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Lepidic predominant adenocarcinoma and invasive mucinous adenocarcinoma of the lung exhibit specific mucin expression in relation with oncogenic drivers

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ABSTRACT

Objectives: To evaluate MUC1, MUC2, MUC5B, MUC5AC, and MUC6 expression in invasive lepidic predominant adenocarcinoma (LPA) and invasive mucinous adenocarcinoma (IMA) of the lung, and the impact of oncogenic drivers.

Materials and methods: MUC1, MUC2, MUC5B, MUC5AC, MUC6, TTF1 and Hnf4α immunohistochemistry was performed on surgical samples from 52 patients with IMA (n = 25) or LPA (n = 27). We searched for *EGFR*, *KRAS*, *BRAF*, and *HER2* mutations and *ALK*, *ROS1*, and *NRG1* rearrangements.

Results: MUC1, MUC2, MUC5B, MUC5AC, and MUC6 expression was detected in tumor cells in 77%, 2%, 63%, 36%, and 21% of cases, respectively. MUC1 was significantly more overexpressed in LPA. MUC5B, MUC5AC, and MUC6 were typically detected in goblet cells and overexpressed in IMA. Hnf4α-positive IMA (n = 11) were TTF1-negative and typically did not expressed MUC1 and expressed MUC5AC and MUC6. Hnf4α-negative IMA (n = 14) showed a reverse profile of mucins expression, with MUC1 expression and a lack of MUC5AC and MUC6 expression. *EGFR*-positive status was significantly associated with LPA, MUC1 expression, and no MUC5B, MUC5AC, or MUC6 expression. *KRAS*-positive status was significantly associated with IMA and MUC5B and MUC5AC expression.

Conclusions: LPA and IMA exhibit specific mucin expression profiles, with MUC1 being associated with LPA, while MUC5B, MUC5AC, and MUC6 were associated with IMA. Hnf4α expression and *EGFR* and *KRAS* mutations may play a role in mucin expression profiles of these lung adenocarcinoma subtypes.

1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide, 85% of cases being non-small cell lung cancers (NSCLC) [1]. The most frequent NSCLC type is invasive lung adenocarcinoma (LUAD), further classified into five subtypes: lepidic, acinar, solid, papillary, or micropapillary [1]. Of these, lepidic predominant adenocarcinomas (LPA) demonstrate a unique histological pattern, *i.e.,* "lepidic growth". A lepidic growth is defined as a proliferation of type II pneumocytes growing along the native alveolar structure. Compared to other adenocarcinoma subtypes, LPA affects a specific patient population comprising predominantly non-smokers and females [2].

While LPA is a non-mucinous LUAD, a mucinous variant of LUAD does exist, designated as invasive mucinous adenocarcinoma (IMA). In IMA, tumor cells show a goblet and/or columnar cell morphology, and are tall and well-differentiated with basally-located nuclei, characterized by abundant cytoplasmic mucin. These patients exhibit a worse prognosis than those with LPA, probably due to the aerogenous

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spreading in IMA, accounting for the common multicentric and bilateral lung involvement [3–13]. Distinguishing between LPA and IMA was an aim of the 2015 World Health Organisation (WHO) classification of lung tumours [1]. LPA typically expressed TTF1, which is a marker of type II pneumocytes lineage, whereas IMA do not [14,15]. Interestingly, a large part of IMA are positive for Hnf4α, a nuclear transcription factor important for goblet cell maturation of cells from colonic mucosae [16]. Hnf4α has been proposed as a diagnostic marker of IMA [14,16]. LPA is associated with epidermal growth factor receptor gene (*EGFR*) mutations, whereas IMA is linked to Kirsten-Ras gene (*KRAS*) mutations and oncogenic fusion genes and never display Epidermal Growth Factor Receptor (*EGFR*) mutations [15,17–24].

The human mucin (MUC) family has been sub-classified into secreted and transmembrane mucins [25]. The secreted mucins, termed MUC2, MUC5AC, MUC5B, and MUC6, are encoded by the cluster of mucin genes at locus 11p15.5 [26]. The encoded mucins form a physical barrier for the epithelial cells that line the respiratory and digestive tracts. The transmembrane mucins, such as MUC1, display a single membrane-spanning region, contributing to the protective mucous gel, cell–cell interactions and cell signaling [27].

In normal airways and lung, MUC1, MUC2 and MUC5B mucin genes are expressed in the submucosal glands and MUC1, MUC2, MUC5AC and MUC5B in the surface epithelium [28]. Deregulated mucin expression is observed in carcinomas, potentially enhancing tumor cell survival and growth capacities [25]. Depolarized expression of MUC1 has proven a marker of poor prognosis in lung cancer, its overexpression favoring the proliferation and survival of lung cancer cells, also interacting with EGFR to promote proliferation [25,29–33]. Three studies showed an overexpression of MUC5AC and MUC6 in cancer cells from IMA samples [34–38]. The role of MUC5AC, MUC5B, and MUC6 in lung cancer cell biology is not fully understood [39,40].

In this study, we sought to describe the pattern of MUC1, MUC2, MUC5B, MUC5AC, and MUC6 protein expression in IMA and LPA, along with their relationship with clinical characteristics and common driver oncogenes.

2. Materials and methods

2.1. Patients

Between January 1992 and December 2012, 52 IMA (n = 25) or LPA (n = 27) patients were diagnosed in the respiratory medicine department of Tenon Hospital (AP-HP, Paris, France) and underwent surgery. All diagnoses were reviewed by a lung pathologist (MA) based on the 2015 WHO classification [1]. Clinical findings at diagnosis and follow-up data were recorded. All patients signed an informed consent form for the research, authorizing analysis of their biological samples. This study was approved by our hospital's human research ethics committee.

