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Novel genetic characteristics in low-grade fetal adenocarcinoma of the lung

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Abstract

Background: Low-grade fetal adenocarcinoma of the lung (L-FLAC) is a rare subtype of lung adenocarcinoma with undetermined histological features and genetic abnormalities. In this study, we attempted to investigate the pathological characteristics and genomic profiles of L-FLAC.

Methods: Among 9839 cases of primary lung adenocarcinoma resected at Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College between January 2011 and June 2016, three cases diagnosed with L-FLAC were selected. An immunohistochemical profile and whole exome sequencing (WES) using tumor and normal tissues was conducted. The last follow-up date of patients was January 2021.

Results: Three cases diagnosed with L-FLAC were finally screened, suggesting a percentage of 0.03%. All three patients were male and diagnosed as stage I following radical lobectomy. The missense variant was found to be the major gene mutation type using WES. *CTNN*B1 and *DICER1* were the two most frequent gene mutations. All cases demonstrated positive TTF-1 expression. In addition, two patients showed positive expression of β-catenin (nuclear/cytoplasmic expression), CgA and Sny. Negative expression of PD-L1 in tumor cells was observed in all three cases. One case with a relatively high tumor mutation burden (TMB) (2.18 mut/Mb) had an inferior overall survival of 11.5 months. However, the other two cases with a lower TMB (0.12 and 0.74 mut/Mb) still acquired disease-free status up to the last follow-up date.

Conclusions: L-FLAC has a specific molecular background which is different from lung adenocarcinoma. Furthermore, gene heterogeneity was found and might be the reason for a dramatically different prognosis in these L-FLAC patients.

KEYWORDS

fetal adenocarcinoma, gene mutation, lung cancer

INTRODUCTION

Fetal adenocarcinoma of the lung (FLAC) is a rare subtype of lung adenocarcinoma named for its morphological characteristics which are similar to developing fetal lung, initially described by Kradin in 1982.[1] Due to their different clinicopathological features, biological behavior and clinical outcome, FLACs are classified into low-grade fetal adenocarcinoma (L-FLAC) and high-grade fetal adenocarcinoma (H-FLAC).[2,3] It has been reported that the incidence of FLAC in all pulmonary neoplasms accounts for an estimated 0.1% –0.5%.[4,5] One study recently revealed that the prevalence of L-FLAC and H-FLAC was 0.32% and 0.54% in Chinese patients, respectively.[6]

The pathological feature of L-FLAC is characterized by immature epithelium, and its morphology is similar to that

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of fetal lung at 10–15 weeks.[7,8] At present, it is generally accepted that L-FLAC shows low nuclear atypia and morule formation, and is composed of pure epithelium without other components. On the other hand, H-FLAC typically exhibits at least 50% fetal morphology and is usually associated with other types of lung adenocarcinoma, such as acinar type, papillary type, micropapillary type, lepidic type and solid type, and even high-grade neuroendocrine carcinoma.[6,9] According to several molecular studies, *KRAS, EGFR, PIK3CA* mutation share low rates in L-FLAC which is different from conventional lung adenocarcinoma which harbors a mutation frequency of 32%-64% for *EGFR* and 13% for *KRAS*.[10–12]Based on the above findings, researchers believe that FLAC may have unique molecular characteristics. Other molecular markers have also been found in FLAC, such as the β-catenin gene (*CTNNB1*) mutation and *DICER1* mutations.[13,14] *CTNNB1* mutation has rarely been reported in lung cancer, but a high prevalence has been observed in L-FLAC, which may also be a distinctive molecular feature. Only a few studies with limited cases have been previously reported, and the molecular features of L-FLAC have not yet been clarified. In this study, a detailed molecular investigation of three cases with L-FLAC was carried out using whole-exome sequencing (WES) to provide further information on the molecular features of FLAC in Chinese patients.

METHODS

Clinical samples

Among 9839 cases of primary adenocarcinoma of the lung resected in the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College between January 2011 and June 2016, three cases that met the inclusion criteria for low-grade fetal adenocarcinoma were subjected to further analyses. Clinical samples (significantly elevated TMB) were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) followed by review of two experienced pathologists without prior knowledge of the patients' conditions.

