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Research Article

Impact of Excision Repair Cross Complementation Group 2 Protein Expression on Clinical Outcome in Patients with Colorectal Cancer

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Received: May 01, 2020; **Published:** July 30, 2020

Abstract

Purpose: To investigate the prevalence of excision repair cross-complementation group 2 (ERCC2) protein expression in colorectal cancer (CRC) patients and to evaluate its association with clinicopathological variables and clinical efficacy.

Materials and Methods: Immunohistochemistry from paraffin-embedded tumor tissue blocks was performed to study ERCC2 expression in 80 untreated CRC patients.

Results: ERCC2 cytoplasmic and nuclear staining was observed in 54% and 36% of patients, respectively. A trend of reduced overall survival (OS) was observed in total patients (P = 0.081) and in early stage patients (P = 0.053) with negative cytoplasmic ERCC2 expression. Further, negative nuclear ERCC2 expression was associated with a significant reduced OS in colon cancer patients (P = 0.038). However, in rectal cancer patients, positive nuclear ERCC2 expression correlated with a trend of reduced relapse-free survival (RFS) (P = 0.061) and a significant reduced OS (P = 0.005). Additionally, a significant reduced OS with positive nuclear ERCC2 expression was observed in rectal cancer patients treated with adjuvant therapy (P = 0.009).

Conclusion: Negative cytoplasmic ERCC2 protein expression could be useful biomarker to classify high risk group of CRC patients with poor prognosis. Additionally, nuclear ERCC2 protein expression displayed differential function indicating its possible role as prognostic and predictive marker according to anatomic site of tumor.

Keywords: *ERCC2 Protein Expression; Colorectal Cancer; Immunohistochemistry; Prognosis; Predictive Biomarker*

Abbreviations

CRC: Colorectal Cancer; NER: Nucleotide Excision Repair; 5-FU: 5-Fluorouracil; XPD: Xeroderma Pigmentosum Group D; ERCC2: Excision Repair Cross Complementation Group 2; ERCC1: Excision Repair Cross-Complementation Group 1; ERCC4: Excision Repair Cross Complementation Group 4; GSTPi: Glutathione S-Transferase Pi 1; RFS: Relapse-Free Survival; OS: Overall Survival

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, with 10.2% incidence and 9.2% mortality rate according to Globocan 2018 [1]. Over past half a century, 5-fluorouracil (5-FU) have constituted as the backbone of chemotherapeutic regimens in the treatment of CRC patients. However, the introduction of the next generation drug, such as oxaliplatin in combination

with 5-FU, has undoubtedly proven beneficial in patients with both early and advanced stage disease and become a standard treatment in the management of this malignancy. The principle mechanism of action of oxaliplatin in cancer cells is inhibition of DNA synthesis by the formation of cross links in DNA [2]. After formation of platinum-DNA adducts, cellular repair mechanism gets activated. One of the major DNA repair pathways, nucleotide excision repair (NER) is involved in the repair of damage caused by oxaliplatin. Excision repair cross complementation group 2 (ERCC2) is an important NER mediator which plays a decisive role in repair of platinum-DNA adducts produced by oxaliplatin. During the NER pathway, ERCC2 gene, also known as xeroderma pigmentosum group D (XPD), encodes a protein which possesses an ATP-dependent helicase activity. It is a subunit of TFIIH which helps in maintaining unwound DNA structure at damaged region [3].

XPD is a highly polymorphic gene and 17 SNPs in the XPD gene have been detected. Epidemiological studies on cancer have focused on three common polymorphisms of the XPD gene because of their high frequencies: C→A silent polymorphism (Arg156Arg) in exon 6, the G→A polymorphism leading to Asp312Asn in exon 10 and the A→C polymorphism leading to Lys751Gln in exon 23 [4]. Evidences also suggests the association of these ERCC2 polymorphisms with decreased DNA repair capacity [5,6], which in turn affects its protein expression as well as its function. Moreover, experimental study by Chen., *et al*. 2002 in human cell lines showed that XPD protein levels correlate with resistance to alkylating agents, suggesting the role of ERCC2 protein expression in predicting the response and prognosis [7]. Many reports demonstrated the association of ERCC2 polymorphisms with survival and therapeutic efficacy in CRC. However, rare studies have examined ERCC2 protein expression and found no association with prognosis and treatment response in CRC. Hence, further studies are necessary to confirm the role of ERCC2 protein expression in CRC.

