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ERCC1 predicting chemoradiation resistance and poor outcome in oesophageal cancer

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ABSTRACT

We assessed whether expression of excision repair cross-complementation group 1 (ERCC1) and/or thymidylate synthase (TS) can predict clinical outcome after preoperative chemoradiotherapy (CRT) in patients with localised oesophageal cancer. Paraffin-embedded pretreatment tumour specimens collected by endoscopic biopsy from patients treated with preoperative CRT (5-fluorouracil/cisplatin or capecitabine/cisplatin plus radiation) were analysed by immunohistochemical assay. Between March 1993 and June 2005, 129 patients were treated with preoperative CRT followed by surgery; of these, 108 biopsy specimens were available for analysis, and 40% and 35% were positive for ERCC1 and TS, respectively. Patients with ERCC1-negative ($p < 0.001$) or TS-negative ($p = 0.04$) tumours were significantly more likely to achieve pathologic major response. In multivariate analysis, ERCC1 was the only independent variable predicting pathologic response ($p < 0.001$). Patients with ERCC1-negative tumours showed tendencies toward prolonged overall survival ($p = 0.10$) and event free survival ($p = 0.08$). Prospective studies are required to determine the benefit of preoperative CRT in ERCC1-negative tumours.

1. Introduction

Oesophageal cancer is the eighth most common cancer worldwide, with 462,000 new cases per year, as well as being the sixth most common cause of cancer mortality. Its prognosis is poor, with a 5-year survival rate of less than 10%.[1] A multimodal approach has been used to improve patient outcomes, with concurrent chemoradiotherapy (CRT) followed by oesophagectomy being the standard treatment option.[2–5]

We have performed several prospective studies testing the effectiveness of preoperative CRT in patients with operable oesophageal cancer with squamous cell histology.[4,6,7] We found that pathologic major response was an independent prognostic factor.[6] However, the major obstacle to improved outcome in operable oesophageal cancer is the inability to

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predict response to CRT. If one could predict the response of CRT, treatment could be tailored to chemoradiation or immediate surgery and the survival of patients with operable oesophageal cancer could therefore be improved. In this point of view, it is necessary to investigate the surrogate biomarkers predicting pathologic response to CRT. Although the prognostic effect of biomarkers has been evaluated in operable oesophageal cancer, most of these studies involved relatively small, heterogeneous groups of patients beyond clinical trials. Moreover, there have been few investigations of biomarkers that can predict pathologic response in the preoperative CRT setting.

The nucleotide excision pathway is one of the most important pathways guarding the integrity of the genome, removing a wide variety of DNA lesions including interstrand cross-links caused by cisplatin or radiation.[8,9] The enzyme, excision repair cross-complementation group 1 (ERCC1), plays a rate-limiting role in the nucleotide excision repair pathway.[10,11] In contrast, thymidylate synthase (TS) is the rate-limiting enzyme involved in DNA synthesis,[12] and competitive inhibition of its activity appears to be the major mechanism for the antitumour effect of fluoropyrimidines.[13] The expression of ERCC1 and/or TS has been associated with clinical outcome in patients with various malignancies.[14–16]

We therefore assessed whether expression of ERCC1 and/or TS can predict clinical outcome, such as pathologic response to CRT and survival, in patients with locally advanced oesophageal cancer.

2. Materials and methods

2.1. Patient selection and evaluation

Beginning in March 1993, we conducted three prospective clinical trials in 268 patients, aged 18 to 75 years, with locally advanced but resectable, histologically established oesophageal cancer.[4,6,7] The eligibility criteria for all three trials were identical. Of the 268 patients, 129 underwent preoperative CRT and oesophagectomy. For ERCC1 and TS analysis, each case was required to meet the following criteria: 1) pretreatment pathology available for analysis, 2) completion of the prescribed CRT, and 3) gross complete resection (R0 or R1 resection). The study protocols were approved by the Institutional Review Board for Human Research of Asan Medical Center.

