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Review Article

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Spatial and Temporal Tumor Heterogeneity in Gastric Cancer: Discordance of Predictive Biomarkers

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ABSTRACT

Gastric cancer (GC) is a highly heterogeneous disease that varies in both histological presentation and genetic characteristics. Recent advances in the treatment of metastatic and unresectable GC have made several biomarker tests essential for patient management. Predictive biomarkers such as human epidermal growth factor receptor 2 (HER2), programmed death-ligand 1 (PD-L1), mismatch-repair (MMR) proteins, claudin 18.2, and fibroblast growth factor receptor 2b (FGFR2b) are commonly evaluated using immunohistochemistry. However, the expression levels of these biomarkers may vary across different tumor areas, and the accuracy of biomarker diagnosis can be affected by sample quantity, sample location, and collection method. Therefore, tumor heterogeneity presents substantial challenges for accurate biomarker-based diagnosis and prediction of therapeutic responses. Tumor heterogeneity can be categorized into spatial heterogeneity, which refers to variations within the primary tumor (intra-tumoral) or between primary and metastatic sites, and temporal heterogeneity, which encompasses changes over time. This review addresses the tumor heterogeneity in predictive biomarker expression in GC, focusing on HER2, PD-L1, MMR, the Epstein–Barr virus, claudin 18.2, and FGFR2b.

Keywords: Gastric cancer; Tumor heterogeneity; Biomarker

INTRODUCTION

Intra-and inter-tumoral heterogeneity are frequently observed in the histology of gastric cancer (GC), and histological type is typically determined based on the dominant histological component within the largest tumor area [1]. Mixed adenocarcinoma, for example, is characterized by a combination of distinct glandular and poorly cohesive histological components [1,2]. In addition to histological variability, recent studies have highlighted considerable heterogeneity in biomarker expression in GC.

Tumor heterogeneity can be categorized into spatial and temporal heterogeneity [3,4]. Spatial heterogeneity is further divided as follows: 1) intra-tumoral heterogeneity, which occurs within the same tumor; 2) differences between the primary tumor and its metastases; and 3) heterogeneity among metastatic sites (**Fig. 1A**). Human cancers comprise genetically

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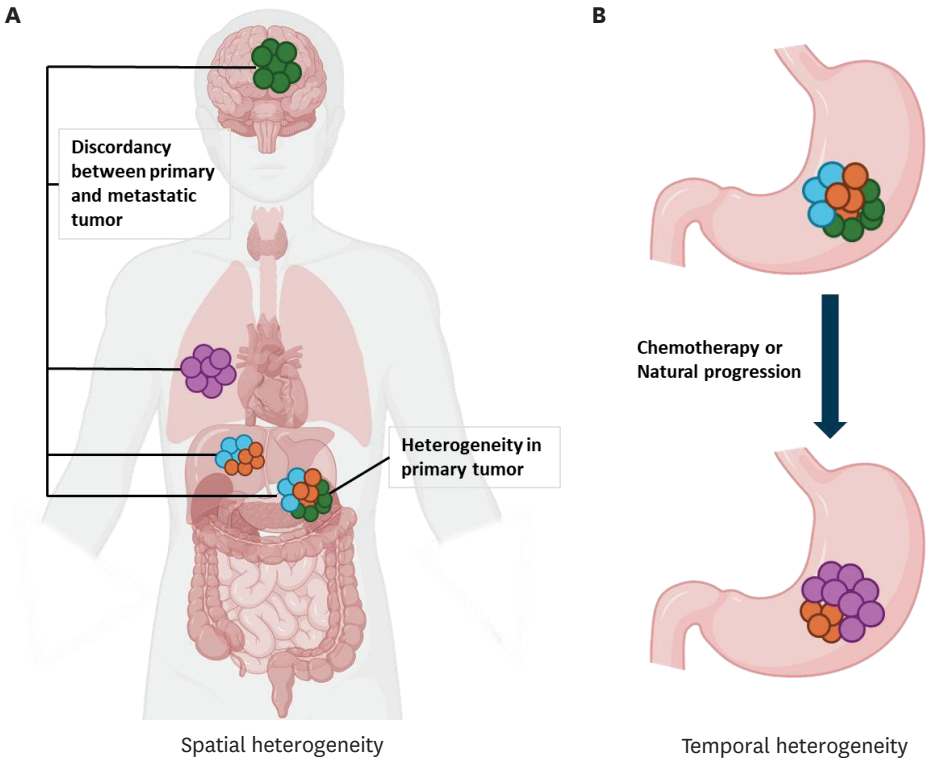

Fig. 1. Spatial and temporal heterogeneity. (A) Spatial heterogeneity refers to heterogeneous positivity within the primary tumor, discordance between the primary and matched metastatic tumors, or discordance among metastatic sites. (B) Temporal heterogeneity refers to changes in genetic features over time, either due to the natural progression of the tumor or response to treatment.

and phenotypically distinct subclones that arise throughout tumor progression [5]. These subclones lead to genetic and phenotypic variability within the same tumor or between multiple sites via diverse mechanisms. Genomic instability, triggered by exogenous or endogenous carcinogenic factors, is suggested to be one of the main drivers of tumor heterogeneity [4,6]. This instability can evoke genetic diversity at the single-nucleotide, long-sequence, or chromosomal level, although other promoting factors in addition to genomic instability are necessary to generate clonal diversity [7].

In contrast, temporal heterogeneity refers to changes over time, either owing to the natural progression of the tumor or as a response to treatment (**Fig. 1B**). Longitudinal tissue sampling has demonstrated clonal evolution and genetic alterations in response to treatment [8,9]. Temporal heterogeneity is believed to stem from the survival and proliferation of pre-existing treatment-resistant subclones or emergence of drug-tolerant cells [4]. This type of heterogeneity is a major cause of resistance to targeted drugs and affects treatment decisions as the genetic profile of the tumor evolves.

Tumor heterogeneity poses considerable challenges for accurate biomarker assessment from small biopsy samples, which may not represent the entire primary tumor or overall tumor burden in the patient. Clinically, heterogeneity complicates biomarker-based diagnoses and contributes to resistance to targeted therapies and immunotherapies [4,10].

