Journal of Oncology

From Molecular Genetics to Diagnosis and Therapy of Gastric Cancer

Lead Guest Editor: Simona Gurzu
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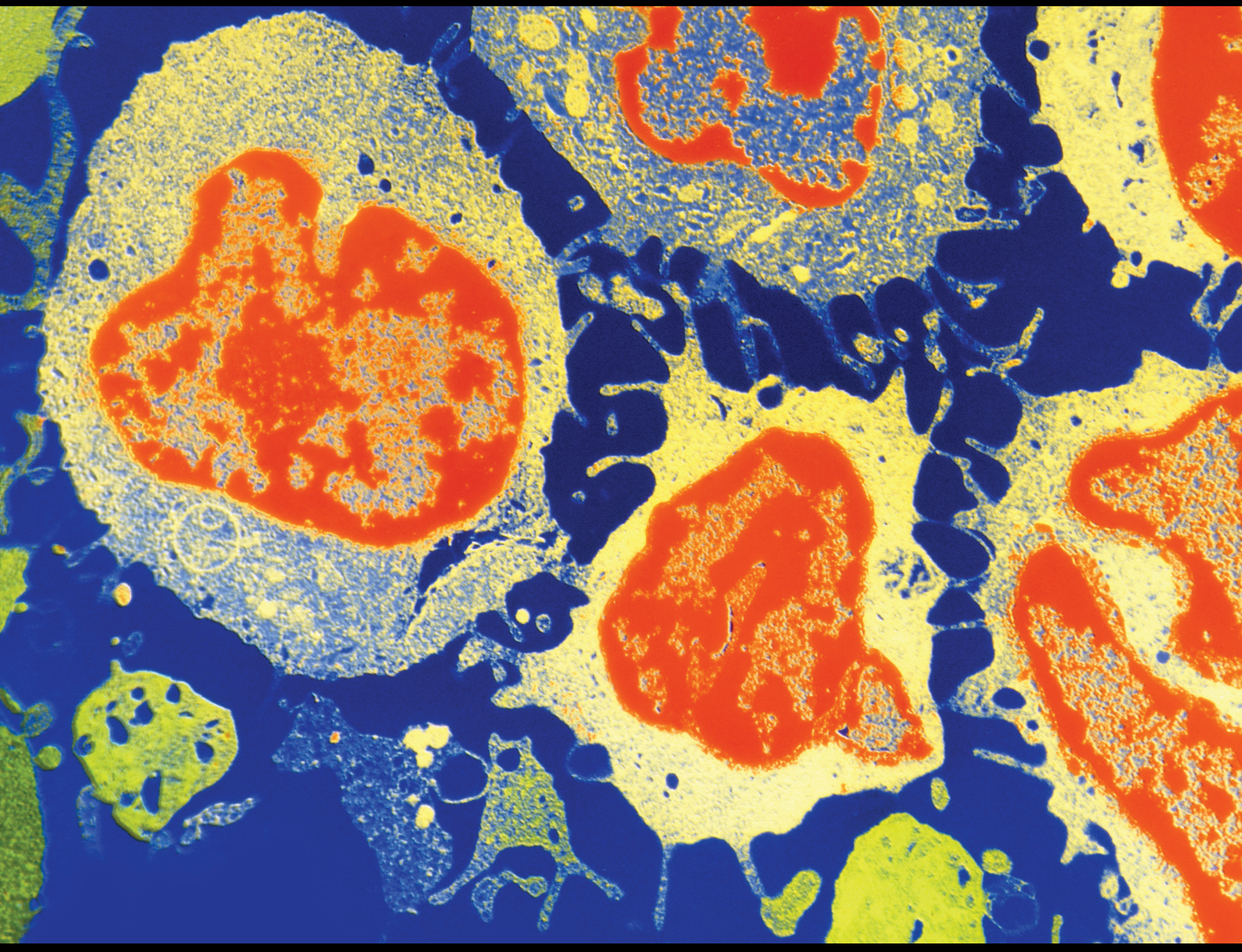

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Research Article

HER2 Heterogeneity in Gastric Cancer: A Comparative Study, Using Two Commercial Antibodies

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Background. Although amplification of the gene encoding human epidermal growth factor receptor 2 (HER2) is used as an indicator for response to trastuzumab, the reported response rate is low, and few patients with gastric cancer (GC) benefit from this individualized therapy. The aim of this study was to examine the expression of c-erbB-2 oncoprotein (HER2), in GC samples, using two commercial immunohistochemical (IHC) antibodies, and to validate the results by checking *HER2* gene amplification by fluorescence in situ hybridization (FISH). *Methods*. We assessed the IHC expression of HER2 using the polyclonal antibody from Dako and CB11 clone from Leica, in 93 consecutive cases of GC samples. In all of the cases, FISH analysis was also performed using the BOND-MAX platform. *Results*. No significant difference was observed between the two HER2 antibodies. Of the 93 cases, 22.58% demonstrated at least focal and 1+ HER2 positivity. Seven cases (7.53%) exhibited 3+ expression, and another 7 carcinomas (7.53%) were equivocal (2+). *HER2* amplification was seen in 11 cases (11.83%), 10 of which were differentiated adenocarcinomas. In 5 of the cases, 2–5 sections were examined, which proved the extremely high intratumorally/intraglandular heterogeneity. FISH heterogeneity was higher in cases with only 2+ positivity on IHC assessment, compared with those showing at least one small focus of 3+ overexpression. *HER2* amplification proved to be an independent negative prognostic factor. *Conclusions*. Due to the highly heterogeneous aspect of GC, at least 3-4 slides should be assessed by IHC, before considering a tumor to be HER2-negative. In cases with small 3+ foci representing less than 5% of tumor and in equivocal (2+) cases, FISH analysis remains the gold standard method.

1. Introduction

Gastric cancer (GC) remains one of the most common causes of cancer-related deaths worldwide, for which the estimation of survival rate, which varies within the same stage, is difficult to be predicted [1]. Although the mortality rate for GC has trended slightly downward in recent decades, it remains a global health problem [2].

Though more than 50 years have passed since the introduction of the Lauren classification, the morphology-based dichotomization of GC into intestinal and diffuse-type carcinoma is still widely used [3]. One of the reasons that an

effective targeted therapy has not yet been found for GC is its high heterogeneity not only between patients but also within tumors [4–6]. Intratumorally heterogeneity refers to both morphological aspects and immunoreactivity of tumor cells to antibodies detecting specific biomarkers, such as human epidermal growth factor receptor 2 (HER2) [7].

*HER*2 is a protooncogene located on the long arm of chromosome 17 (17q21), that encodes the transmembrane tyrosine kinase c-erbB-2 oncoprotein, with roles in cellular growth and differentiation. In patients with metastatic GC, HER2 overexpression is used as an indicator of response to anti-HER2 drugs, such as trastuzumab [8–10]. The reasons for the low number of HER2-positive cases and the lack of response to trastuzumab in some positive cases are still unknown. Many commercial antibodies are used for HER2 immunohistochemistry (IHC), and no guideline indicating the number of slides or tumor cells that should be quantified by IHC has yet been implemented.

In this study, we performed an IHC examination of consecutive cases of GC using two anti-HER2 monoclonal antibodies. In cases with inconclusive results, multiple sections were examined, and *HER*2 gene amplification was assessed.

2. Materials and Methods

2.1. Case Selection. Ninety-three consecutive GC cases diagnosed between 2017 and 2020 in the Department of Pathology of the Clinical County Emergency Hospital, Targu Mures, Romania, were included in the present study. Criteria of inclusion are as follows: patients who received a curative resection, without preoperative adjuvant therapy, with a diagnosis of gastric adenocarcinoma (G1-G3) and a postoperative survival rate of ≥3 months. Poorly cohesive carcinomas, other histological subtypes of carcinomas, nonepithelial or metastatic tumors, and cases from patients receiving palliative surgery were not included. Processing of the cases was approved by the Ethical Committee of the Clinical County Emergency Hospital, Targu Mures, Romania. Written informed consent for publication of clinicopathological data was obtained from patients, who were prospectively included. The follow-up period was between 8 and 42 months.

For all cases, the available slides with tumor cells were reanalyzed. We aimed to establish the staging according to the most recent edition of the American Joint Committee on Cancer tumor staging manual [11]. Tumors were also staged according to the Dukes-MAC-like staging system, proposed in 2017 [12].

2.2. Immunohistochemistry Analysis and Interpretation. In all cases, conventional slides were used for IHC assessment. After reviewing of the hematoxylin and eosin-stained sections, two experienced pathologists chose one representative sample to be used for further IHC processing. For all 93 cases, we performed immunostaining for HER2 using two monoclonal antibodies from two different manufacturers: Dako (DakoCytomation, Glostrup, Denmark) and Leica (Leica Biosystems, Germany). We chose the polyclonal antibody c-ErbB-2 (HER2) from Dako and CB11 clone from Leica. High pH retrieval was performed for the two antibodies. The concentrated antibody from DAKO was diluted (1 : 800), but the Leica antibody was ready to use (RTU). Immunostaining was performed automatically (for both antibodies) using the Bond Max fully automated IHC stainer (Leica).

After developing with diaminobenzidine (DAB) and counterstaining with hematoxylin, the membrane expression of HER2 was independently evaluated by two experienced pathologists based on the HercepTest™ guideline and Ruschoff's criteria [13]: score 0 (negative), tumor cells showed no reactivity or showed reactivity in a site other than the membrane; score 1 (negative), barely visible complete, basolateral, or lateral membranous reaction, visible only at 40x magnification, in ≥10% of cells; score 2 (equivocal), weak to moderate complete, basolateral, or lateral membranous reaction visible at 10–20x magnification, in ≥10% of tumor cells; score 3 (positive), strong complete, basolateral, or lateral membranous staining, in ≥10% of tumor cells. In cases in which the results differed between the two pathologists, the case was reevaluated by both pathologists and by the senior pathologist on the team. When necessary, immunostaining was performed on supplementary slides, for elucidation. For cases showing heterogeneous immunostaining (e.g., small areas with 3+ positivity, below 5–10%, surrounded by areas with 2+ positivity), the percentage of each grade was determined, and *HER*2 gene amplification was assessed by fluorescence in situ hybridization (FISH).

2.3. FISH Analysis and Interpretation. To evaluate the grade of *HER*2 gene amplification and establish an in-house protocol, all HER2-positive tumors, independent of the IHC grade (1+, 2+, and 3+), were further assessed by FISH. FISH analysis was also performed in samples that showed positivity with only one of the two antibodies.

The FISH technique was automatically performed using the Bond Max fully automated IHC and FISH stainer (Leica). It was performed using the PathVysion HER2 DNA Probe Kit according to the manufacturer's instructions. For interpretation, we used the LSI HER2/neu spectrum orange/chromosome 17 centromere probe (CEP17)/spectrum green on a Leica CytoVision system based on a Leica DM4000 fluorescence microscope. The analysis considered 30–50 cells from the hotspot, which were chosen at low magnification and then counted at x1000 magnification. Cases with a HER2/CEP17 ratio under 1.8 were considered negative and those with a ratio ≥2.2 were classified as positive. In cases with a HER2/CEP17 ratio between 1.81 and 2.19 and in negative cases, the count was performed again, first by the same pathologist and then by a pathologist experienced in FISH interpretation, in collaboration with a molecular geneticist, with further correlation of results. In addition, in cases that were either negative or equivocal, 50–100 cells were examined for the second interpretation. When necessary, FISH analysis was performed on supplementary slides, for elucidation.

Table 1: Clinicopathological parameters of the included cases.

Parameter		Values ($n = 93$)
Median age (years)		67 ± 11.45 (range 4783) (%)
Gender	Males	66 (70.96)
	Females	27 (29.04)
Histologic subtype and grade	Well-differentiated adenocarcinoma (G1)	4 (4.30)
	Moderately differentiated adenocarcinoma (G2)	39 (41.93)
	Poorly differentiated adenocarcinoma (G3)	50 (53.76)
Depth of invasion (pT stage)	pT1	8 (8.60)
	pT2	6 (6.45)
	pT3	24 (25.80)
	pT4	55 (59.13)
Lymph node status (pN stage)	pN0	17 (18.27)
	pN1-3	76 (81.73)
Distant metastases (pM stage)	pM0	76 (81.73)
	pM1	17 (18.27)
Dukes-MAC-like Stage	*A1*	7 (7.52)
	A2	1 (1.07)
	B1	6 (6.45)
	B2	—
	C1	4 (4.30)
	C2	20 (21.50)
	D	55 (59.13)

Table 2: Correlation between immunohistochemical results regarding c-erbB-2 oncoprotein (HER2) expression, using two commercial antibodies and gene status.

Immunostains		Total cases (number/%)	FISH assessment-*HER2* gene status (number/%)	
			Amplified cases	Nonamplified cases
HER2 3+	Dako polyclonal	7/33.33	7/100%	0
	Leica CB11 clone	6/28.57	6/100	0
HER2 2+	Dako polyclonal	7/33.33	4/57.14	3/42.86
	Leica CB11 clone	7/33.33	5/71.42	2/28.58
HER2 1+	Dako polyclonal	7/33.33	0	7/100
	Leica CB11 clone	8/38.09	0	8/100

2.4. Statistical Analysis. The results were further analyzed using GraphPad Prism 8 (software-free version). The correlation between the overall survival rate, the clinicopathological parameters, and the grade of IHC staining for HER2 was performed using Fisher's exact test and the chi-square test. For all analyses, p values less than 0.05 were considered statistically significant (95% confidence interval).

3. Results

3.1. Clinicopathological Parameters. The 93 patients included in the present study were diagnosed with GC between the ages of 47 and 83, and the male-to-female ratio was 2.44. Most of the cases were G2 (moderately differentiated) or G3 (poorly differentiated) adenocarcinomas. Half of the cases ($n = 55$; 59.13%) were diagnosed in the advanced stage, pT4N0-3 (Dukes-MAC-like stage D). The other cases were staged as follows: 21.50% ($n = 20$) as C2 (T3N1-3), 4.30 ($n = 4$) as C1 (T3N0), none as B2 (T2N1-3), 6.45% ($n = 6$) as B1 (T2N0), 1.07% ($n = 1$) as A2, and 7.52% ($n = 7$) as A1 (Table 1).

3.2. Immunohistochemical Assessment of HER2. Of the 93 tumors analyzed, 22.58% ($n = 21$) demonstrated focal positivity of at least 1+, independent of the antibody used. Only 7.53% of cases ($n = 7$) was assessed as 3+ (positive) using the Dako antibody, and 6.45% ($n = 6$) was assessed as 3+ using the Leica antibody. In 7.53% of cases ($n = 7$), the IHC assessment showed 2+ positivity (equivocal) using both the Dako and the Leica antibodies. The 2+ category contained the same number of tumors for both clones due to the underscoring tendency of the Leica compared to the Dako antibody: one 3+ case according to Dako assessment was underscored as 2+ using the Leica antibody, and one 2+ case according to Dako was underscored as 1+ using the Leica antibody. In the category of 1+ (negative), we identified 7.53% of cases ($n = 7$) using the Dako and 8.60% ($n = 8$) using the Leica antibodies (Table 2).

