Pulmonary Peripheral Glandular Papilloma and Mixed Squamous Cell and Glandular Papilloma Frequently Harbor the \textit{BRAF} V600E Mutation

\textbf{Running title:} Pulmonary Papillomas Harboring \textit{BRAF} V600E Mutation

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Abstract

Aims: Pulmonary peripheral glandular papilloma (GP) and mixed squamous cell and glandular papilloma (MP) have very similar histological features to pulmonary ciliated muconodular papillary tumor (CMPT) /bronchiolar adenoma (BA). The underlying genetic relationships between GP/MP and CMPT/BA have rarely been characterized. We aimed to reveal the relationship between them.

Methods and results: We performed a clinicopathologic review and next-generation sequencing (NGS) study of 2 GPs and 5 MPs. Histologically, GPs/MPs showed similar cellular and architectural features to CMPTs/BAs, such as bilayered epithelium, bronchiole-associated lesions and skipping (discontinuous) growth pattern. One MP showed partial and inconspicuous endobronchiolar growth and more glandular structures, which was very similar to the appearance of CMPT/BA. The BRAF V600E mutation was detected in 4 papillomas (57.1%, 1 GP and 3 MPs).

Conclusions: Overlapping morphologic features and comparable mutation profiles support that peripheral GPs/MPs and CMPTs/BAs are on the same disease spectrum. We propose expanding the concept of CMPT/BA and including GP and MP in the CMPT/BA family.
Introduction

CMPT is a rare mucin-producing tumor that usually presents as a peripheral nodule. The designation was first proposed by Ishikawa in 2002. The classic description of CMPT is a peripherally located nodular tumor with prominent papillary architecture consisting of mucinous, ciliated, and basal cells. However, the vast majority of cases do not have all of the classic histologic features. Unlike classic CMPTs, many lesions have only focal or absent papillary architecture, and they have variable numbers of ciliated and mucinous cells, with some lesions entirely lacking 1 or both of the luminal components. In addition to mucinous and ciliated cells, Clara cells and some cuboidal cells have been identified in some similar lesions, and a continuous layer of basal cells always exists in all cases. In 2018, Chang et al expanded the concept of CMPT and revised the terminology of these lesions and named them BA.

Like CMPT/BA, GP and MP in the peripheral lung show similar histologic structures and cellular components. Despite their morphologic similarities to papillomas, CMPT/BA was defined as a lesion growing entirely on alveolar walls with no involvement of the bronchial lumen, whereas GP/MP was defined as a lesion at least focally involving bronchioles. Currently, many researchers believe that GP/MP and CMPT/BA represent the same tumor. However, the molecular characteristics of GP/MP remain unclear. Recently, one GP and one MP with a BRAF V600E mutation were reported by Kitawaki et al and Huang et al, respectively. The BRAF V600E mutation was shown to be the most common genetic change in CMPT/BA, which suggests that these tumors belong to the same disease spectrum. Herein, we report 7 cases of GPs/MPs. The morphologic, immunophenotypic, and molecular features were analyzed. We attempt to clarify the relationship between GP/MP and CMPT/BA.

Materials and methods

Patient selection

Peripheral GP/MP was defined as a peripheral lesion with glandular papillary or mixed papillary structure, with the papillary structure completely or partially located in a bronchiole rather than a bronchus. Cartilage plates and submucosal glands were not present in the wall. The presence of glandular papillary structures with superficial glandular cells and basal cells was necessary for the diagnosis. According to the morphology criteria, a total of 7 patients were included in this study dating from 2010 to 2019, in which 6 patients were from the Affiliated Hospital of Qingdao University and 1 patient was from the Affiliated Central Hospital of Qingdao University. All...
clinicopathologic records, including frozen and formalin-fixed paraffin-embedded (FFPE) sections, were reviewed, and the results are summarized in Table 1. In contrast, CMPT/BA was defined as being confined entirely to alveolar walls with no association with the bronchial lumen. Six CMPTs/BAs were also collected for molecular testing as controls (Supplementary Table). One MP (case 2) and two CMPTs/BAs (Supplementary cases 2 and 3) have been described in previous reports. This study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University on October 3, 2019 (No. QYFY WZLL 25703). All patients provided written informed consent.