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Trastuzumab, which targets human epidermal growth factor receptor 2 (HER2), has been approved for use in combination with chemotherapy as a first-line treatment for metastatic or unresectable GC, based on the success of the Trastuzumab for GAstric cancer (ToGA) trial [11]. Recently, immunotherapies that target the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis, such as nivolumab and pembrolizumab, have also been approved for use alongside chemotherapy and/or trastuzumab [12-14]. Therefore, testing for HER2 and PD-L1 expression using immunohistochemistry (IHC) is critical for guiding initial treatment decisions in patients with GC. Additionally, assessing microsatellite instability (MSI)/mismatch-repair (MMR) status and Epstein–Barr virus (EBV) infection via in situ hybridization (ISH) may be essential in determining second-line or subsequent treatment [15]. New biomarkers of GC have recently emerged from clinical trials. Phase-3 trials have shown promising results for zolbetuximab targeting claudin 18.2 (CLDN18.2) in patients with CLDN18.2-positive GC [16,17]. Consequently, CLDN18.2 has become a critical biomarker in GC treatment. A phase-2 study similarly demonstrated the efficacy of an anti-fibroblast growth factor receptor 2b (FGFR2b) monoclonal antibody in patients with FGFR2b-positive GC [18].

This review discusses tumor heterogeneity in relation to predictive biomarkers, including HER2, PD-L1, MSI/MMR, EBV, CLDN18.2, and FGFR2b, focusing on spatial and temporal heterogeneity. The clinical implications of tumor heterogeneity for biomarker-based diagnosis and therapeutic selection are also addressed.

HER2 HETEROGENEITY

Intra-tumoral heterogeneity

In the trastuzumab-based first-line treatment of metastatic or unresectable GC, HER2 positivity is defined as either strong membranous staining on IHC (3+) or weak-to-moderate staining (2+) with *HER2* amplification [1]. A HER2-positive area is defined as positivity in ≥10% of tumor cells in a resected specimen or as a cluster of ≥5 HER2-positive tumor cells in a biopsy sample [1]. HER2 positivity has been observed in approximately 9.8%–23.0% of patients in Asian populations [19]. Initial studies reported controversial results regarding the spatial heterogeneity of HER2 positivity (**Table 1**). For example, one study described discordance rates of 1.5% and 5.1% between primary tumors and matched metastases when assessed using *HER2* gene fluorescence ISH (FISH) and HER2 IHC, respectively [22]. Another study identified intra-tumoral heterogeneity in 2.5% of cases using *HER2* FISH and 14.5% using IHC in a cohort of 325 individuals [20]. Both studies indicated that 80–90% of GC cases were HER2-negative, suggesting a homogeneous expression pattern; however, HER2 heterogeneity was likely underestimated given that it was calculated across all GC cases.

Subsequent studies focused on heterogeneity only among HER2-positive GC cases and demonstrated substantial heterogeneity in HER2 positivity, although their definitions of heterogeneity varied (**Table 1**, **Fig. 2**). In our previous study, intra-tumoral heterogeneity was defined as HER2 overexpression or gene amplification in 5%–50% of the tumor area, which we identified in 74.0% (54) and 41.1% (30) of cases using IHC and FISH, respectively, among 73 cases with IHC scores of 2+ or 3+ [27]. These results are consistent with those of other studies. For instance, in tissue microarray (TMA) analyses, HER2 heterogeneity between different tissue cores was observed in 70.6% (36/51 cases) of GCs with IHC scores of 2+ and 3+ in one study [26] and 33.3% (21/63) in another [21]. Additionally, intra-tumoral

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Table 1. Published results for tumor heterogeneity in HER2 expression

Comparison	Method	Heterogeneity (%)	No. of cases	Cohort	Reference
Intra-tumoral (differences between 3 TMA cores)	FISH	2.5	8/325	Total cases	[20]
Intra-tumoral (differences between 4 TMA cores)	SISH	2.8	14/498	Total cases	[21]
Primary vs. metastatic	FISH	1.5	1/68	Total cases	[22]
Primary vs. metastatic	SISH	7.2	7/97	Total cases	[21]
Biopsy vs. gastrectomy	IHC	3.9	5/128	Total cases	[23]
Biopsy vs. gastrectomy	IHC	12.3	84/702	Total cases	[24]
Intra-tumoral (differences between 4 TMA cores)	IHC	4.2	21/498	Total cases	[21]
Intra-tumoral (differences between 3 TMA cores)	IHC	14.5	47/325	Total cases	[20]
Primary vs. metastatic	IHC	2.1	1/47	Total cases	[25]
Primary vs. metastatic	IHC	5.1	2/39	Total cases	[22]
Primary vs. metastatic	IHC	11.3	11/97	Total cases	[21]
Primary vs. metastatic	IHC	14.3	22/154	Total cases	[26]
Intra-tumoral (differences between 4 TMA cores)	SISH	22.6	14/62	HER2-amplified GC	[21]
Intra-tumoral (5–50%)	FISH	41.1	30/73	IHC 2+/3+ GC	[27]
Intra-tumoral (<50%)	FISH	41.2	21/51	HER2-amplified GC	[28]
Intra-tumoral (differences between 3–9 TMA cores)	FISH	47.3	9/19	HER2-amplified GC	[29]
Primary vs. metastatic	FISH	9.1	1/11	HER2-amplified GC	[22]
Primary vs. metastatic	SISH	38.9	7/18	HER2-amplified GC	[21]
Biopsy vs. gastrectomy	IHC	27.8	5/18	IHC 2+/3+ GC	[23]
Biopsy vs. gastrectomy	IHC	54.1	86/159	IHC 2+/3+ GC	[24]
Intra-tumoral (differences between 4 TMA cores)	IHC	33.3	21/63	IHC 2+/3+ GC	[21]
Intra-tumoral (10%–70%)	IHC	42.9	79/184	IHC 3+ GC	[30]
Intra-tumoral (10%–66%)	IHC	50.0	6/12	IHC 3+ or *HER2*-amplified GC	[31]
Intra-tumoral (2+/3+ in some tumor cells)	IHC	50.0	14/28	HER2-positive GC	[32]
Intra-tumoral (10%–70%)	IHC	70.5	31/44	IHC 2+ GC	[30]
Intra-tumoral (differences between 2–7 TMA cores)	IHC	70.6	36/51	IHC 2+/3+ GC	[26]
Intra-tumoral (5%–50%)	IHC	74.0	54/73	IHC 2+/3+ GC	[27]
Primary vs. metastatic	IHC	6.7	1/15	IHC 2+/3+ GC	[25]
Primary vs. metastatic	IHC	50.0	11/22	IHC 2+/3+ GC	[21]
Primary vs. metastatic	IHC	62.9	22/35	IHC 2+/3+ GC	[26]

