High Expression of Excision Repair Cross-Complementation Group1 Protein Predicts Poor Oucomes in Patients with Nasopharyngeal Cancer

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감사의 글

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저자씀

- ABSTRACT -

High Expression of Excision Repair Cross-Complementation Group1 Protein Predicts Poor Outcome in Patients with Nasopharyngeal Cancer

Purpose: We evaluated the prognostic significance of excision repair cross-complementation group 1 protein (ERCC1), thymidylate synthase (TS) and Bax in patients with nasopharyngeal cancer (NPC) treated with concurrent chemoradiotherapy (CCRT).

Patients and Methods: Pretreatment tumor biopsy specimens from 41 patients with locally advanced NPC (stage I: 1, II: 10, III: 9, IV: 21 patients) were analyzed for ERCC1, TS and Bax expression by immunohistochemistry. All patients were treated with one cycle of induction chemotherapy (5-fluorouracil 1,000 mg/m^2/day and cisplatin 20 mg/m^2/day, days 1-4) followed by CCRT starting on day 22. CCRT consisted of radiotherapy (70Gy/35 fractions for 7 weeks) with cisplatin 20mg/m^2/day for 4 days on weeks 1, 4, 7 of radiotherapy.

Results: High expression of ERCC1, TS and low expression of Bax was observed in 25 (60%), 21 (51%) and 21 (51%) patients, respectively. High expression of ERCC1 was associated with WHO type 1 or 2 histology (p=0.045). Complete response was achieved in 34 patients (83%) after CCRT. With median follow up duration of 106 months (32-152 months) in survivors, 5-year overall survival (OS) of all patients was 53%. In univariate analysis, 5-year OS (75% versus 28%, p=0.005) was significantly inferior in patients with high expression of ERCC1, while high expression of TS and Bax were not correlated with patients

outcome. In multivariate analysis, high expression of ERCC1 was a significant independent prognostic factor for poor OS (p=0.029) along with WHO type 1 or 2 histology.

Conclusion: High expression of ERCC1 protein may be a useful prognostic factor for poor outcome in patients with locally advanced NPC treated with CCRT.

Key Words: Nasopharyngeal cancer, Chemoradiotherapy, ERCC1, Prognostic factor

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I. INTRODUCTION

Although nasopharyngeal cancer (NPC) is rare in most parts of the world including Korea, it is a common type of malignancy in Southeast Asia (Parkin et al., 1997; Chan et al., 2002).

In several phase III studies including the Intergroup 0099 trial and meta-analysis, concurrent chemoradiotherapy (CCRT) with or without adjuvant chemotherapy showed improved survival compared with radiotherapy alone (Al-Sarraf et al., 1998; Lin et al., 2003; Kwong et al., 2004; Langendijk et al., 2004; Chan et al., 2005; Wee et al., 2005). Based on these results, cisplatin-based CCRT is now considered as a standard treatment of locally advanced NPC (Guigay et al., 2006; Guigay, 2008).

Therefore, determining parameters to identify patients who would benefit from cisplatin-based CCRT and who would not has strong clinical implications. The excision repair cross-complementation group 1 (ERCC1) is a drug-resistance related protein that prevents apoptosis of cancer cells by removing platinum-DNA adducts generated by cisplatin and thus repairing damaged cells (Reed, 2005; Olaussen et al., 2006). ERCC1 is also known to be associated with the repair of radiation-induced DNA damage (Murray et al., 1996; Murray et al., 2002). In terms of resistance to 5-fluorouracil (5-FU), thymidylate synthase (TS), which is a critical target of 5-FU, has been widely investigated. TS catalyzes the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, which is an essential process for DNA synthesis (Pinedo and Peters, 1988). A high expression of TS may be associated with 5-FU resistance in a variety of malignancies (Boku et al., 1998; Shirota et al., 2001; Yasumatsu et al., 2009). Lastly, Bax is an apoptosis related protein, which

permeabilizes the mitochondrial membrane, leading to the activation of downstream apoptosis signaling pathways. Because apoptosis is a predominant mechanism of cancer cell death by chemotherapy as well as radiotherapy, abnormal expression of Bax may have clinical implications (Fulda et al., 2006). A low expression of Bax was shown to have poor prognosis in our previous report (Kang et al., 2006). Thus, alterations in these drug-resistance and apoptosis related proteins may be associated with resistance to chemoradiotherapy (CRT), which is the most common cause of treatment failure in locally advanced NPC.

