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Original Article

Objective Quantitation of EGFR Protein Levels using Quantitative Dot Blot Method for the Prognosis of Gastric Cancer Patients

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ABSTRACT

Purpose: An underlying factor for the failure of several clinical trials of anti-epidermal growth factor receptor (EGFR) therapies is the lack of an effective method to identify patients who overexpress EGFR protein. The quantitative dot blot method (QDB) was used to measure EGFR protein levels objectively, absolutely, and quantitatively. Its feasibility was evaluated for the prognosis of overall survival (OS) of patients with gastric cancer.
Materials and Methods: Slices of 2×5 μm from formalin-fixed paraffin-embedded gastric cancer specimens were used to extract total tissue lysates for QDB measurement. Absolutely quantitated EGFR protein levels were used for the Kaplan-Meier OS analysis.
Results: EGFR protein levels ranged from 0 to 772.6 pmol/g (n=246) for all gastric cancer patients. A poor correlation was observed between quantitated EGFR levels and immunohistochemistry scores with ρ=0.024 and P=0.717 in Spearman's correlation analysis. EGFR was identified as an independent negative prognostic biomarker for gastric cancer patients only through absolute quantitation, with a hazard ratio of 1.92 (95% confidence interval, 1.05–3.53; P=0.034) in multivariate Cox regression OS analysis. A cutoff of 208 pmol/g was proposed to stratify patients with a 3-year survival probability of 44% for patients with EGFR levels above the cutoff versus 68% for those below the cutoff based on Kaplan-Meier OS analysis (log rank test, P=0.002).
Conclusions: A QDB-based assay was developed for gastric cancer specimens to measure EGFR protein levels absolutely, quantitatively, and objectively. This assay should facilitate clinical trials aimed at evaluation of anti-EGFR therapies retrospectively and prospectively for gastric cancer.

Keywords: Epidermal growth factor receptor; QDB; Companion assay; Cetuximab; Necitumumab

INTRODUCTION

Anti-epidermal growth factor receptor (EGFR) monoclonal antibody drugs, including cetuximab, panitumumab, nimotuzumab, and necitumumab, have been developed to treat cancer patients in daily clinical practice [1]. These drugs have been approved for

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Conflict of Interest
FT, MY, and JZ are employees of Yantai Quanticision Diagnostics, Inc., a division of Quanticision Diagnostics Inc. who hold patents US9933418, US10295544, and US10788484 covering the QDB method and its applications in clinical diagnostics. LX and BS declare no conflicts of interest.

the treatment of colorectal cancer and head and neck cancers over the last few decades. Meanwhile, there has also been an intense interest being developed with regard to their use in gastric cancer patients.

In 2013, 2 multicenter, phase III clinical trials, the EXPAND and REAL3 trials, published in the same issue of *Lancet Oncology*, evaluated cetuximab and panitumumab, respectively, in combination with chemotherapy to treat advanced gastric cancer [2,3]. However, both trials yielded disappointing results. In EXPAND trial, among 904 participants, patients receiving capecitabine-cisplatin plus cetuximab had mean progression-free survival (PFS) at 4.4 months vs. 5.6 months for those receiving capecitabine-cisplatin alone (hazard ratio [HR], 1.09; 95% confidence interval [CI], 0.92–1.29; P=0.320). For patients enrolled in REAL3 trial, those receiving epirubicin, oxaliplatin and capecitabine (EOC) plus panitumumab had mean overall survival (OS) at 8.8 months vs. 11.3 months for those receiving EOC only (HR, 1.37; 95% CI, 1.07–1.76; P=0.013).

Upon further examination into these 2 trials, it was found that patients were enrolled in the trials without consideration of their EGFR protein levels in tumor tissue (albeit EGFR assessment was required in EXPAND trial). It is counterintuitive to think that a monoclonal antibody drug may work in the absence of its antigen. Perceivably, its efficacy should be tightly associated with the antigen level, in this case, the EGFR protein level in the tumor tissue.

Indeed, there is evidence suggesting that anti-EGFR monoclonal antibody drugs perform better among patients who overexpress EGFR proteins [2-7]. In the FLEX trial, the initial study found that cetuximab in addition to first-line chemotherapy had borderline benefit in patients with advanced non-small-cell lung cancer (NSCLC) with an HR of 0.871; 95% CI, 0.762–0.996; P=0.440 [8]. However, for patients with high EGFR expression (>200 based on immunohistochemistry [IHC]-based H score), the addition of cetuximab led to significantly increased median OS at 12 months vs. 9.6 months for patients receiving chemotherapy alone (HR, 0.73; 95% CI, 0.58–0.93; P=0.011) [2]. Likewise, in SQUIRE trial, there was indication that patients of higher EGFR expression may benefit from Necitumumab treatment [9,10].

Interestingly, a large number of studies have questioned EGFR as an effective predictive biomarker for these drugs [3,11-15]. In both Bonner and EXTREME trials to evaluate cetuximab for squamous cell carcinoma of head and neck cancer patients, cetuximab showed benefits to patients regardless of their EGFR expression levels [16,17]. Consequently, in several clinical trials, including EXPAND and REAL3 trials, these drugs were administered to participants regardless of their EGFR protein levels [4-6,13,18].

There are several potential factors underlying this controversy regarding EGFR protein as a predictive biomarker for anti-EGFR antibody drugs. First, gastric cancer is highly heterogeneous at all levels. It hence remains to be answered whether a tumor slice may be sufficient to provide a holistic view of the disease [7,19,20]. Second, the negative influence of various known or unknown genetic mutations, including KRAS and NRAS mutations, requires further investigation. Third, the effectiveness of IHC, the predominant EGFR assessment method in daily clinical practice, needs to be re-examined.