Aim of the Study

The present study aimed to examine immunohistochemical localization of ERCC2 in CRC patients. Further, its association with clinicopathological parameters along with its prognostic and predictive value has been evaluated in patients with CRC.

Materials and Methods

Patients: A total 80 untreated histologically confirmed CRC patients at The Gujarat Cancer and Research Institute, Ahmedabad, India, between 2012 and 2016, were enrolled in this study. A detailed clinicopathologic history including age, gender, habits, tumor size, lymph node status, histological grade, disease stage, treatment given, etc. was obtained from case files maintained at the institute. TNM classification was done considering World Health Organization (WHO) Grading System for pathological staging. Primary treatment offered to all patients was surgery or surgery followed by adjuvant chemotherapy and/or radiotherapy. The main chemotherapeutic treatment included were 5-FU and leucovorin, oral Capecitabine, or in combination with Oxaliplatin. All 80 patients enrolled in the study, followed up for the period of 24 months or until death within that period, were included for Over-all survival (OS) analysis. Out of 80 patients, 68 patients were alive and 12 patients were dead. For relapse-free survival (RFS) analysis, out of total 80 patients, 2 patients with stage IV cancer and 10 patients with persistence disease were excluded. Hence, 68 patients were included for RFS analysis. Out of these 68 patients, 10 patients had local/distant metastasis, while the rest 58 patients had no recurrence. Survival analysis was also performed in the subgroups of patients with early stage and advanced stage disease; as well as in colon cancer and rectal cancer patients after sub-grouping them according to tumor site. Further, to investigate the predictive value of ERCC2, survival analysis was performed in patients treated with adjuvant 5-FU/oxaliplatin based therapy. Patients' and tumor characteristics were depicted in table 1.

Sample collection: The study has been approved by Institutional Scientific and Ethical Committees and informed consent was obtained from all patients prior to sample collection. To study ERCC2 protein expression, paraffin embedded tumor tissue blocks were retrieved from Histopathology Department of the institute.

Table 1: *Patients' and tumor characteristics.*

Characteristics		N (%)
Age (Range: 20-80 years) (Median age: 55 years)	< 55	43 (54)
	≥ 55	37 (46)
Gender	Female	35 (44)
	Male	45 (56)
Diet	Vegetarian	60 (75)
	Mixed	20 (25)
Habit	No	41 (51)
	Yes	39 (49)
Anatomic site of tumor	Colon	48 (60)
	Rectum	32 (40)
Tumor size	T2	10 (12)
	T3	63 (79)
	T4	07 (09)
AJCC Staging	I	04 (05)
	II	40 (50)
	III	34 (42)
	IV	02 (02)
	Early stage (stage I+II)	44 (55)
	Advanced stage (stage III+IV)	36 (45)
Dukes stage	B	43 (54)
	C	35 (44)
	D	02 (02)
Tumor differentiation	Well	11 (17)
	Moderate	60 (75)
	Poor	09 (11)
Histological type	Adenocarcinoma	59 (74)
	Mucinous adenocarcinoma	21 (26)
Nodal status	Negative	46 (57)
	Positive	34 (43)
Necrosis	Absent	69 (86)
	Present	11 (14)
Lymphatic permeation	Absent	62 (78)
	Present	18 (22)
Vascular permeation	Absent	75 (94)
	Present	05 (06)
Perineural invasion	Absent	66 (83)
	Present	14 (17)
Lymphocytic stromal response	Absent	60 (75)
	Present	20 (25)
Pre-operative serum CEA levels (ng/ml)	< 3.0	23 (29)
	≥ 3.0	57 (71)
Treatment given	Surgery alone	09 (11)
	Surgery + Chemotherapy	49 (61)
	Surgery + Radiotherapy	04 (05)
	Surgery + Chemotherapy+ Radiotherapy	18 (23)