3.3. Fluorescence In Situ Hybridization Assessment of HER2. FISH analysis demonstrated *HER2* gene amplification in all cases assessed as 3+ on IHC, while all cases reported as 1+

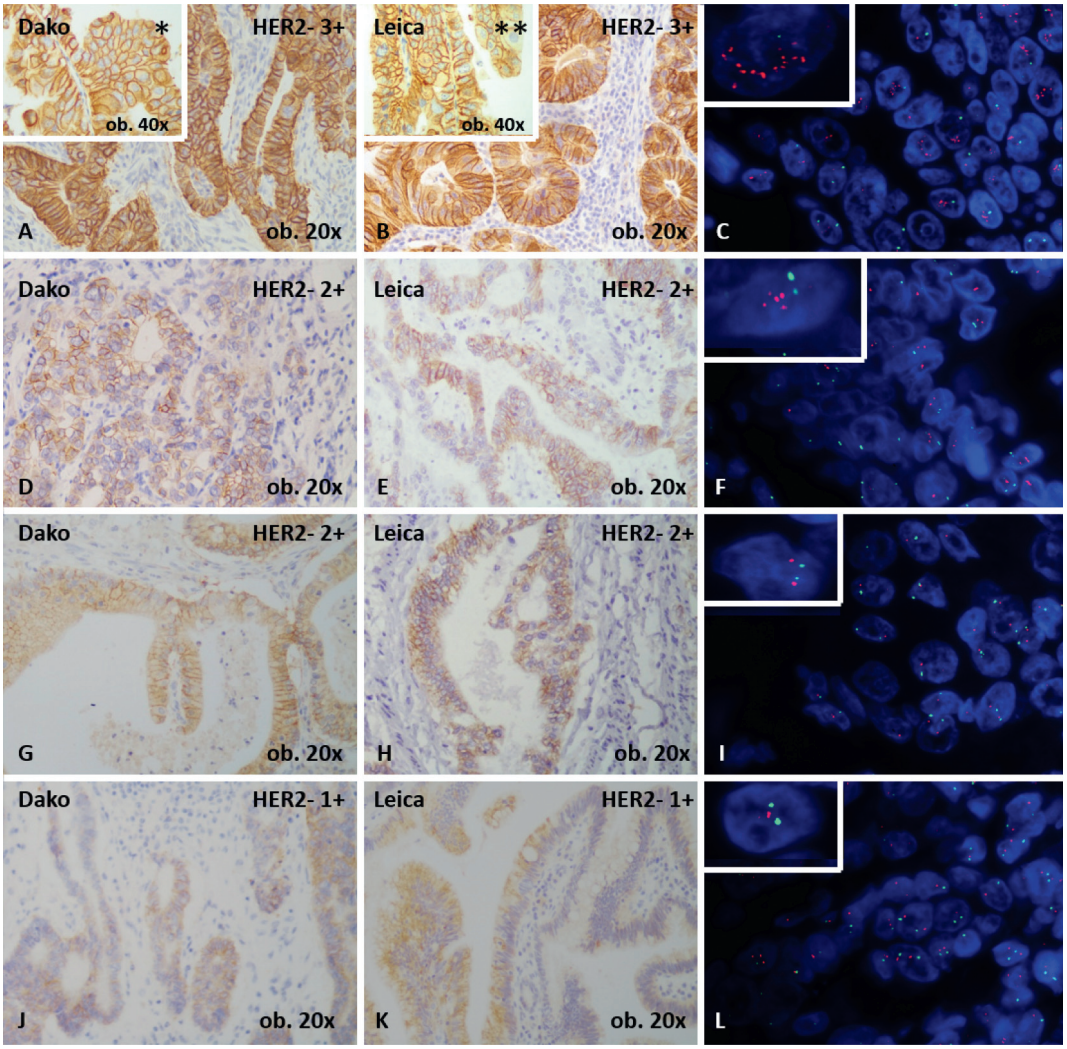

Figure 1: Immunoexpression of c-erbB-2 oncoprotein (HER2), revealed with two commercial antibodies and the corresponding FISH examination. In cases with HER2 3+ positivity (A and B), with strong, evident, complete membranous staining, and well visible on both 20x and 40x magnification (∗ and ∗∗), the *HER*2 gene amplification is clearly proved by FISH analysis (C). In some cases with HER2 2+ positivity (D and E), with moderate complete, membranous reaction visible at 20x magnification, *HER*2 amplification is present (F), whereas other HER2 2+ cases (G and H) do not show amplification (I), same as the cases assessed as 1+ (J-L).

(negative) on IHC were confirmed to lack *HER*2 amplification.

The case reported as 3+ on IHC using the Dako antibody and 2+ using the Leica antibody also showed *HER*2 gene amplification. Of the 7 cases with equivocal (2+) results using the Dako clone, 4 showed *HER*2 gene amplification (Figure 1).

While the IHC assessment confirmed HER2 positivity (3+) in 7.53% ($n = 7$) and 6.45% ($n = 6$) of cases using the Dako and Leica antibodies, respectively, and 2+ positivity (equivocal) in another 7.53% of cases ($n = 7$), *HER*2 gene amplification was demonstrated in 11.82% of cases ($n = 11$). The results are summarized in Table 2.

3.4. Intratumorally Heterogeneity. To assess tumor heterogeneity, for five of the cases, we evaluated HER2 expression by IHC and *HER*2 gene status by FISH on all available slides with viable tumor tissue without extensive necrosis or hemorrhage.

The first two cases were G2 adenocarcinomas with no known distant metastases. Here, 3+ HER2 positivity was found in over 50% of tumor cells on all four slides examined for each case with both of the antibodies. *HER*2 gene amplification was confirmed by FISH analysis.

The third case was a G2 adenocarcinoma with hepatic metastases, from which 4 sections from the primary tumor and one from hepatic metastatic tissue were processed. On IHC assessment of this particular case, the first of four tumor sections from the primary tumor demonstrated 3+ HER2 expression on a single focus, representing less than 5% of the tumor cells, using the antibody from Dako, with the same spot expressing HER2 at a grade of 2+ using Leica assessment. Of the remaining three tumor sections, one demonstrated 2+ HER2 expression using the Dako clone, while the corresponding analysis with the Leica antibody showed

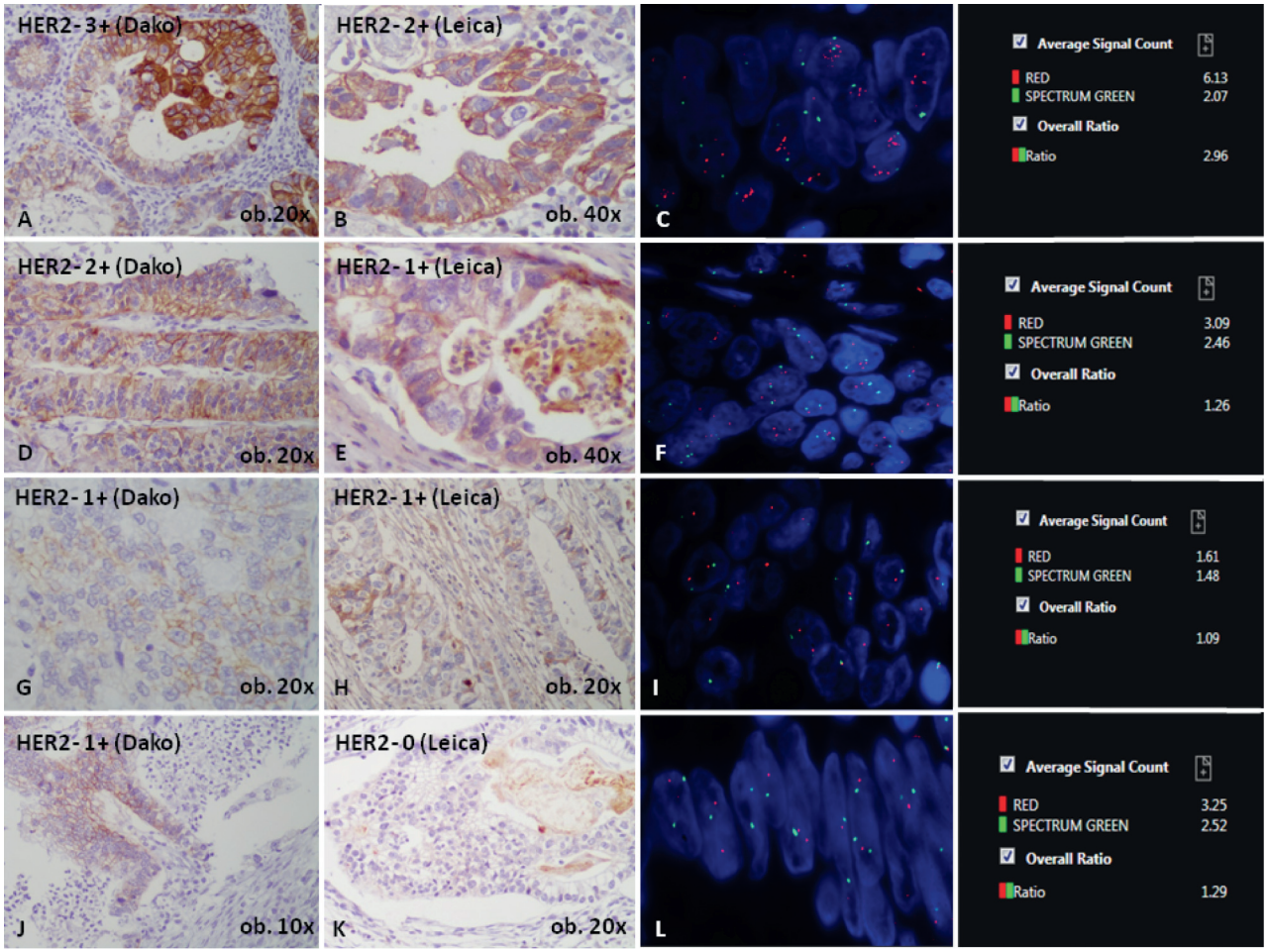

Figure 2: Multiple sections from a representative metastatic gastric adenocarcinoma, showing intratumor/intraglandular heterogeneity for HER2 expression and its corresponding FISH expression. One of the slides from primary tumor shows a 3+ focus with Dako (A) which was equivocal (2+) with Leica stain (B) and confirmed as amplified (C) with a HER2/CEP17 ratio of 2.96. In a second slide (D and E), no gene amplification is seen (F), same as for the third section from primary tumor (G–I). The hepatic metastatic tissue also shows no HER2 positivity (J, K) and no gene amplification (L), independently from the used antibody.

only 1+ expression. The other two sections from the primary tumor were graded as 1+ using both the Dako and Leica antibodies, and the one section derived from metastatic tumor tissue was negative. FISH analysis demonstrated *HER2* amplification only on the first slide that demonstrated positive/equivocal HER2 expression by IHC, with no amplification observed for the remaining slides, including the slide with metastatic tumor tissue (Figure 2).

The fourth case was a G2 adenocarcinoma with no known distant metastases. HER2 assessment by IHC exhibited obviously heterogeneity, with one section showing a focus of 3+ expression, which represent below 5% of tumor cells, proved amplified on FISH analysis. The rest of two assessed sections demonstrated equivocal expression on IHC (2+), and they were certified as nonamplified on FISH assessment (Figure 3).

Last, but not least, the fifth case was a G2 adenocarcinoma with multiple regional lymph node metastases (pN3), but no known distant metastases. IHC assessment demonstrated a heterogeneous pattern, with one slide with foci of 3+ expression, below 5%, which were confirmed as *HER2*-amplified. The second slide showed that multiple areas of 2+ positivity (over 30%) were proved as nonamplified on FISH analysis (Figure 4).

3.5. Correlation of HER2 Expression with Clinicopathological Parameters. Examination of the demographic parameters and tumor-related parameters (such as localization, depth of tumor infiltration, lymph node status, lymphovascular invasion, or presence of distant metastases) did not exhibit correlation with the rate of *HER2* amplification. Most of the amplified cases (10/11) were differentiated adenocarcinomas (G1/2), with only one of the 50 G3 adenocarcinomas exhibiting amplified *HER2* (Table 3). The overall survival rate was also not correlated with the expression of HER2 by

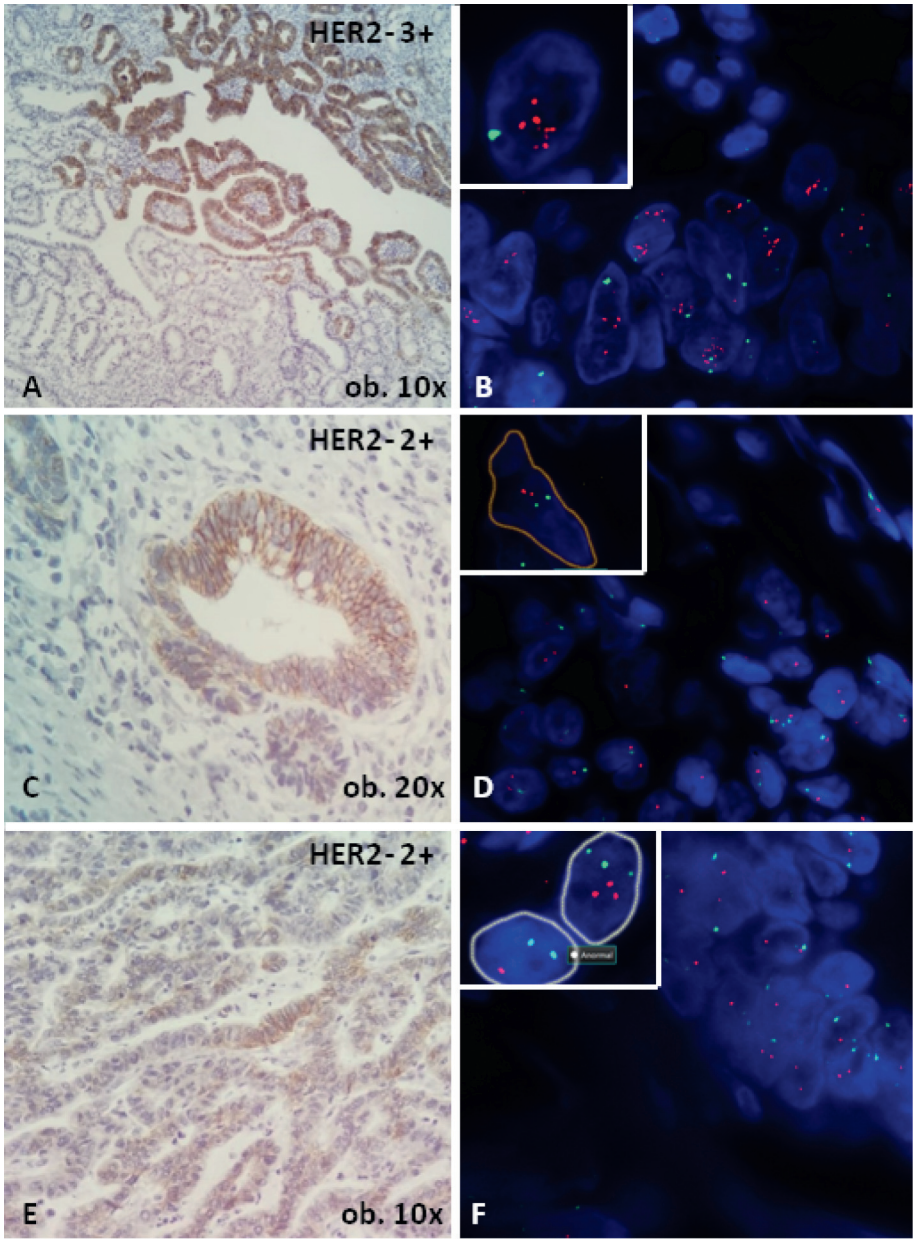

Figure 3: Intratumor heterogeneity, revealed by both immunohistochemical and FISH assessment. In one slide, tumor cells exhibit one focus of 3+ expression (A), confirmed as *HER*2-amplified, with a HER2/CEP17 ratio of 2.41 (B). In other two sections (C–F), extensive areas of 2+ expression can be seen (C and E), with no *HER*2 gene amplification (D and F).

