

# TP53 Co-Mutational Features and NGS-Calibrated Immunohistochemistry Threshold in Gastric Cancer

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**Purpose:** *TP53* is the most frequently mutated gene in gastric cancer and it can be potentially used for gastric cancer diagnosis and screening. However, standardized clinical approaches that could accurately and cost-effectively detect *TP53* mutations in gastric cancer are largely lagged behind.

**Patients and Methods:** We conducted next-generation sequencing (NGS) analysis of 425 cancer-related genes in 42 gastric cancer patients in our cohort. A 1313-patient cohort derived from the cBioPortal database was used for validation. We performed immunohistochemistry (IHC) staining with four commonly used p53 antibodies, and the NGS results were used as the gold standard to optimize the IHC threshold for each antibody.

**Results:** By NGS analysis, we found that around 80% of gastric cancer patients in our cohort harbored *TP53* alterations. Genetic alterations of *BRCA1/2* or *KMT2B* were mostly exclusive with *TP53* mutations, so were the MSI status or low grade of tumors. These results were further validated using the data from cBioPortal. We then used the NGS-derived *TP53* status to optimize four commonly used IHC antibodies for detecting *TP53* mutations. We showed that all antibodies could achieve more than 93% accuracy when proper IHC positivity thresholds were used, especially for the SP5 antibody that could reach 100% sensitivity and specificity with the 20% threshold.

**Conclusion:** Our results indicated that exclusivity between *TP53* and *BRCA* mutations could be potentially used as a cost-effective way to predict *BRCA* status. Also, setting proper IHC thresholds for each specific antibody is critical to accurately detect *TP53* mutations and facilitate disease diagnosis.

**Keywords:** *TP53*, gastric cancer, next-generation sequencing, co-mutation, immunohistochemistry threshold

## Introduction

Tumor protein p53 (*TP53*) is a well-known tumor suppressor gene and its encoded protein p53 is involved in DNA repair, cell cycle arrest, apoptosis, metabolism, differentiation, and various developmental processes.<sup>1</sup> *TP53* is the most frequently altered gene in cancer, and one of the main outcomes of *TP53* mutations is disrupting the normal function of p53 through loss-of-function or nonsense mutations (~10%).<sup>2</sup> In addition, a substantial amount of *TP53* missense mutations (~75%), primarily located at the DNA-binding domain, lose their ability to activate the canonical downstream target genes; instead, they could promote the oncogenic process by repressing the wild-type (WT) p53 or other p53 family members, such as p63 and p73.<sup>3</sup> Multiple studies have shown that tumors could acquire selective advantages by retaining the mutated *TP53*, thus enabling their adaption to stress

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conditions, metabolic imbalance, and tumor microenvironments.<sup>3</sup> Therefore, the *TP53* mutation can serve as a diagnostic/prognostic biomarker and could be potentially used as a treatment target for various cancers.

Gastric cancer is the sixth most common cancer and the second cancer-related mortality globally,<sup>4</sup> with *TP53* being one of the most frequently mutated genes. Busuttil et al found that the intestinal metaplasia, a premalignant lesion of gastric cancer, was associated with elevated WT p53 expression while *TP53* mutations nearly exclusively occurred in the gastric tumors,<sup>5</sup> implying the involvement of *TP53* mutations during the premalignancy to cancer transition and their clinical values for cancer diagnosis. On the other hand, conflicting results were observed between *TP53* mutation status and gastric patient prognosis,<sup>6–9</sup> and several possible explanations have been proposed to address this discrepancy. First, due to the tumor heterogeneity and the complexity of p53-related pathways, *TP53* mutation itself is not enough to accurately predict patient outcomes, and coupling *TP53* mutations with other mutations might better stratify patients with gastric cancer.<sup>10,11</sup> Second, many studies assessed the *TP53* status using immunohistochemistry (IHC), which may detect the accumulation of WT p53 that was induced during stress conditions.<sup>7</sup> In addition, un-optimized IHC could improperly assign the *TP53* status, resulting in inconclusive or contradictory results among studies. It has been estimated that the false positive rate for using p53 IHC ranged from 2% to 45%, and Roshandel et al showed that the traditional threshold of 10% IHC positivity led to only 42% specificity in glioblastoma patients.<sup>12</sup> Although several researchers investigated the correlation between p53 IHC positivity and *TP53* missense mutations in various cancers including glioma and astrocytomas, optimization of the p53 IHC threshold in gastric cancer is largely lagged behind.

In this study, we aimed to further explore the potential usage of the *TP53* mutation as a biomarker for gastric cancer. We performed targeted next-generation sequencing (NGS) to investigate *TP53* mutations and their co-occurred genetic alterations in 42 gastric cancer patients, and the results were further confirmed using the cBioPortal database. In order to achieve reliable p53 IHC results in gastric cancer, we used the *TP53* status derived from the NGS data to optimize the p53 IHC threshold, which could be potentially used for cost-effective and accurate detection of *TP53* mutations and facilitate gastric cancer diagnosis.

## Patients and Methods

### Patients and Samples

Forty-three patients who were diagnosed with gastric cancer in Henan Provincial People's Hospital were included in this study. Each patient had 1 gastric tumor tissue sample, and the adjacent tumor tissues were used as normal controls to filter out germline mutations. A total of 1313 gastric cancer samples from cBio Cancer Genomics Portal (<http://cbioportal.org>) were used as the validation cohort.<sup>13–16</sup> This study was approved by the ethical board of Henan Provincial People's Hospital (ethical number: 2020–89), and this study was conducted in accordance with the Declaration of Helsinki. All patients have signed written informed consent forms prior to sample collection and consented to the publication of related clinical information and data.