HER2 = human epidermal growth factor receptor 2; TMA = tissue microarray; FISH = fluorescence in situ hybridization; SISH = silver in situ hybridization; IHC = immunohistochemistry; GC = gastric cancer.

heterogeneity was detected in 50.0% of patients when defined as 2+ or 3+ HER2 staining in some but not all tumor cells [32]. Regional heterogeneity in HER2 IHC expression—defined as 10%–70% positive cells—was found in 42.9% (79/184) of IHC 3+ and 70.5% (31/44) of IHC 2+ cases [30]. Finally, when characterized as HER2 positivity in 10–66% of tumor cells, heterogeneity was present in 50% of HER2-positive GC cases [31].

HER2 gene amplification has also been investigated, with a study reporting heterogeneity (amplification in <50% of tumor cells) in 41.2% (21/51) of *HER2*-amplified tumors [28]. Additionally, amplification heterogeneity between different TMA cores was observed in 47.3% (9/19) of *HER2*-amplified cases [29].

Comparison between biopsy and surgical resection

In patients with initially metastatic GC, endoscopic biopsy specimens are usually the only tissues available for HER2 testing. However, owing to the considerable heterogeneity in HER2 expression in GC, discrepancies between biopsy and surgical specimens can pose substantial challenges in HER2-targeted therapies. In a study that examined matched biopsy and resection specimens from 128 patients with GC, 5 of the 18 HER2-positive cases (27.8%) had discordant results: two cases were positive only on biopsy and three only on resection [23]. Another study that compared HER2 status between biopsy and resection specimens in a larger cohort of 702 patients with GC identified 86 discrepant cases (12.3% of all cases and 54.1% of 159 HER2-positive cases), with 57 cases positive on biopsy and 29 on resection [24].

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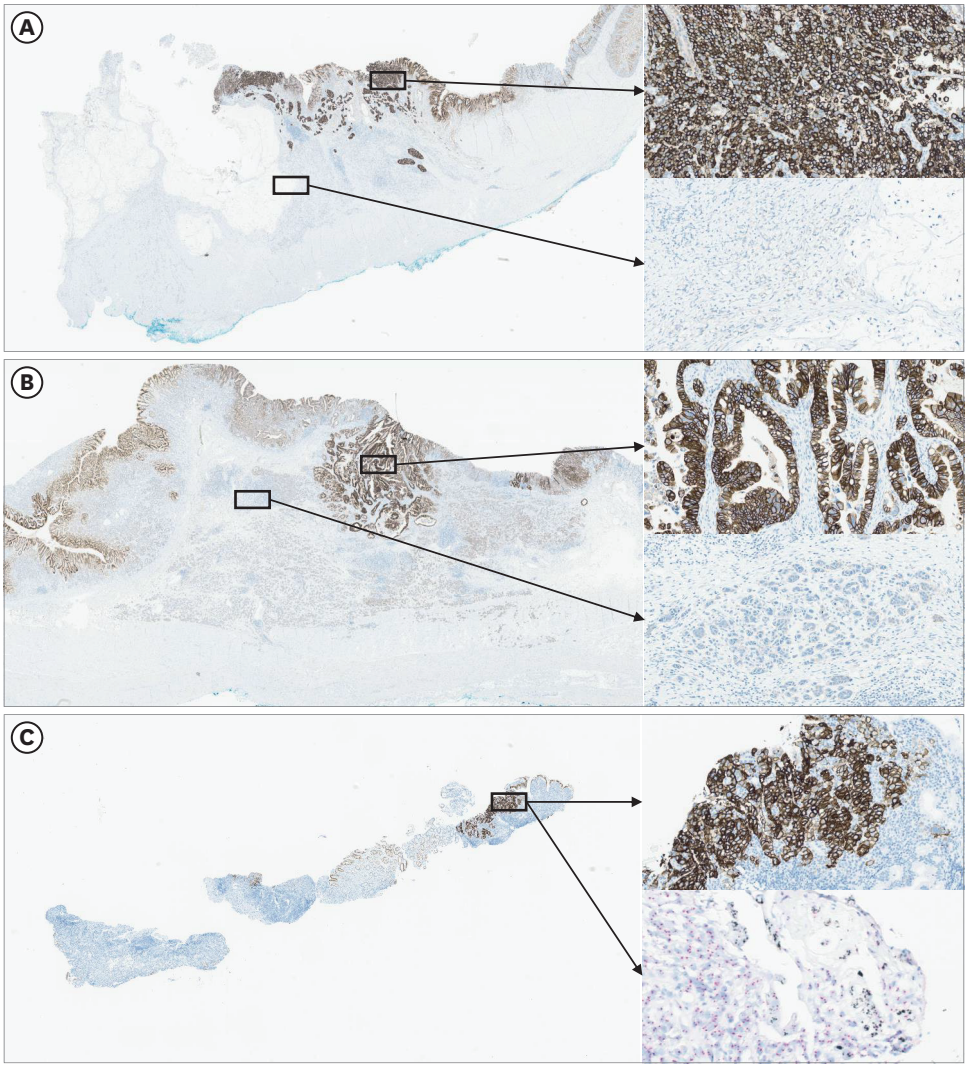

Fig. 2. Intra-tumoral HER2 heterogeneity. (A) A HER2-positive case had a score of 3+ in the poorly differentiated adenocarcinoma area, whereas the poorly cohesive and mucinous adenocarcinoma area was negative. (B) A HER2-positive case had a 3+ score in areas of lower-grade histology, whereas the high-grade areas were negative. (C) HER2 positivity was identified in one of four biopsy fragments, with *HER2* gene amplification observed in the pictured fragment.
HER2 = human epidermal growth factor receptor 2.

