Intra-tumor heterogeneity is a big barrier to precision medicine. To explore the underlying clonal diversity in lung adenocarcinomas, we selected nine individuals with whole-genome sequencing data from primary and matched metastatic tumors as a cohort for study. Similar global pattern of arm-level copy number changes and large variations of somatic single-nucleotide variant between the primary and metastasis are observed in the majority of cases. Importantly, we found breakage-fusion-bridge (BFB) cycles acting as an important mechanism for underlying cancer gene amplification, such as amplification of \(CDK4\), \(CDKN3\) and \(FGFR1\) in early stage. We also identified recurrent focal amplification of gene \(CCNY\) derived from BFB in two metastatic tumors, but not in primary tumor. Clonal analysis of case 236T demonstrated that mutational processes are varying with tumor progression. Collectively, our data provide new insights into genetic diversity and potential therapeutic target in lung adenocarcinoma.

*Journal of Human Genetics (2017) 62, 783–788; doi:10.1038/jhg.2017.40; published online 6 April 2017*
Intra-tumor diversity in lung adenocarcinomas

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RESULTS
Genomic diversity within intra-individual genomes

We analyzed somatic structural variant between primary and metastasis. Great diversity of structural variations was observed across nine cases (Supplementary Figure 2 and Supplementary Table 2), albeit they shared similar mutational mechanisms between primary tumor and metastases. Of note, case 075T has large number of sub-clonal mutations in metastasis compared to primary tumor (Figure 1b). Interestingly, TP53 and PIK3CA mutations areoeaparantly found in primary tumors 23T and 236T, while MET has two missense mutations in metastatic cases 075T and 155T (Figure 1c).

Breakage-fusion-bridge in primary and metastatic genomes

We analyzed somatic structural variant between primary and metastasis. Great diversity of structural variations was observed across nine cases (Supplementary Figure 2 and Supplementary Table 2), albeit they shared similar mutational mechanisms between primary tumor and metastases. Of note, case 075T has large number of structural variations (Supplementary Figure 2) indicating high genomic instability. In addition, EGFR is amplified in 67% samples and ALK-EMLA fusion was found in sample 136 (Figure 1d).

Some of the somatic structural variants were recognized as a single inverted rearrangement exhibiting layers of copy number amplification. These rearrangements may be termed 'fold-back inversions' as previous studies supported,\textsuperscript{12} which is most likely to be generated by BFB cycle. In total, we identified 154 fold-back inversions in primary tumors, as well as 83 in metastases. We observed sporadic distinct arm-level change: gain of chr2 is specific to primary tumor in case 206T; gain of chr4 occurred specifically in metastasis 155T (Supplementary Figure 1). It is worth noting that 4q gain is a metastatic signature in lung cancer.\textsuperscript{14} This data suggests limited copy number change of intra-individual genomes in lung adenocarcinoma.

In contrast, the number of common somatic SNVs varied between primary tumors and metastases and nearly half of mutations are specific to primary genome or metastases (Figure 1a). The proportion of sub-clonal mutation varied across nine cases, of which three case including 236T, 155T and 206T have more sub-clonal mutations in metastasis compared to primary tumor (Figure 1b). Interestingly, TP53 and PIK3CA mutations areoeaparantly found in primary tumors 23T and 236T, while MET has two missense mutations in metastatic cases 075T and 155T (Figure 1c).

Breakage-fusion-bridge detection

We inferred BFB events from the Meerkat results based on three criteria proposed by Peter Campbell et al\textsuperscript{12} (1) Inversion is single inverted that is read as invers _f or invers _r by Meerkat. (2) Inversion segments have copy number change versus the adjacent position. (3) The two ends of the inversions must be separated by <30 kb.

Cancer cell fraction estimation and genome doubling prediction

We applied ABSOLUTE\textsuperscript{13} to quantify copy number change profiles by Patchwork from 24 primary and metastatic tumors, of which nine cases have comparable tumor contents between primary and metastatic tumors (Supplementary Table 1). The remaining 15 cases exhibit nearly no copy number changes in either primary or metastatic tumors, suggesting low proportion of cancer cells in these samples. Therefore, we focused on nine cases with high neoplastic purities that are feasible to characterize intra-tumor heterogeneity in lung adenocarcinoma.

Based on copy number profiling, we found the genomes of 8 cases are nearly diploid except case 236T. All cases shared similar global pattern of arm-level change between primary tumor and metastases (Supplementary Figure 1). Of these eight cases, gene-level copy number correlation ranges from 0.54 to 0.89. However, we still found that are feasible to characterize intra-tumor heterogeneity in lung adenocarcinoma.

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Figure 2 BFB events across nine LC paired primary and metastases. The upper panel shows the intra-arrangements in each chromosome. The bottom panel shows the normalized coverage. (a) CDK4 amplification. (b) CDKN3 amplification. (c) FGFRI amplification. (d) CCNY amplification. A full color version of this figure is available at the Journal of Human Genetics journal online.
72% of fold-back inversions in primary tumors and 75% in metastases are mediated by micro-homology (Supplementary Figure 3).