### Library Preparation and Sequencing

The sample and library preparation procedures follow the methods described by Yang et al.<sup>17</sup> Briefly, the formalin-fixed paraffin-embedded (FFPE) samples were used for DNA extraction using the QIAamp DNA FFPE Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. DNA quality was assessed by spectrophotometry and quantified by Qubit 2.0. Libraries were prepared as previously described.<sup>17</sup> Briefly, 1 µg of fragmented genomic DNA underwent end-repairing, A-tailing and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter). Hybridization-based target enrichment was carried out with GeneseeqOne™ pancancer gene panel (425-cancer-relevant genes, Geneseeq Technology Inc.), and xGen Lockdown Hybridization and Wash Reagents Kit (Integrated DNA Technologies). Captured libraries by Dynabeads M-270 (Life Technologies) were amplified in KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and quantified by qPCR using the KAPA Library Quantification kit (KAPA Biosystems). Target enriched libraries were sequenced on the HiSeq4000 platform (Illumina) with 2×150 bp pair-end reads.

### Mutation Calling and Copy Number Alteration Analysis

Sequencing data were demultiplexed by bcl2fastq (v2.19), analyzed by Trimmomatic<sup>18</sup> to remove low-quality (quality<15) or N bases, and mapped to the reference hg19 genome (Human Genome version 19) using the Burrows-Wheeler

Aligner.<sup>19</sup> PCR duplicates were removed by Picard (available at <https://broadinstitute.github.io/picard/>). The Genome Analysis Toolkit (GATK)<sup>20</sup> was used to perform local realignments around indels and base quality reassurance. Single-nucleotide polymorphisms (SNPs) and indels were called by VarScan2<sup>21</sup> and HaplotypeCaller/UnifiedGenotyper in GATK, with 0.5% mutant allele frequency as the cutoff for tissue samples and a minimum of three unique mutant reads. Common variants were removed using dbSNP and the 1000 Genome project. Germline mutations were filtered out by comparing to patient's normal controls. The resulting somatic variants were further filtered through an in-house list of recurrent sequencing errors that were generated from over 10,000 normal control samples on the same sequencing platform. Gene fusions were identified by FACTERA<sup>22</sup> and copy number variations (CNVs) were analyzed with ADTEX.<sup>23</sup> The log2 ratio cut-off for copy number gain was defined as 2.0 for tissue samples. A log2 ratio cut-off of 0.67 was used for copy number loss detection in all sample types. The thresholds were determined from previous assay validation using the absolute CNVs detected by droplet digital PCR (ddPCR).

## Immunohistochemistry (IHC)

Unstained FFPE sections from tumor specimens collected at diagnosis were subjected to IHC staining with p53 antibodies of SP5 (rabbit monoclonal antibody; Thermo Fisher Scientific), MX008 (mouse monoclonal antibody; MXB<sup>®</sup> Biotechnologies), BP-53-12 (mouse monoclonal antibody; Thermo Fisher Scientific), or DO-7 (mouse monoclonal antibody; Thermo Fisher Scientific), according to the current protocols of the Department of Pathology of Henan Provincial People's Hospital. FFPE sections were also stained with Ki-67 antibody, the clone of which was MIB-1 (Dako), according to the protocol.

## The Concordance Analysis Between p53 IHC and NGS Data

We analyzed the relationship between the *TP53* missense mutations derived from the NGS data and the p53 IHC data using four different antibodies (SP5, MX008, BP-53-12, and DO-7) for a total of 42 samples from the discovery cohort. For different IHC positivity thresholds (0%, 10%, 20%, 30%, 50%, 60%, 90%, and 100%), we calculated their sensitivity and specificity using the NGS results as the reference, and the one with the highest accuracy was selected as the optimal IHC threshold for the specific antibody.<sup>12</sup>

## Statistical Analysis

The *TP53* co-occurring and mutually exclusive mutations were analyzed using the somatic Interactions function available in maftools; specifically, odds ratios (Fisher's exact test) were used to distinguish the co-occurring and mutually exclusive mutations, with odds ratios larger than 1 indicating co-mutation while odds ratios smaller than 1 indicating mutual exclusivity.<sup>24</sup> Comparisons of mutation frequency between different groups were done using Fisher's exact test. For survival data, Kaplan–Meier curves were analyzed using the Log rank test. Two-sided p values of less than 0.05 was considered as statistically significant (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001). All statistical analyses were done in R (R 3.6.2).

## Results

### Patient and Sample Characteristics

A total of 43 gastric cancer patients were included in our study and their tumor tissue samples underwent targeted NGS of 425 cancer-related genes. One sample did not pass the NGS quality control, leaving 42 patient samples for further analysis (discovery cohort). We also analyzed NGS data from 1313 gastric cancer patients, which were obtained from the cBioPortal database and used as the validation cohort. Of the 42-patient discovery cohort, the median age was 60.5 years old, which is comparable with the validation cohort (67 years) (Table 1 and Supplementary Table 1). There are more male patients than female patients (78.6% vs 21.4%), and similar gender bias was also seen in both previous studies<sup>25</sup> and the validation cohort (Supplementary Table 1). Around half of the 42 gastric cancer patients had a smoking history and one-third of them had a drinking history (Table 1). Also, the majority of their tumors were at stage III, with high Ki-67 expression,<sup>26</sup> stable microsatellite, and lymph node metastasis (Table 1). According to the Cancer Genome Atlas (TCGA) molecular subtyping approach, 2.4%, 7.1%, 19%, and 71.4% of the patients in the discovery cohort were classified as Epstein-Barr virus-positive (EBV-positive), microsatellite instable (MSI), chromosomal instable (CIN), and genomically stable (GS), respectively (Table 1), whereas the majority of gastric cancer subtype (71.7%) in the validation cohort was not available (Supplementary Table 1).