Immunohistochemistry

For the study of ERCC2 protein expression, 4 µm thick sections were cut from the formalin fixed paraffin embedded tumor tissue blocks and were mounted on 3-aminopropyletriethoxy silane coated glass slides. The immunohistochemical staining was carried out using anti-XPD rabbit monoclonal antibody (clone EPR9675:ab167418, abcam; 1:50 dilution) and using Mouse and Rabbit specific HRP/DAB (ABC) Detection IHC kit (Abcam), as per manufacturer's protocol recommendations. Antigenicity was retrieved by heating the tissue sections in 10mM tri-sodium citrate buffer (pH-6.0) solution for 20 minutes in a pressure cooker prior to application of the primary antibody. A semiquantitative scoring method ranging from negative (no staining or, 10% of cells stained) to 3+ (1+ staining for 11 - 30% of cells: weak, 2+ staining for 31 - 50% of cells: moderate, and 3+ staining for > 50% of cells: intense) was used [8].

Statistical analysis

The statistical data analysis was performed using SPSS (Statistical Package for the Social Sciences) software. Two tailed χ^2 test was used to determine the association of ERCC2 protein expression with clinicopathological variables. RFS and OS were calculated using Kaplan-Meier method and Log rank test. Correlation between two parameters was calculated using Spearman's correlation coefficient (r) method. P value ≤ 0.05 was considered significant.

Results

Incidence of ERCC2 protein expression in CRC patients

In total CRC patients, the combined immunoreactivity of ERCC2 in cytoplasm and/or nucleus was 70% (56/80). For statistical evaluation, cytoplasmic and nuclear expressions were scored independently and compared separately. Fifty-four percent patients (43/80) showed positive ERCC2 cytoplasmic expression and 36% (29/80) patients showed positive nuclear ERCC2 expression (Figure 1).

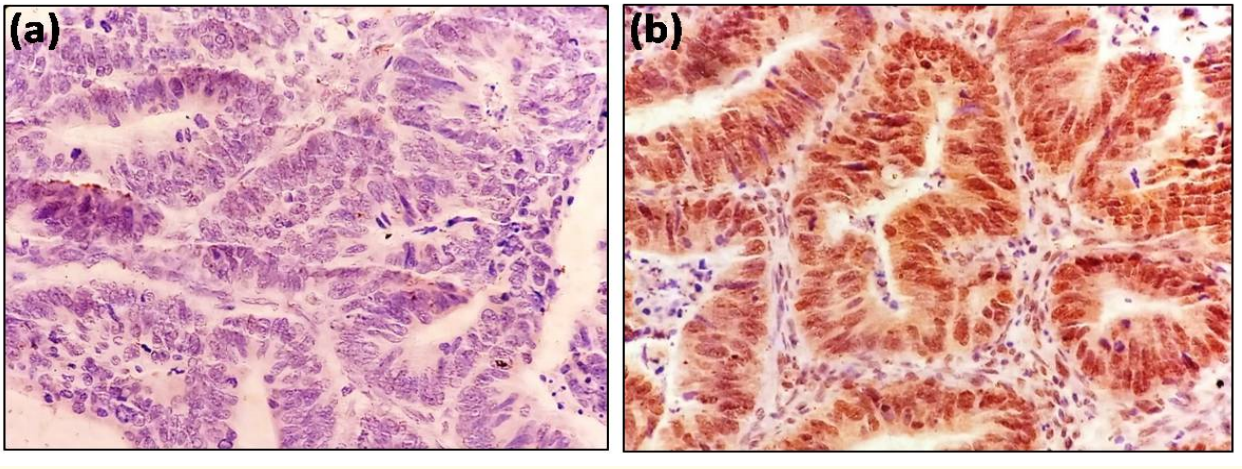

Figure 1: *The representative photomicrographs (40X) for ERCC2 immunostaining in colon tumor tissue (a) negative expression (b) cytoplasmic/nuclear expression.*

Association of cytoplasmic and nuclear ERCC2 protein expression with clinicopathological parameters

A significantly higher positive cytoplasmic ERCC2 expression was noted in patients with T2 tumor size (80%) as compared to T3 (52%) and T4 (29%) tumor size ($\chi^2 = 4.604$, r = -0.240, P = 0.032); and in patients with adenocarcinoma (63%) as compared to those with mucinous adenocarcinoma (29%; $\chi^2 = 7.262$, r = -0.301, P = 0.007). Further, a trend of positive nuclear ERCC2 protein expression was ob-

served in vegetarian patients (42%) as compared to those having mixed diet (vegetarian + non- vegetarian) (20%; $\chi^2 = 3.047$, r = -0.195, P = 0.083); and in habitual patients (46%) as compared to non-habitual patients (27%; $\chi^2 = 3.230$, r = +0.201, P = 0.074).