2.2. Treatment protocols

From March 1993 to March 1995, neoadjuvant chemotherapy consisted of two cycles of cisplatin (60 mg/m^2 i.v. infusion over 5 h on day 1) and 5-FU (1000 mg/m^2 daily as continuous i.v. infusion for 5 days from day 2 to day 6). Concurrent radiotherapy was delivered twice daily, to a dose of 48 Gy, in 40 fractions of 1.2 Gy each, with a minimum of 6 h between treatments. From March 1995 to January 2003, patients were treated with 5-FU for 4 days, from day 2 to day 5, and omitted during the second cycle of chemotherapy. Concurrently, 38 fractions of 1.2 Gy each were delivered twice daily for a total dose of 45.6 Gy. Beginning in January 2003, 5-FU was replaced by capecitabine, and patients received induction cisplatin (60 mg/m^2 i.v.) on day 1 and capecitabine (2000 mg/m^2/day p.o.) on days 1 to 14, followed by 1 week of rest. This was followed by cisplatin (30 mg/m^2 i.v.) on days 22, 29, 36, and 43, plus capecitabine (1600 mg/m^2/day p.o.) 5 days per week, concurrent with radiotherapy. The fractionation schedule was modified to 46 Gy in 23 fractions over 4 weeks (2 Gy per fraction per day).

Surgical resection was performed 4–6 weeks after the end of radiotherapy, using a transhiatal, abdominal-right thoracic (Ivor Lewis) or right thoracic-abdominal-cervical (McKeown) approach. The proximal and distal margins had to be at least 6–8 cm from the gross tumour. A frozen section of the resection margin of each tumour was examined by a pathologist before completion of the surgery. En bloc lymph node dissection included the perioesophageal, infracardial, posterior mediastinal and paracardial lymph nodes, as well as those located along the lesser gastric curvature and at the origin of the left gastric artery, celiac trunk, common hepatic artery and splenic artery. Resections were considered incomplete when microscopic examination revealed positive margins (R1) or when there was residual gross disease (R2).

2.3. Follow-up evaluation and assessment of response

Endoscopy with biopsy, oesophagography, chest CT, abdominal CT, and all blood tests were repeated prior to surgery. Responses were classified as CR, partial response (PR), stable disease (SD), or progressive disease (PD) according to WHO criteria. Patients with no residual viable tumour cells in the surgical specimen (pT0N0M0) were classified as having achieved pathologic CR (pCR); those with residual tumour smaller than 1 cm in the greatest dimension and limited to the mucosa or submucosa without evidence of lymph node involvement or pCR in the oesophagus with microscopic clusters of neoplastic cells in a single regional lymph node were classified as having microscopic residual disease (mRD); and those with macroscopic tumour remnants were classified as having gross residual disease (gRD). Patients with pCR or mRD were designated as the pathologic major response group.

2.4. Immunohistochemical analysis for ERCC1 and TS

Tumour specimens were collected by endoscopic biopsy from the 116 patients with oesophageal cancer before treatment, fixed in formalin and embedded in paraffin tissue blocks. All immunohistochemical analyses were performed in a Benchmark automatic immunostaining device (Ventana Medical System, Tucson, AZ, USA). Five-micrometer-thick tissue sections on poly L-lysine-coated slides were exposed to 10 mM citrate buffer (pH 6.0) and heated for 30 min in a water bath for antigen retrieval. Tumour sections were incubated for 60 min with 1:100 dilutions of mouse monoclonal antibodies against human TS (clone TS106, Zymed, South San Francisco, CA, USA) and ERCC1 (clone 8F1, Neomarkers, Fremont, CA, USA). Antibody binding was detected using the streptavidin-biotin complex method with peroxidase conjugate, and the peroxidase reaction was developed using diaminobenzidine as the chromogen. The sections were counterstained with Meyer's haematoxylin solution and

mounted in a nonaqueous mounting medium. Proliferating cells in tonsils served as a positive control for TS and ERCC1. Two independent pathologists blinded to clinical outcome analysed the results, with discrepancies resolved by consensus. Only nuclear immunoreactivity was considered positive for ERCC1. Nuclear or cytoplasmic reactivity was considered positive for TS.

Staining intensities of TS and ERCC1 were each graded on a scale of 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). The percentages of tumour cells in each grade were calculated for each specimen with 0 indicating 0% staining, 1 indicating 1–9% staining, 2 indicating 10–49% staining, and 3 indicating 50–100% staining. The proportion score was multiplied by the staining intensity to obtain a semiquantitative score ($0 \sim 9$). The median value of each score was *a priori* chosen as the cutoff point for separating positive from negative tumours.