### Mutation Profile and *TP53* Mutation Features

As shown in Figure 1A, *TP53* was the most frequently altered gene within the discovery cohort (78.6%) while the

**Table I** The Demographic Characteristics of 42 Gastric Cancer Patients in the Discovery Cohort

	No. of Patient	Percentage (%)
<b>Median age, years (range)</b>	60.5 (40–77)	
<b>Gender</b>		
Male	33	78.6
Female	9	21.4
<b>Tumor differentiation level</b>		
Poorly differentiated	19	45.2
Moderate to well differentiated	23	54.8
NA		
<b>Ki-67</b>		
Low (<20%)	3	7.1
High (≥20%)	39	92.9
<b>Microsatellite status</b>		
MSI	3	7.1
MSS	39	92.9
<b>Smoking history</b>		
Yes	18	42.9
No	24	57.1
<b>Drinking history</b>		
Yes	13	31
No	29	69
<b>Stage</b>		
I	6	14.3
II	5	11.9
III	29	69
IV	2	4.8
<b>Lymph node metastasis</b>		
Yes	28	66.7
No	11	26.2
NA	3	7.1
<b>Subtypes</b>		
EBV-positive	1	2.4
MSI	3	7.1
CIN	8	19
GS	30	71.4

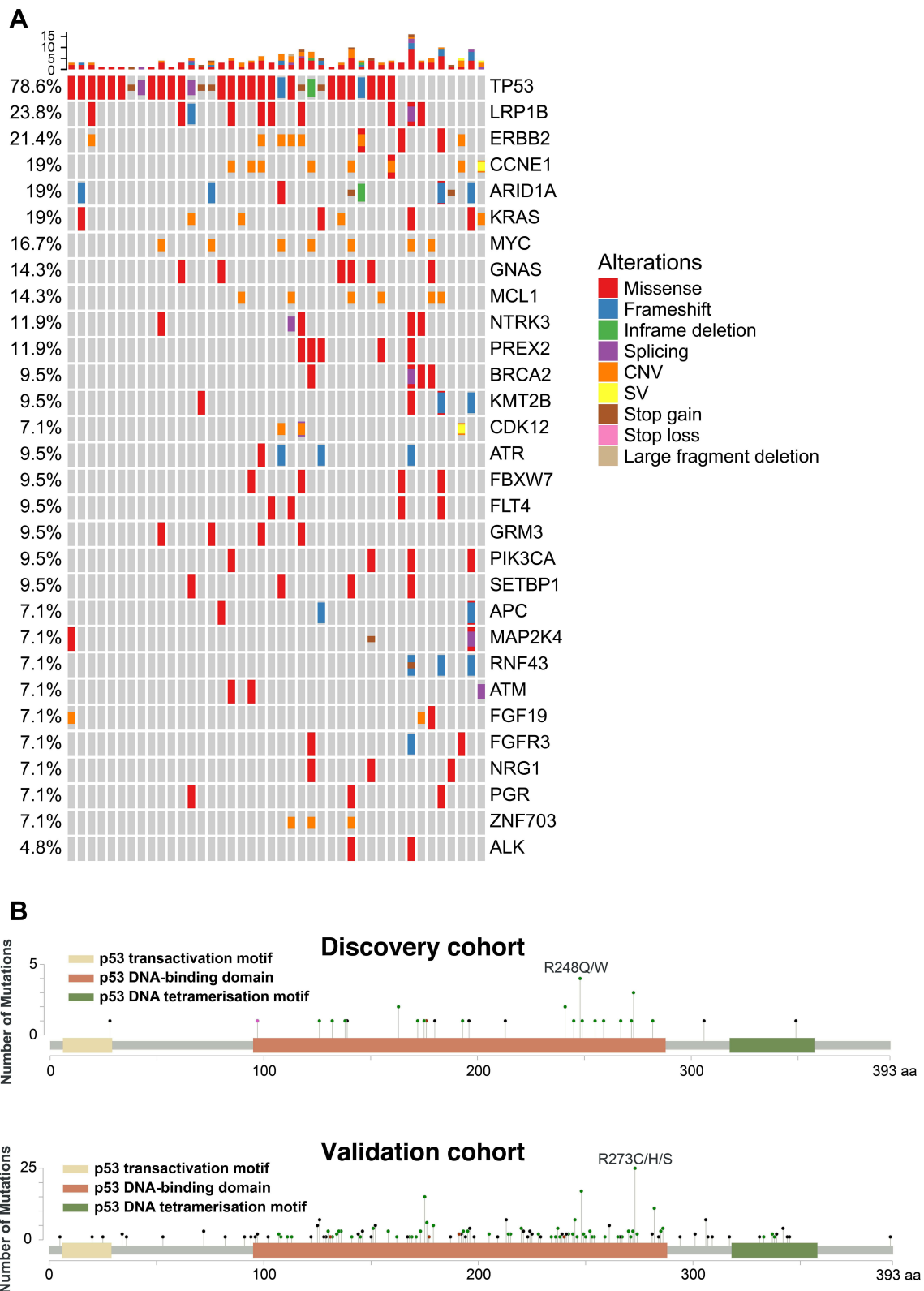
**Abbreviations:** EBV, Epstein-Barr Virus; MSI, Microsatellite Instability; GS, Genomically Stable; CIN, Chromosomal Instability; MSS, Microsatellite Stable.