IHC. In contrast, FISH-verified amplification of the *HER*2 gene was an independent indicator of worse survival (Figure 5).

4. Discussion

Despite improvements in the diagnosis and treatment of patients with GC, the 5-year survival rate is still poor, only 30%–35% [9, 10]. With many GCs diagnosed every year, the need for standardized prognostic and predictive markers is emphasized in many studies published on this subject; nonetheless, much remains unknown [14, 15]. Amongst the markers studied in GC, HER2 seems to have the greatest importance not only as a prognostic marker but also because it has therapeutic importance due to the development and use of anti-HER2 therapy [16, 17]. Trastuzumab is the only anti-HER2 target therapy approved in GC [17], but the selection of patients that could benefit from this treatment is not as straightforward as it is in breast cancer.

The main reason of the difficulty in assessing HER2 in GCs is the intratumorally heterogeneity of its expression, which occurs in 69%–75% of cases [4–7, 9, 18, 19]. In this paper, we emphasize and confirm this heterogeneity, which is present in the same tumor, between primary tumor and metastatic tissue and even in the same tumor gland. It is difficult to manage this aspect, as usually only one tumor

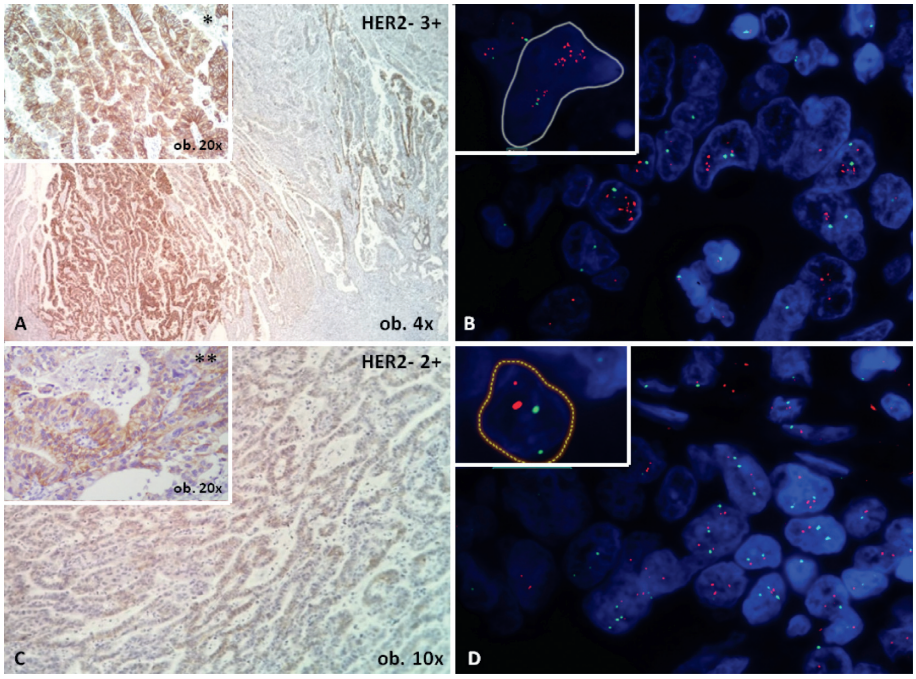

Figure 4: Two sections from a gastric adenocarcinoma, with HER2 heterogeneity. On the sample with a single focus of 3+ expression on immunohistochemistry (a), the *HER2* gene is amplified, with a HER2/CEP17 ratio of 2.21 (b). On the second sample, with larger areas of 2+ positivity (c), no gene amplification is proved on FISH (d).

Table 3: Correlation between clinicopathological factors and HER2 gene status.

Parameter		HER2 gene status		p value
		Number of amplified cases	Number of nonamplified cases	
Median age (years)		73 ± 11.13	67 ± 15.32	0.57
Gender	Male	7	59	0.72
	Female	4	23	
Localization	Proximal stomach	5	38	1.00
	Distal stomach	6	44	
Histological grade	G1	3	1	**<0.001**
	G2	7	32	
	G3	1	49	
pT stage	pT1-2	1	13	0.64
	pT3	4	20	
	pT4	6	49	
pN stage	pN0	3	18	0.71
	pN1-3	8	64	
pM stage	pM0	9	67	1.00
	pM1	2	15	
Dukes-MAC-like stage	A1 (T1N0) + B1 (T2N0) + C1 (T3N0)	3	14	0.58
	A2 (T1N1-3) + C2 (T3N1-3)	3	18	
	D (T4N0-3)	5	50	
Lymphovascular invasion	Present	9	51	0.32
	Absent	2	31	

section is used for diagnosis, and the cutoff is 10%. This paper highlights the importance of testing HER2 expression in at least 3-4 slides, especially for differentiated carcinomas that do not show 3+ positivity on the first slide. Moreover, if tumor cells express 3+ or 2+ HER2 at any extent, even under 5%, on the first slide, it is worth analyzing additional tumor slides for larger foci of HER2 positivity. No cases should be considered HER2-negative without IHC examination of at least 3-4 slides. Biopsy specimens should not be interpreted as negative in any cases, and at least 5 different fragments should be analyzed [9, 20].

In this study, 15% of cases were HER2-positive (2+ and 3+), in line with literature data that showed relatively wide ranges of HER2 protein expression (between 5% and 42%) [21]. The amplification rate was 11.83% in this cohort, the reported rate being from 4% to 13% [21]. These relatively

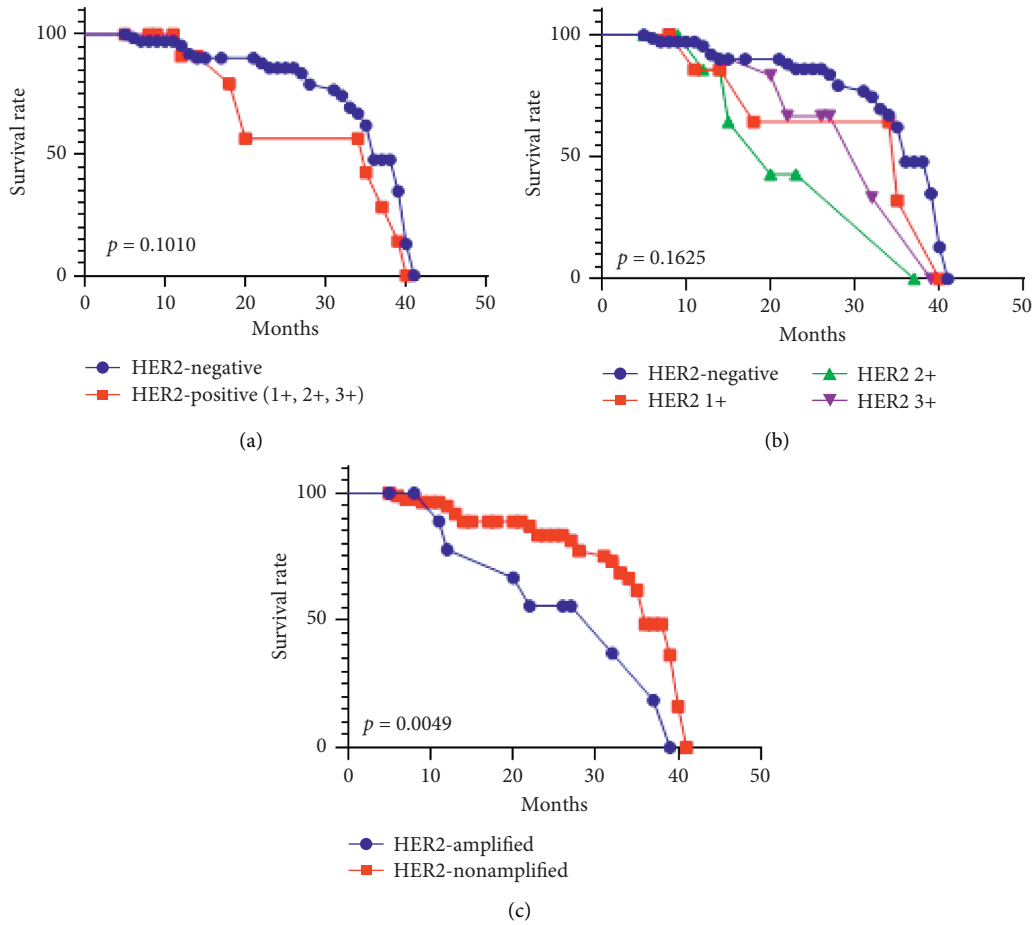

Figure 5: Kaplan–Meier survival curves demonstrate no independent prognostic role of the HER2 immunoexpression (a) and (b), but cases confirmed by FISH with *HER2* gene amplification have a considerably lower survival rate, compared to the nonamplified cases (c).

wide ranges are due to the different protocols using, beginning with discrepancies in fixation, use of different antibodies and, maybe most important, use of nonstandardized scoring protocols, especially on FISH analysis. Like our data, it was previously emphasized that the percentage of HER2 overexpression is consistently higher in tumors with well- or moderately differentiated morphology compared to poorly differentiated carcinomas [22–26]. As poorly cohesive carcinomas rarely express HER2, we did not include such cases in this study. However, their inclusion might significantly decrease the reported rate of HER2 positivity.

With the well-known possibility of false-positive/false-negative results on IHC assessment, we simultaneously evaluated the cases under the study using two different commercial HER2 antibodies. The positivity rate was similar, with only one of the 3+ cases using the Dako antibody showing equivocal positivity (2+) with the Leica antibody. However, this case showed *HER2* amplification. Moreover, all 1+ cases identified using the Dako antibody were assessed as negative using the Leica antibody. As the correlation between the results obtained using the antibodies from the two manufacturers is over 90% [21, 26–29], both clones can be safely used in daily diagnosis, but an in-house standardization is mandatory.

An interesting aspect arose regarding the impact of IHC heterogeneity on FISH analysis, which should be performed by an experienced pathologist. In cases that showed at least one focus of 3+ overexpression on IHC with either of the clones used, with larger areas of 2+ IHC positivity nearby, FISH analysis demonstrated a relatively homogeneous number of amplified *HER2* copies in both the 3+ and the 2+ areas. In comparison, in FISH-confirmed positive cases with only 2+ positivity on IHC assessment, FISH analysis demonstrated intercellular heterogeneity in the number of amplified *HER2* copies. From our perspective, this could have two possible explanations: either the IHC assessment was performed with too much vigilance, with 3+ areas being misinterpreted as 2+ areas due to technical difficulties, or the subcellular mechanisms responsible for *HER2* amplification are slightly different in cases with 3+ IHC results compared to those with only 2+ expression. These aspects should raise the possibility of other molecular signaling pathways acting as positive modulators of classic *HER2* gene expression. It was even suggested that a better response to trastuzumab could be obtained in patients whose tumors exhibited 3+ HER2 (quantified using IHC methods and confirmed with *HER2* amplification) compared with equivocal (2+) amplified cases [30].

One molecular mechanism involved in GC cell heterogeneity could be polysomy, whole chromosomal multiplication [31–34], but this subcellular alteration cannot be responsible for molecular modification in all cases, as FISH analysis does not always report equal amplification of the centromeric region of chromosome 17 (CEP17) and the *HER*2 gene.

5. Conclusions

In GC, the *HER*2 gene is more frequently amplified in differentiated adenocarcinomas, but the rate of intratumorally heterogeneity is extremely high. To prove HER2 positivity, at least 3-4 slides should be examined, and FISH analysis should be performed in any case that shows clusters of HER2 3+ positivity, even they represent less than 5% of tumor cells. A gastrointestinal pathologist with experience in FISH analysis should perform interpretation of immunostaining. Both Dako and Leica clones can be successfully used in daily practice. When possible, for FISH analysis, the samples with at least one small focus of 3+ should be chosen, rather than those with extensive 2+ positivity. *HER*2 amplification is an independent negative prognostic indicator in GC.

Data Availability

The clinicopathological data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

SCB drafted the paper, performed the FISH examination, contributed to the interpretation of immunohistochemical data, and established the study design; JI supervised the immunohistochemical interpretation and contributed to the study design; SVSRI provided the financial support of the study and participated at samples collections; KZ participated at FISH assessment; MC and BTJ participated at surgical interventions and collecting of clinical data; FZZ participated at collection of clinical data and tissue samples and performed the clinical follow-up; GS supervised the experiment, interpretation of the immunohistochemical and FISH correlations, and gave the final consent for publication.

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Research Article

Significance of Detection of the HER2 Gene and PD-1/PD-L1 in Gastric Cancer

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Objective. To explore the relationship between the HER2 gene and PD-1/PD-L1 in gastric cancer and its significance. *Methods*. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) were used to detect HER2 protein expression, HER2 gene amplification, and PD-1/PD-L1 expression in 78 cases of gastric cancer. *Results*. The expression rate of HER2 protein was 43.6% (34/78), of which 19.4% (14/78) were HER2 3+, 14.1% (11/78) were HER2 2+, and 11.5% (9/78) were HER2 1+. The results showed that 19.2% (15/78) of samples had HER2 gene amplification, 3.8% (3/78) of samples had a HER2/CEP17 ratio <2.0, and 19.2% (15/78) of samples had HER2 gene amplificationf and HER2 copy/cell ≥6.0, as detected by FISH. The positive rate of PD-L1 was 38.5% (30/78) in gastric cancer cells and 50.0% (39/78) in interstitial lymphocytes. The expression of the HER2 gene, PD-L1, and PD-1 in gastric cancer was correlated with the stage and lymph node metastasis of gastric cancer ($P < 0.05$). *Conclusions*. The combined detection of the HER2 gene and PD-1/PD-L1 in gastric cancer provides an important reference index for the prognosis of gastric cancer and the benefit of targeted antitumor drugs.

1. Introduction

Although the incidence of gastric cancer (GC) has fallen significantly in the United States and across the world over the past few decades, it still remains the fifth most common malignancy and the third leading cause of cancer death [1, 2]. There are approximately 900,000 new cases of GC per year and 723,000 registered deaths worldwide. Most patients with gastric cancer have reached the middle and late stages at the time of diagnosis. Even if perioperative chemotherapy or adjuvant chemotherapy is used, the survival rate of these patients is still low [3, 4]. Therefore, the search for new therapeutic approaches, such as molecular targeted therapy, has become a hot topic in gastric cancer research. The human epidermal growth factor receptor type 2 (HER2, also referred to as HER2/neu) gene is located on chromosome 17q2l and encodes a transmembrane tyrosine kinase receptor with a relative molecular weight of 185000 Da. At present, the probes for the detection of HER2 gene status are mostly double probes containing the HER2 gene and the centromeric region of chromosome 17 (CEP17), where the gene is located. It was found that HER2 protein was expressed in breast cancer, ovarian cancer, gastric cancer, and other cancers [5–7]. The inducing factors of gastric cancer are mainly related to the activation of carcinogenic factors and the deactivation of tumor suppressor factors. Meanwhile, the tumor cells' immune escape is accompanied by the apoptosis reaction, in which the coregulatory system is involved.