We hypothesized that the subjective and semi-quantitative nature of the IHC method at least partly contributed to this confusion [2,11,18,21]. In a majority of the studies, the results of IHC analysis, or IHC scores, of EGFR protein expression were categorized as

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0/1+/2+/3+ based on the number of positively stained cells and the intensity of staining. A more complicated method (H score) was also used in several retrospective clinical studies by multiplying the intensity (from 0 to 3) of the signal with the corresponding portion of the cells (from 0% to 100%) to yield a grading score ranging from 0 to 300 [2,11,12,22,23].

The IHC method provides valuable information about the distribution of protein biomarkers at the tissue level. However, as summarized by Rudbeck, this method is aqualitative assay in nature and it is not optimized for accurate quantitation of protein biomarker levels [24]. Moreover, the IHC result was interpreted by individual pathologists, thus unavoidably introducing subjectivity in the analysis. In fact, the issues associated with IHC-based biomarker assessment are well epitomized by a decade's efforts to standardize Ki67 protein levels in breast cancer tissues [25].

For the same reason, while the H score is developed to improve the accuracy of biomarker assessment, it remains a method based on image analysis. Thus, its result is still plagued by subjectivity and inconsistency. In fact, it might raise more challenge for pathologists to standardize this method, as its broad scoring range (0 to 300 vs. 0 to 3+ in traditional IHC method) might amplify the differences among individual pathologists.

Another issue underlying the IHC-based method is that the categorized results are not proportional variables. The staining intensity in the IHC method is defined qualitatively rather than quantitatively. Thus, it would be difficult to define a precise threshold for the separation of EGFR-positive (EGFR+) from EGFR-negative (EGFR−) patients. In most cases, patients with any expression of EGFR protein were defined as EGFR+ (IHC score ≥1+). In other studies, the cut-offs were set around 200, 220, and 240 in the 0–300 scale based on limited outcome analyses [5,7-9].

Clearly, a novel method offering objective quantitation of EGFR protein levels is needed to define EGFR as a predictive biomarker for these monoclonal antibody drugs. These quantitative results will also help us to develop an optimized cutoff objectively through outcome analysis to further guide patients with these drugs.

Recently, the quantitative dot blot method (QDB) has been used to measure Her2 and Ki67 protein levels in breast cancer specimens. This is an enzyme-linked immunosorbent assay (ELISA)-like method that can be adopted in routine clinical practice [26-29]. In the analytical process, the signal is quantitated by a microplate reader rather than visual scoring, thus eliminating subjectivity. Similar to ELISA, all samples are measured in triplicate, and internal control of known EGFR protein levels is included in the assay to ensure consistency of the results. Thus, compared with the IHC method, the QDB method is simpler, more objective, and consistent. It also provides absolute quantitated protein levels with unmatched accuracy over the IHC method.

As a proof of concept (POC) study, we introduced a QDB-based assay to measure EGFR protein levels absolutely and quantitatively in gastric cancer tissues. The feasibility of this method was evaluated by exploring EGFR as a prognostic biomarker for gastric cancer patients through OS analysis based on its absolutely quantitated protein levels.

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MATERIALS AND METHODS

Human subjects

A total of 248 formalin-fixed paraffin-embedded (FFPE) gastric cancer resection specimens as 2×5 μm slices between January 2015 and December 2017 were provided sequentially and non-selectively by Yantaishan Hospital at Yantai, China. The inclusion criterion was patients with histologically confirmed gastric cancer at stage I, II, or III (M_0) of the TNM staging system with FFPE tissue specimens available at Yantaishan Hospital at Yantai, China. Patients with stage IV (M_1) were excluded from the study. None of the patients received neoadjuvant treatment. The specimen must have had more than 50% tumor tissue based on hematoxylin and eosin staining and should be suitable for IHC analysis. Follow-up data were available for 245 patients (98.8%) at the last follow-up on November 10th, 2020. A flow chart is shown in **Fig. 1**.

General reagents

All chemicals were purchased from Sinopharm Chemicals (Beijing, China). Recombinant human EGFR protein was purchased from Sino Biological Inc. (Cat# 10001-H20B2; Beijing, China). Rabbit anti-EGFR antibody (clone EP22) was purchased from ZSGB-BIO (Beijing, China). Horseradish peroxidase-labeled donkey anti-rabbit IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The BCA protein quantification kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The QDB plate was provided by Quanticision Diagnostics, Inc. (Research Triangle Park, NC, USA).

Preparation of FFPE tissue

Two FFPE tissue slices of 5 μm each (2×5 μm) from each specimen were placed into 1.5 mL Eppendorf tubes and de-paraffinized before being solubilized using lysis buffer (50 mM HEPES, 137 mM NaCl, 5 mM EDTA, 1 mM $MgCl_2$, 10 mM $Na_2P_2O_7$, 1% TritonX-100, 10% glycerol). FFPE tissue slices of a gastrointestinal stromal tumor (GIST) with an EGFR score of 1+ based on IHC analysis (**Supplementary Fig. 1**) were also lysed in the same lysis buffer to serve as the internal control throughout the study. Supernatants were collected after centrifugation, and the total amount of protein was determined using a BCA protein assay kit.