Survival analysis for RFS (N = 68) and OS (N = 80) in relation to cytoplasmic and nuclear ERCC2 protein expression in CRC patients

In total patients, Kaplan-Meier univariate survival analysis showed a trend of reduced OS in patients with negative cytoplasmic ERCC2 expression (24%) as compared to those with positive expression (9%; P = 0.081; Figure 2a). In early stage patients also, a similar trend of reduced OS was noted with negative cytoplasmic ERCC2 expression (25%) as compared to positive expression (1%; P = 0.053; Figure 2b).

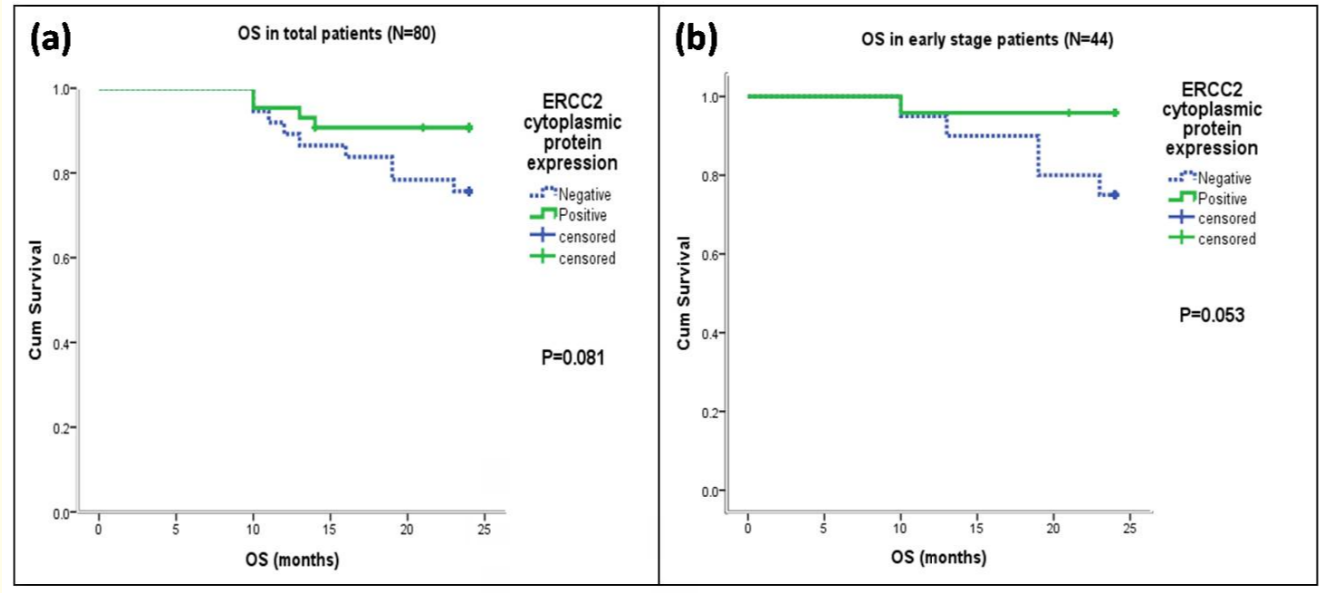

Figure 2: *Kaplan-Meier survival curves for OS in relation to ERCC2 cytoplasmic protein expression in (a) total patients (b) early stage patients.*

According to tumor site, in colon cancer patients, a significant reduced OS was observed with negative nuclear ERCC2 expression (21%) as compared to positive nuclear ERCC2 expression (0%; P = 0.038; Figure 3a). Contradictorily, in rectal cancer patients, a trend of reduced RFS was observed with positive nuclear ERCC2 expression (33%) as compared to negative nuclear expression (5%; P = 0.061; Figure 3b). Additionally, a significant reduced OS was noted in rectal cancer patients with positive nuclear expression (50%) as compared to those with negative nuclear ERCC2 expression (9%; P = 0.005; Figure 3c).

