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ORIGINAL PAPER

Comparison of gene mutation profile in different lung adenocarcinoma subtypes by targeted next-generation sequencing

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Abstract

Background Disease prognosis after resection of lung cancer could be affected by pathological subtypes. In this study, we investigated the difference of gene variation and significantly altered pathways between adenocarcinoma in situ (AIS)/microinvasive adenocarcinoma (MIA) and invasive adenocarcinoma (IAC) subtypes to reveal the molecular mechanism of prognosis differences.

Methods Sixty one tumor tissues were subjected to DNA extraction and customized 136 gene targeted next-generation sequencing. Comparisons between groups were performed with two-sided Fisher's exact test for categorical variables and two-tailed unpaired t test for numerical variables.

Results A total of 402 somatic mutations involved in 70 genes were detected in all these samples, and 74.29% of these genes were mutated in at least two samples. *PMS2*, *ARID1A*, *EGFR,* and *POLE* were the most frequently mutated genes. *ALK_EML4* fusion was observed in one IAC patient and *RET_ KIF5B* fusion in one AIS patient. A significant higher proportion of patients with *TP53* gene mutation was observed in the IAC group ($P=0.0057$). The average onset age in IAC group is 62.48 years, which is greater than other subtypes ($P=0.0166$). It revealed that mutations in genes involved in the mTOR signaling pathway (56.52% vs 26.32%, $P=0.0288$) and Hippo signaling pathway (34.78% vs 10.53%, $P=0.0427$) were significantly enriched in IAC subtypes, suggesting the key involvement of mTOR and Hippo signaling pathways in lung tumor development and malignant progression.

Conclusions This study revealed the heterogeneity of gene mutations and significantly altered pathways between different lung cancer subtypes, suggesting the potential mechanism of different prognosis.

Keywords Lung adenocarcinoma (LUAD) · Next-generation sequencing (NGS) · Gene mutation Profile · mTOR signaling pathway · Hippo signaling pathway

Abbreviations

IAC	Invasive adenocarcinoma
MIA	Microinvasive adenocarcinoma
AIS	Adenocarcinoma in situ
LMAC	Lung mixed adenocarcinoma
NSCLC	Non-small cell lung cancer
LUAD	Lung adenocarcinoma
IASLC	International Association for the Study of Lung Cancer
ATS	American Thoracic Society

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ERS European Respiratory Society
FFPE Formalin-fixed, paraffin-embedded
IGV Integrative Genomics Viewer

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. And non-small-cell lung cancer (NSCLC) is the most common type, which accounts for approximately 85% of lung cancer patients [2]. Lung adenocarcinoma (LUAD) is a subtype of NSCLC, which accounts for 50% of all the diagnosed lung cancer patients [2, 3]. LUAD classification sponsored by the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) has been adopted for many years [4], which has been refined based on the invasion and predominant growth pattern in 2015 [3]. According to the invasion pattern, LUAD is composed of adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), invasive adenocarcinoma (IAC), etc. [3].

In general, surgery is unnecessary for AIS patients because of no interstitial invasion and regular follow-up is recommended [5]. It is a conventionally surgical approach to perform segmentectomy for AIS and MIA patients, which could reserve pulmonary function maximally. And IAC patients undergo lobectomy more often. Furthermore, lymph node dissection is necessary for IAC patients because of the higher probability of regional and distal metastasis. The clinical outcome of LUAD has been significantly improved based on the advances in surgery, radiotherapy, and systemic treatment [6, 7]. AIS and MIA patients have a similar surgical outcomes after complete resection [8], and the five-year survival rate after surgery is almost 100% [9–11]. However, the five-year survival rate after surgical resection for IAC patients decreases substantially to 75% [9–12]. Therefore, it is significantly valuable to distinguish the differences between AIS/MIA and IAC.

Mounting evidence has suggested that disease recurrence and prognosis after resection could be affected by pathological subtypes of lung cancer [13, 14]. Tremendous efforts have been focused on the molecular features of LUAD [15–17] in these years, which could help researchers understand its molecular heterogeneity. Meanwhile, large-scale sequencing studies have elucidated the complex genomic landscape of preinvasive LUAD subtypes [18–20], which benefited from the widespread implementation of the enhanced radiological techniques in lung cancer diagnosis.