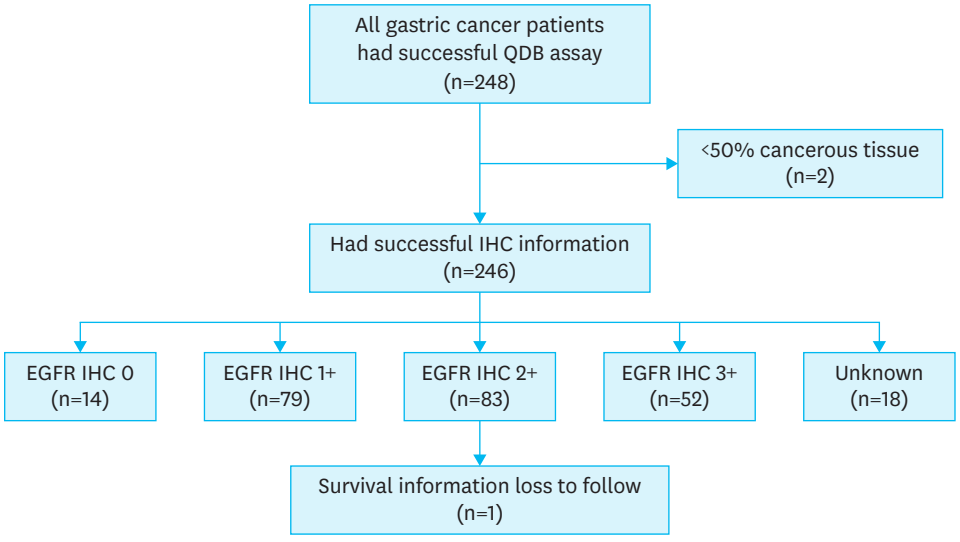

Fig. 1. Flow chart of the gastric cancer specimens.
EGFR = epidermal growth factor receptor; QDB = quantitative dot blot method; IHC = immunohistochemistry.

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Developing QDB-based EGFR assay

Total FFPE tissue lysates from 60 to 80 gastric cancer specimens (0.5 μg) were loaded onto the QDB plate. The quality of the detection antibody (EP22 at 1:1,000 dilution) was evaluated first by screening the signals of these specimens over the blank control (bovine serum albumin at 0.5 μg/unit). After screening, total tissue lysates from 4 to 6 specimens of middle range expression were pooled together and were used to develop a dose curve, along with the serially diluted purified EGFR proteins, as shown in **Supplementary Fig. 2**. The total protein amount loaded for QDB analysis was used for the absolute quantitation of EGFR protein levels in gastric cancer tissues.

IHC-based EGFR scoring

All EGFR scores from the IHC analysis were obtained from medical records. IHC analysis was performed by one of the 3 pathologists in the pathology department of Yantaishan Hospital in Yantai, China, from January 2015 to December 2017. For detection of EGFR protein levels, the slide was stained with standard streptavidin–biotin complex method with 3, 3′-diaminobenzidine as the chromogen using the 5B7 clone from Roche. EGFR scores were assessed based on a report with minor modifications [30]. More specifically, like the H score, the EGFR score was the product of intensity of staining multiplied by the percentage of stained cells. The intensity was categorized as follows: no staining as 0, light yellow as 1, brownish yellow, 2; and brown, 3. The percentage of positive cells was categorized as <10% as 0, 10%–25% as 1, 25%–50% as 2, and >50% as 3. For each specimen, the EGFR score was determined by converting the calculated product into 0 (0–1), 1+ (2–3), 2+ (4–6), and 3+ (>6).

QDB analysis

The QDB process has been described previously with minor modifications [28]. In brief, the final concentration of the FFPE tissue lysates was adjusted to 0.25 μg/μL, and 2 μL/unit was used for QDB analysis in triplicate. The loaded QDB plate was dried at room temperature (RT) for an hour before being blocked in blocking buffer (4% non-fat milk in TBST) for an hour. The plate was inserted into a 96-well microplate filled with 100 μL/well primary antibody (for clone EP22, 1:1,000 in blocking buffer) and incubated overnight at 4°C. Afterwards, the plate was rinsed twice with TBST and washed three times, each time in 10 minutes before it was incubated with a donkey anti-rabbit secondary antibody for 3 hours at RT. The plate was rinsed twice with TBST, washed 5×10 minutes and then inserted into a white 96-well plate pre-filled with 100 μL/well ECL working solution for 3 minutes. The chemiluminescent signals of the combined plate were quantified using a Tecan Infinite 200 Pro Microplate reader with the option “plate with cover” The results represent the average of 3 independent experiments.

The GIST lysate with pre-documented EGFR levels was included as an internal control in all assays to ensure consistency of the results. The individual assay was accepted only when the measured EGFR level of GIST internal control was within 80%–120% of the pre-determined level. Samples with chemiluminescent reading less than 2 fold over the blank were defined as non-detectable and entered as 0 for data analysis.

Developing putative EGFR cutoffs for prognosis of gastric cancer patients

The “surv_cutpoint” function of the “suvminer” R package in combination with the OS of these patients was used to identify the putative cutoff for prognosis of gastric cancer patients. A cutoff of 207.7 pmol/g was identified to have the best performance. For applicability, the cutoff was adjusted to 208 pmol/g.

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Youden's index was used to identify the putative EGFR cutoff based on EGFR scores using the IHC method. The patients were stratified either using 1+ as a cutoff to include 1+, 2+, and 3+ as EGFR+ and 0 as EGFR−; or 2+ as a cutoff to include 2+ and 3+ as EGFR+ and 0 and 1+ as EGFR−. Receiver operating characteristic (ROC) analysis was performed using absolutely quantitated EGFR levels in EGFR+ and EGFR subgroups to create an ROC curve. The Youden index was calculated as the maximum value of sensitivity and specificity of −1. The corresponding value from the QDB analysis was identified as the putative cutoff. An area under the ROC curve (AUC) of less than 0.5 was considered to indicate no discriminative capacity.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software Inc., San Diego, CA, USA) and R version 4.0.4, using a 2-sided statistical test. Missing values in the discrete data were defined as new categories. The results were reported as mean±standard deviation. Statistical significance was set at $P<0.05$. The endpoint of the OS analysis was defined as the time from surgery to death or the last follow-up. Patients who were lost to follow-up were excluded from analysis. Survival data for patients who were still alive at the date of the last follow-up were treated as censored.

Univariate Cox proportional hazard models fitted to OS were employed for HR and corresponding 95% CIs. Multivariable Cox models were utilized to examine the association between subtypes and OS, after adjusting for other clinical variables, such as age, tumor size, and TNM stage. Residuals that were analogous to Schoenfeld residuals in Cox models were used to check the proportionality assumption.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, following a protocol approved by the Ethics Committee of Yantaishan Hospital (YanshanLunZhun2021017 to Lei Xin, China). Informed consent was waived due to the use of archival tissues with retrospective, anonymized clinical data.