We evaluated the prognostic significance of ERCC1, TS and Bax in locally advanced NPC patients treated with 5-FU and cisplatin induction chemotherapy followed by CCRT with cisplatin.

II. PATIENTS AND METHODS

A. PATIENTS

Among 57 patients with locally advanced NPC (stage II-IV) who underwent CRT at Ajou University Medical Center between October 1996 and October 2006, 41 patients with available pre-treatment tumor specimen were included in this retrospective study. All tumor tissues were obtained from nasopharynx, except 1 patient from cervical lymph node. The treatment results for part of this cohort were previous reported (Kang et al., 2006). Each patients underwent physical examination, chest X-ray, magnetic resonance imaging (MRI) with or without CT of the nasopharynx and neck, FDG-positron emission tomography (PET) scan and hematologic and biochemical profiles for initial staging work up. Patients were staged according to the American Joint Committee on Cancer(AJCC) staging.

This research protocol was approved by the Institutional Review Board of the Ajou University Medical Center, Suwon, Korea.

B. METHODS

1. Chemoradiotherapy

After completion of staging work up, patients were treated with one cycle of induction chemotherapy with 5-FU 1,000 mg/m^2/day and cisplatin 20 mg/m^2/day administered on day 1-4. On day 22, CCRT consisted of radiotherapy (70Gy/35 fractions for 7 weeks) with cisplatin 20 mg/m^2/day continuous infusion for 4 days on weeks 1, 4, 7 of radiotherapy was started. Radiotherapy was administered on linear accelerator using conventional bilateral

portals or multiple portals of conformal or intensity modulated radiation therapy techniques. After completion of CCRT, two cycles of adjuvant chemotherapy with the same regimen as in the induction chemotherapy were given if possible.

2. Evaluation

The response to treatment was assessed 8 weeks after completion of CCRT using MRI with or without FDG-PET scan along with physical examination including flexible nasopharyngoscopy. MRI and FDG-PET scan were done alternatively every 3 months up to 2 years after initial posttreatment evaluation. Then, the patient was evaluated with FDG-PET scan every 6 months for 3 years, and yearly thereafter. Response was evaluated according to the RECIST criteria (Therasse et al., 2000).

3. Immunohistochemical staining for ERCC1, TS and Bax

Formalin-fixed, paraffin-embedded tumor tissues were used for immunohistochemical staining of mouse anti-human monoclonal antibodies against ERCC1 (dilution 1:100, Neomarkers, Fremont, CA, USA) and TS (dilution 1:100, Zymed, San Francisco, CA, USA), and rabbit anti-human polyclonal antibody against Bax (dilution 1:1000, DAKO, Carpinteria, CA, USA). Sections were deparafifnized in xylene and rehydrated in graded alcohols and water. For antigen retrieval, specimens were exposed to 10mM citrate buffer (pH 6.0) and heated for 15 minutes in a water bath (120°C). Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxidase for 10 minutes. Sections were treated with protein-blocking solution and then with primary antibodies overnight at 4℃. After several

rinses in phosphate-buffered saline, the sections were incubated in the biotinylated secondary antibodies. Bound antibodies were detected by the streptavidin-biotin method with a Cap-Plus detection kit (Zymed, CA, USA). Slides were rinsed in phosphate-buffered saline, exposed to diaminobenzidine, and counterstained with Mayer's hematoxylin. Negative controls for these proteins were made by the omission of the primary antibody during the process of immunohistochemical staining. For positive control for TS and ERCC1, a tissue section of colon adenocarcinoma known to have high expression of TS and endothelial cells in tonsil were used, respectively, while lymphocytes in the germinal center was used as the positive control for Bax.