At present, emerging immunotherapy against the programmed death-1 (PD-1)/PD-1 ligand (PD-L) pathway is attracting much attention. The immune escape mechanism of the PD-1/PD-L1 pathway is that the combination of PD-L1 on the surface of tumor cells and PD-1 on the surface of

T lymphocytes inhibits the activity of T cells so that tumor cells escape from the attack of T lymphocytes [8–10]. PD-1/PD-L1 immunotherapy aims to treat many kinds of tumors by blocking the PD-1/PD-L1 signaling pathway and restoring the human autoimmune system. Monoclonal antibodies against blocking the PD-1/PD-L1 pathway have entered the clinical stage, and it has been indicated that the therapeutic effect of gastric cancer is significant [11, 12]. In this paper, immunohistochemistry and fluorescence in situ hybridization (FISH) were used to detect HER2 gene amplification and expression of PD-L1 and PD-1 in gastric cancer tissue and to explore the target and correlation of antitumor drug therapy for gastric cancer to provide help for the prognosis and targeted antitumor drug therapy of gastric cancer.

2. Materials and Methods

2.1. Gastric Cancer Tissue Specimen Selection. From November 2015 to February 2019, 78 cases of radical gastrectomy specimens were collected from the Department of Pathology of 989 Hospital, 990 Hospital, and Shenzhen Hospital of Southern Medical University. There were 12 cases of papillary adenocarcinoma, 23 cases of tubular adenocarcinoma, 11 cases of mucinous adenocarcinoma, 7 cases of poorly cohesive carcinoma, and 25 cases of mixed adenocarcinoma. The average age was 57.4 years.

All surgical specimens were processed by pathologists. Specimens were fixed within 30 minutes after surgery and fixed with 10% neutral buffered formalin (NBF) for 8–48 hours. The volume ratio of the fixative to tissue was 10 : 1. Four to six specimens were cut from the central area, and the surrounding area of the tumor (the proximal and distal margin of the tumor, the tumor, and the adjacent gastric mucosa were not included), one at the deepest infiltrating point and one at the closest serosa layer, and all lymph nodes and cancer nodes were cut in different areas. Hematoxylin-eosin (HE) staining, immunohistochemistry, and gene detection were performed.

2.2. Immunohistochemistry (IHC). Monoclonal antibodies of PD-L1 (ab230369), PD1 (ab230369), and HER2 (3b5, ab16901) were bought from Abcam company. Using the Envision method, the operation steps were performed strictly in accordance with the product manual, PBS was used as the negative control instead of the primary antibody, and placental villi and lymph nodes were used as the positive controls for PD-L1 and PD-1, respectively. The ready-to-use kit and the primary antibody were purchased from Fuzhou MaiXin Company.

The percentage of tumor cells exhibiting cell-surface staining for PD-L1 was scored by two independent pathologists who were unaware of outcomes. First, the tumor cell area was determined under 4× low-power microscope; then, under 10–40× microscope, the nuclear staining site of PD-L1 and the percentage of PD-L1 positive tumor cells were identified. Tumor cells with ≥5% positive localization of PD-L1 in the cell membrane and/or cytoplasm were considered positive, and tumor stromal cells with ≥5% positive localization of PD-1 in the cell membrane and/or cytoplasm were considered positive [9, 13]. HER2 positive results were considered as follows: The positive localization was on the cell membrane. There was no staining on the cell membrane, and the result was 0; if tumor cells were faint in membrane staining, the result was 1+; if the basement membrane, side membrane, or integrity membrane of tumor cells had weak to moderate staining, the result was 2+; if there was strong positive staining on basement membrane, side membrane, or integrity membrane of tumor cells, the result was 3+. The positive staining area was evaluated as follows: if the membrane of tumor cells had no staining, the sample was considered negative; if ≥80% of cells were stained, the sample was considered extensive type; if 21%~79% of cells were stained, the sample was considered partial type; and if ≤20% of cells were stained, the sample was considered focal type. Two pathologists were employed to read the film.

2.3. Fluorescence In Situ Hybridization. Paraffin Pretreatment Kit II (mainly including pretreatment solution and protease solution) and the Path Vysion TM HER2 Probe Kit were purchased from Vysis. The pretreatment procedure and FISH procedure of paraffin-embedded gastric cancer tissue sections were carried out in accordance with the literature [14, 15] and the instructions on the kit.

The gastric cancers with IHC grade HER 2+ results underwent FISH analysis. First, the positive area of gastric adenocarcinoma cells was confirmed on IHC, and then, the same field of view from IHC stain was assessed by FISH under a 10× objective lens, and the whole section was observed under 40× objective lens. More than 75% of the cancer cell nuclei had a hybridization signal, which was regarded as a satisfactory result; the lens was replaced with a 100 × objective lens to count at least 30 cancer cells with complete boundaries, isolated and nonoverlapping. The evaluation criteria for HER2 gene amplification were as follows: HER2/CEP17 ratio ≥2.0, and average HER2 copies/cells ≥4.0: FISH positive; HER2/CEP17 ratio <2.0, average HER2 copies/cells <4.0: FISH negative. If the ratio of HER2/CEP17 was less than 2.0 and the average copy number/cell of HER2 was more than 4.0 and less than 6.0, the signals in at least 20 nuclei were counted again. If the results changed, the two results were comprehensively judged and analyzed. In this group, if the ratio of HER2/CEP17 was less than 2.0 and the average copy number of HER2/cell was more than 6.0, the sample was judged as FISH positive (referred to as high multibody cell for short). If many HER2 signals were connected into clusters, they were directly judged as FISH positive. This group was divided into cluster amplification, large granular amplification, and dot amplification.

2.4. Statistical Analysis. SPSS 22.0 software was used for statistical analysis of all data. The $\chi 2$ test was used to analyze the relationship between the expression of HER2, PD-1, and PD-L1 and the clinicopathological characteristics of gastric cancer. The correlation between the two methods was

analyzed by Pearson correlation analysis, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Relationship between HER2 Gene Amplification and the Protein Expression Rate. HER2 protein was positively expressed in the cell membrane. In our search, the positive expression rate was 43.6% (34/78), in which the expression of HER2 protein 3+ accounted for 19.4% (14/78), including 8 cases of 3+ extensive staining (Figure 1(a)), 4 cases of partial staining (Figure 1(b)), and 2 cases of focal staining (Figure 1(c)); the expression of HER2 protein 2+ accounted for 14.1% (11/78), including 8 cases of 2+ extensive staining, 2 cases of partial staining, and 1 case of focal staining; the expression of HER2 protein 1+ accounted for 11.5% (9/78), including 7 cases of 1+ extensive type, 1 case of partial type, and 1 case of focal type. Forty-four cases were negative, accounting for 56.4% (44/78) of all cases. The amplification rate of the HER2 gene was 19.2% (15/78) by FISH technology, including 3 cases of HER2 gene cluster amplification (Figure 2(a)), 5 cases of large granule amplification (Figure 2(b)), 4 cases of dot amplification (Figure 2(c)), and 3 cases of high polymorph amplification (Figure 2(d)). The relationship between HER2 gene protein expression and HER2 gene amplification in gastric cancer is shown in Table 1.

3.2. Relationship of PD-1/PD-L1 Expression. The expression of PD-L1-positive cells in gastric cancer was multifocal and flaky (Figure 3); exfoliated cancer cells in the glandular cavity were expressed in different degrees. The total positive rate was 38.5% (30/78); PD-1 positive cells in tumor stroma were scattered or clumped: they were mainly located in the area of lymphocyte aggregation with focal distribution between adenocarcinoma cells and in the area of single lymphocyte between gastric adenocarcinoma cells (Figure 4). The total positive rate was 50.0% (39/78). The difference of PD-1/PD-L1 expression in tumor cells and tumor stroma was statistically significant ($P < 0.05$), and it was correlated with gastric cancer stage and lymph node metastasis ($P < 0.05$).

3.3. HER2 Gene Amplification and PD-1/PD-L1 Expression and Their Relationship with Clinicopathological Parameters. The amplification rate of the HER2 gene and the expression of PD-1/PD-L1 were not related to the sex and age of patients ($P > 0.05$); the amplification rate of the HER2 gene and the expression of PD-1/PD-L1 were related to the depth of invasion and lymph node metastasis of gastric cancer, with significant differences ($P < 0.05$), as shown in Table 2.

4. Discussion

The status of the HER2 gene determines the benefit of targeted treatment for gastric/GEJ adenocarcinoma. Therefore, accurate detection of the HER2 gene is very important for the selection of patients for targeted treatment. At present, detection of the HER2 gene in gastric/GEJ adenocarcinoma has become a routine project in the pathology department, and many countries have formulated HER2 detection guidelines [16, 17]. The detection of HER2 in gastric cancer is different from that in breast cancer, which has a wide range of morphological heterogeneities. The detection results are affected by many factors, as well as the quality control of the laboratory and the interpretation of staining results. Some studies indicated that the expression rate of HER2 protein was significantly different from 6% to 34% [18–21]. In our research, HER2 protein was detected by combining the positive intensity and the positive area, which was divided into extensive staining, partial staining, and focal staining. The total positive expression rate of HER2 protein was 43.6% (34/78), of which HER2 protein 3+ expression accounted for 19.4% (14/78), including 8 cases of 3+ extensive staining, 4 cases of partial staining, and 2 cases of focal staining; HER2 protein 2+ expression accounted for 14.1% (11/78), including 8 cases of 2+ extensive staining, 2 cases of partial staining, and 1 case of focal staining; HER2 protein 1+ expression accounted for 11.5% (9/78), including 7 cases of 1+ extensive staining, 1 case of partial staining, and 1 case of focal staining. Forty-four cases were negative, accounting for 56.4% (44/78) of all cases. The amplification rate of the HER2 gene was 19.2% (15/78), as detected by the FISH technique; amplifications included 3 cases of HER2 gene cluster amplification, 5 cases of large granule amplification, 4 cases of dot amplification, and 3 cases of high polymorph amplification. The results showed that HER2 amplification was correlated with gastric cancer stage and lymph node metastasis ($P < 0.05$).

There are many reasons for human tumors, which are closely related to the body's autoimmune function. Immunostimulatory molecules have become a hot spot in immunology in recent years. PD-1 and its ligand PD-L1 play an important role in tumor progression. PD-1 is a type I transmembrane protein composed of 288 amino acids, and PD-L1 is a type I transmembrane protein composed of 290 amino acids [11]. PD-1 binds to PD-L1 through the IGV domain of the extracellular domain. PD-1 is expressed on the surface of T cells, B cells, natural killer cells, and tumor infiltrating lymphocytes. PD-L1 is widely expressed in tumor cells. PD-L1 induces the binding of PD-1 on the surface of T-lymphocytes to transmit inhibitory signals to T cells, which inactivates T lymphocytes and suppresses the anti-tumor immune response [12]. Therefore, blocking the PD-1/PD-L1 pathway with drugs can enhance the function of T cells and cause tumor cell death, which will open a new window for tumor treatment. The expression of PD-1/PD-L1 in lung cancer, breast cancer, and malignant melanoma is higher than that in gastric cancer. The positive rate of PD-L1 expression in [13] tumor cells was 23.87%. The positive rate of PD-1 expression was 53.76%. PD-L1 was not expressed in normal gastric tissues but was detected in 42% of gastric cancer tissues. The positive rates of PD-L1 and PD-1 expression in gastric cancer cells were 38.5% and 50.0%, respectively. This study found that the expression of PD-1/PD-L1 was not related to the age of patients with gastric cancer but was related to the stage, lymph node metastasis, and prognosis of gastric cancer.

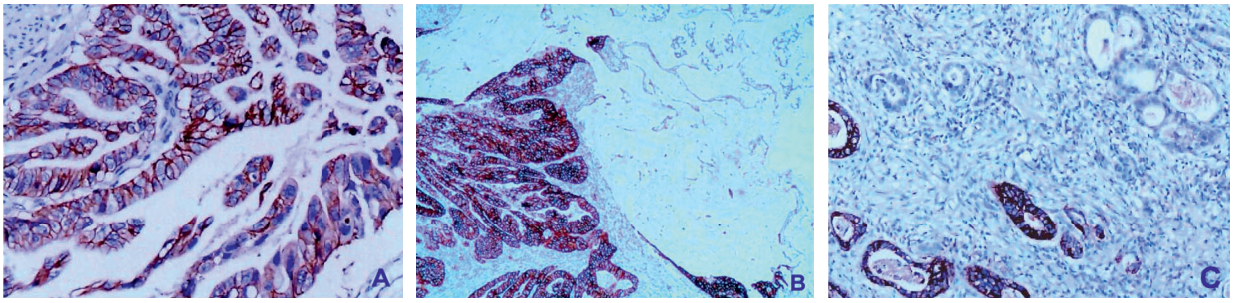

Figure 1: (a) Gastric papillary adenocarcinoma, HER2 positive 3+ extensive staining (X200); (b) Gastric mixed adenocarcinoma, mucinous adenocarcinoma, and papillary adenocarcinoma, HER2 positive 3+ partial staining (X40); (c) gastric tubular papillary adenocarcinoma, HER2 positive 3+ focal staining (X200), Envision method.

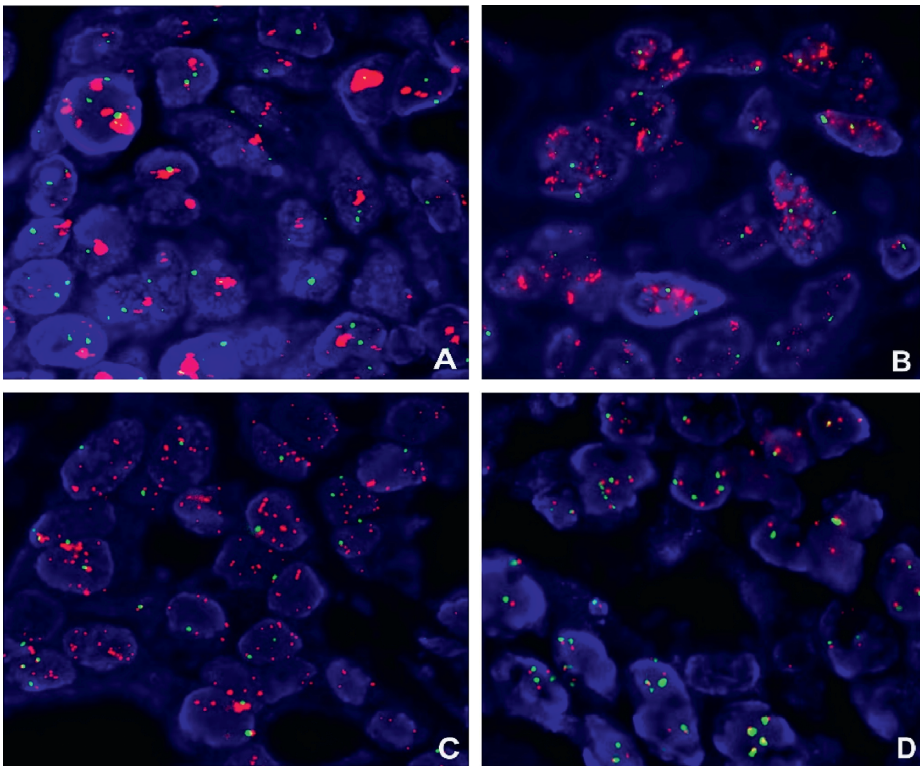

Figure 2: FISH method to detect HER2 gene amplification, red as probe signal, green as chromosome 17: (a) HER2 gene cluster amplification; (b) HER2 gene large particle amplification; (c) HER2 gene dot amplification; (d) HER2 gene high polymorph amplification.

Table 1: Comparison of HER2 protein expression and HER2 gene amplification in 78 cases of gastric cancer.