2.5. Statistical analysis

Statistical analyses of 2×2 contingency tables of categorical variables were performed using Pearson's χ^2 test or Fisher's exact test, where appropriate. Survival probability analyses were performed using the Kaplan–Meier method. Survival was calculated from the date of start of chemotherapy to the date of death or most recent follow-up. Event free survival (EFS) was defined as the time from the date of first chemotherapy to the date of first observation of disease progression, or relapse, or death due to any cause. Significance between group differences was assessed by the log-rank test. Multivariate analyses were performed using a logistic regression model for response and Cox regression models for EFS and overall survival (OS). Factors with p-values <0.1 in univariate analyses were examined with multivariate regression models. All statistical tests were two-sided, with significance defined as $p < 0.05$. Analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) and SigmaPlot version 9.0 (Systat Software Inc., San Diego, CA, USA).

3. Results

3.1. Patient characteristics

Of 116 samples tested, 108 were assessable for both TS and ERCC1 expression. The 108 patients (93 men, 15 women) had a median age of 63 years (range, 43 to 73 years). Histologically, all primary tumours were squamous cell carcinomas. Most tumours originated from the middle and lower oesophagus. Patient baseline demographic and clinical characteristics are shown in Table 1.

Table 1 – Patient characteristics according to pathologic response

	No. of patients ($n = 108$)	PMR ($n = 63$)	<PMR ($n = 45$)
Age, years			
⩽63	48	29	19
>63	60	34	26
Primary tumour length			
⩽5 cm	77	45	32
>5 cm	31	18	13
Tumour location			
Upper third	4	2	2
Middle third	50	32	18
Lower third	54	29	25
Histological grade			
Well differentiated	9	3	6
Moderately differentiated	75	45	30
Poorly differentiated	17	9	8
Missing	7	6	1
Dysphagia			
No ~ dysphagia to solid food	66	47	35
Dysphagia to liquid food	42	16	10
Weight loss			
⩾10%	13	5	8
<10%	93	56	37
Missing	2	2	0
ECOG performance score			
0	13	8	5
1	83	50	33
2	12	5	7
Clinical stage			
Stage IIA	31	18	13
Stage IIB	27	20	7
Stage III	50	25	25

Abbreviations: PMR, pathologic major response.

3.2. Association of biological marker expression with patient and tumour characteristics

The median scores (proportion stained × staining intensity) of ERCC1 and TS were 4 and 3, respectively. Based on this, patients were dichotomised as ERCC1-negative (ERCC1 ⩽ 4) or ERCC1-positive (ERCC1 > 4) and as TS-negative (TS ⩽ 3) or TS-positive (TS > 3). Positive staining for ERCC1 (Fig. 1a) and TS (Fig. 1b) was observed in 43 (40%) and 38 (35%) tumours, respectively. Expression of these biologic markers was not associated with patient and tumour characteristics such as age, sex, tumour differentiation, tumour sized clinical stage, and lymph node metastasis (data not shown).

3.3. Association of biological marker expression with chemoradiotherapy response

Forty-nine of 108 patients (45%) achieved pCR after preoperative CRT, and 14 (13%) achieved mRD; thus, 63 patients achieved PMR (pathologic major response). The median scores of ERCC1 and TS in these 63 patients were 2 (interquartile $1 \sim 4$, mean 3, range $0 \sim 9$) and 3 (interquartile $1 \sim 4$, range $0 \sim 9$), respectively. In contrast, the median scores of ERCC1 and TS in the 45 patients who achieved < PMR were 6 (interquartile $2 \sim 6$, mean 5, range $0 \sim 9$) and 3 (interquartile $1 \sim 5$, mean 3, range $0 \sim 9$), respectively (Fig. 2). Table 2 shows the relationship between biologic markers and pathologic response to CRT. ERCC1 ($p < 0.001$) and TS ($p = 0.042$) expression were significantly correlated with major pathologic response. In multivariate analysis, ERCC1 was the only independent variable predicting pathologic response ($p < 0.001$), although TS expression ($p = 0.060$) and clinical stage ($p = 0.056$) showed a tendency to predict pathologic response (Table 3).