RESULTS

Clinicopathological characteristics of the patients

The clinicopathological characteristics of all the FFPE specimens are listed in **Table 1**. A total of 246 gastric cancer specimens were collected sequentially and non-selectively from local hospitals, with a median follow-up of 51 months (interquartile range, 50–53 months). All patients were at the M_0 stage. Patients with cancer spreading to distant organs or tissues (M_1) were excluded from the study. There were 73.17% male and 26.83% female. The majority of patients were poorly differentiated (89.84%). More than half of the patients were at the T3 stage based on the TNM staging system. Most patients had lymph node metastasis (64.23%).

None of the patients received neoadjuvant therapy or targeted therapy. There was insufficient information about the treatments received by individual patients. In general, in 50 patients with pathological T1 stage (pT1), tegafur/gimeracil/oteracil potassium capsule (S-1) were used for patients with lymph node metastasis, while others were closely monitored. Patients in stages II and III of the TNM staging system before 2016 received the FOLFOX regimen (leucovorin calcium [folinic acid], fluorouracil, and oxaliplatin). Patients from 2016 received

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Table 1. Clinicopathological characteristics of gastric cancer specimens

Characteristics	Cases (n=246)
Age (yr)	
<60	88 (35.77)
≥60	158 (64.23)
Sex	
Male	180 (73.17)
Female	66 (26.83)
Histologic grade	
Well/moderately differentiated	25 (10.16)
Poorly differentiated	221 (89.84)
Tumor size (cm)	
Small (<5)	120 (48.78)
Large (≥5)	120 (48.78)
Unknown	6 (2.44)
Lymph node metastasis	
No metastasis	88 (35.77)
Metastasis	158 (64.23)
TNM stage*	
1	62 (25.20)
2	43 (17.48)
3	141 (57.32)
4	0 (0)
Vascular cancer embolus	
Yes	123 (50.00)
No	119 (48.37)
Unknown	4 (1.63)
Nerve invasion	
Yes	102 (41.46)
No	140 (56.91)
Unknown	4 (1.63)
Lauren's classification	
Intestinal type	50 (20.33)
Diffuse type	85 (34.55)
Mixed type	84 (34.15)
Unknown	27 (10.98)
$EGFR_{IHC}$	
0	14 (5.69)
1+	79 (32.11)
2+	83 (33.74)
3+	52 (21.14)
Unknown	18 (7.32)
Survival	
Yes	156 (63.41)
No	89 (36.18)
Unknown	1 (0.41)

Values are presented as number (%).
EGFR = epidermal growth factor receptor; IHC = immunohistochemistry.
*Based on the American Joint Committee on Cancer (AJCC) 8th edition.

the SOX regimen (oxaliplatin and S-1). Some stage III patients received the DOS regimen later (docetaxel, oxaliplatin, and S-1).

Measuring EGFR protein levels in gastric cancer specimens

The distribution of EGFR protein levels (n=246) is shown in **Fig. 2A**. The EGFR protein levels ranged from 0 (undetectable level) to 772.6 pmol/g, with median at 94 pmol/g, and mean at 108.7±6.07 pmol/g. EGFR levels were plotted against their respective IHC scores (**Fig. 2B**). In the 0 group (n=14), EGFR levels were from 0 to 197 pmol/g, with average at 96.54 pmol/g; in the 1+ group (n=79), EGFR levels were from 0 to 374.4 pmol/g, with mean

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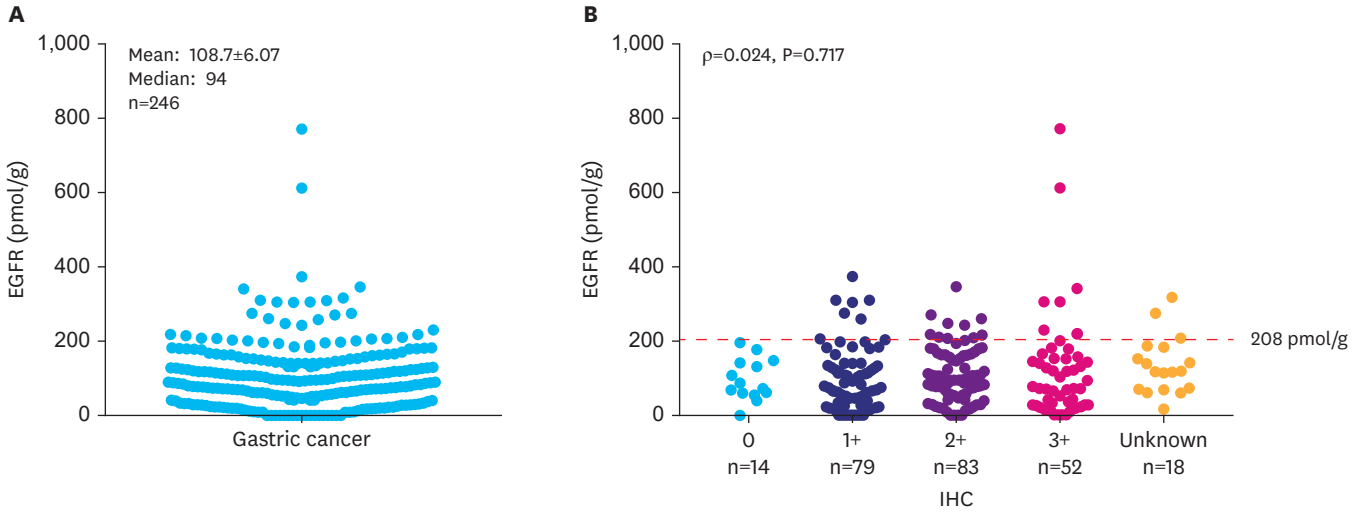