The slides were examined independently by two observers (JHH, JHK) blinded to both clinical and pathologic data. Expression of the drug resistance-related proteins was quantified using visual grading system based on the extent of staining (by percentage of positive tumor cells graded on a scale of 0 to 3; 0 = none, 1 = 1%-10%, 2 = 11%-50%, 3 = 51%-100%) and the intensity of staining (graded on a scale of 0 to 3; 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). A semiquantitative H-score was obtained by multiplying the grades of extent and intensity of staining. The median value of all the H scores was chosen *a priori* as the cutoff value for dividing the expression of proteins into high and low.

4. Statistical analysis

A comparison of clinicopathologic characteristics was evaluated with the Fisher's exact test. Overall survival (OS) was calculated using the Kaplan-Meier method (Kaplan and

Meier, 1958). OS was defined as the time from start of treatment to death. Data on survivors were censored at the last follow-up. The differences between the survival curves were tested by using the log-rank test. The Cox proportional-hazards regression model was used to determine the joint effects of several variables on survival (COX, 1972). Factors with P values <0.1 in univariate analysis were included in the Cox proportional-hazards regression model. All statistical analysis was performed with SPSS for Windows 12.0 software.

III. RESULTS

1. Patients characteristics

The clinicopathologic characteristics of 41 patients are listed in Table 1. One stage I disease patient, according to the 1997 AJCC staging, was included in this study because he had been staged and treated as a stage II patient using 1992 AJCC staging. While 12 patients (29%) completed three cycles of cisplatin chemotherapy during RT as planned, 22 (53%) and five patients (12%) received two and one cycles, respectively, mainly due to the toxicity of CCRT. In addition, two patients did not receive cisplatin during RT because of severe toxicity during the induction chemotherapy. Although two cycles of adjuvant chemotherapy were recommended after completion of CCRT, only five patients (12%) received it.

Table 1. Characteristics of patients.

Characteristics	Number (%)
Age (years)	
Median	50
Range	19-66
Gender	
Male	29 (71)
Female	12 (29)
Performance status (ECOG*)	
0-1	40 (98)
2	1 (2)
WHO histologic type	
I	7 (17)
II	6 (15)
III	28 (68)
Stage	
I	1 (2)
II	10 (25)
III	9 (22)
IV	21 (51)

*Eastern Cooperative Oncology Group scale.

2. Treatment response

Complete remission (CR) was achieved in 34 patients (83%), partial response (PR) in six patients (15%) and progressive disease (PD) in one patient (2%), based on post-CCRT imaging.

3. Association of expression of drug resistance-related proteins with clinicopathologic characteristics

The median values of the H-scores of ERCC1 and TS were both six, while that of Bax was one. High expression of ERCC1, TS and low expression of Bax was observed in 25 (60%), 21 (51%) and 21 (51%) patients, respectively, and tumors with H-scores higher than these median values were classified as high expression (Fig. 1). There was no significant correlation among expression of ERCC1, TS and Bax. High expression of ERCC1 was significantly associated with WHO type 1 or 2 histology (p=0.045), while expression of TS and Bax were not correlated with any clinicopathologic characteristics (Table 2).

