Deconstructing the intra-tumor subclonal heterogeneity of lung synchronous ground-glass nodules using whole-genome sequencing

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Dear Editor,

The persistent presence of ground-glass opacity nodules (GGN) on thin-section computed tomography (CT) usually suggests the presence of lung adenocarcinoma,1 which include atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma, and invasive adenocarcinoma (AD).2 Recent data indicate that up to 20% of GGN patients (3% of minimally invasive adenocarcinoma, and invasive adenocarcinoma adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), recommended strategy for the identi

auxiliary tests that could assist in differential diagnosis nor recommended strategy for the identification and treatment of GGNs exist in the clinical practice guidelines for lung cancer, although clinical evidence suggests that SM-GGNs are more complicated, making treatment decisions difficult.1 Recent studies using next generation sequencing suggest differences in the mutational landscape not only among inter-tumors but also intra-tumor.

In this study, we first performed whole-genome sequencing (WGS) on 15 samples from five patients with each having two GGNs and one normal control (Fig. 1a, Supplementary Fig. S1). Detailed clinical features are summarized in Supplementary Table S1. All patients were non-smoking and disease free within the 24–30 months after surgery. We first explored the somatic copy number variations (CNV) patterns using deep WGS. We found 2184 somatic CNVs (26 deletions and 2158 amplifications) in the early stages of lung AD and 2280 CNVs (72 deletions and 2208 amplifications) in AAH. The CNV heatmap and correlation analyses revealed two CNV patterns. The independent subtype was characterized by independent lineage relationships (patients 1 and 3). Primary lung cancers and their matched AAHs shared considerably fewer global CNV patterns (Fig. 1b). The CNVs across the genomes between primary lung AD and AAH were found to correlate less with each other in two patients (Fig. 1b). However, the parallel subtype was characterized by parallel lineage relationships (patients 2, 4, 5). As indicated by the CNV heatmap, primary lung ADs and their matched AAHs generated similar global CNV patterns (Fig. 1c). The CNVs across the genomes between primary lung ADs and AAHs correlated positively with each other, with a correlation coefficient >0.8 for all three patients (Fig. 1c). The amplifications or deletions did not share the patterns among different patients. Interestingly, the histologic characteristics of the two lesions of each patient of independent lineage relationships (patients 1, 3) showed differences. In patient 1, AAH did not show thickened alveolar walls or more-marked atypical pneumocytes proliferation, whereas AIS showed both characteristics. In patient 3, AAH showed only thickened alveolar walls, whereas primary lung AD showed both characteristics (Fig. 1a).

We used the TITAN program4 for intra-tumor subclone analysis. The germline heterozygous SNP loci (HET) across the genome were detected and the CNV and loss of heterozygosity (LOH) segments were jointly inferred from read depth and digital allele ratios at the HETS of the tumor WGS data. The new CNV and LOH types were reassessed at the multi-clone state that might be different from the previous single clone detection (Fig. 1d, Supplementary Fig. S2). As seen in Fig. 1e, the AAH4 and AD4 had ~30% normal cellular estimate based on the number of Het retained as normal sample. Each sample had two subclones. The subclone C2 of AAH4 shared variation features (copy number GAIN) with subclone C1 of AD4 (Fig. 1f). As seen in Fig. 1d, the AAH4 and AD4 had ~30% normal cellular estimate based on the number of Het retained as normal sample. Each sample had two subclones. The subclone C2 of AAH4 shared variation features (copy number GAIN) with subclone C1 of AD4 (Fig. 1f). Subclone C5 of AAH5 shared features with C5 of AD5 (red, copy number GAIN). Though we did not see a similar CNV pattern between AAH1 and AIS1 in CNV overview, the small C3 (0.28) of AAH1 shared features with the small C4 (0.26) of AIS1 in copy number GAIN. The SM-GGNs of patient 3 did not show co-occurring events in any subclone after the CNV/LOH deconstruction.

For verification, low-depth WGS DNA sequencing was performed on an additional 30 samples from 10 SM-GGN patients, two SM-GGNs and one paired normal controls of the lung tissues for each patient. The overview of somatic CNV shows a strong pattern in SM-GGN pairs of patients 1, 2, 4, and 10 (Fig. 1g). Interestingly, the LOH types showed more even pattern than did CNV types, as almost all SM-GGNs pairs shared ALOH except those in patient 10 (Fig. 1h). Two to five subclones were detected in the 20 GGN samples. Patients 1, 2, 3, 5, 6, and 10 had more common features in the top 1 or 2 subclones compared to patients 7, 8, and 9. Patient 1 showed a parallel CNV pattern between MG1AAH and MG1AIS (Fig. 1i, j). This evidence supported a parallel lineage among the SM-GGNs of patients 1, 2, 3, 5, 6, and 10 and an independent lineage among the SM-GGNs of patients 7, 8, and 9.

Based on our data, we propose two models for the evolution of lung SM-GGNs. Supplementary Figure S3a depicts a model of independent lineage relationship in which two cancer-initiating cell clones (CIC) independently generate synchronous SM-GGNs. SM-GGNs can also arise in parallel from a single primary CIC clone

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(parallel lineage relationship) (Supplementary Fig. S3b). SM-GGNs arising via parallel lineage relationships may share high-impact variants (as these are more likely to be tissue-specific driver mutations), but their continued and separate evolution are expected to produce low-frequency, low-impact variants that would be unique to each lesion. The model also predicts that SM-GGNs with a parallel lineage relationship will have a high proportion of shared copy number aberrations and structural
variants as well as a common subclone structure. Importantly, a shared subclone structure implies that many cells are from the same ancestor clone. Likewise, we acknowledge that various of analysis including RNA-seq, proteogenomics will be required for deeper research on SM-GGNs.

In summary, SM-GGNs in lung tumors present clinical challenges in surgery setting, therapy decision, and prognosis prediction.\textsuperscript{5} Comparing to our understanding of multiple metastasis tumors which were often found to share somatic driver mutations, we have much less knowledge about the molecular biology of how the multiple nodules occur. By deconstructing two types of genomic data (deep WGS:100X, and low-depth WGS:5X), our discoveries include shared CNV patterns and clonal structures between SM-GGNs. Our data support CNV correlation and cellular subclone estimation being an alternative and potentially more accurate and specific route for lineage determination.

DATA AVAILABILITY
All the sequencing data have been deposited at the Sequence Read Archive (SRA, http://submit.ncbi.nlm.nih.gov/sra/), which is hosted by the NCBI under the accession code SUB2009369.

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ADDITIONAL INFORMATION
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Competing interests: The authors declare no competing interests.

Ethics: The institutional Ethics Committee of the Shanghai Pulmonary Hospital approved the study (k16-264).

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