Therefore, several studies have sought to identify the optimal number of biopsy fragments required to reduce the amount of false-negative and -positive results. Ahn et al. [24] recommended the use of at least four biopsy fragments containing tumor cells to accurately determine HER2 status in GC, whereas another study suggested a minimum of five fragments for reliable assessment [33]. Additionally, some researchers have proposed that the use six to eight viable fragments is optimal [34]. Based on these findings, the guidelines of the College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology recommend obtaining a minimum of five biopsy specimens (ideally six to eight) to ensure sufficient tumor sampling for accurate diagnosis and biomarker testing [35].

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Discordance between primary and metastatic tumors

In addition to intra-tumoral heterogeneity, discordance in HER2 positivity between primary and metastatic GCs has been observed, with reported rates ranging from 1% to 14% [15]. Fassan et al. [25] identified a high concordance rate between the HER2 statuses of primary tumors and matched lymph-node metastases, reporting only a 2.1% discordance. Another study found a 14.3% discordance in HER2 status between primary tumors and synchronous metastases (22/154 cases) [26]. Notably, when HER2-negative and 1+ cases were excluded, the discordance rate increased to 62.9% (22/35), with six cases showing positive conversion (negativity in primary tumor but positivity in lymph-node metastasis) and sixteen exhibiting negative conversion [26]. In another study, discordance between primary tumors and paired metastatic lymph nodes was observed in 11.3% of cases assessed using HER2 IHC and 7.2% via silver ISH [21]. These discordance rates increased considerably to 50.0% and 38.9%, respectively, when calculated only for cases with HER2 IHC scores of 2+ or 3+ or those with *HER2* amplification [21].

Kim et al. [20] suggested that these discrepancies between primary and metastatic tumors may originate from heterogeneity within the primary tumor. Additionally, genetic drift or clonal selection during tumor progression might contribute to these differences [36]. Given the spatial heterogeneity observed within primary tumors and between primary and metastatic lesions, incorporating additional HER2 testing of both site types could enhance diagnostic accuracy and reliability.

Clinical significance of HER2 heterogeneity

GC with intra-tumoral HER2 heterogeneity tends to exhibit diffuse or mixed histological types according to the Lauren classification, and generally has low levels of HER2 expression (IHC 2+) or *HER2* gene amplification [27,30]. HER2 is a key oncogenic driver associated with aggressive disease. Although some studies have found correlations between HER2 positivity and poor outcomes in GC, others have failed to confirm its prognostic value [19,21]. Therefore, the predictive significance of HER2 positivity in GC remains unclear. Furthermore, whether patients with heterogeneous HER2-positive GC experience better or worse outcomes than those with homogeneous HER2-positive GC is still unknown [27,37].

In contrast, heterogeneous HER2 positivity in patients with GC that received trastuzumab with chemotherapy as the first-line treatment has been linked to limited clinical benefits, including reduced response and shorter survival times [38]. The median progression-free (PFS) and overall survival (OS) were notably longer in patients with homogeneous HER2-positive GC than in those with heterogeneous HER2-positive GC, and multivariate analyses indicated significant associations between HER2 heterogeneity and poor PFS and OS [32,39,40]. Regarding treatment response rate, one study identified no significant difference between homogeneous and heterogeneous HER2-positive disease [32]; however, other studies have reported greater responses in patients with homogeneous HER2 positivity [39,40]. Therefore, HER2 heterogeneity potentially contributes to trastuzumab resistance.

In the GASTric cancer HER2 re-assessment study 1 (GASTHER1), "rescued" HER2 positivity was identified in 16 of 183 (8.7%) and 10 of 175 (5.7%) patients with initial HER2 negativity via repeated endoscopic biopsy and re-assessment of metastatic or recurrent lesions, respectively [41]. Compared with patients that were HER2-positive on the initial testing of primary tumors, those with rescued HER2 positivity achieved similar overall responses to trastuzumab-based first-line chemotherapy and comparable PFS [41]. However, a subsequent

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study that included a larger cohort and longer follow-up reported significantly worse PFS and OS in patients with rescued HER2 positivity than in those with initial positivity, likely due to HER2 heterogeneity in the rescued group [42].

Circulating tumor DNA tests using digital polymerase chain reaction (PCR) or next-generation sequencing have been explored as alternative HER2 assessment methods, and the sensitivity of digital PCR ranged from 37.5% to 76.5% in previous studies [43-45]. Despite their potential as alternative HER2 tests, these techniques are not used in standard practice because of their relatively low sensitivity and high costs. Recently, antibody-drug conjugates such as trastuzumab-deruxtecan have been introduced to treat GC [46]. These drugs may improve responses in HER2-heterogenous GC by utilizing a bystander effect, in which HER2-targeted agents affect HER2-negative cells adjacent to positive cells [47]. In addition, pembrolizumab-based immunotherapy has been approved as a first-line treatment for HER2-positive GC, and a PD-L1 combined positive score (CPS) ≥1 was observed in 85% of patients with HER2-positive GC that underwent this treatment in the phase-3 KEYNOTE-811 trial [13]. As new treatment regimens continue to emerge for patients with HER2-positive GC, the relevance of HER2 heterogeneity is expected to grow.

Temporal heterogeneity

Trastuzumab targets and kills HER2-positive cancer cells, allowing for the proliferation of HER2-negative cells, which may contribute to the acquired resistance to trastuzumab-based treatments [15]. In a study that compared IHC results from the pre-treatment biopsies and post-treatment surgical samples of individuals with HER2-positive GC, HER2-negative conversion following trastuzumab-based chemotherapy was reported in three of the seven patients (42.9%) [48]. However, since pre- and post-treatment samples were obtained from biopsies and resections, respectively, the observed loss of HER2 expression in this study reflects either treatment effects or pre-existing heterogeneity [48].

Several studies have investigated the loss of HER2 positivity by examining pre- and post-treatment biopsies following tumor progression. In one study, 31.6% (6/19) of patients lost HER2 positivity after trastuzumab-based first-line chemotherapy, and this loss was more frequent in initial IHC 2+ than in IHC 3+ cases (80.0% vs. 14.3%, respectively) [49]. Similarly, in the GASTHER3 study, 14 of 48 (29.2%) patients lost HER2 positivity according to post-progression biopsies, and the median H-score for these samples was significantly lower than that for pre-treatment biopsies [50]. Another research group reported an even higher rate of HER2 positivity loss, with 60.6% (20/33) of patients with refractory disease having exhibited decreased HER2 expression after treatment [51].