2.2. EGFR, KRAS, BRAF, and HER2 mutation analyses

For each formalin-fixed paraffin-embedded (FFPE) specimen, a 3 µm tissue section was stained with hematoxylin and eosin (H and E), and the percentage of tumor cells was assessed by light microscopy. Following DNA isolation (Qiagen, Courtaboeuf, France) from three 20 µm tissue sections, *EGFR* mutations pG719S, pT790M, and pL858R (exons 18, 20, and 21, respectively), *KRAS* mutations pG12S, pG12R, pG12C, pG12A, pG12V, and pG13D (exon 2), as well as *BRAF* mutations pV600E and pV600K (exon 15) were detected by means of allele specific genotyping using TaqMan® assays (Life Technologies SAS, Saint Aubin, France). *EGFR* exon 19 deletions, and *EGFR* and human epidermal growth receptor 2 (*HER2*) exon 20 insertions were detected by means of fragment analysis after capillary gel electrophoresis on an ABI 3100® genetic analyser (Appliedbiosystems, Saint Aubin, France) and size estimation of amplified DNA fragments by Gene Mapper® Software v 3.7 (Appliedbiosystems, Saint Aubin, France).

2.3. ALK and ROS1 immunohistochemistry

Immunostainings of the ALK and ROS1 proteins were performed on 3 µm tissue sections by means of a Benchmark Ventana staining module (Roche Diagnostics, Meylan, France), using either a primary monoclonal ALK antibody (Clone 5A4, Ab 17127; Abcam, Paris, France) or primary monoclonal ROS1 antibody (Clone D4D6, #3287, Cell Signaling Technology, Danvers, MA, USA), as previously described. Positive external controls were performed using a LUAD specimen that had previously been validated for *ALK* rearrangement by fluorescent *in situ* hybridization (FISH) and the *ROS1*-rearranged cell line HCC78. The staining scores were categorized as follows: 0:no staining; 1+:faint cytoplasmic staining; 2+:moderate cytoplasmic staining; 3+:intense granular cytoplasmic staining. If 10% of cells stained with an intensity of ≥2, the staining was considered positive. Specimens with a positive staining score were tested for *ALK* or *ROS1* rearrangement by FISH.

2.4. 2.4. ALK, ROS1, and NRG1 break-apart FISH assay

FISH was performed on unstained 4 µm FFPE tumor-tissue sections using an *ALK* break-apart probe set (Abbott Molecular, Rungis, France) or ZytoLight® SPEC *ROS1* Dual Color Break Apart Probe (ZytoVision, Bremerhaven, Germany), along with a paraffin-pretreated reagent kit (Abbott Molecular), according to the manufacturer's instructions. Tumor tissue was considered *ALK*- or *ROS1*-FISH positive if > 15% of the cells exhibited split orange and green signals and/or single orange signals for *ALK*-FISH and single green signals for *ROS1*-FISH.

Given that *NRG1* fusions have previously been described in tumors without *EGFR/KRAS/BRAF/HER2* mutations or *ALK/ROS1* rearrangements, *NRG1* break-apart FISH assays were only performed in pan wild-type samples. An *NRG1*-specific fluorescent DNA probe was used, generously provided by ZytoVision (ZytoVision, Bremerhaven, Germany). Tumor tissues were considered *NRG1*-FISH positive when > 15% of the nuclei harbored either a split pattern with 3′ and 5′ signals, separated by a distance superior to the diameter of the largest signal, or isolated 3′ (orange) signals.

2.5. Mucin, TTF1 and Hnf4α immunohistochemistry

Immunostaining of MUC1, MUC2, MUC5B, MUC5AC, MUC6, Hnf4α and TTF1 proteins was performed on 3 µm tissue sections, processed by means of a BenchMark ULTRA Ventana® staining module (Roche, Tucson, AZ).

For mucins, antigens were retrieved using the CC1pH 8.8 ethylenediamine-tetraacetic acid (EDTA) antigen retrieval solution (Ventana Medicals System). The primary monoclonal antibodies against MUC1 (clone Ma695, 1/400, Novocastra), MUC2 (clone cp58, 1/50, Novocastra), MUC5B (LUM-5B1 clone, 1/800), MUC5AC (clone CLH2, 1/50, Novacastra), and MUC6 (clone CLHH5, 1/50, Novacastra) were incubated for 36 min at 37 °C. We used the Ultra View DAB detection kit® (Ventana Medicals System, Roche Group).

For Hnf4α, antigens were retrieved using the DAKO pH9 EDTA antigen retrieval solution. The primary monoclonal antibodies against Hnf4α (mouse anti-human Hnf4α, clone H1415, 1/200, Novex Life Technologies) was incubated for 90 min at room temperature. We used DAKO mouse Envision+ System-HRP Labelled Polymer for detection [16]. A positive HNF4a reaction was marked and unequivocal, and we classified the immunoreaction as either positive or negative.

For TTF1, heat-induced epitope retrieval at pH 6 was used. We used the primary monoclonal antibodies against TTF1 (clone 8G7G3/1, 1/100, DAKO) and the Ultra View DAB detection kit® for TTF-1 detection (Ventana Medicals System, Roche Group). TTF-1 staining was scored as the percentage of positive tumor cells per slide, from 0 to 100%, and a score of 10% was chosen as the threshold for positivity.