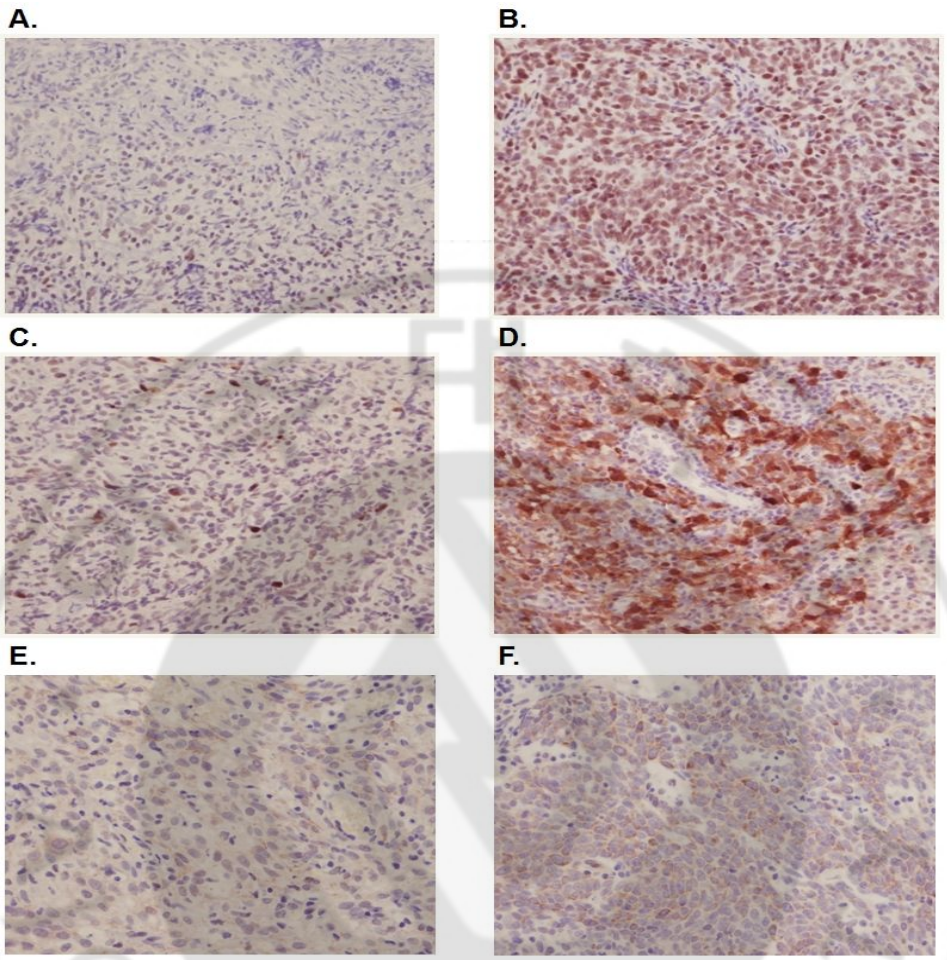

Fig. 1. Immunohistochemical staining of ERCC1, TS and Bax (x400).

(A) Low expression of ERCC1: grade 1 in the extent of staining and weak staining. (B) High expression of ERCC1: grade 3 in the extent of staining and strong staining. (C) Low expression of TS: grade 1 in the extent of staining and weak staining. (D) High expression of TS: grade 3 in the extent of staining and strong staining. (E) Low expression of Bax: grade 1 int the extent of staining and weak staining. (F) High expression of Bax: grade 3 in the extent of staining and moderate staining.

Table 2. Association between expression of ERCC1 and characteristics of patients.

Characteristics	ERCC1 expression		*P*
	Low (%)	High (%)	
Age (y)			0.530
<50[a]	9 (56)	11 (44)	
≥50	7 (44)	14 (56)	
Gender			0.734
Male	12 (75)	17 (68)	
Female	4 (25)	8 (32)	
Stage			0.287
I, II	6 (37)	5 (20)	
III, IV	10 (63)	20 (80)	
WHO histologic type			0.045
I, II	2 (12)	11 (44)	
III	14 (88)	14 (56)	
Response			0.215
Complete	15 (94)	19 (76)	
Others	1 (6)	6 (23)	
TS expression			0.751
Low	7 (44)	13 (52)	
High	9 (56)	12 (48)	
Bax expression			0.058
Low	5 (31)	16 (64)	
High	11 (69)	9 (36)	

[a]Median.

4. Association between expression of drug resistance-related proteins with patient outcome

The median follow-up duration of the survivors was 106 months (range: 32-152 months) and no patient was lost to follow-up. Nineteen patients were alive at the time of analysis. The median OS of total patients was 69 months with 5-year OS of 53%, respectively. In univariate analysis, 5-year OS (75% versus 28%, p=0.005) was significantly inferior in patients with high expression of ERCC1 (Fig. 2, Table 3). High expression of TS and Bax were not associated with OS of patients (p=0.817 and p=0.125) (Fig. 2) (Table 3). In multivariate analysis, high expression of ERCC1 was a significant independent prognostic factor for poor OS (p=0.029) along with WHO type 1 or 2 histology (p=0.008) (Table 4).