	HER2 protein expression rate (%)	Amplification rate of HER2 gene (%)
−	44/78 (56.4)	0
+	9/78 (11.5)	0
++	11/78 (14.1)	1/11 (9.1)
+++	14/78 (19.4)	14/14 (100.0)
Total	34/78 (43.6)	15/78 (19.2)

In recent years, it was reported that the status of the HER2 gene in gastric cancer is related to the expression of p53 protein and the number of Ki67-positive cells in cell proliferation [22, 23]. In this study, we found that HER2 gene status was related to gastric cancer stage, lymph node metastasis, and prognosis, which was consistent with the literature. The PD-1/PD-L1 pathway is an important mechanism of immunosuppression in the tumor microenvironment. Drugs are used to block this pathway and enhance immune function. By detecting the expression of PD-1/PD-L1 in gastric cancer, we can evaluate the prognosis of patients and provide some basis for immunotherapy of

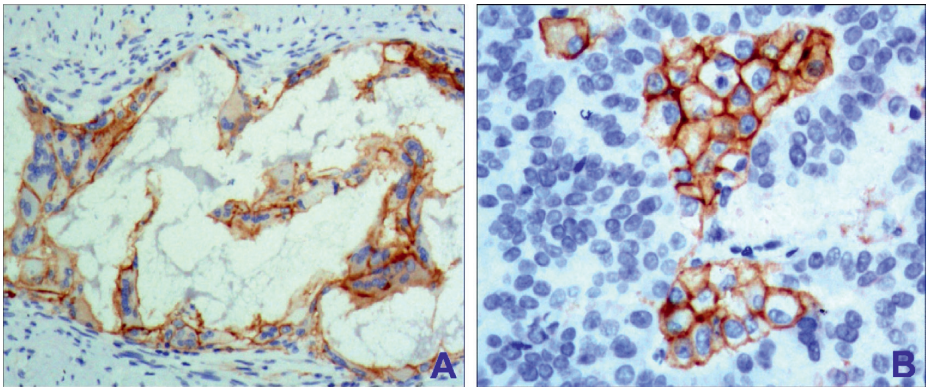

Figure 3: Gastric adenocarcinoma. (a) Gastric mucinous adenocarcinoma shows positive PD-L1 expression (X200). (b) Poorly differentiated gastric adenocarcinoma shows positive PD-L1 expression (X200), Envision method.

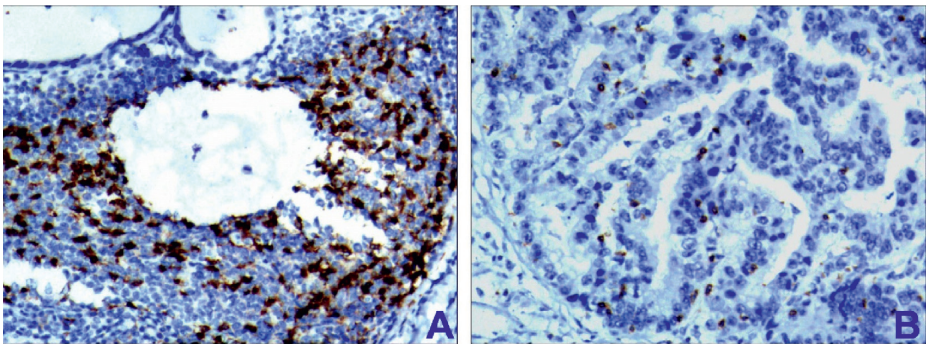

Figure 4: Gastric adenocarcinoma. (a) Interstitial lymphocytes of mucinous adenocarcinoma show positive expression of PD-1 (X200). (b) Interstitial lymphocytes of papillary gastric adenocarcinoma show negative expression of PD-1 (X200), Envision method.

Table 2: Relationship between HER2 gene amplification and PD-1/PD-L1 expression and clinicopathological parameters in 78 cases of gastric cancer.

Type	*n*	HER2 gene Positive, negative	*P* value	Expression of PD-L1 in tumor cells Positive, negative	*P* value	Expression of PD-1 in tumor stromal lymphocytes Positive, negative	*P* value
Sex							
Male	53	11 (26.4), 42 (79.2)	0.619	22 (41.5), 32 (60.4)	0.516	29 (54.7), 24 (45.43)	0.377
Female	25	4 (16.0), 21 (84.0)		8 (32.0), 17 (68.0)		11 (44.0), 14 (56.0)	
Age							
≤60	31	6 (19.4), 25 (80.0)	0.982	9 (29.0), 22 (70.8)	0.164	13 (41.9), 18 (58.1)	0.247
>60	47	9 (19.1), 38 (80.9)		21 (44.7), 26 (55.3)		26 (55.3), 21 (44.7)	
Histological classification							
Papillary adenocarcinoma	12	3 (25.0), 9 (75.0)	0.924	5 (41.6), 7 (58.3)	0.991	6 (50.0), 6 (50.0)	0.977
Tubular adenocarcinoma	23	5 (21.7), 18 (78.3)		9 (39.1), 14 (60.9)		11 (47.8), 12 (52.2)	
Mucinous adenocarcinoma	11	1 (9.1), 10 (90.0)		4 (36.4), 7 (63.6)		5 (45.5), 6 (54.5)	
Low adhesion cancer	7	1 (14.3), 6 (85.7)		2 (28.6), 5 (71.4)		3 (42.9), 4 (57.1)	
Mixed adenocarcinoma	25	5 (20.0), 20 (80.0)		10 (40.0), 15 (60.0)		14 (56.0), 11 (44.0)	
Stage							

Table 2: Continued.

Type	n	HER2 gene Positive, negative	P value	Expression of PD-L1 in tumor cells Positive, negative	P value	Expression of PD-1 in tumor stromal lymphocytes Positive, negative	P value
T1/T2	30	2 (6.7), 28 (93.3)	0.026	8 (26.7), 22 (73.3)	0.129	9 (30.0), 21 (70.0)	0.005
T3/T4	48	10 (20.8), 38 (79.2)		21 (43.8), 27 (56.3)		30 (62.5), 21 (37.5)	
Lymph node metastasis							
With	56	12 (21.4), 44 (78.6)	0.787	26 (43.4), 30 (53.6)	0.021	32 (58.2), 23 (41.8)	0.037
Without	22	3 (13.6), 9 (40.9)		4 (18.2), 18 (81.8)		7 (31.8), 15 (68.2)	

gastric cancer. It was also found that the detection of the HER2 gene and PD-1/PD-L1 in gastric cancer was related to gastric cancer stage and lymph node metastasis ($P < 0.05$).

In conclusion, HER2 protein is heterogeneous in gastric cancer, and the criterion and refinement of the HER2 test is the guarantee of correct medication guidance. This study emphasizes the intensity and scope of HER2 detection. HER2 protein expression can be divided into extensive staining, partial type, and focal staining. HER2 gene expression can be divided into cluster staining, large granule staining, dot staining, and high pleomorphism, which helps clinicians grasp the results of detection strategy, guide drug use, and predict prognosis. The PD-1/PD-L pathway is an important mechanism of immunosuppression in the tumor microenvironment. Drugs are used to block this pathway and enhance immune function. In this study, the HER2 gene and PD-1/PD-L1 were jointly detected in gastric cancer. All three genes were related to gastric cancer stage and lymph node metastasis ($P < 0.05$). This conclusion provides an important reference for the prognosis of gastric cancer and the benefit of targeted antitumor drugs.

Data Availability

All the data generated or analyzed during this study are included within this article.

Disclosure

This work was performed as part of the employment of Tian Yun, Changsong Wang, Nianlong Meng, Xutao Yuan, and Yangkun Wang.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

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Research Article

Genes Involved in the PD-L1 Pathway Might Associate with Radiosensitivity of Patients with Gastric Cancer

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The PD-1/PD-L1 pathway plays an important role in the treatment of cancers as immune checkpoint. However, the association of genes involved in the PD-L1 pathway and radiosensitivity of gastric cancer has not been fully characterized. This study aims to explore the relationship between the expression levels of genes involved in the PD-L1 pathway and radiosensitivity for gastric cancer patients. A total of 367 patients with clinical survival information and radiotherapy information were obtained in The Cancer Genome Atlas (TCGA). Genes involved in the PD-L1 pathway were categorized into high and low expression level groups according to the median value. The Cox proportional hazards model was used to find the association between gene expression level and radiosensitivity. The results show that high expression levels of CD274, EGFR, RAF1, RPS6KB1, PIK3CA, MTOR, CHUK, NFKB1, TRAF6, FOS, NFATC1, and HIF1A were associated with radiosensitivity of gastric cancer. While low expression level of HRAS was also associated with radiosensitivity in gastric cancer. The rates of a new tumor event and disease progression were lower for radiosensitivity patients than other patients. The relationship between the expression level of CD274 and other genes involved in the PD-L1 pathway is significant. GO (Gene Ontology) analysis shows that the biological process of 13 genes was mainly related to innate immune response activating the cell surface receptor signaling pathway. KEGG analysis demonstrated that 13 genes in gastric cancer are mainly related to the PD-L1 expression and PD-1 checkpoint pathway in cancer. The correlation between the expression level of CD274 and other genes involved in the PD-L1 pathway is significant. The present study offered more evidence for using PD-L1 and genes involved in the PD-L1 pathway as potential biomarkers to predict radiosensitive patients with gastric cancer.

1. Introduction

Gastric cancer (GC) is one of the most common and malignant digestive system tumors; it has the second highest incidence and mortality rate of all cancers [1]. According to Chinese cancer statistics, gastric cancer is the second leading cause of cancer death in China and one of the top five malignant tumors [2]. At present, the treatments of gastric cancer mainly include surgical treatment, radiotherapy, chemotherapy, gene therapy, targeted molecular therapy, and comprehensive treatment. Previous studies have reported that radiotherapy is an effective treatment for patients with different stages of gastric cancer [3]. In recent years, radiation therapy in gastric cancer has received increasing attention. However, how to use radiotherapy to improve the patient's quality of life is an urgent problem to be solved.

In the era of precision medicine, searching potential biomarkers and genes to predict radiosensitive patients plays an important role in personalized medicine. One of these examples is the radiosensitivity gene signature, and PD-L1

status of breast cancer patients can be used to select patients [4].

Programmed cell death-ligand encoded by the CD274 gene, also called PD-L1, is the ligand of programmed death-1 (PD-1). PD-L1 is often expressed in T cells, B cells, and other cell types such as DCs, macrophages, mesenchymal stem cells, and a variety of nonhematopoietic cells [5]. The PD-1/PD-L1 pathway activates T cells by delivering costimulatory molecules as the second signal. On the other hand, the PD-1/PD-L1 pathway plays a vital role in maintaining the balance between tolerance and autoimmunity by regulating the degree of activation of T and B cells amongst other immune cell types as critical regulatory immune checkpoints [6]. In solid tumors, the PD-1/PD-L1 inhibitory pathway can be used to suppress the T cell response to promote immune evasion and growth of the tumor by increasing the expression of PD-L1 [7]. PD-L1 expression in the tumor microenvironment has also been studied in multiple solid tumor types including gastric cancer [8], prostate cancer [9], lung cancer [10], and melanoma [11]. In these cancers, PD-L1 overexpression is an indicator of poor prognosis for patient survival.

In this situation, it is essential to understand the regulation mechanism of PD-L1 in cancer. Studies have shown that PD-L1 expression is regulated by transcription factors, signaling pathways, and epigenetic factors [12]. Several signaling pathways include the JAK/STAT pathway, PI3K/Akt signaling pathway, Ras/MEK/ERK pathway, JAK/STAT pathway, HIF-1 signaling pathway, and Toll-like receptor signaling pathway. At the transcriptional level, PD-L1 expression is regulated by several transcriptional factors such as HIF-1, NFκB, and AP-1. HIF-1 directly binds to the hypoxia-response element in the PD-L1 promoter to regulate PD-L1 expression [13]. Additionally, the late induction of PD-L1 expression of IFN-c is achieved by the regulation of interferon regulatory factor-1 (IRF-1) through Janus-activated kinase (JAK) signal transductor and transcriptional activator (STAT) pathways [14]. Activation of common oncogenic pathways such as the PI3K/Akt signaling pathway and the Ras/MEK/ERK pathway has also been shown to affect tumoral PD-L1 expression. Tristetraprolin (TTP) phosphorylation is caused by Ras activating RAF-MEK-ERK downstream signaling cascade and increased PD-L1 protein expression [15]. The PI3K/Akt pathway is also a key signaling pathway of regulating PL-L1 expression in tumor cells. PD-L1 protein expression may be due to the loss of PTEN in cancer cells [16]. Meanwhile, the Toll-like receptor signaling pathway also regulates the PD-L1 expression.

Most research studies have focused on PD-L1 expression and prognosis, and the relationship between the expression level of PD-L1 and radiosensitivity of gastric cancer patients remains unclear. In addition, genes that regulate PD-L1 expression in cancer might be useful biomarkers for predicting radiosensitive of gastric cancer. Therefore, we investigated the correlation between genes involved in the PD-L1 pathway and radiosensitivity in patients with gastric cancer. For precision medicine, our work offered more evidence for using PD-L1 expression levels and other genes as potential biomarkers to predict radiosensitive for gastric cancer patients.

2. Materials and Methods

2.1. Data Sources. All gene expression dataset of the gastric cancer patients was downloaded from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/). 443 patients were collected in the TCGA on October 17, 2019. Effective patient survival information was obtained after excluded samples of no survival time or no survival outcome. Clinical data including age, gender, histologic type, pathological stage, and tumor-node-metastasis (TNM) stage were collected from the clinical dataset. Then, the data were obtained by combining clinical data and normalized mRNA sequencing data. Furthermore, the final data were collected with radiotherapy and deleted repeated information. Finally, 367 samples were obtained. The cleaned clinical data are summarized in Table S1. There were 239 men and 128 women in our study. The number of patients who received the radiotherapy was 76, and the number of patients who did not receive radiotherapy was 291.

2.2. Analysis Method. In the present study, radiosensitive patients were defined as a group of patients who had better overall survival after receiving radiotherapy compared with nonradiotherapy. Radiosensitive gene was defined as the genes which are associated and could be used to identify the radiosensitive patients [17]. The genes involved in the PD-L1 pathway in cancer were found in the Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 27 genes involved in the present study. Each gene was categorized into high and low groups according to the median value. Kaplan–Meier curves were used to show the survival curves in the high expression group and low expression group. The data clean procedure and radiotherapy sensitive gene selection procedure are shown in Figure 1. The relationship between genes expression levels and radiosensitivity was analyzed by the univariate and multivariate Cox proportional hazards models. The stage divided by the 8th edition of the AJCC Cancer Staging Manual [18]. We also used the Dukes-MAC-like staging system of gastric cancer [19]. The clinical and pathological characteristics of this group were compared by the chi-square test. The functions and pathway of genes were analyzed by GO and KEGG. All statistical analyses were performed by using the *R* packages. In addition, *R* package mice were used to impute the missing values. p value 0.05 was considered significant, and all statistical tests were two-sided.