However, the gene variation differences and the molecular mechanism of prognosis differences between AIS/MIA and IAC subtypes are still unclear. To achieve this, we focused on investigating the difference of gene alterations and significantly altered pathways between AIS/MIA and IAC subtypes in this study.

Materials and methods

Patient collection

A total of 61 tumor tissues (involved in 50 lung adenocarcinoma patients) from Renmin Hospital of Wuhan University between October 2021 and July 2022 were included in this study. Patients eligible for inclusion in this study were those with (a) 18–80 years old, (b) histologically proven IAC, or MIA, or AIS, or mixed AIS with MIA, (c) no other serious comorbidities of other organs. All pathology specimens were reviewed by experienced pathologists before analysis. Among these samples, individuals diagnosed with multiple lesions sharing the same pathological type were simultaneously included in this study. Informed consent was obtained from all participants. This study was approved by the Ethical Committee of Renmin Hospital of Wuhan University (approval number: WDRY2023-K027) and was performed in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

Targeted DNA sequencing

DNA samples were collected from formalin-fixed, paraffin-embedded (FFPE) tumor tissues with the QIAamp DNA FFPE Tissue Kit (56,404, Qiagen, Hilden, Germany). Then DNA libraries were constructed with KAPA Library Preparation Kit (Kapa Biosystems Inc., Wilmington, USA). The customized Agilent SureSelect^{XT} DNA 136 panel was used to capture targeted region for 61 samples. Finally, high throughput sequencing was performed on the Illumina X10 platform (Illumina Inc., San Diego, CA, USA). Genes of 136 panel sequencing were shown in Supplementary file 1.

Putative somatic mutation calling and filtering

Low-quality sequencing reads were discarded, including reads containing adaptor sequences, > 5% unknown base 'N,' and > 15% bases with quality < = 19. The average sequencing depth was more than 2900×. After filtration, we obtained 2.75 G high-quality clean bases on average, and the Q_{30} value was > 90% for samples. Then high-quality paired-end reads underwent mutation analysis and human genome build hg19 was used as the reference genome, which mapped to hg19 by Burrows-Wheeler Aligner (BWA version 0.7.15, default parameters, BWA-MEM algorithm). Software GATK MuTect2 (version 4.1, default parameters) was used to identify nonsilent somatic mutations. And mutations were

annotated with software ANNOVAR (version 2016-02-01, default parameters).

Because of the unavailability for paired normal samples, the following criteria were utilized to filter the somatic variants: (a) retain nonsilent coding mutations that mutated in exonic or splicing region; (b) retain mutations that not included in public 1000 Genomes databases or the frequency of the alternative allele was < = 0.01; (c) retain mutations that the allele fraction in the tumor was > = 0.02; (d) retain mutations that called as damaging/deleterious for protein structure by at least one of two used prediction software algorithms, SIFT [21] and PolyPhen2 [22]; (e) remove mutations that recorded in dbSNP database; (f) retain mutations registered in the COSMIC database (version 94) [23] (while those mutations noted with 'SNP' in COSMIC needed to be discarded); and (g) retain mutations that contain > = 10 variant reads and detected in both forward and reverse DNA strands. The remaining mutations were identified as putative somatic mutations and were subsequently used for further analysis.

Gene fusion analysis

GeneFuse (version 0.6.0) [24] was used to verify critical gene fusions. A gene fusion was considered as true: (a) unique reads > 4, (b) verified as true by Integrative Genomics Viewer (IGV) software.

Statistical analysis and graphics drawing

Statistical analysis was performed by GraphPad Prism software (version 8.0). Comparisons between groups were performed with two-sided Fisher's exact test for categorical variables and two-tailed unpaired *t* test for numerical variables. Data were presented as 'Mean with Standard Deviation (SD)' or as percentages of patients. In all analyses, *P* value < 0.0500 was considered statistically significant. Histogram and scatter diagram were plotted by GraphPad Prism software (version 8.0). Gene mutation spectrum was drawn with R package 'maftools' (version 2.12.0) or 'ComplexHeatmap' (version 2.12.1).

