Dear Editor,

Gastric cancer (GC) is a highly heterogeneous disease with a dismal prognosis at both phenotypical and molecular levels.1 The Lauren type is currently the most useful and widely used in GC.2 However, the prognosis and molecular characteristics of Lauren type have not been fully described. Additionally, although several molecular classifications have been proposed,3,4 clinically-relevant subtypes are still urgently needed. Here, we systematically investigated the molecular landscape and evolution features of 169 primary GC samples among Lauren type. We identified a prognostic-relevant subtype based on clone number (CN). Patients with high CN showed high tumour mutation burden (TMB) and significantly enriched in Adherens junction, ERBB2 regulates cell motility, and signaling by WNT pathway, indicating CN may have potential risk of tumour metastasis and benefit from immunotherapy. Our findings may inform the exploration of patient stratification and personalized therapy, as well as new clinical trials designed for the selection of combination therapy strategies.

We enrolled 169 patients with formalin-fixed paraffin-embedded samples of matched tumour and adjacent tissue, including extended Lauren type: intestinal type (IT, n = 26), mixed type (MT, n = 35), non-signet ring diffuse type (NSRD, n = 77), and signet ring type (SRT, n = 31) with signet ring cells accounting for more than 10%.5 The median sequencing depth was 747× (range, 373–1186×) for tumour and was 428× (range, 221–960×) for adjacent tissue (Figure S1). We obtained 2847 Single Nucleotide Variations (SNVs) and 44 indels. The median TMB was 10.56 per Mb (mean 16.43 per Mb). The most recurrent mutant genes included TP53 (39.1%), CDH1 (28.4%), ARID1A (24.9%), TTN (24.3%), and MUC16 (19.5%), which was consistent with previous studies (The Cancer Genome Atlas (TCGA): 48.1%, 8.4%, 25.8%, 54.9%, 33.9%; ACRG: 40%, 4%, 17.8%, 38.2%, 25.8%; oncosg: 47.6%, 9.5%, 13.6%, 38.1%, 19.7%; respectively. Figure S2A).3,4,6 MUC16- and TTN-mutated samples showed higher TMB value (Figure S2B,C,E). Only Adenomatous Polyposis Coli (APC) (p = .038) showed significant difference (Figure 1, Figures S3–S4), suggesting high-frequency mutated genes have similar variation pattern in the four types. There was no significant difference in sex, age, venous invasion, perineural invasion, treatment regimen, TNM stage, tumour site, tumour size, and MSI status except CEA (p = .0221) and CA199 index (p = .03) (Table S1, Figure S5C,D). Patients with high tumour markers generally have worse prognosis (Figure S5A,B). However, we found only patients in NSRD with CEA-high or CA199-high had significantly shorter outcomes (Figure S5E,F), suggesting the importance of stratified management for patient care. The mutational signature analysis also showed only signature 3 and signature 17 were not enriched in NSRD and SRT, respectively (Figure 1, Figure S6).

In terms of comprehensive indicators, we compared the TMB score, mutant-allele tumour heterogeneity (MATH) score,7 and variant allele frequency (VAF) from three dimensions: mutational burden, mutation heterogeneity, and allelic mutation frequency. We found no significant difference in TMB and MATH values between the four types except VAF (Figure 2A–C), suggesting VAF may influence histological types. We then preformed pyclone analysis.8 We defined the highest cell prevalence cluster as clone and the other as subclone. Pyclone inferred 379 clones (IT, 59, 13.9%; MT, 70, 9.9%; NSRD, 176, 12.9%; SRT, 74, 18.8%) and 2514 subclones (IT, 366, 86.1%; MT, 640, 90.1%; NSRD, 1188, 87.1%; SRT, 320, 81.2%). The median CN was 7 (range, 1–54). Highly specific clonal genes were observed in each type (unique ratio: IT, 30.5%; MT, 22.9%; NSRD, 32.9%; SRT, 28.4%) (Figure 2D), and subclonal gene has similar phenomenon (Figure 2E). The results indicated the differences of gene mutation process among the four types.

To clarify the functional role of clonal and subclonal genes, we performed pathway enrichment analysis. The result showed clonal genes in IT were significantly enriched in TP53 regulates transcription of DNA repair

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2022 The Authors. Clinical and Translational Medicine published by John Wiley & Sons Australia, Ltd on behalf of Shanghai Institute of Clinical Bioinformatics
FIGURE 1  Comparison of molecular landscape in four histological types. The genes with a mutation frequency greater than 10% were shown in heatmap. Each column represents a single sample. All 169 samples were divided into four groups according to the expanded Lauren type and were presented separately. The numbers next to the heatmap represent mutation frequency. The percentage stack histogram on the right shows the proportion of each group. The rightmost bar represents the variation type of gene. The upper bar represents mutation number. The bottom pie chart shows the proportion of mutation signature in the four types. Representative immunohistochemistry of Lauren type is displayed below the pie chart at different resolutions of 10X, 20X and 40X, respectively. The immunohistochemistry of the signet ring cells, which account for 10%, 50% and 90%, is also shown respectively.

genes pathway, and subclonal genes were in DNA repair pathways. We also found clonal and subclonal genes in MT were enriched in the PI3K-Akt signaling pathway and c-type lectin receptor signaling pathway, respectively. Several classical tumour-related pathways were enriched in the NSRD, such as pre-NOTCH transcription and translation, Ras signaling pathway, and p53 signaling pathway. Additionally, signaling by receptor tyrosine kinases and ErbB signaling were significantly enriched in the SRT, respectively (Figure 2F). The different functional enrichment features among the four types suggest the clonal evolution patterns may be related to histological phenotypes.

