Higher nuclear EGFR expression is a better predictor of survival in rectal cancer patients following neoadjuvant chemoradiotherapy than cytoplasmic EGFR expression

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Abstract. The aim of the present study was to investigate the prognostic value of cytoplasmic (-C) and nuclear epidermal growth factor receptor (EGFR-N) expression in rectal cancer patients following neoadjuvant concurrent chemoradiotherapy (CCRT). A total of 172 newly diagnosed rectal cancer patients post-neoadjuvant CCRT and curative surgery, treated between January 1998 to December 2008, were included. Pathological tissues used for evaluation were biopsy specimens obtained prior to CCRT, and specimens collected at surgery. EGFR expression in the nucleus and cytoplasm was assessed by immunohistochemistry tests. An intensity of 3+ EGFR reactivity in the cytoplasm (and/or membrane) of tumor cells was defined as overexpression of EGFR-C. The cutoff percentage of immunoreactive tumor cells for EGFR-N overexpression was 50%. Expression levels of EGFR-C and EGFR-N were further analyzed by clinicopathological features for 5-year survival disease-specific survival (DSS), local recurrence-free survival (LRFS) and metastasis-free survival (MeFS). The results revealed that 20.9 and 23.3% of the cohort had high EGFR-N and EGFR-C expression, respectively. EGFR-N overexpression was significantly associated with advanced pre-treatment tumor stage (T3 and 4; P=0.017) and post-treatment tumor stage (T3 and 4; P<0.001). In univariate analysis, EGFR-N overexpression was significantly associated with poorer DSS (P=0.0005), MeFS (P=0.0182), and LRFS (P=0.0014). Furthermore, it remained an independent prognosticator of worse DSS [P=0.007, hazard ratio (HR)=2.755] and LRFS (P=0.0164, HR=3.026) in multivariate analysis. Overexpression of EGFR-N, and not EGFR-C, may help identify rectal cancer patients who have an increased risk of local recurrence and poor survival following neoadjuvant CCRT.

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Abbreviations: CCRT, concurrent chemoradiotherapy; AJCC, American Joint Committee on Cancer; TNM, tumor-node-metastasis; EGFR, epidermal growth factor receptor; EGFR-C, cytoplasmic EGFR; EGFR-N, nuclear EGFR; Pre-Tx, pre-treatment; Post-Tx, post-treatment; DSS, disease-specific survival; LRFS, local-recurrence-free survival; MeFS, metastases-free survival; HR, hazard ratio; CI, confidence interval

Key words: epidermal growth factor receptor, nuclear, rectal cancer, chemoradiotherapy, survival analysis

Introduction

Rectal cancer is an increasing, and important disease worldwide (1). For patients with American Joint Committee on Cancer (AJCC) stage II or III disease, neoadjuvant concurrent chemoradiotherapy (CCRT) followed by radical proctectomy remains the preferred therapy as it has been shown to provide better local control, greater anal preservation, and lesser toxicity (2). However, up to 10-20% of these patients will eventually develop local or distal disease recurrence (3).

Rectal cancer post neoadjuvant CCRT or not are all staged by using the AJCC tumor-node-metastasis (TNM) staging system, and further adjuvant treatment is recommended. There is no modification of staging for patients who receive neoadjuvant CCRT. Prediction of prognosis using TNM staging is suboptimal, and survival of patients with the same stage disease can vary markedly. Therefore, additional prognostic biomarkers to aid in risk stratification and identify patients who may benefit from more intensive treatment may help to improve outcomes.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the HER family of receptor tyrosine kinases, and promotes multiple signaling cascades for cellular survival, such as RAS/MAPK, PI3K/Akt, Stat, and Src (4). Overexpression of cytoplasmic EGFR (EGFR-C) is associated with tumor differentiation, proliferation, and migration, and has been extensively studied as the most important cancer molecular target in recent decades (5,6). Recently, studies have reported that EGFR family members can be transported from the plasma membrane to the nucleus, and regulate target genes involved in cell proliferation and angiogenesis (7,8). The prognostic significance of nuclear EGFR (EGFR-N) has been demonstrated, and is highly associated with tumor progression and worse overall survival (OS) (9,10). It has also been shown to be associated with enhanced resistance to systemic and radiation therapy (7). Therefore, overexpression of EGFR-N is frequently a poor prognostic factor. However, little is known about the relevance of EGFR-N in rectal cancer patients treated with neoadjuvant CCRT.