**Targeted next-generation sequencing**

All H&E slides were carefully examined and the sections with the most abundant tumor component were selected. Based on the morphological findings, other non-tumorous components, such as the adjacent lung tissue and the bronchus tissue, were cut off to the greatest extent possible. Tumor areas from unstained slides were manually microdissected. Targeted next-generation sequencing (NGS) was performed on an Illumina MiniSeq platform (Illumina, San Diego, USA). Genomic DNA was extracted from the formalin-fixed paraffin-embedded tumor blocks using the QIAamp DNA FFPE Tissue Kit (50) (Qiagen, Valencia, CA, Germany), and the concentration was determined using the Qubit dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Libraries of the technical testing cohort were prepared with the GENEIS® Human EGFR/ERBB2/KRAS/NRAS/BRAF/PIK3CA/ALK/ROS1/RET/MET Mutation Test Kit (Geneis, Beijing, China), examined for quantity and quality using the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, USA), and then subjected to clean up, normalization and pooling exactly following the manufacturer’s protocol. Sequencing runs were performed with 15–18 pooled libraries using the MiniSeq High Output Reagent Kit (300 cycles) (Illumina) and paired-end sequencing with 2 × 151 bp. Mutations were identified and annotated through BWA, Freebayes and Annoval and direct visual inspection of the binary sequence alignment/map (BAM) file using the Broad Institute’s Integrative Genomics Viewer (IGV). With sufficient DNA input, the limit of detection is dictated by the depth of coverage (or number of sequencing reads). During our validation of this NGS assay, a cutoff of background noise at 1% was chosen for single nucleotide variations. Approximately 1000 reads are needed to detect a heterozygous mutation at a 99% confidence in a specimen with 50% tumor cellularity. Amplification-refractory mutation system (ARMS) (AmoyDx, Xiamen, China) analysis was performed to confirm the results of NGS.

**Immunohistochemistry**

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Immunohistochemical analysis was performed on paraffin-embedded sections using the following primary antibodies: thyroid transcription factor-1 (TTF-1) (SPT 24, Novocastra, Newcastle, UK), cytokeratin (CK) 7(OV-TL 12/30, DAKO Cytomation, Glostrup, Denmark), CK20 (Ks 20.8, DAKO Cytomation, Glostrup, Denmark), CK5/6 (D5/16B4, Dako Cytomation, Glostrup, Denmark), p40 (BC28, Biocare Medical, Concord, CA, USA), p63 (4A4, Dako Cytomation, Glostrup, Denmark), napsin A (1P64, Novocastra, Newcastle, UK), BRAF (VE1, Ventana Medical Systems, Tucson, AZ, USA), and Ki-67 (MIB1, Dako Cytomation, Glostrup, Denmark). BRAF V600E (VE1) staining was performed using a VENTANA Benchmark® XT automated system (Ventana Medical Systems, Inc., Tucson, AZ, USA). The immunohistochemical stainings for other markers were performed using a Leica BOND-MAX™ fully automated system (Leica Microsystems GmbH, Wetzlar, Germany).

Results

Clinical findings

All patients were Chinese, including 5 women and 2 men with a median age of 56 years (range: 49 to 70 years). One patient had a history of smoking. There are 5 MPs and 2 GPs included in our study. The detailed locations were the right upper lobe (1 case), the right lower lobe (2 cases), the left upper lobe (1 case), and the left lower lobe (3 cases). The median size of the tumors was 1.5 cm (range: 1.0 to 5.0 cm). Three patients appeared asymptomatic, and four patients were symptomatic, including cough (3 cases), chest pain (2 cases) and bloody phlegm (1 case).

Surgical resection was performed in all patients, and an intraoperative frozen section diagnosis was made in 4 patients, of which 2 were misdiagnosed as mucoepidermoid carcinoma (MEC). The lesions were excised by lobectomy (6 cases) or wedge resection (1 case). The mean follow-up was 48.9 months, ranging from 6 months to 89 months, and none of the patients experienced local recurrence or distant metastasis.