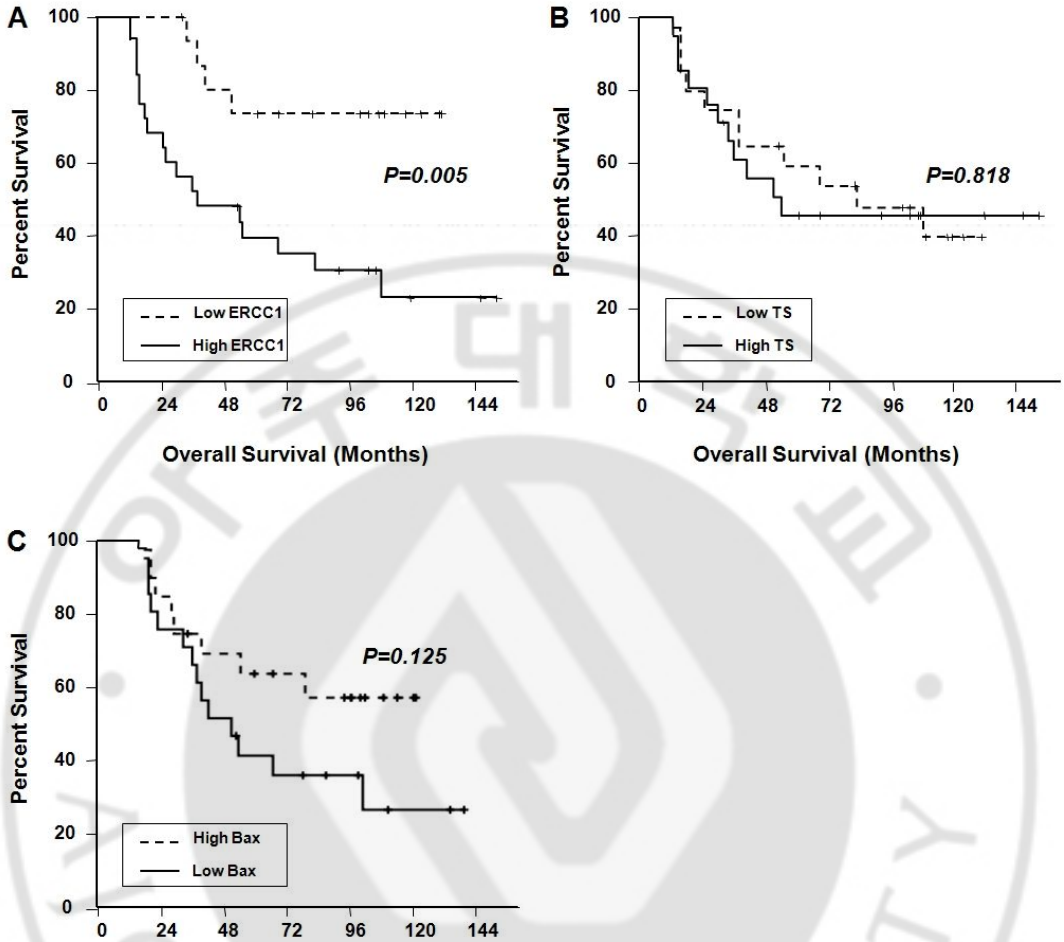

Fig. 2. Overall survival according to the expression of ERCC1 (A), TS (B) and Bax (C).

Table 3. Overall survival of the patients according to various characteristics

Characteristics	No. of Patients	5-year overall survival (%)	*P*
Age (y)			0.020
<50*	20	69	
≥50	21	38	
Gender			0.343
Male	29	51	
Female	12	57	
WHO histologic type			0.0001
I or II	13	23	
III	28	67	
Stage			0.016
II†	11	82	
III	9	56	
IV	21	35	
ERCC1 expression			0.005
Low	16	73	
High	25	39	
TS expression			0.817
Low	20	60	
High	21	46	
Bax expression			0.125
Low	21	42	
High	20	64	

*Median.

†including one patient with stage IB.

Table 4. Multivariate analysis of overall survival.

Prognostic factor	Hazard ratio	95% CI	p value
Age			0.055
<50*	1.00		
≥50	2.43	0.98 – 6.01	
WHO histologic type			0.008
III	1.00		
I or II	3.21	1.35 – 7.63	
Stage			0.060
ERCC1 expression			0.029
Low	1.00		
High	3.42	1.14 – 10.30	

Abbreviations: CI, confidence interval

*Median.