The aim of this study was to evaluate the clinical correlates of EGFR-C and EGFR-N expression in rectal cancer patients after neoadjuvant CCRT, and also to compare the impact of EGFR-N and EGFR-C expression on prognosis.

Materials and methods

Ethics statement. The present study was reviewed and approved by the Institutional Review Board of Chi-Mei Medical Center in Taiwan (Tainan, Taiwan; IRB, CMFHR10501-008). The requirement for informed consent was waived as all identifying information was removed from the dataset prior to analysis.

Patient demographic characteristics and tumor specimens. This retrospective study was performed on formalin-fixed paraffin-embedded (FFPE) tissue specimens from 172 newly diagnosed rectal adenocarcinoma patients treated at Chi-Mei Medical Center between 1998 and 2004. The data was collected and analyzed in February 2014. All patients received preoperative 5-fluorouracil-based chemotherapy concomitant with radiotherapy (45-50 Gy), followed by radical proctectomy with total mesorectal excision. The clinical stage was determined by endoscopic ultrasound (EUS), abdominal computed tomography (CT), or magnetic resonance imaging (MRI) findings. Adjuvant systemic chemotherapy was given if the post-treatment (Post-Tx) tumor or nodal status was beyond T3 or N1. All patients were regularly monitored after diagnosis until death or last follow-up. Patients who had a previous cancer history, distal metastasis at diagnosis, or were unable to complete a full course of CCRT were excluded. The histopathological parameters were reviewed by our pathologist (Dr. Li) who was blind to the patients' clinical data.

The data extracted from the medical records included the date of diagnosis, age, sex, clinical TNM stage, pathological characteristics such as lymphovascular and perineural invasion, chemotherapy regimen and timing, and cause of death. All patients were re-staged and re-graded according to the 7th edition of the AJCC staging system, and World Health Organization (WHO) classification of Tumors of the Colon and Rectum. The primary endpoints were 5-year disease-specific survival (DSS), local recurrent-free survival (LRFS), and metastases-free survival (MeFS) rates. Deaths due to cancer were defined as valid events, and deaths secondary to other causes were censored.

Immunohistochemistry analysis. Immunohistochemistry of tissue microarray (TMA) slides and whole sections were prepared as previously described (11). Briefly, tissue sections from Pre-Tx rectal tumor biopsies were deparaffinized and rehydrated for EGFR immunostaining. Overexpression of EGFR-C was defined as an intensity of 3+ EGFR reactivity seen in the cytoplasm (and/or membrane) of tumor cells (12). To define overexpression, the cutoff percentage of immunoreactive tumor cells was 50% for EGFR-N in this analysis (13).

Statistical analysis. The results are presented as the mean ± standard deviation. All statistical analyses and graphics were performed using IBM SPSS statistical software for Windows, v14.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. Examination of the associations of EGFR expression with clinicopathological features was conducted using the chi-square test. The Kaplan-Meier method was used to analyze the cumulative 5-year DSS, LRFS, and MeFS rates, and rates were compared using the log-rank test to evaluate prognostic differences between subgroups. The primary event was defined as disease-specific mortality, and survival curves were calculated from the date of diagnosis. Those parameters that demonstrated prognostic significance in the univariate analysis were input into the Cox multivariate regression analysis.

Results

Patient demographic and clinical characteristics are summarized in Table I. A total of 172 rectal cancer patients who underwent neoadjuvant CCRT and radical proctectomy with total mesorectal excision from January 1998 to December 2008 were included. The median age was 63 years (range: 22-88 years). The percentages of patients with initial stage I, II, and III disease were 41.9, 29.9, and 28.1% and all stage II, III patients received adjuvant chemotherapy Fifteen (8.7%) patients presented with lymphovascular invasion, and 5 (2.9%) with perineural invasion.