Results

Clinical characteristics and mutational profiles of lung adenocarcinoma patients

A total of 61 tumor samples from 50 lung adenocarcinoma patients were investigated in our study. All the clinical characteristics were shown in Supplementary file 2. All the patients were confirmed as unique pathological subtype, including IAC (23 samples/21 patients), MIA (6 samples/6 patients), AIS (29 samples/20 patients), or LMAC (3 samples/3 patients). All the clinical characteristics and somatic mutations are summarized in Fig. 1. Of these patients, the onset age ranges from 28 to 78 years (median, 59). The number of female patients was more than the number of male patients (31 vs 19). After filtering the mutations, a total of 402 somatic mutations (range 1–54, median 4) involved in 70 genes were detected in all these samples and 74.29% (52/70) of these genes were mutated in at least two samples. Of these mutations, 98.51% (396/402) mutations were observed as a SNV, whereas 1.49% (6/402) as an InDel type.

As shown in Fig. 1, frequently mutated genes detected from this cohort included *PMS2* (65.57%, 40/61), *ARID1A* (37.70%, 23/61), *POLE* (31.15%, 19/61), *EGFR* (27.87%, 17/61), *MSH2* (24.59%, 15/61), *ATM* (19.67%, 12/61), *TSC1* (18.03%, 11/61), *FGFR1* (14.75%, 9/61), *KRAS* (14.75%, 9/61), *AR* (11.48%, 7/61), *DNMT3A* (11.48%, 7/61), *CHEK2* (9.84%, 6/61), *ERBB3* (9.84%, 6/61), *MYC* (9.84%, 6/61), *ATR* (8.20%, 5/61), *BRCA2* (8.20%, 5/61), *KIT* (8.20%, 5/61), *RB1* (8.20%, 5/61), *TP53* (8.20%, 5/61), *ERBB2* (6.56%, 4/61), *MET* (6.56%, 4/61), and *SMO* (6.56%, 4/61). Among them, *PMS2*, *ARID1A*, *EGFR,* and *POLE* were the most frequently mutated genes in this study. Additionally, two critical gene fusions were observed in one IAC patient (Pt17, *ALK*.exon20.chr2:29446349_*EML4*.intron19.chr2:42,546,586) and one AIS patient (Pt44, *RET*.intron11.chr10:43610997_*KIF5B*.intron15.chr10:32,316,372) (Supplementary file 3 and Supplementary Fig. 1).

Gene mutation and clinical feature comparison in hierarchical subgroups with different prognosis (IAC vs AIS/MIA/LMAC)

To explore the possible mechanism that attributes the difference of different lung adenocarcinoma group, gene mutations and clinical characteristics were compared among different subtypes. A significant higher proportion of patients with TP53 gene mutation was observed in the IAC group than that in the AIS/MIA/LMAC group (21.74% vs 0.00%, $P = 0.0057$) (Fig. 2A). There was no significant difference about other gene mutations between these two groups, including *EGFR*, *KRAS*, *ERBB2*, etc. (Supplementary Fig. 2). The average age of onset in IAC group is 62.48 years, which is greater than that 54.41 years in the AIS/MIA/LMAC group ($P = 0.0166$) (Fig. 2B).

Significantly altered pathways between IAC and AIS/MIA/LMAC

Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze the pathways that are distinct between the two groups. The results demonstrate that the major signaling pathways affected in IAC samples were