The GASTHER3 study further highlights the clinical impact of HER2 loss, having observed that although the reduction was not significantly associated with patient outcomes, individuals with reduced HER2 positivity in post-progression samples had no objective response to second-line treatment with trastuzumab emtansine [50]. Therefore, trastuzumab-induced changes in HER2 expression in GC may not only contribute to drug resistance, but also influence the choice of second-line treatment. Consequently, re-assessment of HER2 status prior to the initiation of second-line or subsequent HER2-targeted therapy is recommended if post-progression samples are available [52].

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PD-L1

PD-L1 expression is highly heterogeneous within tumors in patients with GC (**Fig. 3**). According to the guidelines for PD-L1 IHC interpretation, the CPS should be averaged across the entire tumor area to determine the final score [53]. Therefore, the consideration of tumor heterogeneity is crucial when diagnosing PD-L1 positivity and making immunotherapy decisions for patients with GC.

Previous studies have reported higher PD-L1 positivity rates in surgically resected samples than in biopsies, with false negatives being more common in biopsy samples. In a study that compared PD-L1 expression between 112 gastric biopsy samples and matched gastrectomy specimens, CPS ≥1 was observed in 32.1% of biopsies versus 47.3% of gastrectomy samples, with biopsy sensitivity and specificity of 62% and 73%, respectively, in predicting PD-L1 expression in the gastrectomy specimens [54]. Similarly, in 99 patients with advanced GC, biopsy and gastrectomy pairs exhibited 86% concordance in PD-L1 CPS ≥1 when assessed

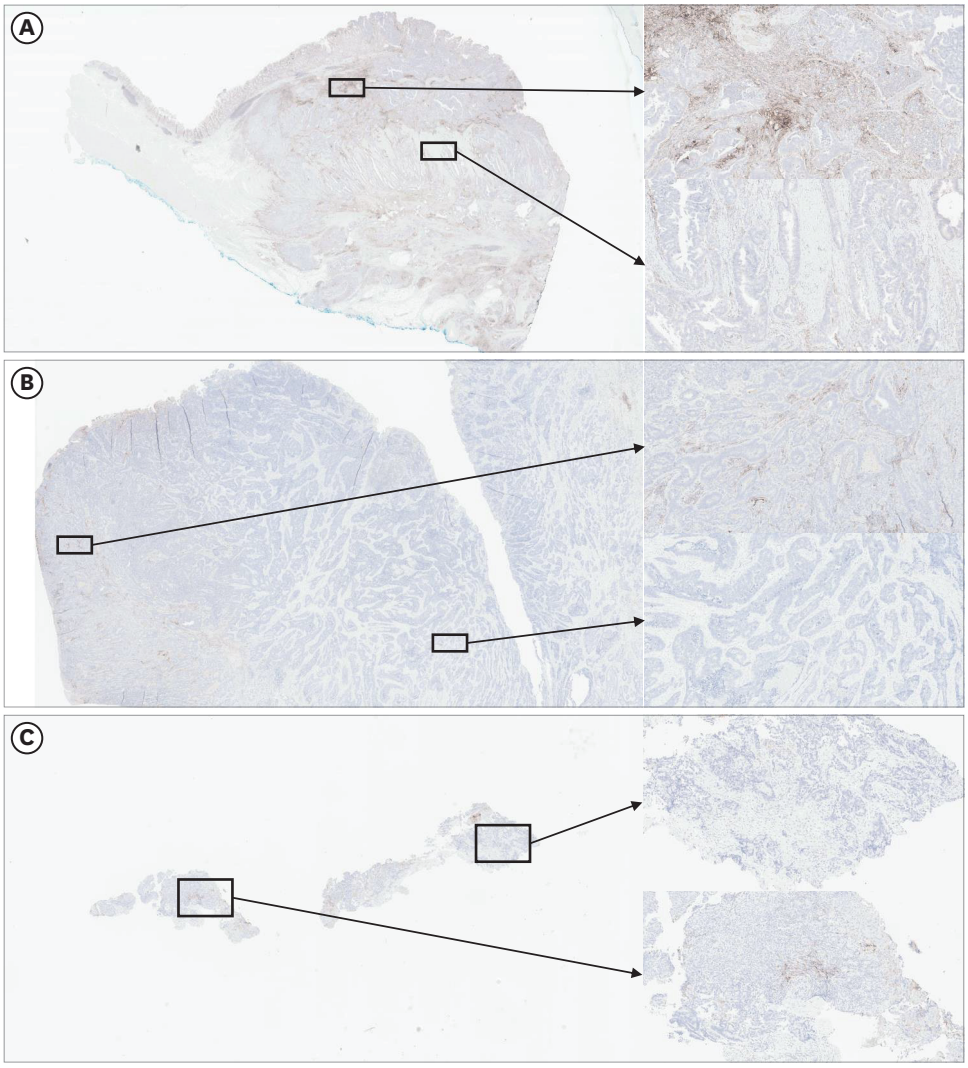

Fig. 3. Intra-tumoral PD-L1 heterogeneity was observed in gastric cancer, with PD-L1 combined positive scores ≥10 (A) and ≥1 and <5 (B and C).
PD-L1 = programmed death-ligand 1.

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using the 22C3 pharmDx assay, with 13 false-negative and no false-positive cases [55]. Another study reported PD-L1 positivity in 46.6% of biopsies and 70.1% of resected samples, with false-negative and -positive biopsy rates of 29.8% and 5.8%, respectively [56].

To improve the accuracy of PD-L1 testing, an adequate number of viable endoscopic biopsies is necessary. This is because the concordance between biopsy and resected specimens is reportedly lower in cases with single-fragment biopsy (48.8%) than in those with multiple fragments (68.9%) [56]. In TMA analyses, combining the CPS for four TMA cores (1.2-mm diameter) yielded results closely aligned with those for resected samples, although the inclusion of fewer cores reduced sensitivity and increased the number of false negatives [57]. In another study that assessed six TMA cores from each gastrectomy specimen, the consistency of PD-L1 positivity between cores and resected samples improved as the number of cores increased, with five identified as being optimal for representational accuracy [58]. These findings suggest that at least four to five biopsy fragments are required to reliably assess PD-L1 expression in GC [59].