*TP53* mutation frequency in the validation cohort was 47%, and this difference might be due to the variation in the tumor stage, tumor differentiation level, ethnicity, gastric cancer subtype, and treatment history between the two patient cohorts.<sup>27,28</sup> The majority of *TP53* mutations were missense mutations, followed by some nonsense mutations

including frameshift and stop gain mutations (Figure 1A). By examining details of these *TP53* mutations, we found that most of them were located within the p53 DNA-binding domains in both discovery and validation cohorts, and a large proportion of them were *TP53* hotspot mutations<sup>9</sup> that were associated with tumor progression and/or recurrences, such as R175, G245, R248, R273, and R282 (Figure 1B and [Supplementary Table 2](#)). In addition to *TP53* mutations, multiple other genes were also frequently mutated in our cohort, including mutations in *LRP1B*, *ARID1A*, and *GNAS*, as well as amplification of *ERBB2*, *CCNE1*, *MYC*, and *KRAS* (Figure 1A), which is in line with previous reports.<sup>29</sup>

## Characterizing *TP53* Co-Occurring and Exclusive Mutations in Gastric Cancer

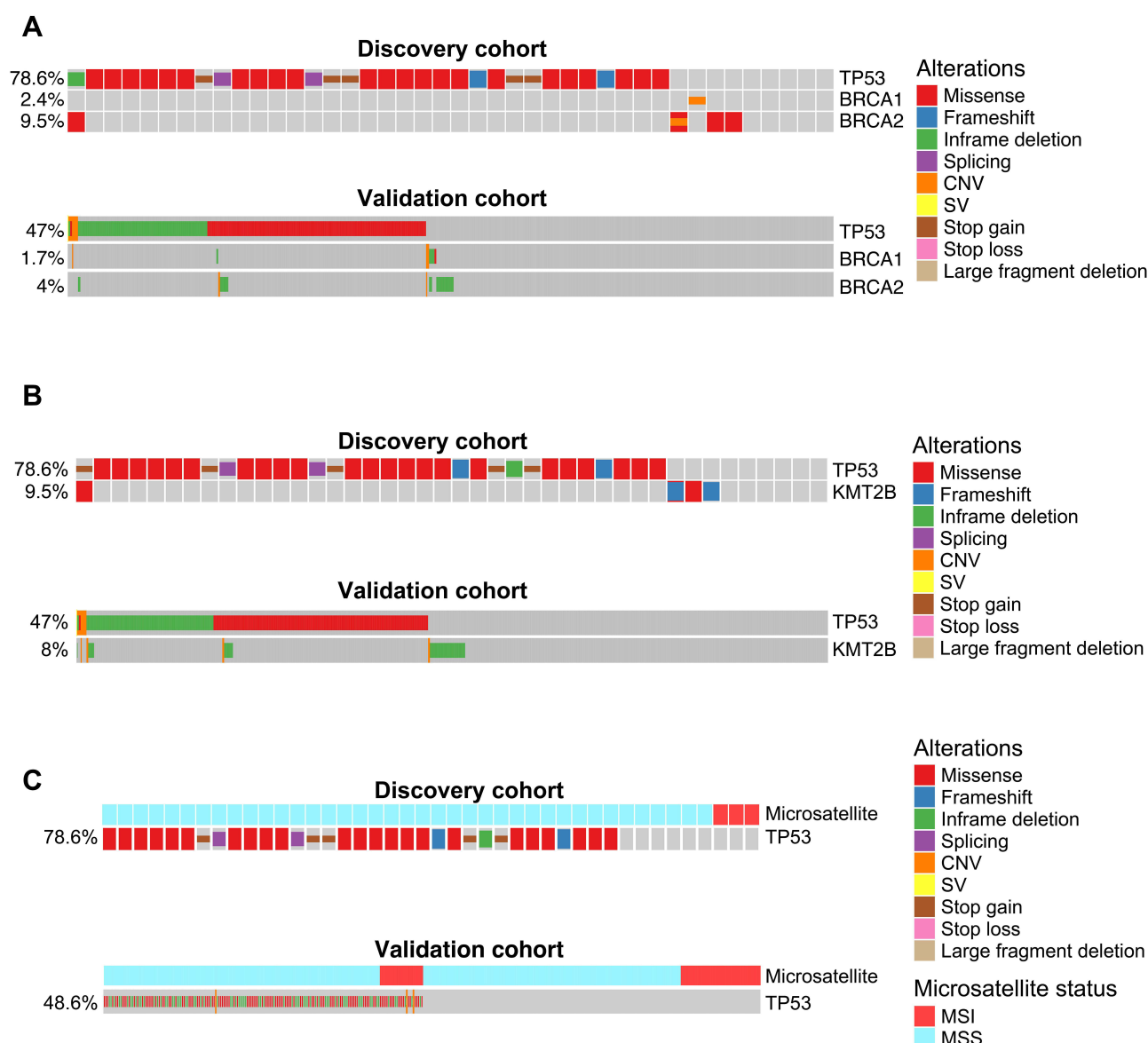
We then investigated the co-occurrence of *TP53* mutations with other genetic alterations. As shown in [Supplementary Table 3](#), three mutated genes were found to be exclusive with *TP53* mutations in the discovery cohort, including *RNF43*, *BRCA2*, and *KMT2B*. To eliminate any sampling bias of the discovery cohort, we validated these results using the 1313-patient results derived from the cBioPortal database. We also included *BRCA1* in the analysis because it is closely related to *BRCA2* in the DNA double-strand break repair pathway and its mutations were also relatively common in gastric cancer. *TP53* mutations demonstrated statistically significant exclusivity with mutations in both *BRCA1* and *BRCA2* in the validation cohort (p value=0.012 and 0.048, respectively) (Figure 2A), implying that there might be some pathway-overlaps between *TP53* and *BRCA* in terms of gastric cancer tumorigenesis and progression. Similarly, the exclusivity between *TP53* mutations and *KMT2B* mutations was confirmed in the validation cohort (p value=0.001) (Figure 2B). On the other hand, the result for *TP53* and *RNF43* mutations were not statistically significant (p value=0.082), thus being excluded for further analysis. Besides gene mutations, we also checked the co-existence of *TP53* mutations with other molecular and clinical features. Microsatellite instability (MSI) was usually associated with impaired DNA mismatch repair pathways, and we found MSI was more likely to be found in patients with WT *TP53*, which is statistically significant in both the discovery cohort and the validation cohort (p value=0.0073 and 0.0139, respectively) (Figure 2C). Intriguingly, we also found that well-differentiated tumors tended to be associated with more