IV. DISCUSSION

We evaluated the expression of ERCC1, TS and Bax under the assumption that abnormalities in these drug-resistance and apoptosis related proteins may be associated with resistance to CRT with 5-FU and cisplatin, ultimately leading to poor outcome in patients with locally advanced NPC.

The 5-year OS (46%) in the current study was somewhat inferior compared with other phase III trials in the endemic area (Lin et al., 2003; Chan et al., 2005; Wee et al., 2005). Although direct comparison is difficult because of different median follow-up duration and treatment scheme, a relatively higher proportion of patients with WHO type 1 or 2 histology in this study is a possible explanation for the difference in outcomes (Ou et al., 2007).

In terms of the relationship between drug-resistance related proteins and the characteristics of patients, high expression of ERCC1 was significantly associated with WHO type 1 and 2 histology. WHO type 3, undifferentiated carcinoma, is prevalent in the endemic area with a strong association with Epstein-Barr virus, while smoking and alcohol consumption are considered as important etiologic factors for WHO type 1 histology (Vaughan et al., 1996; Nakao et al., 2006). Therefore, differences in etiologic factors between type 3 and type 1 or 2 histology could explain distinct genetic alterations, such as high expression of ERCC1, although further validation is required for this supposition. High expression of TS did not correlate with any clinicopathologic characteristics, including outcomes for patients in the current study. In the present cohort, 88% of patients received only one cycle of 5-FU containing chemotherapy, which may explain the failure of high TS expression to predict

patient outcomes. In the case of Bax, although low expression was not significantly associated with patient outcomes, it showed a trend toward poor prognosis. Small number of cohort patients and low median H-score of Bax (one) in our study probably resulted this insignificance and it may necessitate further validation.

The most important finding of the current study was the prognostic significance of the high expression of ERCC1 in NPC patients treated with CRT. The correlation between the expression of ERCC1 and resistance to platinum-based chemotherapy or prognosis has been reported in various malignancies, such as non-small and small cell lung, esophageal, gastric, colorectal, and ovarian cancer (Metzger et al., 1998; Shirota et al., 2001; Joshi et al., 2005; Olaussen et al., 2006; Lee et al., 2008). In head and neck squamous cell carcinoma, high expression of ERCC1 was associated with poor survival in patients with a locally advanced disease treated by CRT (Handra-Luca et al., 2007; Jun et al., 2008).

The poor outcome for NPC patients with high expression of ERCC1 most likely results from a resistance to cisplatin, which is an integral component of CRT for locally advanced NPC. Resistance to radiotherapy is also a possible explanation for poor outcome in patients with a high expression of ERCC1, since ERCC1 is associated radiation-induced DNA damage in some in vitro studies, although the mechanism is not well understood (Murray et al., 1996; Murray et al., 2002). Alternatively, high expression of ERCC1 may reflect the intrinsic aggressiveness of tumor, considering the significant correlation between high expression of ERCC1 and type 1 or 2 histology, which has been suggested as a poor prognostic factor for NPC (Ou et al., 2007).

The present study has several potential limitations. First, this study is a retrospective

analysis with a relatively small sample size. Second, because a significantly small proportion of patients received 5-FU/cisplatin adjuvant chemotherapy after CCRT, it was difficult to evaluate the role of TS as a prognostic factor. Finally, unlike the CCRT with cisplatin in the Intergroup study, which is considered as a standard of care in locally advanced NPC, one cycle of 5-FU/cisplatin induction chemotherapy was administered before the start of CCRT in the present study (Al-Sarraf et al., 1998). This modification was necessary to avoid the delay of treatment due to pretreatment dental care and RT planning. According to this protocol, patients could undergo these procedures during or after induction of chemotherapy.

Despite these limitations, to our knowledge, the present study is the first report investigating the expression of ERCC1 in patients with NPC. Moreover, the role of ERCC1 as an independent poor prognostic factor was demonstrated in a patient cohort treated with uniform CRT regimen in the current study. The present results suggest the possibility that high expression of ERCC1 could be useful as one of the prognostic factors in patients with NPC treated with CCRT, because only a few small studies have been performed in NPC for molecular markers (Kang et al., 2006).