Discordance of PD-L1 expression between primary and metastatic tumors is also evident. In paired baseline primary and metastatic tumors, concordance in PD-L1 CPS ≥1 and ≥10 was observed in 61.3% (38/62) and 83.9% of cases, respectively [60]. Another study identified a PD-L1 CPS ≥ 1 in 52.2% of primary tumors (12/23) but only 4.3% of distant metastases (1/23); however, this analysis was limited by the small sample size and inclusion of metachronous metastasis [61]. Interestingly, the PD-L1 expression in tumor-infiltrating immune cells was higher in lymph-node metastases (54.4%) than in primary tumors (41.6%) [62], with a similar trend reported in another study (60.5% vs. 41.9%, respectively) [63].

Temporal heterogeneity, or post-chemotherapeutic variation in PD-L1 expression, has also been observed in GC. In one study, the PD-L1 immunoreactivity scores (IRS) decreased following chemotherapy; positivity (IRS ≥2) was reduced from 58.3% (42/76) in pre-treatment samples to 41.7% (30/72) in post-treatment samples, with a 50% concordance rate between pre-and post-treatment samples [64]. Another study revealed that the PD-L1 status (CPS ≥1) of pre-treatment primary tumors was concordant with those of post-treatment primary or metastatic samples in 62.7% (52/83) of cases, with an increased rate of 74.7% (62/83) for CPS ≥10 [60]. The concordance rates were not affected by the chemotherapeutic regimens used. PD-L1 positivity with a cutoff of CPS = 1 was identified in 54.2% of pre- and 53.0% of post-treatment samples in this study [60].

Therefore, the spatial and temporal heterogeneity in PD-L1 expression should be carefully considered in clinical practice. The re-evaluation of PD-L1 IHC in recurrent or metastatic tumors may be helpful for accurate PD-L1 assessment in patients with GC.

MSI/MMR

In daily practice, MSI testing is performed using PCR with five microsatellite markers [65]. For molecular testing, DNA is typically extracted from tumor cells after macro-dissection of the entire tumor area, with a sensitivity or limit of detection of approximately 10% for MSI analysis [66]. Therefore, PCR-based MSI analysis is restricted in detecting intra-tumoral heterogeneity. In contrast, MMR status can be assessed using IHC in four MMR proteins—mutL homolog 1 (MLH1), mutS homologs 2 (MSH2) and 6 (MSH6), and post-meiotic

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segregation increased 2 (PMS2)—allowing the estimation of heterogeneity in MMR protein expression. The IHC analysis of MMR proteins is advantageous due to its cost-effectiveness and availability; however, it also has limitations, including indeterminate diagnoses due to ambiguous staining [67]. Thus, the heterogeneity in MMR expression identified on IHC may be underestimated.

A previous study reported no intra-tumoral heterogeneity in the MMR status between the tumor front and center in 415 GC cases [68]. Another study described a high level of instability (MSI-H) detected using PCR in 187 of the 3,723 cases (5.1%) and heterogeneous MMR protein expression within the primary GC area in 11 cases (0.3%) [69]. Additionally, a single instance of heterogeneous MSH2 expression loss in 452 GC cases (0.2%) was reported, corresponding to 2.9% of 34 MSI-H GC cases [70]. In our previous study, we observed subclonal loss of MLH1 expression in 0.4% of all GC cases (24/5676) and 4.3% of 549 MSI-H cases [67]. In contrast, Kim et al. [71] conducted multi-regional sampling of 79 deficient MMR (dMMR) or MSI-H tumors from patients with advanced GC and revealed a heterogeneous MSI status in 8.9%, suggesting that a subset of MSI-H or dMMR tumors exhibits significant heterogeneity. Interestingly, one of six patients with MSI-H GC showed no response in a small phase-2 trial of single-agent pembrolizumab treatment, which was associated with the heterogeneous loss of MLH1 expression [71]. Overall, intra-tumoral heterogeneity in MSI/MMR is rare in GC, occurring in less than 1% of all cases; however, among MSI-H cases, its incidence may be as high as 8.9%.

A few studies have examined the MSI/MMR concordance between primary and metastatic tumors (**Fig. 4**). Recently, a discordant case with MSS in the primary tumor and MSI-H in an ovarian metastasis was identified [69]. Another study documented a GC case with dMMR at the primary site and proficient MMR in synchronous skin metastases; overall, dMMR was more common in primary (8.7%, 2/23) than in paired metastatic GC (4.3%, 1/23) [61]. However, full concordance in MSI/MMR status was observed in a study that analyzed 269 primary GCs with matched metastatic lymph nodes and 98 with matched distant metastases [68]. Although rare, discordance between primary and metastatic tumors should not be overlooked.

EBV

ISH for EBV-encoded RNA (EBER) is considered the gold standard for detecting and localizing latent EBV in GC tissue samples. Typically, EBER ISH exhibits diffuse nuclear positivity in EBV-associated GC, supporting the hypotheses that EBV infection may influence GC carcinogenesis and that EBV-associated GC is a monoclonal proliferation originating from a single cell persistently infected with EBV [72]. Further substantiating these hypotheses, consistent EBV status was observed between the tumor front and center in 415 cases [68]. Additionally, the EBV status of primary tumors was preserved in metastatic lymph nodes (n=284) and distant metastases (n=103), although only 11 cases were EBV-positive [68].