**Figure I** The genetic alterations in gastric cancer patients. **(A)** The co-mutation plot of the top 30 mutated genes from the discovery gastric cancer patient cohort was illustrated and the genes were ranked based on their mutational frequency. CNV: copy-number variation; SV: structural variation. **(B)** Lollipop plot for *TP53* mutations identified in the discovery gastric cancer patient cohort (upper panel) or the validation gastric cancer patient cohort (lower panel).

**Abbreviations:** CNV, copy number variation; SV, structural variant; aa, amino acid.





**Figure 2** Cross-validated *TP53* co-occurring or mutually exclusive genetic alterations in gastric cancer. **(A–C)** Co-mutation plot of *TP53* mutations with *BRCA1/BRCA2* mutations **(A)**, *KMT2B* mutations **(B)**, or microsatellite stability status **(C)** in the discovery patient cohort (upper panel) or the validation patient cohort (lower panel). For the validation patient cohort, only the samples with known *BRCA1/BRCA2/KMT2B* mutation status or microsatellite stability status were included in the analysis.

**Abbreviations:** MSI, microsatellite instability; MSS, microsatellite stable; CNV, copy number variation; SV, structural variant.

*TP53* mutations in both patient cohorts ([Supplementary Figure 1](#)), although the result was not statistically significant as most samples in the validation cohort had unknown tumor differentiation status ([Supplementary Table 1](#)). Intriguingly, because both MSI and well-differentiation were associated with better prognosis in gastric cancer,<sup>30,31</sup> their opposite relationship to *TP53* mutation frequency might be one of the reasons for the conflicting results of using *TP53* mutation as the prognostic biomarker.

As patient overall survival data is available for the validation cohort, we examined whether different *TP53* co-mutated genes would have distinct clinical outcomes. As illustrated in [Supplementary Figure 2A](#), gastric cancer patients with mutated *TP53* had similar overall survival to those with WT *TP53*. Patients with only *BRCA1* mutations had worse survival rates, while patients with both *TP53* and *BRCA1* mutations, although very rare, had the worst overall survival among all the gastric cancer patients in the validation cohort ([Supplementary Figure 2A](#)). On the other

hand, patients with only *BRCA2* mutations or with co-occurred *TP53* and *BRCA2* mutations had indistinguishable overall survival compared with the rest of gastric cancer patients (Log-rank  $p$  value=0.19; [Supplementary Figure 2B](#)). Besides, although *KMT2B*-mutant patients had similar clinical outcomes when compared with patients with only *TP53* mutations or with *TP53*-WT/*KMT2B*-WT, patients with co-existed *TP53* and *KMT2B* mutations had better overall survival ([Supplementary Figure 2C](#)). Therefore, these results indicated that even though some mutations tended to be excluded in *TP53* mutated gastric tumors, a few patients could still harbor both mutations and their clinical outcomes seem to be various and dependent on the specific genetic context.