To evaluate the clonal heterogeneity process, the distribution of high-frequently mutant genes was tested. We found that the same gene can be either a clone gene or a subclone gene in different samples, indicating gene clonal heterogeneity (Figure 3A). Furthermore, CN was significantly correlated with the maximum VAF (Figure 3B). To further illustrate the clinical relevance of CN, we observed the characteristics distribution of CN with the overall sur-
Clonal evolution analysis of four histological types. Violin plot shows the comparison of tumour mutation burden (TMB) (A), mutant-allele tumour heterogeneity (MATH) (B) and variant allele frequency (VAF) (C), respectively. The ns above horizontal line indicates the $p$ value of Mann–Whitney $U$ test greater than .05, and the number above horizontal line indicates $p$ value. Venn diagrams show the sharing gene number of clone (D) and subclone (E) in four types. (F) Clonal and subclonal gene enrichment pathways in four types.
Correlation analysis of clone number stratification and prognosis. (A) The density distribution of the top 10% mutant genes with clonal and subclonal attribute. (B) Correlation analysis between clone number and variant allele frequency (VAF). (C) Comprehensive display of overall survival, VAF and clone number distribution. (D) The prognosis of clonal number stratification.

Detailed mutational landscape analysis of CN stratification showed the mutation frequencies of \textit{ARID1A}, \textit{TTN}, \textit{MUC16}, \textit{MLL4}, \textit{MLL2}, \textit{GRIN2A}, \textit{NRGI}, \textit{ERBB2}, \textit{SPTA1}, \textit{SLIT2}, and \textit{RHOA} genes were significantly higher in CN-high group (Figure 4D). Higher TMB was also observed in CN-high group (Figure 4E). Patients with high CN may benefit from immunotherapy. Functional enrichment analysis showed that Adherens junction, ERBB2 regulates cell motility, and signaling by WNT were significantly enriched in CN-high group (Figure 4F), indicating patients with high CN may have a high risk of metastasis. We also found the prognosis of IT CN \( \geq 7 \) subgroup was significantly worse than that of CN \(< 7 \) subgroup, and consistent trend was observed in NSRD and SRT (Figure 4G). Thus, combining CN and histological phenotype may be an actionable marker for clinical prognosis stratification.

In summary, we highlight the molecular, evolutionary and prognostic heterogeneity of GC phenotype at multidimensionally levels. The clonal evolution patterns of the four histological types showed different characteristics, and CN may be a molecular classification indicator for patient stratification.
**FIGURE 4**  Comparative analysis of the clone number stratification. (A) Multivariate Cox hazard analysis of clinical information and clone number stratification. (B) The proportion of pathological stage in the clone number stratification with Fisher's exact test. (C) The distribution of clone number stratification in the four types, ns means Fisher's exact test p value greater than .05. (D) Comparison of high-frequency mutant genes in clone number stratified. Each column represents a single sample. Different color indicates mutation types. The red asterisk on the left indicates the p value interval (*, .05, .01; **, .01, .001; ***, .001, 0). (E) The violin plot shows the tumour mutation burden (TMB) value of clone number stratification. (F) Enrichment of differential gene pathways based on clone number stratification. (G) Prognostic analysis of clone number stratification in four histological types
ACKNOWLEDGEMENTS
The authors would like to thank the patients, their families and caregivers, data managers and all study investigators for their contributions to study conduct. This study was supported by the National Natural Science Foundation of China (grant number: 8197103463) and the Hunan Natural Science Foundation (grant number: 2012FJ6088).

CONFLICT OF INTEREST
The authors declare there is no conflict of interest.

Jie Ge¹,⁴
Xuan Li¹
Zhenghao Deng¹
Xuan Gao⁷,⁸
Yaoyao Liu⁶
Xingui Xiong⁷
Xianhui Zhao¹
Huan Peng¹
Xin Yi⁶
Xuefeng Xia⁶
Zihua Chen¹,⁴,⁵
Lifeng Li⁶
Haiyan Zhou²
Heli Liu¹,⁴

¹Department of Gastrointestinal Surgery, Xiangya Hospital, Central South University, Changsha, China
²Department of Pathology, Xiangya Hospital, Central South University, Changsha, China
³Institute of Combined Traditional Chinese and Western Medicine, Xiangya Hospital, Central South University, Changsha, China
⁴The Hunan Provincial Key Laboratory of Precision Diagnosis and Treatment for Gastrointestinal Tumor, Changsha, China
⁵International Joint Research Center of Minimally Invasive Endoscopic Technology Equipment and Standardization, Changsha, China
⁶Geneplus-Beijing, Beijing, China
⁷State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
⁸GenePlus-Shenzhen Clinical Laboratory, Shenzhen, China

ORCID
Lifeng Li © https://orcid.org/0000-0002-9813-5688

REFERENCES
1. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. Lancet. 2020;396(10251):635-648.
2. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31-49.
3. Anonymous. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202-209.
4. Cristescu R, Lee J, Nebozhyn M, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21(5):449-456.
5. Mariette C, Carneiro F, Grabsch HI, et al. Consensus on the pathological definition and classification of poorly cohesive gastric carcinoma. Gastric Cancer. 2019;22(1):1-9.
6. Guo YA, Chang MM, Huang W, et al. Mutation hotspots at CTCF binding sites coupled to chromosomal instability in gastrointestinal cancers. Nat Commun. 2018;9(1):1520.
7. Mroz EA, Rocco JW. MATH, a novel measure of intratumor genetic heterogeneity, is high in poor-outcome classes of head and neck squamous cell carcinoma. Oral Oncol. 2013;49(3):211-215.
8. Roth A, Khattra J, Yap D, et al. PyClone: statistical inference of clonal population structure in cancer. Nat Methods. 2014;11(4):396-398.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.