Univariate survival analysis for RFS (N = 57) and OS (N = 67) in relation to cytoplasmic and nuclear ERCC2 protein expression in CRC patients treated with 5-FU/oxaliplatin based adjuvant therapy

When RFS and OS were evaluated in the subgroup of patients treated with 5-FU/oxaliplatin based adjuvant therapy, Kaplan-Meier univariate survival analysis showed no significant difference in the incidence of disease relapse or death between negative and positive cytoplasmic ERCC2 protein expression in total patients or in subgroups of early stage, advanced stage, colon cancer and rectal cancer patients. However, in case of nuclear ERCC2 expression, in colon cancer patients, a trend of reduced OS was observed with negative nuclear ERCC2

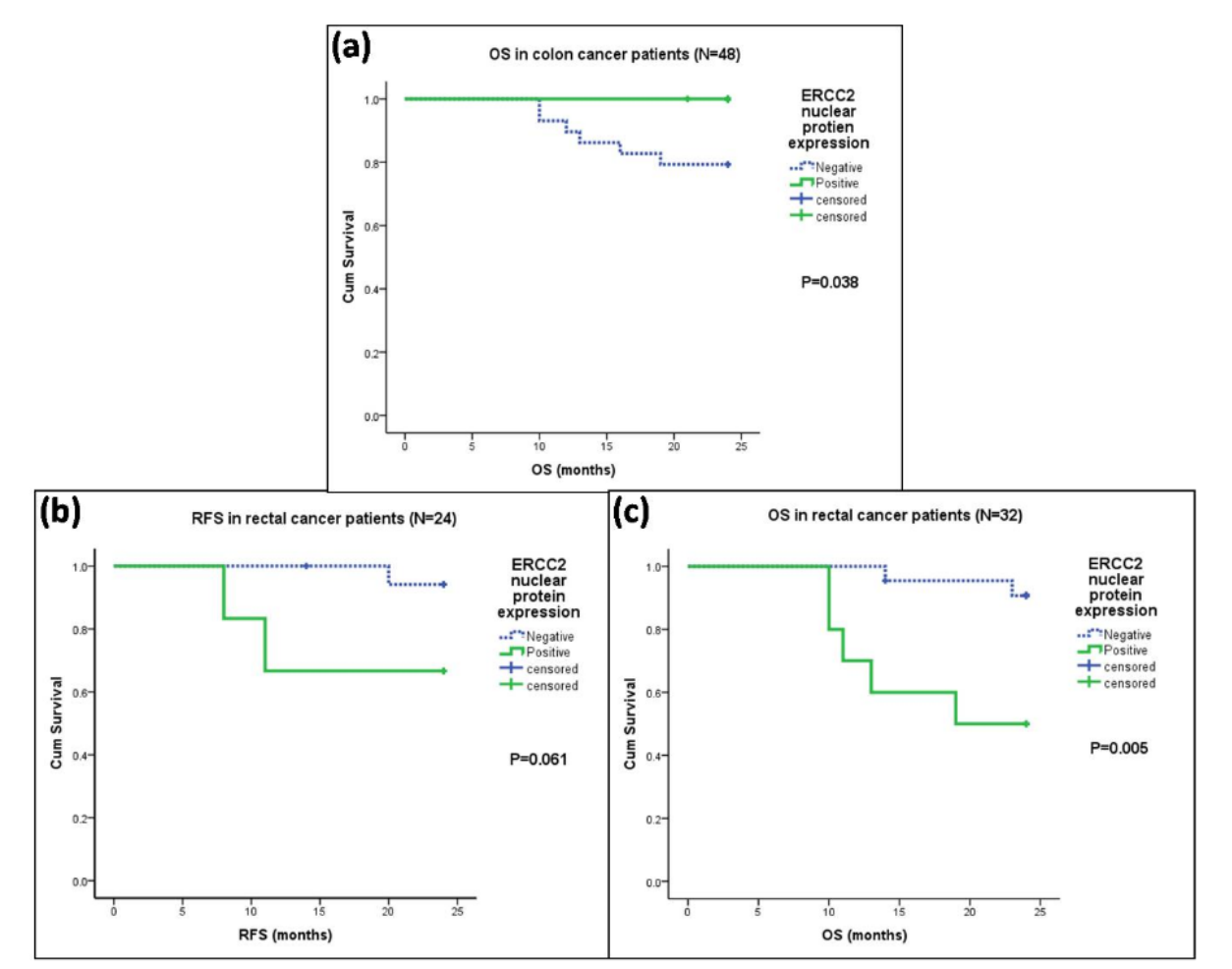

Figure 3: *Kaplan-Meier survival curves for in relation to ERCC2 nuclear protein expression (a) OS in colon cancer patients, (b) RFS and (c) OS in rectal cancer patients.*