### Optimization of the IHC Antibody for Detecting *TP53* Missense Mutations

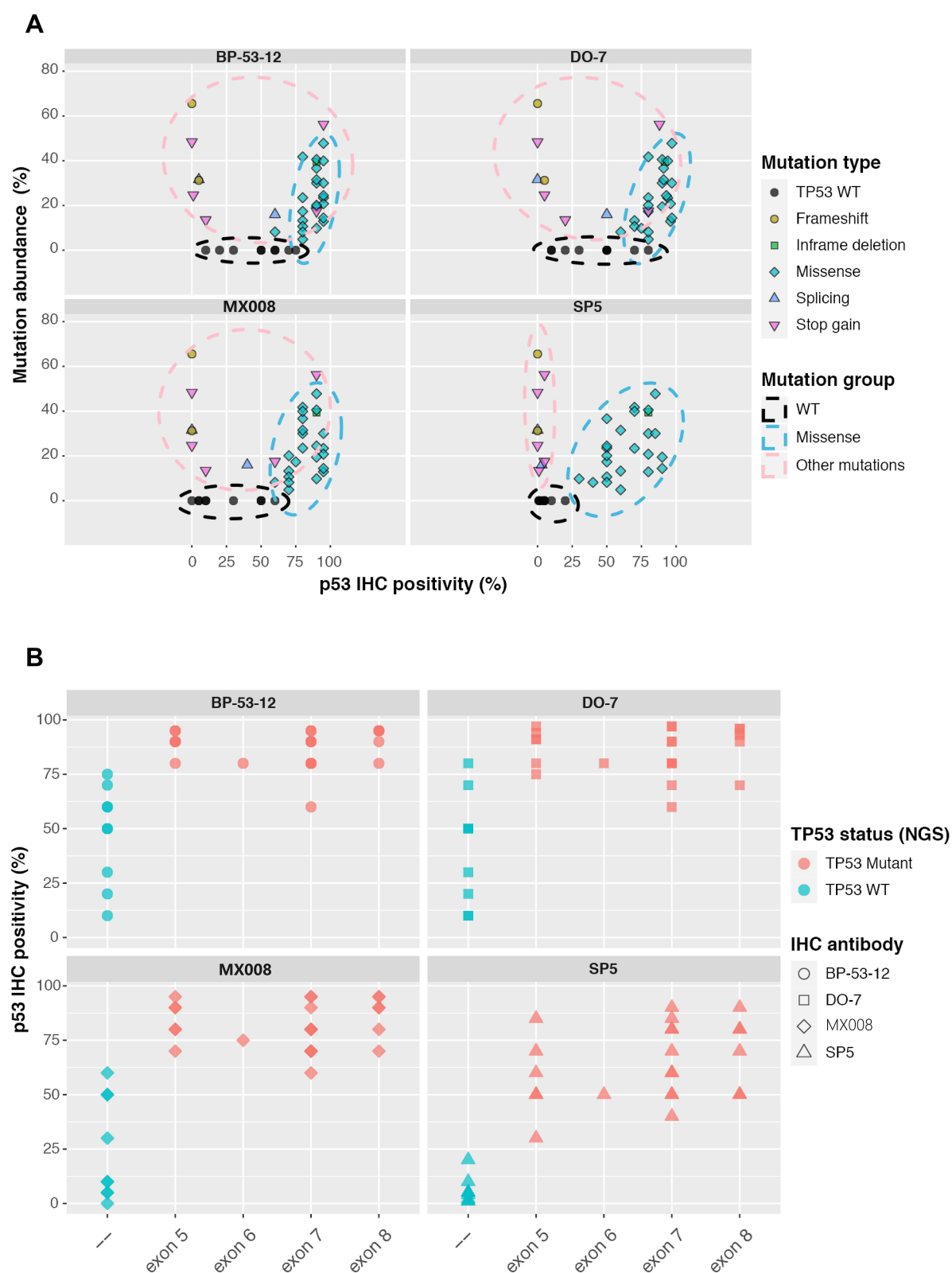
Given that IHC is still widely used in clinical and experimental practices when determining the *TP53* mutation status, we tried to optimize the *TP53* IHC results using the NGS data as the reference. For 42 patients within the discovery cohort, each patient sample underwent IHC staining with four commonly used p53 antibodies, including SP5, MX008, BP-53-12, and DO-7. Multiple *TP53* missense mutations have been reported to promote tumor survival, and cancer cells tended to selectively retain or amplify the mutant *TP53*. On the other side, cells with WT *TP53* might have various p53 protein expression levels depending on the cell stress conditions, and cells with nonsense *TP53* mutations (eg, frameshifts and stop gains) usually had various *TP53* DNA copy number and very low to none p53 protein expression levels.<sup>3</sup> As a result, it is potentially possible for IHC to distinguish different types of *TP53* mutations. As we investigated the relationship between p53 IHC positivity and mutant allele frequency for different *TP53* mutations, we found that all four p53 antibodies could generally separate most *TP53* missense mutations from other *TP53* genotypes ([Figure 3A](#)). In addition, the SP5 antibody could clearly divide *TP53* mutations into three groups, that is, WT, missense mutations, and other mutations, which is superior to the other three p53 antibodies ([Figure 3A](#) and [Supplementary Figure 3](#)). This indicates that the SP5 antibody might have higher specificity to missense mutated p53 compared with the WT p53 or other truncated p53 that resulted from frameshift and stop gain mutations. Also, as the *TP53* missense mutations frequently occurred in exon 5 to

exon 8, we verified that the four p53 antibodies could detect mutations located in all of these exons ([Figure 3B](#)). Lastly, we used the *TP53* NGS data to select the IHC positivity threshold in order to get the highest accuracy of detecting *TP53* missense mutations. All four antibodies could achieve more than 93% accuracy with the optimized IHC threshold, especially for the SP5 antibody that had 100% sensitivity and specificity when setting the positivity threshold to 20% ([Table 2](#)). Overall, these results suggest that a proper IHC positivity threshold is crucial to accurately predict the *TP53* mutation status and SP5 was the best p53 IHC antibody among all the tested antibodies in our patient cohort.

### Discussion

Globally, more than one million people were diagnosed with gastric cancer annually, and gastric cancer accounts for around 782,685 patient deaths in the year 2018.<sup>4</sup> The poor overall survival in gastric cancer is partially attributed to the late diagnosis of the disease. *TP53* is the most frequently mutated gene in gastric cancer, and *TP53* mutations are important for the premalignancy to cancer transition, suggesting the great potential of using *TP53* mutations as a diagnostic marker for gastric cancer. The frequently used techniques to detect *TP53* mutations include NGS and IHC. The NGS approach has a high detection accuracy, but it is also relatively expensive and time-consuming. On the other hand, the traditional IHC approach could rapidly detect *TP53* aberrations at a very low cost; however, IHC cannot easily distinguish between the *TP53* missense mutation and the accumulation of WT p53 under stress conditions, thus limiting its detection accuracy and clinical utility. In the current study, we used NGS-determined *TP53* missense mutations as the gold standard to optimize the IHC threshold for four commonly used p53 antibodies. Of note, all antibodies achieved more than 93% accuracy after threshold optimization, especially for the SP5 antibody. Our results suggest that setting proper IHC thresholds and choosing appropriate p53 antibodies are important to accurately detect *TP53* missense mutations, thereby, facilitating early gastric cancer diagnosis.

Recently, Schoop et al<sup>32</sup> found that p53 immunohistochemical evaluation cannot be used to predict *TP53* mutations in gastric cancer, which seems to be different from our conclusion; however, there were several crucial differences between the two studies. Firstly, Schoop et al tested only one p53 antibody (ie DO-7), while we tested four



**Figure 3** Characterizing different p53 IHC antibodies. **(A)** The relationship between the *TP53* mutated allele frequency and p53 IHC positivity for 4 commonly used p53 antibodies, including SP5, MX008, BP-53-12, and DO-7. Each dot represents a *TP53* genetic change from the 42-patient cohort. **(B)** p53 IHC positivity in different *TP53* exon regions for the 4 tested p53 IHC antibodies. Based on the NGS results, *TP53* WT samples were shown in green while samples harboring *TP53* missense mutations were shown in red. WT, wild type.