Fig. 2. Distribution of absolutely quantitated EGFR protein levels in gastric cancer specimens (A), and their distribution grouped by their IHC scores among gastric cancer specimens (B). Total protein lysates were prepared from 2×5 μm FFPE slices for QDB measurement using a rabbit monoclonal anti-EGFR antibody (EP22). As shown in **Fig. 2B**, the EGFR protein levels of individual FFPE gastric cancer specimens are grouped based on their IHC scores. A putative cutoff at 208 pmol/g to stratify patients of EGFR+ and EGFR subgroups based on OS survival analysis of the patients is marked as a dashed line in the figure. There was no significant difference between any 2 IHC groups when they were analyzed using the Student's t-test. P-values may be found in **Supplementary Table 1**.
EGFR = epidermal growth factor receptor; IHC = immunohistochemistry; FFPE = formalin-fixed paraffin-embedded; QDB = quantitative dot blot method; OS = overall survival.

at 97.9 pmol/g; in the 2+ group (n=83), EGFR levels were from 0 to 347.5 pmol/g, with mean at 109 pmol/g; and in the 3+ group (n=52), EGFR levels were from 0 to 772.6 pmol/g, with mean at 118.9 pmol/g. No statistical difference was detected between any 2 IHC groups when they were analyzed using Student's t-test (**Supplementary Table 1**). When analyzed with Spearman's correlation analysis, the results from the QDB method were poorly associated with IHC scores (ρ=0.024, P=0.717).

We also evaluated the correlations of EGFR levels, measured using IHC and QDB methods, with other clinical pathological factors (**Supplementary Table 2**). EGFR levels from either IHC or QDB methods were not associated with any of these clinicopathological factors. In contrast, there were correlations among multiple clinicopathological factors (**Supplementary Table 3**). More specifically, TNM stage was found to be correlated with tumor size, Lauren's classification, histological tumor grade, vascular tumor embolus, and nerve invasion with statistical significance.

EGFR as an independent prognostic factor of OS

Next, we investigated the prognostic role of EGFR in gastric cancer using both univariate and multivariate OS analyses (**Table 2**). The EGFR protein levels were negatively associated with OS, with HR of 2.15 (95% CI, 1.23–3.75; P=0.007), and 1.92 (95% CI, 1.05–3.53; P=0.034) by univariate and multivariate Cox regression OS analysis, respectively. In addition, among traditional clinical factors, tumor size, Lauren's classification, TNM stage, vascular tumor embolus, and nerve invasion were all found to be statistically significant in univariate Cox regression analysis. However, only TNM stage and vascular tumor embolus were found to be statistically significant in the multivariate Cox regression OS analysis. It should be mentioned that IHC scores were not found to be independent prognostic factors in either univariate or multivariate Cox regression OS analysis (**Table 2** and **Supplementary Table 2**).

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Table 2. Univariate and multivariate Cox regression of overall survival with EGFR levels

Variables	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender	1.44	0.86–2.41	0.168	-	-	-
Age	1.11	0.72–1.73	0.634	-	-	-
Tumor size	1.74	1.13–2.68	0.012	1.24	0.77–1.99	0.371
Lauren's classification	1.39	1.04–1.85	0.025	0.98	0.70–1.36	0.892
Histologic grade	1.80	0.79–4.12	0.165	-	-	-
TNM stage	2.87	1.96–4.20	<0.0001	1.94	1.16–3.24	0.011
Vascular cancer embolus	3.45	2.12–5.61	<0.0001	1.86	1.06–3.25	0.030
Nerve invasion	3.03	1.95–4.72	<0.0001	1.46	0.84–2.56	0.184
$EGFR_{QDB}$	2.15	1.23–3.75	0.007	1.92	1.05–3.53	0.034
$EGFR_{IHC}$	0.76	0.33–1.75	0.524	-	-	-

EGFR = epidermal growth factor receptor; HR = hazard ratio; CI = confidence interval; QDB = quantitative dot blot method; IHC = immunohistochemistry.

Comparison of EGFR levels assessed with QDB and IHC respectively as prognostic factor of OS

A putative cutoff at 208 pmol/g was identified using "surv_cutpoint" function of the "suvminer" R package in combination with the OS of these patients to convert them into EGFR− (≤208 pmol/g) and EGFR+ (>208 pmol/g) subgroups. Its effectiveness was used to predict 3-year survival probability (3y SP) between EGFR+ and EGFR− subtypes using Kaplan-Meier analysis, as shown in **Fig. 3**. As shown in **Fig. 3A**, EGFR− patients had 3y SP at 68% (95% CI, 62%–75%) vs. 42% (95% CI, 26%–67%) for EGFR+ patients. P=0.002 from the log rank test.

Kaplan-Meier OS analysis was also performed based on the IHC scores in **Fig. 3B**. The 2+ group had the best 3y SP at 73% (95% CI, 64%–83%), while those in the 0, 1+, and 3+ groups were 57% (95% CI, 36%–90%), 54% (95% CI, 44%–67%), and 73% (95% CI, 64%–83%), respectively. The 3y SP of 3+ patients was highly similar to that of 2+ patients, with a sudden drop in the next 2 years. When analyzed together, we achieved P=0.056 from the log rank test.

In many clinical trials, all patients with any expression of EGFR protein (IHC scores ≥1+) were considered EGFR+ patients. Kaplan-Meier OS analysis was performed accordingly (**Fig. 3C**). We obtained P=0.520 from the log rank test, with 3y SP for EGFR− at 57% (95% CI, 0.36–0.90), and for EGFR+ at 66% respectively (95% CI, 0.60–0.72).

Alternatively, we also used 2+ as the cutoff in **Fig. 3D** to stratify patients into EGFR+ (2+ and 3+) and EGFR− (0 and 1+) groups. We were able to obtain P=0.010 from log rank test with 3y SP for EGFR+ at 72% (95% CI, 0.65–0.80) and for EGFR− at 55% (95% CI, 0.46–0.66) based on the Kaplan-Meier OS analysis. It should be noted that in both cases, contradictory to the results based on the QDB method, patients with EGFR+ based on IHC analysis were shown to have better prognosis than those with EGFR-.