3. Results

3.1. CD274 and Genes Involved in the PD-L1 Pathway in Cancer Expression Levels. Following the procedure in Figure 1, out of initial 27 genes, 13 genes (CD274, EGFR, HRAS, RAF1, MTOR, RPS6KB1, CHUK, NFKB1, TRAF6, FOS, HIF1A, NFATC1, and PIK3CA) were selected and considered as potential biomarker of radiosensitivity. We investigated each expression level of these genes. As illustrated in Figure S1, the expression levels of 13 genes in gastric cancer were different. The PD-L1 expression level was the lowest among the 13 genes, in which the median expression

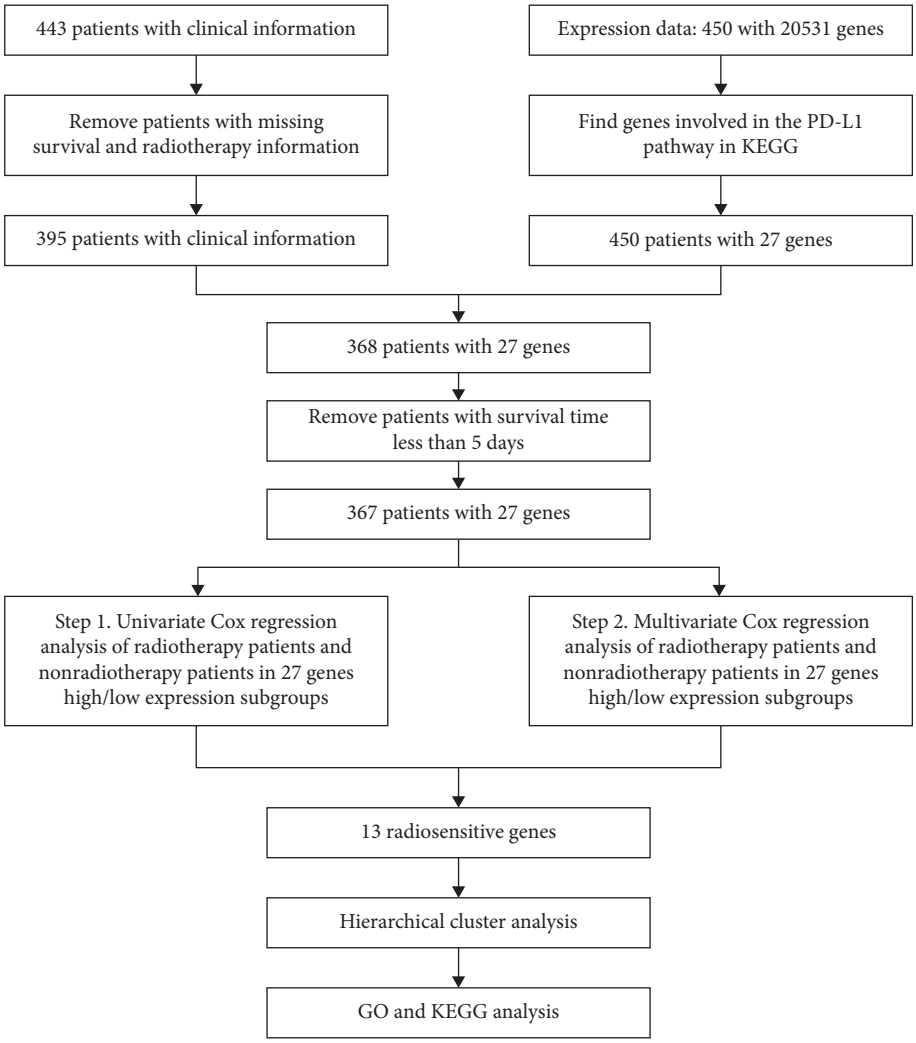


Figure 1: The flow chart of data cleaning and analysis steps.

level was 36.65, and the distribution was mostly concentrated in 19.61–67.74. However, the maximum and minimum values of CD274 expression level were 5728.60 and 1.10. The highest expression level gene of gastric cancer patients was FOS, in which the median expression level was 5007.70, and the maximum and minimum values were 73122.70 and 198.40.

3.2. Correlation Analysis of 13 Genes Expression Levels and Clinical Indicators with Survival. In the present study, the Cox proportional hazard model was used to analyze the association between 13 genes expression levels and clinical factors with survival. Table 1 illustrates the analysis results that univariate analysis and multivariate analysis of clinical indicators and 13 genes expression levels included CD274. The multivariate analysis of each gene expression level is the outcome of gene and clinical factors. The results showed that radiotherapy can improve the patient's overall survival. For clinical indicators, the univariate analysis revealed that T stage ($p = 0.004$), M stage ($p = 0.010$), N stage ($p = 0.001$), pathological stage ($p = 0.001$), targeted therapy ($p = 0.022$), and chemotherapy ($p = 0.034$) were significant factors for overall survival; the multivariate analysis revealed that only N stage ($p = 0.041$) was a significant factor for overall survival, and N stage could relate with survival. However, the univariate analysis and multivariate analysis revealed that the relationship between each expression level of 13 genes and overall survival is not significant.

3.3. Relationship between Expression Levels of 13 Genes and Clinical Indicators. To identify the relationship between each expression level of 13 genes and clinical factors, we next determined which factors were associated with CD274 and other genes via the chi-square test.

Tables 2 and S3–S13 show that each gene expression level of CD274, EGFR, RAF1, PIK3CA, RPS6KB1, CHUK, TRAF6, FOS, NFKB1, and HRAS has no significant associations with clinical indicators. In addition, the relationship between each gene expression level of NFATC1, HIF1A, MTOR, CHUK, and histologic type was statistically significant, which indicated that the expression levels of these four genes are not identical in different pathological types. RPS6KB1 expression level is

Table 1: Associations of clinical indicators and 13 genes expression levels with total survival.

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p values	HR (95%CI)	p values
Radiotherapy				
Yes	0.407 (0.255–0.651)	<0.001	0.417 (0.245–0.710)	0.001
No	1.0000		1.000	
Gender				
Male	1.271 (0.897–1.801)	0.178	1.308 (0.919–1.862)	0.136
Female	1.0000		1.000	
Age				
≥60	1.361 (0.945–1.96)	0.097	1.402 (0.958–2.053)	0.084
<60	1.0000		1.000	
Histologic type				
NOS	1.199 (0.788–1.823)	0.397	1.274 (0.831–1.954)	0.264
DT/MT/SRT	0.903 (0.555–1.47)	0.682	1.112 (0.682–1.840)	0.655
PT/TT	1.0000		1.000	
T Stage				
T3/T4	1.854 (1.215–2.827)	0.004	1.381 (0.855–2.230)	0.187
T1/T2	1.0000		1.000	
M Stage				
M1	1.909 (1.165–3.128)	0.010	1.674 (0.999–2.805)	0.050
M0	1.0000		1.000	
N stage				
N1/N2/N3	1.953 (1.301–2.931)	0.001	1.753 (1.023–3.004)	0.041
N0	1.0000		1.000	
Pathological stage				
III/IV	1.859 (1.309–2.639)	0.001	1.481 (0.897–2.446)	0.125
I/II	1.0000		1.000	
Targeted therapy				
Yes	0.680 (0.489–0.946)	0.022	0.894 (0.430–1.861)	0.765
No	1.0000		1.000	
Chemotherapy				
Yes	0.703 (0.508–0.973)	0.034	0.804 (0.401–1.610)	0.537
No	1.0000		1.000	
CD274				
High	0.813 (0.588–1.123)	0.209	0.734 (0.524–1.027)	0.071
Low	1.0000		1.000	
EGFR				
High	1.098 (0.794–1.518)	0.573	1.209 (0.867–1.686)	0.262
Low	1.0000		1.000	
RAF1				
High	1.046 (0.757–1.445)	0.786	1.249 (0.896–1.741)	0.190
Low	1.0000		1.000	
MTOR				
High	0.906 (0.656–1.252)	0.552	1.008 (0.725–1.401)	0.962
Low	1.0000		1.000	
RPS6KB1				
High	1.154 (0.836–1.595)	0.384	1.283 (0.920–1.789)	0.142
Low	1.0000		1.000	
CHUK				
High	0.865 (0.625–1.197)	0.382	0.941 (0.676–1.309)	0.717
Low	1.0000		1.000	
NFKB1				
High	0.873 (0.632–1.205)	0.409	1.016 (0.729–1.416)	0.926
Low	1.0000		1.000	
TRAF6				
High	1.268 (0.917–1.753)	0.151	1.358 (0.974–1.893)	0.071
Low	1.0000		1.000	
FOS				
High	1.124 (0.813–1.554)	0.478	1.287 (0.923–1.794)	0.137
Low	1.0000		1.000	
HIF1A				

Table 1: Continued.

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p values	HR (95%CI)	p values
High	1.256 (0.909–1.737)	0.168	1.184 (0.838–1.672)	0.339
Low	1.0000		1.000	
NFATC1				
High	1.291 (0.932–1.787)	0.124	1.364 (0.962–1.934)	0.081
Low	1.0000		1.000	
PIK3CA				
High	1.196 (0.864–1.656)	0.280	1.228 (0.876–1.722)	0.233
Low	1.0000		1.000	
HRAS				
High	0.881 (0.638–1.217)	0.441	0.882 (0.635–1.225)	0.454
Low	1.0000		1.000	

Abbreviations.HR: hazard ratio; NOS: not otherwise specified; DT: diffuse type; MT: mucinous type; SRT: signet ring type; PT: papillary type; TT: tubular type.

Table 2: Relationship between expression level and clinical indicators.

	CD274				HRAS			
	High	Low	$\chi\chi2$	p Values	High	Low	$\chi\chi2$	p Values
Gender			0.135	0.714			1.049	0.306
Female	66	62			69	59		
Male	117	122			114	125		
Age			0.763	0.184			0.034	0.854
<60	50	64			56	58		
≥60	130	120			127	123		
Histologic type			2.061	0.357			2.872	0.238
PT/TT	35	44			35	44		
DT/MT/SRT	53	43			44	52		
NOS	95	94			102	87		
T Stage			2.666	0.103			0.067	0.796
T1/T2	56	42			50	47		
T3/T4	124	141			133	133		
N stage			0.566	0.452			1.473	0.225
N0	59	52			62	50		
N1/N2/N3	122	131			121	132		
M Stage			1.233	0.267			0.000	1.000
M0	171	165			168	168		
M1	12	19			15	16		
Pathological stage			0.104	0.747			3.733	0.053
I/II	88	83			97	74		
III/IV	89	92			83	98		
Dukes-MAC stage			4.992	0.082			5.454	0.065
D	50	45			56	39		
C	71	92			72	91		
A/B	55	41			45	51		

Abbreviations. HR: hazard ratio; NOS: not otherwise specified; DT: diffuse type; MT: mucinous type; SRT: signet ring type; PT: papillary type; TT: tubular type.

associated with gender, and NFATC1 expression level is associated with the T stage. There is a significant relationship between each gene expression level of NFATC1, PIK3CA, and pathological stage.

3.4. Relationship between Radiotherapy and Survival in the Two Expression Groups. The study focused on whether the relationship between genes involved in the PD-L1 pathway in cancer and radiosensitivity. The main idea was whether the overall survival of patients with high or low expression level was increased after radiotherapy. For each gene, the gastric cancer patients were categorized into two groups according to their median score and performed survival analysis, respectively. One was a high expression level group and other was a low expression level group. High expression levels of CD274, EGFR, RAF1, RPS6KB1, PIK3CA, MTOR, CHUK, NFKB1, TRAF6, FOS, NFATC1, and HIF1A were sensitive to radiotherapy. However, the low expression level of HRAS was associated with radiosensitivity.

Figure 2 demonstrates that the hazard ratio (HR) of each high expression level group of CD274, EGFR, RAF1, PIK3CA, RPS6KB1, MTOR, CHUK, NFKB1, TRAF6, FOS, NFATC1, and HIF1A was still significant after multivariate adjustment was analyzed compared with the low expression level group.

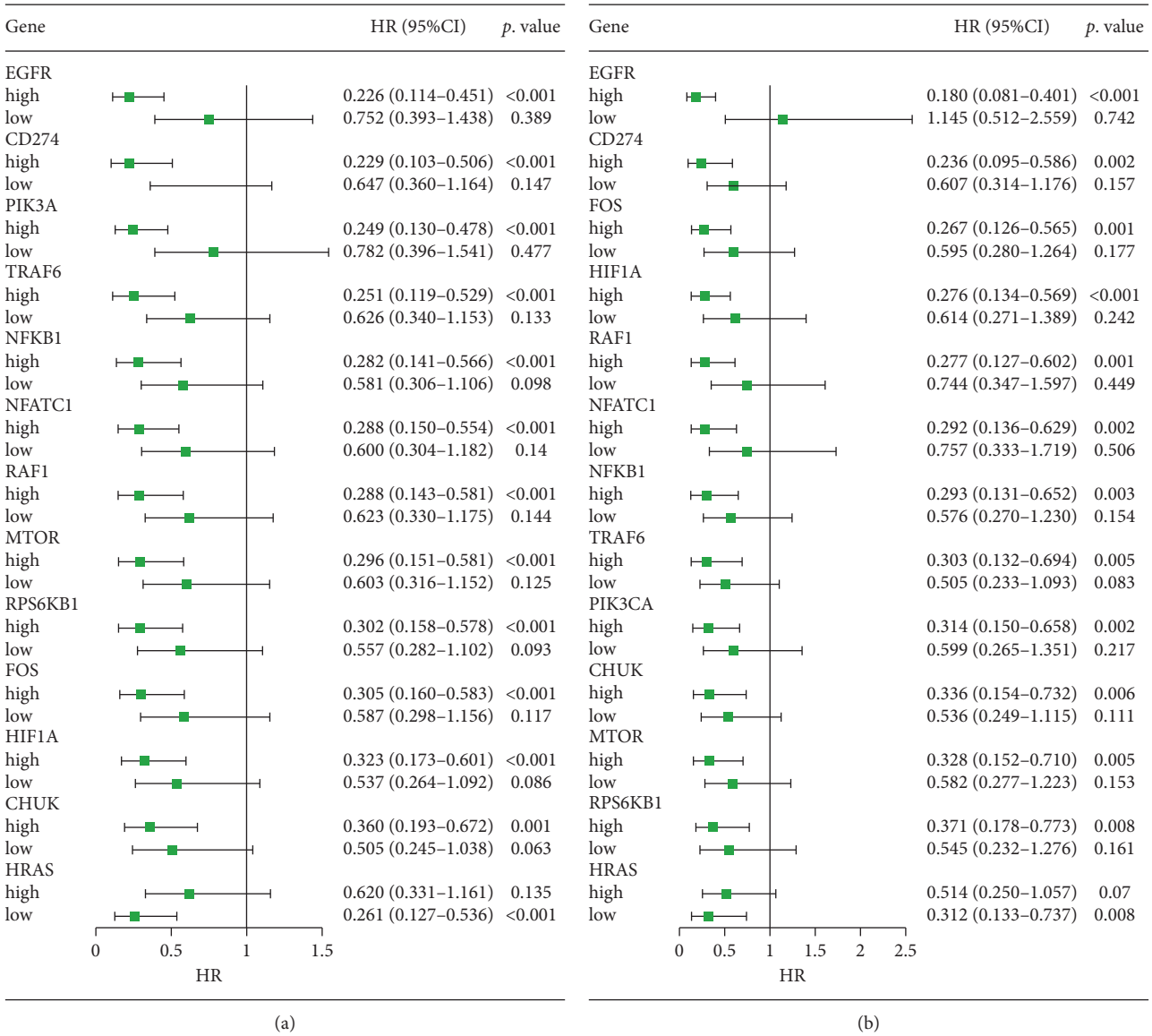


Figure 2: Forest plots for the association analysis between radiotherapy and survival under different expression levels of 13 genes. (a) The forest plot of univariate analysis. (b) The forest plot of multivariable analysis. The adjusted factors include age, gender, histologic type, tumor-node-metastasis (TNM) stage, pathological stage, chemotherapy, and targeted therapy.