Gross findings

All 7 tumors appeared gray-white, soft, and well-demarcated with a gelatinous quality. One tumor was located adjacent to the pleura without pleural retraction. Four tumors (cases 3, 4, 6 and 7) were intracystic, with a cauliflower-like appearance. The surgical margin was definitively negative for all tumors.

Histologic findings

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All 7 cases showed a partially endobronchiolar lesion with abundant mucin production (Figure 1A). Five presented with a nearly pure papillary structure. The epithelial components of the tumor consisted of a mixture of ciliated columnar cells, mucous cells and basal cells (Figure 1B) in GPs. All 5 peripheral MPs showed a similar histomorphology and had a cystic structure. Three had bronchiolar squamous metaplasia in some areas (Figure 1C). Bronchiolar squamous metaplasia was not found in GPs. In the 5 cases of MPs, ciliated columnar cells, mucous cells and basal cells were also observed. Three MPs (cases 2, 4 and 6) showed an almost pure papillary structure (Figure 1D). Cases 1 and 5 had some glandular structure. Transitional papillae with histologic features between glandular and squamous papillae were also present in all MPs indicating that MP represents GP with squamous metaplasia. In case 5, the endobronchiolar growth was partial and inconspicuous (Figure 1E), which was very similar to that in CMPT/BA. All tumors had varying amounts of chronic inflammatory cells, and intraepithelial microabscesses were frequently observed in papillary growth architectures in 6 cases (Figure 1F). Tumor cells lacked nuclear atypia and mitotic figures. Necrosis was not observed in all cases. The peripheries of all tumors showed a skipping pattern of growth of tumor cells (discontinuous tumor cells extending away from the main tumor for more than one alveolus), which showed micropapillary, papillary, and budding/lepidic patterns (or structures) (Figures 2A and 2B). More representative images of each case are provided in the supplementary figures.

Immunohistochemistry and NGS

The immunohistochemistry and molecular findings are summarized in Table 2. Immunohistochemical staining highlighted the bilayered epithelium and multiple cell populations in these cases. The basal cells were highlighted by p63 and p40 in all 7 tumors (Figure 3A). The skipping tumor cells also contained basal cells according to immunohistochemical staining. The cilia of the ciliated columnar cells were highlighted by BRAF, which revealed a variable number of ciliated columnar cells in each patient. BRAF showed cytoplasmic granular staining in 4 cases (Figure 3B). All types of cells, including squamous cells and a variable number of glandular cells stained positively for TTF-1 (case 3 was an exception, TTF-1 was focally positive only in some basal cells) (Figure 3C). Tumor staining of TTF-1 was weaker than that in pneumocytes and similar to that in basal cells in bronchi (Figure 3D). All types of cells, including squamous cells, were positive for CK7 (Figure 3E). Basal cells and squamous cells were always positive for CK5/6, and a small number of superficial glandular cells also showed immunoreactivity for CK5/6 in all cases (Figure 3F). Napsin-A and CK20 were negative in all 7 cases. The Ki67 proliferative index ranged from 1% to 2%. A bilayered epithelium was identified by immunohistochemical staining in
all 7 CMPTs/BAs.

The *BRAF* V600E mutation was detected in 4 (57.1%) cases of GPs/MPs that exhibited cytoplasmic granular staining of BRAF by immunohistochemistry. Four cases of CMPTs/BAs demonstrated genetic changes, including the *BRAF* V600E mutation (2 cases, 33.3%), *EGFR* mutation (1 case, 16.7%) and *KRAS* mutation (1 case, 16.7%) (Supplementary Table). All mutations were confirmed by ARMS analysis.

**Discussion**

Solitary pulmonary tumors with papillary structures were identified in 1998, including squamous cell papilloma, GP, and MP. According to their locations, these tumors can be divided into a central endobronchial type and a peripheral bronchiolar type. The peripheral GPs and MPs display similar clinicopathologic features to CMPTs/BAs. GPs/MPs usually arise in middle-aged and older patients, similar to CMPTs/BAs. All 7 patients we presented fall into the same age range. The presence of only one smoker suggested that smoking may not be a causative factor for these lesions.