We also explored putative cutoffs using Youden's index based on EGFR scores from IHC analysis. The ROC curve was generated using 1+ and 2+ as cutoffs. When patients were stratified using 1+ as the cutoff for EGFR+ (1+, 2+, and 3+) and EGFR− (0), we achieved an AUC of the ROC curve at 0.499, indicating that 1+ as the cutoff lacked discriminative capacity for prognostic purposes.

Alternatively, we used 2+ as a cutoff to stratify patients into EGFR+ (2+ and 3+) and EGFR− (0 and 1+). We were able to achieve the AUC of the ROC curve at 0.537 and identified the putative cutoff at 143 pmol/g based on Youden's index. When patients were stratified using this cutoff,

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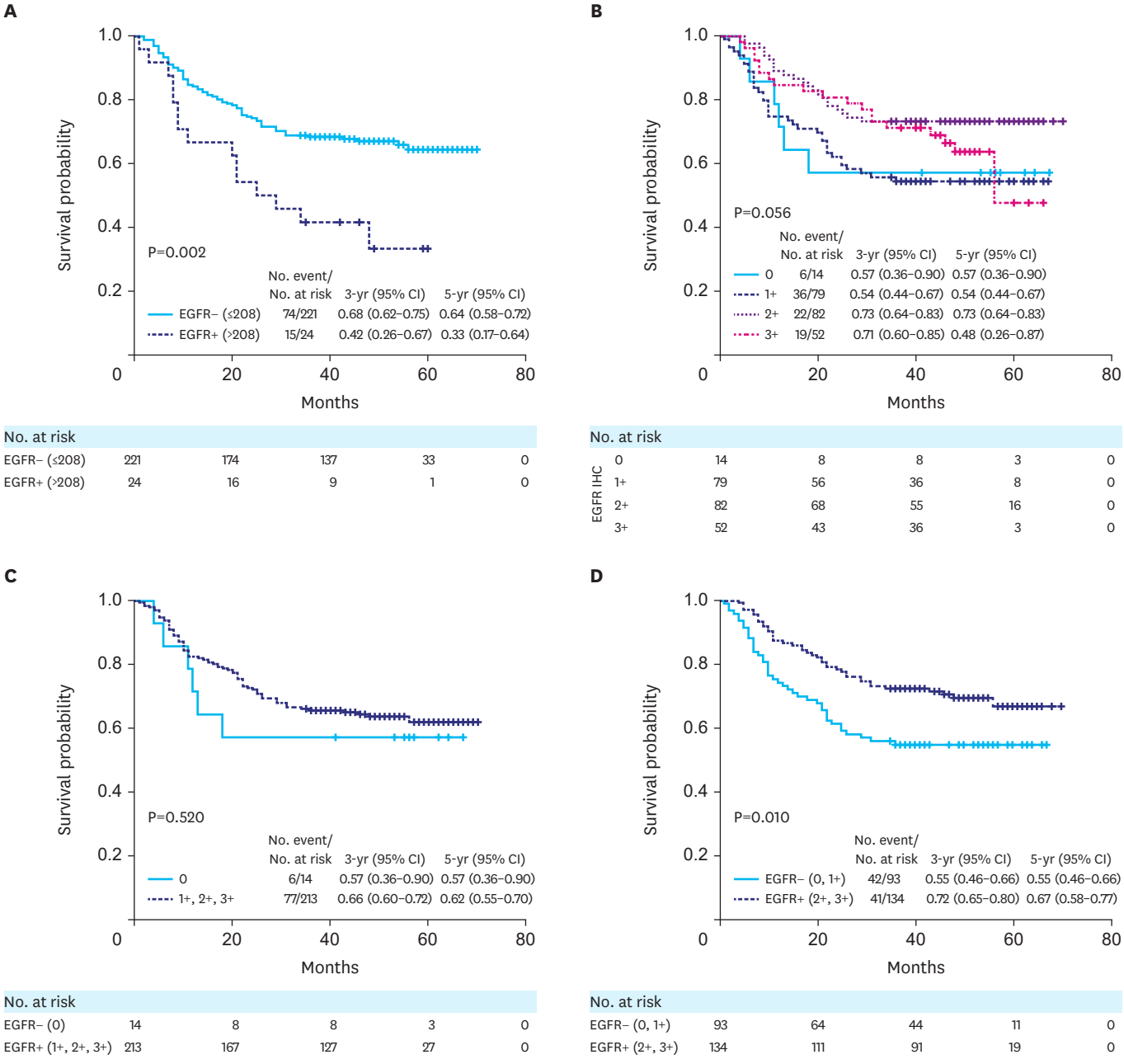

Fig. 3. Kaplan-Meier OS analysis of gastric cancer patients based on their EGFR protein levels measured using QDB (A) or IHC methods (B-D). (A) Absolutely quantitated EGFR protein levels were used to stratify patients using a cutoff of 208 pmol/g into EGFR+ (n=24) and EGFR– groups (n=221) for Kaplan-Meier OS analysis, with 3y SP at 42% (95% CI, 26%–67%) for EGFR+ vs. 68% (95% CI, 62%–75%) for EGFR– patients (log rank test, P=0.002); (B) IHC scores of EGFR protein were used to stratify patients into 0 (n=14), 1+ (n=79), 2+ (n=82), and 3+ (n=52) groups for Kaplan-Meier OS analysis, with 3y SP at 57% (95% CI, 36%–90%), 54% (95% CI, 44%–67%), 73% (95% CI, 64%–83%) and 71% (95% CI, 60%–85%), respectively (log rank test, P=0.056). (C) The patients were stratified using IHC-based EGFR scores of 1+ as cutoff into EGFR– (0 only) and EGFR+ (1+, 2+ and 3+) subgroup for Kaplan-Meier OS analysis. The 3y SP for EGFR– was at 57% (95% CI, 36%–90%) vs. 66% (95% CI, 60%–72%) for EGFR+ patients (log rank test, P=0.520). (D) The patients were stratified using IHC-based EGFR score of 2+ as cutoff into EGFR– (0 and 1+) and EGFR+ (2+ and 3+) subgroups for Kaplan-Meier OS analysis. The 3y SP for EGFR– was 55% (95% CI, 46%–66%) vs. 72% (95% CI, 65%–80%) for EGFR+ patients (log rank test, P=0.010).
OS = overall survival; EGFR = epidermal growth factor receptor; QDB = quantitative dot blot method; IHC = immunohistochemistry; FFPE = formalin-fixed paraffin-embedded; 3y SP = 3-year survival probability; CI = confidence interval.