All patients provided written informed consent, and studies were conducted in accordance with the principles of the Declaration of Helsinki. This study was approved by the Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

Immunohistochemistry analysis

Thick sections 4 μm in size were cut from paraffin-embedded tissue blocks for multiple immunohistochemistry analyses, including TTF-1 (clone 8G7G3/1, ZECA), Napsin A (polyclonal, MXB), β-catenin (clone UMAB15,BIO), Chromogranin A (clone EP38, BIO), synaptophysin (polyclonal, MXB), AFP (clone 2ZA06, BIO), and PD-L1 (clone 22C3, DAKO). All staining was performed on Leica Bond-Max or Roche Ventana. Omission or replacement of the primary antibody with PBS was routinely used as a negative control.

DNA isolation

DNA was extracted from paraffin-embedded tissue blocks using GeneRead DNA FFPE Kit (Qiagen) according to the manufacturer's instructions. Germline DNA was isolated from blood lymphocytes using QIAamp DNA Blood Mini kit (Qiagen). The concentration of DNA was determined using a Qubit fluorometer from Invitrogen (Thermo Fisher Scientific).

Gene mutation analysis

Whole exome sequencing was performed by the Department of Pathology, National Cancer Center (Beijing, China). The libraries were made according to the KAPA Hyper Prep Kit (Roche) manufacturer's instructions. Briefly, Genomic DNA was randomly fragmented to 200 bp using Covaris technology, and adaptors were then added at both ends of the fragments after End repairing and A-tailing. The ligated constructs were amplified through nine reaction cycles. Solution hybridization was applied to the libraries using the Agilent Sure Select Human All ExonV6 Kit (Agilent). Indexed libraries were then sequenced on an Illumina HiSeq platform (Illumina). The sequencing reads were aligned to the human reference genome (UCSC hg19; http://genome.ucsc.edu) using the Burrows-Wheeler Alignment tool. Duplicate reads were removed using Picard, and candidate somatic mutation variant (SNV), insert and deletion sites and annotation were carried out using SAMtools and ANNOVAR. Finally, each candidate site was checked by an integrated genomics viewer (IGV), and unreliable candidate sites were removed. The average target rate of tumor and normal tissues in the three cases for WES was 0.53% and 0.58%, respectively. The average tumor tissue sequencing depth of three patients was 206x. In normal tissues of three cases, the average sequencing depth was 128x.

RESULTS

Clinical characteristics

Among the 9839 primary lung adenocarcinomas screened, three cases were identified as meeting the criteria of L-FLAC, constituting a percentage of 0.03%. The detailed clinical features are summarized in Table 1.

The three patients shared some characteristics in common. All were male without any other diseases or family history of tumor. The mean age of the three patients was 33.7 years old. Furthermore, they were all diagnosed with IA

TABLE 1 Clinical features

Cases	Age	Gender	Stage	Treatment	Smoking history	Relapse
WY25TQ	30	Male	pT2aN0M0	Surgery, adjuvant chemotherapy	Yes	No
WY26TQ	39	Male	pT2aN0M0	Surgery	Yes	No
WY27TQ	32	Male	pT1bN0M0	Surgery, radiotherapy	Yes	Yes