In contrast, rare instances of heterogeneous EBER positivity have been reported. In one study, heterogeneous EBV positivity was identified in four cases, representing 0.8% of 484 GC cases and 18.2% of 22 EBV-positive cases; moreover, heterogeneity was also observed in paired lymph-node metastases [73]. In a more recent study, heterogeneous EBV positivity was identified in four GC cases, accounting for 0.1% of 3,499 consecutive surgical cases and 1.9% of 214 EBV-positive cases [74]. Among these four cases, three exhibited two

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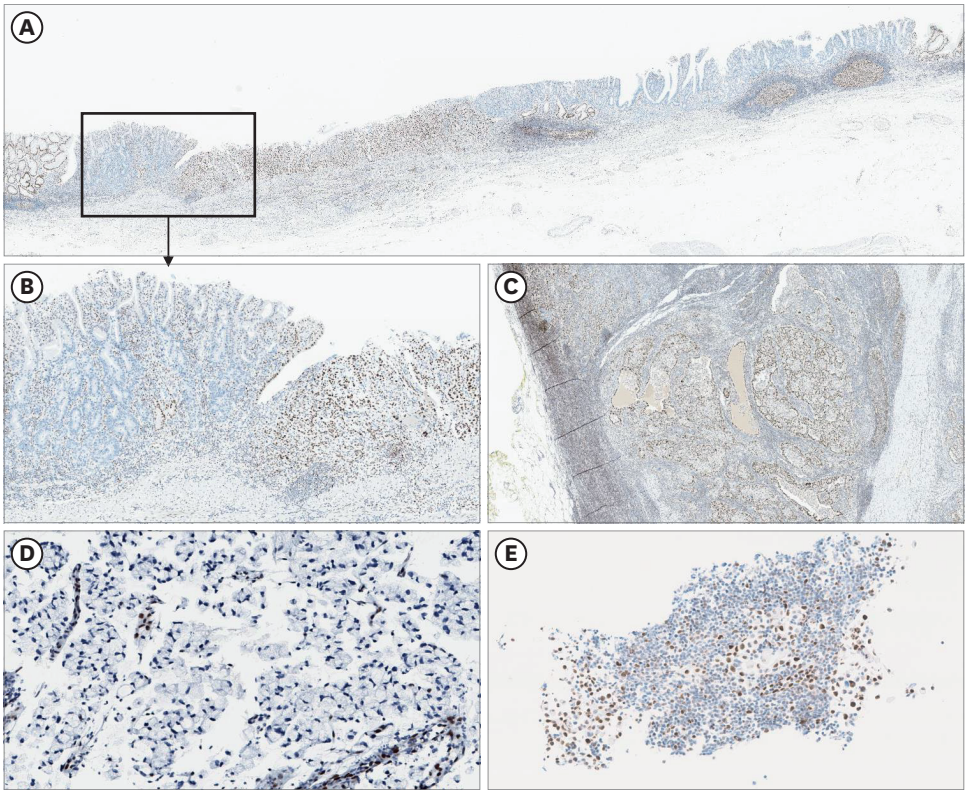

Fig. 4. Tumor heterogeneity in MLH1 expression. A focal area with MLH1 loss was identified (A and B), whereas MLH1 expression was retained in the lymph-node metastasis of the same patient (C). In another patient, MLH1 expression was lost in an endoscopic biopsy sample (D) but present in a metastatic tumor (supraclavicular lymph node) (E). MLH1 = mutL homolog 1.

histologically distinct regions, with the EBV-positive area being poorly differentiated and displaying lymphocytic infiltration [74]. Therefore, although heterogeneous EBV positivity in GC is rarely observed in clinical practice, it raises questions about whether this phenomenon reflects a genuine loss of EBV infection, reduced EBV transcription, or a mix of EBV-positive and -negative tumor populations [73].

CLDN18.2

Positivity rates for CLDN18.2 in GC have been reported to vary widely, ranging from 14% to 88% depending on the testing protocols and positivity criteria applied [75]. Currently, CLDN18.2 positivity is defined as ≥75% of tumor cells showing moderate-to-strong membranous staining with the 43-14A antibody on IHC, as established in the SPOTLIGHT and GLOW clinical trials [75]. CLDN18.2 expression in the GC tumor area varies considerably: 27% of cases in clinical trials had no expression, 22% had low expression (≥1% and <40%), 13% had moderate expression (≥40% and <75%), and 38% had high expression (≥75%) [76], indicating substantial heterogeneity in CLDN18.2 expression within a single tumor (**Fig. 5**).

Defining intra-tumoral CLDN18.2 heterogeneity is challenging because of its positivity threshold of 75%, which is stricter than those for other biomarkers. In comparison, the respective definitions of HER2 heterogeneity in previous studies were positivity in 10%–50%, 33%–66%, or 10%–90% of the tumor area. In a recent study, 299 stage I–III GC cases were

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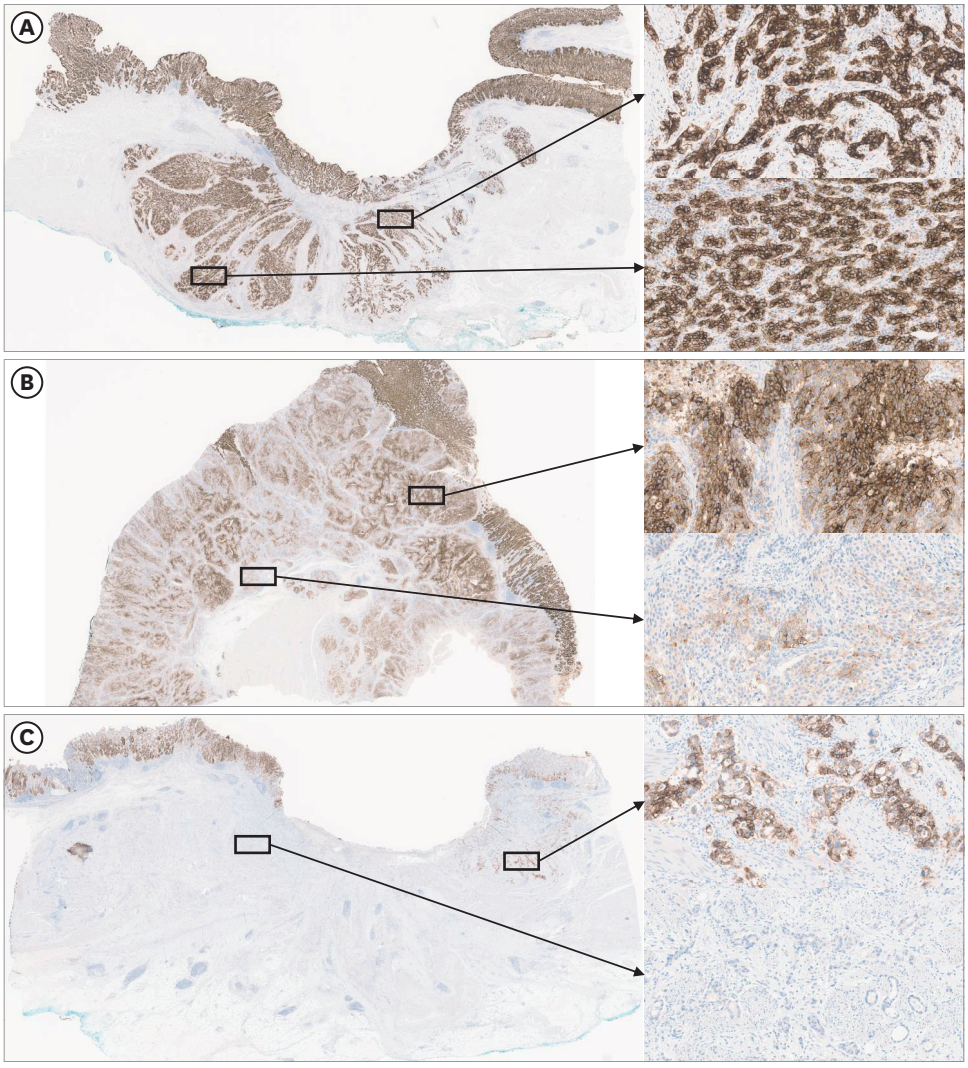