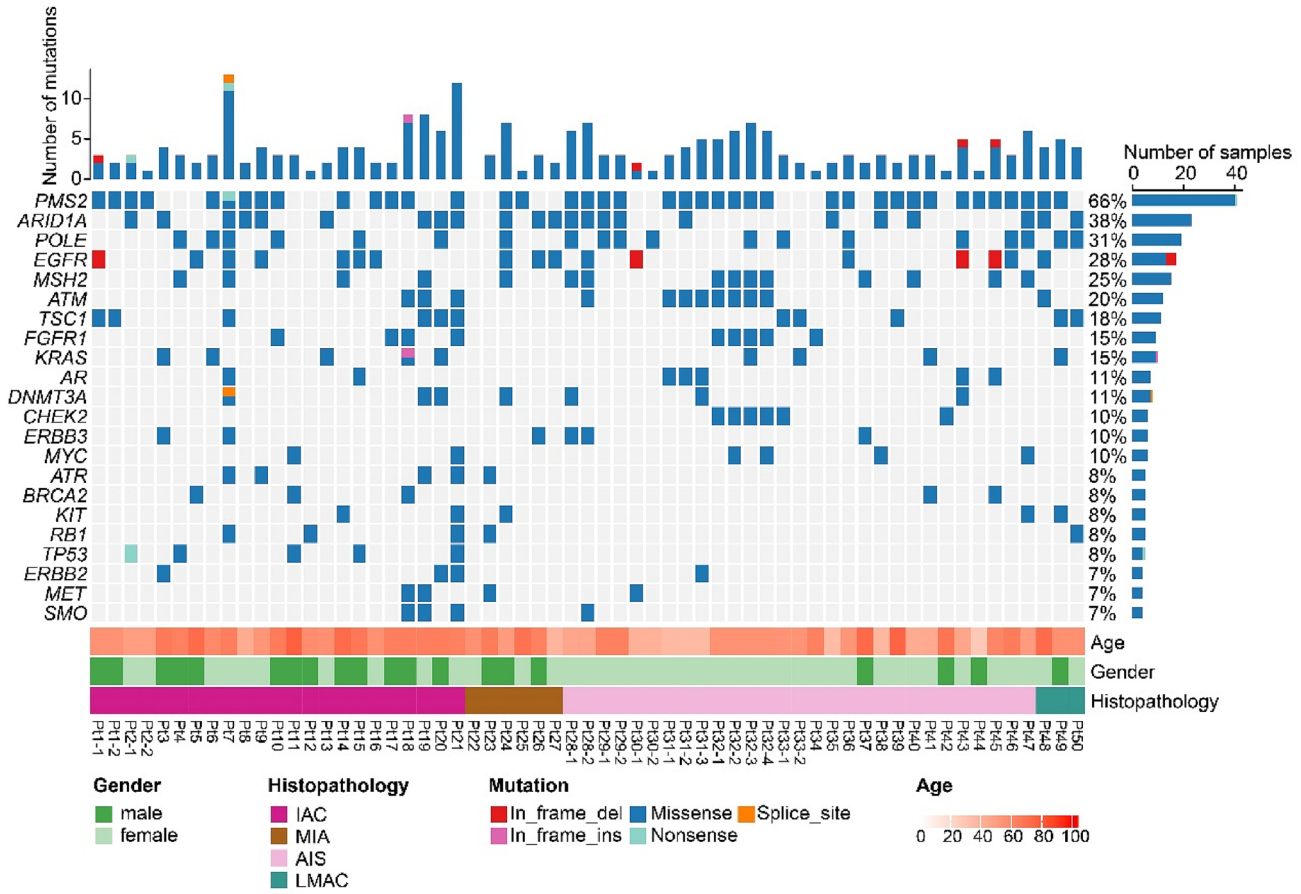

Fig. 1 Clinical attributes and mutational profiles of 61 tumor tissues. Only genes with mutations in > = 4 samples are shown. Top column: mutation number represented in bottom landscape for each sample. Right column: number of mutated samples and gene mutation frequency for each gene. Bottom column: each column represents one sample, and each row shows one gene. IAC, invasive adenocarcinoma. MIA, minimally invasive adenocarcinoma. AIS, adenocarcinoma in situ. LMAC, lung mixed adenocarcinoma