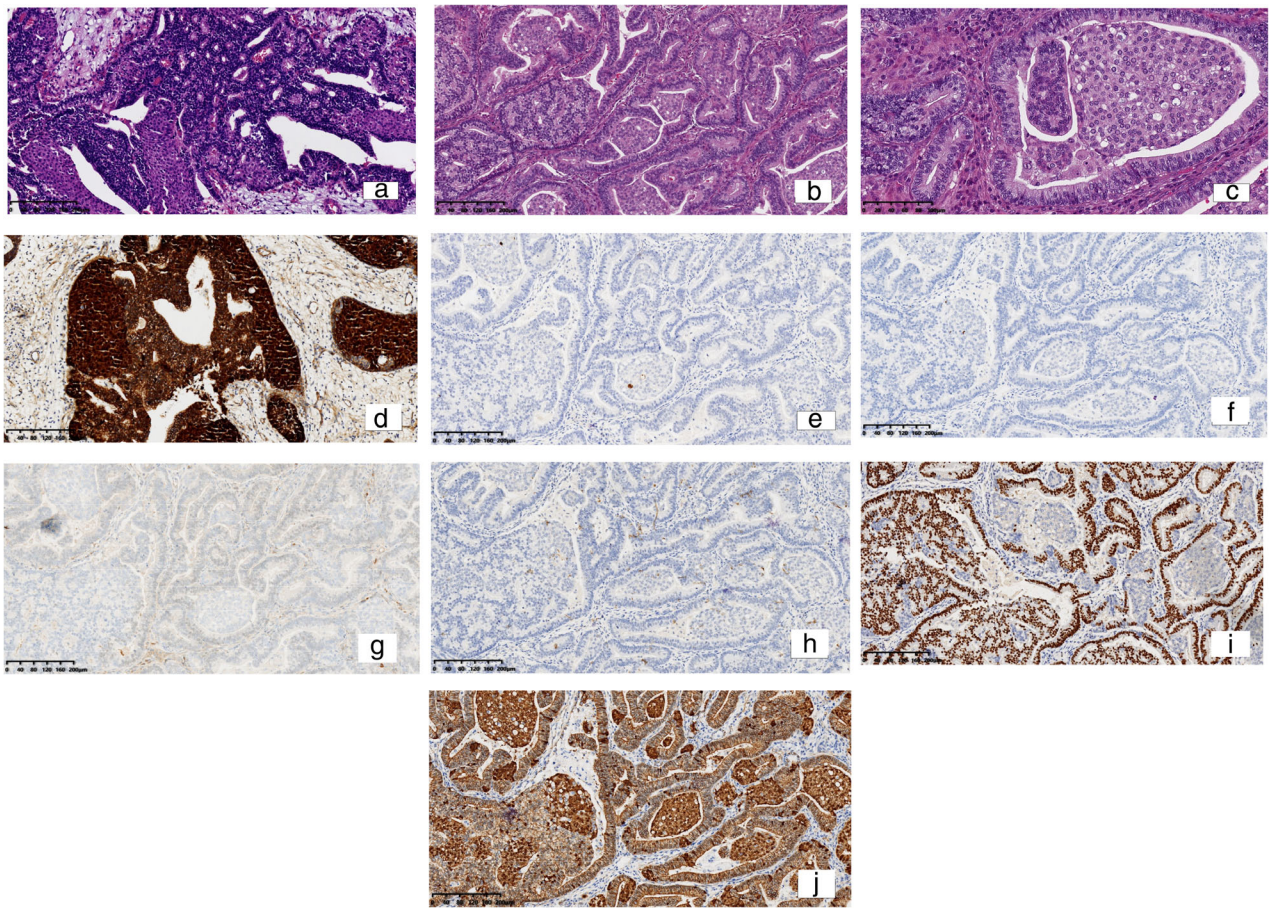

FIGURE 1 Pathological features. (a) Microphotographs of case WY27TQ (hematoxylin & eoson [H&E] magnification ×100). (b) and (c) Microphotographs of case WY26TQ (H&E magnification ×100, H&E. magnification ×200, respectively). (d) Expression of β-catenin in case WY27TQ (immunohistochemistry [IHC] magnification ×100). (e) Expression of AFP in case WY26TQ (IHC magnification ×100). (f) Expression of CgA in WY26TQ (IHC magnification ×100). (g) Expression of SALL4 in WY26TQ (IHC magnification ×100). (h) Expression of Syn in WY26TQ (IHC magnification ×100). I: expression of TTF-1 in WY26TQ (IHC magnification ×100). (j) Expression of β-catenin in case WY26TQ (IHC magnification ×100)

stage, but only one had relapsed 7 months after surgery. While case WY27TQ had an inferior overall-survival of 11.5 months, the other two cases remained disease-free up to the date of last follow-up.