In general, CMPTs/BAs are restricted to peripheral non-endobronchial lesions. However, all reported CMPTs/BAs have invariably been adjacent to bronchioloarterial bundles or penetrated by unpaired medium-sized arteries, supporting the idea that they are bronchiole-associated tumors like papillomas. Although endobronchiolar growth and papillary architecture are typically more conspicuous in GPs/MPs than in CMPTs/BAs, it is difficult to identify whether the tumor is an endobronchiolar lesion in some cases (as in case 5). The overall arrangement of GPs/MPs can be otherwise similar to that of CMPTs/BAs, exhibiting a bilayered epithelium consisting of variable glandular cells and basal cells. A few cases of “papilloma” in the peripheral lung have been reported, and some of them conformed to the CMPT/BA appearance judging from published illustrations. Thus, sometimes it is difficult to distinguish one from another. Squamous metaplasia was found in 1 case of CMPT/BA we presented and in some CMPTs/BAs in the literature. Except for differences in squamous cells, there was no significant difference between GP and MP. MP can be recognized as GP with squamous metaplasia.

Because of their similar histomorphologies and tendency to mimic malignancies, MEC and adenocarcinoma should be listed in the differential diagnoses for GP/MP. Two patients in our study were misdiagnosed with MEC in the initial intraoperative diagnoses. MECs may be confused with GP/MP due to the presence of mucinous cells, intermediate cells, and a papillary...
configuration. However, identifiable atypia is usually present in MEC, and pulmonary MEC is usually a lesion located in the central lung. Recently, a case of ciliated MEC was reported. Similar to classic MEC, the ciliated case also had MAML2 rearrangement, and the presence of this rearrangement can distinguish MEC from GP/MP. A skipping growth pattern can be observed in some CMPTs/BAs. This morphology has been frequently mentioned in GP/MP. All 7 cases presented with skipping growth, which may make GP/MP and CMPT/BA difficult to differentiate from adenocarcinomas. Considering that pathologists are often uncertain about the biological nature of these tumors, some researchers have termed them well-differentiated adenocarcinomas. Judging from the published illustrations in the articles, we believe that those entities represented GPs/MPs or CMPTs/BAs with skipping growth. The presence of skipping growth likely led to a misdiagnosis of adenocarcinoma. GPs/MPs usually lack nuclear atypia and mitotic activity and have basal cells and ciliated cells, which can distinguish them from adenocarcinoma. In some of the cases, the number of ciliated cells was quite low, so pathologists should examine this parameter carefully. However, ciliated cells can be absent in some CMPTs/BAs, creating a substantial challenge for pathologists, especially in diagnoses based on frozen sections. The most reliable diagnostic clue in frozen sections is the basal cell layer, which is universally present in all GPs/MPs and CMPTs/BAs and consistently absent in lung adenocarcinomas. In some cases, the basal cells were inconspicuous. Fortunately, a lack of ciliated cells and unremarkable basal cells are usually not present in the same case. Intraepithelial microabscesses were frequently observed in the papillary structures in 6 cases, providing a potential clue for accurate diagnosis. In our experience, intraepithelial microabscesses are not a common phenomenon in pulmonary papillary adenocarcinoma and mucinous adenocarcinoma.

Immunohistochemically, p63 and p40 highlighted basal cells in all cases. Basal cells and superficial cells displayed some similar immunophenotypes: TTF-1 and CK5/6 staining showed immunoreactivity in both the basal cells and some superficial glandular cells. Squamous cells were also positive for TTF-1 and CK7. These similarities indicate that basal cells have stem cell characteristics and can differentiate into various superficial cells, including ciliated cells, mucous cells and squamous cells. BRAF V600E immunohistochemistry is a very useful marker for these tumors; it can not only highlight cilia, but also be used as a surrogate marker for the presence of an underlying BRAF V600E mutation.