The results of EGFR immunostaining to investigate EGFR expression in the cytoplasm and the nucleus of rectal tumor tissues are shown in Fig. 1. Of the patients, 20.9% had EGFR-N overexpression, 23.3% EGFR-C overexpression and 5.2% have both high EGFR-N and high EGFR-C expression. EGFR-N overexpression was significantly related to advanced pre-Tx tumor T-stage (T3, 4; $P=0.017$) and post-Tx tumor T-stage (T3, 4; $P<0.001$). EGFR-C overexpression was significantly related to pre-Tx tumor T-stage (T3, 4; $P=0.013$), post-Tx tumor

Table I. Associations and comparisons between Nuclear/Cytoplasmic epidermal growth factor receptor expression and clinico-pathological factors in 172 rectal cancer patients.

			EGFR-N (n)			EGFR-C (n)		
Parameter	Variable	No. of cases	Low Exp.	High Exp.	P-value	Low Exp.	High Exp.	P-value
Gender	Male	108	84	24	0.0768	82	26	0.741
	Female	64	51	13		50	14	
Age	<60	65	51	14	0.995	49	16	0.742
	≥60	107	84	23		83	24	
Pre-Tx tumor status (Pre-T)	T1-T2	81	70	11	0.017[a]	69	12	0.013[a]
	T3-T4	91	65	26		63	28	
Pre-Tx nodal status (Pre-N)	N0	125	101	24	0.229	103	22	0.004[b]
	N1-N2	47	34	13		29	18	
Post-Tx tumor status (Post-T)	T1-T2	86	77	9	<0.001[c]	71	15	0.071
	T3-T4	86	58	28		65	25	
Post-Tx nodal status (Post-N)	N0	123	99	24	0.312	99	24	0.066
	N1-N2	49	36	13		33	16	
Lymphovascular invasion	Absent	157	123	34	0.881	124	33	0.025[a]
	Present	15	12	3		8	7	
Perineural invasion	Absent	167	131	36	0.933	130	37	0.048[a]
	Present	5	4	1		2	3	
Cytoplasmic EGFR expression	Low Exp.	132	105	27	0.540	n/a	n/a	n/a
	High Exp.	40	30	10		n/a	n/a	

[a]P<0.05; [b]P<0.01; [c]P<0.001. EGFR-C, cytoplasmic epidermal growth factor receptor; EGFR-N, nuclear epidermal growth factor receptor; Pre-Tx, pre-treatment; Post-Tx, post-treatment; Exp., expression; n/a, not applicable.