**Table 2** The Sensitivity, Specificity, and Accuracy of Various p53 IHC Antibodies

IHC Antibody	IHC Staining Threshold	WT(NGS) and WT(IHC)	WT(NGS) and MT(IHC)	MT(NGS) and WT(IHC)	MT(NGS) and MT(IHC)	Sensitivity	Specificity	Accuracy
SP5	0	0	9	0	23	100% (23/23)	0% (0/9)	71.88% (23/32)
	10%	8	1	0	23	100% (23/23)	88.89% (8/9)	96.88% (31/32)
	20%	9	0	0	23	100% (23/23)	100% (9/9)	<b>100% (32/32)</b>
	30%	9	0	1	22	95.65% (22/23)	100% (9/9)	96.88% (31/32)
	50%	9	0	9	14	60.87% (14/23)	100% (9/9)	71.88% (23/32)
	60%	9	0	12	11	47.83% (11/23)	100% (9/9)	62.5% (20/32)
	90%	9	0	23	0	0% (0/23)	100% (9/9)	28.13% (9/32)
MX008	0	1	8	0	23	100% (23/23)	11.11% (1/9)	75% (24/32)
	10%	5	4	0	23	100% (23/23)	55.56% (5/9)	87.5% (28/32)
	20%	5	4	0	23	100% (23/23)	55.56% (5/9)	87.5% (28/32)
	30%	6	3	0	23	100% (23/23)	66.67% (6/9)	90.63% (29/32)
	50%	8	1	0	23	100% (23/23)	88.89% (8/9)	96.88% (31/32)
	60%	9	0	1	22	95.65% (22/23)	100% (9/9)	<b>96.88% (31/32)</b>
	90%	9	0	18	5	21.74% (5/23)	100% (9/9)	43.75% (14/32)
	100%	9	0	23	0	0% (0/23)	100% (9/9)	28.13% (9/32)
BP-53-12	0	0	9	0	23	100% (23/23)	0% (0/9)	71.88% (23/32)
	10%	1	8	0	23	100% (23/23)	11.11% (1/9)	75% (24/32)
	20%	2	7	0	23	100% (23/23)	22.22% (2/9)	78.13% (25/32)
	30%	3	6	0	23	100% (23/23)	33.33% (3/9)	81.25% (26/32)
	50%	5	4	0	23	100% (23/23)	55.56% (5/9)	87.5% (28/32)
	60%	7	2	1	22	95.65% (22/23)	77.78% (7/9)	90.63% (29/32)
	<b>70%</b>	8	1	1	22	95.65% (22/23)	88.89% (8/9)	<b>93.75% (30/32)</b>
	80%	9	0	8	15	65.22% (15/23)	100% (9/9)	75% (24/32)
	90%	9	0	15	8	34.78% (8/23)	100% (9/9)	53.13% (17/32)
	100%	9	0	23	0	0% (0/23)	100% (9/9)	28.13% (9/32)
DO-7	0	0	9	0	23	100% (23/23)	0% (0/9)	71.88% (23/32)
	10%	2	7	0	23	100% (23/23)	22.22% (2/9)	78.13% (25/32)
	20%	3	6	0	23	100% (23/23)	33.33% (3/9)	81.25% (26/32)
	30%	4	5	0	23	100% (23/23)	44.44% (4/9)	84.38% (27/32)
	50%	7	2	0	23	100% (23/23)	77.78% (7/9)	<b>93.75% (30/32)</b>
	60%	7	2	1	22	95.65% (22/23)	77.78% (7/9)	90.63% (29/32)
	90%	9	0	13	10	43.48% (10/23)	100% (9/9)	59.38% (19/32)
	100%	9	0	23	0	0% (0/23)	100% (9/9)	28.13% (9/32)

**Note:** The bold values in the column of "Accuracy" were the maximum accuracy that can be reached by each p53 antibody at the optimal IHC threshold.

commonly used p53 antibodies. Based on our results, by choosing the optimal IHC threshold, the accuracy of DO-7 could reach as high as 93.75%, which is similar to that of BP-53-12 antibody (accuracy=93.75%) but lower than that of MX008 (accuracy=96.88%) or SP5 (accuracy=100%) antibodies. Therefore, the p53 antibody used in Schoop et al's study was not the best one to predict the mutational status of *TP53*. Secondly, Schoop et al pre-selected IHC threshold and/or optimized the thresholds of IHC staining by their correlation with clinical characteristics (eg, EBV positivity, microsatellite instability, and HER2 and MET status); they then used sequencing data to validate their IHC algorithms and found the prediction results were not

ideal. On the other hand, we directly used NGS data to optimize the IHC threshold. According to our results of the DO-7 antibody, the accuracy of mutation prediction ranged from 59.38% to 93.75%, so choosing an improper IHC threshold could lead to a significant decrease in accuracy. Thirdly, Schoop et al's algorithm tempted to distinguish WT *TP53* from all of the mutated *TP53*, including missense, frameshift, inframe deletion, stop gain, etc. As shown in Figure 3A, *TP53* missense mutations had the highest level of IHC positivity and they separated well from other genotypes, so it is possible to choose a proper IHC threshold for each specific p53 to identify *TP53* missense mutations. However, it seems to be unrealistic

to separate WT *TP53* from other non-missense *TP53* mutations as their levels of IHC positivity were largely overlapped (Figure 3A). Overall, all of these differences in methods and research scopes are likely to contribute to the different conclusions between Schoop et al's study and our study.