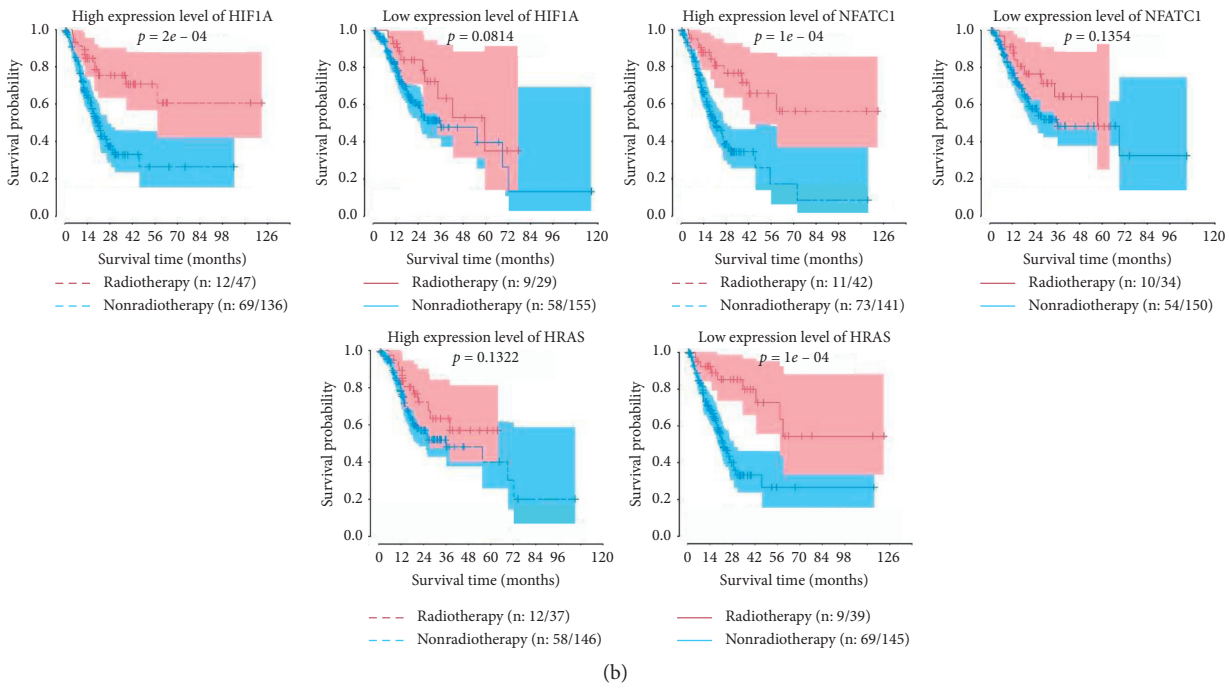
For the CD274 gene, the HR and the HR after multivariate adjustment for radiotherapy vs nonradiotherapy were 0.229 (0.103–0.506) and 0.236 (0.095–0.586), respectively. These results indicated that in the high expression level group of these 12 genes, patients who received radiotherapy would have a significant survival benefit and improved their survival rates. For HRAS, the HR after multivariate adjustment was still significant in the low expression level group.

The Kaplan–Meier curves for overall survival are graphed as shown in Figure 2. Survival curves of the radiotherapy and nonradiotherapy groups were based on differences in the high and low expression levels of each gene (Figure 3). For CD274, EGFR, RAF1, RPS6KB1, PIK3CA, MTOR, CHUK, NFKB1, TRAF6, FOS, NFATC1, and HIF1A genes, the survival rate of a patient who received radiotherapy in the high expression level group was significantly prolonged compared with patients who did not receive radiotherapy (a). Meanwhile, there was no difference between those who received radiotherapy and those who did not in the low expression group (b). For the HRAS gene, the overall survival rate was significantly higher in the low expression level group than patients who received nonradiotherapy.

3.5. Associations among Expressions Levels of 13 Genes and New Tumor Event Rate and Disease Progression Rate. Figures 4 and S2–S12 illustrate the associations among the new tumor event and progressive disease on these two clinical assessment indexes and 13 genes. For new tumor event index and progressive disease rate of CD274, EGFR, RAF1, PIK3CA, MTOR, NFKB1, TRAF6, HIF1A, and NFATC1 genes, the patients in the high expression level group, new tumor event rate, and disease progression rate

(a)

Figure 3: Continued.



(b)

Figure 3: Survival curves under different expression levels of genes involved in the PD-L1 pathway.

were not increased and even decreased by received radiotherapy. Meanwhile, the rate of disease progression was also reduced in the highly expressed group after radiotherapy. This result further verified the high expression level of CD274, and these genes associated with radiosensitivity of patients with gastric cancer.

However, for progressive disease rate of RSPS6KS1 gene, under radiotherapy, there was no difference in the new tumor event index between these two expression level group patients. For CHUK and FOS genes, only the rate of disease progression was reduced in the highly expressed group after radiotherapy. For the HRAS gene, in the low expression level group, these two indexes were lower in patients who received radiotherapy compared with the high expression level group.

3.6. GO and KEGG Analysis of 13 Genes in Gastric Cancer. In the present work, we conducted GO and KEGG analysis of 13 genes in gastric cancer to obtain the biological process, molecular function, cellular component, and pathways. The biological process of 13 genes was mainly related to innate immune response activating the cell surface receptor signaling pathway and the stimulatory C-type lectin receptor signaling pathway (Figure 5). We observed that the molecular functions of 13 genes were mainly involved in protein. Cellular component indicted that 13 genes mainly exit the CD40 receptor and mitochondrial outer membrane. KEGG pathway analysis showed that 13 genes in gastric cancer mainly related to PD-L1 expression and PD-1 checkpoint pathway in cancer and further validated genes from the PD-L1 pathway.

3.7. The Correlation between CD274 and Genes Involved in the PD-L1 Pathway in Cancer and Cluster Analysis. We explored the correlation between CD274 expression level and each expression level of the other 12 genes, and the result is as shown in Figure 6. There are strongly positive correlations in HRAS and HIF1A and FOS and HIF1A. There is a strongly negative correlation in CD274 and CHUK. As shown in Figure 7, all patients are divided into two groups according to the outcome of cluster analysis, cluster 1 includes 195 patients, and cluster 2 includes 172 patients. Furthermore, we plotted the survival plot in these two clusters (Figure 8), for patients in the cluster 1, significantly better survival was observed for patients who received radiotherapy, compared with nonradiotherapy patients.

4. Discussion

Radiotherapy is attracting increased attention as a crucial adjuvant therapy for gastric cancer. In past studies, preoperative radiotherapy has progressed in treating gastric cancer, and patients with D1 or D1 plus lymphadenectomy can benefit from postoperative radiotherapy [3]. In the local palliation of gastric cancer, radiation therapy is an effective and well-tolerated modality [20]. However, patients and doctors are concerned about the side effects and long-term effects of radiotherapy. Radiotherapy has more grade adverse events [21]. Therefore, as the development of individualized treatment, finding potential biomarkers, and validating radiation sensitivity, genes in cancer will be of utmost importance for defining its role as a predictive marker and optimizing strategies for cancer radiotherapy.

PD-L1 is encoded by the CD274 gene and binds to the PD-1 receptor expressed on the surface of T cells [5]. Some research studies demonstrated that the CD274 high expression level was associated with a significantly better patient outcome [22], and PD-L1 mRNA levels were

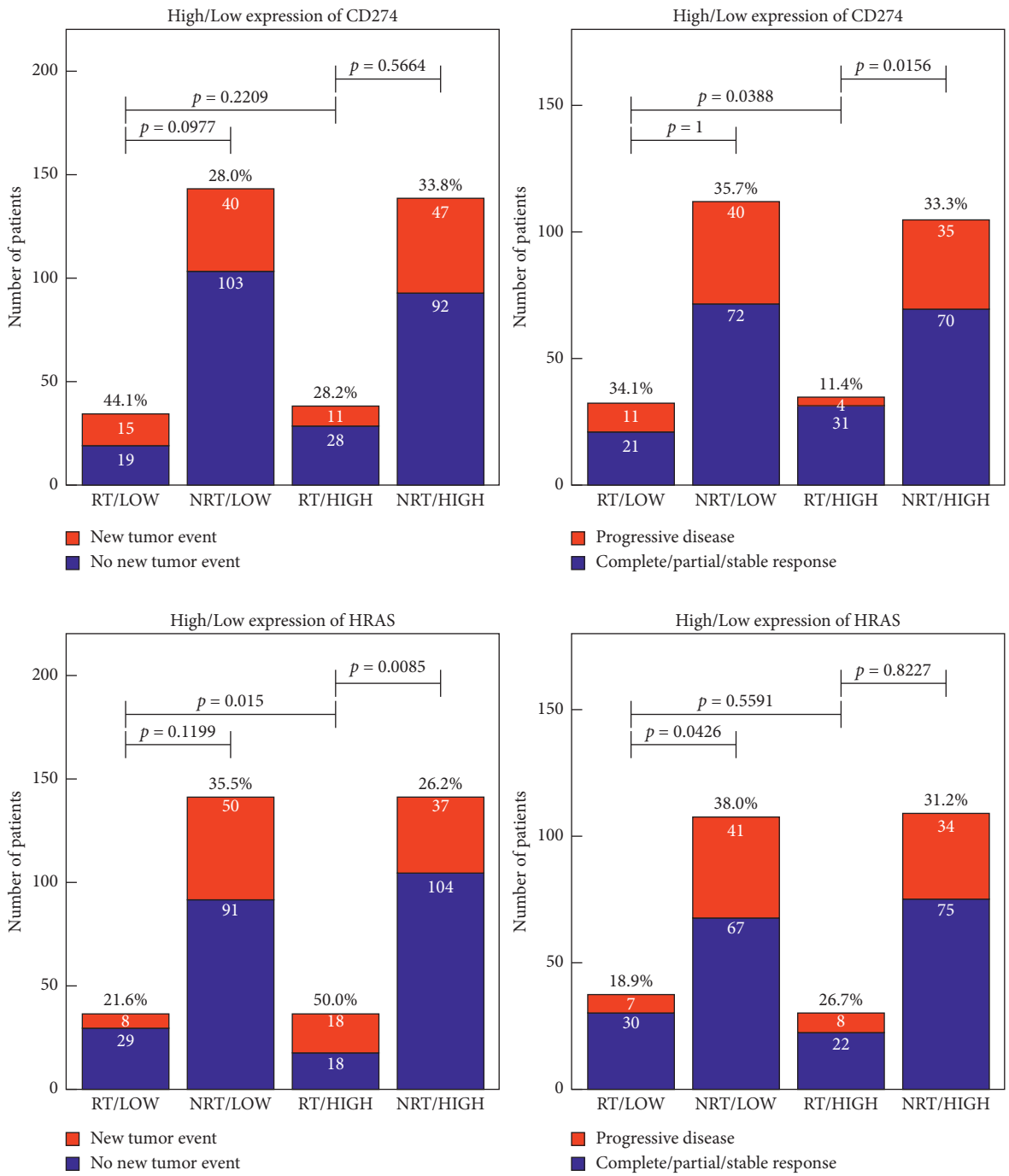


Figure 4: The associations between gene expression level and new tumor event and disease progression rate. The chi-square test was used for comparison of rates of different groups. RT: radiotherapy; NRT: nonradiotherapy; HIGH: high expression level of the gene; LOW: low expression level of gene.

upregulated in gastric cancer [8], which are the same as our study. The PD-1/PD-L1 interaction plays a critical role in antigen, and autoimmunity serves as a regulatory checkpoint. A large number of data suggest that, for many cancers, the PD-L1 pathway may be an active immune checkpoint [23]. The PD-1/PD-L1 pathway provided the second signal for effective activation of T cells as costimulatory molecules. Under normal conditions, when the immune system detects cancer cells, the PD-1/PD-L1 pathway can activate T lymphocytes and recognizes tumor cells and kills them. In solid tumors, the silencing of the immune system can be accomplished by increasing the expression of PD-L1 on the surface of tumor cells [7]. Nowadays, the safety and efficacy of anti-PD-1/PD-L1 treatment drugs in melanoma [11], nonsmall cell lung cancer [24], and colorectal carcinoma [25] have been confirmed. The activity and safety of pembrolizumab monotherapy was demonstrated in advanced gastric patients [26].

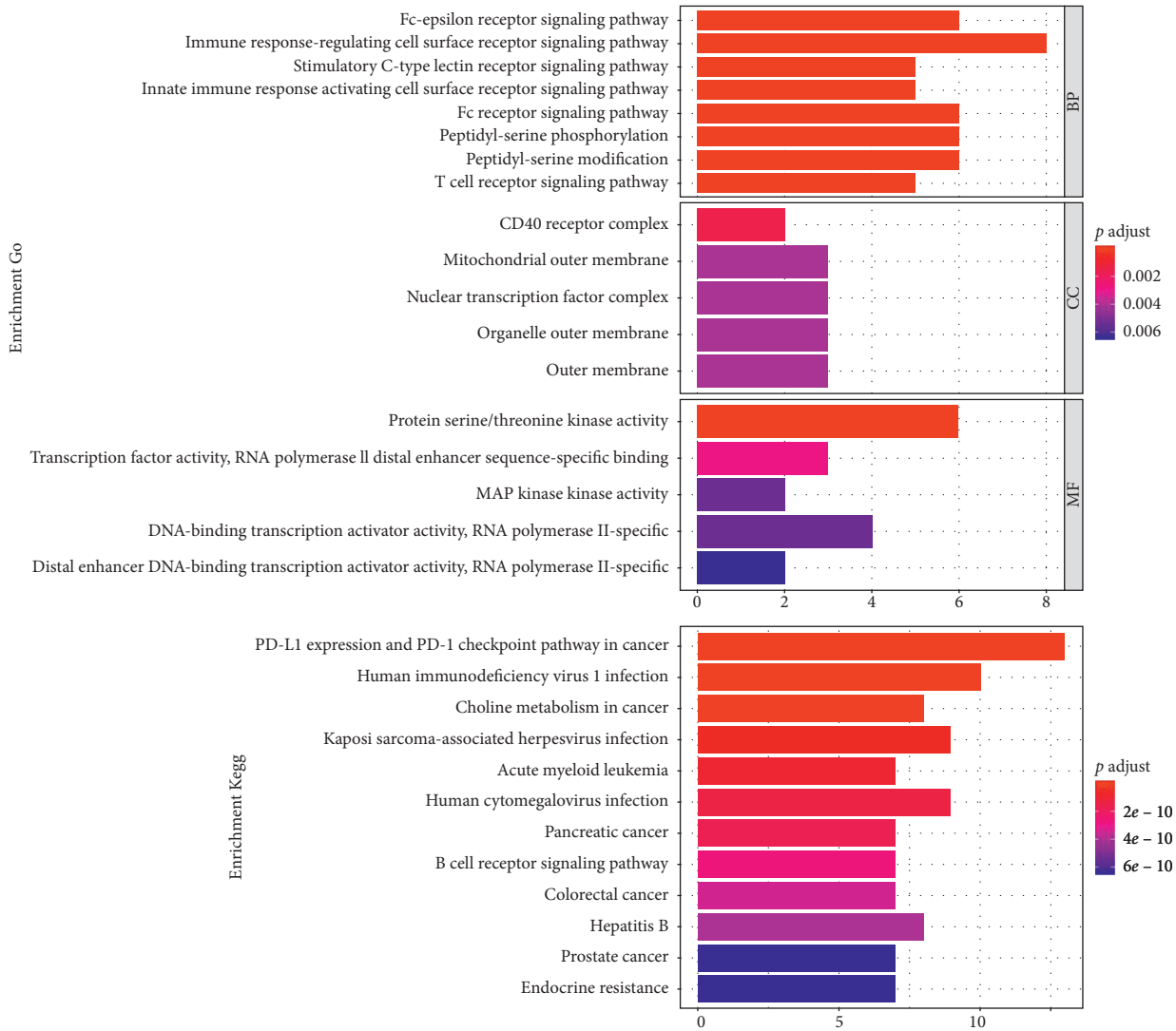


Figure 5: Bar plot for GO and KEGG analysis. BP: biological process; MF: molecular function; CC: cellular component. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Studying the relationship between PD-L1 and genes involved in PD-L1 pathways in cancer and radiosensitivity provides important insights into the precision treatment of patients with gastric cancer. In our study, the gastric cancer patients were obtained from TCGA. The median gene score was chosen to categorize the patients into two groups after cleaning the data. One is a high expression group and other is a low expression group. We also used other cutoffs of CD274 such as upper quartile and lower quartile to perform analysis (Figure S13). The results suggested that, when the cutoff is 1/4, the HR of radiotherapy was not significant in these two groups between radiotherapy and non-radiotherapy by multivariate survival analyzed. We also selected 3/4 that larger than 1/2 as other cutoffs, and the HR of radiotherapy in patients with low expression was statistically significant, which may be because of too many higher expression levels patients in the low expression group. These results are consistent with our conclusion that the high expression level of CD274 is related to the radiotherapy sensitivity of gastric cancer.