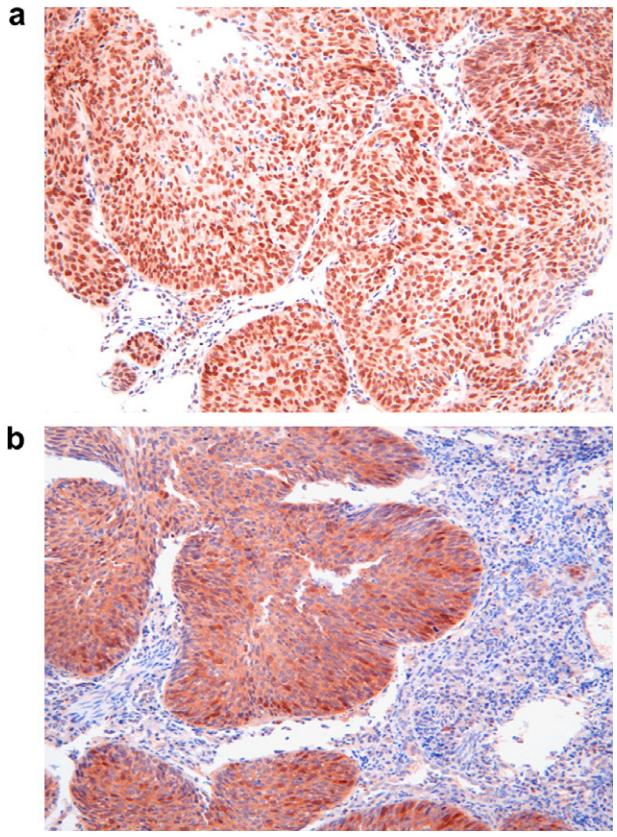

Fig. 1 – Immunohistochemical positive findings of ERCC1 and TS expression (×200). a, high expression of ERCC1; nuclear staining (score 9). b, high expression of TS; nuclear or cytoplasmic staining (score 9).

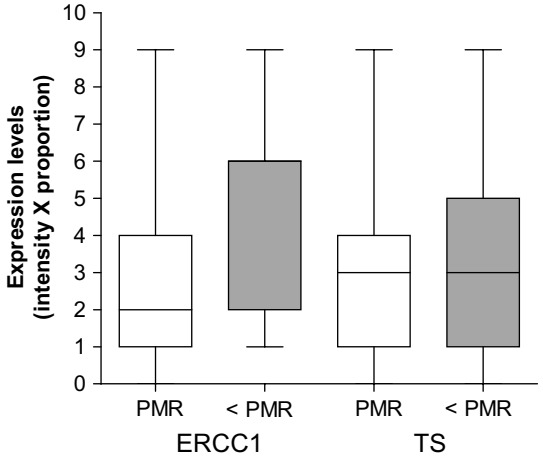

Fig. 2 – Box plot diagram of ERCC1 and TS expression levels according to pathologic response. PMR, major pathologic response.

3.4. *Association of biological marker expression with survival*

At median follow-up of 34.6 months (range, 15.4–152.2 months) for surviving patients, the median overall survival (OS) was 46.5 months (95% CI, 29.1–63.9 months) and the median event free survival (EFS) was 38.2 months (95% CI, 27.4–48.9 months). OS and EFS were each significantly associated with pathologic response and lymph node positivity after chemoradiotherapy. Median OS of patients with ERCC1-negative tumours was 101.9 months (95% CI, 34.8–169.0 months), whereas median OS of patients with ERCC1-positive tumours was 39.1 months (95% CI, 31.8–46.5 months; $p = 0.10$). Median EFS of patients with ERCC1-negative tumours was 58.9 months (95% CI, 16.0–101.8 months), whereas median EFS of patients with ERCC1-positive tumours was 26.6 months (11.5–41.8 months; $p = 0.076$) (Fig. 3). Multivariate analysis showed that pathologic response was an independent prognostic predictor of both OS ($p = 0.014$) and EFS ($p = 0.046$). Although non-expression of ERCC1 tended to predict survival, the expression of TS was not significantly correlated with OS or EFS.

Table 2 – Immunohistochemical results according to pathologic response (PMR versus < PMR)

	No. of patients (%)			*p* value
	Total	PMR	<PMR	
ERCC1				<0.001
⩽4	65	50	15	
>4	43	13	30	
TS				0.042
×⩽3	70	46	24	
×>3	38	17	21	

Abbreviations: PMR, pathologic major response; ERCC1, excision repair cross-complementation group 1; TS, thymidylate synthase.

Table 3 – Logistic regression analysis for pathologic response (pMR versus < pMR)

Factors	No. of patients	Relative risk	95% CI	*p* value
Clinical stage				
II	58	0.39	0.15 ~ 1.11	0.056
III	50	1		
Dysphagia				
No ~ to solid food	66	0.84	0.27 ~ 2.57	0.760
To liquid food	42	1		
Primary tumour				
⩽5 cm	77	0.69	0.24 ~ 2.0	0.503
>5 cm	31	1		
ERCC1				
⩽4	65	0.075	0.03 ~ 0.22	<0.001
>4	43	1		
TS				
⩽3	70	0.36	0.12 ~ 1.04	0.060
>3	38	1		

Abbreviations: ERCC1, excision repair cross-complementation 1; TS, thymidylate synthase.