expression (16%) as compared to positive nuclear ERCC2 expression (0%, P = 0.099; Figure 4a). In contrast, in rectal cancer patients, a significant reduced OS was observed in patients with positive nuclear ERCC2 expression (56%) as compared to negative nuclear ERCC2 expression (12%; P = 0.009; Figure 4b).

Intercorrelation between cytoplasmic and nuclear ERCC2 protein expression

The nonparametric Spearman's correlation revealed that cytoplasmic and nuclear ERCC2 protein expression was not significantly correlated with each other (r = 0.074, P = 0.516).

Discussion

Colorectal cancer is a heterogeneous process as revealed by the interaction of a number of genetic, epigenetic as well as environmental factors like diet and lifestyle. All of these processes constitute biomarkers, which are modifications in genes and associated proteins,

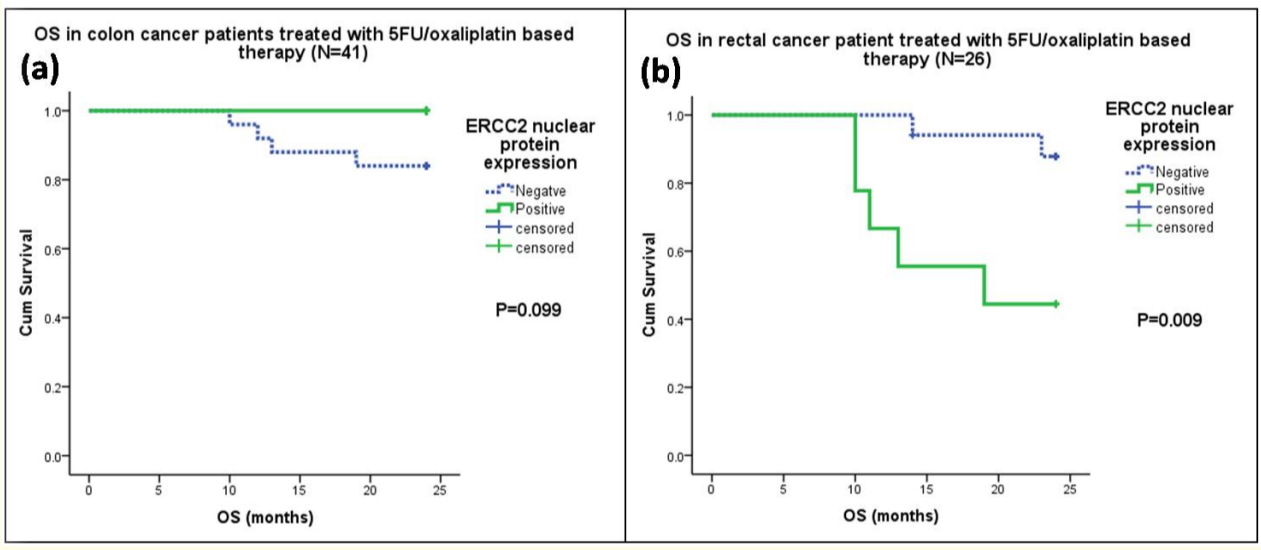

Figure 4: *Kaplan-Meier survival curves for OS in relation to nuclear ERCC2 protein expression in patients treated with 5-FU/oxaliplatin based adjuvant therapy (a) colon cancer patients (b) rectal cancer patients.*

affecting prognosis or therapeutic response [9]. Therefore, identification of CRC molecular markers related to therapeutic response or failure would identify the patients with risk of recurrence and persistent disease and selecting the best treatment for them.