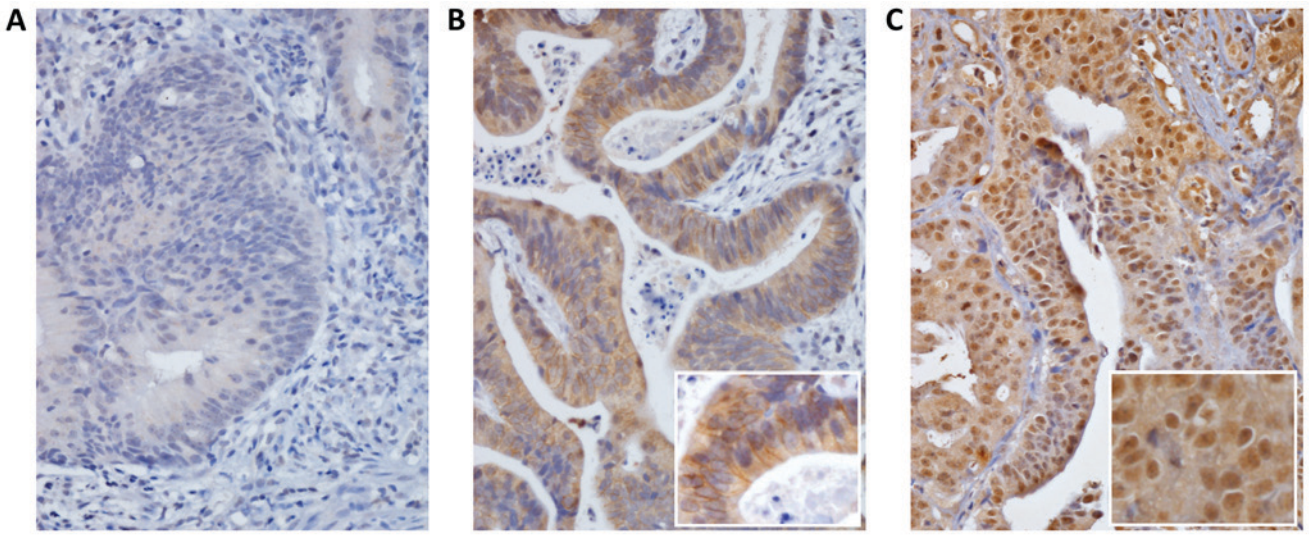

Figure 1. EGFR-C and EGFR-N expression by staining intensity using immunohistochemistry in rectal cancer tissue microarray. (A) EGFR low expression (magnification, x400); (B) EGFR-C high expression; (magnification: Main, x400; insert, x600) and (C) EGFR-N high expression (magnification: Main, x400; insert, x600). EGFR-C, cytoplasmic epidermal growth factor receptor; EGFR-N, nuclear epidermal growth factor receptor.

T-stage (T3, 4; P=0.004), lymphovascular invasion (P=0.025), and perineural invasion (P=0.048).

Univariate and multivariate survival analyses for factors predicting survival. In univariate analyses (Table II), post-Tx tumor status and nuclear EGFR expression were significantly correlated with poorer 5-year DSS, LRFS, and MeFS rate (all, P<0.05).

Pre-Tx nodal status, lymphovascular invasion, and perineural invasion were negatively associated with 5-year DSS and LRFS (all, P<0.05). Pre-Tx tumor status was significantly associated with poorer 5-year DSS only (P=0.0484). Multivariate analyses

Table II. Univariate log-rank analysis for important clinicopathological variables and epidermal growth factor receptor expression.

			DSS		LRFS		MeFS	
Parameter	Variable	No. of cases	No. of events	P-value	No. of events	P-value	No. of events	P-value
Gender	Male	108	20	0.6027	5	0.3096	14	0.1047
	Female	64	11		17		15	
Age	<60	65	13	0.3259	7	0.7575	17	0.0052[b]
	≥60	107	18		15		12	
Pre-Tx tumor status (Pre-T)	T1-T2	81	10	0.0484[a]	7	0.0836	10	0.1288
	T3-T4	91	21		15		19	
Pre-Tx nodal status (Pre-N)	N0	125	19	0.0059[b]	12	0.0025[b]	18	0.0866
	N1-N2	47	21		10		11	
Post-Tx tumor status (Post-T)	T1-T2	86	12	0.0006[c]	5	0.0056[b]	8	0.0123[a]
	T3-T4	86	34		17		21	
Post-Tx nodal status (Post-N)	N0	123	31	0.3442	15	0.6267	20	0.8403
	N1-N2	49	15		7		9	
Lymphovascular invasion	Absent	157	38	0.0107[a]	17	0.0023[b]	26	0.7236
	Present	15	8		5		3	
Perineural invasion	Absent	167	43	0.0297[a]	20	0.0083[b]	28	0.8157
	Present	5	3		2		1	
Cytoplasmic EGFR expression	Low Exp.	132	33	0.6480	16	0.6884	20	0.3363
	High Exp.	40	13		6		9	
Nuclear EGFR expression	Low Exp.	135	28	0.0005[c]	12	0.0014[b]	18	0.0182[a]
	High Exp.	37	18		10		11	

[a]P<0.05; [b]P<0.01; [c]P<0.001. EGFR-C, cytoplasmic epidermal growth factor receptor; EGFR-N, nuclear epidermal growth factor receptor; Pre-Tx, pre-treatment; Post-Tx, post-treatment; DSS, disease-specific survival; LRFS, local-recurrence-free survival; MeFS, metastases-free survival; Exp., expression.

results are shown in Table III. EGFR-N, but not EGFR-C, was an independent prognostic factor for 5-year DSS (hazard ratio [HR]=2.755, 95% confidence interval [CI]: 1.313-5.783) and LRFS (HR=3.026, 95% CI: 1.224-7.281), but not for MeFS (HR=1.853, 95% CI: 0.852-4.032). Pre-Tx nodal status and post-Tx tumor status were independent prognostic factors for 5-year DSS only (HR=2.640, 95% CI: 1.317-5.290; HR=2.210, 95% CI: 1.084-4.503, respectively).

