis via the mTOR protein complex [28]. To date, mutations in *ERBB4, ATR*, and *STK11* have not been previously reported to be associated with PE.

We acknowledge several limitations in our study. First, due to its retrospective design, the specimen types used for NGS were not uniform. Nonetheless, all samples were processed in a CLIA- and CAP-certified laboratory, and mutation detection followed standardized thresholds specific to each specimen type. Although such heterogeneity may introduce potential bias, the binary classification of mutation presence likely mitigated its impact. Future prospective studies employing matched sample types are needed to validate our findings. Second, survival data were not included in this study, as the primary endpoint was the presence of pleural metastasis (PM). Consequently, we

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could not assess the prognostic impact of PM. Future studies with extended follow-up and survival-related endpoints are warranted to evaluate the clinical significance of PM in advanced NSCLC. Third, the observed association between *ALK* or *ERBB4* mutations and PM were based on a limited number of cases and should therefore be interpreted with caution. These results are hypothesis-generating and require validation in larger, independent cohorts. Finally, the study lacked functional validation of exploratory findings, including the inverse association between *ALK/ERBB4* mutations and PM. As these genes have not been extensively examined in the context of pleural metastasis, mechanistic studies are essential to clarify their biological roles and establish causality.

Conclusion

In summary, this study identified potential clinical and molecular factors associated with the occurrence of PM in advanced NSCLC. Five molecular characteristics, including *ALK* mut., *ERBB4* mut., *KRAS* mut., *RB1* mut., and *EGFR* driver mutation, were linked to the development of PM. These findings enhance our understanding of the potential molecular mechanisms underlying PM development, deepen our knowledge of advanced NSCLC biology, and offer valuable insights and directions for future research aimed at elucidating the mechanisms driving PM formation.

Availability of Data and Materials

The data underlying this study are available from the corresponding author upon reasonable request.

Author Contributions

MC and YF conceived and designed the study. JY, and MW were in charge of sequencing data processing. LG, ZH, KC, SY, JY, and MW analyzed data and interpreted results. LG wrote the original draft. KC, SY and YF supervised the whole project. All authors contributed to substantial editorial revisions of the manuscript, have read and approved the final version, participated sufficiently in the work, and agreed to be accountable for all aspects of the study.

Ethics Approval and Consent to Participate

The study was performed according to the Good Clinical Practice guidelines and the Declaration of Helsinki, and the study protocol was approved by independent ethics committees at Zhejiang Cancer Hospital (approval number: IRB-2025-275 (11T)). Written informed consent was obtained from all participants.

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Conflict of Interest

Junrong Yan and Mengyun Wang are employees of Nanjing Geneseeq Technology Inc., which provided sequencing services for this study. The company had no role in the decision to submit the article for publication. These affiliations did not influence the objectivity of the work. The remaining authors declare that they do not have any competing interests.

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