The prevalent genetic alterations in CMPTs/BAs involve BRAF, KRAS, EGFR, and ALK. The BRAF V600E mutation was shown to be the single most common driver mutation in CMPTs/BAs. In our study, 4 of 7 papillomas (57.1%) and 2 of 6 CMPTs/BAs (33.3%) showed the BRAF V600E mutation.
mutation, strongly indicating that GPs/MPs belong to the CMPT/BA family. Unlike in CMPT/BA, there was no other driver mutation except for the \textit{BRAF} V600E mutation in GPs/MPs in our study, and the same has been found in the literature \textsuperscript{4, 5}, which means that GP/MP may represent a subgroup of the CMPT/BA family. \textit{BRAF} is a well-known driver oncogene that is mutated in many types of malignant tumors, such as malignant melanoma \textsuperscript{26}, thyroid papillary carcinoma \textsuperscript{27}, lung adenocarcinoma and colonic adenocarcinoma \textsuperscript{28, 29}. The V600E point mutation is the most common mutation. The \textit{BRAF} V600E mutation is also frequently found in various types of benign tumors. For example, acquired melanocytic nevi and metanephric adenomas of the kidney have this mutation \textsuperscript{30, 31}. The presence of \textit{BRAF} mutations in CMPTs/BAs merely represents evidence for a neoplastic process and is not synonymous with malignancy. Kim et al suspected that oncogene-induced senescence might be the pathogenic mechanism of CMPT, which shows a benign course \textsuperscript{25}.

In conclusion, this study shows that peripheral GPs/MPs have a very similar morphology to CMPTs/BAs. Peripheral GPs/MPs have frequent \textit{BRAF} V600E mutations, similar to CMPTs/BAs. We propose expanding the concept of CMPT/BA and including peripheral GP and MP in the CMPT/BA family. Moreover, it is worth reevaluating the central type MPs and GPs as to whether they represent central type CMPTs/BAs. \textit{BRAF} mutational analysis should be helpful in judging such central lesions in the future.

**Acknowledgements and Author Contributions**

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**References**


15. Abe J, Ito S, Takahashi S *et al.* Mixed squamous cell and glandular papilloma of the lung resembling

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<table>
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<tr>
<th>Case No.</th>
<th>Age (years)/Sex</th>
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<th>Size (cm)</th>
<th>Clinical Presentation</th>
<th>Histologic Finding</th>
<th>Diagnosis</th>
<th>Intraoperative frozen/Postoperative Treatment</th>
<th>Follow-up (months)</th>
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F, female; GP, glandular papilloma; LLL, left lower lobe; LUL, left upper lobe; M, male; MEC, mucoepidermoid carcinoma; MP, mixed squamous cell and glandular papilloma; NED, no evidence of disease; RLL, right lower lobe; RUL, right upper lobe.

* Synchronous acinar adenocarcinoma (sized 1.8cm, T1N0M0) with EGFR-ex19del in the same lobe.
Table 2. Summary of immunohistochemical and molecular findings of GPs and MPs

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+, positive; -, negative.

*<10% of cells positive
Figure legends

**Figure 1.** GPs and MPs. A, The GP showed a partially endobronchial lesion with abundant mucin production. B, Ciliated columnar cells, mucous cells and basal cells in GP. C, Cystic dilated bronchiole with squamous metaplasia was presented in MP (case 2). D, An endobronchiolar lesion and almost a pure papillary structure were observed in case 6. E, Some glandular structures were observed in case 5, and the endobronchiolar growth was partial and inconspicuous, which was very similar to CMPT/BA. F, Intraepithelial microabscesses were frequently observed in papillary growth architectures.

**Figure 2.** Skipping growth patterns in GP and MP. A, Skipping tumor cells with papillary constructions in GP (case 3). B, Skipping tumor cells in MP (case 1).

**Figure 3.** The immunophenotype of this case series. A, The basal cells were highlighted by p63 (case 3). B, BRAF immunopositivity showed a cytoplasmic granular staining (case 2). C, Squamous cells were positive for TTF-1 (case 4). D, Some glandular cells were also positive for TTF-1, but the immunointensity was weaker than the background pneumocytes (case 5). E, CK7 was positive in all cell types (case 4). F, Basal cells, squamous cells and a varying number of superficial glandular cells showed immunopositivity for CK5/6 (case 5).