EGFR-N overexpression as an independent prognostic factor. Overexpression of EGFR-N, but not EGFR-C, was significantly correlated with poor DSS, LRFS, and MeFS in univariate analyses (all, P<0.05) (Table II; Fig. 2). Higher expression of EGFR-N remained an independent predictor of worse DSS and LRFS in multivariate analyses, as shown in Table III (HR=2.755, 95% CI: 1.3137-5.783; HR=3.026, 95% CI: 1.224-7.281, respectively).

Discussion

There have been great advances in the treatment of rectal cancer in recent decades, and neoadjuvant CCRT followed by radical proctectomy has become primary treatment. However, the long-term survival of rectal cancer patients has remained stagnant. Therefore, it is necessary to find effective predictive biomarkers to stratify high-risk patients for more intensive therapy. In this study, we demonstrated that high expression of EGFR-N, but not EGFR-C, was correlated with aggressive tumor behavior and poor outcome. These results suggest that EGFR-N may be useful for outcome prediction in rectal cancer patients after CCRT, and may possibly be a potential therapeutic target.

Molecular methods usually provide reliable information for cancer patients. However, studies that have evaluated prognostic biological markers for rectal cancer have yielded inconclusive results (14,15). A variety of studies have suggested that overexpression of EGFR was positively correlated with aggressive tumor behavior and poor prognosis in colorectal cancer (12,16,17). But, there was considerable discrepancy in the frequency and distribution of EGFR expression in previous immunohistochemical studies (18,19). Many of these studies provided speculative information on the association of protein expression and clinicopathological features. Furthermore, in clinical trials evaluating the efficacy of cetuximab, treatment response was not related to the level of EGFR expression; many patients with colorectal cancer expressing EGFR failed to respond to treatment, and those with EGFR negative tumors responded to therapy. A possible reason that EGFR levels are a poor predictor of response to anti-EGFR therapies is disparity between the form or epitope of EGFR detected by immunohistochemistry and the one targeted by anti-EGFR monoclonal antibodies (7).

Table III. Multivariate analysis.

		DSS			LRFS			MeFS		
Parameter	Category	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
EGFR-N	Low Exp.	1	-	0.0061[b]	1	-	0.0164[a]	1	-	0.1200
	High Exp.	2.418	1.287-4.543		3.026	1.224-7.281		1.853	0.852-4.032	
Pre-Tx nodal status (Pre-N)	N0	1	-	0.0062[b]	1	-	0.0948	-	-	-
	N1-N2	2.640	1.317-5.290		2.308	0.865-6.157		-	-	-
Post-Tx tumor status (Post-T)	T1-T2	1	-	0.0291[a]	1	-	0.1217	1	-	0.0587
	T3-T4	2.210	1.084-4.503		2.305	0.800-6.637		2.257	0.971-5.247	
Age, years	≥60	1	-	0.0203[a]	-	-	-	1	-	0.0112[a]
	<60	0.205	1.183-3.765		-	-		2.608	1.243-5.469	
Lymphovascular invasion	Absent	1	-	0.0651	1	-	0.0683	-	-	-
	Present	2.213	0.951-5.149		2.780	0.926-8.345		-	-	
Pre-Tx tumor status (Pre-T)	T1-T2	1		0.8048	-	-	-	-	-	-
	T3-T4	1.091	0.460-1.824		-	-		-	-	
Perineural invasion	Absent	1		0.8397	1	-	0.4012	-	-	-
	Present	1.145	0.236-3.249		1.999	0.397-10.075		-	-	

[a]P<0.05; [b]P<0.01. EGFR-C, cytoplasmic epidermal growth factor receptor; EGFR-N, nuclear epidermal growth factor receptor; Pre-Tx, pre-treatment; Post-Tx, post-treatment; DSS, disease-specific survival; LRFS, local-recurrence-free survival; MeFS, metastases-free survival; HR, hazard ratio; CI, confidence interval.