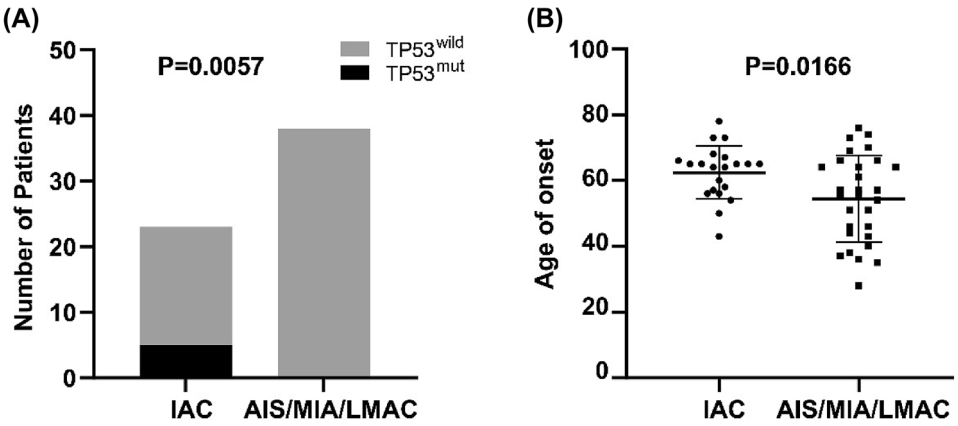

Fig. 2 Comparison of gene mutation (**A**) and age of onset (**B**) in different hierarchical subgroups with lung adenocarcinoma. Note: only genes mutated in > = 4 samples (frequency > = 5%) were considered. *TP53*^{wild}, without *TP53* mutation. *TP53*^{mut}, *TP53* mutation. IAC, invasive adenocarcinoma. MIA, minimally invasive adenocarcinoma. AIS, adenocarcinoma in situ. LMAC, lung mixed adenocarcinoma. Data in B were shown as 'Mean with SD.' Statistics in A: Fisher's exact test. Statistics in B: unpaired t test

mTOR signaling pathway (Fig. 3A, 56.52% vs 26.32%, $P=0.0288$) and Hippo signaling pathway (Fig. 3B, 34.78% vs 10.53%, $P=0.0427$). Genes involved in mTOR signaling pathway detected from our cohort included *TSC1*, *KRAS*, *PIK3CA*, *TSC2*, *STK11*, *MAP2K1*, *BRAF,* and *MTOR*. Mutations in *TSC1* ($n=6$), *KRAS* ($n=5$), *PIK3CA* ($n=1$), *TSC2* ($n=2$), *STK11* ($n=2$), *MAP2K1* ($n=1$), *BRAF* ($n=1$), and *MTOR* ($n=1$) were detected in 13 patients with IAC samples. Five samples in IAC group had concurrent mutations in at least two genes. Genes involved in the Hippo signaling pathway detected from our cohort included *MYC*, *SMAD4*, *CTNNB1*, *APC,* and *YAP1*. Taken together, these results