ERCC2 is one of the core genes involved in transcription- coupled NER pathway, essential for transcription initiation, nucleotide excision repair, cell cycle control, and apoptosis [10]. Many studies demonstrated that polymorphisms identified in the coding regions of ERCC2 gene proposed to predict responses as well as survival to platinum-based chemotherapy in CRC patients [11,12]. These polymorphisms in the XPD gene may cause defects in NER pathway, in consequence may alter XPD mRNA secondary structure and reduce constitutive mRNA levels [13], associated with a decreased XPD protein expression [14]. However, scarce data is available regarding role of ERCC2 protein expression in CRC. Therefore, present study examined ERCC2 protein expression by immunohistochemistry in CRC patients and showed cytoplasmic and/or nuclear immunoreactivity. Cytoplasmic ERCC2 expression was observed in 54% of tumors while, nuclear ERCC2 expression was present in 36% of tumors. Likewise, cytoplasmic over expression of ERCC2 protein was observed in 65% of stage III CRC patients treated with FOLFOX4 adjuvant chemotherapy [15]. However, a report by Lai., *et al*. 2009 displayed intense nuclear immunoreactivity of ERCC2 protein in 59% of Asian patients with CRC [14].

In relation to clinicopathological parameters, present study revealed that a significant higher positive cytoplasmic ERCC2 expression was observed in patients with T2 tumor size and adenocarcinoma as compared to larger tumor size (T3 and T4) and mucinous adenocarcinoma, respectively, suggesting association of loss of ERCC2 cytoplasmic expression with aggressive phenotype. However, nuclear ERCC2 expression was not significantly associated with any of the clinicopathological parameters except with diet and habit. Vegetarian patients and patients with habits showed a trend of higher positive nuclear ERCC2 expression as compared to their respective counterparts. Although no reports are available for correlation between ERCC2 protein expression and clinicopathological parameters in CRC, studies in healthy individuals indicate the association of ERCC2 mRNA expression with age and smoking. Wolfe., *et al*. 2007 included 110 participants and examined the relationship between three SNPs in the XPD gene (R156R in exon 6, D312N in exon 10 and K751Q in exon

23) and mRNA level using quantitative real-time polymerase chain reaction [13]. They studied that the decrease in XPD mRNA levels was significantly greater in smokers and was exacerbated by smoking duration and intensity. They also found that the decrease in XPD mRNA levels was more pronounced in older than in younger subjects. Moreover, another report showed that decrease in ERCC2 mRNA level might be associated with extreme longevity [16].

In relation to prognostic role of cytoplasmic ERCC2 protein expression, present study demonstrated that a trend of reduced OS was observed in patients with negative cytoplasmic ERCC2 expression as compared to those with positive expression in total patients (P = 0.081) as well as in early stage patients (P = 0.053). Association between negative cytoplasmic ERCC2 expression and poor clinical outcome in present study could be explained by reduced repair capacity of genomic DNA, which probably could result in more biologically aggressive tumors due to susceptibility to greater genetic aberrations over the time, thereby resulting in early recurrence and worse outcomes [17] and subsequently to poor survival. However, in relation to adjuvant treatment, cytoplasmic ERCC2 expression failed to predict response/failure to given 5FU/oxaliplatin based treatment in studied patients.

On the other hand, when nuclear ERCC2 expression correlated with prognosis, colon cancer patients with negative nuclear ERCC2 expression was associated with a significant reduced OS (P = 0.038), whereas rectal cancer patients with positive nuclear ERCC2 expression was associated with a significant reduced OS (P = 0.005). Furthermore, supporting the above data, in patients treated with adjuvant therapy, subgroup of colon cancer patients with negative ERCC2 expression also showed reduced OS (P = 0.099), conversely rectal cancer patients with positive ERCC2 expression was associated with a significant reduced OS (P = 0.009). These results indicated differential role of nuclear ERCC2 expression according to anatomic site of the tumor. Upon considering that rectal cancer is biologically more aggressive tumor site than colon cancer, nuclear localization of ERCC2 might have functioned differentially in both the tumor sites.