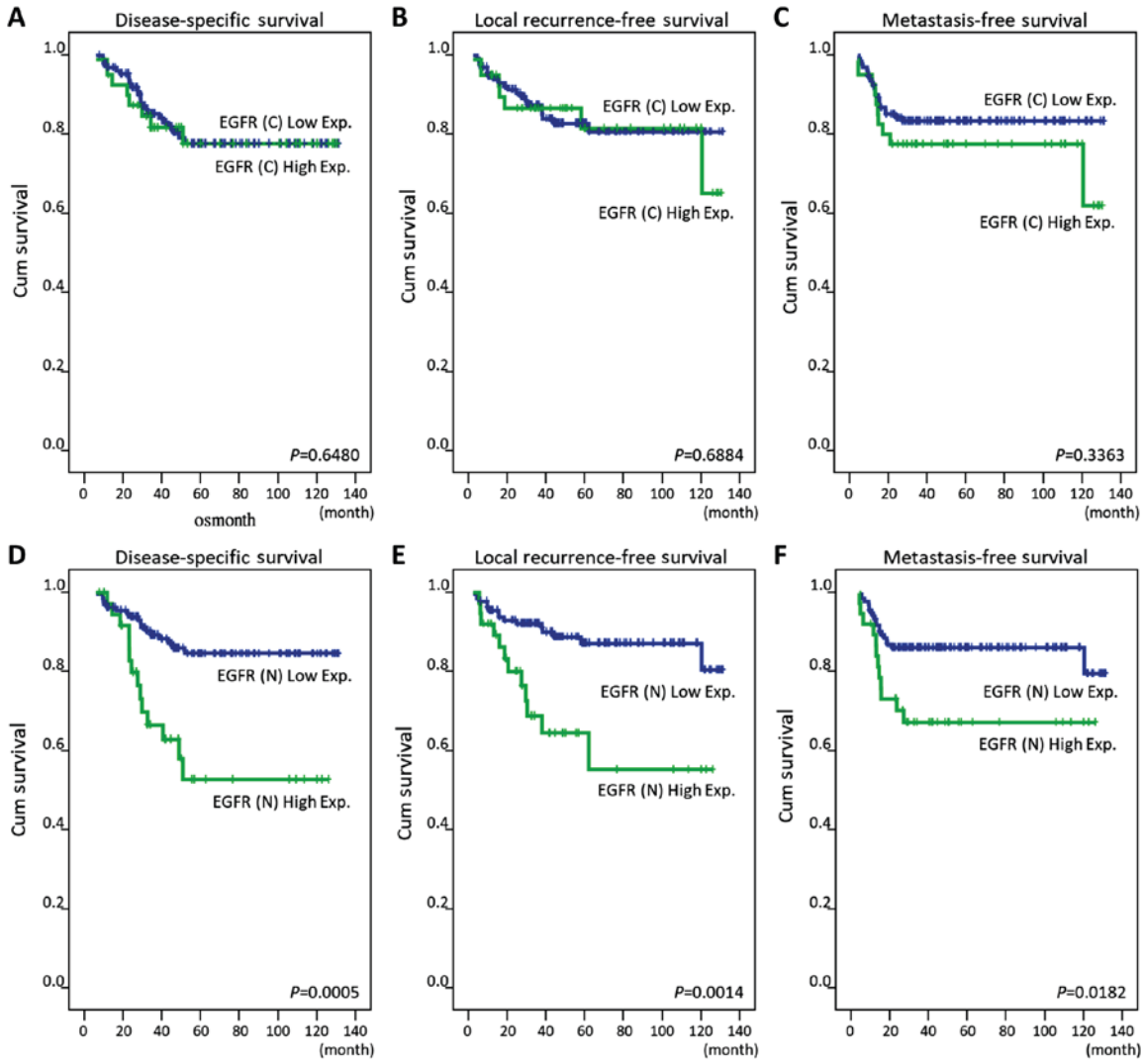

Figure 2. Kaplan-Meier analysis of the 5-year disease-specific survival, local recurrence-free survival and metastasis-free survival for rectal cancer patients with (A-C) EGFR-C and (D-F) EGFR-N expression. EGFR-C, cytoplasmic epidermal growth factor receptor; EGFR-N, nuclear epidermal growth factor receptor.