Pathological features and immunohistochemistry profile

On microscopic examination, the three cases had similar histological characteristics. As shown in Figure 1(a)–(c), low power microphotographs showed that the tumor was mainly composed of glandular and tubular structures. The local gland showed a complex structure. The morules body formed by squamous cells was visible at the base of the gland. Under medium magnification, the glands were seen to be lined by columnar cells and were small with relatively uniform nuclei. Clear cytoplasm and subnuclear glycogen-rich vacuoles were observed. The three specimens showed a similar expression of immunohistochemical staining. Tumor cells presented a diffuse nuclear expression of TTF-1. In all patients, both columnar cells and morules presented an aberrant nuclear/cytoplasmic expression of β-catenin, and showed a positive expression of β-catenin, CgA and Sny. All cases demonstrated a negative expression of AFP, glypican3, and PD-L1. Furthermore, SALL4 positive expression was only observed in case WY26TQ (Table 2).

TABLE 2 Immunohistochemistry profile

CASES	TTF-1	β-catenin (nuclear+)	ERβ	CgA	Syn	AFP (α-fetoprotein)	GPC-3 (glypican3)	SALL4	PD-L1
WY25TQ	3+	3+	–	1+	1+	–	–	–	–
WY26TQ	3+	2+	–	1+	1+	–	–	1+	–
WY27TQ	3+	3+	–	–	–	–	–	–	–

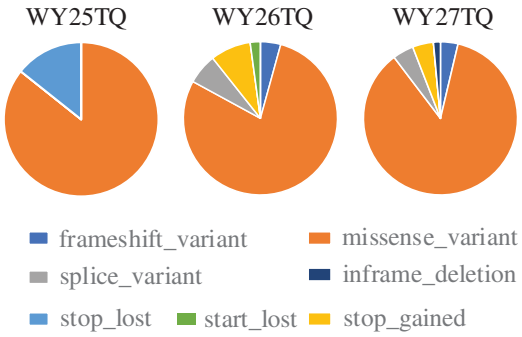

FIGURE 2 Variant classification

Molecular analysis

The missense variant was found as the major gene mutation type in WES testing (Figure 2). *CTNNB1* and *DICER1* were the most two frequently altered genes found in two cases (Figure 3). Missense mutations of *CTNNB1* (the exon 3, c.100G > C, p. Gly34Arg, with an abundance of 26.7%; and c.98C > G, p. Ser33Cys with an abundance of 29%) were found in WY26TQ and WY27TQ cases, respectively. In addition, missense mutations of *DICER1* (the exon 14, c.5428G > T, p. Asp1810Tyr with an abundance of 25.3%; and c.5438A > G, p. Glu1813Gly with an abundance of 37.3%) were observed in WY26TQ and WY27TQ, respectively. Moreover, other missense mutants of *MYCN* (the exon 2, c.173C > T, p. Thr58Met) with an abundance of 14.1% were determined in case WY26TQ. Case WY27TQ had a relatively higher level of tumor mutation burden (TMB) (2.18 mut/Mb). However, the other two cases shared a relatively lower TMB (0.12 and 0.74 mut/Mb, respectively).

DISCUSSION

Low-grade fetal adenocarcinoma of the lung is a rare malignant lung cancer accounting for approximately 0.1% or less of all lung tumors.[15] An initial diagnosis of L-FLAC occurs during the early stages (I–II) of the disease,[16] a finding which is consistent with our results. In 1991, Koss et al. reported 28 cases of well-differentiated FLAC in patients with a mean age of 33 years.[7] Zhang et al. found the mean age of 45 L-FLAC patients at diagnosis was 35 ± 15 years old.[17] In this study, we report on three cases of L-FLAC who were all male patients with an average age of 33.7 years and a history of smoking. In addition to being of relatively younger age and male gender, smoking history might therefore be an influencing factor.