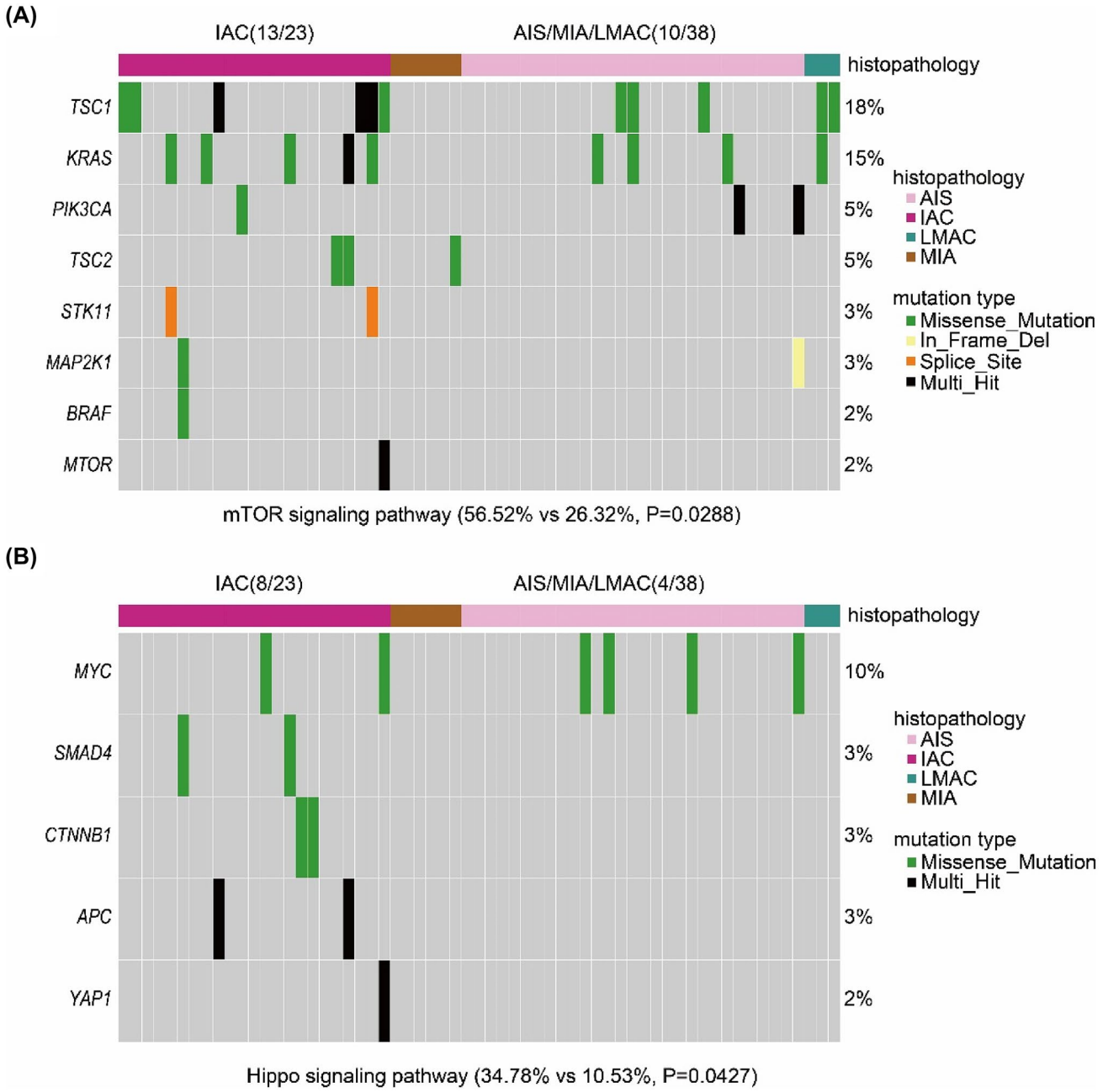

Fig. 3 KEGG analysis reveals distinct pathways in different hierarchical subgroups with lung adenocarcinoma. Two pathways with the significant enrichments in IAC samples as compared to AIS/MIA/LMAC samples. **A:** mTOR signaling pathway (56.52% vs 26.32%, $P=0.0288$). **B**: Hippo signaling pathway (34.78% vs 10.53%, $P=0.0427$). Mutated genes participating in any of the two pathways were listed. Each column represents a sample and each row represents a gene. Mutation types were denoted in different colors. Samples were grouped according to the histopathology. *IAC* invasive adenocarcinoma, *MIA* minimally invasive adenocarcinoma, *AIS* adenocarcinoma in situ, *LMAC* lung mixed adenocarcinoma. Data were shown as percentages of patients. Statistics: Fisher's exact test

indicate that IAC and AIS/MIA/LMAC subtypes are molecularly distinct based on the difference in the number and distribution of somatic mutation types detected in these tumors as well as the major pathways affected by these mutations.