According to the IHC results (Figure 3 and Supplementary Figure 3), although all four antibodies could achieve high accuracy of detecting *TP53* mutations when appropriate IHC thresholds were chosen, there were still some subtle differences in their IHC staining patterns. Unlike some detection assays where proteins are usually denatured and the structure of the proteins are disrupted (eg, Western blot), p53 protein in the IHC assay has been fixed. As a result, p53 protein in IHC staining will reserve most of its original protein structures, either WT or mutated p53. Based on previous studies,<sup>33</sup> mutated p53 had some structural changes that were different from the WT counterparts, which could confer the differential binding affinity of various p53 antibodies. Although none of the four antibodies we used were originally designed to specifically recognize mutated p53 structures, our IHC results provided indirect evidence of the differential binding capacity of these p53 antibodies to various forms of p53. For example, compared with the other three antibodies, SP5 antibodies had the lowest level of WT p53 staining, whereas the four antibodies stained the p53 missense mutation sections similarly (Supplementary Figure 3). Nevertheless, our results need to be further investigated by future structural studies.

Multiple studies have reported the insignificant impact of *TP53* mutation status on treatment response or overall survival.<sup>6,7</sup> On the other hand, some researchers found *TP53*-mutated patients had improved response to chemotherapy,<sup>8</sup> while others reported that patients harboring *TP53* hotspot mutations had worse overall survival and recurrence-free survival.<sup>9</sup> Given that these contradictory results might be partially due to unreliable detection of *TP53* mutations, properly determining *TP53* mutations status is important for predicting patient prognosis. In addition, most *TP53* missense mutations gained capacities to repress some tumor suppressors, such as WT p53, p63, and p73, and could promote tumorigenesis and progression. As a result, targeting mutated *TP53* becomes a hot area for anti-cancer therapies. Multiple small molecule drugs have been found to promote proper folding of mutant p53 and restore normal p53 function, and some of these drugs have achieved promising pre-clinical and

clinical results.<sup>34</sup> Therefore, accurately detecting the *TP53* mutation has potential therapeutic values to direct targeted therapies in gastric cancer.

We characterized the co-existence of *TP53* mutations with other clinical and molecular features. We found that *TP53* mutations tended to be associated with microsatellite stable (MSS) and more differentiated tumors, which might partially explain the conflicting clinical results of using *TP53* mutations as a prognostic biomarker because patients with MSS and well-differentiated gastric tumors were found to have opposing expectations in prognosis.<sup>30,31</sup> Besides the tumor differentiation level and MSI status, we found that *TP53* mutations occurred mutually exclusive with *BRCA1* mutations in gastric tumors. Previous studies demonstrated that *BRCA1* and p53 could exist in the same protein complex and p53 mediated homologous recombination through inhibiting *BRCA1* over-function in response to DNA repair.<sup>35,36</sup> Also, *BRCA1* mutations were found to co-occur with *TP53* mutations in breast cancer,<sup>37,38</sup> and restoring the WT p53 function could potentially target these tumors.<sup>39</sup> The difference in *BRCA1* and *TP53* co-mutational behavior in gastric and breast cancer might be due to the fact that the co-mutation is crucial for the progression and/survival of breast cancer cells while *BRCA1* and *TP53* mutation could independently promote gastric cancer progression, thus resulting in less co-occurring frequency. Given that PARP inhibitors showed promising clinical responses in patients with *BRCA* mutations and detection of *BRCA* mutation status is relatively expensive,<sup>40</sup> our results imply that *TP53*-positive gastric cancer patients were less likely to harbor *BRCA* mutations, thus providing a cost-effective approach to direct the treatment of anti-PARP drugs. In addition, in some rare cases where *BRCA1* and *TP53* mutations co-occurred in the same gastric tumor, these patients tended to have worse overall survival. On the other hand, patients harboring both *KMT2B* and *TP53* mutations were likely to have better overall survival. All these results suggest that combining *TP53* mutations with other clinical and molecular features might better predict the prognosis of gastric cancer patients. Similar results were also observed by Park et al who found that co-mutation of *NRXN1* and *TP53* were associated with distinct drug responses in gastric cancer patients.<sup>11</sup>

Our study had several limitations. First of all, we optimized the p53 IHC threshold using the discovery cohort; however, as the IHC results for the validation cohort were not available, we cannot further confirm

these results. Secondly, the cohort size of the discovery cohort was limited. Thereby, future large cohort studies are still needed to further validate the IHC thresholds identified in our study. Thirdly, although *TP53/BRCA1* co-mutations and *TP53/KMT2B* co-mutations were found to be correlated with patient survival, they were mutually exclusive mutation pairs in our cohort. As a result, there were a limited number of gastric cancer patients harboring these co-mutations, which limited the clinical utility of these results.

## Conclusion

Overall, our results illustrated that *TP53* mutations tended to co-occur with certain molecular and clinical features in gastric tumors, such as WT *BRCA* and WT *KMT2B* as well as stable microsatellite and high level of tumor differentiation. We also optimized the IHC threshold for commonly used p53 antibodies to enable feasible and reliable detection of *TP53* missense mutations, which could further promote the diagnostic, prognostic, and therapeutic values of using the *TP53* mutation as a gastric cancer biomarker.

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## Disclosure

Yang Xu, Kaihua Liu, Yuqian Shi, Xue Wu, and Yang Shao are employees of Nanjing Geneseeq Technology Inc. Xue Wu reports personal fees from Nanjing Geneseeq Technology Inc., outside the submitted work. The authors report no other potential conflicts of interest for this work.

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