Recent evidence has suggested that EGFR can translocate to the nucleus (7). After activation, the nuclear translocation of EGFR has been shown to be dependent on phosphorylation events by many intracellular kinases such as SRC family and AKT (9). The nuclear transport of EGFR is guided by COPI-mediated vesicular trafficking from the Golgi to the endoplasmic reticulum (ER) (20). After being shuttled into the ER membrane, EGFR and importin β1 interface in the nuclear pore complex, and shuttle EGFR from the outer to the inner nuclear membrane. Once in the nucleus, the function of EGFR is widely different from its plasma membrane bound counterpart. EGFR-N is involved in many cellular signaling pathways such as cell proliferation, tumor behavior, DNA synthesis, and DNA repair (21-24). EGFR-N also plays a functional role in the response to molecular therapeutic agents, and has been linked to poor prognosis in a variety of malignancies (9,10,25). Hadzisejdic *et al* (10), reported that higher EGFR-N staining was associated with shorter OS in breast cancer patients. Overexpression of EGFR-N carried a 3.4-times greater mortality risk as compared to EGFR-N negative patients (HR=3.402; P=0.0026). Hoshino *et al* (25), also demonstrated that EGFR-N was associated with an increase in the malignant potential of esophageal squamous cell carcinoma that may affect the prognosis these patients. These intriguing findings emphasize the relevance of evaluating both EGFR-C and EGFR-N expression in colorectal cancer in order to provide additional independent prognostic information.

Our results showed that EGFR-N overexpression was significantly related to advanced pre-Tx tumor T-stage (T3, 4; P=0.017) and post-Tx tumor T-stage (T3, 4; P<0.001). In multivariate survival analysis, EGFR-N expression was also correlated with worse 5-year DSS and LRFS. These findings are different than those of prior studies where EGFR-C was associated with aggressive tumor behavior and poor outcomes (17,19). Similar to our study, Doger *et al* (16) evaluated 60 colon tumor specimens and reported that EGFR-C expression did not predict lymph nodes metastasis or survival. Moreover, EGFR-N overexpression was significantly associated with worse 5-year DSS (HR=2.755, P=0.007) and LRFS (HR=3.026, P=0.0164), but not MeFS (HR=1.85, P=0.12). The possible reasons for these findings may be that nuclear translocation of EGFR decreases

cell survival, and is associated with enhanced resistance to radiation and chemotherapy (7). EGFR-N is correlated with increased DNA-dependent protein kinase (DNA-PK) activity, which plays an essential role in DNA damage repair and radioresistance (26). Thus, the overexpression of EGFR-N in rectal cancers could contribute to the identification of patients who have an increased risk of local recurrences and poor outcomes after neoadjuvant CCRT.

The present study has some limitations. First, the number of patients was relatively small, and the findings should be verified by larger-scale studies. Second, our study cohort included some early-stage rectal cancer patients because of the intention of organ preservation via neoadjuvant CCRT. Further studies focused on locally advanced rectal cancer should be performed. Finally, our results are applicable to only patients with non-metastatic rectal cancer who undergo CCRT.

In summary, the present study revealed that overexpression of EGFR-N, but not EGFR-C, may help identify and stratify high-risk patients after neoadjuvant CCRT. The strong inverse correlation with DFS and LFRS suggested that EGFR-N may be a potential prognostic biomarker and promising therapeutic target.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CY, LL, YL, YT, CYL, MS, CFL and MT conceived and designed the experiments. CY, LL and CFL participated in the design of the study and performed the statistical analysis. CY, LL, CFL and MT drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Review Board of Chi-Mei Medical Center in Taiwan (Tainan, Taiwan; IRB, CMFHR10501-008). The requirement for informed consent was waived as all identifying information was removed from the dataset prior to analysis.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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