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we measured 3y SP of EGFR− patients at 66% (95% CI, 60%–74%) vs. 64% (95% CI, 54%–77%) for EGFR+ patients, with P=0.760 from the rank test. Thus, we were unable to dichotomize patients based on IHC scores using Youden's index, possibly on account of the poor correlation between results from QDB and IHC analyses (**Supplementary Fig. 3** and **Fig. 2B**).

DISCUSSION

In this study, we presented the first practical method to measure EGFR protein levels in gastric cancer tissues, objectively and quantitatively, for daily clinical practice. Its effectiveness was demonstrated preliminarily by showing that EGFR was a negative prognostic factor for OS of gastric cancer patients through statistical analysis, a conclusion that cannot be repeated using IHC scores provided by the local hospital.

EGFR is the first member of the HER family to be cloned from A431, a squamous carcinoma cell line. Overexpression of this protein has been observed in multiple types of cancer, including breast, lung, colorectal, and head and neck cancers [31]. The prognostic role of EGFR has also been well demonstrated in these types of cancers, with the approval of cetuximab and panitumumab for treatment of colorectal and head and neck squamous cancer.

The prognostic role of EGFR in gastric cancer has also been extensively investigated. Multiple studies have supported the prognostic role of EGFR in gastric cancer, including studies in which EGFR amplification status was demonstrated to be a strong negative prognostic factor for gastric cancer [32-34]. However, when assessed using IHC, the ratio of EGFR+ patients in these studies varied from 9% to 16.5% to 27% [32-34]. Overall, the percentage of gastric cancer patients overexpressing EGFR proteins was reported to vary from 2% to 81% among several studies [32]. Clearly, while studies at the DNA level strongly support the prognostic role of EGFR in gastric cancer [32,35-37], the lack of standardization of IHC methods poses a significant challenge to the interpretation of these results.

The performance of IHC-based EGFR assessment in gastric cancer is further challenged by the following factors: First, gastric cancer is known to be more heterogeneous than breast cancer [38]; second, EGFR expression is also found to be more heterogeneous than Her2 [39]. Third, the overall situation is further aggregated by a narrow range of EGFR protein expression levels among gastric patients, making visual scoring difficult. As a comparison, the distribution of EGFR levels was from 0 to 772.6 pmol/g (n=246) in gastric cancer specimens vs. 0 to 31,310 pmol/g for Her2 in breast cancer specimens [27].

All these factors might have contributed to the inadequacy of the IHC method in this study. First, when EGFR levels were measured absolutely and quantitatively, there were no statistically significant differences between any 2 of the 4 IHC groups (0/1+/2+/3+) using Student's t-test (**Supplementary Table 1**); second, the IHC scores of EGFR protein were not an independent prognostic factor when analyzed with Cox regression OS analysis (**Supplementary Table 2**); and third, the survival probability was not related to the IHC scores of patients when analyzed with Kaplan-Meier OS analysis at all. These observations are explained in **Fig. 2B**. For prognostic purposes, a large number of EGFR- specimens (those with EGFR levels ≤208 pmol/g) within each IHC group would overwhelm any EGFR+ related events in clinical studies.

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However, the seemingly contradictory roles of EGFR in the prognosis of gastric cancer patients from IHC and QDB analyses in this study should also not be over-interpreted (**Fig. 3**). The IHC scores used in this study were collected from documented medical records. These were the accumulated results analyzed by several pathologists in the same hospital over a 3-year period. In other words, these IHC scores were presented as real-world data. Thus, they are negatively impacted by multiple factors, including inter-and intra-personal subjectivity over time. For example, our study listed as much as 21.14% of the specimens categorized as 3+. However, in most reported studies, the percentage of 3+ patients was reported to be less than 10% [32-34,40]. It is very likely that the difference between IHC and QDB-based EGFR assessment was exaggerated for the same reason. Perceivably, re-analyzing all these specimens by an individual pathologist in a short time should significantly improve the performance of IHC. Nonetheless, this effort cannot change the qualitative nature of the IHC method, and it cannot eliminate the inherent subjectivity and inconsistency associated with this technique.

Needless to say, necessary steps must be taken before the QDB method can be adopted in clinical practice. Above everything else, a validation study is urgently needed to evaluate this method for clinical use. The current study was a retrospective observational study. It is limited by multiple factors, including the small sample size and lack of detailed treatment information for individual patients. The proposed cutoff also needs to be validated in an independent cohort of patients with gastric cancer. Thus, the conclusions of this study should not be interpreted. Rather, this study should be considered as a POC study only.

Fortunately, the QDB method is an ELISA-like high-throughput method. This requires a minimum amount of starting material for analysis. For most biomarkers, including EGFR, two 5 μm FFPE slices are sufficient for the absolute quantitation of these biomarkers in tumor tissues. Considering the stability of FFPE slices and their convenience of transportation, a few central laboratories should support the clinical needs of most countries.

In this regard, a retrospective prospective study may be initiated with specimens of past clinical trials to re-evaluate the prognostic and predictive roles of EGFR based on QDB analysis [41,42]. For example, EGFR protein levels may be measured blindly using specimens available from various clinical trials of cetuximab or other anti-EGFR drugs to evaluate their predictive role for these drugs.