Histologically, L-FLAC is similar to that of fetal lung, being composed of well-differentiated glands showing a pure histological pattern with low nuclear atypia.[18] Consistent with previous studies, patients with L-FLACs in our study expressed nuclear and cytoplasmic β-catenin and traditional markers of lung adenocarcinoma, such as TTF-1. β-catenin is a key signal molecule in the Wnt signaling pathway. The Wnt signaling pathway is curtailed in embryonic development, but once this pathway is changed, the formation of tumors can occur. Under normal circumstances, β-catenin is located on the cell membrane. If it remains non-phosphorylated, it can migrate to the nucleus and recruit transcription factors. It is reported that the expression of β-catenin in adrenocortical carcinoma is negatively correlated with PD-L1 expression.[19] PD-L1 negative expression was also observed in our study. In addition, the missense mutation of *CTNNB1* gene (β-catenin gene) located on chromosome 3p15.1 was also detected. This gene mutation is considered to be a significant mutation in exon 3 of L-FLAC15 and exon 16[20,21] and our case fully meets the criteria. SALL4 expression was only seen in case WY26TQ. Several studies[22,23] have previously demonstrated that *DICER1*-related malignancies show SALL4 expression. However, more research is needed to clarify whether SALL4 can be used as a relevant marker for diagnosis.

It is worth highlighting in the present study that two L-FLAC cases were not only found to have *DICER1* mutation, but also *CTNNB1* mutation. In a previous study[24] the *CTNNB1* mutation was detected in L-FLAC, and the *EGFR*, *KRAS*, *ALK*, *BRAF*, *MET*, *ROS-1* were observed as wild-type, which is consistent with our study. The *DICER1* mutation was observed in different kinds of tumors, and could contribute to the development of various cancers. It has been reported that the *DICER1* mutation was the origin of the *DICER1* syndrome of familial disease. However, a family history of *DICER1* syndrome was not observed in these two patients. L-FLAC is considered to be the precursor of pulmonary blastoma (PB). PB is a biphasic tumor composed of FLAC (typically low-grade malignancy) and primitive mesenchymal stroma. Both L-FLAC and PB are closely related to *CTNNB1* gene mutation.[25] In some studies, mutations in both *DICER1* and *CTNNB1* have been found in PB and L-FLAC.[26,27] In these two cases of PB, β-catenin was also mainly expressed in membrane and multifocal cytoplasmic/nuclear localization. These cases further support the genetic abnormalities shared by L-FLAC and PB, and the mutation of *DICER1* may be related to the abnormal expression