Discussion

This pilot study aimed to illustrate the difference of gene variants and clinical parameters between IAC and AIS/MIA/LMAC subtypes, an effort to help us perform precise medicine and treatment for different LUAD subtypes. In this study, a total of 402 somatic mutations were detected in all these 61 samples, and 74.29% genes were mutated in at least two samples. Of these mutations, *PMS2* (mismatch repair system component) accounts for the highest percentage (65.57%), which is one of the critical genes in DNA mismatch repair (MMR) pathway with potential crucial roles in carcinogenesis [25, 26]. A high mutation rate of *PMS2* demonstrates that the dysfunction in MMR pathway might impact the tumorigenesis of LUAD and may increase patients' sensivivity to DNA damaging agents. *ARID1A* and *EGFR* genes were also detected with a high mutation rate. *ARID1A* gene, as a critical component of the switch/sucrose nonfermentable (SWI/SNF) complex, plays a role in cell cycle regulation, metabolic reprogramming, and epithelial–mesenchymal transition [27]. In recent years, potential ARID1A mutation-based therapeutic targets have been focused, including Poly (ADP-ribose) polymerase (PARP) inhibitors, enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) inhibitors, and immune checkpoint inhibitors [27]. EGFR gene encodes the epidermal growth factor receptor (EGFR) tyrosine kinase, and EGFR-mutated patients could benefit from EGFR tyrosine kinase inhibitors (TKIs). Besides, other new treatment strategies to overcome EGFR-TKIs resistance have also been researched in these years [28]. Additionally, our results showed that a significant higher mutation rate of *TP53* was observed in IAC than that in AIS/MIA/LMAC, which was consistent with previous study [29]. It suggests the potential oncogenic activity of *TP53* in IAC subtypes. Compared with AIS/MIA/LMAC, patients with IAC harbored an older onset age, which is in line with previous researches [29]. Besides, similar result that elder LUAD patients carried more *TP53* mutations than young LUAD cohorts was observed in Yang's study [30].

To further understand the difference of critical pathways involved in the disease progression for different LUAD subtypes, we performed pathway enrichment analysis. The results revealed that mutations in genes involved in the mTOR signaling pathway (56.52% vs 26.32%) and Hippo signaling pathway (34.78% vs 10.53%) were significantly enriched in IAC subtypes, suggesting the key involvement of mTOR and Hippo signaling pathways in lung tumor development and malignant progression. mTOR signaling pathway integrates a variety of biological cues, including intracellular and extracellular signals, to serve as a central regulator of cell metabolism, growth, proliferation and survival and to regulate organismal homeostasis [31]. mTOR signaling pathway can regulate many biological processes and is also associated with many pathological conditions, including cancer. Several reasons could be summarized for the importance of mTOR pathway in cancer pathogenesis [31]: (a) genes involved in PI3K signaling pathway (upstream of mTOR pathway) are often mutated in cancer, (b) *TP53* mutation could promote mTOR activation [32], (c) mutations in genes of mTOR pathway always result in a superfluous phosphorylation, which could promote ribosome biogenesis, protein synthesis, and angiogenesis to support cell growth and proliferation or regulates cell cycle progression and survival [33, 34]. Hippo signaling pathway exerts critical roles in modulating cell proliferation, cell apoptosis [35], drug resistance [36] and has been demonstrated to contribute to the progression of various diseases including cancer [35]. Mutations or altered expression of gene components in Hippo pathway could promote the migration, invasion, and malignancy of cancer cells [37–39].

It will be very interesting to study how these pathways contribute to lung tumorigenesis, and it might be of great significance to development of small molecule or antibody drugs targeting core components in these pathways to provide new therapeutic strategies for future successful treatment of lung cancer. At present, there are many therapeutic agents that are designed to target the mTOR [40–42] and Hippo signaling pathway [43–46] that could serve as treatment options. Our results suggest the potential emerging therapeutic targets for IAC patients with mutations in genes involved in the mTOR and Hippo pathway.

Conclusion

In summary, we analyzed the differences in somatic mutations and clinical characteristics among different LUAD subtypes. Our results revealed the heterogeneity of gene mutations and significantly altered pathways between IAC and AIS/MIA/LMAC subtypes, suggesting the potential mechanism of different prognosis in different LUAD cohorts and individualized clinical management of patients is needed among different subtypes.

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Author contributions ZM: conceptualization, methodology, investigation, project administration, resources, funding acquisition, supervision, validation, writing-review and editing. SZ, PD and ZP: data curation, formal analysis, writing-original draft, writing and review and editing, visualization. QC and JZ: software, visualization. All the authors contributed to the article and approved the final version of the manuscript submitted for publication.

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Data availability The data that support the findings of this study are not publicly available due to their containing information that could compromise the privacy of research participants but are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This study was approved by the Ethical Committee of Renmin Hospital of Wuhan University (approval number: WDRY2023-K027), and was performed in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

Informed consent Informed consent was obtained from all individual participants included in the study.

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