Since the standard treatment for locally advanced NPC patients is CCRT with cisplatin, patients with high expression of ERCC1 might benefit from alternative strategies such as incorporation of new chemotherapeutic agents or different sequencing of CRT. However, a confirmatory study is essential to prove this speculation.

V. **CONCLUSION**

In conclusion, high expression of ERCC1 was significantly associated with the poor outcome of patients with locally advanced NPC treated by CRT. If the prognostic significance of high expression of ERCC1 is validated by further prospective studies with larger numbers of patients, a relatively simple immunohistochemical staining for ERCC1 with a pretreatment biopsy specimen may provide valuable information to the oncologist in selecting an appropriate CRT regimen.

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-국문 요**약**-

비인강암 환자에서 Excision Repair Cross-Complementation Group1 단백의 과발현이 예후에 미치는 영향

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연구 목적: 비인강암은 동남아시아 지역에서는 유병률이 높으나 국내에서는 드문 종양으로 알려져 있으며, Intergroup 0099 trial을 포함한 다수의 연구에서 cisplatin을 근간으로 한 동시항암화학방사선 요법이 국소진행성 비인강암 환자의 표준치료로 인정받고 있다. 따라서 cisplatin을 근간으로 한 동시항암화학방사선 요법을 시행할 때, 반응을 예측할 수 있는 예후인자를 찾는 것은 임상적으로 중요한 의미를 갖는다. 본 연구에서는 excision repair cross-complementation group 1 protein(ERCC1), thymidylate synthase(TS), Bax 단백의 면역조직화학염색을 통하여 각 단백의 발현 정도에 따른 예후인자로서 임상적 유의성을 알아보고자 하였다.

연구 방법: 41명의 국소진행된 비인강암 환자(stage I: 1명, II: 10명, III: 9명, IV: 21명)를 대상으로 치료 전 시행된 종양의 조직생검 검체를 이용하여 ERCC1, TS, Bax 단백의 발현 정도를 분석하기 위하여 면역조직화학염색을 시행하였다. 모든 환자는 1주기의 유도항암화학요법을(5-fluorouracil 1,000 mg/m^2/day and cisplatin 20 mg/m^2/day, 항암일 1일-4일) 시행 받았고, 이어서 항암일 22

일에 동시항암화학방사선요법을 시행 받았다. 동시항암화학방사선요법은 7주 동안 70Gy/35fr의 방사선치료와 방사선치료 1, 4, 7주에 4일간의 cisplatin ($20mg/m^2/day$) 정맥 투여로 시행하였다.

연구 결과: ERCC1 단백의 과발현은 25명(60%), TS 단백의 과발현은 21명(51%), Bax 단백의 저발현은 21명(51%)에서 관찰되었다. ERCC1의 과발현은 WHO 조직형 1, 2와 연관성이 있었다(p=0.045). 동시항암화학방사선요법 후 완전관해는 34명(83%)의 환자에서 관찰되었다. 중앙 관찰기간은 생존자군에서 106개월(32-152개월)이었으며, 모든 환자군에서 5년 생존률은 53%였다. 단분량분석에서 5년 생존률은 ERCC1 단백이 과발현된 경우 유의하게 낮았으며(75% 대 28%, p=0.005), TS 단백, Bax 단백은 생존률과 임상적으로 유의한 관계를 보이지 못했다. 다분량분석에서 ERCC1이 과발현된 경우 WHO 조직형 1, 2와 더불어 불량한 생존을 예측하는 통계적으로 유의한 예후인자(p=0.029)임이 확인되었다.

결론: 본 연구 결과는 동시항암화학방사선요법을 시행 받은 국소진행성 비인강암 환자에서 ERCC1 단백이 과발현된 경우 불량한 예후와 유의한 연관성이 있음을 보여주었다. 추후 다수의 환자를 대상으로 한 전향적인 연구를 통하여 ERCC1 단백 과발현의 예후인자로서 임상적 유의성이 확인된다면, 치료 전 조직생검 검체를 이용하여 간단한 조직화학염색인 ERCC1 염색을 통하여 적절한 동시항암화학방사선요법을 선택하는데 도움을 줄 것으로 기대된다.

핵심어 : 비인강암, 항암화학방사선요법, ERCC1, 예후인자