4. Discussion

Despite efforts to identify factors predicting pathologic responses to preoperative CRT in patients with locally

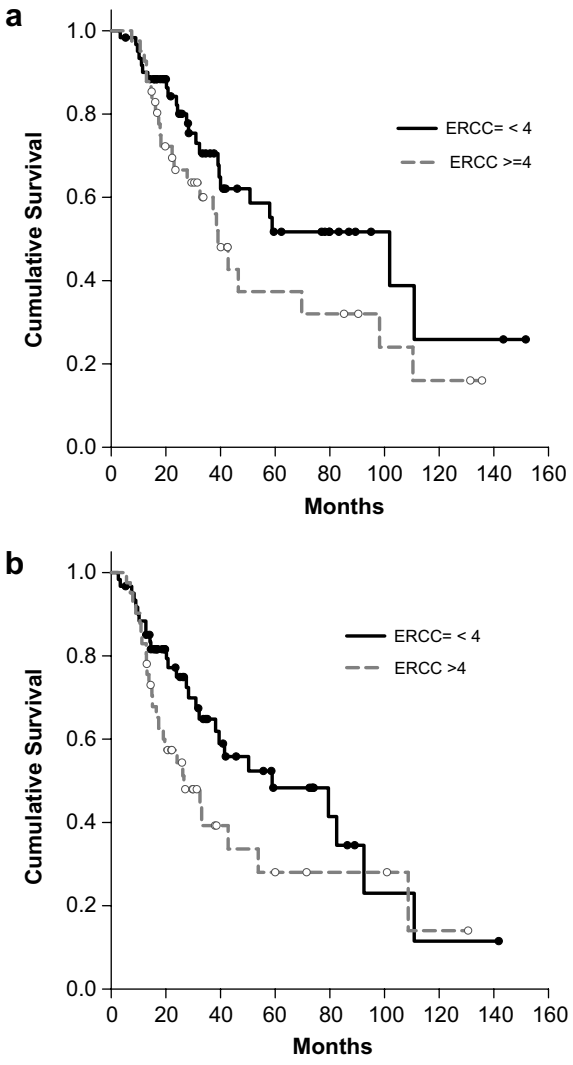

Fig. 3 – Survival differences according to ERCC1 positivity. (a) Overall survival (101.9 months versus 39.1 months, $p = 0.10$); (b) Event free survival (58.9 months versus 26.6 months, $p = 0.076$).

advanced oesophageal cancer, no suitable markers have been identified to date. In considering the mechanism of CRT, we hypothesised that ERCC1 and TS expression may be associated with response, and we therefore assayed whether the expression levels of TS and ERCC1 could act as biomarkers to predict clinical outcome in these patients. We found that lower ERCC1 expression (ERCC1 $\leqslant$ 4) was an independent marker predicting pathologic response to preoperative CRT.

At least four main, partly overlapping damage repair pathways have been investigated in mammals, namely nucleotide-excision repair (NER), base-excision repair (BER), homologous recombination and end joining .[17] The NER and BER systems are multi-step enzymatic complexes involved in repairing nonspecific damage to DNA, including that caused by radiation, cross-linking, and platinum-DNA adduct formation. ERCC1 is the key, rate-limiting enzyme of the NER pathway. ERCC1 expression level has been associated with response to cisplatin-based therapy and/or overall survival in various malignancies, including, ovary, stomach and non-small cell lung cancer.[8,14,16] Regardless of tumour types, however, the expression level of ERCC1 was not correlated with tumour aggressiveness, as determined by T/N stage, histological type or histological grade. Our results were consistent with these earlier findings. In a study of the association between ERCC1 mRNA expression and histopathological response to preoperative CRT in 36 patients with operable oesophageal cancer, Warnecke-Eberz and colleagues found that ERCC1 was expressed at higher levels in patients with squamous cell carcinoma than in those with adenocarcinoma. Moreover, the sensitivity of response prediction was higher in squamous cell carcinoma. Survival analysis was not performed, however, due to the short follow-up time and small numbers of patients.[18] In the present study, we found that the sensitivity and specificity of ERCC1 expression detected by immunohistochemical staining for predicting a major pathologic response were 79% (50/63) and 67% (30/45), respectively, in squamous cell carcinoma.