A standard operation procedure is also required before it can be used in clinical trials and clinical practice. The key reagents for QDB analysis were antibodies and recombinant EGFR protein. In this study, both the antibody and recombinant protein were obtained commercially, thus significantly reducing the difficulty of standardization. In addition, the EP22 antibody used in this study has already been designated as the Analyte-Specific Reagent by the U.S. Food and Drug Administration and in vitro diagnostic by both Canada and the European Union.

The limitations of IHC methods have been well acknowledged, and efforts have been devoted to finding a better alternative. The fluorescence in situ hybridization (FISH) technique is suggested to be a more consistent method for identifying EGFR-positive patients for cetuximab or other anti-EGFR antibody drugs in several studies [10,14,22,43,44]. Selective reaction monitoring-mass spectrometry has also been used successfully to measure EGFR protein levels absolutely and quantitatively in NSCLC tissues [45]. However, both these methods pose technical challenges for daily clinical practice. In addition, FISH method only detects changes

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at the DNA level, and it is not a true quantitative method to allow definition of an optimized threshold through outcome analysis to guide patients for anti-EGFR therapies [14].

While a putative cutoff of 208 pmol/g through outcome analysis for prognosis of gastric cancer patients was proposed in this study, cautious interpretation of this putative cutoff is warranted. Its validity remains to be confirmed in an independent cohort of patients. It also remains to be seen if the above-mentioned cutoff is the same as the one we were seeking to guide patients for these monoclonal anti-EGFR antibody drugs. These questions can only be answered through outcome analysis (PFS, OS, or overall response rate [ORR]) of patients receiving these drugs in much better controlled retrospective and/or prospective clinical trials in the near future.

Nonetheless, we showed in this study that an optimized cutoff can be identified through outcome analysis using absolutely quantitated EGFR levels. An optimized threshold to stratify patients will be identified from continuous variables through outcome analysis (PFS, OS, or ORR) of patients receiving anti-EGFR therapies. The absolute nature of the QDB results also enables seamless integration of multiple clinical studies over time to fine-tune the putative cutoffs for patient selection to achieve maximum benefit of anti-EGFR therapies in the near future.

The issue of tumor heterogeneity remains a challenge for the development of targeted therapies for gastric cancer patients, even after validation of the QDB method. Similar to IHC, the QDB method is used to analyze tissue resection or biopsies. Considering tumor heterogeneity at all levels, analysis limited to tumor per se, or more specifically, a few slices of tumor tissues, may only provide partial information. In this regard, the blood-based circulating tumor DNA (ctDNA) technique provides an unmatched advantage over both IHC and QDB to provide a comprehensive assessment of EGFR mutations at all levels. Thus, ctDNA techniques may be combined with the QDB method to offer a holistic view of the tumor status of individual patients in the future.

The potential impact of KRAS and NARS mutations on the prognostic role of EGFR was not investigated in this study. These mutations are known to undermine the benefits of anti-EGFR monoclonal antibody drugs in colorectal cancer patients [46]. They are also considered as one of the contributing factors to the failure of the EXPAND trial [47]. However, there are limited studies on their roles in gastric cancer. A new anti-EGFR antibody drug was also found to be effective regardless of the KRAS status in several cell lines [48,49]. Future studies are needed to evaluate their impact on the prognostic role of EGFR protein in gastric cancer and to explore the putative relationship between their ctDNA levels and EGFR protein expression for clinical use.

In conclusion, a QDB-based immunoassay was introduced to measure EGFR protein levels absolutely, quantitatively and objectively in gastric cancer specimens. The adoption of this assay may help define EGFR protein as a predictive biomarker to accelerate clinical trials aiming to expand cetuximab, panitumumab, and necitumumab use in gastric cancer patients.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Comparison of EGFR levels between different IHC groups using Student's t-test

Click here to view

Supplementary Table 2
Multivariate Cox regression OS analysis of gastric cancer patients using EGFR protein IHC scores and QDB level

Click here to view

Supplementary Table 3
Correlation between clinicopathologic features and EGFR expression levels measured by IHC and QDB, respectively

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Supplementary Fig. 1
IHC results of GIST (1+) used as internal control for QDB analysis. A 5 μm slice of the GIST specimens used as internal control of QDB analysis was stained with standard streptavidin–biotin complex method with 3, 3′-diaminobenzidine as the chromogen using 5B7 clone from Roche. The image was magnified ×100 in (A) and ×200 in (B).

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Supplementary Fig. 2
Defining the linear range of QDB measurements with anti-EGFR antibody, EP22. (A) Recombinant protein from commercial source was serially diluted as indicated in the figure. Total of 2 μL/unit was loaded to QDB plate for QDB measurement using EP22 clone. The linear range of QDB measurement was defined as the region where the coefficient of determination (r) was above 0.99. (B) Pooled specimen prepared from 2×5 μm FFPE slices obtained from 4 patients of mid-range EGFR expression based on initial QDB screening was used to define the linear range of QDB method for EGFR measurements using EP22 clone. The linear range was defined as the region where the coefficient of determination (r) was above 0.99.

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Supplementary Fig. 3
Using Youden's index to identify putative cutoff based IHC-based EGFR assessment using 2+ as cutoff. The patients were stratified based on their IHC-based EGFR scores, using 2+ as cutoff, into EGFR− (0 and 1+) and EGFR+ (2+ and 3+) subgroups. (A) The absolutely quantitated EGFR levels of these 2 subgroups were used for ROC analysis to calculate the specificity and sensitivity of the assay. Youden's index was calculated as the maximum value of specificity + sensitivity − 1. The absolute quantitated EGFR levels matching the Youden's index was identified as the optimized cutoff at 143 pmol/g. (B) The patients were stratified using 143 pmol/g as cutoff to stratified as EGFR− (≤143) and EGFR+ (>143) subgroups for Kaplan-Meier OS analysis, with P=0.760 from log rank test.

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