Fig. 5. Intra-tumoral CLDN18.2 heterogeneity. (A) Homogeneous expression of CLDN18.2. (B) A CLDN18.2-positive case with a heterogeneous expression pattern. (C) A CLDN18.2-negative case with a heterogeneous expression pattern.
CLDN18.2 = claudin 18.2.

analyzed, 46.5% (139 cases) of which were positive for CLDN18.2 [77]. Of the 139 CLDN18.2-positive cases, 31.0% (43) had heterogeneous expression (75%–90% positivity), with most (40) exhibiting a random expression pattern [77]. In a study on gastrectomy samples, intra-tumoral CLDN18.2 heterogeneity (again defined as 75%–90% positivity) was identified in 38.5% of 32 CLDN18.2-positive samples [78]. When comparing biopsy and surgical specimens, the concordance rate of CLDN18.2 positivity was 81.3%, with a tendency for increased positivity in superficial areas than in the invasive front [79].

In our recent study, we constructed TMA blocks with four 2-mm diameter cores for each case and compared CLDN18.2 positivity across these four cores [80]. Heterogeneous intra-tumoral CLDN18.2 expression among the cores was identified in 68 cases, representing 23.8% of 286 total GC cases and 61.3% of 111 cases with CLDN18.2 positivity in at least one core. However, when we compared CLDN18.2 positivity between whole-tissue sections and the combined results for all four TMA cores, discordance was observed in only 2.4% (2/85) of

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cases. These findings suggest that while CLDN18.2 expression is heterogeneous throughout GC tumors, a combined analysis of four TMA cores may provide a representative assessment for each case.

CLDN18.2 expression differences between primary and metastatic sites have also been examined. The concordance rate between primary tumors and peritoneal metastases was 75.0%, with higher positivity rates in primary tumors than in peritoneal metastases (28.6% [24/84] vs. 20.2% [17/84], respectively) [79]. The concordance across various metastatic sites (peritoneum, liver, lung, bone, and distant lymph nodes) was 74.8% (101/135), with peritoneal metastases showing the highest CLDN18.2 positivity (44.3%) and liver metastases the lowest (17.9%) [78].

Standardized protocols and interpretation guidelines for CLDN18.2 IHC testing were introduced recently, leaving CLDN18.2 heterogeneity in GC yet to be fully elucidated. Further studies are needed to clarify these patterns according to the prescribed guidelines.

FGFR2b

Bemarituzumab, a fucosylated, humanized immunoglobulin G1 anti-FGFR2b monoclonal antibody, was combined with chemotherapy in patients with HER2-negative GC and FGFR2b overexpression, detected via IHC and/or *FGFR2* gene amplification assessed using a circulating tumor DNA assay [18,81,82]. This phase-2 study reported clinically significant outcomes. Although protocols and interpretation guidelines for FGFR2b IHC or *FGFR2* gene testing in routine practice have not yet been standardized, the heterogeneity in FGFR2b expression and *FGFR2* amplification have been explored in a few studies. An earlier study identified FGFR2b overexpression via IHC in 2.5% (9/362) of GC cases, with intra-tumoral heterogeneity—defined as discordance among three TMA cores—observed in five of nine FGFR2b-positive cases (55.5%) [83]. Additionally, discordant FGFR2b expression between primary tumors and paired lymph-node metastases was identified in four of nine cases (44.4%), comprising three cases of negative conversion (positive in primary tumors but negative in lymph-node metastases) and one of positive conversion [83]. In an independent study, FGFR2b overexpression was detected in 8.0% (7/88) of cases, with a higher prevalence in metastatic lymph nodes than in primary tumors (8.0% vs. 3.4%, respectively) [84]. Further, a translational study that used cell lines and patient-derived xenograft models demonstrated durable responses only in tumors with high-level clonal *FGFR2* amplification; conversely, patients with subclonal heterogeneous *FGFR2* amplification did not respond to treatment [85].

CONCLUSION

Biomarker expression is not consistent throughout the entire tumor burden, including primary tumors and metastatic sites, and can change after treatment. Spatial and temporal heterogeneity in the expression of key biomarkers is frequently observed in patients with GC. Among these biomarkers, heterogeneity in HER2 is the most extensively studied, with reports estimating intra-tumoral heterogeneity rates of 2.5%–14.5% across all GC cases [20,21,23,24] and 22.6%–74.0% in those with HER2 positivity or gene amplification [21,26-28,30-32]. Negative conversion of HER2 expression has been observed in post-progression

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biopsies from 29.2%–60.6% of patients with GC that received trastuzumab-based therapies [48-51]. Intra-tumoral heterogeneity in MSI/MMR is less common than HER2 heterogeneity in GC; however, patients with MSI heterogeneity have experienced rapid disease progression following immunotherapy [71]. Additionally, heterogeneity has been studied in emerging biomarkers such as CLDN18.2 and FGFR2b. This variability in biomarker expression may lead to diagnostic inaccuracies and limit biomarker-based treatment efficacy. Therefore, tumor heterogeneity should be carefully considered in the management of patients with metastatic or unresectable GC, particularly in the establishment of standardized guidelines for biomarker testing and interpretation.

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