Notably, we observed multiple cancer genes involved in BFB cycle. High-level of CDK4, CDKN3 and FGFR1 amplification derived from BFB cycle could be observed in both primary and metastatic tumors (Figures 2a–c), implying these BFB events occurred early in tumor development before tumor-cell dissemination. CDK4 encodes a Ser/Thr protein that is important for cell cycle G1 phase, amplification of CDK4 gene has been found in various human tumors including lung tumors, which can lead to tumorigenesis.15 CDKN3 is a cyclin-dependent kinase inhibitor, and this gene was reported to be overexpressed in most human cancer tissues and may have association with poor survival in lung cancer.16 FGFR1 amplification is common in lung and other human cancer and it was predicted as a predictive prognostic maker.17 Interestingly, we found recurrent CCNY BFB-derived amplification specifically in metastatic lung cancer (Figure 2d). CCNY is a member of cyclins controlled cell division cycles and regulated cyclin-dependent kinases. Overexpression of CCNY can result in tumor proliferation, and has been reported to be a therapeutic target in non-small-cell lung cancer.18 However, the underlying mechanism of CCNY amplification in metastatic lung cancer has not been elucidated before. Further functional study is needed to recapitulate its role in metastases. Collectively, our results confirmed an important role of BFB cycles derived gene amplifications in lung cancer, and targeted therapy may benefit lung cancer patients with these amplified oncogenes.

Branched evolution in case 236T

Even though high concordant genomic alterations were found in most primary and metastatic genomes, an exception was found in patient 236, wherein a large difference of arm-level change was observed with 15% common SNVs among them. Clustering with arm level copy number variant change showed that primary sample 236T and metastasis sample 236C had a much greater genetic evolution distance which confirmed the tremendous genomic differences between them (Supplementary Figure 4). Specifically, metastatic genome was predicted to undergo genome doubling using ABSOLUTE that provides reasonable explanation for large genomic changes from primary tumor. To further explore the characteristics of mutational processes, we further inspected its genomic evolution in detail. During the study, ABSOLUTE13 was employed to estimate the cancer cell fraction in both somatic copy number aberration and somatic single nucleotide variant (Figure 3c). On the basis of the cancer cell fraction of each mutation, the mutations were classified.
into three categories: early, middle and late (Figure 3b). It was presumed that the clonal mutation belonged to the early events and sub-clonal mutation belonged to the late events. Mutations in the clonal group were further classified into mutations occurring before metastasis (mutations appear in both primary and metastasis) and mutations that occur after metastasis (mutations appear only in primary or metastasis). Those mutations that happen after metastasis were defined as middle events and the mutations that occurred before metastasis were considered to be early events. Hence in our data, it was observed that primary tumor and metastases of the case 236 and other eight cases were all derived from a common ancestor clone, and then each slowly evolves into two independent clones.

Also, we compared the copy number changes in case 236 (Figure 3a). The 3p deletion was present in both primary and metastasis, indicating it belonged to an early event. Further, the 3p deletion that frequently occurred in lung cancer has been reported to influence the telomerase activity and accelerate the tumor evolution.19 Similar partial deletions in 5q and 6q were present in primary and metastasis. Amplification of chr17q and partial chr19 was observed whereas the whole genome-doubling event occurred only during metastasis. The relative late genome-doubling event in metastasis, which caused many copy number loss and gain has drastically altered the metastatic genomic structure leading to an immense genomic divergence with primary cancer.

We also analyzed the non-synonymous mutations in this sample, and found that LRPIB (p.K2632N) and KMT2C (p.R380L, p.C391*) shared missense mutations in both primary and metastatic tumors (Figure 3b). LRPIB frequently mutated in lung adenocarcinoma and it could be associated with lung carcinogenesis.7 Besides, the PIK3CA H1047R mutation which has been reported in COSMIC (v62) was also observed in LB236. PIK3CA is a well-established oncogene in lung adenocarcinoma, and this mutation can increase the downstream kinase activity, decrease cell apoptosis and promote tumor-cell invasion. Further, recent studies showed that PIK3CA mutation tends to emerge in sub-clones associated with the APOBEC signature environment.20 In consistent, H1047R mutation was a later event to emerge in sub-clones associated with the APOBEC signature

**DISCUSSION**

In summary, in the process of this study we have demonstrated the genomic diversity among primary and metastatic tumors. As arm-level change is relatively stable with few copy number changes during genome evolution, somatic SNV are largely differentiated in the majority of cases. These findings suggest differential evolutionary rate in different time points for copy number change and somatic mutations.

Chromosomal instability is defined as continual loss or gain of whole chromosome or sub-chromosome and numerical chromosome instability and structural instability.22 Mostly, CIN is associated with multi-drug responses and poor prognosis.23,24 Genome doubling have the ability to increase genome instability in a cancer genome.25 Based on our data, we found genome doubling occurred specifically in the metastasis of case 023, and also uncovered recurrent BFBE-derived amplification of gene CCNY specific to metastases. The biological role of genome doubling and BFBE events in metastasis needs further investigation.

Finally, we demonstrated the branched evolutionary process through representative case 023T. The data showed most of mutations occurred in middle or later stage with PIK3CA hotspot mutation in middle stage. These results are consistent with intra-tumor heterogeneity report in kidney cancer that most of the driver or passenger mutations occurred in branched or private stage, and not in the founding clone. Besides, a change of mutational mechanisms was observed with tumor progression. These results indicated that distinct functions of driver mutations were involved in cancer initiation and progression stage.

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank all of the patients and families that contributed to this study. This work is supported by the funding from the Jilin Department of Health (3D5144CQ13430). This work is supported by grants of Guangzhou Health-Medical Collaborative Innovation Project (NO. 201508020247). We also thank The First Affiliated Hospital of Guangzhou Medical University for sharing the whole-genome data.

Author contributions: BY and YZ designed and managed the research. WL and CY performed the bioinformatics analysis. YZ, WL, CY, TX and XM wrote the manuscript. XL, XZ and WZ revised the manuscript.

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