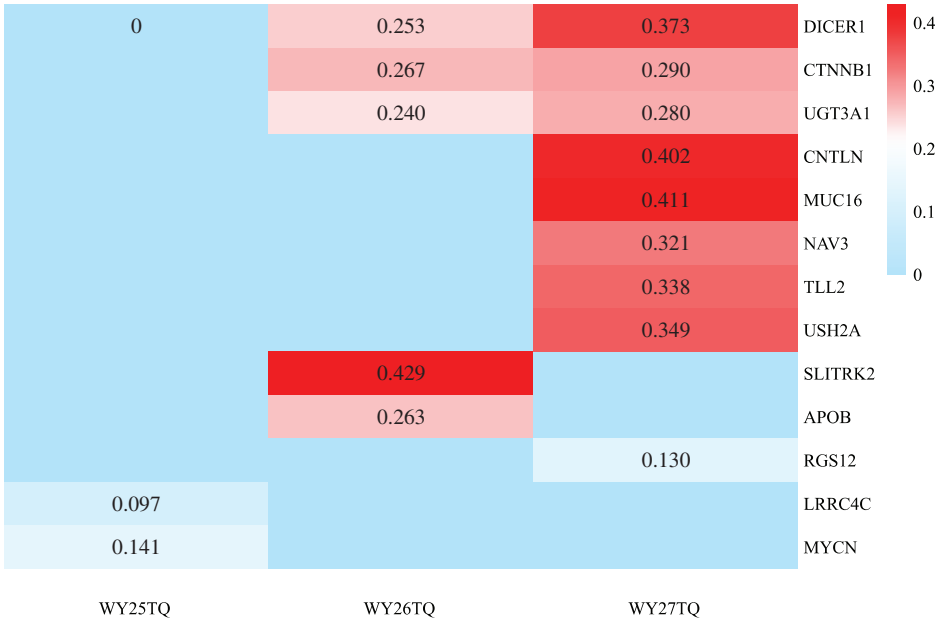

FIGURE 3 Gene mutation type

pattern of β-catenin. In addition, the significance of combined *DICER1* and *CTNNB1* mutations in tumorigenesis have also been reported in liver cancer. In hepatocellular carcinoma, using a conditional knockout mouse model, Sekine et al.[28] found that the expression of growth-promoting genes and fetal stage-specific genes was increased in *DICER1* deficient liver. The Dicer elimination led to increased hepatocyte proliferation and massive apoptosis. Reactivation of the fetal gene expression program might be a key mechanism of hepatocarcinogenesis induced by the loss of *DICER1*. Caruso et al.[29] found a significant association between the *DICER1* mutation and *CTNNB1* mutation. Germline mutations of *DICER1* are correlated with abnormal liver zonation and the excessive activation of β-catenin. These two pathways may have a synergistic effect in liver tumorigenesis.

MYCN is a member of the *MYC* family of proto-oncogenes. It encodes a transcription factor, *MYCN*, that controls the basic process of embryonic development. MYCN protein is located downstream of several signaling pathways that promote the growth, proliferation and metabolism of progenitor cells in different developmental organs and tissues. In contrast, unregulated *MYCN* signals support the development of several different tumors. During the development of mouse embryo, MYCN can be detected in developing lungs, kidneys and intestines.[30] During the 12–24 weeks of gestation, it has been found that MYCN is highly expressed in undifferentiated nerve cells in the neuroepithelial cells of the brain, retina and lungs of human embryos.[31] Our research found missense mutants of *MYCN* in one case, as mentioned above, and this missense variant may correlate with the development of L-FLAC.

Among these three patients, only one patient had distant metastasis shortly after surgery. This patient underwent upper lobectomy of the left lung in 2010, and was finally diagnosed with stage IB adenocarcinoma. Adjuvant treatment was given after surgery with gemcitabine plus carboplatin for four cycles. One year later, the patient returned to the clinic with a further episode of hemoptysis. A left main bronchial nodule was found on imaging, and L-FLAC was diagnosed after total resection of the left lung. Five months after adjuvant mediastinal radiotherapy, lung metastasis was found to be present and the patient's DFS was only 7 months. Although the postoperative stage was early, this patient progressed rapidly and had a short overall survival. A long history of smoking and presence of an ipsilateral lung tumor may therefore be poor prognostic factors. In addition, his TMB was relatively higher than the other two cases. As is already known, TMB is a measure of the number of mutations within a tumor genome, sometimes defined as the total number of nonsynonymous point mutations per coding area of a tumor genome.[32] A previous study has reported that TMB is associated with a poor prognosis in postoperative NSCLC patients.[33] It revealed that in patients with stage I NSCLC, higher TMB was associated with a worse prognosis for both OS and DFS. Multivariate analysis showed poor prognosis with high TMB. High TMB in NSCLC is a poor prognostic factor which may be the reason for rapid disease progression.

This patient also harbored *MUC16* mutation, which has been found to be the third most frequently mutated gene in tumors encoding a high molecular weight membrane-spanning mucin.[34] This protein, thought to be the precursor of CA125 used as a biomarker for ovarian cancer, has been found to suppress the natural killer cell function which regulates tumor cell proliferation, metastasis and innate immune response.[35,36] A recent study indicated that the mutational frequency of *MUC16* is 42.76% in lung adenocarcinoma, and 38.84% in lung squamous cell carcinoma, respectively.[37] Patients with *MUC16* mutation have been found to have

significantly elevated TMB compared to those without them. CD8A and PD-L1 positive is also higher in *MUC16* mutated cases than in wild-type mutations. In addition, patients with *MUC16* mutation receiving ICI treatment have also been associated with superior outcomes. The patient with *MUC16* mutation had a relatively high TMB which is consistent with the above findings, suggesting he would benefit from immunotherapy.

In conclusion, since most FLACs previously described in the literature have been in case studies, data on the molecular characteristics of this rare malignancy remain very limited. In our study, we report on three cases of L-FLAC with more detail of genetic characteristics. L-FLAC is a special clinicopathological malignant entity with unique genetic alterations, and with the use of the WES technique, the rare biological features of this neoplasm and molecular phenotypes might be revealed in the near future, thereby providing the potential genes for targeted therapy.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

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