The methodologies used to measure ERCC1 expression have included immunohistochemical staining, reverse transcription-polymerase chain reaction (RT-PCR) and single nucleotide polymorphism (SNP). Retrospective analyses using RT-PCR or immunohistochemical staining of tissue samples from previously collected paraffin blocks had some limitations in accuracy and reproducibility. Distinct patterns of functional genomic polymorphisms in the ERCC1 gene, related to response to cisplatin or radiotherapy, have been reported in head and neck cancer and in non-small cell lung cancer,[19,20] suggesting that these polymorphisms may predict the effects of preoperative CRT. Techniques such as RT-PCR or SNP, however, are difficult to perform, expensive, and time-consuming, and these genetic variations may be dependent on ancestry. In contrast, immunohistochemical analysis can be performed by almost every pathology laboratory.

The antitumour mechanism of 5-FU suggests that TS expression is a determinant of sensitivity to fluoropyrimidine, and TS expression in colorectal and stomach cancer has been reported to predict clinical outcome.[21,22] In agreement with these findings, our results suggest that high TS expression may correlate with poor response to CRT in oesophageal cancer. However, the predictability of TS expression was not demonstrated by multivariate analysis.

Despite the correlation between ERCC1 expression and response to CRT, we found that patients with ERCC1-negative tumours showed only a tendency toward prolonged OS and EFS. Three hypotheses may explain the inconsistent effect of ERCC1 expression on response and survival. First, all patients in this study underwent curative surgery after CRT. Thus, survival may have been prolonged in some patients with residual disease. That is, the addition of oesophagectomy may have confounded the effect of ERCC1 on survival. Second, the relatively small number of patients assessed in this study may have been insufficient to detect a significant effect on survival. Finally, and most importantly, ERCC1 may have another biologic effect on survival. ERCC1 expression has been shown to have a different prognostic significance in treated and untreated patients.[16,23] In addition, large prospective studies in non-small cell lung cancer have shown

that patients with ERCC1-negative tumours had shorter OS than did patients with ERCC1-positive tumours,[24,25] which may have been due to the role of ERCC1 in preventing mutagenesis. In fact, DNA repair may not only prevent cancer but may also retard molecular events related to progression in established tumours.[26]

A recent phase III trial, in which cisplatin was customised based on quantitative ERCC1 mRNA expression, showed that assessment of ERCC1 mRNA expression in lung cancer tissue was feasible in the clinical setting to predict responses to docetaxel and cisplatin.[27] Our findings suggest that ERCC1 expression level can also help decide between preoperative or definitive CRT and immediate oesophagectomy in patients with operable oesophageal cancer.

Izzo and colleagues demonstrated that nuclear factor kB (NF-kB) expression detected by immunohistochemical analysis is an independent predictor of complete response and survival in preoperative CRT setting.[28] In that study, however, the sample size was relatively small, NF-kB expression was measured before CRT in some patients and after CRT in others, and only patients with adenocarcinoma were enrolled; further studies are therefore required. Additionally, the authors proved the association between pathologic complete response and NF-kB negativity. However, patient survival in the preoperative CRT setting may be associated more with the ability to predict major pathologic response than with the ability to predict complete pathologic response because patients may benefit from surgery after CRT. Early metabolic response, as determined by positron emission tomography (PET), may also predict pathologic response and survival in adenocarcinoma of the oesophagogastric junction. For example, FDG-PET, performed within 14 days after initiation of preoperative chemotherapy, identified patients with a very low response and a poor prognosis.[29] However, metabolic response determined by FDG-PET is still inadequate as a surrogate marker because FDG-PET response is detectable after 2 weeks of CRT treatment, and failure to respond early to chemotherapy may also be a marker of biologically aggressive tumours, which may be associated with poor outcome irrespective of the applied therapy.

The limitations of this study include the limitations of immunohistochemical staining, such as its semiquantitative nature, tissue aging effects, and interobserver variation, all of which may have affected the association between ERCC1 expression and survival. A second limitation is that protein expression in endoscopic biopsy tissue may not be representative of the entire tumour, due to tumour heterogeneity. Considering about 70% concordance between ERCC1 expression and the pathologic response to CRT, additional prospective studies are needed to standardise and optimise methodologies for ERCC1 analysis, to establish a predictive biological profile. Despite these limitations, however, our results are the first to indicate that ERCC1 expression can predict the pathologic response to CRT in operable oesophageal cancer with squamous cell histology.

In conclusion, we have shown here that high ERCC1 expression is associated with CRT resistance in patients with locally advanced oesophageal cancer. The benefit of preoperative CRT in ERCC1-negative tumours should be determined.

Conflict of interest statement

None declared.

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