We divided the patients in the subgroup into patients who received radiotherapy and those who received non-radiotherapy. Then, we performed survival analysis in these two groups and plotted Kaplan–Meier curves for overall survival. Our study found that patients with high CD274 expression level had higher sensitivity, while low expression level suggested that patients were not sensitive to radiotherapy.

Furthermore, we researched genes involved in the PD-L1 pathway which include EGFR, HRAS, RAF1, ALK, PIK3CA, PTEN, AKT3, EGF, MAP2K1, MAPK1, MTOR, RPS6KB1, CHUK, NFKBIA, NFKB1, IFNG, IFNGR1, STAT1, TLR9, TIRAP, MYD88, TRAF6, TICAM1, TICAM2, FOS, HIF1A, and NFATC1 in gastric cancer. Each gene was analyzed by univariate survival and multivariate survival analyzed. Through multivariate analysis, we found that high

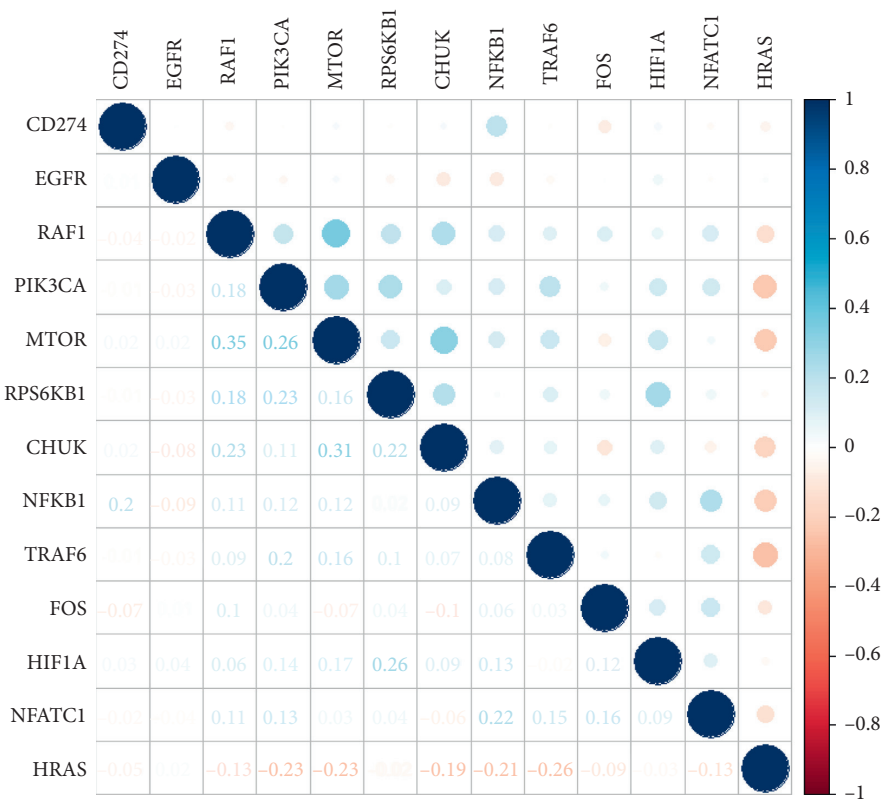


Figure 6: The plot for the correlation of expression levels of genes involved in the PD-L1 pathway.

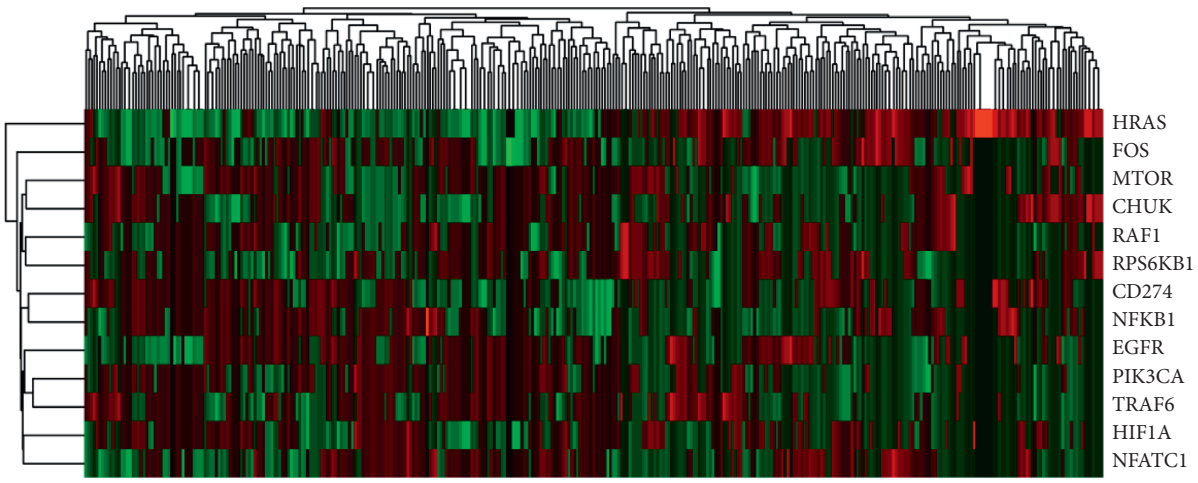


Figure 7: The heatmap of cluster analysis.

expression levels of the EGFR, RAF1, RPS6KB1, PIK3CA, CHUK, NFKB1, TRAF6, FOS, NFATC1, and HIF1A genes and the low expression level of the HRAS gene were associated with radiosensitivity. We also studied the relationship between each gene expression level and clinical indicators, and our assumption supported that new tumor event rate and disease progression rate did not significantly increase by receiving radiotherapy.

In recent years, the Dukes-MAC-like staging system was proposed for gastric cancer [19].We also used the Dukes-MAC-like staging system to analyze the relationship between Dukes-MAC-like stage and survival (Table S2). Univariate analysis showed that Dukes-MAC-like stage itself was associated with survival. However, multivariate analysis demonstrated that Dukes-MAC-like stage itself was not associated with survival. Next, we analyzed the associations among expressions levels of 13 genes and Dukes-MAC-like stage. The results showed that each gene expression level of TRAF6, NFATC1, NFKB1, and PIK3A is associated with the Dukes-MAC-like stage.

Shohei Eto et al. demonstrated that disease-free survival (DFS) and overall survival (OS) were significantly poorer in

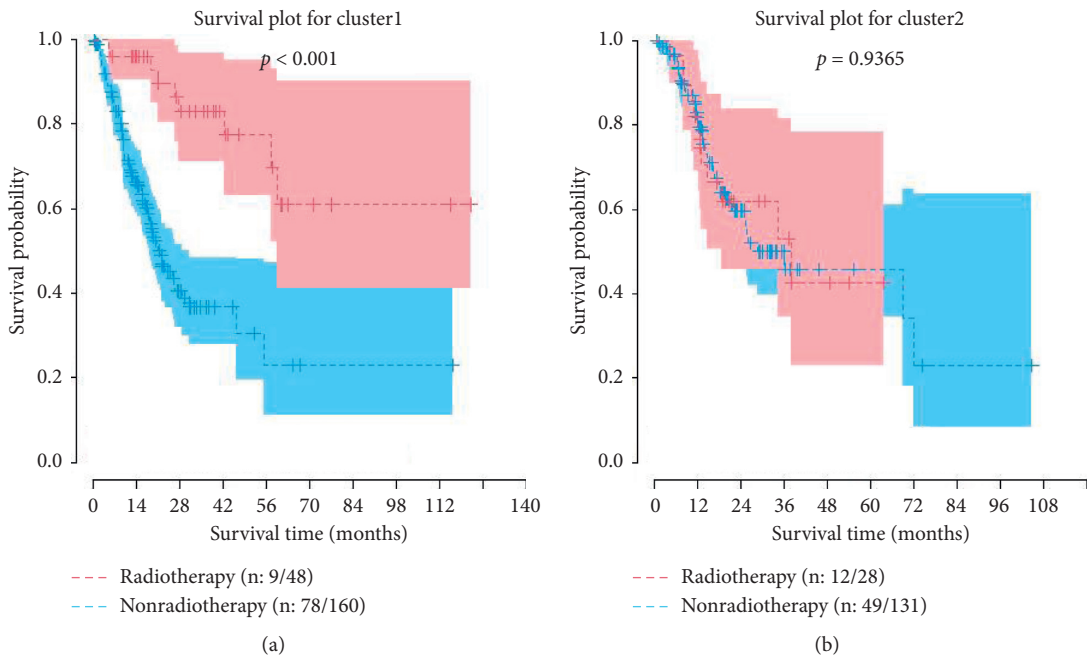


Figure 8: Survival curves under different clusters.

PD-L1-positive patients in gastric cancer [27]. Geng et al. also revealed that the expression levels of PD-L1 are higher and considered as poor prognosis in gastric cancer [28]. However, in this study, there was no significant difference in survival between the high expression group and low expression group.

The genes involved in the PD-L1 pathway in cancer related to radiosensitivity have not been systematically discussed. Preclinical and clinical evidences have demonstrated the activation of antitumor immunity by radiotherapy. The PD-1/PD-L1 pathway plays an important role in immune escape as one of the major mechanisms of cancer. Therefore, the PD-1/PD-L1 pathway after radiotherapy may appropriate systemic antitumor immune activation to improve the curative effect of radiotherapy. In recent years, immunotherapy such as PD-1/PD-L1 immune checkpoint and radiotherapy combined with immunotherapy for gastric cancer have been considered as promising approaches [29]. In cancer treatment, tumor microenvironment is sensitive to treatment with immune checkpoint such as the PD-1/PD-L1 pathway because of radiotherapy. Anti-PD-1/PD-L1 antibodies have potential to relieve immunosuppression caused by radiotherapy in combination therapy [30].

The epidermal growth factor receptor (EGFR) is overexpressed in gastric cancers. The AKT pathway was blocked by the EGFR signaling to suppress the invasion and growth of gastric cancer cells [31]. EGFR expression is associated with the response to radiation. The interaction of EGFR and radiotherapy is complex. Some research studies demonstrated that EGFR inhibitors are related to radiosensitization. EGFR inhibitors may limit tumor repopular through the cytostatic effect [32]. Raf-1 kinase feedback regulation might be associated with radiotherapy sensitivity by enhancing its antiapoptotic function in cancer cells [33]. In glioblastoma, Ras has high activity and sensitivity to radiotherapy [34]. Nuclear factor-kappa B (NF-κB) transcription factors are a key participant in innate and adaptive immune responses as fundamental regulators. The release of NF-κB and activation of the IKK complex were directly involved by the Ras/Raf/MEK/ERK and AKT pathways [35]. The IKK complex enhances the sensitization of ionizing radiation by downregulating either IKK in radiosensitivity [36]. Therefore, Ras/Raf/MEK/ERK and AKT pathways involved CHUK, and RPS6KB1 genes might enhance the sensitizing effect of radiation by downregulation of IKK complex. For RPS6KB1, knockdown of RPS6KB1 increased their sensitivity toward radiation-induced survival inhibition in prostate cancer cells [37]. The activation of the PI3K/AKT pathway involved PI3KCA, and MTOR is linked to radioresistance [38]. Thus, it is supposed that PI3K may overcome radioresistance as a suitable target. Activator protein-1 (AP-1) is encoded by the FOS gene and is related to the control of a variety of cancer cells, such as breast cancer [39] and gastric cancer [2]. The expression level of NFATC1 was decreased in HCC tissues [40] and was significantly upregulated in ovarian cancer [41]. However, more recent findings shed light on the mechanism between FOS, NFATC1, and radiotherapy, which have not been systematically discussed. HIF-1α plays an important role in gastric cancer progression and development as a key transcription factor and its overexpression in gastric cancer [42]. Radiotherapy inhibits cervical cancer cell growth through downregulating HOTAIR to inhibit the expression of HIF-1α [43]. The reason why various solid tumors are sensitive to radiation is affecting the tumor microenvironment by targeting HIF-1 to reduce the antioxidant capacity of tumors. [44]. We speculated that radiotherapy may control the expression of TRAF6 to influence cell growth and apoptosis.

Only the low expression level of HRAS was associated with radiosensitivity in genes involved in the PD-L1 pathway. HRAS is an attractive target associated with radiation resistance. HRAS gene mutations or EGFR amplification activation can reduce the growth delay of postradiation tumors [45]. Ras signaling to the PI3 kinase-Akt pathway is an important contributor to radiotherapy.

One limitation of our study is that the sample size was small in the TCGA cohort. In this study, there was no external validation study, such as clinical verifications. Then, the study did not consider differences in patient radiation doses. In summary, our study demonstrated the potential predictive and prognostic values of genes involved in the PD-L1 pathway of radiotherapy patients in gastric cancer. These findings provided new insights into the treatment of gastric cancer in the field of radiotherapy, particularly with individualized treatment of cancer patients.

5. Conclusion

Genes involved in the PD-L1 pathway might associate with the radiosensitivity of patients with gastric cancer. For precision medicine, our work offered more evidences for using PD-L1 and other genes as potential biomarkers to predict radiosensitive patients for gastric cancer patients.

Data Availability

The data used to support this study are available within this article.

Disclosure

The funding body did not play any roles in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zixuan Du, Derui Yan, and Zaixiang Tang were involved in study conception and design. Zixuan Du, Zhongyang Li, and Derui Yan were involved in data collection and clean. Zixuan Du, Jincheng Gu, and Zaixiang Tang were involved in real data analysis and interpretation. Zixuan Du, Derui Yan, Ye Tian, and Jianping Cao were involved in drafting of the manuscript. All authors reviewed the manuscript.

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Supplementary Materials

Table S1: the information of basic patient characteristics. Table S2: associations of clinical indicators and 13 genes expression levels with total survival. Tables S3–S13: the relationship between expression level and clinical indicators. Figure S1: the box plot for expression levels of genes involved in the PD-L1 pathway. Figures S2–S12: associations among each gene expression level and clinical assessment factors. The chi-square test was used for comparison of rates of different groups. RT: radiotherapy; NRT: nonradiotherapy; HIGH: high expression level of the gene; LOW: low expression level of the gene. Figure S13: the HR values of radiotherapy along with different cutoffs. (*Supplementary Materials*)

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