Importantly, the mechanism of action of chemotherapy drug is to induce DNA damage in cancer cell. The mainstay of treatment in CRC patients is chemotherapy alone or combination with radiotherapy. Since ERCC2 is involved in the repair of DNA damage and majority of the patients in present study were treated with adjuvant chemotherapy, the observation of negative nuclear ERCC2 as a poor prognosticator in colon cancer and positive nuclear ERCC2 expression as a poor prognostic factor in rectal cancer patients could be explained as follows. In case of patients with colon cancer, negative nuclear ERCC2 expression might have led to decreased DNA repair activity in cancer cell, which might have caused higher probability of genetic aberrations and hence leading to aggressiveness of tumor, leading to poor survival. On the other side, it can be hypothesized that in patients with rectal cancer, positive nuclear ERCC2 expression might have led to better DNA repair activity compared to negative expression and hence could resist the oxaliplatin based therapy by repairing the platinum mediated DNA adduct formation, ultimately resulting to less sensitivity or lower response to chemotherapeutic drug. This could be one reason as why rectal cancer patients with positive nuclear ERCC2 protein had poor survival.

In CRC, there is very little information found regarding prognostic and predictive role of ERCC2 expression. A rare report by Huang., *et al*. 2013 suggested that ERCC2 protein expression had no predictive value in stage III CRC patients receiving adjuvant FOLFOX4 treatment [15]. Further, Kassem., *et al*. 2017 studied the expression levels of ERCC1 and ERCC2-mRNA and proteins in 80 CRC patients who received first line oxaliplatin based chemotherapy. Patients with low mRNA ERCC1 levels showed significantly longer OS (P = 0.011) and EFS (p < 0.001). However, no significant relation was found between ERCC2 protein levels and OS or EFS [18]. Additionally, one study did not find the relationship between tumor response and ERCC2 overexpression in rectal cancer patients receiving FOLFOX-based preoperative CRT [19]. Based on prior *in-vitro* study by Xu., *et al*. 2002, a correlation of XPD protein levels with anticancer drug resistance including alkylating agents was detected in human tumor cell lines suggesting that XPD is implicated in the development of this resistance [20]. In ovarian cancer patients also, high levels of ERCC2 mRNA expression were associated with drug resistance [21]. The differences in response to platinum-based chemotherapy in different studies may be related to the changes in ERCC2 protein folding properties, rather than protein expression levels [14]. Further, several studies also showed the role of ERCC2 expression with clinical outcome in other malignancies.

One report examined ERCC1, ERCC2, ERCC4, or GSTPi protein expression in 34 unresectable pancreatic cancer patients and indicated that tumor expression of ERCC1, ERCC2, ERCC4, and GSTPi does not predict the safety or efficacy of FOLFIRINOX in patients with pancreatic cancer [22]. Moreover, previous study by Dabholkar., *et al*. 1992 studied ERCC1 and ERCC2 gene expression levels from tumor tissues of 26 ovarian cancer patients by slot blot analysis, confirmed by polymerase chain reaction analysis, and found that patients who were clinically resistant to platinum-based therapy had a 2.6-fold higher expression level of ERCC1 in their tumor tissue than did patients who responded to that therapy (P = 0.015). However, relative levels of expression of ERCC2 did not differ significantly between responders and non-responders. Finally, they concluded that ERCC1 expression levels in human tumor tissue may have a role in clinical resistance to platinum compounds [23]. These data appear to be consistent with the assertion that ERCC1 serves as an excision nuclease, whereas ERCC2 serves as a helicase.

To the best of our knowledge, this is the first study which examined association of ERCC2 immunohistochemical localization with clinicopathological parameters, prognosis and treatment response in CRC patients. Hence, further studies in larger set of patients are needed to better understand the mechanism underlying the cytoplasmic/nuclear immunoreactivity of ERCC2, its association with prognosis and further its correlation with ERCC2 polymorphism in CRC patients.

Conclusion

Negative cytoplasmic ERCC2 protein expression could be a useful biomarker to classify high risk group of CRC patients with poor prognosis. Additionally, negative nuclear ERCC2 immunohistochemical localization emerged as a poor prognostic and predictive marker in colon cancer patients, while it was associated with better clinical outcome in rectal cancer patients. Hence, nuclear ERCC2 protein expression displayed differential function indicating its possible role as prognostic and predictive marker according to anatomic site of tumor.

Acknowledgement

We are extremely thankful to Medical Oncology Department and Surgical Oncology Department, The Gujarat Cancer and Research Institute, for providing their support to fulfill the present study.

Conflict of Interest

The authors report no conflict of interest.

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Volume 7 Issue 8 August 2020